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Maximizing fish detection with eDNA metabarcoding

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Abstract

Fish biodiversity can be measured by capturing and then sequencing free DNA present in water. Such environmental DNA (eDNA) methods offer an effective, noninvasive tool for species diversity measurement, although standardized protocols are not yet developed. We investigate how metrics of fish biodiversity revealed through eDNA analysis of water are influenced by sampling volume. Water samples were collected from the intertidal reef of Browse Island, a tropical, remote island in the Timor Sea. Aliquots from a single 20,700-ml sample and multiple 2,000-ml samples were filtered in various volumes (25–2,000 ml) across two membrane sizes (0.20 and 0.45 μm). A fish metabarcoding assay was used to characterize the fish diversity within aliquots. All samples, except one, yielded fish DNA sequences. Two hundred and nine operational taxonomic units (cf. species) representing 48 fish families were identified from the complete collection of DNA contained in all samples, comparable to the 200 fish species detected using conventional surveys at this location. Notable additions from eDNA methods were cryptic and nocturnal fish species. Nevertheless, large differences in taxonomic composition (<60% species overlap) between aliquots of identical volumes demonstrate that eDNA in seawater is patchy and that estimates of biodiversity are strongly influenced by the volume of water filtered. We suggest that eDNA studies maximize water volumes as much as logistically possible if the aim is to detect the greatest number of taxa and that species accumulation curves be provided as an indication of sampling adequacy.

KEYWORDS
biomonitoring, Browse Basin, intertidal reef, survey

1 | INTRODUCTION

Fish biodiversity management relies on an accurate understanding of species identity. Biomonitoring of marine fishes conventionally involves observational identification and counts of species using an assortment of techniques including fishing, trapping, baited or unbaited remote underwater video, diver-operated stereo-video, or underwater visual census. Each biomonitoring technique has strengths and weaknesses, but all rely on expertise in fish taxonomy or, at a minimum, observers skilled in fish identification (Harvey et al., 2012; Holmes et al., 2013; Logan, Young, Harvey, Schimel, & Ierodiaconou, 2017). Recently, molecular genetic techniques have

been employed to describe fish assemblages by extracting DNA from the marine environment (Jeunen et al., 2019a; Stat et al., 2017; Thomsen et al., 2012). These environmental DNA (eDNA) techniques remove the need for repeated use of taxonomic expertise as DNA sequences can be taken from samples identified once by an expert and thereafter placed in a reference database of fish DNA—so-called DNA barcoding (Taberlet, Bonin, Zinger, & Coissac, 2018).

Environmental DNA is continuously released into the water by fishes. This shedding of DNA occurs through a variety of avenues, including excreted cells, tissue, feces, or dead individuals leaking genetic material, or as microscopic eggs and larvae (see review by Thomsen & Willerslev, 2015). Fish eDNA detected in water is positively associated with the fish species present in that water body (Thomsen et al., 2012). The spatial and temporal relationship between the presence of a fish and its DNA signal in the water varies, but is potentially useful for surveying fish assemblages in a range of circumstances (Jerde, Wilson, & Dressler, 2019).

Fish eDNA metabarcoding (the use of universal primers and barcoding to identify DNA from a mixture with multiple species) promises to be a noninvasive tool for species diversity measurement that is time- and cost-effective. However, eDNA methodologies are relatively new, diverse, and under continuous development (Jeunen et al., 2019b; Taberlet et al., 2018; Zinger et al., 2019). The precise methods chosen for eDNA metabarcoding affect fish species detection rates. For example, the substrate chosen from which to extract DNA, filter type and pore size used when filtering water samples, preservation method, DNA extraction method, primer assay, sequencing depth, and bioinformatic processing can all affect the ultimate range of taxa detected in a sample (Alberdi et al., 2018; Diaz et al., 2012; Elbrecht & Leese, 2015; Frøslev et al., 2017; Hinlo, Gleeson, Lintermans, & Furlan, 2017; Koziol et al., 2019; Li, Handley, Read, & Hanfling, 2018; Majaneva et al., 2018; Singer, Fahner, Barnes, McCarthy, & Hajibabaei, 2019; Spens et al., 2016). Furthermore, fish behavior and environmental conditions can also affect detection rates. Increased feeding behavior, for example, has been associated with increased eDNA shedding rates (Klymus, Richter, Chapman, & Paukert, 2015). Warmer water is also associated with increased eDNA shedding rates (Jo, Murakami, Yamamoto, Masuda, & Minamoto, 2019), yet eDNA degradation rates are elevated by water temperature (Nevers et al., 2018). The degradation of eDNA in marine systems can occur within days (Collins et al., 2018; Thomsen et al., 2012), thereby increasing the probability of species detection within close proximity to the DNA source. A broad range of taxa have been shown to be spatially discrete using eDNA techniques (Jeunen et al., 2019b), although it is possible that sea currents could transport eDNA beyond areas where the species occur and that predators (e.g., seabirds that prey upon fish) could also redistribute DNA widely through defecation. Given these many variables, the distribution of eDNA is potentially complex and not uniform in space and time, which makes it vital to adopt sampling protocols that are least subject to signal noise.

The volume of water that is filtered for eDNA analysis and the method of collecting it are important considerations in survey design to avoid potential under sampling biases (Zinger et al., 2019). Sample volumes for fish studies range widely (400–6,000 ml reported in review by Turner et al., 2014; Wilcox et al., 2013), although both 1,000 ml (Koziol et al., 2019; Stat et al., 2017) and 2,000 ml volumes are frequently sampled (in review by Turner et al., 2014). Sample replication also varies widely, from studies using triplicate 250-ml samples (Singer et al., 2019), eight replicate 1,000-ml samples (Koziol et al., 2019), to five replicate 2,000-ml samples (Jeunen et al., 2019a) per site. For many species, such as amphibians and macroinvertebrates, detection rates are higher with increased sampling volumes (Lopes et al., 2017; Mächler, Deiner, Spahn, & Altermatt, 2016). For fish species, recent studies in freshwater and temperate oceans indicate that 20,000 ml or more (up to 68,000 ml) may be needed to detect total fish diversity (Cantera et al., 2019; Koziol et al., 2019 appendix; Hanfling et al., 2016). Indeed, the volume needed to describe fish communities in the ocean may vary widely due to diversity of species in a particular location (e.g., tropical vs. temperate systems) and occurrences of rare and transient species.

Here, we test how water sampling volumes influence metrics of marine fish biodiversity as characterized by eDNA metabarcoding at a remote, offshore tropical island in northern Australia. We also compare the fish taxa recovered through eDNA analysis with those observed previously by conventional survey techniques. We show that the patchiness of eDNA in seawater makes water sampling volume of critical importance. Provided sample volume and replication are sufficient, eDNA-based biodiversity surveys can document comparable fish taxa to conventional survey methods, while

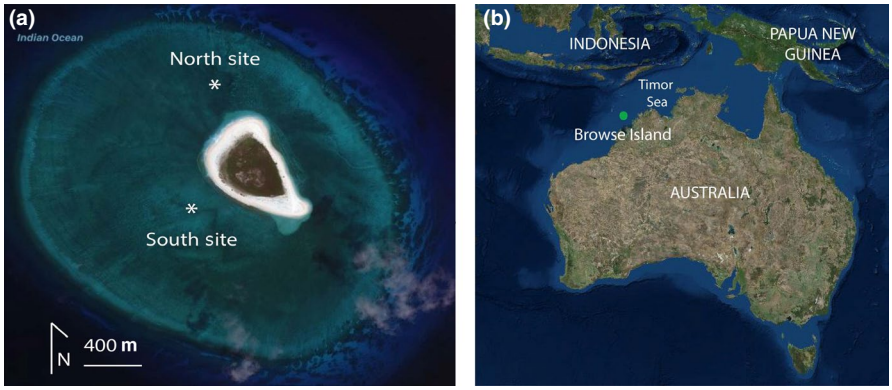

FIGURE 1 (a) Satellite image of Browse Island showing the north and south locations where water samples were collected from the intertidal reef habitat during high tide. (b) Map of Browse Island's location in the Timor Sea, in relation to Australia and Indonesia

revealing the presence of distinct cryptic guilds that are poorly characterized by other methods.

2 | MATERIALS AND METHODS

2.1 | Study site

Water samples were collected during high tide on the intertidal reef of Browse Island, a tropical, remote offshore island in the Browse Basin, Timor Sea (Figure 1). Browse Island is 200 km from the Australian coast and >150 km from the next nearest reef. This remote location offered an ideal site to evaluate the patchiness of DNA in water where the likelihood of reef fish DNA being transported from elsewhere was low. Additionally, recent data on fish diversity using conventional methods are available for the waters surrounding Browse Island (Bessey et al., 2019; Olsen, Bessey, McLaughlin, & Keesing, 2017; Rosser et al., 2014; G.J. Edgar pers. comm., 30 January, 2019). The island is surrounded by a planar platform reef, an extensive reef flat that is conspicuously absent to the northeast of the island, and a well-defined reef crest and slope (Figure 1).

2.2 | Sample collection

To evaluate the distribution of DNA in a single sample, 20,700 ml of seawater was collected at a single location (north site 14.10443°S, 123.54690°E; Figure 1 on 7 October, 2017) by submerging a sterile 20,000-ml plastic container just below the surface until full. Aliquots from the 20,700-ml sample were immediately filtered in various volumes (25, 50, 100, 250, 500, 750, 1,000, 1,500, and 2,000 ml) across both 0.20-µm and 0.45-µm PALL mixed cellulose ester (MCE) Universal Membrane Disc Filters (47 mm) in duplicate (except for the 2,000-ml sample) using a peristaltic Sentino® Microbiology Pump. Previous studies suggest that these MCE 0.45-µm filters perform the best in terms of total DNA yield, probability of fish species detection, and repeatability (Li et al., 2018). The single ~20,000-ml sample was mixed between sample aliquots by shaking the container three times. To prevent contamination, gloves were worn and sterile tweezers were used to handle each filter paper. The membrane disks were immediately frozen after filtering and stored at −20°C until further processing back in the laboratory.

To further evaluate the distribution of DNA using alternative collecting procedures (replicate samples of a set volume), we consecutively collected six 2,000-ml samples of seawater from one location (within a 2 m radius) just below the surface at a second site (south site 14.11160°S, 123.54565°E; Figure 1 on 4 October, 2017). As was done at the north site, various quantities (25, 50, 100, 250, 500, 750, and 1,000 ml) from the south samples were filtered across both 0.20-µm and 0.45-µm membrane disks in duplicate, using water from each sample bottle consecutively.

All filtration apparatus was cleaned before use and between sites by soaking in 10% bleach solution for at least 15 min. To account for possible contamination in the field, we also filtered 50 ml of deionized water across both 0.20-µm and 0.45-µm membrane disks in duplicate prior to filtering marine water samples at both the north and south sites (field controls). It was unnecessary to clean equipment between aliquots at the same site, as our main objective was to collect all DNA from the entire site sample.

2.3 | DNA extraction

Total nucleic acid was extracted from one half of each filter membrane using the standard DNeasy Blood and Tissue Kit (Qiagen), but with the addition of 40 µl of proteinase K. DNA was eluted into 200 µl AE buffer. All extractions took place in a dedicated DNA extraction laboratory using a QIAcube (Qiagen), where benches and equipment were routinely bleached and cleaned.

2.4 | Molecular analysis

A single fish metabarcoding assay approach was used to investigate the fish diversity between samples at the study site. PCR was performed in duplicate on all DNA extractions using a primer set containing template-specific oligonucleotides targeting fish taxa in the mitochondria 16S rDNA region (16SF/D 5′ GACCCTATGGAGCTTTAGAC 3′ and 16S2R-degenerate 5′ CGCTGTTATCCCTADRGTAACT 3′; Berry et al., 2017), with the addition of fusion tag primers unique to each sample that included Illumina P5 and P7 adaptors. Performing a single round of PCR in an ultraclean PCR designated laboratory helped reduce the potential for chimera production, cross-contamination, and index-tag switching. Although recent studies suggest that one-step PCR approaches may introduce bias and inconsistencies in species detection due to the long nucleotide tail altering binding conditions (O'Donnell, Kelly, Lowell, & Port, 2016; Zizka, Elbrecht, Macher, & Leese, 2019), we rotated ten different reverse fusion tag primers (R311–R320) throughout all our samples without detecting any patterns between species occurrence and primers (Figure S1; see Section 2.8). In addition, the large biases reported by O'Donnell et al., (2016) refer to variation in the abundance of sequence reads and therefore would impact our study to a lesser degree as we report only on the presence of species. Our PCR reagents included 12.5 µl SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories), 0.4 µM forward and reverse primer, 2 µl of DNA, and Ultrapure™ Distilled Water (Life Technologies) made up to 25 µl total volume. Mastermix was dispensed using a QIAgility liquid handler (Qiagen), and PCR was performed on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad) using the following conditions: initial denaturation at 95°C for 5 min, followed by 40 cycles of 30 s at 95°C, 30 s at the primer annealing temperature 54°C, and 45 s at 72°C, with a final extension for 10 min at 72°C. All duplicate PCR products from the same subsample were combined prior to library pooling. The library for sequencing was made by mini-pooling amplicons based on similar qPCR Ct values, which were then quantified (QIAxcel; Qiagen) and combined into equimolar ratios (range: 1.4–36.88 ng/µl).

The library was size-selected using a Pippin Prep (Sage Science) and purified using the QIAquick PCR Purification Kit (Qiagen). The volume of purified library added to the sequencing run was determined using qPCR against DNA standards of known molarity (Murray, Coghlan, & Bunce, 2015). The library was unidirectionally sequenced using a 300-cycle MiSeq® V2 Reagent Kit on an Illumina MiSeq platform (Illumina) using standard Illumina MiSeq default settings.

Blank laboratory extraction controls were included on each PCR plate, as well as positive controls (dhufish, *Glaucosoma hebraicum*). Dhufish are a subtropical species of fish inhabiting rocky outcrops and ledges from Shark Bay south to Esperance in Western Australia, and were an appropriate control because their distribution does not extend to Browse Island. Analyses of blank controls revealed no amplification of DNA sequences, while positive controls all amplified multiple reads (mean = 53,104) identifying dhufish with 100% identity (*Glaucosoma hebraicum*, BLAST accession number MH813293.1; see Section 2.6 for further details).

2.5 | Data processing

Data generated by Illumina sequencing were filtered through a series of quality control steps prior to taxonomic assignment. Only reads matching 100% to Illumina adaptors, index barcodes, and template-specific oligonucleotides identified using Geneious® 8.1.4.73 were kept for downstream analyses. Reads below minimum sizes of 170 bp were discarded. Potential chimeras were identified using VSEARCH command uchime_denovo (Rognes et al., 2016; https://github.com/torognes/vsearch) and removed (parent sequences were at least 1.5 times more abundant than chimeras). Samples were collapsed into unique sequence reads and abundance filtered: A combined minimum of five identical reads were required to be considered for taxonomic assignment. Reads were clustered into centroids using the cluster_fast command (97% similarity threshold) in VSEARCH. A postclustering algorithm LULU (Frøslev et al., 2017; https://github.com/tobiasgf/lulu) was then used for curation of amplicon data in R (version 2.14.0; R Development Core Team, 2011). This treatment of amplicon data was implemented to reduce the number of erroneous operational taxonomic units (OTUs) to obtain a conservative biodiversity metric. The final curated OTU table was queried against the NCBI (Benson et al., 2014) nucleotide database using BLASTN (Altschul, Gish, Miller, Myers, & Lipman, 1990). The search set used in BLASTN was the nucleotide collection (nr/nt), with the program selection optimized for highly similar sequences.

2.6 | Taxonomic assignment

The taxonomic assignment of BLAST search results for each OTU were resolved to species, genus, family, or higher, based on the percent similarity to taxa alignments. A summary of BLAST accession number, maximum bit scores, and identities is provided for the most closely matched species to provide transparency in taxonomic assignment (Appendix S1). The top 10 taxa alignments (unless fewer are sufficient) are included, provided they match online database records for fauna known to the region (e.g., Atlas of Living Australia; http://www.ala.org.au, Fishes of Australia; http://fishesofaustralia.net.au/, and FishBase; http://www.fishbase.org). Codes for Australian Aquatic Biota (CAAB number) are also included. All available data on fish taxa observed in the region from previous studies were collated for use as ground truth and for a comparison to our current eDNA study. Species alignments that were discarded by our conservative postclustering analysis (LULU) are noted.

2.7 | Detecting commonly occurring taxa

Although the number of sequence reads assigned to an OTU enables a limited estimation of the relative species abundance within a particular sample/treatment (Deagle et al., 2019; Jarman et al., 2013), of most interest was comparing the number of OTUs detected between different volumes sampled. Therefore, frequency of occurrence (the number of samples containing each OTU) was used to identify the most commonly detected OTUs (hereafter, OTU and species are used interchangeably).

2.8 | Statistical analysis

To characterize the relationship between the number of OTUs detected (y) and volume of water filtered (x), an asymptotic regression model was fitted, of the form $y = b0 + b1 \times (1 - \exp(-\exp(lrc) \times x)$, where b0 is the estimated intercept on the y-axis, b1 is the estimated difference between the asymptote and the y-intercept, and lrc is the estimated logarithm of the rate constant. We use this model because, in theory, only when an asymptote is reached, can the species assemblage be assumed to be effectively sampled (Gotelli & Colwell, 2001). Therefore, forcing an asymptotic regression model allowed us to graphically determine if our sampling was adequate. We then use species accumulation curves to compare total OTUs to cumulative water volume (specaccum in package "vegan"; https://github.com/vegandevs/vegan). We use a random accumulator function and weights giving the sampling effort as a proportion of the total volume sampled, as our sample volumes gradually increased. For these model settings, effort refers to the average sum of weights corresponding to the number of samples.

Permutational multivariate analysis of variance (PERMANOVA), analogous to redundancy analysis (Legendre & Anderson, 1999), was used to determine if quantity of water filtered, filter paper size, site, or their interactions were significant sources of variation in fish community composition. Species lists were subjected to nonmetric multidimensional scaling (nMDS) ordination using a Bray–Curtis dissimilarity matrix based on presence/absence of taxa (vegan and RVAideMemoire packages; Dixon, 2003; Herve, 2018) and grouped by site and volume filtered, or by reverse fusion tag primers (Figure S1). All statistics and graphics were produced using R (version 2.14.0;

R Development Core Team, 2011), with some graphics further edited using Adobe Illustrator (CC 2017).

2.9 | Comparisons to existing data

Fish species detections from the current study were compared to those previously obtained on the intertidal reef around Browse Island using conventional survey methods. Fish observations were obtained from an unbaited remote underwater video survey conducted on the intertidal reef of Browse Island during 2016 and 2017 at the exact north and south locations used in the current study (Bessey et al., 2019; Olsen et al., 2017). Additionally, fish observations from an intertidal pool on the north–northeast reef crest of Browse Island from 2006 were incorporated (Rosser et al., 2014). Finally, we included unpublished observations by Edgar using underwater visual census (UVC) and the Reef Life Survey methodology (Edgar & Stuart-Smith, 2014) along a 5-m-wide transect, as well as along a 1-m-wide transect specifically looking for cryptic fishes (data at www.reeflifesurvey.com). These surveys were conducted at two sites on the northeast side of the island, in reef flat (14.10958°S, 123.55655°E; 4–5 m depth) and reef edge habitat (14.10193°S, 123.5508°E; 5–7 m depth).

3 | RESULTS

3.1 | Taxonomic assignment

All samples yielded DNA sequences, regardless of volume of water filtered or filter paper pore size, with the exception of one sample (100 ml with a 0.20-µm filter membrane from the south site). Our single library generated 12,952,151 sequence reads. After sequence processing, a total of 1,071,824 (excluding positive controls) were used in the creation of the OTU table and the average number of reads per sample was 17,287 (max = 39,367, min = 4,605; 0 sample excluded). We found no correlation between sample volume and number of reads generated (R^2 = .02, p = .326). A total of 209 OTUs representing 48 fish families were assigned from the complete collection of samples. The closest taxa alignment for reads within each OTU is provided (Appendix S1). One OTU was excluded from the dataset because it was detected in the south site field controls (Carangidae, *Trachurus* sp.) and therefore could be interpreted as contamination.

3.2 | Detection of taxa by volume of water filtered, membrane size and site

A total of 176 unique OTUs were detected from north site water aliquots: 152 and 130 when using the 0.45 and 0.20 µm filter member pore size, respectively (Figure 2a,b). Water volumes of even 25 ml revealed several taxa, but with very few overlapping detections (<30%) between replicates (Figure 2; number of overlapping taxa displayed above each replicate) of smaller water volumes (≤100 ml). Samples showed an increased number of overlapping taxa between replicates as larger volumes of water were filtered. Nevertheless, even the largest aliquot (2,000 ml) represented <43% (75/176) of the total taxa detected at the northern site (Figure 1; see inset of percentage by volume filtered). These trends apply when water was filtered across either 0.45- or 0.20-µm filter membranes.

A total of 118 unique OTUs were detected at the south site: 90 and 88 when using the 0.45 and 0.20 µm filter member size, respectively (Figure 2c,d). These south site samples represent true field replicates (multiple collections from one site), and overlapping detections were ≤30% for all quantities of water filtered regardless of filter membrane pore size (Figure 2 inset).

In a similar trend to the north site, samples showed an increase in number of overlapping taxa between replicates as larger volumes of

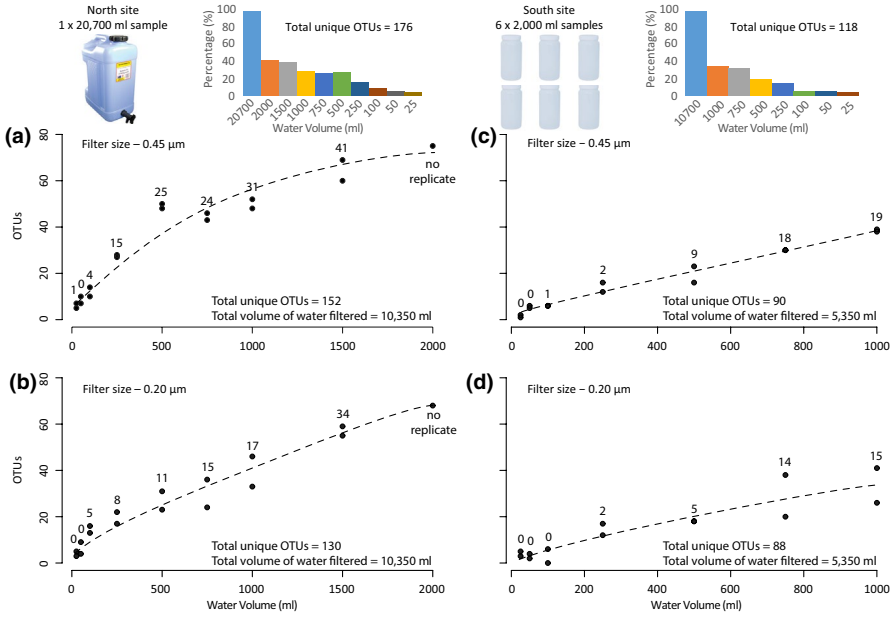

FIGURE 2 Number of fish OTUs identified in water samples collected from the north site using (a) 0.45-µm filter papers and (b) 0.20-µm filter papers, and from the south site using (c) 0.45-µm filter papers and (d) 0.20-µm filter papers. One 20,000-ml container was used to collect water from the north site, while six 2,000-ml containers were used to collect water from the south site. The numbers above the data points represent the number of shared OTUs between replicates. Inset histogram graphics display the maximum percentage of OTUs detected per sample volume (ml)

water were filtered. Nevertheless, the largest aliquot (1,000 ml) only represented 35% (41/118) of the total taxa detected at the south site.

For all aliquots at both sites, there was a positive relationship between volume of water filtered and number of OTUs detected. Only the north site aliquots filtered across 0.45-µm membranes showed evidence of an asymptotic relationship (b0 = 5.00, b1 = 70.98, lrc = −6.64). Species accumulation curves indicated that eight samples (with a total volume of ~4,400 ml) would be needed to approach an asymptote for the north site, whereas seven samples (with a total volume of ~3,850 ml) would be needed for the south site (Figure 3).

Although there was overlap in the presence/absence of fish taxa between sample locations (Figure 4), both site and volume of water filtered, as well as their interactions, were significant sources of variation in the detected fish community (Table 1; PERMANOVA, $p < .01$). Larger volume aliquots clustered closer together than did the smaller volume aliquots (Figure 4; nMDS where stress = 0.187).

3.3 | Frequently detected taxa

At the north site, we detected 38 taxa in at least 30% or more of the sample aliquots (Figure 5). Five of these taxa could only be identified to the order Perciformes. The remaining 33 taxa fell within 15 families (Acanthuridae—5; Balistidae—2; Belonidae—1; Blenniidae—5; Cirrhitidae—1; Gobiidae—2; Labridae—4; Lutjanidae—2; Microdesmidae—2; Muraenidae—3; Pomacentridae—2; Pseudochromidae—1; Scaridae—1; Scorpaenidae—1; and Serranidae—1). Among the most frequently detected taxa were multiple species of blennies and gobies, all of which are small (generally <15 cm), and could be classified as cryptic. Three species of moray eels were also frequently detected. All frequently detected species are typically found on tropical coral reefs, and most have been observed around Browse Island in previous studies (Appendix S2).

At the south site, we detected 17 taxa that were present in at least 30% or more of the sample aliquots (Figure 6). All 10 of the most frequently detected taxa in the south site were also frequently detected in the north site sample, the top two of which were gobies from the genus *Eviota*. *Eviota* species have been documented at Browse Island during surveys for cryptic fishes, conducted by an experienced fish taxonomist (Appendix S2).

Frequently detected taxa from both sites have been previously detected with conventional methods. For example, from the family Acanthuridae, *Acanthurus triostegus* have been previously observed on unbaited remote underwater visual surveys and during diver-based underwater visual census on the reef flat (Appendix S2). Two unknown taxa from the family Blenniidae were frequent in the current eDNA study, coinciding with multiple species of blennies previously observed during cryptic fish surveys in the tide pools at Browse Island (Rosser et al., 2014). Several, more conspicuous taxa, from the families Labridae, Lutjanidae, Balistidae, and Serranidae, specifically *Thalassoma hardwicke*, *Lutjanus decussatus*, *Balistoides viridescens*, and *Cephalopholis argus*, were observed in most previous studies. Indeed, species within the genus *Thalassoma* were the most abundant during underwater visual surveys conducted on the northern reef flat (Edgar, pers. com.).

We summarized a total of 317 fish taxa that have been observed/detected in the intertidal and edge area surrounding Browse Island (Appendix S2) through a combination of eDNA and conventional survey methods. Previous surveys conducted on the intertidal reef using conventional methods (Bessey et al., 2019; Rosser et al., 2014, and unpublished data from Edgar) observed 98, 33, and 119 fish species, respectively. The current eDNA study has expanded the list of fish taxa using the Browse Island intertidal reef flat, especially in regard to the families Muraenidae, Scorpaenidae, Blenniidae, and Gobiidae. Of the 14 taxa of eels

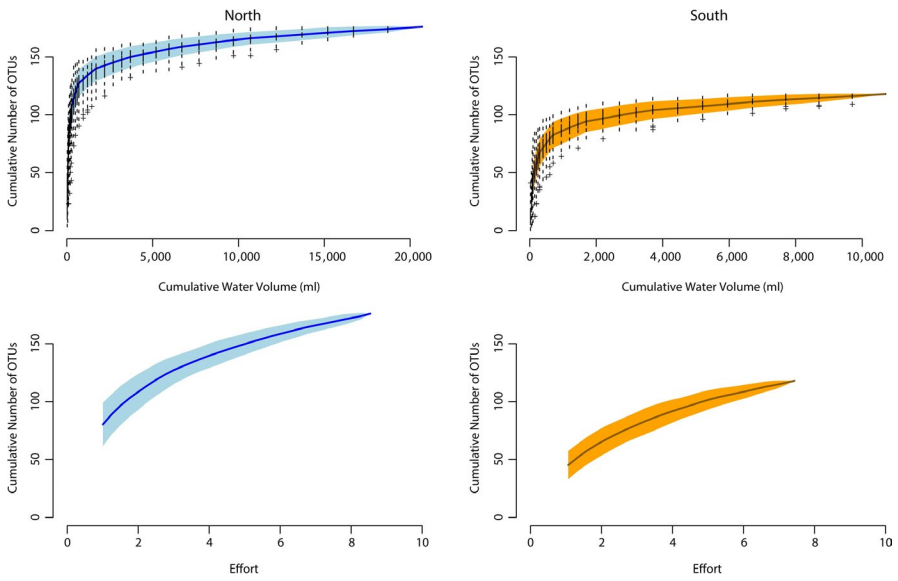

FIGURE 3 Species accumulation curves (overlaid with boxplots) for the north and south sites by cumulative water volume and sampling effort. Shading indicates confidence interval

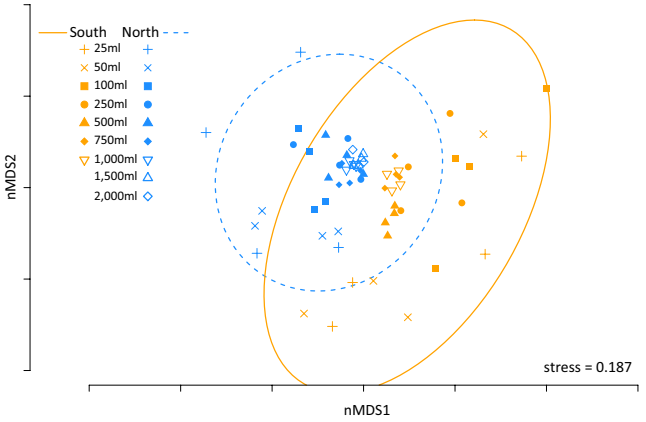

FIGURE 4 Nonmetric multidimensional scaling plot of fish OTUs for each site by volume of water filtered. Ellipses represent 99% confidence bands for each site

(Muraenidae) detected in the current study, only five had been previously reported in the study area.

4 | DISCUSSION

Our results support mounting evidence that eDNA analysis can characterize fish biodiversity in comparable detail to conventional survey methods. Furthermore, they show that eDNA detects fish species poorly represented by conventional methods. Yet, they also demonstrate that the potential of this new biodiversity surveying tool will be maximized if greater attention is applied to sampling protocols. Specifically, we show that DNA molecules can be patchy within seawater samples. As a consequence, the volume of water sampled becomes critical if the aim is to maximize the number of taxa detected. We suggest that water volumes be maximized where possible and that studies provide species accumulation curves by sampled volume as an indication of sampling sufficiency.

4.1 | Patchiness of DNA in seawater

The diversity of fish taxa detected within a seawater sample was significantly influenced by the volume of water filtered. Assurance that sufficient volume of water was sampled to characterize alpha diversity at a location could be provided by demonstrating an asymptotic relationship between volume of water filtered and number of OTUs detected. This was not achieved in the current study. Even the largest volume of water filtered (2,000 ml) only accounted for <43% of the taxa detected in the entire 20,700-ml sample. This means that studies that filter insufficient water volumes inevitably will yield many false-negative detections. Fish studies conducted in a temperate seawater system indicated that ~20,000 ml was required before family accumulation curves showed asymptotic tendencies (Koziol et al., 2019). Indeed, species richness estimates are sensitive to sampling effort in eDNA metabarcoding surveys, and suggested sampling recommendations are site specific (Grey et al., 2018). Yet, conventional reef fish survey techniques face similar challenges, as no standardized survey method has emerged that would allow comparability of data across studies (Caldwell, Zgliczynski, Williams, & Sandin, 2016). We suggest that eDNA studies maximize water volumes as much as logistically possible, that sites be repeatedly sampled, and that species accumulation curves with water sampling volume be provided as an indication of sampling adequacy. One novel approach to maximizing the volume of seawater filtered is to extract DNA from the tissue of sponges (Mariani, Baillie, Colosimo, & Riesgo, 2019), as these animals naturally ingest and retain large quantities of waterborne eDNA.

4.2 | Validating detected taxa

Conventional fish surveys conducted on the intertidal reef surrounding Browse Island validated approximately half of the fish taxa detected through eDNA methods (Appendix S2). Although limited, quantitative fish data from the area (Edgar, unpublished data) found that many of the most abundant genera observed during

TABLE 1 Results of permutational multivariate analysis of variance (PERMANOVA) for determining sources of variation in presence/absence of fish community at intertidal reef survey sites

	Df	Sums of Sqs	Mean Sqs	*F* value	*P* (perm)
Site	1	2.13	2.13	8.64	**.001**
Filter size	1	0.33	0.33	1.35	.138
Quantity filtered	1	1.89	1.89	7.68	**.001**
Site × Filter size	1	0.20	0.20	0.81	.655
Site × Quantity filtered	1	0.62	0.62	2.53	**.002**
Filter size × Quantity filtered	1	0.12	0.12	0.49	.974
Site × Filter size × Quantity filtered	1	0.27	0.27	1.08	.347
Residuals	53	13.07	0.25		
Total	60	18.64			

Note: Results significant at $\alpha = .05$ are shown in bold.

FIGURE 5 The most commonly re-occurring OTUs in water samples from the north site with representative images of species. Only OTUs detected in ≥30% of samples are shown, by quantity filtered (ml) and size of filter paper (µm). Species are listed in order of most commonly re-occurring

FIGURE 6 The most commonly re-occurring OTUs in water samples from the south site with representative images of species. Only OTUs that showed up in ≥30% of samples are shown by quantity filtered (ml) and size of filter paper (µm). Species are listed in order of most commonly re-occurring

conventional surveys (e.g., *Acanthurus*, *Ctenochaetus*, *Chrysiptera*, *Halichoeres*, *Pomacentrus*, and *Thalassoma*) were also the most frequently detected using eDNA methods. During cryptic fish surveys, the genera *Blenniella*, *Cirripectes*, *Salarias*, and *Eviota* were abundant, all of which feature predominately in our eDNA detections. Additionally, all frequently detected fish species are known to use tropical intertidal habitats. These data contribute to mounting evidence that eDNA analysis can characterize fish biodiversity in comparable detail to conventional methods (Jeunen et al., 2019b; Stat et al., 2017).

4.3 | Extension of taxa lists at remote locations

Our eDNA analysis has extended the list of taxa known to inhabit Browse Island by detecting cryptic species, such as blennies, gobies, and eels. Conventional fish surveys are conducted during daylight hours and are subject to the behavioral traits of the fish (e.g., avoidance or attraction to diver; Prato, Thiriet, Di Franco, & Francour, 2017). eDNA methods require only DNA particles from a species to be collected for detection. Consequently, we detected a much greater proportion of cryptic fish species than observed by visual survey methods. This result concurs with a recent suggestion that the abundance and productivity of cryptic fishes is underappreciated and that they provide most of total fish production in reef ecosystems and play a critical trophic role (Brandl et al., 2019).

We also detected 14 taxa of eels (Muraenidae), of which only five had been previously reported for the study area (a 180% increase in diversity of this group), although all are known to occur throughout the northwest of Western Australia and the Indo-Pacific. Many species of eels spend their days hiding in crevices and become more active at night, making them more difficult to detect during conventional methods. Indeed, their abundance and ecological significance are often underestimated due to their cryptic behavior, even though some studies using ichthyocides have demonstrated that they can comprise up to 47% of the carnivorous fish biomass in an area (Brock, Lewis, & Wass, 1979). eDNA methods allow for a nondestructive way to detect cryptic species. Employing these noninvasive eDNA methods and maximizing detection rates are especially useful when surveying remote locations that are costly to reach, are logistically difficult to survey, or are constrained by safety considerations.

4.4 | Caveats

All eDNA metabarcoding studies have limitations or potential biases (see Zinger et al., 2019 for an overview), and our study demonstrates some of these. We use only half of each filter paper, and it is unknown how processing the entire filter paper would increase fish species detections. It is also possible that the DNA in the 2-µl aliquot used for each PCR amplification is patchy. Additionally, biases and inconsistencies introduced from our one-step PCR approach are difficult to test for as all fusion tag primer combinations are unique. The preferential amplification of certain taxa over others is unknown. Our conservative postclustering method also combined some morphologically distinct species into one group that would otherwise be multiple species. Ultimately, as with all eDNA studies, taxonomic identification of DNA sequences relies on species being accurately represented and present in reference databases. Although we incorporated field sampling controls, PCR controls, and technical replicates, looked for primer biases, and employed consistent downstream processing of all samples, we acknowledge that unknown biases could have occurred during many stages of the eDNA workflow.

5 | CONCLUSION

eDNA fish surveys complement and enhance conventional fish surveys. While eDNA biodiversity estimates benefit from the ground-truthing of conventional studies, they can extend our knowledge of taxa for certain locations. The use of this new biodiversity tool for monitoring marine fish will benefit from rigorous sampling design that accounts for the displayed patchiness of DNA molecules in seawater.

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DATA AVAILABILITY STATEMENT

Raw sequences and the final dataset will be available on the CSIRO Data Access Portal (https://data.csiro.au/dap) after manuscript acceptance. A spatial representation of the eDNA survey results will be available through the Atlas of Living Australia after manuscript acceptance at https://collections.ala.org.au/public/show/dr14581 (www.ala.org.au).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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