

FINAL REPORT

Detection and Identification of Environmental DNA from Beaked
Whales on the Navy's Atlantic Undersea Test and Evaluation
Centre (AUTC)

SERDP Project RC19-1038

JANUARY 2021

Charles Scott Baker
Oregon State University

Diane Claridge and Charlotte Dunn
**Bahamas Marine Mammal Research
Organization**

Tom Fetherston
Naval Undersea Warfare Center

D. Nevé Baker
University of California, Santa Cruz

Holger Klinck
Cornell University

Debbie Steel (Technical Support)
Oregon State University

Distribution Statement A

This document has been cleared for public release



This report was prepared under contract to the Department of Defense Strategic Environmental Research and Development Program (SERDP). The publication of this report does not indicate endorsement by the Department of Defense, nor should the contents be construed as reflecting the official policy or position of the Department of Defense. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise, does not necessarily constitute or imply its endorsement, recommendation, or favoring by the Department of Defense.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE (DD-MM-YYYY) 24-01-2021		2. REPORT TYPE SERDP Final Report		3. DATES COVERED (From - To) Jul 2019 to Jan 2021	
4. TITLE AND SUBTITLE Detection and identification of environmental DNA from beaked whales on the Navy's Atlantic Undersea Test and Evaluation Centre (AUTEC)				5a. CONTRACT NUMBER RC19-1038	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Baker, CS, Steel, D, Claridge, D, Dunn, C, Fetherston, T, Baker, DN, Klinck, H.				5d. PROJECT NUMBER RC19-1038	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Oregon State University Hatfield Marine Science Center, 2030 SE Marine Science Dr, Newport, Or 97365				8. PERFORMING ORGANIZATION REPORT NUMBER RC19-1038	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) Strategic Environmental Research and Development Program 4800 Mark Center Drive, Suite 16F16 Alexandria, VA 22350-3605				10. SPONSOR/MONITOR'S ACRONYM(S) SERDP	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S) RC19-1038	
12. DISTRIBUTION / AVAILABILITY STATEMENT Distribution A: Approved for public release; distribution is unlimited.					
13. SUPPLEMENTARY NOTES Prepared in cooperation with NUWIC, BMMRO and Cornell University					
14. ABSTRACT Detection and identification of species, subspecies or stock of whales, dolphins and porpoises at sea remain challenging obligations for monitoring activities by the Department of Defense (DoD), particularly for cryptic or elusive species like beaked whales. The primary goal of this Limited Scope project was to quantify the detection of environmental (e)DNA from beaked whales on the Navy's Atlantic Undersea Test and Evaluation Centre (AUTEC), using an acoustically assisted sampling design. The specific technical objectives of the research were to: 1) conduct small-boat surveys on the AUTEC range to locate beaked whales using the acoustic array; 2) collect seawater from the proximity of the beaked whales using a serial and spatial sampling design and filter seawater on location; 3) extract eDNA from filters and quantify eDNA of beaked whales or other cetacean species using droplet-digital (dd) PCR; 4) confirm species identity and novel haplotype variants by conventional sequencing of eDNA barcodes and next-generation sequencing of eDNA metabarcodes, using primers from the mtDNA control region; and 5) relate the quantification of eDNA by ddPCR to the presence or absence of beaked whales detected by acoustic monitoring.					
15. SUBJECT TERMS AUTEC, beaked whales, eDNA,					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT Unclassified	18. NUMBER OF PAGES 26	19a. NAME OF RESPONSIBLE PERSON Charles Scott Baker
a. REPORT Unclassified	b. ABSTRACT Unclassified	c. THIS PAGE Unclassified			19b. TELEPHONE NUMBER (include area code) 541-272-0560

TABLE OF CONTENTS

ACRONYMS, ABBREVIATIONS, AND UNITS	3
KEYWORDS AND ACKNOWLEDGMENTS	3
ABSTRACT	4
EXECUTIVE SUMMARY	5
OBJECTIVES	10
BACKGROUND	10
MATERIALS AND METHODS	12
RESULTS AND DISCUSSION	15
CONCLUSIONS AND IMPLICATIONS FOR FUTURE RESEARCH	20
LITERATURE CITED	21
ATTACHMENTS	23
Appendix i: Metadata for collection of individual samples	

LIST OF FIGURES

Figure 1: An overview of the workflow for the eDNA collection	12
Figure 2: Primer sequences used in eDNA barcoding and metabarcoding	14
Figure 3: An example of acoustic localization on the AUTECH range	16
Figure 4: Average DNA concentration in copies/ul from ddPCR	17
Figure 5: The variable sites resolving eDNA barcodes	18
Figure 6: Summary of assembled reads of the 5 haplotype variants	19
Figure 7: Evolutionary relationships of mtDNA control region haplotypes	19

Acronyms, Abbreviations, and Units

AUTEC - Atlantic Undersea Test and Evaluation Centre

bp - base pair

BMMRO - Bahamas Marine Mammal Research Organisation

CGRB - Center for Genome Research and Computing

ddPCR - droplet-digital polymerase chain reaction

DNA - deoxyribonucleic acid

DOD - Department of Defense

eDNA - environmental deoxyribonucleic acid

GPS - global position system

kHz - kilohertz

L - liter

μL - micro-liter

M3R - Marine Mammal Monitoring on Navy Ranges program

m - meters

mtDNA - mitochondrial deoxyribonucleic acid

NOAA – National Oceanographic and Atmospheric Administration

NUWIC - Navy Undersea Warfare Center

OSU - Oregon State University

PCI - phenol/chloroform/isoamyl alcohol

PCR - polymerase chain reaction

PCTE - polycarbonated track edge

photoID - photographic identification

PMRF – Pacific Missile Range Facility

qPCR – quantitative PCR

QC- quality control

RHIB - rigid-hull inflatable boat

SCORE – Southern California Offshore Range

SNP - Single Nucleotide Polymorphism

TE - tris buffer and ethylenediaminetetraacetic acid

USGS – U.S. Geological Survey

Keywords:

eDNA, eDNA barcoding, eDNA metabarcoding, PCR, ddPCR, mtDNA, haplotypes, beaked whales, vocalization

Acknowledgements:

We thank the staff of the AUTEC range for their hospitality during our fieldwork and Peter Dugan, of Cornell University for assistance with acoustic monitoring.

Abstract

Detection and identification of species, subspecies or stock of whales, dolphins and porpoises at sea remain challenging obligations for monitoring activities by the Department of Defense (DoD), particularly for cryptic or elusive species like beaked whales. The primary goal of this Limited Scope project was to quantify the detection of environmental (e)DNA from beaked whales on the Navy's Atlantic Undersea Test and Evaluation Centre (AUTEC), using an acoustically assisted sampling design.

The specific technical objectives of the research were to: 1) conduct small-boat surveys on the AUTEC range to locate beaked whales using the acoustic array; 2) collect seawater from the proximity of the beaked whales using a serial and spatial sampling design and filter seawater on location; 3) extract eDNA from filters and quantify eDNA of beaked whales or other cetacean species using droplet-digital (dd) PCR; 4) confirm species identity and novel haplotype variants by conventional sequencing of eDNA barcodes and next-generation sequencing of eDNA metabarcodes, using primers from the mtDNA control region; and 5) relate the quantification of eDNA by ddPCR to the presence or absence of beaked whales detected by acoustic monitoring.

During 12 days of August, 2019, we conducted 9 small-boat surveys and collected 56 samples of seawater (paired samples of 1L each) using both a spatial collection design, in the absence of visual confirmation of the whale, and serial collection design in the proximity of the whales. There were 7 sightings of singles or small groups of whales, including 11 Blainville's beaked whales (*Mesoplodon densirostris*). All whales were located initially with the assistance of information from the acoustic array. Quantification by ddPCR showed very low levels (<0.1 copies/ μ l) of eDNA from the 4 spatial design collections. None of these 20 samples yielded amplicons for conventional or next-generation sequencing with the MiSeq. Quantification by ddPCR of the 31 serial samples identified 11 likely positive detections (>0.1 copies/ μ l). eDNA barcoding by conventional sequencing and eDNA metabarcoding by next-generation sequencing confirmed species identification of 9 of these samples from 3 of the 4 serial collections. We further resolved 5 intra-specific variants (i.e., haplotypes), two of which showed an exact match to previously published haplotypes and three that have not been reported previously to the international repository, GenBank. An evolutionary reconstruction of the 5 eDNA haplotypes with all other published haplotypes of Blainville's beaked whales showed clear differences between oceanic populations and the potential for further resolution of stock difference within oceans.

The Limited Scope project confirmed the power of eDNA barcoding and eDNA metabarcoding to identify species and intra-specific diversity of beaked whales when sampled in the proximity of a terminal dive. This promises to greatly enhance the potential for genetic sampling of beaked whales and other species that are sensitive to the approach of vessels for biopsy sampling. The characterization of intra-specific diversity, as represented by haplotype variants, also shows promise for resolving stock structure at least at the oceanic scale, and perhaps within oceans using frequency differences. The collection of eDNA for species identification and haplotype diversity could be included in routine monitoring of beaked whales and other cetacean species on the Navy's ranges.

Executive Summary

Introduction. Advances in analyses of environmental (e)DNA now offer an alternative to acoustic recording and biopsy sampling for detection and identification of rare, cryptic or vulnerable cetacean species. Here, the DNA that is shed or excreted from individuals during normal activity can be collected from the environment, filtered, and amplified via the Polymerase Chain Reaction (PCR) using primers targeted for specific taxonomic groups. eDNA has been used widely in freshwater systems (Ficetola *et al.* 2008; Jerde *et al.* 2011; Stewart *et al.* 2017), and is now finding a growing number of applications in the marine environment (Thomsen *et al.* 2012), including detection and identification of marine megafauna (Foote *et al.* 2012; Sigsgaard *et al.* 2016). Whales, dolphins and porpoises are good candidates for eDNA sampling given their known tendency to release cellular DNA in their shed skin, fecal plumes and their ‘spout’ or blow.

The methodology for eDNA sampling is advancing rapidly as the number and range of application increases. One of these advances is droplet-digital (dd)PCR, a powerful new technology for quantifying low levels of DNA by fractionating a PCR reaction into more than 20,000 droplets using an oil emulsion (Doi *et al.* 2015). Amplification of the target DNA is quantified by incorporating a fluorescent dye directly into the amplicon reaction or into a molecular probe designed to target a specific sequence bracketed by the PCR primers. The target-positive and target-negative droplets are individually counted by passing them in a single stream through a fluorescence detector similar to a flow cytometer. The ratio of the target-positive to the target-negative droplets is used to estimate the number of copies of the target DNA in the sample, under the assumptions that the target molecules are distributed among the 20,000 droplets according to a Poisson function. Thus, unlike conventional quantitative (q)PCR, ddPCR allows for direct quantification without the need for standard curves, eliminating the variance associated with creating and running standards with each batch (Cao *et al.* 2016).

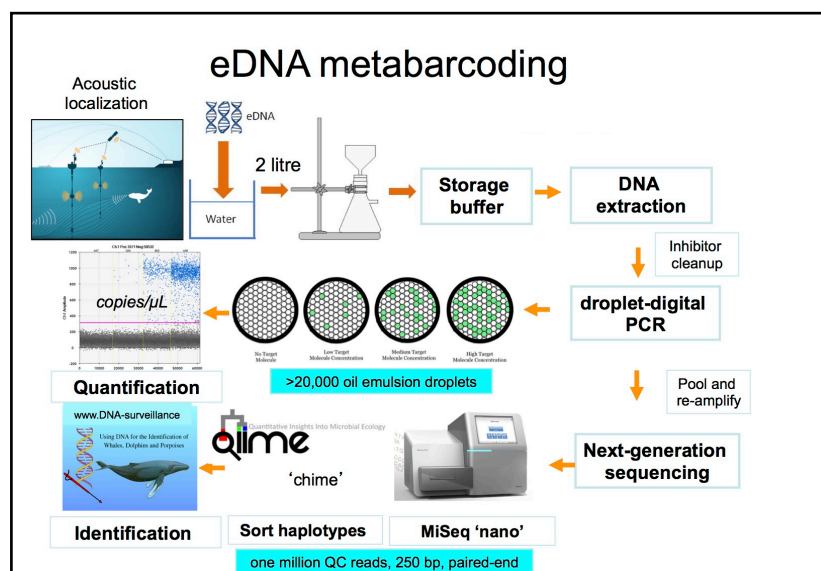
Previously, we demonstrated the power of ddPCR to detect eDNA from seawater collected following the passage of killer whales in coastal waters and of conventional eDNA barcoding to identify mtDNA haplotypes specific to the ‘southern resident’ community (Baker *et al.* 2018). Our sampling design was intended to quantify eDNA from the ‘wake of the whale’, i.e., from directed sampling assisted by visual or acoustic localization. The result of our serial sampling also provided some insight into the potential for spatial sampling of eDNA for the purposes of describing habitat use or estimating ‘occupancy’ in the marine environment.

Objectives. In keeping with the Statement of Need, RCSON-19-C2, the primary objective of our Limited Scope project was to quantify the detection of environmental eDNA from beaked whales on the Navy’s Atlantic Undersea Test and Evaluation Centre (AUTEC) range, using an acoustically assisted sampling design. This included quantification of eDNA by ddPCR and sequencing by conventional eDNA barcoding and next-generation eDNA metabarcoding (Figure 1). For this project, we assembled an extremely experienced team of co-performers, including acoustic support from the Navy Undersea Warfare Center (NUWC) and Cornell University, logistic support and local knowledge of the beaked whales population from the Bahamas Marine Mammal Research Organisation (BMMRO), and support of genetic analysis from the Marine Mammal Institute, Oregon State University.

Technical Approach. The Technical Approach of the project was to:

- 1) Conduct small-boat surveys on the AUTEK range during a 12-day field season, to locate beaked whales using the acoustic array;
- 2) Collect seawater from the proximity of the beaked whales using a serial and a spatial sampling design and filter seawater on location;
- 3) Extract eDNA from filters and quantify eDNA of beaked whales or other cetacean species using droplet-digital (dd) PCR;
- 4) Confirm species identity and novel haplotype variants by re-amplification and conventional sequencing of eDNA barcodes and next-generation sequencing of eDNA metabarcode; and,
- 5) Relate the quantification of eDNA by ddPCR to the presence or absence of beaked whales detected by acoustic monitoring.

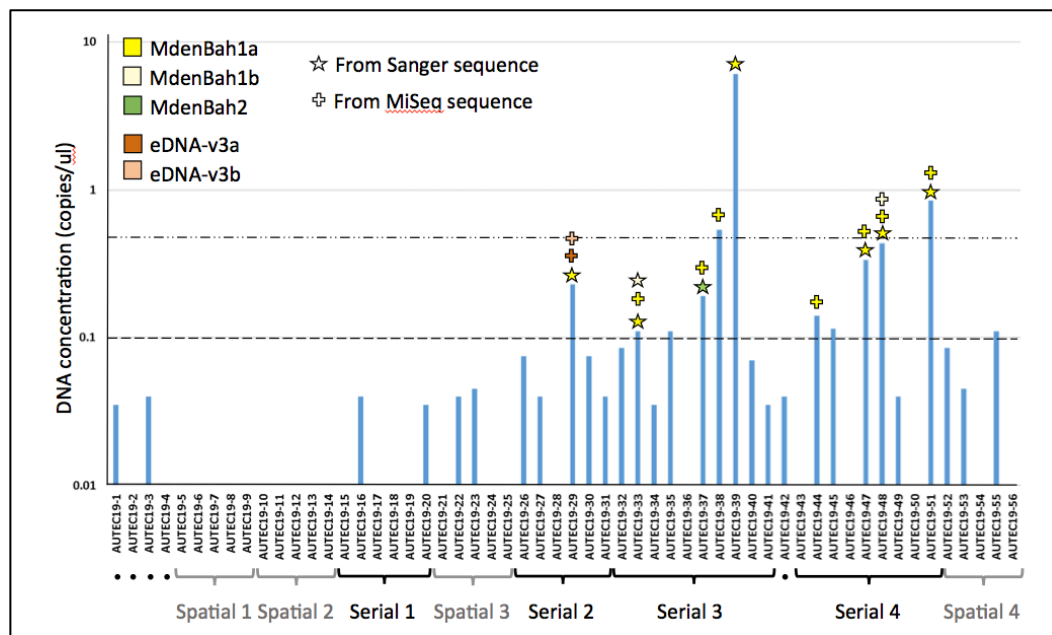
Figure 1: An overview of the technical approach for the eDNA collection, with acoustic localization, quantification by ddPCR and species identification by eDNA barcoding and eDNA metabarcoding.



Results and Discussion. During the 12 days available for fieldwork on the AUTEK range, there were 9 small-boat surveys undertaken on 8 days. Records from the 9 small-boat surveys documented 508 km of survey tracks during a total of 51 hours on the water. There were 7 sightings of singles or small groups, totaling 14 individuals. This included 11 Blainville's beaked whales (*Mesoplodon densirostris*), 2 unknown cetaceans and a single sperm whale. Of the 11 individual Blainville's beaked whales encountered, 10 were identified from photographs and 6 of these were new to the BMMRO catalogue. The 4 known individuals had sighting records dating back to 2016, 2012 and 2005. During the 9 surveys, we were successful in collecting 56 paired samples of 1L each (i.e., 102 L total) within the vicinity of beaked whales. This included 4 spatial design collections (20 paired samples each) in the absence of any visible proximity to the whales and 4 serial design collections (31 paired samples total) in the visible proximity of the whales. An additional 12 samples were included in relevant analyses as positive and negative controls for either field or laboratory handling.

Following cleanup of PCR inhibitors, all samples were submitted for ddPCR. The average values of the ddPCR, run in duplicate, were low for most samples, with many having 95% Poisson confidence limits overlapping with zero. Only 12 of the 56 samples exceeded the relaxed threshold of 0.1 copies/ μ l, considered a likely positive for detection, and only 3 of these met the strict threshold of 0.5 copies/ μ l (see Baker *et al.*, 2018). Only one of the 12 likely positive samples was collected in a spatial design. Many of the other samples collected in the 4 spatial designs showed average values of 0.0 copies/ μ l.

Figure 2: Average DNA concentration in copies/ μ l as determined by droplet digital (dd) PCR. The dash/dot horizontal line represents a concentration of 0.5 copies/ μ l, previously determined to be a strict threshold (i.e., high probability) for a subsequent positive detection by eDNA barcoding (Baker *et al.*, 2018). The dashed line represents a concentration of 0.1 copies/ μ l, previously determined to be a relaxed threshold (i.e., a low probability) for positive detection. Stars represent samples that were identified as *Mesoplodon densirostris* via conventional eDNA barcoding and crosses represent samples identified via MiSeq. The colors refer to the sequence variant identified (i.e., mtDNA haplotypes).

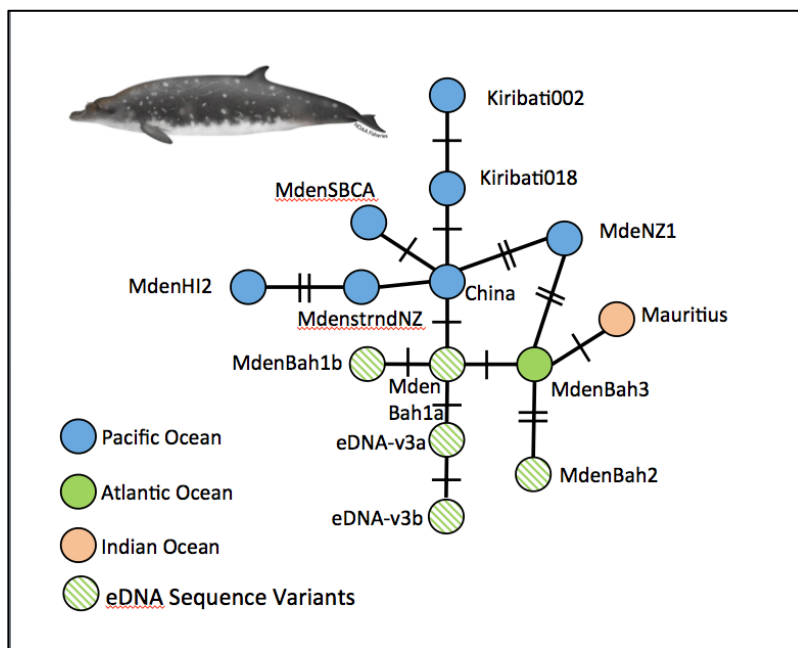


A third run of the ddPCR was reserved from the droplet counter and used, instead, to harvest amplifiable products for subsequent conventional PCR and sequencing. This protocol was intended to help overcome any residual inhibitors and to confirm species identification by conventional eDNA barcoding. Of the 12 likely positive samples from the ddPCR, 7 yielded amplicons suitable for conventional sequencing. These 7 sequences confirmed species identification of Blainville's beaked whale and resolved two sequence variants, i.e., haplotypes. A BLAST search of GenBank confirmed identity with two mtDNA haplotypes reported previously from the Atlantic Ocean, referred to here as MdenBah1a and MdenBah2 (Morin *et al.*, 2012).

To assess the potential to detect further haplotype variants with next-generation sequencing, we submitted the 12 likely positive samples for eDNA metabarcoding on the Illumina MiSeq as both a series of individual and pooled libraries. To reduce sequencing errors, we used a high fidelity polymerase (Kapa HiFi) for amplification of the primary eDNA extractions (not the ddPCR reactions). As a further validation of quality control, all PCR reactions, Illumina libraries and MiSeq runs were repeated three times. Of the 12 samples submitted, 8 provided sequences identified as Blainville's beaked whale, including two samples that had not provided results from amplification of the ddPCR reaction for conventional sequencing (Figure 2).

Considering both the conventional sequencing and the MiSeq results, 9 of the 12 samples considered to be likely positives from the ddPCR provided species identification of Blainville's beaked whales. A review of the sequencing variants provided evidence of 5 haplotypes among the 9 samples (Figure 2). These 5 haplotypes were found in at least two independent runs of the MiSeq or supported by conventional PCR and sequencing. Two of the haplotypes, discussed above, were an exact match to sequences reported previously from the North Atlantic and available on GenBank (Morin et al., 2012). The other three haplotypes have not, to our knowledge, been reported previously. An evolutionary network of all previously reported haplotypes suggests the potential for detection of population structure from haplotype identity, at least at the oceanic scale, and perhaps within oceans using frequency differences (Figure 3).

Figure 3: Evolutionary relationships of mtDNA control region haplotypes from available public sources (e.g., GenBank) and the 5 haplotypes detected by eDNA barcoding and metabarcoding on the AUTECH range. The lines connect the most closely related haplotypes. The short, perpendicular lines indicate the number of nucleotide differences or insertion/deletion events resolving the haplotypes.



Implications for Future Research and Benefits. The Limited Scope effort confirmed the power of eDNA barcoding and metabarcoding to identify species and intra-specific diversity of beaked whales when sampled in the proximity of the terminal dive. This promises to greatly enhance the potential for genetic sampling of beaked whales and other species that are sensitive to the approach of vessels for biopsy sampling. The characterization of intra-specific diversity, as represented by haplotype variants, also shows promise for resolving stock structure at least at the oceanic scale, and perhaps within oceans using frequency differences. The absence of eDNA detection for the spatial sampling design was disappointment but not surprising. The precision of the acoustic localization was highly variable and weather conditions were often marginal during the spatial sampling, as evidenced by the absence of any visual confirmation during these sampling events.

To enhance and standardize eDNA methods for detection and identification of beaked whales on the Navy ranges, future research could include,

- 1) Conducting additional spatial and directed serial sampling of eDNA on the AUTC range and contributing to the catalogue of mtDNA haplotypes from the resident beaked whales. This would be an extension of the previous project, taking advantage of additional field time and continued support from BMMRO and NUWIC. The research team learned a lot from the first efforts in 2019 and could further refine field methods to improve sensitivity of detection and precision of localization. Additional research capacity would also allow us to design species-specific primers to amplify Single Nucleotide Polymorphism (SNPs) from eDNA. These nuclear genomic markers could be used for individual identification and perhaps genotype capture-recapture for estimating census abundance and effective population size (e.g., Hamner et al. 2017).
- 2) Sampling of eDNA at depth for both predator and prey species, with support from NUWC for acoustic localization of the whales in 3 dimensions. NUWIC partners have confirmed that including depth in the current localization was possible in the Whiskey range where the density of hydrophone is high. With this capability, we would sample seawater at depth (with Niskin bottles or similar) to identify the whales and include additional identification of prey species using “universal primers” for eDNA metabarcoding. We anticipate that these methods would provide additional information on habitat quality and whale distribution, building on the previous hydro-acoustic surveys (Benoit-Bird et al., 2020).
- 3) Extending either option 1 or 2 to other species and to other Navy ranges with acoustic support, including the Pacific Missile Range Facility (PMRF) and Southern California Offshore Range (SCORE), and, eventually to areas without acoustic arrays. Initial sample collection (with assistance of Marine Ecology & Telemetry Research and supplemental funding from ONR) confirms the potential for directed sampling of eDNA from Cuvier’s beaked whales (*Ziphius cavirostris*) on the SCORE range.

Objectives

Detection and identification of species, subspecies or stock of whales, dolphins and porpoises at sea remain challenging obligations for monitoring activities by the Department of Defense (DoD), particularly for cryptic or elusive species like beaked whales. In keeping with the Statement of Need, RCSON-19-C2, the primary goal of this Limited Scope project was to quantify the detection of environmental (e)DNA from beaked whales on the Navy's Atlantic Undersea Test and Evaluation Centre (AUTC), using an acoustically assisted sampling design. The specific objectives of the research were to:

- 1) Conduct small-boat surveys on the AUTC range during a 12-day field season, to locate beaked whales using the acoustic array;
- 2) Collect seawater from the proximity of the beaked whales using a serial and spatial sampling design and filter seawater on location;
- 3) Extract eDNA from filters and quantify eDNA of beaked whales or other cetacean species using droplet-digital (dd) PCR;
- 4) Confirm species identity and novel haplotype variants by re-amplification and conventional sequencing of eDNA barcodes and next-generation sequencing of eDNA metabarcode; and
- 5) Relate the quantification of eDNA by ddPCR to the presence or absence of beaked whales detected by acoustic monitoring.

All of these objectives have now been met and the experimental design is ready for further refinement and export to other Navy ranges with acoustic support, including the Pacific Missile Range Facility (PMRF) and Southern California Offshore Range (SCORE), and, eventually to areas without acoustic arrays.

Background

Acoustic monitoring is widely used to detect the presence of cetaceans for the purposes of monitoring and mitigation of anthropogenic disturbance. Although highly effective in many contexts, acoustic monitoring has several known limitations. First, detections are limited to individuals that are calling and calling in many species is seasonal, sex-specific or infrequent. Second, cetaceans (e.g., porpoises and pygmy sperm whales) producing ultra-high frequency calls >100 kHz are difficult to acoustically monitor because of current technical limitations. Third, species identification can be difficult to validate, particularly for the diverse but poorly described family of beaked whales (Baumann-Pickering *et al.*, 2014).

DNA 'barcoding' is now a well-accepted tool in taxonomy and conservation of cetaceans (Baker 2008), providing an alternative, but complementary, method for identification of species and, in some species, for delimitation of stocks, Distinct Population Segment or subspecies (Taylor *et al.*, 2017). A comprehensive reference database representing mitochondrial (mt) DNA sequences of all known cetaceans is available through a web-based program for species identification, www.dna-surveillance.auckland.ac.nz, curated by one of the PIs (Baker *et al.*, 2003). This includes validated reference sequences from all known species of beaked whales, several of which are represented by sequences from the holotypes (Dalebout *et al.*, 2004). However, non-lethal genetic sampling for identification of whales, dolphins and porpoises (cetaceans) at sea remains difficult. Most samples have been collected with some form of a biopsy dart projected with a crossbow or a modified veterinary capture rifle (Noren and Mocklin, 2011). This requires a close approach of a vessel, usually within a few 10s of meters, while the individual is at the surface. It is also limiting because of access, distribution or behavior of cetaceans. Some species

are rare, cryptic or both, e.g., beaked whales (Dalebout *et al.*, 2004). Others species are difficult to approach because of their elusive behavior, e.g., the pygmy and dwarf sperm whale. Finally, some species are considered vulnerable to disturbance from the close approach of a vessel or the biopsy sample itself (Noren and Mocklin 2011).

Advances in analyses of environmental (e)DNA now offer an alternative for detection and identification of rare, cryptic or vulnerable cetacean species. Here the DNA that is shed or excreted from individuals during normal activity can be collected from the environment, concentrated, and amplified via the Polymerase Chain Reaction (PCR) using primers targeted for specific taxonomic groups. eDNA has been used widely in freshwater systems (Ficetola *et al.*, 2008; Jerde *et al.*, 2011; Stewart *et al.*, 2017), and is now finding a growing number of applications in the marine environment (Thomsen *et al.*, 2012), including detection and identification of marine megafauna (Eva Egelyng Sigsgaard 2016; Foote *et al.*, 2012). Whales, dolphins and porpoises would seem good candidates for eDNA sampling given their known tendency to release cellular DNA in shed skin, fecal plumes and the ‘spout’ or blow (Hunt *et al.*, 2013).

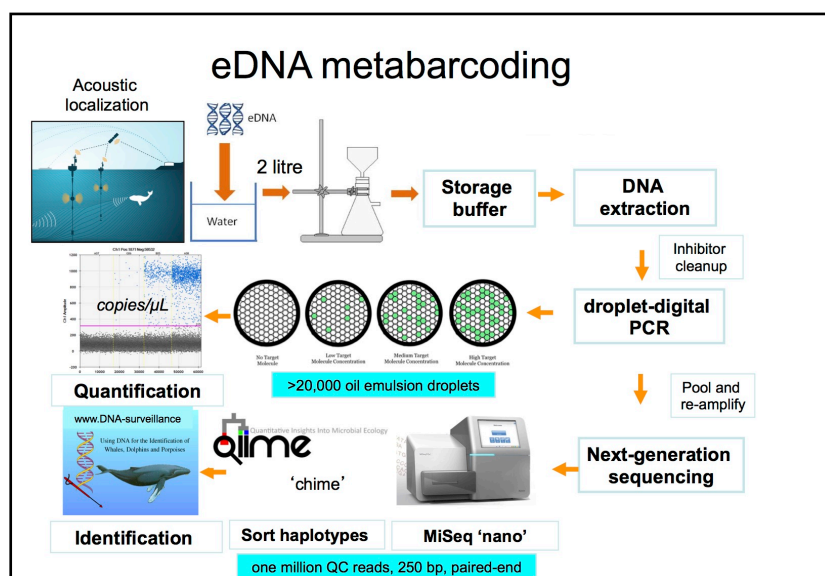
The methodology for eDNA sampling is advancing rapidly as the number and range of application increases. One of these advances is droplet-digital (dd)PCR, a powerful new technology for quantifying low levels of DNA by fractionating a PCR reaction into more than 20,000 droplets using an oil emulsion (Doi *et al.*, 2015). Amplification of the target DNA is quantified by incorporating a fluorescent dye directly into the amplicon reaction or into a molecular probe designed to target a specific sequence bracketed by the PCR primers. The target-positive and target-negative droplets are individually counted by passing them in a single stream through a fluorescence detector similar to a flow cytometer. The ratio of the target-positive to the target-negative droplets is used to estimate the number of copies of the target DNA in the sample, under the assumptions that the target molecules are distributed among the 20,000 droplets according to a Poisson function. Thus, unlike conventional quantitative (q)PCR, ddPCR allows for direct quantification without the need for standard curves, eliminating the variance associated with creating and running standards with each batch (Cao *et al.*, 2016). Owing to reaction partitioning, ddPCR is also thought to show an increased tolerance to inhibitors, making it an attractive alternative to qPCR for detection and quantification of eDNA (Dingle *et al.*, 2013).

Previously, we have demonstrated the potential for ddPCR to detect eDNA from seawater collected following the passage of killer whales in Puget Sound. For this, we took advantage of methods previously developed for species identification of cetacean products sold in Japanese and Korea markets (e.g., Baker *et al.*, 2006; Baker and Palumbi 1994), including a comprehensive reference database of mitochondrial (mt)DNA sequences from most recognized species of cetaceans (Dalebout *et al.*, 2004; Ross *et al.*, 2003). We chose killer whales in Puget Sound for this initial investigation because their well-described habits allowed us to sample efficiently and because their distribution includes multiple ‘communities’ and ecotypes, identifiable by distinct vocalizations and distinct mtDNA haplotypes. Our sampling design was intended to quantify eDNA from the ‘wake of the whale’, i.e., from directed sampling assisted by visual or acoustic localization. However, the result of our serial sampling also provided some

insight into the potential for spatial sampling of eDNA for the purposes of describing habitat use or estimating ‘occupancy’ in the marine environment.

In brief, the intent of our Limited Scope study was to evaluate the potential for an acoustically assisted sampling design for the AUTC range, to continue to refine laboratory methods for eDNA extraction and amplification by ddPCR and to improve confidence in determinations of presence/absence of eDNA by improving the ‘signal to noise’ ratio of the ddPCR. For the latter, we validated the limits of quantification with conventional and next-generation sequencing methods. A flowchart of our technical approach is given in Figure 1. For this, we have assembled an extremely experienced team of co-performers, including acoustic support from the Navy Undersea Warfare Center (NUWC) and Cornell University, logistic support and local knowledge of the beaked whales population from the Bahamas Marine Mammal Research Organisation (BMMRO), and support of genetic analysis from the Marine Mammal Institute, Oregon State University.

Figure 1: An overview of the workflow for the eDNA collection, with acoustic localization, quantification by ddPCR and species identification by eDNA barcoding and metabarcoding.



Materials and Methods

AUTC field effort and sampling design

The sampling of seawater for environment (e)DNA was conducted during residency at the AUTC range, Andros Island, the Bahamas from 31 July to 13 August, 2019. These dates were chosen in consultation with co-performers at NUWC and BMMRO to maximize the number of days that the AUTC range was available for research (referred to as ‘green days’). Small-boat surveys were conducted on the AUTC range aboard a 23-foot, Rigid Hull Inflatable Boat (RHIB) operated by BMMRO. The real-time acoustic localization from the Marine Mammal Monitoring on Navy Ranges (M3R) program was used to direct the RHIB to the vicinity of acoustically active whales. If the location of the whales could be visually confirmed, the vessel moved into the position of the “terminal dive” and initiate a *serial* sampling series.

After initial positioning, seawater samples were collected from the surface in a 1L, wide mouth, sterile Nalgene bottle. All serial samples were collected in pairs, one from the starboard and one from the port side of the boat, for a total of 2L for each sample. Seawater samples were collected from the surface at the time of the terminal dive and at 15-minute intervals for up to two hours. A shipboard GPS was used to maintain position of the terminal dive. If the location of the whale could not be visually confirmed, the boat was moved to the best approximation from the acoustic detection. We then initiated a localized *spatial* sampling series. For this, seawater samples (2 x 1L bottles) were collected at 100 m intervals for 400 m along both north-south and east-west axes (i.e., 9 paired samples), intersecting at the primary coordinates from the acoustic localization. The shipboard GPS was used to position the boat for the prescribed distances.

M3R system for acoustic localization

The M3R program has developed a system to allow real-time continuous, automated detection, classification, localization and monitoring of marine mammals on the U.S. Navy's undersea test and training ranges (Jarvis et al., 2014; AUTECH 2015). The AUTECH M3R system consists of the M3R cluster and three monitoring stations. The cluster runs the detection/classification, data association, and localization software, records the raw data, and sends it to the display machines in the monitoring stations. The display machines run various display and monitoring tools and archive the processed data. The display used during the eDNA experiment was M3RWorldView based upon NASA's open-source World Wind program with marine mammal detection software overlaid. The hydrophones are color-coded to represent the acoustic activity as the number of detections per hydrophone relative to the mean number of detections across the range, with red representing the highest activity, and black being no activity at all. Localizations, or 'posits', are optionally displayed as small whale and dolphin icons. The different icons and colors represent different whale types in the 'Whdetect' detector output, which characterizes click detections by frequency band. Clicking on a hydrophone will launch a 'WChart' window, which is a real-time scrolling hard-limited spectrogram display for that hydrophone. Blainville's beaked whale (*Mesoplodon densirostris*) vocalizations characteristically show a floor of 24 kHz. M3R software computes localizations from time difference of arrival data using a hyperbolic, multilateration algorithm, providing position data. Clicking on a position displays a window with information on the type, latitude and longitude, and time. It also shows the master hydrophone it was calculated from, an 'Equal' field which indicates the number of posits that are exactly the same as this one, and a 'Neighbors' field which displays the number of posits within a certain preset radius of this posit. The more 'Neighbors' a position has, the more likely it is to be accurate.

Standardization of eDNA collection, filtering and extraction

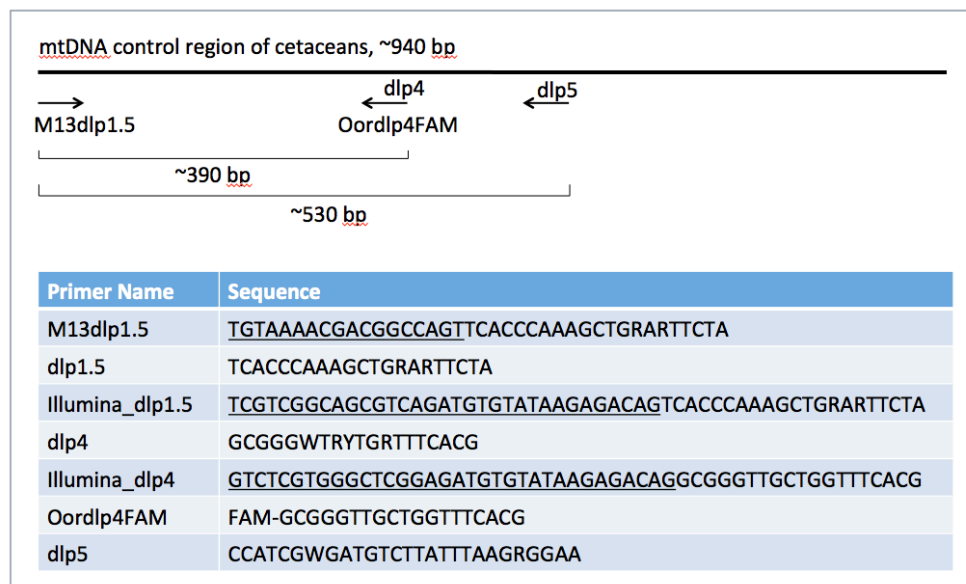
Seawater samples were collected in paired samples of 1L each and stored on ice until return to the AUTECH housing each evening. The seawater samples were then filtered on site in the AUTECH housing, through a 0.4 micron, polycarbonate track-etched filter (PCTE, GE Lifesciences, USA) using a portable Nalgene™ filter apparatus and low-pressure vacuum pump. The filters were stored in Longmire's solution for transport back to a 'clean room' at the Hatfield Marine Science Centers, Oregon State University. eDNA was extracted from the filters by a conventional phenol/chloroform/isoamyl alcohol (PCI) method (Baker et al., 2018). The PCI extraction was chosen because chloroform dissolves the PCTE filters, optimizing the recovery of the eDNA (Renshaw et al., 2015). Initial efforts to amplify eDNA showed evidence of inhibition to conventional PCR, presumably resulting from co-extraction of biological compounds from the surface seawater samples. PCR inhibitors were removed or

reduced using the OneStepTM PCR Inhibitor Removal Kit (Zymo Research). Following the OneStep cleanup, all samples were standardized to approximately 50µl volume in TE, i.e., the eDNA from a 1L sample was recovered in a final volume of 50µl.

eDNA barcoding primers, conventional PCR and sequencing

Molecular identification of species, or ‘DNA barcoding’ is now a well-accepted tool in taxonomy and conservation of cetaceans (Baker 2008). A comprehensive reference database representing mitochondrial (mt) DNA sequences of all known cetaceans is available through a web-based program for species identification, www.dna-surveillance.auckland.ac.nz, curated by the PI (Baker *et al.*, 2003). Using this database, we chose two primer pairs to amplify and sequence an overlapping fragment of the cetacean mtDNA control region or d-loop (Figure 2). The primers Dlp1.5 to Dlp5 amplify a fragment of about 530 base pairs (bp) in length and primers Dlp1.5 to Dlp4 amplify a fragment of 390 bp, nested within Dlp1.5 to 5 (Dalebout *et al.*, 2004). The modified primer, Oordlp4FAM was used as the molecular probe in the ddPCR reactions (Baker *et al.*, 2018). The sequence length of either combination of primers is sufficient for confident identification of cetacean species, and in some cases identification geographic variants (i.e., haplotypes), while allowing for amplification of degraded eDNA (Dalebout *et al.*, 2004). We attempted to amplify the eDNA of each sample using both primer pairs, Dlp1.5 to 5 and 1.5 to 4. For this 1µl of the eDNA extraction was added to 20µl of standard PCR reaction (Platinum *Taq*), following standard conditions described in Baker *et al.*, 2018. The success of the conventional PCR was judged by the visualization of the amplicon on an agarose gel. Any visible amplicons were sequenced in both directions with Big Dye terminator chemistry (ThermoFisher Scientific, USA) on an ABI3730xl. Sequences were aligned to known haplotypes of Blainville’s whales (Dalebout *et al.*, 2004; Morin *et al.*, 2012) and visually inspected with the software Sequencer 4.1 (Gene Code).

Figure 2: Primer sequences used in eDNA barcoding and metabarcoding and approximate locations on the control region of the cetacean mtDNA. Some taxon-base primer were modified to include adaptors used in library preparation or attachment of fluorescent probe (FAM), shown as underline.



ddPCR, DNA barcoding and metabarcoding

The extracted eDNA was quantified by droplet digital (dd)PCR using the BioRad QX200 Droplet Digital™ instrument and a ddPCR™ Supermix for Residual DNA Quantification (BioRad). For this we used the primers Dlp1.5 to 5 with a molecular probe designed from the nested primer, Dlp4 (Figure 2). The ddPCR amplification of each sample was run in triplicate. Two of the three replicates were used for ddPCR quantification with the droplet reader. The target-positive and target-negative droplets of the ddPCR reactions were visualized and analyzed using the manufacturer's software, QuantaSoft (Bio-Rad Laboratories Inc., 2014). Quantification of target DNA, in copies/μL of reaction and associated 95% confidence limits for measurement error, are based on an assumption of a Poisson distribution of the target DNA among the more than 20,000 droplets from a typical 20 μL reaction. The third ddPCR reaction was set aside for conventional PCR and Sanger sequencing. After ddPCR amplification, samples showing positive detection (i.e., a relaxed threshold of >0.1 copies/μL, see Baker et al., 2018) were harvested from the oil emulsion and reamplified by conventional PCR. This two-step amplification protocol was intended to help overcome the potential for inhibitors in the conventional PCR.

For eDNA metabarcoding on the Illumina MiSeq, we used a high-fidelity polymerase (Kapa Hifi, Roche) to amplify the Dlp1.5 to 4 fragment from the eDNA extractions. The amplicon from each sample was then prepared for sequencing on an Illumina MiSeq using a Nano flowcell following a standard library preparation with indexing. The metabarcoding library was run as a multiplex of 48 samples with the expectation of >20,000 reads per sample using 250 bp, paired-end reads. All samples were repeated three times, with independent Illumina libraries, on independent sequencing runs, to identify and subsequently remove polymerase or sequencing artifacts. All ddPCR and MiSeq runs were conducted by the OSU core facility, the Center for Genome Research and Computing (CGRB).

Quality control and informatics

Conventional PCR and sequencing included the usual no-template control and review of electropherograms for identification of haplotypes and variants (e.g., Baker et al., 1996). The output of the MiSeq was de-multiplexed and the paired-end reads were assembled using the program Qiime2 (Bolyen et al., 2019). The assembled reads, or 'haplotypes', were then submitted to a BLAST search of GenBank for initial species identification and to the curated database maintained on *DNA-surveillance* for confirmation (Ross et al., 2003). Sequences arising from host-cell contamination of molecular reagents (e.g., mouse, pig and cow) were identified by Qiime2 and removed (Leonard et al., 2007). Within-species sequence variants (i.e., haplotypes) of cetaceans were evaluated for assembly or polymerase errors. We accepted haplotype variants only if they were included in at least two independent runs of the MiSeq or supported by conventional PCR and sequencing.

Results and Discussion

Vessel surveys and photo-identification

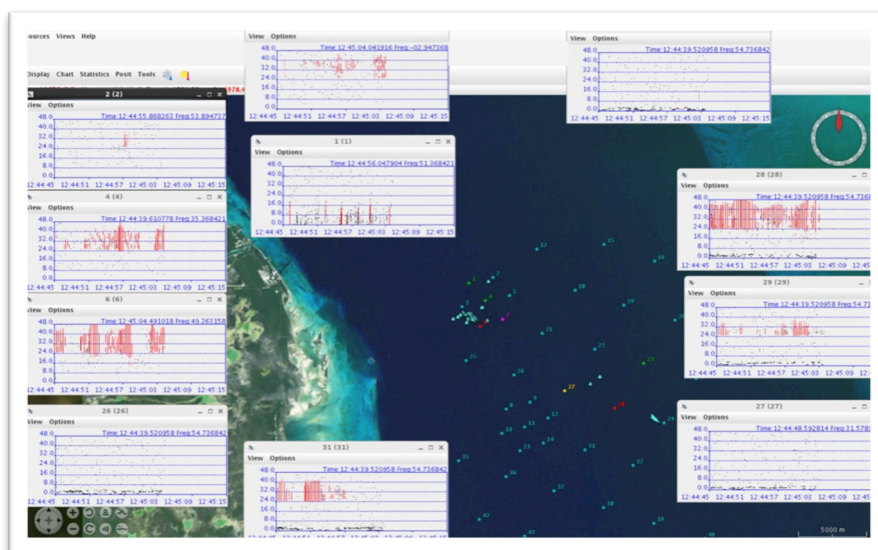
During the 12 days available for fieldwork on the AUTEK range, there were 9 small-boat surveys undertaken on 8 days. Of this total, two ended early due to weather and one ended early because of a range closure for a Naval exercise (referred to as a 'red day'). There was no acoustic support for one of the surveys, as a consequence of unscheduled maintenance of the hydrophone array. No surveys were conducted on the other five days due to unfavorable weather

or to range closure. Records from the 9 small-boat surveys documented 508 km of survey tracks during a total of 51 hours on the water. There were 7 sightings of singles or small groups, totaling 14 individuals. This included 11 Blainville's beaked whales (*Mesoplodon densirostris*), 2 unknown cetaceans and a single sperm whale. Of the 11 individual Blainville's beaked whales encountered, 10 were identified from photographs and 6 of these were new to the BMMRO catalogue. The 4 known individuals had sighting records dating back to 2016, 2012 and 2005 (2 individuals). Two of the known individuals had been sampled previously with a biopsy dart and one new individual was sampled, allowing for the potential to match mtDNA haplotypes with eDNA. PhotoID codes and notes on individual resighting records are included in Appendix i.

Acoustic localization and sample collection

All whales were located initially with the assistance of information from the acoustic array (see Figure 3). However, as beaked whales only vocalize at depth (presumably while foraging), the M3R operator cannot provide precise localization for the whales as they surface, or while at the surface. Once vocalizations stop, the M3R operator can only assume the whales are surfacing and provide the last position at depth to guide the vessel. Unfortunately, the whales do not always come straight up but rather travel at an angle and so can be some distance away from their foraging location. Despite this limitation, we were successful in collecting 56 paired samples of 1L each (i.e., 102 L total) within the vicinity of beaked whales. This included 4 spatial design collections (20 paired samples each) in the absence of any visible proximity to the whales and 4 serial design collections (31 paired samples total) in the visible proximity of the whales (Figure 4). An additional 12 samples were included in relevant analyses as positive and negative controls for either field or laboratory handling (see Appendix i).

Figure 3: An example of acoustic activity and localization of beaked whales on the AUTECH range, from August 8, 2019, taken as screenshots from M3RWorldView (courtesy, T. Fetherston, NUWIC). Acoustic activity is indicated by the color of the hydrophone, with red indicating highest activity. The insets show the time (x axis) and frequency in kHz (y axis) of vocalizations on selected hydrophones. Blue triangles indicate positions of vocalizing beaked whales (e.g., > 24 kHz). A cluster of beaked whale positions near hydrophones 3, 4 and 6 was used to locate the whales at the surface and collect eDNA for the third serial sample collection (see Figure 4).



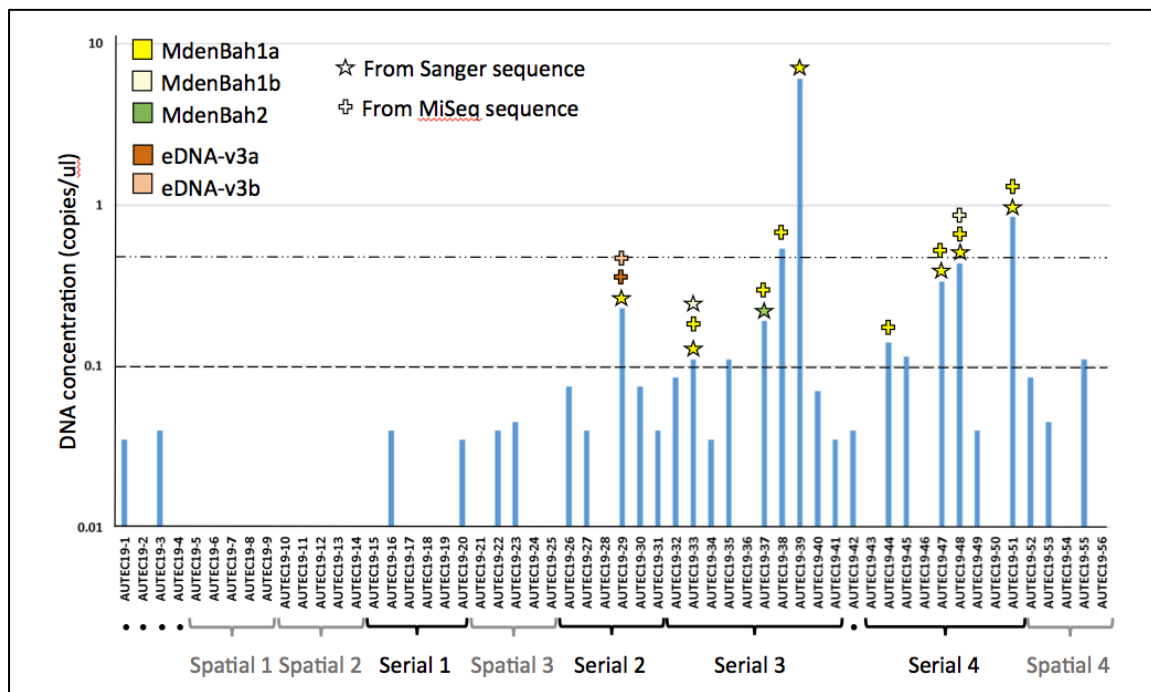
Conventional PCR and inhibitors

Following the PCI extraction, all 56 samples were tested for inhibitors by convention PCR, using a two-stage nested amplification protocol with Dlp1.5 to 5, followed by 1.5 to 4. No samples yielded a visible amplicon, suggesting either inhibitors to the PCR reaction or very low concentrations of eDNA in the samples, or both.

ddPCR quantification and conventional sequencing of eDNA barcodes

Following clean-up of inhibitors with the Zymo OneStep, all samples were submitted for ddPCR in triplicate, using Dlp4 as a florescent probe. The average values of the ddPCR, run in duplicate, were low for most samples, with many having 95% Poisson confidence limits overlapping with zero (see Appendix i). Only 12 of the 56 samples exceeded the relaxed threshold of 0.1 copies/ μ l, considered a likely positive for detection, and only 3 of these met the strict threshold of 0.5 copies/ μ l (see Baker et al., 2018). Only one of the 12 likely positive samples was collected in a spatial design. Many of the other samples collected in the 4 spatial designs showed average values of 0.0 copies/ μ l (Figure 4).

Figure 4: Average DNA concentration in copies/ μ l as determined by droplet digital (dd) PCR. The dash/dot horizontal line represents a concentration of 0.5 copies/ μ l, previously determined to be a strict threshold (i.e., high probability) for a subsequent positive detection by eDNA barcoding (Baker et al., 2018). The dashed line represents a concentration of 0.1 copies/ μ l, previously determined to be a relaxed threshold (i.e., a low probability) for positive detection. Stars represent samples that were identified as *Mesoplodon densirostris* via conventional eDNA barcoding and crosses represent samples identified via MiSeq. The colors refer to the sequence variant identified (i.e., mtDNA haplotypes).



A third run of the ddPCR was reserved from the droplet counter and used, instead, to harvest amplifiable products for subsequent conventional PCR and sequencing. This protocol was intended to help overcome any residual inhibitors and to confirm species identification by conventional eDNA barcoding. Of the 12 likely positive samples from the ddPCR, 7 yielded amplicons suitable for conventional sequencing. These 7 sequences confirmed species identification of Blainville's beaked whale and resolved two sequence variants, i.e., haplotypes. A BLAST search of GenBank confirmed identity with two mtDNA haplotypes reported previously from the Atlantic Ocean, referred to here as MdenBah1a and MdenBah2 (Figure 5; Morin et al., 2012).

Figure 5: The variable sites resolving eDNA barcodes (i.e., mtDNA haplotypes) from Blainville's beaked whales on the AUTECH range compared to those available on the international repository, GenBank. Note that trimming the length of the reference sequences to the fragment identified in eDNA resulted in collapse of some variant represented in GenBank. Match-to-first shows the reference sequence on the first line and a period (.) to indicate identity. An insertion/deletion is considered a fifth character and indicated by two dashes (--). Sequences of the 5 haplotypes reported here have been submitted to GenBank (as a PopSet).

GenBank Number	Haplotype Code	76	105	106	109	135	136	148	155	161	177	293	315	321
KF032867	MdenBah1a	A	A	T	T	A	G	C	G	A	G	C	G	G
	MdenBah1b	.	--	.	.	.	.	.	.	.	.	.	.	.
KF032874	MdenBah3	.	.	.	.	.	.	.	.	.	.	T	.	.
AB610396	Mauritius	T	.	.	.	.	.	.	.	.	.	T	.	.
AB610397	Mauritius	T	.	.	.	.	R	.	.	.	.	T	.	.
KF032873	MdenBah2	.	.	C	C	.	.	.	.	.	.	T	.	.
AY579513	MdenNZ1	.	.	.	.	.	.	T	.	G	.	T	.	.
KF032862	MdenHI2	.	.	.	.	.	.	.	C	G	A	T	.	.
KF032860	MdenSBCA	.	.	.	.	G	.	.	.	G	.	.	.	.
KY542115	China	.	.	.	.	.	.	.	.	G	.	.	.	.
KC540691	Kiribati002	.	.	.	.	.	.	.	.	G	.	.	A	A
KC540694	Kiribati018	.	.	.	.	.	.	.	.	G	.	.	A	.
KF032861	MdenstrndNZ	.	.	.	.	.	.	.	C	G	.	.	.	.
	eDNAv3a	.	.	.	.	.	A	.	.	.	.	.	.	.
	eDNAv3b	.	--	.	.	.	A	.	.	.	.	.	.	.

eDNA metabarcoding and novel haplotype variants

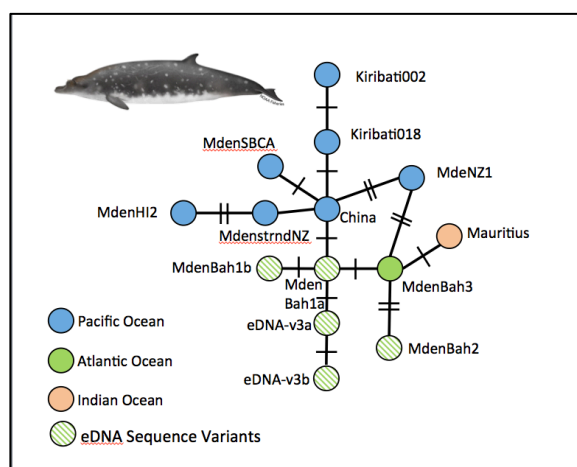
To assess the potential to detect further haplotype variants with next-generation sequencing, we submitted the 12 likely positive samples for eDNA metabarcoding on the Illumina MiSeq as both a series of individual and pooled libraries. To reduce sequencing errors, we used a high fidelity polymerase (Kapa HiFi) for amplification of the primary eDNA extractions (not the ddPCR reactions). As a further validation of quality control, all PCR reactions, Illumina libraries and MiSeq runs were repeated three times. Of the 12 samples submitted, 8 provided sequences identified as Blainville's beaked whale, including two samples that had not provided results from amplification of the ddPCR reaction for conventional sequencing (Figure 6).

Figure 6: Summary of assembled reads of the 5 haplotype variants from the triplicate runs of the MiSeq (250 bp, paired-end), for samples judged to be likely positives (> 0.1 copies/ μ l) from ddPCR quantification for beaked whales eDNA. The first row provides the sample code for each column (see Figure 4). The second row provides associated identification of haplotypes by conventional Sanger sequencing.

	AUTEC19 -29	AUTEC19 -33	AUTEC19 -35	AUTEC19 -37	AUTEC19 -38	AUTEC19 -39	AUTEC19 -44	AUTEC19 -45	AUTEC19 -47	AUTEC19- 48	AUTEC19 -51	AUTEC19 -55
Sanger	Mden Bah1a	Mden Bah1a,b	-	Mden Bah2	-	Mden Bah1a	-	-	Mden Bah1a	Mden Bah1a	Mden Bah1a	-
MdenBah1a	-	9387	-	-	61	-	127	-	1224	1069	44	-
MdenBah1b	-	-	-	-	-	-	-	-	-	183	-	-
MdenBah2	-	-	-	-	-	-	-	-	-	-	-	-
MdenBah3a	126	-	-	-	-	-	-	-	-	-	-	-
MdenBah3b	3011	-	-	-	-	-	-	-	-	-	-	-
Other	70	196	55	42	32	39	1705	361	260	148	224	150
Total	3207	9583	55	42	93	39	1832	361	1484	1400	280	150

Considering both the conventional sequencing and the MiSeq results, 9 of the 12 samples considered to be likely positives from the ddPCR provided species identification of Blainville's beaked whales. A review of the sequencing variants provided evidence of 5 haplotypes among the 9 samples (Figure 5). These 5 haplotypes were found in at least two independent runs of the MiSeq or supported by conventional PCR and sequencing. Two of the haplotypes, discussed above, were an exact match to sequences reported previously from the North Atlantic and available on GenBank (Morin et al., 2012). The other three haplotypes have not, to our knowledge, been reported previously. An evolutionary network of all previously reported haplotypes suggests the potential for detection of population structure from haplotype identity, at least at the oceanic scale, and perhaps within oceans using frequency differences (Figure 7).

Figure 7: Evolutionary relationships of mtDNA control region haplotypes from available public sources (e.g., GenBank) and the 5 haplotypes detected by eDNA barcoding and metabarcoding on the AUTEC range (see Figure 5). The lines connect the most closely related haplotypes. The short, perpendicular lines indicate the number of nucleotide differences or insertion/deletion events resolving the haplotypes.



Conclusions and Implications for Future Research

The Limited Scope effort confirmed the power of eDNA barcoding and metabarcoding to identify species and intra-specific diversity of beaked whales when sampled in the proximity of the terminal dive. This promises to greatly enhance the potential for genetic sampling of beaked whales and other species that are sensitive to the approach of vessels for biopsy sampling. The characterization of intra-specific diversity, as represented by haplotype variants, also shows promise for resolving stock structure at least at the oceanic scale, and perhaps within oceans using frequency differences. The absence of eDNA detection for the spatial sampling design was disappointment but not surprising. The precision of the acoustic localization was highly variable and weather conditions were often marginal during the spatial sampling, as evidenced by the absence of visual confirmation.

To continue enhancing and standardizing eDNA methods for detection and identification of beaked whales on the Navy ranges, future research could include,

- 4) conducting additional spatial and directed serial sampling of eDNA on the AUTC range and contributing to the catalogue of mtDNA haplotypes from the resident beaked whales. This would be an extension of the previous project, taking advantage of additional field time and continued support from BMMRO and NUWIC. The research team learned a lot from the first efforts in 2019 and could further refine field methods to improve sensitivity of detection. Additional research capacity would also allow us to design species-specific primers to amplify Single Nucleotide Polymorphism (SNPs) from eDNA. These nuclear genomic markers could be used for individual identification and perhaps genotype capture-recapture for estimating census abundance and effective population size (e.g., Hamner et al., 2017).
- 5) sampling of eDNA at depth for both predator and prey species, with support from NUWC for acoustic localization of the whales in 3 dimensions. NUWIC partners have confirmed that including depth in the current localization was possible in the Whiskey range where the density of hydrophone is high. With this capability, we would sample seawater at depth (with Niskin bottles or similar) to identify the whales and include additional identification of prey species using “universal primers” for eDNA metabarcoding. We anticipate that these methods would provide additional information on habitat quality and whale distribution, building on the previous hydro-acoustic surveys (Benoit-Bird et al., 2020).
- 6) extending either option 1 or 2 to other species and to other Navy ranges with acoustic support, including the Pacific Missile Range Facility (PMRF) and Southern California Offshore Range (SCORE), and, eventually to areas without acoustic arrays. Initial sample collection (with assistance of Marine Ecology & Telemetry Research and supplemental funding from ONR) confirms the potential for directed sampling of eDNA from Cuvier’s beaked whales (*Ziphius cavirostris*) on the SCORE range.

Literature cited

- Atlantic Undersea Test and Evaluation Center (AUTEC) Marine Mammal Monitoring on Navy Ranges (M3R) Program User's Manual, Version 1.0 05 August 2015, Naval Undersea Warfare Center, Newport, RI 02841
- Baker, C.S. 2008. A truer measure of the market: the molecular ecology of fisheries and wildlife trade. *Molecular Ecology* 17:3985–3998.
- Baker, C.S., D. Steel, S. Nieukirk, and H. Klinck. 2018. Environmental DNA (eDNA) From the Wake of the Whales: Droplet Digital PCR for Detection and Species Identification. *Frontiers in Marine Science* 5:133.
- Baker, C.S., F. Cipriano, and S.R. Palumbi. 1996. Molecular genetic identification of whale and dolphin products from commercial markets in Korea and Japan. *Molecular Ecology* 5:671-685.
- Baker, C.S., M.L. Dalebout, S. Lavery and H.A. Ross. 2003. www.DNA-surveillance: applied molecular taxonomy for species conservation and discovery. *Trends in Ecology & Evolution* 18:271-272.
- Baker, C.S. and S.R. Palumbi. 1994. Which whales are hunted? A molecular genetic approach to monitoring whaling. *Science* 265:1538-1539.
- Baumann-Pickering, S., M.A. Roch, R.L. Brownell Jr, A.E. Simonis, M.A. McDonald, A. Solsona-Berga, E.M. Oleson, S.M. Wiggins and J.A. Hildebrand. 2014. Spatio-temporal patterns of beaked whale echolocation signals in the North Pacific. *PLoS ONE* 9:e86072.
- Benoit-Bird, K. J., B. L. Southall, M. A. Moline, D. E. Claridge, C. A. Dunn, K. A. Dolan, and D. J. Moretti. 2020. Critical threshold identified in the functional relationship between beaked whales and their prey. *Marine Ecology Progress Series* 654:1-16.
- Bio-Rad Laboratories Inc. 2014. Droplet Digital™ PCR applications guide. Hercules:Bio-Rad Laboratories Inc., https://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6407.pdf
- Bolyen E., Rideout J.R., Dillon M.R., et al., 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology* 37: 852–857.
- Cao, Y., J.F. Griffith and S.B. Weisberg. 2016. The Next-Generation PCR-Based Quantification Method for Ambient Waters: Digital PCR. Pages 113-130 *Marine Genomics: Methods and Protocols*. Springer New York, New York, NY.
- Dalebout, M. L., C. S. Baker, V. G. Cockcroft, J. G. Mead, and T. K. Yamada. 2004. A comprehensive molecular taxonomy of beaked whales (Cetacea: Ziphiidae) using a validated mitochondrial and nuclear DNA database. *Journal of Heredity* 95:459-473.
- Dingle, T.C., R.H. Sedlak, L. Cook and K.R. Jerome. 2013. Tolerance of Droplet-Digital PCR vs Real-Time Quantitative PCR to Inhibitory Substances. *Clinical Chemistry* 59:1670.
- Doi, H., T. Takahara, T. Minamoto, S. Matsushashi, K. Uchii and H. Yamanaka. 2015. Droplet Digital Polymerase Chain Reaction (PCR) Outperforms Real-Time PCR in the Detection of Environmental DNA from an Invasive Fish Species. *Environmental Science & Technology* 49:5601-5608.
- Ficetola, G.F., C. Miaud, F. Pompanon and P. Taberlet. 2008. Species detection using environmental DNA from water samples. *Biology Letters* 4:423-425.
- Foote, A.D., P.F. Thomsen, S. Sveegaard, M. Wahlberg, J. Kielgast, L.A. Kyhn, A.B. Salling, A. Galatius, L. Orlando and M.T.P. Gilbert. 2012. Investigating the Potential Use of Environmental DNA (eDNA) for Genetic Monitoring of Marine Mammals. *PLoS ONE* 7:e41781.
- Jarvis, S. M., R. P. Morrissey, D. J. Moretti, N. A. DiMarzio, and J. A. Shaffer. 2014. Marine Mammal Monitoring on Navy Ranges (M3R): A Toolset for Automated Detection, Localization, and Monitoring of Marine Mammals in Open Ocean Environments, *MTS Journal*,

- Hamner, R. M., R. Constantine, R. Mattlin, R. Waples, and C. S. Baker. 2017. Genotype-based estimates of local abundance and effective population size for Hector's dolphins. *Biological Conservation* **211, Part A**:150-160.
- Hunt, K.E., M.J. Moore, R.M. Rolland, N.M. Kellar, A.J. Hall, J. Kershaw, S.A. Raverty, C.E. Davis, L.C. Yeates, D.A. Fauquier, T.K. Rowles and S.D. Kraus. 2013. Overcoming the challenges of studying conservation physiology in large whales: a review of available methods. *Conservation physiology* 1:cot006-cot006.
- Leonard, J.A., O. Shanks, M. Hofreiter, E. Kreuz, L. Hodges, W. Ream, R.K. Wayne, and R.C. Fleischer. 2007. Animal DNA in PCR reagents plagues ancient DNA research. *Journal of Archaeological Science* 34:1361-1366.
- Morin, P.A., S. Duchene, N. Lee, J. Durban, and D. Claridge. 2012. Preliminary analysis of mitochondrial genome phylogeography of Blainville's, Cuvier's and Gervais' beaked whales. Report to the Scientific Committee of the International Whaling Commission, SC/64/SM14.
- Noren, D.P. and J.A. Mocklin. 2011. Review of cetacean biopsy techniques: Factors contributing to successful sample collection and physiological and behavioral impacts. *Marine Mammal Science* 28:154-199.
- Parsons, K. M., M. Everett, M. Dahlheim, and L. Park. 2018. Water, water everywhere: environmental DNA can unlock population structure in elusive marine species. *Royal Society Open Science* **5**.
- Renshaw, M. A., B. P. Olds, C. L. Jerde, M. M. McVeigh, and D. M. Lodge. 2015. The room temperature preservation of filtered environmental DNA samples and assimilation into a phenol/chloroform/isoamyl alcohol DNA extraction. *Molecular ecology resources* **15**:168-176.
- Ross, H. A., G. M. Lento, M. L. Dalebout, M. Goode, P. McLaren, A. G. Rodrigo, S. Lavery, and C. S. Baker. 2003. DNA surveillance: web-based molecular identification of whales, dolphins and porpoises. *Journal of Heredity* **94**:111-114.
- Sigsgaard, E. E., I. B. Nielsen, S. S. Bach, E. D. Lorenzen, D. P. Robinson, S. W. Knudsen, M. W. Pedersen, M. A. Jaidah, L. Orlando, E. Willerslev, P. R. Møller, and P. F. Thomsen. 2016. Population characteristics of a large whale shark aggregation inferred from seawater environmental DNA. *Nature Ecology & Evolution* **1**:0004.
- Stewart, K., H. Ma, J. Zheng and J. Zhao. 2017. Using environmental DNA to assess population-wide spatiotemporal reserve use. *Conservation Biology* 31:1173-1182.
- Taylor, B.L., F.I. Archer, K.K. Martien, P.E. Rosel, B.L. Hancock-Hanser, A.R. Lang, M.S. Leslie, S.L. Mesnick, P.A. Morin, V.L. Pease, W.F. Perrin, K.M. Robertson, K.M. Parsons, A. Viricel, N.L. Vollmer, F. Cipriano, R.R. Reeves, M. Krützen and C.S. Baker. 2017. Guidelines and quantitative standards to improve consistency in cetacean subspecies and species delimitation relying on molecular genetic data. *Marine Mammal Science* 33:132-155.
- Thomsen, P.F., J. Kielgast, L.L. Iversen, P.R. Møller, M. Rasmussen and E. Willerslev. 2012. Detection of a Diverse Marine Fish Fauna Using Environmental DNA from Seawater Samples. *PLoS ONE* 7:e41732.
- Wegleitner, B.J., C.L. Jerde, A. Tucker, W.L. Chadderton and A.R. Mahon. 2015. Long duration, room temperature preservation of filtered eDNA samples. *Conservation Genetics Resources* 7:789-791.

Appendix 1: AUTC 2019 sampling from the vessel 'Steno' with Diane Claridge, Charlotte Dunn, Nevé Baker and Scott Baker, with acoustic support from Tom Fetherston and Peter Dugan.											
All samples are 2L of seawater filtered through 0.4 micron polycarbonate filter, unless otherwise noted.											
Samples shaded in yellow were chosen for ddPCR sequencing and metabarcoding based on exceeding 0.1 copies/µl from ddPCR quantification.											
Date	Time	Sample type	sample event	eDNA sample code	ddPCR copies/ul	lower 95% CL	upper 95% CL	group size	haplotypes	photoID	comment
1-Aug-2019		Control		AUTC-Co1	0	0.00	0.11				seawater from harbor breakwater
				AUTC-Co2	0.04	0.00	0.18				
2-Aug-2019		Spot	Spot01	AUTC19-1	0.04	0.00	0.21				seawater from Whisky range but no acoustic support
				AUTC19-2	0	0.00	0.13				
3-Aug-2019	10:40	Spot	Spot02	AUTC19-3	0.04	0.00	0.19				sample in proximity to acoustic detection
				AUTC19-4	0	0.00	0.12				
4-Aug-2019	8:48	Spatial proximity	Spatial01	AUTC19-5	0	0.00	0.12				above click-spot of foraging dive but poor conditions
				AUTC19-6	0	0.00	0.12				N200
				AUTC19-7	0	0.00	0.10				E200
				AUTC19-8	0	0.00	0.12				S200
				AUTC19-9	0	0.00	0.11				W200
	11:30	Spatial proximity	Spatial02	AUTC19-10	0	0.00	0.13				above click-spot of foraging dive but poor conditions
				AUTC19-11	0	0.00	0.12				N200
				AUTC19-12	0	0.00	0.11				E200
				AUTC19-13	0	0.00	0.11				S200
				AUTC19-14	0	0.00	0.13				W200
	13:38	Serial flukeprint	Serial01	AUTC19-15	0	0.00	0.12	2		Md617, Md623	collected where 2 whales circling circling
				AUTC19-16	0.04	0.00	0.19			Md617 or Md623	fluke print proximity
				AUTC19-17	0	0.00	0.11			Md617	fluke print proximity
				AUTC19-18	0	0.00	0.11				fluke print proximity from sample 16 location
				AUTC19-19	0	0.00	0.11				fluke print proximity from sample 16 location+15
				AUTC19-20	0.04	0.00	0.18				fluke print proximity from sample 16 location+30
5-Aug-2019	16:12	Spatial proximity	Spatial03	AUTC19-21	0	0.00	0.13				above click-spot of foraging dive
				AUTC19-22	0.04	0.00	0.21				N200
				AUTC19-23	0.04	0.00	0.18				E200
				AUTC19-24	0	0.00	0.11				S200
				AUTC19-25	0	0.00	0.12				W200
	16:52	Serial flukeprint	Serial02	AUTC19-26	0.07	0.01	0.24	3		Md624 or Md625 or Md626	fluke print
				AUTC19-27	0.04	0.00	0.20			Md624 or Md625 or Md626	fluke print
				AUTC19-28	0	0.00	0.11			Md624, Md625, Md626	whales circled the boat, collected sample in circle
				AUTC19-29	0.23	0.09	0.47		MdenBah1a, eDNA-v3a, eDNA	Md624 or Md625 or Md626	fluke print
				AUTC19-30	0.08	0.01	0.24			Md626	terminal dive fluke print
				AUTC19-31	0.04	0.00	0.17			Md626	terminal dive fluke print+15
8-Aug-2019	8:44	Serial flukeprint	Serial03	AUTC19-32	0.08	0.01	0.24	2		Md627	fluke print
				AUTC19-33	0.11	0.02	0.28	1	MdenBah1a, MdenBah1b	Md628	fluke print
				AUTC19-34	0.04	0.00	0.19	1		Md628	fluke print proximity/near terminal dive location
				AUTC19-35	0.11	0.03	0.30	1		Md628	near terminal dive location+15
				AUTC19-36	0	0.00	0.10	1		Md628	near terminal dive location+30
				AUTC19-37	0.2	0.08	0.40	1	MdenBah1a, MdenBah2	Md627	fluke print
				AUTC19-38	0.52	0.29	0.85	2	MdenBah1a	Md628	fluke print
				AUTC19-39	6.1	5.10	7.00	2	MdenBah1a	Md628	terminal dive fluke print, post biopsy sample
				AUTC19-40	0.07	0.01	0.23	1		Md627	fluke print
				AUTC19-41	0.03	0.00	0.16	1		Md627	fluke print
9-Aug-2019	13:00	Spot	Spot03	AUTC19-42	0.04	0.00	0.18				spatial proximity
	13:44	Serial flukeprint	Serial04	AUTC19-43	0	0.00	0.12	mature females		Md501 or Md504 or Md598	fluke print proximity
				AUTC19-44	0.14	0.04	0.34		MdenBah1a	Md504 or Md598	fluke print
				AUTC19-45	0.11	0.03	0.30			Md504	fluke print
				AUTC19-46	0	0.00	0.12			Md504	fluke print
				AUTC19-47	0.33	0.16	0.60		MdenBah1a	Md598	fluke print

				AUTEC19-48	0.49	0.25	0.85		MdenBah1a, MdenBah1b	Md598	terminal dive fluke print
				AUTEC19-49	0.04	0.00	0.17			Md598	terminal dive fluke print+15
				AUTEC19-50	0	0.00	0.11			Md598	terminal dive fluke print+30
				AUTEC19-51	0.84	0.52	1.27		MdenBah1a	Md501 or Md504 or Md598	terminal dive fluke print
10-Aug-2019	13:20	Spatial proximity	Spatial04	AUTEC19-52	0.08	0.01	0.27				above click-spot of foraging dive
				AUTEC19-53	0.04	0.00	0.19				N200
				AUTEC19-54	0	0.00	0.11				E200
				AUTEC19-55	0.12	0.03	0.32				S200
				AUTEC19-56	0	0.00	0.12				W200
		negative control		AUTEC19-blank	0	0.00	0.18				
		negative control		AUTEC19-Tap1	0.04	0.00	0.2				
		negative control		AUTEC19-Tap2	0.04	0.00	0.11				
		negative control		AUTEC19-Tap3	0.03						
		negative control		blank10Sept19	0.035						
		negative control		blank11Sept19	0						
		negative control		blank27Aug19	0						
		negative control		blank6-7Jan20	0						
		negative control		blank9Sept19	0						
		positive control		Che1000	34.495						