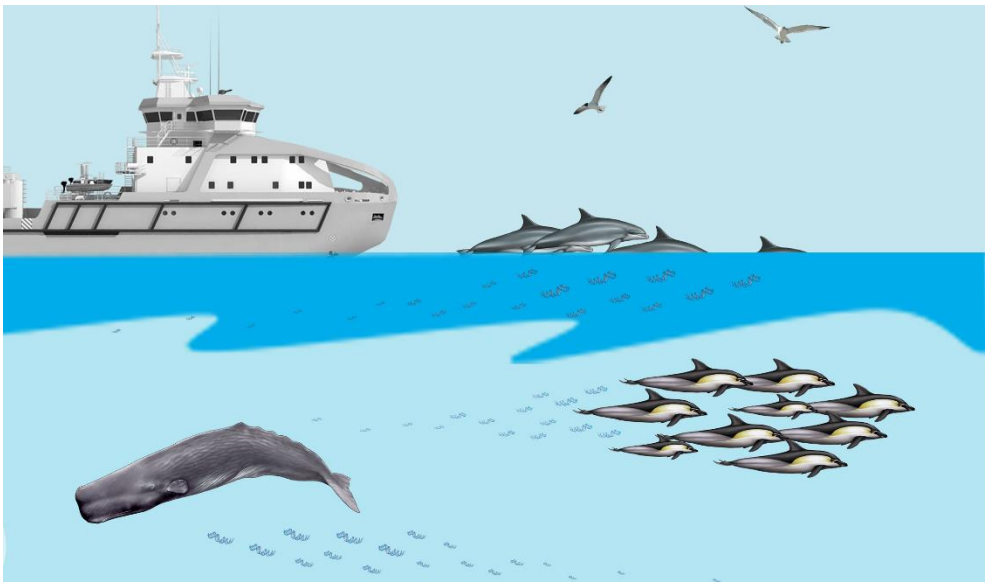
The Potential and Pitfalls of Environmental DNA in Cetacean Research



The advantages and disadvantages of using environmental DNA for research on cetacean biodiversity

Shiva Jalalizadeh (S5334780)

rijksuniversiteit groningen

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Image front page: (Suarez-Bregua et al., 2022)

Shiva Jalalizadeh (S5334780)
s.jalalizadeh@student.rug.nl

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Thesis supervisor: Per Palsbøll

Second supervisor: Martine Bérubé

Pre-MSc Biology

Groningen, Netherlands

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Rijksuniversiteit Groningen

Zernike Campus
Duisenberg Building
Nettelbosje 2
9747 AE Groningen

T: +31 50 363 4624
E: reception-fszw@rug.nl

Foreword

This thesis was written for a pre-masters program in Biology at Rijksuniversiteit Groningen. The topic of this thesis is environmental DNA and its claimed benefits vs. potential drawbacks when researching cetaceans in their marine habitat. This topic has been controversial among marine biologists since its capabilities and promise are hindered by its shortcomings and the potential errors that accompany its results. While the jury is still out on whether and to what extent eDNA is reliable to use in the field of marine biology, this literature review provides insight on both sides of the debate. As an aspiring marine biologist, I found it interesting and informative to deepen my knowledge of this surveying technique that I might come into contact with in my future career.

In this foreword, I would like to thank Bregje Wertheim, for introducing me to the topic of eDNA during her lectures and providing me with my first small impression of what it can do and be used for. Most importantly, I would like to thank my supervisor Per Palsbøll for his time, guidance, and constructive/informative feedback. Finally, I would like to thank my second supervisor Martine Bérubé for taking the time to read and review my bachelor's thesis. I am grateful; for this writing opportunity that taught me the more academic and scientific aspects of research and writing a literature review and also to all the people who played a part in this learning experience.

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Summary

Environmental DNA (eDNA) is genetic material released from an organism into its environment. It is used as a surveying tool in marine biology and has been attributed to numerous alleged capabilities over the years. Nevertheless, eDNA is still an emerging field of study, and the research on its validity is currently limited and potentially influenced by publication bias. This literature review is an examination of the claimed benefits and potential drawbacks or misinterpretations of eDNA. This review aims to provide an important reality check on the use of eDNA, through the question: "*To what extent can environmental DNA be implemented to promote understanding of cetacean biodiversity?*" An analysis of articles shows that while in some cases eDNA samples can result in positive identifications, many factors (e.g. oceanographic or environmental) need to be considered when interpreting its results. This review explores the current state of research conducted on eDNA and highlights the ad hoc nature of these studies. It critically discusses the eDNA-successes obtained and emphasizes the importance of not overselling eDNA as a substitute for well-established traditional surveying methods. Finally, this review highlights the gap in the literature for eDNA-failures and false-negative results. Hereby, it emphasizes that additional field research is necessary to enable a direct comparison between eDNA and traditional methods to reach a conclusive determination on the superior approach to sampling and surveying. Until then, it advises the use of eDNA as a complementary tool to traditional surveying methods as opposed to a replacement.

Keywords: eDNA, cetaceans, biodiversity, biomonitoring, detection, identification

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1 Introduction

To discover more about the marine environment and understand what's going on within it, marine biologists have been using traditional survey methods. These methods involve physically catching, tagging, or biopsying an organism (Pinfield et al., 2019). They are often invasive and disturbing to the animals because they require the proximity of a vessel to the individual while it is at the surface (Baker et al., 2018). In 1999, the first environmental DNA, or eDNA, study was conducted (Willerslev et al., 1999). Since then DNA obtained from environmental samples such as sediments, ice, or water has been claimed to represent an important source of information on past and present biodiversity (Pedersen et al., 2015).

eDNA is DNA expelled into the environment in forms including feces, sloughed skin, scales, blood, hair, and mucus (Pinfield et al., 2019). Since its introduction into the field of marine biology two decades ago, the validity and accuracy of eDNA have been a topic of ongoing discussion. With validity, eDNAs' ability to measure what it is supposed to measure (Middleton, 2023) is questioned. With accuracy, the question is the extent to which the measure reflects reality. While several papers argue that eDNA has beneficial uses and proposes a lot of promise in the field of genetics and marine biology, others try to draw attention to its pitfalls/shortcomings. Thus, urging the researcher to make an informed decision on whether its use is reliable and effective for a specific type of study.

1.1 Environmental DNA

eDNA is an emerging tool in marine biology and it is claimed that it aids in biodiversity, abundance, and distribution assessments. eDNA-based approaches aim to identify and characterize organisms in an environment through the analysis of the genetic material (e.g., mucus and feces) that they leave behind (Ruppert et al., 2019). These sources of eDNA can then be collected through water sampling and amplified using one of several genetic techniques. Hereby allowing for the species to be detected and studied without the need to directly see or sample them (Ficetola et al., 2008). A simplified schematic overview of the workflow associated with eDNA sampling, sample processing, and data generation can be seen below in Figure 1.

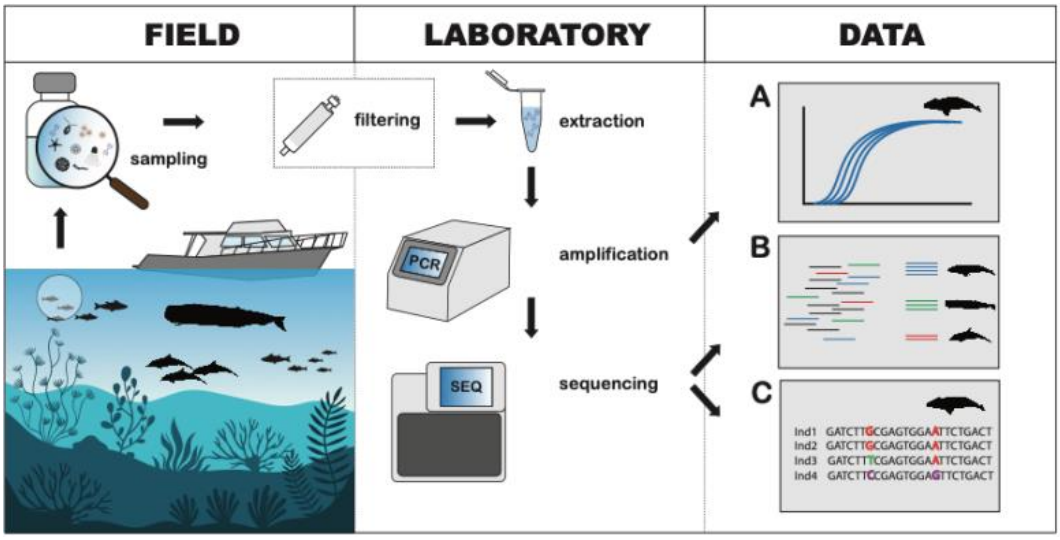

Figure 1 Overview of eDNA sampling and analysis (Székely et al., 2022)

1.2 The use of eDNA

Initial aquatic eDNA research was limited to determining the presence or absence of a species in freshwater ecosystems. Since then, new applications of eDNA emerged and eDNA was adopted to sample in more challenging marine environments (Dejean et al., 2012; Ficetola et al., 2008; Jerde et al., 2011 cited in Pinfield et al., 2019). eDNA has been used to determine;

- species abundance/biomass and ecological assemblages in a habitat,
- population structure and trophic interactions,
- organism behavior (such as migratory patterns, habitat preferences, spawning timing/locations) and asses;
- the diet of marine species (Deagle et al., 2010, Carr, 2017 and Palsbøll et al., 2007; Palsbøll et al., 2013; Waples & Gaggiotti, 2006 cited in Székely et al., 2021).

Additionally, eDNA has increasingly been used as a monitoring tool to; detect the presence of rare/endangered, elusive, or invasive species. It is also claimed to demonstrate species biodiversity and obtain estimates of population genetic diversity in aquatic environments (Thomsen et al. 2012b cited in Valsecchi et al., 2020).

1.3 eDNA in the field of marine biology

Over the years, several eDNA studies have accumulated in the field of marine biology. A study by Sigsgaard et al. (2017) demonstrated the use of eDNA to provide estimates of genetic diversity in a whale shark *(Rhincodon typus)* aggregation. A study by Parsons et al. (2018) on harbor porpoises *(Phocoena phocoena)* revealed indications of significant genetic differentiation within a currently recognized single stock of harbor porpoises. It also identified two previously undocumented mitochondrial haplotypes from seawater samples. Another eDNA study by Baker et al. (2018) confirmed killer whale *(Orcinus orca)* presence in Puget Sound, North America, and correctly identified the killer whale ecotype present at the time of seawater sampling.

Inferring about; species detections, abundance estimation, genetic diversity, and population structure using traditional direct sampling via skin biopsies, is claimed to be a difficult and expensive task. Especially for highly mobile marine species such as cetaceans. This is why researchers have promoted eDNA as an innovative, relatively simple, non-invasive, and cost-effective alternative (Parsons et al., 2018). However, eDNA is an emergent field of study, and research on its validity is limited. Additionally, its effectiveness is dependent on several factors (Pinfield et al., 2019) and it is subject to publication bias (Fediajevaite et al., 2021) since eDNA-failures are less likely to be published than eDNA-successes. To map these points and weigh the possible misinterpretations and drawbacks of eDNA against its claimed benefits, the following research question was formulated:

To what extent can environmental DNA be implemented to promote understanding of cetacean biodiversity?

This literature review will discuss the current view on the use of eDNA as well as its potential and future implications. To narrow the review down, the focus will be on cetaceans and the use of eDNA in biodiversity assessments. Cetaceans represent good candidates for eDNA sampling given their known tendency to release cellular DNA in shed skin, fecal plumes, and their “spout” or blow (Amos et al., 1992; Parsons et al., 1999 and Hunt et al., 2013 cited in Baker et al., 2018). This review aims to provide an important reality check on the use of eDNA and draw attention to the need to exercise caution when interpreting its results. Ultimately, increasing the reliability of the facts added to the library of marine biology through eDNA research.

2 The benefits of eDNA

In 2018, Baker et al. conducted research on the detection and species identification of cetaceans and confirmed the potential to detect eDNA in the wake of whales for up to two hours. eDNA sampling was done from 25 encounters with killer whales in Puget Sound off the northwest mainland Seattle. The re-amplification and sequencing of this eDNA barcode resulted in the detection of a "southern resident community" of killer whales. These results were consistent with the calls from hydrophone recordings and visual observations. Baker et al. (2018), citing Dalebout et al. (2004), claimed that traditional sampling methods were limiting and accompanied by methodological challenges. He associated these challenges with access, (for example, to cetacean foraging zones in the extreme deep-sea environment, Visser et al., 2021), distribution, and behavior of cetaceans. Some species are rare, cryptic, or both, while other species are difficult to approach because of their elusive behavior. Parsons et al. (2018) claimed that elusive and highly mobile animals confound traditional approaches to collecting tissue samples and that near-shore cetaceans are highly vulnerable to fisheries by-catch and the effects of habitat degradation. It is important to remember that Baker et al. (2018) and Parsons et al. (2018) focused on beaked whales *(Ziphius cavirostris)* and harbor porpoises respectively. The statements made were aimed at these species and may not be generalized reliably. Beaked whales are typically deep-ocean species (Madsen et al., 2014) and as opposed to coastal species, are not often sighted. In the study by Parsons et al. (2018), the shallow coastal distribution, habitat and prey preferences of harbor porpoises made them highly vulnerable to incidental capture during net-fishing operations and other anthropogenic impacts (Jefferson & Curry, 1994; Read, 1994; Barlow et al., 1995 cited in Parsons et al., 2018). These factors have led researchers to believe and further claim that traditional surveying methods have limitations and risks that eDNA does not.

Additionally, research conducted by Miller et al. (2022) claimed that some cetacean species, such as the Northern bottlenose whales *(Hyperoodon ampullatus)*, are sensitive to disturbance. Research conducted by Lambertsen (1987) and Krützen et al. (2002) stated that most samples collected by traditional methods require a biopsy dart to be shot at close range with a crossbow or a modified veterinary capture rifle. Baker et al. (2018) claimed that this method is invasive and possibly dangerous/lethal to the target animal (based on the experience of the shooter) and also disturbing to the pod and other species that may be swimming around in the vicinity. The vessel from where the dart is projected usually gets within 10-20 meters of the individual animal that is meant to be sampled while it's at the surface (Dalebout et al., 2004). Some species are sensitive to the close approach of this vessel or to the biopsy sample itself (Noren and Mocklin, 2012). These conceptions are why, Baker et al. (2018) claimed that using traditional methods for genetic sampling was a difficult, limiting, and disruptive task. Consequently, leading them to adopt droplet digital (dd)PCR technology for the detection and species identification of cetaceans using eDNA (more information on ddPCR is given in Chapter 2.1). However, usually, the researchers collecting the biopsy samples are experienced. There is not enough evidence to suggest that the biopsies collected have resulted in any damage, let alone be lethal. As reported by Cantor et al. (2010), biopsy sampling is unlikely to have long-term effects, and studies aiming at testing, if there were anything other than short-term effects, have failed to find any.

A meta-analysis by Fediajevaite et al. (2021) that compared traditional- and eDNA survey methods, suggested that eDNA outperforms traditional surveys. Fediajevaite et al. (2021) claimed that eDNA is a faster and cheaper alternative and that it allows the monitoring of species abundance and biodiversity. Including endangered and elusive species, with the advantage of being non-destructive. They claimed that eDNA methodologies are less prone to morphological identification bias and spatial autocorrelation due to sequence comparisons to an existing database. Hereby increasing their ability

to demonstrate an accurate picture. Carr (2017) also claimed that eDNA can be more comprehensive since it allows the researcher to infer the presence of species that have passed through but are no longer present. Fediajevaite et al. (2021) do caution that these "pro" eDNA arguments are based on just a fraction of available eDNA papers and that these papers might be subject to publication bias.

Research conducted by Baker et al. (2018) was able to make species-specific detections of southern resident killer whales following their passage using ddPCR. They were able to sample eDNA without imposing any disturbance and remained outside of the 200-yard limitation (182 m) of current vessel-approach regulations for killer whales (Noren & Mocklin, 2012). The eDNA samples obtained were compared with a comprehensive reference database of mitochondrial (mt)DNA sequences from the most recognized species of cetaceans (Ross et al., 2003; Dalebout et al., 2004). From these reference sequences, they designed primers for short fragments of the mtDNA, referred to as "mini-barcodes" and used them to target killer whales and improve the amplification of their DNA degraded in the ocean. Using eDNA sampling, Baker et al. (2018) were able to detect the eDNA of killer whales in 17 of the 25 encounters (68%) and were able to confirm for two encounters, that the sequences matched the mtDNA haplotype of the southern resident killer whales. Chapter 2.1, elaborates on the collection and analysis of eDNA, thereby providing insight into concepts such as ddPCR used in the study by Baker et al. (2018).

2.1 Biomonitoring: from sampling to interpretation

eDNA is commonly used in monitoring and hereby sustaining biodiversity. Biodiversity is an indicator of the actual state, health, and prosperity of an ecosystem. Anthropological actions such as climate change and habitat destruction have major impacts on this biodiversity. Therefore protecting, preserving, and restoring an ecosystem increasingly gains importance. This is realized by biomonitoring (Baird & Hajibabaei, 2012 cited in Rodríguez-Ezpeleta et al., 2021).

Biomonitoring is used for developing biotic indices that aid in assessing ecological status, measuring impacts of anthropogenic activities in natural ecosystems, evaluating biodiversity loss, surveying nonindigenous species, and identifying cryptic species (Balvanera et al., 2006; Fišer et al., 2018 cited in Rodríguez-Ezpeleta et al., 2021). Thus, biomonitoring activities aid in the implementation of regional, national, and international regulations, thereby directly contributing to management and conservation efforts. However, in biomonitoring, access to remote locations, limited specialist taxonomic knowledge, and low sensitivity for the detection of rare and elusive species have served as obstacles (Zinger et al., 2020 cited in Rodríguez-Ezpeleta et al., 2021). Rodríguez-Ezpeleta et al. (2021) claimed that advances in eDNA have increased opportunities to overcome these obstacles and that eDNA has increasingly been implemented in the field of genetics and marine biology (Pawlowski et al., 2020; Taberlet et al., 2012 cited in Rodríguez-Ezpeleta et al., 2021).

Working with eDNA starts with its *sampling*. There are a variety of techniques that could be used for this purpose but the when, where, and how all depend on the type of study and research question. It stays imperative in all cases that the sampling strategy produces a representative picture of the sampled geographical range and time frame. It is also important that it accounts for environmental and species distribution heterogeneity, adheres to practices for preventing external and cross-contamination among samples, and stays true to the unique ecological conditions and goals of individual sampling programs. The sampling techniques that can be applied vary from simple (e.g., bottle or bucket of water) to more sophisticated gear (e.g., Niskin bottles). They also include artificial, biological, or even automatic on-site sampling devices. Before sampling, it is important to consider; sample collection coordinates, the amount of collected material, and storage conditions. These considerations enable study replication and support correct data interpretation (Rodríguez-Ezpeleta et al., 2021).

Figure 2 eDNA water sample (Crane, 2020)

Once a water sample (as can be seen in Figure 2) has been collected, the laboratory work and eDNA analysis commence. Just like sampling, there are multiple ways to amplify and sequence the environmental DNA extracted. eDNA can be:

1) Interrogated for the presence and quantification of a given taxon through a detection assay such as quantitative PCR (qPCR) or digital droplet PCR (ddPCR),
2) Enriched for a given taxonomic group before sequencing through PCR or capture (metabarcoding) or
3) Directly sequenced (metagenomics, Rodríguez-Ezpeleta et al., 2021).

In the field of eDNA research, DNA metabarcoding is a rapidly developing approach. In combination with high throughput sequencing, it allows eDNA to assess and monitor marine biodiversity (Valsecchi et al., 2020). Water samples can be analyzed using high-throughput sequencing followed by comparison with DNA databases. Hereby determining what types of organisms are or were recently in the vicinity of the sampling location (Carr, 2017). Through the use of universal primer sets targeting taxa of interest, eDNA can detect communities of species from a single sample by metabarcoding. Valsecchi et al. (2011) claimed that eDNA has hereby improved the spatiotemporal resolution of biodiversity surveys. DNA metabarcoding identifies multiple species from a mixed sample (bulk DNA or eDNA) based on high-throughput sequencing (HTS) of a specific DNA marker (Liu et al., 2020). With high-throughput sequencing, many fragments of DNA can be sequenced in parallel and enable the ability to read hundreds of millions of DNA fragments at the same time. Generating more data, with less time and costs (Cabuzu, 2022). It differs from conventional DNA barcoding because the amount of DNA sequence data derived by HTS allows taxonomy to be rapidly assigned to many species present in a sample (Liu et al., 2020). It is important that primers and/or probes used for amplification, detection assays, and metabarcoding, should be carefully chosen to target the desired taxon in an unbiased way. Each step of the laboratory work must include negative- and positive controls. Negative controls can help to identify potential (cross-) contaminants. Positive controls (e.g., the target species), are useful to enable verification that the laboratory work is not compromised (Rodríguez-Ezpeleta et al., 2021).

One possible way of translating raw data to interpretable data, a process known as bioinformatics, is droplet digital PCR (ddPCR, Rodríguez-Ezpeleta et al., 2021). In the study by Baker et al. (2018), as mentioned in Chapter 2, this eDNA amplification method was used in the field. It detected a southern resident community of killer whales in Puget Sound. ddPCR can quantify low levels of DNA (which can often be the case in eDNA sampling) by fractionating a PCR reaction into more than 20,000 droplets using an oil emulsion. Each water droplet separates template DNA molecules into individual PCR reactions. As a result, thousands of independent amplification events can take place within a single

sample, hereby amplifying low levels of DNA. These amplification events are then analyzed individually on a droplet reader which counts whether droplets are positive or negative for the mutation of interest (Bio-Rad, 2023).

The sequences extracted from the obtained DNA are then aligned to known haplotypes of the taxonomic groups of interest. These sequences are visually inspected with software and compared to reference databases. Once this has been done, the results can start being interpreted. Deriving biomonitoring conclusions from eDNA data requires being aware of potential sampling, data generation, and data analysis biases. The analysis can result in phenomena that are known to occur but the manner or magnitude in which they affect each data set might be unclear. It is also possible that some of the expected species are absent in reference databases or that the primers used do not amplify a given taxon (Rodríguez-Ezpeleta et al., 2021).

3 The potential drawbacks of eDNA

As opposed to the claimed benefits of eDNA reported by Baker et al. (2018), in 2019, Pinfield et al. conducted a research where they observed a killer whale community from fishing trawlers. They tried to capture their eDNA by collecting water samples in the pelagic waters west of Scotland and Ireland. The killer whales closely approached the fishing vessels and samples were collected in close proximity in inshore and offshore waters. However, none of the recovered samples returned positive detections of killer whale eDNA. Pinfield et al. (2019) stated that the killer whales were visually observed during 60% of the sampling events and the animals approached within ten meters (estimated by eye) of the vessel during four of these events. However, they were unable to conclusively amplify or enrich any killer whale eDNA, resulting in false-negative detections rather than true-positive detections. Therefore, in their article Pinfield et al. (2019) caution that while eDNA is increasingly being adopted as a biodiversity monitoring tool, the use of eDNA surveys in the marine environment is still in its infancy. Next to its possibilities and benefits, its shortcomings are also just starting to be understood. The fact that Pinfield et al. (2019) reported false-negative detections demonstrates that eDNA may not be accurate. The researchers' inability in detecting killer whale eDNA when the whales were directly in front of them, raises the question of whether visual observations could be a more reliable alternative. It is important to remember that many factors affect species detection and biodiversity monitoring by eDNA and that it is not always able to give an accurate impression of reality.

Accuracy in research captures bias. For a surveying method like eDNA to be accurate, the variable estimated/measured by it needs to correspond to the true state of the variable that is being estimated. When there is an error in these estimates, meaning they aren't similar, this means that the results and following conclusions are biased/inaccurate. To lower bias, validation of the results by complementary methods is necessary (Martinez & Dumer, 2014). In the research by Pinfield et al. (2019), eDNA samples were collected in varying sea conditions, from the surface and subsurface of the water. Research conducted by, Pinfield et al. (2019) discussed both the influence of abiotic and biotic factors on the persistence of eDNA in aquatic systems and addressed the issues in methodology as reasons for the false negative detections. In this research, possibly that of Baker et al. (2018) and many other eDNA success publications, weather conditions and calm vs. choppy sea states caused differences in dilution and dispersion rates of eDNA (Pinfield et al., 2019). Pinfield et al. (2019) reported that there were a higher number of killer whale-positive detections from eDNA when sampling from the air/surface interface (surface water) compared to the subsurface. They believed that the advection of sloughed skin or feces at the surface contributed to this. Pinfield et al. (2019) also concluded that animal behavior during sampling is an important factor to consider. Killer whales may defecate less during foraging than during traveling, resting, or socializing, and lack of fecal matter from the target organism may reduce the successful capture of target DNA (Pinfield et al., 2019). In addition, Durban & Pitman (2012) cited by Pinfield et al. (2019), found that water temperatures correlate with skin turnover rate in killer whales. They suggested that colder sea surface temperatures in higher latitudes may be linked with a reduced rate of skin shedding. These (a)biotic factors impact the sample volume, cause difficulties in methodology during eDNA analysis and consequently impact the accuracy of results (Pinfield et al., 2019).

Pinfield et al. (2019) citing Alberdi et al., (2018); Harper et al., (2019); Schultz & Lance, (2015); Spens et al., (2017) and Stewart, (2019) discussed that the probability of eDNA detection and the reliability of the results obtained can vary. They depend on the number of samples, the volume of water collected, the timing of sampling (e.g., breeding/spawning season), and sample concentration. However, while the sample is so imperative in eDNA research, the distribution and collection of the sample depend on many factors. For example, the amount of time that DNA remains in an area and

how widely it disperses depends on environmental conditions such as currents and sedimentation rates (Carr, 2017). A study by Collins et al. (2018) estimated that eDNA detections (freshwater or marine) may only be reliable for up to 48 hours. However, the time until total degradation or dilution beyond detectability in marine ecosystems can range from hours to days (Dejean et al., 2011). This varies between studies and among target species based on;

- oceanographic conditions such as salinity, mixing of larger water masses, and tide/current actions of the ocean/sea. Hereby, causing dispersal and dilution of the eDNA (Thomsen et al., 2012a),
- the environment and weather conditions, including UV radiation and bacterial action (Pinfield et al., 2019; Strickler et al., 2015),
- location, with the probability of detecting eDNA, expected to rapidly decrease with distance from its shedding source (Thomsen et al., 2012a cited in Pinfield et al., 2019), and
- sensitivity of laboratory methodologies used (Pinfield et al., 2019).

From a methodological point of view, the detection of eDNA can also be affected by multiple factors. For example, the presence of polymerase chain reaction (PCR) inhibitors (Goldberg et al. 2016 cited in Fediajevaite et al., 2021), the amplification methodologies used, preservation methods of the sample, and potential contamination from sampling, laboratory, human or microbial sources (Alberdi et al., 2018; Harper et al., 2019; Schultz & Lance, 2015; Spens et al., 2017; Stewart, 2019; Laurence, et al., 2014 cited in Pinfield et al., 2019). Cross-contamination of the eDNA with, among others, traces of human DNA can cause the analysis to be inconclusive (Rodríguez-Ezpeleta et al., 2021). These factors can tamper with the results obtained through eDNA sampling and result in inference errors and false negatives (Fediajevaite et al., 2021), as it did in research conducted by Pinfield et al. (2019). Supporting this, multiple eDNA studies targeting cetaceans using species-specific primers have suggested that cetacean eDNA is hard to detect. Even in close spatial or temporal proximity to the source animal (Valsecchi et al., 2021). In the study by Foote et al. (2012), the molecular detection of harbor porpoise eDNA was observed to diminish at distances greater than ten meters. Research by Székely et al. (2021), found that bowhead whale *(Balaena mysticetus)* eDNA diminished substantially in water samples collected ten minutes after the whale presence. While time since and distance from the shedding source are significant when it comes to collecting eDNA, research shows that there is no reference or standard. The speed at which eDNA degrades and the amount of the remaining eDNA that is collected can change depending on the target animal and the environment. Hereby decreasing the repeatability/reliability of this surveying method (Dejean et al., 2011 cited in Foote et al., 2012)**.**

The previously elaborated research by Pinfield et al. (2019) demonstrates that at present, not all populations and types of environments may be suitable for eDNA research. In open water systems, where large bodies of water masses are constantly exchanging and adverse weather conditions can occur frequently, eDNA fragmentation and dispersion are likely to be more rapid. Thus, capturing cetacean DNA is more challenging in open water or deep-ocean systems than in more sheltered inshore regions. Therefore, caution must be exercised in the interpretation of eDNA results and in distinguishing between true negatives and false negatives (Pinfield et al., 2019).

4 Discussion

Research conducted by Pinfield et al. (2019) and Baker et al. (2018) showed that the claims about the reliability of eDNA are divided and that its use can provide conflicting results. Where Baker et al. (2018) were able to detect a southern resident community of killer whales from eDNA samples and support these findings with acoustic and visual observations, Pinfield et al. obtained only false negatives despite actual encounters and visual sightings. Valsecchi et al. (2021) stated that results from eDNA are generally more accurate when the habits of the investigated species are well-defined and the data collection is tuned to this. In the research conducted by Baker et al. (2018) it is mentioned that the researchers chose killer whales in Puget Sound for their investigation because their well-described habits allowed them to locate and sample individuals or groups efficiently (Hauser et al., 2007). It is crucial to note here that the habits being discussed, were initially described through visual sightings, which are part of the traditional surveying method that Baker et al. (2018) challenge in favor of promoting eDNA. Baker et al. (2018) also stress that the efficiency of genetic sampling can substantially increase and elusive species can be detected when the approximate location of a dive and the behavior of the target species is known. This observation might explain the positive eDNA results obtained, raising doubts about the reliability of eDNA as a detection method and questioning its overall effectiveness. It also indicates that the predictability of habitat uses and high-frequency occurrence rates of target species may be key to the success of capturing target DNA for cetaceans (Pinfield et al., 2019).

Both Pinfield et al. (2019) and Baker et al. (2018) obtained eDNA samples from the air/surface interface (surface water) of the ocean. Baker et al. (2018) inferred that the higher number of positive detections in this area could be attributed to the advection of sloughed skin or feces at the surface and/or surface tension retaining DNA from exhalation blows. However, the lack of positive detections in the study by Pinfield et al. (2019), despite also sampling from the (sub)surface in calm conditions, would suggest that there may be other factors that determine the successful capture of target DNA. For example, the sample volume (available feces, skin, etc.) may influence the detection rates. Supporting this notion, Baker et al. (2018) discovered a notable positive correlation between the number of samples collected in an encounter and the pod size of the encounter. Indicating that animal behavior at different times, such as reduced defecation during foraging, may also play a vital role in eDNA detection. The abundance of fecal matter from the target organism may have increased the successful capture of target DNA during sampling for Baker et al. (2018). However, Port et al. (2016), cited by Pinfield et al. (2019), stress that eDNA can provide only a snapshot of organisms recently present in the local area. Where eDNA is sampled may generate spatial sampling bias. When analyzing suspended genetic material, it is not possible to determine several factors. Including; whether the animals were present in the area recently, whether the multiple samples originate from the same animal if the eDNA originated elsewhere and was transported by sea currents or advection, or if the eDNA derived from the remains of a deceased animal (Foote et al., 2012). This raises doubts about the reliability of abundance estimations made using eDNA, calling into question the accuracy of assessments that rely solely on eDNA as a method. There is not enough evidence to conclude that eDNA would be less sensitive to spatial bias than traditional surveying methods.

It can be noticed that studies so far have been conducted in an ad hoc manner. They often serve the purpose of promoting eDNA by collecting samples and making analyses where traditional surveying methods would have sufficed. Recent studies may be overselling the superior benefits of eDNA. In the study by Baker et al. (2018), the positive detections of killer whales during their two-hour wake should not be deemed surprising considering the high abundance of genetic material (e.g. sloughed skin and feces) in this time frame. Furthermore, when planning data collection for a survey, factors such as

cetacean abundance, movement, and behavior are typically taken into consideration by researchers. The likelihood of sampling water directly in the wake of surfacing species that are never observed is relatively low. In such cases, researchers often leverage their expert knowledge of the species to identify locations where the animals are known to the surface, aiming to optimize eDNA results. These considerations, made before conducting research, may be the reason for eDNA-successes. However, in these scenarios, eDNA analysis may not provide any additional or novel insights beyond what can be obtained through traditional surveys that incorporate visual observation and acoustic methods. For a study, selecting a location where cetaceans are known to be present and sampling there can diminish the utility of eDNA. If researchers can get close enough to visually observe the animals or capture their images through aerial or ship-based photography, they can already identify the species and even the individuals present, using photo identification software. In such cases, the use of eDNA becomes redundant and costly. Given these arguments, the contribution of eDNA to the field of marine biology becomes subject to debate. It is advisable to conduct simultaneous eDNA surveys and traditional surveys for the same location and species to facilitate a direct comparison of their effectiveness. This approach would enable researchers to evaluate and compare the performance of each method in detecting the target species and assess their respective strengths and limitations.

A meta-analysis conducted by Fediajevaite et al. (2021), compared multiple studies to assess the efficacy of eDNA against traditional surveying methods. The general claims regarding the effectiveness of eDNA surveys in the existing literature are founded on limited evidence. Many published works on eDNA emphasize its applications and potential uses in various contexts. These papers aim to present compelling evidence for eDNA analysis as a viable alternative to traditional genetic sampling methods that involve physical handling, biopsying, and tagging of individuals. The research tries to demonstrate that eDNA is a simple and cheap alternative to traditional surveys since it doesn't require a direct encounter with the target species. However, it is important to consider that while eDNA analysis may offer savings in terms of cost and effort during data collection, comparable resources are often necessary for data analysis. In reality, the cost-effectiveness of eDNA sampling is offset by the increase in DNA extraction and sequencing costs (Taberlet et al., 1999 cited in Foote et al., 2012). Additionally, the majority of the results published on eDNA in marine biology are based on positive results. A researcher is less likely to publish negative results and report eDNA-failures. Therefore, the existing literature on eDNA may be reflecting a publication bias and give a distorted impression of the research done and knowledge obtained on the "promise" and capabilities of eDNA (Beng & Corlett, 2020 cited in Fediajevaite et al., 2021).

In conclusion, it can be said that currently there is a gap in the literature when it comes to studies with false-negative results for eDNA. This means that the field of marine biology is missing an important reality check. Currently, it cannot inform; conservationists, management bodies, and researchers of the potential pitfalls of eDNA and help them when working toward optimizing their workflow and ensuring the successful capture of target DNA (Pinfield et al., 2019). While eDNA's ability to detect and monitor cetaceans is documented and published numerous times, the shortcomings and errors that occur with its use underestimate the reliability of the results that are obtained through it.

A potential middle ground to continue using eDNA, while critically assessing its additional value and realistically interpreting its findings, is to integrate traditional survey methods with eDNA survey methods. It would be beneficial to use eDNA as an additional tool to tried and tested techniques such as visual inspection, acoustics, etc. rather than an outright replacement and method standing on its own (Pinfield et al., 2019). Research conducted by Foote et al. (2012) using static acoustic monitoring devices (which log detected echolocation click trains of harbor porpoises) provided a record of occurrence and relative density at each site. Next to this, reliable field validation was done by eDNA-

based tests. The successful genetic detection of harbor porpoises at a location where they were also acoustically detected demonstrates that, with optimization, eDNA has the potential to complement existing visual and acoustic methods. This integration could enhance sampling efficiency and improve the ability to detect a diverse range of marine taxonomic groups, including cryptic species that are otherwise challenging to observe (Foote et al., 2012). It is crucial to approach the use of eDNA with caution and acknowledge that its full potential is realized when combined with traditional surveying methods to validate its results. This integration allows for improved accuracy of eDNA analysis while minimizing financial and time losses associated with ineffective study designs. Meanwhile, the publication of unsuccessful eDNA studies can continue to better inform the eDNA research community (Pinfield et al., 2019).

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