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**Exploring the Application of eDNA for Intraspecific Studies of
the Shannon Bottlenose Dolphins (*Tursiops truncatus*
Montagu, 1821): a Preliminary Study**

Thesis presented by

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for the degree of

Masters of Zoology

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Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism and intellectual property."

Abstract

With the recent advancements in molecular technologies, environmental DNA (eDNA) has become an especially useful tool for biodiversity monitoring. More recently, eDNA has been investigated as a tool to obtain fine scale molecular information such as individuals' mitochondrial DNA (mtDNA) haplotypes. This study examines the capabilities of eDNA in enhancing our understanding and monitoring of the well-studied Shannon Estuary bottlenose dolphin (*Tursiops truncatus*) population. Water samples taken from the fluke print of dolphins were filtered using two different filter types Sterivex (~1.5L) and Waterra (~50L) species-specific qPCR was used to detect bottlenose dolphins' eDNA, as well as conventional PCR to amplify a 543bp haplotype region to identify known haplotypes from eDNA samples. These same water samples were also used for a metabarcoding analysis of fish species in an attempt to better understand potential prey species. Bottlenose dolphin eDNA was successfully detected in 44% of the samples using Sterivex filters and 61% using Waterra filters. The two haplotypes known from the Shannon estuary population (Ire1 and 2) were also successfully identified using Dlp1.5 and Dlp8G primers, and putative fish prey species were successfully obtained by both filter methodologies. The effectiveness of both sample acquisition approaches was compared and their implementation in further eDNA studies considered. The results of this study add to the evidence that eDNA is a promising non-invasive method for monitoring both presence/absence and genetic diversity of bottlenose dolphins.

Introduction

In recent years environmental DNA (eDNA) has emerged as useful tool for biodiversity monitoring (Beng and Corlett, 2020; Bohmann *et al.*, 2014). The basis of eDNA is that organisms leave behind trace amounts of DNA in their environment, whether that be soil, water, or even air (Ficetola *et al.*, 2008; Lynggaard *et al.*, 2022). The capture, extraction, and amplification of this eDNA allows for the detection of species without the need for visual observation. This makes eDNA especially useful for monitoring of rare and cryptic species or the identification of invasive species (Sepulveda *et al.*, 2019; Thomas *et al.*, 2020). eDNA is especially useful in the study of marine mammals as they tend to be mobile and elusive species that can be difficult to study using traditional survey methods (Foote *et al.*, 2012). The ability of eDNA to detect cetacean species has been demonstrated in a variety of species and environments (Alter *et al.*, 2022; Haderlé *et al.*, 2023; Juhel *et al.*, 2021; Székely *et al.*, 2022). A recent study showed that it was possible to detect the elusive, deep diving Cuvier's beaked whale (*Ziphius cavirostris*) from eDNA collected from the water surface (Boldrocchi *et al.*, 2024). Aside from single species detections, eDNA in combination with high-throughput next generation sequencing can be used to detect whole communities (Closek *et al.*, 2019), and therefore has the potential to be used to identify predator prey-dynamics in marine ecosystems (Berger *et al.*, 2020).

As yet there is no standardised methodology to detect eDNA in cetacean species. In terms of sampling, water samples can be taken in transects in areas the target species are expected to be (Boldrocchi *et al.*, 2024; Székely *et al.*, 2021), in areas where the target species were recently observed (Baker *et al.*, 2018; Greiner-Ferris, 2020; Pinfield *et al.*, 2019), or directly from the fluke print (the patch of calm water left after a cetacean as dived) of the target species (Parsons *et al.*, 2018; Székely *et al.*, 2021). While most studies collect 1L of water (Alter *et al.*, 2022; Baker *et al.*, 2018; Zhang *et al.*, 2023), the volume of water collected can range from 15ml to 30L (Foote *et al.*, 2012; Juhel *et al.*, 2021;

Parsons *et al.*, 2018). Filter types used also vary between studies, with some studies using open cellulose filter discs (Greiner-Ferris, 2020; Parsons *et al.*, 2018), while others use capsule filters (Alter *et al.*, 2022; Juhel *et al.*, 2021; Székely *et al.*, 2021). The volume of water collected influenced the choice of filter type. In the lab there are various preexisting DNA extraction kits to choose from or eDNA can be extracted by protocols such as phenol-chloroform extraction (Greiner-Ferris, 2020; Parsons *et al.*, 2018; Székely *et al.*, 2021). For target eDNA, two widely used methods are qPCR and metabarcoding (Closek *et al.*, 2019; Parsons *et al.*, 2018; Székely *et al.*, 2021; Valsecchi *et al.*, 2020), and more recently droplet digital PCR has also been used as an especially sensitive method of eDNA detection (Baker *et al.*, 2018; Scriver *et al.*, 2024). The lack of consistent field and lab protocols makes it difficult to select the optimum methodology for a specific study.

Understanding the genetic variation within populations is a valuable tool when assessing species distribution, connectivity, and diversity, which is essential for the implementation of effective management and conservation of vulnerable species (Palsbøll *et al.*, 2007, 2013; Waples and Gaggiotti, 2006). Generally, population genetic information for cetacean species is obtained through biopsy samples taken from both dead and live animals (Krützen *et al.*, 2002; Meschersky *et al.*, 2008). Biopsy sampling live animals is challenging and may cause undue stress on the animal. eDNA has the potential to be used as a simpler and non-invasive method to obtain this same genetic information. While the detection of species using eDNA is reasonably well studied, the potential population level inferences from eDNA are yet to be discovered (Adams *et al.*, 2019; Sigsgaard *et al.*, 2020). Thanks to advancements in molecular tools, it is becoming increasingly possible to obtain genetic data at the intraspecific level using indirect (yet targeted) sampling approaches, such as individual pinnipeds' DNA from scat at haul out sites (Steinmetz *et al.*, 2023) and baleen whales' individual DNA from blows using drones (O'Mahony *et al.*, 2024). However, disentangling individual eDNA from composite matrices such as

water, which may contain eDNA from multiple individuals, is still particularly challenging. In recent years, a few studies have used eDNA to investigate intraspecific diversity in cetacean species such as killer whales (*Orcinus orca*), harbour porpoise (*Phocoena phocoena*) and bowhead whales (*Balaena mysticetus*) (Baker *et al.*, 2018; Parsons *et al.*, 2018; Székely *et al.*, 2021). This study investigates the applicability and capabilities of eDNA for monitoring bottlenose dolphins (*Tursiops truncatus*), focusing on the well-studied resident Shannon Estuary bottlenose dolphin population.

The outer Shannon Estuary is a Special Area of Conservation (SAC) located on the West coast of Ireland. The estuary originates from the River Shannon, the longest river in Ireland, the estuary spans approximately 120km from Limerick City to the Atlantic Ocean. The Shannon estuary encompasses a wide variety of marine environments and is home to many different species. The habitat heterogeneity offered by the Shannon Estuary makes it an ideal location for bottlenose dolphins to feed, breed and socialise (Ingram and Rogan, 2002). The Shannon bottlenose dolphins have been consistently studied over the past 30 years, with ongoing monitoring of the population using photo-identification and individual recognition (e.g. Englund *et al.*, 2008; Rogan *et al.*, 2018). In the most recent population size estimate in 2018, it was reported that there were between 121-160 individuals in the population (Rogan *et al.*, 2018). Unlike other dolphin populations which tend to be mobile and migratory, the Shannon dolphins demonstrate a high level of site-fidelity (Mirimin *et al.*, 2011; Nykänen *et al.*, 2018). While the population itself and how it uses the estuary is well studied, apart from some stomach content data, little is known about what these dolphins are feeding on (Dunshea *et al.*, 2013). A better understanding of the feeding preferences of these dolphins is needed as it has important implications for both dolphin and fisheries management.

Population genetic studies based on biopsies of dolphins in Irish waters and those that have stranded in Ireland have identified that the Shannon dolphins are genetically distinct

from other Irish coastal and pelagic dolphin populations (Mirimin *et al.*, 2011; Nykänen, Louis, *et al.*, 2019). While there is evidence of dispersal and interactions between Shannon dolphins and other populations, Shannon dolphins appear to be both genetically and geographically isolated. This isolation, combined with a relatively small population size, raises concerns about their vulnerability to anthropogenic pressure and environmental changes (Mirimin *et al.*, 2011; Nykänen, Louis, *et al.*, 2019).

Although the estuary is recognised as an important habitat for these dolphins it also functions as a major shipping route with as many as one thousand ships utilising the estuary annually (O'Brien *et al.*, 2016; SIFP, 2013). The Shannon Estuary is also a key area for industrial and economic developments and is frequently used by locals and tourists for recreational activities (SIFP, 2013). The heavy use of the estuary puts a lot of anthropogenic stress on the bottlenose dolphin population. Ongoing photo-identification and acoustic surveys provide valuable information about how the Shannon dolphins use their environment and how this may be affected by anthropogenic stress (Barker and Berrow, 2016; Carmen *et al.*, 2021; Rogan *et al.*, 2018). However, to effectively manage and conserve the Shannon dolphins continued monitoring strategies must be implemented.

This study evaluates the use of eDNA based techniques to effectively monitor the Shannon bottlenose dolphin population. By taking water samples from the fluke prints of dolphins we investigate how the use of two different filter types and methodologies can effectively capture bottlenose dolphin eDNA. The aims of this study were to determine if 1. eDNA can be used to detect bottlenose dolphins. 2. eDNA can be used to identify previously reported bottlenose dolphin mitochondrial DNA haplotypes (mtDNA) haplotypes (Mirimin *et al.*, 2011). 3. inferences can be made about potential bottlenose dolphin prey in the Shannon Estuary and 4. to evaluate the performance of two commercially available filters in this study.

Methods

Study site

This study took place in the Shannon Estuary SAC situated on the West coast of Ireland, which connects the River Shannon to the Atlantic Ocean. The general study area and survey route followed that of Ingram and Rogan (2002) (Figure 1). However, this route varied depending on weather conditions and dolphin encounters. Surveys were conducted using a 6.5m rigid inflatable boat (RIB).

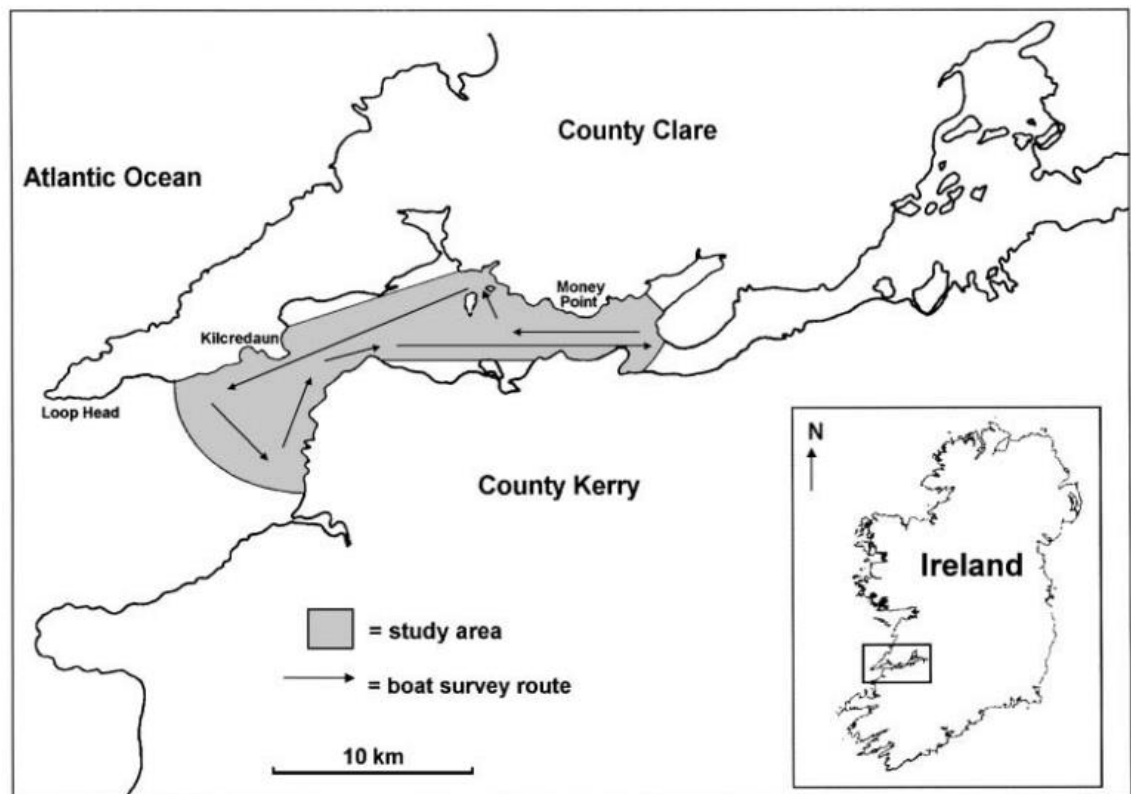


Figure 1: Map of Shannon Estuary and survey route (from Ingram and Rogan, 2002)

Sample collection

When a group of dolphins were spotted the group was approached carefully and the following information was recorded: Date and time of encounter, GPS coordinate, group size, composition, and behaviour. Following this photo ID was conducted and eDNA samples were taken.

Photo ID

An effort was made to photograph all individuals in a group. These photographs were used where possible to identify which individuals were being sampled on each sampling occasion. The photographs were also compared to those in UCC's catalogue of known Shannon dolphins. This was to verify that the dolphins being sampled were known resident Shannon dolphins and to cross reference whether any of the individuals encountered had previously been biopsied (Mirimin *et al.*, 2011; Nykänen *et al.*, 2018), as well as providing an update to the long running UCC Shannon Dolphin Catalogue.

eDNA sampling

Where possible, samples were taken from the fluke prints of individual dolphins, otherwise samples were taken at random from where group members recently surfaced. Surface water samples were taken using buckets and collected in a 60L barrel. Once a sample was collected the water was subsampled and filtered using Sterivex-HV (0.45µm pore size; Millipore) and Waterra 0.45-micron eDNA filters (Waterra USA Inc.). Between 1 and 1.5L were pushed through Sterivex filters using a clean, disposable 50ml syringe. Once filtration was finished, filters were dried by pushing air through the filter. For Waterra filters an ARGALY electronic pump (purpose built for use with Waterra filters) was attached to the filter before it was placed in the water and ~50L of water were pumped through the filter. Again, after filtration was completed, the filters were dried by allowing the pump to pull air through the filter until there was no water remaining in the filter capsule. A storage buffer consisting of TES buffer (0.1M TRIS, 10mM EDTA, 2% sodium dodecyl sulfate: pH 8) and proteinase K (20mg/ml) in a ratio of 190:1 was added to each filter's casing (1.5ml for Sterivex and 50ml for Waterra). Initially buffer was added to the outlet side of the Waterra filters (per Waterra instructions) however, this prevented the addition of 50ml into the filter. Thus, for the remainder of the samples buffer was added to the inlet side of the filters. After the addition of the buffer, filters were shaken vigorously for one minute. Filters were stored in a cooler on ice before storage in a -20°C

freezer upon return to the lab. Between each sample buckets and barrels were sprayed and wiped down with 20% bleach, rinsed three times with sea water and dried.

Field controls

As a field control to test if bottlenose dolphin eDNA persists in the estuary, seawater samples were taken in the absence of dolphins. The RIB remained in one position for 15-minutes and the area was constantly monitored for dolphins. After 15-minutes had elapsed - if no dolphins were observed a seawater sample was taken. The sample was filtered using both filter types and stored in the same manner as above. Additionally, field negatives were taken to test that no eDNA was being carried over from one sample to another. After a sample was taken and equipment was cleaned, 10 litres of clean water i.e. from bottled or tap water, was poured into the sampling bucket, swirled around and emptied into the collection barrel and swirled around again. Five litres of this water was filtered through a Waterra filter, and 1.5 litres was filtered through a Sterivex filter, the filters were then stored as above.

eDNA extraction

Sterivex filters were removed from the freezer and incubated at 56°C for 3 hours, shaking every 30 minutes. The contents of the filters were then removed from the capsule inlet using a 3ml syringe. These lysates and an extraction negative which contained only ATL buffer were extracted using a DNeasy Blood & Tissue Kit (QIAGEN) according to the manufacturer's instructions, with a 1-hour incubation after addition of ATL buffer and proteinase K.

To extract eDNA from Waterra samples a modified version of the precipitation protocol described by Pont *et al.* (2018) was used. Waterra filters were removed from the freezer and left to thaw at room temperature for at least 3 hours. Once thawed, filters were placed horizontally on a mini orbital shaker at max speed (300rpm) for 15 minutes, rotating filters 180° halfway through. The content of each filter was emptied into a 50ml falcon

tube and centrifuged for 15 minutes at 10,178g (maximum speed). Supernatant was removed until there was 15ml remaining in the tube, then 33ml of ethanol and 1.5ml of 3M sodium acetate was added to each tube before storing the tubes in a -20°C freezer overnight. The next day tubes were removed from the freezer and allowed to return to room temperature. The tubes were centrifuged again at 10,178g at 6°C, then all supernatant was removed leaving only the pellet. In the case of filters where buffer was initially added to the outlet side, after precipitation, an additional 50ml of buffer was added to the inlet side of each filter and they were precipitated again following the same protocol.

Lysates obtained from the outlet side were extracted using a DNeasy Blood & Tissue Kit (QIAGEN) with a modified first step: 720µl of ATL buffer was added to the lysate tubes, the tubes were then vortexed briefly and centrifuged for 1 minute at 1000g. This mixture was then pipetted into a 2ml tube containing 20µl of proteinase K and incubated at 56°C for 1 hour. The remaining protocol was completed according to manufacturer's instructions. The lysates obtained from the inlet side of the filters contained much more sediment than those obtained from the outlet side. Therefore, lysates obtained from the inlet side of the filters were extracted using a DNeasy PowerSoil Pro Kit (QIAGEN) following the manufacturer's instructions. Extraction negatives containing only ATL buffer were also included in the extraction process to ensure there was no contamination during the extraction process.

Total eDNA quantification

The total eDNA concentration of all extracts including those from bottlenose dolphin samples, field controls and field/extraction negatives were measured using a Qubit fluorometer 3.0 and a Qubit dsDNA Quantitation, High Sensitivity (HS) Assay Kit (Life Technologies). Measurements were carried out according to manufacturer's instruction using 5µl of each extract and fresh Qubit standards were prepared for every sample.

eDNA concentrations for each sample were measured in triplicate. The eDNA concentration for some samples was too high to be measured by HS Qubit so the measurements were repeated using a Qubit dsDNA Quantitation, Broad Range (BR) Assay Kit (Life Technologies).

To determine if there was a significant difference between the total eDNA concentration obtained from Sterivex and Waterra filters, a Wilcoxon Signed Rank test was performed in SPSS (IBM SPSS Statistics 27).

Species specific primer selection for qPCR assay

The species specificity of eight primer pairs was evaluated before the most appropriate set of primers were selected for eDNA quantification using qPCR. Three bottlenose dolphin specific primer pairs were taken from existing literature (Greiner-Ferris, 2020; Stoeckle *et al.*, 2018). Three primer pairs were selected using the publicly available assayID tool (<https://github.com/jammc313/assayID>) which designs species specific primers for qPCR assays based on target and non-target DNA sequences obtained from GenBank. Additionally, as the primers used by Mirimin *et al.* (2011) to identify Irish bottlenose dolphin haplotypes produced an amplicon too long for qPCR, two primer pairs were designed using PrimerQuest (IDT) to amplify subsections of this haplotype region containing informative polymorphisms (Table 1).

The specificity of all primers was assessed using primer BLAST (Altschul *et al.*, 1990). Specificity was also tested by running all primers in PCR reactions with a selection of marine mammal DNA obtained from tissue samples from a range of species: Bottlenose dolphin (5 ng/ μ L, 0.5 ng/ μ L and 0.05 ng/ μ L), common dolphin (*Delphinus delphis*), harbour porpoise (*Phocoena phocoena*), killer whale (*Orcinus orca*), pilot whale (*Globicephala melas*), sperm whale (*Physeter macrocephalus*), fin whale (*Balaenoptera physalus*) and grey seal (*Halichoerus grypus*). PCR blanks were also included in each PCR run. The following PCR conditions were used for all primer pairs: Initial denature

was carried out at 94°C for 2 minutes, followed by 35 cycles of denature at 94°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 30 seconds. This was followed by a final extension at 72°C for 5 minutes. The PCR products were then isolated by gel electrophoresis to identify which species were amplified by each primer set. Based on these results the Dloop primers designed by Greiner-Ferris (2020) appeared to be the most species specific. However, after using AliView (Uppsala University) to align these primers with bottlenose dolphin sequences sampled in Ireland (Nykänen, Kaschner, *et al.*, 2019) obtained from GenBank (NCBI) (Sayers *et al.*, 2022) the primers were modified to better match the genotype of bottlenose dolphins found in Irish waters.

Bottlenose dolphin eDNA quantification

qPCR assays were evaluated using both TaqMan and SYBR Green chemistry with the modified Greiner-Ferris primers. For the TaqMan assay a corresponding probe was designed using PrimerQuest (IDT). Both methods were tested extensively using a 1:10 dilution series of bottlenose dolphin DNA extracts (from skin samples) in molecular grade water. When using TaqMan chemistry only DNA concentrations $> 0.01\text{ng}/\mu\text{l}$ were detected, whereas DNA concentrations $< 0.001\text{ ng}/\mu\text{l}$ were detected using SYBR Green chemistry. Subsequently, all eDNA samples, field negatives and field controls were run in triplicate using SYBR Green master mix. Each qPCR plate also contained standard dilutions of bottlenose dolphin DNA (between 5 and 7 magnitudes of dilution), and one negative technical control, these were also run in triplicate.

Two sets of criteria were set to define positive detections: i) Conservative criteria, where all replicates of a sample amplified bottlenose dolphin eDNA and the sample was not flagged for high standard deviation between samples and ii) Relaxed criteria, where bottlenose dolphin eDNA was amplified in at least one replicate.

Again, to determine if there was a significant difference between the mean total eDNA concentration obtained from paired samples using Sterivex and Waterra filters, a

Wilcoxon Signed Rank test was performed in SPSS. Additionally, a Generalised Linear Model (GLM) was used to determine if the flow of the tide (ebb or flow) or the group size when samples were taken influenced the amount of target eDNA obtained in Waterra (DNeasy PowerSoil Pro) samples.

To test for sample inhibition, all extracts were also subject to a qPCR where they were spiked with an internal laboratory positive control and associated primers (Kavlick, 2018). A sample was deemed to be inhibited if the mean ct for the sample was three or more cycles greater than the ct of the IPC on its own (Hartman *et al.*, 2005).

Table 1: Primer sequences tested for qPCR

Source	Target Region	Primer Name	Primer Sequence	Amplicon Length
Greiner Ferris (2020)	Cytb	TtCytbF	5'-CGAGTGAATCTGAGGTGGATTT-3'	94
		TtCytbR	5'-CAATGCTGTGATGATGAATGGAAGA-3'	
	Dloop	TtDloopF	5'-CACACGTGCATGCTAATATTAG-3'	203
		TtDloopR	5'-GAGTGACCATAGGATATAATGGAGA-3'	
This study	Dloop	TtDloopF (MOD)	5'-CACATGTGCATGCTAATATTAG-3'	203
		TtDloopR (MOD)	5'-GAGCGACCATAGGATATAATGGAG-3'	
Stoeckle <i>et al.</i> (2018)	12S	M13bodoF	5'-TGTAACGACGCCAGTTGACCCAACTAATAGACAC-3'	187
		M13bodoR	5'-CAGGAAACAGCTATGACTCTTAGTTGTCGTGATTTCAG-3'	
This study	Dloop	TtDloop152F	5'-TGGTCGCTCCATTAGATCACG-3'	152
		TtDloop152R	5'-TGGTCCTGAAGTAAGAACCAGA-3'	
		TtDloop156F	5'-TCCATACGATAAGTTAAAGCTCGT-3'	156
		TtDloop156R	5'-CACGCGCATGGTGATTAAG-3'	
Assay ID Tool	ND1	TtND1F	5'-CGACCAGCCACATCTTCAAC-3'	107
		TtND1R	5'-TTGATGAGTGGGTGCGGTAT-3'	
	ND4	TtND4F	5'-GGTCACACTTCAAGTCTCCTA-3'	141
		TtND4R	5'-CCTGCGTTAAGTCGTTCTGT-3'	
	ND5	TtND5F	5'-CCCCAATCCTCCCTCTCGTA-3'	125
		TtND5R	5'-TGAGTGGAGTAGGGCTGAGA-3'	

eDNA sequencing

Two sets of primers were tested to obtain sequences that could be used to infer haplotype information from the eDNA extracts based on the haplotypes identified by Mirimin *et al.* (2011). The first were modified versions of the primers L15926 and H00034 designed by Rosel *et al.* (1994) which Mirimin *et al.* (2011) used to identify “Irish” bottlenose dolphin haplotypes. The second set of primers Dlp1.5 and Dlp8G was developed by Baker *et al.* (1998) and Dalebout *et al.* (2005), and previously used by Louis *et al.* (2014) to identify the same bottlenose dolphin haplotypes. Preliminary results from tissue extracts and

selected eDNA samples suggest that the Louis *et al.* (2014) primers were more suitable for sequencing.

All eDNA extracts underwent PCR with the second set of primers following the same PCR conditions as Louis *et al.* (2014). Subsequently the PCR products were run on a 2% agarose gel for 1.5-2 hours. A UV lightbox (Vilber Lourmat TFX 20M) was used to visualise the resulting bands. Any bands that matched the expected fragment length (~700bp) were cut out from the gel using a scalpel and were purified using a QIAquick Gel Extraction Kit (QIAGEN). The Dlp8G primer was then added to the products before they were sent to an accredited laboratory (Eurofins) for Sanger sequencing.

Table 2: Primers used to obtain bottlenose dolphin haplotypes

Source	Target Region	Primer Name	Primer Sequence	Amplicon Length
Baker <i>et al.</i> (1998)	D-loop	Dlp1.5	5'-TCACCCAAAGCTGGAATTCTA-3'	748
Dalebout <i>et al.</i> (2005)		Dlp8G	5'-GGAGTACTATGTCCTGTAACCA-3'	
This study	D-loop	L15926 (MOD)	5' -ACCTCGGTCTTGTAACCA-3'	667
Rosel <i>et al.</i> (1994)		H00034	5'-TACCAAATGTATGAAACCTCAG-3'	

Haplotype assignment

Once sequence files were received, they were viewed in AliView. The sequences were manually trimmed of primers and low-quality ends, based on the accompanying chromatograph provided by Eurofins. These trimmed sequences were then aligned against the Irish bottlenose dolphin haplotype sequences (Mirimin *et al.*, 2011) in MEGA 11 (Tamura *et al.*, 2021). Once aligned, sequences from eDNA samples were compared by eye using the highlight difference from trace function in AliView to the known haplotypes. eDNA sequences were assigned a haplotype if they matched one exactly and if it was not possible to assign a single haplotype all possibilities were noted. If an eDNA sequence did not match any of the known haplotypes the sequence was submitted to BLAST (NCBI) (Altschul *et al.*, 1990) to verify the taxa, and any mismatches were checked against the chromatograph to identify possible sequencing errors. If the BLAST

(Altschul *et al.*, 1990) results returned bottlenose dolphin and the mismatched bases showed evidence of sequencing error a maximum parsimony approach was implemented to assign haplotypes, while noting the possibility of error in the assignment.

Metabarcoding

To examine potential prey composition, aliquots of ten extracts were sent to AllGenetics (AllGenetics; Spain) for library prep and high-throughput sequencing using MiFishU primers (Miya *et al.*, 2015). Bioinformatic analysis was performed on the sequences received from AllGenetics using the Dada2 (Callahan *et al.*, 2016) metabarcoding pipeline in Rstudio (R Core Team, 2024). Primers were removed using cutadapt, sequences trimmed where phred score dropped below 35, and then forward and reverse reads were merged. Only sequences between 180-200bp were retained as this was the expected amplicon length (Miya *et al.*, 2015). Taxonomy was assigned manually by submitting sequences to BLAST (Altschul *et al.*, 1990). Species were assigned based on 100% percentage identity and 100% query cover. If a sequence matched to more than one species the sequence was assigned to the species most likely to be present in the Shannon and if there was more than one species that could possibly be found in the Shannon a higher taxonomic rank was assigned e.g. genus, family etc.

Results

Samples collected

Throughout the sampling period 20 Sterivex and 18 Waterra samples were collected across 19 sampling events (Table 3). Additionally, two field control samples in the absence of dolphins and three field negative samples using clean water were taken, all of which were filtered using one Sterivex and one Waterra filter per sample. Samples were taken in a variety of locations ranging from the inner estuary to the outer mouth of the estuary, with seven samples being taken from single individuals (Figure 2). Most samples were taken on a flood tide, with two samples taken just at high water. The mean group size was 2.5 individuals. On most occasions, the main behaviour was recorded as “slow travelling”, while foraging was suggested on only two occasions. On some occasions dolphin encounters ended before the target volume of water could be collected.

Table 3: Table detailing the dates, times and conditions at which samples were taken as well as the behaviour and group composition of the dolphins, and the volume of water filtered on each sampling event.

Date	High Tide	Low Tide	Sampling Event	Time	Sea State	Behaviour	Adults	Juveniles	Sterivex Vol. (L)	Waterra Vol. (L)
09/08/2023	12:35	18:41	1	12:13	1	Slow Travelling	2	1	1.5 x3	0
16/08/2023	19:03	12:44	2	13:37	0	Foraging/Milling	1	0	1.5	51
			3	13:45	1	Slow Travelling	1	1	2	50
			4	16:00	1	Slow Travelling	1	1	0	42
			5	18:09	1	Slow Travelling	4	1	1.5	50
08/09/2023	13:17	19:39	6	13:58	1	Milling/Slow Travelling	1	0	1.5	53
			7	14:37	1	Feeding	5	0	1.5	50
			8	16:02	1	Slow Travelling	2	0	1.5	50
29/11/2023	19:01	12:36	9	11:40	1	Travelling	1	0	1	36
			10	13:30	2	Slow Travelling	3	3	1	38
			11	14:40	2	Travelling	1	0	1	42
01/05/2024	12:24	18:23	Control 1	11:20		N/A	0	0	1.5	50
			12	12:20	2	Travelling	2	1	1.5	31
			13	17:00	2	Travelling	4	0	1.5	19
			Control 2	15:45		N/A	0	0	1.5	50
08/05/2024	19:22	12:51	14	13:50	1	Slow Travelling	1	0	1.5	50
			15	14:15	1	Slow Travelling	1	0	1.5	50
			16	15:20	1	Travelling	2	1	1.5	50
			17	16:10	1	Milling/Slow Travelling	5	0	1.5	50
			18	16:40	1	Slow Travelling	2	0	1.5	50
			19	17:44	1	Slow Travelling	0	1	1.5	50

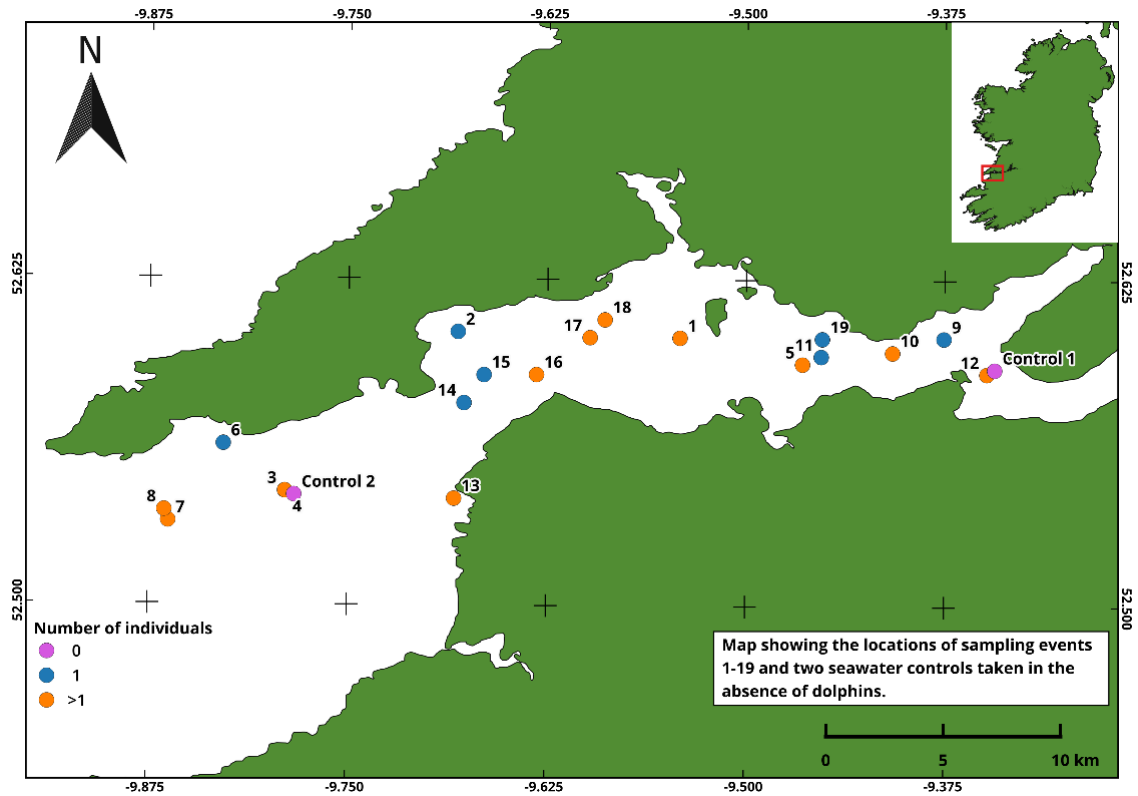


Figure 2: Map showing the locations where samples were taken and how many dolphins were sampled in the sample area.

Total eDNA quantification

To avoid repetition and bias due to some Waterra filters being extracted via two different extraction methods only eDNA quantities for Waterra filters extracted using the DNeasy PowerSoil Pro kit were used to compare the amount of eDNA obtained from Sterivex and Waterra filters. The amount of total eDNA ranged from 0.119ng/μl to 11.867ng/μl (Sterivex) and from 0.024ng/μl to 132ng/μl (Waterra). Across all sampling events Waterra yielded both the highest and the lowest concentrations of total eDNA whereas Sterivex filters extracted using the DNeasy Blood and Tissue Kit were much more consistent (Figure 3).

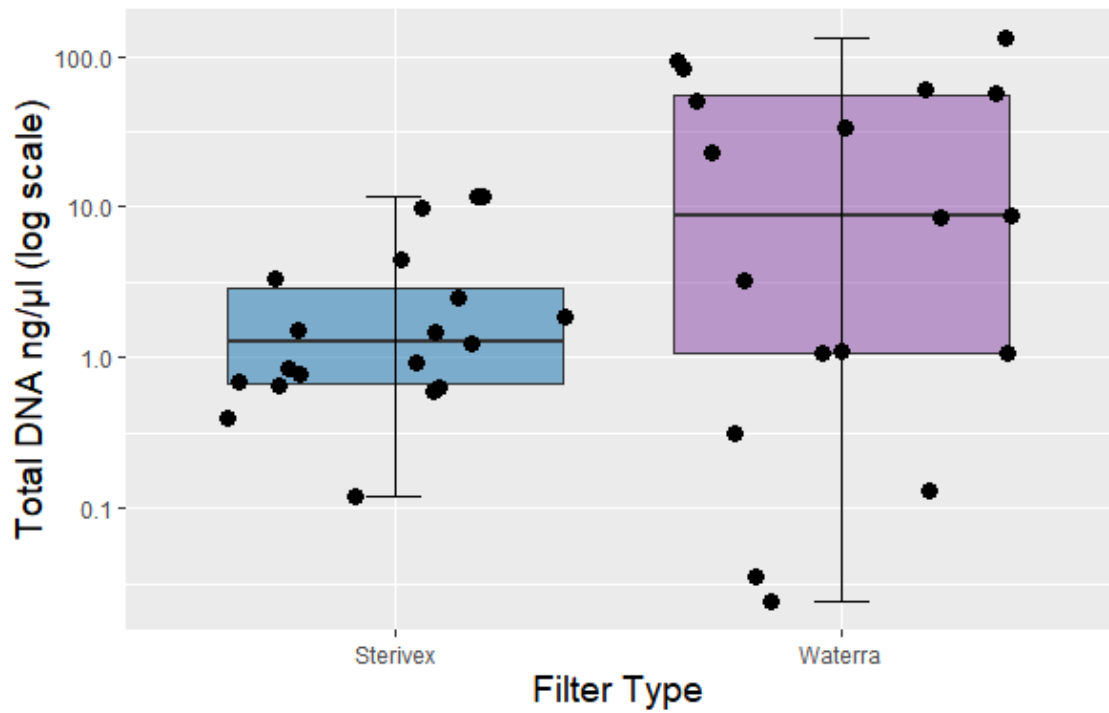


Figure 3: Boxplot showing total eDNA obtained by Sterivex and Waterra filters presented on a base 10 log scale. Each point represents one filter.

Further analysis comparing only sampling events where both Sterivex and Waterra samples were taken shows large variability in the amount of eDNA obtained from each sample (Figure 4). Despite Waterra filters yielding more eDNA in 11 out of 17 sampling events, no statistically significant difference was found between the mean total eDNA obtained using these two methods (Wilcoxon Signed Rank, p -value = 0.076).

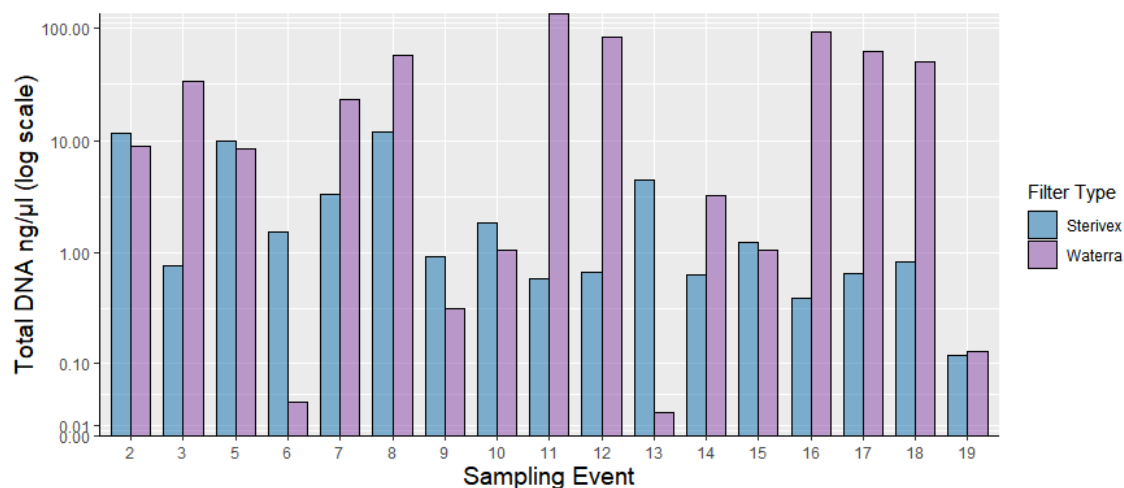


Figure 4: Bar chart showing the total eDNA obtained per sample from Sterivex and Waterra filters presented on a base 10 log scale.

The total eDNA obtained from Waterra filters was compared as a function of the extraction method, as across six samples extracts from the same filter can exhibit high levels of variation (Figure 5). A Wilcoxon Signed Rank test indicates that there is no statistically significant difference between the quantity of eDNA obtained from Waterra filters extracted using DNeasy Blood & Tissue and DNeasy PowerSoil Pro kits.

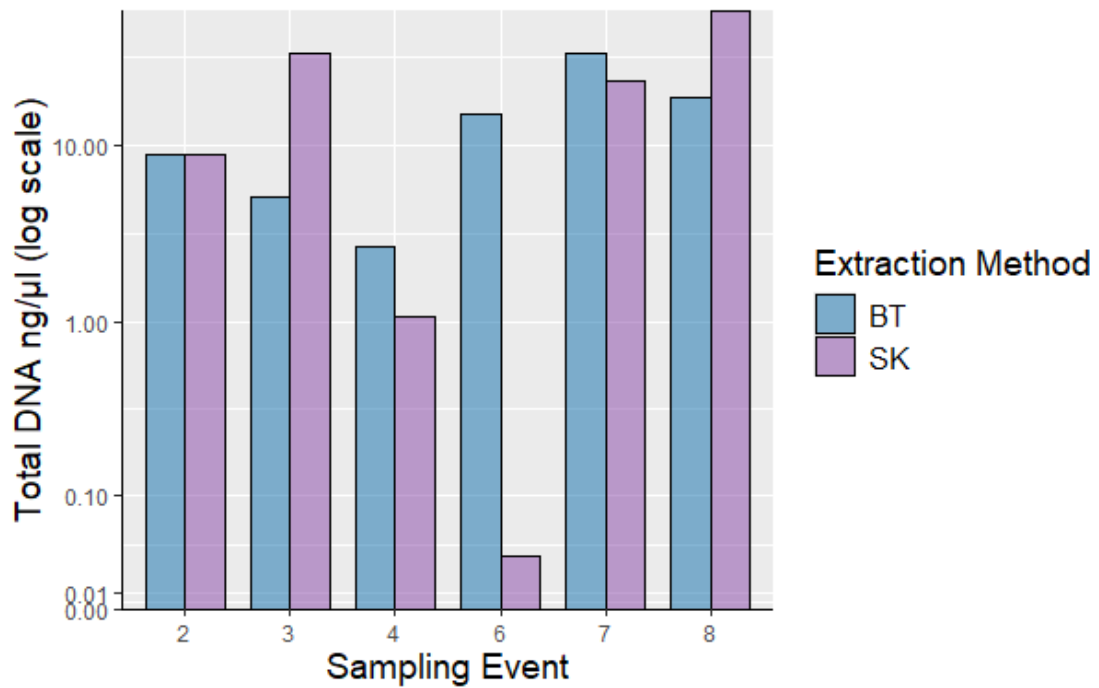


Figure 5: Bar chart showing the total eDNA yield of Waterra filters extracted using DNeasy Blood and Tissue and DNeasy PowerSoil Kits. Presented on a base 10 log scale.

Bottlenose dolphin eDNA quantification

When using conservative criteria to determine whether bottlenose dolphin eDNA was present in each sample, two Sterivex samples were deemed positive, one Waterra sample extracted using DNeasy Blood & Tissue kit was positive and eight Waterra samples extracted using DNeasy PowerSoil Pro Kit were positive (Table 2, Figure 6). No bottlenose dolphin eDNA was detected in any of the seawater or clean water controls. The amount of target eDNA extracted from the positive detections was very variable, ranging from $5.67 \times 10^{-6} \text{ ng/}\mu\text{l}$ to $7.504 \times 10^{-5} \text{ ng/}\mu\text{l}$ (Sterivex) and $0.000128 \text{ ng/}\mu\text{l}$ to $0.00152 \text{ ng/}\mu\text{l}$ (Waterra). For the one sample where target eDNA was found using both Sterivex and Waterra filters (#7) the amount of eDNA extracted was considerably lower in the Sterivex filter (Figure 6). However, on one occasion, the target eDNA was detected using the Sterivex filter but not on the Waterra filter.

Table 3: Table showing bottlenose dolphin eDNA detections in each sample using conservative criteria. Triplicate Sterivex samples were taken during one sampling event, hence the three ticks. BT: DNeasy Blood and Tissue kit; SK: DNeasy PowerSoil kit; ✓: Present; X: absent; -: N/A

Sampling Event	Sterivex	Waterra (BT)	Waterra (SK)
1	XXX	-	-
2	X	X	X
3	X	X	✓
4	X	X	X
5	X	X	X
6	X	✓	X
7	✓	X	✓
8	X	X	✓
9	X	-	X
10	X	-	X
11	X	-	✓
12	X	-	✓
13	X	-	X
14	X	-	X
15	X	-	X
16	X	-	✓
17	X	-	✓
18	X	-	✓
19	✓	-	X

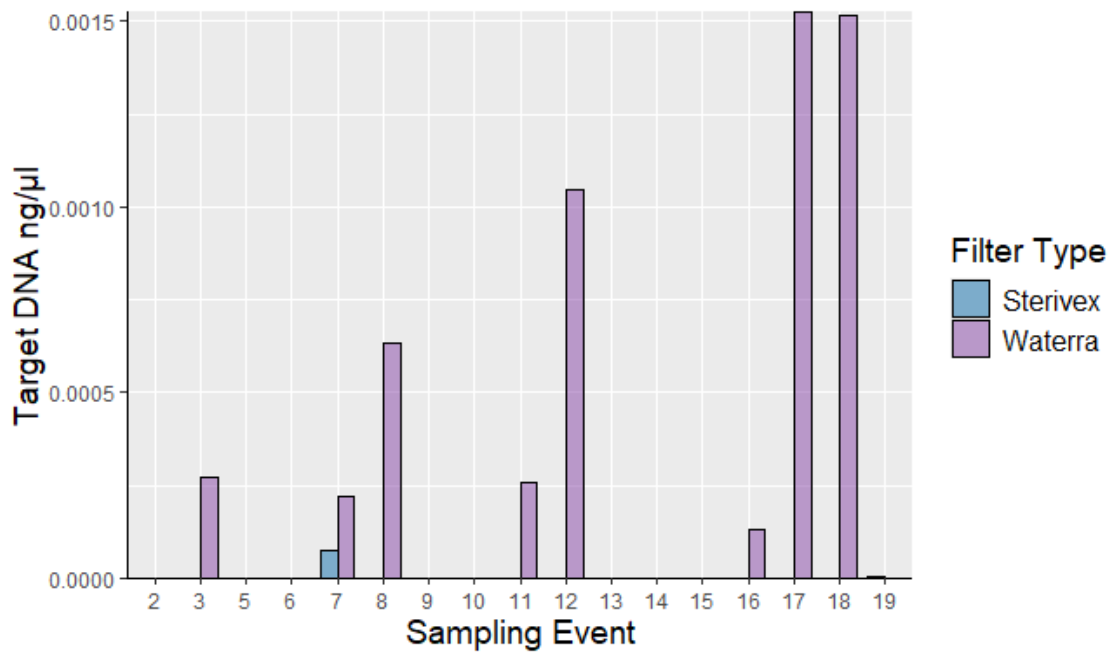


Figure 6: Bar chart showing conservative estimates of the amount of target eDNA obtained from Sterivex and Waterra filters per sampling event.

When the relaxed criteria were used to determine whether bottlenose dolphin eDNA was present in samples, positive detections were seen in eight Sterivex samples, three (DNeasy Blood and Tissue) and 11 (DNeasy PowerSoil Pro) Waterra samples (Table 3, Figure 7). The amount of target eDNA extracted from the positive detections was very variable, ranging from $3.42 \times 10^{-6} \text{ ng/}\mu\text{l}$ to $0.000167 \text{ ng/}\mu\text{l}$ (Sterivex) and $1.867 \times 10^{-5} \text{ ng/}\mu\text{l}$ to $0.00152 \text{ ng/}\mu\text{l}$. One positive detection of very low quantity ($1.42 \times 10^{-6} \text{ ng/}\mu\text{l}$) was seen in a single replicate of seawater control 1 extracted from a Waterra filter.

A Wilcoxon Signed Rank test revealed that median target eDNA quantity obtained by Waterra filters was significantly greater than that of Sterivex filters ($p\text{-value} = 0.013$). The results from the GLM found that neither the state of the tide or the group size sampled had a statistically significant effect on the quantity of target eDNA obtained in Waterra (DNeasy PowerSoil Pro) samples.

Table 4: Bottlenose dolphin eDNA detections in each sample using relaxed criteria.

Triplicate Sterivex samples were taken during one sampling event, hence the three ticks.

BT: DNeasy Blood and Tissue kit; SK: DNeasy PowerSoil kit; ✓: Present; X: absent; -:

N/A

Sampling Event	Sterivex	Waterra (BT)	Waterra (SK)
1	✓✓✓	-	-
2	✓	X	✓
3	X	X	✓
4	-	✓	✓
5	X	X	X
6	✓	✓	X
7	✓	✓	✓
8	✓	X	✓
9	X	-	X
10	X	-	X
11	X	-	✓
12	X	-	✓
13	X	-	X
14	X	-	✓
15	X	-	X
16	X	-	✓
17	✓	-	✓
18	✓	-	✓
19	✓	-	X

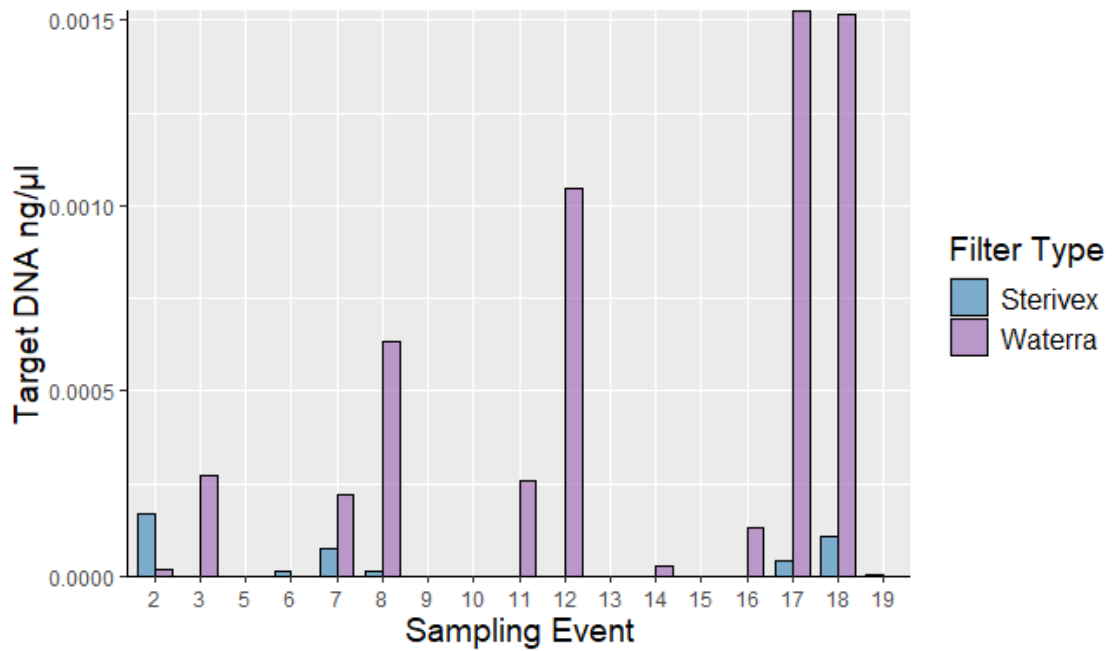


Figure 7: Bar chart showing relaxed estimates of the amount of target eDNA obtained from Sterivex and Waterra filters per sampling event.

PCR inhibition

In eDNA samples, PCR inhibition was detected in Sterivex filters on five sampling events (#3, 9, 11, 12 and 13). In contrast, no inhibition was detected in any of the Waterra extracts. However, inhibition was present in all clean water controls for both filters and the Sterivex extract for seawater control 1.

Haplotype assignment

It was possible to identify haplotypes in eight out of 19 sampling events, Ire1, 2 and 7 were detected in the samples (Table 4). Haplotypes were obtained from two Sterivex filters and seven Waterra filters (DNeasy PowerSoil Pro Kit). It was not possible to determine a haplotype for Sterivex #17 as the sequence obtained matched perfectly to both Ire2 and Ire7 due to the sequence not being long enough to contain the SNP that

differentiates the two haplotypes. Sequences obtained from sample #7 are inconsistent across filter types and sequencing primers. After maximum parsimony haplotype assignment, the sequence obtained from Sterivex #7 did not match any of the existing haplotypes, there were two mismatches with all bottlenose dolphin haplotypes in non-polymorphic sites (Mirimin *et al.*, 2011; Nykänen, Kaschner, *et al.*, 2019). When this sequence was submitted to NCBI BLAST (Altschul *et al.*, 1990) it returned only bottlenose dolphin (query cover 100%, percentage identity 99%). If the sequence for Sterivex #7 were to be cut after these mismatches it would match to Ire2 and Ire7 with insufficient length to distinguish the two. Waterra #7 when sequenced with the H00034 primer returned the Ire1 haplotype however, when sequenced with the Dlp8G primer returned the Ire2 haplotype. In total, the Ire1 haplotype appeared three times and the Ire2 haplotype appeared six times across eight sampling events.

Table 5: Bottlenose dolphin haplotypes obtained from each sampling event and the associated primer used for sequencing

Sampling Event	Filter Type	Haplotype Otained per Sequencing Primer		
		L15926	H00034	Dlp8G
2	Sterivex	Ire1		
3	Waterra (SK)			Ire1
7	Sterivex			No match
7	Waterra (SK)		Ire1	Ire2
8	Waterra (SK)		Ire2	Ire2
11	Waterra (SK)			Ire2
16	Waterra (SK)			Ire2
17	Sterivex			Ire2 or Ire7
17	Waterra (SK)			Ire2
18	Waterra (SK)			Ire2

Metabarcoding

In total, 33 fish species (including two elasmobranchs) and three fish families were identified across 10 extracts (Table 5). The most common species appearing in nine samples were the European sprat (*Sprattus sprattus*) followed by European pilchard (*Sardina pilchardus*) and conger eel (*Conger conger*) which appeared in four and three extracts, respectively. Extracts from sampling events #7 and #8 show that there is variability in the species identified between extracts from the same sampling event and even the same filter. In Waterra #7 (DNeasy Blood and Tissue) eight species were identified of which five were not identified in Waterra #7 (PowerSoil Pro Kit). Similarly, one unique species occurred in Waterra #8 (DNeasy Blood and Tissue) not present in Waterra #8 (PowerSoil Pro Kit) or Sterivex #8, and Waterra #8 (PowerSoil Pro Kit) identified two species not present in Sterivex #8 or Waterra #8 (DNeasy Blood and Tissue). Waterra #11 (PowerSoil Pro Kit) contained the most species diversity with 20 species recorded, of which 13 were unique. These 13 unique species account for 36% of fish species across all samples. In addition to fish, both human and bottlenose dolphin eDNA was present in all 10 samples.

Table 6: The fish species presence/absence identified by metabarcoding per sample and per filter

Species	Common name	Sampling Event										Instances
		2	3	5	6	7	7*	8	8	8*	11*	
<i>Sprattus sprattus</i>	European spratt											9
<i>Sardina pilchardus</i>	European pilchard											4
<i>Conger conger</i>	Conger eel											3
<i>Pollachius pollachius</i>	Atlantic pollock											2
<i>Scomber scombrus</i>	Atlantic mackerel											2
Triglinae (subfamily)	Gurnard sp.											2
<i>Labrus bergylta</i>	Ballan wrasse											2
<i>Pomatoschistus pictus</i>	Painted goby											2
<i>Oncorhynchus mykiss</i>	Rainbow trout											2
<i>Merlangius merlangus</i>	Whiting											2
<i>Symphodus melops</i>	Corkwing wrasse											2
<i>Pomatoschistus microps</i>	Common goby											2
<i>Raja clavata</i>	Thornback ray											2
<i>Scomberesox saurus</i>	Atlantic saury											1
<i>Gadus morhua</i>	Atlantic cod											1
<i>Dicentrarchus labrax</i>	European seabass											1
<i>Pholis gunnellus</i>	Rock gunnel											1
<i>Platichthys flesus</i>	European flounder											1
<i>Clupea harengus</i>	Atlantic herring											1
<i>Gasterosteus aculeatus</i>	Three-spined stickleback											1
<i>Atherina boyeri</i>	Big-scale sand smelt											1
<i>Solea solea</i>	Common sole											1
<i>Micromesistius poutassou</i>	Blue whiting											1
<i>Taurulus bubalis</i>	Longspine bullhead											1
<i>Gobius paganellus</i>	Rocky goby											1
<i>Lophius piscatorius</i>	Common monkfish											1
<i>Galeorhinus galeus</i>	School shark (Tope)											1
<i>Trisopterus luscus</i>	Pouting (poor cod)											1
<i>Anguilla anguilla</i>	Atlantic eel											1
<i>Trachurus trachurus</i>	Atlantic horse mackerel											1
<i>Syngnathus rostellatus</i>	Lesser pipefish											1
Ammodytidae (family)	Sand eel sp.											1
<i>Ciliata septentrionalis</i>	Northern rockling											1
Lotidae (family)	Rockling sp.											1
<i>Lipophrys pholis</i>	Common blenny											1
<i>Callionymus lyra</i>	Common dragonet											1

	Sterivex
	Waterra
*	Soil Kit

Discussion

eDNA as a tool for species specific detection

This study has demonstrated that it is possible to use eDNA for species-specific bottlenose dolphin qPCR detection. Using a relaxed threshold for positive detections, bottlenose dolphin eDNA was detected in 14 of 19 samples (~74%). However, when using a conservative threshold for positive detections, bottlenose dolphin eDNA was detected in nine samples (~47%). These detection rates are an improvement on the results of Greiner-Ferris (2020), the study which provided the basis for the qPCR primers used in this study. In the Greiner-Ferris (2020) study, positive detections for bottlenose dolphin were only obtained in biopsy and aquarium control samples when using the TtDloopF/TtDloopR primers. Similar to this study, Greiner-Ferris (2020) used the DNeasy Blood and Tissue kit for extraction and similar qPCR conditions. However, in this study, sample volumes were larger, 1.5L and 50L, and collected from the fluke print of dolphins. Compared to 1L collected by Greiner-Ferris (2020) within 30 seconds of dolphins surfacing. Samples collected by Greiner-Ferris (2020) were also filtered upon return to the lab using mixed cellulose ester filter paper (0.45µm) rather than onboard like in this study, therefore it is possible that eDNA may have degraded by the time it was filtered and extracted or the use of an open filter caused the eDNA to degrade (Spens *et al.*, 2017).

In contrast to other studies which sampled from the fluke prints of other cetacean species the detection rates of this study are quite low. Two similar studies by Székely *et al.* (2021) and Parsons *et al.* (2018) successfully detected bowhead whale (*Balaena mysticetus*) and harbour porpoise, respectively, in 100% of their fluke print samples. Székely *et al.* (2021) collected 1L water samples and extracted onboard using Sterivex filters, though with a smaller pore size in this study, 0.22µm opposed to 0.45µm. The extraction method used

by Székely *et al.* (2021) was a modified version of the DNeasy Blood and Tissue protocol detailed by Spens *et al.* (2017) which was optimised for Sterivex filters, this may contribute to more positive detections. Parsons *et al.* (2018) filtered between 2.3 and 3.1L of water through a nitrocellulose filter (0.45µm) and did not use a preexisting DNA extraction kit, rather a phenol-chloroform extraction protocol was used. Both of these studies also used TaqMan assays with species-specific probes for qPCR (Parsons *et al.*, 2018; Székely *et al.*, 2021), whereas this study used SYBR Green chemistry without probes. A lack of uniformity in study design and methodology makes it difficult to pinpoint what causes differences in positive detections between studies.

Variation in the success of using eDNA for species specific detection is common and there is no clear consensus on what causes this variation. A study by Pinfield *et al.* (2019) took water samples within 20m metres of killer whales yet was unable to detect killer whale eDNA in any of their samples. Conversely, another study by Baker *et al.*, (2018) also working with killer whales took samples from the wake of the whales and got positive detections in 17 of 25 encounters (68%). They also reported positive detections up to 2 hours after an encounter. The source of this variation could come from any point of the experimental methodology as there is, as yet no standard methodology.

In the field variation could be caused by environmental variables, including season, habitat, and tides. In this study no correlation was found between the direction of the tide (ebb and flow) and eDNA concentration, however this could be due to a low sample number (n = 4 ebb, 14 flow) and that samples were taken opportunistically, not according to tide. A recent study by Baetscher *et al.* (2024) investigated the effect of tides on the rate of eDNA dispersal and found that higher concentrations of eDNA were collected on flow tide and higher rates of dispersal were observed on ebb tide. Variation may also be

caused by differences in eDNA shedding rates, group size and degradation of eDNA and the target species (Andruszkiewicz Allan *et al.*, 2021; Gendron and Mesnick, 2001; Harrison *et al.*, 2019; Whitehead *et al.*, 1990). Interestingly, both Greiner-Ferris (2020) and Baker *et al.* (2018) found that there was a positive correlation between group size and eDNA concentration. However, this study found that there was no statistically significant correlation. Greiner-Ferris (2020) goes on to state that more active behaviours were observed in larger groups such as breaching, physical interaction and tail slapping, whereas the most frequently recorded behaviour in this study was slow travelling. This may suggest that behaviour rather than group size affects eDNA concentrations.

Sample collection also introduces other sources of variation, making comparisons between studies difficult, including where samples are collected i.e. fluke prints, transects, at the surface or at depth (Boldrocchi *et al.*, 2024; Pinfield *et al.*, 2019; Székely *et al.*, 2021); or how much water is collected and filtered. Generally, eDNA studies filter ~1L of water (Alter *et al.*, 2022; Baker *et al.*, 2018; Székely *et al.*, 2021; Zhang *et al.*, 2023), but this can range from 15ml to 50L depending on the type of filter used (Foote *et al.*, 2012; Juhel *et al.*, 2021).

In the lab variation can stem from DNA extraction methods or primers, probes and master mixes used for qPCR. This study used SYBR Green chemistry for qPCR whereas other studies use TaqMan chemistry with associated species-specific probes which may lead to a more sensitive assay (Parsons *et al.*, 2018; Székely *et al.*, 2021). qPCR is generally used for species specific detections however some studies use Droplet Digital PCR (ddPCR) (Baker *et al.*, 2018; Scriver *et al.*, 2024). Metabarcoding can also be applied to the study of marine mammals and can also be used to identify multiple species at once (Valsecchi *et al.*, 2020, 2021). There is scope for further work to investigate in more detail the effects

of any of these variables on the successful species-specific detection of marine mammals using eDNA.

Population level genetic information from eDNA

In this study it was possible to obtain haplotype sequences in 8 of 19 sampling events (~42%). Of these 8 sampling events, at least one of the two haplotypes expected to be present in the Shannon Estuary (Mirimin *et al.*, 2011) were identified in all sampling events. While it was not possible to differentiate Ire2 and Ire7 for Sterivex samples #7 or #17, it is likely that the correct haplotype for these samples is Ire2 because it is known to be found in the Shannon and Ire7 was only identified in 1 of 82 individuals by Mirimin *et al.* (2011). Waterra #7 (DNeasy PowerSoil Pro) returning both Ire1 and Ire2 depending on which primers were used for sequencing suggests that the individuals sampled on this occasion were a mixture of individuals with the Ire1 haplotype and individuals with the Ire2 haplotype. One of the difficulties when sampling eDNA from bottlenose dolphins is that they tend to occur in groups (average group size here was 2.5 individuals), so in cases where groups are made up of individuals of multiple haplotypes perhaps it is more appropriate to use a metabarcoding approach to discern multiple haplotypes from one sample (Parsons *et al.*, 2018).

While the use of eDNA to acquire population level genetic information from marine mammals is still in its infancy, eDNA is proving to be a very useful tool in population genetic studies. At present there are a few key aspects of eDNA sampling to consider to maximise the effectiveness of a study. Sampling in the fluke print of cetaceans yields significantly more eDNA than transect sampling (Székely *et al.*, 2021). However, fluke print sampling may not be possible due to limitations of the sampling vessel (Pinfield *et al.*, 2019) or when approach distances are limited (Baker *et al.*, 2018). In this case, as

described by Baker *et al.* (2018), likely the best methodology is to sample from areas where the target species were recently observed and if possible, track currents to minimise the effects of dispersal. Sampling this way Baker *et al.* (2018) were able to obtain haplotype sequences up to an hour after the animals were observed. When sampling smaller cetaceans such as small delphinids or phocoenids where it is difficult to accurately sample a single individual, techniques such as species specific metabarcoding can be used to identify multiple haplotypes in the same sample. As seen in this study, the amount of genetic information that can be inferred from eDNA samples may be linked to the volume of water filtered, therefore it is recommended, where possible, to take as much water as possible/logistically feasible when sampling.

The successful identification of bottlenose dolphin haplotypes in this study provides a starting point for population genetic studies of other resident dolphin populations. Only two haplotypes were present in the Shannon bottlenose dolphin population, it would be interesting to investigate how eDNA could be applied to populations with greater haplotype diversity as this has been achieved in other species (Parsons *et al.*, 2018; Székely *et al.*, 2021). If a large-scale investigation across multiple populations within European waters were to be carried out, it is important to ensure validity and comparability across research groups working in different areas. This could be achieved by conducting a “ring test” where samples are exchanged and extracted using similar methodologies, for inter-lab calibration.

While this study, among others has shown that cetacean mtDNA haplotypes can be identified (Baker *et al.*, 2018; Parsons *et al.*, 2018; Székely *et al.*, 2021), the extent of eDNA’s potential in population genetic studies is still to be discovered. The next steps for investigating the potential of eDNA is sequencing multiple mtDNA haplotype regions.

Székely *et al.* (2021) attempted to shotgun sequence the whole mitochondrial genome of bowhead whales from eDNA but could not assemble the mitogenome due to excessive amounts of non-target amplification. An alternative sequencing technique such as capture sequencing with species-specific probes could potentially allow for full mitogenomes to be sequenced (Aylward *et al.*, 2018; Reinholdt Jensen *et al.*, 2021). Additionally, the next step from identifying mtDNA is identifying nuclear DNA. Microsatellite arrays targeting multiallelic nuclear eDNA markers could be used to estimate allele frequencies in cetacean species and provide higher resolution population structure information (Andres *et al.*, 2021), although discriminating genotypes could be difficult in mixed samples. eDNA stands to be an instrumental tool in the future of cost effective and non-invasive population genetic studies.

Potential prey of Shannon bottlenose dolphins

Though metabarcoding was only done on ten extracts across seven sampling events, 36 fish species were identified, including two critically endangered species Atlantic eel (*Anguilla anguilla*) and school shark (*Galeorhinus galeus*), and one non-native species (Rainbow Trout). Previous stomach content analysis of Irish bottlenose dolphins identified that over 50% of prey species were of the Gadiformes family. The most frequently recorded species found in over 60% of stomachs were whiting (*Merlangius merlangus*), blue whiting (*Micromesistius poutassou*), pollack (*Pollachius pollachius*), saithe (*P. virens*) and haddock (*Melanogrammus aeglefinus*). Other species recorded included dogfish (*Scyliorhinus canicula*), conger eel (*Conger conger*) and horse mackerel (*Trachurus trachurus*) (Hernandez-Milian *et al.*, 2015). Of these species whiting, blue whiting, pollack, conger eel and horse mackerel were identified in this study via eDNA. However, bottlenose dolphins are reported to be generalist species and the majority of

species identified in this study could be potential prey. The generalist nature of bottlenose dolphins (Barros and Wells, 1998; Dunshea *et al.*, 2013; Hernandez-Milian *et al.*, 2015; Santos *et al.*, 2007) coupled with the high number of species identified by metabarcoding may suggest that metabarcoding alone is not the most suitable approach when investigating the diet of bottlenose dolphins.

Interestingly, salmon (*Salmo salar*) has been reported to be an important prey item for bottlenose dolphins in the Shannon Estuary (Ingram, 2000). However, salmon was not identified in any of the metabarcoding samples. This could be due to insufficient sample size or that there actually were no salmon in the area at the time of sampling. In line with this, fish stock surveys in the Shannon Estuary conducted by Inland Fisheries Ireland in September and October of 2008 and 2014 recorded only three salmon in 2008 and none in 2014 (Kelly *et al.*, 2015). However, all samples in this study used for metabarcoding were collected between the 16th of August and the 29th of November 2023 which overlaps with the ESB Fisheries Conservation Census which recorded Salmon returning to rivers upstream of the Shannon estuary from March to December 2023 with a peak of 461 individuals recorded at Parteen Weir in November (ESB, 2023). These discrepancies in salmon counts and a general shortage of literature available on salmon populations in the Shannon estuary warrant further dedicated studies.

The prey investigation in this study was very much preliminary. A dedicated metabarcoding study with samples taken both in the presence and absence of feeding bottlenose dolphins may yield better results.

Sterivex vs Waterra filters

This study compared two filter types, 0.45µl Sterivex filter capsules and 600cm² 0.45µl Waterra filter capsules. The results show that Waterra filters obtained significantly more

target eDNA. In this section the benefits and drawbacks of both filter types will be discussed (Table 8).

The Sterivex filters were able to filter between 1 and 1.5L, the Waterra filters filtered between 19 and 53L. The reason that some Sterivex filtered only 1L was excessive clogging prematurely, likely due to higher levels of sediment in the water. However, while premature filter clogging was an issue in a few Waterra filters the more common reason for lower samples volumes was failure to collect a full 50L of water. For logistical purposes, if it was not possible to collect 50L of water from a single fluke print of a bottlenose dolphin, multiple fluke prints had to be sampled. This made it very easy to lose track of the dolphins before 50L of water was collected especially when they were travelling quickly or generally uninterested in the RIB. Additionally, the only additional equipment required when using Sterivex filters are disposable 50ml syringes whereas a pump is required to pull water through Waterra filters. Waterra filters also require a larger container to hold 50L of water, these pieces of additional equipment result in higher costs and larger space requirements onboard the sampling vessel.

In terms of extraction protocols, Sterivex filters can be extracted very quickly and easily, the lysate can be easily removed from the filter using just a disposable syringe and the eDNA extraction can be done by using a preexisting DNA extraction kit. The process of isolating eDNA from Waterra filters is more complicated. The precipitation protocol used in this study is time consuming as it includes freezing overnight and contains more steps before the lysates are ready to be extracted using a preexisting DNA extraction kit. A recent study using Waterra filters used a similar but more streamlined precipitation method which may be a quicker alternative as it does not include an overnight step (Roblet *et al.*, 2024).

Waterra filters captured significantly more target eDNA and had a much higher maximum eDNA quantity for total and target eDNA than Sterivex. It has been suggested that greater water volumes obtain more eDNA (Bessey *et al.*, 2020). However, there was a large variation in eDNA quantities obtained by Waterra filters. A possible explanation for the variations in quantity from Waterra filters may be due to the extraction method rather than the filter itself. The PowerSoil Pro kit has been shown to be capable of obtaining high amounts of eDNA, but the inhibitor removal steps may cause a reduced eDNA yield (Feng *et al.*, 2023; Galla *et al.*, 2024; Ushio, 2019). This is also evidenced in this study, albeit in only a few samples that were extracted from Waterra filters using both the DNeasy PowerSoil Pro kit and the DNeasy Blood and Tissue kit that the eDNA quantities obtained from the DNeasy Blood and tissue kit were more consistent. So, perhaps this variation in the amount of eDNA obtained by Waterra filters can be remedied by optimising the extraction method used (Sellers *et al.*, 2018).

Whether conservative or relaxed criteria were used to determine what was classed as a positive detection, Waterra filters out performed Sterivex. This is likely due to the larger amount of water filtered by Waterra filters increasing the likelihood of capturing target eDNA (Bessey *et al.*, 2020). Likewise, more haplotype sequences were obtained from Waterra filters, suggesting that possibly the quality of eDNA obtained by Waterra filters is higher than that of Sterivex. With this in mind it would seem that Waterra filters are better suited for eDNA studies especially when trying to obtain population level genetic information. However, other studies have also obtained similar if not better population level genetic information by using smaller volumes of water (Parsons *et al.*, 2018) and using Sterivex filters (Székely *et al.*, 2021).

Inhibition was found to be present in five of the Sterivex eDNA samples and one seawater control, however there was no evidence of inhibition in any of the Waterra eDNA samples or seawater controls. This could be due to the extraction method used for the Waterra filters as the DNeasy PowerSoil Pro kit contains an inhibitor removal step whereas the DNeasy Blood and Tissue kit used for Sterivex samples does not. Contrary to this, inhibition was detected in Sterivex sample #3 and the associated Waterra sample which was extracted with both the DNeasy Blood and Tissue kit and DNeasy PowerSoil Pro kit and showed no sign of inhibition regardless of the extraction method used. It is possible that as that the higher volume of water being filtered through the Waterra filters resulted in any PCR inhibitors that may have been present being diluted to a level where they did not affect eDNA detection (Peixoto *et al.*, 2020; Wang *et al.*, 2017). Interestingly, inhibition was detected in all clean water controls across both filters, all of which were extracted using the DNeasy Blood and Tissue kit. Previous studies have reported that drinking water can have sufficient levels of PCR inhibitors such as humic acid, chlorine and iron and cause inhibition in qPCR reactions (Dalecka and Mezule, 2018; Schrader *et al.*, 2012). This, coupled with presumably no bottlenose dolphin DNA for the qPCR reaction to amplify, could cause the reaction to be deemed inhibited. Treatment of all samples that were inhibited with inhibitor removal kits would be beneficial to determine more accurately whether eDNA is present or not in these samples (Capo *et al.*, 2020).

For this study, Waterra filters seem to be better suited than Sterivex. However, it is worth considering the aims and logistics of a project and choosing a filter type based on the specific needs. In order to determine more accurately if there is an objectively superior filter type to use for eDNA sampling an increased number of paired samples would be required. It would also be beneficial to investigate if the volume of water filtered through

a filter affects the amount of eDNA obtained. This would then allow for the determination of whether eDNA content increases linearly with water volume or if other variables become the limiting factor of eDNA capture. While in this study 50L of water was filtered using Waterra filters, if the optimum volume of water were less it would reduce the time and effort involved in collecting large volumes of water.

Table 7: A summary of the pros and cons of Sterivex and Waterra filters

	Sterivex	Waterra
Pros	• Small • Easy to use • Quick and easy to extract • Little additional sampling equipment required • More consistent eDNA quantities	• Can filter large volumes of water • Potential to acquire large quantities of eDNA • More positive detections • More haplotype sequences
Cons	• Generally lower eDNA quantities • Low sample volumes • Less positive detections • Less haplotype sequences	• Highly variable eDNA quantities • Bulky – requires a lot of storage space • Requires additional sampling equipment i.e. pump, tubing, collection barrel • Complex and lengthy extraction process • Extraction more susceptible to human error

Conclusion

eDNA is proving to be a viable method to both detect bottlenose dolphins as well as identifying mtDNA haplotypes. Both Sterivex and Waterra filters are promising options for sample acquisition, although for population genetic studies it seems that the high-volume capacity of Waterra filters makes them more suitable. Further work needs to be carried out to optimise eDNA sampling methods and lab protocols.

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References

- Adams, C.I.M., Knapp, M., Gemmell, N.J., Jeunen, G.J., Bunce, M., Lamare, M.D. and Taylor, H.R. (2019), “Beyond biodiversity: Can environmental DNA (eDNA) cut it as a population genetics tool?”, *Genes*, MDPI AG, Vol. 10 No. 3, doi: 10.3390/GENES10030192.
- Alter, S.E., King, C.D., Chou, E., Chin, S.C., Rekdahl, M. and Rosenbaum, H.C. (2022), “Using Environmental DNA to Detect Whales and Dolphins in the New York Bight”, *Frontiers in Conservation Science*, Frontiers Media SA, Vol. 3, doi: 10.3389/fcosc.2022.820377.
- Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990), “Basic local alignment search tool”, *Journal of Molecular Biology*, J Mol Biol, Vol. 215 No. 3, pp. 403–410, doi: 10.1016/S0022-2836(05)80360-2.
- Andres, K.J., Sethi, S.A., Lodge, D.M. and Andrés, J. (2021), “Nuclear eDNA estimates population allele frequencies and abundance in experimental mesocosms and field samples”, *Molecular Ecology*, John Wiley & Sons, Ltd, Vol. 30 No. 3, pp. 685–697, doi: 10.1111/MEC.15765.
- Andruszkiewicz Allan, E., Zhang, W.G., C. Lavery, A. and F. Govindarajan, A. (2021), “Environmental DNA shedding and decay rates from diverse animal forms and thermal regimes”, *Environmental DNA*, John Wiley & Sons, Ltd, Vol. 3 No. 2, pp. 492–514, doi: 10.1002/EDN3.141.
- Aylward, M.L., Sullivan, A.P., Perry, G.H., Johnson, S.E. and Louis, E.E. (2018), “An environmental DNA sampling method for aye-ayes from their feeding traces”, *Ecology and Evolution*, John Wiley & Sons, Ltd, Vol. 8 No. 18, pp. 9229–9240, doi: 10.1002/ECE3.4341.
- Baetscher, D.S., Pochardt, M.R., Barry, P.D., Larson, W.A., (2024), “Tide impacts the dispersion of eDNA from nearshore net pens in a dynamic high-latitude marine environment”, *Environmental DNA*, John Wiley & Sons, Ltd, Vol. 6 No. 2, p. e533, doi: 10.1002/EDN3.533.
- Baker, C.S., Flórez-González, L., Abernethy, B., Rosenbaum, H.C., Slade, R.W., Capella, J. and Bannister, J.L. (1998), “Mitochondrial DNA variation and maternal gene flow among humpback whales of the southern hemisphere”, *Marine Mammal Science*, John Wiley & Sons, Ltd, Vol. 14 No. 4, pp. 721–737, doi: 10.1111/J.1748-7692.1998.TB00758.X.
- Baker, C.S., Steel, D., Nieukirk, S. and Klinck, H. (2018), “Environmental DNA (eDNA) from the wake of the whales: Droplet digital PCR for detection and species identification”, *Frontiers in Marine Science*, Frontiers Media S. A, Vol. 5 No. APR, doi: 10.3389/fmars.2018.00133.
- Barker, J. and Berrow, S. (2016), “Temporal and spatial variation in group size of bottlenose dolphins (*Tursiops truncatus*) in the Shannon Estuary, Ireland”, *Biology*

and Environment: Proceedings of the Royal Irish Academy, Project Muse, Vol. 116B No. 1, pp. 63–70, doi: 10.1353/bae.2016.0023.

- Barros, N.B. and Wells, R.S. (1998), “Prey and Feeding Patterns of Resident Bottlenose Dolphins (*Tursiops truncatus*) in Sarasota Bay, Florida”, *Journal of Mammalogy*, Oxford Academic, Vol. 79 No. 3, pp. 1045–1059, doi: 10.2307/1383114.
- Beng, K.C. and Corlett, R.T. (2020), “Applications of environmental DNA (eDNA) in ecology and conservation: opportunities, challenges and prospects”, *Biodiversity and Conservation*, Springer, Vol. 29 No. 7, pp. 2089–2121, doi: 10.1007/S10531-020-01980-0/FIGURES/2.
- Berger, C.S., Bougas, B., Turgeon, S., Ferchiou, S., Ménard, N. and Bernatchez, L. (2020), “Groundtruthing of pelagic forage fish detected by hydroacoustics in a whale feeding area using environmental DNA”, *Environmental DNA*, Blackwell Publishing Inc., Vol. 2 No. 4, pp. 477–492, doi: 10.1002/edn3.73.
- Bessey, C., Jarman, S.N., Berry, O., Olsen, Y.S., Bunce, M., Simpson, T., Power, M., *et al.* (2020), “Maximizing fish detection with eDNA metabarcoding”, *Environmental DNA*, John Wiley & Sons, Ltd, Vol. 2 No. 4, pp. 493–504, doi: 10.1002/EDN3.74.
- Bohmann, K., Evans, A., Gilbert, M.T.P., Carvalho, G.R., Creer, S., Knapp, M., Yu, D.W., *et al.* (2014), “Environmental DNA for wildlife biology and biodiversity monitoring”, *Trends in Ecology & Evolution*, Elsevier Current Trends, Vol. 29 No. 6, pp. 358–367, doi: 10.1016/J.TREE.2014.04.003.
- Boldrocchi, G., Conte, L., Galli, P., Bettinetti, R. and Valsecchi, E. (2024), “Cuvier’s beaked whale (*Ziphius cavirostris*) detection through surface-sourced eDNA: A promising approach for monitoring deep-diving cetaceans”, *Ecological Indicators*, Elsevier, Vol. 161, p. 111966, doi: 10.1016/J.ECOLIND.2024.111966.
- Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A. and Holmes, S.P. (2016), “DADA2: High-resolution sample inference from Illumina amplicon data”, *Nature Methods* 2016 13:7, Nature Publishing Group, Vol. 13 No. 7, pp. 581–583, doi: 10.1038/nmeth.3869.
- Capo, E., Spong, G., Königsson, H. and Byström, P. (2020). Effects of filtration methods and water volume on the quantification of brown trout (*Salmo trutta*) and Arctic char (*Salvelinus alpinus*) eDNA concentrations via droplet digital PCR. *Environmental DNA*, 2(2), pp.152–160.
- Carmen, M., Berrow, S.D. and O’Brien, J.M. (2021), “Foraging behavior of bottlenose dolphins in the Shannon estuary, Ireland as determined through static acoustic monitoring”, *Journal of Marine Science and Engineering*, MDPI AG, Vol. 9 No. 3, pp. 1–17, doi: 10.3390/jmse9030275.
- Closek, C.J., Santora, J.A., Starks, H.A., Schroeder, I.D., Andruszkiewicz, E.A., Sakuma, K.M., Bograd, S.J., *et al.* (2019), “Marine Vertebrate Biodiversity and Distribution Within the Central California Current Using Environmental DNA (eDNA)

- Metabarcoding and Ecosystem Surveys”, *Frontiers in Marine Science*, Frontiers Media S.A., Vol. 6, doi: 10.3389/fmars.2019.00732.
- Dalebout, M.L., Robertson, K.M., Frantzis, A., Engelhaupt, D., Mignucci-Giannoni, A.A., Rosario-Delestre, R.J. and Baker, C.S. (2005), “Worldwide structure of mtDNA diversity among Cuvier’s beaked whales (*Ziphius cavirostris*): implications for threatened populations”, *Molecular Ecology*, John Wiley & Sons, Ltd, Vol. 14 No. 11, pp. 3353–3371, doi: 10.1111/J.1365-294X.2005.02676.X.
- Dalecka, B., Mezule, L. (2018), “Study of potential PCR inhibitors in drinking water for *Escherichia coli* identification”, *Agronomy Research*, Vol. 16(S2), 1351-1359.
- Dunshea, G., Barros, N.B., McCabe, E.J.B., Gales, N.J., Hindell, M.A., Jarman, S.N. and Wells, R.S. (2013), “Stranded dolphin stomach contents represent the free-ranging population’s diet”, *Biology Letters*, The Royal Society, Vol. 9 No. 3, doi: 10.1098/RSBL.2012.1036.
- Englund, A., Ingram, S. and Rogan, E. (2008), An Updated Population Status Report for Bottlenose Dolphins Using the Lower River Shannon SAC in 2008. National Parks and Wildlife Services
- ESB. (2008), “ESB Fisheries Conservation Annual Report: A report on ESB Fisheries Conservation activities to the Department of Communications, Energy & Natural Resources.” esb.ie.
- Feng, S., DeKlotz, M. and Taş, N. (2023), “Comparison of three DNA extraction methods for recovery of microbial DNA from Arctic permafrost”, *MicroPublication Biology*, California Institute of Technology, Vol. 2023, doi: 10.17912/MICROPUB.BIOLOGY.000834.
- Ficetola, G.F., Miaud, C., Pompanon, F. and Taberlet, P. (2008), “Species detection using environmental DNA from water samples”, *Biology Letters*, Biol Lett, Vol. 4 No. 4, pp. 423–425, doi: 10.1098/RSBL.2008.0118.
- Foote, A.D., Thomsen, P.F., Sveegaard, S., Wahlberg, M., Kielgast, J., Kyhn, L.A., Salling, A.B., *et al.* (2012), “Investigating the Potential Use of Environmental DNA (eDNA) for Genetic Monitoring of Marine Mammals”, *PLoS ONE*, Vol. 7 No. 8, doi: 10.1371/journal.pone.0041781.
- Galla, G., Praeg, N., Rzehak, T., Sprecher, E., Colla, F., Seeber, J., Illmer, P., *et al.* (2024), “Comparison of DNA extraction methods on different sample matrices within the same terrestrial ecosystem”, *Scientific Reports*, Nature Research, Vol. 14 No. 1, doi: 10.1038/s41598-024-59086-4.
- Gendron, D. and Mesnick, S.L. (2001), “Sloughed skin: a method for the systematic collection of tissue samples from Baja California blue whales”, *IWC Journal of Cetacean Research and Management*, Vol. 3 No. 1, pp. 77–19.

- Greiner-Ferris, K. (2020), Testing the Potential of Environmental DNA: Genetic Monitoring of Estuarine and Coastal Bottlenose Dolphin (*Tursiops truncatus*) Stocks in South Carolina (Masters Thesis). Coastal Carolina University
- Haderlé, R., Valentini, A., Ung, V. and Jung, J.-L. (2023), “Improving Marine Megafauna Knowledge with eDNA: A French Story”, *Biodiversity Information Science and Standards*, Vol. 7, doi: 10.3897/biss.7.111435.
- Harrison, J.B., Sunday, J.M. and Rogers, S.M. (2019), “Predicting the fate of eDNA in the environment and implications for studying biodiversity”, *Proceedings of the Royal Society B*, The Royal Society, Vol. 286 No. 1915, doi: 10.1098/RSPB.2019.1409.
- Hartman, L.J., Coyne, S.R. and Norwood, D.A. (2005), “Development of a novel internal positive control for Taqman® based assays”, *Molecular and Cellular Probes*, Academic Press, Vol. 19 No. 1, pp. 51–59, doi: 10.1016/J.MCP.2004.07.006.
- Hernandez-Milian, G., Berrow, S., Santos, M.B., Reid, D. and Rogan, E. (2015), “Insights into the Trophic Ecology of Bottlenose Dolphins (*Tursiops truncatus*) in Irish Waters”, *Aquatic Mammals*, European Association for Aquatic Mammals, Vol. 41 No. 2, pp. 226–239, doi: 10.1578/AM.41.2.2015.226.
- Ingram, S. (2000), The Ecology and Conservation of Bottle- Nose Dolphins in the Shannon Estuary, Ireland (Doctoral Dissertation)., University College Cork, Cork, Ireland.
- Ingram, S.N. and Rogan, E. (2002), “Identifying critical areas and habitat preferences of bottlenose dolphins *Tursiops truncatus*”, *Marine Ecology Progress Series*, Vol. 244, pp. 247–255, doi: 10.2307/24866379.
- Juhel, J.B., Marques, V., Polanco Fernández, A., Borrero-Pérez, G.H., Mutis Martinezguerra, M., Valentini, A., Dejean, T.. (2021), “Detection of the elusive Dwarf sperm whale (*Kogia sima*) using environmental DNA at Malpelo island (Eastern Pacific, Colombia)”, *Ecology and Evolution*, John Wiley and Sons Ltd, Vol. 11 No. 7, pp. 2956–2962, doi: 10.1002/ece3.7057.
- Kavlick, M.F. (2018), “Development of a universal internal positive control”, *BioTechniques*, Future Science Ltd, Vol. 65 No. 5, pp. 275–280, doi: 10.2144/BTN-2018-0034.
- Kelly, F.L., Connor, L., Matson, R., Coyne, J., Feeney, R., Morrissey, E. and Rocks, K. (2015) “Water Framework Directive Fish Stock Survey of Transitional Waters in the Shannon International River Basin District – Shannon Estuary, Fergus Estuary and Limerick Docks 2014.” Inland Fisheries Ireland, 3044 Lake Drive, Citywest Business Campus, Dublin 24, Ireland.
- Krützen, M., Barré, L.M., Möller, L.M., Heithaus, M.R., Simms, C. and Sherwin, W.B. (2002), “A biopsy system for small cetaceans: Darting success and wound healing

- in *Tursiops spp.*”, *Marine Mammal Science*, John Wiley & Sons, Ltd, Vol. 18 No. 4, pp. 863–878, doi: 10.1111/J.1748-7692.2002.TB01078.X.
- Louis M., Viricel A., Lucas T., Peltier H., Alfonsi E., Berrow S., Brownlow A., Covelo P., Dabin W., Deaville R., de Stephanis R., Gally F., Gauffier P., Penrose R., Silva M., Guinet C., Simon-Bouhet B. (2014), “Habitat-driven population structure of bottlenose dolphins, *Tursiops truncatus*, in the North-East Atlantic”, *Molecular Ecology*, Vol. 23, pp. 857–874, doi: 10.1111/mec.12653.
- Lynggaard, C., Bertelsen, M.F., Jensen, C. V., Johnson, M.S., Frøslev, T.G., Olsen, M.T. and Bohmann, K. (2022), “Airborne environmental DNA for terrestrial vertebrate community monitoring”, *Current Biology*, Cell Press, Vol. 32 No. 3, pp. 701-707.e5, doi: 10.1016/J.CUB.2021.12.014.
- Meschersky, I.G., Kholodova, M. V. and Zvychnaynaya, E.Y. (2008), “Molecular genetic study of the beluga (*Delphinapterus leucas*: Cetacea, Monodontidae) summering in the southern Sea of Okhotsk as compared to North American populations”, *Russian Journal of Genetics*, Vol. 44 No. 9, pp. 1105–1110, doi: 10.1134/S1022795408090147.
- Mirimin, L., Miller, R., Dillane, E., Berrow, S.D., Ingram, S., Cross, T.F. and Rogan, E. (2011), “Fine-scale population genetic structuring of bottlenose dolphins in Irish coastal waters”, *Animal Conservation*, Vol. 14 No. 4, pp. 342–353, doi: 10.1111/j.1469-1795.2010.00432.x.
- Miya, M., Sato, Y., Fukunaga, T., Sado, T., Poulsen, J.Y., Sato, K., Minamoto, T., *et al.* (2015), “MiFish, a set of universal PCR primers for metabarcoding environmental DNA from fishes: detection of more than 230 subtropical marine species”, *Royal Society Open Science*, The Royal Society Publishing, Vol. 2 No. 7, doi: 10.1098/RSOS.150088.
- Montagu, G. (1821). Description of a species of *Delphinus*, which appears to be new. *Memoirs of the Wernerian Natural History Society*. 3: 75-82
- Nykänen, M., Dillane, E., Englund, A., Foote, A.D., Ingram, S.N., Louis, M., Mirimin, L., *et al.* (2018), “Quantifying dispersal between marine protected areas by a highly mobile species, the bottlenose dolphin, *Tursiops truncatus*”, *Ecology and Evolution*, John Wiley & Sons, Ltd, Vol. 8 No. 18, pp. 9241–9258, doi: 10.1002/ECE3.4343.
- Nykänen, M., Kaschner, K., Dabin, W., Brownlow, A., Davison, N.J., Deaville, R., Garilao, C., *et al.* (2019), “Postglacial Colonization of Northern Coastal Habitat by Bottlenose Dolphins: A Marine Leading-Edge Expansion?”, *Journal of Heredity*, Oxford University Press, Vol. 110 No. 6, pp. 662–674, doi: 10.1093/JHERED/ESZ039.
- Nykänen, M., Louis, M., Dillane, E., Alfonsi, E., Berrow, S., O’Brien, J., Brownlow, A., *et al.* (2019), “Fine-scale population structure and connectivity of bottlenose dolphins, *Tursiops truncatus*, in European waters and implications for conservation”,

- Aquatic Conservation: Marine and Freshwater Ecosystems*, John Wiley and Sons Ltd, Vol. 29 No. S1, pp. 197–211, doi: 10.1002/aqc.3139.
- O'Brien, J.M., Beck, S., Berrow, S.D., André, M., van der Schaar, M., O'Connor, I. and McKeown, E.P. (2016), “The Use of Deep Water Berths and the Effect of Noise on Bottlenose Dolphins in the Shannon Estuary cSAC”, *Advances in Experimental Medicine and Biology*, Adv Exp Med Biol, Vol. 875, pp. 775–783, doi: 10.1007/978-1-4939-2981-8_95.
- O'Mahony, É.N., Sremba, A.L., Keen, E.M., Robinson, N., Dundas, A., Steel, D., Wray, J., *et al.* (2024), “Collecting baleen whale blow samples by drone: A minimally intrusive tool for conservation genetics”, *Molecular Ecology Resources*, John Wiley & Sons, Ltd, p. e13957, doi: 10.1111/1755-0998.13957.
- Palsbøll, P.J., Bérubé, M. and Allendorf, F.W. (2007), “Identification of management units using population genetic data”, *Trends in Ecology & Evolution*, Elsevier Current Trends, Vol. 22 No. 1, pp. 11–16, doi: 10.1016/J.TREE.2006.09.003.
- Palsbøll, P.J., Zachariah Peery, M., Olsen, M.T., Beissinger, S.R. and Bérubé, M. (2013), “Inferring recent historic abundance from current genetic diversity”, *Molecular Ecology*, John Wiley & Sons, Ltd, Vol. 22 No. 1, pp. 22–40, doi: 10.1111/MEC.12094.
- Parsons, K.M., Everett, M., Dahlheim, M. and Park, L. (2018), “Water, water everywhere: Environmental DNA can unlock population structure in elusive marine species”, *Royal Society Open Science*, Royal Society Publishing, Vol. 5 No. 8, doi: 10.1098/RSOS.180537.
- Peixoto, S., Chaves, C., Velo-Antón, G., Beja, P. and Egeter, B., 2021. “Species detection from aquatic eDNA: Assessing the importance of capture methods.”, *Environmental DNA*, Vol. 3(2), pp.435-448.
- Pinfield, R., Dillane, E., Runge, A.K.W., Evans, A., Mirimin, L., Niemann, J., Reed, T.E., *et al.* (2019), “False-negative detections from environmental DNA collected in the presence of large numbers of killer whales (*Orcinus orca*)”, *Environmental DNA*, Blackwell Publishing Inc., Vol. 1 No. 4, pp. 316–328, doi: 10.1002/edn3.32.
- Pont, D., Rocle, M., Valentini, A., Civade, R., Jean, P., Maire, A., Roset, N., *et al.* (2018), “Environmental DNA reveals quantitative patterns of fish biodiversity in large rivers despite its downstream transportation”, *Scientific Reports 2018 8:1*, Nature Publishing Group, Vol. 8 No. 1, pp. 1–13, doi: 10.1038/s41598-018-28424-8.
- R Core Team. (2024), “A Language and Environment for Statistical Computing”, R Foundation for Statistical Computing, Vienna, Austria.
- Reinholdt Jensen, M., Eva, |, Sigsgaard, E., Liu, S., Manica, A., Steffen, |, Bach, S., *et al.* (2021), “Genome-scale target capture of mitochondrial and nuclear environmental DNA from water samples”, *Molecular Ecology Resources*, John Wiley & Sons, Ltd, Vol. 21 No. 3, pp. 690–702, doi: 10.1111/1755-0998.13293.

- Roblet, S., Priouzeau, F., Gambini, G., Dérjard, B. and Sabourault, C. (2024), “Primer set evaluation and sampling method assessment for the monitoring of fish communities in the North-western part of the Mediterranean Sea through eDNA metabarcoding”, *Environmental DNA*, John Wiley & Sons, Ltd, Vol. 6 No. 3, p. e554, doi: 10.1002/EDN3.554.
- Rogan, E., Garagouni, M., Nykänen, M., Whitaker, A., Ingram, S., Rogan, E., (2018), Bottlenose Dolphin Survey in the Lower River Shannon SAC 2018. National Parks and Wildlife Services
- Rosel, R.E., Dizon, A.E., Heyning, J.E. and Rose, R.E. (1994), Genetic Analysis of Sympatric Morphotypes of Common Dolphins (Genus *Delphinus*)., *Marine Biology*, Vol. 199, pp. 159-167
- Santos, M.B., Fernández, R., López, A., Martínez, J.A. and Pierce, G.J. (2007), “Variability in the diet of bottlenose dolphin, *Tursiops truncatus*, in Galician waters, north-western Spain, 1990–2005”, *Journal of the Marine Biological Association of the United Kingdom*, Cambridge University Press, Vol. 87 No. 1, pp. 231–241, doi: 10.1017/S0025315407055233.
- Sayers, E.W., Bolton, E.E., Brister, J.R., Canese, K., Chan, J., Comeau, D.C., Connor, R., *et al.* (2022), “Database resources of the national center for biotechnology information”, *Nucleic Acids Research*, Nucleic Acids Res, Vol. 50 No. D1, pp. D20–D26, doi: 10.1093/NAR/GKAB1112.
- Schrader, C., Schielke, A., Ellerbroek, L. and Johne, R. (2012). “PCR inhibitors - occurrence, properties and removal.” *Journal of Applied Microbiology*, Vol. 113(5), pp.1014–1026.
- Scriver, M., von Ammon, U., Youngbull, C., Pochon, X., Stanton, J.-A.L., Gemmell, N.J. and Zaiko, A. (2024), “Drop it all: extraction-free detection of targeted marine species through optimized direct droplet digital PCR”, *PeerJ*, Vol. 12, p. e16969, doi: 10.7717/peerj.16969.
- Sellers, G.S., Di Muri, C., Gómez, A. and Hänfling, B. (2018), “Mu-DNA: A modular universal DNA extraction method adaptable for a wide range of sample types”, *Metabarcoding and Metagenomics*, Pensoft Publishers, Vol. 2, doi: 10.3897/mbmg.2.24556.
- Sepulveda, A.J., Schabacker, J., Smith, S., Al-Chokhachy, R., Luikart, G. and Amish, S.J. (2019), “Improved detection of rare, endangered and invasive trout in using a new large-volume sampling method for eDNA capture”, *Environmental DNA*, John Wiley & Sons, Ltd, Vol. 1 No. 3, pp. 227–237, doi: 10.1002/EDN3.23.
- SIFP. (2013), Strategic Integrated Framework Plan (SIFP) for the Shannon Estuary.
- Sigsgaard, E.E., Jensen, M.R., Winkelmann, I.E., Møller, P.R., Hansen, M.M. and Thomsen, P.F. (2020), “Population-level inferences from environmental DNA—

- Current status and future perspectives”, *Evolutionary Applications*, Wiley-Blackwell, Vol. 13 No. 2, pp. 245–262, doi: 10.1111/EVA.12882.
- Spens, J., Evans, A.R., Halfmaerten, D., Knudsen, S.W., Sengupta, M.E., Mak, S.S.T., Sigsgaard, E.E., *et al.* (2017), “Comparison of capture and storage methods for aqueous microbial eDNA using an optimized extraction protocol: advantage of enclosed filter”, *Methods in Ecology and Evolution*, John Wiley & Sons, Ltd, Vol. 8 No. 5, pp. 635–645, doi: 10.1111/2041-210X.12683.
- Steinmetz, K., Murphy, S., Cadhla, O., Carroll, E.L., Onoufriou, A.B., Russell, D.J.F., Cronin, M., *et al.* (2023), “Population structure and genetic connectivity reveals distinctiveness of Irish harbour seals (*Phoca vitulina*) and implications for conservation management”, *Aquatic Conservation: Marine and Freshwater Ecosystems*, John Wiley & Sons, Ltd, Vol. 33 No. 2, pp. 160–178, doi: 10.1002/AQC.3909.
- Stoeckle, M.Y., Mishu, M. Das and Charlop-Powers, Z. (2018), “GoFish: A versatile nested PCR strategy for environmental DNA assays for marine vertebrates”, *PLOS ONE*, Public Library of Science, Vol. 13 No. 12, p. e0198717, doi: 10.1371/JOURNAL.PONE.0198717.
- Székely, D., Cammen, K.M. and Tange Olsen, M. (2022), “Needles in an ocean haystack: using environmental DNA to study marine mammals in the North Atlantic”, *NAMMCO Scientific Publications*, UiT The Arctic University of Norway, Vol. 12, doi: 10.7557/3.6482.
- Székely, D., Corfixen, N.L., Mørch, L.L., Knudsen, S.W., McCarthy, M.L., Teilmann, J., Heide-Jørgensen, M.P., *et al.* (2021), “Environmental DNA captures the genetic diversity of bowhead whales (*Balaena mysticetus*) in West Greenland”, *Environmental DNA*, John Wiley & Sons, Ltd, Vol. 3 No. 1, pp. 248–260, doi: 10.1002/EDN3.176.
- Tamura, K., Stecher, G. and Kumar, S. (2021), “MEGA11: Molecular Evolutionary Genetics Analysis Version 11”, *Molecular Biology and Evolution*, Oxford Academic, Vol. 38 No. 7, pp. 3022–3027, doi: 10.1093/MOLBEV/MSAB120.
- Thomas, A.C., Tank, S., Nguyen, P.L., Ponce, J., Sinnesael, M. and Goldberg, C.S. (2020), “A system for rapid eDNA detection of aquatic invasive species”, *Environmental DNA*, John Wiley & Sons, Ltd, Vol. 2 No. 3, pp. 261–270, doi: 10.1002/EDN3.25.
- Ushio, M. (2019), “Use of a filter cartridge combined with intra-cartridge bead-beating improves detection of microbial DNA from water samples”, *Methods in Ecology and Evolution*, John Wiley & Sons, Ltd, Vol. 10 No. 8, pp. 1142–1156, doi: 10.1111/2041-210X.13204.
- Valsecchi, E., Arcangeli, A., Lombardi, R., Boyse, E., Carr, I.M., Galli, P. and Goodman, S.J. (2021), “Ferries and Environmental DNA: Underway Sampling From Commercial Vessels Provides New Opportunities for Systematic Genetic Surveys of

- Marine Biodiversity”, *Frontiers in Marine Science*, Frontiers Media S.A., Vol. 8, doi: 10.3389/fmars.2021.704786.
- Valsecchi, E., Bylemans, J., Goodman, S.J., Lombardi, R., Carr, I., Castellano, L., Galimberti, A., *et al.* (2020), “Novel universal primers for metabarcoding environmental DNA surveys of marine mammals and other marine vertebrates”, *Environmental DNA*, John Wiley & Sons, Ltd, Vol. 2 No. 4, pp. 460–476, doi: 10.1002/EDN3.72.
- Wang, H., Qi, J., Xiao, D., Wang, Z. and Tian, K., 2017. “A re-evaluation of dilution for eliminating PCR inhibition in soil DNA samples.”, *Soil Biology and Biochemistry*, Vol. 106, pp.109-118.
- Waples, R.S. and Gaggiotti, O. (2006), “What is a population? An empirical evaluation of some genetic methods for identifying the number of gene pools and their degree of connectivity”, *Molecular Ecology*, John Wiley & Sons, Ltd, Vol. 15 No. 6, pp. 1419–1439, doi: 10.1111/J.1365-294X.2006.02890.X.
- Whitehead, H., Gordon, J., Mathews, E.A. and Richard, K.R. (1990), “Obtaining skin samples from living sperm whales”, *Marine Mammal Science*, John Wiley & Sons, Ltd, Vol. 6 No. 4, pp. 316–326, doi: 10.1111/J.1748-7692.1990.TB00361.X.
- Zhang, S., Cao, Y., Chen, B., Jiang, P., Fang, L., Li, H., Chen, Z., *et al.* (2023), “Assessing the potential use of environmental DNA for multifaceted genetic monitoring of cetaceans: Example of a wandering whale in a highly disturbed bay area”, *Ecological Indicators*, Elsevier B.V., Vol. 148, doi: 10.1016/j.ecolind.2023.110125.