Marine Mammal Science

ARTICLE

Evaluating the Use of Environmental DNA for Pinniped Detection and Population Genetics at Haulout Sites

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ABSTRACT

Population genetics is an important tool in marine resource management, but the collection of genetic data for marine mammals can be physically invasive and logistically challenging. Environmental DNA (eDNA) provides a non-invasive method for monitoring the presence of marine mammals. However, effective applications are limited by gaps in our knowledge about how eDNA behaves in the marine environment, and its use for population genetics is an emerging area. Here, we test the application of eDNA methods for detection and characterization of intraspecific genetic diversity in gray seals (*Halichoerus grypus atlantica*) in the Western North Atlantic. Our objectives were to quantify gray seal eDNA concentration around haulout sites and to test the ability of eDNA sampling to detect mitochondrial control region haplotypes. Gray seal eDNA was consistently detected 50 m from seal haulouts and could be detected up to 150 m from shore. Up to 14 haplotypes were detected in a single water sample, and we observed a positive correlation between the frequency of haplotypes detected in our eDNA samples and the frequency of those haplotypes in the population, as determined by previous tissue sampling. This work demonstrates the ability of eDNA sampling to capture broad population genetic patterns from seals at haulout sites, with important considerations for future use in non-invasive monitoring and management.

1 | Introduction

Across protected and exploited species, wildlife management hinges on the successful identification of appropriate management units. Termed stocks, conservation units, distinct population segments, or otherwise, depending on the defining management structure, these units are intended to reflect populations of species that are demographically independent (Dizon et al. 1992; Moritz 1994; Waples 1991). Such population structure can be particularly challenging to define in marine systems without apparent physical barriers. Population genetics has therefore become one of the primary tools used in marine resource management, from fisheries to marine mammals (Morin and Dizon 2009; Waples et al. 2008).

Conventional methods for obtaining genetic data often involve collecting samples of tissue, blood, or feces. However, the collection of such samples can be physically invasive, cause stress, or be difficult to achieve, particularly for species that are rare, elusive, or protected (Adams et al. 2019; Sigsgaard

et al. 2020). Additionally, a single blood, tissue, or fecal sample provides genetic information on only a single individual, and thus a large number of samples must be collected to sufficiently represent the diversity present in a population (Phillips et al. 2019). For many marine mammals, the logistical challenges of locating, capturing, or biopsying individuals make conventional collection methods laborious, limited in scope, or constrained to stranded or dead individuals (Parsons et al. 2018; Van Cise et al. 2024). The permitting and technical demands of such work also limit participation in monitoring (Adams et al. 2019). The development of non-invasive sampling techniques may expand participation and sampling capacity for obtaining genetic data on marine mammals while minimizing impacts on individuals.

Environmental DNA (eDNA) methods have emerged over the past decade as a promising alternative or supplementary approach to traditional tissue-based sampling of marine mammals (Suarez-Bregua et al. 2022; Székely et al. 2022). eDNA approaches use highly sensitive molecular methods to analyze genetic material isolated from environmental samples of water, soil, or air (Thomsen and Willerslev 2015). To date, species-specific eDNA assays have been developed and vetted for the detection of several species of marine mammals (e.g., Mediterranean monk seal, *Monachus monachus*, Valsecchi et al. 2022; humpback whale, *Megaptera novaeangliae*, Andruszkiewicz et al. 2020; manatees, *Trichechus spp*, Hunter et al. 2018), and many marine mammal species have been detected in metabarcoding studies of vertebrate assemblages (Closek et al. 2019; Djurhuus et al. 2020; Sevellec et al. 2021; Stoeckle et al. 2021). In addition, emerging research suggests the potential utility of eDNA-based approaches for population genetics (Adams et al. 2019; Parsons et al. 2018, 2024; Sigsgaard et al. 2020; Von Duyke et al. 2023). A few studies have sequenced mitochondrial control region haplotype variants in water samples collected in the near vicinity of diving cetaceans, including bowhead whales (*Balaena mysticetus*; Székely et al. 2021), killer whales (*Orcinus orca*; Baker et al. 2018), and harbor porpoises (*Phocoena phocoena*; Parsons et al. 2018; Parsons et al. 2024). While most of these studies have focused on capturing genetic information from a single individual, Parsons et al. (2018) reported detecting multiple haplotypes from a single water sample collected in the vicinity of a group of harbor porpoises, and thus demonstrated the potential for species-specific eDNA approaches to capture genetic information from multiple individuals.

Here, we expand eDNA sampling to pinniped haulouts to further explore the extent to which eDNA can be encountered and used to detect haplotype diversity from water samples. Pinnipeds, including seals, sea lions, and walruses, spend time both on land and at sea, and many species aggregate in large numbers at land-based haulouts. These large aggregations are often predictable and accessible, and as such, they also provide an opportunity to improve our understanding of the dynamics of eDNA (e.g., persistence and transport) in marine and coastal environments, uncertainty around which continues to hamper the application of eDNA data to management scenarios in many contexts (Mathieu et al. 2020).

In this study, we analyzed eDNA samples collected near several large aggregations of gray seals (*Halichoerus grypus atlantica*) on the coast of Cape Cod, Massachusetts, USA. Gray seals in the Western North Atlantic exemplify a complicated conservation scenario where population growth following historical depletion has provoked both celebration and concerns for ongoing management (Cammen et al. 2019). Following a decade of exponential growth facilitated by immigration from Canadian breeding colonies, increased habitat availability for pupping, and high dispersal capabilities (Wood et al. 2020), the ecological role of gray seals as high trophic predators has prompted questions about how this species should be managed moving forward (Cammen et al. 2019). Effective management of this species requires understanding its genetic diversity and population structure, which to date have been studied using extensive tissue-based sampling at haulouts across the Western North Atlantic (Cammen, Schultz, et al. 2018; Cammen, Vincze, et al. 2018; Wood et al. 2011). These genetic sampling efforts provide a strong foundation against which we can compare eDNA-based haplotyping efforts to better understand how these emerging tools could supplement traditional methods for characterizing intraspecific diversity.

Quantifying levels of intraspecific genetic diversity within and between populations is fundamental to population genetic approaches used in marine mammal management, but eDNA-based metrics of population diversity have only recently begun to be developed and employed (notably in Bennington et al. 2024 and Parsons et al. 2024). Here, we evaluate the potential for eDNA-based approaches to accurately capture population-scale summaries of genetic diversity, including haplotype diversity and frequency, in a well-studied population of gray seals. Specifically, the objectives of the current study are (1) to develop a gray seal quantitative PCR (qPCR) assay and use it to quantify spatial variation in gray seal eDNA around haulouts in relation to sampling distance from the haul out and seal abundance; and (2) to identify gray seal mitochondrial haplotypes captured via eDNA sampling and compare these to haplotype diversity obtained from past tissue and concurrent fecal sampling. Based on our findings, we discuss the capacity and limitations of eDNA-based tools to support decision-making for pinniped management, as well as offer evidence-based recommendations for eDNA sampling designs around pinniped haulouts.

2 | Methods

2.1 | Sample Collection

Surface seawater samples were collected around gray seal haulout sites on Cape Cod, Massachusetts, USA during the summers of 2020 and 2021, and fall 2022 (Tables 1 and 2, Figure S1). Pre-sterilization of collection equipment was performed following the "CCS Seal eDNA collection standard protocols" (Supporting Information: Protocols). Water samples were collected in paired replicates consisting of two 1 L bottles filled by hand with surface seawater from 4 to 7 m survey vessels (Supporting Information: Protocols). At each haulout, water samples were collected 50 m away from the haulout at the beginning and end of a transect that ran parallel to the shore. Transects were typically run opposite to the primary alongshore direction of water flow, collecting the first pair of samples immediately downstream and the second pair immediately

TABLE 1 | Characteristics of each haulout surveyed. At dry haulouts, seals were resting on sandy beaches. At wet haulouts, seals were observed resting in the water.

Site name	Site abbreviation	Latitude/longitude (°)	Date/time	Seal abundance	Wet/dry haulout
Chatham Harbor	CH1	41.6863, −69.94731	6/23/2020 9:13	280	Wet
Provincetown Harbor	PTH1	42.03114, −70.18263	6/25/2020 8:35	140	Wet
Chatham Harbor	CH2	41.68631, −69.94718	7/6/2020 8:24	290	Wet/dry
Pleasant Bay	PB	41.70627, −69.94272	7/6/2020 9:07	240	Dry
Provincetown Harbor	PTH2	42.02909, −70.18018	7/21/2020 6:30	44	Wet
Tern Island	TI1	41.689166, −69.94972	8/3/2020 7:02	125	Dry
Strong Island	SI1	41.71555, −69.94194	8/3/2020 8:05	140	Dry
Strong Island	SI2	41.71482, −69.94431	8/19/2020 8:25	95	Dry
Long Point, Provincetown	LPT1	42.034983, −70.16783	8/25/2020 10:16	325	Dry
Tern Island	TI2	41.69925, −69.94979	9/17/2020 7:08	107	Dry
Provincetown Harbor, West End	PTWE	42.0327, −70.1851	6/30/2021 9:50	119	Wet
Long Point, Provincetown	LPT2	42.0356, −70.16762	7/13/2021 6:40	337	Dry
Long Point, Provincetown	LPT3	42.0354, −70.1669	7/24/2021 5:48	222	Dry
Billingsgate Shoals	BGS	41.8665, −70.05921	8/25/2021 6:50	85	Dry
Long Point, Provincetown	LPT4	42.03551, −70.16766	9/6/2021 7:38	146	Dry
Provincetown Harbor	PTH3	42.03205, −70.18263	9/7/2021 17:25	35	Wet
Long Point, Provincetown	LPT5	42.0352, −70.16841	9/14/2021 11:07	269	Dry
Tern Island	TI3	41.689133, −69.9499	9/21/2021 7:39	165	Dry
Long Point, Provincetown	LPT6	42.02447, −70.17324	11/15/2022 9:27	18	Wet

upstream of the haulout. Following the transect, the haulout was approached, and a third pair of samples was collected near the shoreline (within 1 m, hereafter referred to as 0 m samples) from the approximate center of the haulout, often as or after seals flushed from the beach into the water. In 2021, a sample pair was also collected 30 m from shore at the midpoint of the haulout after the shoreline sampling was completed. Sampling was further expanded during two surveys in August and early September 2021 to include an additional sample pair collected 100 m downstream of the haulout along the initial 50 m transect and three samples taken along each of two additional transects running parallel to shore at 100 and 150 m from the haulout. In 2020, fecal samples from individual seals were also opportunistically collected from the sand at terrestrial haulouts after eDNA

TABLE 2 | Number of eDNA filters, fecal samples, and filtration controls collected during each survey and at various distances from shore.

Site name	Site abbreviation	eDNA filters by distance from shore					Fecal samples	Filtration controls
		0 m	30 m	50 m	100 m	150 m		
Chatham Harbor	CH1	4[a]		4			4	2
Provincetown Harbor	PTH1	3[b]		4			1	2
Chatham Harbor	CH2	2		4			2	2
Pleasant Bay	PB	2		4			5	
Provincetown Harbor	PTH2	2		4				2
Tern Island	TI1	2		4			4	2
Strong Island	SI1	2		4			3	
Strong Island	SI2	2		4				2
Long Point, Provincetown	LPT1	2		4			5	2
Tern Island	TI2	2[a]		6[c]			8	2
Provincetown Harbor, West End	PTWE	2	2	4				2
Long Point, Provincetown	LPT2	2	2	4				2
Long Point, Provincetown	LPT3	2	2	4				2
Billingsgate Shoals	BGS	2	2	5[c]	6	6		2
Long Point, Provincetown	LPT4	2	2	6	6	6		2
Provincetown Harbor	PTH3	2	2	4				2
Long Point, Provincetown	LPT5	2	2	2[c]				2
Tern Island	TI3	2	2	4				2
Long Point, Provincetown	LPT6	2		4				2
Total filter sample size		41	16	79	12	12	32	34

[a]In some cases, water samples collected near shore had to be split across more than two filters due to high particle content in the water that clogged filters.
[b]More than two samples were taken close to shore at a haulout when there were multiple groupings of animals separated by short distances on the beach.
[c]Issues during filtering (e.g., due to human error or technical malfunction) occasionally altered the number of eDNA filters per site.

sampling (Hudak and Sette 2019). Water and fecal samples were stored on ice in separate sealed bags.

After each survey, water samples were filtered within 12 h at a shore-based lab through 0.45 μm cellulose nitrate filters on a three-cup vacuum manifold (Baker et al. 2018; see Filtration Protocols in Supporting Information: Materials). Up to 1 L of water was processed per filter, using approximately equal portions of the replicate 1 L samples collected at each transect location. Each sampling event thus typically resulted in two filters, each integrating up to 500 mL of water from each 1 L bottle. Filtration controls were collected on each survey day by filtering 1 L of tap water before and after filtering the seawater samples. Filters and fecal samples were frozen at −20°C until extraction.

2.2 | Sample Processing

Water and fecal samples were processed in parallel to extract, quantify, and characterize gray seal DNA (Figure 1). Water samples yielded information about the concentration of gray seal DNA and the number and types of genetic variants (i.e., haplotypes) in the nearshore environment. In complement, fecal samples were each associated with a single genetic variant, representing individual gray seals present on the beach. Water samples, which may contain DNA from multiple individuals, require highly parallel (next-generation) amplicon sequencing, while fecal samples that contain DNA from a single individual can be characterized with traditional Sanger sequencing.

DNA was extracted from a total of 194 filters, including 160 environmental filters and 34 filtration controls, and 32 fecal samples. Extractions from filters were performed using the Qiagen DNeasy Blood and Tissue kit in a molecular lab dedicated to pre-PCR eDNA processes, following the manufacturer's protocol for tissue extraction with the following modifications. To ensure filters were fully submerged in extraction reagents, volumes of lysis Buffer ATL and Proteinase K were doubled during the initial incubation at 56°C for 1.5 h. Following incubation, filters were transferred from the lysis tube to Qiagen Lyse and Spin Baskets and centrifuged at 16,000 rcf (xg) for 5 min in order to extract all remaining liquid that was pooled with the lysis extract. The lysis extract was then purified using double volumes of Buffer AL

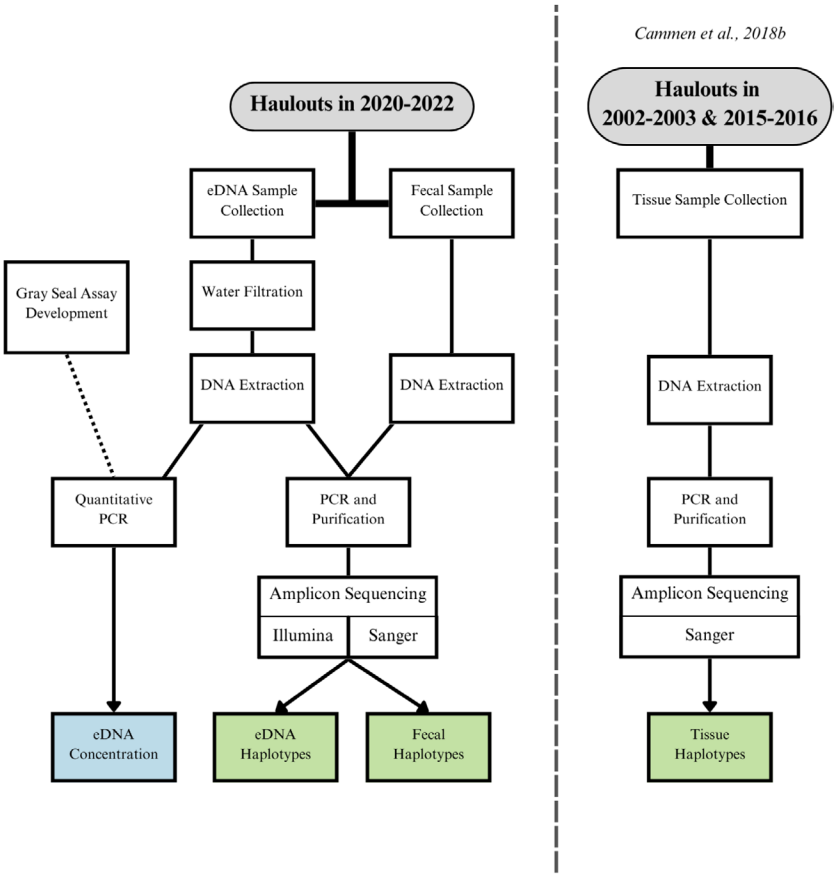

FIGURE 1 | Workflow overview, including sample collection, laboratory processing, and data generation. Prior work to identify tissue-based haplotypes in this population is further described in Cammen, Vincze, et al. (2018).

and ethanol on QIAamp Mini spin columns. Following lysis and purification steps, 100 μL of Buffer AE was added to each mini spin column and incubated at 56°C for 5 min prior to final elution. Fecal samples ($n = 32$) were extracted using the Qiagen QIAamp Fast DNA Stool Mini Kit in a separate lab. Extractions followed the manufacturer's protocol with the following modification: samples were centrifuged at full speed for 2 min following the addition of InhibitEX buffer and 1-min vortex. Extraction controls with no filters or fecal material were included with each set of extractions ($n = 31$) to detect contamination introduced during the extraction process, such as contamination of reagents or pipettes. DNA extracts were stored at −20°C until further processing.

2.3 | qPCR Assay Development

To develop a gray seal qPCR assay for use in eDNA, we designed species-specific primers through comparison of publicly available mitogenome sequences for all seal species present in the Gulf of Maine and North Atlantic Arctic (gray seal: NCBI Accession No. KU963302; bearded seal [*Erignathus barbatus*]: AM181027, harbor seal [*Phoca vitulina*]: X63726, harp seal [*Pagophilus groenlandicus*]: AM181030; hooded seal [*Cystophora cristata*]: AM181028; ringed seal [*Pusa hispida*]: NC_008433). We performed *in silico* design and initial testing using Benchling, a free, browser-based program. We surveyed all mitochondrial gene regions, selecting by eye primer regions that contained the most nucleotide differences between target (gray seal) and non-target species, while limiting self or cross complementarity issues within the primers. We selected two best primer set candidates: a 125 base pair region of NADH dehydrogenase 6 (ND6) and a 106 base pair region of NADH dehydrogenase 4 (ND4). We tested for primer specificity *in silico* using the seal mitogenome sequences listed above as well as NCBI Primer-BLAST (Version 2.10.0) against all records (Altschul et al. 1990; Ye et al. 2012). Primer-BLAST was used for basic complementarity testing, and more in-depth oligo testing was performed using the IDT OligoAnalyzer tool.

Candidate primers were tested in vitro for basic specificity against DNA extracts from tissues of gray seals and the most common non-target species, harbor seals. PCR reactions were composed of 10 μL Taqman Environmental Master Mix 2.0 (Applied Biosystems), 500 nM primers, 1 μL of template DNA, and nuclease-free water to bring the volume to 20 μL. The thermal protocol was 95°C for 10 min, followed by 36 cycles of 95°C for 10 s and 60°C for 30 s. Amplification was checked on a 1.5% agarose gel. Following this test, the ND6 primer set was rejected for cross-amplification of non-target species; the ND4 primer set (Forward: 5′-CCCACGGTTTAACATCATCCGTAT-3′; Reverse: 5′-GGGAGGAGCACTTGCAGA-3′; located at positions 12,063 and 12,128, respectively, on the gray seal mitochondrial genome KU963302) was retained with good target amplification and no non-target amplification. A Taqman probe was also designed using the same process used to design the primers, where sequence

alignments were used to maximize base pair differences. The resulting probe (5′-TTCCAACTACGAACGCACCCAC-3′; located at position 12,101 on the gray seal mitochondrial genome KU963302) used FAM for the fluorescent dye and a NFQ quencher.

The qPCR protocol for the ND4 primer set was further optimized through testing of multiple primer and probe concentrations and annealing temperatures. We tested a series of four concentrations using a six-member dilution series of gBlock synthetic standard (31,250, 6250, 1250, 250, 50, 10 copies/μL). The gBlock consisted of a 133 basepair fragment of the ND4 gray seal gene exactly matching the qPCR assay target region: 5′-AGCCCTAATAATCGCCCACGGTTTAACATCAT CCGTATTATTCTGCCTAGCCAATTCCAACTACGAACGC ACCCACAGTCGAACTATAATCCTCGCACGCGGTCTGCA AGTGCTCCTCCCCTTAATAGCAGCC-3′. Each qPCR reaction was composed of 10 μL Taqman Environmental Master Mix 2.0 (Applied Biosystems), 2 μL of primer-probe mix at variable concentration (Primers 2 μM–250 nM, Probe 1 μM–125 nM), 1 μL of gBlock (Integrated DNA Technologies), and nuclease free water to bring the volume to 20 μL. The TaqMan Environmental Master Mix is optimized for detecting low-concentration targets and reducing the effects of inhibitors that may be present in environmental samples. qPCRs were run on a Bio-Rad CFX-96, with the following thermal protocol: 95°C for 10 min, followed by 50 cycles of 95°C for 10 s and 60°C for 30 s. We further tested an annealing temperature gradient of 53°C–62°C. The optimal protocol was that which yielded the lowest Cq values (defined here as the PCR cycle at which fluorescence rises above a baseline, indicating amplification) while maintaining amplification in all members of the standard curve and appropriate qPCR efficiency (95%–105%). This optimum was achieved in a 20 μL qPCR reaction composed of 10 μL Taqman Environmental Master Mix 2.0 (Applied Biosystems), 1 μM primers, 500 nM probe, 1–3 μL template DNA, and nuclease free water to bring the volume to 20 μL, run with an annealing temperature of 60°C. qPCR efficiency for this primer-probe set was determined to be 98.83%, with y-intercept of 39.42 and R^2 of 0.9957, and detections were successful in the laboratory down to the lowest concentration tested, 10 copies/reaction (as such, limits of detection and quantification were not computed).

Finally, the specificity of the optimized primer and probe set was confirmed using DNA extracts from all seal species present in the Gulf of Maine. We used DNA (20–25 ng/μL) previously extracted from skin samples collected from wild ($n = 3$ gray seals from Muskeget Island, Massachusetts, US; $n = 3$ gray seals from Sable Island, Canada; $n = 3$ harbor seals from Gulf of St. Lawrence, US) or bycaught animals ($n = 3$ harbor seals and 1 harp seal from the Northeast US), as well as DNA extracted from filtered 1 L water samples collected in pools holding harp ($n = 3$), hooded ($n = 1$) and harbor seals ($n = 4$) at captive rehabilitation centers in the Northeast US.

2.4 | qPCR

We used our qPCR assay to quantify the concentration of gray seal DNA in all water samples collected in 2020 and 2022, as well as during the two extended surveys in 2021 and all corresponding filtration and extraction controls. Samples were analyzed in triplicate using the master mix, thermal protocol, and six-member dilution series of gBlock synthetic standard described above, with 3 μL of eDNA template and 1 μL of synthetic seal-sequence DNA (gBlocks). Triplicate no-template controls were included on each plate to detect contamination introduced during the qPCR process. The gBlock dilution series provided positive controls for each plate and a means for correcting for interassay variation between samples run on different plates (Bustin et al. 2009). The concentration of gray seal eDNA in copies per mL seawater was estimated using each sample's mean Cq value, elution volume, filter volume, and the standard curve obtained from the gBlocks run on each plate. Positive detection was defined as any sample where at least one of three qPCR replicates amplified before cycle 45, a highly sensitive threshold designed to capture detections even of environmentally relevant low concentrations of target DNA. A lack of false positive detections in numerous no-template qPCR controls affirms that detections even at this high cycle threshold were not due to primer dimers or other PCR artifacts.

2.5 | Sequencing

All eDNA and fecal samples from 2020 and the short-range eDNA samples from 2021 were selected for amplicon sequencing. For samples collected in 2021, this included samples collected 0, 30, and 50 m from shore in the immediate vicinity of the haulout. A fragment of the mitochondrial control region, ranging in length from 381 to 384 bp due to indel mutations, was sequenced in eDNA and fecal samples using next-generation and Sanger sequencing, respectively. This region was amplified using the following primers (HgCReF2: 5′-CTGACATCCACTCAACCCCC-3′; HgCReR2: 5′-TGGATTCTAGGACTTTCATGGC-3′; located at positions 16,361 and 16,743, respectively, on the gray seal mitochondrial genome KU963302) that were designed from the visual alignment of gray and harbor seal mitochondrial control region sequences previously described in this population by Cammen, Vincze, et al. (2018). Aiming to be species-specific, these primers were located in regions that maximized differences between gray seals and harbor seals and minimized variation within gray seals. Primer3 (release 0.4.0) was used to evaluate the suitability of multiple primer pairs in this region, and the final primers were selected after trialing two forward and two reverse primers on DNA extracted from filtered 1 L water samples collected in pools at captive rehabilitation centers, using the process described above for qPCR assay optimization to ensure strong target amplification and a lack of non-target amplification.

Amplification of fecal samples was conducted via PCR in a single 20 μL reaction per sample, with 1× Colorless GoTaq Flexi Buffer (Promega), 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.3 μM of each primer, 0.10 μL of GoTaq Flexi DNA Polymerase (Promega), 0.6 mg/mL BSA, and 10 μL of the fecal DNA extract. The PCR used the following temperature profile: initial denaturation step at 94°C for 5 min; 10 initial touchdown cycles of 94°C for 30 s, 65°C decreasing by 1°C each cycle for 1 min, and 72°C for 1 min; an additional 50 amplification cycles with an annealing temperature of 55°C; and a final elongation step at 72°C for

45 min. Fecal PCR products were visualized on agarose gels, purified using Exonuclease I and Antarctic Phosphatase, and sequenced in both directions by Eurofins Genomics (Louisville, Kentucky, USA).

Amplification of eDNA samples was similarly conducted via PCR, except with 35 post-touchdown amplification cycles instead of 50, using the same primers as fecal samples, but with the addition of TruSeq Illumina adapters. To test for variation among PCR reactions and sequencing runs, half of the 2021 samples were amplified in triplicate to be sequenced on a single run, and all samples from 2020 were amplified twice to be sequenced on separate runs. Otherwise, each sample was amplified as a single reaction. eDNA PCR products were purified with Beckman Counter Agencourt AMPure XP beads at 1.8× concentration using the KAPA Pure Beads (KR1245-v3.16) magnetic bead protocol, and purified products were visualized on an agarose gel. Samples with visible bands were sequenced at the Hubbard Center for Genome Studies (University of New Hampshire, USA) on an Illumina NovaSeq (2 × 250 bp). Positive controls consisting of gray seal tissue extract and negative no-template PCR controls were also included in each amplicon sequencing run. All PCR products for filtration and extraction controls from 2021 surveys (which were not analyzed via qPCR) were observed to be negative via gel visualization and therefore were not sequenced.

Mock haplotype pools and resequencing of environmental samples were used to assess how sequence composition may be impacted by PCR and sequencing processes. Four mock haplotype pools were created using tissue samples of known haplotypes and concentrations, as described in the Supporting Information: Methods and Table S1. The mock haplotype pools and half of the samples from the 2021 surveys were amplified and sequenced in triplicate on a single sequencing run. Additionally, samples from 2020 were amplified and sequenced twice on separate runs.

2.6 | Sequence Processing

Haplotypes obtained from fecal samples via Sanger sequencing were manually edited in Codon Code Aligner v 8.0.1 in order to remove primer sequences and correct mismatches. Sequences obtained from water samples via next-generation sequencing were analyzed using the DADA2 pipeline (Callahan et al. 2016) in RStudio (R version 4.2.2; R Core Team 2023). Primer sequences were removed using Cutadapt (Martin 2011), and then sequences were quality checked, filtered, and trimmed in DADA2. Any reads shorter than 175 nucleotides were removed, reads were truncated after 220 nucleotides, and no ambiguous bases were allowed. Sequences were then dereplicated and, following error rate estimation, sample inference was conducted in DADA2 to infer amplicon sequence variants (ASVs), merge paired reads, and remove bimeras. Sequencing runs were analyzed separately and then the resulting ASV tables were merged. The final list of ASVs and fecal sequences were compared to haplotypes identified in this region (GenBank: KY793030–KY793067) from previous tissue sampling in order to identify matches between sequences obtained from eDNA sampling, fecal sampling, and tissue sampling.

The presence and proportion of erroneous sequences in the four mock haplotype pools were used to define read proportion thresholds for an ASV to be considered a detected haplotype within each sample. To do this, we conducted a sensitivity analysis by plotting how the number of true positives, false positives, and false negatives within each mock haplotype pool changed as increasingly stringent read thresholds were applied (1%–5%, see Supporting Information: Results and Table S2). Based on these plots, we defined two read proportion thresholds, 2% and 5%, where the filtering was most effective at removing errors while still retaining true haplotypes. These specific thresholds were chosen because the lower 2% threshold served to remove as many errors as possible while retaining all or almost all of the true positives, while the more conservative 5% threshold removed every erroneous sequence from the mock haplotype pools at the expense of also removing true positives present at low frequencies. ASVs that exceed the given read threshold are referred to as eDNA haplotypes throughout the manuscript, though we acknowledge that they may also include erroneous sequences. Unless otherwise specified, the results presented here are from analyses run using the 2% threshold. Additionally, following the comparison of PCR triplicates, all further analyses were conducted on a single, randomly selected PCR replicate for samples in which multiple replicates were sequenced. For samples from 2020 that were sequenced twice, sequences from the first sequencing run were used due to more equal partitioning of sequencing reads among samples.

2.7 | Data Analyses

The occurrence of each eDNA haplotype was calculated as the number of samples in which the proportion of reads attributed to that ASV surpassed the read threshold. The overall frequency of each eDNA haplotype was calculated by summing the proportion of reads attributed to that ASV across a randomly selected replicate of each sample. ASV accumulation curves were constructed using the specaccum function (method = "random") in the R package vegan (v2.6-10) (Oksanen et al. 2024) to assess haplotype detection saturation under our sampling scheme. Alpha diversity in the form of haplotype richness (total number of ASVs that surpass the read threshold) and Shannon's diversity index (measure of richness and evenness that evaluates number of ASVs and relative read frequency) was calculated for each sample. Diversity metrics were compared between samples collected at varying distances from shore using a Tukey multiple comparisons of means test, implemented in the stats package (v3.6.2) in R.

Haplotype composition was further compared between samples, within replicates, and within and between haulouts using Bray-Curtis pairwise dissimilarity calculated using the vegan package in R. To evaluate the degree of variation found within replicate types (PCR, sequencing, and field), average pairwise dissimilarity was compared using a Tukey multiple comparisons of means test, as described above. Similarly, average pairwise dissimilarity was compared among samples collected within and between haulouts at varying distances from shore, and a non-metric multidimensional scaling (NMDS) ordination based on Bray-Curtis distance was generated in the phyloseq (v1.16.2) package in R

(McMurdie and Holmes 2013) to visually assess clustering by haulout site or distance.

Linear models built in the stats R package were used to test the extent to which variation in log-transformed eDNA concentration could be explained by distance from shore and seal abundance at each haulout site. The log-transformed eDNA concentrations were not significantly different from a normal distribution (Shapiro–Wilk normality test, $W = 0.97$, p value $= 0.21$), and a traditional linear model more closely met the assumptions of homoscedasticity and normally distributed residuals than a comparable generalized linear model on untransformed data with a log link function. Generalized linear models were used to test the explanatory power of the same covariates on haplotype richness and diversity. Generalized linear models were used here as these response variables were more appropriately represented by Poisson and Gamma distributions than Gaussian, and were determined to sufficiently meet model assumptions through the review of residual diagnostic plots. Generalized linear models predicting haplotype richness used a Poisson distribution with a log link function, and those predicting haplotype diversity used a Gamma distribution with an inverse link function. Models of each type were compared by their AIC values, with the best model having the lowest AIC. Models within two AIC of each other were considered comparable, and covariates with p values less than 0.05 were considered significant.

Finally, our eDNA haplotypes were compared to those obtained from our fecal samples and previous tissue sampling (Cammen, Vincze, et al. 2018). We calculated each eDNA haplotype's occurrence and cumulative frequency as described above. These metrics were compared against the frequency of each haplotype across tissue samples collected in the area in 2002–2003 and 2015–2016 (Cammen, Vincze, et al. 2018). Concordance between the two eDNA metrics and tissue-based haplotype frequency was assessed with non-parametric Spearman rank correlation tests. eDNA haplotypes were also compared to haplotypes obtained from fecal samples collected concurrently during surveys in 2020. Non-parametric Wilcoxon rank sum tests were used to compare the prominence of haplotypes that were detected by both methods at the same haul-out against those that were not.

3 | Results

3.1 | Quality Control

We tested for evidence of systematic field- or lab-based contamination of eDNA samples at multiple stages in the process (pre- and post-filtration, DNA extraction, PCR and qPCR). Potential contamination was detected in 0 pre-filtration controls ($n = 17$), 2 of 17 post-filtration controls (0.021 copies/mL detected for survey SI2 on 8/19/2020; 0.040 copies/mL detected for survey LPT1 on 8/25/2020), 0 qPCR no template controls ($n = 11$), and 1 of 31 extraction controls (0.18 copies/mL, standardized to a 1 L filter volume, detected for survey CH1 on 6/23/2020). The filtration controls reflect a subsampling of contamination that may be present on the filtering equipment, but given the low detection rate and low concentrations present when detected, we assume they do not indicate survey-wide contamination of samples and these detections were not used to exclude surveys from the dataset. In contrast, the extraction controls test for procedural contamination during extractions (e.g., of reagents or pipettes), and therefore all environmental samples ($n = 8$) from the potentially contaminated extraction set were removed from the dataset.

3.2 | eDNA Concentration

Following removal of samples that did not pass quality control (see above), gray seal eDNA amplified in all 2020 environmental samples collected up to 50 m from shore, with mean Cq values ranging from 25.05 to 48.77 (mean $= 35.01 \mp 5.45$). Some variation between qPCR replicates was observed, and this intraassay variation (Bustin et al. 2009) was quantified as the standard deviation in Cq values for the three qPCR replicates of each sample. The mean and median standard deviation in Cq value for samples collected in 2020 were 0.65 and 0.38, respectively. Fifty-five (96.5%) samples from 2020 passed the positive detection threshold (at least one of three qPCR replicates amplified before cycle 45) with concentrations ranging from 0.01 to 701.50 (mean $= 64.31 \mp 159.65$) copies per mL. Among the environmental samples, there was a significant difference in gray seal eDNA concentration between samples collected 50 m away from haulout sites (mean concentration $= 0.77 \mp 1.41$ copies per mL seawater) and samples taken near the shoreline after seals had flushed into the water (184.62 ∓ 230.48 copies per mL seawater) (Wilcoxon rank sum test $p = 7.14 \times 10^{-8}$). This pattern was consistent across all haulout sites, despite variation in gray seal eDNA concentrations among sites (Figure 2A).

Environmental samples ($n = 43$) from the two extended surveys (Billingsgate and Long Point) conducted in 2021 were also analyzed with qPCR to further test the spatial range of detection. From these surveys, gray seal eDNA was positively detected in 10 of 21 (47.62%) samples from the survey at Billingsgate Shoals (85 seals present) and 22 of 22 samples (100%) from the survey at Long Point (146 seals present); mean Cq values in positive samples ranged from 25.95 to 41.76 (mean $= 35.89 \mp 5.85$) at Billingsgate Shoals and 25.93 to 45.14 (mean $= 35.08 \mp 4.30$) at Long Point. The mean and median standard deviation in Cq value for samples collected in 2021 were 0.65 and 0.32, respectively. Concentrations ranged from 0.01 to 215.85 (mean $= 32.93 \mp 72.83$) copies per mL at Billingsgate Shoals and < 0.01 to 429.03 (mean $= 26.40 \mp 92.64$) copies per mL at Long Point. Positive detections were obtained from samples collected at the furthest distance from shore (150 m) in both surveys. Again, the highest eDNA concentration from both surveys was obtained from samples taken at the shoreline (Figure 2B).

Gray seal eDNA was detected in three of the six (50%) environmental samples collected in 2022 at the smallest sampled haulout (LPT6—18 seals observed), specifically both samples collected 50 m offshore downstream of the haulout, and in one of the two samples collected at the shoreline. Mean Cq values ranged from 33.31 to 41.92 (mean $= 36.56 \mp 4.67$), mean and median standard deviation in Cq value were 0.54 and 0.33, respectively, and

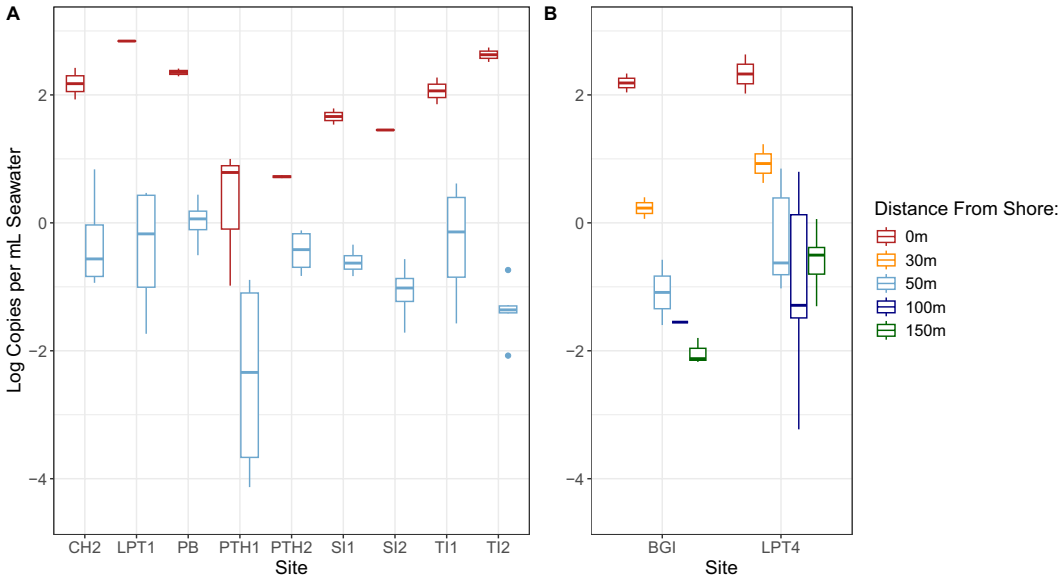

FIGURE 2 | Log-transformed gray seal eDNA concentration (copies/mL) by haulout site and distance from shore. Box A depicts haulout sites sampled in 2020 at the shoreline and 50 m offshore. Box B depicts the attenuation of eDNA signal with distance during two extended surveys in 2021.

TABLE 3 | Model selection for gray seal eDNA concentration, haplotype richness, and haplotype diversity. ΔAIC is in reference to the lowest AIC of any model in that group.

						AIC	ΔAIC	*R*-squared
eDNA concentration, $n = 50$								
log (eDNA) ~	**distance***	+	**seal abundance***			96.97	0.00	75.82%
log (eDNA) ~	**distance***	+	**seal abundance***	+	dist × seal	98.09	1.12	76.27%
log (eDNA) ~	**distance***					108.59	11.61	67.70%
log (eDNA) ~			seal abundance			160.18	63.21	3.17%
						AIC	**ΔAIC**	**% deviance explained**
Haplotype richness, $n = 69$								
Richness ~	**distance***	+	seal abundance	+	**dist × seal***	364.35	0.00	10.57%
Richness ~	**distance***					366.27	1.92	5.03%
Richness ~	**distance***	+	seal abundance			367.60	3.26	5.66%
Richness ~			seal abundance			371.49	7.15	0.15%
Haplotype diversity, $n = 69$								
Diversity ~	distance					196.87	0.00	1.05%
Diversity ~			seal abundance			197.65	0.78	0.038%
Diversity ~	distance	+	seal abundance			198.87	2.00	1.05%
Diversity ~	distance	+	seal abundance	+	**dist × seal***	199.64	2.77	2.62%

*Indicates statistical significance of covariates ($p < 0.05$) and the bold is meant to highlight the significant terms.

concentrations ranged from < 0.01 to 2.65 (mean $= 1.29 \mp 1.32$) copies per mL.

Linear models were used to evaluate the impacts of distance to shore and seal abundance on log-transformed eDNA concentration (Table 3). eDNA concentration was found to decrease with distance from shore ($p < 1 \times 10^{-14}$) and increase with seal abundance ($p < 0.001$) (Figure 3A), with distance having a larger effect (estimate $= -0.045$) than the number of seals present (estimate $= 0.004$). Together, these factors explained 75.82% of observed variance in eDNA concentration (Figure 3A). A second top model ($\Delta AIC = 1.12$) explained 76.27% of variance and had seal abundance and proximity to shore as covariates, as well as an interaction term. When

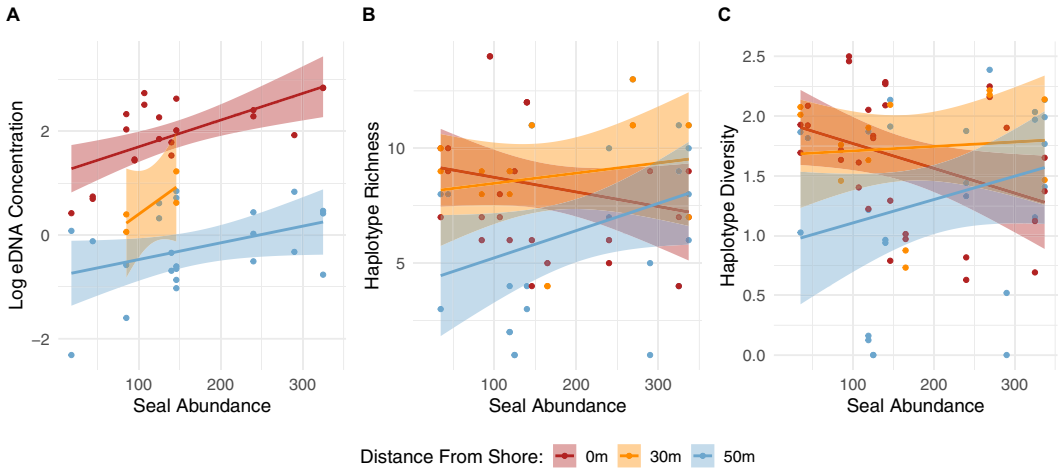

FIGURE 3 | Influence of gray seal abundance and sampling distance from shore on (A) eDNA concentration, (B) eDNA haplotype richness, and (C) eDNA haplotype diversity.

used as the sole predictor in a model, distance from shore explained more of the observed variance in eDNA concentration (67.70%) than seal abundance (3.17%).

3.3 | Gray Seal eDNA Diversity

3.3.1 | Haplotype Detection

PCR amplification for amplicon sequencing was attempted for 57 environmental samples from 2020 (shoreline and 50 m samples) and 61 samples from 2021 (shoreline, 30, and 50 m samples). In total, this consisted of 195 attempted amplifications, as approximately half ($n = 32$) of the 2021 samples were amplified in triplicate and all samples from 2020 ($n = 35$) were amplified in duplicate. In total, gray seal mitochondrial control region sequences were successfully obtained from 142 amplifications, representing 73 (62%) of the 118 environmental samples collected in 2020 and 2021. We also sequenced three positive controls and four mock haplotype pools. No sequences remained in any PCR negative controls following bioinformatic processing. Results from the mock haplotype pools are presented in the Supporting Information: Materials.

Across all samples, a total of 864 ASVs were initially identified. Once read thresholds were applied, this number was reduced to 47 ASVs under the 2% threshold and 29 ASVs under the 5% threshold. ASV accumulation curves suggest that these totals likely represent most of the dominant ASVs in the population, but that additional rare ASVs may be captured with further environmental sampling (Figure S2). The number of eDNA haplotypes detected in a single water sample ranged from 1 to 14 (mean = 7.51 ∓ 3.34) under the 2% threshold (Figure 4), and 1 to 10 (mean = 4.37 ∓ 2.05) under the 5% threshold (Figure S3).

3.3.2 | Haplotype Variation Within and Between Sites

Haplotype composition was found to be highly replicable among PCR triplicates, sequencing replicates, and field replicates (paired water samples), confirming consistency in the implementation of field and lab methods (Figure 5). Among replicates, the mean pairwise dissimilarity was lowest among PCR replicates and highest among paired water samples. Dissimilarity within each of the replicate types (PCR, sequencing, and field replicates) was significantly lower than that observed within and between haulout groups (Tukey multiple comparisons of means, adjusted p value < 0.05), with no significant difference at the within- and between-haulout scales (Figure 5). Within technical replicate groupings (sequencing or PCR replicates), we observed an increase in the number of replicates a haplotype was detected within as the average read proportion of that haplotype in that replicate group increased (Figure S4). We observed no visible clustering by haulout site or distance using an NMDS ordination based on Bray-Curtis distance (Figure S5).

3.3.3 | Factors Impacting Haplotype Diversity

As described above for eDNA concentration, we used generalized linear models to explore two primary factors that may impact haplotype richness and Shannon diversity: distance from shore and the number of seals present at the haulout site (Table 3, Figure 3B,C). Distance from shore (effect size estimate = -1.31×10^{-2}) was identified as the largest effect predictor in the best model for haplotype richness, which also included seal abundance (estimate = -7.43×10^{-4}) and the interaction of the two terms. Together with their interaction term, these factors explained 10.57% of the observed deviance in haplotype richness. A second top model ($\Delta AIC = 1.92$) included only distance as a significant term and explained 5.03% of deviance in haplotype richness. The mean number of haplotypes observed per sample was higher in samples collected closer to shore after flushing the seals (0 m: 8.33 ∓ 2.81; 30 m: 8.77 ∓ 2.65) than in samples collected during the initial transect 50 m offshore (6.46 ∓ 3.71), though the differences were not significant (Tukey multiple comparisons of means, $p > 0.05$).

Neither distance from shore nor seal abundance was significant in the top two models for haplotype diversity, which each explained less than 2% of the observed deviance in diversity. Similar to richness, Shannon diversity was nominally higher in samples collected closer to the shore after flushing the seals (0 m: 1.64 ∓ 0.54; 30 m: 1.73 ∓ 0.49) than in samples collected

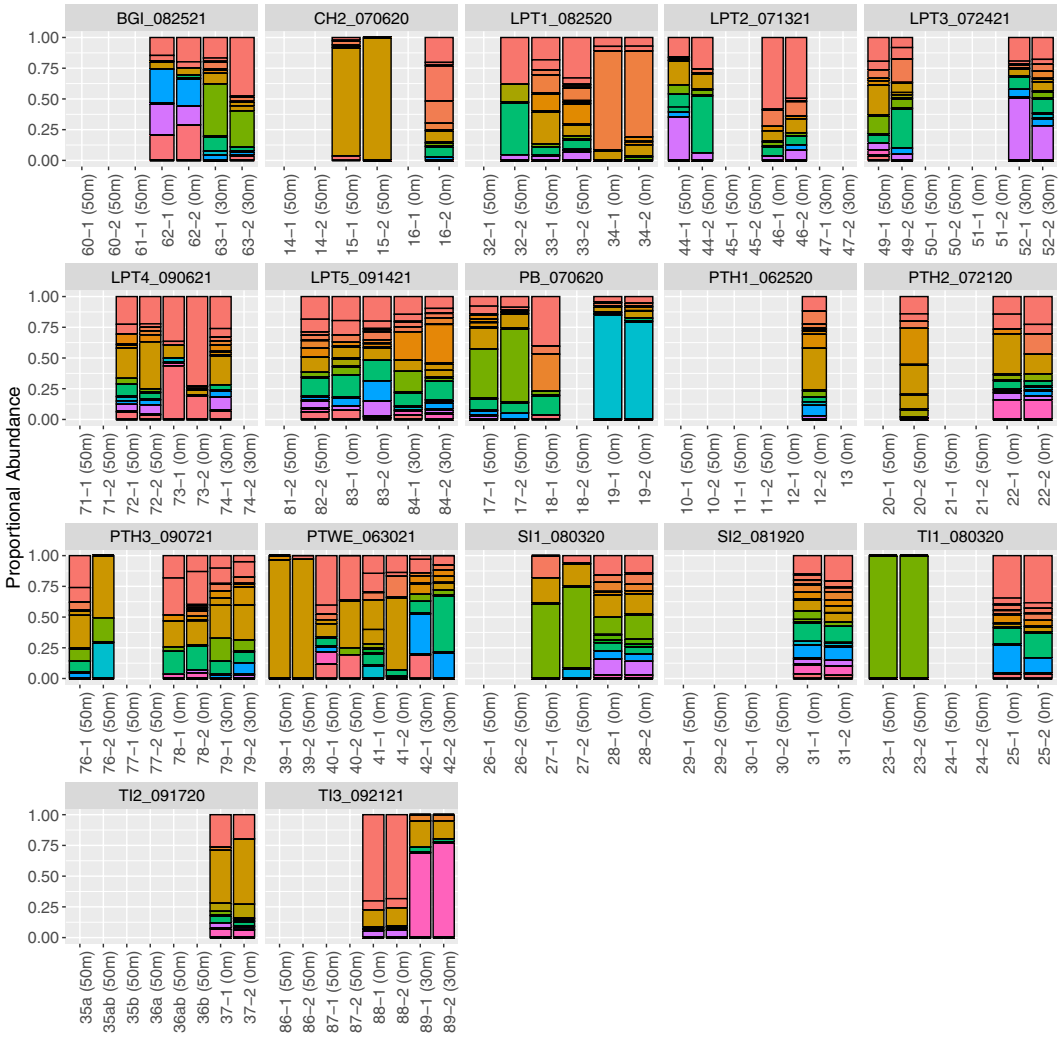

FIGURE 4 | Haplotypic composition of water samples under the 2% detection threshold, organized by survey. Each color represents a unique gray seal amplicon sequence variants (ASV). Surveys are labeled by site name abbreviation and date, as listed in Tables 1 and 2. Sample IDs are followed by a number indicating the replicate in paired collections (e.g., ID-1 and ID-2), as well as the distance from shore at which they were collected. Samples without data are those that failed to amplify gray seal eDNA.

during the initial transect 50 m offshore (1.31 ∓ 0.76), though the differences were not significant (Tukey multiple comparisons of means, $p > 0.05$). Model selection for haplotype richness and diversity was repeated with haplotypes detected under the 5% read threshold, and no terms in any of the models were significant (Table S3).

3.3.4 | Method Comparisons

We compared the results of our eDNA sampling to the results of previous tissue-based sampling in the region and to fecal sampling conducted concurrently at the gray seal haulouts. Of the 47 and 29 haplotypes detected in our eDNA samples under the 2% and 5% thresholds, respectively, 25 (53.19%) and 21 (72.41%) matched one of the 38 haplotypes previously reported in the Western North Atlantic population of gray seals (Cammen, Vincze, et al. 2018; Wood et al. 2011). The remaining 22 and 8 eDNA haplotypes either represent erroneous sequences retained after the read thresholds were applied, or new haplotypes that were not previously identified in the population.

The occurrence and overall frequency of each eDNA haplotype passing the 2% read threshold was compared with the matching haplotype proportion reported at nearby haulouts sampled in 2002–2003 and 2015–2016 through traditional tissue sampling of 113 individuals (Cammen, Vincze, et al. 2018). Both eDNA haplotype occurrence and overall frequency were positively correlated with haplotype frequency identified from tissue sampling according to Spearman's rank correlation tests (Occurrence: $\text{rho}^2 = 0.65$, $p < 1 \times 10^{-6}$; Frequency: $R^2 = 0.67$, $p < 1 \times 10^{-6}$; Figure S6).

In addition to previous tissue-based sampling, we also compared the result of our eDNA samples to data collected from concurrent fecal sampling. Mitochondrial control region haplotypes were successfully sequenced in 25 of 32 fecal samples collected onshore during 8 surveys in 2020. From these samples, 16 haplotypes were identified, of which 15 matched a haplotype previously identified in this population via tissue sampling (Cammen, Vincze, et al. 2018; Wood et al. 2011). The single haplotype identified in these fecal samples that did not match a previously identified haplotype did match an ASV identified via eDNA sampling.

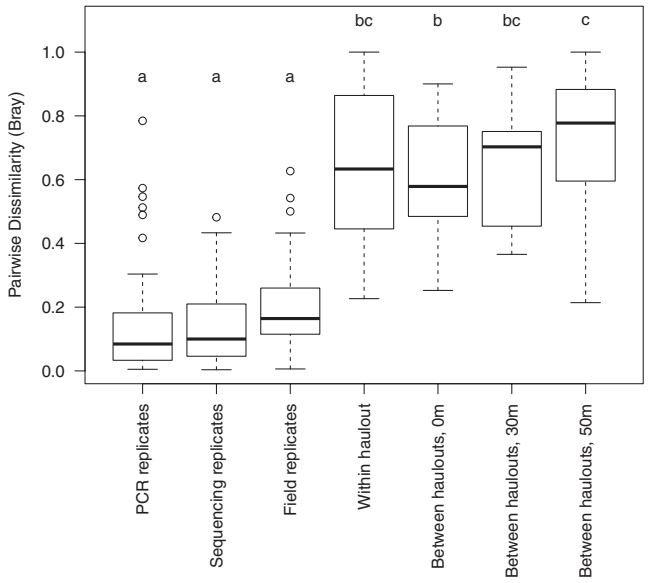

FIGURE 5 | Bray-Curtis pairwise dissimilarity of gray seal eDNA haplotype composition within replicate types and sample groups. The "within haulouts" group refers to samples taken during the same survey collected at 50 m offshore verses at the shoreline. Boxplots labeled with the same lowercase letter are not significantly different (Tukey multiple comparisons of means, $p < 0.05$).

Across 26 eDNA samples collected during these surveys that were successfully sequenced, 33 eDNA haplotypes were detected under the 2% read threshold across both sequencing runs. After removing three fecal samples collected during a single survey without eDNA sequences, haplotypes from 59% (13/22) of the fecal samples were captured in these eDNA samples collected at the same haulout on the same day. The frequency of occurrence of these matching ASVs was significantly higher than the occurrence of all other ASVs both across the entire dataset ($N = 17$ surveys; mean survey occurrence of matching ASVs: 14.92 ∓ 2.61; non-matching ASVs: 3.63 ∓ 3.85; Wilcoxon rank sum test, $p < 0.001$) and the subset of seven surveys with concurrent fecal and eDNA sampling (mean survey occurrence of matching ASVs: 6.08 ∓ 1.24; non-matching ASVs: 0.75 ∓ 1.49; Wilcoxon rank sum test, $p < 0.001$).

4 | Discussion

Our investigation of eDNA dynamics around gray seal haulouts yields several new insights of relevance to eDNA's potential for protected species monitoring. The new qPCR assay we report here demonstrated consistent detection of gray seal eDNA at sampling distances unlikely to significantly disturb resting animals on the beach, suggesting its potential for non-invasive monitoring. Our results further suggest that eDNA-derived haplotype data may accurately reflect population-level, though not necessarily site-specific, haplotype frequencies, with a bias towards capturing dominant haplotypes. Finally, we find that both distance from shore and seal abundance have significant effects on eDNA concentration and haplotype richness and little to no effects on haplotype diversity. Taken together, these results are a promising first step in developing eDNA-based tools for pinniped research and management, and they suggest multiple new avenues of research to fully understand eDNA dynamics along coastal beaches in order for these eDNA-based tools to be used in support of decision-making processes for pinniped management.

4.1 | Gray Seal eDNA Detection

Sampling for gray seal eDNA around haulout sites on Cape Cod, Massachusetts yielded consistent positive detections from 50 m offshore, and in some cases further. This high detection rate is likely enabled by the abundance of seals (average: 167; range: ~18–337) at these haulouts. The spatial limit of detection, defined here as the distance from shore at which the eDNA signal is no longer detectable by our methods, is likely greater than 150 m for groups of seals of this size.

The ability of eDNA sampling to detect species presence at distance is influenced by a variety of factors. These include the target species and its density, abundance, and behavior; the sampling methodology used (e.g., sample volume, sampling location, and sampling depth); the sensitivity of the assay and the laboratory methods used; and environmental conditions (Baker et al. 2018; Foote et al. 2012; Pinfield et al. 2019; Székely et al. 2022). We found that gray seal eDNA concentration increased with seal abundance across our dataset, although to a lesser degree than proximity to shore. Together, the two covariates explained ~75% of the variation in eDNA concentration. Remaining unexplained variation could be attributed to site bathymetry, wind and current patterns, seal movement patterns, or various unaccounted environmental factors that are known to impact eDNA transport and persistence (Harrison et al. 2019). Laboratory processes, including variation in the template matrix between samples and stochastic priming that may occur at low concentrations, may also contribute to unexplained variation. We had limited capacity to test for these factors, due to small sample sizes. However, during one survey of a haulout with only 18 seals in 2022, the effect of currents was evident, with no detection of seal eDNA upcurrent of the haulout along the 50 m transect but successful detection downcurrent. These results demonstrate spatial variation in eDNA signal and the likely influence of current direction.

Overall, our study provides proof of concept that measurable quantities of eDNA can be captured from the water surrounding gray seal haulout sites, including from distances sufficiently removed from animals to allow for non-disruptive sampling. We also describe some of the factors that influence gray seal eDNA concentration in these environments, which can aid in future interpretation of eDNA-based detection of seals and in sampling design for future eDNA studies. However, practical applications of this method rely more on what additional information can be obtained from this genetic material beyond detection, as seals can be easily visually surveyed at these sites from these distances.

4.2 | Gray Seal eDNA Diversity

A few researchers have recently suggested eDNA's potential utility for population genetics (Adams et al. 2019; Sigsgaard et al. 2020). This application is similar to community metabarcoding but targets genetic loci with informative intraspecific variation, rather than loci (e.g., COI, 16S, 12S) with highly

conserved primer regions around species-informative sections (Adams et al. 2019). Accordingly, here we amplified and sequenced a fragment of the mitochondrial control region, which had been previously well characterized in this gray seal population (Cammen, Vincze, et al. 2018; Wood et al. 2011). We demonstrate that multiple haplotypes can be detected in a single eDNA sample, even at distances of 50 m offshore and in samples with low eDNA concentrations. Moreover, we found evidence that eDNA sampling at non-disturbance distances may be sufficient for population-scale summaries of genetic diversity.

We detected up to 14 haplotypes in a single water sample, demonstrating the ability of eDNA methods to capture genetic material from many individuals at once. Indeed, it is likely some of our samples contained eDNA from > 14 individuals, though the exact number cannot be determined given mitochondrial haplotypes are not unique genetic fingerprints. Other eDNA studies in marine mammals that have attempted eDNA haplotyping have primarily focused on sampling eDNA shed from a single animal in lieu of remote biopsy sampling (Baker et al. 2018; Székely et al. 2021), though Parsons et al. (2018) detected up to two haplotypes in surface seawater samples collected near small groups of harbor porpoises. Our work builds on these studies, as well as other non-mammalian eDNA haplotyping research (eels, Halvorsen et al. 2023; abalone, Adams et al. 2023; fish, Tsuji et al. 2020; frogs, Zanovello et al. 2023; and whale sharks, Sigsgaard et al. 2016) to support that much more diverse haplotypic pools from larger groups of individuals can be sampled and characterized via eDNA.

While we were able to detect many haplotypes via eDNA sampling, we did not detect all of the previously described haplotypes present in the population, and the concurrent fecal sampling shows that eDNA sampling did not capture all variation present at a particular haulout. We suggest this is likely not a problem with an eDNA approach per se, as much as an issue of insufficient eDNA sampling effort (e.g., number of spatially and temporally distinct samples, volume of water filtered, sequencing depth, etc.). Among haplotypes, it was those that are known to be relatively dominant in the population that were most likely to be detected in both eDNA and fecal samples when collected concurrently. Similarly, Adams et al. (2023) compared haplotypes obtained from eDNA and tissue sampling and found that eDNA sampling successfully captured common haplotypes but failed to detect two out of three known rare haplotypes. These limitations are likely akin to the read-depth and swamping effects that limit detecting rare species in eDNA community metabarcoding (Gold et al. 2021; Peixoto et al. 2021; Stoeckle et al. 2022). In this sense, the application of haplotype rarefaction analysis could provide a ready means to evaluate the probable adequacy of eDNA sampling effort in any given survey. Likewise, given limited survey time and resources, future studies should be conducted to assess which particular survey design refinements (e.g., number of samples, water volumes, read depths) provide the greatest scope to improve the proportion of captured haplotypes.

A large amount of the variation in haplotype composition among samples was unexplained by distance from shore or seal abundance, despite finding significant relationships of both factors with eDNA concentration. It is possible that this lack of an observed relationship may be the result of saturation of common haplotypes at the seal densities we sampled (average: 167; range: ~18–337 seals per haulout) and masking effects associated with swamping of rare haplotypes at our read depths. Nonetheless, we found a general concordance between the detection frequency of our eDNA-derived haplotypes and the frequency of these haplotypes as previously measured in tissue samples collected at nearby haulouts (Cammen, Vincze, et al. 2018). This finding fits with other studies that have reported comparable haplotype frequencies obtained from eDNA sampling and tissue sampling (Sigsgaard et al. 2016; Tsuji et al. 2020).

Despite its putative bias against detecting rare haplotypes, our eDNA sampling did detect at least one new haplotype that had not previously been detected by tissue sampling, suggesting that eDNA has a capacity to improve coverage of seal haplotype variation. After the application of our more conservative read filter (5%), which removed all erroneous sequences from our mock haplotype pools, eight eDNA haplotypes remained that did not match a previously known haplotype in this population. Though these eDNA haplotypes could still represent erroneous sequences (e.g., from PCR or sequencing error), additional evidence supported that some of the sequences may be new haplotypes that had not been previously identified for gray seals in the Western North Atlantic. One of these novel eDNA haplotypes (Accession Number PV261950) matched a sequence also identified in a fecal sample. A second eDNA haplotype (Accession Number PV261949) was the most abundant sequence in both water samples of a sample pair in both sequence replicates (read proportion: 67.39% ∓ 3.38%). This haplotype was also present in 10/142 (7.11%) other eDNA samples across our dataset when using the 5% read filter. The remaining sequences (Table S4) that did not match a previously known haplotype in this population neither matched a fecal haplotype nor were the most abundant sequence in a sample, and their identity as possible new haplotypes is less certain.

Errors and bias during the PCR and sequencing processes continue to be a challenge for eDNA haplotyping, and if not addressed could lead to erroneous conclusions (Nichols et al. 2018; Shelton et al. 2023). We identified a substantial number of erroneous sequences in our data, an issue that has also been documented in other eDNA haplotyping studies (e.g., Bennington et al. 2024). Various approaches for separating real and erroneous sequences have been demonstrated across eDNA haplotyping studies with varying observed error rates, including assessing positive controls (Parsons et al. 2018) and LULU approaches (Frøslev et al. 2017; Dugal et al. 2022; Bennington et al. 2024). The latter uses sequence similarity and co-occurrence with known haplotypes to identify likely erroneous sequences. Here, we demonstrate another method utilizing mock haplotype pools (Supporting Information: Results) to assess eDNA sequencing error rates, an approach that has been effective in other settings (Banchi et al. 2020). From the analysis of our mock haplotype pools, we designed and evaluated multiple analysis pipelines that accepted different levels of error for haplotype detection, and we acknowledge that the methods described here may not be suitable for research aims that have little room for low levels of sequence error (e.g., identifying rare haplotypes or new genetic variants). The 5% read threshold that removed all errors from our mock communities is higher than the error rate of

1.7% determined in Parsons et al. (2018), and lower than that of 19.2% observed in Bennington et al. (2024). In addition to these insights into error rates, our mock haplotype pools further revealed variation in the extent to which haplotype composition was preserved. Some haplotypes in our mock haplotype pools were over- or under-represented (Supporting Information: Results), and thus future studies might seek to measure and correct for possible PCR bias driving such deviations as a means to more closely approximate actual haplotype frequencies in nature, much the same as suggested in community metabarcoding (Shelton et al. 2023).

Beyond these recommendations to address sequence errors, we also utilize our structured sampling design to offer additional recommendations for future studies. Our sampling captured seal haplotypes from samples collected at the shoreline of haulouts as well as 50 m offshore and found relatively little, if any, effect of distance on haplotype richness and diversity. This suggests that sampling around haulouts at distances unlikely to disturb seals may be adequate for estimating population-level genetic diversity. Our dissimilarity analysis further demonstrated that broad spatial sampling will capture the greatest diversity, and thus, with limited resources, researchers may benefit from distributing environmental samples in space (or time) more than increasing replication at a given location or time. Although a higher number of PCR replicates might be used to better capture stochastic variation in sequence composition and detect rare variants that may otherwise drop out due to detection stochasticity, as observed in our comparison of rare and abundant haplotypes among sample replicates, the high consistency observed among PCR replicates relative to field replicates and environmental groups in our study suggests that fewer PCR replicates might be sufficient for analyses targeting the most-dominant ASVs. This conclusion is similar to those that have been drawn from community metabarcoding eDNA studies (Nichols et al. 2018). Techniques such as occupancy modeling can be used to help estimate the number of replicates needed to achieve a research aim given taxa or haplotype rarity (Doi et al. 2019; Nichols et al. 2018).

Finally, when planning future eDNA studies, it is critical that field, lab, and computational approaches are designed around the specific ecological contexts and research questions being studied. For example, the eDNA concentrations and associated haplotypic variation seen in this study of seals at large haulout sites may not be readily captured from seals in less abundant or concentrated settings (e.g., smaller haulouts, in the vicinity of single individuals, or in estuaries or open ocean with more complex flow regimes). Therefore, eDNA sampling with the goal of population-level assessment may be most effectively conducted around large haulouts or breeding colonies as assessed in this study. Other studies likely provide methods more appropriate to sampling individuals or small groups for alternative objectives, such as identifying cryptic species (Baker et al. 2023) or substituting for the collection of individual tissue biopsies (Székely et al. 2021).

Overall, our results demonstrate the ability of eDNA water sampling to capture genetic material from seals and estimate population-level allele frequencies. The ability to estimate population-level allele frequencies from non-invasive sampling around seal haulout sites supports the ongoing development of eDNA-based population diversity indices. Future studies investigating the application of eDNA approaches for characterizing haplotype diversity between populations may further enable the use of these tools to describe the structure and diversity of previously uncharacterized populations. With this work, and many other emerging studies in this field, both the broad extent and realistic limitations of eDNA-based approaches for marine mammal management are coming further into light.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Raw quantitative PCR (qPCR) data and the associated sample metadata can be found on Dryad (https://doi.org/10.5061/dryad.djh9w0wb1). Raw sequencing reads are available at the NCBI Sequence Read Archive at accession number PRJNA1240662.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.