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Don't Hold Your Breath: Limited DNA Capture Using Non-Invasive Blow Sampling for Small Cetaceans

Chloe Victoria Robinson[1,2] and Hanna Katariina Nuuttila[1]

[1]*SEACAMS2, Swansea University, Singleton Park, Swansea, SA2 8PP, Wales, UK*
E-mail: chloer@uoguelph.ca
[2]*Centre for Biodiversity Genomics, University of Guelph, 50 Stone Road East, Guelph, ON, N1G 2W1, Canada*

Abstract

Non-invasive conservation strategies currently used to monitor small cetaceans in the United Kingdom mainly focus on photo identification of individuals as a method to generate estimates of population status. With increasing anthropogenic pressures in the form of renewable energy developments, fishing, and tourism, there is a need for molecular techniques to more accurately measure population structure and health status of free-ranging cetaceans. Previous non-invasive DNA sampling has consisted of faecal, skin, or exhaled breath (blow) sampling. To date, blow sampling has been implemented primarily with an emphasis on microbial information, and field applications of this methodology have primarily targeted large mysticete cetaceans. In this study, we attempted to optimise an existing blow sampling protocol to enhance field sample collection and DNA recovery for both *Tursiops truncatus* and *Delphinus delphis*. Following previous recommendations, we streamlined the sampling approach to reduce the occurrence of avoidance behaviour and improve the precision for individual targeting. Overall, we collected 37 blow samples from *T. truncatus* (n = 7) and *D. delphis* (n = 30). Cetacean mtDNA was successfully extracted from one *D. delphis* sample, which is the first reported DNA detection of a small free-ranging cetacean from blow to our knowledge. Herein, we highlight the difficulty obtaining DNA from wild cetacean blow samples, despite improved collection success, and the uncertainty of determining the effects of storage on sample degradation prior to DNA extraction. We suggest future studies to improve DNA detectability through the use of robust preservatives such as an ethanol-sodium acetate solution. While promising, further optimisation is required to employ blow sampling as a conservation tool for estimates of respiratory microbial loads and individual identification for the purposes of genotype capture-recapture of small cetaceans.

Key Words: DNA, non-invasive, conservation, conservation genetics, *Tursiops truncatus*, *Delphinus delphis*

Introduction

Cetaceans (whales, dolphins, and porpoises) are valuable indicators of the health status of the marine environment (Azzellino et al., 2014; Peltier et al., 2014). Biological samples collected from both dead and free-living cetaceans can provide vital information on the level of chemical toxins in seawater (Belante et al., 2012; Peltier et al., 2014; Murphy et al., 2015); can determine genetic parameters including effective population size (Hoelzel, 1998); and can allow characterisation of pathogenic microbial communities in the skin, gut, and respiratory microbiomes (Acevedo-Whitehouse et al., 2010; Blacklaws et al., 2013; Russo et al., 2018). There is currently limited information on baseline microbe communities in wild cetaceans (Johnson et al., 2009; Sanders et al., 2015; Raverty et al., 2017) due to licence and permitting restrictions for collecting samples. In both humans and animals, it has been found that the microbiome plays a significant role in host immune function, and external stressors can cause a shift in the species-specific microbial communities (Nelson et al., 2015; Bahrndorff et al., 2016).

The emergence of pathogenic diseases (Van Bressem et al., 1999; Law et al., 2012) is a primary threat to species such as the bottlenose dolphin (*Tursiops truncatus*) and short-beaked common dolphin (*Delphinus delphis*), in conjunction with additional threats, including increased disturbance from vessel activity (Kelly et al., 2004), effects of contaminant accumulation (e.g., polychlorinated biphenyls [PCB]), and renewable energy developments (Gill, 2005; Dolman & Simmonds, 2010; Bonar et al., 2015; Roche et al., 2016). Infectious respiratory diseases caused by microbes, including *Brucella* spp. (Foster et al., 2002; Gaydos et al., 2004), morbillivirus (Acevedo-Whitehouse et al.,

2010), and influenza A (Ramis et al., 2012), have contributed to a number of small cetacean mass mortalities within the last three decades (Duignan et al., 1992; Fernandez et al., 2008; Venn-Watson et al., 2012). Currently, monitoring the prevalence of infectious agents in wild cetaceans in the UK has been limited to *postmortem* analyses (e.g., Blacklaws et al., 2013; Gkafas et al., 2017), which is problematic as it does not allow for assessment of extant bacterial communities in presumably healthy living individuals (Apprill et al., 2017; Raverty et al., 2017).

Similarly, studies concerning host molecular analyses of cetacean populations in the UK have largely relied on available tissue from cetacean *postmortems* (e.g., Parsons et al., 2002). As genetic tools are increasingly being utilised to identify distinct units for conservation of cetaceans worldwide (European Commission, 2011; Constantine et al., 2012; Foote et al., 2012; Alves et al., 2013; Gaspari et al., 2013; Sveegaard et al., 2015; Chen et al., 2017; Taylor et al., 2017; Attard et al., 2018; Parsons et al., 2018), there is an increased necessity to sample from living individuals to evaluate the existing population status of different cetacean species (Frankham, 2010).

For biological sample collection, there has been a shift in the primary methodology used for collecting DNA from cetaceans—from tissue biopsy and swab sampling (Harlin et al., 1999) to non-invasive techniques such as fecal sampling (Parsons, 2005) and the collection of blow from individuals (Hogg et al., 2009; Acevedo-Whitehouse et al., 2010; Hunt et al., 2014; Thompson et al., 2014; Geoghegan et al., 2018). Blow sampling has been used to determine the respiratory microbiome of larger species of whale such as humpback (*Megaptera novaeangliae*) and gray (*Eschrichtius robustus*; Acevedo-Whitehouse et al., 2010; Geoghegan et al., 2018) whales and has been successfully employed in captive facilities with smaller species, including bottlenose dolphins (Frère et al., 2010) and harbour porpoises (*Phocoena phocoena*; Borowska et al., 2014). Larger whales are a preferred target cetacean species for this methodology due to the large volume of exhaled air produced (202 L/sec for *E. robustus* vs 70 L/sec for *T. truncatus*; Wartzok, 2002), which is more likely to harbour enough lung surfactant for successful DNA detection (Apprill et al., 2017; Richard et al., 2017). While captive studies provide valuable baseline information necessary for interpreting data from wild populations (Bik et al., 2016), development and optimisation of methods to enable such analyses in wild small cetacean species is needed (Parsons et al., 2002; Mirimin et al., 2009; Frankham, 2010; Frère et al., 2010).

This project aimed to pilot a streamlined version of equipment used to collect blow from cetaceans in previous studies (Acevedo-Whitehouse et al., 2010; Raverty et al., 2017) to (1) test the potential of collecting DNA from the blow from target cetaceans (*T. truncatus* and *D. delphis*) and (2) identify mitochondrial haplotypes of target species and determine presence/absence of known pathogenic microbial species in the respiratory tract of target cetaceans if DNA detection is successful.

Methods

Study Locations and Sample Collection

Between August 2017 and August 2018, blow sample collection was attempted from both *T. truncatus* and *D. delphis* from a variety of vessel platforms. For collecting *T. truncatus* samples, we joined Sea Watch Foundation (hereafter SWF) on their *T. truncatus* scientific surveys from New Quay, Aberystwyth, and Pwllheli as part of the Cardigan Bay Monitoring Project and undertook dedicated "blow" surveys using the Swansea University rigid hull inflatable boat (RHIB) from New Quay (Figure 1). *D. delphis* samples were collected from a chartered vessel (RHIB) out of Swansea, and a personal vessel (sailing boat) was used for collecting samples from Lundy, Pembrokeshire, and Isles of Scilly (Table S1; supplementary materials for this article are available on the "Supplemental Material" page of the *Aquatic Mammals* website: https://www.aquaticmammalsjournal.org/index.php?option=com_content&view=article&id=10&Itemid=147). Blow sampling was carried out under licence to disturb and photograph wild animals and possess derivatives for scientific or educational purposes. The license was granted by Natural Resource Wales (Licence Number 79487:OTH:SA:2018), conforming to UK legislation under the Animals (Scientific Procedures) Act 1986 Amendment Regulations (SI 2012/3039).

During cetacean encounters, blow samples were collected from the bow of the boat when individuals were exhibiting "bow riding" behaviour and positively associating with the boat (i.e., no avoidance). We targeted individuals without calves to limit disturbance and avoid collecting blow from multiple individuals. To collect samples, we used a blue/grey telescopic pole (colour chosen to reduce the level of adverse behaviour as observed previously by *T. truncatus* and *D. delphis*; Acevedo-Whitehouse et al., 2010) with a single sterile Petri dish affixed to the end via a suction cup, which was extended out from the bow when an individual was seen coming to the surface to exhale. Petri dish lids remained on prior to deployment of the pole to limit airborne and seawater contamination. Samples were collected 30 cm to 1 m above the blowhole of each

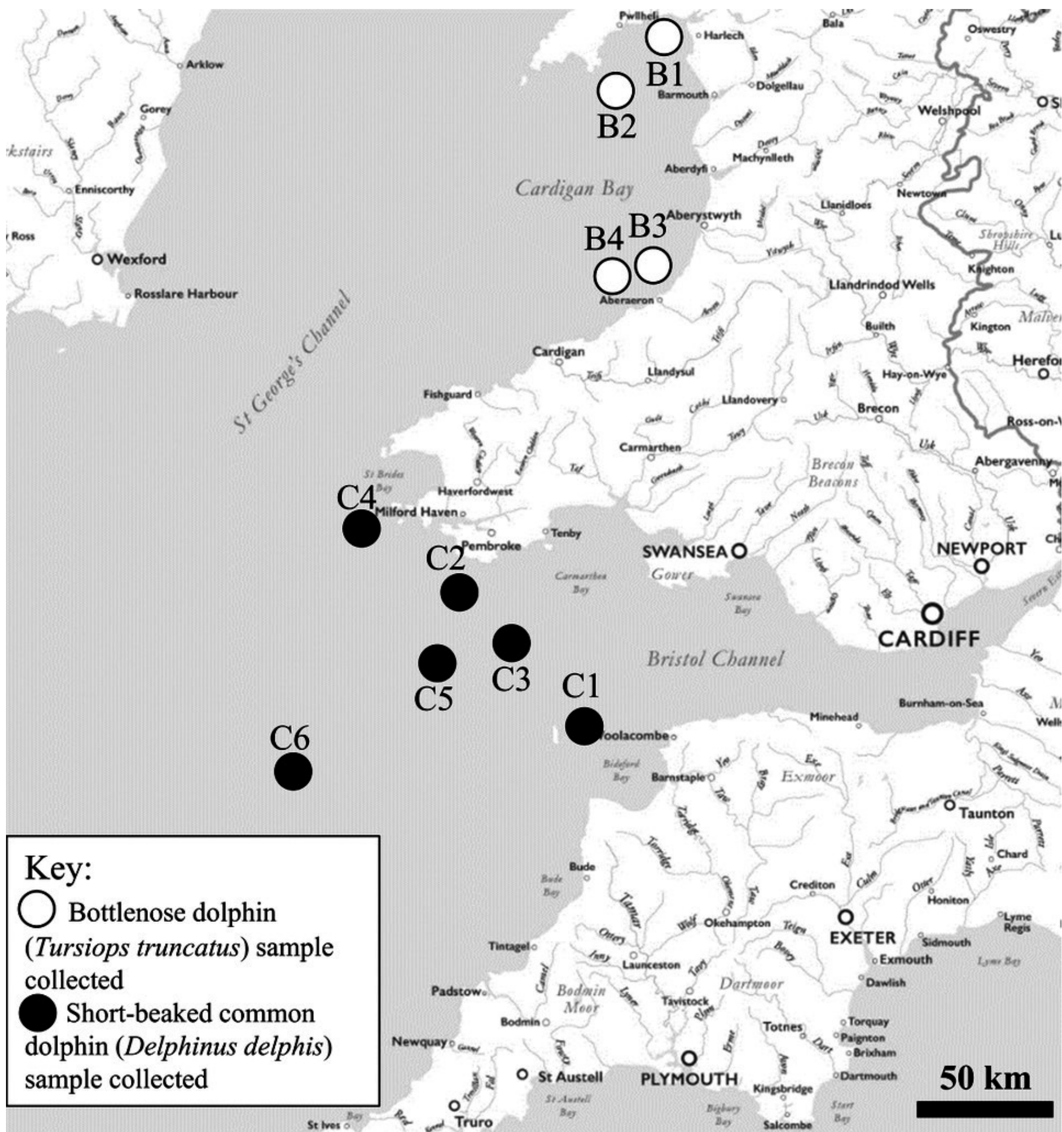

Figure 1. Map of sites around Wales and southwest England (UK) where exhaled breath (blow) samples were collected for both bottlenose dolphins (*Tursiops truncatus*) and short-beaked common dolphins (*Delphinus delphis*). ©Maproom

individual, and the pole was immediately retracted after each sampling attempt (Figure S1).

To determine which individuals had been sampled, a GoPro HERO 4 was attached to the sampling pole to allow video recording concurrent with the sample collection to capture footage of dorsal fins. In addition, a dedicated member of the staff collected photo-identification pictures of sampled individuals (where possible) using a Canon EOS 7D with a Canon EF-S 55-250 mm lens for identification of individuals from photo-identification catalogues.

Petri dishes were retrieved and assessed for evidence of blow (i.e., wet condensate), and positive dishes were covered and taken inside the wheelhouse to be swabbed using a sterile swab (Isohelix, Kent, UK) and snapped into a 1.5-mL Eppendorf tube pre-filled with 100% molecular-grade ethanol. Eppendorf tubes (1.5 mL) were placed temporarily in an ice box before being stored at -20ºC until processing. To avoid contamination, Petri dishes were disposed of and new sterile dishes affixed after each sampling attempt, even if blow was not visually detected. Metadata included time, date of

sample collection, GPS coordinates, and camera codes for photo-identification purposes. After each cetacean encounter for which ≥ 1 blow sample was collected, a single 15-mL Falcon tube of surface seawater was collected as a negative control for downstream respiratory microbiome analyses (Raverty et al., 2017). Seawater samples were only processed from cetacean encounters which produced a positive blow sample (i.e., positive 12S amplification) for the target cetacean species.

Primer Design

Dolphin-specific primers were designed and checked in silico for non-target amplification using *NCBI-BLAST* (Ye et al., 2016). The primer pair was designed to be complementary to *D. delphis* and *T. truncatus* (Cetacea12S_F: 5'-ACTATCGGCAACAGCCCAAA-3' and Cetacea12S_R 5'-AGGGTTTGCTGAAGATGG CG-3') to avoid amplification of non-Delphinidae mammals (i.e., humans and seals), and to enable the amplification of a 144 base pair (bp) product of the 12S mtDNA gene. Primers were assessed *in vitro* using positive control tissue for both species (mouth swabs: *D. delphis* [SW2018/531]; skin tissue: *T. truncatus* [SW2018/756]) collected *post-mortem* by the Cetacean Strandings Investigation Programme (CSIP). DNA was extracted from positive control samples using a Qiagen DNeasy Blood and Tissue Kit (Qiagen, Sussex, UK) following manufacturer's standard protocol for tissue extractions. The 12S fragment was amplified in a Bio-Rad T100 Thermal Cycler (Bio-Rad, Watford, UK) in a total reaction volume of 30 µL, with 15 µL BioMix™ mastermix (Bioline, London, UK) containing Taq DNA polymerase, 1.5 µL each forward and reverse primer at 10 µM, 5 µL template DNA at between 0.1 and 5.6 ng/µL concentration, and 7 µL ultrapure water utilising the following Cetacea12S PCR profile: 95ºC for 3 min followed by 40 cycles of 95ºC for 30 s, 60ºC for 30 s, and 72ºC for 45 s, with a final elongation step of 72ºC for 10 min. Following amplification, products were run on a 1% agarose gel and purified using a QIAquick PCR Purification kit (Qiagen). Samples were sequenced in both directions on an ABI3130xl Genetic Analyser, the quality of sequences (i.e., no contamination/bp overlap and correct bp size for fragment) were checked using the software program *Chromas* (Technelysium, South Brisbane, Queensland, Australia), and resulting sequences which passed the quality check were analysed using *NCBI-BLAST* to confirm target species (Ye et al., 2016).

DNA Extraction and Amplification

Total genomic DNA was extracted from blow samples (ethanol preserved swabs; $N = 36$) and a seawater sample ($N = 1$) using a Qiagen Powerlyzer PowerSoil DNA extraction kit (Qiagen), which has proven to be effective at obtaining sufficient quantities of DNA for analysis from low template environmental samples (Hermans et al., 2018). DNA from ethanol was pelleted by first centrifuging 1.5-mL Eppendorf tubes at 20,000 xg for 10 min (Macher et al., 2018) followed by removal of supernatant. Swab and ethanol DNA were recombined after eluting the cellular debris from the Eppendorf tube in 20 µL of PowerBead solution. The remaining DNA extraction process was carried out following manufacturer's instructions with swab and ethanol DNA combined, apart from a reduction in elution volume from 100 to 50 µL in two elution steps (2 × 25 µL) to increase the total DNA concentration (Rohland & Hofreiter, 2007). Quality and quantity of extracted DNA was assessed using a Nanodrop 2000 (Thermo Fisher Scientific, Loughborough, UK). Samples were amplified using designed Cetacea12S primers following the Cetacea12S PCR protocol above.

To assess the viability of DNA from blowholes for use with mtDNA primers designed for haplotype analysis, any DNA samples which amplified using the Cetacea12S primers were amplified using M13Dlp1.5 (5'-TGTAAAACGACAGCCAGTTCA CCCAAAGCTGRARTTCTA-3') and Dlp8G (5'-GGAGTACTATGTCCTGTAACCA-3'; Dalebout et al., 1998) to amplify a 650-bp portion of Control Region (D-loop), along with Tglu (5'-TGACCTGAARAACCAYCGTTG-3') and CB2 (5'-CCCTCAGAATGATATTTGTCCTCA-3'), which amplify a 400-bp region of Cytochrome b (Cytb; Farías-Curtidor et al., 2016). DNA was amplified in a total reaction volume of 30 µL, with 15 µL BioMix™ mastermix containing Taq DNA polymerase, 1.5 µL each forward and reverse primer at 10 µM, 5 µL template DNA at between 0.5 and 5 ng/µL concentration, and 7 µL ultrapure water using PCR utilising the following conditions for both primer pairs: three cycles of 94ºC for 30 s, 45ºC for 30 s, 72ºC for 90 s; followed by 35 cycles of 94ºC for 30 s, 55ºC for 30 s, and 72ºC for 90 s; and finishing with a single extension of 72ºC for 5 min (Palumbi, 1995). All PCR products were run on a 1% agarose gel. PCR products for samples that produced a band of expected sizes were purified using QIAquick PCR Purification kit (Qiagen), sequenced on an ABI3130xl Genetic Analyser; the quality of sequences (i.e., no contamination/bp overlap and correct bp size for fragment) were checked using *Chromas*, and resulting sequences which passed the quality check were analysed using *NCBI-BLAST* to confirm target species (Ye et al., 2016).

Sex Determination and Microbial PCR Screening
To identify the sex of individuals that produced a positive sample for the Cetacea12S primers, samples were amplified along with positive controls of known sex using cetacean-specific sex primers SRY593 (5'-AAGCGACCCATG AACGCATT-3') and SRY764 (5'-GTATTTC TCTCTGTGCATGG-3') with BioMix™ mastermix containing Taq DNA polymerase utilising the following PCR cycling conditions: 94ºC for 2 min, followed by 40 cycles of 94ºC for 1 min, 55ºC for 2 min, and 7ºC for 1 min (Vader et al., 1992). Samples were co-amplified with the Cetacea12S primers, which act as a positive control for the PCR reaction. PCR products were visualised on a 1% agarose gel. A male-specific band at 170 bp (in addition to a band at 144 bp for the Cetacea12S product) corresponds to a male sample, and lack of a band at 170 bp (but presence of band at 144 bp for the Cetacea12S product) corresponds to a female sample (Vader et al., 1992).

Blow samples that produced a positive sample for the Cetacea12S primers were amplified using previously designed microbial primers (Raverty et al., 2017) to test for presence of key respiratory microbial species, including *Brucella* spp., morbillivirus, canine distemper virus, influenza virus, and Mollicutes (*Mycoplasma* spp.), using PCR protocols as stated in Raverty et al. (2017). In addition, seawater samples from each encounter which produced a positive for the cetacean species in the corresponding blow sample were amplified using the microbial PCR method described above to confirm that any positive microbial DNA originated from the cetacean lungs as opposed to the sea surface microbiota (Raverty et al., 2017).

Results

Sampling Success
Between August 2017 and August 2018, 37 blow samples were collected from both *T. truncatus* (n = 7) and *D. delphis* (n = 30) during 13 dedicated cetacean surveys (Table S1). Out of 13 surveys, 11 of these yielded samples from either *T. truncatus* or *D. delphis*. The reduced number of *T. truncatus* samples reflects a combination of some avoidance behaviour and the reduced rate of encounter compared to the *D. delphis* as reflected in Table S1. DNA concentrations of the 37 samples were low (range: 0.5 to 1 ng/µL; Table S2). Out of 37 samples, only one sample successfully amplified using the Cetacea12S, D-loop (GenBank Accession Number MK425687), and Cytb primers; the remaining samples failed to amplify for all three primer sets. The corresponding seawater sample for a successful blow sample encounter failed to amplify using Cetacea12S, D-loop, and Cytb primers, suggesting the DNA detected was directly from the blowhole rather than the seawater. PCR products were sequenced (12S [144 bp], D-loop [650 bp], and Cytb [400 bp]) for both *T. truncatus* and *D. delphis*; positive controls matched 100% with respective species on *NCBI-BLAST* after sequencing (GenBank Accession Numbers for D-loop: MK425686 for *T. truncatus*, and MK425688 and MK425689 for *D. delphis*; see supplementary information for 12S sequences). The positive blow DNA sample matched 100% with *D. delphis* on *NCBI-BLAST* with all sets of primers. Footage from the GoPro camera for the successful sample was cross-checked with identification photos taken by the dedicated person during the encounter to ensure the sampled individual had been documented. Please see the "Supplemental Material" section on the *Aquatic Mammals* website for example MP4 files of sample collection.

Sex Determination and Microbial Screening
Positive controls (*D. delphis* female; *T. truncatus* male) were amplified with the SRY sex determination primers; the female positive control resulted in a band at 144 bp, and the male positive control produced two bands (144 and 170 bp). The blow sample produced one band at 144 bp and, therefore, was determined to be female (Vader et al., 1992). For all five primer sets, the blow and seawater samples failed to amplify any of the target microbial species. The successful co-amplification of the Cetacea12S primers indicated that the lack of amplification was likely due to the absence of targeted microbiota rather than a failed PCR (Vader et al., 1992).

Discussion

In this study, we were able to detect mitochondrial DNA from the blow of a small cetacean; however, low DNA yield and detection success remain a problem for this methodology despite optimisation. We attempted to streamline a previous blow sampling technique (Raverty et al., 2017) to increase the sample capture success from a variety of vessels for both *T. truncatus* and *D. delphis*. In comparison to Acevedo-Whitehouse et al. (2010), neither *T. truncatus* nor *D. delphis* displayed avoidance behaviour (see Stensland & Berggren, 2007) when presented with the pole and, instead, maintained their position on the bow throughout the majority (for *T. truncatus*) or entirety (for *D. delphis*) of the attempted sample collection period. The approach of using a single Petri dish on a camouflaged pole appeared to limit avoidance behaviour, which enabled us to collect a greater number of samples per species than the previous study (Acevedo-Whitehouse et al., 2010).

The primary aim for non-invasive methods is to avoid disturbing, harming, or adversely affecting the target species during sample collection (Taberlet & Luikart, 1999). There are many factors to consider when collecting biological samples directly from cetaceans, including group composition (Fruet et al., 2016), behaviour exhibited (Fruet et al., 2016; Geoghegan et al., 2018), proximity to vessel (Acevedo-Whitehouse et al., 2010), and environmental conditions such as sea state and wind direction (Burgess et al., 2018). During the *T. truncatus* sample surveys, despite low levels of wind and calm sea state, most encounters involved groups containing mothers with calves and/or individuals which did not approach the boat to bow ride. This highlights why it is not possible to attempt sample collection for every encounter.

In comparison with *T. truncatus*, *D. delphis* are considered to exhibit a stronger attraction to boats, presumably for bow riding (Perrin, 2009), which, combined with lack of avoidance behaviour, resulted in more opportunities for collecting blow samples. In addition, *D. delphis* often aggregate in larger social groups (~20 to 200; Evans et al., 2003; Perrin, 2009) than *T. truncatus* (~6 to 30; Evans et al., 2003), presenting a greater number of available individuals for sampling; however, these larger groups may also increase the risk of cross-contamination of blows from multiple animals. The highly active behaviour of *D. delphis* at the surface makes targeting those individuals more difficult than *T. truncatus*; however, using a single Petri dish allowed for a more focussed approach, thus reducing the chance of collecting blow from numerous individuals simultaneously.

Despite many samples appearing to contain lung surfactant, identifiable by dark, mucosal residue on the surface of the Petri dishes, we were unable to recover DNA from most of the samples. The observed low DNA recovery suggests inconsistency regarding volume of lung surfactant produced, difficulty collecting blow from optimal heights and position above the blow hole for each sample attempt, and/or DNA degradation post-sample collection. To address the problem of recovering sufficient DNA from Petri dishes for downstream analysis, alternative DNA storage and resuspension methods could be employed such as storing swabs on ice and subsequently resuspending them in TE buffer, which has been reported to avoid the issue of DNA adhesion to 1.5-mL Eppendorf tubes (and subsequent loss of yield during extraction) when stored in ethanol for prolonged periods of time (Frère et al., 2010).

During this study, an unforeseen issue with sample storage arose, meaning it was difficult to consistently have access to cold (< 4ºC) storage, particularly on surveys which lasted in excess of 12 hours. Ultimately, this may have resulted in DNA degradation between sample collection and extraction (Richard et al., 2017). To overcome this in the future, sodium acetate could be added to swabs stored in ethanol as EtOH-NaAc solution has been shown to be effective in preserving highly fragmented environmental DNA (eDNA) at room temperature for up to 7 days (Ladell et al., 2019), which would reduce the necessity for cold (< 4ºC) storage on small vessels. Additionally, placing swabs directly into lysis buffer would also prevent DNA degradation for prolonged periods of time (Zainabadi et al., 2019).

It is unclear overall whether the lack of DNA detection was a result of degradation or insufficient quantities of DNA released in the blow. However, the one positive blow sample for *D. delphis* was among the samples, highlighting positive DNA recovery despite less than optimum storage conditions. To address the latter, there is potential to increase the amount of lung surfactant collected from *D. delphis*. Due to the absence of adverse behaviour exhibited by this species in comparison with the *T. truncatus*, it would be possible to utilise a Petri dish with a fractionally larger surface area or add a second Petri dish parallel to the pole, thus increasing the capture quantity while maintaining a focussed approach. Finally, using quantitative PCR (qPCR) or digital droplet PCR (ddPCR) platforms could enhance detection due to the increased sensitivity of these techniques (Taylor et al., 2017).

Overall, we have shown this optimised blow sampling methodology still falls short of replacing more invasive methodology (i.e., biopsy sampling and skin swabs) as a viable method for obtaining DNA from wild small cetaceans. We have concluded that a combination of increased survey effort, further optimisation of blow capture methodology, and utilising more effective DNA preservation techniques (EtOH-NaAc solution) could increase the likelihood of successful cetacean and microbial DNA detection. For non-invasive biological sampling of cetaceans to be non-intrusive yet yield enough samples to produce meaningful results, the behaviour and physiology of the target species needs to be taken into consideration so that methodology can be altered to be species-specific. Moving away from the "one size fits all" approach to non-invasive DNA sampling is necessary for ensuring the greatest chance of successful sample collection. Collecting genetic data from wild cetaceans is increasingly necessary for conservation management strategies. Until non-invasive methodology is optimised for application in locations where licenses for invasive sampling are difficult to acquire, accurate population and health status estimates remain unknown.

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