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Hide 'n seq: Direct versus indirect metabarcoding of coral reef cryptic communities

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Abstract

Ecological patterns in biodiversity are primarily based on conspicuous organisms. Few methods are used to survey the taxonomically rich cryptobiome, which is made up of inhabitants from within microhabitats. One way that cryptic marine biodiversity can be non-invasively surveyed is by analyzing environmental DNA (eDNA) present in seawater. Using coral reefs as a model system, here we compare estimates of cryptic diversity among community biomass and eDNA metabarcoding sampling methods with a broad eukaryotic marker (COI). First, contributions to eDNA were investigated across cryptobiomes through a comparison of community metabarcoded biomass from standardized autonomous reef monitoring structures (ARMS) to eDNA acquired from seawater in which individual ARMS were soaked. Second, we compared these results to those from eDNA samples taken from within reef crevices and the ambient water column. Metabarcoding of community biomass from ARMS and eDNA from the two types of water samples revealed significantly different communities of cryptic coral reef habitat with little overlap between methods. Taxa that were unique to metabarcoding of ARMS biomass were predominantly from chitinous and calcifying groups (polychaetes, palaemonid shrimp, mollusks, brittle stars, and red algae), which suggests that these taxa are underrepresented in eDNA surveys. Other than the corals themselves, sponges and red algae were significant drivers of reef crevice community differences, while ambient seawater samples detected mostly planktonic organisms and reef fishes. Our data indicate that both eDNA and ARMS provide incomplete accounting of cryptic diversity. Direct sampling of biomass is best suited for building taxonomies and improving databases, whereas eDNA methods offer rapid insights into the composition of cryptobiomes. Because each method likely captures different taxa, multiple targeted assays can be used to provide the greatest estimates of metazoan and macroalgal richness.

KEYWORDS
biodiversity, cytochrome c oxidase I, coral reefs, eDNA methods, marine metazoans

1 | INTRODUCTION

The global patterning of biodiversity is a direct result of numerous complex phenomena that continually act across multiple spatial scales resulting in dynamic species ranges within geographic and ecological space (Blowes et al., 2019; Descombes et al., 2015; Tittensor et al., 2010). With many different biomonitoring protocols implemented across diverse ecosystems, our biodiversity estimates are unavoidably biased by observational methods (Heisenberg, 1958). Measuring local biodiversity is widely recognized as a crucial step in conservation planning (Margules & Pressey, 2000; Noss, 1990). However, when biodiversity data are sparse, monitoring cannot be performed rapidly on a routine basis (Dale & Beyeler, 2001; Hirst, 2008; Parrish et al., 2003), or different methods uncover contrasting patterns of biodiversity (Whitworth et al., 2017), conservation planning becomes increasingly difficult. Marine conservation lags behind terrestrial efforts given limited marine protection status (Gleason et al., 2006; Mouillot et al., 2020; Sala et al., 2021) and islands in particular face elevated levels of habitat destruction and biodiversity loss (Tershy et al., 2015). Coastal reef ecosystems make up a small percentage of the ocean, yet contribute disproportionately to the overall abundance and diversity of marine life (Costanza et al., 2014; Fisher et al., 2015). Local and global stressors threaten coral reefs and the ecological goods and services they provide (Moberg & Folke, 1999). With the rapid decline of coral reefs on a global scale (Descombes et al., 2015; Hughes et al., 2003, 2017; Pandolfi et al., 2003), efficient and effective assessments of biodiversity are vital for understanding ecological processes and supporting conservation efforts (Knowlton et al., 2010; Margules & Pressey, 2000; Parrish et al., 2003).

The surface-dwelling corals and fishes that are overtly associated with reef habitats account for a fraction of the total species richness of metazoans, whereas the coral reef cryptobiota (microflora and fauna, cavity-dwellers, or coelobites) living in the crevices of the coral limestone structure make up an estimated 91% of known species in this ecosystem (Hartman, 1970; Jackson & Buss, 1975; Jackson et al., 1971; Stella et al., 2010). These organisms support key ecological processes, such as nutrient cycling (Depczynski & Bellwood, 2003; Richter et al., 2001) and carbonate cementation (Wulff & Buss, 1979; Zundelevich et al., 2007), and can aid in deterring coral predators (Glynn, 1980, 1983). Cavity-dwellers consist of important trophic groups such as suspension feeders (Scheffers et al., 2010; Wunsch & Richter, 1998), predators (Glynn, 2006; Reaka, 1987), herbivores (Coen, 1988; Klumpp et al., 1988), and detritivores (Rothans & Miller, 1991) that sustain ecosystem function on coral reefs (reviewed by Brandl et al., 2019). Yet, our knowledge and exploration of these cryptic habitats are restricted by the limitations of traditional survey methods and taxonomic expertise. As a result, large portions of overall coral reef biodiversity are missed or remain undocumented, as so-called 'dark diversity' (Pärtel et al., 2011).

Few methods currently exist for surveying cryptic reef habitat and its dark diversity. Autonomous reef monitoring structures (ARMS) are one tool that has been used widely in long-term benthic settlement monitoring of coral reef cryptobiota. The primary advantage of ARMS is that they provide a standardized, replicated habitat similar to the structural complexity of surrounding reef cavities (Brainard et al., 2009; Leray & Knowlton, 2015; Ransome et al., 2017). ARMS also allow for non-destructive sampling of coral habitat interstices and have helped fill large gaps in surveying cryptobiota which otherwise would remain largely inaccessible (Wunsch & Richter, 1998). By incorporating morphometric sorting and voucher-based DNA barcode sampling of both sessile and motile taxa, the use of ARMS has contributed significantly to DNA libraries that currently house a limited number of barcoded sequences from this vast community (Timmers et al., 2020). With the advent of high-throughput DNA sequencing, the DNA metabarcoding of ARMS biomass has facilitated standardized community-level biodiversity comparisons across spatial and temporal scales (e.g., Leray & Knowlton, 2015; Hurley et al., 2016; Pearman et al., 2016, Pearman et al., 2019; Pennesi & Danovaro, 2017; Carvalho et al., 2019; David et al., 2019).

However, ARMS sampling has several limitations. First, although ARMS circumvent destructive sampling of the reef, they require deployment in the field for years to allow for adequate colonization, particularly for diverse communities with potentially long exclusion times and few dominant competitors (Buss & Jackson, 1979; Pearman et al., 2016; Ransome et al., 2017). Second, they require SCUBA diving and specialized tools to install and recover the units. Third, they require extensive pre-processing upon recovery and many hours of skilled labor to capture the sessile and motile communities that colonized each ARMS unit. Although ARMS have become a standard method to survey cryptic taxa, the time required for adequate settlement means that rapid surveys are not possible.

Recent advances in high-throughput DNA sequencing technologies have led to the implementation of biodiversity assays targeting environmental DNA (eDNA), genetic material shed by organisms in the form of metabolic waste or sloughed cells. Environmental DNA assays offer fast and efficient insights into reef-scale metrics of biodiversity (Alexander et al., 2020; DiBattista et al., 2017, 2020; Nguyen et al., 2020; Sawaya et al., 2019) and can detect both abundant and rare taxa (Balasingham et al., 2018; Borrell et al., 2017; Boussarie et al., 2018; Jerde et al., 2011; Nichols & Marko, 2019; Port et al., 2016; Thomsen et al., 2012b) at a fraction of the cost of visual survey methods (Brown et al., 2004; Harper et al., 2018). Environmental DNA surveys require minimal field effort yet allow researchers to detect taxa across the tree of life (Stat et al., 2017). The relative simplicity of eDNA protocols allows for surveying field sites that would otherwise be difficult to access (DiBattista et al., 2019; Everett & Park, 2017) and can easily be adapted for citizen science to further increase public awareness of biodiversity loss (Deiner et al., 2017).

However, one of the main disadvantages of eDNA surveys is that detection depends on capture methods (Bessey et al., 2020; Deiner et al., 2015; Freeland, 2017; Piggott, 2016) and rates of eDNA production, transport, and degradation (Andruszkiewicz

et al., 2019; Dell'Anno & Corinaldesi, 2004; Jo et al., 2019), which may vary across taxa (Klymus et al., 2015; Sassoubre et al., 2016) and habitat types (Deiner & Altermatt, 2014; Kelly et al., 2018; O'Donnell et al., 2017). These limitations may ultimately lead to patchiness of eDNA signals in seawater samples (Bessey et al., 2020) and therefore bring into question the capability of eDNA samples from the water column to contain DNA from cryptobiota living inside the reef structure. While eDNA from filtered seawater has been used extensively in studies of plankton (Berry et al., 2019; Djurhuus et al., 2018; Kelly et al., 2014; Massana et al., 2015), nekton (Bakker et al., 2017; Berry et al., 2019; Boussarie et al., 2018; DiBattista et al., 2017; Thomsen et al., 2012a), and dominant epifauna (Alexander et al., 2020; Everett & Park, 2017; Nichols & Marko, 2019; Port et al., 2016), much less is known about its effectiveness for surveying reef cryptobiomes. As eDNA targets genetic material sloughed from tissue or as waste, sampling eDNA in the water column likely favors mucus-producing taxa, plankton, such as micro-algae and copepods, and other abundant benthic taxa living near or on the surface of the reef.

Here, we compare and contrast metazoan cryptobiota communities directly detected in ARMS units to those indirectly sampled from eDNA extracted from water samples using DNA metabarcoding of a 313 base pair fragment of the mitochondrial DNA (mtDNA) cytochrome c oxidase subunit I (COI) gene (Geller et al., 2013; Leray et al., 2013), the standard universal barcode for identifying most animal groups (Hebert et al., 2003). Our study's objectives were first to compare direct estimates of cryptic biodiversity using community-based ARMS metabarcoding to indirect estimates of eDNA released by intact ARMS communities soaking in filtered seawater before being disassembled. Ideally, eDNA from soaking ARMS should capture the same cryptic diversity as the direct metabarcoding of accumulated biomass. However, we predict that due to the variation in eDNA shedding among invertebrate taxa, ARMS biomass will capture different communities than eDNA sampling. Second, we compare these two sampling methods to eDNA collected from natural reef crevices and the ambient water column to assess whether eDNA can be used to survey reef cryptobiomes. Due to differences in water flow and the patchiness of eDNA in seawater, we do not expect that eDNA from the water column will reveal the same cryptic diversity when compared to samples taken from inside reef interstices. Although both metabarcoding of ARMS biomass and eDNA share certain biases (see Murray et al., 2015), a comparison assumes that ARMS attract similar settlers to natural reef habitat (Pennesi & Danovaro, 2017; Plaisance et al., 2011). This is the first study to our knowledge to compare cryptobiota from ARMS biomass with eDNA collections.

2 | MATERIALS AND METHODS

Our study compared different assessment methods for detecting cryptobiota using DNA metabarcoding (Figure 1). First, we examined filtered water from soaking individual ARMS units (referred to here as 'soaking') with subsequent bulk extractions of ARMS biota (from here on referred to as 'biomass'). Second, we compared these two sampling methods to eDNA surveys of the surrounding reef using filtered seawater taken from within reef crevices (from here on referred to as 'crevices') and the ambient seawater above the reef (from here on referred to as 'ambient').

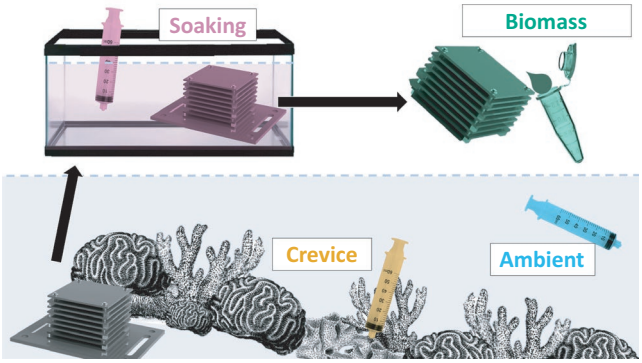

FIGURE 1 The four methods used in the DNA metabarcoding of cryptic coral reef habitat: biomass (accumulated biomass from ARMS fractions, pooled by unit, $n = 6$), soaking (aqueous DNA collected from soaking ARMS units in seawater aquaria, $n = 6$), crevices (eDNA collected from within reef crevices, $n = 12$), and ambient (eDNA from surface seawater collected from the back reef, $n = 6$)

2.1 | ARMS deployment

Six Autonomous Reef Monitoring Structures (ARMS) units (made from 23 cm × 23 cm PVC, forming a tiered stack of open and semi-closed layers) were deployed subtidally (Figure S1), at a depth of approximately 3–4 m, on the reef at Moku o Loʻe (Hawaiʻi Institute of Marine Biology, HIMB), located on the Hawaiʻian island of Oʻahu (Figure S2). ARMS units were left out on the reef over 23 months starting in July 2016 to allow settlement and colonization of marine taxa.

2.2 | ARMS retrieval, soaking, and metabarcoding

Upon retrieval (in June 2018), a 106-μm Nitex-lined crate was placed over the ARMS to limit the loss of motile organisms during transport to the surface. Once back at HIMB, ARMS units were submerged for two hours in aerated 75-l aquaria containing 45 μm filtered seawater prior to sample processing. Immediately after ARMS units were removed from the aquaria, water in each tank was homogenized by stirring with a sterile aluminum wand. Water samples were collected from each aquarium using two new 60-ml Luer-lock syringes (to avoid clogging the filter), which would later be pooled by ARMS (soaking, $n = 6$).

For each of the six ARMS units, plates were disassembled and scraped cleaned of all the accumulated biomass. Biomass was then

homogenized in a sterilized blender, poured into a 45-μm filter, and 10 g of the homogenate was subsampled for DNA metabarcoding. Water holding ARMS were then filtered through 500-μm and 100-μm sieves to collect any mobile specimens. These 500-μm and 100-μm sieved fractions underwent a decantation process to separate the sediments from the biological tissue (Leray & Knowlton, 2015). The resulting biological material was then crushed using a mortar and pestle, and 10 g was subsampled to be analyzed via DNA metabarcoding (biomass, n = 6). All metabarcoding samples were then preserved for high-throughput sequencing of the cytochrome c oxidase I (COI) gene.

2.3 | Reef crevice and ambient eDNA sampling

Ambient water samples (n = 6) were collected from the adjacent back reef within 10 m of the original ARMS deployment location (depth 2–4 m, distance >10 m from the reef) using new 60-ml syringes. Six reef crevices (depth 2–4 m, Figure S1) were sampled twice using new 60-ml syringes (sampling spaced by 10-min intervals, n = 12), which were partially inserted into each crevice (approximately 3–5 cm). Crevices were selected based on the following criteria: (1) were completely contained within the dominant mounding coral, *Porites compressa*, which forms the majority of the back reef; (2) had crevice opening diameters limited to <7 cm and a crevice depth of at least 15 cm; and (3) had no visible outlet; all crevices represented a common size range in the back reef habitat. As reef crevices at this site were generally <500 ml in volume, sampling of crevice water was limited to small volumes (120 ml) to avoid 'contamination' of crevice water with ambient water, thereby limiting the sample volumes used in method comparisons.

2.4 | Environmental DNA filtration

Following the collection of eDNA syringes, all samples were immediately filtered through mixed cellulose ester filters (Millipore; diameter 13 mm, pore size 0.22 μm) attached to Luer-lock filter holders. Collection blanks (DI water filtered through new syringes, filter, and Luer-lock filter holder, see Contamination prevention, below) were filtered in the field alongside biological samples. Filters and filter holders were placed on ice and transported back to the laboratory for DNA extraction. Using sterile forceps, filters were placed into individual 2-ml screw-cap microcentrifuge tubes with 720 μl ATL buffer (Qiagen). Tubes were shaken vigorously for 5 minutes in a tissue lyser (Retsch), then incubated at 56°C for 30 minutes, followed by an additional shaking and 30-min incubation step. Each filter was then digested with 80 μl Proteinase K (Qiagen) and incubated at 56°C for 1 hr. After transferring 600 μl of the supernatant to new tubes, the manufacturer's protocol for DNeasy Blood & Tissue Kit (Qiagen) was followed with minor adjustments: 600 μl AL buffer, 600 μl ethanol, and two final elution steps of 50 μl AE buffer.

2.5 | DNA metabarcoding and sequencing

Amplifications of the COI gene were conducted in 13 μl volumes targeting a 313-bp fragment using mlCOIintF/jgHCO2198 primers (Leray et al., 2013) plus the Illumina overhang adapters. Reactions consisted of 6.3 μl MyTaq 2x (Bioline), 0.65 μl BSA (Thermo Fisher Scientific, 20 mg/ml), 4.45 μl nuclease-free water (Growcells), 1.0 μl template DNA (21.3 ± 2.76 ng/μl SE), and 0.3 μl of each forward and reverse primers (10 μM). Thermal cycling parameters were as follows: initial denaturation step of five minutes at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 50°C, and 45 s at 72°C, with a final extension of 10 min at 72°C. The quality of all amplifications was assessed using gel electrophoresis, running PCR products through a 1.5% agarose gel stained with GelRed (Biotium), and visualized on an ultraviolet imaging platform. Libraries were prepared for sequencing by purification with a 1:1.12 ratio (DNA:beads) of Agencourt Ampure XP beads (Beckman Coulter), followed by indexing using Nextera XT v2 (Illumina) adapters (following the manufacturer's protocol) and an additional bead purification step. Amplicons were then assessed for DNA concentration with a Qubit fluorometer and dsDNA HS detection kit (Thermo Fisher Scientific). Pooled equimolar amplicons (including no template controls) were then pair-end sequenced on an Illumina MiSeq platform using the V3 600-cycle reagent kit in one MiSeq flow cell at the Advanced Studies in Genomics and Proteomics facility at the University of Hawai'i at Mānoa.

2.6 | Contamination prevention

All laboratory surfaces and equipment were sterilized using a 10% bleach solution before and after processing samples. Amplification products (as well as negative controls) were prepared for sequencing in a post-PCR work area physically separated from pre-PCR areas (using dedicated post-PCR pipettors, plastics, and reagents) to prevent contamination. Template controls (PCR negatives, n = 2, and collection blanks, n = 2) were sequenced alongside biological samples as a necessary precaution to monitor cross-contamination of samples and carry-over from any potential equipment contamination. Syringes, filters, and filter holders used in eDNA sampling were used only once.

Thirty-nine MOTUs were present in negative control samples, 34 of which were present in the collection blank, and only one was able to be identified to phylum (Nemertea). Molecular operational taxonomic units (MOTUs) present in controls (Figure S3) were removed from the dataset prior to downstream analysis. The positive PCR control (n = 1) was spiked with 1 μL template DNA extracted from *Porites compressa* tissue (1.2 ng/μl).

2.7 | Bioinformatics

After sequencing, a total of 8.5 million demultiplexed reads (83,285 ± 6680 *SE* reads from ARMS extractions and eDNA samples) were

pre-processed by paired-end merging followed by primer, adapter, and quality trimming in Geneious Prime v2021.0.3 (Biomatters, Ltd.). Next, 3.2 million reads were then processed using the R modular package for metabarcoding bioinformatics: *Just Another Metabarcoding Pipeline* (JAMP—https://github.com/VascoElbrecht/JAMP), which integrates USEARCH v10.0.240 (Edgar, 2010), VSEARCH v2.4.3 (Rognes et al., 2016), and CutAdapt v1.9 (Martin, 2011). More specifically, sequence lengths were filtered (CutAdapt; min = 295, max = 340), and any remaining low-quality sequences were filtered (UPARSE; fastq_filter with maxee = 0.25 and qmax = 60) and discarded (Edgar & Flyvbjerg, 2015). A total of 2,910,394 million sequences were dereplicated (min. unique size = 2) and clustered with simultaneous *de novo* removal of chimeras using USEARCH (cluster_otus 97% identity). The remaining 2,826,190 million dereplicated reads of all samples, including singletons, were then matched to their respective clustered MOTUs with a minimum match of 97% (USEARCH; usearch_global and strand plus).

MOTUs were then translated into amino acids and aligned to the BIOCODE reference data set using Multiple Alignment of Coding Sequences (MACSE; Ranwez et al., 2011). MACSE detects interruptions in open reading frames from nucleotide substitutions that can result in stop codons which are likely to be pseudogenes. MOTUs that did not pass through MACSE (n = 15) were removed from the MOTU table and only MOTUs with a read abundance above 0.01% (MOTU sequences divided by the sample sequence sum) were considered in downstream analysis to reduce the number of false positives due to PCR and sequencing errors (Bista et al., 2017; Bokulich et al., 2013; Elbrecht et al., 2017).

The paucity of reference barcode data in public databases hinders the interpretation of metabarcoding data, with large numbers of MOTUs being classified as 'unidentified' (Leray & Knowlton, 2015). Therefore, we cross-checked MOTU sequence classifications against five reference databases to maximize taxonomic assignments for cryptic reef metazoans. First, we ran two local BLASTn searches, one against the DNA barcodes taken from a set of ARMS plates used in settlement mesocosms at HIMB, identified using a combination of 28S RNA and COI DNA barcoding and morphology (Timmers et al., 2020), and another against a curated reference database containing 16,679 COI sequences of coral reef fauna from the Moʻorea BioCode project (Meyer, 2017). Second, we classified sequences using the ecotag algorithm (Boyer et al., 2016), which uses a lowest common ancestor classification against a local COI database consisting of 192,929 sequences from GenBank and BOLD (Wangensteen et al., 2018). Third, we used the R package, Informatic Sequence Classification Trees (INSECT), which uses a hidden Markov model approach to assign sequences against a classification tree built from 396,413 sequences from the MIDORI database and GenBank (Wilkinson et al., 2018). Finally, we classified sequences against 1,054,973 COI-derived sequences (~500,000 of which are not in BOLD or Midori databases) from GenBank with CO-ARBitrator, which provides taxonomic classification based on sequence properties to reduce error rates (Heller et al., 2018).

Taxonomic assignments were only accepted if the approach matched at ≥85% identity, ≥85% coverage, and ≥200-bp alignment length (Ransome et al., 2017), due to the limited number of marine invertebrates within reference databases. Ecotag assignments were accepted if the "best identity" was ≥80% and INSECT full assignments set at a probability of ≥0.80 were accepted while anything less than a 0.80 probability was assigned to the phylum level. Annotated MOTUs were then examined across competing methods, and the final classification was based on the assignment which achieved the single greatest identity from the five databases. We accepted class, order, family, and genus annotations if the sequence identity was ≥90%, ≥92%, ≥96%, and ≥98%, respectively (Timmers et al., 2020; Yang et al., 2017). Only metazoan and macroalgal MOTUs were kept for downstream analysis, and sequences assigned to the classes Aves, Mammalia, and Insecta were removed from the dataset. While a handful of marine insect and DNA has been detected in reef fish gut contents (Brandl et al., 2020), most marine insect species are found on surface waters and mainly within mangrove swamps, estuaries, saltmarshes, and intertidal zones (Cheng, 1976). Thus, we removed MOTUs identified to the class Insecta because these MOTUs are most likely from contamination due to land-based processing of ARMS rather than from inside reef interstices.

2.8 | Statistical analyses

Data were analyzed using R v4.0.2 (R Development Core Team, 2011). The resulting MOTU table was rarefied to a mean sequence depth of 7,900 reads (rrarefy.perm, n = 100) using the EcolUtils package (Salazar, 2018) to control for the effects of library size estimates (Gotelli & Colwell, 2001; Weiss et al., 2017). We then examined community composition by phylum, class, order, and family based on the DNA read abundances and number of MOTUs assigned to each taxonomic rank. We ran a generalized linear model (GLM, family = quasipoisson) on DNA read abundances among phyla for each of the detection methods. A permutational analysis of variance (PERMANOVA, one-factor design, permutations = 9999) was performed first on all the metazoan and macroalgal MOTUs, second on only those MOTUs that were identified to order using the reference sequence databases, and third on MOTUs identified to order with scleractinians (corals) removed to compare only crevice-dwellers. The latter analysis limited the signal from the dominant mounding *Porites* corals which formed the majority of the reef structure at this site (Nichols & Marko, 2019 and references within) and does not form the structure of ARMS. Pairwise comparisons were calculated using the adonis.pair function in the EcolUtils package (Salazar, 2018). Non-metric multidimensional scaling (NMDS) was used to visualize community data from the three MOTU tables (all metazoans and macroalgae, all identified to order, and identified to order with corals removed) using the metaMDS (Hellinger standardization of Bray–Curtis dissimilarities) and envfit (permutations = 9999) functions of the

vegan package (Oksanen et al., 2013) to examine which taxa were correlated with communities among sampling methods. All graphs were produced using ggplot2 (Wickham, 2011).

3 | RESULTS

Across all sampling methods, the majority of DNA sequence reads were from metazoans and macroalgae (66.2%, Figure S4), with 25.2% of reads belonging to microbes and 8.6% that were unclassified. After removal of microbes and unclassified reads, the rarefaction curve of MOTU accumulation showed that our sequence depth adequately captured metazoan and macroalgal richness present in each sample (Figure S5). Among the retained 893 metazoan and macroalgal MOTUs, most DNA reads were assigned to arthropods (27.6%), followed by cnidarians (18.1%), annelids (15.9%), mollusks (11.9%), sponges (8.8%), red algae (8.4%), and chordates (7.2%). For the three eDNA sampling methods (soaking, ambient, and crevice), cnidarians were most abundant in crevice samples (*Porites* making up 82% of scleractinian DNA reads and 48% of total crevice reads), whereas arthropods were most abundant in ambient and soaking samples (Figure 2). The most abundant MOTUs were attributed to arthropods detected across all methods (biomass 35%, soaking 32%, ambient 23%, and crevice 19%) and sponges in crevices (27%). There were significant differences in DNA read abundances for cnidarians from crevices (GLM quasipoisson; p = 0.004, Figure 3, Table S1), arthropods from ambient and soaking (p = 0.042 and p = 0.047, respectively), and annelid worms and mollusks from biomass samples (p = 0.033 and p = 0.013, respectively).

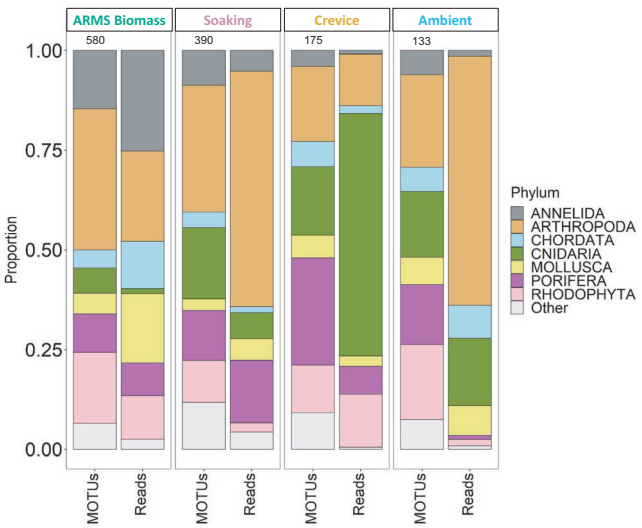

FIGURE 2 Proportional number of metazoan and macroalgal molecular operational taxonomic units (MOTUs, totals marked above each method) and DNA reads detected by each sampling method (biomass, soaking, crevice, and ambient) that were identified to the phylum level using the five reference sequence databases. Phyla that had <1.0% relative abundance (Echinodermata, Nemertea, Nematoda, Ochrophyta, Chlorophyta, Platyhelminthes, Gastrotricha, Xenacoelomorpha, Bryozoa, Rotifera, and Entoprocta) were grouped as "Other"

Across all four sampling methods used to survey reef cryptobiota, ARMS biomass detected the greatest percentage of total metazoan and macroalgal MOTUs (65%), with over a third of all 893 MOTUs (35.4%) being unique to this method. Upon closer examination of the MOTUs detected by ARMS biomass, unique DNA read abundances were mainly distributed across palaemonid shrimp, brittle stars, 15 families of annelid worms (14 within the class Polychaeta), and seven families of mollusks (five of which were calcifying, Figure S6). All but one pairwise comparison (biomass and soaking) shared fewer than 20 MOTUs (Figure 4), with only seven detected in all methods (Figure S7). The seven taxa shared across all samples included *Porites* (order Scleractinia, 64.1% relative DNA read abundance), *Dendostrea* spp. (order Ostreida, 17.4 ± 3.6%), and the following with minor contributions (<0.5% relative DNA read abundance), Acinetosporaceae (order Ectocarpales), hydroids (order Leptothecata), *Callyspongia* (order Haplosclerida), and anemones (order Actiniaria, Figure S7). Biomass and soaking shared the greatest number of MOTUs (180, 20.2%) and had similar DNA read abundances across phyla (GLM quasipoisson: χ^2 = 12.2, df = 12, p = 0.43, Figure S8).

The eDNA samples were much more variable, detecting high relative abundances of fewer taxonomic groups, such as planktonic copepods, corals, oysters, sponges, and red algae when compared to ARMS methods (Figure S9). Sampling the ambient water column recovered 64 unique MOTUs across ten phyla, but unique read abundances were predominantly from single occurrences of polychaete worms (order Terebellida), planktonic copepods, and reef fish (Figure S10). Targeting crevice eDNA detected 30 unique MOTUs with red algae from a single family (Rhodomelaceae) and sponges in greatest relative abundance (Figure S11).

Significant community compositional dissimilarities for identifiable MOTUs (Bray–Curtis) were detected by each sampling method for all metazoan and macroalgal MOTUs (PERMANOVA: k = 2, stress = 0.09, $F_{3,23}$ = 5.13, p < 0.001, Figure 5a), metazoan and macroalgal MOTUs that were identified at the order level (PERMANOVA: k = 3, stress = 0.07, $F_{3,23}$ = 10.4, p < 0.001, Figure 5b), and MOTUs identified to order with stony corals removed (PERMANOVA: k = 4, stress = 0.08, $F_{3,23}$ = 3.55, p < 0.001, Figure 5c). Community differences were driven by significant pairwise differences between all sampling methods at all levels tested (α = 0.05, Holm corrected, Tables S2–S4).

Crevice and ambient eDNA samples from the reef were dominated by stony corals (order Scleractinia, Figure 5b). Biomass and soaking of ARMS were distinct (from ambient and crevice eDNA methods) mainly due to the abundance of polychaete worms (order Phyllodocida and Capitellida), sponges (orders Dendroceratida, Haplosclerida), oysters (order Ostreoida), decapods (order Decapoda), scyphozoans (order Semaeostomeae), tunicates (order Enterogona), mollusks (order Littorinimorpha), and red algae (order Palmariales, Table S5). Using a subset of the data to omit the dominant coral taxa, crevice and ambient samples were instead driven by red algae (order Ceramiales) and sponges (order Homosclerophorida, Figure 5c, Table S6).

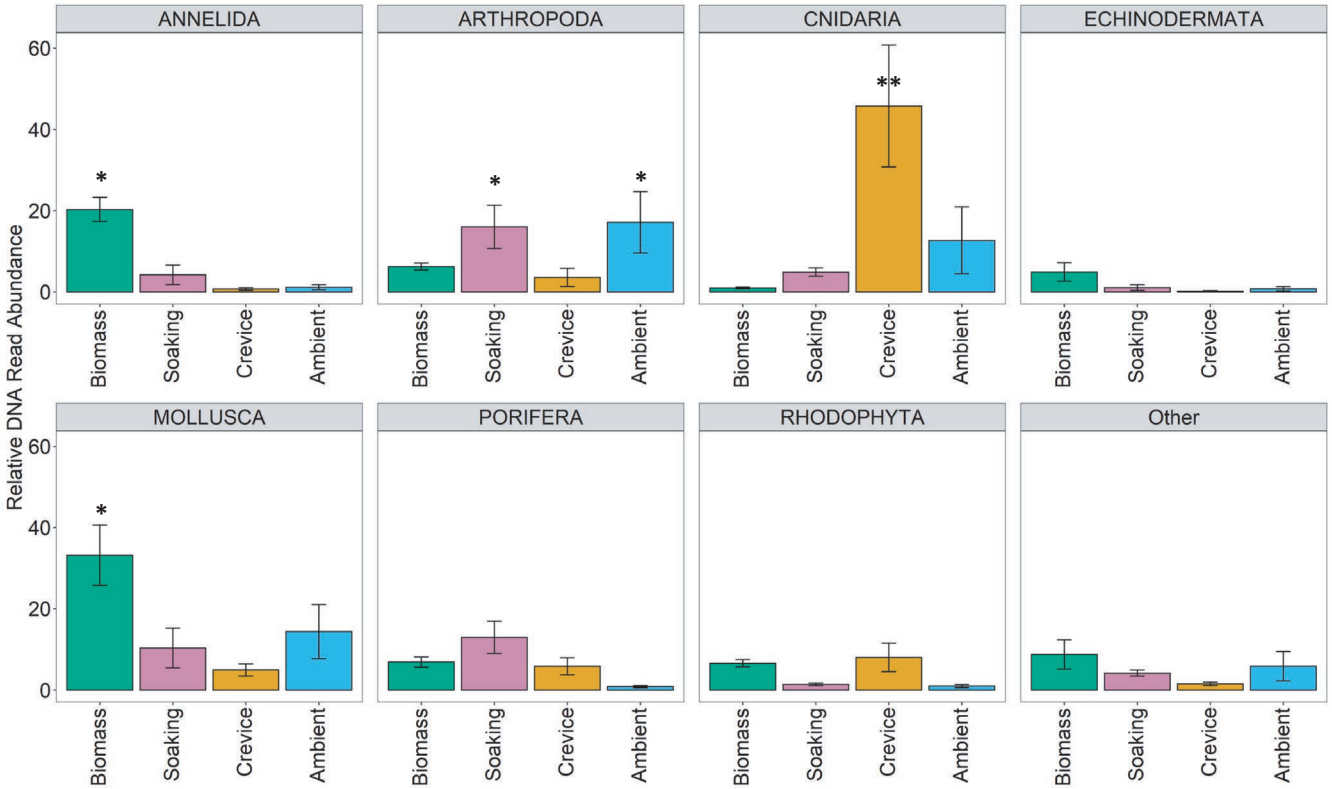

FIGURE 3 Relative DNA read abundances from phyla detected using the four methods (biomass, soaking, ambient, crevice). Phyla that had <1.0% relative abundance (Echinodermata, Nemertea, Nematoda, Ochrophyta, Chlorophyta, Platyhelminthes, Gastrotricha, Xenacoelomorpha, Bryozoa, Rotifera, and Entoprocta) were grouped as "Other". Significance codes from a generalized linear model (Phylum:Method): $p < 0.001$ = '***'; <0.01 = '**'; <0.05 = '*'

4 | DISCUSSION

Based on the data from our four survey methods, patterns of MOTU richness and abundance of DNA reads suggest that broad eDNA surveys from ambient seawater do not fully capture reef cryptobiome diversity. Ambient and crevice communities only shared 5% of total MOTUs and were significantly distinct from each other. Ambient eDNA samples also had some of the fewest shared MOTUs with cryptobiota from ARMS (Figure 4), partly due to dilution of cryptobiota eDNA and our limited sampling volume, which could be maximized to increase detection of rare taxa (Bessey et al., 2020; Turner et al., 2014). Nevertheless, the MOTUs detected by soaking ARMS indicate that chitinous and calcareous taxa will be undersampled using any eDNA method, as they may contribute less to the aqueous eDNA pool. Ambient eDNA was dominated by copepods and taxa that live close to the surface of the reef structure, mostly corals and other suspension feeders (Figure S9). While targeted sampling of reef cryptobiomes using eDNA methods may generally be more accessible, metabarcoding of ARMS biomass outperformed eDNA in several regards and adds to the existing literature that eDNA surveys overlook certain benthic taxa (Antich et al., 2020; Leduc et al., 2019), particularly chitinous and calcifying invertebrates. However, given the extensive time and resources needed to process the ARMS, incorporating multiple metabarcoding substrates (settlement plates, water column, crevices, sediments) is perhaps the optimal approach for broad detection of reef-associated cryptobiota (Koziol et al., 2019). Hence, soaking is likely inadequate as a standalone replacement for disassembling and metabarcoding of ARMS biomass.

Detecting taxa across the tree of life with eDNA depends on each taxon's relative contribution to the eDNA pool. While shedding rates vary among taxonomic groups, information on specific eDNA shedding rates is currently limited to a handful of species (Jo et al., 2019; Klymus et al., 2015; Sansom & Sassoubre, 2017; Sassoubre et al., 2016; Wood et al., 2020). By comparing metabarcoding ARMS biomass to eDNA, we aimed to quantify which cryptofaunal groups contribute the most to the eDNA pool, and conversely, which taxa are most likely missed. Among all four sampling methods, metabarcoding of ARMS biomass detected the greatest number of unique MOTUs, sharing the majority of MOTUs with the soaking of ARMS settlement plates. Soaking shared 42% of taxa detected by ARMS biomass, indicating that eDNA may miss the majority of cryptobiota, which may be an effect of soaking times. ARMS biomass taxa that were missed by the soaking method were chitinous arthropods (predominantly of the class Malacostraca), polychaetes, brittle stars, and calcifying mollusks (Figure S6), suggesting that the eDNA contribution is limited among these groups and may lead to underrepresentation in eDNA surveys of cryptic diversity. Unique MOTUs are expected across methods due to random sampling of rare taxa during DNA sequencing (Leray & Knowlton, 2017), but considering that biomass had such a large proportion of unique MOTUs from

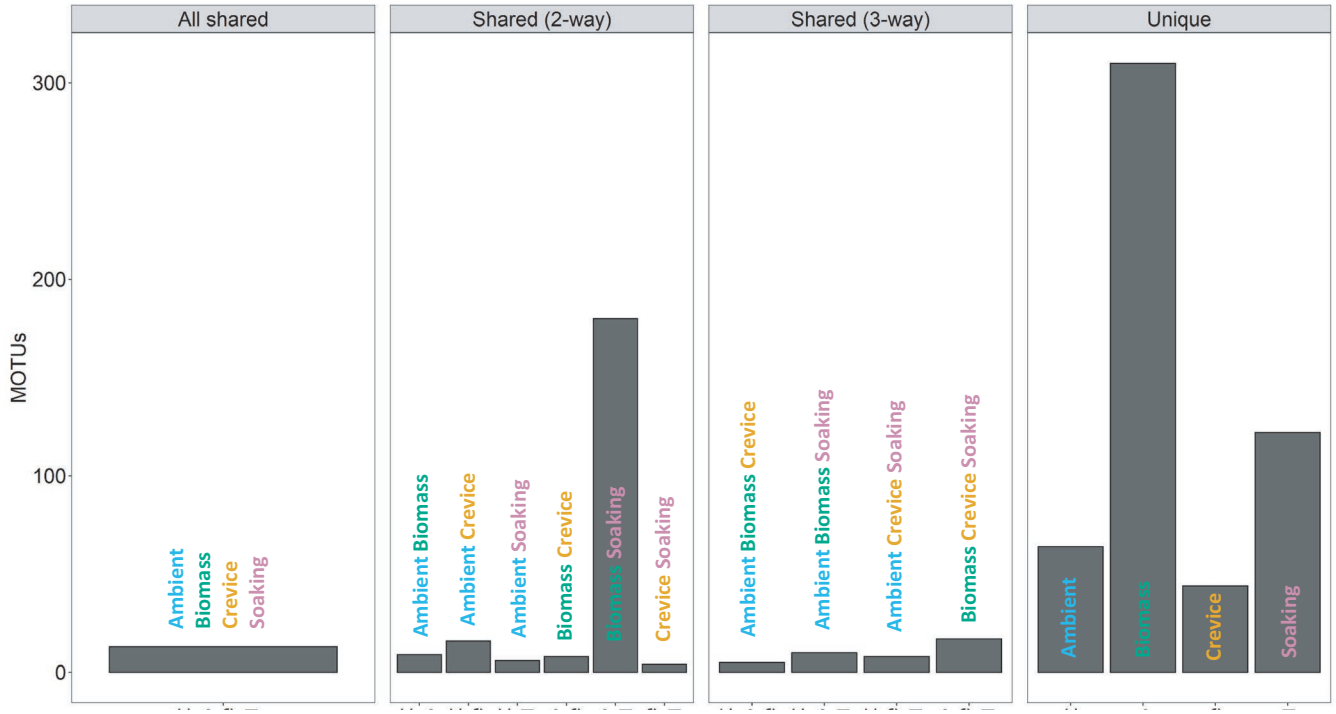

FIGURE 4 Total shared and unique metazoan and macroalgal molecular operational taxonomic units (MOTUs) detected by each sampling method (biomass, soaking, crevice, and ambient), identified using the five reference sequence databases

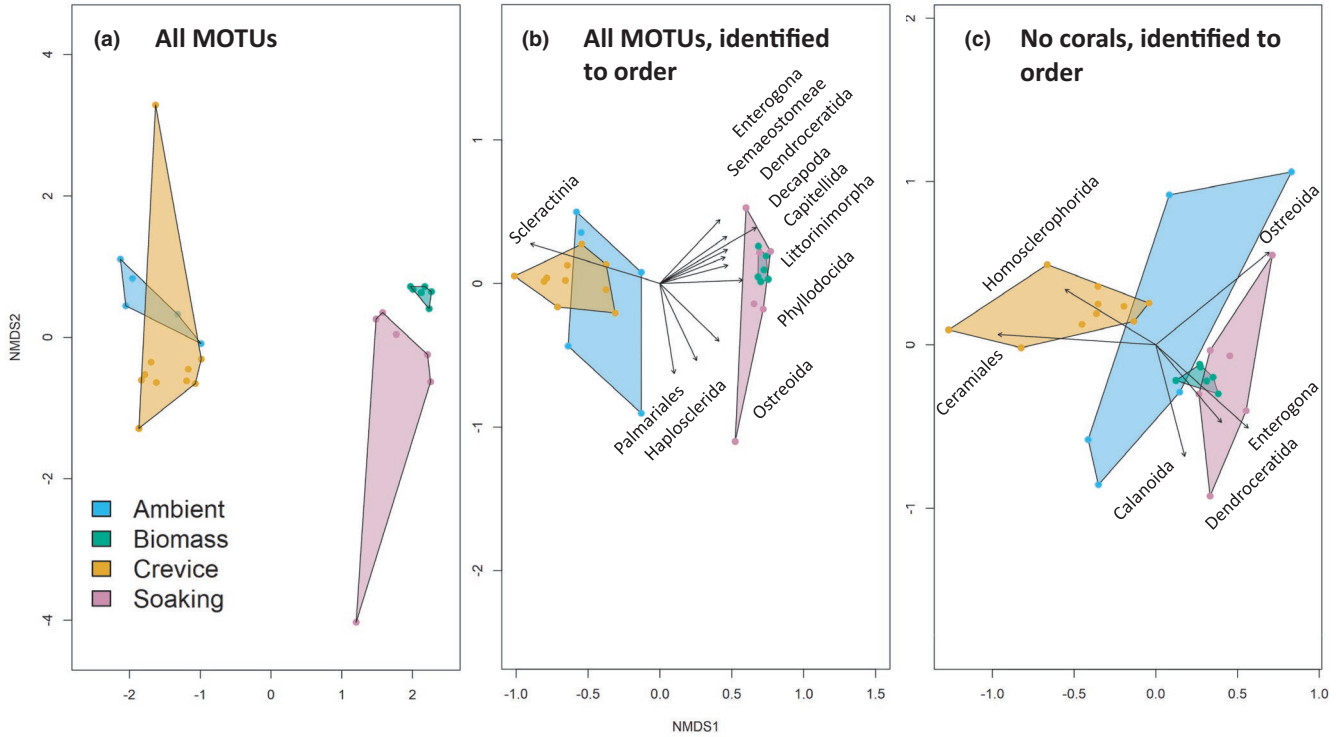

FIGURE 5 (a) Non-metric multidimensional scaling (NMDS, Bray–Curtis dissimilarity, relative abundance ≥0.01%) of all metazoan and macroalgal molecular operational taxonomic units (MOTUs) from cryptic habitat communities. (b) NMDS (Bray–Curtis dissimilarity, relative abundance ≥0.01%) of MOTUs identified to order level using the five reference sequence databases. (c) NMDS (Bray–Curtis dissimilarity, relative abundance ≥0.01%) of MOTUs identified to order level, but with order Scleractinia removed. The direction and length of vectors are proportional to the degree of correlation as determined by the envfit function (permutations = 9999, pmax = 0.05, Tables S4 and S5) in the vegan package (Oksanen et al., 2013). One ambient sample was dropped, as it did not contain any metazoan or macroalgal MOTUs

soaking indicates that its contribution to aqueous eDNA is not comparable among taxa.

As eDNA surveys are becoming increasingly commonplace, determining whether cryptic taxa are represented in seawater samples is an important consideration when evaluating marine biodiversity. By using a single universal marker (COI) for eukaryotes, our sampling from one coral reef detected 893 metazoan and macroalgal taxa, approximately 15% of all described Hawaiian marine species (Miller & Eldredge, 1996), nearly doubling previous estimates of subtropical eukaryotic coral reef diversity using the combined results from multiple metabarcoding assays (Stat et al., 2017). Although two or more MOTUs could belong to the same species, the large number of detected taxa is remarkably high, considering the stringent sequence filtering parameters, limited spatial distribution of samples, and relatively small volumes that were employed. While multiple primers are suggested for broad detection of taxa across the tree of life, eDNA estimates of biodiversity have been focused on what we designate here as "ambient" samples (Alexander et al., 2020; DiBattista et al., 2017, 2020; Stat et al., 2017). Ambient eDNA detected only 15% of MOTUs at our site, but by adding crevice samples, we were able to detect an additional 80 MOTUs (9%) that were not detected in ambient samples. While our sampling volumes were limited to match the volumes of reef crevices, larger volumes (either by pooling many crevices, e.g., Kelly et al., 2014) or through passive eDNA collection (Bessey et al., 2021) may narrow the differences between ARMS and eDNA sampling. In the absence of ARMS biomass, a combination of targeted eDNA approaches using varying sampling substrates and metabarcoding assays may be needed to uncover the greatest non-destructive estimates of coral reef biodiversity.

On the other hand, soaking of ARMS detected 116 unique MOTUs (Figure S12), which demonstrates a potential pitfall of all eDNA methods—the exact source of macrobial eDNA is unknown. For instance, extracellular DNA signals remain orders of magnitude longer in sediment than in the water column (Dell'Anno & Corinaldesi, 2004; Turner et al., 2015) and sediment accumulation on ARMS (Figure S1a) may have contributed to unique MOTUs detected by soaking, but not in the accumulated ARMS biomass. Biomass-rich specimens can also dominate read abundances thereby preventing smaller and less abundant taxa from being detected (Elbrecht et al., 2017). Thus, the unique MOTUs uncovered from the soak might also be a result of sequencing artifacts with the metabarcoding of ARMS biomass. Even if some unique MOTUs from the soaking method did not actually originate from organisms in the ARMS, the presence or absence of exogenous DNA has no bearing on the relatively low number of taxa shared by biomass and the soaking method.

Sampling from the water column and from soaking of ARMS clearly misses a substantial number of taxa that were directly detected on the ARMS, but this approach might not be accurately capturing the nearby cryptic reef communities given that the ARMS communities detected from both the biomass and soaking samples were significantly different from reef crevice sampling. Habitat type is a predominant driver of niche differentiation in coral reef invertebrate communities (Knowlton & Jackson, 1994) and differs depending on whether coral heads are alive or dead (Gibson et al., 2011), with degraded reef frameworks exhibiting greater diversity (Enochs & Manzello, 2012; Nelson et al., 2016). Larval settlement of sessile reef invertebrates can be greater on artificial substrates (Higgins et al., 2019), in which community composition is affected by the orientation and composition of plates (Siddik et al., 2019), presence of microtopographies (Whalan et al., 2015), and grazing pressure (Hixon & Brostoff, 1985). The organisms that settle on plastic settlement plates such as those that comprise an ARMS structure may not be representative of the dominant organisms inhabiting coral carbonate structures (Zimmerman & Martin, 2004), although similarities have been found in prior surveys using ARMS (Pennesi & Danovaro, 2017; Plaisance et al., 2011).

The significant differences between the crevice and soaking samples were driven largely by the amplification of corals, sponges, and red algae (order Ceramiales) in crevices and greater abundances of annelids and chitinous and calcifying taxa found on the ARMS. These differences are likely not merely due to differences in eDNA production, as crevice and soaking samples shared few taxa. The ARMS community difference could be due to settlement plates attracting more annelid larvae (Pinochet et al., 2020) or as a result of the short residence times (minutes) of water through reef crevices (Richter & Wunsch, 1999), which may flush low-abundance eDNA (compared to hours for soaking of confined ARMS). If that interpretation is correct, more intensive crevice sampling may detect greater annelid richness, which makes up a substantial proportion of reef cryptobiota (Enochs & Manzello, 2012; Milne & Griffiths, 2014).

The crevice community differences could be due to the proximity of *Porites compressa* colonies. Most DNA reads from crevice samples were from hard corals (Figure S9) which regularly shed mucus (Brown & Bythell, 2005), especially under the high sediment exposure within Kāneʻohe Bay (Bessell-Browne et al., 2017; Hunter & Evans, 1995). Similarly, the high sponge richness found in the crevice samples (Figure 2), particularly among those belonging to the order Homosclerophorida (Figure 5c), could be a result of sponge dominance within reef interstices (Jackson & Winston, 1982; Richter et al., 2001). Sponge contribution to eDNA signals may be inflated by high pumping rates of seawater (Weisz et al., 2008) and the continuous shedding of cells (Goeij et al., 2013; Rix et al., 2018). As a result, the eDNA collected from the cavities may be reflecting this biological phenomenon rather than capturing the overall community within the cavities. Regardless, even limited sample volumes from targeted eDNA sampling from within reef crevices show potential as a tool to increase diversity estimates of cryptobiota which are clearly not represented in ambient eDNA collections. Furthermore, as the number of taxa detected is highly affected by the volume of water filtered and sampling effort (Bessey et al., 2020; Grey et al., 2018), filtering larger volumes of water from crevices (see Kelly et al., 2014) may increase eDNA-based diversity estimates.

While understanding the contribution to eDNA across taxa is vital for implementing surveys of cryptic diversity on reefs, all metabarcoding assays are influenced by the patchiness of reference sequence databases. Species with inherent societal, scientific, or

governmental interest are taxonomically biased in diversity data (Troudet et al., 2017) and can lead to lacking, or poor-quality reference data for cryptobiota, which by definition are themselves underrepresented. Although we tried to maximize the number of assigned MOTUs by querying five different reference sequence databases, the efficacy of DNA metabarcoding relies heavily on having robust reference sequence databases and markers that amplify taxa across many phyla (Deagle et al., 2014; Leray & Knowlton, 2016). Differences in primer binding affinity across taxa, as well as biases associated with PCR amplification, can also result in inaccurate estimates of relative abundances (Elbrecht & Leese, 2015; Piñol et al., 2018). However, there are challenges and caveats to every survey technique attempting to measure and assess biological diversity (Magurran & McGill, 2011). As a result, it is more important to choose an appropriate method that targets the system being studied based on the question of interest. For capturing diversity estimates in cryptic habitats, metabarcoding of biomass has proven to be a powerful tool (Leray & Knowlton, 2016). For comparing biodiversity among habitats, eDNA surveys may still be the method of choice, especially in resource-limited programs.

With coral reefs in decline from numerous threats, incorporating surveys of cryptobiomes is vital for management and conservation initiatives (Knowlton et al., 2010) and will provide better insights into how reef-associated biodiversity will be impacted by environmental conditions associated with climate change and other disturbances (Baker et al., 2008; Descombes et al., 2015; Edmunds et al., 2014; Hughes et al., 2003, 2018; Przeslawski et al., 2008). Further emphasis must be placed on understanding patterns of biodiversity, especially for groups of organisms that are underrepresented in barcoding databases, yet are critical to ecosystem function. The widespread use of molecular techniques, such as DNA metabarcoding, has allowed comprehensive surveys of entire marine ecosystems with high sensitivity (Deiner et al., 2017; Leray & Knowlton, 2015, 2016; Stat et al., 2017), particularly among taxonomic groups that would otherwise be missed through traditional survey methods (Nichols & Marko, 2019; Pearman et al., 2016). In addition to its non-destructive nature, eDNA surveys are relatively simple and can be applied to resource-limited programs, or to increase the scope of existing initiatives. Careful consideration must be given to soaking times and sample volumes, while maximizing both would be ideal for future assays of benthic cryptobiome communities. We demonstrate that metabarcoding remains a comprehensive and thorough survey tool for the reef cryptobiome, but water column eDNA assays are not the most effective method in capturing cryptic biodiversity. Our results highlight the importance of direct species collections, with eDNA providing a complementary and more rapid approach for characterizing cryptic communities.

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CONFLICTS OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

MT, PN, and PM contributed to conceptualization; MT contributed to ARMS set-up; MT, PM, and PN contributed to field sampling; PN and MT contributed to laboratory work; MT and PN made formal analysis; PN prepared the draft; PM made funding acquisition and project administration. All authors contributed to writing, review, and editing and gave final approval for publication.

DATA AVAILABILITY STATEMENT

All R code and materials used in the analysis will be made available in some form to any researcher for purposes of reproducing or extending the analysis. Sequence data that support the findings of this study have been deposited to datadryad.org (https://doi.org/10.5061/dryad.9s4mw6mgf).

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SUPPORTING INFORMATION

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

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