









# Field collections and environmental DNA surveys reveal topographic complexity of coral reefs as a predictor of cryptobenthic biodiversity across small spatial scales

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## Abstract

Coral reefs represent some of the most biodiverse ecosystems in the world but are currently undergoing large-scale degradation due to anthropogenic stressors. Such degradation usually begins with coral bleaching, and if the stress condition is inflicted for too long may eventually result in loss of structural complexity (or “flattening”) of the reef, dramatically changing habitat availability for reef-associated fauna. Despite having been linked to important ecosystem functions, cryptobenthic organisms are often overlooked in ecological monitoring programs, and their microhabitat dependencies are poorly understood. Here, we combined collection-based biodiversity monitoring techniques with five different environmental DNA (eDNA) sampling techniques (Reef water, sediment, crevice water, slurp gun, and bulk sediment) to survey cryptobenthic fishes and annelids on a Maldivian fringing coral reef. Collectively, 176 fish and 140 annelid taxa were detected with eDNA across 14 surveyed sites, more than doubling the reported annelid taxa in the region with 88 new occurrences. Water filtered near the reef structure revealed the highest species richness out of the five eDNA sampling techniques tested. Furthermore, we found correlations between fish species richness and topographic complexity for both collection- and eDNA-based techniques. This suggests that detection by eDNA may be linked to site-specific predictors and reveal community differences across small spatial scales (tens of meters). We also report that reef flattening (going from structural complex to less complex sites) can cause a 50% reduction in fish diversity and that cryptobenthic fish species richness was highly associated with branching corals. In contrast, annelid communities showed no clear correlations with environmental predictors, but co-amplification of non-target, non-annelid taxa may have distorted such correlations if present. This suggest that the predictive powers of eDNA for environmental gradients may be dependent on the targeted taxa.

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## KEYWORDS

annelids, coral reef fish, eDNA, meiofauna, metabarcoding, microhabitat

## 1 | INTRODUCTION

Humans are exerting strong negative impacts on coral reef ecosystems at a global scale (Hoegh-Guldberg et al., 2017; Hughes et al., 2017; Hughes, Kerry, et al., 2018). For example, marine heat waves caused by climate change have already resulted in severe mass coral bleaching events (Hughes, Anderson, et al., 2018). These are often followed by some level of coral decline, loss of structure and ecosystem degradation (Brodnicke et al., 2019; Heron et al., 2017; Miller et al., 2006; Moore et al., 2012), which, in turn, can induce system-wide phase shifts from coral to rubble or algal dominated reefs (Graham et al., 2015; Hughes et al., 2010; Hughes, Kerry, et al., 2018; Wild et al., 2011). The loss of dominant, fast-growing, reef building corals, such as acroporids, can lead to reduced structural complexity (Alvarez-Filip et al., 2009), a feature of reefs that has been shown to be vital in sustaining high biodiversity (Graham & Nash, 2013). In the wake of these changes, reef-associated consumer communities, such as coral reef fishes, are exhibiting marked changes in their community composition and functional structure (Brandl et al., 2016; Darling et al., 2017; Newman et al., 2015; Richardson et al., 2018). This has often been attributed to the varying dependence of fish species on the coral reef benthos (Brandl et al., 2018). Indeed, many coral reef fish have distinct microhabitat preferences (Ahmadia et al., 2012; Cadena-Estrada et al., 2019; Munday, 2004; Wilson et al., 2008), and rely on subtle variation in coral reef topography for shelter and/or foraging opportunities (Brandl et al., 2015; Rogers et al., 2014). While the responses of mobile, conspicuous fish communities to coral reef degradation are relatively well documented, potential effects on cryptic fishes and invertebrate communities are poorly resolved (Nelson et al., 2016; Stella et al., 2011; Yan & Bellwood, 2023). Given the tendency of these taxa to be highly associated with specific microhabitats (Kramer et al., 2014), establishing a better understanding of how these communities may react to reef-wide changes is critical.

Biodiversity monitoring on coral reefs is often undertaken through the use of visual surveys and with a focus on easily discernible taxa such as larger fish species and the corals themselves (Beisiegel et al., 2017; Costello et al., 2017; Sweet et al., 2021). However, more recently, molecular techniques such as the use of environmental DNA (eDNA) sampling of seawater have gained substantial traction (Alexander et al., 2020; Lafferty et al., 2021; West et al., 2020). Environmental DNA has been touted as a particularly effective method for surveying or characterizing rare, endangered or otherwise elusive species (Agersnap et al., 2022; Beng & Corlett, 2020; Carvalho et al., 2019; Thomsen & Willerslev, 2015). Community detection through the use of eDNA also appears to be able to detect changes in species richness of even the smallest animals across space and time (Atherton & Jondelius, 2020; Carvalho

et al., 2019; Gielings et al., 2021; Jeunen et al., 2019). However, care needs to be taken when choosing the optimal sampling method, especially when the focus of a study is on benthic communities, which are prone to shed DNA more locally and might not be traceable in the water column alone (Antich et al., 2021; Brodnicke, Meyer, et al., 2023; Gómez-Buckley et al., 2023).

Despite the common focus on larger fish during surveys, the majority (abundance and diversity) of coral reef fauna are small-bodied and cryptobenthic (Brandl et al., 2018; Stella et al., 2011). Annelid worm abundance, for example, can reach into their hundreds per 100 cm<sup>2</sup> of reef, whilst cryptobenthic reef fish make up approximately half of the total fish diversity (Brandl et al., 2018; Kramer et al., 2012). Naturally, the small size and inconspicuousness of cryptobenthic fishes makes them dramatically underrepresented in visual surveys and difficult to monitor without destructive sampling (Ackerman & Bellwood, 2002; Glynn & Enochs, 2011; Knowlton et al., 2010), necessitating the use of invasive sampling methods such as weighted net-enclosures and the application of anesthetics or ichthyocides such as clove oil or rotenone (Ackerman & Bellwood, 2002; Ahmadia et al., 2012; Alzate et al., 2014). These methods require several skilled divers and are both time consuming and invasive. Similarly, benthic annelids need to be extracted from the substrate – a process which can be quite destructive on coral reefs (Veeramuthu et al., 2013; Worsaae et al., 2021). Recent attempts to use eDNA on reefs specifically for detection of cryptobenthic fish resulted in low eDNA detection rates when compared to physically collected detection of these small and elusive taxa (Gómez-Buckley et al., 2023). It could also be that eDNA sampling methodology and downstream analysis perhaps can be improved and result in higher detection rates to better complement conventional surveys for monitoring the diversity of cryptobenthic fauna. Cryptobenthic reef fish and invertebrates of which annelids constitute a major group, are integral components of the coral reef food web, and as such survey methods that can monitor the presence of these important fauna are critically needed (Brandl et al., 2019; Brodnicke et al., 2022; Casey et al., 2019; O'Shea et al., 2013).

In the present study, we surveyed fish and annelid communities on a highly biodiverse coral reef atoll in the southern Maldives. Specifically, we performed net-enclosed biodiversity surveys of cryptobenthic fishes, physical collections of annelids (for DNA barcodes), and eDNA sampling with the aim to: (I) assess which of the five eDNA sampling methods had highest species detection, which is highly relevant for future applied eDNA biomonitoring of cryptobenthic reef diversity (II) compare the physically and eDNA detected communities of cryptobenthic reef fish across sites and association with the microhabitats, and (III) examine the annelid and fish diversity detected through eDNA surveys and the association with the environment.

## 2 | METHODS

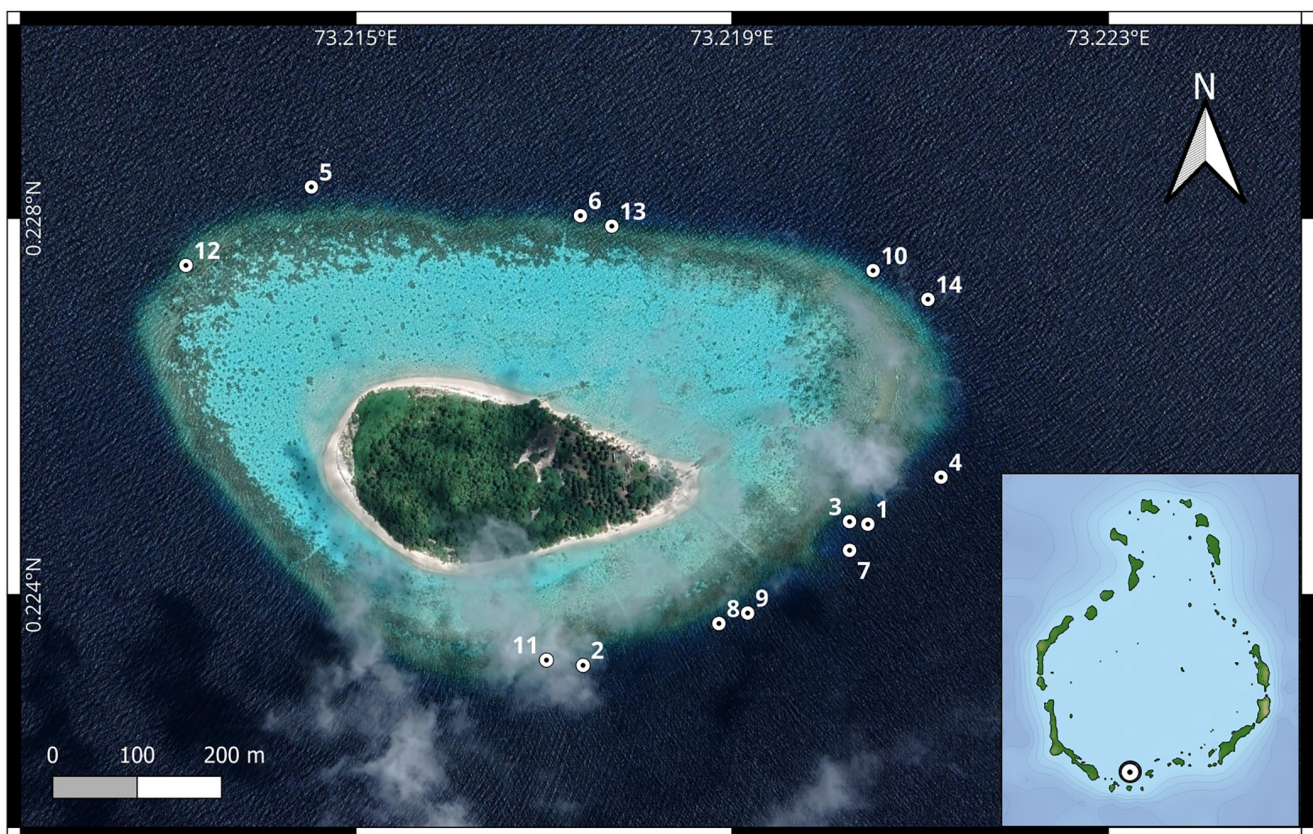
### 2.1 | Study location

Over a three-week expedition (from 15th November to 6th December 2021), we studied 14 sites with varying microhabitats on a fringing reef around the island Kandahalagalaa in the Huvadhu atoll of the Maldives in the Indian Ocean (Figure 1). All sites were sampled at mid-day and at depths of approximately 10 m (8.8–13.0 m). Site microhabitat characteristics were calculated as proportions or for topographic complexity scored on a scale from 0 to 5 (Graham et al., 2015), from five 40 cm × 40 cm quadrats photographed perpendicular to the reef plane at each site (for analytical details see “Microhabitat assessment” methods in Appendix S1). Collected samples were processed at the Small Islands Research Centre, on Faresmaathoda Island. All sampling and export of sample material was performed in collaboration with the Maldives Marine Research Institute and with the collection permit (OTHR)30-D/INDIV/2021/270.

### 2.2 | Environmental DNA sampling

At each site, three replicate eDNA samples were collected before physical collection of fish and annelids, using five distinct

collection methods while SCUBA diving. These included: (1) reef water: 1 L of water taken from within 1–10 cm distance of the reef structure and filtered into a 0.22 µm Sterivex-GP filter (Merck Life Science, henceforth referred to as eDNA filters). (2) sediment: 5 g sediment collected by scraping the top sediment next to the corals (upper 5 mm of the substrate) into a 5 mL cryotube. (3) crevice water: 1 L of water taken from within the coral reef structure (crevices and inside corals) and filtered into an eDNA filter using sterile syringes with a 15 cm steel extension for extended reach. (4) slurp gun: 15 L was extracted using a slurp gun with a 45 cm rubber extension. This water was filtered through a 63 µm mesh and the mesh stored in a sterile container. In the laboratory, the mesh was rinsed into the water (0.5 L) in the same container and that water was filtered through an eDNA filter. (5) bulk sediment: at 10 sites an additional sediment sample (0.5 L) was collected in a half full sterile container. In the laboratory, the sediment particles were resuspended into the collection water (0.5 L), which was then filtered through an eDNA filter. Sterile 50 mL syringes were used while filtering for all samples and controls. All filters were transported to the laboratory in zip-lock bags and within an hour, 2 mL of premade Longmire's solution (Longmire et al., 1997) was added to each filter (which was drained of seawater) as well as to the sediment to conserve the DNA. Field controls were taken at each site on the boat, filtering 1 L of commercial drinking water through



**FIGURE 1** Map of the study location around the island Kandahalagalaa and the location of the 14 study sites along the fringing reef. The site numbers are the order in which the sites were surveyed. The inset shows the sheltered location (circle) of the study reef within the Huvadhu Atoll of the Maldives in the Indian Ocean.

an eDNA filter. An eDNA filter was also filled with the Longmire buffer as a control in the laboratory.

### 2.3 | Cryptobenthic reef fish collection

To collect cryptobenthic reef fish, we enclosed a coral reef area of approximately 5 m<sup>2</sup> with fine, bell-shaped mesh (mosquito netting), weighted by a chain (Ackerman & Bellwood, 2002; Brandt et al., 2020). This netted enclosure was put in place by three to four scuba divers. An impermeable tarp (also weighed down with a chain around the perimeter) was then added on top of the net to restrict the clove oil from dispersing. Afterwards, we inundated the site underneath the tarp with clove oil anesthetic (1:5 clove oil: 75% ethanol solution). After applying the anesthetic, the divers collected all anesthetized fish within the site in plastic bags using forceps. When all fish were collected, they were brought to the surface and placed in ice water. In the laboratory, the fish were photographed, measured, weighed and identified. They were then placed in 99% ethanol for cataloguing in the Natural History Museum of Denmark and the Maldives Marine Research institute. The fish identifications were also later validated by experts and relevant literature where necessary. A subset of the total number of species identified were de novo sequenced (Table S1), seeking to complement existing reference DNA barcode repositories. For cryptobenthic reef fish detection comparison with the first site (site 1) was omitted as this was used as a training site for fish collection.

### 2.4 | Annelid collection

Meiobenthic annelids were collected from the sediment at the same sites as above by scooping the top layer (approximately 5 cm) of sediment into a sample container. In the laboratory, specimens were anesthetized by suspending them in MgCl<sub>2</sub> solution with gentle rotation. The solution was then decanted through a 63 μm mesh following the protocol outlined in Worsaae et al. (2021). The annelids in the samples were sorted under a stereo-microscope and placed individually in either 99% ethanol for later DNA extraction or fixed in 3% Trialdehyde (in 0.15M cacodylate buffer) for morphological analysis. After detailed microscopical examinations in the laboratory, 53 morphotypes were identified to genus or family level and the morphologically corresponding, ethanol-preserved specimens were used for de novo sequencing to obtain DNA barcodes (see Table S1).

### 2.5 | Laboratory DNA methods

The molecular work was performed in ultra-clean laboratories, specifically designed for minimizing contamination of samples, in which rigorous cleaning procedures with 5% bleach, ethanol and

UV light treatments in place. Pre- and post-PCR laboratories are separated to minimize the risk of cross contamination between samples and previous PCR products. DNA was extracted from the water eDNA sample filters in a modified version (Spens et al., 2017; Thomsen et al., 2016; Turon, Antich, et al., 2020) of the standard protocol for DNeasy Blood and Tissue kit (QIAGEN) and DNeasy Power Soil kit for the sediment and sediment-based filters (see “eDNA extraction” method in Appendix S1 for more details). For each set of extractions one extraction control was also included ( $n=12$ ). DNA concentrations of extracted eDNA samples were measured using Qubit. Each extraction was split into two aliquots, each comprising a volume of 20 μL, where one aliquot was stored at -80°C for later use, and the second aliquot was pooled with the extracts of the two other environmental replicates of the same sampling method for each site, to help reduce site variability in eDNA detection and reduce processing costs (Goldberg et al., 2016). Negative extraction controls, buffer controls and field controls were pooled separately and in total this produced 88 extraction pools. These pools were amplified in triplicates with mitochondrial 12S MiFish-U primers (Miya et al., 2015) and nuclear 18S primers (V1-V2 region), as described in Appendix S1 under “PCR methodologies”. Targeting the V1-V2 region had previously shown acceptable amplification of annelid DNA (Brandt et al., 2021; Martinez et al., 2020). Each replicate pool of amplicons was adjusted to contain 250 ng μL<sup>-1</sup> of 12S DNA and 300 ng μL<sup>-1</sup> for 18S DNA for commercial sequencing using NovaSeq technology by Novogene (Cambridge, UK). We aimed for 200,000 reads per pooled sample of 150bp paired-end data for 12S and 250bp paired-end data for 18S, respectively.

To supplement the NCBI database, we created 12S DNA barcodes from 38 cryptobenthic fishes and 18S DNA barcodes from 53 specimens of annelids also collected at our sites (see “PCR methodologies” in Appendix S1 for details).

### 2.6 | Bioinformatics

Raw sequence data for both 12S and 18S were processed using the MetaBarFlow pipeline (Jensen et al., 2022; Sigsgaard et al., 2022), which makes use of DADA2 (Callahan et al., 2016) and demultiplex scripts (Frøslev et al., 2017). For specific pipeline settings see “Metabarflow settings” in methods in Appendix S1. For the 12S data, we supplemented the blast search against the full NCBI database (Altschul et al., 1990) with a local blast search against the 38 newly generated 12S DNA barcodes.

Taxonomic identification was assigned based on a last common ancestor approach. The 12S sequences were further filtered so that we only included ASVs with ≥98% similarity to a reference sequence and used the last common ancestor as the taxon ID (Jensen et al., 2022, 2023). Non-target species (mammals, birds and bacteria) were removed from the data. Taxonomic identification assigned to ASVs were further delineated when a species assignment resulted in matches with species that have a geographic

range outside the Indian Ocean, or with junior synonyms or sister species known to occur outside the Indian Ocean and not having the potential target species available in the reference database. In this geographic delineation, the local taxon was chosen if ID's with identical sequence similarity but diverging geographic regions were detected. Geographic range was assessed using personal field observations (video and photos), literature on the fish diversity of the Maldives (Kuitert, 2014), FishBase (Froese & Pauly, 2021) and iNaturalist (Ueda, 2021). In the rare case that a species could not be assigned this way (but the genus was present in the Indian Ocean), we used the genus followed by sp. for that ASV. Lastly, two freshwater fish (*Gobio gobio* and *Oncorhynchus nerka*), native to Northern Europe and North America were detected and removed as (despite all the procedures in place) they are most likely lab-contaminants. The raw files and metadata for the 12S dataset is publicly available at dryad [Zenodo.org](https://doi.org/10.5281/zenodo.8374070) (DOI: [10.5281/zenodo.8374070](https://doi.org/10.5281/zenodo.8374070)).

We downloaded all ~7600 sequences matching the search term "Annelida '18S'" (a conservative search term) in the NCBI database on December 13th, 2022, and supplemented these with newly generated 18S barcodes from our 53 taxa of microscopic annelids collected on site. We also conducted a local blast search against these sequences requiring matches to have 100% query coverage, alignment lengths  $\geq 300$ bp and  $\geq 90\%$  similarity. We then extracted all sequences from the MetaBarFlow output, which also had hits in our local blast search, and updated taxonomic identifications where the local blast had higher % similarity. Each sequence with ambiguous taxonomic assignment was scrutinized and (like the fish dataset), 98% similarity was used as a cut-off for species assignment and 95% and 90% for genus and family, respectively. Pelagic annelid taxa ( $n=3$ ) were removed as we focused on benthic annelids in this study. The annelid taxa were further manually examined and edited based on ASV sequence similarity, the number of unique species, genera and families noted and their previous detection checked against the GBIF occurrence list for the Maldives (GBIF.org, 2023).

All controls (field, only Longmire buffer and extraction blank) had six or fewer total reads per sample assigned to fish species (three to six) for 12S. There were no annelid sequences in the controls for 18S. The number of reads (highest value) for a taxon found in the controls was deducted from all occurrences of that taxon in the sample dataset to reduce any potential impact of contaminants. The raw files and metadata for the 12S and 18S datasets are publicly available at [Zenodo.org](https://doi.org/10.5281/zenodo.8374070) (DOI: [10.5281/zenodo.8374070](https://doi.org/10.5281/zenodo.8374070) and [10.5281/zenodo.8372346](https://doi.org/10.5281/zenodo.8372346)).

## 2.7 | Statistical analyses

PCR replicates were merged, summing the read counts of ASVs detected in replicates of the same sample pool (three environmental extracts described above), after which singletons were removed. ASVs were then collapsed according to their taxonomic identification and cryptobenthic reef fish grouped by the following families:

Apogonidae, Blenniidae, Gobidae, Pseudochromidae, Syngnathidae and Tripterygiidae, following the definition of Brandl et al. (2018). Physically detected fish (individuals collected with clove oil enclosures) from the cryptobenthic families had their abundance converted to presence/absence. For the comparison between sites the sequence data was rarefied to median sequence depth to mitigate bias caused by uneven sequence depth. For both fish and annelid dataset 100 rarefy iterations were performed to test if any taxa were lost, which was not the case. For further details see [Figures S4 and S5](#) for rarefaction curves of fish species and annelid species/genera. For each method at each site the data from the four sampling methods were merged (reef water, crevice water, sediment and slurp gun). Here, we omitted the bulk sediment as it was not sampled at all sites. For comparisons among methods, sample reads were also rarefied to the median sequencing depth for each method at each site, using *vegan* (Oksanen et al., 2018) in R v4.0.2 (R Core Team, 2022). The rarefaction did not lead to the complete removal of any taxa in the 12S or 16S dataset. To further, eliminate the effect of unequal sequence effort and for the further statistical analysis, all data was converted to presence/absence and species richness was calculated as the total number of species detected at each site and in each eDNA detection method. This was deemed the most conservative approach as the number of taxonomically assigned sequences varied across methods and sites ([Figure S1](#)), despite the relative number of obtained sequences being relatively similar across methods ([Figure S2](#)). For comparisons of eDNA sampling methods, only sites with all five methods were used ( $n=10$ ). Venn diagrams of species overlaps were produced in R v4.0.2 with the *venn.diagram* function from the *VennDiagram* package (Chen & Boutros, 2011). Due to the heteroscedastic structure of the data, eDNA sampling methods (fish, cryptobenthic reef fish and annelids) and fish detection method (eDNA and physical detection) comparisons were performed with a non-parametric Kruskal-Wallis test followed by a Dunn's test with the Holm-Bonferroni adjustment for multiple comparisons to establish difference between groups. These were performed with the *kruskal.test* and *dunn.test* functions from base R and the *dunn.test* package (Dinno, 2017; R Core Team, 2022).

To visualize the microhabitat and community differences between sites, a non-metric multidimensional scaling ordination was performed with the *metaMDS* function in the *vegan* package using Bray-Curtis dissimilarities on the microhabitat proportions (Oksanen et al., 2018). We calculated correlations (based on Pearson's product moment) and their significance between species richness and the 13 microhabitats, depth and topography with the *cor* and *cor.test* function from the *vegan* package (Oksanen et al., 2018). These were visualized with linear regressions in *ggplot* (Wickham, 2016). Further, to find species-specific correlations between the physically detected cryptobenthic fish (using abundance) and microhabitats, depth and topography we used the *rcorr* function from the *Hmisc* package and the *corrplot* function from the *corrplot* package for visualization (Harrell, 2021; Wei & Simko, 2021). All other figures were made using the *ggplot2* package (Wickham, 2016).

### 3 | RESULTS

#### 3.1 | Sequencing output and microhabitat characterization

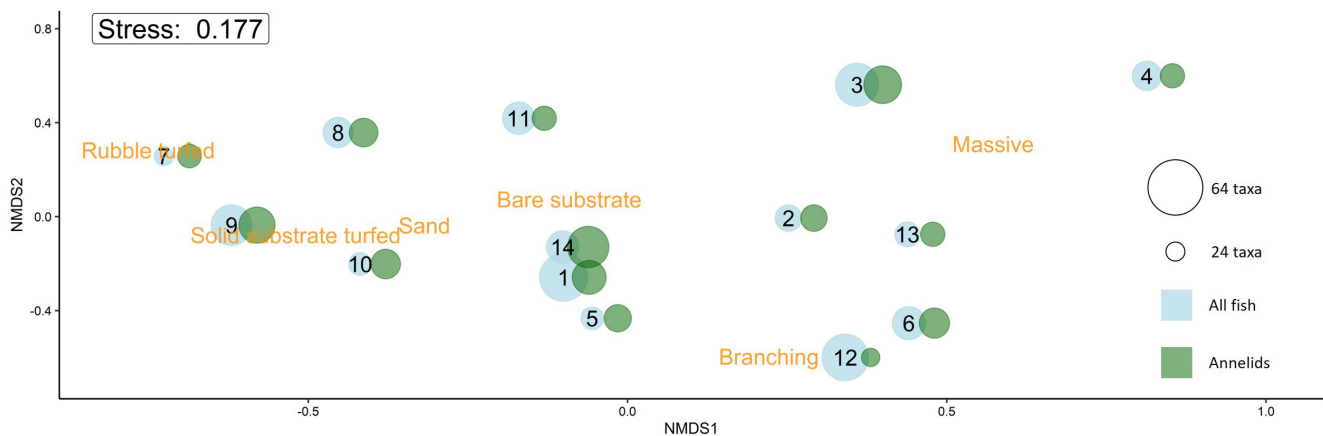
We obtained 126.8M taxonomically assigned fish sequences spanning 176 taxa (species and genus level) across the 14 sites from the 65 pooled samples. The 38 barcodes of 12S obtained from our own collections increased the taxonomically assigned sequences by 9.6% and added eight previously unassigned fish taxa to the eDNA dataset. Mean fish taxon richness per site (across all eDNA sampling methods) was  $42.8 \pm 12.3$ . From the 18S ASVs, we obtained approximately 74.2M taxonomically assigned sequences of which benthic annelids were represented with 140 taxa from 94 genera and 37 families (Table S2). This diversity was detected even though annelid sequences only made up 0.7M of the 18S sequences. Of the recorded taxa, 88 had no records in the GBIF occurrence list (Table S2). The 18S barcodes from our annelid collection updated 18 sequence identifications and again added eight new taxa to the dataset. The mean species richness of annelid taxa across eDNA sampling methods was  $36.3 \pm 8.0$  taxa per site and the species richness was more similar among sites than for the fishes (Figure 2). For 12S, most of the ASVs were assigned to fish (at class level) (Figure S3a) and species detection was saturated for most sampling methods at each site (Figure S4). For the 18S data, relatively few of the total sequences were assigned to Annelida (2.0%), with the majority coming from Arthropoda and Apicomplexa (Figure S3b). The broad specificity of the primers were a challenge when targeting the annelid phyla and this drove a reduction in the sequencing depth across many of the sites/methods deployed for Annelida specifically (Figure S5). This as well as the unexpectedly high prevalence of Apicomplexa in the substrate may have influenced the substantial difference in annelid community composition found across sites (Figure S1b).

The microhabitats dominating the surveyed sites were (in ascending order): massive coral (23.6%), branching coral (22.9%), turf-covered substrate (10.1%), bare substrate (9.2%), turf-covered rubble (9.1%) and sand (7.2%). The non-metric multidimensional scaling ordination revealed variation along the first dimension. The negative side was dominated by turf-covered microhabitats, while the positive side was dominated by sites with high proportions of living corals (branching and massive). Only a few sites were dominated by just one microhabitat, such as site seven which was dominated by turf-covered rubble.

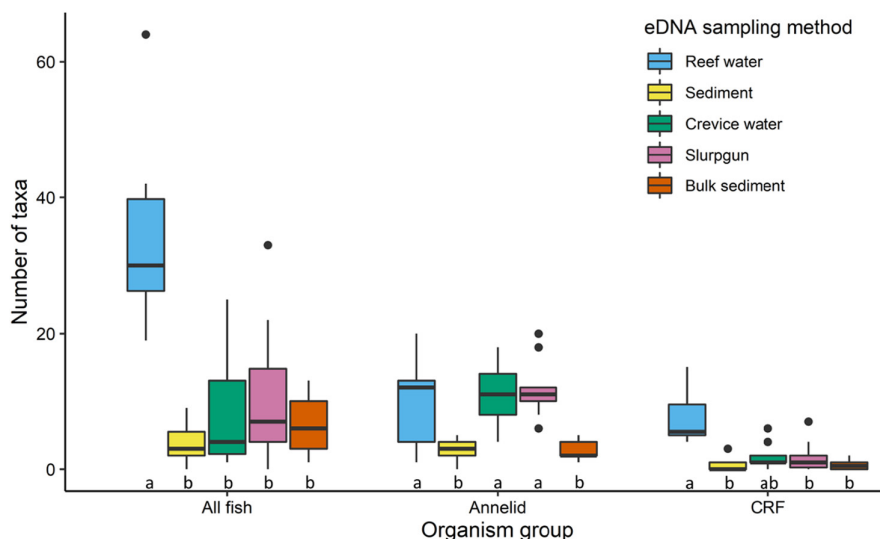
#### 3.2 | Comparisons of eDNA sampling

The reef water samples consistently yielded the highest detection rates of the five eDNA methods surveyed for all fish (Figure 3). This was significantly higher than all the other eDNA sampling methods ( $p < 0.01$ ), ranging between 21 and 64 fish species across sites. The same sample method (reef water) also detected the highest total number of fish (129 species) compared to any of the other eDNA methodologies. This corresponds to 73.7% of the total fish taxa detected using eDNA. Only eight taxa out of 175 (4.6%) were detected consistently across all five eDNA methods assessed (Figure S6a). The slurp gun, bulk sediment and crevice water detected ten, ten, and nine taxa across all sites, respectively, which were not detected by reef water. Sediment samples detected no unique fish taxa and only two which the reef water did not detect.

A similar pattern was observed for cryptobenthic reef fishes only, that is, the reef water eDNA detected between five and 16 cryptobenthic reef fish species across sites. This was significantly higher than all but the crevice water method ( $p < 0.01$ ). Reef water further detected 93.8% of all cryptobenthic species (30 out of 32), while one additional species was detected by the slurp gun and

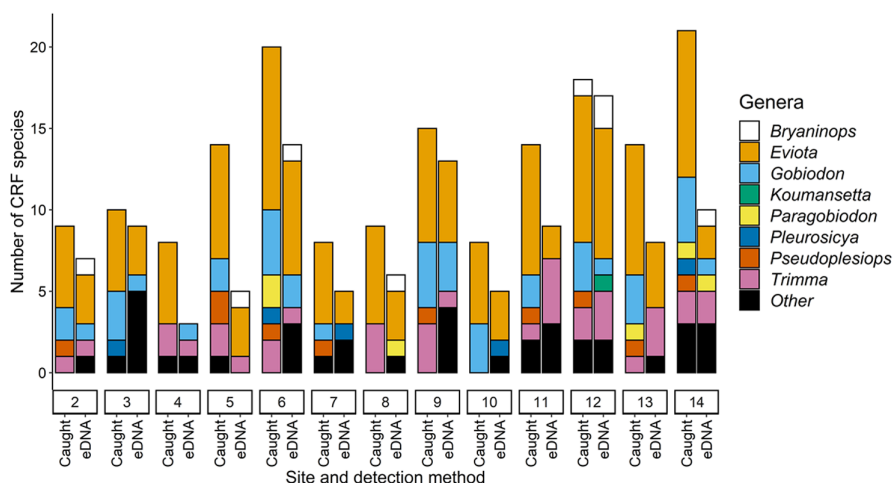


**FIGURE 2** Ordination of the 14 sampling sites (numbers) using a non-metric multidimensional scaling (NMDS) based on the Bray-Curtis dissimilarities of the 13 microhabitats. This means the 14 sites are placed in respect to their similarity in microhabitats of which the six most abundant across sites are displayed in orange. Sites (numbers) closer together are more similar in microhabitat composition and are characterized by the nearby microhabitats. The size of the blue circles at each site depicts fish species richness and green is annelid richness at each site. The richness of the two groups is on the same scale and circle size range from 24 taxa to 64 taxa. Branching and massive refer to live coral with those growth forms.



**FIGURE 3** Mean species richness detected by each eDNA sampling method separated by target group (All fish, Annelid, CRF: cryptobenthic reef fish). Means and quantiles were calculated from the 10 sites, which were sampled with the five eDNA methods. Samples were rarefied to median sequence depth prior to converting it to presence/absence. Different letters depict significant differences between methods for each group (different colors) (e.g., for cryptobenthic reef fish the reef water method (a) is significantly different from sediment, slurp gun and bulk sediment method (b), but not from the crevice water method (ab). Black dots are outliers.

**FIGURE 4** Number of cryptobenthic reef fish (CRF) species detected at each site using physical or eDNA collections. The size of the bars depicts the number of species within each genus. Note that site 1 was excluded as a training site for cryptobenthic reef fish collection.



bulk sediment methods and another one from the crevice water (Figure S6b).

For annelids, the slurp gun method had the highest detection rate, ranging from six to 21 taxa across sites. However, the reef water and crevice water sampling methods were not significantly lower regarding taxon detection ( $p > 0.05$ ). Regardless, all three methods consistently detected a greater number of taxa than the sediment and/or the bulk sediment ( $p < 0.001$ ). In annelid taxon overlap, reef water detected the most unique taxa (23), followed by crevice water (16) and slurp gun (10). In total, the reef water detected 62 out of the 101 taxa (61.4%) included in the method comparison. When combined with the crevice water, 89.1% of all benthic annelid taxa identified were detected by the two methods. The bulk sediment contributed only four additional taxa and the sediment two (Figure S6c).

### 3.3 | Detection by eDNA versus physically detected fish communities

We physically collected 900 specimens of fish from the clove oil stations, of which 812 were classified as cryptobenthic reef fish. Cryptobenthic reef fishes comprised 50 different species with a mean species richness of  $14.5 \pm 4.8$  species per site (Figure 4). Environmental DNA sampling detected 38 cryptobenthic reef fish species and the mean richness per site was  $10.2 \pm 4.1$  species. Comparison of the communities inferred by eDNA at each site and the two detection methods highlighted some overlap in the genera present, as both methods were dominated by the goby genera *Eviota* and *Gobiodon* and to a lesser degree *Trimma*. However, this was overshadowed by high discrepancy between the two methods (only 34.8% or eight out of 23 genera overlapped), with physical detection revealing a significantly

higher total species richness than eDNA (Kruskal–Wallis test,  $p=0.004$ ). However, the use of eDNA more commonly detected rare genera (21) when compared to the physical collections (15). Species accumulation curves flattened for eDNA and traditional methods across the 14 sites, suggesting that the application of various complementary techniques yielded a good representation of the biodiversity of cryptobenthic fishes at the study location (Figure S7).

### 3.4 | Linking community diversity with environmental predictors

For all fish as well as for cryptobenthic reef fish, we found correlations between species richness and environmental predictors recorded at each site. The species richness of physically collected cryptobenthic reef fishes had a significant and strong positive correlation with both topography ( $r=0.84$ ,  $p<0.01$ , correlation based on Pearson's product moment) and branching corals ( $r=0.7$ ,  $p<0.01$ ; Figure 5a,b). While cryptobenthic reef fish detected by eDNA only showed a significant and strong positive correlation with topography ( $r=0.66$ ,  $p=0.01$ ), there was a general positive correlation with branching coral which was not significant ( $r=0.47$ ,  $p=0.09$ ) (Figure 5b). For the species richness of all fishes detected via eDNA, we also found a significant positive correlation with site topography ( $r=0.63$ ,  $p=0.016$ ) (Figure 5c). When comparing the fish diversity from the highest structurally complex site (topography 5; 62 species) with the lowest complexity sites (topography 1; 25 species), fish diversity was approximately twice as high. For annelid taxon richness there were no significant correlations with the environmental parameters.

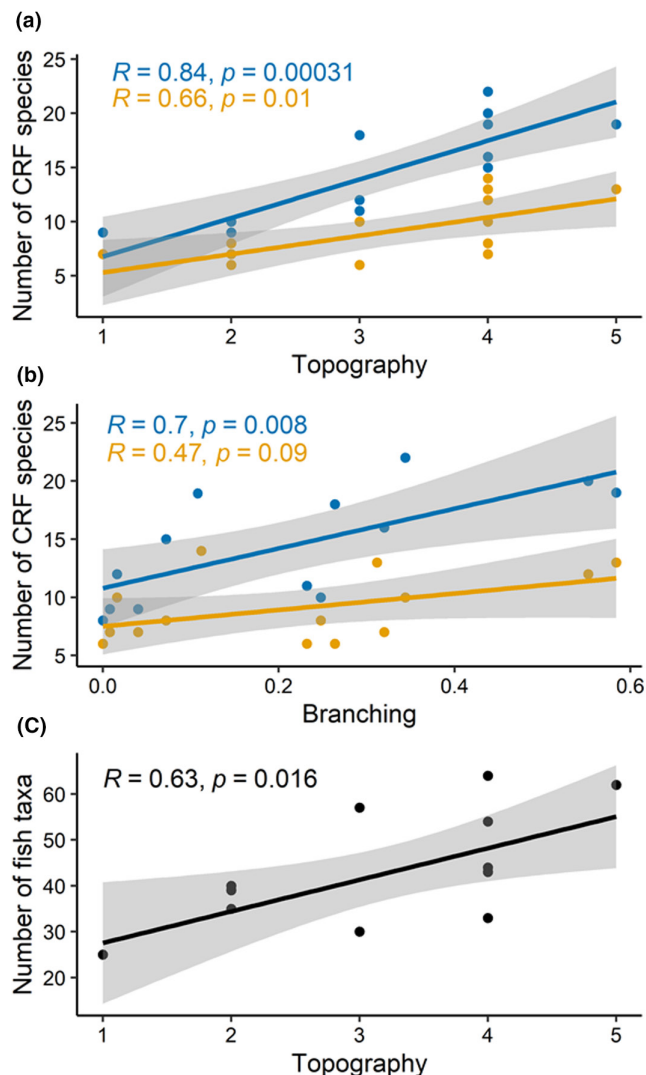
### 3.5 | Microhabitats of cryptobenthic reef fishes

For the physically collected cryptobenthic reef fish species, we found distinct correlations with the microhabitat availability at our sites (Figure 6). Notably, branching corals had a strong positive association with seven species, spanning six genera. Relationships were strong for the obligate coral-dwelling genera *Gobiodon* and *Paragobiodon*. The genus *Eviota* also displayed differential habitat preferences, with *E. mikiae* associated with branching corals and deeper depth, and *Eviota* sp.1 positively associated with turf-algal dominated hard substrates and fungia (free-living) corals. Both of which had negative correlations with other *Eviota* (*E. mikiae*, *E. sebreei* and *E. sp.2*).

## 4 | DISCUSSION

### 4.1 | eDNA sampling method comparison

By employing five eDNA sampling methods, we were able to identify the most efficient method to assess cryptobenthic fish in tropical coral reef ecosystems. For fish, the reef water samples

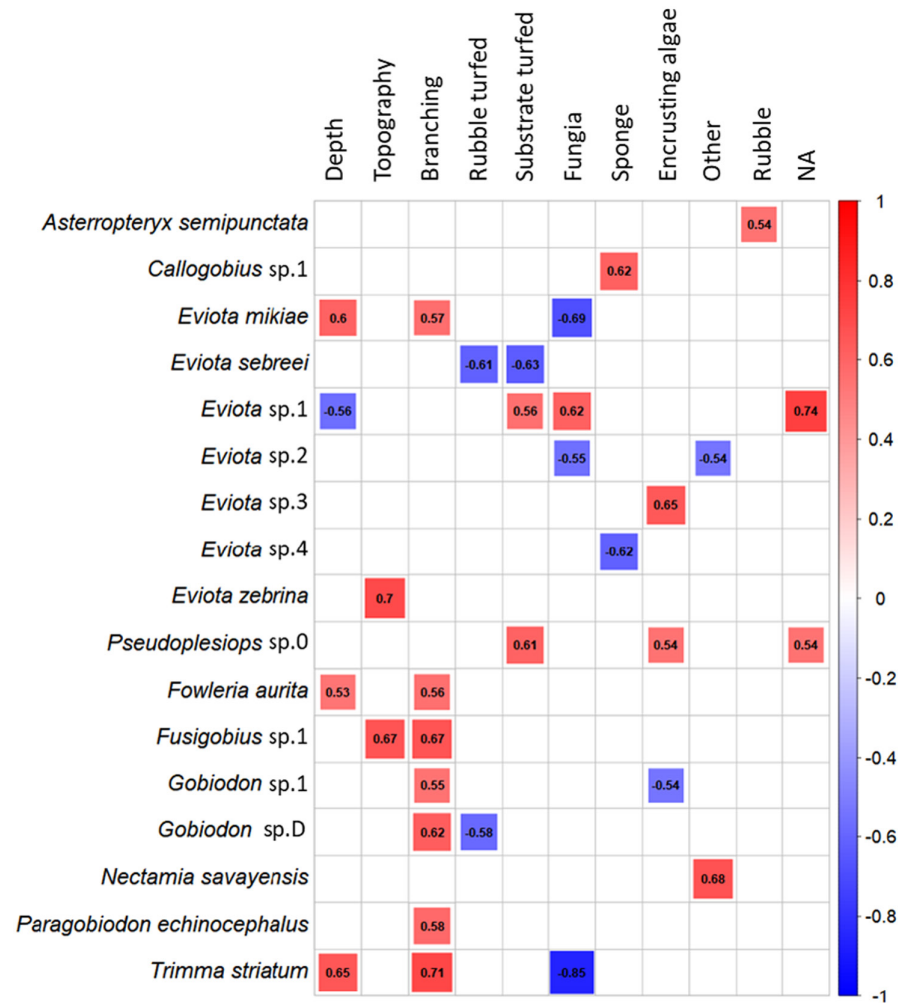


**FIGURE 5** Scatterplots with fitted correlations (linear regressions) between species richness and the microhabitats and topography (site structural complexity). (a) Cryptobenthic reef fish (CRF) detected either via physical collections (blue) or eDNA (yellow), both positively correlated with topography. (b) A positive correlation with proportion of branching corals, which is only significant for cryptobenthic reef fish (CRF) physically detected. (c) All fishes and topography (black) revealing a positive significant correlation. The gray area is the 95% confidence level interval of the linear regression models fitted by the `stat_smooth` function in the `ggplot2` package. Note different y-axis ranges.

detected the most taxa across sites and had the highest number of unique taxa, making this the best eDNA sampling method of the ones tested. While several other studies have shown similar results (Dugal et al., 2023; Miya, 2022; West et al., 2020), some have suggested sediment to be superior for eDNA-based diversity detection (Holman et al., 2019; Sakata et al., 2020). Others have also highlighted the importance of sampling proximity to the benthic environment when sampling for organisms residing there (Antich et al., 2021). In support of the importance of localized sampling, our results show that water collected close to the reef site (within 10 cm) allowed for the detection of 74% of the total fish taxa and



**FIGURE 6** Heatmap of the correlations between the abundance of cryptobenthic reef fishes and environmental predictors. Only significant correlations are shown ( $p < 0.05$ , spearman correlations) and fish species and environmental predictors not displayed did not have any significant correlations. The numbers displayed are the correlation coefficients. NA represents not identifiable microhabitat (usually too dark in a crevice), sp.0–4 were morphologically and genetically different, but could not be assigned to a described species.



94% of all cryptobenthic reef fish detected across all methods. Methodologically, our results highlight the importance of the right eDNA sampling method for the target group, and this is especially true for cryptobenthic reef fish (Gómez-Buckley et al., 2023; Nichols et al., 2022).

For benthic annelids, the slurp gun, crevice water and reef water methods were all equally efficient at recovering eDNA. The slurp gun detected the most taxa (at a single site), and reef water detected the most unique taxa (23). Importantly, in both cases, we may have also obtained annelid eggs from the water column. Indeed, the most abundantly detected annelid family (Syllidae) has a pelagic spawning life history strategy (Aguado et al., 2012), suggesting that obtained reads may be from different ontogenetic pelagic stages and not necessarily closely associated to the site where sampled. That said, we recommend that when targeting this group, a combination of eDNA sampling methods are utilized, as the reef water and the crevice water collectively detected 89% of the benthic annelids encountered. It would be interesting to now test if benthic annelids (and other benthic invertebrates) can be assessed in water borne eDNA from other marine ecosystems. Such data would greatly advance our knowledge of these important groups in years to come.

The methods that relied on sediment (sediment and bulk sediment) detected the fewest annelid taxa. Based on the relatively high

number of reads recovered in these samples, we suspect the lack of detection was not due to PCR inhibitors in the sediment samples, instead we regard this as being a consequence of the diversity of other invertebrates and microorganism occupying the reads. For 18S, our major challenge was that relatively few of the returned ASVs could be assigned to annelid taxa. The same region (18S V1–V2) has previously been used in eDNA monitoring studies (Atherton & Jondelius, 2020; Castro et al., 2021; Fonseca et al., 2014; Haanel et al., 2017), and detected a relative higher proportion of Annelida in their sequences. In our study, only 2% of the assigned sequences were annelids likely caused by co-amplified eDNA from invertebrate taxa which might be more dominant in the environment. This indicates that caution should be taken when comparing sites or methods in further analysis and conclusions. For 12S MiFish-U primers, we predominantly recovered acanthurids (a pelagic family) in the sediment samples. A study on carp (*Cyprinus carpio*) showed that their eDNA can concentrate up to 1000 times in the sediment compared to the water column (Turner et al., 2015), and since acanthurids were not over-represented in the water-based sampling methods, it could be that DNA from this family accumulates in the sediment where the DNA degradation is slower than in water (Harrison et al., 2019; Sakata et al., 2020). Nevertheless, the stochasticity observed in eDNA detection for annelids and, to some degree, for fish makes

the sediment-based sampling method appear unreliable for diversity assessments of these taxa among other benthic taxa of the highly diverse coral reefs.

## 4.2 | Coral reef fish detection comparison

Across all sites and eDNA sampling methods, we detected 176 taxa of fish on a relatively small spatial scale (just one depth, one reef, in one atoll, in one country). Other studies have detected similar fish richness patterns, but across larger spatial scales, for example in Indonesia (189 genera) (Marwayana et al., 2022), Japan (291 species) (Oka et al., 2021), or Qatar (148 species) (Sigsgaard et al., 2019). When visually investigating the diversity of a more northerly atoll in the Maldives, 349 bony fish species were found across several marine habitats (Chabanet et al., 2012). Our richness detection then equals 176/349 (around 50%) and may be incapable of covering all the other species because of our spatially restricted sampling (one reef), and our conserved habitat selection (coral reef) and the consistent depth and time of day of sampling.

Our eDNA sampling effort identified 38 cryptobenthic reef fish species. This is high, especially when considering this group is often overlooked and as such has a reduced reference database to compare against (Gómez-Buckley et al., 2023). The number of species appear to conform with other studies based on conventional surveys (Ahmadia et al., 2012; Cadena-Estrada et al., 2019). We detected more fish via physical detection (50 species) compared to eDNA sampling (38) and there was only a 34.8% overlap in genera. Detecting more cryptobenthic reef fish species by enclosure compared to eDNA was also observed in a recent study from Tonga (Gómez-Buckley et al., 2023). However, this is contrary to Mathon et al. (2022) who detected more cryptobenthic fish taxa via eDNA compared to their visual surveys. That said, visual surveys have been criticized for often missing cryptobenthic species (Ackerman & Bellwood, 2000) so eDNA will be an obvious benefit if clove oil sampling is not possible. Combined, this evidence suggests eDNA surveys are more representative for assessing cryptobenthic reef fish than visual surveys, but (to date) less useful than the highly field intensive and invasive enclosure detection method. Further, we show that eDNA analysis appears to reveal a broader range of taxa across any given surveyed ecosystem (Mathon et al., 2022). This trend will likely increase as more DNA barcodes become publicly available. Indeed, when we included an additional 38 new barcodes from local cryptobenthic reef fish, we increased the taxonomic assignment of eDNA sequences substantially.

## 4.3 | Importance of reef topography and microhabitats for cryptobenthic reef fish richness

Positive correlations were found for cryptobenthic fish species richness and topography with both eDNA and physical collection data, suggesting that the two different detection methods can yield

similar associations between species richness and the environment. This is a major finding since currents and wave action can disperse eDNA effectively across large spatial scales with degradation rates of weeks (Goldberg et al., 2016; Thomsen et al., 2012). In addition, recent eDNA monitoring studies on shallow water tropical coral reefs have often detected the presence of deep sea species (Turon, Angulo-Preckler, et al., 2020; West et al., 2020), suggesting vagrant movement of eDNA. This phenomena was not observed in the present study, suggesting a more local signal. Such local signals have likewise been reported in other systems (Eble et al., 2020; Jensen et al., 2022). West et al. (2020) were able to highlight fine-scale differences in coral reef communities using eDNA with only a few kilometers between sites. Variations in communities or species richness has also been resolved on even smaller scales (i.e., hundreds of meters), but these studies were not focusing on reefs per se (Jeunen et al., 2019; Oka et al., 2021; Thomsen et al., 2012). Here, our data lends support for the use of monitoring eDNA on very small spatial scales (tens of meters) revealing differences in very local communities, as site-specific variation showed clear differences in the cryptobenthic fish present.

## 4.4 | Correlations between reef fish and the benthos

The positive correlation between topography and taxon richness, across all fish data (caught or detected via eDNA), supports that site structural complexity has a positive influence on diversity of tropical fish (Darling et al., 2017; Friedlander et al., 2003; Graham & Nash, 2013). Rugosity (roughness of the site) is commonly highlighted as a good predictor for coral reef fish diversity (Gratwicke & Speight, 2005) and the process of reef flattening (a reduction in structural complexity) has been argued to impact fish communities negatively (Darling et al., 2017; Newman et al., 2015; Syms & Jones, 2000). This has been shown to especially be the case for the small-bodied fishes (Graham et al., 2007). A relationship between diversity in refugia size for prey and coral reef fish diversity has been suggested be due to reduced predation mortality (Rogers et al., 2014). With larger reef site structures, the size range of refugia improves, ultimately promoting fish diversity. From our comparison of highly complex sites with almost completely flat sites we observed a 50% decrease in fish taxon richness. This supports the hypothesis that reef complexity is vital for sustaining high diversity (Dalben & Floeter, 2012; Darling et al., 2017; Graham & Nash, 2013), and this underlines that conservation of coral reef complexity should be a top priority for coral reef managers, especially when protecting fish biodiversity is the goal. As we found the same positive correlations between diversity and coral reef complexity using either a conventional detection techniques or eDNA analysis, we see this as support of how non-invasive eDNA surveys can become a major player in future monitoring programs of reef fish species richness.

The correlations we found between cryptobenthic reef fish species richness and branching corals could be explained (at least

in part) by the close association of especially one genus (*Gobiodon*) with acroporid corals (Brandl et al., 2018; Duchene et al., 2013; Hing et al., 2019). The convoluted structure of branching corals provides shelter from predators that many fish species can take advantage of (Coker et al., 2009; Rogers et al., 2014). The positive association suggests that a reduction in branching corals, a result ever more likely as climate change continues (Hughes, Kerry, et al., 2018), will result in a marked reduction in this important group of fishes (Munday, 2004). In particular, the species abundance correlation analysis revealed that six genera, including the coral-dwelling *Gobiodon* and *Paragobiodon* are highly associated with topographic complexity. In a future setting exhibiting more severe heatwaves (Frölicher et al., 2018), coral degradation and resulting reef flattening (Alvarez-Filip et al., 2009; Brodnicke et al., 2019; Coker et al., 2009), we hypothesize that these genera are at particular risk of local or even regional extinctions (Coker et al., 2009; Munday, 2004). Collectively, these findings highlight the importance of species-specific identification when inferring habitat specialization and ultimately inferences of local extinction risks.

#### 4.5 | High annelid diversity despite 18S assignment challenges

We detected 140 annelid taxa, which is some of the highest annelid diversity reported on a coral reef to date (Newman et al., 2015; Stella et al., 2011). We were also able to resolve these at much higher taxonomic levels than previously undertaken for this phylum (Atherton & Jondelius, 2020). Previously, only 82 marine annelid taxa had been recorded (including many unverified taxa) for the Maldives based on the available, but incomplete database (GBIF.org, 2023). We detected 88 new records, which equates to more than doubling of the known annelid diversity for this region from this single study. In addition to adding to the total diversity of the Maldives, these findings vastly increase the known distribution of a substantial number of annelid taxa, and while many molecular identifications were only at genus level, the data suggests many of these species are not scientifically described yet. Furthermore, this extraordinarily high annelid diversity was detected despite the majority of 18S sequences originating from other phyla (98%) in our samples as well as encountering challenges incomplete annelid DNA reference databases. This emphasizes the importance of locally derived barcodes/libraries, which, in our case, resulted in a substantial improvement of detection/identification rates. The design of more annelid specific primers (which do not co-amplify as many non-target taxa), could greatly improve the usability of eDNA for this group, allowing for detailed downstream analyses such as the exploration of microhabitat preferences. The lack of a relationship between annelid communities and microhabitat in the present study may have arisen from these shortcomings. A previous study, which only found 45 oligochaetes (physical detection) revealed high species-specific habitat preference (Yildiz, 2016). Some annelids are well known as bioindicators of ecosystem health and pollution

(Dean, 2008; Pinheiro et al., 2020), highlighting another key usage of such data moving forward.

#### 4.6 | Concluding prospects

Our combined extractive and eDNA surveys provide a comprehensive view of cryptic biodiversity on a Maldivian reef. The localized detection of fish communities through eDNA suggests its utility for monitoring local biodiversity changes in response to environmental impacts, aiding marine management and conservation. Scalable eDNA methods such as automated eDNA sampling, can offer cost-effective expansion to larger and remote geographic ranges (Hendricks et al., 2023), atoll or ecoregion-scale diversity detection, crucial for effective protective measures and sustaining coral reef ecosystem services on which millions rely (Robinson et al., 2019).

#### AUTHOR CONTRIBUTIONS

The research was designed and conceptualized by OBB, KW, MJS and SJB. The fieldwork was performed by OBB, KW, PRM, TB and BLA, while the eDNA molecular and fish barcode work was done by OBB guided by SWK and KP. Annelid ID and barcodes were made by KW and by TB and BLA under guidance of KW. Fish ID was done by PRM and OBB. Bioinformatics and statistics were performed by MRJ and OBB. Data analysis and visualization was done by OBB who also wrote the main body of the manuscript with substantial input and interpretive insights from KW, MJS, SJB, PRM, MRJ, PFT, KP and SWK. All authors have read and agreed to the final version of the manuscript.

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#### CONFLICT OF INTEREST STATEMENT

All authors confirm that there is no conflict of interest.

#### DATA AVAILABILITY STATEMENT

The raw eDNA sequences for 12S and 18S are publicly available and deposited together with the metadata, and PCR tag files at [Zenodo](#).

org. For 12S sequences DOI: [10.5281/zenodo.8374070](https://doi.org/10.5281/zenodo.8374070) (Brodnicke, Reinholdt Jensen, et al., 2023a) and 18S sequences DOI: [10.5281/zenodo.8372346](https://doi.org/10.5281/zenodo.8372346) (Brodnicke, Reinholdt Jensen, et al., 2023b).

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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