Passive eDNA capture by SCUBA divers and snorkellers for monitoring inshore fish biodiversity

November 2023

Natural England Commissioned Report NECR506

www.gov.uk/natural-england

NATURAL ENGLAND

About Natural England

Natural England is here to secure a healthy natural environment for people to enjoy, where wildlife is protected and England's traditional landscapes are safeguarded for future generations.

Further Information

This report can be downloaded from the Natural England Access to Evidence Catalogue. For information on Natural England publications or if you require an alternative format, please contact the Natural England Enquiry Service on 0300 060 3900 or email enquiries@naturalengland.org.uk.

Copyright

Catalogue code: NECR506

Report details

Author(s)

Erika F. Neave M.Sc. and Professor Stefano Mariani

Natural England Project Manager

Dr. Sarah Meek

Contractor

Biological and Environmental Sciences Department, Liverpool John Moores University

Keywords

eDNA, metabarcoding, passive capture, metaprobes, fish, biodiversity, citizen science

Acknowledgements

We would like to thank the Natural England Dive Unit, particularly Fiona Crouch and Carolyn Waddell for collecting samples at South Beach, Studland Bay. We thank Halton Charters Ltd., particularly Bob Halton and Natasha Yates for providing boat and crew time in kind and facilitating communication with volunteer divers. We thank Lisa Shafe, Micheal Moore and an anonymous diver for collecting samples from Scapa Flow. We thank Karen Boswarva for help with sample preservation and water filtration. We also thank Seasearch volunteer snorkellers, Fiona Crouch, Charlotte Bolton and Angus Jackson for sample collection. We are grateful for the feedback and guidance from Sarah Meek and Lucy May on the contents of this report, and to Natural England for commissioning the study.

Citation

Neave, E. F., Mariani, S., Meek, S. 2023. Passive eDNA capture by SCUBA divers and snorkellers for monitoring inshore fish biodiversity. NECR506. Natural England.

Foreword

Natural England commission a range of reports from external contractors to provide evidence and advice to assist us in delivering our duties. The views in this report are those of the authors and do not necessarily represent those of Natural England.

Natural England has been exploring ways of utilising the quickly developing eDNA methods for marine monitoring. The metaprobes used in this project show great potential for expanding our eDNA monitoring using citizen scientists, allowing us to collect more broadscale data on species presence without expensive surveys. The metaprobes provide a simpler alternative to labour intensive filtering, often used in eDNA sampling, and have other potential uses outside of Divers (i.e., attachment to other structures) which can be explored in later projects.

Summary

Passive capture of marine eDNA presents a solution to an evidence gap in the routine monitoring of inshore fish communities. Records of coastal marine fish biodiversity can be data deficient due to the requirements of high technical and taxonomic skills of surveyors, difficult and varied conditions of underwater habitats, and the mobile, elusive, or cryptic nature of fish.

A novel DNA based method used metaprobes, passive aquatic environmental DNA collection devices, to explore data acquisition on inshore marine fish communities whilst utilising the technical expertise of citizen scientists and existing Natural England survey operations.

Metaprobes were used during dives at Mount Batten, Plymouth; Studland Bay, Dorset; and Orkney, Scotland. All samples (after PCR and PCR purification) except for one (a negative control) had a measurable DNA concentration, 67 OTUs were assigned to bony and cartilaginous fish species with the vast majority falling into the class Actinopterygii (Top 3 represented species; *Salmo salar*, *Trisopterus minutus*, *Trisopterus esmarkii*).

Metaprobes performed well in comparison to active eDNA collection, providing a simpler alternative to labour intensive filtering, showing potential uses outside of Divers (i.e., attachment to other structures) and for expanding our eDNA monitoring using citizen scientists, allowing us to collect more broadscale data on species presence without expensive surveys.

Contents

1. Introduction

Biodiversity monitoring, particularly in the marine environment, poses many challenges such as sourcing the technical expertise required (i.e., correct SCUBA diving qualifications, medical requirements for scientific divers, and adhering to the Diving at Work Regulations 1997) and expert taxonomic knowledge (i.e., Fisheries observers, visual census training). Moreover, methods that are used to assess fish stocks offshore, like hydroacoustics or trawling, are not viable inshore given more complex and fragile ecosystems such as seagrass beds, rocky reefs, and kelp forests. While Natural England's Dive Unit undertake some routine inshore surveys, these surveys generally focus on seafloor habitat, so any records of fish are either made serendipitously or during one-off commissioned efforts.

Routine monitoring of inshore fish communities is usually not carried out by statutory bodies to inform site condition assessments of Natura 2000 sites. Occasional fish surveys are undertaken to provide information on MCZ FOCI, e.g. black seabream, smelt. Therefore, when sourcing knowledge on coastal marine fish biodiversity, Natural England relies on databases such as the Global Biodiversity Information Facility (GBIF) that amalgamate different data sources such as fisheries catch assessments or citizen science records, e.g. Seasearch visual census records from volunteer divers. In many cases these records can be data deficient. However, since fish are often mobile, elusive, or cryptic by nature, it is often not practical to monitor fish biodiversity long-term using a single technique (e.g. underwater visual census, baited/unbaited underwater cameras, remote sensing such as echosounder data).

In recent years, Natural England has worked with contractors to test whether DNA based methods improve their capacity to monitor aquatic biodiversity. Non-invasive DNA methods work around some of the difficulties regarding marine biodiversity surveys mentioned previously: namely technical skills, taxonomic skills, and difficult and varied conditions of underwater habitats. Additionally, the non-invasive aspect of DNA monitoring in some cases is more suitable for adhering to licenses and permissions (e.g. marine licences for coring in MPAs, harbour authority permissions). As DNA based technology matures, these approaches are lessening in cost, improving in reliability, and expanding in scope. Particularly since traditional marine surveys can be costly and DNA sequencing is becoming cheaper, it will be important to consider genetic-based techniques for fish surveying. In this report, we explore a novel DNA based method that leans on the technical expertise of citizen scientists and existing Natural England survey operations to provide data on inshore marine fish communities.

1.1 Environmental DNA

Environmental DNA (eDNA) is DNA gathered from nature in a non-invasive way, such that the target organisms of interest are not isolated in the sample (Taberlet et al., 2012).

Research on environmental DNA monitoring of macrobial organisms is exponentially increasing (Pawlowski et al., 2020).

1.1.1 Active aquatic eDNA capture

eDNA capture methods historically have required some sort of active component, whether that be filtering water manually with a syringe or using an electric pump. Automated water filtration has been tested by Natural England, first in a pilot study (NECR287) and in NECR330 using a programmable large-volume marine sampler deployed at depth (Mynott 2019; Mynott and March 2020). This method was quite successful relative to traditional methods, such that many more species were detected compared to historical fisheries catch data provided by the Marine Management Organisation (Mynott and March 2020). However, filtration of eDNA regardless of whether it is done manually or automatically requires an investment in either the labour required, equipment or both.

1.1.2 Passive eDNA capture methods to date

Passive, filtration-free eDNA capture has only been explored in the past few years. Various materials for passive eDNA capture have been tested (Bessey et al., 2022; Kirtane et al., 2020). For example, filters that would normally be used for eDNA filtration were exposed to the water column for at least four hours and in some cases detected similar species richness to active eDNA capture by filtration (Bessey et al., 2021).

1.2 Metabarcoding

Metabarcoding is an analysis that can be performed on an eDNA sample. It involves using high-throughput sequencing and algorithms to assign taxonomic information to sequences, thus estimating species richness (Deiner et al., 2017). eDNA metabarcoding of fishes has been shown to be comparable to traditional methods of fish community surveying (Keck et al., 2022).

1.3 Metaprobes

Metaprobes are passive aquatic environmental DNA collection devices. They are simply hollow, perforated plastic spheres filled with sterile cotton gauze (Figure 1A,1B) and have been used in commercial trawling nets to assess fisheries catch by metabarcoding analysis (Maiello et al., 2022). Here, we instead attached the metaprobes to snorkellers and SCUBA divers which were already in the water for survey purposes (Figure 1C) or for recreation. The plastic sphere casing protects the sterile cotton gauze from getting lost or unravelling while being deployed. The plastic design is freely available to 3-D print (Maiello et al., 2022).

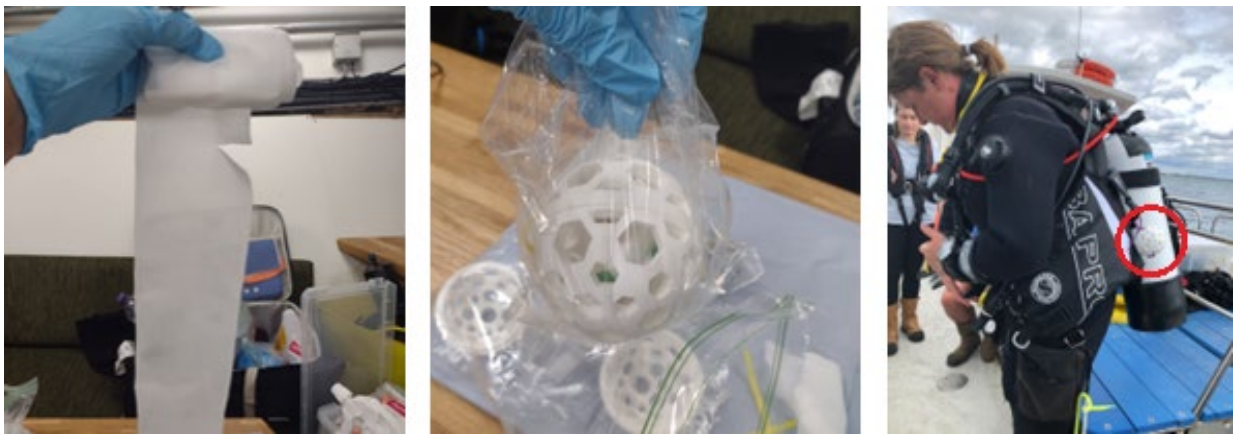

Figure 1. Pictures of metaprobe assembly and attachment to a SCUBA diver. Left: Sterile medical gauze being prepared to place inside a metaprobe. Middle: A prepared metaprobe, ready to cable tie to a diver's equipment. Right: Metaprobe on Natural England diver.

1.4 Aims and Objectives

The overall aim of this study was to test the efficacy of eDNA capture using a passive sampling technique: attaching metaprobes to SCUBA divers and snorkellers.

Specifically, we did a series of pilot tests as a first effort towards the following objectives:

1. Understanding how passive eDNA capture compares to active filtration of aquatic eDNA for metabarcoding analysis.

2. Optimising the conditions for extracting eDNA from passive capture material (i.e. cotton gauze) which involved testing for the best:
    a. Preservation methods
    b. Amount of input material for extractions
    c. DNA extraction method and reagents

2. Materials and Methods

2.1 Sample Collection

The sample collection events took place at four different dive sites, across three locations: Mount Batten, Plymouth; Studland Bay, Dorset; and Orkney, Scotland (Table 1).

Table 1. Summary of sample collection sites.

Location	Site	Latitude	Longitude	Date
Mount Batten, Plymouth	North side	50°21,480'N	04°07,899'W	17/06/2022
Studland Bay, Dorset	South Beach 1	50°38,537'N	01°56,461'W	05/07/2022
Studland Bay, Dorset	South Beach 2	50°38,546'N	01°56,482'W	06/07/2022
Orkney, Scotland	SMS Bayern	58°53,920'N	03°10,615'W	16/11/2022

Three volunteer snorkellers, each wearing a metaprobe, went for a 60-minute snorkel on the North side of Mount Batten, Plymouth. Two SCUBA divers, both on surveys for the Natural England dive team, wore metaprobes on two dives in in Studland Bay for 73 and 77 minutes, respectively. Three volunteer recreational SCUBA divers wore metaprobes while diving on the wreck of the SMS Bayern, Orkney, whilst four 1.5 L bottles of seawater were filtered using 50 mL syringes through Sterivex filters. Seawater samples were only collected at the Orkney sampling site. Detailed sampling methods and material preparation can be found in Appendix 2A.

The gauzes from the metaprobes collected were stored in either 100% molecular grade ethanol or silica beads and stored at -20°C where possible (i.e., During transit of the samples to Liverpool John Moores University the samples were insulated but exposed to room-temperature for ~24 hrs). Sample information is provided in tables 5-7 (Appendix 1).

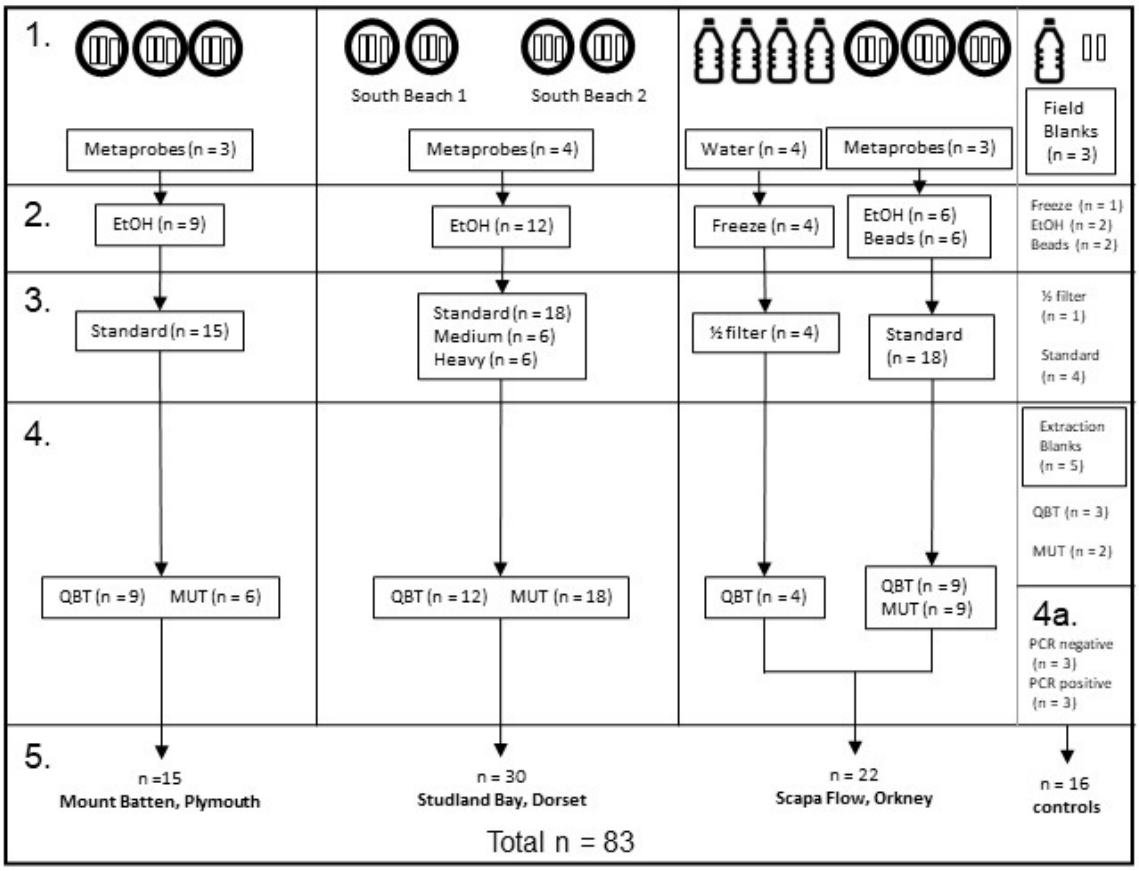

Figure 2. Visual schematic of the sample collection and downstream processing plan, which ultimately results in a total of 83 samples for sequencing. Row 1 shows a visual representation of the types of sampled collected: metaprobe gauzes, eDNA filters, and respective field blanks. Row 2 shows the preservation treatment used: freeze (-20°C), EtOH (100% molecular grade ethanol) or silica beads. Row 3 shows how the preserved samples were then subsampled for DNA extraction. The gauze weight ranges added to extractions were as follows: 'heavy' indicates between 0.9 g to 1.1 g, 'medium' indicates between 0.6 g to 0.8 g, and 'standard' indicates between 0.2 g to 0.4 g. Row 4 shows a breakdown of how many samples were treated with different DNA extraction protocols: QBT (Qiagen Blood and Tissue kit) and MUT (MuDNA Tissue). Row 5 summarizes the sample totals per location.

2.2 Laboratory Standards and Quality Control Measures

2.2.1 Laboratory Decontamination

The samples were processed following a unidirectional workflow. The DNA extractions for all the samples occurred in a laboratory allocated for eDNA analysis with dedicated materials and PPE. All equipment was sterilised in a 10% bleach solution for at least 10 minutes, followed by a 5% detergent solution and rinsed with UV purified water. Before use, all equipment and consumables are sterilised in 30 minutes of UV radiation. PCR products were handled in a separate laboratory.

2.2.2 Control Samples

Field blanks were collected in Orkney for the aquatic eDNA analysis (i.e., 1.5 L of bottled drinking water) and for the metaprobe sampling (i.e., Gauze from a metaprobe exposed to the air.) (Figure 2). Field blanks for the metaprobe sampling events in Plymouth and Dorset were not collected since those sample collection events were volunteer led. DNA extraction blanks (i.e., lysis buffer) were collected for each day of extraction and for each extraction method performed. PCR controls, three positives (i.e., tissue extractions of a fish not native to the UK, the freshwater catfish *Pangasianodon hypophthalmus*) and three negatives (Molecular grade water) were also sequenced (Figure 2, Box 4a).

2.3 DNA Extraction and PCR

DNA from the metaprobes and the water samples was extracted using either the DNeasy Blood and Tissue kit (Quiagen) (Appendix 2B) or a non-commercial published protocol namely 'Mu-DNA', which has modular components that have been customized to purpose and are detailed in this report (Sellers et al., 2018) (Appendix 2D). The reagents for the Mu-DNA method were prepared fresh for these extractions following the published protocol which is kept updated by the author on protocols.io but have been summarized in this report (Appendix 2C).

Aquatic eDNA filters were halved so a portion could be archived. The metaprobe guaze was cut, blotted dry if preserved in ethanol and weighed, aiming for various weight ranges:

- Heavy: 0.9 – 1.1 g
- Medium: 0.6 – 0.8 g
- Standard: 0.2 – 0.4 g

Three replicates of each DNA extract were amplified by PCR using a thermocycler (Bio-Rad) and with a primer set universal to Teleost fishes (Tele02) (Taberlet et al., 2018). Details of the PCR protocol are in Appendix 2E.

DNA extracts and PCR product were quantified using a Qubit dsDNA HS Assay kit (Invitrogen) (Table 2).

2.4 Sequencing Library Preparation

2.4.1 Purifying PCR product

PCR replicates were combined and visualized on a 2% agarose gel (150 ml 1X TBE buffer with 3 g agarose powder) stained with 1.5 µl SYBRsafe dye (InVitrogen). PCR products were individually purified using 1:1 ratio of PCR product to Mag-Bind® Total Pure NGS magnetic beads (Omega Bio-Tek) following the manufacturer protocol (Appendix 2F).

Products were visualised on an agarose gel again to assure purity (i.e., target length (167 bp) bands on agarose gels were visible with minimal to no other bands present).

2.4.2 Library building and Adapter Ligation

Purified PCR products were quantified using a Qubit dsDNA HS Assay kit (Invitrogen), and pooled equimolar (i.e., each sample has a unique 8-bp dual barcodes). 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 (Appendix 2G). 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.

2.4.3 Sequencing

The dual-indexed library and PhiX control was then quantified by qPCR using the NEBNext® Library Quant Kit for Illumina (New England Biolabs). The library was loaded at a final molarity of 70 pM with a 10% PhiX spike-in. The library was sequenced at Liverpool John Moores University on an Illumina iSeq100 using iSeq i1 Reagent v2 (300 cycles).

2.5 Bioinformatic Pipeline

The raw data consisted of two ‘FASTA’ files containing the forward and reverse sequences from the run. The sequences were quality controlled through the following series of steps using Python v2 with the OBITOOLS 1.2.11 package (Boyer et al., 2016). 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 (Q<30) quality scores were removed. The remaining paired-end alignments were demultiplexed (i.e., the DNA tags used as unique identifiers for each sample are identified and labelled with sample IDs provided) 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 with ‘d-value’ = 1. Taxonomy was assigned using the ‘sintax’ command in VSEARCH against a reference database of UK fish species generated using the METAFISHLib protocol (Collins et al., 2021) and available code. This resulted in taxonomic assignments where each level (i.e., family, genus) was associated with a percent probability of correct assignment.

2.6 Analysis and Statistics

All analyses and statistics were performed in R v.4.1.3.

2.6.1 Fish Detections and Contamination control

Analyses were carried out with taxonomies that had a ≥ 98% identity to the reference sequences (i.e., named species in this study had a ≥98% identity at the species level and 100% identity at all higher levels of assignment). Fish reads were removed from samples if sequencing reads were present in sample blanks and negative controls (Appendix 1, Table 7).

2.6.2 Community Composition (Beta-Diversity)

The sequencing reads were transformed with Hellinger's transformation and a dissimilarity matrix was created with the Bray-Curtis method using the 'vegdist' function from the R package vegan. The dissimilarity matrix was used to calculate the relative beta-diversity of the samples using the 'metaMDS' function from vegan and plotted with functions from the R package ggplot2 (Figure 3). A permutational multivariate analysis of variance (PERMANOVA) was performed to test whether there was a correlation between sampling location and beta-diversity using the function 'aov' from base R (Table 4). Additional analysis of fish community composition can be found in Appendix 2H (Tables 8-10).

2.6.3 Metaprobe Extraction Optimisation (Alpha-Diversity)

For brevity, the influence of preservation, gauze amount and extraction type were assessed statistically by using species richness (alpha-diversity) as the dependant (response) variable. Kruskal-Wallis tests were used to assess the variances in richness as a result of extraction treatments. Additional analyses are described in Appendix 2H.

3. Results

3.1 DNA Extraction and PCR

The DNA extracts and PCR products as well as purified PCR products were quantified (Table 2). 47 of the DNA extracts were below the limit of detection, which is not unusual for aquatic environmental samples. After PCR and PCR purification, all samples except for one (a negative control) had a measurable DNA concentration.

Table 2. DNA concentrations of each sample (n = 83): water, metaprobe gauze, and controls.

Sample ID	Type	DNA extract (ng/µl)	PCR product (ng/µl)	Purified PCR product (ng/µl)
1A	metaprobe	0.36	21	19.8
1B	metaprobe	0.254	24.4	19.4
1C	metaprobe	2.7	20.6	23
1D	metaprobe	0.442	17.5	20.6
1E	metaprobe	BLD	16.9	18.3
1F	metaprobe	1.4	19.4	23.4
1G	metaprobe	0.152	8.34	8.04
1H	positive PCR	1.5	79.8	too high
2A	metaprobe	0.106	24.8	11.9
2B	metaprobe	BLD	14.7	9.74
2C	metaprobe	0.162	25	17.4
2D	metaprobe	BLD	12.1	8
2E	metaprobe	BLD	12.5	7.1

Sample ID	Type	DNA extract (ng/µl)	PCR product (ng/µl)	Purified PCR product (ng/µl)
2F	metaprobe	0.3	18	11.4
2G	metaprobe	BLD	7.92	7.24
2H	metaprobe	BLD	11.2	8.04
12A	metaprobe	0.306	33.8	25.6
12B	metaprobe	0.228	21.6	24
12C	metaprobe	0.846	26	28.8
12D	metaprobe	BLD	21.2	17.1
12E	metaprobe	BLD	12.2	2.92
12F	metaprobe	0.148	23	19.7
12G	metaprobe	0.306	5.04	0.394
4A	metaprobe	0.776	29.4	19.8
12H	negative PCR	BLD	4.64	BLD
4B	metaprobe	0.168	11.9	4.1
4C	metaprobe	0.364	43.6	42.2
4D	metaprobe	BLD	11.4	9.08
4E	metaprobe	0.608	23.2	12.9
4F	metaprobe	BLD	19.7	12.6
4G	metaprobe	BLD	5.86	2.4

Sample ID	Type	DNA extract (ng/µl)	PCR product (ng/µl)	Purified PCR product (ng/µl)
4H	metaprobe	BLD	3.02	0.644
5A	metaprobe	0.82	28.4	8.6
5B	metaprobe	BLD	13.1	2.68
5C	metaprobe	BLD	9.66	3.7
5D	metaprobe	0.134	8.92	2.82
5E	metaprobe	BLD	9.58	2.48
5F	metaprobe	0.31	9.86	2.5
5G	metaprobe	BLD	18.6	2.9
5H	metaprobe	0.3	14.8	2.34
6A	negative PCR	BLD	9.8	0.288
6B	metaprobe	0.202	13.4	5.5
6C	metaprobe	BLD	4.72	0.556
6D	metaprobe	0.108	18.5	21.6
6E	metaprobe	BLD	6.36	3.7
6F	metaprobe	BLD	8.22	3.14
6G	metaprobe	BLD	5.26	1.05
6H	metaprobe	BLD	3.86	2.04
7A	water	3.76	26.6	18.6

Sample ID	Type	DNA extract (ng/µl)	PCR product (ng/µl)	Purified PCR product (ng/µl)
7B	water	5.94	16.9	6.23
7C	water	2.84	20.6	18.1
7D	water	6.28	16.9	11.4
7E	metaprobe	BLD	28.6	30
7F	metaprobe	BLD	12.3	6.96
7G	metaprobe	BLD	24.2	27.2
7H	metaprobe	BLD	8.74	3.54
8A	positive PCR	1.5	87.8	34.2
8B	metaprobe	BLD	19.7	8.6
8C	metaprobe	BLD	8.1	0.202
8D	metaprobe	BLD	26.4	8.1
8E	metaprobe	BLD	18.2	7.56
8F	metaprobe	BLD	35.2	23
8G	metaprobe	BLD	19.9	3.56
8H	metaprobe	0.128	20.2	8.14
9A	positive PCR	1.5	77.6	85.2
9B	metaprobe	BLD	6.64	0.58
9C	metaprobe	0.128	42.2	29

Sample ID	Type	DNA extract (ng/µl)	PCR product (ng/µl)	Purified PCR product (ng/µl)
9D	metaprobe	BLD	8.1	2.02
9E	metaprobe	0.228	27	7.56
9F	metaprobe	BLD	13.4	1.01
9G	metaprobe	0.176	28.6	15.5
9H	metaprobe	BLD	5	0.206
10A	metaprobe	BLD	6.16	1.42
10B	metaprobe	BLD	9.06	0.484
10C	metaprobe	BLD	15.9	0.942
10D	metaprobe	BLD	13.4	0.574
10E	negative PCR	BLD	15.8	6.76
10F	1601	BLD	10.4	0.27
10G	EB	BLD	2.96	0.11
10H	EB	BLD	3.8	0.22
11A	EB	BLD	11.2	6.3
11B	water	0.268	64.8	75.6
11C	EB	BLD	5.28	0.12

3.2 Fish Detections

After the reads were decontaminated using controls, and all controls such as the positive control (freshwater iridescent catfish) were removed from the analysis (Appendix 1, Table 7), a total of 67 OTUs were assigned to bony and cartilaginous fish species (Table 3).

65 OTUs matched to the class Actinopterygii, one matched to the class Elasmobranchii (*Galeus melastomus*; Blackmouth catshark) and another matched to the class Holocephali (*Chimaera monstrosa*; Rabbit fish). The top five OTUs with the most sequencing reads were as follows:

1) *Salmo salar* (Atlantic salmon) 97,528
2) *Trisopterus minutus* (Poor cod) 87,496
3) *Trisopterus esmarkii* (Norway pout) 72,678
4) *Dicentrarchus labrax* (European bass) 49,193
5) *Ammodytes* spp. (Sand eels) 26,006

Table 3. Fish detections are indicated by a +, no detections are indicated by a -. Columns indicate order level taxonomic assignment and rows have been organised alphabetically by order. Species level taxonomic assignment is provided in every case, except for one which could not be confidently assigned to species level (*Ammodytes* ssp.). The total reads pIOTU are provided. Each sample category (e.g., Mounth Batten, Studland offshore, etc.) has a different sampling effort which is noted at the bottom of the table under the heading 'Total Samples per Sample Category'. The total OTUs per sample category is also provided at the bottom of the table.

Order	Species	Mount Batten	Studland Beach 1	Studland Beach 2	Orkney water	Orkney metaprobe	Total Reads
Anguilliformes	*Anguilla anguilla*	+	-	+	-	-	702
Anguilliformes	*Conger conger*	+	-	+	+	+	403
Atheriniformes	*Atherina boyeri*	+	+	-	+	+	17853
Carcharhiniformes	*Galeus melastomus*	-	+	-	-	-	348
Chimaeriformes	*Chimaera monstrosa*	+	-	-	+	-	61

Order	Species	Mount Batten	Studland Beach 1	Studland Beach 2	Orkney water	Orkney metaprobe	Total Reads
Clupeiformes	*Clupea harengus*	-	+	+	+	+	2651
Clupeiformes	*Sprattus sprattus*	+	+	+	+	+	1973
Cypriniformes	*Phoxinus phoxinus*	+	+	-	-	-	341
Gadiformes	*Gadiculus argenteus*	-	+	-	-	-	120
Gadiformes	*Gadus morhua*	-	-	-	+	+	3922
Gadiformes	*Melanogrammus aeglefinus*	-	-	-	+	+	288
Gadiformes	*Micromesistius poutassou*	-	-	-	+	+	342
Gadiformes	*Pollachius pollachius*	+	+	+	+	+	3181
Gadiformes	*Pollachus virens*	+	-	-	+	+	5
Gadiformes	*Raniceps raninus*	-	-	-	+	-	157
Gadiformes	*Trisopterus esmarkii*	+	+	+	+	+	72678
Gadiformes	*Trisopterus luscus*	+	+	-	-	-	1169

Order	Species	Mount Batten	Studland Beach 1	Studland Beach 2	Orkney water	Orkney metaprobe	Total Reads
Gadiformes	*Trisopterus minutus*	+	+	+	+	+	87496
Gadiformes	*Ciliata mustela*	+	-	-	+	-	2031
Gadiformes	*Ciliata septentrionalis*	+	-	-	+	-	185
Gadiformes	*Molva molva*	-	-	-	-	+	199
Gadiformes	*Merluccius merluccius*	-	+	-	-	-	151
Gasterosteiformes	*Spinachia spinachia*	+	+	-	-	-	557
Gobiesociformes	*Diplecogaster bimaculata*	-	-	-	+	-	131
Mugiliformes	*Chelon auratus*	+	+	+	+	-	11147
Mugiliformes	*Chelon labrosus*	+	+	-	-	-	6405
Osmeriformes	*Osmerus eperlanus*	-	-	-	+	-	83
Perciformes	*Ammodytes* ssp.	+	+	+	+	+	26006
Perciformes	*Ammodytes tobianus*	+	+	+	-	-	10
Perciformes	*Coryphoblennius galerita*	+	-	-	-	-	585

Order	Species	Mount Batten	Studland Beach 1	Studland Beach 2	Orkney water	Orkney metaprobe	Total Reads
Perciformes	*Lipophrys pholis*	+	-	-	-	-	1782
Perciformes	*Parablennius gattorugine*	+	+	-	-	-	184
Perciformes	*Callionymus lyra*	-	+	+	-	-	107
Perciformes	*Trachurus trachurus*	-	+	-	-	-	7
Perciformes	*Crystallogobius linearis*	-	-	-	+	-	11
Perciformes	*Gobius niger*	+	-	+	-	-	117
Perciformes	*Gobius paganellus*	+	+	-	-	-	1942
Perciformes	*Gobiusculus flavescens*	+	+	+	+	-	1215
Perciformes	*Pomatoschistus microps*	+	+	-	-	-	771
Perciformes	*Pomatoschistus minutus*	+	+	+	+	+	7249
Perciformes	*Pomatoschistus pictus*	+	-	-	-	-	28
Perciformes	*Ctenolabrus rupestris*	+	+	-	-	+	5663
Perciformes	*Labrus bergylta*	+	+	+	+	+	11616

Order	Species	Mount Batten	Studland Beach 1	Studland Beach 2	Orkney water	Orkney metaprobe	Total Reads
Perciformes	*Labrus mixtus*	-	-	-	-	+	382
Perciformes	*Symphodus bailloni*	+	+	+	-	+	3509
Perciformes	*Symphodus melops*	+	+	+	-	-	9258
Perciformes	*Dicentrarchus labrax*	+	+	+	+	+	49193
Perciformes	*Pholis gunnellus*	-	-	+	+	+	3621
Perciformes	*Scomber scombrus*	-	-	-	+	-	676
Perciformes	*Sparus aurata*	-	+	+	-	-	455
Perciformes	*Chirolophis ascanii*	-	+	-	-	+	2476
Perciformes	*Zoarces viviparus*	-	-	-	+	+	447
Pleuronectiformes	*Limanda limanda*	-	-	-	+	-	338
Pleuronectiformes	*Scophthalmus rhombus*	-	+	-	-	-	509
Pleuronectiformes	*Zeugopterus punctatus*	-	-	-	+	-	121
Pleuronectiformes	*Solea solea*	-	+	-	-	-	270

Order	Species	Mount Batten	Studland Beach 1	Studland Beach 2	Orkney water	Orkney metaprobe	Total Reads
Salmoniformes	*Salmo salar*	+	+	+	+	+	97528
Scombriformes	*Katsuwonus pelamis*	-	-	-	-	+	56
Scorpaeniformes	*Agonus cataphractus*	-	-	-	+	-	150
Scorpaeniformes	*Myoxocephalus scorpius*	-	-	-	+	+	401
Scorpaeniformes	*Taurulus bubalis*	+	+	-	+	-	5018
Scorpaeniformes	*Cyclopterus lumpus*	-	+	-	+	-	872
Scorpaeniformes	*Chelidonichthys cuculus*	+	-	-	-	-	81
Syngnathiformes	*Nerophis ophidion*	-	+	-	-	-	496
Syngnathiformes	*Syngnathus acus*	-	+	-	+	+	817
Syngnathiformes	*Syngnathus rostellatus*	-	+	-	-	-	200
Syngnathiformes	*Syngnathus typhle*	-	+	-	-	-	1486
Total samples per sample category							

Order	Species	Mount Batten	Studland Beach 1	Studland Beach 2	Orkney water	Orkney metaprobe	Total Reads
		15	24	6	4	18	N/A
Total OTUs per sample category							
		35	39	21	35	26	N/A

3.3 Fish Community Composition

All samples were compared using beta-diversity (i.e., Bray-Curtis distance), a metric which describes the relative community composition of samples. The four locations, noting that each location had a variety of sample types (Appendix 1, Table 6), had a significantly different fish community composition (R^2 = 0.36, p<0.001) (Table 4). The community composition of each pair of locations was compared (post-hoc tests) to understand whether all locations were different from each other, or whether only certain pairs of locations were driving the difference (Appendix 3A, Table 10). Almost all pairs of locations were significantly different (p<0.05) except for the dive sites that are both located in Studland Bay (p = 0.822) (i.e. South Beach 1 and South Beach 2).

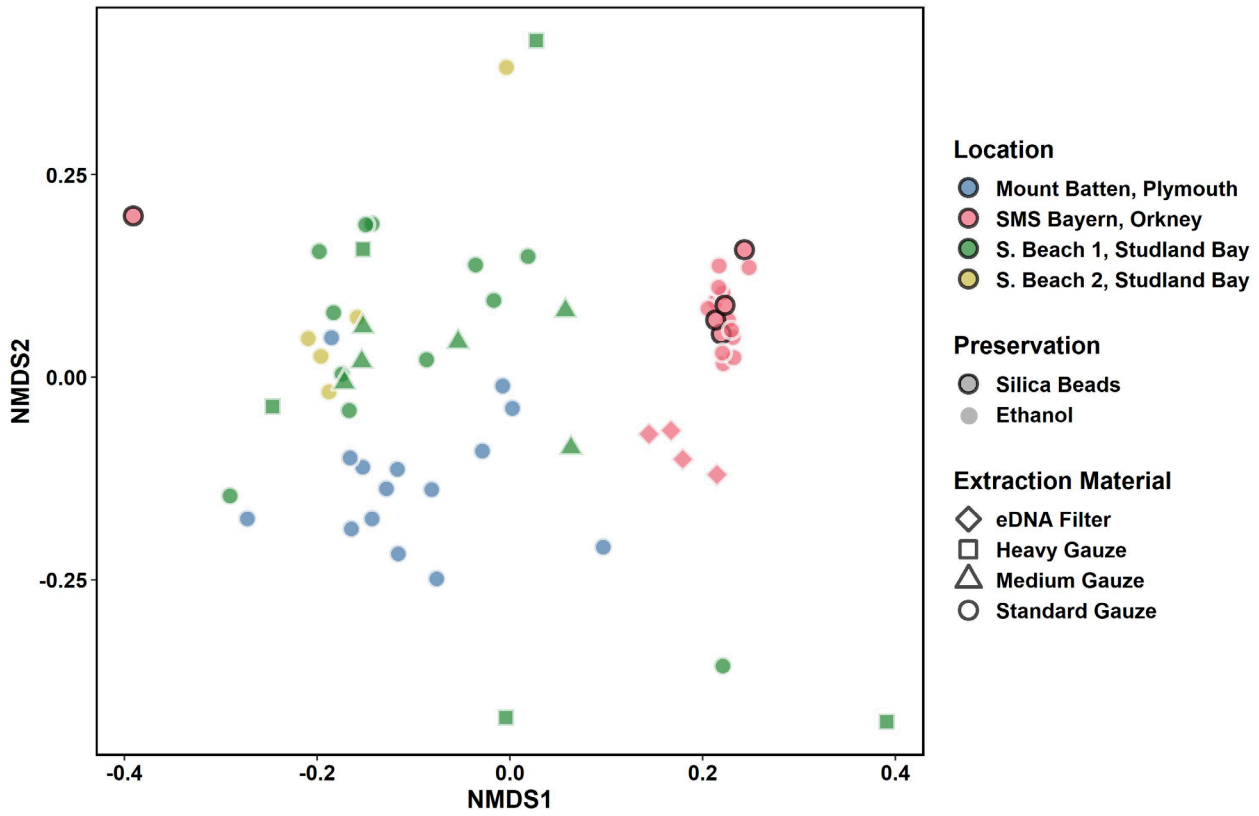

Figure 3. Non-metric multidimensional scaling (NMDS) plot displaying all samples, with location differentiated by colour, preservation method differentiated by outline colour and extraction material differentiated by shape of the points. All points outlined in black were preserved with silica beads while the rest of the samples were preserved in ethanol. The gauze weight ranges added to extractions were as follows: 'heavy' indicates between 0.9 g to 1.1 g, 'medium' indicates between 0.6 g to 0.8 g, and 'standard' indicates between 0.2 g to 0.4 g.

Table 4. Results of the permutational multivariate analysis of variance (PERMANOVA) testing for differences in beta-diversity between locations: Mount Batten, Plymouth; SMS Bayern, Orkney; South Beach 1, Studland Bay; South Beach 2, Studland Bay. Note: some cells left blank.

Source	DF	Sum of Sqs	Mean Sq	F Value	R^2	P value
Groups	3	8.0615	2.68715	11.186	0.35868	0.000999
Residuals	60	14.4138	0.24023		0.64132	
Total	63	22.4753			1.00000	

3.4 Metaprobe Extraction Optimisation

3.4.1 Preservation Effect

To gain an understanding of the influence of different preservation techniques on eDNA recovery from metaprobes, three metaprobes from SMS Bayern dive site were compared. From each of the three metaprobes, four samples preserved in ethanol and two preserved in silica beads were extracted, half with the Qiagen Blood and Tissue kit and the other with the Mu-DNA method. This resulted in 18 samples in total. One of the samples which had been preserved in silica beads and extracted with the Mu-DNA method did not have any OTU detections, and overall there appeared to be greater differences in preservation techniques when samples were extracted using the Mu-DNA method compared to the Qiagen Blood and Tissue kit (Appendix 3B, Figure 11).

When the samples were pooled together (i.e., Six bead preserved verses 12 ethanol preserved) the ethanol samples detected 11 OTUs that were not detected by beads, while bead samples had three unique OTU detections (Figure 4). Just under 50% of detections or 12 OTUs were shared between the sampling preservation methods (Figure 4).

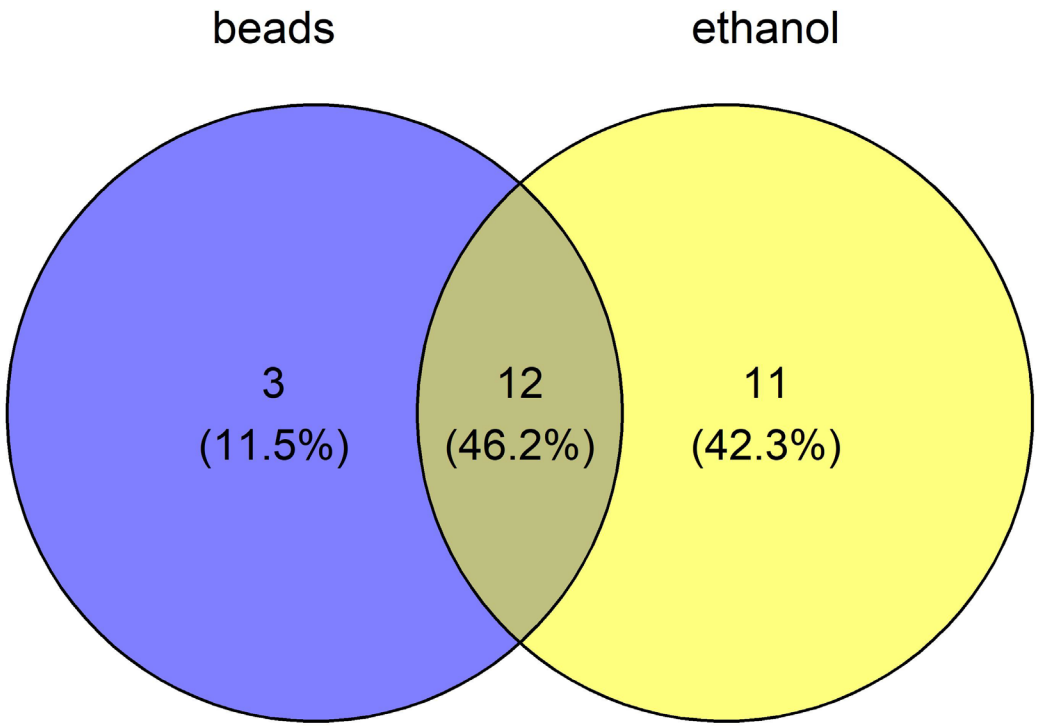

Figure 4. Venn diagralomparing OTU detections in samples from SMS Bayern, Orkney preserved in silica beads verses ethanol.

3.4.2 Weight of Input Gauze Effect

Two metaprobes from the same location (South Beach 1, Studland Bay) were used to perform repeat extractions maintaining the same method (Mu-DNA Tissue) but varying the

weight of the input gauze. Exact weights were recorded (Appendix 1, Table 6). The weights fit into the following ranges:

- Heavy: 0.9 – 1.1 g
- Medium: 0.6 – 0.8 g
- Standard: 0.2 – 0.4 g

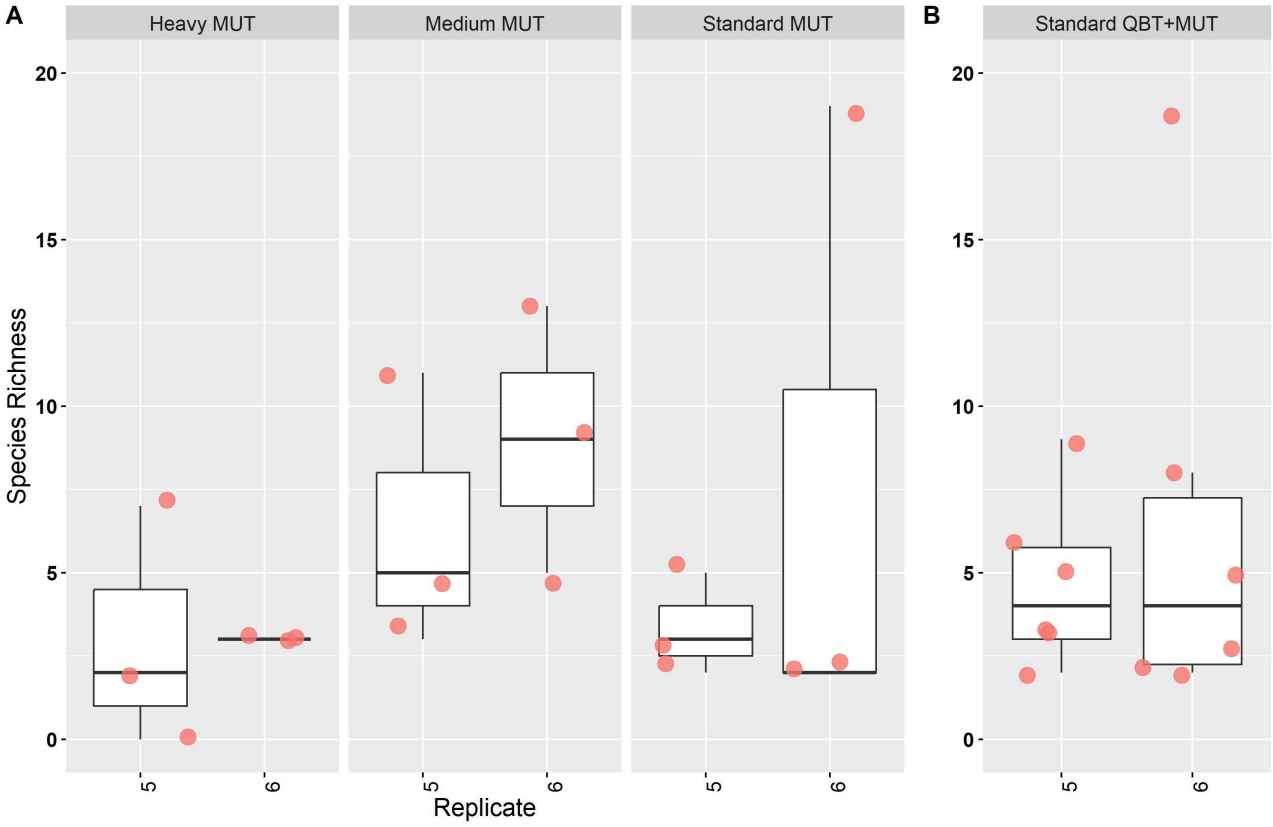

Figure 5. Boxplots of samples from South Beach 1, Studland Bay (replicates 5 & 6) sorted by gauze weight ranges added to extractions. A) Comparisons of the input gauze weight ranges extracted using the Mu-DNA Tissue method (MUT). B) Extraction replicates at the standard weight range, with the Qiagen Blood and Tissue kit (QBT) extractions added.

The differences between the weight range treatments were assessed using Kruskal-Wallis tests, with species richness as the dependant variable. Despite the data suggesting that the medium input weight could increase species detections (Figure 5A), there was no significant difference between the input weights ($p = 0.086$). Moreover, when analysing the Iposition of OTU detections, the medium input weight detected a total of 22 species while the standard input weight detected a total of 23 OTUs with an overlap of 15 (Figure 6). Additional extraction replicates, which had been extracted with the Qiagen Blood and Tissue kit, were added to the standard weight range to see if extraction replication changed this result, but still there was no significant difference ($p = 0.080$) (Figure 5B) (Appendix 3B, Table 11).

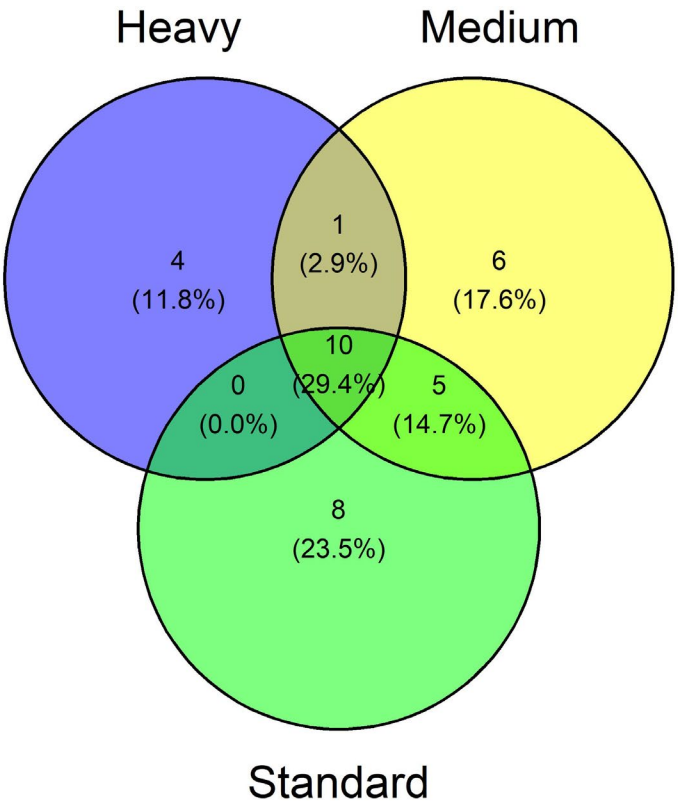

Figure 6. Venn Igram comparing OTU detections in samples from South Beach 1, Studland Bay with various gauze input weights.

3.4.3 Extraction Method Effect

The same samples from Mount Batten, Plymouth, SMS Bayern, Orkney, and South Beach 1, Studland Bay were extracted twice; once with the Mu-DNA Tissue (MUT) method and once with a Qiagen Blood and Tissue kit (QBT) (Figure 7). There was no significant difference found between the MUT and QBT methods when comparing alpha-diversity (species richness) of the individual extractions (Appendix 3B, Table 12). However, when combining the extraction replicates from Orkney together, the total species richness of the QBT was 21 while it was 13 for MUT (Figure 8). Moreover, QBT had 10 species which were also found in the active capture eDNA samples, while the MUT samples had 3 (Appendix 3C, Figure 12).

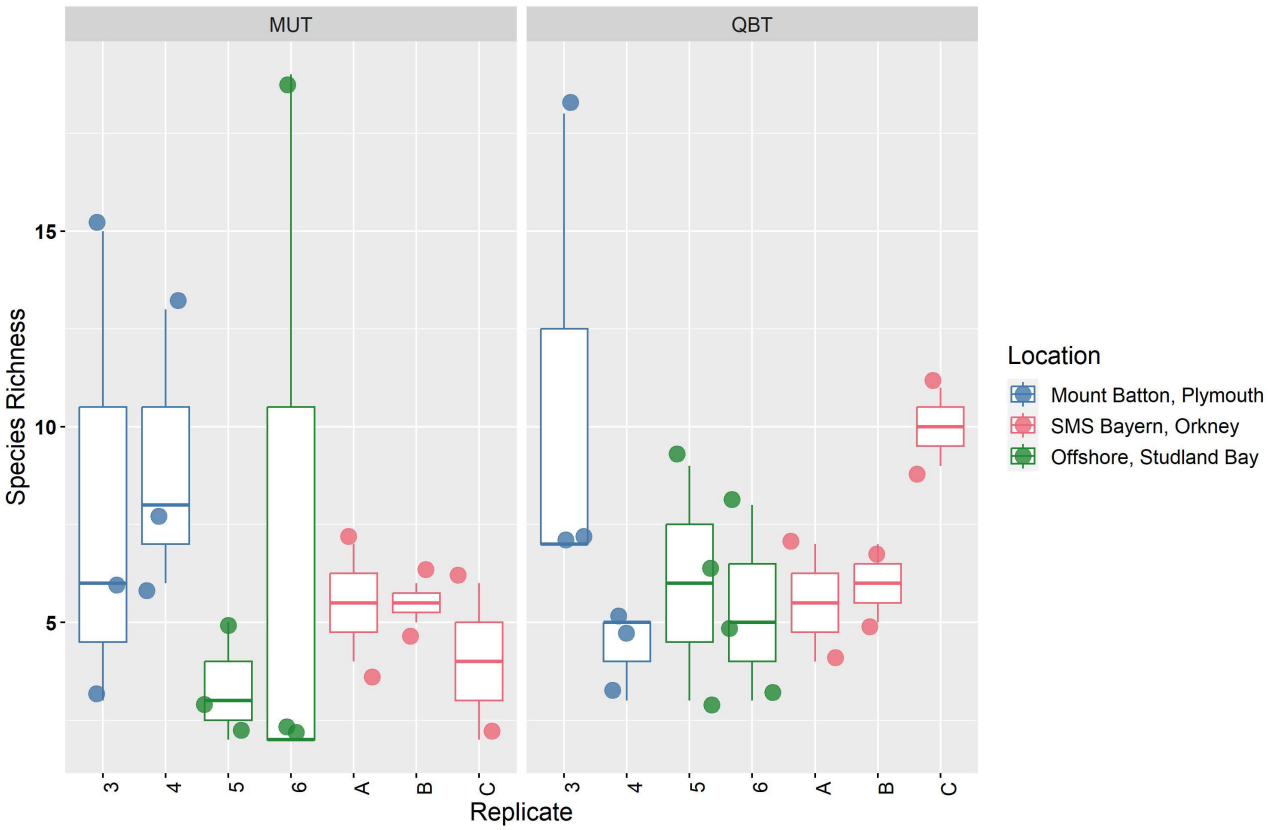

Figure 7. Boxplots of metaprobe samples from Mount Batten, Plymouth, SMS Bayern, Orkney, and South Beach 1, Studland Bay. Samples were extracted using either the Mu-DNA Tissue method (MUT) or the Qiagen Blood and Tissue kit (QBT).

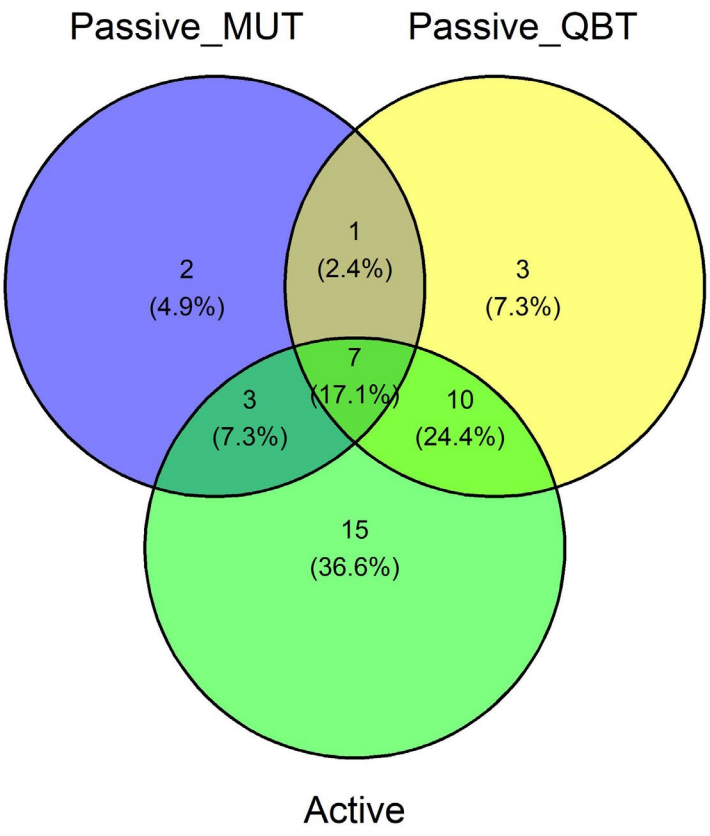

Figure 8. Venn diagram of OTU detections in samples from from SMS Bayern, Orkney comparing active eDNA capture to passive eDNA capture separated by extraction method.

3.5 eDNA Capture Comparison

Twenty species were detected by both active and passive eDNA capture methods (Figure 9). Active eDNA sampling consisted of four 1.5 L water samples which were filtered onto Sterivex filters, while passive samples were 17 DNA extractions (one extraction had no detections) resulting from three metaprobes deployed on divers (Figure 2).

Six fish were unique to the passive metaprobe samples: *Molva molva* (Common ling), *Chirolophis ascanii* (Yarrell's blenny), *Ctenolabrus rupestris* (Goldsinny wrasse), *Labrus mixtus* (Cuckoo wrasse), *Sym'hodus bailloni* (Baillon's wrasse), and *Katsuwonus pelamis* (Skipjack tuna). 15 fish species were unique to the active capture samples (Table 3).

Despite the passive samples containing some unique species, the species accumulation curves show that in two samples, active eDNA capture detected over 20 species while passive eDNA capture required ten samples to detect over twenty species (Figure 10).

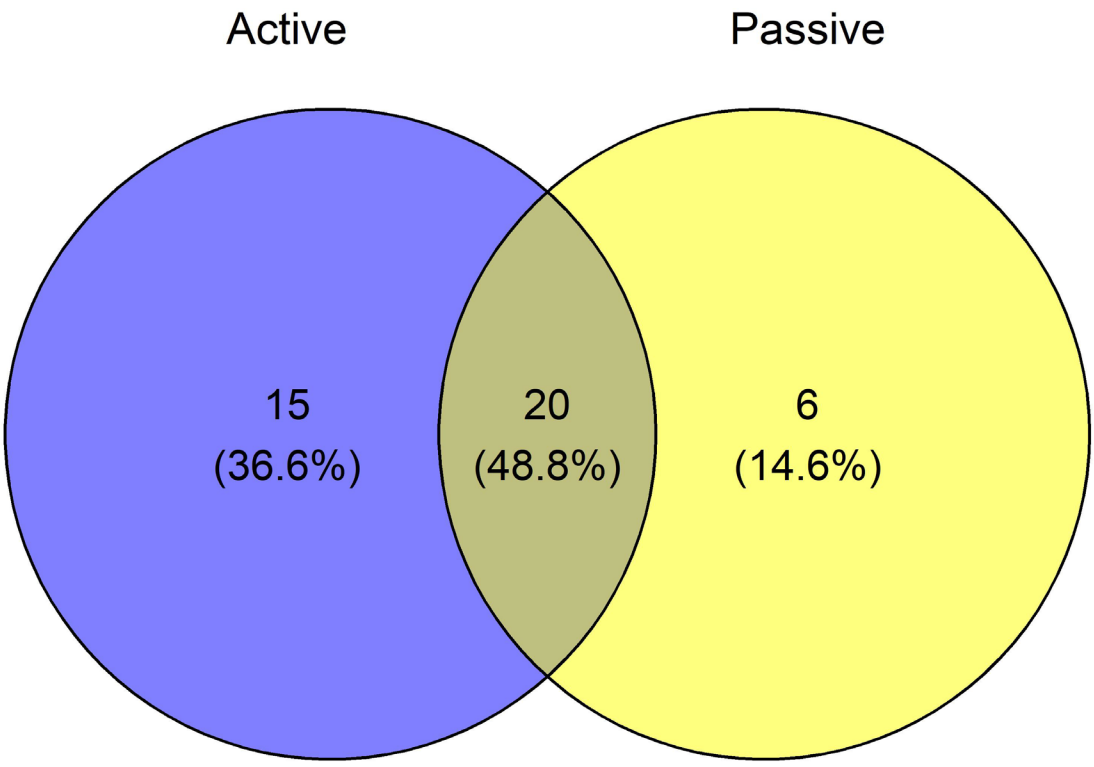

Figure 9. Venn diagram comparing OTU detections in samples from SMS Bayern, Orkney where either active eDNA capture or passive eDNA capture was used.

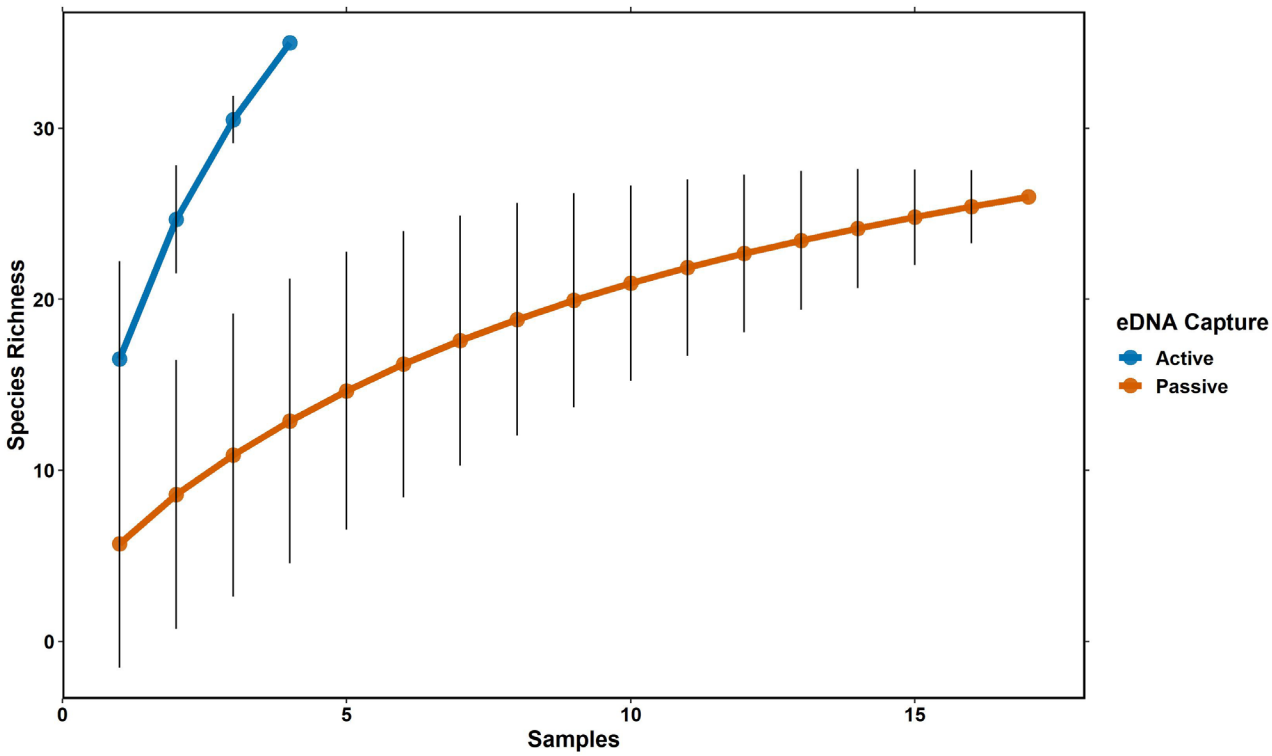

Figure 10. Species accumulation curves comparing samples from SMS Bayern, Orkney where either active eDNA capture or passive eDNA capture was used.

4. Discussion

4.1 Recommended Metaprobe Methods

Based on this pilot study, the following methods are recommended when using cotton gauze in metaprobes for passive eDNA capture by divers and snorkellers:

1. Preservation in 100% molecular grade ethanol
2. Extracting 0.2 g – 0.4 g piece of medical gauze
3. Using the Qiagen Blood and Tissue extraction kit

4.2.1. Preservation: Ethanol

Direct comparisons were made between samples collected on the SMS Bayern dive, where some gauze was preserved in 100% molecular grade ethanol while other gauze from the same metaprobe was preserved by dehydration in silica beads. Ethanol worked well for DNA preservation which was expected based on prior research (Minamoto et al., 2016).

Silica bead preservation varied in success depending on the extraction method used. Of the six samples preserved with silica beads, three that were extracted with the MuDNA Tissue method (MUT) did not perform as well as those extracted with the Qiagen Blood and Tissue kit (QBT) (Appendix 3B, Figure 11). One of the bead preserved samples extracted with MUT did not have any detections. The remaining three extracted with QBT had comparable species richness to that preserved in ethanol. While this result was promising, more replication is needed to fully understand whether silica beads could be a viable alternative preservation method. It's possible that more biological replication as well as extraction replication of silica bead preserved samples could result in comparable fish community composition to ethanol preserved gauze.

Until preservation methods can be tested with more replication and considering that the silica bead preserved samples were stored in -20°C where possible, 100% ethanol is the most reliable choice for preserving eDNA captured on gauze.

4.2.2. Weight: 0.2 g – 0.4 g

Direct comparisons of samples collected South Beach 1, Studland Bay were made, where the amount of gauze sub-sampled for DNA extractions varied in weight. The standard weight (0.2 g – 0.4 g) was considered 'standard' because the lysis step of the DNA extraction could be contained within a 1.5 mL Falcon tube (i.e., a convenient sized tube for most laboratory equipment).

Since there was no significant difference between the input amounts of gauze when species richness was used as a predictor, we recommend that the standard weight of 0.2

g – 0.4 g be used. Moreover, when comparing the composition of the fish detections, the standard weight had the highest species richness (i.e., 23), eight of which were unique (Figure 6). The medium weight had 22 detections of which 15 were shared with the standard weight and six were unique (Figure 6). The lack of significant difference could have been due to the modest sample size since there appears to be a trend of increased species detections for the medium input weight, but there was also an outlier in the standard weight that could suggest that it is more to do with the randomness of where the eDNA collects on the gauze and therefore this could be resolved by extraction replication (Figure 5).

Extracting with the standard weight of gauze is the most cost effective as it uses the least amount of reagents per extraction. The standard weight is also the least awkward to deal with in the laboratory since it suites standard equipment. For these reasons, we recommend using 0.2 g – 0.4 g dry-weight of gauze per passive eDNA extraction.

4.2.3. Extraction: Qiagen Blood and Tissue Kit

Direct comparisons of the DNA extraction protocols were made between metaprobe samples from three locations. No significant difference was found when simply testing whether the average number of species detected differed by extraction protocol.

However, when using active eDNA capture as a reference to compare to, the fish communities detected using the Qiagen Blood and Tissue kit (QBT) more closely resembled the active eDNA capture method than did the samples extracted with Mu-DNA tissue (MUT) method (Figure 8). MUT is much cheaper cost per sample but has more health and safety as well as contamination risks since the user must mix the reagents themselves. For these reasons, where possible the QBT is the recommended DNA extraction protocol for gauzes.

4.2 Passive eDNA Capture by divers and snorkellers

Passive eDNA capture by divers and snorkellers worked remarkably well considering how much less effort passive capture takes relative to active capture by water filtration. A total of 67 species were detected across locations with very low sampling effort or biological replication (2-3 snorkellers or divers per site). Technical replication (i.e. DNA extraction replicates) was prioritised over biological replication to establish appropriate laboratory methods. Considering the low biological replication, passive detection worked amazingly well, detecting ~50% of species that were detected by active filtration from the Orkney sampling site. Passive eDNA capture by divers and snorkellers also generated extensive species lists for Studland Bay and Plymouth Sound.

In a previous study, tropical and temperate habitats were monitored both by using active eDNA filtration and passive eDNA collection by submerging filters in the water column (Bessey et al., 2021). The study found that in the temperate habitat, the passive eDNA capture was able to match active eDNA filtration in terms of species richness. However, 78

passive eDNA filters were deployed and 9 active eDNA samples were filtered to achieve a congruence in eDNA capture methods. The passive eDNA capture from the Bessey study was achieved without divers and snorkellers, and it's likely that the eDNA capture via metaprobes and swimming action behaves differently to suspended filters; it's possible that the chances of eDNA capture are increased by swimmers' movements. Still comparing what is known about passive eDNA capture with the results of this report is helpful in the sense that other studies have been able to detect similar species richness, hinting that with increased sampling effort the temperate ecosystems of the UK may be particularly suited to passive eDNA capture.

In the future, more SCUBA divers could be used to easily increase biological replication and this could be achieved with citizen scientist volunteers from existing programmes such as Seasearch and/or with British Sub-Aqua Club (BASC). A citizen science approach would not only lower survey costs for statutory bodies but could also provide opportunities for public outreach and engagement. Citizen science programmes and engagement with diving and snorkelling communities could result in long-term monitoring data sets, or bio-banked (i.e. frozen) samples that could be processed as needed and when budgets allow. In this context, the potential for passive eDNA capture by metaprobes becomes quite powerful because it requires so much less technical expertise, time and effort compared to active water eDNA filtration.

5. Recommendations for Further Work

5.1 Comparisons to other survey data

For brevity, this report only provides fish detections in the results section, but does not discuss them at length. Fish detections from passive eDNA capture could be compared to fish occurrence data from prior traditional surveys or other data sources. Specific analyses on species of interest could be explored.

5.2 Other Vertebrates

This data was analysed with a curated reference database of UK fish species (Materials and Methods, Section 2.5). However, due to the wider specificity that the Tele02 primers are noted to have (Taberlet et al., 2018), the data could be reanalysed to check whether unassigned reads assign to other vertebrates of interest. The DNA extracts could also be returned to using different primer sets for PCR.

5.3 Passive eDNA preservation

Preservation was explored in this pilot study, but not with enough replication. In the few samples we tested, dehydration by silica beads worked better than expected. Further exploration of silica beads for preservation is worthwhile for a few reasons:

1. 100% molecular grade ethanol is not easy to access
2. 100% molecular grade ethanol presents health and safety concerns, particularly in the context of working on boats and/or with volunteers
3. 100% molecular grade ethanol is not easily transported, mailed, or shipped
4. Silica beads are cheaper than 100% molecular grade ethanol
5. Silica beads can be sterilized in an oven and reused while 100% molecular grade ethanol cannot be reused

For future work, we recommend that more samples preserved in beads should be directly compared to those preserved in ethanol. While ethanol works well, silica beads may extend the context in which passive eDNA capture by snorkeler or SCUBA divers is suitable.

5.4 Time and passive eDNA capture by divers and snorkellers

An aspect of passive eDNA capture that remains unclear is to what extent time spent swimming with a metaprobe affects the eDNA capture ability. Only two previous studies have tested time, but did not find it to be a significant driver of difference in species

detection (Bessey et al., 2021, 2022). However, the mechanism for eDNA adherence to materials is unknown and so understanding the relationship of time within the specific context of cotton gauze and swimmers will be important for understanding the potential of this method in citizen science activities. It's possible that the action of swimming is a key component of capture, and this should be directly compared to submersion.

5.5 Sensitivity and Cost-Benefit Analysis

It would be useful to increase the passive eDNA sampling effort by having more snorkellers or divers surveying to test whether more biological replication is needed to reach a comparable sensitivity to eDNA that has been captured by filtration. A Bioblitz, in which tens of volunteers participate could be an ideal context to test this. Samples from a Bioblitz and deeper sequencing would produce a bigger dataset from which a rigorous cost-benefit analysis comparing the eDNA capture methods could be undertaken.

List of Tables

List of Figures

References

Bessey, C., Gao, Y., Truong, Y. B., Miller, H., Jarman, S. N., & Berry, O. (2022). Comparison of materials for rapid passive collection of environmental DNA. *Molecular Ecology Resources*, *22*(7), 2559–2572. https://doi.org/10.1111/1755-0998.13640

Bessey, C., Neil Jarman, S., Simpson, T., Miller, H., Stewart, T., Kenneth Keesing, J., & Berry, O. (2021). Passive eDNA collection enhances aquatic biodiversity analysis. *Communications Biology*, *4*(1), Article 1. https://doi.org/10.1038/s42003-021-01760-8

Boyer, F., Mercier, C., Bonin, A., Le Bras, Y., Taberlet, P., & Coissac, E. (2016). obitools: A unix-inspired software package for DNA metabarcoding. *Molecular Ecology Resources*, *16*(1), 176–182. https://doi.org/10.1111/1755-0998.12428

Collins, R. A., Trauzzi, G., Maltby, K. M., Gibson, T. I., Ratcliffe, F. C., Hallam, J., Rainbird, S., Maclaine, J., Henderson, P. A., Sims, D. W., Mariani, S., & Genner, M. J. (2021). Meta-Fish-Lib: A generalised, dynamic DNA reference library pipeline for metabarcoding of fishes. *Journal of Fish Biology*, *99*(4), 1446–1454. https://doi.org/10.1111/jfb.14852

Deiner, K., Bik, H. M., Mächler, E., Seymour, M., Lacoursière-Roussel, A., Altermatt, F., Creer, S., Bista, I., Lodge, D. M., de Vere, N., Pfrender, M. E., & Bernatchez, L. (2017). Environmental DNA metabarcoding: Transforming how we survey animal and plant communities. *Molecular Ecology*, *26*(21), 5872–5895. https://doi.org/10.1111/mec.14350

Keck, F., Blackman, R. C., Bossart, R., Brantschen, J., Couton, M., Hürlemann, S., Kirschner, D., Locher, N., Zhang, H., & Altermatt, F. (2022). Meta-analysis shows both congruence and complementarity of DNA and eDNA metabarcoding to traditional methods for biological community assessment. *Molecular Ecology*, *31*(6), 1820–1835. https://doi.org/10.1111/mec.16364

Kirtane, A., Atkinson, J. D., & Sassoubre, L. (2020). Design and Validation of Passive Environmental DNA Samplers Using Granular Activated Carbon and Montmorillonite Clay. *Environmental Science & Technology*, *54*(19), 11961–11970. https://doi.org/10.1021/acs.est.0c01863

Maiello, G., Talarico, L., Carpentieri, P., De Angelis, F., Franceschini, S., Harper, L. R., Neave, E. F., Rickards, O., Sbrana, A., Shum, P., Veltre, V., Mariani, S., & Russo, T. (2022). Little samplers, big fleet: EDNA metabarcoding from commercial trawlers enhances ocean monitoring. *Fisheries Research*, *249*, 106259. https://doi.org/10.1016/j.fishres.2022.106259

Minamoto, T., Naka, T., Moji, K., & Maruyama, A. (2016). Techniques for the practical collection of environmental DNA: Filter selection, preservation, and extraction. *Limnology*, *17*(1), 23–32. https://doi.org/10.1007/s10201-015-0457-4

Mynott, S., and Marsh, M., 2020. Development of a novel (DNAbased) method for monitoring inshore fish communities using a programmable large-volume marine eDNA sampler. Natural England Commissioned Reports, Number NECR330

Mynott, S., 2019 ‘Pilot study to validate an environmental DNA sampler for monitoring inshore fish communities’. Natural England Commissioned Report number NECR287

Pawlowski, J., Apothéloz-Perret-Gentil, L., & Altermatt, F. (2020). Environmental DNA: What’s behind the term? Clarifying the terminology and recommendations for its future use in biomonitoring. *Molecular Ecology*, *29*(22), 4258–4264. https://doi.org/10.1111/mec.15643

Rognes, T., Flouri, T., Nichols, B., Quince, C., & Mahé, F. (2016). VSEARCH: A versatile open source tool for metagenomics. *PeerJ*, *4*, e2584. https://doi.org/10.7717/peerj.2584

Sellers, G. S., Muri, C. D., Gómez, A., & Hänfling, B. (2018). Mu-DNA: A modular universal DNA extraction method adaptable for a wide range of sample types. *Metabarcoding and Metagenomics*, *2*, e24556. https://doi.org/10.3897/mbmg.2.24556

Taberlet, P., Bonin, A., Zinger, L., & Coissac, E. (2018). *Environmental DNA: For Biodiversity Research and Monitoring*. Oxford University Press.

Taberlet, P., Coissac, E., Hajibabaei, M., & Rieseberg, L. H. (2012). Environmental DNA. *Molecular Ecology*, *21*(8), 1789–1793. https://doi.org/10.1111/j.1365-294X.2012.05542.x

Appendices

Appendix 1. Sample Information

Table 5. Samples and controls sequenced (n = 83).

Sample ID	Type	Replicate	Dive Site
1A	metaprobe	2	Mount Batten north side
1B	metaprobe	2	Mount Batten north side
1C	metaprobe	2	Mount Batten north side
1D	metaprobe	3	Mount Batten north side
1E	metaprobe	3	Mount Batten north side
1F	metaprobe	3	Mount Batten north side
1G	metaprobe	4	Mount Batten north side
1H	positive PCR	control	NA
2A	metaprobe	4	Mount Batten north side
2B	metaprobe	4	Mount Batten north side
2C	metaprobe	5	South Beach 1
2D	metaprobe	5	South Beach 1
2E	metaprobe	5	South Beach 1
2F	metaprobe	6	South Beach 1
2G	metaprobe	6	South Beach 1
2H	metaprobe	6	South Beach 1

Sample ID	Type	Replicate	Dive Site
12A	metaprobe	7	South Beach 2
12B	metaprobe	7	South Beach 2
12C	metaprobe	7	South Beach 2
12D	metaprobe	8	South Beach 2
12E	metaprobe	8	South Beach 2
12F	metaprobe	8	South Beach 2
12G	metaprobe	3	Mount Batten north side
12H	metaprobe	3	Mount Batten north side
4A	negative PCR	control	NA
4B	metaprobe	3	Mount Batten north side
4C	metaprobe	4	Mount Batten north side
4D	metaprobe	4	Mount Batten north side
4E	metaprobe	4	Mount Batten north side
4F	metaprobe	5	South Beach 1
4G	metaprobe	5	South Beach 1
4H	metaprobe	5	South Beach 1
5A	metaprobe	6	South Beach 1
5B	metaprobe	6	South Beach 1
5C	metaprobe	6	South Beach 1

Sample ID	Type	Replicate	Dive Site
5D	metaprobe	5	South Beach 1
5E	metaprobe	5	South Beach 1
5F	metaprobe	5	South Beach 1
5G	metaprobe	6	South Beach 1
5H	metaprobe	6	South Beach 1
6A	negative PCR	control	NA
6B	metaprobe	6	South Beach 1
6C	metaprobe	5	South Beach 1
6D	metaprobe	5	South Beach 1
6E	metaprobe	5	South Beach 1
6F	metaprobe	6	South Beach 1
6G	metaprobe	6	South Beach 1
6H	metaprobe	6	South Beach 1
7A	water	A	Orkney Bayern
7B	water	B	Orkney Bayern
7C	water	C	Orkney Bayern
7D	water	D	Orkney Bayern
7E	metaprobe	A	Orkney Bayern
7F	metaprobe	A	Orkney Bayern

Sample ID	Type	Replicate	Dive Site
7G	metaprobe	A	Orkney Bayern
7H	metaprobe	A	Orkney Bayern
8A	positive PCR	control	NA
8B	metaprobe	A	Orkney Bayern
8C	metaprobe	A	Orkney Bayern
8D	metaprobe	B	Orkney Bayern
8E	metaprobe	B	Orkney Bayern
8F	metaprobe	B	Orkney Bayern
8G	metaprobe	B	Orkney Bayern
8H	metaprobe	B	Orkney Bayern
9A	positive PCR	control	NA
9B	metaprobe	B	Orkney Bayern
9C	metaprobe	C	Orkney Bayern
9D	metaprobe	C	Orkney Bayern
9E	metaprobe	C	Orkney Bayern
9F	metaprobe	C	Orkney Bayern
9G	metaprobe	C	Orkney Bayern
9H	metaprobe	C	Orkney Bayern
10A	metaprobe	field blank	Orkney Bayern

Sample ID	Type	Replicate	Dive Site
10B	metaprobe	field blank	Orkney Bayern
10C	metaprobe	field blank	Orkney Bayern
10D	metaprobe	field blank	Orkney Bayern
10E	negative PCR	control	NA
10F	1601	extraction blank	NA
10G	EB	extraction blank	NA
10H	EB	extraction blank	NA
11A	EB	extraction blank	NA
11B	water	field blank	Orkney Bayern
11C	EB	extraction blank	NA

Table 6. Sample metadata.

Sample ID	Dive Site	Preservative	Weight	Weight (g)	Extraction Method
1A	Mount Batten north side	Ethanol	standard	0.25	QBT
1B	Mount Batten north side	Ethanol	standard	0.2	QBT
1C	Mount Batten north side	Ethanol	standard	0.4	QBT
1D	Mount Batten north side	Ethanol	standard	0.31	QBT
1E	Mount Batten north side	Ethanol	standard	0.27	QBT
1F	Mount Batten north side	Ethanol	standard	0.31	QBT
1G	Mount Batten north side	Ethanol	standard	0.21	QBT
2A	Mount Batten north side	Ethanol	standard	0.22	QBT
2B	Mount Batten north side	Ethanol	standard	0.36	QBT
2C	South Beach 1	Ethanol	standard	0.2	QBT
2D	South Beach 1	Ethanol	standard	0.26	QBT
2E	South Beach 1	Ethanol	standard	0.2	QBT
2F	South Beach 1	Ethanol	standard	0.28	QBT
2G	South Beach 1	Ethanol	standard	0.4	QBT
2H	South Beach 1	Ethanol	standard	0.21	QBT
12A	South Beach 2	Ethanol	standard	0.38	QBT
12B	South Beach 2	Ethanol	standard	0.22	QBT
12C	South Beach 2	Ethanol	standard	0.23	QBT
12D	South Beach 2	Ethanol	standard	0.38	QBT
12E	South Beach 2	Ethanol	standard	0.2	QBT
12F	South Beach 2	Ethanol	standard	0.4	QBT
12G	Mount Batten north side	Ethanol	standard	exact weight not recorded	MUT
12H	Mount Batten north side	Ethanol	standard	exact weight not recorded	MUT
4B	Mount Batten north side	Ethanol	standard	exact weight not recorded	MUT
4C	Mount Batten north side	Ethanol	standard	exact weight not recorded	MUT
4D	Mount Batten north side	Ethanol	standard	exact weight not recorded	MUT
4E	Mount Batten north side	Ethanol	standard	exact weight not recorded	MUT
4F	South Beach 1	Ethanol	standard	0.31	MUT
4G	South Beach 1	Ethanol	standard	0.21	MUT
4H	South Beach 1	Ethanol	standard	0.2	MUT
5A	South Beach 1	Ethanol	standard	0.24	MUT
5B	South Beach 1	Ethanol	standard	0.24	MUT
5C	South Beach 1	Ethanol	standard	0.32	MUT
5D	South Beach 1	Ethanol	medium	0.79	MUT
5E	South Beach 1	Ethanol	medium	0.61	MUT
5F	South Beach 1	Ethanol	medium	0.78	MUT
5G	South Beach 1	Ethanol	medium	0.61	MUT
5H	South Beach 1	Ethanol	medium	0.64	MUT
6B	South Beach 1	Ethanol	medium	0.7	MUT
6C	South Beach 1	Ethanol	heavy	1.05	MUT

Sample ID	Dive Site	Preservative	Weight	Weight (g)	Extraction Method
6D	South Beach 1	Ethanol	heavy	1.03	MUT
6E	South Beach 1	Ethanol	heavy	1	MUT
6F	South Beach 1	Ethanol	heavy	1.04	MUT
6G	South Beach 1	Ethanol	heavy	1.1	MUT
6H	South Beach 1	Ethanol	heavy	1.01	MUT
7A	Orkney Bayern	freeze	NA	NA	QBT
7B	Orkney Bayern	freeze	NA	NA	QBT
7C	Orkney Bayern	freeze	NA	NA	QBT
7D	Orkney Bayern	freeze	NA	NA	QBT
7E	Orkney Bayern	Ethanol	standard	0.23	QBT
7F	Orkney Bayern	Ethanol	standard	0.2	MUT
7G	Orkney Bayern	Ethanol	standard	0.27	QBT
7H	Orkney Bayern	Ethanol	standard	0.2	MUT
8B	Orkney Bayern	Silica beads	standard	0.08	QBT
8C	Orkney Bayern	Silica beads	standard	0.06	MUT
8D	Orkney Bayern	Ethanol	standard	0.27	QBT
8E	Orkney Bayern	Ethanol	standard	0.23	MUT
8F	Orkney Bayern	Ethanol	standard	0.32	QBT
8G	Orkney Bayern	Ethanol	standard	0.29	MUT
8H	Orkney Bayern	Silica beads	standard	0.08	QBT
9B	Orkney Bayern	Silica beads	standard	0.08	MUT
9C	Orkney Bayern	Ethanol	standard	0.2	QBT
9D	Orkney Bayern	Ethanol	standard	0.2	MUT
9E	Orkney Bayern	Ethanol	standard	0.36	QBT
9F	Orkney Bayern	Ethanol	standard	0.26	MUT
9G	Orkney Bayern	Silica beads	standard	0.15	QBT
9H	Orkney Bayern	Silica beads	standard	0.13	MUT
10A	Orkney Bayern	Ethanol	standard	0.26	QBT
10B	Orkney Bayern	Ethanol	standard	0.2	MUT
10C	Orkney Bayern	Silica beads	standard	0.12	QBT
10D	Orkney Bayern	Silica beads	standard	0.6	MUT
10F	NA	NA	NA	NA	QBT
10G	NA	NA	NA	NA	QBT
10H	NA	NA	NA	NA	QBT
11A	NA	NA	NA	NA	MUT
11B	Orkney Bayern	NA	NA	NA	QBT
11C	NA	NA	NA	NA	MUT

Table 7. Sample blanks and negative controls with a description of the reads removed for decontamination.

Sample ID	Type	OTU	Reads	Reads removed
4A	PCR negative	NA	0	NA
6A	PCR negative	NA	0	NA
10A	Field blank	NA	0	NA
10B	Field blank	*Symphodus melops*	1	0; not detected in any Orkney samples
10C	Field blank	*Merluccius merluccius*	1287	0; not detected in any Orkney samples
10D	Field blank	NA	0	NA
10E	PCR negative	*Salmo salar*	1	1; removed from all samples
10F	Extraction blank	NA	0	NA
10G	Extraction blank	NA	0	NA
10H	Extraction blank	NA	0	NA
11A	Field blank	*Trisopterous minutus*	1	1; removed from samples 4B-6H
11A	Field blank	*Trisopterus esmarkii*	1	1; removed from samples 4B-6H
11A	Field blank	*Phycis blennoides*	4	0; not detected in any samples

Sample ID	Type	OTU	Reads	Reads removed
11A	Field blank	*Helicolenus dactylopterus*	11	0; not detected in any samples
11B	Extraction blank	NA	0	NA
11C	Extraction blank	NA	0	NA

Appendix 2. Detailed Materials and Methods

A. Material Preparation and Field Sampling Methods

These passive eDNA capture methods have been written for a non-technical audience and provide instructions for how volunteers can prepare metaprobes. The cotton gauze should be certified sterile and can be found on various medical supply websites.

Preparing Metaprobes

1. Rinse dirty metaprobes with tap water, removing any residue or salt.

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.

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.

Aquatic eDNA Filtration

This protocol is modified from the SeaDNA protocols.

1. Before sample collection, filter any filtration blanks. Store-bought water was purchased for filtration blanks and the bottles were kept and used for sampling. Store eDNA samples in the fridge (or cool box if fridge unavailable) to be filtered after the dives finish and once the metaprobes are preserved.
2. Expose field blanks to the air for 10-20 seconds in the same place where the eDNA samples will be filtered.
3. With clean gloves, clean the outside of the eDNA bottles with 10% bleach.
4. Change gloves and remove a Sterivex filter from its packaging. Place in open packaging on sterilized surface. Remove syringe from packaging and separate the plunger from the barrel.
5. Secure the barrel of the syringe by twisting on to the inlet end (wide end) of the Sterivex filter which should now be placed over a waste area (e.g. sand away from sampling processing area). Make sure you only touch the sides of the Sterivex filter – do not touch the inlet or outlet ends at any time.
6. Pour ~50 ml of aqueous eDNA sample into the barrel of the syringe. Now attach the plunger and filter into a waste area (e.g., a bucket).
7. Remove Sterivex by twisting, then remove plunger. Reattach Sterivex and repeat step 5. Repeat steps 5 and 6 until all seawater has been filtered.
8. Remove Sterivex by twisting, then remove plunger. Reattach Sterivex. Pump air through the Sterivex until the filter is visibly dry. Remove and reattach Sterivex as necessary. Usually takes 2-3 attempts before filter is visibly dry.
9. Change gloves to retrieve labelled Whirl-pak sample bag and place the Sterivex filter unit inside. Place the bag/tube inside a labelled resealable bag and place the resealable bag into a coolbox with ice packs. Transfer to -20°C as soon as possible and for long-term storage.

B. 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.

C. 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.

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.

D. Mu-DNA Method

The following methods are a combination of the ‘Tissue’ and ‘Water’ protocols adapted from the ‘Mu-DNA: a modular universal DNA extraction method adaptable for a wide range of sample types V.2’ document on protocols.io.

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.

E. PCR protocol

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 (Taberlet et al., 2018). 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 DNA extract.

Three replicates per sample were amplified 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.

F. Mag-Bind® Total Pure NGS magnetic beads Clean-up Protocol

The following methods have been modified from the 'Mag-Bind® Total Pure NGS - 96-well Plate Protocol' in the Mag-Bind® Total Pure NGS product manual published in November 2018. Unless otherwise stated, the manufacturer protocol should be followed.

1. Read the manufacturer's instruction manual for the magnetic separation device, if provided.

2. Measure the volume of the PCR product or library. Determine the volume of beads that will be added to the reaction (i.e., for a 1x bead clean-up the same volume of beads to sample will be combined).

3. Shake or vortex the beads to resuspend any particles that may have settled. Allow the beads to come to room temperature before use.

4. Add the desired volume of beads to each well based upon desired fragment size to recover. Let sit at room temperature for 5 minutes.

5. Place the sample(s) on a magnetic separation device to magnetize the beads. Let sit at room temperature until the beads are completely cleared from solution. This normally takes between 2-3 minutes but can be longer for larger volumes.

6. Aspirate and discard the cleared supernatant. Do not disturb the beads.

7. With the sample(s) remaining on the magnet, add 200 µL 80% ethanol to each well.

8. Let sit at room temperature for 30 seconds.

9. Aspirate and discard the cleared supernatant without disturbing the beads.

10. Repeat Steps 8-9 for a second 80% ethanol wash step.

11. Leave the sample(s) on the magnetic separation device for 2-5 minutes to air dry the beads. Remove any residual liquid with a pipettor.

12. Remove the sample(s) from magnetic separation device.

13. Add 20 µL (for clean PCR product) or 40 µL (for a library in preparation for adapter ligation) Elution Buffer (see Section C. Mu-DNA Method Reagents) to each well.

14. Pipet up and down 20 times or vortex for 30 seconds.

15. Let sit at room temperature for 5 minutes.

16. Repeat step 5.

17. Transfer the cleared supernatant containing purified DNA to a new tube and store at 2-8°C if storage is only for a few days. For long-term storage, samples should be kept at -20°C.

G. Adapter Ligation Protocol (NEXTFLEX® Rapid DNA-Seq)

The following methods have been modified from the ‘Option 2: size selection’ protocol in the NEXTFLEX® Rapid DNA-Seq Kit 2.0 (1 ng – 1 µg) NOVA-5188-01 published in 2019. Unless otherwise stated, the manufacturer protocol should be followed.

End Repair

1. Thaw NEXTFLEX® End-Repair & Adenylation Buffer Mix on ice, and vortex for 5-10 seconds.

2. For each library (Note: one in this report), combine the following reagents on ice in a microsample tube:

- 15 µL NEXTFLEX® End-Repair & Adenylation Buffer Mix 2.0
- 3 µL NEXTFLEX® End-Repair & Adenylation Enzyme Mix 2.0
- 32 µL library

3. Run the following thermocycler programme: 30 min at 20 °C, then 30 min at 65 °C, followed by a 4 °C hold. The heated lid should be on at 105 °C.

Adapter Ligation

4. Thaw NEXTFLEX® Ligase Buffer Mix 2.0 to room temperature, and vortex for 5-10 seconds. Do not spin down tube, as this may cause components of the mix to separate and affect performance.

5. Based on the amount of input DNA (calculated from the concentration of your library prior to end repair) dilute the adapters. See the manufacturer protocol for details on how to do this.

6. Combine the following in a new microsample tube and mix thoroughly by pipette (pipetting up and down 15 times):

- 50 µL End Repaired & Adenylated DNA
- 44.5 µL NEXTFLEX® Ligase Buffer Mix 2.0
- 2.5 µL NEXTFLEX® Barcoded Adapter (diluted if necessary)
- 3.0 µL NEXTFLEX® Ligase Enzyme 2.0

7. Incubate in a thermal cycler with heated lid turned off or open for 15 minutes at 20°C, followed by a 4°C hold.

8. Store at 2-8°C if storage is only for a few days. For long-term storage, samples should be kept at -20°C.

H. Additional Analysis

While a PERMANOVA is non-parametric and does not require homogeneity of variance, homogeneity of group dispersions can indicate the confidence of a PERMANOVA, therefore this was tested using the ‘betadisper’ function from the R package vegan. A post-hoc test to understand the group dispersions was done by implementing the ‘TukeyHSD’ function.

Comparisons of fish community composition by extraction treatments were visualized on venn diagrams using the R package ggvenn.

Species accumulation curves were calculated using the ‘accucomp’ function from the R package BiodiversityR.

Appendix 3. Additional Results

A. Fish Community Composition

Table 8. Results of the multivariate homogeneity of group dispersions test, where groups are sampling locations.

Source	DF	Sum of Sqs	Mean Sq	F Value	P value
Groups	3	0.32244	0.107479	2.8231	0.04627
Residuals	60	2.28426	0.038071		

Table 9. Results of the post-hoc Tukey test following the multivariate of group dispersions test on locations.

Groups	diff	lwr	upr	P adj
Orkney Bayern; Mount Batten north side	-0.085535725	-0.25984146	0.08877001	0.5685872
South Beach 1; Mount Batten north side	0.085751294	-0.08536760	0.25687019	0.5513744
South Beach 2; Mount Batten north side	0.009476332	-0.25678008	0.27573274	0.9996985
South Beach 1; Orkney Bayern	0.171287019	0.01566592	0.32690812	0.0254901
South Beach 2; Orkney Bayern	0.095012057	-0.16155904	0.35158316	0.7621258
South Beach 2; South Beach 1	-0.076274962	-0.33069177	0.17814185	0.8576938

Table 10. Results of PERMANOVA for pairwise comparisons between each combination of sampling locations.

Source	DF	Sums of Sqs	Mean Sq	F value	R^2	P value	Adjusted P Value
Orkney Bayern; Mount Batten north side	1	4.5870	4.587	24.013	0.41329	0.000999	0.001498
Residuals	34	6.4948	0.191		0.58608		
Total	35	11.0818			1.00000		
South Beach 1; Mount Batten north side	1	1.9787	1.97873	7.2167	0.16699	0.000999	0.001498
Residuals	36	9.8708	0.27419		0.83301		
Total	37	11.8495			1.00000		
South Beach 2; Mount Batten north side	1	0.6991	0.69915	2.9872	0.14233	0.002997	0.003596
Residuals	18	4.2129	0.23405		0.85767		
Total	19	4.9121			1.00000		
South Beach 2; Orkney Bayern	1	2.2652	2.26525	11.967	0.33272	0.000999	0.001498
Residuals	24	4.5430	0.18929		0.66728		

Source	DF	Sums of Sqs	Mean Sq	F value	R^2	P value	Adjusted P Value
Total	25	6.8083			1.00000		
South Beach 1; Orkney Bayern	1	4.4878	4.4878	18.477	0.30553	0.000999	0.001498
Residuals	42	10.2009	0.2429		0.69447		
Total	43	14.6887			1.00000		
South Beach 2; South Beach 21	1	0.1881	0.18813	0.61768	0.02321	0.8222	0.822177
Residuals	26	7.9190	0.30458		0.97679		
Total	27	8.1072			1.00000		

B. Metaprobe Extraction Optimisation

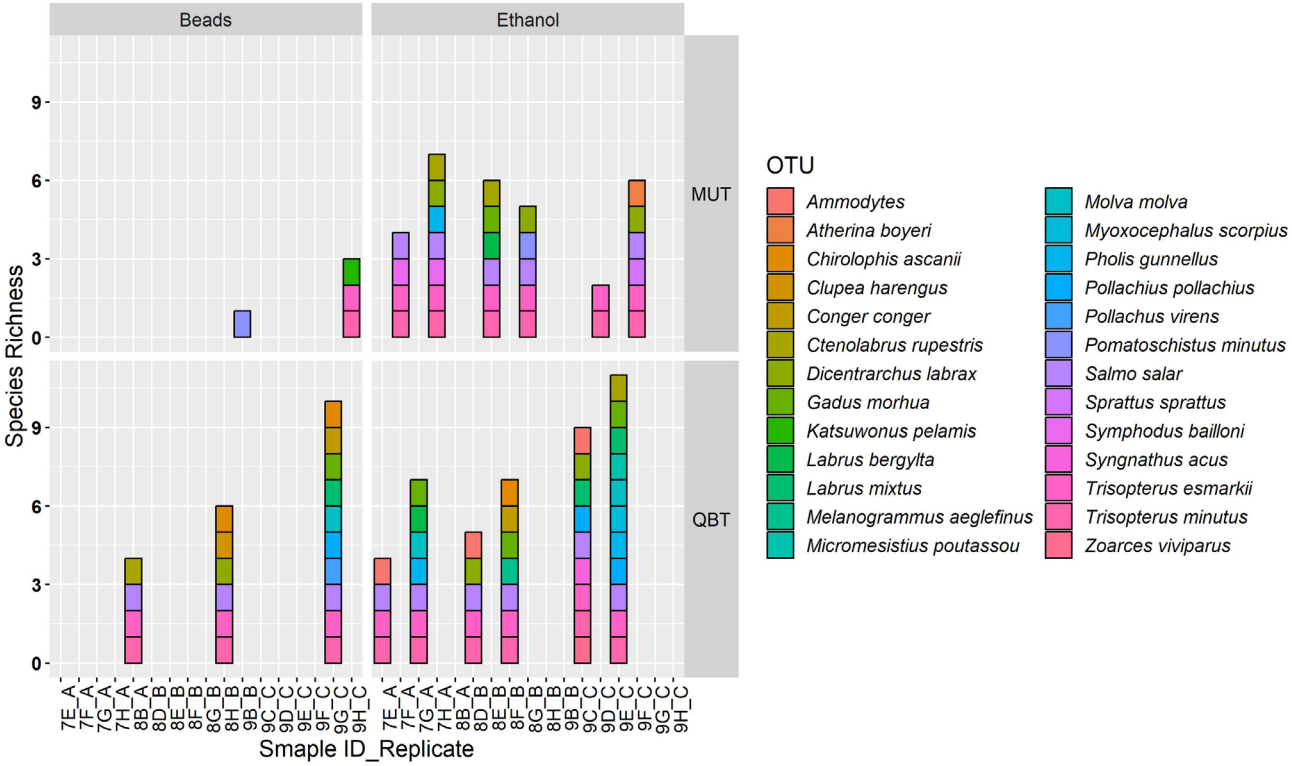

Figure 11. Stacked bar charts comparing the detected species richness at the SMS Bayern dive site based on differences between preservation and extraction methods.

Table 11. Kruskal-Wallis tests of differences between species richness detected by different gauze weight ranges.

Kruskall-Wallis Test	Chi-squared	DF	P value
Richness by weight range (MUT)	4.9182	2	0.08551
Richness by weight range (MUT & QBT)	5.0594	2	0.07968

Table 12. Kruskal-Wallis tests of differences between species richness detected by different extraction methods.

Kruskall-Wallis Test	Chi-squared	DF	P value
Richness by extraction method	1.1055	1	0.2931

C. eDNA Capture

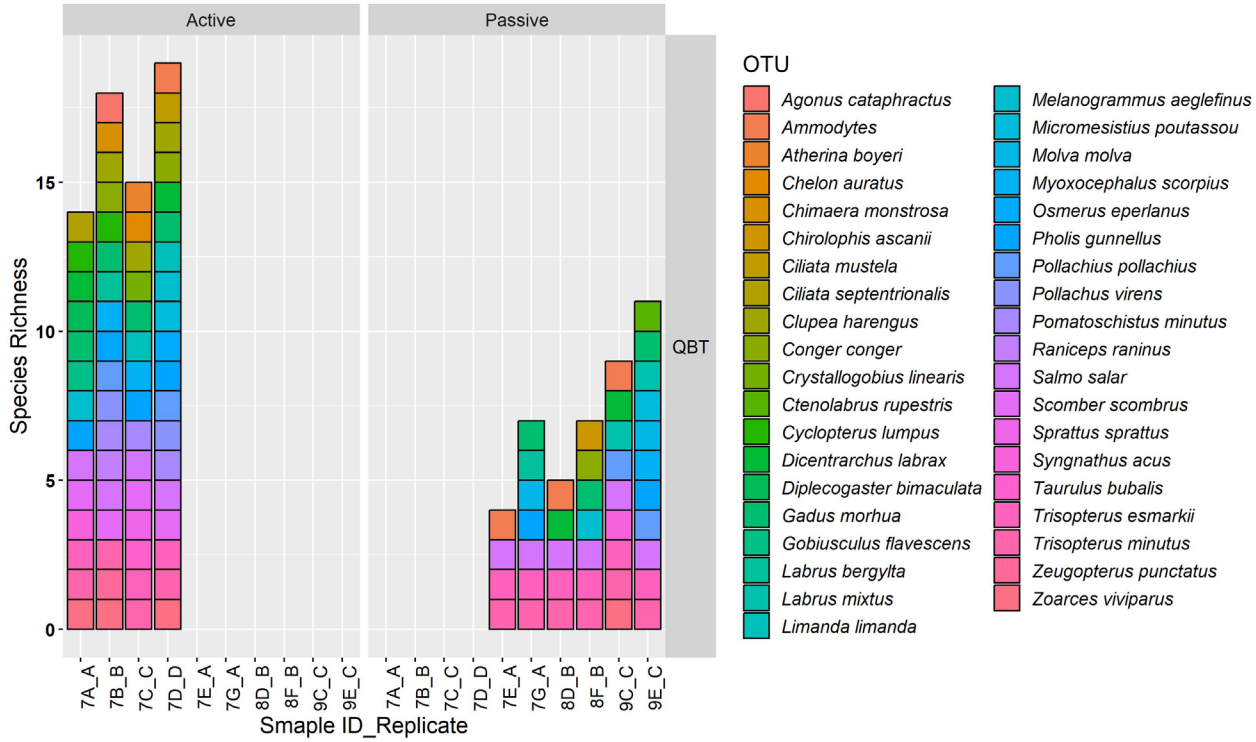

Figure 12. Stacked bar charts comparing the detected species richness at the SMS Bayern dive site based on differences between eDNA capture method.

www.gov.uk/natural-england