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BRIEF COMMUNICATION

The challenge of implementing environmental DNA metabarcoding to detect elasmobranchs in a resource-limited marine protected area

Marcelo Merten Cruz[1] | Thomas Sauvage[2] | Anthony Chariton[3] | Thales Renato Ochotorena de Freitas[1]

[1]Programa de Pós-graduação em Genética e Biologia Molecular, Departamento de Genética, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil

[2]Programa de Pós-graduação em Ecologia, Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil

[3]School of Life Sciences, Macquarie University, Sydney, Australia

Correspondence
Marcelo Merten Cruz, Programa de Pós-Graduação em Genética e Biologia Molecular, Departamento de Genética, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9500, 91501-970 Porto Alegre, Brazil.
Email: marcelomcruz4@gmail.com

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Abstract

Elasmobranchs are threatened and eDNA metabarcoding is a powerful tool that can help efforts to better understand and conserve them. Nevertheless, the inter-calibration between optimal methodological practices and its implementation in resource-limited situations is still an issue. Based on promising results from recent studies, the authors applied a cost-effective protocol with parameters that could be easily replicated by any conservationist. Nonetheless, the results with fewer elasmobranchs detected than expected reveal that endorsed primers and sampling strategies still require further optimization, especially for applications in resource-limited conservation programmes.

KEYWORDS
biomonitoring, conservation tools, eDNA, elasmobranchs, metabarcoding, MPA

In the 21st century, the ocean has been facing unprecedented impacts from human activities (Luypaert *et al*., 2020). To this extent, several elasmobranch populations (*e.g*., sharks, rays, skates and sawfish) are declining and changing their distribution due to the wide variety of threats they are facing (Dulvy *et al*., 2014, 2017, 2021). Overfishing is the main threat for all threatened elasmobranch species evaluated by Dulvy *et al*. (2021). Elasmobranchs also face additional issues such as the climate crisis, pollution, biomagnification, by-catch, and habitat loss (Dulvy *et al*., 2014; Pacoureau *et al*., 2021). Unfortunately, their naturally slow reproductive characteristics exacerbate these threats and hamper population recovery (Denney *et al*., 2002; Field *et al*., 2009).

The loss of elasmobranch diversity may lead to irreversible environmental collapses because they (as high-level predators) help regulate and maintain the balance of marine ecosystems by structuring the dynamics of food webs (Heupel *et al*., 2014; Navia *et al*., 2017). Considering the ecological role of elasmobranchs, there is a particular concern in establishing defined geographical areas for their protection (Heithaus *et al*., 2012; Stevens, 2010). To this extent, marine protected areas (MPA) are the main tool used in spatial management (Rigby *et al*., 2019). The establishment of MPAs is a common policy strategy to provide a refuge against anthropogenic-related threats and is considered vital to ocean health and resilience (Crear *et al*., 2020). That is because well-managed MPAs can act as a recovery space, where species of particular importance, such as fishes, substantially increase in size, density, species richness and biomass (Lester *et al*., 2009).

To effectively monitor elasmobranch species within MPAs, it is necessary to investigate their presence, distribution and critical habitats within the protected range (Rigby *et al*., 2019). In such efforts, environmental DNA (eDNA) metabarcoding, an innovative molecular approach that allows identifying the presence of species from water

samples to track the genetic material released by species in their environments, was previously developed to target elasmobranchs with specific primers (Bakker *et al*., 2017). This non-invasive technique demonstrated that elasmobranch species diversity and eDNA read abundance directly reflects the known degree of marine anthropogenic impact (Bakker *et al*., 2017; Boussarie *et al*., 2018).

The eDNA application gained a promising avenue when sequencing technologies improved the capacity to analyse fragmented genetic traces (Fraija-Fernández *et al*., 2020; Guardiola *et al*., 2015). With the aid of recent advancements, elasmobranch biomonitoring studies rapidly advanced from the eDNA detection of target species (Gargan *et al*., 2017; Simpfendorfer *et al*., 2016; Weltz *et al*., 2017) to the differentiation between species through high-throughput sequencing – metabarcoding (Bakker *et al*., 2017; Boussarie *et al*., 2018; Collins *et al*., 2019; Miya *et al*., 2015). The metabarcoding approach has been proven to be an efficient tool for marine monitoring and ecosystem health assessment (Kelly *et al*., 2014; Leray & Knowlton, 2015; Thomsen *et al*., 2012). Furthermore, recent studies provided a proof of concept that sharks, rays and their relatives can be monitored using eDNA metabarcoding (Dunn *et al*., 2022; Fraija-Fernández *et al*., 2020; Ip *et al*., 2021; Liu *et al*., 2022; Mariani *et al*., 2021; Marwayana *et al*., 2021; Monuki *et al*., 2021).

Based on recent positive testimonies of eDNA metabarcoding to detect elasmobranch species, the authors expect that this technique is ready to be implemented in realistic situations. Also, eDNA metabarcoding is often recommended as a cost-effective and easily replicated technique (Boussarie *et al*., 2018; Fraija-Fernández *et al*., 2020; Marwayana *et al*., 2021; Miya *et al*., 2015; Monuki *et al*., 2021). Those characteristics are essential to monitor elasmobranchs in MPAs in very funding-limited situations (Rigby *et al*., 2019).

For example, eDNA metabarcoding could be beneficial to monitor elasmobranchs in a remote and resource-limited MPA known as Fernando de Noronha archipelago (FNA) in the equatorial Atlantic. Elasmobranchs are one of the main groups of FNA's vertebrate populations. Historical records indicate FNA shelters at least 26 elasmobranch species (Garla & Garcia, 2008; Petean *et al*., 2020; Soto, 2001). The use of FNA as a nursery habitat was reported for at least three shark species: *Carcharhinus perezi* (Poey, 1876), *Ginglymostoma cirratum* (Bonnaterre 1788) and *Negaprion brevirostris* (Poey 1868) (Garla *et al*., 2006, Garla *et al*., 2009). These have been well-studied by acoustic telemetry and underwater observations (Garla *et al*., 2006; Garla *et al*., 2009, Garla, Gadig, Garcia, *et al*., 2017, Garla, Gadig, & Garrone-Neto, 2017).

Here, the authors tested the capability of using eDNA as a routine tool for monitoring elasmobranchs in MPAs using the FNA as a case study system. Their expectation is to detect the three permanent shark residents and beyond that provide further information on the elasmobranch biodiversity in FNA. Choosing FNA as a model also bears the goal of evaluating the approach as a baseline for future policy implementation by the Brazilian Ministry of Environment to better protect and monitor the archipelago.

eDNA collections were made under research permit no. 62360 from the Brazilian Ministry of Environment (SISBIO). The sampling area was a previously classified nursery area for sharks in FNA. An assessable ("realistic") protocol gathering the most practical aspects from other successful elasmobranch metabarcoding studies is presented here and further methodology details are provided in (Supporting Information S1).

A total of 24 samples (eight stations sampled in triplicate—Boussarie *et al*., 2018), consisting of 2 l per sample, were collected at surface levels (West *et al*. 2020) using sterile whirl-pack bags. The water samples were filtered in a sterilized environment, and the resulting filters were kept in Longmire's solution for DNA extraction (Wegleitner *et al*., 2015). Following the suggestion of Mariani *et al*. (2021), the water samples were filtered at the end of the collection day. To ensure that no contamination occurred during the filtering process, three field negative control samples were also filtered (see Supporting Information S1). In addition, a muscle tissue sample from *Rhizoprionodon terraenovae* (not reported in FNA) was used as a positive control.

DNA extracts were sent to EcoMol Consultoria for amplification, library preparation and sequencing. Amplification was performed using the Elas02 primer, which targets 170–185 bp of the mitochondrial 12S rRNA gene. The raw sequences were processed in a custom pipeline using DADA2 (version 1.6; Callahan *et al*., 2016) in R version 4.0.2 (R Core Development team, 2020).

The eDNA amplicons yielded a total of 1,311,609 reads, including the positive control as well as amplification and sampling negative controls that were removed after the applied pipeline. After bioinformatic filtering, 85,313 merged, paired-end, non-chimeric and assembled reads were obtained. Of these, 23,105 belonged to marine vertebrate species. This number represents only 1.51% of the total reads, as the vast majority of the reads were discarded because they corresponded to non-specific (prokaryotic) amplicons. These exceeded the sequencing length for successful merging and explain the important data loss. The Elas02 primer pair appeared to cross-amplify a longer section of the bacterial 16S mitochondrial rDNA (Supporting Information S2). The sequencing reads of this gene fragment were longer than the originally targeted 12S rRNA reads, which indicates successful merging during bioinformatic processing and explains the large data loss.

The targeted survey for elasmobranchs resulted in three detected species that matched reference database sequences to 100%: *Hypanus berthalutzae* (Naylor and Lima 2020), *Ginglymostoma cirratum and Prionace glauca* (Linnaeus 1758). The used reference database from FNA's elasmobranchs is presented in Supporting Information S3. In addition to the identification of elasmobranchs, this analysis also identified 37 marine vertebrate species (Table 1), most representing reef fish species, even mammals, such as, the spinner dolphin (*Stenella longirostris*). The filtered sequencing data can be found at https://github.com/marcelomcruz4/Fernando-de-Noronha-filtered-sequencing-data.

The metabarcoding approach confirmed the presence of *G. cirratum* in their previously described nursery ground (Garla *et al*., 2009). *G. cirratum* was the only resident detected in this probe.

TABLE 1 Class and family of surveyed marine vertebrate species

Class	Family	Species	Number of reads	Total sites
Chondrichthyes	Ginglymostomatidae	*Ginglymostoma cirratum*	626	3
Chondrichthyes	Carcharhinidae	*Prionace glauca*	662	2
Chondrichthyes	Dasyatidae	*Hypanus berthalutzae*	3470	6
Actinopterygii	Muraenidae	*Gymnothorax vicinus*	10	1
Actinopterygii	Clupeidae	*Harengula jaguana*	117	5
Actinopterygii	Myctophidae	*Myctophum brachygnathum*	10	1
Actinopterygii	Myctophidae	*Diaphus brachycephalus*	273	1
Actinopterygii	Holocentridae	*Sargocentron ensifer*	245	2
Actinopterygii	Holocentridae	*Myripristis kochiensis*	171	1
Actinopterygii	Gobiidae	*Priolepis hipoliti*	24	1
Actinopterygii	Sphyraenidae	*Sphyraena jello*	5	1
Actinopterygii	Scombridae	*Thunnus obesus*	293	1
Actinopterygii	Scombridae	*Acanthocybium solandri*	280	3
Actinopterygii	Istiophoridae	*Istiophorus platypterus*	84	1
Actinopterygii	Carangidae	*Selar crumenophthalmus*	34	1
Actinopterygii	Carangidae	*Seriola rivoliana*	26	1
Actinopterygii	Carangidae	*Elagatis bipinnulata*	52	1
Actinopterygii	Carangidae	*Caranx crysos*	66	1
Actinopterygii	Carangidae	*Caranx latus*	11	1
Actinopterygii	Labrisomidae	*Malacoctenus triangulatus*	12	1
Actinopterygii	Pomacentridae	*Abudefduf saxatilis*	182	1
Actinopterygii	Belonidae	*Tylosurus crocodilus*	135	1
Actinopterygii	Belonidae	*Platybelone argalus*	9	1
Actinopterygii	Labridae	*Xyrichtys novacula*	108	1
Actinopterygii	Labridae	*Halichoeres radiatus*	139	1
Actinopterygii	Labridae	*Doratonotus megalepis*	31	1
Actinopterygii	Labridae	*Thalassoma lunare*	62	1
Actinopterygii	Scaridae	*Cryptotomus roseus*	11	1
Actinopterygii	Scaridae	*Sparisoma chrysopterum*	12,293	5
Actinopterygii	Scaridae	*Sparisoma axillare*	102	3
Actinopterygii	Scaridae	*Sparisoma rubripinne*	359	1
Actinopterygii	Acanthuridae	*Ctenochaetus tominiensis*	162	3
Actinopterygii	Serranidae	*Cephalopholis sexmaculata*	45	1
Actinopterygii	Kyphosidae	*Kyphosus pacificus*	61	1
Actinopterygii	Kyphosidae	*Kyphosus vaigiensis*	1583	5
Mammalia	Delphinidae	*Stenella longirostris*	1352	2

Nonetheless, the survey has contributed to reporting the presence of the recently identified *H. berthalutzae* in FNA (Petean *et al*., 2020). This stingray species is endemic to Brazilian waters, yet little is known about its ecological aspects (Branco-Nunes *et al*., 2021). In addition, the authors detected blue shark (*P. glauca*), an elasmobranch previously reported in the archipelago; nonetheless, their habitat use in the region has not yet been investigated (Soto, 2001). The blue shark is pelagic; its distribution is the widest among all large sharks, and it is common throughout the coast of continental Brazil (Hazin and Lessa, 2005). Due to their highly migratory nature, the presence of blue sharks in Fernando de Noronha's waters is probably occasional (Hazin *et al*., 1994).

Beyond elasmobranchs, additional biodiversity could be catalogued in this manner. In particular, the authors detected both reef and pelagic teleost biodiversity (Figure 1a), which suggests that eDNA metabarcoding can be used for biomonitoring this group of fishes in FNA.

Even though the application of elasmobranch eDNA metabarcoding brings significant results to elasmobranch research, the optimal balance between gold-standard protocols and the feasibility of

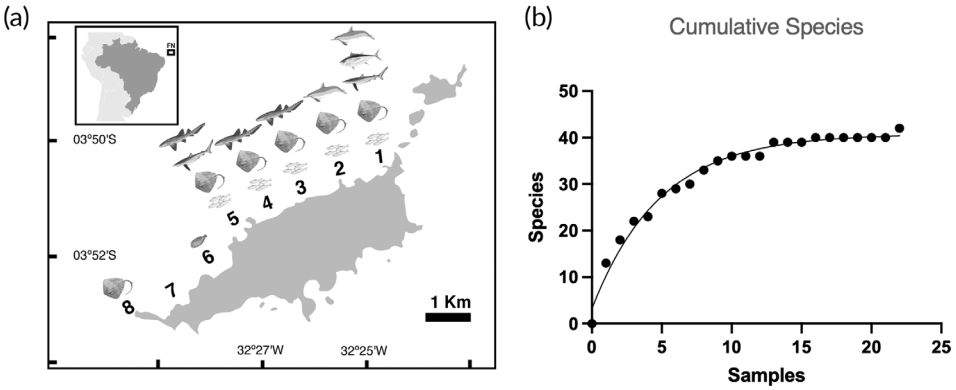

FIGURE 1 (a) Distribution of the major species (*Ginglyomostoma cirratum*, *Prionace glauca*, *Hypanus berthalutzae*, *Thunnus obesus*, *Harengula jaguana*, *Xyrichtys novacula*, and *Stenella longirostris*) across the north-western coast of Fernando de Noronha Archipelago. (b)Accumulation curve of identified species in water samples from the north-western coast of Fernando de Noronha Archipelago. , *Ginglymostoma cirratum*; , *Prionace glauca*; , *Hypanus berthalutzae*; , *Thunnus obesus*; , *Harengula jaguana*; , *Xyrichtys novacula*; , *Stenella longirostris*

their implementation in some MPAs is a fine-balance and requires further research. In this case study, the authors could detect elasmobranchs through metabarcoding, but encountered some important limitations such as cross-amplification and lack of detection power (Figure 1b).

Although elasmobranch metabarcoding appears quite established in literature (Dunn *et al*., 2022; Fraija-Fernández *et al*., 2020; Ip *et al*., 2021; Liu *et al*., 2022; Mariani *et al*., 2021; Marwayana *et al*., 2021; Monuki *et al*., 2021), the results of this study suggest that it is not mature enough to be applied in more challenging situations. For example, the authors expected that their protocol would detect at least a greater number of elasmobranch species than a recent baited remote underwater video stations (BRUVS) survey in the same area (5; Schmid *et al*., 2020) and potentially increase the total number of known species (*i.e*., reduce the phantom diversity of sharks and rays; Ip *et al*., 2021). The main constraint in capturing elasmobranch diversity in the Fernando de Noronha case study was the cross-amplification of prokaryotic DNA by the Elas02 primer pair, representing a large waste of sequencing effort. Interestingly, Elas02 was chosen in this study because according to the literature, it supposedly performed substantially better in comparison to other primers for the detection of this specific taxonomic group (Collins *et al*., 2019; Liu *et al*., 2022; Mariani *et al*., 2021).

It is possible that previous studies did not consider the use of Elas02 primer in a realistic sampling strategy – similar to what MPAs' managers in tropical areas would use (*i.e*., lack of refrigeration, logistical constraints, and prolonged periods of time before the filtering procedure). Recently (only after the authors of this study sequenced their samples), Dunn *et al*. (2022), suggested that this primer pair lacks specificity and may be inefficient for the amplification of elasmobranch eDNA. In addition, warm surface waters are known for increased microbial activity (Bruce *et al*., 2021). Low specificity of the Elas02 primer and an overabundance of bacterial DNA in the authors' samples together may explain the high cross-amplification rates they encountered (interference phenomenon as explained by Wilcox *et al*., 2013).

Indeed, the mitochondrial 12S rRNA gene shows several regions of significant homology to bacterial 16S rRNA sequences (Eperon *et al*., 1980). Previous metabarcoding studies faced the issue of cross-reactivity of a metazoan 12S rRNA primer with bacterial 16S rRNA sequences (Machida *et al*., 2012; Lim and Thompson, 2021), and the opposite also occurs (Huggins *et al*., 2020).

In essence, capturing DNA fragments from elasmobranchs in sea water can be challenging, as they usually occupy the upper-level trophic position, so naturally their density is reduced compared to species occupying lower trophic levels (Postaire *et al*., 2020). Moreover, elasmobranch populations are in decline and, consequently, these species are a difficult target as the density will potentially reflect the sensitivity of eDNA detection (Adams *et al*., 2019; Furlan *et al*., 2016). The authors recognized that 2 l of the environmental sample in an open-water habitat is insufficient to capture eDNA from elusive species. In turn, maximizing water volumes as much as logistically possible will potentially increase fish eDNA detection (Bessey *et al*., 2020).

Additional sampling strategies that maximize the detection of elusive species are certainly important, but the feasibility of their implementation in MPAs is not fully discussed in previous studies. To expand the detectability, it would be preferable to intensify the sampling effort by: increasing the number of samples (Bruce *et al*., 2021), sampling throughout different seasons/habitats (Jeunen *et al*., 2019; Postaire *et al*., 2020) and replicating across water column depths (Marwayana *et al*., 2021). What should be carefully considered is that almost all amendments raised above could potentially hamper the two main eDNA promises: to be cost-effective and repeatable (Dickie *et al*., 2018; Evans *et al*., 2017).

Marine eDNA technologies are frequently pointed out as a cost-effective tool, mostly because they are more economical than underwater visual census (UVCs) and BRUVS operations (Boussarie *et al*., 2018; Stat *et al*., 2019; Ip *et al*., 2021). Future investigators should be aware that an eDNA metabarcoding study is not inexpensive. Moreover, metabarcoding research is an expensive investment for middle and low-income economies (Leese *et al*., 2018). Logistical challenges are imposed on scientists who are not from wealthy countries, *e.g*., the exchange rate from dollars or euros to home currencies that is often unfavourable (Valenzuela-Toro & Viglino, 2021). Then, as the extraction, amplification, and sequencing materials are usually priced in dollars by international

companies, an intensification of sampling in metabarcoding surveys is not always practical in economic turmoil realities.

Biomonitoring programmes in developing/least-developed countries operate with very limited funding (Weigel *et al*., 2011). This coupled with restricted access to the newest biotechnological infrastructure hinders the optimal step of testing different parameters (Leese *et al*., 2018). Then, a realistic and calibrated protocol that considers sampling design, primer choice and costs is needed by policy-makers, without the risk of failure at any stage, which would waste more time and increase cost (Helmy *et al*., 2016; Leese *et al*., 2018). In truth, academic scientists sometimes tend to overlook socio-economic constraints, which regulators must consider routinely (Leese *et al*., 2018). Therefore, logistical, and methodological parameters that are not established should be proposed with caution by studies that evaluated a single geographic region or protocol.

The costs of "ideal" sampling strategies will potentially cause a social gap between who is able to use such technology and consequently a detachment of fishers, managers, community members, and local people in elasmobranch conservation (Rigby *et al*., 2019). Without diversifying the elasmobranch metabarcoding narrative, scientists from developing and least developed countries are in danger of being passive spectators rather than active contributors to this revolutionary method (Ahmadia *et al*., 2021). In conclusion, the field of eDNA metabarcoding in a conservation context would strongly benefit from more research that tests and reports on field sampling methods that are realistic and cost-efficient all over the world – including in situations with very limited funding.

AUTHOR CONTRIBUTIONS

M.M.C., A.C. and T.R.O.F. conceived and the study; M.M.C. conducted the sampling; M.M.C. and T.S. conducted all experiments; M.M.C. and T.S. analysed the data; M.M.C. wrote the manuscript; A.C., T.S. and T.R.O.F. reviewed the manuscript; all authors read and approved the final version.

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ETHICS STATEMENT

The care and use of experimental animals complied with Brazil animal welfare laws, guidelines and policies as approved by the research permit no. 62360 from the Brazilian Ministry of Environment (SISBIO).

ORCID

Marcelo Merten Cruz https://orcid.org/0000-0001-8374-5144

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