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False-negative detections from environmental DNA collected in the presence of large numbers of killer whales (*Orcinus orca*)

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Abstract

While environmental DNA (eDNA) is becoming increasingly established in biodiversity monitoring of freshwater ecosystems, the use of eDNA surveys in the marine environment is still in its infancy. Here, we use two approaches: targeted quantitative PCR (qPCR) and whole-genome enrichment capture followed by shotgun sequencing in an effort to amplify killer whale DNA from seawater samples. Samples were collected in close proximity to killer whales in inshore and offshore waters, in varying sea conditions and from the surface and subsurface but none returned strongly positive detections of killer whale eDNA. We validated our laboratory methodologies by successfully amplifying a dilution series of a positive control of killer whale DNA. Furthermore, DNA of Atlantic mackerel, which was present at all sites during sampling, was successfully amplified from the same seawater samples, with positive detections found in ten of the eighteen eDNA extracts. We discuss the various eDNA collection and amplification methodologies used and the abiotic and biotic factors that influence eDNA detection. We discuss possible explanations for the lack of positive killer whale detections, potential pitfalls, and the apparent limitations of eDNA for genetic research on cetaceans, particularly in offshore regions.

KEYWORDS

eDNA, environmental DNA, metagenomics, Orcinus orca, PCR, Scomber scombrus, whole-genome enrichment

1 | INTRODUCTION

Environmental DNA (eDNA) is increasingly used as a monitoring tool to detect the presence of rare or invasive species in aquatic environments. Macro-organisms expel DNA into the environment in many forms, including feces, sloughed skin, scales, blood, hair, and mucus. These sources of eDNA can then be collected through water sampling and amplified using one of several genetic techniques (Ficetola, Miaud, Pompanon, & Taberlet, 2008; Thomsen & Willerslev, 2015). In many freshwater systems, eDNA has proven to be cheaper, faster, and more sensitive than traditional survey methods that involve physically catching, tagging, or biopsying an organism (Ficetola et al., 2008; Thomsen et al., 2016). As a result, eDNA sampling has enabled researchers to study endangered, cryptic, or elusive species (Thomsen, Kielgast, Iversen, Wiuf et al., 2012). In some cases, eDNA has led to the detection of species (including alien invasive species) in areas where they had previously gone unreported, underpinning its significance as a conservation monitoring tool (Dejean et al., 2012; Jerde, Mahon, Chadderton, & Lodge, 2011; Thomsen, Kielgast, Iversen, Møller et al., 2012). Furthermore, eDNA techniques have been successful in detecting an array of fauna including arthropods (Alberdi et al., 2018; Thomsen & Sigsgaard, 2019), amphibians (Dejean et al., 2012; Pilliod, Goldberg, Arkle, & Waits, 2013), cnidarians (Minamoto et al., 2017), reptiles (Hunter et al., 2015), fish (Jane et al., 2015; Jerde et al., 2011; Sigsgaard et al., 2016), and mammals (Foote et al., 2012; Thomsen, Kielgast, Iversen, Wiuf et al., 2012) making it an attractive tool with little to no species limitations.

Initial aquatic eDNA research was limited to determining the presence or absence of a species in freshwater ecosystems (Dejean et al., 2012; Ficetola et al., 2008; Jerde et al., 2011). Subsequently, many applications have started to emerge, including the use of eDNA to determine species abundance (Pilliod et al., 2013; Thomsen et al., 2016), biomass (Jane et al., 2015; Nevers et al., 2018; Takahara, Minamoto, Yamanaka, Doi, & Kawabata, 2012), and population structure (Sigsgaard et al., 2016; Parsons, Everett, Dahlheim, & Park, 2018). Additionally, eDNA research has developed into sampling in the more challenging marine environment (Baker et al., 2018; Bakker et al., 2017; Foote et al., 2012; Minamoto et al., 2017; Parsons et al., 2018; Sigsgaard et al., 2016; Thomsen, Kielgast, Iversen, Møller et al., 2012; Thomsen et al., 2016; Thomsen & Willerslev, 2015).

Degradation of eDNA in marine ecosystems ranges from hours to days (Murakami et al., 2019; Thomsen, Kielgast, Iversen, Møller et al., 2012) compared to weeks in freshwater systems (Dejean et al., 2011; Thomsen, Kielgast, Iversen, Wiuf et al., 2012). This has been attributed to salinity, mixing of larger water masses, and tide/current actions causing dispersal and dilution of the eDNA, with the probability of detecting eDNA in marine waters expected to rapidly decrease with distance from its source (Thomsen, Kielgast, Iversen, Møller et al., 2012). In addition, significant seasonal variability in the persistence of marine dissolved eDNA from several hours to over a month has been correlated with higher temperatures, subsequent enhancement of microbial metabolism, and low concentrations of bioavailable phosphate, resulting in increased microbial utilization of dissolved eDNA as an organic phosphorus substrate (Salter, 2018). One contrasting study by Collins et al. (2018) found that degradation rates are slower in the marine environment compared to freshwater, but estimated that eDNA (freshwater or marine) may only be reliably detected for up to 48 hr. While the time until total degradation or dilution beyond detectability of eDNA from seawater samples varies between studies and is dependent on the environment, weather conditions, location, and the sensitivity of laboratory methodologies used, the common conclusion is that eDNA from the marine environment provides a snapshot of organisms recently present in the local area (Port et al., 2016).

Several eDNA studies have targeted specific animal populations to acquire genetic material through seawater sampling, as direct genetic sampling remains challenging at sea. A study by Sigsgaard et al. (2016) demonstrated the use of eDNA to provide estimates of genetic diversity in a whale shark (*Rhincodon typus*) aggregation off Qatar in the Arabian Gulf. In this study, the first of its kind, similar mitochondrial haplotype frequencies were recovered from seawater eDNA compared to tissue samples, expanding the applications of eDNA to encompass population genetics of aquatic organisms (Sigsgaard et al., 2016). A similar study by Parsons et al. (2018) on harbor porpoises (*Phocoena phocoena*) in the inland waters of southeast Alaska revealed indications of significant genetic differentiation within a currently recognized single stock of harbor porpoises and identified two previously undocumented mitochondrial haplotypes from seawater samples. Another, recently published, marine mammal eDNA study confirmed killer whale (*Orcinus orca*) presence in Puget Sound, North America, and correctly identified the killer whale ecotype present at the time of seawater sampling (Baker et al., 2018). Furthermore, positive killer whale detections were found for up to two hours following an encounter, despite movement of the water mass by several kilometers due to tidal currents (Baker et al., 2018). Determining population structure of highly mobile marine species such as cetaceans, from traditional direct sampling via skin biopsies is often a very difficult and expensive task. Thus, the promise shown by previous studies in the use of eDNA to generate population-specific mitochondrial sequence data from seawater samples prompted us to attempt eDNA sampling on an offshore cetacean population.

Large numbers of killer whales aggregating around vessels during fish hauling have been observed from pelagic fishing vessels targeting the Northeast Atlantic mackerel (*Scomber scrombus*, Linnaeus, 1758) stock, along their spawning migration route from the Norwegian Deeps to the northwest coast of Ireland (Pinfield et al., 2011). Foraging interactions between killer whales and commercial mackerel trawlers and purse seiners have previously been described with this fishery (Bloch & Lockyer, 1988; Couperus, 1994; Luque, Davis, Reid, Wang, & Pierce, 2007). However, no dedicated research has been carried out on this killer whale community, and thus, knowledge of their ecology is limited to opportunistic efforts. To bridge this gap, dedicated research was conducted from fishing trawlers during the Northeast Atlantic mackerel (NEAM) fishery in pelagic waters west of Scotland and Ireland. DNA from free-ranging

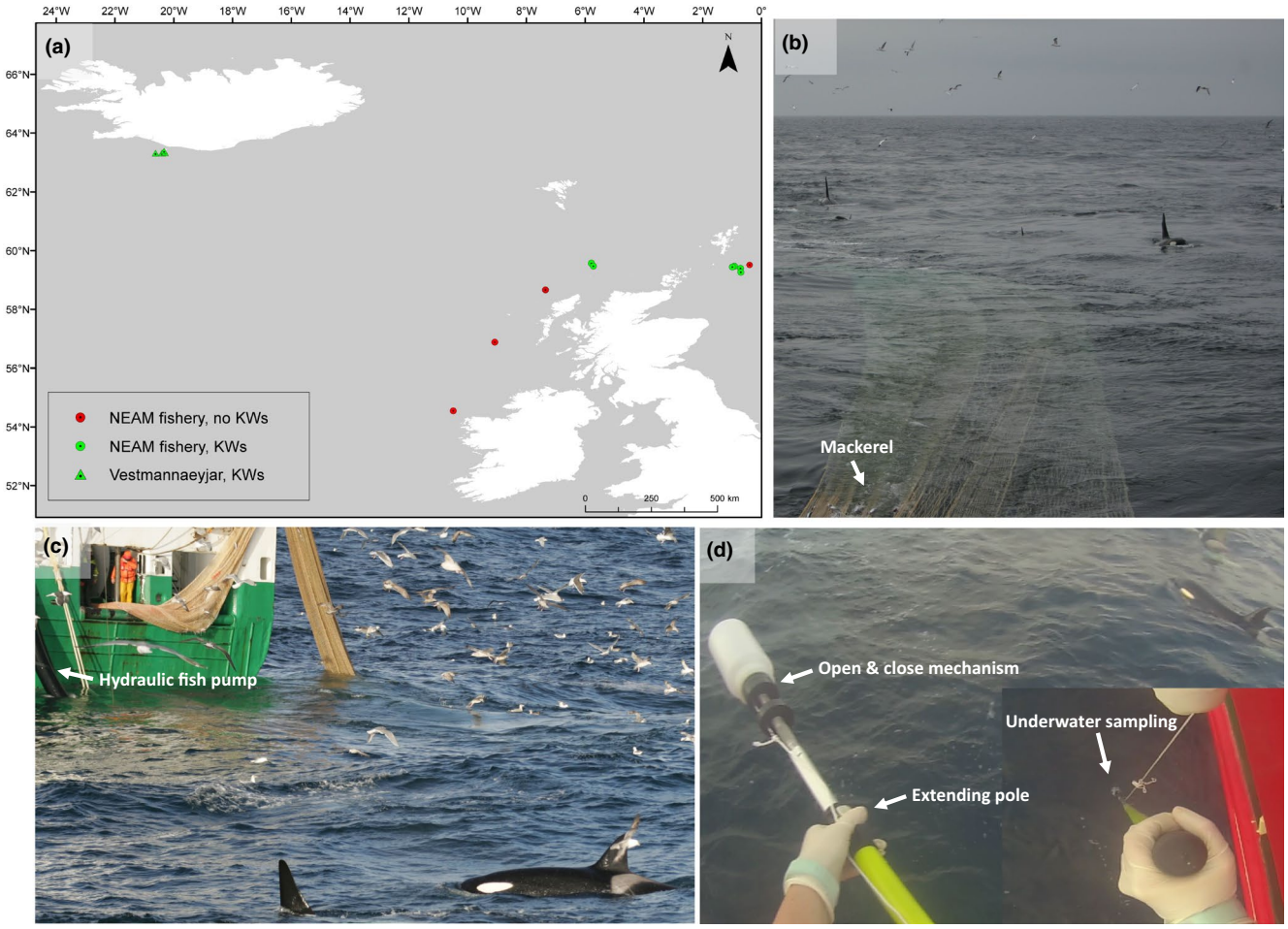

FIGURE 1 (a) Seawater sampling sites and associated presence of killer whales (KWs) along the Northeast Atlantic mackerel (NEAM) fishery route and around Vestmannaeyjar, Iceland (Map: ArcMap v10.4.1, basemap source: ESRI, GEBCO20140). (b) Killer whales approaching a NEAM fishing vessel during net hauling. (c) Killer whales circling a trawl net during hydraulic pumping of fish onboard the vessel. (d) Deployment and seawater sampling using the Veggerby eDNA sampler pole in the presence of killer whales

cetaceans is typically collected by remote biopsy sampling of epithelial tissue (e.g., Krützen et al. 2002). However, biopsy sampling opportunities are rare from fishing vessels in the prevailing adverse winter weather conditions of the North Sea and Northeast Atlantic and become even more difficult with the lack of a dedicated biopsy vessel. Given the close approaches of these killer whales to the fishing vessels (Pinfield et al., 2011), it was expected that there would be a high likelihood of capturing eDNA from this offshore killer whale population. To test this assumption, seawater sampling was carried out to detect killer whale presence and determine the genetic affiliation of these killer whales to neighboring populations.

2 | MATERIALS AND METHODS

2.1 | Seawater sampling: NEAM Fishery

Ten seawater samples were taken along the NEAM migration route (one per site) from the North Sea into Irish waters in the autumn and winter months of 2017 (Figure 1a). Sampling was carried out from Irish pelagic trawl fishing vessels in various weather conditions (Beaufort seastate 4–6) using two methods: (a) a rope and bucket method and (b) a Veggerby eDNA sampler (Veggerby & Veggerby 2018) supplied by Wildco®, Yulee, Florida. The Veggerby eDNA sampler allows water to be sampled from a predetermined depth and may be raised from this depth without contamination from other depths of water or from travel through air following surfacing (Veggerby & Veggerby 2018). The sampler has an extendable pole (9–17 ft) with an attachment end for a sample bottle to be attached. The bottle is filled underwater via a suction-based method, whereby a line (rope) running up the length of the pole is pulled to open the attachment end (when at the desired depth), water flows into the sample bottle, the line is then released, a spring closes the lid and seals the sample bottle before bringing it back to the surface.

Prior to sampling, all equipment was washed with soapy water, rinsed, sterilized by immersion in 10% diluted household bleach for 10 min, rinsed with distilled water, and finally wiped clean with 80% ethanol. All samples were collected from the subsurface (≤1 m) during hydraulic pumping of the fish catch from the trawl net into the vessel, as this is when killer whales come in close to the vessels to forage on dropouts from the net (Figure 1b,c). However, samples

were collected regardless of whether killer whales were present or not.

For the rope and bucket method, a 2.5 L bucket was lowered over the side to approximately 1 m depth, filled, and emptied twice to ensure any bleach or ethanol residue from sterilization was removed, and the third fill was taken onboard. From the bucket, a 900 ml eDNA sample was taken and stored in 5 × 180 ml sterile containers. To test for cross-contamination during sampling, another 180 ml sterile container was filled with distilled water on the deck straight after seawater sampling and used as a field-negative control.

For the Veggerby eDNA sampler method, the pole was extended over the side with a 1 L, wide mouth, sterile Nalgene™ bottle attached (Figure 1d). Prior to each sampling event, the eDNA sampler pole was washed down with freshwater, and the attachment end was removed and sterilized with the Nalgene sampler bottles in the manner described above. The extendable part of the pole was cleaned with disinfectant spray and wiped with 80% ethanol. During sampling, the sample bottle was filled underwater at approximately 1 m depth and closed before resurfacing (Figure 1d). Like the previous method, the sample bottle was filled and emptied twice, and the third sample was brought onboard and sealed.

All samples were frozen onboard at −20°C before being transferred (on ice packs) initially to a −20°C freezer in the laboratory and subsequently to a −80°C freezer until filtration was carried out.

2.2 | Seawater sampling: Vestmannaeyjar, Iceland

Four seawater samples (one per site) were collected in August 2017 around Vestmannaeyjar, South Iceland, in calm weather conditions, Beaufort seastate 2 (Figure 1a). This area is part of the home range of a population of killer whales which predominantly predate on the Icelandic summer-spawning stock of Atlantic herring, *Clupea harengus* (Samarra et al., 2017). During a killer whale encounter, a 1,080 ml seawater sample was collected from the sea surface from a small rigid inflatable boat (RIB) by hand, using sterilized containers (6 × 180 ml) and clean disposable nitrile gloves. In addition, a 100 ml bottle of molecular grade water was transferred to a 180 ml sterilized container at two of the sample sites directly after seawater sampling and used as a field-negative control to test for contamination. While onboard, the samples were stored with icepacks in an insulated bag and transferred to a fridge (4°C) or freezer (−20°C) that day depending on whether filtration could be carried out the same day (fridge) or not (freezer).

2.3 | Filtration and eDNA extraction

Prior to filtration and extraction, bench surfaces and all equipment were wiped with bleach (5%) and laboratory-grade ethanol (70%). In addition, a disposable face mask and coverall was worn during the filtration process to reduce the risk of contamination. Each sample was filtered separately, and filtration was carried out in a laminar flow hood in a separate building away from a modern DNA laboratory to avoid cross-contamination. Seawater samples collected from the NEAM fishery were defrosted at room temperature and filtered through Sterivex-GP capsule filters (polyethersulfone 0.22 µm pore size with luer-lock outlet (Merck KGaA) using a prepacked sterile 50-ml luer-lock syringe following Spens et al. (2017). Remaining water inside the capsule was removed by using the luer-lock syringe to push air through it until dry. Following filtration, the ends of the capsules were sealed with parafilm and stored in a −80°C freezer until extraction.

The Icelandic samples were filtered on the same day as collection or straight after they were defrosted. Filtration was carried out as previously described (without the flow hood, face mask, and coverall), in a building that was not previously exposed to cetacean tissue samples. Following filtration, a sterile solution of 1 M Longmire's buffer (Longmire, Maltbie, & Baker, 1997) was pushed through the capsule using a new sterile luer-lock syringe and each capsule was stored open-ended in a 20 ml sterile container also containing Longmire's buffer. All samples were stored at room temperature for transportation, and after 9.5 weeks, the samples were transferred to a −80°C freezer until extraction.

2.4 | Extraction

All extractions took place in a laminar flow hood away from other modern DNA work. Extractions from the filters with no buffer (NEAM fishery) and from the Icelandic samples with buffer were carried out in batches. Samples from the NEAM fishery were not extracted in the order they were collected in, in order to reduce bias in the molecular workflow with respect to extraction day (i.e., batch effects). However, all Icelandic samples were extracted in one batch. Extractions were performed using the DNeasy® Blood & Tissue kit (QIAGEN) and MinElute spin columns (QIAGEN), with slight modifications to the standard protocol as described in Spens et al. (2017). Following Spens et al. (2017), two extractions were carried out on each sample that was stored in buffer (Icelandic samples): an extraction from the filter within the capsule after removal of the buffer (indicated by a "c") and an extraction from the removed buffer (indicated by a "T"). To test for contamination within the extraction kit or from the laboratory environment, a laboratory-negative control was included by placing all of the kit reagents into an Eppendorf tube without any sample. This was subsequently extracted and subjected to analysis in the same manner as the rest of the samples. The extracted DNA was eluted with 70 µl of molecular grade water, which was allowed to incubate on the spin column for 10 min prior to centrifugation. During extraction and purification, MinElute spin columns (QIAGEN) were used as they retain small fragments of DNA down to 70 bp; any fragments greater than or equal to this should be retained by the columns. A total of 30 samples were extracted; 18 experimental samples (10 NEAM and 8 Icelandic eDNA), 9 field-negative controls, and 3 laboratory-negative controls. The DNA was then placed in a −20°C freezer until transportation (in insulated packaging) to a dedicated PCR laboratory at the Centre for GeoGenetics, Denmark. Upon arrival, the samples were placed back in a −20°C freezer until PCR amplification.

2.5 | Primer design and PCR optimization

Our initial goals were twofold: to detect the presence of killer whales using eDNA and to infer population structure from our eDNA data. To achieve the first goal, we employed qPCR, which has been found to be suitably sensitive by previous studies to detect species presence from eDNA. Five sets of oligonucleotide primers (*Orca_01* to *Orca_05*, Table S1) to target short fragments (≤ 175 bp) of the mitochondrial (mtDNA) control gene region (CO1) of North Atlantic killer whales were designed using the online primer design tool, *Primer-BLAST* (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Information available through GenBank® was used to maximize base pair mismatches between killer whales and closely related species. Short amplicon sizes were deemed necessary, as it was anticipated that the samples would likely contain more short fragments of DNA rather than longer fragments due to eDNA degradation (Jo et al., 2017; Thomsen & Willerslev, 2015). However, a previously published set of primers targeting a longer fragment at the 5' of the mtDNA control region, H16498 (Rosel, Dizon, & Heyning, 1994) and L15812 (Zerbini et al., 2007) were also included to detect potential longer DNA fragments.

Species specificity of the primer pairs was tested on extracted DNA from non-target cetacean species which occur in the study area (bottlenose dolphin (*Tursiops truncatus*), Risso's dolphin (*Grampus griseus*), Atlantic white-sided dolphin (*Lagenorhynchus acutus*)) and on Atlantic mackerel, using standard PCR. A positive control of high molecular weight and high concentration (100 ng/µl) of killer whale DNA extracted from blood was included. Reactions were performed in 20 µl volumes, consisting of 10 µl of 2x TopBio PP Combi Mastermix, 1 µM each of forward and reverse primers and 2 µl of template DNA. The PCR was run under the following thermal cycling parameters of 95°C (3 min) followed by 40 cycles of 95°C (30 s), 48°C (45 s), 72°C (1 min), and finally 72°C (5 min). The non-target cetacean species did generate amplification signals; however, the primers did not amplify mackerel. Thus, PCR could be susceptible to false-positive detections of killer whale DNA due to the presence of other cetacean species in our study area but should not be triggered by the presence of mackerel. The most sensitive primer sets (*Orca_05* and H16498 and L15812) were selected and used in all subsequent reactions. Following this, the optimal primer annealing temperature for qPCR was determined using a gradient of 4°C increments between 48°C and 68°C on a high-quality positive control sample (100 ng/µl DNA extracted from killer whale blood). The most effective annealing temperature for amplifying killer whale DNA was found to be 68°C, which would be expected to further increase species specificity and was applied to all subsequent reactions.

We utilized the same approach to design five primers to target short fragments (≤162 bp) of the COX1 gene in Atlantic mackerel and found that *Scom_01* at 60°C was the most sensitive. All primer sequences are included in Table S1.

2.6 | Quantitative PCR

Quantitative PCR took place in the laboratories at the Centre for GeoGenetics, University of Copenhagen, Denmark. The facilities are designed for handling environmental samples requiring the most stringent precautions to avoid contamination. Prior to any work in the laboratory, all surfaces are washed with 5% bleach and 70% ethanol and laboratory coats were changed between pre-PCR and PCR rooms.

Quantitative PCR was performed on a Stratagene Mx3005P (Thermo Fisher Scientific Inc.) using thermal cycling parameters of 95°C (5 min) followed by 40 cycles of 95°C (30 s), 68°C (30 s), 72°C (30 s), and finally 72°C (7 min). Both the long primers (H16498 and L15812) that target 426-bp fragments and the short primers (*Orca_05*) that target 175-bp fragments of killer whale mtDNA were used in separate qPCRs to determine whether long and/or short fragments of killer whale DNA were present in the eDNA extracts, respectively. Each eDNA extract was run in triplicate alongside a positive control dilution series, the field- and laboratory-negative controls and two negative PCR controls (UV-treated laboratory-grade water). The positive control used was the ~ 100 ng/µl concentration of DNA extracted from killer whale blood in a dilution series (1:1, 1:10, 1:100, 1:1,000, 1:10,000, and 1:100,000). The dilution series was used to aid quantification of killer DNA concentrations present in the extracts and also to check for PCR inhibition within the qPCR (Jane et al., 2015). The qPCR mastermix was made in a final reaction volume of 25 µl containing the following; 2.5 mM $MgCl_2$, 2.5 µl 10x reaction buffer, 0.4 µM each of forward and reverse primer, 0.8 mM dNTPs, 1 U Taq, 1 µl SYBR green, and 1 µl DNA and 15.6 UV-treated laboratory-grade water. As the goal of this step was to detect species presence, we did not design taxon-specific TaqMan probes. Further steps were planned to validate source taxa.

The above steps were repeated using the *Scom_01* primer set designed to amplify 112-bp fragments of Atlantic mackerel DNA and included a dilution series of a positive control of mackerel DNA extract from fin clips (Appendix S1).

2.7 | Whole-genome enrichment capture

To maximize the potential to leverage population-level information from any killer whale DNA isolated from the seawater samples (see Adams et al., 2019; Jones & Good, 2016), the eDNA extracts were enriched for killer whale DNA using targeted whole-genome capture with killer whale RNA baits (Enk et al., 2014). Our goal here was to map the sequencing reads generated from eDNA libraries enriched for killer whale DNA to the killer whale reference genome (Foote et al., 2015) and then compare to a global dataset of killer whale genomes (Foote et al., 2019) setting the minor allele frequency, so that only SNPs (single nucleotide polymorphisms) also present in the global dataset would be called in the eDNA datasets. This would therefore identify the potential source populations of any killer whale eDNA detected, based on the sharing of rare alleles.

Nine of the eDNA samples (01, 13, 14, 15, 17c, 17T, 20T, 20c, 21c) were randomly selected for whole-genome enrichment capture. Dual-indexed DNA libraries were built on 16.3 µl of extract using the BEST library build method of Carøe et al. (2018) and then individually amplified for 15 cycles with an annealing temperature of

TABLE 1 Summary of eDNA samples collected during the Northeast Atlantic mackerel fishery and around Vestmannaeyjar, Iceland, in 2017

Sample	Date	Sampling site	Latitude	Longitude	Beaufort seastate	Sampling method	Volume (ml)	No. KWs	Closest KW from vessel (m)
7	23/01/17	NEAM fishery	59.56965	−5.78242	5	RB	900	15–20	5
8	30/01/17	NEAM fishery	59.4761	−5.71468	6	RB	900	4–5	5
1	04/02/17	NEAM fishery	58.65952	−7.34317	5	RB	900	0	–
10	10/02/17	NEAM fishery	56.88175	−9.07112	5	RB	900	0	–
2	16/02/17	NEAM fishery	54.54722	−10.4862	6	RB	900	0	–
12	25/10/17	NEAM fishery	59.39928	−0.71467	5	P	1,000	3	Unknown Seen by crew
3	26/10/17	NEAM fishery	59.51683	−0.40833	6	P	1,000	0	–
13	30/10/17	NEAM fishery	59.47365	−0.91898	5	P	1,000	9	10
14	30/10/17	NEAM fishery	59.44285	−0.98578	4	P	1,000	60–70	1
15	07/11/17	NEAM fishery	59.26367	−0.70128	6	P	1,000	1–3	Unknown Seen by crew
17	13/08/17	Vestmannaeyjar	63.31997	−20.40092	2	RIB	1,080	9	≤20
19	13/08/17	Vestmannaeyjar	63.31365	−20.61658	2	RIB	1,080	11	≤20
20	16/08/17	Vestmannaeyjar	63.32983	−20.29463	2	RIB	1,080	8–15	≤20
21	16/08/17	Vestmannaeyjar	63.38752	−20.32883	2	RIB	1,080	20	≤20

Notes: Includes number of killer whales sighted and closest distance of killer whales from the vessel during seawater sampling.
Abbreviations: KW, Killer whale; NEAM, Northeast Atlantic mackerel; P, eDNA sampler pole; RB, rope and bucket; RIB, rigid inflatable boat.

55°C using AmpliTaq Gold DNA Polymerase (Applied Biosystems). Libraries were pooled and subjected to one round of whole-genome enrichment capture using genome-wide biotinylated RNA baits (two reactions) built from modern DNA by Arbor Biosciences, with a hybridization period of 24 hr at 55°C. The postcapture pool of libraries was then re-amplified for 10 cycles using KAPA HiFi HotStart ReadyMixPCR kit (KAPA Biosystems) and sequenced across a partial (~1/10) lane of an Illumina HiSeq4000.

Before investigating at the population level, the sequence data were screened to determine species presence in the captured eDNA pool, and an initial custom database was constructed using the BLAST + module to include the available cetacean reference genomes in the RefSeq repository (killer whale, bottlenose dolphin, and minke whale (*Balaenoptera acutorostrata*)). In addition, human (*Homo sapiens*), Atlantic herring, and a set of unplaced scaffolds of the Atlantic bluefin tuna (*Thunnus thynnus*) genome (the closest available related genome to Atlantic mackerel) were included. After removing indexes and adapters using AdapterRemoval (Lindgreen, 2012), sequences were converted from fastq to fasta format using seqtk (https://github.com/lh3/seqtk). Sequences were then compared against the custom database and the results were downloaded and visualized in MEGAN v6.14.2 (Huson et al., 2016) where a naive LCA (lowest common ancestor) algorithm with stringent parameter settings (Min score = 60, Max expected = 2e-10, Min percent identity = 100, Top percent = 1, Min support percent = 0, Min support = 1, Min complexity = 0) was applied to explore the data. Given the paucity of confirmed killer whale reads (see results), investigating at the population level was not possible.

3 | RESULTS

Ten seawater samples were collected from the Irish NEAM fishing vessels during eight trips using the sterilized rope and bucket method for the first five samples collected and the Veggerby eDNA sampler for the other five samples (Table 1). Killer whales were visually observed during 60% of sampling events and group size ranged from 1 to 70 individuals. The animals approached within 10 m (estimated by eye) of the vessel during four of these events, the other two were unconfirmed distances as sightings were reported by a crew member afterward, but the animals were likely within 20 m. In Iceland, four seawater samples were collected during four small boat-based surveys around Vestmannaeyjar. These samples were collected on the 15 and 16th of August 2017 at the end of photo-ID/biopsy encounters (1–2 hr) with killer whales. The killer whales were within 20 m during seawater sampling and group size ranged from 8 to 20 individuals.

3.1 | Extraction

Altogether, DNA was extracted from 18 seawater samples, 10 from the NEAM fishery, and 8 from Vestmannaeyjar, Iceland (4 samples duplicated; 4 × capsule (“c”), 4 × buffer (“T”)). The eDNA extracts (no field- or laboratory-negative controls) were run unamplified on an electrophoresis gel with a 100 bp and 1 kb ladder to determine the fragment size of the DNA captured. Bright bands appeared on the gel at >10 kb length (Figure S1), indicating that long fragments of DNA were present; however, the source of the DNA was unknown. The presence of high molecular weight

DNA could stem from either DNA extraction from whole organisms (e.g., planktonic) present in the seawater sample, or from intracellular DNA (incl. mackerel or killer whale) shed just prior to seawater sampling.

3.2 | Quantitative PCR

For the long primer set, the positive control dilution series performed as expected, identifying killer whale DNA reliably up to 35 cycles. After 38 cycles, fluorescence started to appear in all samples including the negative controls. To investigate this further, the qPCR products were run on an electrophoresis gel. Bright bands appeared in all eDNA samples at various lengths, none matching the target amplicon length, indicating that the fluorescence was from primers binding to each other and to other non-target PCR artifacts and was unlikely to result from amplification of killer whale DNA. In contrast, the positive control PCRs produced bands at the expected amplicon length. This suggested that the high molecular weight DNA present in the eDNA samples was not killer whale DNA, but most likely from plankton and/or mackerel. Thus, if any killer whale DNA was present, it was likely <500 bp in length.

For the *Orca_05* primer set that targets a 175 bp fragment, the cycle threshold (C_t) of the positive control dilution series corresponded well with the relative concentrations (Figure 2). However, only one eDNA sample (02) had a C_t of 35 cycles or less (Figure 2), and this was a sample-collected when no killer whales were visually detected (Table 1). All other samples failed to amplify, or the C_t was >37 cycles, less than that of the 1 in 10^5 dilution of the positive control, and potentially, fluorescence was again due to PCR artifacts (Figure 2).

In contrast, qPCR using primer set *Scom_01* designed to amplify a 112 bp amplicon of mackerel DNA resulted in positive detections in 10 out of the 18 eDNA extracts (Figure S2). This equated to 9 of the 14 sampling sites (8 NEAM, 1 Icelandic) with positive detections of Atlantic mackerel (Table S2). Each eDNA extract was run in triplicate alongside the positive control dilution series, the field- and laboratory-negative controls and two negative PCR controls. The C_t standard deviation (*SD*) of the replicates of each of the positive control dilution series was used to define the cycle threshold. C_t *SD* values >0.25 were considered unreliable. The cycle threshold was determined to be 30 cycles, and a sample was considered positive for the presence of Atlantic mackerel if any one of the three replicates had a C_t value of 30 cycles or less. One of the 9 field-negative controls had a positive detection of a small quantity (<0.01 ng/μl) of mackerel DNA, although no amplification was observed in any of the laboratory-negative controls.

3.3 | Whole-genome enrichment capture

The successful building of DNA libraries from eDNA extracts was confirmed pre-capture through visualization on a gel and post-capture using an Agilent 2200 TapeStation with a High Sensitivity D1000 screentape and ladder. This confirmed a post-capture DNA peak of 8,280 pmol/L with a distribution around 192 bp in length. However, comparing the sequencing reads against our custom database using the blastn program in BLAST+ module, one sample (17c) failed to assign reads to any of the included genomes, while in the remaining samples just 16–67 reads were assigned to the killer whale genome (Table 2). Comparison of reads that mapped to the killer whale genome with a published global dataset of killer whale genomes (Foote et al., 2019) found no shared SNPs between the eDNA samples and the global datasets. Thus, it was not possible to make any inference of population structure from this data, and there was no further support for the veracity of the species-level assignment. From the same eight samples, 4–621 reads were assigned to the herring genome, while 5–439 reads were mapped to the bluefin tuna genome, suggesting that the enrichment process had removed most of the mackerel DNA. However, allowing for two mismatches (Min percent identity = 97.5) in the identifications resulted in an increase in reads aligning to bluefin tuna (21–1341 reads). Contamination from human DNA was found in 8 of the 9 samples, with between 86 and 32,186 reads being assigned to the human genome.

4 | DISCUSSION

Despite the majority of eDNA samples in this study being collected within 20 m of killer whales, we were unable to conclusively amplify or enrich for any killer whale eDNA. We had anticipated that given the close approaches of the killer whales to the NEAM fishing vessels, we would amplify small fragments of killer whale DNA, resulting in strong positive detections. The successful amplification of the serial dilution of the positive control of killer whale DNA indicates that the ambiguous or negative detections of killer whale DNA from the seawater samples were not due to qPCR failure. Furthermore, we successfully amplified Atlantic mackerel DNA from the same eDNA extracts using qPCR, suggesting that the false-negative detection of killer whale DNA was not due to issues with the DNA extraction steps. Atlantic mackerel was present at all sites during sampling, including in Iceland where they were observed feeding in large aggregations at the water surface. Thus, our qPCR analyses indicated that while the seawater samples contained DNA, killer whale DNA was an undetectably small component of the environmental metagenome.

The qPCR results were supported by the results of the whole-genome enrichment (WGE), where only a few reads were assigned to killer whale despite enriching eDNA libraries using RNA baits designed on the killer whale genome. The database applied to this dataset was intended as a first step in a longer chain of analyses; however, it led to highlighting some of the inherent challenges of metagenomic analyses, where the lack of appropriate references can confound results (prevents the classification of source species that are not represented) and incomplete databases lead to false-positive identifications, that is, the program falsely assigns reads to the represented species because a better match cannot be found. To limit this, only reads with 100% similarity to the references were included, but even then, there is a

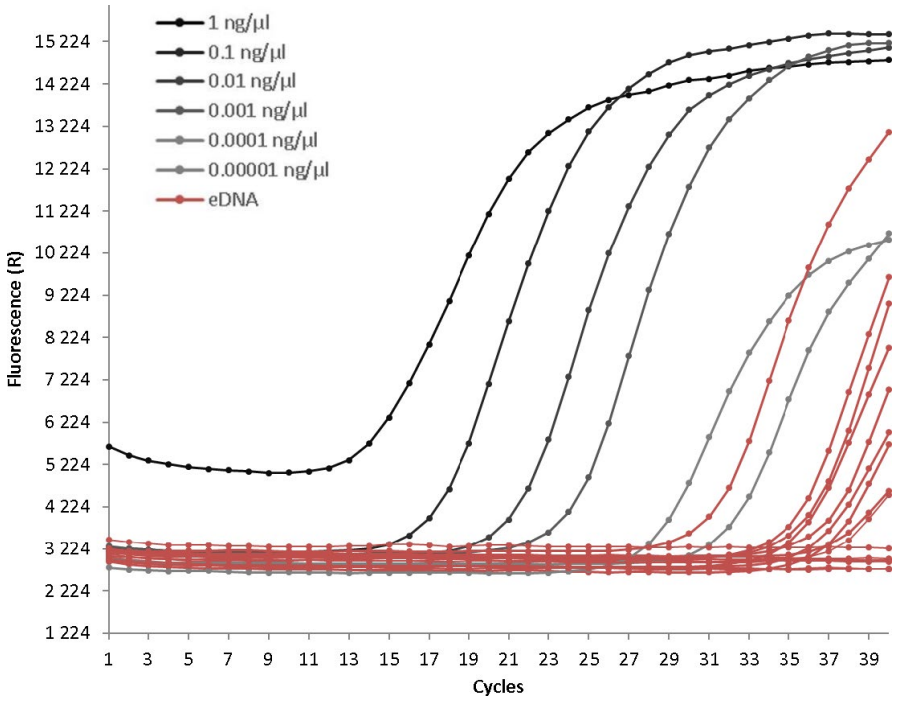

FIGURE 2 Quantitative PCR to estimate the quantity of short fragments (nanograms per microliter (ng/µl)) of killer whale DNA in eDNA samples collected during the Northeast Atlantic mackerel fishery and around Vestmannaeyjar, Iceland, in 2017. Includes a serial dilution of a positive control of killer whale DNA extract

possibility of false-positives, and this limits the detection of degraded DNA. However, allowing for mismatches did not particularly increase the number of reads assigned to killer whale, which indicates that even degraded DNA is not detected. Furthermore, comparing all reads in the enriched eDNA libraries that mapped to the killer whale genome to a global dataset (Foote et al. 2019) found no shared SNPs. Thus, there is a strong possibility that these reads originate from another species, but map to conserved regions of the killer whale genome.

The need to consider potential contamination from sampling or laboratory sources is especially relevant when working with complex samples expected to contain low quantities of DNA. Trace human contamination of sequencing data that were found in 8 of the 9 samples is an inescapable issue in modern high-throughput sequencing facilities (see Hooper et al., 2019), as well as microbial contamination (Laurence, Hatzis, & Brash, 2014). Ultimately, both methods (qPCR and WGE) should have been susceptible to false-positives, as both approaches could conflate detections of DNA from closely related cetacean species and even more distantly related mammalian species such as humans, with detections of killer whale. Thus, we feel confident in our assessment that killer whale DNA was either absent or present in quantities too low to unequivocally confirm species presence, despite positive close-range visual detections. Given this, we consider which abiotic and biotic factors could have played a role in these false-negative genetic detections. In doing so, it also seems fitting to discuss some of the key differences in the sampling and laboratory methodologies used in this study and in the recently published Baker et al. (2018) study which successfully amplified killer whale eDNA.

In this study, samples were collected offshore (NEAM fishery) and inshore (Iceland) in non-sheltered areas, in both calm (Beaufort 2, Iceland) and adverse weather conditions (Beaufort 4–6, NEAM fishery). Weather conditions were not ideal for eDNA collection from the mackerel fishing vessels, choppy seastates of Beaufort 4–6 are likely to dilute and disperse eDNA at a faster rate than calm seas. This coupled with the fact that killer whales only approach the vessels during hauling and were not present beforehand likely contributed to the lack of killer whale DNA captured. However, in Iceland, conditions were calm and seawater samples were collected from a RIB following long encounters (1–2 hr) with killer whales, but again killer whale DNA was not successfully amplified. In contrast, Baker et al. (2018) carried out sampling in a sheltered estuary where the predictable nature of the killer whale movements is known. Unlike Baker et al. (2018), we did not drive into the wake of the whales to carry out seawater sampling in Iceland, but the whales were present and in close proximity to the boat during sampling (<20 m). Taking the samples directly behind the whales may have yielded positive results. For the NEAM samples, it was not possible to move the vessel during killer whale encounters as it was engaged in hauling operations. Seawater sampling at any other time was not possible as the vessel was constantly moving in search of fish.

Baker et al. (2018) found a higher number of killer whale positive detections from eDNA when sampling from the air/surface interface compared to the subsurface. The authors inferred that this could be contributed to the advection of sloughed skin or feces at the surface and/or surface tension retaining DNA from exhalation blows. Our samples collected in Iceland using handheld containers were taken from the air/surface interface and the samples collected using the rope and bucket method from the NEAM fishing vessels would also have contained surface water; however, the eDNA sampler pole collected subsurface water at approximately 1 meter depth. Despite sampling from both surface and subsurface, and in calm conditions off Iceland, the lack of positive detections would suggest that other factors may determine the successful capture of target DNA from

TABLE 2 Metagenomic identifications showing number of identified reads and percentage of identified reads assigned as matching to killer whale, Atlantic herring, bluefin tuna, and human, in a subset of eDNA extracts subjected to a whole-genome enrichment capture experiment

	Total	*Killer whale (O. orca)*		*Atlantic herring (C. harengus)*		*Atlantic bluefin tuna (T. thunnus)*		*Human (H. sapiens)*	
Sample	Number	Number	% of total	Number	% of total	Number	% of total	Number	% of total
1	158,520	29	0.0183	42	0.02649	215	0.1356	440	0.2776
13	627,741	26	0.0041	47	0.0075	52	0.0083	867	0.1381
14	622,471	31	0.0050	68	0.0109	38	0.0061	2,806	0.4508
15	2,266,335	42	0.0019	621	0.0274	439	0.0193	1873	0.0826
17c	10	0	0	0	0	0	0	0	0
17T	10,258	42	0.4094	4	0.0390	5	0.0487	86	0.8384
20c	212,810	16	0.0075	37	0.0174	7	0.0033	32,186	15.1242
20T	13,709	25	0.1823	4	0.0291	5	0.0365	433	3.1581
21c	359,154	67	0.0187	146	0.0407	5	0.0014	1,772	0.4939

Note: Sample details are provided in Table 1. Two extractions were carried out on each of the Icelandic samples (17–21); an extraction from the filter within the capsule after removal of Longmire's storage buffer (indicated by a "c"), and an extraction from the removed buffer (indicated by a "T").

seawater samples. For example, animal behavior during sampling may be an important factor to consider. Killer whales may defecate less during foraging than during traveling, resting, or socializing. Feces are considered one of the highest sources of eDNA in environments (Alberdi et al., 2018; Baker et al., 2018; Klymus, Richter, Chapman, & Paukert, 2015), particularly for marine mammals whose feces are known to float (Gillett, Frasier, Rolland, & White, 2010; Stewart, 2019). Lack of fecal matter from the target organism may reduce the successful capture of target DNA. In addition, colder sea surface temperatures in higher latitudes may be linked with a reduced rate of skin shedding. Humpback whales (*Megaptera novaeangliae*) and sperm whales (*Physeter macrocephalus*) are thought to lose skin less frequently at higher latitudes compared to tropical waters (Whitehead et al., 1990), while blue whales (*Balaenoptera musculus*) off Baja California sloughed skin independently of season and sea surface temperature; however, a tendency for a decrease in shed skin in cooler water was noted (Gendron & Mesnick, 2001). There is also evidence of water temperature influencing skin turnover rate in killer whales; for example, Antarctic killer whales make "skin maintenance migrations" of thousands of kilometers to warm waters to remove dead skin layers and associated diatom and microbial communities (Durban & Pitman, 2012; Hooper et al., 2019). While the sea surface temperature of the Northeast Atlantic waters sampled in this study are not as extreme as the frigid Antarctic waters, they may still be sufficiently cold to restrict blood flow to the outer skin layers, reducing turnover time and shedding rate, thus having an impact upon eDNA detection.

While mackerel was present in Iceland, only low concentrations of mackerel were amplified in one of the samples despite large mackerel schools being present at the time of sampling. The amplification efficiency of the serial dilutions in each qPCR was assessed, and these were near the expected 100%, and therefore, there was no evidence of PCR inhibition. Values well above or below 100% could indicate inhibition, sub-optimal primers or reaction conditions, pipetting errors, or formation of non-specific products or primer dimers (Collins et al., 2018). The probability of eDNA detection also depends on the number of samples, volume of water collected, timing of sampling (e.g., breeding/spawning season), sample concentration, preservation methods, number of PCR replicates, and amplification methodologies used (Alberdi et al., 2018; Harper et al., 2018; Schultz & Lance, 2015; Spens et al., 2017; Stewart, 2019). We collected between 900 and 1,080 ml at each site but sampling a larger volume of water may have improved our detection probability (Harper et al., 2018; Hunter, Ferrante, Meigs-Friend, & Ulmer, 2019; Schultz & Lance, 2015). Foote et al. (2012) also highlighted that small sample volume may have affected their ability to successfully detect harbor porpoises in seawater samples collected around acoustic dataloggers, with just one sample from eight sites amplifying porpoise DNA despite positive acoustic detections at four of the sites. Foote et al. (2012) were also unable to detect porpoise eDNA beyond 10 m of a sea pen in a sheltered area containing four captive harbor porpoises. This further implies that a close approach or sampling in the wake of cetaceans may be necessary to increase eDNA detection probability. In addition, increasing the number of PCR replicates may also have increased our chances of finding killer whale DNA, as PCR replicates counteract the effects of PCR stochasticity (Leray & Knowlton, 2015; Taberlet et al., 1996). When the starting number of DNA templates is small, PCR stochasticity can result in variable amplification success across replicate PCRs (Taberlet et al. 1996). PCR stochasticity is evident in many eDNA studies whereby a positive detection may only be found in one of several PCRs and/or not in all replicates in the PCR (Alberdi et al., 2018; Biggs et al., 2015; Dejean et al., 2012; Foote et al., 2012; Sigsgaard et al., 2016). This can occur even in those studies that use more sensitive methods such as the digital droplet (dd)PCR used by Baker et al. (2018). To counteract this effect, increasing the number of PCR replicates, sample replicates,

and the number of field replicates is advised in order to achieve a reliable result (Leray & Knowlton, 2015; Piggott, 2016; Schultz & Lance, 2015; Taberlet et al., 1996; Willoughby, Wijayawardena, Sundaram, Swihart, & DeWoody, 2016). Ficetola et al. (2015) advised at least eight replicates per PCR to reduce false-positives when the occupancy (presence/absence) of a species is unknown. In this study, occupancy of the target species is known from the visual observations, and therefore, false-positives were not considered an issue and increasing the number of replicates is unlikely to have changed the outcome of this study.

The sensitivity of the PCR method used is also likely to influence the probability of detection. While qPCR is considered sensitive to low concentrations of target DNA as was evident in its ability to detect mackerel DNA in the Icelandic eDNA sample and to amplify highly diluted positive controls of killer whale DNA, the lack of killer whale eDNA suggests that ultra-sensitive methods such as ddPCR (Baker et al., 2018; Doi et al., 2015; Nathan, Simmons, Wegleitner, Jerde, & Mahon, 2014) or CRISPR-Cas (Williams et al. 2019) may be essential, unless the biomass of the target species and/or environmental conditions allows for detection using less sensitive methods. Further comparison of the field and laboratory methodologies used by Baker et al. (2018) and the current study is provided in Table S3.

4.1 | eDNA and conservation

Much of the published eDNA literature highlights the use and applications of eDNA as an important conservation tool for the monitoring of animal populations and provides compelling evidence for eDNA analysis as a potential replacement for traditional genetic sampling methods, for example, physical handling, biopsying and tagging of individuals (Deiner et al., 2017; Harper et al., 2018; Minamoto et al., 2017; Stewart, Ma, Zheng, & Zhao, 2017; Thomsen & Willerslev, 2015). While there have been many reviews and studies on best practices for eDNA studies (Alberdi et al., 2018; Spens et al., 2017) and on the influence of abiotic and biotic factors on the persistence of eDNA in aquatic systems (Barnes et al., 2014; Coble et al., 2018), very little of the eDNA published literature contain studies with false-negative results for target DNA, and thus, there is a gap in the literature. Such studies yield key information regarding those species and environments where eDNA studies were not successful, providing an important reality check to inform conservationists, management bodies and researchers on potential pitfalls in their planned studies and help to work toward optimizing their workflow to ensure successful capture of target DNA.

5 | CONCLUSION

While novel technologies and developments in the eDNA field are moving toward identifying presence, and quantifying abundance of aquatic species through water sampling, as a cost-effective alternative to non-genetic techniques, eDNA remains an emergent field of study and its effectiveness is dependent on a number of variables. For example, the type of environment (freshwater/marine, stream/lake, inshore/offshore, sheltered/non-sheltered), weather conditions, sea surface temperatures, animal behavior, body size, age, density, along with habitat use/frequency, sampling, and storage techniques are all important factors that are known to influence the production and degradation of eDNA. This study provides evidence that, at present, not all populations and types of environments may be suitable for eDNA research. In open water systems, where large bodies of water masses are constantly exchanging and adverse weather conditions can occur frequently, eDNA fragmentation and dispersion are likely to be more rapid, and thus, capturing cetacean DNA is more challenging than in more sheltered inshore regions. Predictability of habitat use and high-frequency occurrence rates of target species in a given area may be key to the success of capturing target DNA for cetaceans. However, this requires baseline data, which is scarce for cetacean populations offshore. This coupled with cold seawater temperatures in higher latitudes with potentially lower skin shedding rates, may make these regions particularly difficult for cetacean eDNA research. Further research using ultra-sensitive methods of DNA amplification such as ddPCR or CRISPR-Cas on cetacean eDNA samples would reveal if these methods would be more appropriate for non-sheltered areas.

To conclude, eDNA has proven to have many useful applications in both marine and freshwater ecosystems, but our study highlights that caution must be exercised in the interpretation of eDNA results, as one may not always be able to distinguish between true-negatives and false-negatives as we were here. Undoubtedly, eDNA has great potential in the context of cetacean monitoring and management, but as a complimentary additional tool rather than an outright replacement of tried and tested techniques. We support current recommendations that advise pilot studies be performed on new systems/organisms before implementing eDNA monitoring (Hansen, Bekkevold, Clausen, & Nielsen, 2018; Harper et al., 2018), and furthermore, we encourage the publication of unsuccessful eDNA studies to better inform the eDNA research community, reduce financial and time losses due to ineffective study designs, and thereby aid the continued success of eDNA applications in future studies.

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CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

R.P., A.E., E.D., L.M., E.E.S., E.R., T.E.R., and A.D.F. conceived the study; R.P. and F.I.P.S. carried out sample collection; R.P., E.D. and A.D.F. conducted the lab work; R.P., E.D., A.W.K.R., J.N., and A.D.F. analyzed the data; and R.P. and A.D.F. wrote the manuscript with input from all co-authors.

DATA AVAILABILITY STATEMENT

Sequence data will be deposited in Dryad upon acceptance.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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