Creative eDNA capture strategies for measuring marine biodiversity.

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A thesis presented to Liverpool John Moores University in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the School of Biological and Environmental Sciences.

June 2024

This thesis is dedicated to Rosalina Baldeo Oliveras, my Grandma Rose. Thank you Loleng for always encouraging me to take education seriously.

Maraming salamat po.

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Acknowledgements

When I was undergraduate, someone told me, “Doing a PhD is selfish, you should know that before you decide to do one”. I believe they were right. The shear amount of people that have supported me professionally and personally to help me achieve this goal are a testament to that. I will forever be grateful to everyone that carried me along the way to this doctorate.

Stefano, I cannot thank you enough for your endless support and mentorship over the years. Maybe you’ll remember that I was over the moon to start as a research assistant for the SpongeDNA project, but when you asked me if I wanted to register as a PhD student, I initially said no. Truthfully, it was because I was scared. I knew research was hard, I was not sure a PhD was right for me, and let’s face it, I can be a bit risk adverse. Your enthusiasm, quirkiness and positivity quickly made me realize that I would be stupid not to take up the opportunity to do a PhD with you. Thank you for helping me develop my writing and always reading everything; this support has allowed me to enjoy this process through to the end. Most importantly, thank you for giving me confidence as a scientist, by taking the time to give me lots of critical feedback but equally lots of validation when I did things well.

There is a small army of post-docs that have made this thesis possible. A massive thank you to all of these legends: Lynsey Harper, Belén Arias, Peter Shum, Wang Cai, and Giulia Maiello. Lynsey, thank you for introducing me to world of eDNA, and for your time and patience to teach me everything I know about laboratory work. Belen, thank you for being wonderful company in the lab and always available for questions. Peter, thank you so much for being essentially a co-supervisor and for sacrificing your time shortly after Enya was born to help with the Greece fieldwork; chapter 3 would have been impossible without you, and you might be the only person I trust to help force my torso underwater for long periods of time. Wang, thank you so much for having an amazing sense of humor and for being so masterful at bioinformatics. It was so much fun getting to travel for field work and to conferences with you. Giulia, thank you for letting me ‘stand on the shoulders’ of your thesis and for always being willing to help me troubleshoot. Half of this thesis would not have been possible without you.

There are many other researchers, collaborators, and university staff that I am grateful to have worked with. Thank you so much to Ana Riesgo for sharing the amazing world of sponges with me and always being supportive even though our relationship was almost completely remote. Thank you to Kostas Kiriakoulakis for serving as my co-supervisor. And thank you to many others who have supported me through the PhD: Andrea Waeschenbach, Daniel Graham, Thomas Byrne, Steven Longworth, Lucia Bravo, Rupert Collins, as well as helpful staff in BES and the LJMU doctoral academy. There is a long list of volunteers and collaborators that were integral to the DNA divers project. A huge thank you to Ivvet Modinou for meeting regularly with me in the early days and for helping me get it off the ground. I cannot thank Bob Anderson, Natasha Yates and Fiona Crouch enough for believing in me and donating boat-time and personal time; the project would have been impossible without you. Thank you to all of the SCUBA divers and snorkellers for participating in this research.

I am so grateful for the support I’ve had from my peers and the wider Mariani lab group. Chris, thank you for always being super encouraging and teaching me about British banter and pub culture. Thank you to Courtney and Annie for being amazing company whether it be in the lab, field or outside of work. Alice, I feel so incredibly lucky to have had someone as brilliant as you to go through this PhD journey with. When I was feeling my lowest during this process you always helped me remain level-headed and could get me out of slump, whether that involved letting me talk you through all of my figures, helping me trouble-shoot and make sense of lab results, getting coffees with me, celebrating when progress was made, trying new beers at ship & mitre, the list feels endless. This thesis would not have been possible without making these lifelong friendships.

There is no way I could have done this without my family. Callum, thank you so much for your love, gentleness, and seemingly endless patience. Thank you for keeping me sane, listening to me talk about research, technical support, photo-shop tutorials, making me hot drinks, and for being my biggest cheerleader. Mom, thank you for always wanting me to be happy, and for supporting my education and my choice to pursue a subject out of curiosity more than anything else. Dad, thank you for teaching me to love nature and for taking me snorkelling and later diving as a child. You always pushed me out of my comfort zone on those adventures and were fully supportive (emotionally and financially) of my dream to be a marine biologist. Micheal, thank you for always telling me that you think it’s cool that I’m a scientist, and for having the best sense of humour; you always lift me up. Thank you to the Spiers family, Pete and Annie, for always asking about how my PhD was going and having me over for dinner. Thank you to my friends and ultimate frisbee teammates who successfully kept me distracted on the weekends, providing the stamina needed to complete this thesis. And finally, thank you to my cat Calvin for the snuggles. I love you all so much.

“Even in the vast and mysterious reaches of the sea we are brought back to the fundamental truth that nothing lives to itself.”

—**Rachel Carson**, *Silent Spring*

General Abstract

The ocean is the largest habitat on our planet and it remains the least explored, with 75% of the seafloor unmapped and an estimated 90% of marine species left undiscovered. Already from the fraction of marine species and ecosystems that we do know about, it is overwhelmingly clear that ocean life is interconnected to all life on Earth and provides vital services to society. Despite an inherent link between ocean life and the well-being of people, many of our measurements of biodiversity are occasional, focus on single species, can be invasive and counter-intuitive to conservation efforts, and are not feasible for marine managers to routinely implement. Now more than ever before, a rapid, broadly applicable, non-invasive, low-cost technology exists to generate multi-species observations of marine life, which are critical for their management and conservation. Environmental DNA (eDNA) metabarcoding should make marine biodiversity discovery and monitoring an easy feat, but a major challenge that slows its widespread uptake is the cumbersome and limiting processes required to capture and concentrate eDNA from vast marine habitats. This thesis examines mechanisms for overcoming those challenges by investigating two new and creative strategies for capturing eDNA: i) by leveraging the filtration capacity of marine sponges as natural eDNA sampling accumulators; ii) by deployment of simple, quasi-passive eDNA samplers in association with SCUBA diving activities; and explores how these strategies could extend the reach of current marine biodiversity monitoring efforts. Two chapters are dedicated to each eDNA capture technique, where the first displays a proof-of-concept for the method while the second provides a fine-tuned and targeted application of the approach. The thesis highlights how working in partnership, particularly to share resources already existing in the worlds' biological collections and to include non-professional scientists and communities in the research process, significantly enhances scientific discovery. Life below water exists over vast ocean habitats that can be difficult to measure but we now have technologies that spark optimism. Creativity and resourcefulness are necessary ingredients that will help us

overcome any remaining challenges and make effective stewardship possible everywhere.

Authorship Declaration

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

Author contributions to each data chapter are explained below and formatted using Contribution Roles Taxonomy (CRediT).

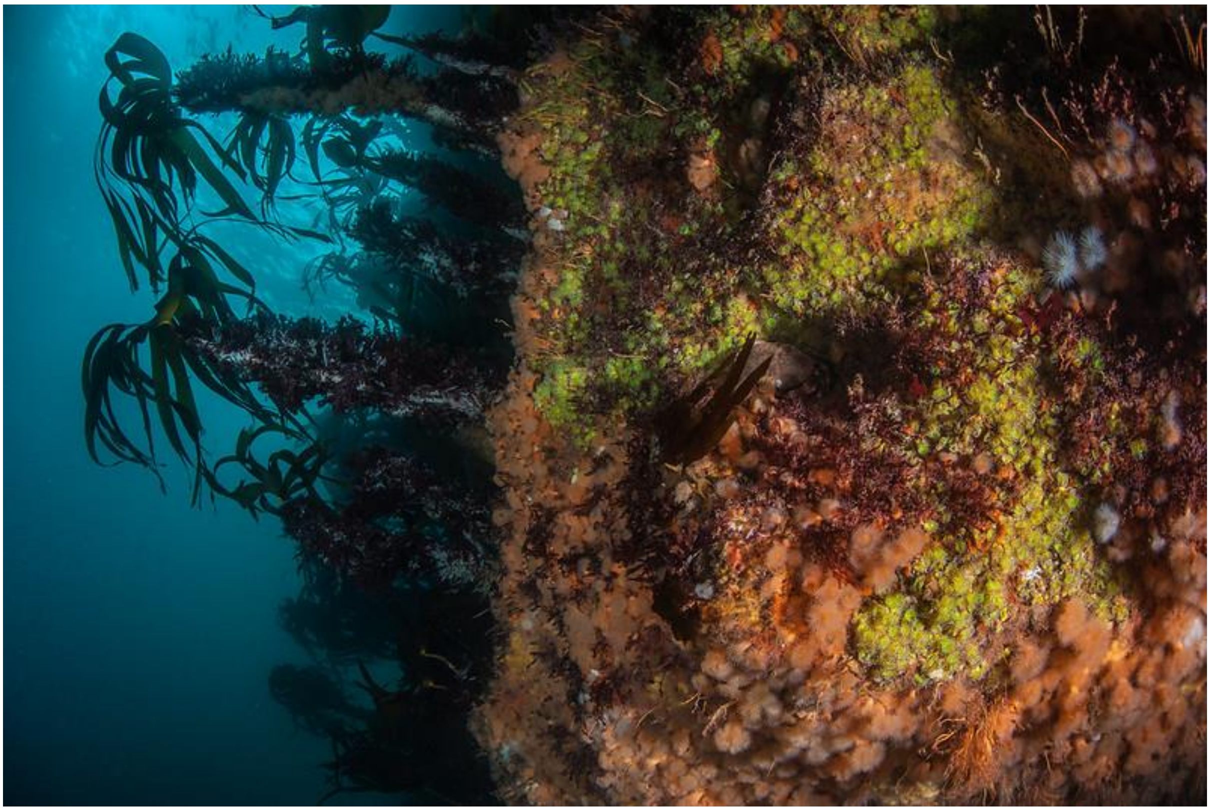
Chapter 2: Conceptualization, E.F.N., M.B.A., L.R.H., A.R. and S.M.; Formal Analysis, E.F.N., W.C., and L.R.H.; Investigation, E.F.N, M.B.A and L.R.H.; Visualization, E.F.N.; Writing – Original Draft, E.F.N. and S.M.; Writing – Review & Editing, E.F.N., W.C., M.B.A., L.R.H., A.R. and S.M.; Supervision and Funding Acquisition, A.R. and S.M.

Chapter 3: Conceptualization, E.F.N., S.M.; Methodology: E.F.N, C.D., P.S., A.R., S.M. Formal Analysis, E.F.N.,S.M.; Investigation, E.F.N, C.D., P.S., A.R., S.M.; Data Curation: E.F.N., Visualization, E.F.N.; Writing – Original Draft, E.F.N., C.D., and S.M.; Writing – Review & Editing, E.F.N., C.D., P.S., A.R. and S.M.; Supervision and Funding Acquisition, A.R. and S.M.

Chapter 4: Conceptualization, E.F.N., F.C, S.Meek., S.Mariani.; Methodology: E.F.N, G.M., S.Mariani. Formal Analysis, E.F.N.; Investigation, E.F.N, A.W., A.C., N.Y., C.J.P., F.C., H.J.N.C., P.S., W.C., R.A., K.B., M.C., R.H., W.N., C.B., B.A., E.J.; Data Curation: E.F.N., G.M., Visualization, E.F.N.; Writing – Original Draft, E.F.N., S.Mariani.; Writing – Review & Editing, E.F.N., A.W., A.C., G.M., N.Y., C.J.P., F.C., H.J.N.C., P.S., W.C., R.A., K.B., M.C., R.H., W.N., F.B., C.B., B.A., E.J., S.Meek, G.S., S.Mariani; Supervision S.Mariani; Project Administration: E.F.N., S.Meek; Funding Acquisition, E.F.N. and S.Mariani.

Chapter 5: Conceptualization, E.F.N., N.Y.; Methodology: E.F.N. Formal Analysis, E.F.N.; Investigation, N.Y., B.A.; Data Curation: E.F.N., Visualization, E.F.N., S. M.; Writing – Original Draft, E.F.N., S.M.; Writing – Review & Editing, E.F.N., S.M.; Supervision S.Mariani; Project Administration: E.F.N.; Funding Acquisition, E.F.N. and S.M.

Chapter 1
General Introduction



Sea anemones covering a vertical cliff below the kelp line in St. Kilda, Scotland.

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1.1 People & biodiversity in the ocean

Life in the ocean is inextricably linked to the wellness of people. More than 3.3 billion people depend on ocean life for at least 20% of their daily protein (FAO, 2020); seafood can be part of a nutrient-rich sustainable diet (Koehn et al., 2022); countless medicines, including many cancer treatments, have been derived from ocean life (Dyshlovoy and Honecker, 2019); and blue spaces and ocean recreation support mental (White et al., 2021) and physical health (Carreño et al., 2023; Lloret et al., 2023). The vastness of the ocean on our planet and how it connects us is reflected by a staggering statistic—over 80% of global trade occurs via ocean shipping (WWF, 2024). Given these services it may come as no surprise that the market value for marine industries and resources is estimated at $2.5 trillion USD annually; meaning that if the ocean was a country, it would have the equivalent GDP of the world's 7th largest economy (WWF, 2015). Aside from monetary value, there is the priceless cultural value that marine ecosystems provide and have provided for generations before us (Allison et al., 2020). Finally, and perhaps most importantly, life in the ocean regulates global climate (Behrenfeld et al., 2006), therefore reducing ocean warming will mitigate the likelihood of extreme weather events such as flooding (Alifu et al., 2022; Mousavi et al., 2011), which is a major hazard to people, given that over 50% of Earth's population live within 3 km of freshwater (e.g., river deltas) (Kummu et al., 2011) and that 1 billion people live on coastal land less than 10 meters above high tide lines (Kulp and Strauss, 2019). These services, people's livelihoods and sustenance, all provided by the ocean, rely on maintaining the integrity of healthy ocean ecosystems.

The health of the ocean is often assessed by observing ocean life. Biodiversity, arising from the phrase 'biological diversity', is an all-encompassing term covering the variety of ecosystems, the species that reside in them, and even the genetic variation within species (Secretariat of the Convention on Biological Diversity, 2000). The simplest and most common way of measuring biodiversity, however, is focused on the species-level, by counting the number of different species in an area to determine the richness of a biological community. At the time of writing this thesis there are currently 245 911 accepted marine eukaryotic species in the WoRMS (World Register of Marine Species; www.marinespecies.org) database, but experts predict that the total number of existing marine eukaryotic species could be 10-fold higher than the current accepted amount (Appeltans et al., 2012; Mora et al., 2011). These species all have unique functions within marine ecosystems and rely on different functions of other species for survival. Losing species can cause ecosystem functions to diminish (Gamfeldt et al., 2015), especially if only one species is performing a function that no others can fill (e.g., ecosystem engineers) (Olden et al., 2004). On the other hand, high species richness increases ecosystem functions even when marine ecosystems are under pressure from human-caused disturbances, such as rapid ocean warming (Benkwitt et al., 2020). In fact, there is much evidence to suggest that higher species richness increases the productivity of ecosystems, playing a role akin to abiotic stressors (e.g., temperature, nutrients) (Duffy et al., 2017), which underpins biodiversity as highly important for healthy ocean ecosystems.

Despite obvious links between marine biodiversity and the services it provides to people (Palumbi et al., 2009), there are many human-caused threats that put marine species at risk of extinction. These include but are not limited to direct exploitation, habitat destruction or modification, pollution, climate change and invasive species (Lotze, 2021). People used to have a prevailing sense that the ocean was “too big to fail” and that it had an inexhaustible abundance of seafood; but there is more awareness now that this is not the case, with estimates that marine fish abundance declined by 38% between the years 1970 and 2007 (Hutchings et al., 2010). There is still a high degree of uncertainty around exactly how many marine species exist (Appeltans et al., 2012), especially in less explored environments such as the deep-sea (Danovaro et al., 2017). The International Union for the Conservation of Nature (IUCN) red list is the most comprehensive database of species at risk of extinction, but many marine species remain unassessed due to data deficiency (Luypaert et al., 2020). Acquiring species occurrence data at sea can require expensive surveys relative to land-based surveys, and many marine species are cryptic and not visible with the naked eye (Leray and Knowlton, 2016), or elusive and rare (Magurran and Henderson, 2003). Modern conservation efforts take a whole ecosystem approach, trying to conserve as much biodiversity as possible to maintain healthy ecosystem functioning and services to humans and our planet (Lotze, 2021). Now more than ever before, we have the means necessary to monitor multiple marine species in congruence, using a fast, non-invasive, sensitive, cost-effective tool– environmental DNA analysis.

1.2. Environmental DNA and metabarcoding analyses

Environmental DNA (eDNA) refers to DNA that is isolated from an environment without first separating any target organisms from the sampled environmental medium (i.e., soil, water, sediment, air, etc.) (Taberlet et al., 2012a). The precise definition of eDNA is debated. Recent opinion articles defend this Taberlet et al., 2012 definition, stating that eDNA should be the total pool of DNA that is isolated from any environmental sample (Pawlowski et al., 2020), while others prefer to reserve 'eDNA' for referring to the traces of DNA belonging to macro-organisms found in environmental samples which they are not physically present in. The former definition better reflects the history of eDNA research. For example, the concept of extracting DNA from an environmental sample initially came about in 1987 by soil microbiologists that wanted to isolate microbial DNA from samples, and intuitively saw no need to first separate whole microorganism communities from the sediment containing them (Ogram et al., 1987). The latter definition, restricting eDNA to macro-organisms, reflects the often-desired target taxonomic groups of eDNA analyses, rather than the total DNA that is isolated from the environment— a large fraction of which is usually of microbial origin. Adding to the confusion, collecting whole organisms from an environment and extracting the DNA from a bulk mixture of specimens is often termed 'community DNA'; and therefore, when filtering water for macro-organism eDNA, whole non-target microorganisms (e.g., phytoplankton) are also inevitably captured, making this terminology difficult to navigate in practice (Deiner et al., 2017a).

This thesis focuses on the analysis of eDNA of macro-organismal origin through the capture of both intracellular and extracellular DNA isolated from marine sources, followed by laboratory processes that amplify and sequence vertebrate DNA. Understanding the physical states of eDNA is an area of ongoing research (Jo and Minamoto, 2021; Mauvisseau et al., 2022) but, put simply, eDNA can be in either intracellular form (e.g., marine mammals' molt process releases whole cells from skin and fur (Yochem and Stewart, 2009)) or the extracellular form (e.g., the mucus layer that covers a fish's epidermis sheds mitochondrial DNA (Ignatavičienė et al., 2023)). Sloughing of mucus and molting are both examples of eDNA resulting from the presence of a living macro-organism. An important caveat of eDNA is that it can also come from non-living sources such that the recency of life is uncertain (i.e., prey DNA from faeces), or from decomposing tissue (Deiner et al., 2017a). Moreover, the state of the eDNA (e.g., intracellular or extracellular) including whether it is dissolved in the environment or adsorbed onto particles can influence its rate of decay as well as abiotic factors which speed up (e.g., UV exposure) or slow down (e.g., cold water temperatures) eDNA degradation. The interaction between eDNA states, abiotic factors, and decay rates all require further investigation (Barnes et al., 2014; Mauvisseau et al., 2022). Given the various states and processes which eDNA undergo in the environment, many molecular biology discoveries have honed our ability to deal with highly degraded DNA.

Since eDNA consists of genomic DNA at low concentrations and with relatively low quality, polymerase chain reaction (PCR) or the method used to make copies of (i.e., amplify) DNA (Mullis and Faloona, 1987) is largely relied on for making eDNA analyses possible. Early eDNA studies confirmed the presence of a species by using a species-specific primer to amplify DNA using either PCR (Deagle et al., 2003; Martellini et al., 2005) or real-time quantitative PCR (qPCR) (Biggs et al., 2015; Kirshtein et al., 2007), eventually leading to DNA amplification and subsequent sequencing with 454 pyrosequencing (Ficetola et al., 2008). Development of universal primer sets (Epp et al., 2012; Kocher et al., 1989) (i.e., targeting a group of taxa rather than a single species) was critical for DNA barcoding standardization (Hebert et al., 2003) as was the establishment of coordinated initiatives (e.g., CBOL, Consortium for the Barcode of Life) all of which contributes to reference sequences from specimens that are necessary for the identification of species. This gave rise to a technique called DNA metabarcoding from which the goal is to identify taxa from a bulk community sample that generally consists of high-quality DNA and of longer lengths compared to eDNA

(Taberlet et al., 2012b). Metabarcoding became possible in conjunction with the development of massively parallel sequencing technology, allowing for the high-throughput species identification required for ecological research.

One of the first uses of metabarcoding analysis on modern aquatic eDNA samples was to amplify a fragment of the 16S gene (from filtered seawater) revealing deep-sea microbial communities with 454 pyrosequencing (Sogin et al., 2006). Technologies have improved so much that amplicon libraries can be built even from low concentration, fragmented eDNA, and sequenced with high-throughput Illumina technology, and even ‘third generation’ methods, such as Single-Molecule Sequencing in Real Time (SMRT, PacBio) and Oxford Nanopore Technologies (ONT, MinION). Further eDNA research showed that eDNA metabarcoding can detect higher species richness than DNA metabarcoding of bulk community samples (J.-N. Macher et al., 2018), emphasizing its applicability for biodiversity assessments.

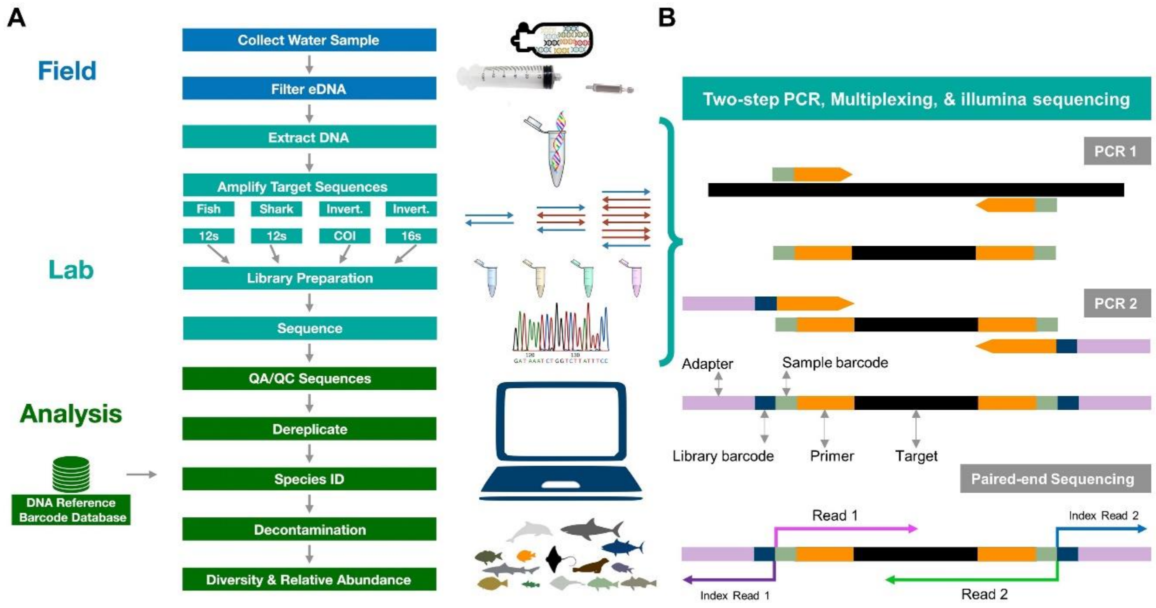

Figure 1.1 A typical eDNA metabarcoding workflow, modified from a figure in (Gold et al., 2022) (A), and a diagram showing the two-step PCR and library process used for preparing amplicons for paired-end Illumina sequencing (B).

Despite progressive improvement of ‘third generation’ sequencers, the projects in this thesis make use of the established ‘gold standard’ approach for eDNA metabarcoding analyses, implemented in Illumina platforms. Briefly, the typical aquatic eDNA metabarcoding workflow comprises of: 1) collecting and filtering water in the field; 2) extracting the eDNA in the laboratory; 3) amplifying the desired gene and target region of the eDNA; 4) building libraries and carrying out sequencing; 5) analysing the data and applying quality control steps before ecological analysis (**Figure 1.1** A). This thesis will use a two-step PCR process to amplify eDNA and prepare libraries for paired-end sequencing on Illumina platforms (**Figure 1.1** B). In the past decade, marine eDNA metabarcoding analyses have rapidly expanded, exploring a wide range of taxa (Andruszkiewicz et al., 2017; Nguyen et al., 2020; Stat et al., 2017) as well as monitoring and conservation applications (Gilbey et al., 2021; Gold et al., 2021; Kelly et al., 2014; Miya, 2022).

1.3. A succinct history of marine environmental DNA studies

Marine aquatic macro-organismal eDNA analyses are expanding the way people monitor and manage marine ecosystems (Kelly et al., 2024, 2014). Over the last decade published marine eukaryotic eDNA research, especially involving multiple species detection, has rapidly increased (Eble et al., 2020). Marine macro-organismal eDNA research has borrowed ideas and progressed from knowledge discovered in different research areas such as ancient eDNA analyses of ice cores (Willerslev et al., 1999), sediment cores and permafrost (Willerslev et al., 2003); microbial metabarcoding (Sogin et al., 2006); eDNA metabarcoding of soil (Epp et al., 2012), sediment (Fonseca et al., 2010) and wastewater (Martellini et al., 2005); and of course, freshwater modern eDNA research (Ficetola et al., 2008; Jerde et al., 2011). One of the earliest studies targeting macro-organismal eDNA from the marine environment, published in 2006, was the detection of nematode DNA from sediment by amplifying nematode specific fragments of the 18S gene with PCR, and separating the fragments using gel electrophoresis (Austen et al., 2006). The establishment of BOLD (i.e., Barcode of Life Data System, https://www.boldsystems.org/) (Ratnasingham and Hebert, 2007), the development of metabarcoding with multiplexed samples (Parameswaran et al., 2007), and primer optimization (Cristescu and Hebert, 2018), subsequently led to the advancement of aquatic macro-organismal eDNA metabarcoding analyses of freshwater (Deiner et al., 2015) and seawater (Thomsen et al., 2012).

The first proof-of-concept studies showcasing the promise of collecting modern seawater eDNA from macrofauna were arguably the detection of northern Pacific sea stars (*Asterias amurensis*) in ballast water (Deagle et al., 2003) and the detection of harbor porpoise from a sea pen housing the animals (Foote et al., 2012). Both studies were controlled and used species-specific primers to amplify eDNA. In 2012, the first seawater macro-organism eDNA metabarcoding study was published, where Thomsen and colleagues sampled a coastal marine environment and detected 15 fish and four bird species, which were also represented in congruent conventional fish-surveys and in national biodiversity records, respectively (Thomsen et al., 2012). Over time, primer development, increasing reference sequences and improved sequencing technology has allowed marine researchers to survey a wide variety of taxonomic groups (**Figure 1.2**Figure 1.2). Marine eDNA metabarcoding studies now commonly target multiple genetic loci allowing for biodiversity assessments of taxa across multiple kingdoms (Stat et al., 2017a), spanning trophic levels (Djurhuus et al., 2017), and detecting changes in community composition with seasonality (Djurhuus et al., 2020). Moreover, community composition recovered from marine eDNA has been shown to reflect differences over depth stratification (Andruszkiewicz et al., 2017; Canals et al., 2021; Jeunen et al., 2020; Monuki et al., 2021), between areas of protection (Gold et al., 2021b), along varying amounts of urbanized coastline (Kelly et al., 2016), between habitats and salinity gradients (García-Machado et al., 2022; Jeunen et al., 2019a), and even at fine spatial scales (O'Donnell et al., 2017; Port et al., 2016; West et al., 2020). More is being understood about the degradation of marine eDNA for example, the rate of decay of inshore eDNA is 1.6 times faster than offshore eDNA, it can persist for up to 48 hours, and abiotic and biotic factors can simultaneously influence decay rates (Collins et al., 2018). Marine eDNA continues to be explored as a tool for quantitative biodiversity assessment (Shelton et al., 2022), for biological invasions (Duprey et al., 2023), for fisheries and aquaculture management (Gilbey et al., 2021), and even as a means to capture population genetic information (Adams et al., 2019; Parsons et al., 2018; Sigsgaard et al., 2016). The realization of the utility for eDNA analyses in marine ecosystem management is encouraging knowledge exchange between the academic and public sectors to figure out the best course for standardization and implementation (Jarman et al., 2018; Kelly et al., 2024).

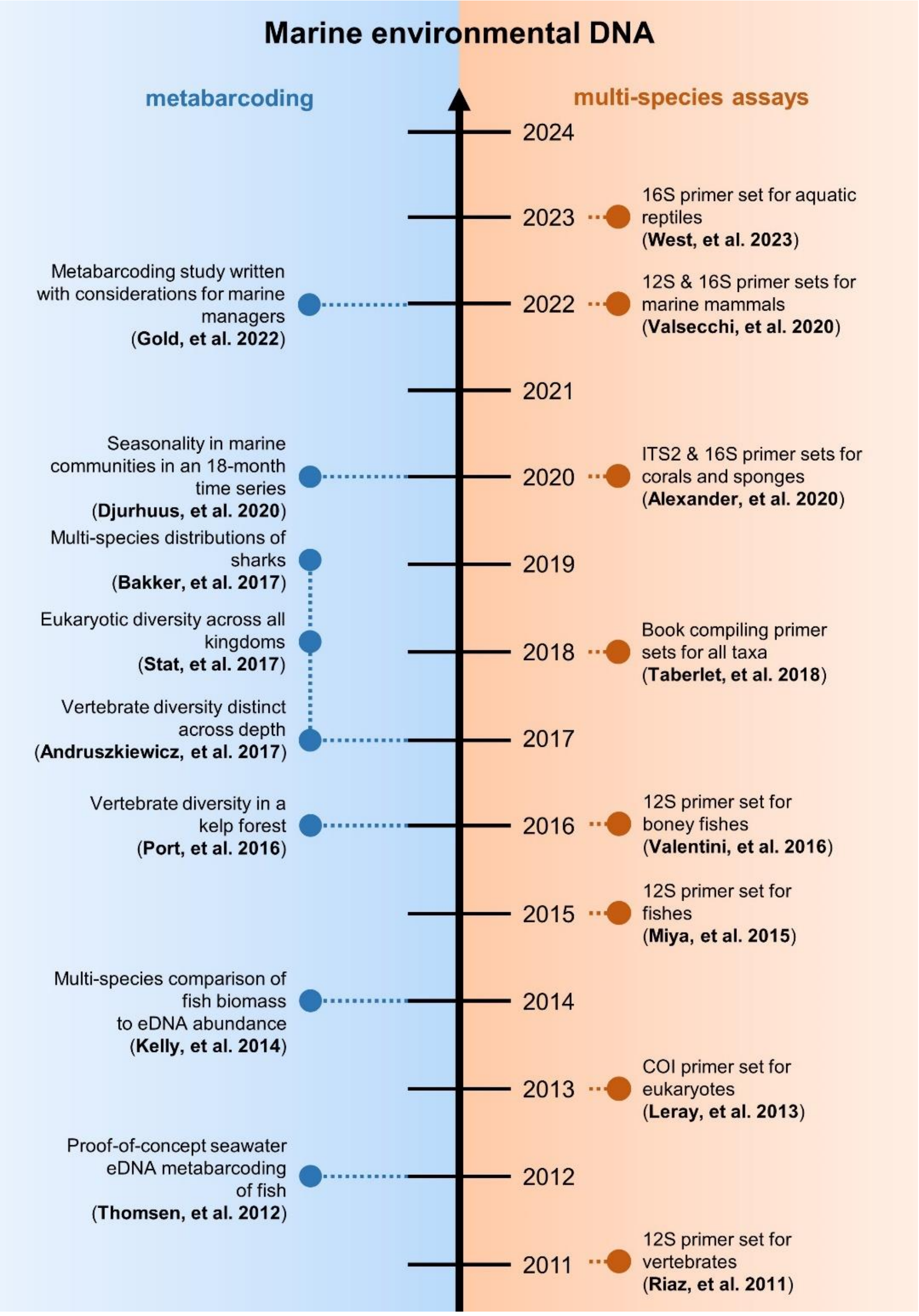

Figure 1.2 Timeline of selected marine eDNA studies that use metabarcoding analyses (blue) and/ or develop primer sets for multi-species assays (orange).

1.4. environmental DNA Capture Methodology

An important consideration for implementing eDNA analyses into ecological research and biodiversity monitoring strategies is choosing an appropriate DNA capture method. Here, eDNA capture is defined as the concentration of intracellular and/or extracellular DNA. While this may seem rather straightforward, eDNA capture methods are technical considerations that can have a strong influence on biodiversity detection (Deiner et al., 2015), and therefore eDNA capture presents an area of ongoing research and development. Aquatic eDNA capture methodologies can be broadly categorized into four main approaches: filtration, precipitation, association, and adsorption. Filtration and precipitation are the most well-studied and conventional methods to date. Association and adsorption methods have become increasingly explored over the last few years and will feature as the main eDNA capture strategies explored in this thesis.

1.4.1 Filtration & Precipitation

Macro-organismal eDNA is often at low concentrations and can become more dilute in marine habitats relative to freshwater due to the vastness of ocean environments. Early research efforts on marine environmental DNA tested small volumes (15 ml to 500 ml) of seawater to determine whether eDNA analysis works in the marine environment by using the same volumes of water collected in freshwater studies for direct comparisons (Foote et al., 2012; Thomsen et al., 2012); as expected, these early works concluded that marine eDNA was more dilute than freshwater eDNA but that the method was still promising. Therefore, marine eDNA capture often requires filtration of anywhere from 1 L per replicate in the coastal ocean to ~10 L in the open ocean (Eble et al., 2020). Depth is also an important consideration since eDNA is less concentrated in deep seawater (McClenaghan et al., 2020b). A range of filters and filter pore-sizes have been used for eDNA capture; glass fiber filters (GF/F) and cellulose filters are common materials and pore sizes between 0.20 µm to 0.45 µm are also common. However, many different volumes of water, filters, and pore sizes are used beyond what I have mentioned here. The variance in pore size can be great due to the states of eDNA, which can be dissolved or often are particle-bound and, in such cases, can be captured using relatively large pore sizes; therefore, turbidity is also an important consideration in coastal environments (Kumar et al., 2022). Filtration is often done by hand with a syringe, but can also be assisted mechanically with a vacuum or peristaltic pump, and there are even large-volume water samplers capable of pumping up to 60 L of water *in situ* (Govindarajan et al., 2022). Precipitation of eDNA can be done by performing liquid-liquid extraction techniques (e.g., phenol: chloroform) that separate nucleic acids from other organic matter; samples can then be frozen until centrifugation, where the precipitant containing the DNA can be isolated (Ficetola et al., 2008). Filtration remains more practical that precipitation for marine eDNA studies due to the larger volumes of seawater required for samples, relative to freshwater, and because precipitation involves hazardous reagents which can be precarious to use in the field. Choosing to use precipitation over filtration and even the type of filter employed (i.e., capsules, disc filters) can all have a significant effect on eDNA capture (Peixoto et al., 2021).

1.4.2 Association

A growing branch of eDNA research is exploring methodology for sequencing eDNA that has been ingested by (Calvignac-Spencer et al., 2013a; Carvalho et al., 2022) or adhered to other organisms (Al-Wahaibi et al., 2023). This research initially stemmed from studying ectoparasite-host interactions (Kent, 2009) and has since focused on invertebrate organisms, such as mosquitos (Ligon et al., 2009), ticks (Gariepy et al., 2012) and carrion flies (Calvignac-Spencer et al., 2013b) that concentrate vertebrate DNA through ingestion of blood or tissue. This type of eDNA collection, sometimes referred to as iDNA[1] or 'invertebrate-derived DNA', is not without limitations; for instance, the broad applicability of invertebrate eDNA samplers is dependent on their distribution and optimal when they have generalist diets (Bohmann et al., 2013; Carvalho et al., 2022). Yet, the analysis of eDNA from associated species interactions can be an extremely powerful tool for

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[1] Confusingly, iDNA can also be an abbreviation for 'intracellular DNA'.

conservation ecology. Analyzing ~30,000 leech bloodmeals has led to vertebrate ecological data used for landscape-level insights—by calculating the site-occupancy of mammals, birds and amphibians, researchers discriminated the effectiveness of a protected area (Ji et al., 2022). And eventually, inspiration taken from these terrestrial studies, such as analyzing the blood of terrestrial leeches (Schnell et al., 2015, 2012) (**Figure 1.3**), progressed the field to apply these ideas in aquatic environments such as collecting freshwater leeches to detect mammal communities (Lynggaard et al., 2022). Leeches' bloodmeals have even led to the discovery of species interactions we were unaware of, such as confirmation of a leech (*Haementeria acuecueyetzin*) found parasitising on a Antillean manatee in Chiapas, Mexico (Pérez-Flores et al., 2016).

While invertebrate-derived eDNA research for biodiversity assessment began with a focus on insects (Calvignac-Spencer et al., 2013b), it has expanded to include a wide variety of organisms from aquatic macroinvertebrates (Mariani et al., 2019) and terrestrial vertebrates (Montauban et al., 2021). In the marine realm, parasitic copepods (*Pandarus rhincodonus*) have been sampled to see if copepod collection could function as a minimally invasive technique for sampling whale shark mitochondrial DNA; this technique has successfully been used to study the population genetics of whale sharks (Meekan et al., 2017). For species-level biodiversity assessment, eDNA from the gut contents of brown shrimp (*Crangon crangon*) has been analysed to reconstruct fish and invertebrate communities in estuaries (Siegenthaler et al., 2019b) (**Figure 1.3**). Brown shrimp are opportunistic feeders that will scavenge dead carcasses but will also hunt juvenile fish and invertebrates, and passively ingest other microorganisms and likely particulate organic matter (Siegenthaler et al., 2019a). This begs the question: what makes organisms samplers of eDNA, and, similarly, when are diet metabarcoding analyses no longer eDNA metabarcoding analyses? For instance, the faecal material of juvenile bull sharks (*Carcharhinus leucas*) has been collected by swabbing their cloacal opening with cotton and metabarcoding the residue, revealing the prey assemblage of the sharks (van Zinnicq Bergmann et al., 2021). The shark cloacal swabbing study specifically makes sure that eDNA from the surrounding water does not affect the detections recovered on the cotton swab by sampling both simultaneously. Do diet metabarcoding studies become eDNA analyses when the prey has been digested past the point of morphological identification? Following that logic, both the brown shrimp (Siegenthaler et al., 2019b) and shark cloacal swabbing (van Zinnicq Bergmann et al., 2021) studies would be classified as natural eDNA samplers, although it is arguable that the authors of those studies may agree and/ or disagree with this categorization. There is clearly a grey area that exists along the spectrum of studies that classify as the analysis of eDNA ingested by organisms, sometimes referred to as nsDNA (natural sampler DNA), and the analysis of diet from DNA metabarcoding organisms' gut contents.

Filter feeders such as mussels (Jeunen et al., 2021; Weber et al., 2023), clams (Kim et al., 2023), anemones (Cunnington et al., *in press*) and sponges (phylum: Porifera) (Mariani et al., 2019) have recently been explored as natural samplers of aquatic eDNA. The sessile nature of these filter-feeders, in contrast to flies, shrimps or sharks, perhaps make them especially suited for biodiversity assessments since the detection of taxa may be more closely associated with the geographic location where the invertebrate filter-feeder specimen was collected. In 2019, whole DNA from sponge tissue was used for targeting vertebrates and the taxa detected (i.e., chinstrap penguin and rock goby) reflected the Antarctic and Mediterranean communities from where the sponges were sampled (Mariani et al., 2019). This quickly sparked further research on the utility of sponges as eDNA samplers (**Figure 1.3**). Studies have been carried out on tropical sponge specimens (Turon et al., 2020), on DNA extraction strategies (Harper et al., 2023), on controlled experiments with captive sponges (Cai et al., 2022), and comparisons have even been made between conventional water eDNA sampling and sponge-derived eDNA (Jeunen et al., 2021). Sponges as natural eDNA samplers are explored in chapter 2 and chapter 3 of this thesis.

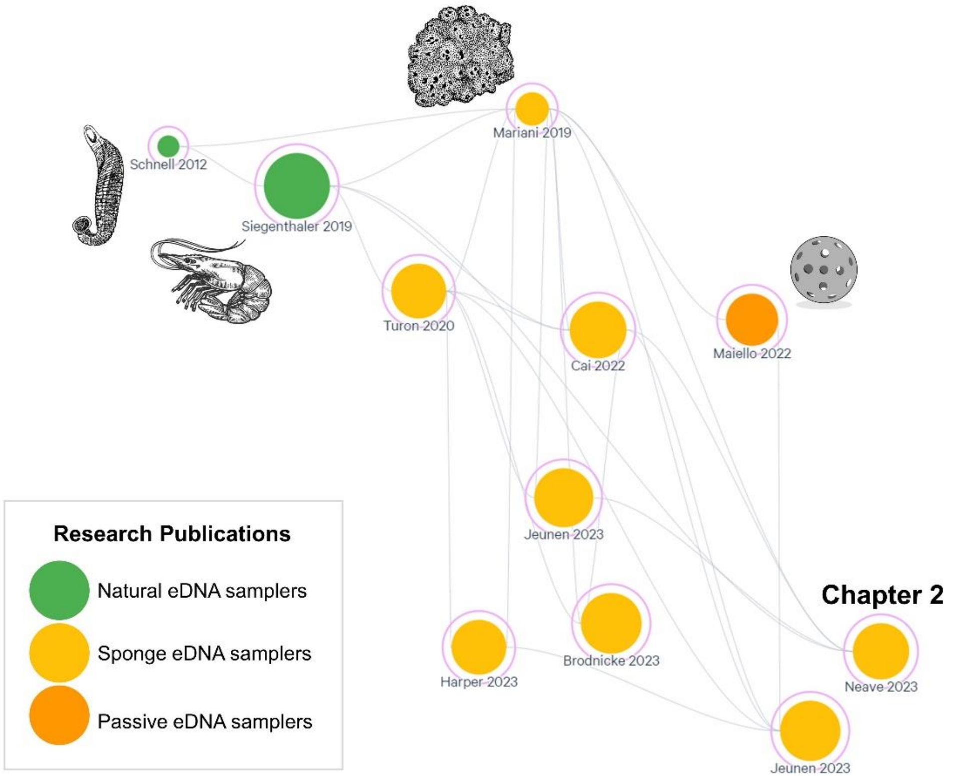

Figure 1.3 Publications leading to and resulting from Mariani et al. 2019. Grey lines indicate citations. Map created and edited in Litmaps (https://www.litmaps.com/).

Ingestion is not the only process by which eDNA can become associated with organisms; in some instances, it can naturally adhere to or collect on living things. In terrestrial systems, collecting eDNA from leaves (Valentin et al., 2020) and flowers (Thomsen and Sigsgaard, 2019), tree trunks (Valentin et al., 2020), and rainwater run-off from trees (Macher et al., 2023) has been used to detect arthropods and other associated fauna. While not the organism itself, spider webs have been collected and analysed for various taxa including mammal eDNA (Gregorič et al., 2022; Newton et al., 2024) as well as pollen (Bell et al., 2023), while honey (Bovo et al., 2018) from bee hives has been collected and analyzed for plants, insects, etc.— these are more examples of studies that may extend the definition of eDNA; are webs and hives considered part of the wider environment or the extension of a 'natural sampler' organism, and when does this categorization matter? It may be important when interpreting biodiversity detections, to consider how the adhesion of eDNA to a particular natural substrate is influenced by both a ‘natural sampling’ organism and the target taxa. In aquatic environments, eDNA adheres onto the substrates that microorganisms excrete as they form biofilms (Al-Wahaibi et al., 2023) and this eDNA has been successfully analyzed revealing macroinvertebrate (Rivera et al., 2021) and fish communities (Rivera et al., 2022). Growth of biofilms can naturally lead to biofouling, and in temperate marine kelp forests the biofouling on naturally occurring cobbles has been collected for community DNA metabarcoding (Shum et al., 2019). The metabarcoding of bulk marine invertebrate tissue would not strictly be considered eDNA metabarcoding; however, if some of those marine invertebrates were filter feeders could eDNA be targeted? Questions like this may be more useful for the advancement of biodiversity surveillance with eDNA analysis, rather than worrying about the semantics of what is and is not termed environmental DNA.

1.4.3 Adsorption

In the last few years there have been efforts to understand how eDNA absorbs onto inanimate surfaces and materials (**Table 1-1**). This research is particularly motivated by difficulties met when filtering water, such as filters clogging, the need to sterilize equipment between filtering, and that large volumes of water and biological replication are needed to sufficiently measure habitats with high species diversity (Bessey et al., 2020; Zinger et al., 2019). Experimenting with the adsorption of eDNA onto submerged materials, sometimes called ‘passive’ eDNA sampling, could present a simple solution to the time and effort required by filtration. Some materials may have a composition which increases the entrapment of DNA; certain surfaces can be positively charged and therefore attract negatively charged DNA molecules. The first passive eDNA capture experiment was published in 2020 by Kirtane and colleagues, where they showed that for the substrates they tested (i.e., montmorillonite clay and granular activated carbon) the highest net DNA yield was reached prior to a submersion time of 7 days, due to the nature of eDNA to go through both adsorption and desorption over time (Kirtane et al., 2020). Cellulose ester filter membranes, typically used for water filtration, were submerged in the water column on two different reefs (tropical and temperate);

97% of the submerged filters contained fish detections and revealed different fish community compositions from each distinct habitat (Bessey et al., 2021). Fourteen different materials have been tested for passive eDNA adsorption, with cellulose, cotton and nylon being the most popular materials (**Table 1-1**). Some studies have tried a combination of approaches. Instead of filtering water, researchers have used adsorbent materials to 'swab' bodies of water (McDonald et al., 2023) or have placed materials in a position to be assisted by another mechanism (i.e., inside a trawl fishing net) (Albonetti et al., 2023; Maiello et al., 2024, 2022) (**Figure 1.3**), enhancing eDNA collection. A similar approach, using innate materials for quasi-passive eDNA capture, is explored in chapter 4 and 5 of this thesis.

Table 1-1 Studies (N=12) testing passive eDNA adsorption onto different materials in aquatic environments.

	Material/ Particle or Pore size	Housing or Structure	Environment	Time	Target Taxa	Gene	Author	Year
1	**Activated Carbon**							
	granular activated carbon/ 0.3−2 mm	NA	lab	1-240 min & 24 hr	Oncorhynchus keta (chum salmon) sperm DNA	control region	Kirtane et al.	2020
	granular activated carbon/ 0.3−2 mm	polyester mesh (178 µm)	microcosm	1-21 days	Lampsilis siliquoidea (fatmucket mussel)	NADH	Kirtane et al.	2020
	granular activated carbon/ 0.3−2 mm	polyester mesh (178 µm) + PVC housing	microcosm	7,21 days	fish	12S	Kirtane et al.	2020
	granular activated carbon/ 1.18−3.35 mm	teabag	mesocosm	0.5-72 hr	Andrias davidianus (Chinese giant salamander)	NADH	Chen et al.	2022
	powdered activated carbon/ 0.30−0.85 mm	teabag	mesocosm	0.5-72 hr	Andrias davidianus (Chinese giant salamander)	NADH	Chen et al.	2022
2	**Cellulose**							
	cellulose/ 0.45 µm	pearl oyster aquaculture frame	mesocosm	0.83-18 hr	fish	16S	Bessey et al.	2022
	cellulose - 1% chitosan/ 0.45 µm*	pearl oyster aquaculture frame	mesocosm	0.83-18 hr	fish	16S	Bessey et al.	2022
	cellulose - 3% chitosan/ 0.45 µm*	pearl oyster aquaculture frame	mesocosm	0.83-18 hr	fish	16S	Bessey et al.	2022
	cellulose - electrospun nanofibres/ 0.45 µm*	pearl oyster aquaculture frame	mesocosm	0.83-18 hr	fish	16S	Bessey et al.	2022
	cellulose - electrospun nanofibres, 1% chitosan/ 0.45 µm*	pearl oyster aquaculture frame	mesocosm	0.83-18 hr	fish	16S	Bessey et al.	2022
	cellulose ester membrane/ 0.45 µm	passive depolyment device (specifics undisclosed)	ocean	~24 hr	fish	16S	Bessey et al.	2023

	non-charged, cellulose ester/ 0.45 µm	pearl oyster aquaculture frame	ocean	4-24 hr	fish	16S	Bessey et al.	2021
	cellulose nitrate/ 0.45 µm	teabag	mesocosm	0.5-72 hr	Andrias davidianus (Chinese giant salamander)	NADH	Chen et al.	2022
	mixed cellulose acetate and nitrate/ 0.45 um	teabag	mesocosm	0.5-72 hr	Andrias davidianus (Chinese giant salamander)	NADH	Chen et al.	2022
	mixed cellulose acetate and nitrate/ 1.20 um	teabag	mesocosm	0.5-72 hr	Andrias davidianus (Chinese giant salamander)	NADH	Chen et al.	2022
	cellulose nitrate filters/ 0.45 µm	fishing net	mesocosm	12 hr	fish	16S	Jeunen et al.	2022
	cellulose nitrate filters/ 0.45 µm	fishing net	ocean	12 hr	fish	16S	Jeunen et al.	2022
3	**Cotton**							
	absorbent cotton/ NA	teabag	mesocosm	0.5-72 hr	Andrias davidianus (Chinese giant salamander)	NADH	Chen et al.	2022
	cotton fibres/ NA	nylon bag, pearl frame	mesocosm	0.83-18 hr	fish	16S	Bessey et al.	2022
	cotton medical-grade gauze/ NA	plastic 3-D printed sphere (metaprobe)	trawl net	NA	fish	12S	Maiello et al.	2022
	cotton medical-grade gauze/ NA	plastic 3-D printed sphere (metaprobe)	trawl net	NA	elasmobranchs	12S	Albonetti et al.	2023
	cotton medical-grade gauze/ NA	plastic 3-D printed sphere (metaprobe)	trawl net	NA	fish	12S	Maiello et al.	2023
	cotton medical-grade gauze/ NA	plastic 3-D printed sphere (metaprobe)	trawl net	NA	invertebrates	COI	Maiello et al.	2023
4	**Glass**							

	glass fiber/ 0.70 µm	teabag	mesocosm	0.5-72 hr	Andrias davidianus (Chinese giant salamander)	NADH	Chen et al.	2021
	glass fiber/ 0.70 µm	teabag	lake	0.5-72 hr	fish	12S	Chen et al.	2022
5	**Hemp**							
	hemp fibers/ NA	nylon bag, pearl frame	mesocosm	0.83-18 hr	fish	16S	Bessey et al.	2022
6	**Hydroxyapatite**							
	hydroxyapatite/ 80 µm	teabag	mesocosm	0.5-72 hr	Andrias davidianus (Chinese giant salamander)	NADH	Chen et al.	2022
	hydroxyapatite/ NA	NA	lab	120 min	Asellus aquaticus (isopod)	16S	Verdier et al.	2022
7	**Montmorillonite clay**							
	montmorillonite clay/ 2−60 µm	NA	lab	1-240 min & 24 hr	Oncorhynchus keta (chum salmon) sperm DNA	control region	Kirtane et al.	2020
	montmorillonite clay/ 2−60 µm	two cell strainers (40 µm)	microcosm	1-21 days	Lampsilis siliquoidea (fatmucket mussel)	NADH	Kirtane et al.	2020
	montmorillonite clay/ 2−60 µm	two cell strainers (40 µm) + PVC housing	microcosm	7,21 days	fish	12S	Kirtane et al.	2020
8	**Nylon**							
	nylon/ 0.45 µm	teabag	mesocosm	0.5-72 hr	Andrias davidianus (Chinese giant salamander)	NADH	Chen et al.	2022
	nylon filters/ 5 µm	fishing net	mesocosm	12 hr	fish	16S	Jeunen et al.	2022
	nylon filters/ 5 µm	fishing net	ocean	12 hr	fish	16S	Jeunen et al.	2022

	positively charged electrostatic nylon/ 0.45 µm	pearl oyster aquaculture frame	ocean	4-24 hr	fish	16S	Bessey et al.	2021
	positively charged nylon membranes [+ve Nylon]/ 0.45 µm	fishing net	mesocosm	12 hr	fish	16S	Jeunen et al.	2022
	positively charged nylon membranes [+ve Nylon]/ 0.45 µm	fishing net	ocean	12 hr	fish	16S	Jeunen et al.	2022
9	**Polyethersulfone**							
	Supor PES membrane/ 0.45 µm	NA	rock pool	15 sec.	mammals	12S	McDonald et al.	2023
	Supor PES membrane/ 0.45 µm	NA	rock pool	15 sec.	mammals	16S	McDonald et al.	2023
	Supor PES membrane/ 0.45 µm	NA	cattle trough	15 sec.	mammals	12S	McDonald et al.	2023
	Supor PES membrane/ 0.45 µm	NA	cattle trough	15 sec.	mammals	16S	McDonald et al.	2023
10	**Polycarbonate**							
	polycarbonate/ 0.20 µm	teabag	mesocosm	0.5-72 hr	Andrias davidianus (Chinese giant salamander)	NADH	Chen et al.	2022
11	**Resin**							
	macroporous weak alkaline acrylic anion exchange resin/ 0.32−1.25 mm	teabag	mesocosm	0.5-72 hr	Andrias davidianus (Chinese giant salamander)	NADH	Chen et al.	2022
12	**Silica gel**							
	silica gel/ 1−3 mm	teabag	mesocosm	0.5-72 hr	Andrias davidianus (Chinese giant salamander)	NADH	Chen et al.	2022

13	**Sponge (artificial)**							
	sponge - active carbon/ NA	pearl frame	mesocosm	0.83-18 hr	fish	16S	Bessey et al.	2022
	sponge - zeolite/ NA	pearl frame	mesocosm	0.83-18 hr	fish	16S	Bessey et al.	2022
	whirl-Pak® Speci-sponges®/ NA	fishing net	mesocosm	12 hr	fish	16S	Jeunen et al.	2022
	whirl-Pak® Speci-sponges®/ NA	fishing net	ocean	12 hr	fish	16S	Jeunen et al.	2022
14	**Unknown Material**							
	fishing net/ NA	NA	mesocosm	12 hr	fish	16S	Jeunen et al.	2022
	fishing net/ NA	NA	ocean	12 hr	fish	16S	Jeunen et al.	2022

1.5. Aims and Objectives

Marine species are among the least well known and most poorly understood species in the world. Ocean biodiversity loss has tangible effects globally and poses threats to the health and livelihood of humans. This thesis aims to investigate unconventional aquatic eDNA capture techniques and understand their usefulness, if any, for monitoring marine vertebrates. Specifically, two creative capture techniques were investigated: 1) leveraging the filtration capacity of marine sponges (Phylum: Porifera) as natural eDNA sampling accumulators 2) deployment of simple, quasi-passive eDNA samplers to adsorb eDNA through the swimming motion of SCUBA divers and snorkellers. A number of questions have been explored, such as: What is the efficacy of sponge tissue and collection as a means for capturing marine vertebrate assemblages in different habitats?; How does sponge-derived eDNA compare to conventional filtered aquatic eDNA and visual census surveys?; What is the effectiveness of quasi-passive eDNA collection by SCUBA divers and how does this technique compare to water filtration? The objectives of each chapter are as follows:

- To investigate whether eDNA could be isolated from North Atlantic deep-sea sponges, previously collected for other research purposes, specifically targeting marine bony fish (**chapter 2**)
- To compare sponge-derived eDNA, aquatic eDNA, and visual census data from a Mediterranean island, and understand their effectiveness to characterise coastal fish assemblages (**chapter 3**)
- To test a quasi-passive eDNA adsorption technique in an aquarium and in nature across a variety of habitats, cold temperate to tropical, specifically targeting bony fish and elasmobranchs (**chapter 4**)
- To apply quasi-passive eDNA adsorption, assisted by volunteer SCUBA divers, on fine scales to test whether it can differentiate communities of vertebrates: mammals, birds, bony fish and elasmobranchs (**chapter 5**)

Chapter 2

Trapped DNA fragments in marine sponge specimens unveil north Atlantic deep-sea fish diversity

This chapter was published in *Proceedings of the Royal Society B*

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A Sharpchin rockfish (*Sebastes zacentrus*) swims above a glass sponge assemblage (Porifera: Hexactinellida).

2.1 Abstract

Sponges pump water to filter feed and for diffusive oxygen uptake. In doing so, trace DNA fragments from a multitude of organisms living around them are trapped in their tissues. Here we show that the environmental DNA retrieved from archived marine sponge specimens can reconstruct the fish communities at the place of sampling and discriminate North Atlantic assemblages according to biogeographic region (from Western Greenland to Svalbard), depth habitat (80-1600 m), and even the level of protection in place. Given the cost associated with ocean biodiversity surveys, we argue that targeted and opportunistic sponge samples – as well as the specimens already stored in museums and other research collections – represent an invaluable trove of biodiversity information that can significantly extend the reach of ocean monitoring.

2.2 Introduction

The worrying and widespread trend of ocean biodiversity loss that typifies the Anthropocene calls for increasingly powerful and accurate approaches to expose the nuances of this loss, understand its main drivers, and inform mitigation strategies. One such recent scientific advance has been ‘environmental DNA’ (eDNA) analysis, an approach by which collecting DNA fragments shed by organisms in their habitat allows researchers to generate biodiversity data at unprecedented scales (Deiner et al., 2017a) and granularity (Jeunen et al., 2019a), redefining the way we observe and understand ocean life.

Biological research collections are critical for eDNA analyses. Apart from expanding DNA taxonomic reference databases from tissues (de Santana et al., 2021), they also provide untapped genomic insights that have become more accessible with the advancement of molecular techniques (Raxworthy and Smith, 2021). Metabarcoding in particular allows for ecological insights, such as detecting multi-decadal community shifts from eDNA in ethanol-preserved ichthyoplankton samples (Gold et al., 2022), or tracking micro-evolutionary changes in the gut microbiome of 100-year-old fish specimens (Heindler et al., 2018). These are prime applications of the extended specimen concept (Webster, 2017), that is, a comprehensive approach to biodiversity collections that extends beyond the physical object to multiple other uses made possible by efforts such as digitization, and new attitudes towards phenotypic description such as considering holobionts (Lendemer et al., 2020)· (Teixeira-Costa et al., 2023).

Filter-feeding marine sponges (phylum: Porifera) were recently found to act as natural eDNA samplers, able to retain eDNA fragments reflective of their surrounding biological communities (Mariani et al., 2019). Sponges are ideal extended specimens, in that exploring beyond the host DNA provides an understanding of the environment from which the sponge was collected. Experimental studies subsequently found that sponge species differ in their ability to retain eDNA, with some species likely to trap DNA for longer intervals than what is usually observed in water samples (Cai et al., 2022; Jeunen et al., 2023). Given the urgent need to measure trajectories of biodiversity changes, we explored whether this sponge natural sampler approach could characterise fish assemblages across the North Atlantic by leveraging sponge specimens previously collected for other scientific purposes from vulnerable and underexplored deep-sea habitats.

2.3 Results

We detected natural sampler DNA (nsDNA) from three sponge species (*Geodia barretti, Geodia hentscheli,* and *Phakellia ventilabrum*) (N = 54, retained from 64 samples sequenced – see Methods) across varied benthic habitats in the North Atlantic (**Figure 2.1**). The specimens were between 3-10 years old, spanning the continental shelf down to the bathyal slope (~80-1900 m), and cover large biogeographic regions such as the Northeast Atlantic, North American Boreal, and Norwegian-Arctic Seas (**Figure 2.1** B, C, D) (Costello et al., 2017) (S2 Table 1). We amplified a fish-specific 12S mitochondrial rRNA marker (tele02) (Taberlet et al., 2018) from the previously extracted total DNA of the sponge specimens, and sequenced the targeted amplicons on an Illumina iSeq 100, resulting in 5,269,740 raw reads. After quality filtering (see 2.5 Methods), we retained 4,565,067 reads for downstream analyses (S2 Table 2), resulting in a median of 12,992 reads per sample (N = 74) (S2 Figure 1), including controls (N = 10) and samples that were later removed (N = 10) for having low reads (mostly *G. hentscheli*).

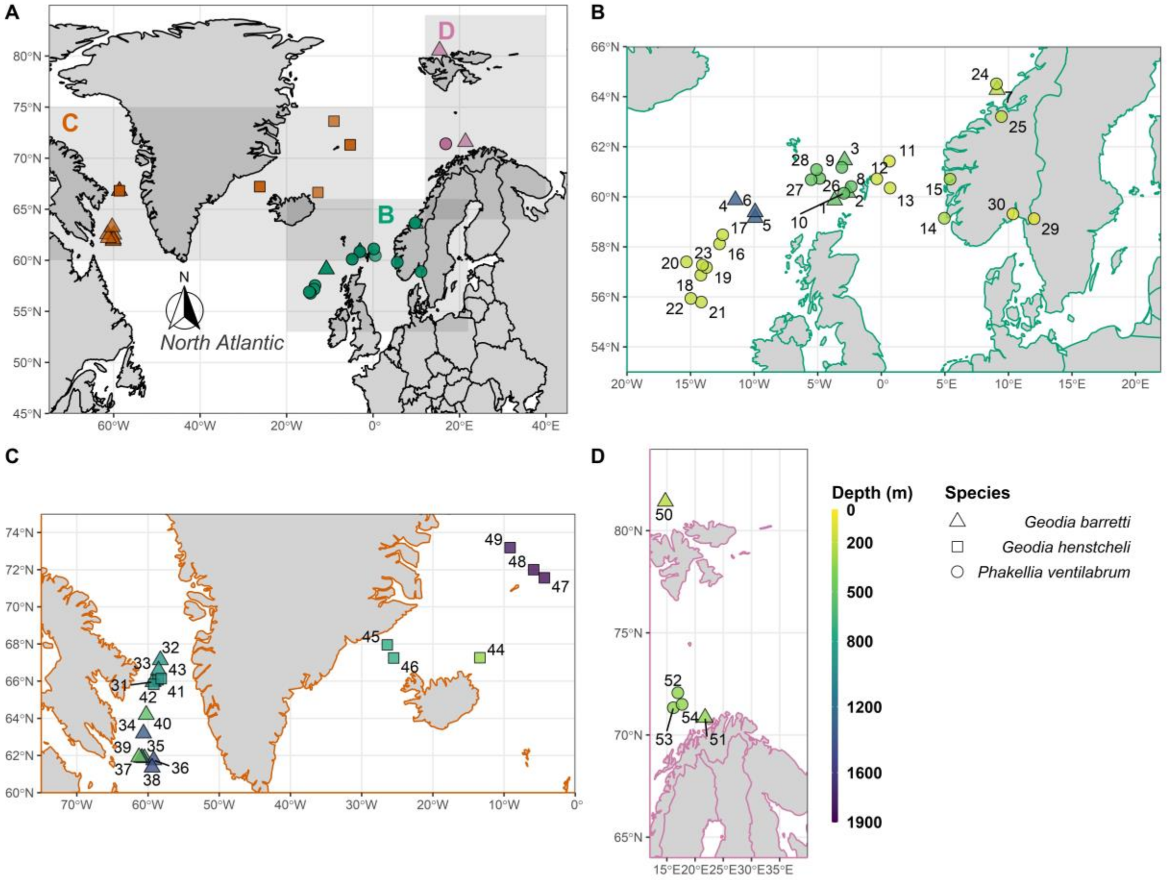

Figure 2.1 Maps showing locations of sponge specimen retrieval. Depth is indicated by the colour bar and sponge species is indicated by the shape of the points. Panel showing the North Atlantic study area (A). Panel showing the Northeast Atlantic region (B). Panel showing the North American Boreal Atlantic region (C). Panel showing the Norwegian-Arctic Seas Atlantic region (D). Sponge specimens in B, C and D are jittered for visibility and labelled 1-30 (Northeast Atlantic), 31-49 (North American Boreal) and 50-54 (Norwegian-Arctic Seas).

2.3.1 Vertebrate Biodiversity

After bioinformatic processing, the sponges yielded 142 eukaryote molecular operational taxonomic units (MOTUs), resulting in 125 non-human, contaminant free, marine MOTUs, which could be identified confidently to at least the taxonomic rank of class. Among these, we detected 119 fish MOTUs of which 65 were identified to species level at ≥99% identity to reference sequences, excluding contaminants (S2 Table 2, 3). The following species were removed from downstream analysis: our positive control (the tropical freshwater catfish *Pangasianodon hypophthalmus*), two species (ie. *Amphiprion ocellaris*, *Pomacanthus imperator*) from a different project processed at a similar time (Cai et al., 2022), and one Indo-Pacific fish heavily traded as seafood (*Nemipterus zysron*). The fish MOTUs, spread over the classes Actinopterygii and Chondrichthyes, comprised 28 orders, 54 families and 94 genera. A sand sea star (*Astropecten irregularis*) common in deep sea benthos was also detected, while sponge DNA was never detected and likely not amplified, due to their phylogenetic distance from vertebrates. We also removed domestic animals (e.g., *Sus scrofa*, *Bos taurus*) and terrestrial mammals such as caribou (*Rangifer tarandus*), native to the Northern Hemisphere, whose putatively leached DNA was found in a *G. barretti* specimen from the Davis straight, west of Greenland. After these removals, we detected five 'bonus' non-fish vertebrate species, including three marine mammals (harbour porpoise (*Phocoena phocoena*), Atlantic white-sided dolphin (*Lagenorhynchus acutus*), and Bryde's whale (*Balaenoptera brydei*), detected in both the west and east North Atlantic) as well as two seabirds (pelagic cormorant (*Phalacrocorax pelagicus*) and glaucous gull (*Larus hyperboreus*)).

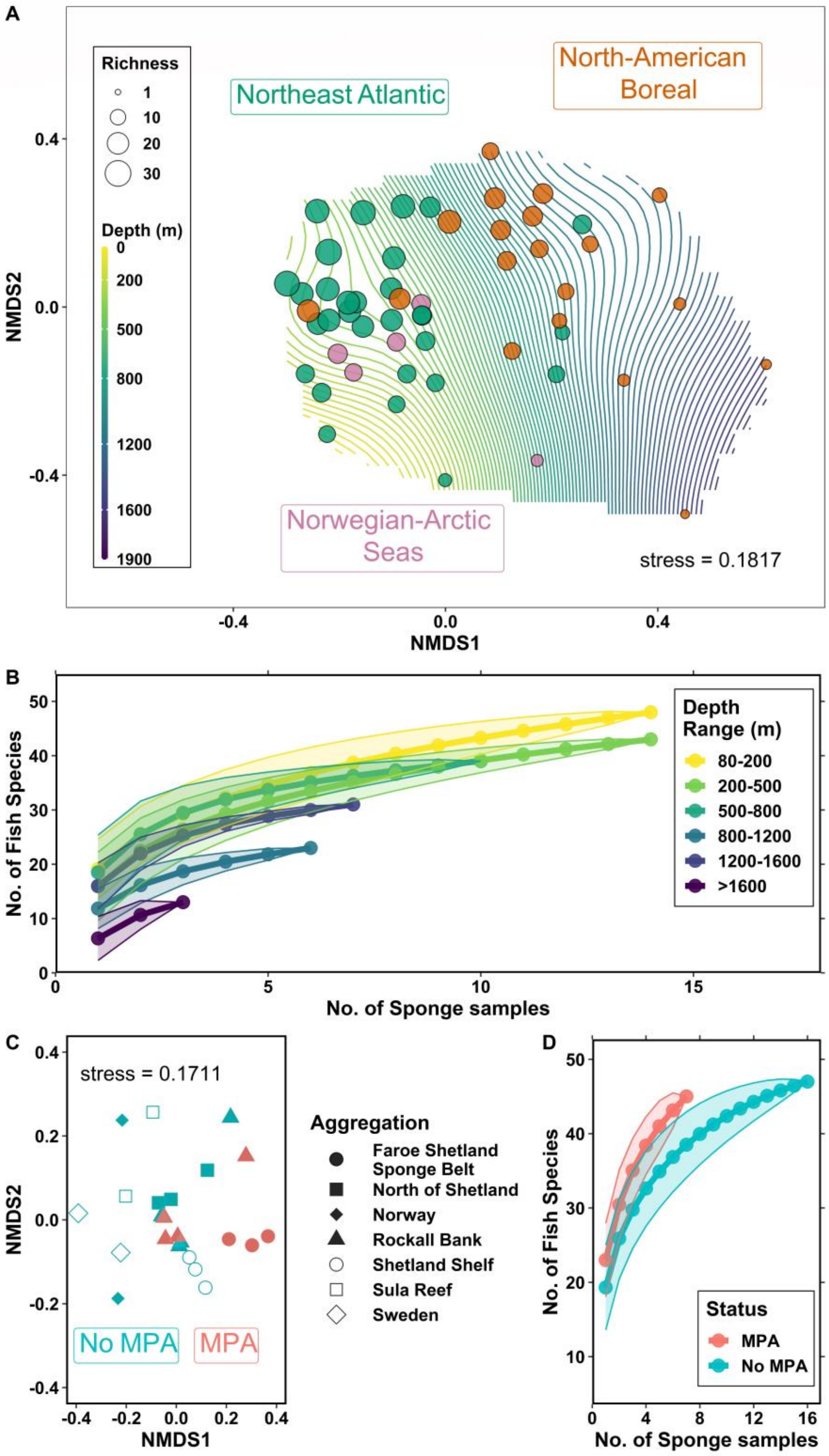

Figure 2.2 Plots conveying alpha and beta diversity from species-level teleost and elasmobranch detections. Non-metric Multi-Dimensional Scaling (NMDS) plot of a Jaccard dissimilarity species matrix, where points are coloured by North Atlantic region and size indicates species richness. Depth is plotted as a surface, where each line denotes a 20 m interval (A). Fish species accumulation curve, grouped by depth range (B). NMDS plot of a Jaccard dissimilarity species matrix of P. ventilabrum samples from the Northeast Atlantic region. Points are coloured by MPA status and shapes represent different sponge aggregations (C). Fish species accumulation curve of the P. ventilabrum samples from the Northeast Atlantic region, grouped by MPA status (D).

2.3.2 Biogeography and depth-associated Fish assemblages

Fish communities significantly differed between biogeographic regions of the North Atlantic ($R^2 = 0.16$, $p < 0.001$, **Figure 2.2** A, S2 Table 4). Beta diversity was examined through Non-metric Multi-Dimensional Scaling (NMDS) of a Jaccard dissimilarity matrix based on teleosts and elasmobranchs detected across sponge samples (comprising of only MOTUs identified to the species level, though the same pattern was observed when including genus level detections, S2 Figure 2), and by permutational multivariate analysis of variance (PERMANOVA) testing. The effects of region and sponge species, as well as the potential interactive effect between these two factors, on fish communities were also tested by PERMANOVA, but the dispersions of each group of sponge samples were not homogenous which PERMANOVA testing is sensitive to. Bearing in mind this caveat, the effect of sponge species was significant ($R^2 = 0.08$, $p < 0.05$) and independent from the effect of biogeographic regions (i.e. the interaction was not significant), but the regional effect explained more of the variation in fish community composition than the effect of sponge species ($R^2 = 0.16$, $R^2 = 0.08$, respectively) (S2 Table 4).

Sponge samples appeared broadly grouped into the biogeographic regions previously determined from global distribution data of marine taxa (Costello et al., 2017), emphasizing the effectiveness of sponge nsDNA to capably distinguish between marine realms (**Figure 2.2** A). Pairwise comparisons of beta-diversity revealed that all regions significantly differed, with the North American Boreal region showing greater divergence from both the Northeast Atlantic ($R^2 = 0.14$, $p < 0.001$) and the Norwegian-Arctic Seas ($R^2 = 0.13$, $p < 0.001$), compared to the divergence observed between the regions located in the eastern North Atlantic ($R^2 = 0.06$, $p = 0.025$) (S2 Table 4).

Latitude, depth and sampling year were all significant correlates of fish beta-diversity. Depth had the strongest correlation ($R^2 = 0.58$, $p < 0.001$) followed by latitude ($R^2 = 0.35$, $p < 0.001$) and year ($R^2 = 0.17$, $p = 0.018$) (S2 Table 4). We attribute the weaker correlation with sampling year to be a by-product of the different regions being sampled in separate months. Depth was plotted as a smooth surface over the NMDS ordination plane (**Figure 2.2** A), particularly highlighting how the composition of the Northeast Atlantic sites correspond with shallower continental shelf depths, while the North American Boreal samples follow the gradient of the slope into bathypelagic depths. Species richness approached saturation among all depth ranges from which sponges were sampled, with the least variance in the 1200-1600 m range where the standard deviation decreased with relatively less samples. Fish species richness progressively decreased with depth, except between 1200-1600 m depth, which had a higher richness than the 800-1200 m samples, but also approached saturation most robustly. (**Figure 2.2** B).

To further test the extent to which sponge nsDNA data could be used to distinguish between more fine-scale fish assemblages, the *P. ventilabrum* samples from the Northeast Atlantic were analysed as a subset (N = 23) to compare similar habitats and to control for any possible bias introduced by using different sponge species. We observed variance across samples collected in areas with differing levels of marine protection. Species richness appeared to be higher in marine protected area (MPA) sites, and communities detected in MPAs significantly differed from those outside MPAs ($R^2 = 0.09$, $p = 0.026$) (**Figure 2.2** C, 2D). The same subset of sponges was also tested for significant differences in teleost and elasmobranch beta-diversity between various *P. ventilabrum* aggregations (**Figure 2.2** C); however, none of the pairwise comparisons among aggregations were significant after correcting the p-values for multiple testing (S2 Table 5). This was likely due to low replication within each of the several locations (e.g., Sula reef, Shetland Shelf) being compared.

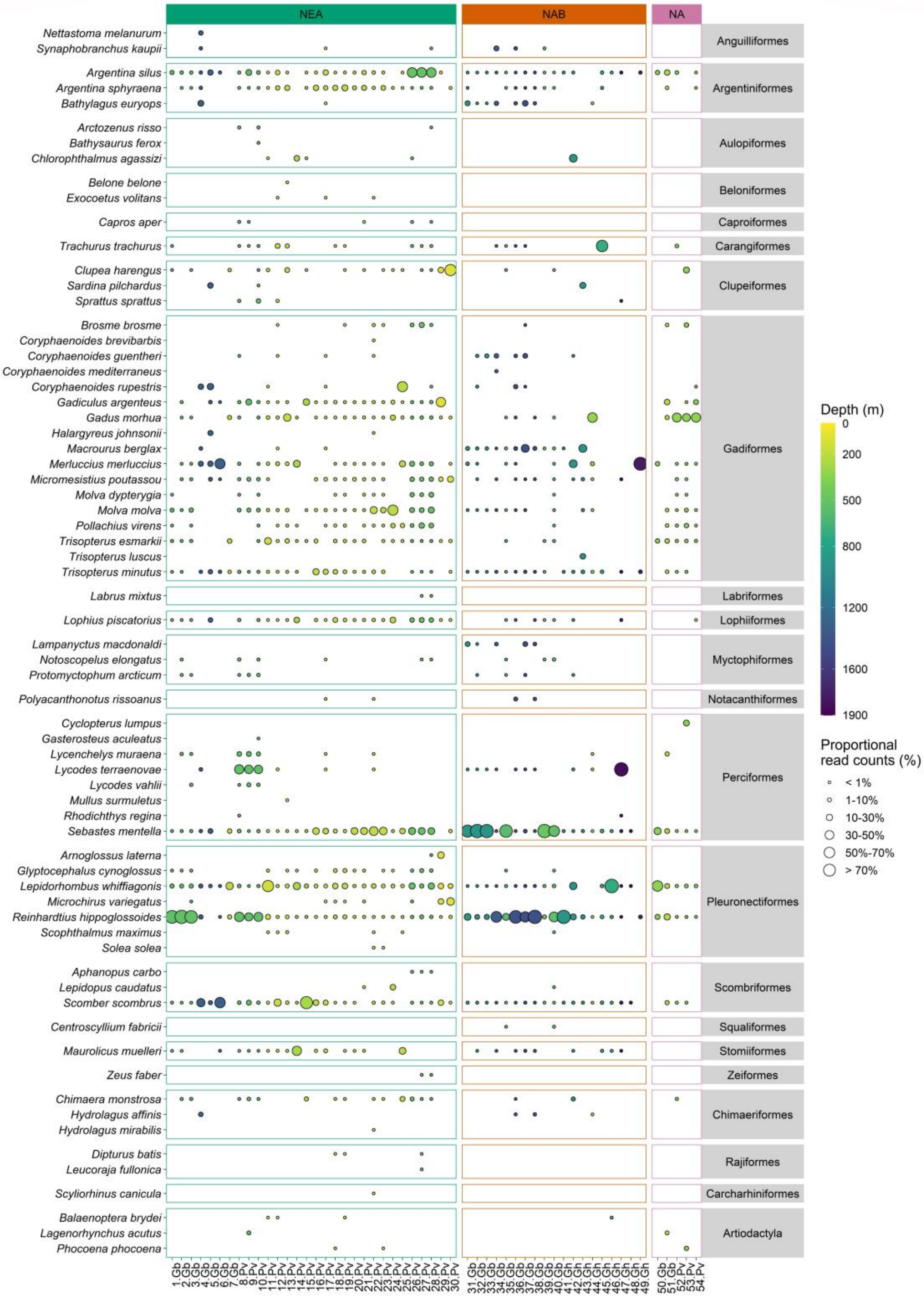

Figure 2.3 Bubble plot showing teleost, elasmobranch and mammal species detected, where the size of the bubble indicates the proportional read counts of that species represented in a sample. Samples are listed at the bottom, where the number refers to the labels in Figure 2.1 and the abbreviations refer to the sponge species (Gb = Geodia barretti, Gh = Geodia hentscheli, and Pv = Phakellia ventilabrum). The bubbles are coloured by the depth at which the sponge specimen was sampled. The vertical panels separate the sponge specimens by the three biogeographic regions: NEA = Northeast Atlantic, NAB = North American Boreal, and NA = Norwegian-Arctic Seas.

2.3.3 Fish detections and indicator species analysis

Greenland halibut (*Reinhardtius hippoglossoides*), beaked redfish (*Sebastes mentella*), and megrim (*Lepidorhombus whiffiagonis*) were detected in almost all 54 samples (i.e., 52, 51, and 50 samples, respectively) (**Figure 2.3**, S2 Table 3). Other frequently detected species included Atlantic mackerel (*Scomber scombrus*), greater argentine (*Argentina silus*) and poor cod (*Trisopterus minutus*) (i.e., 48, 46, and 44 samples, respectively), all of which are known to be abundant organisms in pelagic and demersal habitats of the North Atlantic.

While the 12S marker was designed to pick up teleost fish, six cartilaginous fish (class: Chondrichthyes) were also detected. Three chimaeras, the closest living relatives to sharks and rays, were detected, including the rabbit fish (*Chimaera monstrosa*) which was detected in 17 samples. Two elasmobranchs were from the family Rajidae: the shagreen ray (*Leucoraja fullonica*) which is IUCN red-listed as vulnerable and the blue skate (*Dipturus batis*) which is critically endangered, were both detected in the Northeast Atlantic (**Figure 2.3**).

Indicator value species analysis conducted across biogeographic regions and depth ranges (**Figure 2.4** A, B) detected eight species as biogeographic indicators, and 16 species as depth layer indicators, with seven species identified as indicators for both region and depth (S2 Table 6). Indicator values (A, B, *stat*) were calculated using presence-absence data to conservatively interpret detections. “A” is the estimate probability that samples are associated to a region or depth layer if the indicator species has been detected in the sample (i.e., specificity or predictive value). “B” is the estimate probability of detecting the indicator species in a region or depth layer (i.e., sensitivity). “Stat” is the indicator value index which suggests the strength of the indicator species association and encompasses both “A” and “B” values.

Many species of commercial value had strong significant associations for both region and depth range. Norway pout (*Trisopterus esmarkii*) was positively associated with the Northeast Atlantic and Norwegian-Arctic Seas (*stat* = 0.857, $p < 0.0001$) (**Figure 2.4** A) and had a strong association with depths ranging from 80-800 m (*stat* = 0.903, $p < 0.0001$), such that there was high specificity (A = 1) or likelihood that a Norway pout detection occurred in habitats shallower than 800 m depth (**Figure 2.4** B, S2 Table 6). Pollock (*Pollachius virens*) shared the same region and depth associations as Norway pout, although to a lesser strength. Atlantic cod (*Gadus morhua*) also showed clear associations with the eastern Atlantic between 80–800 m (**Figure 2.4** A, B). Roughhead grenadier (*Macrourus berglax*) and blacksmelt (*Bathylagus euroyops*) had a strong association with the North American Boreal with grenadier having a higher likelihood of detection (B = 0.738) than blacksmelt (B = 0.603). Both species were associated to depths between 800 and 1600 m (**Figure 2.4** B).

The indicator species analysis was repeated with the *P. ventilabrum* subset (N = 23) of the Northeast Atlantic data to identify indicator species of MPA sites. Four species were significant indicators of MPA sites (**Figure 2.4** C). These species included the moray wolf eel (*Lycenchelys muraena*), Atlantic eelpout (*Lycodes terraenovae*), Arctic telescope (*Protomyctophum arcticum*) and Vahl’s eelpout (*Lycodes vahlii*), all of which had high specificity (A = 0.999, 0.999, 1.0, and 1.0, respectively) to MPAs. The moray wolf eel and the Atlantic eelpout both shared the highest association with MPAs (*stat*(s) = 0.756, $p < 0.05$) (S2 Table 6).

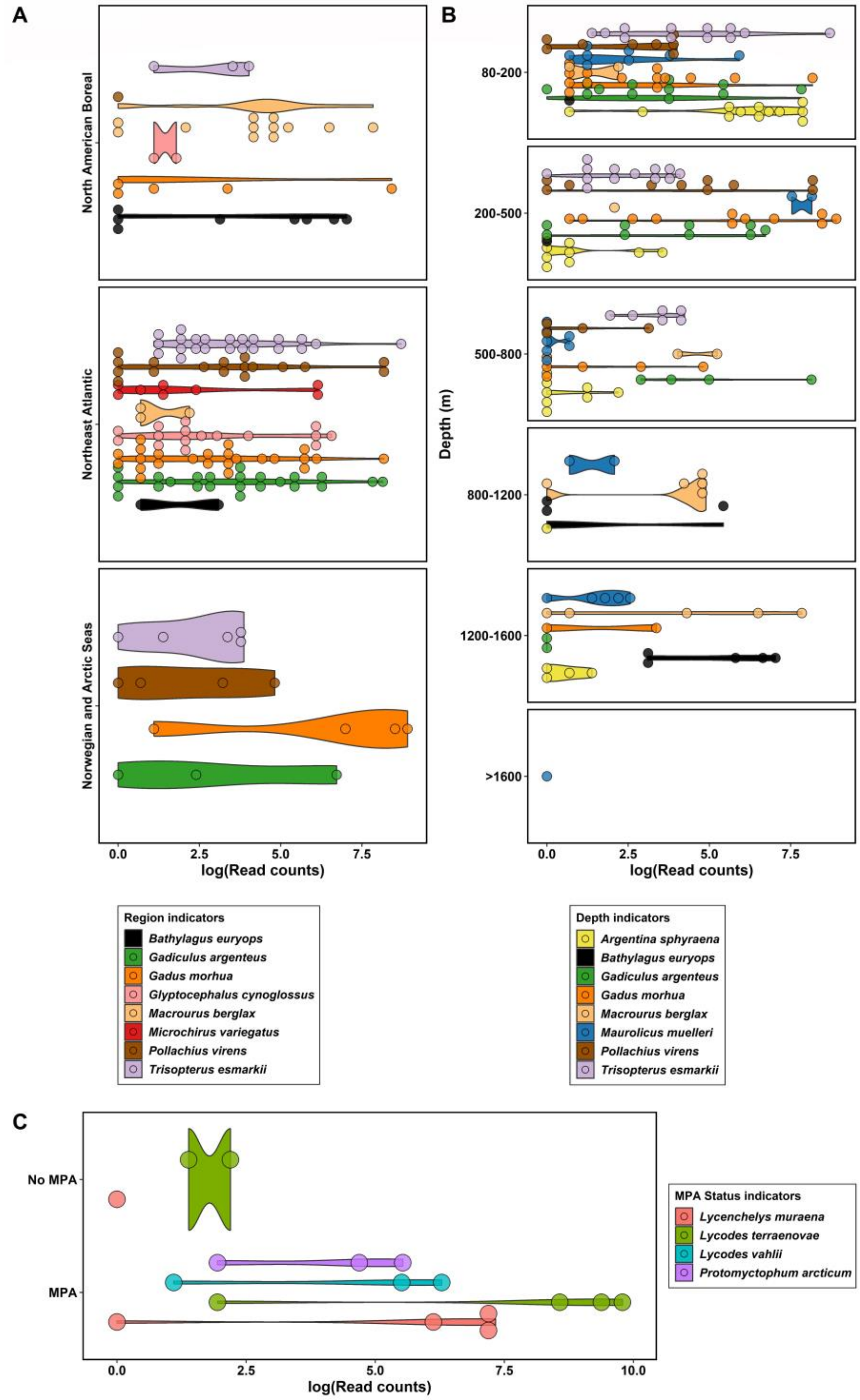

Figure 2.4 Violin dot plots of log-transformed read counts, highlighting identified indicator species. Violin dot plot of indicator species associated with biogeographic regions (A). Violin dot plot of the top eight indicator species associated with depth (B). Violin dot plot of indicator species associated with MPA status (C).

2.4 Discussion

The retrieval of fish sequences from sponge specimens previously collected for other monitoring purposes provides perhaps the most attractive demonstration to date of the role of sponges as practical, cost-effective, universal natural DNA samplers for aquatic biodiversity studies. We confidently detected at least 65 teleost and elasmobranch species that could be used to distinguish fish assemblages and identify indicator species associated with depth and biogeographic regions within the North Atlantic.

Congruent with what we know about sponge nsDNA *ex situ* (Cai et al., 2022), some sponge species appeared to perform better than others. The original experimental design considered 93 sponge specimens; however, only 64 of them were selected for sequencing because they showed amplification of the desired target DNA region (i.e. bands on agarose gels). After bioinformatic quality control, DNA information from 54 individual sponges was retained. Of the 34 *G. hentscheli* samples attempted, only 17 were sequenced and nine were kept after rarefaction. Sample loss occurred, although to a lesser degree, also for *G. barretti* (i.e. 33 attempted, 21 sequenced, 19 kept). *P. ventilabrum* resulted instead in a 100% success rate (N = 26), followed by *G. barretti* (58%) and *G. hentscheli* (26%). Curiously, *P. ventilabrum* likely has higher pumping rates and lower microbial abundance than the *Geodia* species (Kutti et al., 2013; Weisz et al., 2008). It is possible that higher microbial abundance could contribute to increased rates of eDNA decay within sponges due to decomposition by bacteria (Weisz et al., 2008) and less need to derive energy from the uptake of dissolved organic carbon (Bart et al., 2021). Given these observed coincidences, relationships between sponge physiology and nsDNA efficacy would be an exciting area for further investigation.

Sponges are part of a growing list of 'natural sampler' organisms, from which eDNA analysis is possible. High DNA sampling efficiency in some sponge species (i.e. *P. ventilabrum*) is an obvious advantage for biomonitoring, yet the percent success rate of the tetractinellid (*Geodia*) sponges was comparable to or even better than other organisms that have been tested as natural DNA samplers. For example, various leech species have been used to detect prey DNA, with vertebrate detection rates ranging from 9% to 80% of attempted specimens (Lynggaard et al., 2022). Similarly, when gut contents of the European brown shrimp (*Crangon crangon*), a generalist scavenger, were analysed with DNA metabarcoding to reconstruct estuarine fish assemblages (Siegenthaler et al., 2019b), up to eight stomachs had to be pooled, per DNA extraction, to constitute a sufficient sample. Extraction pooling could represent an appropriate methodological solution for favourable and widespread sponge species with moderate amplification success, such as *G. hentscheli* (i.e. 26%).

The detected fish communities significantly differed among biogeographic regions of the North Atlantic (**Figure 2.2** A), and depth was identified as the most important variable in shaping beta-diversity (S2 Table 4). Several fish species seemed to be more associated with either the west or east North Atlantic. Thickback sole (*Microchirus virens*) was unique to the Northeast Atlantic; saithe (*Pollachius virens*) and Norway pout (*Trispoterus esmarkii*) were present in the east Atlantic far more than the North American Boreal. Seven species, most of commercial value, were identified as significant indicators of both region and depth. Fishes known to be deep-sea adapted were indeed significantly associated with greater depths, for instance, Rakery beaconlamp (*Lampanyctus macdonaldi*) from 800-1600 m and small-eyed rabbitfish (*Hydrolagus affinis*) from 1200-1600 m. Moreover, the mesopelagic silvery lightfish (*Maurolicus muelleri*) was significantly associated with all sampled depth layers, except for 200-500 m, suggesting that the nsDNA signal detected their flexible migratory behaviour (Christiansen et al., 2021). Interestingly, Atlantic cod (*Gadus morhua*) was associated with the same region and depth range as the silvery pout (*Gadiculus argenteus*), which could be indicative of their known predator-prey relationship (Link and Garrison, 2002).

Fish assemblages under different MPA status were distinguishable within the subset of *P. ventilabrum* specimens from the Northeast Atlantic, and greater species richness was observed in specimens from MPAs (**Figure 2.2** C, D). Indicator species associated with MPAs were mostly benthic, such as the moray wolf eel (*Lycenchelys muraena*), which preys on crustaceans and other invertebrates that take refuge in sponge grounds (Garcia, 2007). Atlantic eelpout (*Lycodes terraenovae*) and Vahl's eelpout (*Lycodes vahlii*) were also indicators and known to eat sponge remains and cryptofaunal organisms such as brittle stars (Albert, 1993). Notably, with traditional survey methods *Lycodes* sp. have been found to correlate positively with high sponge biomass (Kenchington et al., 2013). Differences between the sponge aggregations were strong ($R^2 = 0.52$) though when pairwise comparisons were made, only one pair, the Faroe Shetland Sponge Belt and Rockall Bank, was identified as a potential driver of the difference. While the significant difference between MPA status was modest ($R^2 = 0.09$), with adequate samples sizes and targeted rather than opportunistic sampling, sponge nsDNA shows promise for more fine-scale biodiversity surveying.

Environmental DNA analysis is an emerging tool for deep-sea biodiversity (Everett and Park, 2018; Sinniger et al., 2016; Thomsen et al., 2016) and ecological studies (Canals et al., 2021; Fujiwara et al., 2022; Visser et al., 2021), yet eDNA is less abundant in the deep-sea, such that larger volumes of water are needed to attain representative samples, and the manual labour required to filter those samples *in situ* can become a limitation (McClenaghan et al., 2020b). Furthermore, remote, deep-sea habitats are expensive to reach in the first place, so leveraging of natural samplers in this context represents a major boost for large scale ocean exploration and monitoring. For instance, the specimens in this study had previously been used to understand sponge phylogenetics and connectivity of deep-sea environments (Taboada et al., 2022a, 2022b). The deep sea and high seas are subject to threats such as overfishing (Pusceddu et al., 2014), deep-sea mining (Van Dover et al., 2017), climate change and pollution (Danovaro et al., 2020); sponges are habitat-forming organisms (Maldonado et al., 2015) that provide shelter for cryptic animals, thereby also attracting larger more mobile predators (Danovaro et al., 2008), and as such play a fundamental role in the structure and functioning of marine ecosystems. Now, the wealth of environmental, biological and molecular data that can be comprehensively obtained from sponges significantly expands their broader value in marine ecology and conservation.

2.5 Methods

2.5.1 Specimen Selection

Three sponge species - *Phakellia ventilabrum* (N = 26, order Bubarida), *Geodia barretti* (N = 21, order Tetractinellida) and *Geodia hentscheli* (N = 17, order Tetractinellida) - from various North Atlantic sponge grounds were selected for sequencing (N = 64 of which 54 were analysed for the study – see Statistical Analysis), all collected previously for the SponGES project (www.deepseasponges.org), which ran until 2020 (S2 Table 1). The sponges were stored in 100% EtOH which was replaced at least once to maintain a high percentage of EtOH, since the water retained by the sponges can significantly dilute the preservative. For each DNA extraction, between 1-1.5 cm^3 of sponge tissue was used. For the *Geodia* spp., we avoided the cortex (with less cells than the choanosome), but for *Phakellia ventilabrum* we used pieces containing both the pinacoderm and choanosome; these decisions were made based on the original purpose of these specimens which was for phylogenetic analysis but recent research has shown that for the purpose of eDNA metabarcoding analysis the particular part of the sponge biopsied does not significantly change the results (Harper et al., 2022). The sponge DNA had been extracted between 6 and 36 months after sampling using the Qiagen DNeasy Blood and Tissue Kit (Hilden, Germany), optimal for sponge nsDNA extraction (Harper et al., 2022) and were stored at the Natural History Museum, London, at -80°C until being transported to -20°C freezers at Liverpool John Moores University.

2.5.2 Library Preparation and Sequencing

DNA extracts were diluted with molecular grade water to between 30-50 ng/µl. DNA was amplified using PCR with the Tele02 primers (Taberlet et al., 2018). The forward sequence Tele02-F (5’-AAACTCGTGCCAGCCACC-3’) and the reverse sequence Tele02-R (3’-GGGTATCTAATCCCAGTTTG-5’), were used to target a 167 bp fragment of the mitochondrial 12S rRNA gene. PCRs were prepared to a total volume of 20 µl for each sample and included 10 µl of 2X MyFi Mix (Meridian Bioscience), 1 µl of each forward and reverse primer, 0.16 µl Bovine Serum

Albumin (Thermo Fisher Scientific), 5.84 µl molecular grade water, and 2 µl of diluted DNA extract. The samples were amplified in triplicate across two libraries using the following conditions: 95°C for 10 min, followed by 35 cycles of 95°C for 30 s, 60°C for 45 s, 72°C for 30 s, and finishing at 72°C for 5 min followed by a 4°C hold. Negative controls (N = 5) and positive controls (N = 5), which were molecular grade water and a single fish species not present in the North Atlantic (iridescent catfish *Pangasionodon hypopthalmus*) respectively, underwent PCR alongside the samples. PCR triplicates were pooled and visualized on a 2% agarose gel (150 ml 1X TBE buffer with 3 g agarose powder) stained with 1.5 µl SYBRsafe dye. PCR products were individually purified using a double-size selection in 1:1 and 0.6:1 ratio of Mag-Bind® Total Pure NGS magnetic beads (Omega Bio-Tek) to PCR product. Products were visualised on an agarose gel again to assure purity (i.e. target length bands on agarose gels were visible with minimal to no other bands present). Purified PCR products were quantified using a Qubit dsDNA HS Assay kit (Invitrogen), and pooled equimolar into their corresponding libraries (i.e. pooled samples each contained unique 8-bp dual barcodes). Pooled libraries were imaged on a Tape Station 4200 (Agilent) to check the purity of the libraries. The libraries were then purified based on the Tape Station results, double-size selecting the target fragment using magnetic beads as explained before. A unique adapter sequence was ligated to each library using the NEXTFLEX® Rapid DNA-Seq Kit for Illumina (PerkinElmer) following the manufacturer protocol. After adapter ligation, the libraries were again imaged on the Tape Station and purified with magnetic beads, this time with a 0.8:1 ratio of beads to sample, as per the NEXTFLEX® Rapid DNA-Seq Kit instructions. The dual-indexed libraries were then quantified by qPCR using the NEBNext® Library Quant Kit for Illumina (New England Biolabs). The libraries were pooled at equimolar concentrations having a final molarity of 50 pM with a 10% PhiX spike-in. The libraries were sequenced at Liverpool John Moores University on an Illumina iSeq100 using iSeq i1 Reagent v2 (300 cycles).

2.5.3 Bioinformatics Pipeline

The sequences were quality controlled through the following series of steps using Python v2 within the OBITOOLS 1.2.11 (Boyer et al., 2016) package. The raw sequences were trimmed to a length of 150 bp using the command 'obicut' to remove low-quality bases from the ends which were determined from the output of the 'fastqc' command. The trimmed reads were then merged using 'illuminapairedend', from which any paired-end alignments with low (<40) quality scores were removed. The remaining paired-end alignments were demultiplexed using 'ngsfilter', filtered by length (130 – 190 bp) and dereplicated using 'obiuniq'. Chimeras were removed de novo using the programme VSEARCH version 2.4.3 (Rognes et al., 2016). The remaining sequences were then clustered using the programme SWARM v2 (Mahé et al., 2015) with 'd-value' = 3. Taxonomy was assigned using the Bayesian LCA-based taxonomic classification method (BLCA) (Gao et al., 2017). We first created a database using 'ecoPCR' from OBITOOLS with the Tele02 primers against the EMBL database (release version r143). This database was combined with a trained BLCA custom database containing fish species, specifically Teleosts and Elasmobranchs, (custom database file can be found here: https://github.com/eneave/Trapped-DNA-fragments-in-marine-sponges-Neave-et-al-2023). The workflow of BLCA was followed and can be found at: https://github.com/qunfengdong/BLCA. This resulted in taxonomic assignments where each level (i.e., family, genus) was associated with a percent probability of correct assignment. Analyses were carried out with taxonomies that had a ≥ 99% probability of correct assignment to reference sequences (i.e. species referenced in this study had a ≥99% identity at the species level and 100% identity at all higher levels of assignment to reference sequences).

2.5.4 Statistical Analysis

All downstream analyses were done using R version 4.1.3 (R Core Team, 2022). The MOTUs were decontaminated by removing the highest number of reads of a contaminant present in either the PCR positive control or PCR negative control from all samples (S2 Figure 3). Ten samples that had less than 100 reads were removed from the dataset based on a rarefaction curve (read counts) suggesting species saturation after 100 reads (S2 Figure 1). Using the R package vegan v 2.5.7 (Oksanen et al., 2013), beta-diversity was examined through multi-dimensional scaling of a Jaccard dissimilarity matrix (presence-absence) of teleosts and elasmobranchs detected from each sponge, comprising of only MOTUs identified to the species level. We tested the homogeneity among the group dispersions of biogeographic regions and sponge species using the functions ‘betadisper‘ and ‘anova‘, then tested for significant differences in beta-diversity between regions, sponge species, and region and sponge species as interacting terms, by permutational multivariate analysis of variance (PERMANOVA) using the function ‘adonis‘. The same tests (excluding sponge species as an explanatory variable) were repeated for all *G.barretti* samples (S2 Figure 4, S2 Table 7) and for the *P. ventilabrum* subset of the Northeast Atlantic. Pairwise comparisons of the biogeographic groups and population groups were performed, and p-values were corrected with the Benjamini-Hochberg method (Benjamini & Yekutieli, 2001). Correlations of fish assemblages with latitude, sampling depth, and sampling year were tested for using the function ‘envfit‘. All tests on beta-diversity were done on Jaccard dissimilarity matrices and underwent 1000 permutations. The ‘accumcomp‘ function from the BiodiversityR package v 2.14.2.1 (Kindt and Coe, 2005) was used to create species accumulation curves. Using the R package indicspecies v 1.7.12 (Cáceres and Legendre, 2009), an indicator value species analysis and multilevel pattern analysis was done using the function ‘multipatt‘ with IndVal.g method on the same Jaccard dissimilarity matrix of species for sampling depth ranges, biogeographic regions and MPA status in the Northeast Atlantic with *P. ventilabrum* samples. Tests underwent 10,000 permutations. All figures were generated using the R packages tidyverse v 1.3.1 and ggplot2 v 3.4.0 (Wickham, 2011; Wickham et al., 2019).

Chapter 3

Refining a molecular tool kit to capture tropicalization in Mediterranean Marine Protected Areas

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Black-colored sponge on a reef in the National Marine Park of Zakynthos, with two female ornate wrasses (*Thalassoma pavo*) swimming in the distance.

Photographer: Peter Shum

3. 1 Abstract

Tropicalization, the process by which tropical species expand their ranges poleward due to global ocean warming, is a prominent threat to Mediterranean marine ecosystems, challenging their effective management and conservation. The arrival of non-indigenous fish is exacerbated by the human-made Suez Canal that allows Indo-Pacific species to enter the region from the Red Sea (Lessepsian migration). While some non-indigenous species (NIS) have already changed the composition and function of native Mediterranean communities, many others continue to arrive but often will go unnoticed without regular surveys or until they are strongly established. We conducted biodiversity surveys using underwater visual census (UVC), aqueous environmental DNA (eDNA) and sponge-derived eDNA (i.e., eDNA accumulated in sponge tissue) at two protected locations in Zakynthos Island (Ionian Sea, Greece). Seven NIS were detected by eDNA, but only one (dusky spinefoot, *Siganus luridus*) was detected with all three methods. Six of the NIS are likely to be of Lessepsian origin, and four species were detected at both locations. The fish assemblages of the two locations could be distinguished based on both UVC and eDNA but not from sponge-derived eDNA data, perhaps given low filtration rates. Of the three methods, aqueous eDNA metabarcoding provided the most comprehensive species list including new NIS detected (with the redtoothed triggerfish, *Odonus niger*, and the houndfish, *Tylosurus crocodilus*, being the first and third record in the Mediterranean Sea, respectively). Our findings highlight the potential value of incorporating molecular methods into regular monitoring as early warning tools for detecting NIS in marine protected areas threatened by ocean warming.

3.2 Introduction

With the current pace of climate change, there is evidence that temperate habitats are prone to tropicalization, a phenomenon defined by poleward distribution shifts of warm-affinity species in response to sea warming (McLean et al., 2021; Osland et al., 2021; Vergés et al., 2014). The Mediterranean Sea is undergoing accelerated warming compared to other ocean basins (Urdiales-Flores et al., 2023), resulting in an influx (i.e., biological invasions) of warm-affiliated non-indigenous species (NIS) (Lejeusne et al., 2010). In addition to warming, tropicalization is amplified in this region due to the Suez Canal (1869) which provides a pathway for NIS to travel from the Red Sea to the Mediterranean Sea (Katsanevakis et al., 2014). This has caused pronounced tropicalization, especially in the eastern Mediterranean Sea (Edelist et al., 2013; Katsanevakis et al., 2014; Raitsos et al., 2010). The impacts of NIS in this region are associated with substantial shifts in the structure of native communities and the deterioration of habitats and ecosystem functioning (Peleg et al., 2020; Vergés et al., 2014; Zarzyczny et al., 2023), impairing significant ecosystem services necessary for human prosperity (Tsirintanis et al., 2022). At present, the Mediterranean Sea, harbors around 1000 NIS, predominantly originating from tropical regions, with newly established species increased by 40% during the period 2011-2021 (Zenetos et al., 2022). Therefore targeted inventory and monitoring of NIS are frequently acknowledged as crucial priorities and in policies (i.e., EU Regulation 1143/2014 for alien species) for this region (European Commission. Joint Research Centre., 2021).

Generating cost-effective, reliable biodiversity evidence remains a challenge for environmental managers, especially at sea. Underwater visual census (UVC) is a standard method for collecting biodiversity information in Mediterranean Marine Protected Areas (MPAs) (Giakoumi et al., 2017). However, this approach is time consuming, requires the expertise of skilled taxonomists, and inevitably introduces observer bias which is difficult to mitigate over the timescales relevant to long-term monitoring. Environmental DNA is complementary to UVC, with higher sensitivity to detect more taxa, such as cryptobenthic (Bessey et al., 2023), highly elusive pelagic species (Bakker et al., 2017) and NIS (Jerde et al., 2011). While eDNA analyses are becoming more cost-effective, they have not been routinely incorporated into coastal monitoring strategies. This is often due to unfamiliarity with the technology or uncertainty as to whether the data can complement or enhance traditional visual information at managed sites. Visual census offers more explicit quantitative data, such as abundance information, which managers are generally more accustomed to relying on. However, several countries are beginning to adopt eDNA analyses in monitoring strategies and biosecurity measures (De Brauwer et al., 2023; Jerde et al., 2013; Stepien et al., 2022.).

Mediterranean MPAs are vulnerable to biological invasions, even after only a few years from the establishment of NIS in the recipient MPA (Dimitriadis et al., 2024), with tropicalization acting as an exogenous threat that challenges conservation efforts and outcomes (Giakoumi et al., 2017). Tropicalization causes functional shifts in fish communities leading to ecosystem phase shifts that can have effects on the behaviours and genotypic composition of both native and non-indigenous species (Vergés et al., 2014; Zarzyczny et al., 2023). These problems have been identified as key conservation issues in Zakynthos MPA (Giakoumi et al., 2019) and other Greek islands with similar habitats (Bianchi et al., 2014). Here we conducted biodiversity surveys at two MPAs of Zakynthos Island in Greece, comparing traditional UVC methods to aqueous eDNA. We also collected biopsies from marine sponges, filter-feeding organisms that have recently been found to collect eDNA, an approach termed ‘natural sampler DNA’ (nsDNA) (Mariani et al., 2019; Turon et al., 2020). Metabarcoding of marine sponge nsDNA has been shown to be highly comparable to aqueous eDNA for detecting fish species of various sizes in artificial (Cai et al., 2023) and natural environments (Jeunen et al., 2021). Together with conventional UVC methods we explored the potential for molecular sampling strategies to expand the tool kit that managers can use to monitor marine sites of ecological and cultural significance.

3.3 Methods

3.3.1 Study Area

Two sampling locations within the marine protected areas of Zakynthos (Eastern Ionian Sea, Greece), were monitored for fish biodiversity using UVC, eDNA, and sponge nsDNA (**Figure 3.1** A). Korakonissi (**Figure 3.1** B), within the protected area of 92/43/EU Directive under code GR2210001, is a popular swimming location consisting of rocky reefs, gravel and sand substratum with depths between ~4-12 m where we sampled. This area has low protection status, with no specific measures for fish conservation in effect. The other sampling location, Dafni beach, has the maximum protection level (i.e., no-take, no-go from May to October, only swimming is allowed) of the National Marine Park of Zakynthos (**Figure 3.1** C), and consists of a mixture of shallow rocky reefs, seagrass beds and sand substrate habitats. At each location, visual census surveys, aqueous eDNA and sponge collection was repeated once in the morning (07:00-10:00) and once in the evening (16:00-19:00) between September 10-14, 2021.

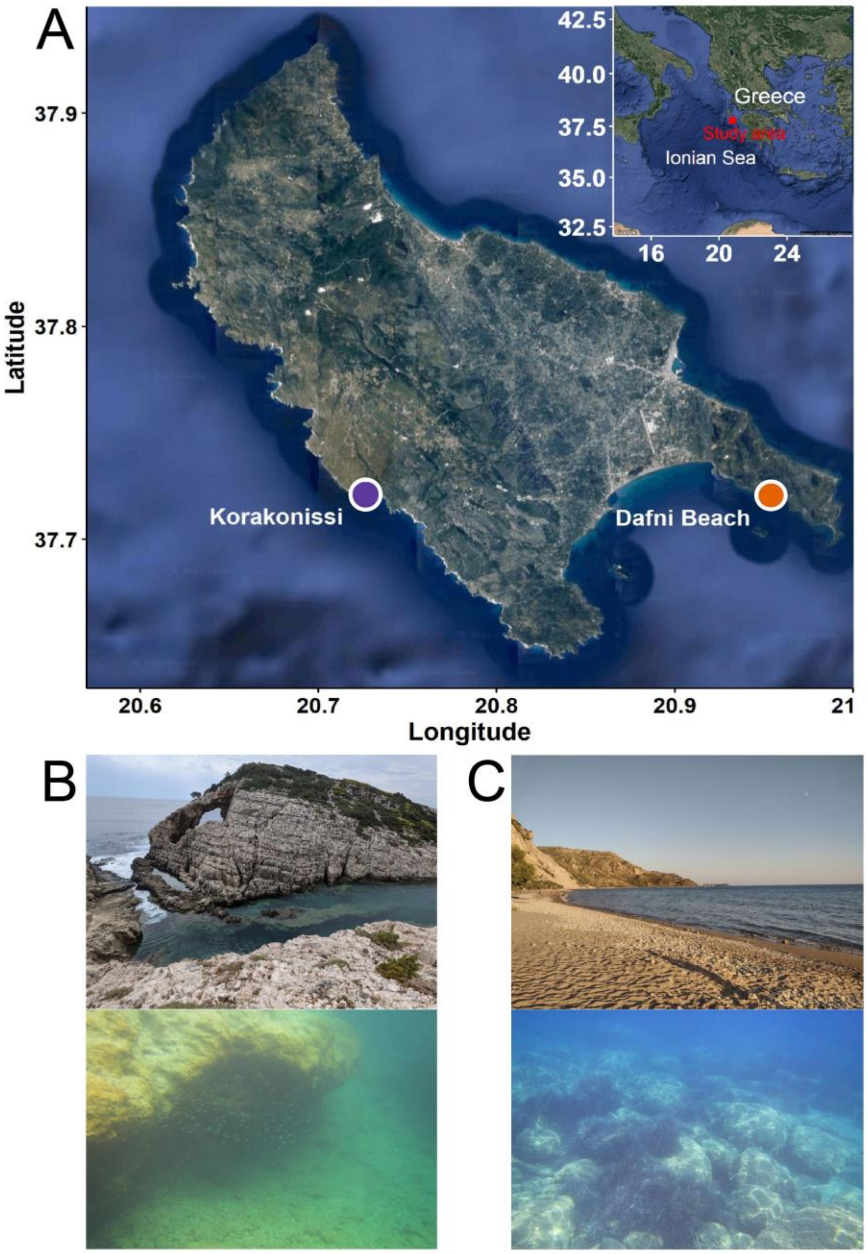

Figure 3.1 Map of Zakynthos Island, Greece showing the two sampling locations (A): Korakonissi (B) and Dafni Beach (C) which is within the National Marine Park of Zakynthos.

3.3.2 Underwater Visual Census

Underwater visual census (UVC) was performed within a ~10 meter sampling radius and using the standard stationary point methods established by the U.S. National Marine Fisheries Service (Bohnsack and Bannerot, 1986). UVC was achieved with two snorkelling observers, each with over 25 years of experience with identifying Mediterranean fish species. Fish were identified to the lowest taxonomic rank possible and counted. However, schooling fish ≥~50 were recorded as an exact abundance of 50.

3.3.3 Aqueous eDNA Sampling and Sponge Collection

Whilst UVC was underway, seawater was collected in 2 L bottles for eDNA metabarcoding analysis. Filtration blanks (2 L of filtered store-bought bottled drinking water) were performed before and at the end of each sampling day (n = 8) to control for the sterility of filtration equipment. Field blanks were collected at each sampling location in the morning and evening (n = 4), also using 2 L of bottled drinking water, which were opened on the beach for 20 seconds to expose to the air, closed, then stored and transported in the same manner as the bottles used for seawater sample collection. Sample bottles were filled 1 meter below the surface. Each triplicate sample was obtained from different areas to get an average representation of the sampling point (n = 36). Aqueous eDNA samples were then brought to shore and stored on ice packs in an insulated box.

Biopsies (~2 cm^3) of two to three sponge species were taken from the inner cortex of the sponge at each site and sampling event (n = 85). Wearing gloves, sponge biopsies were excised using a dive knife and pliers, stored in pre-labelled bags, and taken to shore for storage an insulated box. The seawater was pushed through a 0.45 µm Sterivex filter (PES membrane, Merck Millipore) using a 60 mL syringe (Fischer Scientific). Filters were then sealed into individual bags and stored at -20°C. Sponge biopsies were transferred into 15 ml falcon tubes containing 100% molecular grade ethanol. Sponges were also stored at -20°C.

3.3.4 DNA Extractions and Sponge Identification

Equipment used for DNA extractions was washed in a 10% bleach solution, followed by 5% Lipsol detergent then Milli-Q water, and exposed to ultra-violet light for 30 minutes. DNA was extracted from the sterivex filters using a modular universal DNA extraction protocol (version 2); specifically, all steps of the Mu-DNA tissue extraction protocol with the addition of an inhibitor removal step taken from the mu-DNA soil protocol, which resulted in 100 µl of extracted DNA concentrated (Sellers et al. 2018). Sponge tissue was dried by blotting storage ethanol onto filter paper inside a petri dish using tweezers (Harper et al., 2023). Between 0.25 and 0.35 grams dry-weight of sponge were placed with lysis buffer into a 1.5 ml microtube and incubated at 55°C for 24 hrs. DNA was extracted from the sponge tissue using the same modified Mu-DNA tissue protocol used for the sterivex filters (Harper et al., 2023; Sellers et al., 2018). An extraction blank was processed with each batch of filter and sponge DNA extractions (n = 3, n = 4, respectively).

Sponges are notoriously difficult to identify based on morphology alone, so a fragment of the *cytochrome c oxidase I* (COI) was used to identify and confirm species. Each sponge sample was PCR amplified using the universal primers known for their efficiency in amplifying invertebrate DNA, using LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and matched to HCO2198 (5'-TAAACTTCAGGGTGACCA AAAAATCA-3') (Folmer et al. 1994), to amplify a 658 bp fragment of the COI. The PCR protocol included a 10 min denaturing step at 95°C, followed by 35 cycles of 95°C for 1 min, 48-52°C for 1 min, and 72°C for 30 sec, with a final extension at 72°C for 5 min. Amplified DNA was Sanger sequenced in both directions at Macrogen.

3.3.5 eDNA Library Preparation and Sequencing

Sponge DNA extracts were diluted with molecular grade water to between 30-50 ng/µl, while aqueous eDNA extracts were unaltered (all < 5 ng/µl). DNA was PCR amplified using with the Tele02 8-bp dual barcoded primers (Taberlet et al., 2018):Tele02-F (5'-AAACTCGTGCCAGCCACC-3') and Tele02-R (3'-

GGGTATCTAATCCCAGTTTG-5’), which were used to target a ~167 bp fragment of the mitochondrial 12S ribosomal RNA gene. PCRs were prepared to a total volume of 20 µl for each sample and included 10 µl of 2X MyFi Mix (Meridian Bioscience), 1 µl of each forward and reverse primer (10µM), 0.16 µl Bovine Serum Albumin (Thermo Fisher Scientific), 5.84 µl molecular grade water, and 2 µl of diluted DNA extract. All samples were amplified in triplicate using the following conditions: 95°C for 10 min, followed by 35 cycles of 95°C for 30 s, 60°C for 45 s, 72°C for 30 s, and a final extension at 72°C for 5 min followed by a 4°C hold. Negative controls (molecular grade water, n = 6) and positive controls (template of iridescent catfish, *Pangasionodon hypopthalmus*, which is not present in the Mediterranean, n = 6), underwent PCR alongside the samples. PCR triplicates visualized on a 2% agarose gel stained with SYBRsafe dye and pooled. PCR products were individually purified twice using a 1:1 followed by 0.6:1 ratios of Mag-Bind® Total Pure NGS magnetic beads (Omega Bio-Tek) to PCR product. Products were visualised on an agarose gel again to assure purity (i.e., target length bands on agarose gels were visible with minimal to no other bands present). Purified PCR products were quantified using a Qubit dsDNA HS Assay kit (Invitrogen) and pooled at equimolar concentration into separate aqueous eDNA and sponge nsDNA libraries. The size integrities of the libraries were analyzed on a TapeStation 4200 (Agilent). The libraries were then purified based on the TapeStation results by repeating the magnetic beads procedure with the same ratios explained before. A unique adapter sequence was ligated to each library using the NEXTFLEX® Rapid DNA-Seq Kit for Illumina (Revvity) following the manufacturer protocol. After adapter ligation, the libraries visualized on the TapeStation and purified with magnetic beads with a 0.8:1 ratio of beads to sample. The dual-indexed libraries were then quantified by qPCR using the NEBNext® Library Quant Kit for Illumina (New England Biolabs).

The aqueous eDNA library was pooled at equimolar concentration with a library from another project for sequencing, while the sponge nsDNA library was sequenced on a dedicated separate run. The final libraries were diluted to a molarity of 85pM with a 10% PhiX spike-in and sequenced at Liverpool John Moores University on separate runs (aqueous eDNA - October 2022; sponge nsDNA – December 2022) using an Illumina iSeq100 with iSeq i1 Reagent v2 300 cycles.

3.3.6 Bioinformatics Pipeline

The sequence libraries were analyzed using OBITools 1.2.11 (Python v2) (Boyer et al., 2016). Raw R1 and R2 sequences were trimmed to a length of 149 bp using the command *obicut* to remove bases determined from the per-base quality scores from *fastqc*. The trimmed reads were merged using *illuminapairedend*, keeping paired-end alignments at >30 quality score. Paired-end alignments were then demultiplexed using *ngsfilter* and the identified barcoded samples (aqueous eDNA and sponge DNA) were concatenated into a single fasta file. The sequences were then filtered by length (130 - 190 bp) and dereplicated using *obiuniq*. Chimeras were removed *de novo* using VSEARCH version 2.4.3 (Rognes et al., 2016). The remaining sequences were then clustered using SWARM v2 (Mahé et al., 2015) with d value = 1.

Taxonomy was assigned by searching for consensus between two methods: 1) *ecotag* with a database created using *ecoPCR* with the Tele02 primers querying the EMBL database (release version r143) for non-human vertebrates (taxid settings: -r 7742 -i 9606) and 2) Bayesian LCA-based taxonomic classification method (BLCA) (Gao et al., 2017) with a custom 12S reference database, generated using a custom python script: https://github.com/shump2/Haploconator/blob/main/python_scripts/0.generate_dbBLCA.py. Briefly, accession numbers matching to the mitochondrial 12S ribosomal RNA were downloaded from Genbank and the function *blastdbcmd* was used to extract sequences from the nt_euk and ref_euk NCBI databases (accessed June 2022). A blast database was generated using *makeblastdb* and the taxonomy file used for BLCA was formatted. The taxonomic output was merged by the molecular operational unit (MOTU) IDs. If the taxonomies matched, the assignment with the highest percent identity was used, but if the taxonomies differed (e.g. *ecotag* assigned to family-level while BLCA assigned to genus-level), the taxonomy with the highest percent identity > 90% and at the most informative taxonomic level was retained (e.g. if the family-level assignment has a percent identity of 100% and the genus-level has a percent identity of 98%, the detection was assigned to the genus-level taxonomy). Both assignment methods together (S3 Table 1) supplemented by manual checks using the NCBI BLAST tool (S3 Table 2), resulted in the best taxonomic resolution of the dataset. Taxonomies were collapsed using a 98% similarity method whereby species and genus-level assignments were retained if they had ≥ 98% identity, family-level assignments were retained if they had 90-100% identity, order-level assignments were retained if they had 80-100% identity, and class and phylum level assignments were retained if they had 70-100% identity. Species and genus-level assignments were only retained if they were ≥ 98% to have high confidence in the taxonomy assigned, particularly in the context of non-indigenous species identification.

3.3.7 Statistical Analysis

Statistical analyses were done using R version 4.1.3 (R core team 2022). Any contamination present in controls was removed 10-fold from the samples to decrease the likelihood of false positives (i.e., 1 read of *Thalassoma pavo* was present in a negative control, so 10 reads of *Thalassoma pavo* were removed from each sample in the dataset) (Supplementary Table 3). This strict decontamination protocol was followed since we were particularly interested in non-indigenous species detections but wanted to be conservative in our conclusions. For management purposes, false positives could be more favorable than false negatives, and this should be considered when designing a decontamination protocol. For downstream analysis, only genus or species level assignments were retained. Any sample with less than 100 reads was removed from the dataset based on the assumption that this threshold indicated the sequencing was unsuccessful, since the samples removed had a factor of ~10-100 less reads than the remaining samples. This threshold removed 71 (84% of total) sponge samples and 25 (69% of total) aqueous eDNA samples, of which 53 sponge samples and 16 aqueous eDNA samples had zero reads. While we believe the sample loss for the aqueous eDNA samples was due to an aggressive inhibitor removal step in the extraction process that is typically used for soil samples, the lack of success for certain sponges is likely due to PCR inhibition and is expanded upon in our discussion. Loss of some aqueous eDNA samples were further analyzed by comparing this study to the only other study to our knowledge which includes environmental DNA samples from Zakynthos (Aglieri et al., 2021). Aglieri and colleagues' data was cleaned to retain species level detections with a percent identity ≥ 98%, the same threshold used in our study, so that the datasets could be directly compared. Comparisons between these two datasets regarding species richness were performed using species accumulation curves calculated with the function *accumcomp* in the R package BiodiversityR package v 2.14.2.1 (Kindt, 2023). The function *diversityresult* was used to calculate the Shannon Index and J-evenness for each method (i.e., eDNA, sponge, UVC) at each location. The J-evenness was calculated by dividing the Shannon Index by the log of species richness (Pielou, 1966). These diversity indices for each method were compared at each location using Mann-Whitney U (Wilcoxon rank-sum) tests or Kruskal-Wallis with a post-hoc pairwise Wilcoxon rank-sum test.

The R package vegan v 2.5.7 (Oksanen et al., 2009) was used to compare beta-diversity between sampling locations. Jaccard dissimilarity indices were calculated from genus and species-level presence-absence data using the function *vegdist*, resulting in separate matrices for UVC and aqueous eDNA observations. Matrices were also made of Bray-Curtis dissimilarity, calculated from count and read data (i.e. UVC and aqueous eDNA, respectively) which had been transformed using *decostand* specifying the Hellinger method. Non-metric multi-dimensional scaling (NMDS) plots were created for each distance matrix and sample type using the *metaMDS* function. Stress plots of each ordination were observed and led to the removal of three outlier eDNA samples from Korakonissi (S3 Figure 1, 2). The following tests were performed on both Jaccard and Bray-Curtis indices (S3 Figure 3): homogeneity among the group dispersions of locations (i.e. Dafni Beach and Korakonissi) was tested using the functions *betadisper*, *anova* and *TukeyHSD;* for each sample type, differences in beta-diversity between locations were tested with a permutational multivariate analysis of variance (PERMANOVA) using the function *adonis2*. The sampling location map was made with the following R packages: rstudioapi v 0.15.0 (Ushey et al., 2024) and ggmap v 3.0.2 (Kahle and Wickham, 2013). All remaining figures were generated using the R packages tidyverse v 1.3.1 (Wickham et al., 2019) and ggplot2 v 3.4.0 (Wickham, 2011), except for Figure 3 which was created in Adobe Illustrator. Code used for the statistical analysis is available in this repository: https://github.com/eneave/zakynthos_metabarcoding

3.4 Results

3.4.1 Aqueous eDNA detections and UVC observations

The eDNA sequencing run resulted in 7,749,842 reads total, of which 4,099,666 reads pertained to this project (the aqueous eDNA library was sequenced alongside a separate project), and the sponge sequencing run resulted in 7,052,976 reads total. Both eDNA and sponge runs were combined for a total of 803 MOTUs assigned to the bony fishes (class: Actinopterygii). Of those, 417 MOTUs assigned to genus and species level taxonomy. After collapsing these MOTUs by the percent identity criteria (see Methods: 3.3.6 Bioinformatics pipeline) there remained 42 species, 17 genera, and 8 family-level assignments. After cleaning the data for downstream analysis (see Methods: 3.3.7 Statistical analysis) and once molecular and UVC occurrence data were combined, there was a total of 55 taxa, 39 of which identified to species level, and all included in 38 genera within 25 families (**Figure 3.2**, S3 Table 4). At Dafni Beach 24 taxa were detected by eDNA and UVC, seven of which were detected by both methods. At Korakonissi, 33 taxa were detected by eDNA and 22 by UVC, with 10 being detected by both methods (**Figure 3.2**).

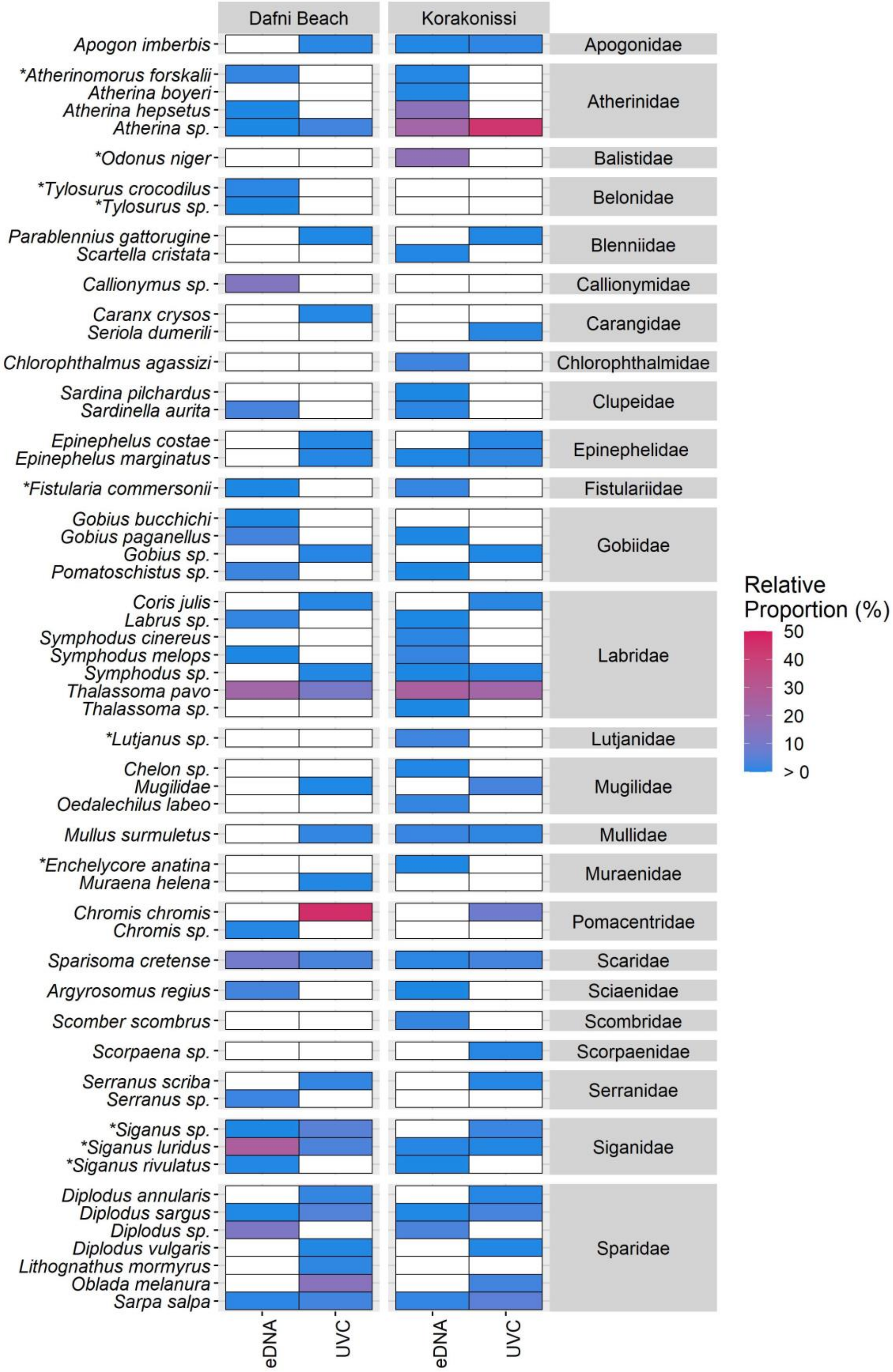

Figure 3.2 Heatmap showing the relative proportion of detections (eDNA) and counts (UVC). (*) Non-indigenous species.

In some instances, the eDNA data helped improve taxonomic granularity. One example of this was evidence of non-indigenous rabbitfish species (genus: *Siganus*). Despite the known presence of two species of *Siganus* in the area, observers could only confidently recognize *Siganus luridus*, recording the remaining rabbitfish observations as *Siganus* sp. at both locations. At Dafni Beach the largest proportion of eDNA detections was of *Siganus luridus* (26.4%; 13,731 reads), while 0.16% (82 reads) belonged to *Siganus rivulatus*. Both *Siganus* species were also detected at Korakonissi making up 0.46% of eDNA read counts. Another instance where eDNA provided greater taxonomic granularity than UVC was for the small schooling sand smelts (Family: Atherinidae). At Korakonissi, *Atherina* sp. made up 44.1% of the visual counts, while eDNA from the same location detected three species from the family Atherinidae (i.e., *Atherina boyeri*, *Atherina hepsetus* and *Atherinomorus forskalii*), where their combined relative proportion including the *Atherina* sp. genus-level assignment was 38.4%.

However, there were also cases where UVC had greater taxonomic resolution than eDNA metabarcoding. Three species of sparidae in the genus *Diplodus*, were identified by UVC (i.e., *Diplodus annularis*, *D. sargus* and *D. vulgaris*) while only one species, *Diplodus sargus*, could be identified to the species level by eDNA (**Figure 3.2**). Damselfish, *Chromis chromis*, made up 46.3% of the visual observations at Dafni beach and 8.4% of UVC at Korakonissi but was not detected at species-level by eDNA, while, the genus *Chromis* was detected at Dafni Beach.

UVC and eDNA both detected 13 genera (34 %), but at the species-level they shared only eight observations or 20 % of species-level detections (**Figure 3.3**, S3 Table 4), emphasizing the complementary nature of different survey methods. eDNA detected six more non-indigenous species than UVC, such as the Red Sea hardyhead silverside (*Atherinomorus forskalii*), a small Lessepsian species which would be difficult to tell apart from native sand smelts using visual census. Other non-indigenous species detected by eDNA included the Indo-Pacific Red-toothed triggerfish (*Odonus niger*), Fangtooth moray (*Enchelycore anatine*), Hound needlefish (*Tylosurus crocodilus*), the marbled spinefoot (*Siganus rivulatus*) and the Bluespotted cornetfish (*Fistularia commersonii*). Ornate wrasse (*Thalassoma* *pavo*) was a considerable component of the community detected by both methods, making up 22.3% and 25.5% of eDNA detections and 10.7% and 22.8% of UVC observations, from Dafni Beach and Korakonissi respectively (**Figure 3.2**). Parrotfish (*Sparisoma cretense*), dusky spinefoot (*Siganus luridus*), white seabream (*Diplodus sargus*), and salema porgy (*Sarpa salpa*) were also detected at both locations with both methods (**Figure 3.2**, **Figure 3.3**).

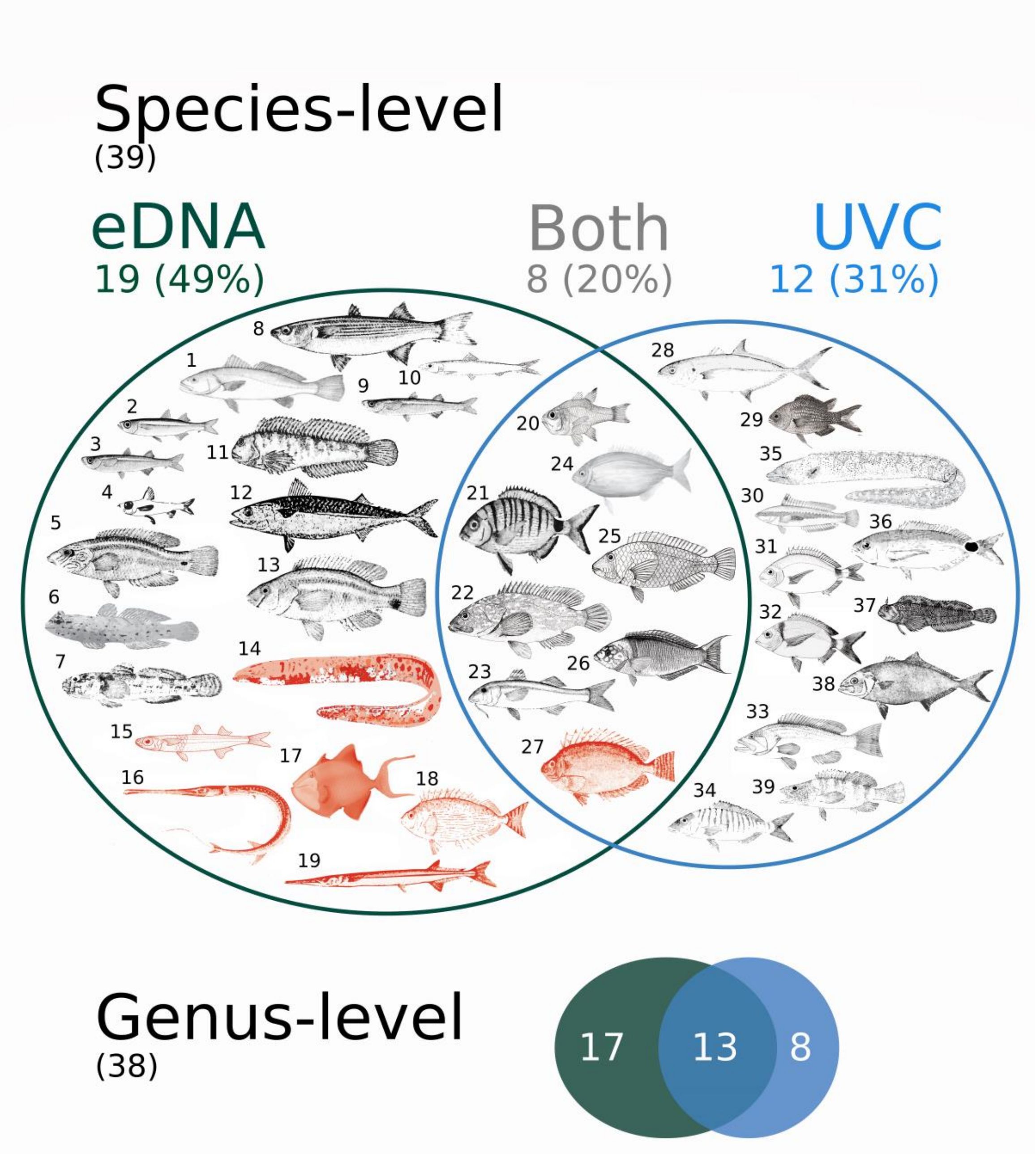

Figure 3.3 Venn diagram illustrating species-level detections and observations from aqueous eDNA and UVC respectively. Fish images and left aligned numbers correspond to the following species: 1) Argyrosomus regius, 2) Atherina boyeri, 3) Atherina hepsetus, 4) Chlorophthalmus agassizi, 5) Symphodus melops, 6) Gobius bucchichi, 7) Gobius paganellus, 8) Oedalechilus labeo, 9) Sardina pilchardus, 10) Sardinella aurita, 11)

Scartella cristata, 12) Scomber scombrus, 13) Symphodus cinereus, 14) Enchelycore anatina, 15) Atherinomorus forskalii, 16) Fistularia commersonii, 17) Odonus niger, 18) Siganus rivulatus, 19) Tylosurus crocodilus, 20) Apogon imberbis, 21) Diplodus sargus, 22) Epinephelus marginatus, 23) Mullus surmuletus, 24) Sarpa salpa, 25) Sparisoma cretense, 26) Thalassoma pavo, 27) Siganus luridus, 28) Caranx crysos, 29) Chromis chromis, 30) Coris julis, 31) Diplodus annularis, 32) Diplodus vulgaris, 33) Epinephelus costae, 34) Lithognathus mormyrus, 35) Muraena Helena, 36) Oblada melanura, 37) Parablennius gattorugine, 38) Seriola dumerili, 39) Serranus scriba. Red fish images are non-indigenous species. The small Venn diagram provides the same information as the illustrated Venn diagram. Additional metadata is in Supplementary Table 4.

3.4.2 Sponge nsDNA as a molecular biomonitoring method

Four sponge species were biopsied, and their total DNA was extracted and prepared for vertebrate metabarcoding and Sanger sequencing. Only *Chondrosia reniformis* was successfully amplified for COI identification, while the other three species were identified based on their skeletal (spongin) arrangements. *Chondrosia reniformis* (**Figure 3.4** A) occurred at both Dafni Beach and Korakonissi. *Sarcotragus* sp. (**Figure 3.4** B) and *Ircinia variabilis* (**Figure 3.4** C) were found only at Dafni Beach, while another *Ircinia* sp. (**Figure 3.4** D) was unique to Korakonissi. *Ircinia* sp. had the highest success rate, such that 44% of specimens collected resulted in over 100 reads with detections of the target taxa, Actinopterygii (**Table 3-1**). When combining sponge samples, seven fish species were detected from nsDNA at Dafni Beach and 11 fish species were detected at Korakonissi (**Figure 3.4** E). Twenty-four unique detections were made at Dafni Beach when combining aqueous eDNA samples compared to seven from sponge samples. Of the seven sponge detections, *Mullus surmuletus* was the only fish not detected in the aqueous eDNA. Thirty-three unique detections were made at Korakonissi, while of the 11 detections from sponges, three were not present in the aqueous eDNA (i.e., *Gobius bucchichi*, *Gobius* sp., *Chromis* sp.). When comparing the total read counts from eDNA samples to the total reads of nsDNA, certain species were detected by the same order of magnitude (i.e., *Siganus luridus*, *Siganus rivulatus*, *Diplodus* sp., *Diplodus sargus* and *Thalassoma pavo*). Some cryptic species that could be found in closer proximity to sponges had more combined total reads in the nsDNA than eDNA, such as two goby species at Korakonissi (i.e. *Gobius bucchichi* and *Gobius paganellus*).

Table 3-1 Sponge species tested as candidates for sponge-derived eDNA.

Species Code	Sponge Species	# Samples	# Samples w/ detections	# Samples over 100 reads	# Samples w/ target species	# Fish Species Detected
A	Chondrosia reniformis	35	20	13	4 (11.4%)	4
B	Sarcotragus sp.	18	18	16	2 (11.1%)	5
C	Ircinia variabilis	12	12	11	0 (0%)	0
D	Ircinia sp.	18	18	16	8 (44.4%)	10

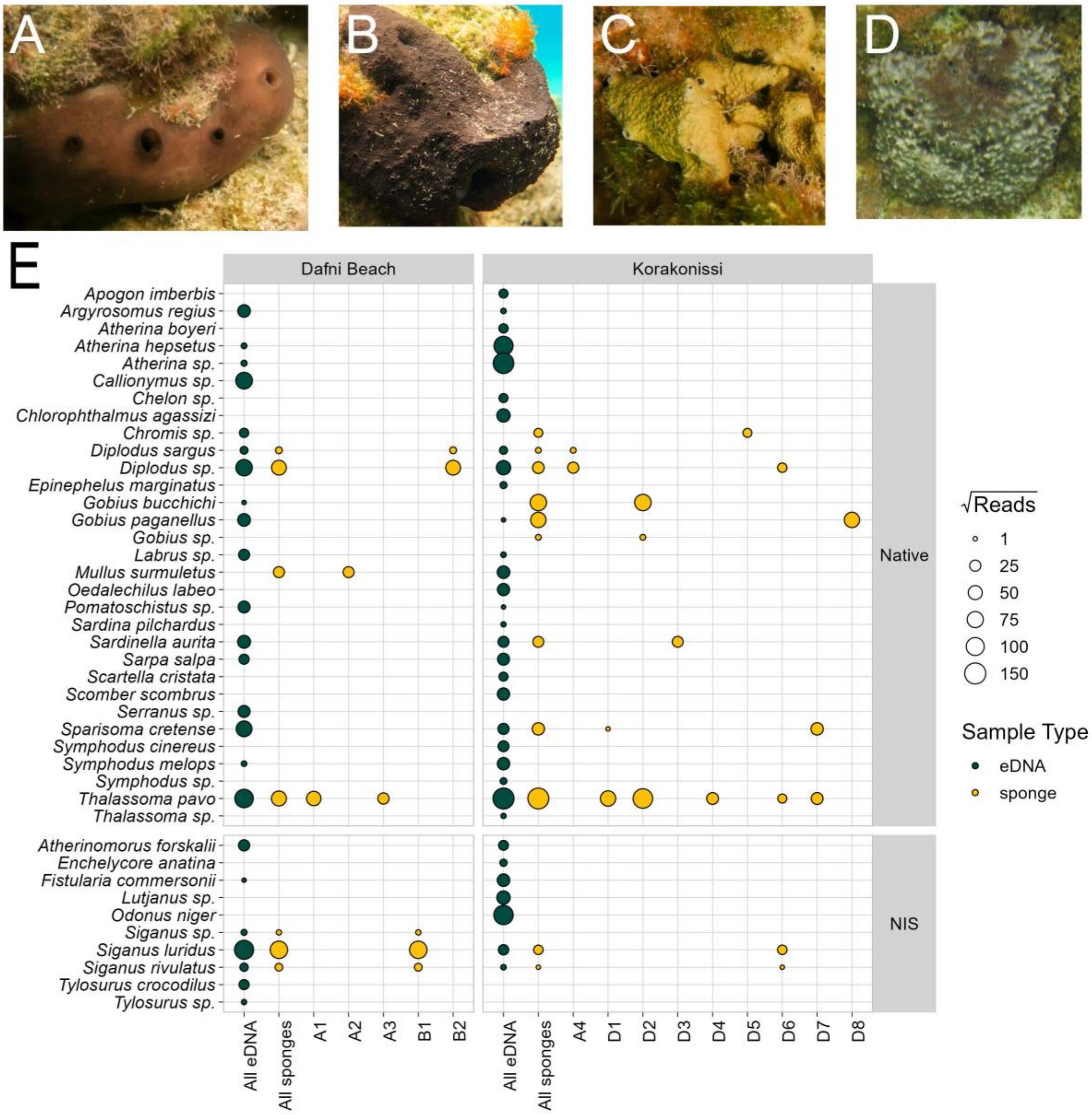

Figure 3.4 Pictures of the sponge species which were biopsied for sponge nsDNA analysis. Chondrosia reniformis (A) Sarcotragus sp. (B) Ircinia variabilis (C) Ircinia sp. (D) Bubble plot showing the square root reads of all eDNA and all sponge nsDNA. Samples combined as well as individual sponge nsDNA samples (E).

3.4.3 Zakynthos biodiversity

The Shannon Index at both Dafni Beach and Korakonissi did not significantly differ between eDNA and UVC sampling methods (Wilcoxon rank-sum: U = 17, p = 0.4306; U = 21, p = 0.0832; respectively). At Dafni Beach, the Shannon Index from sponges could only be calculated for two samples since species richness was low. However, at Korakonissi the sponges could be compared to the other sampling methods and were significantly different from UVC (Wilcoxon rank-sum: U = 21, p = 0.0004) but not eDNA (Wilcoxon rank-sum: U = 21, p = 0.0832) (**Figure 3.5** A). The J-Evenness at Dafni Beach for eDNA and UVC did not significantly differ (Wilcoxon rank-sum: U = 19, p = 0.5853). Similarly, at Korakonissi J-Evenness did not significantly differ between all pairwise combinations of sampling methods (Wilcoxon rank-sum: $p > 0.05$) (**Figure 3.5** B). The group dispersions by sampling location of UVC and eDNA beta-diversity were both homogenous (ANOVA: df = 1, F = 1.468, p = 0.254; df = 1, F = 3.412, p = 0.114, respectively). Beta-diversity (Bray-Curtis dissimilarity) significantly differed between locations in both the UVC data and eDNA data (PERMANOVA: df = 1, $R^2 = 0.60$, F = 15.088, p = 0.004; df = 1, $R^2 = 0.33$, F = 2.937, p = 0.027; respectively). The results remained the same when using the Jaccard index for UVC data but were no longer significant for eDNA (PERMANOVA: df = 1, $R^2 = 0.320$, F = 4.701, p = 0.003; df = 1, $R^2 = 0.221$, F = 1.705, p = 085; respectively).

The aqueous eDNA samples from this study were compared to a previous study on eDNA sampled from inside and outside the National Marine Park of Zakynthos (Aglieri et al., 2021) (**Figure 3.6**). The eDNA samples from these different studies and sites had similar species accumulation curves, suggesting a similar number of fish species expected in the locations. One exception to this observation were samples taken from the MPA at the previous study, which have about half the number of species compared to our samples, despite a similar sample size.

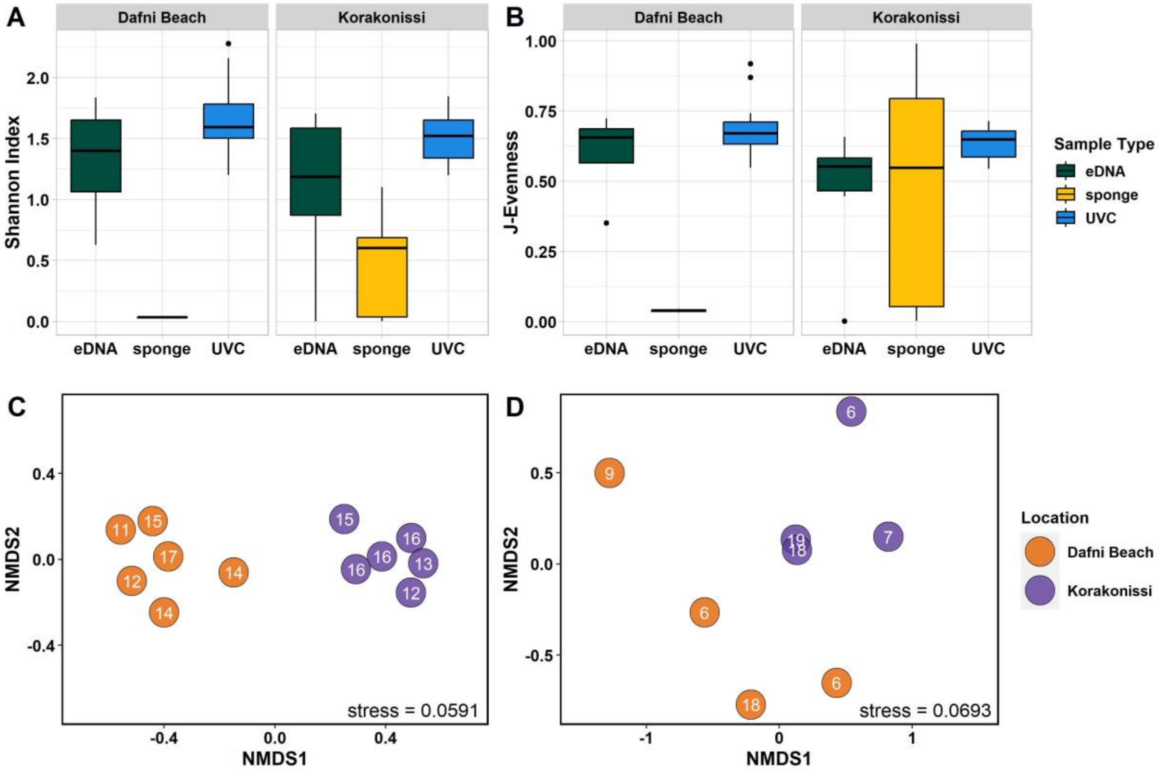

Figure 3.5 Box plots of Shannon indices calculated for the different sampling types at each location (A). Box plots of J-evenness calculated for the different sampling types at each location (B). NMDS plot calculated from Bray-Curtis dissimilarity calculated from UVC, where numbers on points indicate alpha-diversity (C). NMDS plot calculated from Bray-Curtis dissimilarity from eDNA, where numbers on points indicate alpha-diversity (D).

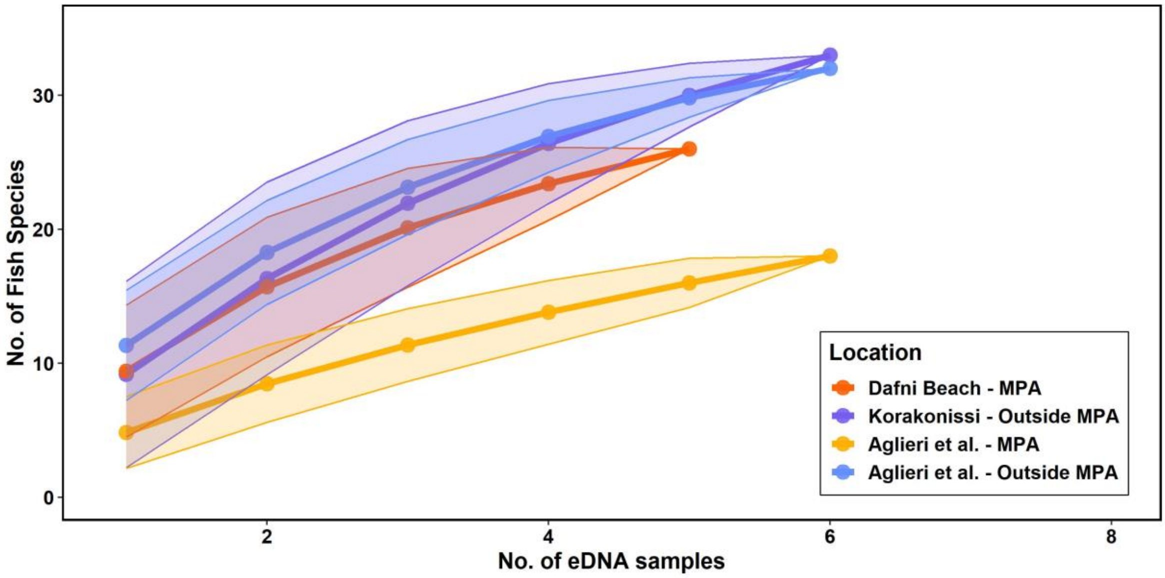

Figure 3.6 Species accumulation curves of aqueous eDNA samples from this study and from Aglieri and colleagues 2021 inside and outside the MPA of the National Marine Park of Zakynthos (NMPZ).

3.5 Discussion

Monitoring biological elements within MPAs over the long term poses a significant challenge to managers and conservation practitioners in terms of effort, cost, capacity, and efficiency. Timely and precise information on the status of protected elements (i.e., species, habitats) is critical for their effective management, particularly when considering how climate change is calling for more adaptive and climate smart management strategies. Environmental DNA could be a solution to obtain a more comprehensive inventory of marine biodiversity information especially in MPAs, while potentially acting as an early warning system for non-indigenous species for which implementing counteracting measures at early invasion stages is crucial for a successful management outcome (Simberloff et al., 2013). Sponge specimen collection for natural sampler DNA (nsDNA) could be promising for establishing baselines, where specimens have been collected and stored prior to the advent of eDNA technology. The present study on fish diversity in Zakynthos offers important insights into the benefits and caveats of implementing monitoring strategies using complementary methods.

3.5.1 Aqueous eDNA Detections vs. UVC observations

Like previous research studies, eDNA was able to provide greater taxonomic granularity than UVC and both methods provided complementary information (Aglieri et al., 2021; Lamy et al., 2021; Valdivia-Carrillo et al., 2021). For instance, at both locations the wrasse genus *Symphodus*, was recorded by UVC, but the species *Symphodus cinereus* and *S. melops* were detected only by eDNA (**Figure 3.2**). Often this lack of species-level identification visually, was what led to a low number of species (21%) present in both data sets, whereas genus-level overlap between the data sets was much greater (37%) (**Figure 3.3**). Yet, eDNA was capable of detecting the presence of fish species inhabiting deep waters as adults and shallow pelagic areas as larvae and juveniles (e.g. *Chlorophthalmus agassizi*)(D'Onghia et al., 2006) that inevitably go unnoticed by UVC methods due to depth limitation and difficulty of visually spotting and identifying species at early life stages. There were exceptions where UVC distinguished more species within a genus than eDNA metabarcoding. While both UVC and eDNA detected the white seabream *Diplodus sargus*, eDNA failed to identify *D. annularis* and *D. vulgaris*, which were instead visually detected. Similarly, *Chromis chromis* was abundant at both locations, however only genus-level (*Chromis* sp.) detections were present in the eDNA from Dafni Beach and the sponge nsDNA from Korakinissi. This ambiguity in the molecular methods, despite obvious visual presence, was due to the inability of the primer we used to resolve the *Chromis* genus, an occasional trade-off of metabarcoding analysis. Although only one *Chromis* species is endemic to the Mediterranean, further damselfish species within this genus could expand their ranges into the Mediterranean and potentially go undetected by this method. This further highlights the importance of treating primers as assays in the sense that even though they are designed to universally amplify fish taxa, there will always be closely-related species that may remain indistinguishable, while others will have sequences with poor affinity with the chosen primers. Despite these minor ambiguities – and the impact of the aggressive extraction protocol – the eDNA metabarcoding analysis still produced a more comprehensive list of species than did the UVC survey (**Figure 3.3**).

When considering individual samples, fish diversity (Shannon index) and evenness detected by eDNA and UVC did not significantly differ (**Figure 3.5** A, B) and both methods were able to distinguish location specific beta-diversity (**Figure 3.5** C, D). Environmental DNA metabarcoding has been used to determine beta-diversity of fish communities on spatial scales relevant to management, and at a regional scale it appears to detect more taxa than UVC (Lamy et al., 2021), which is similar to what we observed in Zakynthos. In tropical environments with high diversity, eDNA metabarcoding is often able to capture a wider variety of fish families than UVC (Polanco Fernández et al., 2021; Zamani et al., 2022) and we also observe this, with eDNA capturing nine (36% of total) unique families, two of which include NIS.

3.5.2 Detection of Non-indigenous species

eDNA and sponge nsDNA showed greater detection ability of non-indigenous species relative to UVC. eDNA metabarcoding detected seven NIS (i.e. *Atherinomorus forskalii*, *Enchelycore anatine, Fistularia commersonii*, *Odonus niger*, *Siganus luridus, Siganus rivulatus*, *Tylosurus crocodilus*), while only one (*Siganus luridus*) was observed with UVC (**Figure 3.2**). Although the fish fauna in Zakynthos protected areas have been systematically and regularly monitored over the last decade by the use of UVC, fisheries catch data and citizen science initiatives with a special emphasis on detecting NIS (Dimitriadis et al., 2021, 2024; Dimitriadis, et al., 2023; Ragkousis et al., 2023). Red Sea hardyhead silverside (*Atherinomorus forskalii*), red-toothed triggerfish (*Odonus niger*), and houndfish (*Tylosurus crocodilus*) to our knowledge have not been previously reported in our study area. However, *Atherinomorus forskalii* is not new to Greek waters (Bariche et al., 2015; Zenetos et al., 2013) and has been reported in Zakynthos on a citizen science platform (i.e., iNaturalist; https://greece.inaturalist.org/taxa/608753-Atherinomorus-forskalii) during 2022, emphasising the power of citizen science. The eDNA detection of red-toothed triggerfish (*Odonus niger*), to the best of our knowledge, is the first record of this species in the Mediterranean region. While *Odonus niger* is native to the Red Sea it is also an ornamental fish species sold in Greek aquarium stores for direct trade (Papavlasopoulou et al., 2014), rendering accidental or deliberate release by aquarists as a possible mode of introduction in the study area. Houndfish (*Tylosurus crocodilus*) was first reported in the North Aegean Sea in 2005 (Sinis, 2005) and more recently in the Adriatic Sea (Fortič et al., 2023), such that the present finding is the third report of the species in the Mediterranean.

The most established non-indigenous taxa in Zakynthos are the rabbitfishes and their herbivory is causing shifts in ecosystem function (Dimitriadis et al., 2021; Golani, 1998). Dusky spinefoot (*Siganus luridus*) was introduced to Zakynthos in 2004 and today comprises the most abundant fish species in the National Marine Park of Zakynthos (NMPZ); it accounts for almost one third of total fish biomass (Dimitriadis et al., 2024) which interestingly mirrored the relative read abundance recorded in this study (26.4%) at Dafni Beach. Marbled spinefoot (*Siganus rivulatus*) was observed for the first time in NMPZ in 2009 (Dimitriadis et al., 2021) and progressively increased its population size (Dimitriadis et al., 2024). Both rabbitfish species were present in the sponge nsDNA from both Dafni Beach and Korakonissi.

Snappers (genus: *Lutjanus*) are known Lessepsian migrants, which we detected at Korakonissi and species from this genus are known to have colonised the Mediterranean since the 1970s (Bariche et al., 2015). Moreover, mangrove red snapper (*Lutjanus argentimaculatus*) was recently detected by eDNA metabarcoding in the NMPZ (Aglieri et al., 2021). While the physical ability of these fish to enter via the Suez Canal has facilitated the Mediterranean to become a NIS hotspot, ocean warming of the region is what ultimately allows these warm-affinity fish to thrive. With eDNA we detected the range expanding fangtooth moray (*Enchelycore anatina*), a subtropical species native to the eastern Atlantic, which since it was first reported in the study area in 2012 (Kapiris et al., 2014) it has been rare to appear in fish monitoring surveys.

3.5.3 Refining the Molecular Tool kit

eDNA metabarcoding analysis has been explored as a method for early and rapid detection of NIS (Duprey et al., 2023; Zaiko et al., 2015). But a more common approach when studying NIS with eDNA is to use species-specific primers with analyses such as PCR (Chucholl et al., 2021), qPCR (LeBlanc et al., 2020) and ddPCR (Chucholl et al., 2021; Doi et al., 2015; Nathan et al., 2014). When comparing species detection using species-specific approaches with metabarcoding, the latter has been able to perform similarly to qPCR and for the same investigator effort delivers multiple species distribution data (Harper et al., 2018). Of course, the outcome of these methods comparisons could differ depending on the species of interest. Metabarcoding analysis is useful in exploratory contexts such as our study, where many of the invasive species detected were not yet observed by UVC in the study area, so they were relatively unexpected.

Sponge nsDNA is an emerging tool for biodiversity census in aquatic systems (Mariani et al., 2019; Riesgo et al., 2024; Turon et al., 2020), and this study provides valuable information on how different sponge species function as natural environmental DNA samplers. In previous studies, captive sponges have been shown to vary in eDNA signal based on species (Cai et al., 2022) and evidence of this in the natural world also exists (Brodnicke et al., 2023; Neave et al., 2023), despite some conflicting observations showing no differences between sponge species, but this could have been due to low replication (Turon et al., 2020). Sponges have different pumping rates which can change based on environmental conditions and contain different levels of microbial symbionts that could inhibit the DNA extraction processes (Brodnicke et al., 2023.; Harper et al., 2023). In particular, our targeted sponge species are notoriously abundant in microbial symbiotic partners, which usually leads to poor filtration rates (Weisz et al., 2008). Only a few studies have compared eDNA signal from sponge nsDNA to that of aqueous eDNA. The results are varied but remarkable, a couple findings highlighting stark contrasts between sponge nsDNA and aqueous eDNA (Brodnicke et al., 2023; Jeunen et al., 2023), while another found no significant difference between the methods (Jeunen et al., 2021). Further, a recent study suggests that sponge nsDNA may detect more complex community structures compared to aqueous eDNA (Cai et al., *in press*). Clearly the sponge species, laboratory processes and environmental factors all could be playing a role in the efficiency of these natural samplers. All of the sponges sampled in this study were relatively ineffective compared to previous efforts using sponge nsDNA for biodiversity surveys (Jeunen et al., 2021; Neave, et al., 2023; Riesgo et al., 2024), but were comparable to some efforts where individual samples consistently detected less than ten species (Brodnicke et al., 2023; Turon et al., 2020). Based on our results, it appears that further research is necessary to thoroughly understand what determines the suitability of sponge species for nsDNA analyses. In previous studies, *Chondrosia reniformis* was also tested and did not have any detections of target taxa (Mariani et al., 2019) but without comparison to water and based on only one sample, it was not certain whether the lack of detections was representative of this species. Despite the suboptimal performance of the sponges in this study, our nsDNA analysis still led to the detection of two non-indigenous species and 13 taxa in total.

Tropicalization involves the range expansions of tropical species and retractions of native, more temperate species. Prior studies have used eDNA to track range expansions of invasive (Larson et al., 2017; Richardson et al., 2016) and endangered species (Harper et al., 2019; Hobbs et al., 2019; Valsecchi et al., 2022). However, many of these studies focus on a single species and have developed species-specific probes, which is why the ability for eDNA to monitor tropicalization needs further study using metabarcoding analysis to monitor the expansion and contraction of native and non-native species. It is interesting to note that the species-specific molecular strategies seem to mirror long-standing traditional conventions of environmental management, which initially placed much emphasis on single species, and only very recently are beginning to move to a more holistic approach. Species-specific probes may be more attractive to management because they may provide a sense of a more controlled, bespoke survey, but in terms of monitoring tropicalization, metabarcoding analysis has more potential. Whilst metabarcoding analysis can lead to false positives (Darling et al., 2021) and false negatives (Jackman et al., 2021), there are methodological strategies to overcome these challenges (McClenaghan et al., 2020a) and overall it provides multiple species distributions simultaneously (Harper et al., 2018) and may be more effective at early detection of non-indigenous fishes (Van Nynatten et al., 2023). While the presence of tropical NIS is evidence of topicalization, long term molecular monitoring coupled with other abiotic measurements could lead to further insights regarding the rate at which this is occurring, and its ecosystem ripple effects. One such study over a longer temporal scale used eDNA metabarcoding of archived samples to show short-term tropicalization of fish communities over a marine heatwave (Gold et al., 2023). Our study presents seven species-level and three genus-level detections of tropical NIS, only two of which (*Lutjanus* sp. and *Siganus luridus*) were detected in eDNA samples collected from the NMPZ only three (i.e. 2018) years prior (Aglieri et al., 2021). This difference highlights that repeat molecular monitoring could lead to insights on tropicalization that UVC does not have the sensitivity for, which would represent the early warning system that MPA managers require for the development of timely and effective NIS mitigation strategies.

Environmental DNA and UVC were both able to distinguish Mediterranean fish communities, from high and low protection status MPAs, less than 30 km apart. Exemplifying the complementary nature of eDNA and UVC, community composition at each location differed such that only seven (13%) and 10 (18%) of 55 taxa were detected by both methods across locations which corroborates many prior studies showing that eDNA and UVC typically detect different sets of species (Aglieri et al., 2021). Still eDNA provided nearly half (49%) of the species detections alone and captured six out of seven non-indigenous species (NIS), from which three are new records in the study area despite the regular, long-standing and NIS focused fish monitoring at Zakynthos islands by visual census and fisheries related methods (Dimitriadis et al., 2023). Sponge nsDNA captured 13 taxa, all of which were also detected by eDNA, except for striped-red mullet (*Mullus surmuletus*), which was highly abundant in the UVC. Despite likely suffering from PCR inhibition, sponge nsDNA successfully detected dominant species including sea breams (*Diplodus* sp.), non-indigenous rabbit fishes (*Siganus* sp.) and ornate wrasses (*Thalassoma pavo*). *Siganus luridus* was the only NIS detected by all three methods, highlighting the strength of molecular tools for determining NIS presence. The redtoothed triggerfish (*Odonus niger*), an Indo-Pacific species present in the Red Sea, was detected at Korakonissi and to our knowledge has never been previously reported in the Mediterranean, while *Tylosurus crocodilus* is only the third record in this region. Overall, this study advocates for a more integrated approach for marine biodiversity monitoring, combining UVC with eDNA metabarcoding. Such a holistic approach is crucial for early detection of ecosystem changes, particularly in the context of increasing tropicalization and the introduction of NIS, enabling more proactive and informed conservation strategies. This approach also offers a comprehensive understanding of marine ecosystems, ensuring a balance between scientific rigor and practical applicability in biodiversity monitoring and management.

Chapter 4

DNA Divers: volunteer-based eDNA capture for local and global marine biodiversity monitoring

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Artistic photo of a 3D-printed metaprobe nestled among sea urchins.

Photographer: Bob Anderson

4.1 Abstract

Volunteer-based biodiversity recording is a powerful source of scalable data that has yet to be used to its full potential by the scientific community. Coastal ecosystems are varied and complex, forcing managers to make difficult decisions on how to best allocate resources for monitoring biological components. Emerging technologies such as environmental DNA (eDNA) analysis are promising for measuring marine biodiversity, yet most management organizations lack personnel and capacity to collect molecular data. Here we investigate, together as professional and non-professional scientists, the efficacy of a novel quasi-passive eDNA capture technique. By working with a small (<30) network of snorkellers and SCUBA divers, we detect 275 unique teleost and elasmobranch species spanning six countries across 90 degrees of latitude. The ease of this technique matched by the eagerness and generosity of volunteers presents an untapped, viable approach for local and global multi-species marine biodiversity monitoring while improving science literacy.

4.2 Introduction

Marine and coastal habitats modulate processes affecting water quality, disease, and climate, as well as provide many ecosystem services that bolster the economy, such as food, tourism, and recreation (Barbier, 2017). With biodiversity loss the health of these habitats diminishes and ecosystem services wane (Palumbi et al., 2009). Therefore, there is a need to understand the biological components of seascapes to enable appropriate policies and stewardship and sustain ecosystem services. Marine environmental DNA (eDNA) analyses have the accuracy and sensitivity necessary to monitor biological components at the species-level and across the tree of life (i.e. microbes to macro-organisms) (Stat et al., 2017a). Yet, despite substantial advances in methodology, one major roadblock for the uptake of eDNA monitoring is the ability to capture eDNA routinely, efficiently and at scale. Inherently, eDNA exists in trace amounts, and in aquatic environments, water filtration, even when assisted mechanically by vacuum pumps or autonomous samplers (Hendricks et al., 2023), still requires a lot of time and/or engineering investment. Lack of time and resources for eDNA capture present a hindrance to the uptake of eDNA analysis for large-scale, routine marine biodiversity monitoring.

Despite the challenge of scalability, eDNA analysis is a promising tool for marine management and conservation because in many cases it offers advantages over conventional methods. For instance, morphological identification methods (e.g., visual surveys, video, plankton tow-net) require expert taxonomists, visible conditions and exclude organisms that cannot be seen with the naked eye or distinguished using microscopy. Moreover, physical survey methods (e.g., trawling, electrofishing), in addition to visual identification constraints, can be detrimental to delicate habitats and cause disturbance or require collecting specimens, which can be counterproductive in conservation contexts. In marine environments, eDNA analysis often results in higher species richness compared to visual dive surveys and can even differentiate communities in habitats over short distances (< 100 m) (Gold et al., 2021b; Port et al., 2016). When comparing tandem trawl data with eDNA, both methods result in similar species richness and the relative abundance of eDNA sequence reads correlates with trawl catch counts (Stoeckle et al., 2021; Thomsen et al., 2016). The scientific community continues to compare conventional monitoring methods with eDNA analyses which is important for understanding how eDNA data can replace or be integrated with traditional measurements (Fediajevaite et al., 2021). Nonetheless, enough evidence exists to support the adoption of eDNA analysis for holistic marine biodiversity monitoring, but strategies to do so have yet to be formalised and implemented (Kelly et al., 2024).

One of the largest and fastest growing sources of biodiversity data comes from the grass-roots efforts of volunteer monitoring programs or citizen science (Chandler et al., 2017). Citizen science, or the inclusion of non-professional scientists in the scientific process, is a methodology employed across a variety of fields to overcome issues of scalability. For example, crowdsourcing, particularly through recruitment on web platforms (i.e., Zooniverse, SciStarter, iNaturalist), encourages volunteers to collect and analyse large volumes of data. This concept has been used to scale up eDNA monitoring, for instance water samples from the entire coastline of Denmark were collected by volunteers at coordinated time points, providing snapshots of coastal fish biodiversity (Agersnap et al., 2022). Similarly, eDNA citizen science projects have emerged in an effort to cover large geographic areas and establish long-term monitoring strategies, such as the CALeDNA program (Meyer et al., 2021) and UNESCO-led expeditions of marine world heritage sites (https://www.unesco.org/en/edna-expeditions).

Since the process of actively filtering eDNA requires time and investment in dedicated personnel, passive eDNA capture techniques have been explored as an alternative (Bessey et al., 2021; Kirtane et al., 2020; Verdier et al., 2022). The first study on passive eDNA capture tested the ability of porous materials (i.e., montmorillonite clay and granular activated carbon) to adsorb extracellular DNA over time, and found that after soaking for days to weeks the materials accrued a similar amount of DNA to 1 L of filtered water (Kirtane et al., 2020). Other sorbent materials, for example, a resin-hydroxyapatite mix (Verdier et al., 2022), a macroporous weak alkaline acrylic anion exchange resin (Chen et al., 2022), and silica gel (Chen et al., 2022) have been tested as potential substrates for circumventing water filtration. In temperate and tropical marine sites, metabarcoding of passively captured eDNA was tested by suspending filter membranes (i.e., positively charged nylon and non-charged cellulose ester) and compared to filtered water, resulting in 172 fish taxa, 109 of which were detected by passive sampling (Bessey et al., 2021).

While studies demonstrate that citizen science and passive eDNA sampling is a solution to the difficulties of scalability in marine eDNA projects (Agersnap et al., 2022; Bessey et al., 2021), this research is still in its infancy. One promising branch of passive eDNA sampling research has involved combining passive sampling, using sterile cotton in a perforated 3D-printed probe (the ‘metaprobe’), with conventional trawl surveys by placing the passive eDNA sampler into the fishing net, from which the metaprobe recovered between 70-90% of the species caught in the net (Maiello et al., 2024, 2022). Here we combine the metaprobe with the power of SCUBA diving citizen scientists to scale up biodiversity monitoring efforts in coastal marine habitats (**Figure 4.1** A). First, we evaluate the influence of metaprobe soaking time in an aquarium; then we assess the influence of different preservation and DNA extraction techniques, testing the approach in natural marine habitats, ultimately comparing the results to conventional water filtration (**Figure 4.1** B-D). Finally, through engagement with existing citizen science networks, independent volunteers, SCUBA diving clubs, the dive charter industry, non-profits, and statutory governing agencies, we scale up, sampling at dive sites spanning 90 degrees of latitude.

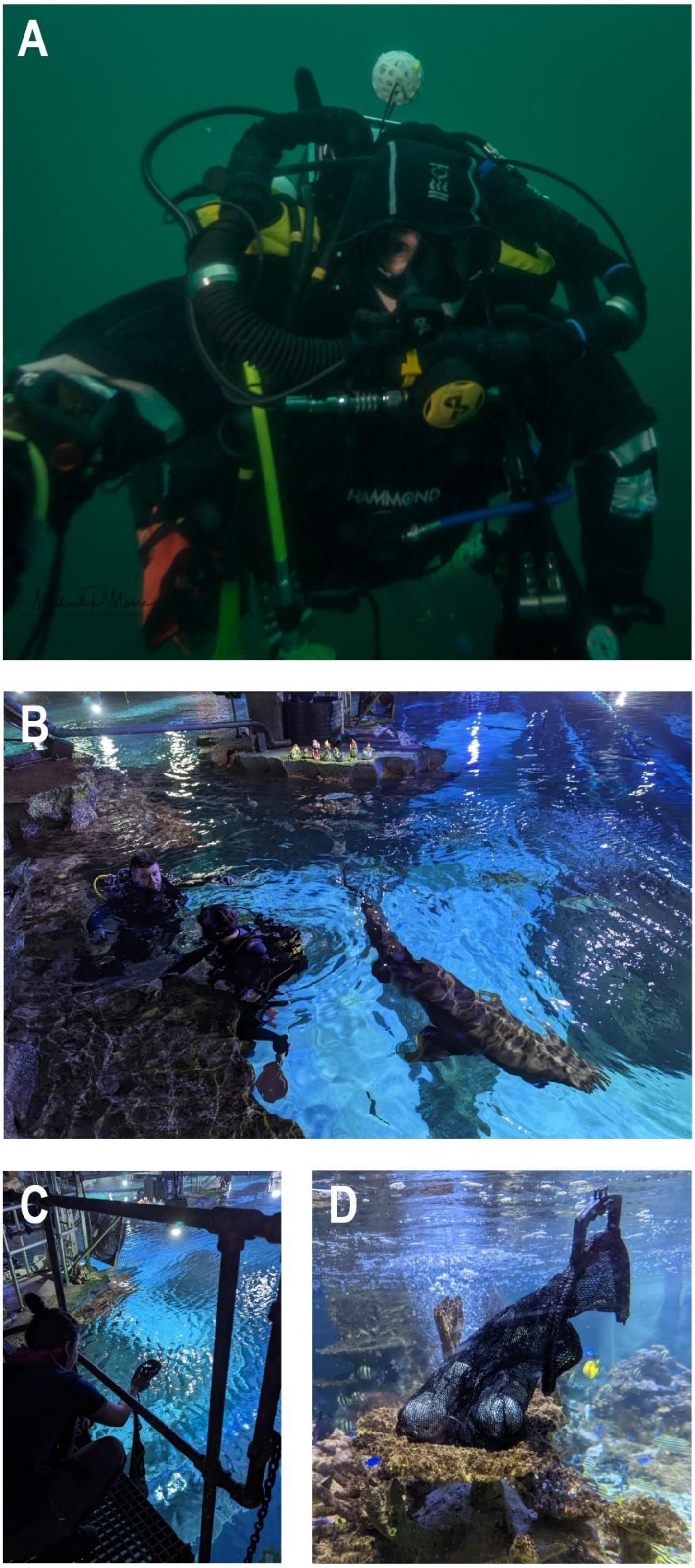

Figure 4.1 Volunteer SCUBA diver diving with a metaprobe in Scapa Flow, Orkney (A). Blue planet aquarium divers preparing for a dive in the tropical ocean exhibit (B). Securing metaprobes in a mesh dive bag to soak in the water column of the ocean exhibit (C). Metaprobes soaking in the coral cave exhibit perched on artificial coral (D). Photo A credit to Micheal Moore and photos B-D credit to Alice Cunnington.

4.3 Results

4.3.1 Sequencing Overview

A total of 190 samples were sequenced, including 16 syringe-filtered aqueous eDNA samples, 10 samples for the metaprobe timed soaking experiments, and 164 diver metabrobe samples (S4 Table 1). The combined sequencing libraries resulted in 6,172,921 reads after bioinformatic quality control, of which 90% (5,579,301 reads) were assigned taxonomy with an identity score ≥98% and retained for analysis. Taxonomic assignment was achieved by using global and local reference databases and searching for consensus between assignment methods (S4 Tables 2-6). Most of the samples were collected in the North Atlantic from coastal dive sites in England, Scotland, and Norway (**Table 4-1**). In total, 39 metaprobes were collected by divers in natural settings (i.e., not the aquarium) and many were sub-sampled for sequencing after undergoing different methodological treatments or for DNA extraction replication. Even with a relatively small number of metaprobes (39) and dives (17), when combining the diver's samples from all countries, 275 unique taxa were identified to either genus or species-level.

Table 4-1 Dive sites sampled.

Organisation	Dive Sites			Sample		Collection Date		Coordinates		Data
	Country	Location	Site	Bottle	MP	Month	Year	Latitude	Longitude	
Blue Planet Aquarium	England, UK	Blue Planet Aquarium	Ocean Exhibit	y	y	Mar	2023	-	-	This Study
			Coral Cave	n	y	Mar	2023	-	-	This Study
Halton Charters Ltd.	Norway	Bergen	Dogfish Walk	n	y	Jul	2022	60 31'54.0"N	5 06'27.8"E	This Study
		Florø	Welheim	n	y	Jul	2022	61 17'47.1"N	4 34'58.7"E	This Study
		Måløy	-	n	y	Jun	2022	61 56'20.0"N	5 07'60.0"E	This Study
		Ørsta	Radbod	n	y	Jun	2022	62 12'01.0"N	6 01'58.0"E	This Study
	Scotland, UK	Scapa Flow, Orkney	SMS Bayern	y	y	Nov	2022	58 53'55.2"N	3 10'36.9"W	NECR506
			SMS Brummer	y	y	Nov	2022	58 53'48.4"N	3 09'12.6"W	This Study
individual volunteers	California, USA	San Carlos Beach	Rock Wall	n	y	Aug	2022	36 36'35.2"N	121 53'41.8"W	This Study
	Jordan	Gulf of Aqaba	الشاطء الجنوبي	n	y	Dec	2022	29 26'59.9"N	34 58'11.9"E	This Study
	Cabo Verde	Boa Vista	Bodega de Choco	n	y	Dec	2022	15 56'11.3"N	22 48'19.8"W	This Study
Natural England	England, UK	Studland Bay	South Beach 1	n	y	Jul	2022	50 38'32.2"N	1 56'27.7"W	NECR506
			South Beach 2	n	y	Jul	2022	50 38'32.8"N	1 56'28.9"W	NECR506
Newcastle University SAC	Scotland, UK	St. Abbs	Wuddy Rocks	n	y	May	2022	55 54'23.9"N	2 07'42.6"W	This Study
			White Heugh	n	y	May	2022	55 54'16.8"N	2 07'53.8"W	This Study
Seasearch	England, UK	Liverpool Docks	Dukes dock	y	y	Dec	2022	53 23'56.0"N	2 59'27.3"W	This Study
		Plymouth Sound	Mount Batten	n	y	Jun	2022	50 21'28.8"N	4 07'53.9"W	NECR506
Sharklife Conservation Group	South Africa	Sodwana Bay	7 mile reef	n	y	Jul	2022	27 26'31.2"S	32 43'01.2"E	This Study
			9 mile reef	n	y	Jul	2022	27 25'01.2"S	32 43'30.0"E	This Study

4.3.2 eDNA capture in an aquarium

Aquarium staff divers wore metaprobes during routine dives to benchmark this eDNA capture technique against the standard method of manual filtration (i.e., pushing water with a syringe through a filter). Additionally, and independent of the divers, metaprobes were soaked to isolate and understand the relationship between time exposed to water and eDNA capture (**Figure 4.1** C,D). In a 3.8 million L tropical ocean display exhibit, a SCUBA diving pair undertook two dives (i.e., Dive 1: Fish feeding show; Dive 2: Routine maintenance), each diver wearing a metaprobe (**Figure 4.1** B). Simultaneously, four 1.5 L bottles of water were collected for filtration and metaprobes were soaked for varying lengths of time up to a maximum of 240 minutes (**Figure 4.2**; S4 Figure 1).

A primer designed for Elasmobranch detection was used to analyse the samples from the ocean display, where 12 Elasmobranch species are housed. Only seven of the 12 elasmobranch taxa could be identified to species-level, due to a lack of 12S reference sequences for four of the species and an inability to confidently (≥98% identity score) assign *Heterodontus zebra* (**Table 4-2**). Despite lacking references sequences for some species, sequences of other species belonging to the same genera were used to assign genus-level taxonomy which resulted in 10 elasmobranch taxa detected overall (i.e., 7 species-level, 3 genus-level) (**Figure 4.2**).

Of the 10 elasmobranch taxa detected overall, nine were detected using active filtration in three of the eDNA bottles (i.e., *Orectolobus* sp. missing) but all 10 were detected in Bottle D (**Figure 4.2** A). All 10 elasmobranch taxa were detected in Dive 1 from both samples, but in Dive 2 *Orectolobus* sp. was not detected at all and *Chilosyllium* sp. was not detected in replicate A (**Figure 4.2**). From the soaking experiment, five elasmobranch species were detected in 10 minutes but by 60 minutes all of the ten identifiable elasmobranchs were detected (i.e., when combining all six samples from 10, 30, and 60 minutes). Without combining time points, the samples that soaked for 240 minutes were the only ones that individually contained all 10 elasmobranch taxa (**Figure 4.2** B).

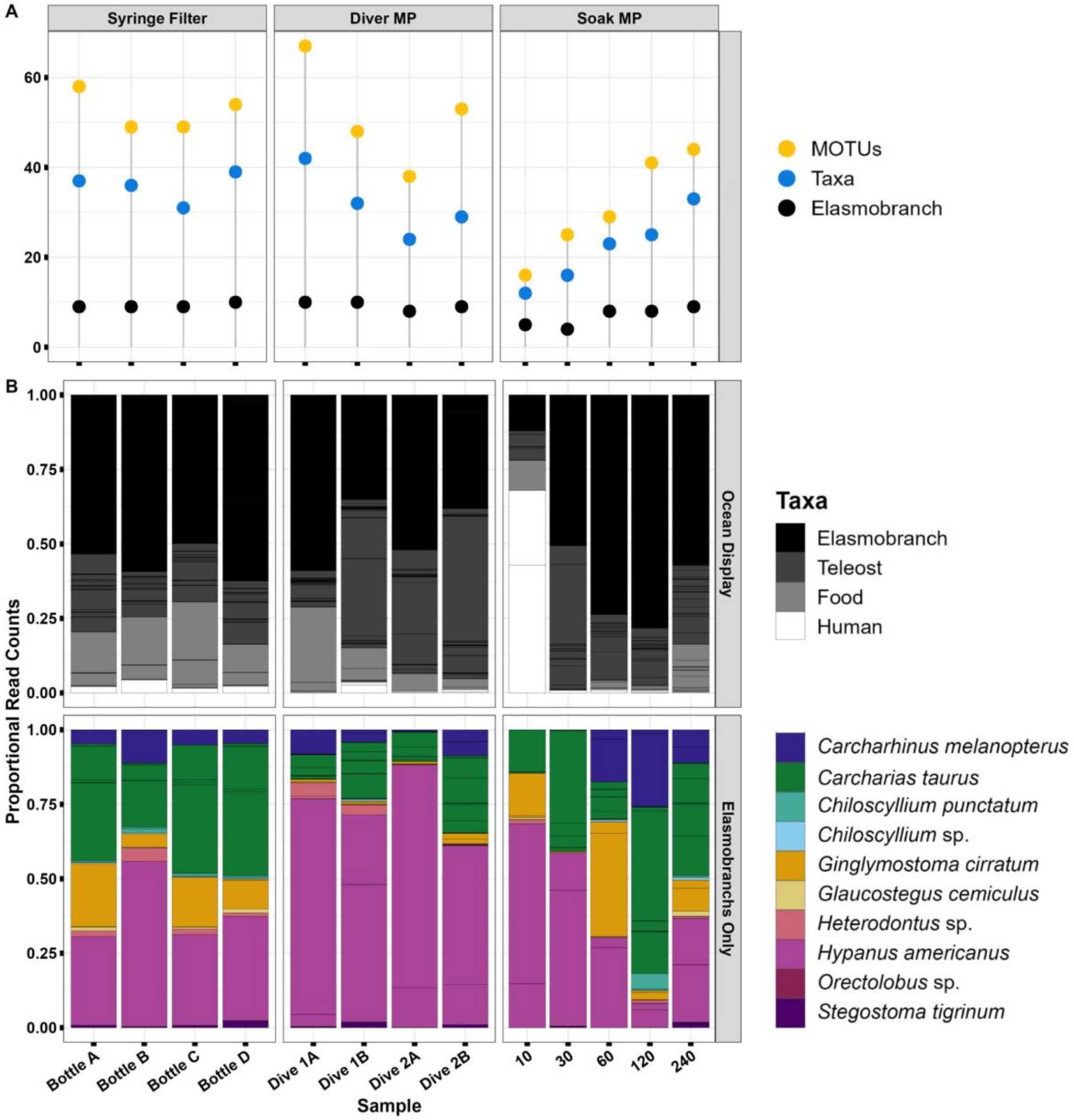

Figure 4.2 Lollipop plot showing the number of unique MOTUs, taxa, and Elasmobrachs from each sample (A). Stacked bar charts (top) showing the proportion of MOTUs assigned to Elasmobranchs or Teleosts listed in the Ocean Display inventory. Stacked bar charts (bottom) showing the proportion of MOTUs assigned to Elasmobranch taxa (B).

The soaking experiment was replicated in a smaller exhibit, referred to hereafter as the 'coral cave', which contained tropical fish species. The teleosts, which were the target taxa of the primer used for metabarcoding of the coral cave samples, also increased in number of detections over time, with the top ten most read abundant taxa detected by 60 minutes soaking time (S4 Figure 2). Combining data from the coral cave and the ocean display, soaking time was found to be a statistically significant factor determining the number of MOTUs ($R^2 = 0.34$, $p = 0.004$) and unique taxa detected per sample ($R^2 = 0.60$, $p < 0.001$) (Figure 3, Supporting Table 9). When adding the samples collected by divers, which were collected from 50-minute and 65-minute dives, the relationship between time and detections weakened, but was still significant (MOTUs: $R^2 = 0.15$, $p = 0.02$ and Taxa: $R^2 = 0.26$, $p = 0.003$) (S4 Figure 3, S4 Table 9).

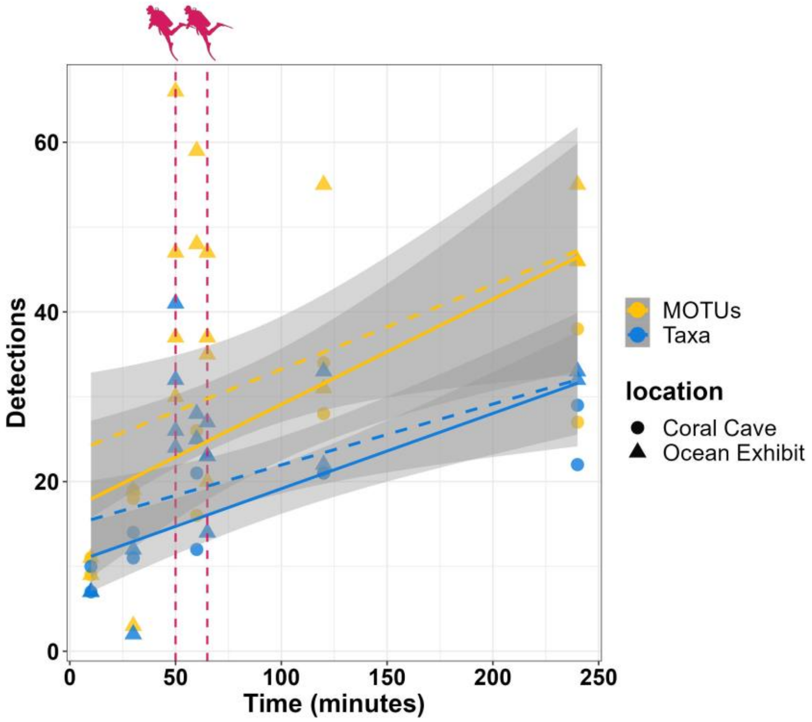

Figure 4.3 Number of MOTUs and number of taxa detected from metaprobes over the course of the soaking experiments (from the Ocean Exhibit and Coral Cave) at the following time intervals: 10 min, 30 min, 60 min, 120 min, and 240 min. Linear regression lines overlayed

(solid line). Diver metaprobes from the Ocean Exhibit added in at 50 min and 65 min (vertical dashed lines). Liner regression models recalculated to include dives overlayed (dashed lines).

Table 4-2 Inventory of elasmobranchs from the Ocean Display (Blue Planet Aquarium), reference sequence availability and detections of the elasmobranchs from eDNA metabarcoding analysis.

Inventory		Reference Sequence Available		Detection*
Scientific name	Common name	species-level	genus-level	
Carcharhinus melanopterus	Blacktip Reef Shark	Yes	Yes	Yes
Carcharias taurus	Sand Tiger Shark	Yes	Yes	Yes
Chiloscyllium arabicum	Arabian Carpetshark	**No**	Yes	genus-level
Chiloscyllium punctatum	Brownbanded Bamboo Shark	Yes	Yes	Yes
Ginglymostoma cirratum	Nurse Shark	Yes	Yes	Yes
Glaucostegus cemiculus	Blackchin Guitarshark	Yes	Yes	Yes
Heterodontus portusjacksoni	Port Jackson Shark	**No**	Yes	genus-level
Heterodontus zebra	Zebra Bullhead Shark	Yes	Yes	genus-level
Hypanus americanus	Southern Stingray	Yes	Yes	Yes
Orectolobus hutchinsi	Western Wobbegong	**No**	Yes	genus-level
Orectolobus maculatus	Spotted Wobbegong	**No**	Yes	genus-level
Stegostoma tigrinum	Zebra Shark	Yes	Yes	Yes

*Detection status was the same for syringe filtered samples and metaprobes worn by SCUBA divers.

4.3.3 Comparing eDNA collection in nature

eDNA capture by filtering 1.5 L of water was compared to eDNA collected on metaprobes worn by SCUBA divers and snorkellers at four locations: the Ocean exhibit at the Blue Planet Aquarium, Dukes Dock in Liverpool, and the SMS Bayern and SMS Brummer dive sites in Orkney (**Figure 4.4**). The syringe filtered eDNA from samples collected in nature resulted in a higher amount of unique taxa detections (i.e., between 65-73%) compared to unique taxa detected in metaprobe eDNA (i.e., 4-10%) (**Figure 4.4** B-D). This differed from the samples collected at Blue Planet Aquarium which had a large proportion of taxa (~65%) detected by both methods, rather than unique taxa from a single method representing a majority (**Figure 4.4** A-D). Regardless of sample type, beta-diversity among dive site locations significantly differed ($R^2 = 0.304$, p = 0.001, **Figure 4.4** E, S4 Table 10), while beta-diversity did not statistically differ between sample types, when controlling for dive site location ($R^2 = 0.045$, p = 0.057, **Figure 4.4** E, S4 Table 10).

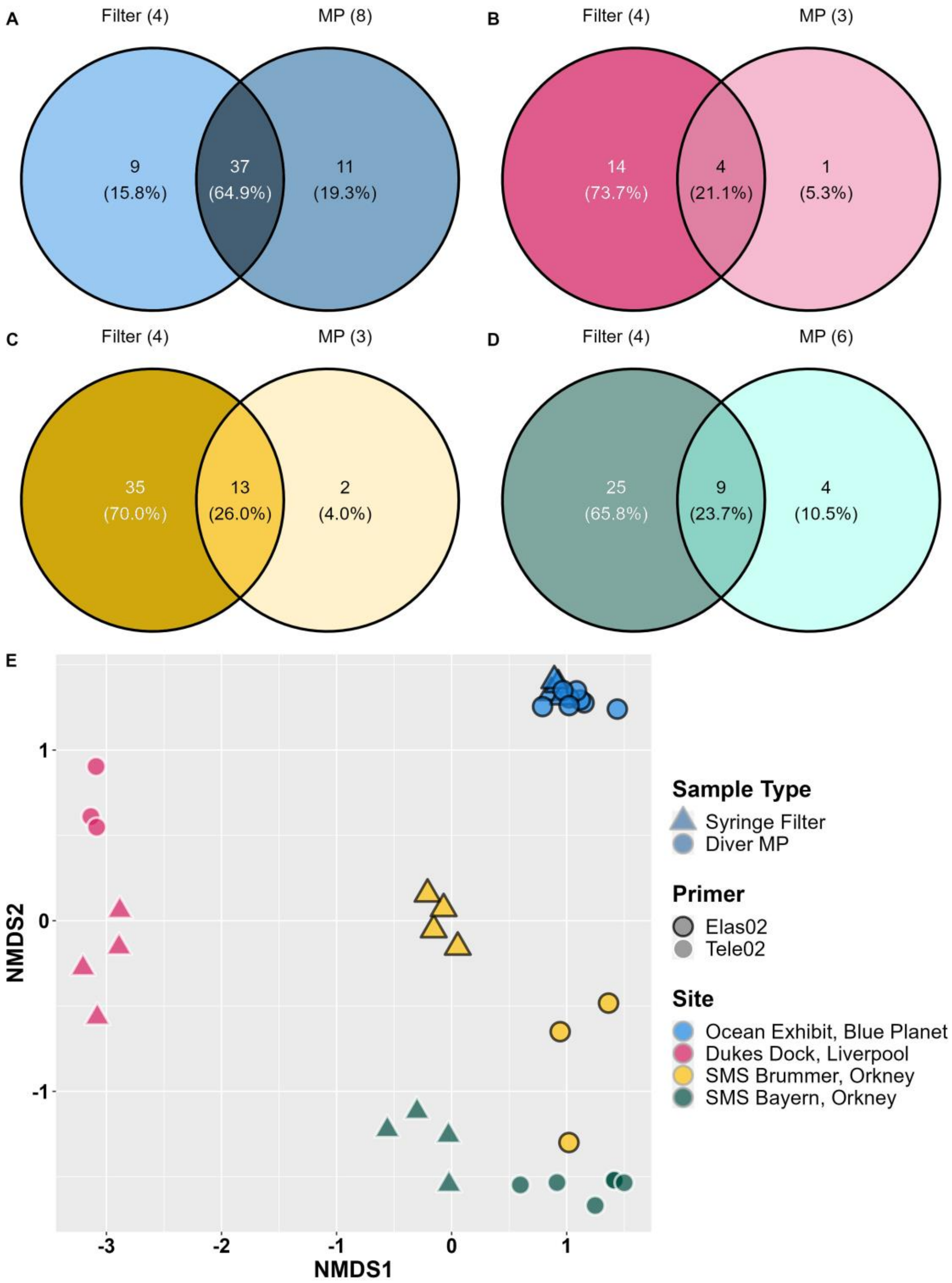

Figure 4.4 Venn diagrams showing number of genus and species-level taxa detected using syringe filtered water verses divers with metaprobes at the Blue Planet Aquarium (A), Dukes Dock in Liverpool (B), SMS Brummer in Orkney (C) and SMS Bayern in Orkney (D). Shading of the sets and unions darken with increasing number of taxa. Sets or unions with the highest number of taxa in each Venn diagram have white text. Non-metric dimensional scaling plot of Jaccard distances for genus and species-level taxa (E).

4.3.4 Optimizing field and laboratory methods

To evaluate which field and laboratory techniques work best, we first tested whether the amount of input gauze material and lysis buffer affected the outcome of the DNA extractions in terms of species richness, given the uncertainty around the degree of randomness by which DNA adheres to the cotton-gauze. We found no significant difference between input amounts of cotton or lysis buffer (p = 0.704, p = 302, respectively; S4 Table 11; S4 **Figure 4.4**). When comparing different DNA extraction methods, we found no significant difference between techniques (i.e., Mu-DNA or Qiagen DNeasy Blood and Tissue kit) (p = 0.671, S4 Table 12). Finally, we tested whether the different preservation techniques influenced species richness and found that there was a strong effect of preservation, with ethanol preserved sub-samples being much more likely to have higher species richness than the corresponding silica gel preserved cotton (p = 0.006, S4 Table 13).

4.3.5 Scaling up

In the UK, strategic partnerships were made with organisations to help recruit volunteers. Specifically, coordinators from an underwater visual survey citizen science program (i.e., Seasearch; https://www.seasearch.org.uk/), were contacted and participants planning visual census dives also wore metaprobes. Natural England, a statutory environmental conservation body, had their dive team wear metaprobes whilst surveying seagrass bed cover. Halton Charters Ltd., a live-aboard boat and dive charter company, had recreational divers wear metaprobes on wreck dives in Scapa Flow, Orkney Islands and Norwegian fjords. University students also took metaprobes when diving in St. Abbs marine protected area, Eastern Scotland. These efforts provided a snapshot of North Atlantic inshore fish biodiversity, resulting in 84 unique genus and species-level taxa detected. The vertebrate communities detected at dive locations were significantly separated according to ICES advisory areas, which are established in part by biogeography but also for fisheries management purposes (p =

0.001, **Figure 4.5**, S4 Table 14). Species that are common and widely distributed in the North Atlantic, such as Atlantic cod (*Gadus morhua*), silvery pout (*Gadiculus argenteus*), butterfish (*Pholis gunnellus*), plaice (*Pleuronectes platessa*), did not cluster near a single set of samples but instead occupied neutral space, indicating their importance across multiple locations.

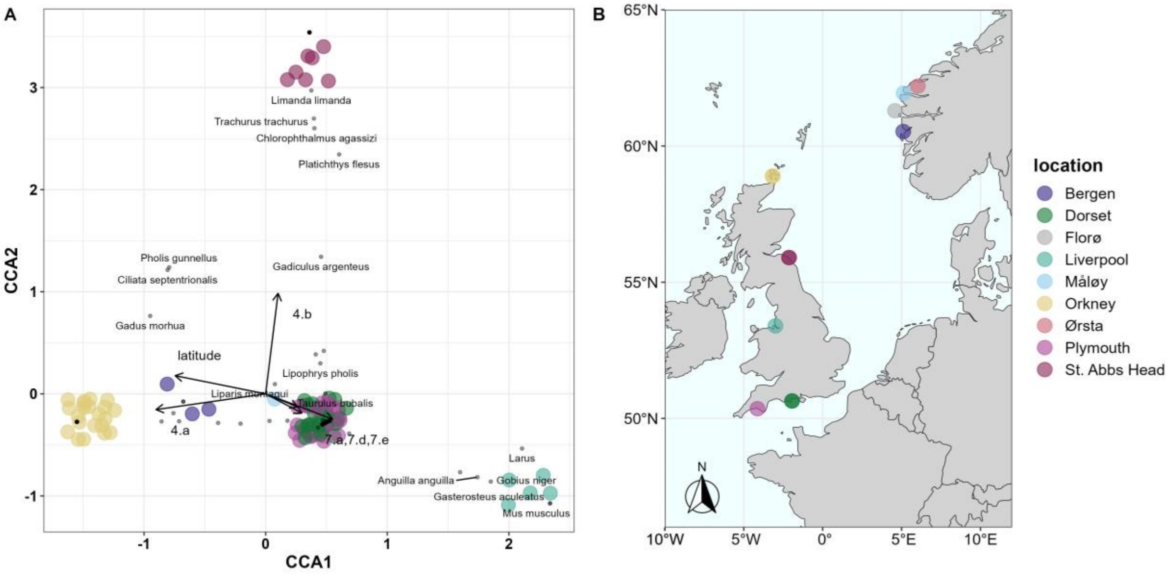

Figure 4.5 CCA plot showing how communities detected North Atlantic dive sites separate by ICES advisory areas and latitude. Location points are randomly jittered by 0.2 for visibility (A). North Atlantic dive site locations. Florø is not included in the CCA plot since samples were preserved in silica beads and failed (B).

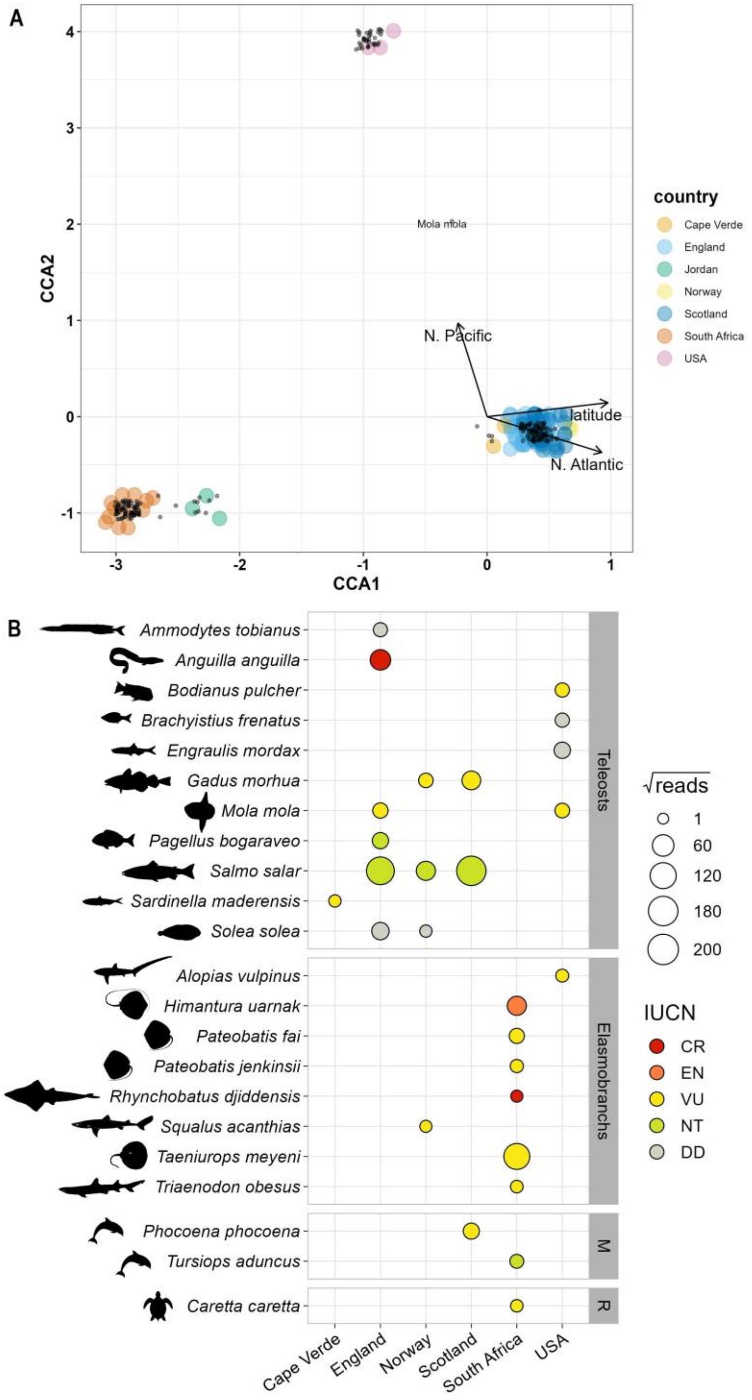

Figure 4.6 CCA plot showing how communities detected at global dive locations separate by ocean basin and latitude. Location points are randomly jittered by 0.2 for visibility (A). Bubble plot showing square-root transformed read counts of detections within the following IUCN red list categories: 'critically endangered' (CR), 'endangered' (EN), 'vulnerable' (VU), 'near-threatened' (NT) and 'data deficient' (DD). Taxa within the 'least concern' and 'not evaluated'

categories are not shown. 'M' and 'R' are abbreviations for Mammals and Reptiles, respectively (B).

Working with individuals traveling outside of the UK and with the conservation group, Sharklife, in South Africa, we were able to collect samples from six countries in total: Cabo Verde, England and Scotland (United Kingdom), Jordan, Norway, South Africa, and United States of America (California). When plotting the samples and species on a CCA ordination, the countries significantly differed along the latitudinal gradient (p = 0.001) and between ocean basins (p = 0.001) (Figure 6A, Supplementary Table 14). Most species points clustered around corresponding samples from each country, with one clear exception: the cosmopolitan pelagic ocean sunfish (*Mola mola*), which was detected in both the North Atlantic and North Pacific basins (**Figure 4.6** A). Out of 180 species-level detections, 18 (10%) were classified as 'near threatened' or more severe, according to the IUCN Red List criteria (Supporting table 15). Two critically endangered fish, the European eel (*Anguilla anguilla*) and the giant guitarfish (*Rhynchobatus djiddensis*) were detected in England and South Africa, respectively (**Figure 4.6** B). An endangered ray, the reticulate whipray (*Himantura uarnak*), was detected in South Africa. In total, there were six Elasmobranchs classed as vulnerable which included for example, the thresher shark (*Alopias vulpinus*) and spurdog or spiny dogfish (*Squalas acanthias*), detected in the USA and Norway, respectively. Of all the countries, samples from South Africa contained the most threatened species detections even though only two dives were performed in the country with only two metaprobes deployed for each, and all of which were preserved in silica beads for ease of transport (S4 table 15).

4.4 Discussion

Monitoring biodiversity is essential for provisioning the necessary stewardship and policies needed to protect the integrity of marine ecosystems and the services they provide. Routine monitoring of coastal habitats, despite proximity to human populations, is not viable in many places due to a lack of resources as well as the complexity of the marine environment and biotas, which necessitate different and costly approaches (Livore et al., 2021). Environmental DNA (eDNA) analysis is one approach that is highly sensitive, non-invasive, and can be universally applied to different marine taxa and habitat types, providing extensive species distribution information (Deiner et al., 2017b; Sigsgaard et al., 2020). Moreover, there are ongoing efforts to implement eDNA collection as a key monitoring strategy internationally. Concentrating eDNA typically involves water filtration, but more recently passive techniques whereby DNA adheres to a surface over time, have been explored. Here we used a quasi-passive eDNA capture technique and a network of snorkelling and SCUBA diving volunteers to collect eDNA from marine environments. The volunteers mainly went diving in the UK, where the project started, but the network of engaged organizations and people rapidly expanded, resulting in samples spanning six countries, and various coastal habitat types.

4.4.1 Protocol Refinement

As samples were collected, methods were tested iteratively, and a sampling protocol was reached based on differences in sequencing results and on what was most convenient for our sampling purposes. The preservation method (i.e., silica gel beads or 100% ethanol) affected species richness detected such that replicates of samples preserved in ethanol performed significantly better. These metaprobe samples were exposed to the same field and laboratory conditions, but with varying preservation techniques, such that we divided the cotton gauze and preserved the sub-samples differently to compare the outcome. We did this 18 times, and nine (50%) sub-samples that had been preserved in silica beads resulted in no detections of target taxa (i.e., non-human vertebrates), whereas the corresponding ethanol preserved sub-samples always had detections. Despite preservation by silica gel dehydration yielding disappointing results compared to ethanol, all of the samples collected from Sodwana Bay, South Africa were preserved in silica beads to ease shipping logistics, and 11 out of those 12 samples contained target taxa. It seems that silica beads can work if the cotton gauze is completely dehydrated, but to practically achieve dry conditions using the beads was difficult. This differed from previous research that placed metaprobes in bottom-trawl nets, such that the efficacy of ethanol and silica gel beads for preservation was not found to be statistically different (Maiello et al., 2024). The commercial extraction protocol (Qiagen Blood and Tissue kit) did not significantly differ from the non-commercial method (Mu-DNA Tissue protocol) (Sellers et al., 2018) and the same was true for different amounts of input cotton gauze and lysis buffer. With increased replication these results could change, but where possible we found that preservation in ethanol, and using the Qiagen Blood and Tissue DNA extraction kit (i.e., with 1000 µl of the provided lysis buffer, Buffer ATL, per DNA extraction) with between 0.2-0.4 g of cotton-gauze, produced the most consistent and reliable results.

4.4.2 Benchmarking against conventional eDNA capture

Diver metaprobes differentiated vertebrate communities from various sampling locations and detected similar communities to corresponding aqueous eDNA samples. In an aquarium setting, diver metaprobes compared to typical filtered aqueous eDNA samples resulted in statistically similar community compositions, where ~65% of the fish detected were the same. When comparing detections of elasmobranchs, at least one sample from each method contained all 10 of the total unique elasmobranch detections (**Figure 4.2**). The diver metaprobes however, contained more unique detections than did the filtered eDNA (i.e., ~20% versus ~15%) (**Figure 4.4**). These observations differed markedly to diver and filtered samples collected in nature, where the overlap in detections by each method was between 20-30%, with filtered eDNA samples containing between 65-75% of unique taxa (**Figure 4.4** A-D). This percentage of overlap was lower compared to a previous study using active filtration and passive eDNA capture on different types of suspended filters where active and passive methods shared between 43%-75% of species detections; however, this was with ~70 passive capture replicates deployed at each site with soaking times between

4-24 hours (Bessey et al., 2021), which is not a feasible scenario with SCUBA diving. The volume of filtered water needed to capture the eDNA necessary for a representative sample of biodiversity can differ depending on the target taxonomic group (Jeunen et al., 2019b) and the habitat being sampled (Bessey et al., 2020; Kumar et al., 2022), and similarly we would anticipate that factors influencing exposure to the environment: such as time in the water, distance covered by a diver and site hydrodynamics as well as habitat type, all could profoundly change the optimal sample size. Clearly, our experiments in an aquarium show that detections increased statistically with time, but that this relationship weakened in strength when adding in the samples collected by divers (**Figure 4.3**), which suggests that the swimming motion of a diver can increase the eDNA accumulation rate in the gauze, compared to a stationarily soaking metaprobe. Previous passive eDNA capture field experiments show that species richness did not increase with time (Bessey et al., 2021) and that the relationship between submersion time remains unclear (Zhang et al., 2024) but it is possible that over longer sampling periods the eDNA has time to desorb from the capture substrate (Kirtane et al., 2020). Establishing these thresholds for diver metaprobes in the future in targeted habitat types could enable further understanding of how to implement this monitoring strategy. At the four locations where we collected both filtered eDNA samples and diver metaprobes, community structure differed across locations, but did not significantly differ between sampling strategies (**Figure 4.4** E). Moreover, when comparing the vertebrate communities detected in temperate North Atlantic habitats (i.e., coastal UK and Norway), the communities at sampling locations could be segregated according to their corresponding ICES advisory area (**Figure 4.5**), demonstrating the capability for this approach to be employed for large-scale monitoring.

4.4.3 Towards a global effort

One-hundred-forty-six samples were collected in six countries by volunteers, resulting in 275 vertebrate taxa assigned to either a genus or species, 180 of which were identified to the species-level. In some cases, such biodiversity records were obtained by a modest number of metaprobes: samples collected in South Africa came from two dives (i.e., by one dive buddy pair), resulting in cotton material from just four individual metaprobes, while the Cape Verde, California, and Jordan sampling events consisted of only one dive at each, with just one diver of a buddy pair wearing a metaprobe. The cotton from these metaprobes was sub-sampled in triplicate for sequencing, representing just under 15% of the sequenced samples (n = 21), but recovering 132 marine taxa (14 genera, 118 species). Therefore, despite samples in the North Atlantic region (i.e., Norway, UK) accounting for the remaining 85% of sequenced samples, they detected 77 species, constituting 42% of all the species detected from nature. This demonstrates that had there been more biological replication (i.e., more dives and more metaprobes deployed) or more technical replication (i.e., more extraction and/or PCR replicates taken from cotton within individual metaprobes) at dive locations outside of the North Atlantic, the species richness detected could have been immensely surpassed. Despite this, the vertebrate communities detected at the dive sites were significantly different between countries and grouped by ocean basins and across latitude (**Figure 4.6** A). There were certain vertebrates that were unique to dive sites and made up a large portion of read counts, for example: 17,933 reads of California sea lion (*Zalophus californianus*) in California, 14,158 reads of Round ribbontail ray (*Taeniurops meyeni*) from 9-mile reef in South Africa, and 47,524 reads of three-spined stickleback (*Gasterosteus aculeatus*) from Dukes dock in Liverpool, UK. Round ribbontail rays are one example of the 12 species we detected which are classed as vulnerable by the IUCN (**Figure 4.6** B). With relatively low effort sampling, we detected important fisheries species that are classed as data deficient by the IUCN including lesser sand eels (*Ammodytes tobianus*) and common sole (*Solea solea*). Some detections were of rare elasmobranchs such as the thresher shark (*Alopias vulpinus*) in California and the giant guitarfish (*Rhynchobatus djiddensis*) in South Africa, highlighting how these data collected by volunteers could meaningfully contribute to global biodiversity data repositories.

This project, conveyed as ‘DNA divers’ to the general public, presents an example of how marine recreationists can be engaged with eDNA studies and in doing so learn about such emerging technologies and about the importance of monitoring biodiversity more broadly. There have been successful citizen science programs working with volunteers either to collect eDNA or to go SCUBA diving for visual surveys. Metaprobes can be 3D printed, cotton medical gauze is widely available, and these materials can be easily assembled and attached to a diver’s BCD (buoyancy control device) or weight belt with cable ties. This has the advantage of there being little to no training required for SCUBA divers or snorkelers to participate in collecting marine eDNA, relative to visual census, which requires taxonomic training. Moreover, in the UK, visual census undertaken by volunteers cannot be executed with industry standard survey methods, such as transects, since this violates The Diving for Work Regulations 1997. This means that any eDNA metabarcoding data collected by volunteers is concomitant with visual census information and could provide two-layers of species presence data associated with the dive site more generally and without violating diving for work laws. At Dukes Dock in Liverpool, volunteer Seasearch divers completed a visual census survey (S4 Figure 7) while collecting eDNA that was ultimately screened for teleost species; both methods contained observations of three-spined stickleback (*Gasterosteus aculeatus*) and black goby (*Gobius niger*). The divers recorded all types of taxa they could see, including invertebrates like mussels (*Mytilus edulis*), European green crab (*Carcinus maenus*) and yellow sun sponge (*Halichondria bowerbanki*), while the metaprobe eDNA was analysed targeting a vertebrate gene therefore capturing urban landscape-level species too, like gulls (*Larus* sp.) and house mice (*Mus musculus*). The second most sequence read abundant fish was the critically endangered European eel (*Anguilla anguilla*), unseen by the divers, but divers saw two-spotted goby (*Gobiusculus flavescens*), undetected in the eDNA. Visual observations will always remain a valuable source of marine biodiversity data, but passive eDNA collection could be a way to crowdsource molecular samples, supplement monitoring efforts, and include a wider demographic of SCUBA divers and marine leisure users who either do not have the time, interest, or resources to engage in taxonomic training.

There is broad interest in passive methods for non-invasive biodiversity surveillance using eDNA metabarcoding (Bessey et al., 2023; Chen et al., 2022; Kirtane et al., 2020; Maiello et al., 2022; Verdier et al., 2022; Zhang et al., 2024). The semantics of what constitutes 'passive surveillance' can differ. Some methods have been labelled as passive due to the automated process of water filtration using marine robotics (Hendricks et al., 2023; Truelove et al., 2022), while other passive techniques have explored DNA binding to submerged materials (Kirtane et al., 2020; Maiello et al., 2022; Verdier et al., 2022). In our case, we rely on the latter as well as the exertion and exploration activities of volunteer SCUBA divers and snorkelers. This study benefitted from the involvement of ~30 divers – some of whom are co-authors of this study – who went on dives in four continents across a range of habitats (i.e., seagrass beds, kelp forests, shipwrecks, fjords, coral reefs), resulting in the detection of 275 different taxa overall. Although the diver metaprobes likely capture fewer species than what it is usually possible using traditional water filtering from a given area, they still proved effective at significantly distinguishing assemblages at regional and global scales and detecting species of conservation importance with relatively low effort. While we are not suggesting that our method excludes the need for engineering autonomous eDNA sampling instruments, the vast potential of the 'DNA divers' approach is undeniable: many coastal marine areas remain poorly monitored, while volunteers seem to be very keen to embrace the method. Through such a cost-effective approach, it is reasonable to expect that eDNA samples from virtually every region of the world can be rapidly amassed, thereby upscaling and boosting marine monitoring, and at the same time raising public awareness and improving science literacy.

4.5 Methods

4.5.1 Sample Collection

Sample collection events took place at various dive sites in the UK, Cape Verde, Jordan, Norway, South Africa, and the USA as well as the Blue Planet Aquarium (**Table 4-1**). In some locations eDNA sampling by water filtration was performed to compare to metaprobes which had been worn by snorkellers or SCUBA divers. Specific details on metaprobe preparation found in the supplemental material (see S4 Methods). When possible, metaprobes were prepared in the laboratory and distributed to snorkellers and SCUBA divers, all of whom were volunteers, but some of which were professional SCUBA divers (e.g., Divers from Blue Planet Aquarium are dive professionals who volunteered to wear the metaprobes, while samples collected by Natural England were done so using their professional dive unit). Metaprobes were filled with various amounts of sterile cotton medical gauze, and through practice we established that up to two whole rolls of medium-sized cotton gauze rolls (10 cm x 3.7 m) could be easily removed from plastic packaging and placed directly in the metaprobe. Water samples were collected in bleach-cleaned 1.5 L bottles and were filtered on site through a 0.45 µm Sterivex filter (PES membrane, Merck Millipore). In the Blue Planet Aquarium, both sample types were collected (**Table 4-1**) alongside a controlled timed experiment, where metaprobes were soaked in display tanks (**Figure 4.1** C, D). The timed experiment was repeated in two independent tanks (the Ocean exhibit and the Coral Cave). In each tank, five metaprobes were placed in a mesh dive bag and allowed to soak at the surface of the tanks; one was removed and preserved at each time point: 10, 30, 60, 120, and 240 minutes. Samples were preserved in different ways depending on sample type and treatment. For instance, sterivex filters were dried by pushing air through the cartridge. Metaprobe gauze was either dried using silica beads or stored in 100% molecular grade ethanol. All samples were transported in an insulated box with ice packs until they could be stored at -20°C.

4.5.2 DNA extraction and sequencing

4.5.2.a DNA extraction

DNA was extracted from the metaprobe gauze and sterivex filters using either the Qiagen DNeasy Blood and Tissue kit following the manufacturer's protocol, but with slight modifications (see S4 Methods), or 'Mu-DNA', a non-commercial published protocol. The reagents for the Mu-DNA method were prepared fresh for these extractions following the published protocol which is kept updated by the authors on protocols.io and we indicate the specific steps we followed from the 'water' and 'tissue' methods in the supplement (Sellers et al., 2018) (see S4 Methods). To remove the sterivex filters from the capsules the plastic was broken open using pliers and the center column holding the filter was emptied onto a petri dish. The filter was then removed from the centre column of the capsule using tweezers. Half of the filter was saved as archival material and the other half was cut into small pieces using dissecting scissors and placed into a 1.5 ml Eppendorf tube. The metaprobe cotton gauze was prepared by cutting off small pieces from various parts of the larger roll. The small pieces were placed onto blotting paper to remove ethanol and were weighed in weighing boats. The various weight ranges were established based on the amount of cotton that could reasonably fit in Eppendorf tubes of various sizes: heavy (0.9 - 1.1 g), medium (0.6 – 0.8 g), and standard (0.2 – 0.45 g). Each weight range was placed into Eppendorf tubes of different sizes for extraction with a proportional volume of lysis buffer (i.e., 5000 µl, 3000 µl, 1000 µl). All non-disposable equipment used for DNA extractions was soaked in a 10% bleach solution, followed by 5% Lipsol detergent then Milli-Q water, and UV treated for at least 30 minutes. Disposable equipment used for DNA extractions was sterile and exposed to ultra-violet light under a hood for 30 minutes before use.

4.5.2.b DNA amplification

Three replicates of each extract were amplified by polymerase chain reaction (PCR). The tele02 (specifically: forward sequence Tele02-F 5'-AAACTCGTGCCAGCCACC-3' and the reverse sequence Tele02-R 3'-GGGTATCTAATCCCAGTTTG-5') or elas02 (specifically: forward sequence Elas02-F 5'-GTTGGTHAATCTCGTGCCAGC-3' and the reverse sequence Tele02-R 3'-CATAGTAGGGTATCTAATCCTAGTTTG-5') primers were used to target 167 and 171 bp regions of the 12S gene, depending on the sample library (Taberlet et al., 2018) (S4 Table 1). The PCR cycles for tele02 were as follows: 95°C for 10 min, followed by 35 cycles of 95°C for 30 s, 60°C for 45 s, 72°C for 30 s, and finishing at 72°C for 5 min followed by a 4°C hold. The PCR cycles for elas02 were as follows: 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 54°C for 1 min, 72°C for 1 min, and finishing at 72°C for 5 min followed by a 4°C hold. Some samples were extraction replicates (i.e., the same DNA extraction), and there were negative controls such as field blanks, extraction blanks, PCR blanks as well as positive controls. Tables detailing samples for each sequencing run are reported in the supplement (S4 Table 2-6). All PCR replicates were visualized on a 2% agarose gel (150 ml 1X TBE buffer with 3 g agarose powder) stained with 1.5 µl SYBRsafe dye (InVitrogen). The sample bands and positive controls were assessed to determine the PCR success, and if unsuccessful the PCR was repeated. The PCR replicates were combined by sample and purified using 1:1 ratio of PCR product to Mag-Bind® Total Pure NGS magnetic beads (Omega Bio-Tek) following the manufacturer's protocol. Samples were visualised on an agarose gel again to assure purity (i.e., target length (~200 bp) bands on agarose gels were visible with minimal to no other bands present).

4.5.2.c Library preparation and sequencing

Purified PCR products were quantified using a Qubit dsDNA HS Assay kit (Invitrogen), and pooled at equimolar concentration. The pooled library was imaged on a Tape Station 4200 (Agilent) to check the estimated target band length and assess purity. Based on the Tape Station results, the library was cleaned a final time using magnetic beads in a 1:1 ratio with library volume. A unique adapter sequence was ligated to each library using the NEXTFLEX® Rapid DNA-Seq Kit for Illumina (PerkinElmer) following the manufacturer protocol. After adapter ligation, the libraries were again imaged on the Tape Station and purified with magnetic beads, this time with a 0.8:1 ratio of beads to sample, as per the NEXTFLEX® Rapid DNA-Seq Kit instructions. Each dual-indexed library and PhiX control was then quantified by qPCR using the NEBNext® Library Quant Kit for Illumina (New England Biolabs). Each library was loaded at a final molarity of 70 pM with a 10% PhiX spike-in. The libraries were sequenced at Liverpool John Moores University on an Illumina iSeq100 using iSeq i1 Reagent v2 (300 cycles).

4.5.3 Bioinformatics Pipeline

The sequence libraries resulting from sequencing runs were demultiplexed by specifying FASTQ generation on the sample run sheets in the Local Run Manager which resulted in forward and reverse FASTQ files for four libraries. All were processed following the same steps, with modifications made based on the primer used. Many functions from the Python (v 2.7.5) package, OBITools (v 1.2.11), were implemented in the following pipeline. First, FastQC (v 0.11.9) software was used to visually assess per base sequence quality. The forward and reverse sequences were trimmed using OBITools::obicut to lengths between 148 to 150 bp (depending on the fastqc results) such that the average quality score was >36 and that all reads had a maximum length of 150 bp. The trimmed reads were paired using OBITools::illuminapairedend and sorted based on quality score such that paired reads >Q30 were processed. The paired reads were demultiplexed using OBITools::ngsfilter and filtered by length (i.e. elas02 libraries 130 to 210 bp; tele02 libraries 130 to 190 bp). Sequences were dereplicated using OBITools::obiuniq. VSEARCH (v 2.15.2) software, specifically VSEARCH::uchime_denovo was used to remove chimeras. SWARM (v 3.0.0) was then used to cluster sequences with the fastidious option and d = 1. Taxonomic assignment was achieved by using a variety of tools and searching for consensus amongst them. First, global reference databases were generated by using OBITools::ecoPCR for both primer pairs (i.e. tele02 or elas02) against the EMBL nucleotide reference database (r143) specifying all vertebrate sequences (taxid=7742) but excluding human and mouse (taxid=9606). Then OBITools::ecotag was used to assign taxonomy referencing the corresponding global databases. Local databases were made using meta-fish-lib to generate custom reference databases (Collins et al., 2021). For the North Atlantic, the latest release of a UK reference database (v258) was downloaded from the github repository (Collins et al., 2021). For each location, a species list was made by supplying the ISO country code for each place: Cape Verde (132), Jordan (400), and South Africa (710) followed by executing the meta-fish-lib pipeline (Collins et al., 2021). For California, a regional 12S reference database called FishCARD was downloaded (Gold et al., 2021a). Ten human 12S sequences were added to each local database, anticipating human sequences that are known to be amplified by these primer sets. Taxonomy was then assigned using a few algorithms: 1) OBITools::ecotag with the corresponding global reference databases and using 2) VSEARCH::sintax with the corresponding local reference databases. The BLAST+ software was also used to perform a blastn with a word length = 7 against the local reference databases. Then final taxonomy was assigned as such: 1) Ecotag best identities < 70% were discarded; 2) MOTUs which had a three-way consensus (i.e. ecotag, sintax and blastn returned the same taxonomy) were assigned accordingly; 3) when three-way consensus was not possible, if MOTUs had a two-way consensus between sintax and the blastn results, and both of which were >95% identity then they were assigned accordingly; 4) Remaining MOTUs which did not have consensus between the methods were assigned with the ecotag given assignment since the global vertebrate database was better suited for these cases; 5) The final assignments were filtered by an identity of ≥98%. The final assignments were manually inspected, after the decontamination process explained under the statistical analysis section, to make sure taxonomic assignments made ecological sense. Some changes were made, for instance in the aquarium samples: an inventory list was used to aid in species assignment and some assignments were assumed to be part of the inventory if there were closely related fish that appeared visually similar (S4 Tables 7-8). For comprehensive details regarding the bioinformatics and statistical analysis, all code is available in a github repository: https://github.com/eneave/DNA-Divers.

4.5.4 Statistical Analysis

Further data processing and statistical analysis were done using R (v.4.1.3). The results of each sequencing library were further decontaminated using the R package decontam (v.1.14.0), specifically by screening the negative controls using the function decontam::isContaminant with the “prevalence” method set to a threshold of 0.5. To control for tag jumping, 0.001% of the total reads for each library was calculated, and any detection lower than 0.001% of the total library reads was removed (i.e. ranging from 2-6 reads). After these thresholds were employed, a small number of species needed to be removed from samples since they did not make ecological sense: *Galeus melastomus* detected in Dorset seagrass beds, *Chimaera monstrosa* a deep-sea species detected in multiple samples (*Galeus melastomus*, *Chimaera monstrosa* could have been laboratory contamination from another project processed at a similar time (Albonetti et al*.,* 2023)), *Salmo salar* was detected in California likely because it’s a commonly consumed species, *Carcharhinus melanopterus* was an aquarium species in an Orkney sample, as well as *Carcharias taurus* and *Gymnothorax kidako* were aquarium species that were both removed from St.Abbs samples. Due to the unnatural biomass in an aquarium, the aquarium samples had higher amounts of DNA which could have caused this contamination. Finally, from all samples, common domestic species: chicken (*Gallus gallus*), sheep (*Ovis aries*), cow (*Bos taurus*), pig (*Sus scrofa*), dog (*Canis lupus*) and cat (*Felis catus*) were removed.

4.5.4.a Aquarium samples

Using the inventories provided by the aquarium, taxa detections were classed as either i) inventory species, ii) species used as food, and iii) contamination (S4 Tables 7, 8). Most detections in the aquarium samples were accounted for. For instance, Dalmatian pelican (*Pelecanus crispus*) was removed from the data, but it was likely a true detection as *Pelecanus crispus* is on display in a different part of the aquarium. However, Atlantic salmon (*Salmo salar*) was detected but not listed by the aquarium as a food item nor was it present in an exhibit; therefore, it was classified as contamination and removed from aquarium samples. Using the R package stats (v.4.1.3), linear models (stats::lm) were calculated to assess the relationship between

MOTUs and detected taxa over time for both soaking experiments as well as the for the dives in the ocean exhibit display.

4.5.4.b Comparisons of Diver metaprobes to filtered eDNA samples

To compare the vertebrate communities detected in eDNA collected by filtration versus that of diver metaprobes, various functions from the R package vegan (v.2.6.4) were used. Multivariate homogeneity of group dispersions (vegan::betadisper) was tested for both dive site location and sample type followed by testing for significance (vegan::permutest). PERmutational Multivariate ANalysis Of VAriance (PERMANOVA) was performed using vegan::adonis2 with the stata parameter set to the dive site location, so that each location was independently considered when comparing the differences in sample type. A second PERMANOVA was carried out to also test for differences in communities by location, independently considering sample type by setting the strata parameter to type.

4.5.4.c Comparisons of preservation, processing and DNA extraction methods

We tested whether the input weight of cotton gauze effected the alpha-diversity by analysing the species richness and Shannon index in a selection of samples (n=18), each extraction belonging to a different weight category: high (0.9 – 1.1 g, requiring 5000 µl of lysis buffer and five centrifugations), medium (0.6 – 0.8 g, requiring 3000 µl of lysis buffer and four centrifugations), and standard (0.2 – 0.4 g, requiring 1000 µl of lysis buffer and three centrifugations). We used a Generalized Linear mixed model (GLMM) implemented with the R packages lme4 (v.1.1.32) and lmerTest (v.3.1.3), specifically the lme4::lemr function. GLMM was also used to test for the effect of different DNA extraction methods (i.e., Mu-DNA or Qiagen DNeasy Blood and Tissue kit) and to test for the effect of preservation strategy on species richness.

4.5.4.d Differentiating dive sites and locations using CCA model building

A model-building process was employed to build a minimum adequate model from metadata variables for a canonical correspondence analysis (CCA). The species matrix of square-root transformed read counts was used as a predictor of the CCA. First, a scoping model was made using all of the metadata variables, by setting the intercept parameter in vegan::cca to '.'. Then a blank model was made by setting the intercept parameter to ‘1’. The function stats::add1 was used to add metadata variables to the model in a stepwise manner, where the object of the function was the blank model, the scope was set to the scoping model, and the test was set to ‘permutation’. This stepwise process was repeated until no more variables were significant and an ANOVA (baseR::anova) test was run on the final CCA model to test the strength of the significance for the final model terms. This process was repeated for a subset of the data, specifically samples from the North Atlantic, for which more metadata variables were available.

Chapter 5

Return to the Edge of the World: SCUBA-assisted, eDNA analysis differentiates marine vertebrate assemblages of a remote archipelago

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Volunteer divers relaxing around the shot line during their safety stop.

Photographer: Micheal Moore

5.1 Abstract

Passive environmental DNA (eDNA) strategies are being explored as a means of increasing biological replication since the time constraints of actively filtering water to collect eDNA have to be factored into survey designs, limiting replication power. The time that filtering requires deters the uptake of eDNA surveys by marine managers, especially when trusted traditional survey techniques provide familiar results. Here we explore a quasi-passive eDNA capture technique assisted by the swimming motion of SCUBA divers to test whether the eDNA collected could produce biodiversity data on a fine scale. Specifically, six volunteer divers performed a dive on two rocky reefs less than 5 km apart in St. Kilda, Scotland, an UNESCO world heritage site. In just two dives over 60 fish, seabirds and mammals were detected comprising of vertebrate assemblages that were significantly different between the reefs and were evident regardless of the chosen genetic assay. This demonstrates that this technique could be practically employed by volunteers or by professional public agencies in tandem with their routine underwater work, as a means to significantly extend the reach of marine survey efforts.

5.2 Introduction

Environmental DNA (eDNA) metabarcoding is a powerful, universal tool for monitoring marine vertebrates (Andruszkiewicz et al., 2017; Eble et al., 2020). More traditional monitoring methods tend to be limited to known species, since *a priori* knowledge (e.g., distribution) is required to successfully design and implement morphological or visual-based surveys (Bohmann et al., 2014). Moreover, the survey techniques and expertise needed to monitor diverse marine vertebrate groups such as cryptobenthic fish, seabirds or marine mammals are varied and typically require the application of multiple survey techniques, such as anesthetized fish collection (Depczynski and Bellwood, 2004), colony counts (Walsh et al., 1995), or aerial surveys (Eberhardt et al., 1979), respectively; this would in turn call for an assembly of experts with completely different training. While traditional methods may still be required for a variety of reasons, like ground truthing or gathering life history data, eDNA analysis is overwhelmingly practical for gathering multi-species occurrence data, since the application of the technology is inclusive of all taxonomic groups (Stat et al., 2017b).

Despite the ability to target a wide range of taxa, eDNA collection for management purposes is only recently coming to the fore, with the primary bottleneck being the collection of quality environmental samples, which demands dedicated personnel to adhere to decontamination protocols necessary for the time-consuming water filtration process which concentrates eDNA. Depending on the volume of water collected and the amount of particulate matter, water filtration can take anywhere from minutes to hours extending far beyond a normal working day. The marine environment has highly dilute eDNA, so typically large volumes of water need to be collected and even with the assistance of automated pumping mechanisms, this process is limited in speed by the fine filter pore-sizes (≤ 0.45 µm) necessary to capture eDNA (Kumar et al., 2022). Furthermore, automated water pumping mechanisms are still under development, many of which are being tested in freshwater rather than more corrosive seawater environments, and can be a costly investment (Hendricks et al., 2023; Sepulveda et al., 2019; Thomas et al., 2018). While traditional surveys are also time-consuming and taxing in other own ways, the time needed to filter water for eDNA is a barrier in the uptake of the method, because managers are not yet in the position to choose to invest in eDNA collection over already routine surveys. In addition, routine surveys, which could require collecting specimens, may not be conducive to eDNA collection occurring in tandem by the same personnel. Management relevant implementation of eDNA analysis for monitoring cannot occur until more confidence is established in the effectiveness of the strategy for the surveys it would hypothetically replace.

An emerging area of research that could overcome this problem is eDNA capture by passive methods. Several studies have tested eDNA adsorption onto submerged materials *ex situ* (Bessey et al., 2022; Chen et al., 2022; Kirtane et al., 2020), and a few studies have used submerged filters or materials in marine contexts

(Bessey et al., 2023; Jeunen et al., 2022; Maiello et al., 2022). Passive eDNA collection has allowed for increased biological replication, necessary for more accurate interpretations of species prevalence from eDNA data (Ficetola et al., 2015), that is often limited due to time constraints when eDNA is collected by active filtration (Bessey et al., 2021). Even though eDNA metabarcoding as a technology is ready to address many management and decision making needs (Darling, 2019; Gold et al., 2021b; Kelly et al., 2024), the capacity to physically collect eDNA samples can be lacking. Given this mismatch in successful navigation of hurdles impeding operational use of eDNA analysis, we explored whether a quasi-passive eDNA capture strategy could be carried out by non-professionals, whilst SCUBA diving for recreational reasons. A group of six volunteer SCUBA divers collected eDNA at two dive sites in St. Kilda, Scotland by wearing a metaprobe, a perforated plastic sphere filled with cotton, that collects eDNA as divers swim. St. Kilda is an uninhabited remote archipelago, roughly 180 km from Scotland's mainland that is managed by Nature Scot, has designated protected areas, and is an UNESCO world heritage site. The motivation for testing this technique was that we expected that it could collect eDNA (based on results of chapter 4), and that it had the potential to gather enough eDNA to distinguish the biodiversity of dive sites on a fine-scale, which is why we chose to sample sites from the same protected area. This technique, if robust, could present survey opportunities for both volunteers as well as professional agency work, since it could be performed all whilst carrying out traditional underwater surveys.

5.3 Methods

5.3.1 Sample Collection

5.3.1.a eDNA samples

Samples were collected by volunteer SCUBA divers from two dive sites in St. Kilda, an archipelago located to the west of the Outer Hebrides, 180 km from mainland Scotland, and a UNESCO world heritage site of ecological and cultural significance (**Figure 5.1** a). The Mina Stac dive site (57°49'33.9"N, 8°34'08.0"W) was located near the coast of the main island Hirta and took place on May 6, 2023, having a maximum depth of 30 meters (**Figure 5.1** b). The Stac Lee dive (57°51'55.7"N, 8°30'38.3"W) was to the west of the island Boreray and occurred on May 5, 2023, having a maximum depth of 46 meters (**Figure 5.1** c). While diving, six SCUBA divers each brought cotton medical gauze housed in a plastic perforated sampling device called a 'metaprobe', previously used for other quasi-passive eDNA capture in fishing nets (Maiello et al., 2022) (**Figure 5.1** d). Both dives began at approximately 9:00 AM and lasted ~45 minutes. Specific details on metaprobe preparation are in the supplemental material (S4 Methods). The metaprobes were prepared in the laboratory; the plastic housing was soaked in 10% bleach for 24 hrs, rinsed in a 5% lipsol detergent solution for 10 min, with a final rinse in deionised water and allowed to air dry; then they were exposed to UV-light for 30 minutes. Each metaprobe was filled with one roll of certified sterile, medium-sized cotton medical gauze (10 cm x 3.7 m). The probes were sealed shut with cable ties, placed in resealable bags and shipped to the divers before their trip to St.Kilda along with wide-rimmed specimen containers of 100% molecular grade ethanol for preservation of the cotton post-dives. All collected samples were transported in an insulated box with ice packs until they could be stored at -20°C.

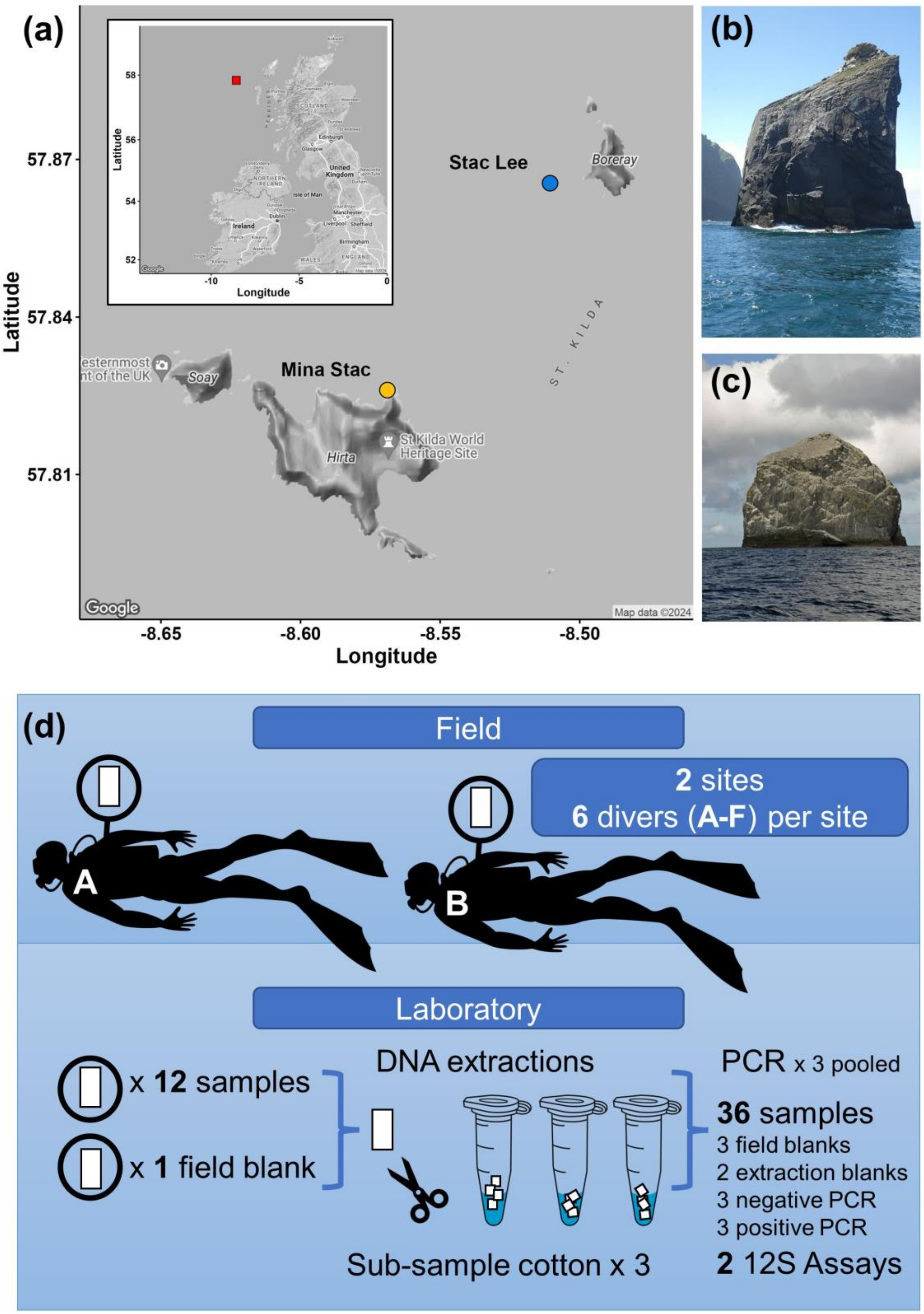

Figure 5.1 Study location and experimental design. (a) Map of St. Kilda, Scotland and eDNA sampling locations (b) Mina Stac and (c) Stac Lee. (d) Diagram showing experimental design.

5.3.1.b Biodiversity records from NBN atlas

Biodiversity occurrence records were downloaded from NBN atlas on June 3, 2024 by using a 0.5 kilometer spatial search radius, covering the dive site and the nearby area, where each dive site was a central point of the radii (Stac Lee: 57.865483 -8.510633; Mina Stac: 57.826083, -8.5689). The records were filtered to include only confirmed identifications.

5.3.2 DNA extraction and sequencing

DNA was extracted from the cotton using the Qiagen DNeasy Blood and Tissue kit following the manufacturer's protocol, but with slight modifications such as using 1000 µl of lysis buffer (S4 Methods). The metaprobe cotton gauze was prepared by cutting off small pieces from various parts of the larger roll. The small pieces were placed onto blotting paper to remove ethanol and were weighed. Between 0.2 – 0.45 g (dry-weight) of cotton were used per DNA extraction and this process was repeated in triplicate per gauze roll (**Figure 5.1** d).

Three replicates of each extract were amplified by polymerase chain reaction (PCR). Two assays targeting different regions of the 12S mitochondrial gene we employed. The Riaz (Riaz-F 5'-ACTGGGATTAGATACCCC-3'; Riaz-R 3'-TAGAACAGGCTCCTCTAG-5') and Elas02 (Elas02-F 5'-GTTGGTHAATCTCGTGCCAGC-3'; Elas02-R 3'-CATAGTAGGGTATCTAATCCTAGTTTG-5') primers targeted respectively 82 bp and 171 bp regions using PCR conditions specified in the original publications (Riaz et al., 2011; Taberlet et al., 2018). For each assay, 3 field blanks, 2 DNA extraction blanks, 3 PCR blanks and 3 positive controls, were amplified alongside the 36 samples (Figure 1d) (S5 Table 1). For positive controls, DNA extractions from a freshwater catfish (*Pangasianodon hypophthalmus*) and from a tropical sting ray (*Hypanus guttatus*) were used for the Riaz and Elas02 primers, respectively. All PCR replicates were visualized on a 2% agarose gel (150 ml 1X TBE buffer with 3 g agarose powder) stained with 1.5 µl SYBRsafe dye (InVitrogen). The PCR replicates were combined by sample and purified using 1:1 ratio of PCR product to Mag-Bind® Total Pure NGS magnetic beads (Omega Bio-Tek) following the manufacturer's protocol. Samples were visualised on an agarose gel again, checking for bands at the anticipated target bp length.

Purified PCR products were quantified using a Qubit dsDNA HS Assay kit (Invitrogen) and pooled at equimolar concentration. The pooled library was imaged on a Tape Station 4200 (Agilent) to check the target band length and assess purity. Based on the Tape Station results, the library was cleaned a final time using magnetic beads in a 1:1 ratio with library volume. A unique adapter sequence was ligated to each library using the NEXTFLEX® Rapid DNA-Seq Kit for Illumina (PerkinElmer) following the manufacturer protocol. After adapter ligation, the libraries were again imaged on the Tape Station and purified with magnetic beads, this time with a 0.9:1 ratio of beads to sample. Each dual-indexed library and PhiX control was then quantified by qPCR using the NEBNext® Library Quant Kit for Illumina (New England Biolabs). Both libraries were prepared with a final molarity of 85 pM with a 10% PhiX spike-in. The Elas02 library, made up half of a sequencing run since it was prepared with another Elas02 library (sequence run C) from Chapter 4 (S4 Table 1). The libraries were sequenced at Liverpool John Moores University on an Illumina iSeq100 using iSeq i1 Reagent v2 (300 cycles).

5.3.3 Bioinformatics

The sequence libraries were processed using the Python (v 2.7.5) package OBITools (v 1.2.11), as well as VSEARCH (v 2.15.2), and SWARM (v 3.0.0) following the same pipeline employed in Chapter 4 up until the taxonomic assignment steps. Software and functions are hereafter reported using the following format: software::function. All forward and reverse sequences for both runs were trimmed using OBITools::obicut to 150 bp; when using OBITools::ngsfilter the paired end reads were filtered by different lengths depending on the assay (i.e. elas02, 130 to 210 bp; Riaz, 80 to 170 bp); and SWARM (v 3.0.0) was employed with the fastidious option and d = 1. For taxonomic assignment, reference databases were generated for each assay using CRABS (v

0.1.4) (Jeunen et al., 2023). Taxonomy was then assigned using VSEARCH::sintax with the corresponding reference databases. The BLAST+ software was also used to perform a blastn with a word length = 7 against the reference databases to check for alignments between the reference and sample sequences. Then final taxonomy was assigned with the results from sintax if the blastn results did not have multiple hits of differing taxa with 100% alignment. In those instances, a higher taxonomic rank was assigned. In the Riaz library, three fish were incorrectly assigned to Pacific species and were reassigned using the same method (sintax) but with a local reference database specifically designed for UK fish species (but not suitable for wider vertebrate communities) (Collins et al., 2021) (S5 Table 2). Percent identity thresholds for assignments and the decontamination process are explained under the statistical analysis section. For details on the taxonomic assignments and any taxa reassigned based on the BLAST alignments see S5 Table 2. For comprehensive details regarding the bioinformatics and statistical analysis, all code is available in a github repository: https://github.com/eneave/stkilda-dna-divers

5.3.4 Statistical Analysis

Further data processing and statistical analysis were done using R (v.4.1.3). R packages are hereafter reported using the following format, Rpackage::function. In cases where the package or function are not reported, the analysis is described and is either a routine function from base R or a routine function from a specified package. The package tidyverse (v.2.0.0) was used for data manipulation and visualization.

Sequence reads resulting from each assay were further quality controlled using the R package decontam (v.1.14.0), to remove contamination detected in the negative controls (i.e., field blank, extraction blank, PCR blanks) screening the negative controls using the function decontam::isContaminant with the “prevalence” method set to a threshold of 0.5. Genus-level detection with a percent identity ≥95% and species-level identifications with a percent identity ≥98% were kept for statistical analysis. All reads belonging to the family Hominidae (i.e., human) or from domestic genera: *Canis*, *Felis*, *Gallus*, *Meleagris*, *Ovis*, and *Sus*, were removed. To control for tag jumping, 0.001% of the total reads for each library was calculated from the total decontaminated read counts, and any detection lower than 0.001% of the total library reads was removed (i.e., 4 and 1 reads for Riaz and Elas02 assays, respectively). After these removals, 241 reads of a freshwater fish, assigned as *Gobio gobio* were removed from the Elas02 results (S5 Table 2).

The beta-diversity of all sequenced samples (n=72), excluding controls, were initially analysed as separate observations to compare vertebrate communities detected in both dive sites and by either assay. Various functions from the R package vegan (v.2.6.4) were used for community diversity analyses. Multivariate homogeneity of group dispersions vegan::betadisper was tested for both dive site and assay followed by testing for significance vegan::anova. A non-metric multidimensional scaling (NMDS) plot was made from converting the read data to binary, followed by the Jaccard index (vegan::vegdist). The NMDS was calculated using the default settings of the vegan::metaMDS function with the distance parameter specified as "jaccard". Permutational multivariate analysis of variance (PERMANOVA) was performed using vegan::adonis2 with dive site and assay tested independently and as interacting terms. Beta-diversity was then analysed again, with the taxa detected from each assay combined to represent the total taxa detected from each DNA extraction. With functions from the R package betapart (v.1.6) differences in beta-diversity were tested for between the dive sites betapart::beta.pair using the Jaccard index and these results were analysed to understand whether those differences were a result of taxon turnover and/or the result of nestedness. Multivariate homogeneity of group dispersions and PERMANOVAs were carried out again for dive site groups using the same functions from the vegan package as described before. This process was repeated with a subset of the community, 19 taxa, that were highly present in biological or technical replicates from either site.

Taxon richness was compared between the dive sites using a Kruskal-Wallis test (stats::kruskal.test). The package iNEXT (v.3.0.0) was used to calculated species accumulation curves, specifically a sample-size-based rarefaction and extrapolation curve. Indicator species of dive sites and assay choice were tested for using the package indicspecies (v.1.7.12) with the function indicspecies::multipatt and the method set to “IndVal.g”. Indicator species were tested for with presence-absence data rather than read counts, so that the varying sample read depths (e.g., between the assays) did not affect the analysis. The values calculated were “A”, “B” and “stat”; where the “A” value is the probability that a species is associated to a dive site or assay if it is detected in a sample (i.e., specificity), and “B” is the overall probability of detecting the species at that dive site or within using a particular assay (i.e., sensitivity). “Stat” encompasses both “A”, specificity, and “B”, sensitivity.

5.4 Results

Negative controls had relatively low contamination with most signs of human contamination occurring in the field (S5 Figure 1). After quality filtering, we retained a total of 3,904,947 reads for downstream analysis resulting in a median of 25,590 (mean = 70,965) reads per sample (S5 Figure 2). After retaining detections which could either be assigned at the genus-level at ≥95% identity or species-level at ≥98% identity, 67 unique taxa resulted. The unique taxa spread across five classes: Actinopteri, Aves, Mammalia, Chondrichthyes, and Branchiopoda; such that, they mainly were finfish, seabirds or marine mammals, with only three taxa belonging to either Chondrichthyes or Branchiopoda. The finfish, seabirds and marine mammals were represented in 19 orders, 30 families, and 46 genera.

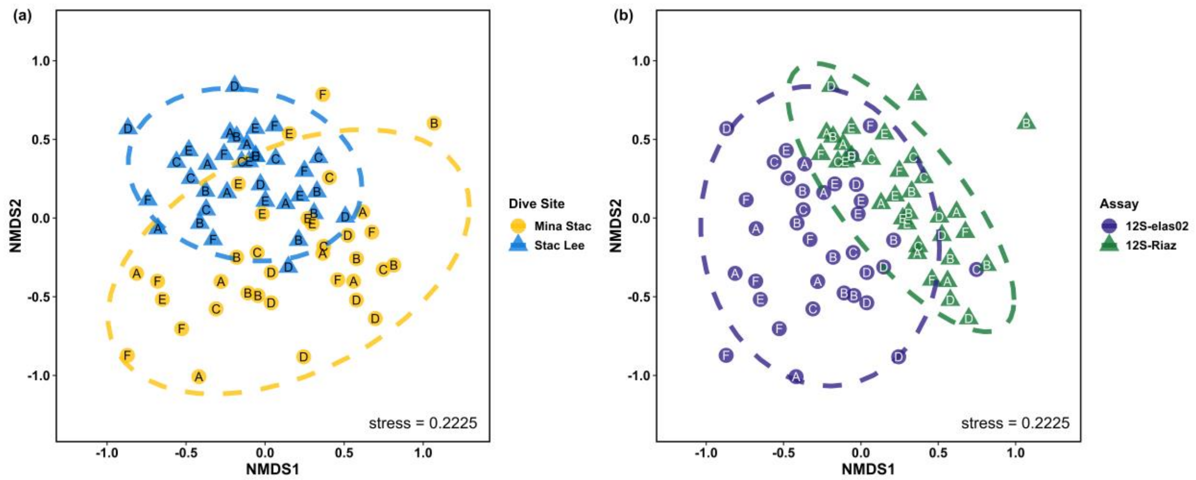

Figure 5.2 Non-metric Multi-Dimensional Scaling (NMDS) plot of beta-diversity represented by a Jaccard dissimilarity matrix, where points and shapes indicate the dive site (a) or assay (b) and letters (i.e., A-F) indicate the metaprobe the community data resulted from. Ellipses show the groupings by (i.e., dive sites or assays) with 95% confidence.

The DNA acquired from the cotton swabs (N=72) resulted in communities that differed between dive sites ($R^2 = 0.098$, $p = 0.001$), and as expected, also resulted in different communities depending on the chosen assay ($R^2 = 0.085$, $p = 0.001$) (**Figure 5.2**, S5 Table 3). However, the interaction of the dive site and assay choice was not significant ($R^2 = 0.018$, $p = 0.094$). An indicator species analysis was used to determine which species were most associated with a specific assay, as well as with either dive site, the latter having ecological implications (S5 Table 4). Six taxa were significant indicators, associated with the Elas02 assay. These taxa, in order starting from highest total read counts, included grey seal (*Halichoerus grypus*), white-beaked dolphin (*Lagenorhynchus albirostris*), pollocks (*Pollachius* sp.), long-spined sea scorpion (*Taurulus bubalis*), Northern fulmars (*Fulmarus glacialis*), and herring (*Clupea* sp.) (**Figure 5.3**). The other four species that were significantly indicative of the Riaz assay were fivebeard rockling (Ciliata mustela), red gunnard (Chelidonichthys cuculus), and righteye flounders (Microstomus sp.) as well as a seal species that had to be reassigned at the family level (Phocidae), after BLAST alignment showed that the short 12S Riaz fragment could not distinguish the reads from other seal species and hooded seals (*Cystophora cristata*), which they originally assigned to. (**Figure 5.3**, S5

Table 4). Northern gannet (*Morus bassanus*) was the indicator species with the strongest association (stat = 0.841, $p < 0.001$) and the only species associated to Stac Lee. The rock cook wrasse (*Centrolabrus exoletus*) displayed high specificity (A = 0.923) to Mina Stac but a relatively modest likelihood of being detected within the Mina Stac samples (B = 0.343) compared to other indicator species. Both the grey seals and the ambiguous seals (Phocidae) were significantly associated with Mina Stac ($p(s) < 0.05$). And finally, common murres (*Uria aalgae*) were the strongest indicator species of Mina Stac (stat = 0.627, p = 0.001).

Considering the taxa detected by both assays, the species richness at Mina Stac was significantly higher than at Stac Lee (χ^2= 7.0059, df = 1, p = 0.008) (**Figure 5.4** a). Mina Stac had a greater number of detections in the finfish, seabird, and mammalian groups, with a total species detected at 57, whilst 38 taxa were detected at Stac Lee (**Figure 5.3**). Rarefaction and extrapolation curves for each dive site do not overlap, suggesting that the difference in alpha-diversity between sites is not due to under sampling and reflects a biological reality (**Figure 5.4** b). Beta-diversity of the communities, with detections from both assays combined into occurrence data, was driven by species turnover rather than nestedness (**Figure 5.4** c, **Table 5-1** a).

Figure 5.3 Stacked bars of total read counts for each taxa detected at either dive site. Colors specify the assay which the read counts were generated from. Read counts are log10

transformed and bars are ordered decreasing from greatest total read counts within each class.

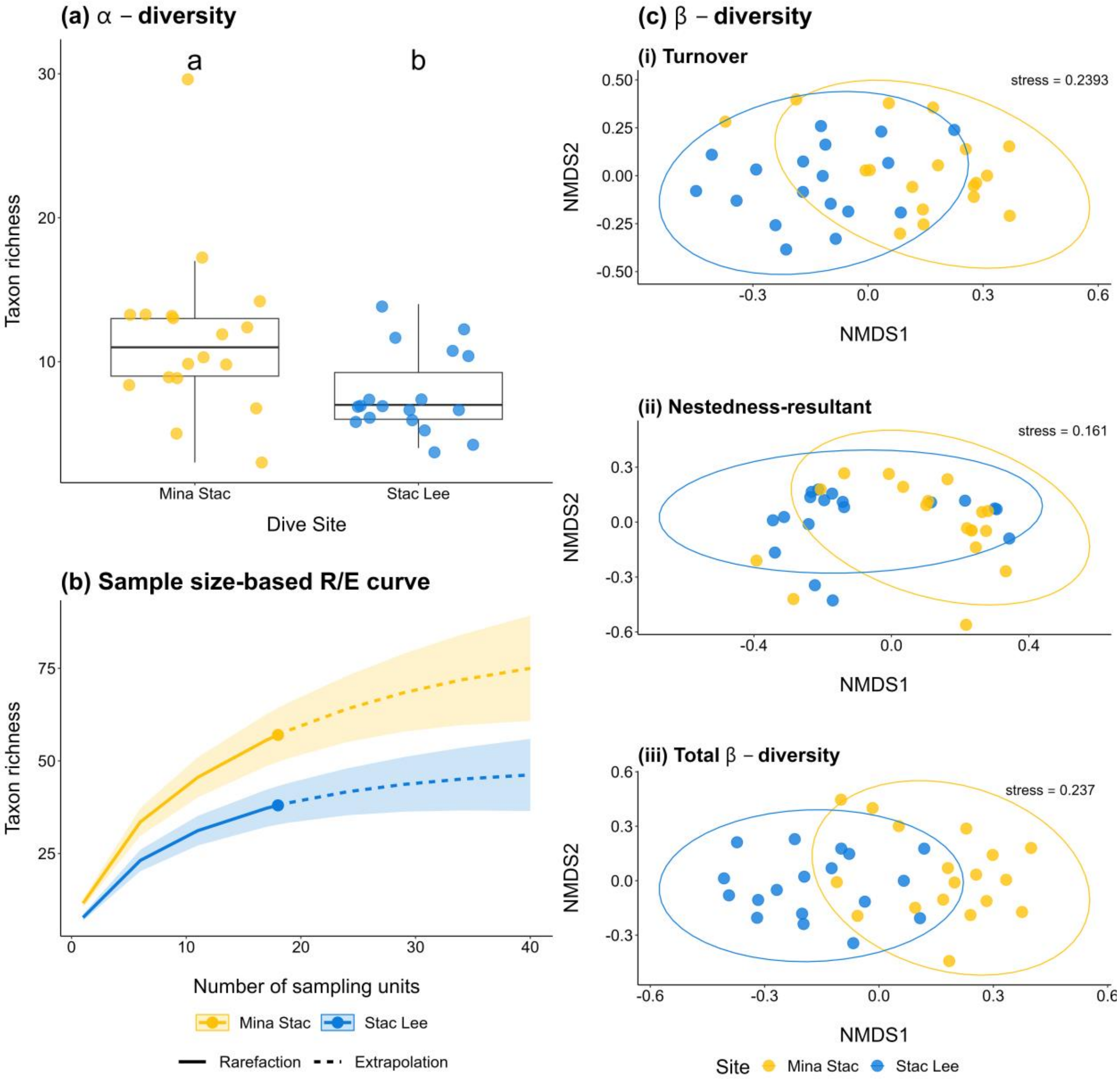

Figure 5.4 Alpha and beta diversity comparisons made between dive site locations, where yellow is Mina Stac and blue is Stac Lee: (a) boxplot showing species richness, (b) sample size-based rarefaction/extrapolation (R/E) for dive site, and (c) non-metric multidimensional scaling (NMDS) plots of dive site communities for each beta diversity component.

Table 5-1 Summary of analyses statistically comparing homogeneity of multivariate dispersions between communities at dive sites (ANOVA), and variation in community composition of sampling locations in each dive site (PERMANOVA). Relative contributions of taxon turnover, nestedness, and total beta-diversity (Jaccard index) for each dive site are given in brackets. The analyses are performed twice, where (a) includes the whole dataset and (b) is calculated with a subset of the taxa.

	Homogeneity of multivariate dispersions (ANOVA)				**Community similarity (PERMANOVA)**			
(a)	**Mean distance to centroid ± SE**	df	F	p-value	df	F	R^2	p-value
Turnover		1	0.6486	0.4262	1	4.7187	0.12187	0.001
Mina Stac (87.77%)	0.351±0.033							
Stac Lee (89.01%)	0.393±0.015							
Nestedness		1	1.016	0.3206	1	-4.1587	-0.13936	0.996
Mina Stac (5.06%)	0.137±0.025							
Stac Lee (3.77%)	0.094±0.008							
Total β-diversity		1	0.0004	0.9845	1	3.937	0.10378	0.001
Mina Stac (92.83%)	0.4813±0.0071							
Stac Lee (92.78%)	0.4809±0.0026							
(b)								
Turnover		1	0.6133	0.439	1	7.4409	0.17956	0.001
Mina Stac (80.58%)	0.285±0.019							
Stac Lee (84.41%)	0.319±0.014							
Nestedness		1	0.3339	0.5672	1	-1.2989	-0.03972	0.885
Mina Stac (8.19%)	0.133±0.023							
Stac Lee (5.55%)	0.107±0.011							
Total β-diversity		1	0.4689	0.4981	1	5.9988	0.14997	0.001
Mina Stac (88.78%)	0.394±0.013							
Stac Lee (89.96%)	0.415±0.004							

Detections at either site were further examined to understand how biological replication (i.e., diver metaprobes, of which there were six collected from either site) and technical replication (i.e., DNA extraction replicates) influenced the dataset (**Figure 5.5**). Two criteria were assessed: 1) for biological replication, whether taxa were detected at a site in three or more metaprobes or 2) for technical replication, whether taxa were detected in all three DNA extracts resulting from one or more metaprobes. A total of 19 taxa met one or both criteria. The dive sites differed in amounts of taxa that met these criteria, for instance, there were 16 and 12 taxa that were detected in half or more of the metaprobes from Mina Stac and Stac Lee, respectively. Mina Stac had twice the number of taxa that met the technical replication criteria than Stac Lee (i.e., 10 and 5, respectively). Eleven taxa met both criteria across the two dive sites (**Figure 5.5**). Interestingly, five of these eleven taxa were all determined to be indicator species pertaining to either dive site (i.e., *Centrolabrus exoletus*, *Halichoerus grypus*, Phocidae, *Uria aalge*, *Morus bassanus*).

The biodiversity records downloaded from NBN atlas resulted in 114 unique observations from the Stac Lee vicinity and 265 unique observations from the Mina Stac vicinity, of which 113 and 165 were marine, respectively. Historical observations from Stac Lee and Mina Stac contained six and seven fish species in total, of which we detected three and six in the eDNA samples. Interestingly, northern gannets (*Morus bassunus*) were determined to be indicator species from the Stac Lee eDNA and in the historical records they are only recorded at Stac Lee. Similarly, grey seals (*Halichoerus grypus*) were indicator species in the Mina Stac eDNA and only have recorded occurrences from Mina Stac. When re-analyzing beta-diversity with the subset of 19 taxa that met the replication criteria (**Figure 5.5**), results mirrored the analysis containing all the detections (**Table 5-1**): turnover was still a significant factor for differences in beta-diversity (**Table 5-1** b). Patterns across historical data, our total eDNA data set and the refined eDNA data set considering replicated detections, show congruence in species detections across the different dive sites.

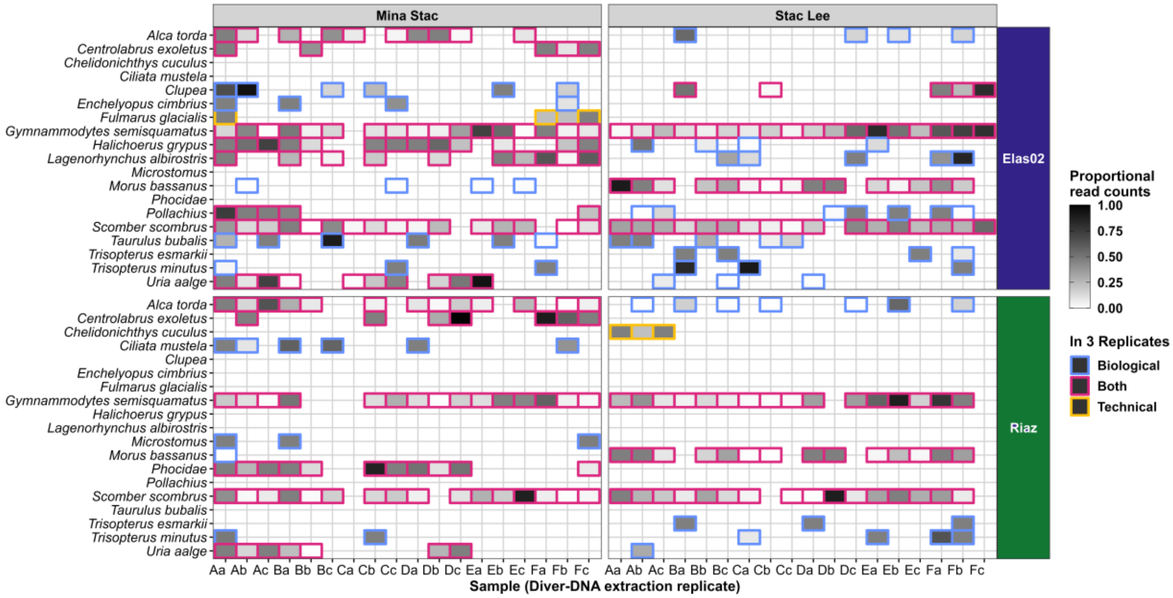

Figure 5.5 Heatmap showing the proportional read counts of taxa that were detected at each dive site by either three separate divers (blue outline), in all three DNA extraction replicates from at least one diver (yellow outline) or satisfy both of those conditions (red outline). Taxa that did not meet at least one of these criteria are not displayed. Proportional read counts generated from the two different 12 assays (Elas02, Riaz) are shown separately.

5.5 Discussion

St. Kilda is a volcanic archipelago featuring towering sea cliffs that host the largest colony of seabirds in Europe and was home to people for 2,000 years who subsisted on a diet of seabirds and agriculture before the islands were left uninhabited from 1930. In addition to their natural and cultural significance as decreed by UNESCO, St. Kilda are also part of Scotland's marine protected area network and designated as a special protection area (SPA) for its sea cave and rocky reefs. The results from this study demonstrate that eDNA capture using metaprobes deployed by SCUBA divers, followed by metabarcoding analysis, is a nimble, effective tool for rapidly surveying marine vertebrates in remote areas of key conservation relevance. In just two dives exploring these cold-temperate reefs, the eDNA captured by recreational SCUBA divers confidently detected 67 taxa to either genus or species-level, belonging primarily to the bony fish, seabirds and mammals. As it currently stands, eDNA collection is rarely employed by marine managers because of the additional resources required for implementation on top of already existing and established surveys. However, collecting this eDNA required little extra time from the volunteers, since no filtration, the typical mode of eDNA capture, was involved. Moreover, the metaprobe is constructed from materials that are user friendly for non-professionals, widely available and low-cost. These practicalities make this method suitable for future citizen science and monitoring in other SPAs, as it could occur alongside recreational or routine underwater survey activities.

The sampling efforts of six SCUBA divers detected significant, ecologically relevant differences in marine vertebrate communities between two rocky reefs situated ~5 km from each other, Mina Stac and Stac Lee (**Figure 5.2** a). Based on an indicator species analysis considering the presence and absence of taxa in the samples combined from both assays, two seabird species were the most strongly associated taxa with either site, such that guillemots (*Uria aalge*) were highly detected at Stac Mina, while Northern gannets (*Morus bassanus*) were a feature of Stac Lee (S5 Table 4). Both grey seals (*Halichoerus grypus*) detected in the elas02 assay, and another earless seal species reassigned to the Family level by the Riaz assay, were significantly associated with Mina Stac. The most likely scenario is that the latter is also a sequence indicative of grey seals, which are known to frequent the shores of St. Kilda, rather than evidence of a separate species. Nevertheless, two species cannot be ruled out, since other seal species, such as harbor seals (*Phoca vitulina*), have distributions in this region. This does however provide clues as to why the assays perform differently, since they target different lengths of the 12S gene, with the Riaz primer targeting a much shorter DNA fragment. A similar pattern occurred such that minke whales (*Balaenoptera acutorostrata*) where detected at both locations by the elas02 primer, but another baleen whale species (*Balaenoptera* sp.), at no finer taxonomic granularity for the group, was detected by the Riaz primer. These primers did however amplify Risso's dolphin (*Grampus griseus*) eDNA, not detected in the elas02 assay, while the elas02 assay contained many fish species that were not present in data resulting from the Riaz primers. Some of those fish, namely pollack

(*Pollachius* sp.), long-spined bullhead (*Taurulus bubalis*) and herring (*Clupea* sp.) were in fact significantly associated with the elas02 primer data (S5 Table 4). The imperfection of these assays can lead to detections from groups that are not being targeted at all, such as zooplankton, a water-flea (*Chydorus sphaericus*), detected at Stac Lee. It comes as no surprise that the difference in communities detected by either assay was significant (**Figure 5.2** b), which was desired since the elas02 primers were designed for elasmobranchs and fish more broadly, while the Riaz primers were designed for vertebrates with less focus on a particular class.

Combining data from both assays, Mina Stac with 57 taxa, was more species rich than Stac Lee, which had 38 taxon detections. While the rarefaction curves from either site are both ascending, from 18 samples each generated with six biological replicates (i.e., divers in water), when those curves are extrapolated, they reach saturation and their standard deviations do not overlap. This suggests that indeed, the sites have differing vertebrate richness and that this is not an artifact of sample size (**Figure 5.4** b). Furthermore, combining the assays still resulted in a significant difference in total beta-diversity (R^2=0.10, p=0.001) where the driving cause of this difference was species turnover (R^2=0.12, p=0.001) (**Figure 5.4** c). Environmental DNA can be transported between sites and particularly when eDNA exists at low concentrations, the nature of the PCR process can preferentially amplify more rare species when there is less eDNA captured from a sampling site (Gold et al., 2021b); this can make sites with lower species richness artificially appear to have higher species richness to due to transported DNA from more pelagic species, for example. To give more robustness to our interpretations, we analysed which species had frequent detections in our replicates, compiling a list of species for each site that either were detected in three or more biological replicates (out of six diver metaprobes) or detected in all three DNA extracts taken from a single metaprobe. This resulted in 19 taxa across sites, that met these replication criteria. Interestingly, all of the indicator species identified as associated to either site met these criteria. We then repeated the analyses of beta-diversity with the subset of these 19 species, only to find that both signals of differences between species turnover (R^2=0.17, p=0.001) and total-beta-diversity (R^2=0.14, p=0.001) between sites were confirmed, and even strengthened. This demonstrates that the differences between sites were unlikely to be caused by detections sourced erroneously; for instance, from eDNA signals reminiscent of pelagic sources or transported via currents.

Currently, very little is known about the efficacy of eDNA adsorption onto materials ($\lessapprox$15 studies) and whether the approach will become a mainstay of biodiversity surveying. This study is perhaps the first practical demonstration that this approach is worth developing and investing in. Seventy out of the 72 (97%) sequenced samples contained target taxa and when combining assays, all 36 samples contained detections. This is commensurate to other experiments testing passive eDNA capture onto filter membranes, where target taxa were also detected in 97% of samples (Bessey et al., 2021). However, the present study offers empirical evidence of a convenient, quasi-passive eDNA sampling device that captures fine-scale ecological heterogeneity in remote marine habitats, without entailing significant additional resources or disruption to the volunteers' activities.

All DNA extracts amplified with the elas02 assay amplified target taxa, but two samples (i.e., one technical replicate from Mina Stac, metaprobe C; one technical replicate from Stac Lee, metaprobe F) did not generate reads from marine vertebrates. It is worth noting that a large proportion of sequences (~50-70%) were identified as human DNA, which is understandable given that the assays targeted vertebrates and the metaprobes were deployed attached to divers that have at least their faces exposed to the seawater (S5 Figure 3). For future work, human blocking primers could be used in the laboratory workflow to minimize this from happening. Yet, even with this occurring, six biological replicates were sufficient to achieve a representative sample of site-specific vertebrate biodiversity (**Figure 5.4**).

The taxa detected had ecological significance and were corroborated by historical records. For instance, St.Kilda has the largest colony of breeding gannets in the world, with an estimated 60,000 breeding pairs living between the island Boreray and its surrounding sea stacks, which include our sampling site, Stac Lee. The gannets (*Morus bassanus*) were the only indicator species associated with Stac Lee and when looking at the sample replicates it is clear that even though the species is detected at both sites, it is a feature of Stac Lee (**Figure 5.5**). Based on the historical biodiversity records accessed from NBN atlas, only five species of fish have been recorded within a 0.5 km radius of the Stac Lee dive site. Three of these species, pollack, three-bearded rockling, and long-spined bullhead were detected by eDNA, but two of the species were not: monkfish (*Lophius piscatorius*) and leopard-spotted goby (*Thorogobius ephippiatus*). Still the eDNA samples from Stac Lee contained 29 fish detections including detections of two elasmobranchs: the small-spotted catshark (*Scyliorhinus canicula*) and *Dipturus* sp.; the latter of which could either be a blue skate or a flapper skate, both of which are critically endangered. Records from 0.5 km radius of the Mina Stac dive site contained seven fish, all of which were detected at Mina Stac during this eDNA survey except for the cryptic snailfish (*Liparis* sp.), although *Liparis montagui* was detected at Stac Lee on this occasion. Both grey seals and harbor seals exist in the historical records from Mina Stac, and the seal detections we had (i.e., *Halichoerus grypus*, Phocidae) were significant indicators of this dive site in particular. Forty-two fish species were detected at Mina Stac in the eDNA samples, highlighting the sensitivity of aquatic eDNA for fish detection. Fish species that were highly detected across both sites include smooth sandeels (*Gymnammodytes semisquamatus*) and mackerel (*Scomber scombrus*). Northern gannets feed on both of these species, and the waters surrounding St.Kilda are known breeding grounds for mackerel, which breed in the UK from April onwards. Sandeels are important in the diet of razorbills (*Alca torda*) and guillemots (*Uria aalge*), both of which were highly detected at both dive sites. Another predator of sandeels notably missing from these eDNA samples, are Atlantic puffins (*Fratercula arctica*), of which half of the UK population breeds in St. Kilda. Despite this notable absence, many protected vertebrate species in St.Kilda were detected (e.g., white-beaked dolphin *Lagenorhynchus albirostris*, harbour porpoise *Phocoena phocoena*, Northern gannets *Morus bassanus*, etc.).

Environmental DNA analyses are becoming increasingly considered for management and conservation applications, but the time-consuming process of water filtration, the typical method of concentrating eDNA, is a hindrance to its widespread uptake. This study shows that with a modest sample size, eDNA can be collected in a quasi-passive manner by using cotton to “swab” a dive site, while a SCUBA diver undergoes their usual activities underwater. The metaprobe, housing the cotton, provides a reusable, low-cost structure that the collection material can be housed in, and has been highly successful in other surveying contexts as well, such as analysing fisheries catch. Here, we have shown how metaprobes can be deployed by recreational SCUBA divers in a remote location that is logistically difficult to monitor simply due to its remoteness from the mainland and its exposure to storms. St. Kilda has some of the most diverse marine life in the United Kingdom, which supports seabird populations of global importance. Now visitors of this uninhabited archipelago can be custodians for its future, and hopefully serve as inspiration for the uptake and upscaling of this approach.

Chapter 6

General Conclusion

The sunset over Korakonissi, Zakynthos, Greece.

Photographer: Peter Shum

6.1 Summary and synthesis of findings

Measuring biodiversity in the ocean has never been easier, but challenges remain. Environmental DNA metabarcoding is a technique that can be applied over a range of different purposes such as biodiversity discovery, invasive species management, routine monitoring for conservation, and even investigating more complex ecological processes (Beng and Corlett, 2020; Deiner et al., 2017a). However, the resources and effort required to concentrate eDNA by filtering water, particularly from marine environments, are at best cumbersome and, at worst, an impassable barrier to practitioners who would be implementing this approach.

Improvements have been made to help overcome the time investment required to capture eDNA from dilute marine environments, and these include various autonomous filtering devices that sample water for analysis in a laboratory or can even be integrated with sensors that perform genetic assays *in situ* (Govindarajan et al., 2022; Hendricks et al., 2023; McQuillan and Robidart, 2017). Sensor integration is still an area of ongoing development and has not achieved analyses commensurate with the complexity of metabarcoding. Seawater filtering robots are continuously improving, but the designs are generally still too bulky, complicated, and expensive to warrant widespread adoption. Even as these designs improve, there will be a period where the cost for these instruments remains high, hindering their viability in many places globally. Until the technology for autonomous eDNA collection is cost-effective and mainstream, eDNA capture will always be a first order challenge for the broad-scale adoption of eDNA metabarcoding as a means for generating species occurrence data.

Letting your creativity flow is an important aspect of the scientific process that often goes underappreciated; at least by the public, since popular culture often portrays scientists as rigid with a propensity to strictly follow logical steps. With that frame of mind, choosing not to focus efforts on engineering eDNA sampling robots could be construed as a waste of time, with alternative, unconventional, low-tech methods perceived as just a distraction. However, searching for creative solutions to capturing eDNA are warranted because low-cost alternatives to autonomous robots could be hiding in plain sight and could help democratize molecular approaches of biodiversity science. This thesis has sought creative strategies for eDNA capture from the ocean, and specifically focused on two avenues. Chapters of this thesis are dedicated to each avenue, first by exploring it in a proof-of-concept manner and on the largest scale available to us at the time, and then by fine-tuning each method with focused studies at local, management relevant scales. The first approach looks to the most accomplished water filterers on the planet. Sponges, or the animal phylum Porifera, have existed on earth since multicellular life organised itself on this planet, and now can be found in freshwater as well as the ocean, pumping up to 10,000 liters of water per day (Kahn et al., 2015). The second approach asks skilled volunteers, namely SCUBA divers, through a community science project titled “DNA divers”, to accumulate eDNA onto passive sampling devices as they dive, overcoming the need for water filtration.

In chapter two, three species of sponges from North Atlantic, mostly deep-sea, sponge grounds were screened for their ability to act as natural samplers of eDNA. We specifically used an assay aimed at detecting bony fish and, in the process, also detected elasmobranchs and marine mammals, the community composition of which discriminated the North Atlantic deep-sea into known and different biogeographic regions. Not all of the sponge samples produced DNA which amplified well, and some that were sequenced had low amounts of sequence read counts, leading to the realization that one of the species, the chalice sponges (*Phakellia ventilabrum*), worked better than the others, *Geodia barretti* and *Geodia hentscheli*. It was noted that the chalice sponges have higher pumping rates compared to the *Geodia* species. Pumping rates in sponges are also correlated with the amount of microbial abundance that they have in their tissues, where high microbial abundance sponges, such as these *Geodia* species, rely on receiving some nutrients directly from their microbial endosymbionts and therefore do not need to filter as much particulate organic matter for energy from the water column. As this study was being completed, sponge species were also shown both *ex situ* (Cai et al., 2022) and *in situ* (Brodnicke et al., 2023) to have different propensities for being good natural eDNA samplers. Despite evidence of sponge species influencing eDNA capture efficiency, the differences in fish communities found across depth layers made ecological sense and biogeographic signals were more pronounced than any variation resulting from the different sponge species. Importantly, this chapter utilized sponge samples previously collected for other research purposes and did not require additional specimen collection from sponge ground habitats. In fact, the sponges were collected through an international collaborative research project consisting of 25 partners from the European Union, the USA, and Canada that ran from 2016 to 2020 (www.deepseasponges.org), which highlights the added value of biological collections of sponges.

A critical part of developing eDNA capture strategies is ground-truthing the recovered data and comparing it to more conventional ways of eDNA collection such as manual water filtration with syringes. In chapter three, in contrast to the opportunistic approach used in chapter two, we employed targeted sampling, such that underwater visual surveys were used to compare fish sightings to eDNA, both from filtered water samples and by taking biopsies from sponges on Mediterranean rocky reefs. This project was carried out in collaboration with the Natural Environment and Climate Change Agency (NECCA), specifically with the management unit of Zakynthos national park in Greece. It is well known that visual surveys and eDNA metabarcoding data do not match perfectly, with each method detecting species undetected by the other. Our results show this, and while there was some species overlap, especially of common and abundant species, ultimately eDNA detected more species that were non-indigenous to the region, emphasizing the sensitivity of eDNA analyses and their usefulness for early detection of alien taxa. We also collected biopsies from four species of sponges: the kidney sponge (*Chondrosia reniformis*), the putative black leather sponge (*Sarcotragus* sp.), the variable loggerhead sponge (*Ircinia variabilis*) and a second *Ircinia* species. Of these four species, the sponge from the genus *Ircinia* (i.e., the species could not be identified with DNA barcoding or microscopy of sponge spicules) had the highest number of samples (44%) containing detections of finfish. But none of these sponges came close to performing as well as the deep-sea sponges from chapter two, many of which we chose not to sequence based on little evidence of the target DNA fragment amplifying, whereas the samples collected from Greece showed faint signs of amplification and we sequenced everything regardless. This does beg the question, do sponges collected from a deep-sea environment work better as natural samplers? The deep-sea has cold temperatures and dark conditions, all of which should slow down the degradation of eDNA. Moreover, there is more particulate organic matter in the deep-sea relative to the shallow seas where it is exported from, so maybe there are more particles for eDNA to bind to before subsequently being captured by a pumping sponge. Though there are many questions left to address about the mechanisms enabling sponges to be natural eDNA samplers, chapter two of this thesis demonstrates that the technique can produce extraordinary results, while the work in chapter three provides more evidence towards which species are most suitable.

Citizen (community) science which involves participation of volunteers in the scientific process can be powerful for scaling up research efforts whilst also improving science literacy among the general public. In chapter four, the community science project “DNA divers” demonstrated a tractable method for eDNA collection using a low-cost device (i.e., the metaprobe) that was assisted by the swimming motion of SCUBA divers and snorkelers. The sampling efforts were varied and guided by the many collaborating organisations in order to match their activities and interests. Initial samples were collected by clients of a small live-aboard dive charter business (Halton Charters Ltd.) and employees of an executive non-departmental public body (Natural England). Natural England was interested to see how this method might enable them to monitor fish (which they do not currently monitor aside from the protected species spiny (*Hippocampus guttulatus*) and short-snouted (*Hippocampus hippocampus*) seahorses) and if it might increase their capacity to implement citizen science projects. These datasets were analysed for a report titled, “Passive eDNA capture by SCUBA divers and snorkellers for monitoring inshore fish biodiversity (NECR506)", which was published in Natural England's Access to Evidence catalogue. More congruent syringe-filtered and metaprobe eDNA samples were collected in natural and controlled (Blue Planet Aquarium) settings and experiments were undertaken showing that submersion time increases the number of MOTUs and detections but also that the swimming motion from divers expedites this process. Where we had overlapping data in nature, ~20-30% taxa were detected by both eDNA capture techniques, but syringe-filtered eDNA contained more unique detections (~65-75%) than did the metaprobes. The ease of implementation of the metaprobes ultimately led to partnering with many more organisations and volunteers (**Table 4-1**), allowing for testing and refining of the technique in a variety of countries and habitats.

From a year of working with these volunteers, we learned the most useful and convenient ways to deploy and extract the eDNA from the cotton gauze. Chapter five of this thesis implements everything learned from chapter four, to see if volunteers could survey a remote archipelago called St. Kilda, located ~60 km west of the Outer Hebrides, which is designated as a special area of conservation in the UK as well as a UNESCO world heritage site. UNESCO has demonstrated interest in eDNA for monitoring their marine heritage sites and have been carrying out an expedition with citizen science volunteers collecting and filtering water samples across 25 of them, of which St. Kilda was not included. The divers in chapter five visited two dive sites, both rocky reefs situated near sea stacks that are habitat for nesting seabirds. Using two assays, we were able to detect nearly 60 vertebrate species, including bony fish, elasmobranchs, seabirds, and marine mammals. Despite being similar habitats, cold-temperate rocky reefs less than 5 km apart, the communities detected were significantly different. Unlike chapter four, which showed the promise of this technique by detecting taxa belonging to extremely divergent marine habitats from different biogeographic regions (e.g., tropical coral reefs vs. temperate kelp forests), chapter five demonstrated the utility of applying this approach for monitoring at fine scales, relevant to local management requirements.

Marine environmental DNA analyses and research capacity have massively expanded since the first marine eDNA metabarcoding experiment taking place on a Danish pier just over 10 years ago (Thomsen et al., 2012). From the efforts of researchers, increasing exponentially over the last decade (Eble et al., 2020), the scientific community has come a long way in optimizing the laboratory techniques and bioinformatic software needed to analyze eDNA metabarcoding datasets. Fewer studies have focused on innovating eDNA capture strategies, particularly those that deviate from reliance on filtration mechanisms. This thesis examined creative avenues of eDNA capture and revealed viable, low-cost workarounds to filtration that produced ecologically relevant biodiversity observations. This was achieved by utilizing already existing biological collections, working in partnership with environmental management agencies, collaborating with eager and generous volunteers, and working with highly skilled SCUBA diving citizen (non-professional) scientists.

6.2 Future research and outlook

Neither eDNA capture strategy presented in this thesis is perfect, nor were the strategies envisioned to entirely replace filtration as a method for concentrating eDNA. Capture of eDNA by capitalizing on its accumulation in sea sponges does not work with every sponge species. While passive eDNA capture aided by snorkellers and divers does not detect as many taxa as does eDNA concentrated by filtration. Yet each strategy explored has strengths. For example, the sponges in chapter two were sourced entirely from already existing museum collections (Neave et al., 2023), requiring no investment in sampling and significantly extending the data retrieved from a specimen to information beyond the physical object— termed the extended specimen concept (Lendemer et al., 2020). Moreover, while this thesis was being carried out, sponges caught as bycatch from long-lines (Jeunen et al., 2024) and removed from oceanographic arrays during routine cleaning of biofouling (Cai et al., *in press*) have been analysed with eDNA metabarcoding, gleaning biodiversity information from animals which would have otherwise died with no scientific purpose. A strength of diver-assisted metaprobes, is the ease with which the strategy was implemented regardless of the activity the volunteer was undertaking in the water. For example, a number of dives in chapter four were carried out by piggybacking off of a long-running citizen science project organized by the Marine Conservation Society called "Seasearch" (https://www.seasearch.org.uk/), which conducts visual census surveys of dive sites around the UK (**Figure 6.1**). Since deploying the metaprobe is hands-free, divers can carry out other activities, such as underwater photography, and collect the eDNA with minimal training required. This was particularly useful when metaprobes were attached to professional Natural England divers who were doing underwater transects and survey work in tandem. For these reasons, metaprobes should not be limited to divers and snorkellers, particularly since they were originally designed for trawl nets (Maiello et al., 2022), as they have the capacity to be extended for deployment with other marine leisure users like kayakers and Stand Up Paddleboarders. While these approaches, sponge-derived eDNA and quasi-passive capture by divers, will need further refinement, the results from this thesis demonstrate success in early efforts and promise as self-contained eDNA capture strategies.

Figure 6.1 Collecting eDNA via syringe-filtration after divers from the citizen science project Seasearch have completed a visual survey whist wearing metaprobes. Photo taken by Alistair Allen.

Marine biodiversity loss presents both tangible problems such as unhealthy and less functional ecosystems, decreased food security, and lowered economic value, as well as intangible problems, such as the extinction of undiscovered life, some of which may produce novel natural compounds that could lead to cures for diseases, or others that may have an immeasurable cultural and evocative significance (Erdmann et al., 1998). But there is much hope because people know how to overcome these problems by implementing effective conservation strategies and policies (Knowlton, 2021). Species distribution and occurrence data is necessary for implementing effective conservation strategies and eDNA metabarcoding provides a powerful means for generating such data. Efforts towards standardizing the way eDNA data is formatted for global sharing are currently being developed by the OBIS (Ocean Biodiversity Information System) Secretariat (Silliman et al., 2023) so that metabarcoding data can meet FAIR data principles (i.e., findable, accessible, interoperable, reusable). This thesis demonstrated the value of museum collections and the integral role that governing agencies and citizens play in shaping applicable biodiversity conservation research. Upon submission of this thesis (June 2024) the United States announced its National Aquatic Environmental DNA Strategy, which calls for federal involvement in aquatic eDNA research and emphasizes both the role museums will play as well as highlights the advantages of engaging the public with citizen (or community) science (Gold & Nichols, 2024). The coordination of public agencies, public facing institutions (i.e., museums, zoos and aquariums, universities), industry stakeholders, NGOs and citizens ought to be continued to create the necessary tools to conserve and protect marine biodiversity.

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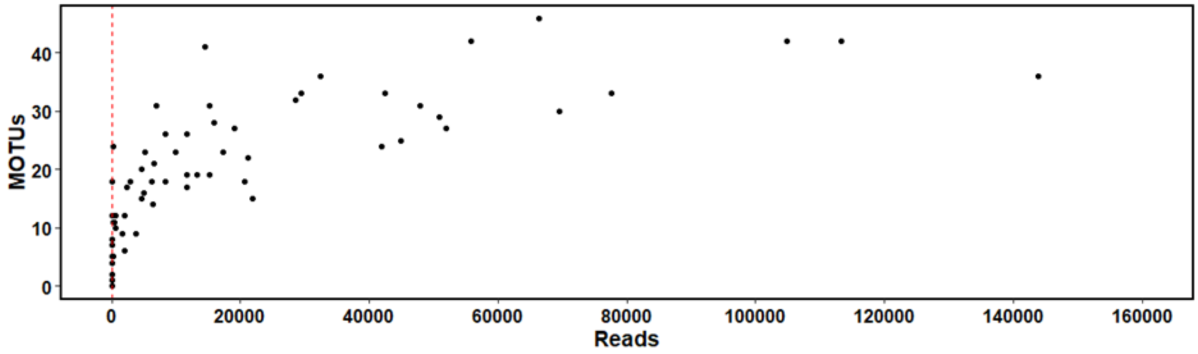
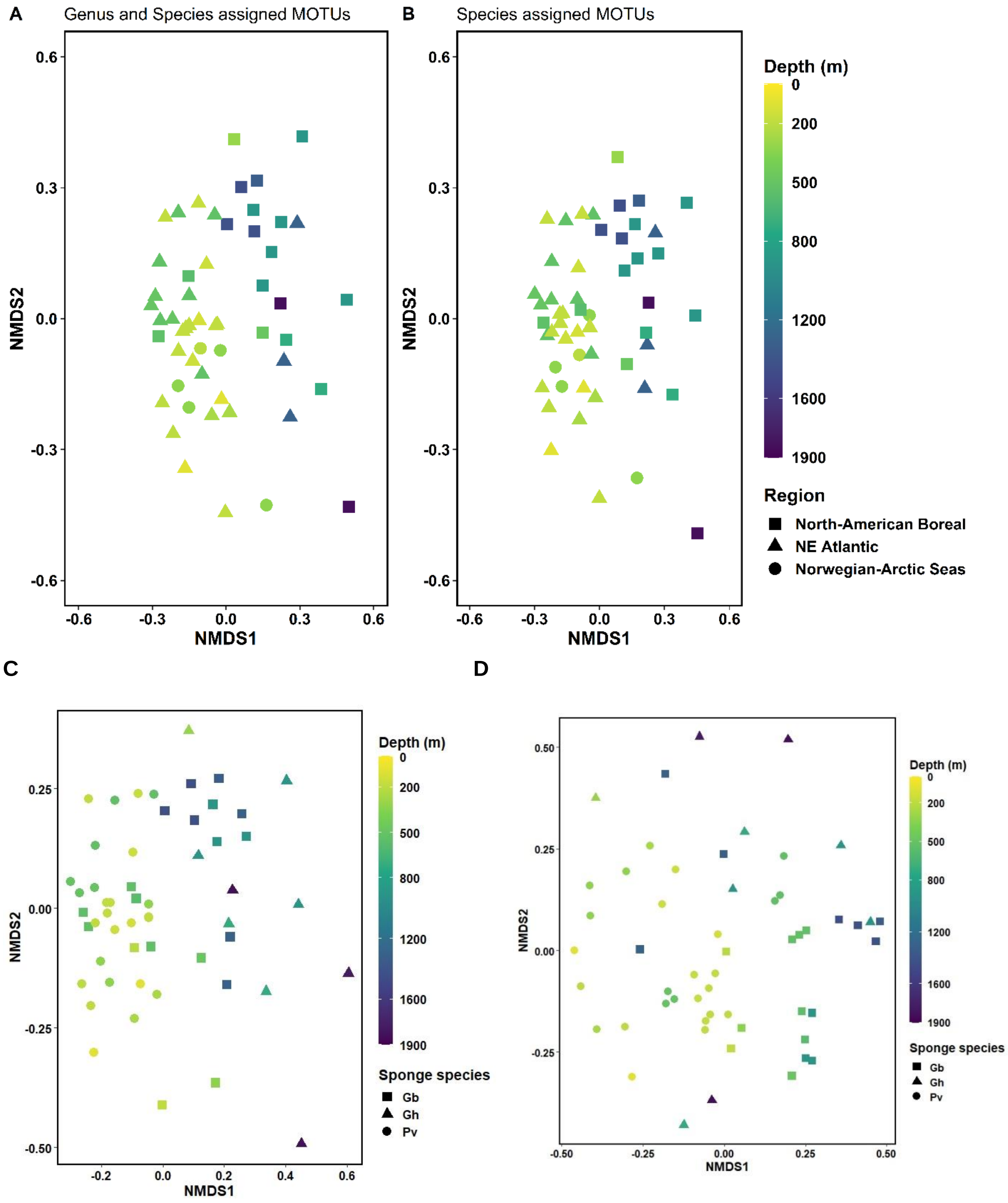
Supplemental Material

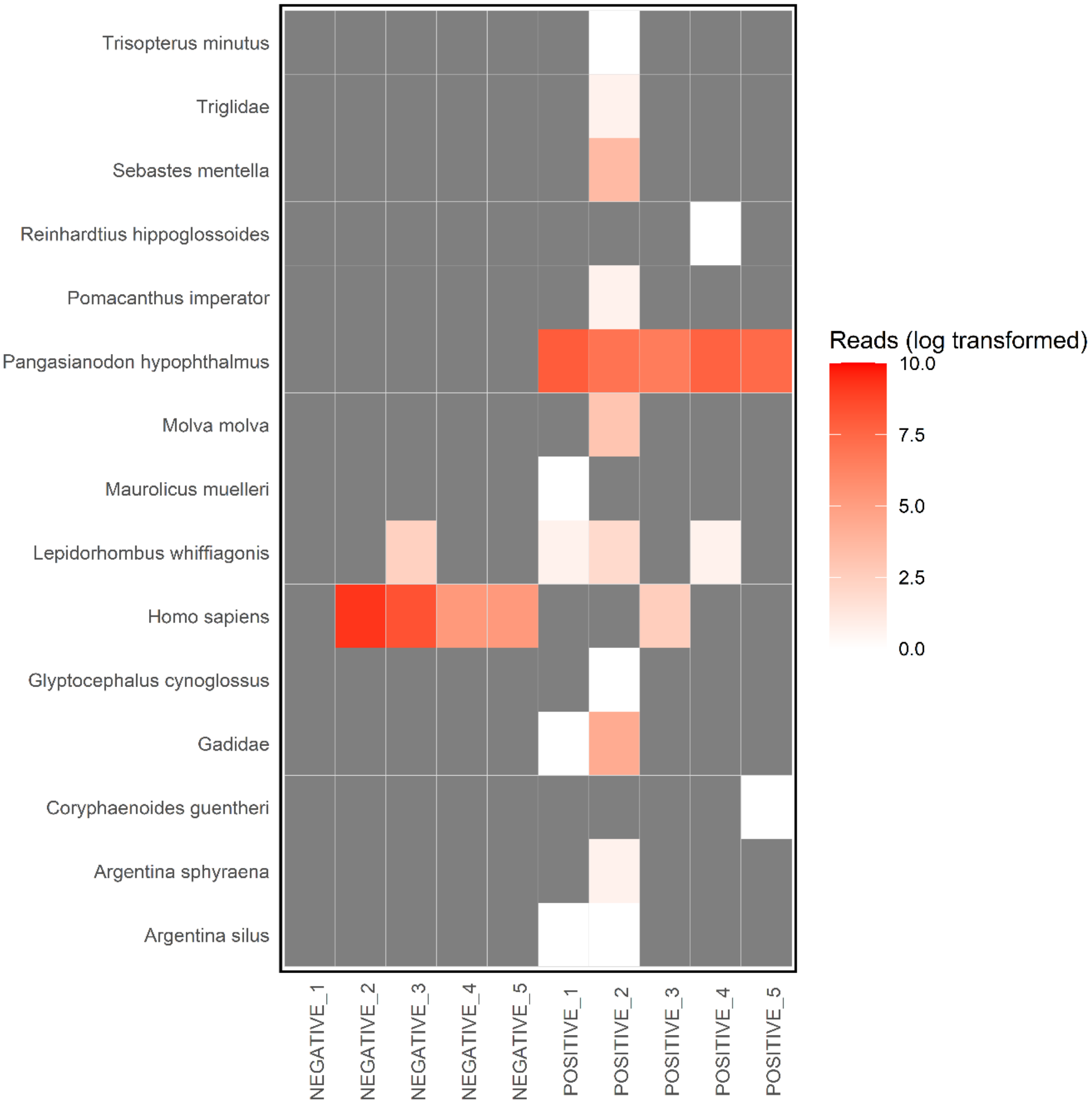
S.2 Chapter 2 Supplement



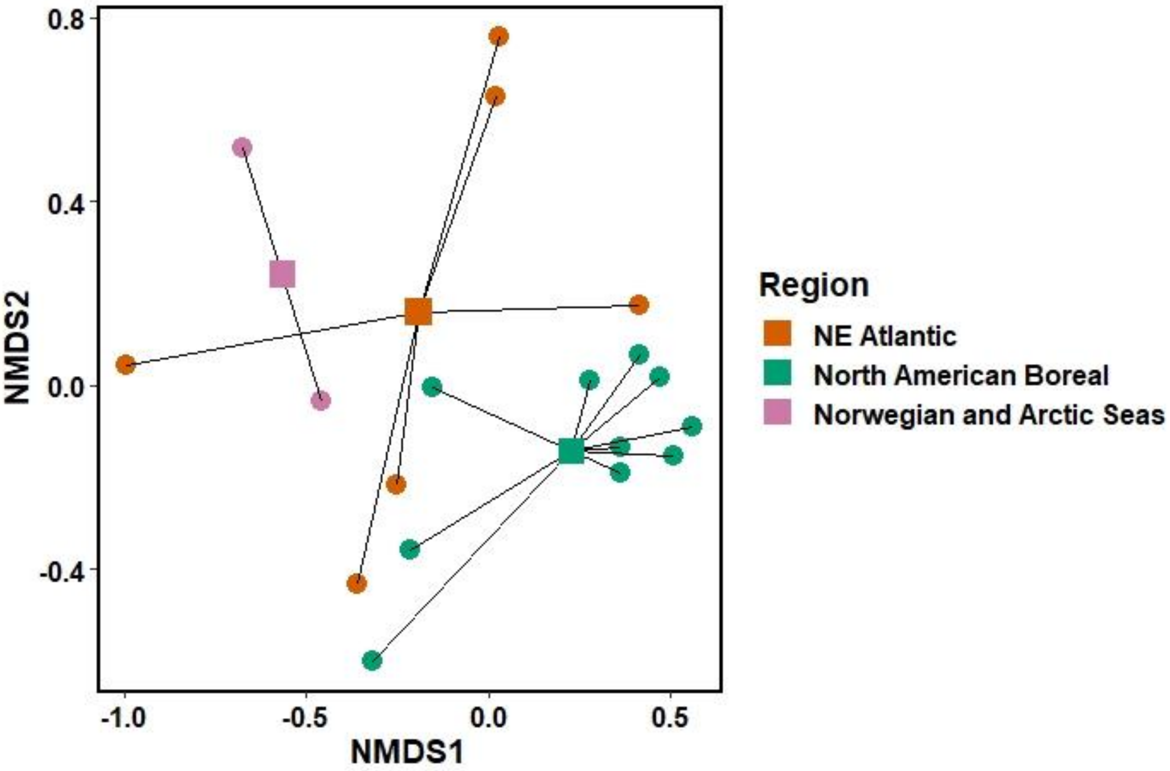
S2 Figure 1. Rarefaction curve with a dashed red line through x = 100 reads.



S2 Figure 2. Non-metric Multi-Dimensional Scaling (NMDS) plots conveying beta diversity from teleost and elasmobranch detections. **A** NMDS plot of a Jaccard dissimilarity matrix containing all teleost and elasmobranch MOTUs which could be assigned to either genus or species level (88). **B** NMDS plot of a Jaccard dissimilarity matrix containing all teleost and elasmobranch MOTUs which could be assigned to species level (65) (i.e., the same data plotted in Figure 2A). **C** NMDS plot of a Jaccard dissimilarity matrix of species-level MOTUs, where shapes are sponge species. **D** NMDS plot of a Bray-Curtis dissimilarity matrix of species-level MOTUs, where shapes are sponge species.



S2 Figure 3. Heat map showing log-transformed reads in controls. An explanation of how contamination was dealt with can be found in the Methods section of the main manuscript. Grey tiles indicate that no reads were present in controls for a given taxon.



S2 Figure 4. Non-metric Multi-Dimensional Scaling (NMDS) plot of a Jaccard dissimilarity species matrix of teleosts and elasmobranchs detected from only *Geodia barretti* samples (N = 21), where points are coloured by North Atlantic biogeographic region. The centroids of each biogeographic region are indicated by squares.

S2 Table 1. List of sponge specimens analysed (N = 54), where Gb = *Geodia barretti*, Gh = *Geodia henstcheli*, and Pv = *Phakellia ventilabrum.* The sponge abbreviations are used both in the 'Short ID' column and the 'Species' column. Other abbreviations: Lat = latitude in decimal degrees, Lon = longitude in decimal degrees, MPA = marine protected area, and UK = unknown.

short ID	long ID	Species	Aggregation	Region	Lat	Lon	Depth (m)	MPA Status	Month	Year
1.Gb	Gb_FS_MPA_14N4_s_50	Gb	Faroe Shetland Sponge Belt	NE Atlantic	60.83583	-3.03717	525	MPA	August	2018
2.Gb	Gb_FS_MPA_20T6_s_49	Gb	Faroe Shetland Sponge Belt	NE Atlantic	60.83583	-3.03717	525	MPA	August	2018
3.Gb	Gb_FS_MPA_22V2_s_51	Gb	Faroe Shetland Sponge Belt	NE Atlantic	60.83583	-3.03717	525	MPA	August	2018
4.Gb	Gb_RSB_MPA_13M4_d_74	Gb	W Rosemary bank	NE Atlantic	59.12883	-10.8078	1304	MPA	September	2016
5.Gb	Gb_RSB_MPA_13M5_d_72	Gb	W Rosemary bank	NE Atlantic	59.12883	-10.8078	1304	MPA	September	2016
6.Gb	Gb_RSB_MPA_18R4_d_73	Gb	W Rosemary bank	NE Atlantic	59.12883	-10.8078	1304	MPA	September	2016
7.Gb	Gb_Sva_20T5_s_25	Gb	Trondheimsfjorden	NE Atlantic	63.58612	9.84555	191	No MPA	UK	2016
8.Pv	Pv_FS_MPA_01601_s_52	Pv	Faroe Shetland Sponge Belt	NE Atlantic	60.83583	-3.03717	525	MPA	August	2018
9.Pv	Pv_FS_MPA_01602_s_26	Pv	Faroe Shetland Sponge Belt	NE Atlantic	60.83583	-3.03717	525	MPA	August	2018
10.Pv	Pv_FS_MPA_01603_s_53	Pv	Faroe Shetland Sponge Belt	NE Atlantic	60.83583	-3.03717	525	MPA	August	2018
11.Pv	Pv_NoS_11452_s_08	Pv	North of Shetland	NE Atlantic	60.454	0.538	131	No MPA	January	2019
12.Pv	Pv_NoS_11456_s_22	Pv	North of Shetland	NE Atlantic	61.116	0.175	155	No MPA	January	2019
13.Pv	Pv_NoS_Notag_s_09	Pv	North of Shetland	NE Atlantic	61.116	0.175	155	No MPA	January	2019
14.Pv	Pv_NwK_ST5_38_s_29	Pv	Norway	NE Atlantic	59.81359	5.603875	259	No MPA	September	2016
15.Pv	Pv_NwK_ST5_39_s_30	Pv	Norway	NE Atlantic	59.81359	5.603875	259	No MPA	September	2016

16.Pv	Pv_RB_11218_s_13	Pv	Rockall Bank	NE Atlantic	57.52475	-13.4195	173.5	No MPA	September	2018
17.Pv	Pv_RB_11221_s_23	Pv	Rockall Bank	NE Atlantic	57.52475	-13.4195	173.5	No MPA	September	2018
18.Pv	Pv_RB_1418_s_15	Pv	Rockall Bank	NE Atlantic	57.1705	-13.638	187	No MPA	May	2018
19.Pv	Pv_RB_1420_s_16	Pv	Rockall Bank	NE Atlantic	57.1705	-13.638	187	No MPA	May	2018
20.Pv	Pv_RB_MPA_11417_s_20	Pv	Rockall Bank	NE Atlantic	56.7755	-14.4681	189.5	MPA	September	2018
21.Pv	Pv_RB_MPA_11419_s_21	Pv	Rockall Bank	NE Atlantic	56.7755	-14.4681	189.5	MPA	September	2018
22.Pv	Pv_RB_MPA_1865_s_24	Pv	Rockall Bank	NE Atlantic	56.905	-14.741	186	MPA	April	2018
23.Pv	Pv_RB_MPA_1866_s_14	Pv	Rockall Bank	NE Atlantic	56.905	-14.741	186	MPA	April	2018
24.Pv	Pv_SRT_046_26_s_11	Pv	Sula Reef	NE Atlantic	63.65222	9.758833	203	No MPA	October	2016
25.Pv	Pv_SRT_046_49_s_12	Pv	Sula Reef	NE Atlantic	63.65222	9.758833	203	No MPA	October	2016
26.Pv	Pv_SSNd_H044_01642_s_45	Pv	Shetland Shelf North	NE Atlantic	60.106	-4.808	496	No MPA	May	2018
27.Pv	Pv_SSNd_H044_01643_s_46	Pv	Shetland Shelf North	NE Atlantic	60.106	-4.808	496	No MPA	May	2018
28.Pv	Pv_SSNd_H044_01645_s_47	Pv	Shetland Shelf North	NE Atlantic	60.106	-4.808	496	No MPA	May	2018
29.Pv	Pv_Sw_11_s_05	Pv	Sweden	NE Atlantic	58.89083	11.09502	90	No MPA	March	2019
30.Pv	Pv_Sw_9_s_04	Pv	Sweden	NE Atlantic	58.89083	11.09502	90	No MPA	March	2019
31.Gb	Gb_Can_M_Trip8_75.1_d_69	Gb	Arctic NAFO 0A_0B (close to Paamiut)	North American Boreal	66.80795	-58.6674	903	No MPA	October	2016
32.Gb	Gb_Can_M_Trip8_75.2_d_70	Gb	Arctic NAFO 0A_0B (close to Paamiut)	North American Boreal	66.80795	-58.6674	903	No MPA	October	2016
33.Gb	Gb_Can_M_Trip8_75.3_d_71	Gb	Arctic NAFO 0A_0B (close to Paamiut)	North American Boreal	66.80795	-58.6674	903	No MPA	October	2016

34.Gb	Gb_Can_S_15O6_d_75	Gb	Davis Strait	North American Boreal	62.5184	-59.9705	1332	No MPA	October	2014
35.Gb	Gb_Can_S_16P6_s_55	Gb	Davis Strait	North American Boreal	62.56012	-61.3943	568	No MPA	October	2014
36.Gb	Gb_Can_S_17Q3_d_77	Gb	Davis Strait	North American Boreal	62.0539	-60.0918	1427	No MPA	October	2014
37.Gb	Gb_Can_S_23W6_d_78	Gb	Davis Strait	North American Boreal	61.8989	-60.1364	1440	No MPA	October	2014
38.Gb	Gb_Can_S_2B6_d_76	Gb	Davis Strait	North American Boreal	62.0539	-60.0918	1427	No MPA	October	2014
39.Gb	Gb_Can_S_5E2_s_44	Gb	Davis Strait	North American Boreal	62.19923	-61.2192	483	No MPA	October	2014
40.Gb	Gb_Can_S_8H6_s_54	Gb	Davis Strait	North American Boreal	63.26382	-60.3552	536	No MPA	October	2014
41.Gh	Gh_Can_M_Trip8_75.5_d_66	Gh	Arctic NAFO 0A_0B (close to Paamiut)	North American Boreal	66.80795	-58.6674	903	No MPA	October	2016
42.Gh	Gh_Can_M_Trip8_75.7_d_67	Gh	Arctic NAFO 0A_0B (close to Paamiut)	North American Boreal	66.80795	-58.6674	903	No MPA	October	2016
43.Gh	Gh_Can_M_Trip8_75.8_d_68	Gh	Arctic NAFO 0A_0B (close to Paamiut)	North American Boreal	66.80795	-58.6674	903	No MPA	October	2016
44.Gh	Gh_Ice_A11_641_B_s_25	Gh	Iceland	North American Boreal	66.63883	-12.7558	308.5	No MPA	UK	2019
45.Gh	Gh_Ice_ME85_3_1123_2_C_d_59	Gh	Iceland	North American Boreal	67.214	-26.1992	723.35	No MPA	September	2011

46.Gh	Gh_Ice_ME85_3_1123_B_d_60	Gh	Iceland	North American Boreal	67.214	-26.1992	723.35	No MPA	September	2011
47.Gh	Gh_JMR_SM_AGT03_26_d_93	Gh	Between Jan Mayen_Schultz Massif (Mohn)	North American Boreal	71.30333	-5.28733	1848.5	No MPA	July	2014
48.Gh	Gh_JMR_SM_AGT03_27_d_94	Gh	Between Jan Mayen_Schultz Massif (Mohn)	North American Boreal	71.30333	-5.28733	1848.5	No MPA	July	2014
49.Gh	Gh_Vst_019TVG_010B_d_91	Gh	Vesterisbanken	North American Boreal	73.61406	-9.03518	1784	No MPA	August	2019
50.Gb	Gb_BS_imp_9I4_s_39	Gb	Barents Sea	Norwegian and Arctic Seas	71.58797	21.37533	333	No MPA	March	2017
51.Gb	Gb_Sva_7G6_s_26	Gb	Svalbard	Norwegian and Arctic Seas	80.53853	15.36161	215	No MPA	September	2011
52.Pv	Pv_SMK_ROV25_12_s_33	Pv	Tromsø shelf	Norwegian and Arctic Seas	71.39517	16.81599	330	No MPA	UK	2017
53.Pv	Pv_SMK_ROV25_13_s_35	Pv	Tromsø shelf	Norwegian and Arctic Seas	71.39517	16.81599	330	No MPA	UK	2017
54.Pv	Pv_SMK_ROV25_14_s_36	Pv	Tromsø shelf	Norwegian and Arctic Seas	71.39517	16.81599	330	No MPA	UK	2017

S2 Table 2. Reads from (N = 74) samples sequenced, including controls (N = 10) and samples which were removed from the statistical analysis for having low reads (N = 10).

	Reads
Total	5,269,740
Total after 'illuminapairedend' step with Q40 threshold	4,565,067
Total after bioinformatic pipeline	3,091,641
Total assigned taxonomy (Eukaryota)	3,040,133
Total assigned to target taxa (Actinopterygii)	2,531,138
Total assigned to species-level (99% simlaity) Actinopterygii	1,778,145
Total assigned to species-level (99% simlaity) Actinopterygii, Elasmobranchii, Chondrichthyes	1,781,183
Total assigned to species-level (99% simlaity) Actinopterygii, Elasmobranchii, Chondrichthyes, Mammalia marine-only	1,782,119

S2 Table 3. Fish identified to species level at a similarity threshold of ≥ 99%, alphabetically by class.

Class	Order	Family	Common name	Scientific name	No. of Samples Detected
Actinopteri	Anguilliformes	Nettastomatidae	Blackfin sorcerer	*Nettastoma melanurum*	1
Actinopteri	Anguilliformes	Synaphobranchidae	Kaup's arrow-tooth eel	*Synaphobranchus kaupii*	6
Actinopteri	Argentiniformes	Argentinidae	Greater argentine	*Argentina silus*	46
Actinopteri	Argentiniformes	Argentinidae	Argentine	*Argentina sphyraena*	34
Actinopteri	Argentiniformes	Bathylagidae	Goiter blacksmelt	*Bathylagus euryops*	10
Actinopteri	Aulopiformes	Bathysauridae	Deepsea lizardfish	*Bathysaurus ferox*	1
Actinopteri	Aulopiformes	Chlorophthalmidae	Shortnose greeneye	*Chlorophthalmus agassizi*	5
Actinopteri	Aulopiformes	Paralepididae	spotted barracudina	*Arctozenus risso*	3
Actinopteri	Beloniformes	Belonidae	Garfish	*Belone belone*	1
Actinopteri	Beloniformes	Exocoetidae	Tropical two-wing flyingfish	*Exocoetus volitans*	3
Actinopteri	Caproiformes	Caproidae	Boarfish	*Capros aper*	5
Actinopteri	Carangiformes	Carangidae	Atlantic horse mackerel	*Trachurus trachurus*	17
Actinopteri	Clupeiformes	Clupeidae	Atlantic herring	*Clupea harengus*	19
Actinopteri	Clupeiformes	Clupeidae	European pilchard	*Sardina pilchardus*	3
Actinopteri	Clupeiformes	Clupeidae	European sprat	*Sprattus sprattus*	4
Actinopteri	Gadiformes	Gadidae	Norway pout	*Trisopterus esmarkii*	31
Actinopteri	Gadiformes	Gadidae	Poor cod	*Trisopterus minutus*	44
Actinopteri	Gadiformes	Gadidae	Blue whiting	*Micromesistius poutassou*	33
Actinopteri	Gadiformes	Gadidae	Pouting	*Trisopterus luscus*	1
Actinopteri	Gadiformes	Gadidae	Atlantic cod	*Gadus morhua*	33
Actinopteri	Gadiformes	Gadidae	Pollock; Saithe	*Pollachius virens*	22
Actinopteri	Gadiformes	Gadidae	Silvery pout	*Gadiculus argenteus*	27
Actinopteri	Gadiformes	Lotidae	Blue ling	*Molva dypterygia*	14
Actinopteri	Gadiformes	Lotidae	Common ling	*Molva molva*	36
Actinopteri	Gadiformes	Lotidae	Cusk	*Brosme brosme*	10

Actinopteri	Gadiformes	Macrouridae	Günther's grenadier	*Coryphaenoides guentheri*	10
Actinopteri	Gadiformes	Macrouridae	Mediterranean grenadier	*Coryphaenoides mediterraneus*	1
Actinopteri	Gadiformes	Macrouridae	Roundnose grenadier	*Coryphaenoides rupestris*	10
Actinopteri	Gadiformes	Macrouridae	Shortbeard grenadier	*Coryphaenoides brevibarbis*	1
Actinopteri	Gadiformes	Macrouridae	Roughhead grenadier	*Macrourus berglax*	15
Actinopteri	Gadiformes	Merlucciidae	European hake	*Merluccius merluccius*	34
Actinopteri	Gadiformes	Moridae	Slender codling	*Halargyreus johnsonii*	2
Actinopteri	Labriformes	Labridae	Cuckoo wrasse	*Labrus mixtus*	2
Actinopteri	Lophiiformes	Lophiidae	Angler	*Lophius piscatorius*	32
Actinopteri	Myctophiformes	Myctophidae	Lanternfish	*Notoscopelus elongatus*	9
Actinopteri	Myctophiformes	Myctophidae	Arctic telescope	*Protomyctophum arcticum*	9
Actinopteri	Myctophiformes	Myctophidae	Rakery beaconlamp	*Lampanyctus macdonaldi*	5
Actinopteri	Notacanthiformes	Notacanthidae	Smallmouth spiny eel	*Polyacanthonotus rissoanus*	4
Actinopteri	Perciformes	Cyclopteridae	Lumpfish	*Cyclopterus lumpus*	1
Actinopteri	Perciformes	Gasterosteidae	Three-spined stickleback	*Gasterosteus aculeatus*	1
Actinopteri	Perciformes	Liparidae	Threadfin seasnail	*Rhodichthys regina*	2
Actinopteri	Perciformes	Sebastidae	Beaked redfish	*Sebastes mentella*	51
Actinopteri	Perciformes	Zoarcidae	Atlantic eelpout	*Lycodes terraenovae*	17
Actinopteri	Perciformes	Zoarcidae	Moray wolf eel	*Lycenchelys muraena*	9
Actinopteri	Perciformes	Zoarcidae	Vahl's eelpout	*Lycodes vahlii*	4
Actinopteri	Pleuronectiformes	Bothidae	Mediterranean scaldfish	*Arnoglossus laterna*	2
Actinopteri	Pleuronectiformes	Pleuronectidae	Witch flounder	*Glyptocephalus cynoglossus*	18
Actinopteri	Pleuronectiformes	Pleuronectidae	Greeenland halibut	*Reinhardtius hippoglossoides*	52
Actinopteri	Pleuronectiformes	Scophthalmidae	Turbot	*Scophthalmus maximus*	6
Actinopteri	Pleuronectiformes	Scophthalmidae	Megrim	*Lepidorhombus whiffiagonis*	50
Actinopteri	Pleuronectiformes	Soleidae	Common sole	*Solea solea*	2
Actinopteri	Pleuronectiformes	Soleidae	Thickback sole	*Microchirus variegatus*	8
Actinopteri	Scombriformes	Scombridae	Atlantic mackerel	*Scomber scombrus*	48
Actinopteri	Scombriformes	Trichiuridae	Black scabbardfish	*Aphanopus carbo*	3
Actinopteri	Scombriformes	Trichiuridae	Silver scabbardfish	*Lepidopus caudatus*	3
Actinopteri	Squaliformes	Etmopteridae	Black dogfish	*Centroscyllium fabricii*	2
Actinopteri	Stomiiformes	Sternoptychidae	Silvery lightfish	*Maurolicus muelleri*	25
Actinopteri	Syngnathiformes	Mullidae	Striped red mulllet	*Mullus surmuletus*	1
Actinopteri	Zeiformes	Zeidae	John Dory	*Zeus faber*	2
Chondrichthyes	Chimaeriformes	Chimaeridae	Rabbit fish	*Chimaera monstrosa*	17
Chondrichthyes	Chimaeriformes	Chimaeridae	Small-eyed rabbitfish fish	*Hydrolagus affinis*	4
Chondrichthyes	Chimaeriformes	Chimaeridae	Large-eyed rabbitfish	*Hydrolagus mirabilis*	1
Chondrichthyes	Carcharhiniformes	Scyliorhinidae	Lesser spotted dogfish	*Scyliorhinus canicula*	1
Chondrichthyes	Rajiformes	Rajidae	Blue skate	*Dipturus batis*	3
Chondrichthyes	Rajiformes	Rajidae	Shagreen ray	*Leucoraja fullonica*	1

S2 Table 4. Results of the multivariate homogeneity of group dispersions (*betadisper* and *anova* functions), permutational multivariate analysis of variance (i.e., PERMANOVA, *adonis* function), including pairwise comparisons and fits of environmental vectors (*envfit* function) of fish OTUs identified to the species level: a-b) between groups; c-d) between groups (i.e., biogeographic region) and treatments (pairwise comparisons); e) goodness of fit of environmental vectors: latitude, depth and sampling year. Tests are based on Jaccard's dissimilarity distances and 1000 permutations. P(BH): P value corrected with the Benjamini-Hochberg method. Significant P values are bolded. All functions are available in the R package vegan.

a) Multivariate homogeneity of group dispersions analysis between biogeographic regions.

Source	DF	Sq	Mean Sq	F Value	P value
Region	2	0.04797	0.023987	1.9433	0.1537
Residuals	51	0.62953	0.012344		

b) Multivariate homogeneity of group dispersions analysis between sponge species.

Source	DF	Sq	Mean Sq	F Value	P value
Sponge	2	0.08152	0.040759	4.8151	**0.01215**
Residuals	51	0.43170	0.008465		

c) PERMANOVA between biogeographic regions, sponge species, and the biogeographic region:sponge species interaction. *****The sponge species had heterogenous group dispersions.

Source	DF	Sums of Sqs	Mean Sqs	F Model	R^2	P value
Region	2	1.7391	0.86955	5.0062	0.16411	**0.000999**
Sponge	2	0.8833	0.44165	2.6795	0.08335	**0.001998***
Region:Sponge	1	0.0635	0.06353	0.3854	0.00599	0.978022*****
Residuals	48	7.9116	0.16482		0.74655	
Total	53	10.5975			1.00000	

d) PERMANOVA for pairwise comparisons between each combination of biogeographic regions.

Source	DF	Sums of Sqs	Mean Sqs	F Model	R^2	P value	P(BH)
Northeast Atlantic; North American Boreal	1	1.3782	1.37824	7.8209	0.14266	**0.000999**	**0.001498501**
Residuals	47	8.2826	0.17622	0.85734			
Total	48	9.6608	1.00000				
Northeast Atlantic; Norwegian and Arctic Seas	1	0.3034	0.30341	1.9246	0.05511	**0.02498**	**0.024975025**
Residuals	33	5.2025	0.15765	0.94489			
Total	34	5.5059	1.00000				
North American Boreal; Norwegian and Arctic Seas	1	0.6566	0.65659	3.4135	0.13432	**0.000999**	**0.001498501**
Residuals	22	4.2317	0.19235	0.86568			
Total	23	4.8883	1.00000				

e) Correlation of environmental vectors to site ordination scores.

Vector	NMDS1	NMDS2	R^2	P value
Latitude	0.91612	-0.40091	0.3496	**0.000999**
Sampling Depth	0.95229	0.30518	0.5756	**0.000999**
Sampling Year	-0.99819	0.06007	0.1679	**0.017982**

S2 Table 5. Results of the multivariate homogeneity of group dispersions (*betadisper* and *anova* functions), permutational multivariate analysis of variance (i.e., PERMANOVA, *adonis* function), including pairwise comparisons: a-b) between groups; c-d) between groups (i.e., populations, marine protected area (MPA) status), e) pairwise comparisons between populations. Tests are based on Jaccard's dissimilarity distances and 1000 permutations. P(BH): P value corrected with the Benjamini-Hochberg method. Significant P values are bolded. All functions are available in the R package vegan.

a) Multivariate homogeneity of group dispersions analysis between *P. ventilabrum* populations.

Source	DF	Sq	Mean Sq	F Value	P value
Groups	6	0.058586	0.0097643	0.927	0.5018
Residuals	16	0.168533	0.0105333		

b) Multivariate homogeneity of group dispersions analysis between *P. ventilabrum* specimens collected within verses outside MPAs.

Source	DF	Sq	Mean Sq	F Value	P value
Groups	1	0.003008	0.0030083	0.4077	0.5301
Residuals	21	0.154968	0.0073795		

c) PERMANOVA between *P. ventilabrum* populations.

Source	DF	Sums of Sqs	Mean Sqs	F Model	R^2	P value
Groups	6	1.5167	0.252779	2.7152	0.50451	**0.000999**
Residuals	16	8.8584	0.17369	0.83589		
Total	22	10.5975	1.00000			

d) PERMANOVA between *P. ventilabrum* specimens collected within verses outside MPAs.

Source	DF	Sums of Sqs	Mean Sqs	F Model	R^2	P value
Groups	1	0.26186	0.26186	2.0038	0.08711	**0.02597**
Residuals	21	2.74436	0.13068	0.91289		
Total	22	3.00622	1.00000			

e) PERMANOVA for pairwise comparisons between each combination of *P. ventilabrum* locations. The abbreviations refer to the following sponge locations: 'fs'= Faroe Shetland Sponge Belt; 's'= North of Shetland; 'nk'= Norway; 'rb'= Rockall Bank; 'ss'= Shetland Shelf; 'tf'= Sula Reef; and 'sw'= Sweden.

Pairwise Comparison	P value	P(BH)	Pairwise Comparison	P value	P(BH)
fs_s	0.100000000	0.1400000	nk_rb	0.050949051	0.1400000
fs_nk	0.100000000	0.1400000	nk_ss	0.100000000	0.1400000
fs_rb	**0.005994006**	0.1258741	nk_tf	1.000000000	1.0000000
fs_ss	0.100000000	0.1400000	nk_sw	0.666666667	0.7000000
fs_tf	0.100000000	0.1400000	rb_ss	0.071928072	0.1400000
fs_sw	0.100000000	0.1400000	rb_tf	0.050949051	0.1400000
nk_s	0.500000000	0.5526316	rb_sw	0.053946054	0.1400000
rb_s	0.155844156	0.2045455	ss_sw	0.100000000	0.1400000
ss_s	0.100000000	0.1400000	ss_tf	0.100000000	0.1400000
tf_s	0.300000000	0.3705882	sw_tf	0.333333333	0.3888889
sw_s	0.100000000	0.1400000			

S2 Table 6. Results of the indicator value species analysis (*multipatt* function) and multilevel pattern analysis (*IndVal.g* method): a-b) sampling depth ranges; c-d) biogeographic regions; e-f) MPA status in the Northeast Atlantic with *P. ventilabrum* samples. Tests underwent 10000 permutations and the number of indicator species, indicator species for both depth range and biogeographic region as well as significant P values are bolded. “A” is the estimate probability that samples are associated to the matched group(s) if the indicator species has been detected in the sample (i.e., specificity or predictive value). “B” is the estimate probability of detecting the indicator species in the matched group(s) (i.e., sensitivity). “Stat” is the indicator value index which suggests the strength of the indicator species association to the group(s). Associations can be positive or negative. Groups separated with a “+” denote an additional group association to the indicator species. All functions are available in the R package Multipatt.

a) Summary table of indicator value and multilevel pattern analysis for sampling depth ranges.

Total number of Species	65
Selected number of Species	**16**
Number of species associated to 1 group	2
“ 2 groups	5
“ 3 groups	5
“ 4 groups	3
“ 5 groups	1

b) Results of the indicator value and multilevel pattern analysis for sampling depth ranges.

Species	Depth Range Group(s) separated by ‘+’	A	B	Stat	P value
Lycodes vahlii	500-800	1.0	0.4	0.632	**0.0181**
Hydrolagus affinis	1200-1600	0.8571	0.4286	0.606	**0.208**
Bathylagus euroyops	800-1200 + 1200-1600	0.8947	0.6154	0.742	**0.0038**
Coryphaenoides guentheri	800-1200 + 1200-1600	0.7471	0.4615	0.587	**0.0401**
Lampanyctus macdonaldi	800-1200 + 1200-1600	1.0000	0.3846	0.620	**0.0247**
Macrourus berglax	800-1200 + 1200-1600	0.7888	0.7692	0.779	**0.0001**
Protomyctophum arcticum	500-800 + 800-1200	0.8673	0.5000	0.659	**0.0068**
Lycodes terraenovae	800-1200 + 1200-1600 + >1600	0.7453	0.6250	0.683	**0.0133**
Clupea harengus	80-200 + 200-500 + 500-800	1.0000	0.5000	0.707	**0.0082**
Glyptocephalus cynoglossus	80-200 + 200-500 + 500-800	1.0000	0.4737	0.688	**0.0135**
Pollachius virens	80-200 + 200-500 + 500-800	1.0000	0.5789	0.761	**0.0015**
Trisopterus esmarkii	80-200 + 200-500 + 500-800	1.0000	0.8158	0.903	**0.0001**
Argentina sphyraena	80-200 + 200-500 + 500-800 + 1200-1600	0.9451	0.7333	0.833	**0.0091**
Gadiculus argenteus	80-200 + 200-500 + 500-800 + 1200-1600	1.0000	0.6000	0.775	**0.0073**
Gadus morhua	80-200 + 200-500 + 500-800 + 1200-1600	1.0000	0.7333	0.856	**0.0003**
Maurolicus muelleri	80-200 + 500-800 + 800-1200 + 1200-1600 + >1600	0.9489	0.5750	0.739	**0.0274**

c) Summary table of indicator value and multilevel pattern analysis for biogeographic regions.

Total number of Species	65
Selected number of Species	**8**
Number of species associated to 1 group	4
“ 2 groups	4

d) Results of the indicator value and multilevel pattern analysis for biogeographic regions.

Species	Group(s) separated by ‘+’	A	B	Stat	P value
Glyptocephalus cynoglossus	Northeast Atlantic	0.8352	0.5333	0.667	**0.0437**
Microchirus variegatus	Northeast Atlantic	1.0000	0.2667	0.516	**0.0381**
Bathylagus euroyops	North American Boreal	0.8633	0.4211	0.603	**0.0414**
Macrourus berglax	North American Boreal	0.8633	0.6316	0.738	**0.0012**

Gadiculus argenteus	Northeast Atlantic + Norwegian and Arctic Seas	1.0000	0.7714	0.878	**0.0001**
Gadus morhua	Northeast Atlantic + Norwegian and Arctic Seas	0.8588	0.8000	0.829	**0.0007**
Pollachius virens	Northeast Atlantic + Norwegian and Arctic Seas	0.9629	0.6000	0.760	**0.0092**
Trisopterus esmarkii	Northeast Atlantic + Norwegian and Arctic Seas	0.9180	0.8000	0.857	**0.0001**

e) Summary table of indicator value and multilevel pattern analysis for MPA status in the Northeast Atlantic with *P. ventilabrum* samples.

Total number of Species	58
Selected number of Species	**4**
Number of species associated to 1 group	4

f) Results of the indicator value and multilevel pattern analysis for MPA status.

Species	Group	A	B	Stat	P value
Lycenchelys muraena	MPA	0.9999	0.5714	0.756	**0.0069**
Lycodes terraenovae	MPA	0.9998	0.5714	0.756	**0.0074**
Protomyctophum arcticum	MPA	1.0000	0.4286	0.655	**0.0176**
Lycodes vahlii	MPA	1.0000	0.4286	0.655	**0.0176**

S2 Table 7. Results of the permutational multivariate analysis of variance (i.e., PERMANOVA, *adonis2* function (*adonis* now deprecated)), including pairwise comparisons of fish OTUs identified to the species level for ONLY *Geodia barretti* samples (N = 21): A) between groups; B) between treatments (i.e. pairwise comparisons). Tests are based on Jaccard's dissimilarity distances and 1000 permutations. P(BH): P value corrected with the Benjamini-Hochberg method. Significant P values are bolded. All functions are available in the R package vegan.

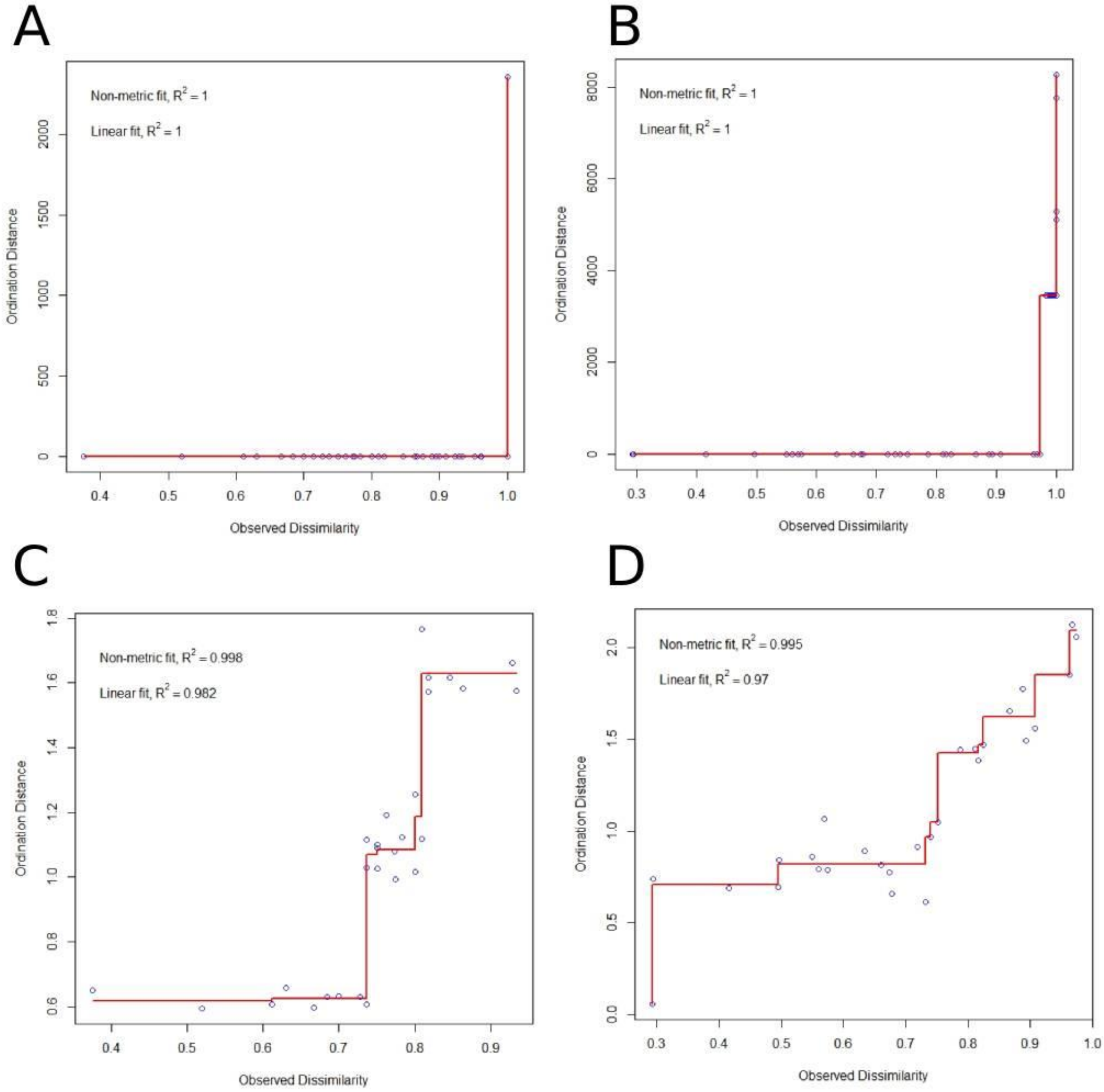
A) PERMANOVA between biogeographic regions from *Geodia barretti* samples.

Source	DF	Sums of Sqs	R^2	F	P value
Groups	2	0.6425	0.19848	1.8572	**0.01898**
Residuals	15	2.5945	0.80152		
Total	17	3.2370	1.00000		

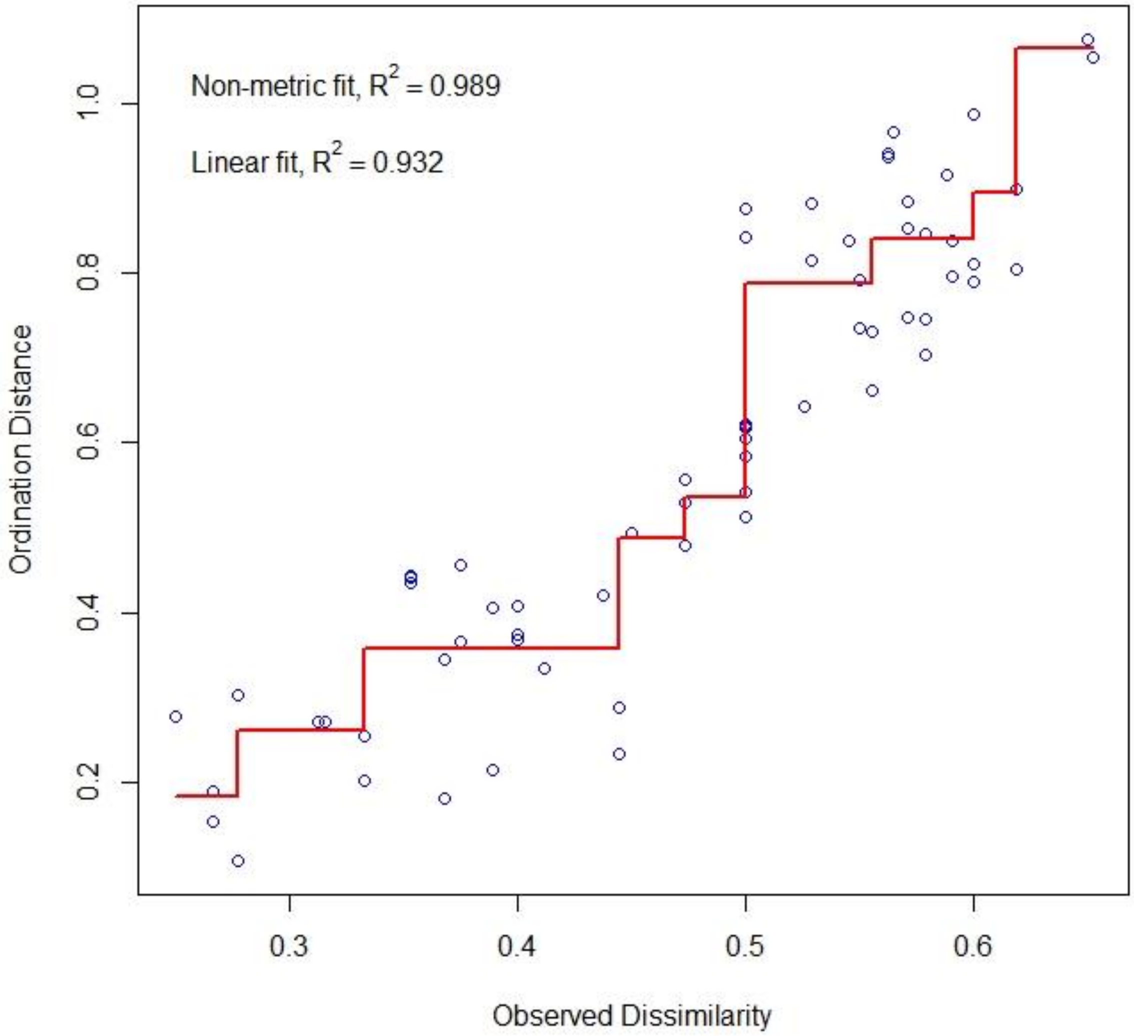
B) PERMANOVA for pairwise comparisons between each combination of biogeographic regions from Geodia barretti samples.

Source	DF	Sums of Sqs	R^2	F	P value	P(BH)
Northeast Atlantic; North American Boreal	1	0.3554	.01303	2.0974	**0.02597**	0.05094905
Residuals	14	2.3723	0.8697			
Total	15	2.7277	1.000			
Northeast Atlantic; Norwegian and Arctic Seas	1	0.1605	0.10525	0.7058	0.8042	0.80419580
Residuals	6	1.3644	0.89475			
Total	7	1.5250	1.00000			
North American Boreal; Norwegian and Arctic Seas	1	0.38285	.020862	2.6362	**0.03397**	0.05094905
Residuals	10	1.45226	0.79138			
Total	11	1.83511	1.00000			

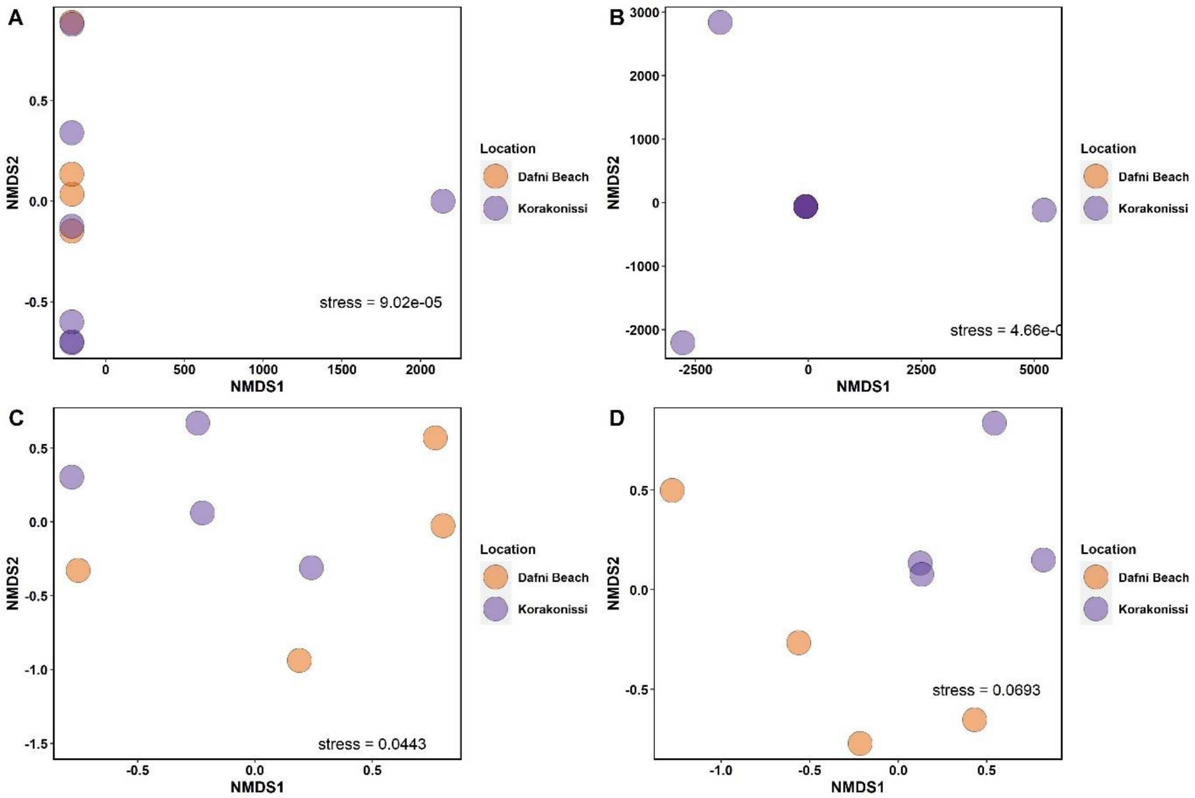
S.3 Chapter 3 Supplement



S3 Figure 1. Stress plots of eDNA ordination data. **A** Stress plot of Jaccard dissimilarity including all eDNA samples. **B** Stress plot of Bray-Curtis dissimilarity including all eDNA samples. **C** Stress plot of Jaccard dissimilarity with the three outlying samples are removed. **D** Stress plot of Bray-Curtis dissimilarity with the three outlying samples are removed.



S3 Figure 2. Stress plot of Jaccard dissimilarity calculated from UVC data.



S3 Figure 3. NMDS plots of eDNA ordination data. All points are made slightly transparent, so darker shades (relative to legend) represent overlapping points. **A** NMDS of Jaccard dissimilarity including all eDNA samples. **B** NMDS of Bray-curtis dissimilarity including all eDNA samples. **C** NMDS of Jaccard dissimilarity with the three outlying samples are removed. **D** NMDS of Bray-Curtis dissimilarity with the three outlying samples are removed (Same data as Figure 5D).

S3 Table 1. Left column showing MOTUs which were unassigned, assigned, assigned to target taxa and target taxa at genus or species level over the three steps of taxonomic assignment. Right column showing number of taxa for each taxonomic level after MOTUs are collapsed over the three steps of taxonomic assignment. The final dataset information is in row three, which combines all taxonomic assignment methods.

	MOTUs				**Collapsed Taxonomy**				
	Unassigned MOTUs	Assigned MOTUs	Target Taxa MOTUs	Target Taxa MOTUs at Genus or Species Level	Species (100% - 98%)	Genus (100% - 98%)	Family (100% - 90%)	Order (100% - 80%)	Class and Phylum (100% - 70%)
1. ecotag w/ EMBL database of target taxa only	537	535	535	285	23	20	42	6	49
2. BLCA w/ custom reference database	560	512	238	217	18	31	33	4	2
3. Combined methods w/ blast	269	803	536	417	42	17	8	3	6

S3 Table 2. MOTUs which were assigned to species level after using the NCBI Blast tool.

MOTU id	best_identity_final	Manual Taxanomic assignment
gre2_000110514	100	*Argyrosomus regius*
gre2_000111343	100	*Atherinomorus forskalii*
gre2_000120754	100	*Diplodus sargus*
gre2_000123570	100	*Epinephelus marginatus*
gre2_000002161	99.4	*Sardinella aurita*
gre2_000110418	99.26	*Sarpa salpa*
gre2_000000080	100	*Siganus luridus*
gre2_000013666	100	*Siganus rivulatus*
gre2_000000578	100	*Sparisoma cretense*
gre2_000110705	99.3939394	*Symphodus cinereus*
gre2_000110841	99.39	*Symphodus melops*
gre2_000000028	99.38	*Thalassoma pavo*
gre2_000111411	99.38	*Tylosurus crocodilus*

S4 Table 3. Contamination found in controls and the corresponding number of reads which were removed from the dataset.

Contaminant	Found In	Reads	Reads removed from Dataset
Atherina hepsetus	sample.DBE_filterstart	2	20
Atherina sp.	sample.DBM_fieldblank	1	10
Dicentrarchus labrax	sample.pcrpositive4	1	not present in other samples
Gobius bucchichi	sample.pcrpositive	1	10
Lagodon rhomboides	sample.pcrnegative4	42	not present in other samples
Odonus niger	sample.DBE_filterend	1	10
Scomber scombrus	sample.DBE_filterstart	2	20
Sprattus sprattus	sample.pcrpositive4	1	10
Symphodus bailloni	sample.pcrpositive4	1	not present in other samples
Thalassoma pavo	sample.pcrnegative	1	10
Thalassoma sp.	sample.pcrnegative4	82	820

S4 Table 4. Species and genus detections displayed in Figure 3.4.

Label #	scientific_name_final	Venn
1	Argyrosomus regius	eDNA
2	Atherina boyeri	eDNA
3	Atherina hepsetus	eDNA
4	Chlorophthalmus agassizi	eDNA
5	Symphodus melops	eDNA
6	Gobius bucchichi	eDNA
7	Gobius paganellus	eDNA
8	Oedalechilus labeo	eDNA
9	Sardina pilchardus	eDNA
10	Sardinella aurita	eDNA
11	Scartella cristata	eDNA
12	Scomber scombrus	eDNA
13	Symphodus cinereus	eDNA
14	Enchelycore anatina	eDNA
15	Atherinomorus forskalii	eDNA
16	Fistularia commersonii	eDNA
17	Odonus niger	eDNA
18	Siganus rivulatus	eDNA
19	Tylosurus crocodilus	eDNA
20	Apogon imberbis	Both
21	Diplodus sargus	Both
22	Epinephelus marginatus	Both
23	Mullus surmuletus	Both
24	Sarpa salpa	Both
25	Sparisoma cretense	Both
26	Thalassoma pavo	Both
27	Siganus luridus	Both
28	Caranx crysos	UVC
29	Chromis chromis	UVC
30	Coris julis	UVC
31	Diplodus annularis	UVC
32	Diplodus vulgaris	UVC
33	Epinephelus costae	UVC
34	Lithognathus mormyrus	UVC
35	Muraena helena	UVC
36	Oblada melanura	UVC
37	Parablennius gattorugine	UVC
38	Seriola dumerili	UVC
39	Serranus scriba	UVC

#	Genus	Venn
1	Argyrosomus	eDNA
2	Atherinomorus	eDNA

3	Callionymus	eDNA
4	Chelon	eDNA
5	Chlorophthalmus	eDNA
6	Enchelycore	eDNA
7	Fistularia	eDNA
8	Labrus	eDNA
9	Lutjanus	eDNA
10	Odonus	eDNA
11	Oedalechilus	eDNA
12	Pomatoschistus	eDNA
13	Sardina	eDNA
14	Sardinella	eDNA
15	Scartella	eDNA
16	Scomber	eDNA
17	Tylosurus	eDNA
18	Apogon	Both
19	Atherina	Both
20	Chromis	Both
21	Diplodus	Both
22	Epinephelus	Both
23	Gobius	Both
24	Mullus	Both
25	Sarpa	Both
26	Serranus	Both
27	Siganus	Both
28	Sparisoma	Both
29	Symphodus	Both
30	Thalassoma	Both
31	Caranx	UVC
32	Coris	UVC
33	Lithognathus	UVC
34	Muraena	UVC
35	Oblada	UVC
36	Parablennius	UVC
37	Scorpaena	UVC
38	Seriola	UVC

S.4 Chapter 4 Supplement

S4 Methods:

Metaprobe Material Preparation and Field Sampling Methods

These methods have been written for a non-technical audience and provide instructions for how volunteers can prepare metaprobes. The cotton gauze (~ 10 cm x 3.7 m) should be certified sterile and can be found on various medical supply websites. The same methods were followed if metaprobes were prepared in a laboratory, except that after preparation they were treated with 30 min of UV.

Preparing Metaprobes

1. Rinse dirty metaprobes with tap water, removing any residue or salt. (If not new)

2a. Prepare a bleach solution to clean the dirty metaprobes and dirty scissors. Wearing medical gloves, fill one wash basin with a solution made up of 2 parts bleach cleaning chemical and 3 parts bottled drinking water. The depth of the solution should be enough to submerge at least one half of a metaprobe. This bleach solution is ideally 10% bleach, however most household cleaning equipment will not be this powerful. Aim to make the bleach solution strong enough to smell if you are standing directly next to it. Proceed to step 3a.

2b. If it is not possible to do step 2a, follow this alternate step. Locate paper towels and a cleaning chemical containing bleach. Proceed to step 3b.

3a. Soak the dirty metaprobes and scissors in the bleach solution. The metaprobes should soak for at least half an hour (30 min.). They can be left overnight. The scissors should soak for ~10 min. since the steel will rust if left too long. Proceed to step 4.

3b. Spray the dirty metaprobes and scissors with the cleaning chemical containing bleach. Wearing gloves, generously apply bleach cleaning chemical to the metaprobes and scissors. Wipe both dry with paper towels. Repeat three times. Proceed to step 4.

4. Prepare a soapy solution to rinse the metaprobes and scissors. Wearing medical gloves, fill the second wash basin with a solution made up of 1 part washing up detergent and 20 parts bottled drinking water. Aim to make a soapy solution that is milder (i.e. not too many bubbles) than what you would use to do the washing up. Transfer the bleached metaprobes and scissors to the soapy wash bath for 5 min. Top tip: Since bleach degrades DNA (cleans the metaprobes) it is critical to remove excess bleach before preparing clean metaprobes. This is an important step!

5. Allow the scissors and metaprobe to air dry on paper towels or a clean surface.

6. Prepare the cotton gauze for the metaprobes. Clean a table or surface with a cleaning product that contains bleach. Wearing gloves, open the gauze and cut it into quarters. Place three of the quarters into a metaprobe half. The quarters should be about the size of a cotton ball (i.e., large enough so that the gauze is too large to fit through the perforations in the metaprobe). Note that the purpose of pre-cutting the gauze is so that they will fit into sample collection tubes. Depending on the size of your sample collection tubes, this may not be an issue, in which case the gauze roll can remain intact and placed directly into the metaprobe.

Important Note: After trial and error, volunteers found that two whole rolls (10 cm x 3.7 m) of cotton gauze fit into the metaprobe and did not need to be altered. Larger sample collection containers were used to accommodate this. See supplementary table, column 'Gau', indicating whether whole or partial rolls of cotton were used.

7. Once the pieces of gauze are in the metaprobes use two to three cable ties to join the opposite halves together**.**

8. Keep the clean metaprobes in a clean storage place until your next dive. This could be a fresh resealable bag or a wash basin that has been wiped clean with bleach cleaning chemicals. Top tip: Make sure this storage area is protected from any seawater spraying into the boat.

Preserving Metaprobe Samples

1. Health and safety is the #1 priority. Please make sure you have safely exited the water and are in a comfortable position on your boat or on land.

2. Preserve the samples within half an hour (30 min.) of completing your dive.

3. Cut the metaprobe free from your dive equipment. Cut the cable tie attaching your metaprobe to your BCD or other equipment. Store the metaprobe in a clean area. If necessary, a resealable bag can be used to keep the metaprobe clean while you take off your dive gear. Top tip: Ask someone wearing medical gloves to assist you with this step. Medical gloves can be difficult to put on wet hands.

4. Wear medical gloves, if not already doing so.

5. Cut the metaprobe open on a clean surface. Do not use a surface that is normally used to gut or clean fish. Clean a table or surface with a cleaning product that contains bleach. Cut the cable ties on the exterior of the metaprobe which hold it closed. Top tip: Place the halves of the metaprobe open-side facing up onto the surface. This prevents the gauze from touching the table and helps prevent sample contamination.

6. Place each medical dressing roll into a plastic screw-cap tube. The screw-cap tubes will contain either silica beads or ethanol. If silica beads, shake the tube so that the beads surround the gauze. The beads will turn green as they adsorb water. If using ethanol, make sure the sample is submerged. Note the number on the tube and cap of the plastic screw-cap tubes. This number will be recorded on the Collection Log Form.

7. Keep the samples in a cool, safe place. Keep the sample tubes in a cold, dark, safe place. Suggested places (in order from best to acceptable): freezer, fridge, cool box, in a plastic bag shieled from sunlight. Once on land, it is highly preferable to keep the samples in a freezer or fridge.

8. Fill out the Collection Log Form. A collection log form should be provided with your DNA Divers sampling materials but it can also be found online.

DNA extraction:

Qiagen DNeasy Blood and Tissue Kit Method with Modifications

The following methods have been modified from the 'Purification of Total DNA from Animal Tissues (Spin-Column Protocol)' in the Qiagen DNeasy Blood and Tissue Handbook published in July 2020. Unless otherwise stated, the manufacturer protocol should be followed.

1. Warm Buffer ATL and Buffer AL to 56°C to fully dissolve any precipitates that may have formed during storage.

2. Prepare input material for lysis:

a. For eDNA filters: Using plyers, break open the plastic filter chamber. Over a petri dish, use dissecting scissors and tweezers cut the filter into small pieces. Place half of the filter pieces in a 1.5 ml Eppendorf tube for lysis, and the other half in a 1.5 ml Eppendorf tube for archive at -20°C.

b. For metaprobe gauze: Cut away small pieces of gauze with dissecting scissors, taking sections from various parts. Using tweezers and blotting paper, blot the gauze, changing blotting paper twice, or until most of the ethanol is gone. Weigh the gauze using a weigh boat and adjust the input to between 0.2 g and 0.4 g. Place gauze into a 1.5 ml Eppendorf tube.

3. Add 720 mL Buffer ATL and 80 mL Proteinase K, which can be premixed for the number of sample extracts accordingly.

4. Mix thoroughly by pulse-vortexing for 5–10 s, and incubate at 56°C in a thermomixer overnight (~16 hours).

5. For eDNA filters, centrifuge at 10,000 xg for 1 min at room temperature. This step can be skipped for metaprobe gauzes as they do not form a pellet.

6. For eDNA filters, without disturbing the pellet, transfer the supernatant to a fresh 1.5 mL Eppendorf tube. Using the pipette tip, press the gauze to the side of the tube and transfer the supernatant to a fresh 1.5 mL Eppendorf tube.

7. Measure the volume of the supernatant and add the same volume of Buffer AL to the sample. Mix thoroughly by pulse-vortexing. Then add the same volume of 100% ethanol. Mix again by pule-vortexing. (e.g., 600 µl supernatant requires the addition of 600 µl buffer AL and then 600 µl ethanol)

8. Pipet the mixture into the DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge at ≥6000 x g (8000 rpm) for 1 min.

9. Empty the collection tube and repeat step 8 until all of the mixture has been passed through the spin column. Then replace the collection tube with a fresh tube.

10. Add 500 µl Buffer AW1, and centrifuge for 1 min at ≥6000 x g (8000 rpm). Discard flow-through and collection tube.

11. Add 500 µl Buffer AW2, and centrifuge for 3 min at 20,000 x g (14,000 rpm) to dry the DNeasy membrane. Discard flow-through and collection tube.

12. Place the DNeasy Mini spin column in a clean 1.5 mL or 2 mL Eppendorf tube, and pipet 100 µl Buffer AE directly onto the DNeasy membrane.

13. Incubate at room temperature for 1 min, and then centrifuge for 1 min at ≥ 6000 x g (8000 rpm) to elute.

14. Pipette the same 100 µl Buffer AE back onto the membrane and repeat step 13 to increase the final DNA concentration in the eluate.

Mu-DNA Method Reagents

The following methods are taken from the 'guidelines' section of 'Mu-DNA: a modular universal DNA extraction method adaptable for a wide range of sample types V.2' document on protocols.io (dx.doi.org/10.17504/protocols.io.qn9dvh6).

Stock solutions

Stock solutions are given as compositions for 100 mL with the exception of PK.

- 1 M Tris HCl (pH 8):

Dissolve 15.7 g of Tris HCl in 75 mL ddH2O. Adjust to pH 8 with 5 M NaOH. Bring to 100 mL with ddH2O.

- 0.5 M EDTA (pH 8):

Dissolve 18.6 g of disodium EDTA dihydrate in 75 mL ddH2O. Adjust to pH 8 with 5 M NaOH. Bring to 100 mL with ddH2O.

- 20% SDS:

Dissolve 20 g sodium dodecyl sulphate in 75 mL ddH2O, bring to 100 mL with ddH2O.

- Proteinase K (PK)*

*The PK solution described in the protocol was not used. Instead, Proteinase K Solution (20 mg/mL), RNA grade (Invitrogen) was used without modification.

- 5 M Ammonium acetate:

Dissolve 38.6 g ammonium acetate in 75 mL ddH2O, bring to 100 mL with ddH2O.

- 180 mM Aluminium etc.:

Dissolve 8.2 g aluminium ammonium sulphate dodecahydrate in 75 mL ddH2O, bring to 100 mL with ddH2O.

- 3% Calcium chloride:

Dissolve 3 g calcium chloride dihydrate in 75 mL ddH2O, bring to 100 mL with ddH2O.

- 5.5 M Guanidine HCl:

Dissolve 52.6 g guanidine hydrochloride in 75 mL ddH2O, bring to 100 mL with ddH2O.

Working solutions

All working solutions are composites of stock solutions. All working solution compositions are given for a 100 mL final volume. The same ratios could be maintained to adjust for different desired final volumes. Note that some working solutions consist of a single stock solution.

- Lysis Solution:

To 75 mL ddH2O add 6.7 mL 1 M Tris HCl (pH 8), 5.3 mL 0.5 M EDTA (pH 8), 1.7 g guanidine thiocyanate, 8.7 g trisodium phosphate dodecahydrate and 0.2 g sodium chloride. Stir mixture until all solids dissolve. Adjust to pH 9.0 with 5 M HCl. Bring to final 100 mL volume with ddH2O.

- Tissue Lysis Additive:

20% SDS

- Flocculant Solution:

To 50 mL 5 M Ammonium acetate add 25 mL 180 mM Aluminium etc. Vortex briefly before adding 25 mL 3% Calcium chloride. Vortex briefly to mix.

- Tissue Binding Solution:

To 50 mL 5.5 M Guanidine HCl add 50 mL 100% ethanol. Vortex briefly to mix.

- Wash Solution:

To 20 mL ddH2O add 80 mL 100% ethanol.

- Elution Buffer:

To 75 mL ddH2O add 1 mL 1 M Tris HCl (pH 8) and 0.2 mL 0.5 M EDTA (pH 8). Bring to 100 mL with ddH2O.

Mu-DNA Method

The following methods are a combination of the 'Tissue' and 'Water' protocols adapted and paraphrased from the 'Mu-DNA: a modular universal DNA extraction method adaptable for a wide range of sample types V.2' document on protocols.io (dx.doi.org/10.17504/protocols.io.qn9dvh6).

Lysis

1. Incubate the Tissue Lysis Additive and Tissue Binding Solution at 55°C to prevent any precipitates that may have formed and until use.

2. Prepare input material for lysis:

a. For eDNA filters: Using plyers, break open the plastic filter chamber. Over a petri dish, use dissecting scissors and tweezers cut the filter into small pieces. Place half of the filter pieces in a 1.5 ml Eppendorf tube for lysis, and the other half in a 1.5 ml Eppendorf tube for archive at -20°C.

b. For metaprobe gauze: Cut away small pieces of gauze with dissecting scissors, taking sections from various parts. Using tweezers and blotting paper, blot the gauze, changing blotting paper twice, or until most of the ethanol is gone. Weigh the gauze using a weigh boat and adjust the input amount for the following weight categories: Heavy: 0.9 – 1.1 g; Medium: 0.6 – 0.8 g; and Standard: 0.2 – 0.4 g. Place Heavy amounts into a 50 ml Falcon tube; Medium amounts into a 5 ml Eppendorf tube; and Standard amounts into a 1.5 ml Eppendorf tube.

3. Add lysis solution master mix to input material. A lysis solution master mix was made by mixing in 13:1:1 ratio, Lysis Solution (13): Tissue lysis additive (1): Protienase-K (1). 1000 µL of the lysis solution master mix was added to eDNA filters and standard gauze weights. 3000 µL of the lysis solution master mix was added to medium gauze weights. 5000 µL of the lysis solution master mix was added to heavy gauze weights.

4. Vortex the tubes and incubate at 55°C for ~16 hours overnight.

5. For eDNA filters, centrifuge at 10,000 xg for 1 min at room temperature. This step can be skipped for metaprobe gauzes as they do not form a pellet.

6. For eDNA filters, without disturbing the pellet, transfer the supernatant to a fresh 1.5 mL Eppendorf tube. Using the pipette tip, press the gauze to the side of the tube and transfer the supernatant to a fresh tube.

Inhibitor Removal

7. Add 0.3 X volume of Flocculant Solution (e.g., if 700 µL of lysis supernatant is transferred then 210 µL of Flocculant Solution should be added). Vortex briefly and incubate at 4°C for 10 minutes.

8. Centrifuge at 10,000 xg for 1 min at room temperature.

9. Without disturbing the pellet, transfer the supernatant to a fresh tube.

Silica Binding

10. Add 2 X volume Tissue Binding Solution (e.g., if 700 µL of solution from the inhibitor removal step is transferred then 1400 µL of Tissue Binding Solution should be added for a total volume of 2100 µL). Vortex briefly to mix.

11. Transfer 700 µL of the mixture to a spin column.

12. Centrifuge at ≥ 10,000 xg for 1 min at room temperature, discard the flow-through.

13. To standardize the extractions and for practical purposes repeat steps 11 and 12 as follows: a maximum of three times for eDNA filters and standard gauze amounts, exactly four times for medium gauze amounts and exactly five times for heavy gauze amounts.

Wash

14. Add 500 µL of Wash Solution to the spin column.

15. Centrifuge at 10,000 xg for 1 min at room temperature. Discard the flow-through.

16. Repeat steps 1 and 2 a second time.

17. Centrifuge at 10,000 xg for 2 min at room temperature, replace collection tube with a fresh 1.5 mL Eppendorf tube.

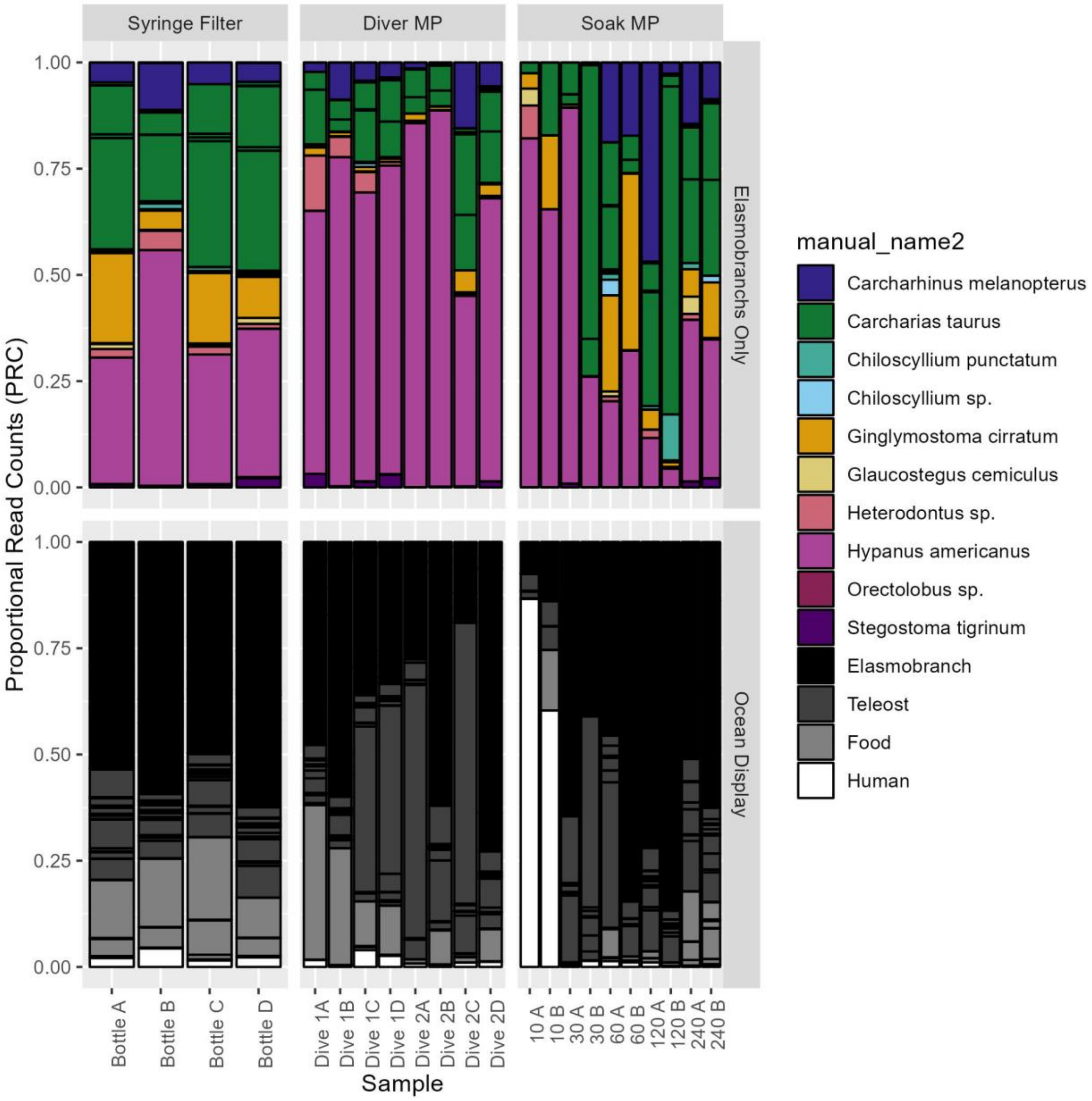
Elution

18. Add 100 µL of Elution Buffer directly to the spin column membrane and incubate for 1 min at room temperature.

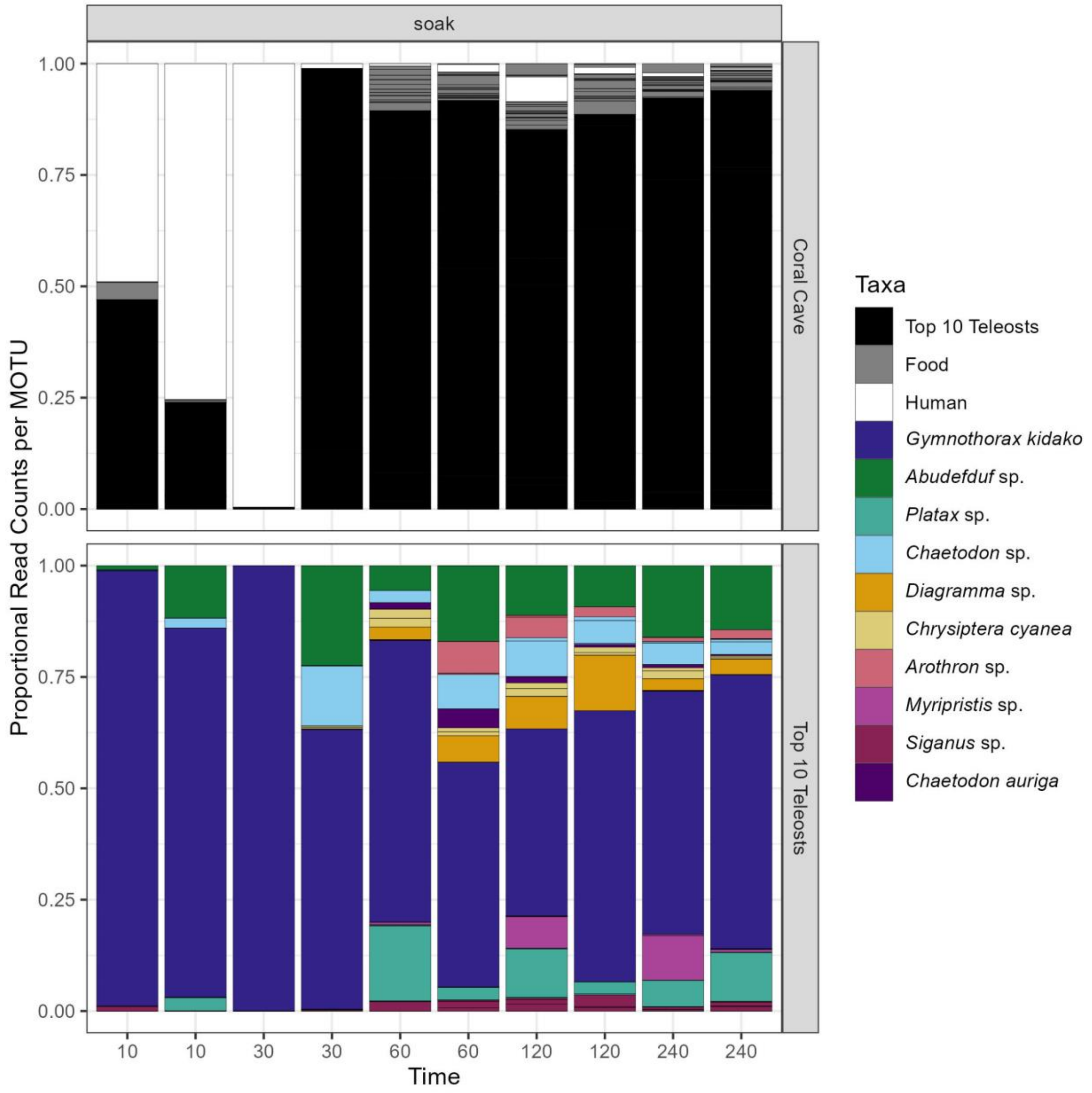
19. Centrifuge at 10,000 xg for 1 min at room temperature.

20. Take the product in the tube and pipette it onto the spin column membrane. Repeat step 19.

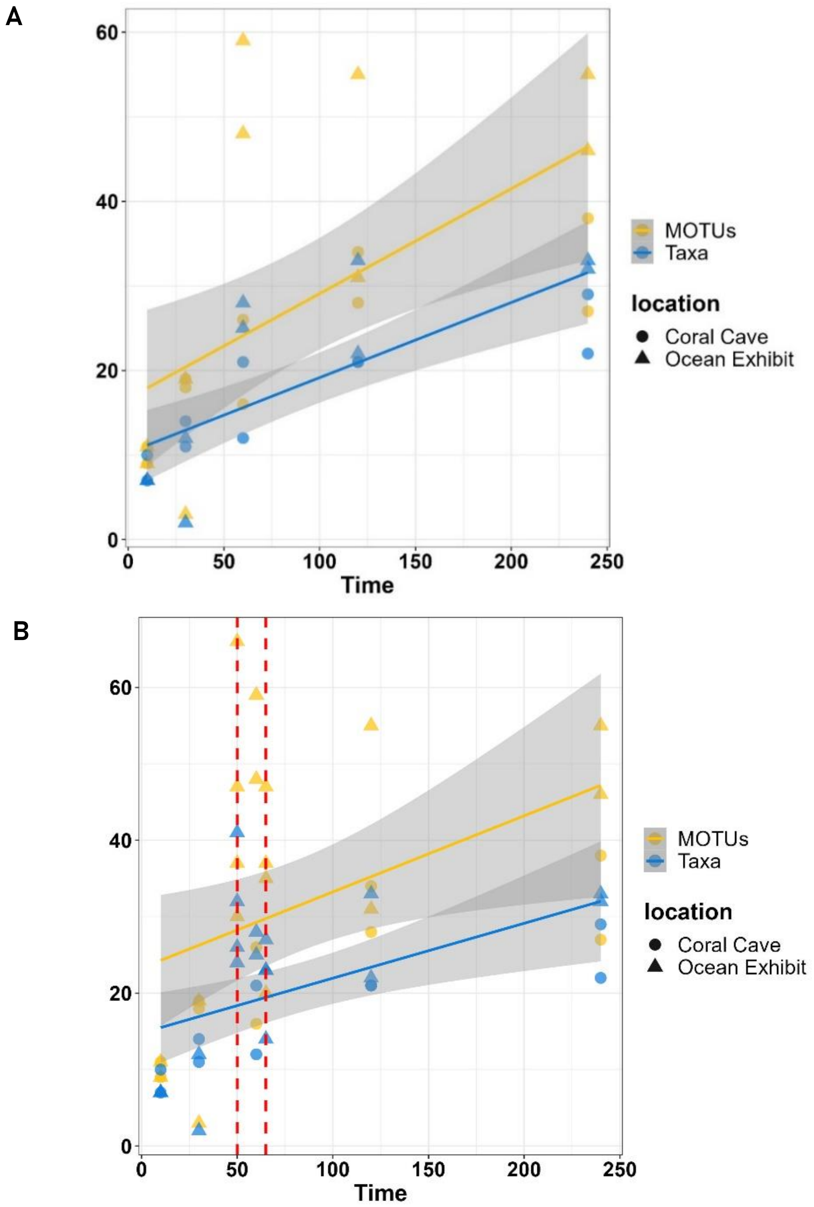
21. The DNA is now in the Eppendorf tube.



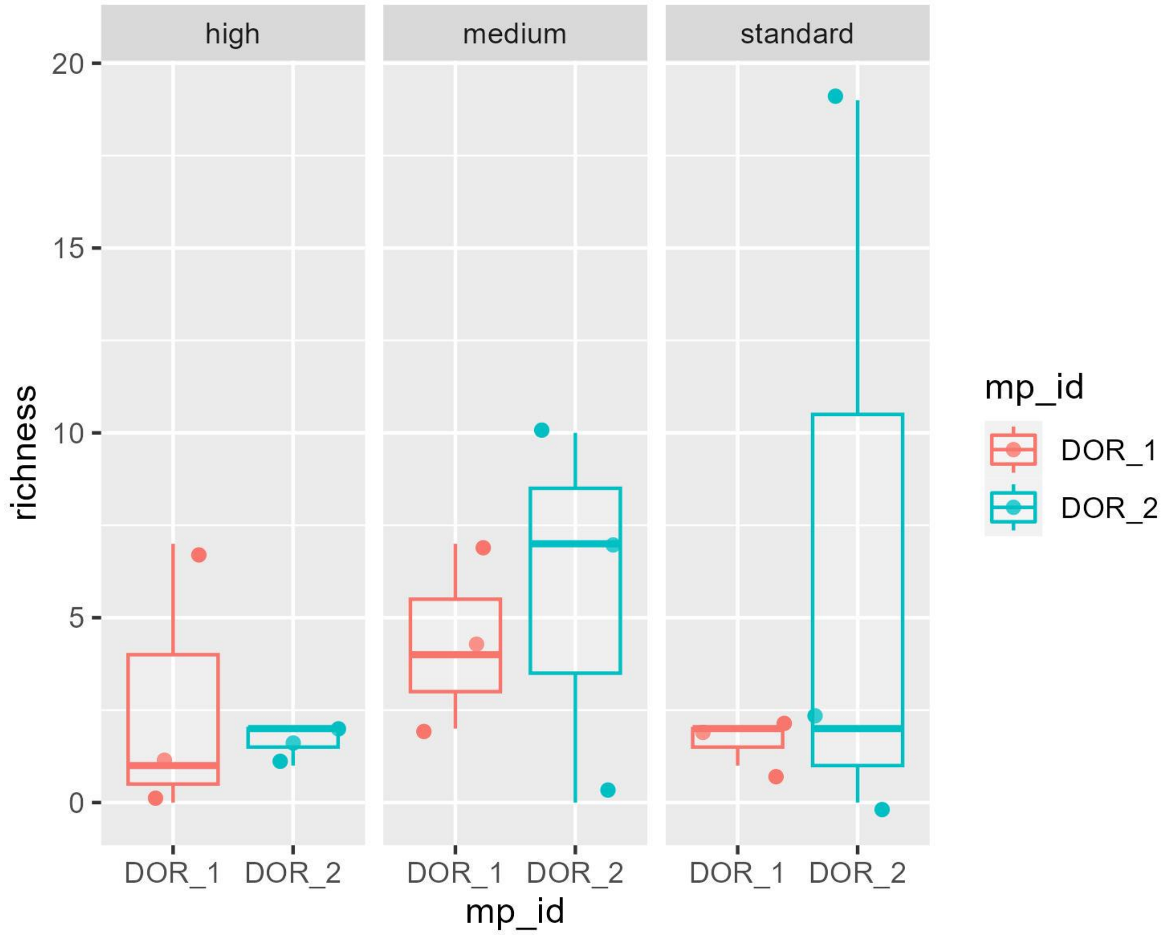
S4 Figure 1. Stacked bar charts (top) showing the proportion of MOTUs assigned to Elasmobranch taxa with sequencing replicates separated. Stacked bar charts (bottom) showing the proportion of MOTUs assigned to Elasmobranchs or Teleosts listed in the Ocean Exhibit inventory with sequencing replicates separated.



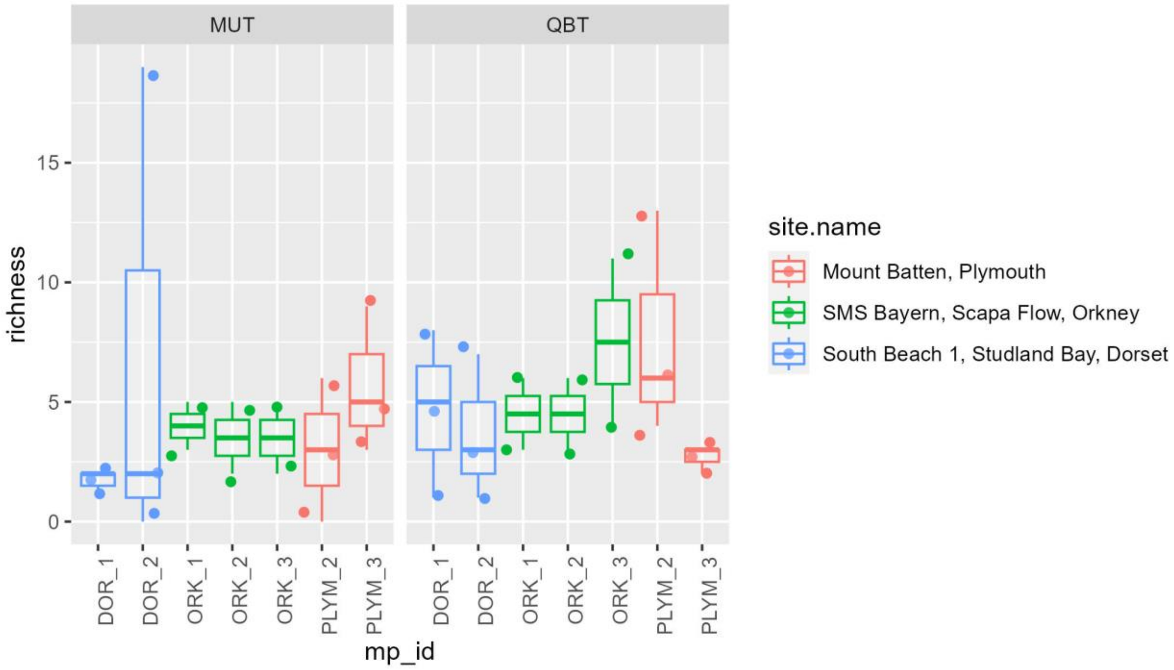
S4 Figure 2. Stacked bar charts (top) showing the proportion of MOTUs assigned to the top 10 most read abundant Teleosts from Coral cave exhbit with sequencing replicates separated. Stacked bar charts (top) showing the proportion of MOTUs assigned to the top 10 most read abundant Teleost taxa with sequencing replicates separated.



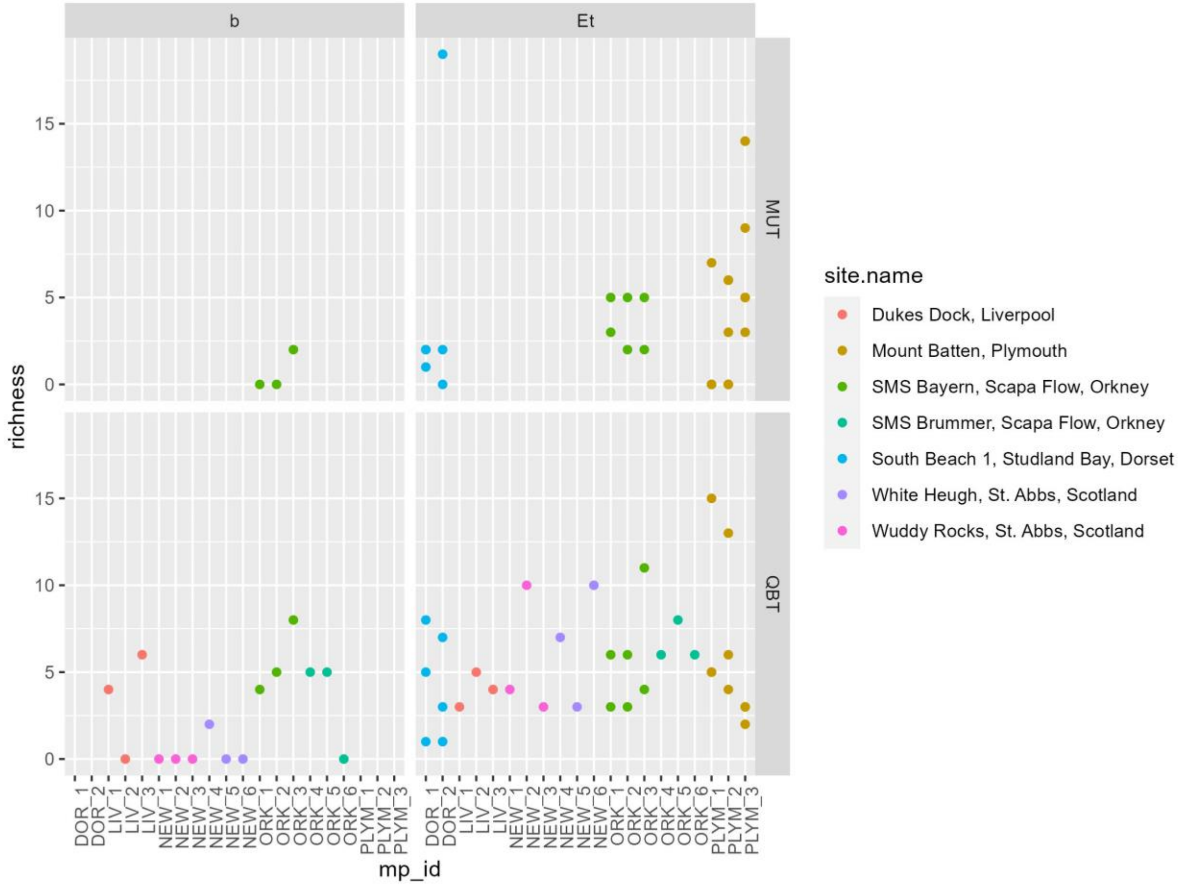
S4 Figure 3. A) Number of MOTUs and number of taxa detected from metaprobes over the course of the soaking experiments (from the Ocean Exhibit and Coral Cave) at the following time intervals: 10 min, 30 min, 60 min, 120 min, and 240 min. Linear regression lines overlayed. **B)** Same plot as S3A, with diver metaprobes from the Ocean Exhibit added in at 50 min and 65 min. Linear regression models recalculated to include dives and lines overlayed.



S4 Figure 4. Box plots of the species richness for samples (DOR_1, DOR_2) collected from Studland Bay, Dorset at the South Beach 1 dive site, sorted by gauze weight ranges.

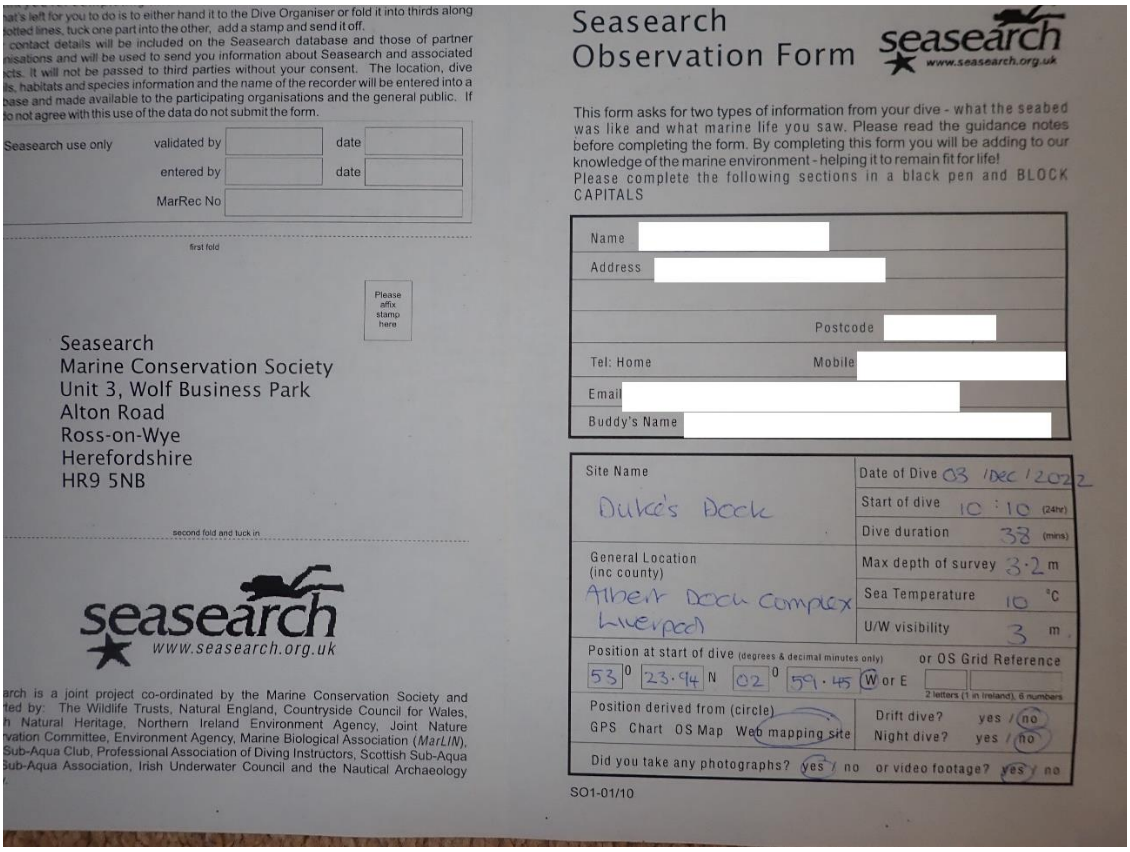


S4 Figure 5. Species richness for samples collected from Mount Batten, Plymouth, SMS Bayern, Orkney, and South Beach 1, Studland Bay. The Mu-DNA Tissue extraction method (MUT, left) is compared to the Qiagen Blood and Tissue kit (QBT, right).



S4 Figure 6. Dot plot comparing the detected species richness for samples from various dive sites which underwent different preservation treatments (vertical columns), where ‘b’ = silica beads and ‘Et’ = ethanol, and different DNA extraction methods, where ‘MUT’ = Mu-DNA Tissue method and ‘QBT’ = Qiagen Blood and Tissue kit. The horizontal QBT panel shows samples used to test for the effects of the different preservation techniques (Supplementary Table 13). Samples that did not contain detections are shown with a species richness of zero, therefore treatment iterations with no data do not exist.

S4 Figure 7. Seasearch Obervation form from the Dukes Dock, Liverpool dive (December 2022). Front side shown below and back side on the following page.



at's left for you to do is to either hand it to the Dive Organiser or fold it into thirds along otted lines, tuck one part into the other, add a stamp and send it off.
contact details will be included on the Seasearch database and those of partner nisations and will be used to send you information about Seasearch and associated cts. It will not be passed to third parties without your consent. The location, dive ls, habitats and species information and the name of the recorder will be entered into a base and made available to the participating organisations and the general public. If o not agree with this use of the data do not submit the form.

Seasearch use only
validated by ☐ date ☐
entered by ☐ date ☐
MarRec No ☐

first fold

Please affix stamp here

Seasearch
Marine Conservation Society
Unit 3, Wolf Business Park
Alton Road
Ross-on-Wye
Herefordshire
HR9 5NB

second fold and tuck in

seasearch
www.seasearch.org.uk

arch is a joint project co-ordinated by the Marine Conservation Society and ted by: The Wildlife Trusts, Natural England, Countryside Council for Wales, h Natural Heritage, Northern Ireland Environment Agency, Joint Nature vation Committee, Environment Agency, Marine Biological Association (*MarLIN*), Sub-Aqua Club, Professional Association of Diving Instructors, Scottish Sub-Aqua Sub-Aqua Association, Irish Underwater Council and the Nautical Archaeology

Seasearch Observation Form

seasearch
www.seasearch.org.uk

This form asks for two types of information from your dive - what the seabed was like and what marine life you saw. Please read the guidance notes before completing the form. By completing this form you will be adding to our knowledge of the marine environment - helping it to remain fit for life!
Please complete the following sections in a black pen and BLOCK CAPITALS

Name [redacted]
Address [redacted]
Postcode [redacted]
Tel: Home
Mobile [redacted]
Email [redacted]
Buddy's Name [redacted]

Site Name
Duke's Dock

Date of Dive 03 /DEC /2022
Start of dive 10 : 10 (24hr)
Dive duration 38 (mins)

General Location (inc county)
Albert Dock Complex
Liverpool

Max depth of survey 3·2 m
Sea Temperature 10 °C
U/W visibility 3 m

Position at start of dive (degrees & decimal minutes only)
53 ° 23·94 N 02 ° 59·45 [✓ W] or E

or OS Grid Reference ☐ ☐
2 letters (1 in Ireland), 6 numbers

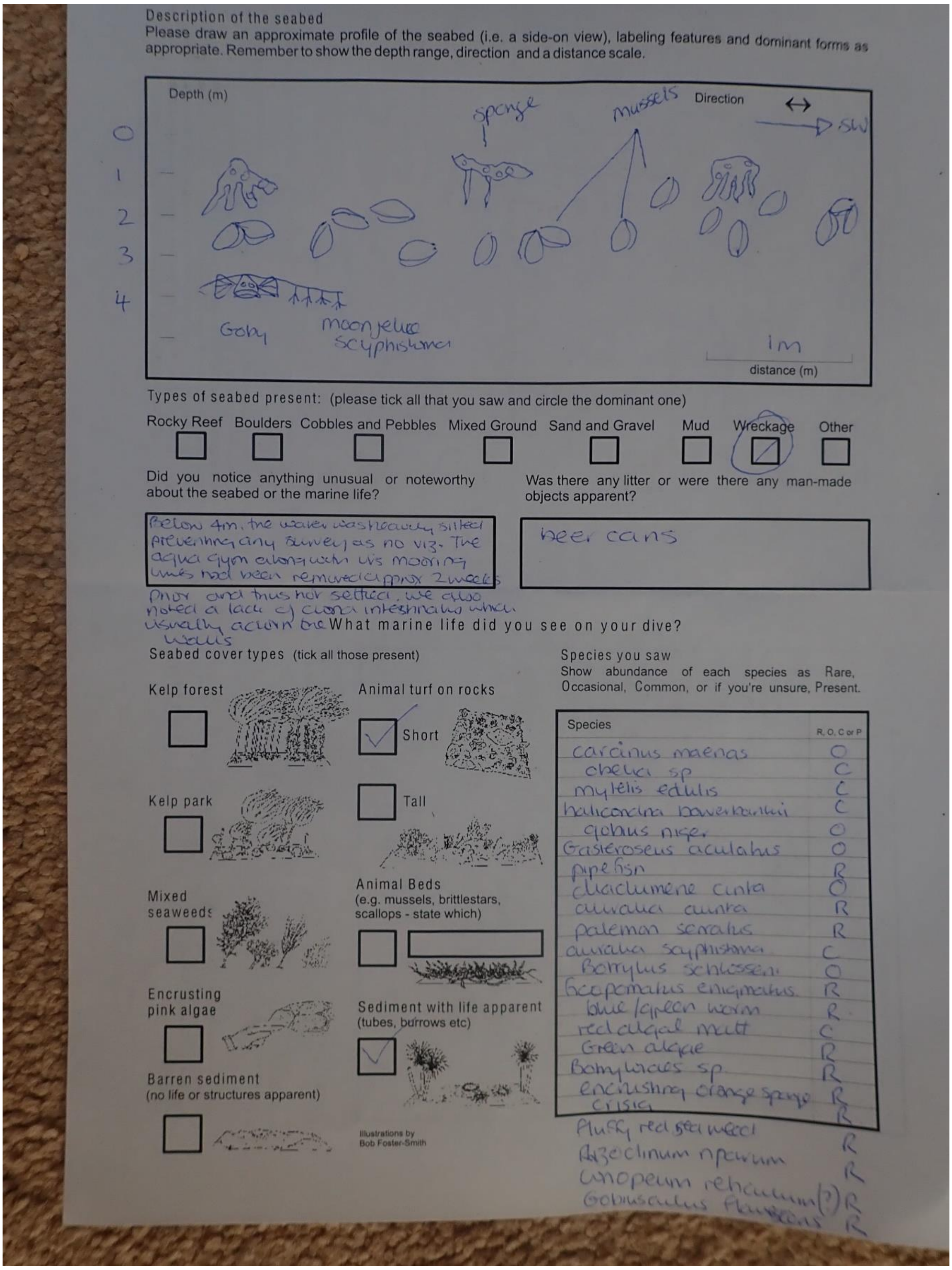
Position derived from (circle)
GPS Chart OS Map [✓ Web mapping site]

Drift dive? yes / [✓ no]
Night dive? yes / [✓ no]

Did you take any photographs? [✓ yes] / no or video footage? [✓ yes] / no

SO1-01/10

S4 Figure 7. (continued)



Description of the seabed

Please draw an approximate profile of the seabed (i.e. a side-on view), labeling features and dominant forms as appropriate. Remember to show the depth range, direction and a distance scale.

Depth (m) Direction ↔ distance (m)

Types of seabed present: (please tick all that you saw and circle the dominant one)

Rocky Reef ☐ Boulders ☐ Cobbles and Pebbles ☐ Mixed Ground ☐ Sand and Gravel ☐ Mud ☐ [✓ Wreckage ☒] Other ☐

Did you notice anything unusual or noteworthy about the seabed or the marine life?

Below 4m. the water was heavily silted preventing any survey as no viz. The aqua gym along with its mooring lines had been removed approx 2 weeks prior and thus not settled. We also noted a lack of ciona intestinalis which usually adorn the walls

Was there any litter or were there any man-made objects apparent?

beer cans

What marine life did you see on your dive?

Seabed cover types (tick all those present)

Kelp forest ☐

Kelp park ☐

Mixed seaweeds ☐

Encrusting pink algae ☐

Barren sediment (no life or structures apparent) ☐

Animal turf on rocks

☑ Short

☐ Tall

Animal Beds (e.g. mussels, brittlestars, scallops - state which) ☐

Sediment with life apparent (tubes, burrows etc) ☑

Species you saw

Show abundance of each species as Rare, Occasional, Common, or if you're unsure, Present.

Species	R, O, C or P
carcinus maenas	O
obelia sp	C
mytelis edulis	C
halicondria bowerbankii	C
gobius niger	O
Gasteroseus aculatus	O
pipe fish	R
diadumene cinta	O
aurelia aurita	R
paleman serratus	R
aurelia scyphistoma	C
Botrylus schlosseri	O
Ficopomatus enigmatus	R
blue/green worm	R
red algal matt	C
Green algae	R
Botryloides sp	R
encrusting orange sponge	R
crisia	R
Fluffy red seaweed	R
Rhizoclinum riparium	R
Conopeum reticulum (?)	R
Gobiusculus flavescens	R

S4 Table 1. Sequenced samples and metadata used for analysis.

S4 Table 1.1. Sample information for all 190 samples sequenced where ‘Num’ = sample number, ‘Run’ = sequencing run, ‘Type’ = sample type (‘MP’ = metaprobe, ‘eDNA’ = eDNA filter), ‘mp_id’ = metaprobe ID, ‘Pres’ = preservation treatment (‘Et’ = 100% ethanol, ‘b’ = silica beads), ‘Ext’ = extraction type, ‘Gau’ = gauze treatment, ‘W’ = weight in grams, and ‘ICES area’ = International Council for Exploration of the Sea advisory areas. The abbreviations in the ‘Gau’ column indicate the different ways gauze had been rolled and placed in the metaprobe: ‘ham’ = cutting rolls in thirds by width (3.3 cm x 3.7 m); ‘hotf’ = cutting rolls in half by length and width (5 cm x 1.85 m); ‘hott’ = cutting rolls in thirds by length (10 cm x 1.23 m); ‘whole’ = no modification (10 cm x 3.7 m).

Num	Run	Type	mp_id	Dive Site Name	Location	Country	Via	Pres	Ext	Gau	W (g)	Weight Category	Primer	Time (min)	ICES area
1	A	MP	PLYM_1	Mount Batten	Plymouth	England	snorkel	Et	QBT	ham	0.25	standard	tele02	60	7.e
2	A	MP	PLYM_1	Mount Batten	Plymouth	England	snorkel	Et	QBT	ham	0.2	standard	tele02	60	7.e
3	A	MP	PLYM_1	Mount Batten	Plymouth	England	snorkel	Et	QBT	ham	0.4	standard	tele02	60	7.e
4	A	MP	PLYM_2	Mount Batten	Plymouth	England	snorkel	Et	QBT	ham	0.31	standard	tele02	60	7.e
5	A	MP	PLYM_2	Mount Batten	Plymouth	England	snorkel	Et	QBT	ham	0.27	standard	tele02	60	7.e
6	A	MP	PLYM_2	Mount Batten	Plymouth	England	snorkel	Et	QBT	ham	0.31	standard	tele02	60	7.e
7	A	MP	PLYM_3	Mount Batten	Plymouth	England	snorkel	Et	QBT	ham	0.21	standard	tele02	60	7.e
8	A	MP	PLYM_3	Mount Batten	Plymouth	England	snorkel	Et	QBT	ham	0.22	standard	tele02	60	7.e
9	A	MP	PLYM_3	Mount Batten	Plymouth	England	snorkel	Et	QBT	ham	0.36	standard	tele02	60	7.e
10	A	MP	DOR_1	South Beach 1, Studland Bay	Dorset	England	SCUBA	Et	QBT	hotf	0.2	standard	tele02	77	7.d
11	A	MP	DOR_1	South Beach 1, Studland Bay	Dorset	England	SCUBA	Et	QBT	hotf	0.26	standard	tele02	77	7.d
12	A	MP	DOR_1	South Beach 1, Studland Bay	Dorset	England	SCUBA	Et	QBT	hotf	0.2	standard	tele02	77	7.d
13	A	MP	DOR_2	South Beach 1, Studland Bay	Dorset	England	SCUBA	Et	QBT	hotf	0.28	standard	tele02	90	7.d
14	A	MP	DOR_2	South Beach 1, Studland Bay	Dorset	England	SCUBA	Et	QBT	hotf	0.4	standard	tele02	90	7.d
15	A	MP	DOR_2	South Beach 1, Studland Bay	Dorset	England	SCUBA	Et	QBT	hotf	0.21	standard	tele02	90	7.d

16	A	MP	DOR_3	South Beach 2, Studland Bay	Dorset	England	SCUBA	Et	QBT	hotf	0.38	standard	tele02	73	7.d
17	A	MP	DOR_3	South Beach 2, Studland Bay	Dorset	England	SCUBA	Et	QBT	hotf	0.22	standard	tele02	73	7.d
18	A	MP	DOR_3	South Beach 2, Studland Bay	Dorset	England	SCUBA	Et	QBT	hotf	0.23	standard	tele02	73	7.d
19	A	MP	DOR_4	South Beach 2, Studland Bay	Dorset	England	SCUBA	Et	QBT	hotf	0.38	standard	tele02	73	7.d
20	A	MP	DOR_4	South Beach 2, Studland Bay	Dorset	England	SCUBA	Et	QBT	hotf	0.2	standard	tele02	73	7.d
21	A	MP	DOR_4	South Beach 2, Studland Bay	Dorset	England	SCUBA	Et	QBT	hotf	0.4	standard	tele02	73	7.d
22	A	MP	PLYM_2	Mount Batten	Plymouth	England	snorkel	Et	MUT	ham	NR	standard	tele02	60	7.e
23	A	MP	PLYM_2	Mount Batten	Plymouth	England	snorkel	Et	MUT	ham	NR	standard	tele02	60	7.e
24	A	MP	PLYM_2	Mount Batten	Plymouth	England	snorkel	Et	MUT	ham	NR	standard	tele02	60	7.e
25	A	MP	PLYM_3	Mount Batten	Plymouth	England	snorkel	Et	MUT	ham	NR	standard	tele02	60	7.e
26	A	MP	PLYM_3	Mount Batten	Plymouth	England	snorkel	Et	MUT	ham	NR	standard	tele02	60	7.e
27	A	MP	PLYM_3	Mount Batten	Plymouth	England	snorkel	Et	MUT	ham	NR	standard	tele02	60	7.e
28	A	MP	DOR_1	South Beach 1, Studland Bay	Dorset	England	SCUBA	Et	MUT	hotf	0.31	standard	tele02	77	7.d
29	A	MP	DOR_1	South Beach 1, Studland Bay	Dorset	England	SCUBA	Et	MUT	hotf	0.21	standard	tele02	77	7.d
30	A	MP	DOR_1	South Beach 1, Studland Bay	Dorset	England	SCUBA	Et	MUT	hotf	0.2	standard	tele02	77	7.d
31	A	MP	DOR_2	South Beach 1, Studland Bay	Dorset	England	SCUBA	Et	MUT	hotf	0.24	standard	tele02	90	7.d
32	A	MP	DOR_2	South Beach 1, Studland Bay	Dorset	England	SCUBA	Et	MUT	hotf	0.24	standard	tele02	90	7.d
33	A	MP	DOR_2	South Beach 1, Studland Bay	Dorset	England	SCUBA	Et	MUT	hotf	0.32	standard	tele02	90	7.d
34	A	MP	DOR_1	South Beach 1, Studland Bay	Dorset	England	SCUBA	Et	MUT	hotf	0.79	medium	tele02	77	7.d
35	A	MP	DOR_1	South Beach 1, Studland Bay	Dorset	England	SCUBA	Et	MUT	hotf	0.61	medium	tele02	77	7.d
36	A	MP	DOR_1	South Beach 1, Studland Bay	Dorset	England	SCUBA	Et	MUT	hotf	0.78	medium	tele02	77	7.d

37	A	MP	DOR_2	South Beach 1, Studland Bay	Dorset	England	SCUBA	Et	MUT	hotf	0.61	medium	tele02	90	7.d
38	A	MP	DOR_2	South Beach 1, Studland Bay	Dorset	England	SCUBA	Et	MUT	hotf	0.64	medium	tele02	90	7.d
39	A	MP	DOR_2	South Beach 1, Studland Bay	Dorset	England	SCUBA	Et	MUT	hotf	0.7	medium	tele02	90	7.d
40	A	MP	DOR_1	South Beach 1, Studland Bay	Dorset	England	SCUBA	Et	MUT	hotf	1.05	high	tele02	77	7.d
41	A	MP	DOR_1	South Beach 1, Studland Bay	Dorset	England	SCUBA	Et	MUT	hotf	1.03	high	tele02	77	7.d
42	A	MP	DOR_1	South Beach 1, Studland Bay	Dorset	England	SCUBA	Et	MUT	hotf	1	high	tele02	77	7.d
43	A	MP	DOR_2	South Beach 1, Studland Bay	Dorset	England	SCUBA	Et	MUT	hotf	1.04	high	tele02	90	7.d
44	A	MP	DOR_2	South Beach 1, Studland Bay	Dorset	England	SCUBA	Et	MUT	hotf	1.1	high	tele02	90	7.d
45	A	MP	DOR_2	South Beach 1, Studland Bay	Dorset	England	SCUBA	Et	MUT	hotf	1.01	high	tele02	90	7.d
46	A	eDNA	NA	SMS Bayern, Scapa Flow	Orkney	Scotland	NA	NA	QBT	NA	NA	NA	tele02	NA	4.a
47	A	eDNA	NA	SMS Bayern, Scapa Flow	Orkney	Scotland	NA	NA	QBT	NA	NA	NA	tele02	NA	4.a
48	A	eDNA	NA	SMS Bayern, Scapa Flow	Orkney	Scotland	NA	NA	QBT	NA	NA	NA	tele02	NA	4.a
49	A	eDNA	NA	SMS Bayern, Scapa Flow	Orkney	Scotland	NA	NA	QBT	NA	NA	NA	tele02	NA	4.a
50	A	MP	ORK_1	SMS Bayern, Scapa Flow	Orkney	Scotland	SCUBA	Et	QBT	hott	0.23	standard	tele02	NR	4.a
51	A	MP	ORK_1	SMS Bayern, Scapa Flow	Orkney	Scotland	SCUBA	Et	MUT	hott	0.2	standard	tele02	NR	4.a
52	A	MP	ORK_1	SMS Bayern, Scapa Flow	Orkney	Scotland	SCUBA	Et	QBT	hott	0.27	standard	tele02	NR	4.a
53	A	MP	ORK_1	SMS Bayern, Scapa Flow	Orkney	Scotland	SCUBA	Et	MUT	hott	0.2	standard	tele02	NR	4.a
54	A	MP	ORK_1	SMS Bayern, Scapa Flow	Orkney	Scotland	SCUBA	b	QBT	hott	0.08	standard	tele02	NR	4.a

55	A	MP	ORK_1	SMS Bayern, Scapa Flow	Orkney	Scotland	SCUBA	b	MUT	hott	0.06	standard	tele02	NR	4.a
56	A	MP	ORK_2	SMS Bayern, Scapa Flow	Orkney	Scotland	SCUBA	Et	QBT	hott	0.27	standard	tele02	NR	4.a
57	A	MP	ORK_2	SMS Bayern, Scapa Flow	Orkney	Scotland	SCUBA	Et	MUT	hott	0.23	standard	tele02	NR	4.a
58	A	MP	ORK_2	SMS Bayern, Scapa Flow	Orkney	Scotland	SCUBA	Et	QBT	hott	0.32	standard	tele02	NR	4.a
59	A	MP	ORK_2	SMS Bayern, Scapa Flow	Orkney	Scotland	SCUBA	Et	MUT	hott	0.29	standard	tele02	NR	4.a
60	A	MP	ORK_2	SMS Bayern, Scapa Flow	Orkney	Scotland	SCUBA	b	QBT	hott	0.08	standard	tele02	NR	4.a
61	A	MP	ORK_2	SMS Bayern, Scapa Flow	Orkney	Scotland	SCUBA	b	MUT	hott	0.08	standard	tele02	NR	4.a
62	A	MP	ORK_3	SMS Bayern, Scapa Flow	Orkney	Scotland	SCUBA	Et	QBT	hott	0.2	standard	tele02	NR	4.a
63	A	MP	ORK_3	SMS Bayern, Scapa Flow	Orkney	Scotland	SCUBA	Et	MUT	hott	0.2	standard	tele02	NR	4.a
64	A	MP	ORK_3	SMS Bayern, Scapa Flow	Orkney	Scotland	SCUBA	Et	QBT	hott	0.36	standard	tele02	NR	4.a
65	A	MP	ORK_3	SMS Bayern, Scapa Flow	Orkney	Scotland	SCUBA	Et	MUT	hott	0.26	standard	tele02	NR	4.a
66	A	MP	ORK_3	SMS Bayern, Scapa Flow	Orkney	Scotland	SCUBA	b	QBT	hott	0.15	standard	tele02	NR	4.a
67	A	MP	ORK_3	SMS Bayern, Scapa Flow	Orkney	Scotland	SCUBA	b	MUT	hott	0.13	standard	tele02	NR	4.a
68	B	MP	BPD_7	Ocean Exhibit, Blue Planet, Ellesmere Port	Blue Planet Aquarium	shark tank	soak	Et	QBT	whole	NR	standard	elas02	60	NA
69	B	MP	BPD_8	Ocean Exhibit, Blue Planet, Ellesmere Port	Blue Planet Aquarium	shark tank	soak	Et	QBT	whole	NR	standard	elas02	120	NA
70	B	MP	BPD_8	Ocean Exhibit, Blue Planet, Ellesmere Port	Blue Planet Aquarium	shark tank	soak	Et	QBT	whole	NR	standard	elas02	120	NA

71	B	MP	BPD_9	Ocean Exhibit, Blue Planet, Ellesmere Port	Blue Planet Aquarium	shark tank	soak	Et	QBT	whole	NR	standard	elas02	240	NA
72	B	MP	BPD_9	Ocean Exhibit, Blue Planet, Ellesmere Port	Blue Planet Aquarium	shark tank	soak	Et	QBT	whole	NR	standard	elas02	240	NA
73	B	MP	BPD_1	Ocean Exhibit, Blue Planet, Ellesmere Port	Blue Planet Aquarium	dive2	SCUBA	Et	QBT	whole	NR	standard	elas02	65	NA
74	B	MP	BPD_1	Ocean Exhibit, Blue Planet, Ellesmere Port	Blue Planet Aquarium	dive2	SCUBA	Et	QBT	whole	NR	standard	elas02	65	NA
75	B	MP	BPD_2	Ocean Exhibit, Blue Planet, Ellesmere Port	Blue Planet Aquarium	dive2	SCUBA	Et	QBT	whole	NR	standard	elas02	65	NA
76	B	MP	BPD_2	Ocean Exhibit, Blue Planet, Ellesmere Port	Blue Planet Aquarium	dive2	SCUBA	Et	QBT	whole	NR	standard	elas02	65	NA
77	B	eDNA	NA	Ocean Exhibit, Blue Planet, Ellesmere Port	Blue Planet Aquarium	shark tank	NA	NA	QBT	NA	NA	NA	elas02	NA	NA
78	B	eDNA	NA	Ocean Exhibit, Blue Planet, Ellesmere Port	Blue Planet Aquarium	shark tank	NA	NA	QBT	NA	NA	NA	elas02	NA	NA
79	B	eDNA	NA	Ocean Exhibit, Blue Planet, Ellesmere Port	Blue Planet Aquarium	shark tank	NA	NA	QBT	NA	NA	NA	elas02	NA	NA
80	B	eDNA	NA	Ocean Exhibit, Blue Planet, Ellesmere Port	Blue Planet Aquarium	shark tank	NA	NA	QBT	NA	NA	NA	elas02	NA	NA
81	B	MP	BPD_3	Ocean Exhibit, Blue Planet, Ellesmere Port	Blue Planet Aquarium	dive1	SCUBA	Et	QBT	whole	NR	standard	elas02	50	NA
82	B	MP	BPD_3	Ocean Exhibit, Blue Planet, Ellesmere Port	Blue Planet Aquarium	dive1	SCUBA	Et	QBT	whole	NR	standard	elas02	50	NA

83	B	MP	BPD_4	Ocean Exhibit, Blue Planet, Ellesmere Port	Blue Planet Aquarium	dive1	SCUBA	Et	QBT	whole	NR	standard	elas02	50	NA
84	B	MP	BPD_4	Ocean Exhibit, Blue Planet, Ellesmere Port	Blue Planet Aquarium	dive1	SCUBA	Et	QBT	whole	NR	standard	elas02	50	NA
85	B	MP	BPD_5	Ocean Exhibit, Blue Planet, Ellesmere Port	Blue Planet Aquarium	shark tank	soak	Et	QBT	whole	NR	standard	elas02	10	NA
86	B	MP	BPD_5	Ocean Exhibit, Blue Planet, Ellesmere Port	Blue Planet Aquarium	shark tank	soak	Et	QBT	whole	NR	standard	elas02	10	NA
87	B	MP	BPD_6	Ocean Exhibit, Blue Planet, Ellesmere Port	Blue Planet Aquarium	shark tank	soak	Et	QBT	whole	NR	standard	elas02	30	NA
88	B	MP	BPD_6	Ocean Exhibit, Blue Planet, Ellesmere Port	Blue Planet Aquarium	shark tank	soak	Et	QBT	whole	NR	standard	elas02	30	NA
89	B	MP	BPD_7	Ocean Exhibit, Blue Planet, Ellesmere Port	Blue Planet Aquarium	shark tank	soak	Et	QBT	whole	NR	standard	elas02	60	NA
90	B	eDNA	NA	SMS Brummer, Scapa Flow	Orkney	Scotland	NA	NA	QBT	NA	NA	NA	elas02	NA	4.a
91	B	eDNA	NA	SMS Brummer, Scapa Flow	Orkney	Scotland	NA	NA	QBT	NA	NA	NA	elas02	NA	4.a
92	B	eDNA	NA	SMS Brummer, Scapa Flow	Orkney	Scotland	NA	NA	QBT	NA	NA	NA	elas02	NA	4.a
93	B	eDNA	NA	SMS Brummer, Scapa Flow	Orkney	Scotland	NA	NA	QBT	NA	NA	NA	elas02	NA	4.a
94	B	MP	ORK_4	SMS Brummer, Scapa Flow	Orkney	Scotland	SCUBA	Et	QBT	hott	NR	standard	elas02	32	4.a
95	B	MP	ORK_5	SMS Brummer, Scapa Flow	Orkney	Scotland	SCUBA	Et	QBT	hott	NR	standard	elas02	32	4.a
96	B	MP	ORK_6	SMS Brummer, Scapa Flow	Orkney	Scotland	SCUBA	Et	QBT	hott	NR	standard	elas02	32	4.a
97	B	MP	ORK_5	SMS Brummer, Scapa Flow	Orkney	Scotland	SCUBA	b	QBT	hott	NR	standard	elas02	32	4.a

98	B	MP	ORK_6	SMS Brummer, Scapa Flow	Orkney	Scotland	SCUBA	b	QBT	hott	NR	standard	elas02	32	4.a
99	B	MP	ORK_4	SMS Brummer, Scapa Flow	Orkney	Scotland	SCUBA	b	QBT	hott	NR	standard	elas02	32	4.a
100	B	MP	BPC_1	Coral Cave, Blue Planet, Ellesmere Port	Blue Planet Aquarium	coral cave	soak	Et	QBT	whole	NR	standard	tele02	10	NA
101	B	MP	BPC_1	Coral Cave, Blue Planet, Ellesmere Port	Blue Planet Aquarium	coral cave	soak	Et	QBT	whole	NR	standard	tele02	10	NA
102	B	MP	BPC_2	Coral Cave, Blue Planet, Ellesmere Port	Blue Planet Aquarium	coral cave	soak	Et	QBT	whole	NR	standard	tele02	30	NA
103	B	MP	BPC_2	Coral Cave, Blue Planet, Ellesmere Port	Blue Planet Aquarium	coral cave	soak	Et	QBT	whole	NR	standard	tele02	30	NA
104	B	MP	BPC_3	Coral Cave, Blue Planet, Ellesmere Port	Blue Planet Aquarium	coral cave	soak	Et	QBT	whole	NR	standard	tele02	60	NA
105	B	MP	BPC_3	Coral Cave, Blue Planet, Ellesmere Port	Blue Planet Aquarium	coral cave	soak	Et	QBT	whole	NR	standard	tele02	60	NA
106	B	MP	BPC_4	Coral Cave, Blue Planet, Ellesmere Port	Blue Planet Aquarium	coral cave	soak	Et	QBT	whole	NR	standard	tele02	120	NA
107	B	MP	BPC_4	Coral Cave, Blue Planet, Ellesmere Port	Blue Planet Aquarium	coral cave	soak	Et	QBT	whole	NR	standard	tele02	120	NA
108	B	MP	BPC_5	Coral Cave, Blue Planet, Ellesmere Port	Blue Planet Aquarium	coral cave	soak	Et	QBT	whole	NR	standard	tele02	240	NA
109	B	MP	BPC_5	Coral Cave, Blue Planet, Ellesmere Port	Blue Planet Aquarium	coral cave	soak	Et	QBT	whole	NR	standard	tele02	240	NA
110	B	eDNA	NA	Dukes Dock	Liverpool	England	NA	NA	QBT	NA	NA	NA	tele02	NA	7.a
111	B	eDNA	NA	Dukes Dock	Liverpool	England	NA	NA	QBT	NA	NA	NA	tele02	NA	7.a

112	B	eDNA	NA	Dukes Dock	Liverpool	England	NA	NA	QBT	NA	NA	NA	tele02	NA	7.a
113	B	eDNA	NA	Dukes Dock	Liverpool	England	NA	NA	QBT	NA	NA	NA	tele02	NA	7.a
114	B	MP	LIV_1	Dukes Dock	Liverpool	England	SCUBA	Et	QBT	hott	NR	standard	tele02	38	7.a
115	B	MP	LIV_2	Dukes Dock	Liverpool	England	SCUBA	Et	QBT	hott	NR	standard	tele02	38	7.a
116	B	MP	LIV_3	Dukes Dock	Liverpool	England	SCUBA	Et	QBT	hott	NR	standard	tele02	38	7.a
117	B	MP	LIV_3	Dukes Dock	Liverpool	England	SCUBA	b	QBT	hott	NR	standard	tele02	38	7.a
118	B	MP	LIV_2	Dukes Dock	Liverpool	England	SCUBA	b	QBT	hott	NR	standard	tele02	38	7.a
119	B	MP	LIV_1	Dukes Dock	Liverpool	England	SCUBA	b	QBT	hott	NR	standard	tele02	38	7.a
120	B	MP	NEW_1	Wuddy Rocks	St. Abbs Head	Scotland	SCUBA	Et	QBT	hotf	NR	standard	tele02	45	4.b
121	B	MP	NEW_1	Wuddy Rocks	St. Abbs Head	Scotland	SCUBA	b	QBT	hotf	NR	standard	tele02	45	4.b
122	B	MP	NEW_2	Wuddy Rocks	St. Abbs Head	Scotland	SCUBA	Et	QBT	hotf	NR	standard	tele02	45	4.b
123	B	MP	NEW_2	Wuddy Rocks	St. Abbs Head	Scotland	SCUBA	b	QBT	hotf	NR	standard	tele02	45	4.b
124	B	MP	NEW_3	Wuddy Rocks	St. Abbs Head	Scotland	SCUBA	Et	QBT	hotf	NR	standard	tele02	45	4.b
125	B	MP	NEW_3	Wuddy Rocks	St. Abbs Head	Scotland	SCUBA	b	QBT	hotf	NR	standard	tele02	45	4.b
126	B	MP	NEW_4	White Heugh	St. Abbs Head	Scotland	SCUBA	Et	QBT	hotf	NR	standard	tele02	37	4.b
127	B	MP	NEW_4	White Heugh	St. Abbs Head	Scotland	SCUBA	b	QBT	hotf	NR	standard	tele02	37	4.b
128	B	MP	NEW_5	White Heugh	St. Abbs Head	Scotland	SCUBA	Et	QBT	hotf	NR	standard	tele02	37	4.b
129	B	MP	NEW_5	White Heugh	St. Abbs Head	Scotland	SCUBA	b	QBT	hotf	NR	standard	tele02	37	4.b
130	B	MP	NEW_6	White Heugh	St. Abbs Head	Scotland	SCUBA	Et	QBT	hotf	NR	standard	tele02	37	4.b
131	B	MP	NEW_6	White Heugh	St. Abbs Head	Scotland	SCUBA	b	QBT	hotf	NR	standard	tele02	37	4.b
132	C	MP	CV_1	Bodega de choco	Boa Vista	Cape Verde	SCUBA	Et	QBT	hott	NR	standard	elas02	40	NA

133	C	MP	CV_1	Bodega de choco	Boa Vista	Cape Verde	SCUBA	Et	QBT	hott	NR	standard	elas02	40	NA
134	C	MP	CV_1	Bodega de choco	Boa Vista	Cape Verde	SCUBA	Et	QBT	hott	NR	standard	elas02	40	NA
135	C	MP	CA_1	San Carlos Beach Wall	California	USA	SCUBA	Et	QBT	hotf	NR	standard	elas02	42	NA
136	C	MP	CA_1	San Carlos Beach Wall	California	USA	SCUBA	Et	QBT	hotf	NR	standard	elas02	42	NA
137	C	MP	CA_1	San Carlos Beach Wall	California	USA	SCUBA	Et	QBT	hotf	NR	standard	elas02	42	NA
138	C	MP	RS_1	South Beach	Gulf of Aqaba	Jordan	SCUBA	Et	QBT	hott	NR	standard	elas02	50	NA
139	C	MP	RS_1	South Beach	Gulf of Aqaba	Jordan	SCUBA	Et	QBT	hott	NR	standard	elas02	50	NA
140	C	MP	RS_1	South Beach	Gulf of Aqaba	Jordan	SCUBA	Et	QBT	hott	NR	standard	elas02	50	NA
141	C	MP	SAD_1	SL9F	Sodwana Bay	South Africa	SCUBA	b	QBT	hotf	NR	standard	elas02	NR	NA
142	C	MP	SAD_1	SL9F	Sodwana Bay	South Africa	SCUBA	b	QBT	hotf	NR	standard	elas02	NR	NA
143	C	MP	SAD_1	SL9F	Sodwana Bay	South Africa	SCUBA	b	QBT	hotf	NR	standard	elas02	NR	NA
144	C	MP	SAD_2	SL9M	Sodwana Bay	South Africa	SCUBA	b	QBT	hotf	NR	standard	elas02	NR	NA
145	C	MP	SAD_2	SL9M	Sodwana Bay	South Africa	SCUBA	b	QBT	hotf	NR	standard	elas02	NR	NA
146	C	MP	SAD_2	SL9M	Sodwana Bay	South Africa	SCUBA	b	QBT	hotf	NR	standard	elas02	NR	NA
147	C	MP	SAD_3	SL7F1	Sodwana Bay	South Africa	SCUBA	b	QBT	hotf	NR	standard	elas02	NR	NA
148	C	MP	SAD_3	SL7F1	Sodwana Bay	South Africa	SCUBA	b	QBT	hotf	NR	standard	elas02	NR	NA
149	C	MP	SAD_3	SL7F1	Sodwana Bay	South Africa	SCUBA	b	QBT	hotf	NR	standard	elas02	NR	NA
150	C	MP	SAD_4	SL7F2	Sodwana Bay	South Africa	SCUBA	b	QBT	hotf	NR	standard	elas02	NR	NA

151	C	MP	SAD_4	SL7F2	Sodwana Bay	South Africa	SCUBA	b	QBT	hotf	NR	standard	elas02	NR	NA
152	C	MP	SAD_4	SL7F2	Sodwana Bay	South Africa	SCUBA	b	QBT	hotf	NR	standard	elas02	NR	NA
153	D	MP	NOR_1	Radbod	Ørsta	Norway	SCUBA	b	MUT	hotf	NR	standard	tele02	40	2.a.2
154	D	MP	NOR_1	Radbod	Ørsta	Norway	SCUBA	b	MUT	hotf	NR	standard	tele02	40	2.a.2
155	D	MP	NOR_1	Radbod	Ørsta	Norway	SCUBA	b	MUT	hotf	NR	standard	tele02	40	2.a.2
156	D	MP	NOR_2	Måløy	Måløy	Norway	SCUBA	b	MUT	hotf	NR	standard	tele02	40	4.a
157	D	MP	NOR_2	Måløy	Måløy	Norway	SCUBA	b	MUT	hotf	NR	standard	tele02	40	4.a
158	D	MP	NOR_2	Måløy	Måløy	Norway	SCUBA	b	MUT	hotf	NR	standard	tele02	40	4.a
159	D	MP	NOR_3	Dogfish Walk	Bergen	Norway	SCUBA	b	MUT	hotf	NR	standard	tele02	41	4.a
160	D	MP	NOR_3	Dogfish Walk	Bergen	Norway	SCUBA	b	MUT	hotf	NR	standard	tele02	41	4.a
161	D	MP	NOR_3	Dogfish Walk	Bergen	Norway	SCUBA	b	MUT	hotf	NR	standard	tele02	41	4.a
162	D	MP	NOR_4	Dogfish Walk	Bergen	Norway	SCUBA	b	MUT	hotf	NR	standard	tele02	41	4.a
163	D	MP	PLYM_1	Mount Batten	Plymouth	England	snorkel	Et	MUT	ham	NR	standard	tele02	60	7.e
164	D	MP	PLYM_1	Mount Batten	Plymouth	England	snorkel	Et	MUT	ham	NR	standard	tele02	60	7.e
165	D	MP	PLYM_1	Mount Batten	Plymouth	England	snorkel	Et	MUT	ham	NR	standard	tele02	60	7.e
166	D	MP	PLYM_2	Mount Batten	Plymouth	England	snorkel	Et	MUT	ham	NR	standard	tele02	60	7.e
167	D	MP	PLYM_2	Mount Batten	Plymouth	England	snorkel	Et	MUT	ham	NR	standard	tele02	60	7.e
168	D	MP	PLYM_2	Mount Batten	Plymouth	England	snorkel	Et	MUT	ham	NR	standard	tele02	60	7.e
169	D	MP	PLYM_3	Mount Batten	Plymouth	England	snorkel	Et	MUT	ham	NR	standard	tele02	60	7.e
170	D	MP	PLYM_3	Mount Batten	Plymouth	England	snorkel	Et	MUT	ham	NR	standard	tele02	60	7.e
171	D	MP	PLYM_3	Mount Batten	Plymouth	England	snorkel	Et	MUT	ham	NR	standard	tele02	60	7.e
172	D	MP	PLYM_4	Mount Batten	Plymouth	England	snorkel	Et	MUT	ham	NR	standard	tele02	60	7.e
173	D	MP	PLYM_4	Mount Batten	Plymouth	England	snorkel	Et	MUT	ham	NR	standard	tele02	60	7.e
174	D	MP	PLYM_4	Mount Batten	Plymouth	England	snorkel	Et	MUT	ham	NR	standard	tele02	60	7.e
175	D	MP	PLYM_5	Mount Batten	Plymouth	England	snorkel	Et	MUT	ham	NR	standard	tele02	60	7.e
176	D	MP	PLYM_5	Mount Batten	Plymouth	England	snorkel	Et	MUT	ham	NR	standard	tele02	60	7.e
177	D	MP	PLYM_5	Mount Batten	Plymouth	England	snorkel	Et	MUT	ham	NR	standard	tele02	60	7.e
178	D	MP	PLYM_6	Mount Batten	Plymouth	England	snorkel	Et	MUT	ham	NR	standard	tele02	60	7.e

179	D	MP	PLYM_6	Mount Batten	Plymouth	England	snorkel	Et	MUT	ham	NR	standard	tele02	60	7.e
180	D	MP	PLYM_6	Mount Batten	Plymouth	England	snorkel	Et	MUT	ham	NR	standard	tele02	60	7.e
181	D	MP	PLYM_7	Mount Batten	Plymouth	England	snorkel	Et	MUT	ham	NR	standard	tele02	60	7.e
182	D	MP	PLYM_7	Mount Batten	Plymouth	England	snorkel	Et	MUT	ham	NR	standard	tele02	60	7.e
183	D	MP	PLYM_7	Mount Batten	Plymouth	England	snorkel	Et	MUT	ham	NR	standard	tele02	60	7.e
184	D	MP	NOR_4	Dogfish Walk	Bergen	Norway	SCUBA	b	MUT	hotf	NR	standard	tele02	41	4.a
185	D	MP	NOR_4	Dogfish Walk	Bergen	Norway	SCUBA	b	MUT	hotf	NR	standard	tele02	41	4.a
186	D	MP	NOR_5	Dogfish Walk	Bergen	Norway	SCUBA	b	MUT	hotf	NR	standard	tele02	41	4.a
187	D	MP	NOR_5	Dogfish Walk	Bergen	Norway	SCUBA	b	MUT	hotf	NR	standard	tele02	41	4.a
188	D	MP	NOR_5	Dogfish Walk	Bergen	Norway	SCUBA	b	MUT	hotf	NR	standard	tele02	41	4.a
189	D	MP	NOR_6	Welheim	Florø	Norway	SCUBA	b	MUT	hotf	NR	standard	tele02	41	4.a
190	D	MP	NOR_6	Welheim	Florø	Norway	SCUBA	b	MUT	hotf	NR	standard	tele02	41	4.a

S4 Table 1.2. Additional geographic information for samples sequenced where Ocean basins are as follows: ‘NAt’ = North Atlantic, ‘NPa’ = North Pacific, and ‘Ind’ = Indian.

Dive Site Name	Location	Country	Ocean Basin	ICES Ecoregion	ICES Area
Mount Batten, Plymouth	Plymouth	England	NAt	Celtic Seas	7.e
South Beach 1, Studland Bay, Dorset	Dorset	England	NAt	Greater North Sea	7.d
South Beach 2, Studland Bay, Dorset	Dorset	England	NAt	Greater North Sea	7.d
SMS Bayern, Scapa Flow, Orkney	Orkney	Scotland	NAt	Celtic Seas	4.a
SMS Brummer, Scapa Flow, Orkney	Orkney	Scotland	NAt	Celtic Seas	4.a
Dukes Dock, Liverpool	Liverpool	England	NAt	Celtic Seas	7.a
Wuddy Rocks, St. Abbs, Scotland	St. Abbs Head	Scotland	NAt	Greater North Sea	4.b
White Heugh, St. Abbs, Scotland	St. Abbs Head	Scotland	NAt	Greater North Sea	4.b
Bodega de choco	Boa Vista	Cape Verde	NAt	NA	NA
San Carlos Beach Wall, CA, USA	California	USA	NPa	NA	NA
South Beach, Gulf of Aqaba, Jordan	Gulf of Aqaba	Jordan	Ind	NA	NA
SL9F, Sodwana Bay, South Africa	Sodwana Bay	South Africa	Ind	NA	NA
SL9M, Sodwana Bay, South Africa	Sodwana Bay	South Africa	Ind	NA	NA
SL7F1, Sodwana Bay, South Africa	Sodwana Bay	South Africa	Ind	NA	NA
SL7F2, Sodwana Bay, South Africa	Sodwana Bay	South Africa	Ind	NA	NA
Radbod, Ørsta, Norway	Ørsta	Norway	NAt	Norwegian Sea	2.a.2
Måløy, Norway	Måløy	Norway	NAt	Greater North Sea	4.a
Dogfish Walk, Bergen, Norway	Bergen	Norway	NAt	Greater North Sea	4.a
Welheim, Florø, Norway	Florø	Norway	NAt	Greater North Sea	4.a

Abbreviations used in S4 Tables 2-6.

Supplementary tables 2-6 show calculations of MOTUs and taxa from each sequencing library analysed in this dataset.

Tables X.1 show the total MOTUs and total reads in the sequencing library; the total MOTUs with taxonomy assigned at ≥70% (**motus70**), the total reads with taxonomy assigned at ≥70% (**reads70**) as well as the percent of each relative to the whole sequencing run (**percentmotus70**, **percentreads70**); the exact same statistics are calculated again but for MOTUs and reads with taxonomy assigned at ≥98% (i.e., **motus98**, **reads98**, **percentmotus98**, **percentreads98**).

Tables X.2 present statistics showing how MOTUs with taxonomy ≥70% were assigned. Specifically, the number of MOTUs with species-level assignments that had consensus between the three taxonomic assignment approaches (See Materials and Methods) (**species_3way**) and the percent of the total sequencing run that they represented (**percentmotuspecies_3way**). The same statistics were calculated at genus-level (**genus_3way**, **percentmotugenus_3way**). The number of MOTUs with species-level assignments that had consensus between two taxonomic assignment approaches (**species_2way**) and the percent of the total sequencing run that they represented (**percentmotuspecies_2way**). The number of MOTUs assigned to *Homo sapiens* (**human**) and the percent of the total sequencing run that they represented (**percentmotu_human**). And finally, the number of MOTUs with species-level assignments that did not have consensus were assigned using ecotag and a global reference database (**ecotag**) and the percent of the total sequencing run that they represented (**percentmotu_ecotag**).

Tables X.3 present statistics showing MOTUs with taxonomy ≥98% (**motus98**) at species (**species_98**) genus (**genus_98**) and higher (**other_98**) levels, the percent of the run they represent (**percentmotuspecies_98**, **percentmotugenus_98**, **percentmotuother_98**), and how they were assigned. Specifically, the number of MOTUs with genus and species-level assignments that had consensus between the three taxonomic assignment approaches (See Materials and Methods) (**method3way98**) and the percent of the total sequencing run that they represented (**percentmotu_3way98**). The number of MOTUs with genus and species-level assignments that had consensus between two taxonomic assignment approaches (**method2way98**) and the percent of the total sequencing run that they represented (**percentmotu_2way98**). The number of MOTUs assigned to *Homo sapiens* (**human98**) and the percent of the total sequencing run that they represented (**percentmotu_human98**). And finally, the number of MOTUs with species-level assignments that did not have consensus were assigned using ecotag and a global reference database (**ecotag98**) and the percent of the total sequencing run that they represented (**percentmotu_ecotag98**).

S4 Tables 2.1-2.3. Sequencing statistics from sequencing run 1, library 1 (tele02 primer).

S4 Table 2.1.

stat_all	value_all	stat_70	value_70	stat_98	value_98
total_MOTUs	483	motus70	451	motus98	243
Library_Reads	**1824604**	reads70	1823704	reads98	**1687590**
-	-	percentmotus70	93.37	percentmotus98	50.31
-	-	percentreads70	99.95	percentreads98	**92.49**

S4 Table 2.2

stat	value
motus70	451
species_3way	64
percentmotuspecies_3way	14.19
genus_3way	7
percentmotugenus_3way	1.55
species_2way	153
percentmotuspecies_2way	33.92
human	31
percentmotu_human	6.87
ecotag	196
percentmotu_ecotag	43.46

S4 table 2.3

stat2	value2
motus98	243
species_98	204
percentmotuspecies_98	83.95
genus_98	27
percentmotugenus_98	11.11
other_98	12
percentmotuother_98	4.94
method3way98	71
percentmotu_3way98	**29.22**
method2way98	92
percentmotu_2way98	**37.86**
human98	6
percentmotu_human98	2.47
ecotag98	74
percentmotu_ecotag98	**30.45**

S4 Tables 3.1-3.3. Sequencing statistics from sequencing run 2, library 1 (elas02 primer).

S4 Table 3.1

stat_all	value_all	stat_70	value_70	stat_98	value_98
total_motus	1324	motus70	704	motus98	450
lib_reads	**954294**	reads70	927707	reads98	**884808**
-	-	percentmotus70	53.17	percentmotus98	33.99
-	-	percentreads70	97.21	percentreads98	**92.72**

S4 Table 3.2

stat	value
motus70	704
species_3way	139
percentmotuspecies_3way	19.74
genus_3way	67
percentmotugenus_3way	9.52
species_2way	349
percentmotuspecies_2way	49.57
human	27
percentmotu_human	3.84
ecotag	122
percentmotu_ecotag	17.33

S4 Table 3.3

stat2	value2
motus98	450
species_98	360
percentmotuspecies_98	80
genus_98	75
percentmotugenus_98	16.67
other_98	15
percentmotuother_98	3.33
method3way98	151
percentmotu_3way98	**33.56**
method2way98	242
percentmotu_2way98	**53.78**
human98	7
percentmotu_human98	1.56
ecotag98	50
percentmotu_ecotag98	**11.11**

S4 Tables 4.1-4.3. Sequencing statistics from sequencing run 2, library 2 (tele02 primer).

S4 Table 4.1

stat_all	value_all	stat_70	value_70	stat_98	value_98
total_motus	828	motus70	820	motus98	385
lib_reads	**1494951**	reads70	1494884	reads98	**1292245**
-	-	percentmotus70	99.03	percentmotus98	46.50
-	-	percentreads70	100.00	percentreads98	**86.44**

S4 Table 4.2

stat	value
motus70	820
species_3way	121
percentmotuspecies_3way	14.76
genus_3way	69
percentmotugenus_3way	8.41
species_2way	177
percentmotuspecies_2way	21.59
human	62
percentmotu_human	7.56
ecotag	391
percentmotu_ecotag	47.68

S4 Table 4.3

stat2	value2
motus98	385
species_98	252
percentmotuspecies_98	65.45
genus_98	114
percentmotugenus_98	29.61
other_98	19
percentmotuother_98	4.94
method3way98	126
percentmotu_3way98	**32.73**
method2way98	127
percentmotu_2way98	**32.99**
human98	9
percentmotu_human98	2.34
ecotag98	123
percentmotu_ecotag98	**31.95**

S4 Tables 5.1–5.3. Sequencing statistics from sequencing run 3, library 1 (elas02 primer).

S4 Table 5.1

stat_all	value_all	stat_70	value_70	stat_98	value_98
total_motus	3784	motus70	2493	motus98	917
lib_reads	**909806**	reads70	895852	reads98	**785300**
-	-	percentmotus70	65.88266	percentmotus98	24.23362
-	-	percentreads70	98.46627	percentreads98	**86.3151**

S4 Table 5.2

stat	value
motus70	2493
species_3way	215
percentmotuspecies_3way	8.62
genus_3way	73
percentmotugenus_3way	2.93
species_2way	651
percentmotuspecies_2way	26.11
human	402
percentmotu_human	16.13
ecotag	1152
percentmotu_ecotag	46.21

S4 Table 5.3

stat2	value2
motus98	917
species_98	716
percentmotuspecies_98	78.08
genus_98	147
percentmotugenus_98	16.03
other_98	54
percentmotuother_98	5.89
method3way98	213
percentmotu_3way98	**23.23**
method2way98	487
percentmotu_2way98	**53.11**
human98	48
percentmotu_human98	5.23
ecotag98	169
percentmotu_ecotag98	**18.43**

S4 Tables 6.1-6.3. Sequencing statistics from sequencing run 4, library 1 (tele02 primer).

S4 Table 6.1

stat_all	value_all	stat_70	value_70	stat_98	value_98
total_motus	1071	motus70	1064	motus98	333
lib_reads	**989266**	reads70	989113	reads98	**929358**
-	-	percentmotus70	99.35	percentmotus98	31.09
-	-	percentreads70	99.98	percentreads98	**93.94**

S4 Table 6.2

stat	value
motus70	1064
species_3way	117
percentmotuspecies_3way	11.00
genus_3way	39
percentmotugenus_3way	3.67
species_2way	301
percentmotuspecies_2way	28.29
human	179
percentmotu_human	16.82
ecotag	428
percentmotu_ecotag	40.23

S4 Table 6.3

stat2	value2
motus98	333
species_98	310
percentmotuspecies_98	93.09
genus_98	20
percentmotugenus_98	6.01
other_98	3
percentmotuother_98	0.90
method3way98	125
percentmotu_3way98	**37.54**
method2way98	175
percentmotu_2way98	**52.55**
human98	16
percentmotu_human98	4.80
ecotag98	17
percentmotu_ecotag98	**5.11**

S4 Table 7. Taxonomic assignments and taxa category of teleosts detected in the Ocean Exhibit.

Teleost Detection	Category
Abudefduf	inventory
Acanthurus	inventory
Anisotremus virginicus	inventory
Balistes vetula	inventory
Caesio caerulaurea	inventory
Caesio cuning	inventory
Chrysiptera cyanea	inventory
Coris gaimard	inventory
Diodon	inventory
Epinephelus	inventory
Epinephelus lanceolatus	inventory
Gnathanodon speciosus	inventory
Grammistes sexlineatus	inventory
Gymnothorax funebris	inventory
Haemulon sciurus	inventory
Heniochus acuminatus	inventory
Labroides dimidiatus	inventory
Lutjanus	inventory
Lutjanus kasmira	inventory
Lutjanus sebae	inventory
Megalops atlanticus	inventory
Megalops cyprinoides	inventory
Naso	inventory
Odonus niger	inventory
Paracanthurus hepatus	inventory
Paranthias colonus	inventory
Platax	inventory
Pomacanthus maculosus	inventory
Pseudobalistes fuscus	inventory
Siganus	inventory
Thalassoma	inventory
Argyrosomus regius	putative inventory
Monodactylus argenteus	putative inventory
Naso elegans	putative inventory
Pseudocaranx dentex	putative inventory
Trachinotus blochii	putative inventory
Trachinotus rhodopus	putative inventory
Ammodytes	food
Auxis	food
Clupea harengus	food
Clupeinae	food
Euthynnus	food

Gadidae	food
Gadus	food
Hippoglossus	food
Melanogrammus	food
Merluccius merluccius	food
Sardina pilchardus	food
Scomber	food
Scomber scombrus	food
Trachurus trachurus	food

S4 Table 8. Taxonomic assignments and taxa category of teleosts detected in the Coral cave Exhibit.

Teleost Detection	Category
Abudefduf	inventory
Acanthurus	inventory
Acanthurus olivaceus	inventory
Arothron	inventory
Arothron hispidus	inventory
Balistoides conspicillum	inventory
Chaetodon	inventory
Chrysiptera	inventory
Chrysiptera cyanea	inventory
Diodon	inventory
Gymnomuraena zebra	inventory
Gymnothorax flavimarginatus	inventory
Holacanthus ciliaris	inventory
Labroides dimidiatus	inventory
Melichthys	inventory
Myripristis	inventory
Odonus niger	inventory
Paracanthurus hepatus	inventory
Parupeneus cyclostomus	inventory
Platax	inventory
Plectorhinchus vittatus	inventory
Pomacanthus	inventory
Pseudanthias squamipinnis	inventory
Siganus	inventory
Thalassoma	inventory
Zebrasoma	inventory
Zebrasoma desjardinii	inventory
Balistapus undulatus	putative inventory
Cephalopholis sexmaculata	putative inventory
Chaetodon auriga	putative inventory
Chromileptes altivelis	putative inventory
Diagramma	putative inventory
Gymnothorax kidako	putative inventory
Hemitaurichthys	putative inventory
Naso elegans	putative inventory
Zebrasoma velifer	putative inventory
Clupea harengus	food
Gadidae	food
Micromesistius poutassou	food
Pollachius virens	food
Scomber	food
Scomberesox saurus	food

S4 Table 9. Results of the linear models run on the timed soaking experiments and controlled aquarium dives.

S4 Table 9.1 Linear model results for change in MOTUs through time for the soaking experiment.

Coefficients				
	estimate	Std. error	t value	Pr(>|t|)
Intercept (MOTUs)	16.68991	4.67073	3.573	0.00217
Slope (time)	0.12402	0.03774	3.287	0.00410
	Multiple R^2: 0.375	Adjusted R^2: 0.3403	F statistic : 10.8 on 1 and 18	p-value: **0.004101**

S4 Table 9.2 Linear model results for change in taxa through time for the soaking experiment.

Coefficients				
	estimate	Std. error	t value	Pr(>|t|)
Intercept (Taxa)	10.28326	2.09996	4.897	0.000116
Slope (time)	0.08877	0.01697	5.232	5.63e-05
	Multiple R^2: 0.6033	Adjusted R^2: 0.5813	F statistic : 27.38 on 1 and 18	p-value: **5.633e-05**

S4 Table 9.3 Linear model results for change in MOTUs through time for the soaking experiment and with Ocean Exhibit dives added in.

Coefficients				
	estimate	Std. error	t value	Pr(>|t|)
Intercept (Taxa)	23.28414	4.47017	5.209	1.94e-05
Slope (time)	0.09958	0.04097	2.431	0.0223
	Multiple R^2: 0.1851	Adjusted R^2: 0.1538	F statistic : 5.908 on 1 and 26 DF	p-value: **0.02228**

S4 Table 9.4 Linear model results for change in taxa through time for the soaking experiment and with Ocean Exhibit dives added in.

Coefficients				
	estimate	Std. error	t value	Pr(>|t|)
Intercept (Taxa)	14.78161	2.40377	6.149	1.68e-06
Slope (time)	0.07179	0.02203	3.258	0.00312
	Multiple R^2: 0.2899	Adjusted R^2: 0.2626	F statistic : 10.62 on 1 and 26 DF	p-value: **0.003116**

S4 Table 10. Results of PERMANOVA and beta-dispersion tests comparing syringe-filter eDNA samples to diver metaprobes (relating to Figure 3E).

S4 Table 10.1 Permutation test (function: permutest) for homogeneity of multivariate dispersions for dive sites.

	DF	Sum Sq	Mean Sq	F	N.perm	Pr(>F)
Groups	3	0.65627	0.218755	9.2787	999	0.001
Residuals	32	0.75443	0.023576			

S4 Table 10.2 Permutation test (function: permutest) for homogeneity of multivariate dispersions for sample type.

	DF	Sum Sq	Mean Sq	F	N.perm	Pr(>F)
Groups	1	0.02022	0.020219	0.8	999	0.386
Residuals	34	0.85925	0.025272			

S4 Table 10.3 PERMANOVA (function: adonis) testing. adonis2(jac_dat ~ site.name, data = cem_jac, permutations = 999, strata = cem_jac$type)

	DF	SumOfSqs	R2	F	Pr(>F)
type	3	3.9786	0.3041	4.6611	0.001
Residual	32	9.1047	0.6959		
total	35	13.0833	1.0000		

S4 Table 10.4 PERMANOVA (function: adonis) testing. adonis2(jac_dat ~ type, data = cem_jac, permutations = 999, strata = cem_jac$site.name)

	DF	SumOfSqs	R2	F	Pr(>F)
type	1	0.5922	0.04526	1.6118	0.057
Residual	34	12.4911	0.95474		
total	35	13.0833	1.00000		

S4 Table 11. GLMM output testing if input weight of gauze and lysis buffer effects alpha-diversity.

REML criterion at convergence		109.9			
Scaled residuauls:					
Min		1Q	Median	3Q	Max
-1.4190		-0.4807	-0.2409	0.3736	2.6272
Random effects:					
Groups		Name	Variance	Standard deviation	Corr
mp_id		(intercept)	2.321e+01	4.8177739	
		Weight_g	2.322e+01	4.8184723	0.00
		lysis	4.791e-07	0.0006922	-0.01 -1.00
		Residual	2.322e+01	4.8185362	
Number of obs:		18	Groups:	mp_id, 2	
Fixed effects:					
	Estimate	Std. Error	df	T value	Pr(>|t|)
(intercept)	-16.332898	48.364102	2.030992	-0.338	0.767
Weight_g	3.878243	8.048875	1.173324	0.482	0.704
lysis	-0.001883	0.001932	4.284934	-0.975	0.382
time	0.255225	0.577383	2.015853	0.442	0.701
Correlation of fixed effects:					
			intercept	Weight_g	lysis
		Weight_g	-0.016		
		lysis	0.003	-0.876	
		time	-0.996	-0.014	0.007

S4 Table 12. GLMM to test for differences in extraction method.

REML criterion at convergence		68.6			
Scaled residuauls:					
Min		1Q	Median	3Q	Max
-1.68974		-0.70852	-0.06431	0.52593	2.90766
Random effects:					
Groups		Name	Variance	Standard deviation	Corr
mp_id		(intercept)	2.062e-10	1.436e-05	
		ExtractionQBT	6.721e-10	2.592e-05	-0.91
		Residual	3.692e-01	6.076e-01	
Number of obs:		36	Groups:	mp_id, 7	
Fixed effects:					
	Estimate	Std. Error	df	T value	Pr(>|t|)
(intercept)	1.02673	0.20254	32	5.069	1.62e-05
extractionQBT	0.08671	0.20254	32	0.428	0.671
Site.names Bayern, Orkney	-0.15793	0.24806	32	-0.637	0.529
Site.names Beach 1, Studland Bay	-0.36166	0.24806	32	-1.458	0.155
Correlation of fixed effects:					
			intercept	extractionQBT	Site.names Bayern, Orkney
	extractionQBT		-0.500		
	Site.names Bayern, Orkney		-0.612	0.000	
	Site.names Beach 1, Studland Bay		-0.612	0.000	0.500

S4 Table 13. GLMM to test differences in preservation method.

REML criterion at convergence	138			
Scaled residuauls:				
Min	1Q	Median	3Q	Max
-1.23162	-0.70790	-0.09452	0.61072	1.92178
Random effects:				
Groups	Name	Variance	Standard deviation	Corr
mp_id	(intercept)	0.0000	0.0000	
	preservationEt	0.7409	0.8608	NaN
	Residual	7.2981	2.7015	
Number of obs:	33	Groups:	mp_id, 15	
Fixed effects:				

	Estimate	Std. Error	df	T value	Pr(>|t|)
(intercept)	-1.4699	6.2688	12.9708	-0.234	0.81827
preservationEt	3.0235	0.9993	25.3678	3.026	**0.00562**
Site.names Bayern, Orkney	3.3037	3.3489	17.9087	0.987	0.33702
Site.names Brummer, Orkney	0.9473	1.6823	25.2057	0.563	0.57836
Site.names White Heugh, St.Abbs	-0.1288	1.5969	24.6572	-0.081	0.93636
Site.names Wuddy Rocks, St.Abbs	-0.9541	1.5969	24.6572	-0.597	0.55565
Sequence.run	1.8409	3.0290	13.0953	0.608	0.55375
Correlation of fixed effects:					

	intercept	preservationEt	Site.names Bayern, Orkney	Site.names Brummer, Orkney	Site.names White Heugh, St.Abbs	Site.names Wuddy Rocks, St.Abbs
preservationEt	-0.260					
Site.names Bayern, Orkney	-0.936	0.128				
Site.names Brummer, Orkney	0.188	-0.060	-0.056			
Site.names White Heugh, St.Abbs	-0.127	0.000	0.238	0.475		
Site.names Wuddy Rocks, St.Abbs	-0.127	0.000	0.238	0.475	0.500	
Sequence.run	-0.981	0.190	0.896	-0.315	0.000	0.000

S4 Table 14. Results of CCA for diver metaprobe data.

S4 table 14.1 Metadata variables included for CCA model building process of all diver metaprobe data.

Metadata variable	Description
latitude	Continuous, decimal degrees
preservation	Categorical, indicating silica beads or ethanol
extraction	Categorical, indicating DNA extraction method
Weight_class	Categorical, indicating input weight range
Primer	Categorical, indicating primer used for PCR
Sequence.run	Categorical, indicating sequence run sample is from
Ocean_basin	Categorical, indicating ocean basin

S4 table 14.2 Significance of the variables selected for the minimum adequate CCA model of all data.

	DF	Chi Square	F	Pr(>F)
Ocean_basin	2	1.9760	5.0265	**0.001**
latitude	1	0.8184	4.1638	**0.001**
residual	108	21.2283		

S4 table 14.3 Metadata variables included for model building process of North Atlantic data.

Metadata variable	Description
latitude	Continuous, decimal degrees
preservation	Categorical, indicating silica beads or ethanol
extraction	Categorical, indicating DNA extraction method
Weight_class	Categorical, indicating input weight range
Primer	Categorical, indicating primer used for PCR
Sequence.run	Categorical, indicating sequence run sample is from
Ices_ecor	Categorical, ICES ecoregion
Ices_area	Categorical, ICES area

S4 table 14.4 Significance of the variables selected for the minimum adequate CCA model of North Atlantic data.

	DF	Chi Square	F	Pr(>F)
Ices_area	5	3.0978	5.1515	**0.001**
latitude	1	0.1656	1.3772	0.190
residual	85	10.2229		

S4 Table 15. Species-level detections from metaprobes worn by divers in nature. IUCN categories for each species are given, where 'CR' = critically endangered, 'EN' = endangered, 'VU' = vulnerable, 'NT' = near threatened, 'DD' = data deficient, 'LC' = least concern, and 'NE' = not evaluated.

Number	Class	Species	Read Counts	IUCN
1	Actinopterygii	*Anguilla anguilla*	2360	**CR**
2	Actinopterygii	*Gadus morhua*	1141	**VU**
3	Actinopterygii	*Mola mola*	198	**VU**
4	Actinopterygii	*Trachurus trachurus*	93	**VU**
5	Actinopterygii	*Bodianus pulcher*	46	**VU**
6	Actinopterygii	*Sardinella maderensis*	4	**VU**
7	Actinopterygii	*Salmo salar*	55188	**NT**
8	Actinopterygii	*Pagellus bogaraveo*	259	**NT**
9	Actinopterygii	*Solea solea*	548	**DD**
10	Actinopterygii	*Engraulis mordax*	267	**DD**
11	Actinopterygii	*Brachyistius frenatus*	43	**DD**
12	Actinopterygii	*Ammodytes tobianus*	39	**DD**
13	Actinopterygii	*Dicentrarchus labrax*	123089	LC
14	Actinopterygii	*Trisopterus minutus*	122708	LC
15	Actinopterygii	*Atherina boyeri*	81556	LC
16	Actinopterygii	*Trisopterus esmarkii*	80690	LC
17	Actinopterygii	*Gasterosteus aculeatus*	47524	LC
18	Actinopterygii	*Symphodus melops*	25903	LC
19	Actinopterygii	*Labrus bergylta*	19641	LC
20	Actinopterygii	*Symphodus bailloni*	16254	LC
21	Actinopterygii	*Pomatoschistus minutus*	15600	LC
22	Actinopterygii	*Sprattus sprattus*	13969	LC
23	Actinopterygii	*Chelon auratus*	13652	LC
24	Actinopterygii	*Pseudanthias squamipinnis*	12871	LC
25	Actinopterygii	*Taurulus bubalis*	12243	LC
26	Actinopterygii	*Atherinopsis californiensis*	10133	LC
27	Actinopterygii	*Ctenolabrus rupestris*	9435	LC
28	Actinopterygii	*Sardinops sagax*	4985	LC
29	Actinopterygii	*Pollachius pollachius*	4073	LC
30	Actinopterygii	*Porichthys notatus*	3534	LC
31	Actinopterygii	*Gobiusculus flavescens*	2083	LC
32	Actinopterygii	*Gobius paganellus*	1942	LC
33	Actinopterygii	*Gymnammodytes semisquamatus*	1729	LC
34	Actinopterygii	*Clupea harengus*	1716	LC
35	Actinopterygii	*Gobius niger*	1580	LC
36	Actinopterygii	*Platichthys stellatus*	1561	LC
37	Actinopterygii	*Syngnathus typhle*	1486	LC
38	Actinopterygii	*Oxylebius pictus*	1455	LC
39	Actinopterygii	*Lutjanus bohar*	1264	LC

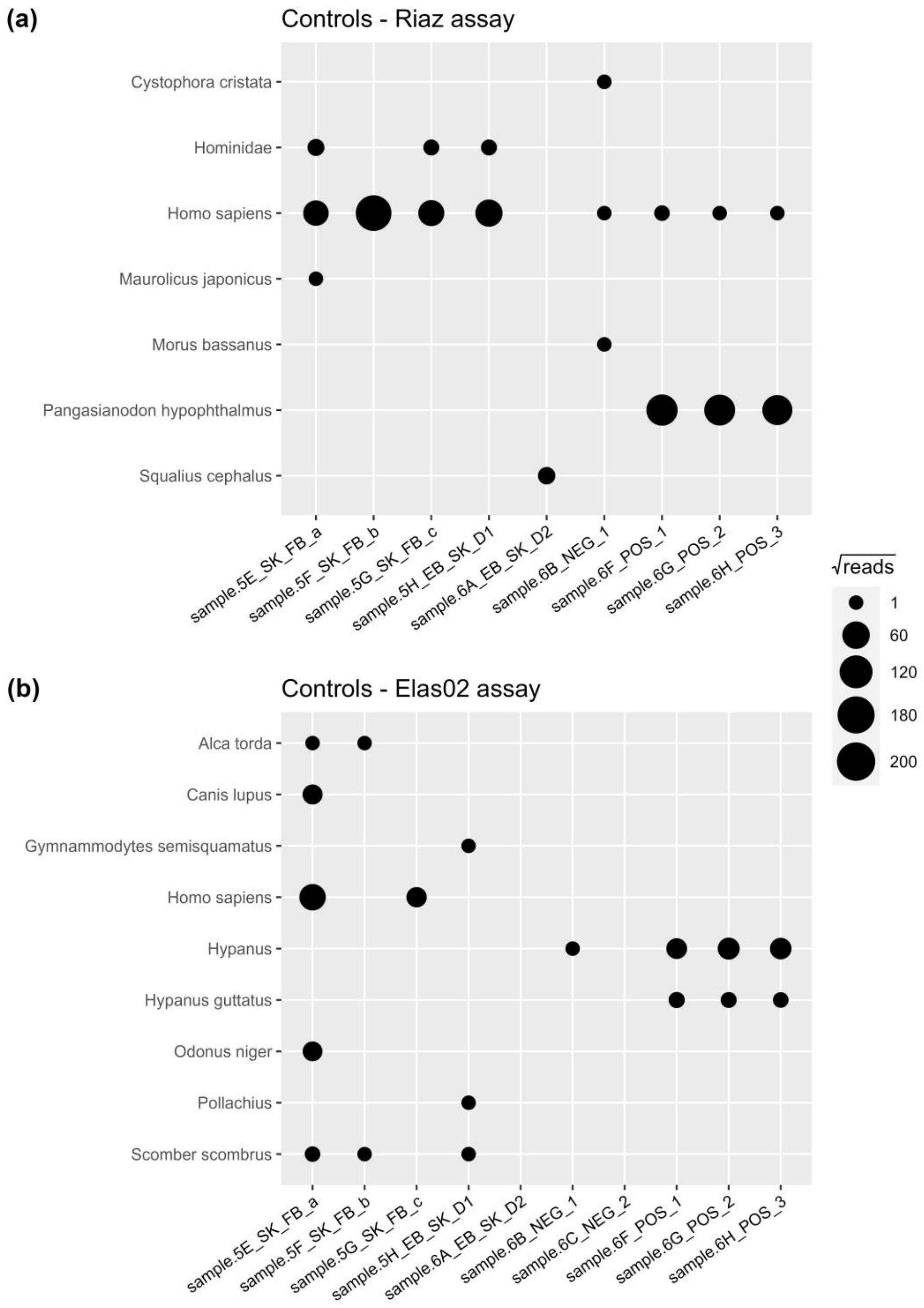
40	Actinopterygii	*Naso lopezi*	1154	LC
41	Actinopterygii	*Scarus rubroviolaceus*	966	LC
42	Actinopterygii	*Nerophis ophidion*	822	LC
43	Actinopterygii	*Syngnathus acus*	785	LC
44	Actinopterygii	*Acanthurus nigrofuscus*	767	LC
45	Actinopterygii	*Parablennius gattorugine*	642	LC
46	Actinopterygii	*Coryphoblennius galerita*	612	LC
47	Actinopterygii	*Ciliata mustela*	587	LC
48	Actinopterygii	*Spinachia spinachia*	557	LC
49	Actinopterygii	*Scophthalmus rhombus*	509	LC
50	Actinopterygii	*Sufflamen chrysopterum*	499	LC
51	Actinopterygii	*Sparus aurata*	452	LC
52	Actinopterygii	*Pterocaesio marri*	427	LC
53	Actinopterygii	*Balistapus undulatus*	420	LC
54	Actinopterygii	*Pomatoschistus pictus*	393	LC
55	Actinopterygii	*Phanerodon vacca*	388	LC
56	Actinopterygii	*Labrus mixtus*	380	LC
57	Actinopterygii	*Pomatoschistus microps*	339	LC
58	Actinopterygii	*Conger conger*	292	LC
59	Actinopterygii	*Pervagor janthinosoma*	289	LC
60	Actinopterygii	*Lipophrys pholis*	288	LC
61	Actinopterygii	*Acanthurus leucosternon*	271	LC
62	Actinopterygii	*Paracaesio sordida*	271	LC
63	Actinopterygii	*Cirrhitichthys oxycephalus*	267	LC
64	Actinopterygii	*Phoxinus phoxinus*	253	LC
65	Actinopterygii	*Merluccius merluccius*	252	LC
66	Actinopterygii	*Pycnochromis nigrurus*	241	LC
67	Actinopterygii	*Embiotoca jacksoni*	226	LC
68	Actinopterygii	*Acanthurus thompsoni*	203	LC
69	Actinopterygii	*Syngnathus rostellatus*	200	LC
70	Actinopterygii	*Anampses caeruleopunctatus*	168	LC
71	Actinopterygii	*Bodianus bilunulatus*	142	LC
72	Actinopterygii	*Callionymus lyra*	125	LC
73	Actinopterygii	*Sardina pilchardus*	125	LC
74	Actinopterygii	*Rhacochilus vacca*	122	LC
75	Actinopterygii	*Myripristis kuntee*	114	LC
76	Actinopterygii	*Sillago sihama*	111	LC
77	Actinopterygii	*Ostorhinchus apogonoides*	109	LC
78	Actinopterygii	*Anarrhichthys ocellatus*	95	LC
79	Actinopterygii	*Odonus niger*	83	LC
80	Actinopterygii	*Gymnothorax nudivomer*	79	LC
81	Actinopterygii	*Chlorophthalmus agassizi*	72	LC
82	Actinopterygii	*Citharichthys stigmaeus*	67	LC
83	Actinopterygii	*Labroides dimidiatus*	66	LC
84	Actinopterygii	*Coris caudimacula*	60	LC
85	Actinopterygii	*Diplodus sargus*	52	LC

86	Actinopterygii	*Lutjanus fulviflamma*	47	LC
87	Actinopterygii	*Liparis montagui*	46	LC
88	Actinopterygii	*Cyclothone alba*	44	LC
89	Actinopterygii	*Belone belone*	42	LC
90	Actinopterygii	*Cantherhines dumerilii*	41	LC
91	Actinopterygii	*Girella nigricans*	40	LC
92	Actinopterygii	*Zoarces viviparus*	38	LC
93	Actinopterygii	*Siganus luridus*	35	LC
94	Actinopterygii	*Parupeneus macronemus*	33	LC
95	Actinopterygii	*Ciliata septentrionalis*	31	LC
96	Actinopterygii	*Chromis weberi*	22	LC
97	Actinopterygii	*Scomber scombrus*	22	LC
98	Actinopterygii	*Acanthurus dussumieri*	21	LC
99	Actinopterygii	*Halichoeres californicus*	20	LC
100	Actinopterygii	*Halichoeres cosmetus*	18	LC
101	Actinopterygii	*Gobio gobio*	17	LC
102	Actinopterygii	*Gymnothorax melatremus*	17	LC
103	Actinopterygii	*Sparisoma cretense*	17	LC
104	Actinopterygii	*Cephalopholis sexmaculata*	16	LC
105	Actinopterygii	*Azurina lepidolepis*	15	LC
106	Actinopterygii	*Micrenophrys lilljeborgii*	15	LC
107	Actinopterygii	*Citharichthys sordidus*	14	LC
108	Actinopterygii	*Lutjanus argentimaculatus*	13	LC
109	Actinopterygii	*Pomacanthus imperator*	12	LC
110	Actinopterygii	*Salmo trutta*	12	LC
111	Actinopterygii	*Cymatogaster aggregata*	11	LC
112	Actinopterygii	*Heterostichus rostratus*	11	LC
113	Actinopterygii	*Caranx crysos*	10	LC
114	Actinopterygii	*Centropyge multispinis*	10	LC
115	Actinopterygii	*Crossorhombus valderostratus*	10	LC
116	Actinopterygii	*Lutjanus kasmira*	10	LC
117	Actinopterygii	*Parupeneus pleurostigma*	10	LC
118	Actinopterygii	*Megalaspis cordyla*	9	LC
119	Actinopterygii	*Caesio varilineata*	8	LC
120	Actinopterygii	*Ptereleotris evides*	7	LC
121	Actinopterygii	*Plectorhinchus orientalis*	6	LC
122	Actinopterygii	*Sargocentron diadema*	6	LC
123	Actinopterygii	*Antennatus tuberosus*	5	LC
124	Actinopterygii	*Stethojulis interrupta*	5	LC
125	Actinopterygii	*Ctenochaetus binotatus*	4	LC
126	Actinopterygii	*Trachinocephalus myops*	4	LC
127	Actinopterygii	*Trachinotus blochii*	4	LC
128	Actinopterygii	*Gnathanodon speciosus*	3	LC
129	Actinopterygii	*Sufflamen fraenatum*	3	LC
130	Actinopterygii	*Pollachius virens*	107912	NE
131	Actinopterygii	*Ammodytes marinus*	7591	NE

132	Actinopterygii	*Pholis gunnellus*	3025	NE
133	Actinopterygii	*Chirolophis japonicus*	2470	NE
134	Actinopterygii	*Trisopterus luscus*	1169	NE
135	Actinopterygii	*Toxabramis swinhonis*	1031	NE
136	Actinopterygii	*Cyclopterus lumpus*	812	NE
137	Actinopterygii	*Molva molva*	341	NE
138	Actinopterygii	*Rhacochilus toxotes*	217	NE
139	Actinopterygii	*Gadiculus argenteus*	184	NE
140	Actinopterygii	*Oplegnathus fasciatus*	158	NE
141	Actinopterygii	*Scorpaenichthys marmoratus*	85	NE
142	Actinopterygii	*Liparis mucosus*	64	NE
143	Actinopterygii	*Echiichthys vipera*	58	NE
144	Actinopterygii	*Plectorhinchus flavomaculatus*	55	NE
145	Actinopterygii	*Esselenichthys carli*	44	NE
146	Actinopterygii	*Sebastes gilli*	40	NE
147	Actinopterygii	*Micrometrus aurora*	35	NE
148	Actinopterygii	*Myoxocephalus scorpius*	34	NE
149	Actinopterygii	*Ophiodon elongatus*	34	NE
150	Actinopterygii	*Sphyraena jello*	30	NE
151	Actinopterygii	*Oncorhynchus mykiss*	28	NE
152	Actinopterygii	*Micromesistius poutassou*	13	NE
153	Actinopterygii	*Anoplopoma fimbria*	12	NE
154	Actinopterygii	*Plotosus lineatus*	6	NE
155	Actinopterygii	*Clinocottus recalvus*	3	NE
156	Actinopterygii	*Micrometrus minimus*	3	NE
157	Aves	*Alca torda*	4729	LC
158	Aves	*Columba livia*	1302	LC
159	Aves	*Pelecanus occidentalis*	125	LC
160	Aves	*Arenaria interpres*	101	LC
161	Aves	*Columba palumbus*	84	LC
162	Aves	*Uria aalge*	63	LC
163	Aves	*Larus occidentalis*	56	LC
164	Elasmobranchii	*Rhynchobatus djiddensis*	3	**CR**
165	Elasmobranchii	*Himantura uarnak*	1309	**EN**
166	Elasmobranchii	*Taeniurops meyeni*	14158	**VU**
167	Elasmobranchii	*Pateobatis fai*	109	**VU**
168	Elasmobranchii	*Pateobatis jenkinsii*	13	**VU**
169	Elasmobranchii	*Alopias vulpinus*	9	**VU**
170	Elasmobranchii	*Triaenodon obesus*	6	**VU**
171	Elasmobranchii	*Squalus acanthias*	3	**VU**
172	Elasmobranchii	*Myliobatis californica*	15	LC
173	Elasmobranchii	*Lamna ditropis*	5	LC
174	Mammalia	*Phocoena phocoena*	227	**VU**
175	Mammalia	*Tursiops aduncus*	31	**NT**
176	Mammalia	*Zalophus californianus*	17933	LC
177	Mammalia	*Mus musculus*	1101	LC

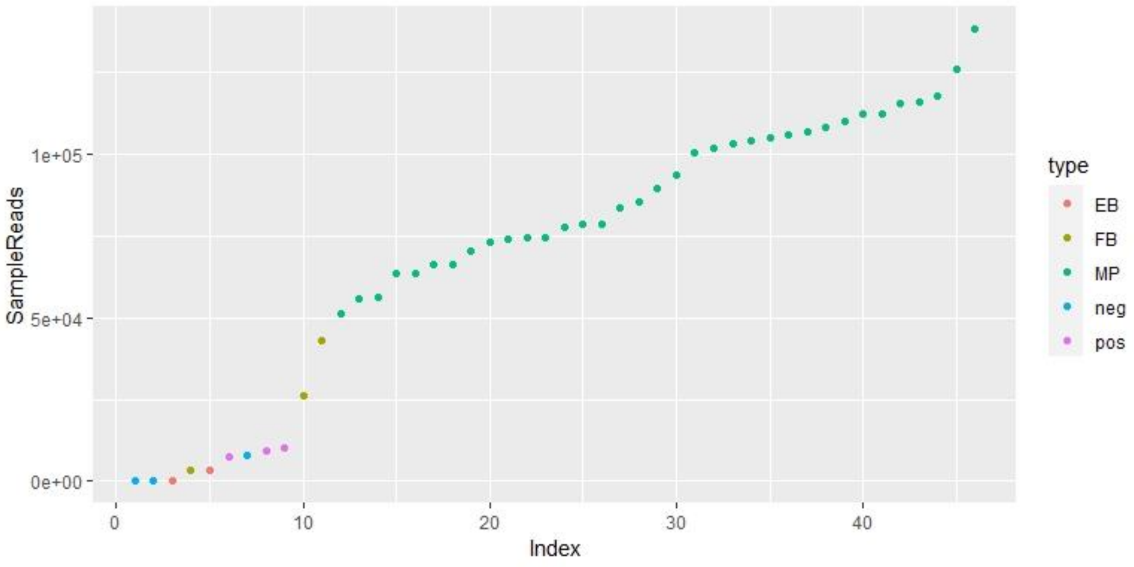
178	Mammalia	*Phoca vitulina*	81	LC
179	Mammalia	*Stenella attenuata*	11	LC
180	Reptilia	*Caretta caretta*	5	**VU**

S.5 Chapter 5 Supplement

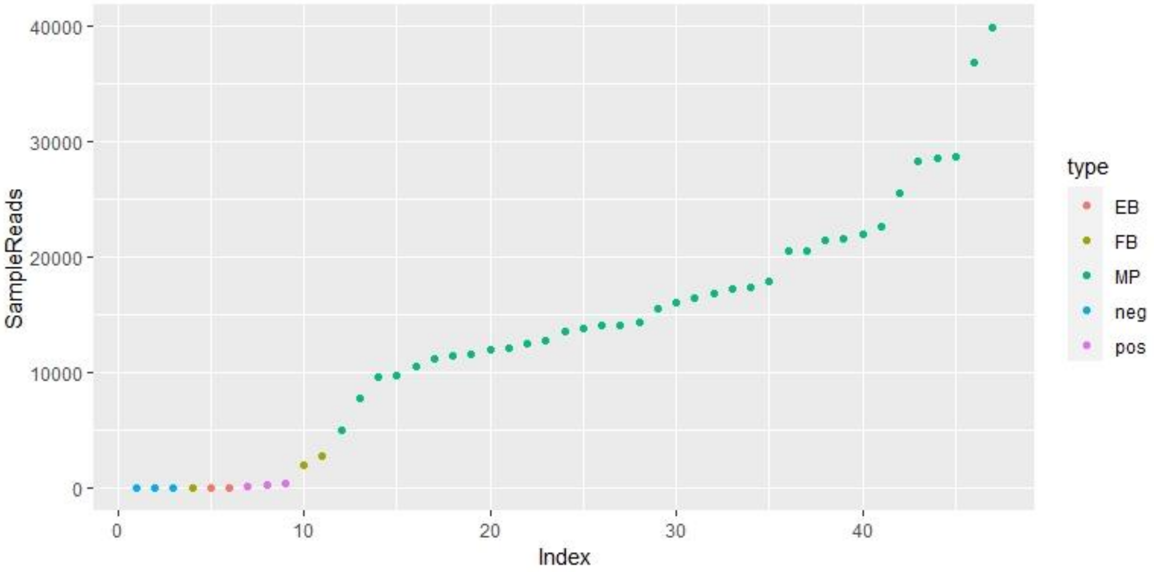


S5 Figure 1. Bubble-plot showing square root transformed reads in controls from the riaz **(a)** and elas02 **(b)** assays. An explanation of how contamination was dealt with can be found in the Methods section of the main manuscript. Two PCR negative controls and one negative control from the riaz and Elas02 assays contained no reads and are not shown.

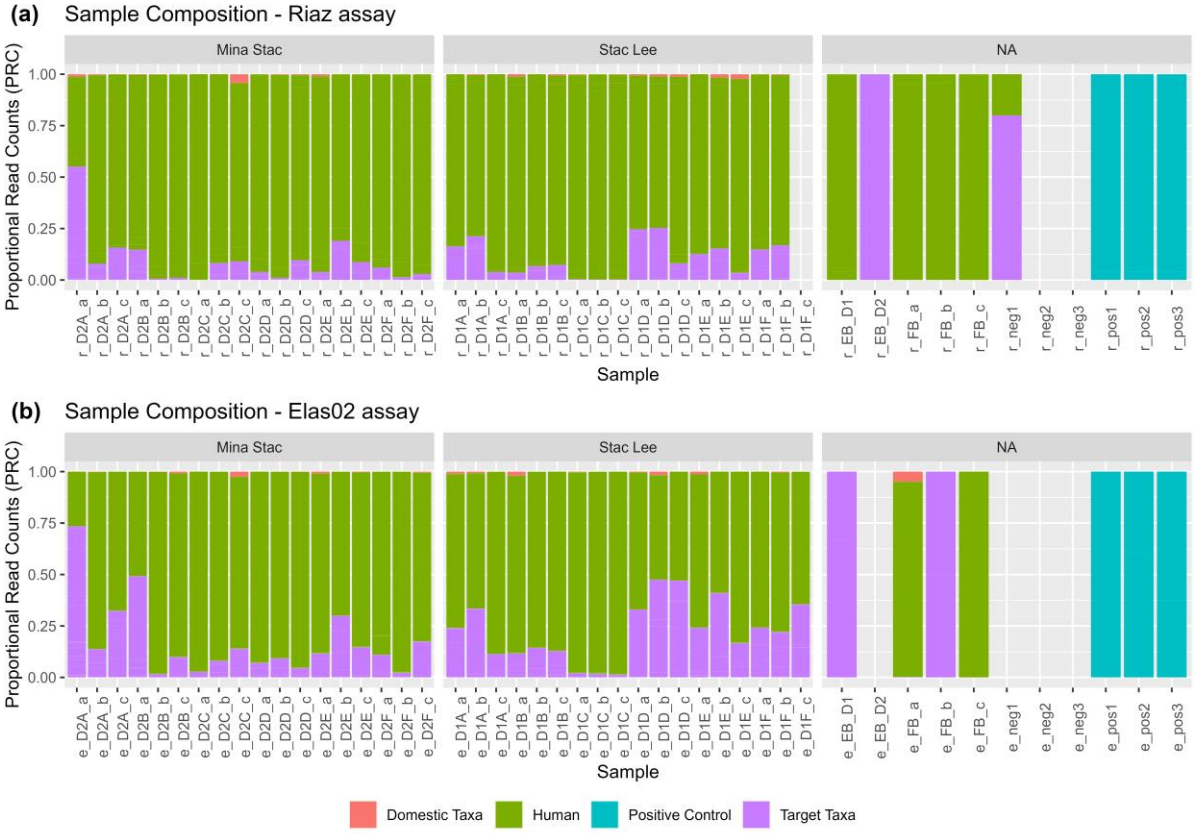
(a)



(b)



S5 Figure 2. Samples and controls and number of reads per sample after all bioinformatic filtering (contaminants, see Supplemental Table 2, are still present) for the Riaz **(a)** and Elas02 **(b)** assays.



S5 Figure 3. Bar-charts showing proportional read counts for samples prepared with the Riaz **(a)** and Elas02 **(b)** assays.

S5 Table 1. List of samples and controls analyzed, where abbreviations mean: MP =metaprobe, FB = field blank, EB = extraction blank, neg = PCR negative control, and pos = PCR positive control.

#	Sequence Sample ID	Primer	Site	Type	MP replicate	DNA extraction replicate	~Dive time(min)	Max. depth (m)
1	sample.1A_SK_D1A_a	elas02, riaz	Stac Lee	MP	A	a	46	46
2	sample.1B_SK_D1A_b	elas02, riaz	Stac Lee	MP	A	b	46	46
3	sample.1C_SK_D1A_c	elas02, riaz	Stac Lee	MP	A	c	46	46
4	sample.1D_SK_D1B_a	elas02, riaz	Stac Lee	MP	B	a	46	46
5	sample.1E_SK_D1B_b	elas02, riaz	Stac Lee	MP	B	b	46	46
6	sample.1F_SK_D1B_c	elas02, riaz	Stac Lee	MP	B	c	46	46
7	sample.1G_SK_D1C_a	elas02, riaz	Stac Lee	MP	C	a	46	46
8	sample.1H_SK_D1C_b	elas02, riaz	Stac Lee	MP	C	b	46	46
9	sample.2A_SK_D1C_c	elas02, riaz	Stac Lee	MP	C	c	46	46
10	sample.2B_SK_D1D_a	elas02, riaz	Stac Lee	MP	D	a	46	46
11	sample.2C_SK_D1D_b	elas02, riaz	Stac Lee	MP	D	b	46	46
12	sample.2D_SK_D1D_c	elas02, riaz	Stac Lee	MP	D	c	46	46
13	sample.2E_SK_D1E_a	elas02, riaz	Stac Lee	MP	E	a	46	46
14	sample.2F_SK_D1E_b	elas02, riaz	Stac Lee	MP	E	b	46	46
15	sample.2G_SK_D1E_c	elas02, riaz	Stac Lee	MP	E	c	46	46
16	sample.2H_SK_D1F_a	elas02, riaz	Stac Lee	MP	F	a	46	46
17	sample.3A_SK_D1F_b	elas02, riaz	Stac Lee	MP	F	b	46	46
18	sample.3B_SK_D1F_c	elas02, riaz	Stac Lee	MP	F	c	46	46
19	sample.3C_SK_D2A_a	elas02, riaz	Mina Stac	MP	A	a	45	30
20	sample.3D_SK_D2A_b	elas02, riaz	Mina Stac	MP	A	b	45	30
21	sample.3E_SK_D2A_c	elas02, riaz	Mina Stac	MP	A	c	45	30
22	sample.3F_SK_D2B_a	elas02, riaz	Mina Stac	MP	B	a	45	30
23	sample.3G_SK_D2B_b	elas02, riaz	Mina Stac	MP	B	b	45	30
24	sample.3H_SK_D2B_c	elas02, riaz	Mina Stac	MP	B	c	45	30

25	sample.4A_SK_D2C_a	elas02, riaz	Mina Stac	MP	C	a	45	30
26	sample.4B_SK_D2C_b	elas02, riaz	Mina Stac	MP	C	b	45	30
27	sample.4C_SK_D2C_c	elas02, riaz	Mina Stac	MP	C	c	45	30
28	sample.4D_SK_D2D_a	elas02, riaz	Mina Stac	MP	D	a	45	30
29	sample.4E_SK_D2D_b	elas02, riaz	Mina Stac	MP	D	b	45	30
30	sample.4F_SK_D2D_c	elas02, riaz	Mina Stac	MP	D	c	45	30
31	sample.4G_SK_D2E_a	elas02, riaz	Mina Stac	MP	E	a	45	30
32	sample.4H_SK_D2E_b	elas02, riaz	Mina Stac	MP	E	b	45	30
33	sample.5A_SK_D2E_c	elas02, riaz	Mina Stac	MP	E	c	45	30
34	sample.5B_SK_D2F_a	elas02, riaz	Mina Stac	MP	F	a	45	30
35	sample.5C_SK_D2F_b	elas02, riaz	Mina Stac	MP	F	b	45	30
36	sample.5D_SK_D2F_c	elas02, riaz	Mina Stac	MP	F	c	45	30
37	sample.5E_SK_FB_a	elas02, riaz	NA	FB	FB	a	NA	NA
38	sample.5F_SK_FB_b	elas02, riaz	NA	FB	FB	b	NA	NA
39	sample.5G_SK_FB_c	elas02, riaz	NA	FB	FB	c	NA	NA
40	sample.5H_EB_SK_D1	elas02, riaz	NA	EB	NA	NA	NA	NA
41	sample.6A_EB_SK_D2	elas02, riaz	NA	EB	NA	NA	NA	NA
42	sample.6B_NEG_1	elas02, riaz	NA	neg	NA	NA	NA	NA
43	sample.6C_NEG_2	elas02, riaz	NA	neg	NA	NA	NA	NA
44	sample.6D_NEG_3	elas02, riaz	NA	neg	NA	NA	NA	NA
45	sample.6F_POS_1	elas02, riaz	NA	pos	NA	NA	NA	NA
46	sample.6G_POS_2	elas02, riaz	NA	pos	NA	NA	NA	NA
47	sample.6H_POS_3	elas02, riaz	NA	pos	NA	NA	NA	NA

S5 Table 2. Contamination in samples or negative controls. Actions taken for each primer assay are specified as remove or reassign. All domestic taxa were removed at the genus and species level, although only genera are listed. Reassignments are in bold.

Primer	Taxa	Action	Notes
elas02	Homo sapiens/Hominidae	remove	human/PCR artifact
elas02	Canis	remove	domestic
elas02	Felis	remove	domestic
elas02	Meleagris	remove	domestic
elas02	Ovis	remove	possibly real, but also domestic
elas02	Sus	remove	domestic
elas02	Gallus	remove	domestic
elas02	Hypanus	remove	positive control, 7 reads
riaz	Homo sapiens/Hominidae	remove	human/PCR artifact
riaz	Canis	remove	domestic
riaz	Felis	remove	domestic
riaz	Ovis	remove	possibly real, but also domestic
riaz	Sus	remove	domestic
riaz	*Gobio gobio*	remove	freshwater contamination
riaz	*Chirolophis japonicus*	**Reassign**	***Chirolophis ascanii***
riaz	*Cystophora cristata*	**Reassign**	BLAST ambiguity, **Phocidae**
riaz	*Lepidotrigla hime*	**Reassign**	***Chelidonichthys cuculus***
riaz	*Maurolicus japonicus*	**Reassign**	***Maurolicus muelleri***

S5 Table 3. Results of the multivariate homogeneity of group dispersions (*betadisper* and *anova* functions) and permutational multivariate analysis of variance (i.e., PERMANOVA, *adonis2* function) between groups. Samples treated with different primer assays have been kept separate. Two samples treated with the riaz primer assay are not included because they did not contain target taxa reads. Tests are based on Jaccard's Index and 1000 permutations. Significant P values are bolded. All functions are available in the R package vegan.

a) Multivariate homogeneity of group dispersions analysis between dive sites.

Source	DF	Sq	Mean Sq	F Value	P value
Dive site	1	0.07326	0.073262	9.3402	0.004
Residuals	68	0.53337	0.007844		

b) Multivariate homogeneity of group dispersions analysis between primer assays.

Source	DF	Sq	Mean Sq	F Value	P value
Assay	1	0.01359	0.0135896	2.4748	**0.112**
Residuals	68	0.37341	0.0054913		

c) PERMANOVA between biogeographic regions, sponge species, and the biogeographic region:sponge species interaction. *****The sponge species had heterogenous group dispersions.

Source	DF	Sums of Sqs	F Model	R^2	P value
Dive site	1	1.8227	8.1591	0.09876	**0.001**
Assay	1	1.5606	6.9857	0.08455	**0.001**
Dive site:Assay	1	0.3292	1.4735	0.01783	0.094
Residuals	66	14.7442		0.79885	
Total	69	18.4566		1.00000	

S5 Table 4. Results of the indicator value species analysis (*multipatt* function) and multilevel pattern analysis (*IndVal.g* method): a-b) dive sites and c-d) primer assays. Tests underwent 10000 permutations and the number of indicator species and significant P values are bolded. “A” is the estimate probability that samples are associated to the matched group(s) if the indicator species has been detected in the sample (i.e., specificity or predictive value). “B” is the estimate probability of detecting the indicator species in the matched group(s) (i.e., sensitivity). “Stat” is the indicator value index which suggests the strength of the indicator species association to the group(s). All functions are available in the R package Multipatt.

a) Summary table of indicator value and multilevel pattern analysis for dive sites.

Total number of Species	67
Selected number of Species	**5**
Number of species associated to 1 group	5

b) Results of the indicator value and multilevel pattern analysis for dive sites.

Species	Dive Site	A	B	Stat	P value
Uria aalge	Mina Stac	0.8095	0.4857	0.627	**0.0010**
Centrolabrus exoletus	Mina Stac	0.9231	0.3429	0.563	**0.0017**
Halichoerus grypus	Mina Stac	0.7647	0.3714	0.533	**0.0238**
Phocidae	Mina Stac	0.7857	0.3143	0.497	**0.0341**
Morus bassanus	Stac Lee	0.8529	0.8286	0.841	**0.0001**

c) Summary table of indicator value and multilevel pattern analysis for primer assay.

Total number of Species	67
Selected number of Species	**10**
Number of species associated to 1 group	10

d) Results of the indicator value and multilevel pattern analysis for primer assay.

Species	Primer Assay	A	B	Stat	P value
Halichoerus grypus	Elas02	1.0000	0.4722	0.687	**0.0001**
Pollachius	Elas02	1.0000	0.3333	0.577	**0.0004**
Clupea	Elas02	1.0000	0.3056	0.553	**0.0003**
Taurulus bubalis	Elas02	1.0000	0.3056	0.553	**0.0003**
Fulmarus glacialis	Elas02	1.0000	0.1667	0.408	**0.0258**
Phocidae	Riaz	1.0000	0.4118	0.642	**0.0001**
Ciliata mustela	Riaz	0.8811	0.2059	0.426	**0.0272**
Chelidonichthys cuculus	Riaz	1.0000	0.1471	0.383	**0.0243**
Microstomus	Riaz	1.0000	0.1471	0.383	**0.0237**

Audentes fortuna juvat,

Lux libertas,

Ad Astra Per Scientiam.

Fortune favours the bold,

Light and liberty,

To the stars through science.