INTRODUCTION

Determining the distribution of organisms is the foundation to many ecological studies and theories, but sampling frequency in space and time often limits our ability to measure broad-scale patterns. To overcome these limitations, ecologists strive to create more efficient methods to enhance sampling coverage. Using environmental DNA (eDNA) to detect macroorganisms within aquatic environments is a promising technique that could broaden the scale of ecological studies measuring the distribution of animals, as it is more cost effective...
than many traditional techniques (Civade et al., 2016; Yamamoto et al., 2017). This has contributed to rapid growth in the use of eDNA to detect the presence (DiBattista et al., 2019; Jeunen et al., 2019; Willerslev et al., 2003) and possibly estimate the relative abundance of organisms (Port et al., 2016; Thomsen et al., 2012).

Environmental DNA is the genetic material left by organisms in their environment. Depending on the goals of a study, environmental DNA can be extracted from many environmental matrices, including water or sediment. DNA can persist for months to millennia in sediment (Turner, Uy, & Everhart, 2015; Willerslev et al., 2014), so that extracting eDNA from sediment potentially offers an unprecedented potential to detect organisms in the ecosystem currently and in the past. The persistence of DNA in sediment results from adsorption to sediment particles (Deere et al., 1996; Lorenz & Wackernagel, 1987). However, sediment characteristics likely affect DNA adsorption (Ogram, Sayler, Gustin, & Lewis, 1988), which in turn can affect the extraction of eDNA. Moreover, the extracted solution of DNA may also include PCR inhibitors, such as humic substances present in silt and clay, which have been shown to inhibit amplification of DNA (Buxton et al., 2017; Lekang, Thompson, & Troedsson, 2015; Schrader, Schielke, Ellerbroek, & Johne, 2012). Thus, sediment characteristics could alter metabarcoding results and may limit the feasibility to compare communities detected by eDNA from different environments.

Studies have looked at how different sediments affect the quality and quantity of extracted DNA, as well as the diversity of organisms identified by metabarcoding. Buxton (Buxton et al., 2017) found that top soil, with high organic content, reduced detection of a species because of PCR inhibition, as compared to sand. Other studies have found that both sediment type and extraction protocol can affect results from eDNA. For example, the amount of extracted DNA was consistent among extraction protocols for sand but not clay, while the community from cloning was not affected by extraction protocol in sand but was affected in clay (Lekang et al., 2015). In addition, sediment type (soil or stream sediment) and commercial extraction kits used affected the quality and quantity of extracted DNA (Hermans, Buckley, & Lear, 2018). Thus, both extraction protocol and sediment type can affect results from eDNA, potentially hindering the use of sediment eDNA as a source of information for species richness across ecosystems.

Our goal was to provide robust procedures for using sediment eDNA to quantify diversity across marine habitats and more specifically to assess how results from eDNA of macroorganisms are affected by sediment type sampled from three tropical marine habitats, coral reefs, seagrass meadows, and mangrove forests. These habitats are important for eDNA studies as they have important ecological and economic roles (Barbier et al., 2011; Costanza et al., 1997), and together account for much of the biodiversity in tropical marine habitats (Gratwicke & Speight, 2005; Gray, 1997). Unlike former studies, we standardized the DNA within sediment as opposed to measuring the DNA within natural sediments, given that the amount of DNA may vary within and among habitats. Specifically, we conducted two experiments to achieve this goal: (a) measured the amount of extracellular DNA extracted from sediments that had DNA removed prior to a controlled DNA addition; (b) quantified the number of sequenced reads in sediments after we added an equal amount of DNA of a species not occurring naturally in the habitats. In addition, we also tested three extraction methods during the second experiment to see if extraction method could interact with sediment type to affect the amount of extracted DNA.

2 | MATERIALS AND METHODS

2.1 | Sample collection

At each sampling, five subsamples were taken at each location by inserting 10 ml modified plastic syringes, with the tips removed (21 mm diameter opening), 5 cm into the sediment. Subsamples were collected haphazardly within a 100 m² area, each separated by at least 2 m. The subsamples were kept on ice, returned to the laboratory, and the top 8 mm of the sediment from each subsample was collected by pushing the core with a plunger until the correct amount was above the syringe and “cut” with a sterile plastic knife. The five subsamples were pooled to make one sample (2.8 cm² of sediment), which was then kept in 50-ml falcon tubes at −80°C until DNA extraction. Nitrile gloves were worn during collection and processing to reduce contamination, and all sampling materials were sterilized before use by soaking them in 10% bleach for at least 30 min. Samples were collected from three different habitats: coral reefs, seagrass meadows, and mangrove forests. Mangrove sediment was collected from moist sediment just above the water level. Collecting samples within the coral reef and seagrass meadow necessitated snorkeling because subtidal sampling was not feasible from the boat because of damaging the habitat-forming species. Sampling protocol with syringe was chosen to minimize contamination from the snorkeler and all precautions were taken to reduce contamination, which included sampling from down current, and quickly collecting and capping the sample. In addition, steps were taken to reduce sample contamination including conducting extraction and pre- and post-PCR preparation in separate areas where surfaces and instruments were deeply decontaminated with bleach and UV light.

2.2 | DNA extraction

We used a commercial extraction method for extracting DNA from sediments with DNA removed and added two additional extraction methods for testing the extraction of novel DNA. All protocols targeted extracellular DNA. The first protocol (referred to here as MO BIO) utilized a phosphate buffer solution which was added to the sediment; then, this buffer was purified using the MO BIO PowerSoil DNA isolation kit (Taberlet et al., 2012) (MoBio Laboratories, Inc.). First, a phosphate buffer solution (1.97 g of Na₂HPO₄ and 14.7 g of Na₂HPO₄ per liter of sterile water) was added to sediment in a 50-ml falcon tube for a 1:1 volume ratio (Taberlet et al., 2012) and gently mixed for 30 min using a HulaMixer (Life Technologies). The mixed samples were then centrifuged at 10,000 g for 10 min, and
To remove existing DNA in the sediment samples, we autoclaved and bleached the samples before we added a known amount of DNA. First, 20 g of a sediment sample from each habitat was autoclaved at 121°C for 80 min. Three samples from each habitat (0.5 ml) were autoclaved at 121°C for 80 min. Three samples from each habitat (0.5 ml) were processed using a MO BIO PowerSoil DNA isolation kit skipping the cell lysis steps (skipped steps 1–12). The second extraction protocol was based on Lever et al. (2015) and now referred to as the Lever extraction. The published protocol was followed using the extracellular extraction with the carbonate removal treatment. This protocol was chosen because it has a carbonate removal step which is not present in the MO BIO protocol. The third protocol was a combination of the two protocols previously described, adding the extracted DNA from the MO BIO protocol back to the sediment (sediment was already treated with phosphate buffer for the MO BIO protocol) before conducting the Lever extraction. This protocol, which extracted DNA twice from the sediment, was accessed to see if combining both protocols improved DNA extraction. An extraction blank (nuclease-free water) for both extraction methods was included through all steps.

### 2.3 | Experiment using sediments with DNA removed

Sediment samples were collected from a seagrass meadow a coral reef and a mangrove forest along the central Saudi Arabian Red Sea coast (see Table 1 for details). Basic characteristics of the sediment were noted (preponderance of mud or sand), and organic and inorganic matter were measured from sediment (not used for the DNA extraction) by the loss-on-ignition method (Luczak, Janquin, & Kupka, 1997). Briefly, the loss-on-ignition method consisted of heating 3 g of the sediment to 375°C for 3 hr, and the lost mass was measured as organic material. The sediment was then heated to 800°C for 12 hr and the lost mass was measured as inorganic material.

To remove existing DNA in the sediment samples, we autoclaved and bleached the samples before we added a known amount of DNA. First, 20 g of a sediment sample from each habitat was autoclaved at 121°C for 80 min. Three samples from each habitat (0.5 ml) from the autoclaved sample were then put into a sterile 2-ml tube. Second, the sediment was washed with 0.5 ml of bleach two times. To remove the bleach, the sediment was then washed with nuclease-free water three times. A separate sample of sediment was then run through the same extraction steps as described for extracellular DNA and analyzed with a Qubit 2.0 fluorometer (Quant-IT dsDNA High Sensitivity Assay kit; Invitrogen) to ensure that the DNA concentration was below detection limit. In agreement with a past study (Otte et al., 2018), initial tests found that DNA was still present after autoclaving (tested with Qubit 2.0 fluorometer), so the bleach step was added. Ultrapure Salmon Sperm (Oncorhynchus keta; 0.5 ml) with a concentration of 100 ng/µl (Invitrogen, USA) was added to the DNA-free sediment samples and mixed for two hours in a HulaMixer. After mixing, tubes were centrifuged and the supernatant was separated into a different 2 ml tube. The DNA in both the sediment (0.5 ml) and supernatant (0.5 ml added to phosphate buffer) were extracted using the MO BIO protocol previously described and analyzed with Qubit 2.0 fluorometer to determine DNA concentrations.

### 2.4 | Sequencing experiment

Sediment samples were collected from three coral reefs: three seagrass meadows, and three mangrove forests along the Saudi Arabian coast of the Red Sea (Table 1). Sites were chosen to broaden the breadth of our findings with different sediment sources both within and among the different habitats. Salmon sperm DNA at a concentration of 10 ng/µl and volume of 1 ml was added per 1 ml of sediment sample. Three grams of sediment was mixed with the salmon DNA for 2 hr using a HulaMixer. The sediment was then divided into individual 1 g aliquots, and DNA was extracted from these aliquots using each of the three different extraction protocols.

For DNA amplification and sequencing, we used primers targeting a 130 base pairs region of the18S rRNA gene (F 5′-TTGTACACCACTCCGCRCTG -3′; R 5′-CCTTCYGAGATGACCATCTCC -3′) (Amaral-Zettler et al., 2009). The primer included an Illumina adapter (F 5′-TCGTCCAGGCACTTCAGATGTATAAGACAG;
R 5’-GTCTCGGTGTCGACGAGATCTTATAAGAGACGG-3’). This primer set was chosen because it is commonly used to detect eukaryotes and amplified salmon DNA based on initial PCR tests, with the goal of making our findings relevant to metabarcoding studies. PCR reactions were conducted using Amplitaq DNA Polymerase kit (Thermo Fisher), using 5 µl of buffer II, 0.2 µl of Taq polymerase, 1 µl of both 10 mM forward and reverse primer, 5 µl of sample, 7.8 µl of PCR grade water, and 1 µl of DNA. Thermal conditions for the PCR were 95°C for 5 min followed by 35 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s, which was followed by a final stage of 72°C for 2 min. Five replicate PCR assays for each sample were run and then pooled to decrease PCR bias. An extraction, a PCR and positive blank were included in all procedures. The positive blanks consisted of four sequentially diluted samples of salmon DNA (1 µl of 1.0, 0.1, 0.01, and 0.001 ng/µl) to measure if DNA concentration was related with the number of reads. PCR products were visualized using gel electrophoresis (1.5%) and cleaned by AMPure XP magnetic bead-based purification (Beckman Coulter) according to the MiSeq library preparation guide.

Dual indices and Illumina sequencing adapters from the Illumina Nextera XT Index Kits v2 (Illumina, Inc.) were added to the target amplicon in a second PCR step using Kapa HotStart HiFi 2× ReadyMix DNA polymerase (Kapa Biosystems Ltd.) following MiSeq library preparation guide (12.5 µl of polymerase, 2.5 µl of each index, 5 µl of sample, and 5 µl of PCR grade water; 95°C for 3 min followed by eight cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s, which was followed by a final stage of 72°C for 5 min). Libraries were again visualized by gel electrophoresis and purified using AMPure XP beads. DNA concentrations were quantified using TapeStation system (Agilent Technologies) following the manufacturers protocol and then pooled using equimolar amounts based on TapeStation results. If a sample had less than 15 ng of DNA, the entire sample was added to the pool. Finally, the library pool was purified by Wizard SV Gel and PCR Clean-Up System (Promega) to remove any remaining contaminants and PCR artifacts and quantified using KAPA SYBR FAST Universal qPCR kit with Illumina Primer Premix (Kapa Biosystems Ltd.) and the average DNA strand size was assessed using a Bioanalyzer (Agilent Technologies). Six pM of the 18S rRNA gene amplicon libraries were sequenced on one lane of the Illumina MiSeq platform with 25% PhiX control at the KAUST sequencing facility. The libraries were sequenced using 2 × 300 bp overlapping paired-end reads following the MiSeq library preparation guide.

2.5 | Sequences analysis

The DADA2 workflow was used for sequence analysis to model and correct substitution errors, and filter and cluster the amplicons (Callahan et al., 2016). The DADA pipeline was more accurate and resulted in fewer spurious reads than other commonly used pipelines based on clustering similar reads (Callahan et al., 2016), and unique reads are called sequence variants (SV). The entire workflow was conducted in R version 3.5.1 (R Development Core Team, 2012) using the DADA2 package (Callahan et al., 2016), except for primer removal. First, the primers were removed from the sequences using CUTADAPT allowing one error for every 10 base pairs in the primer sequence (Martin, 2011). During the DADA2 pipeline, the forward and reverse reads were trimmed to 115 and 95 bp based on visual inspection of the error rates using the fastqPairedFilter function with two expected errors per read. The reads were then de-replicated (derepfastq function), and the SVs were determined using the dada function based on the reads from all samples. Paired reads that matched perfectly were merged (mergePairs function), and chimera reads were removed using the removeBimeraDenovo function. Taxonomy was assigned to the SVs using the RDP classifier (Lan, Wang, Cole, & Rosen, 2012; Wang, Garrity, Tiedje, & Cole, 2007), which implements a naïve Bayesian classifier method to assign taxonomic classification to each SV at successively broader taxonomic levels. The assignTaxonomy function was used to assign taxonomy with the default values, except for minboot which was set at a more conservative value of 70 instead of the default of 50. A reference library was created from the SILVA database (version 132 SSU NR99), and sequences were filtered based on in silico amplification using virtualPCR function from the insect package (Wilkinson, Stat, Bunce, & Davy, 2018). Code used is available for both the pipeline (https://github.com/ngeraldi/dada2-and-insect-pipeline) and creation of the reference library (https://github.com/ngeraldi/Training_datasets).

Additional filters were used to reduce the likelihood of false positives. SVs were removed if they were present in less than two samples to reduce PCR and sequencing errors. To remove errors from contamination and sequencing, SVs were removed if the maximum number of reads from any control (extraction and PCR blanks) was greater than 0.1% of the sum of the number of reads for the SV for all samples, or if the total number of reads was less than 0.1% of the total reads in samples. This balanced the false positives from contamination in the laboratory and sequencing, but allowed a small amount of cross-contamination to reduce false negatives. After filtering, the number reads per sample were rarefied to the smallest number of reads within a sample to standardize the number of reads among samples. The salmon DNA was identified as Oncorhynchus mykiss (>90% of the Salmonidae Family), and we included the reads for all SVs identified as Salmonidae Family. After filtering, the majority of reads in samples was salmon and analysis of the other eukaryotes identified was not conducted because this community was likely not representative of the natural community given the low read counts.

For statistical analysis of the first extraction test using sediment with the DNA removed, the amount of extracted DNA from the three different habitats was tested for heterogeneity with the Levene’s test and for differences among the means with a linear model (LM). For the second extraction test, the difference in organic and inorganic matter was tested with a LM. The difference in salmon reads among sediment from the three habitats and extraction protocol was tested with a 2-way LM with the fixed factors and the interaction between them. Significant differences among the levels within factors were determined from nonoverlapping standard
errors from the model output. Summary statistics were calculated using the ANOVA function from the car package (Fox & Weisberg, 2011) for all LMs.

3 | RESULTS

3.1 | Experiment using sediments with DNA removed

The seagrass sediments were muddy, and the mangrove sediments were primarily mud with some sand, while the coral sediment was almost exclusively coarse carbonate sand. The organic content of the sediment was 2.3% dry weight (DW) from the coral reef and the seagrass meadow, and 1.2% DW from the mangrove forest. The inorganic content of sediment, largely composed of carbonate minerals, was 45% DW from the coral reef, 43% DW from the seagrass meadow, and 25% DW from the mangrove forest. The amount of DNA extracted from the different habitats had homogeneous variance (Levene’s Test, $F_{2,6} = 2.874$, $p = .133$). The extraction of extracellular DNA did not differ among sediment sources (ANOVA, $F_{2,9} = 0.710$, $p = .529$). The amount of DNA recovered from the sediment was $10,300 \pm 363$ ng. A similar amount of DNA was extracted from the liquid surrounding the sediment, $10,181 \pm 746$ ng.

3.2 | Sequencing experiment

For the sequencing experiment, samples were collected throughout the Red Sea, with both coral reef and seagrass meadow sediments being dominated by carbonate sand while the mangrove sediments were primarily mud with some carbonate sand. The organic carbon did not differ among habitats, $2.3 \pm 0.3$% DW organic carbon (ANOVA, $F_{2,6} = 0.094$, $p = .912$), while the inorganic carbon was lower in the mangrove forest ($19.0 \pm 3.2$) compared to the other two habitats ($44.0 \pm 1.1$; ANOVA, $F_{2,6} = 46.51$, $p < .001$). After filtering, the raw number of reads and rarefied reads had a similar pattern (Figure 1). Samples had $2006 \pm 163$ salmon reads with a range of 44 to 3,004 reads. The number of salmon reads in positive blanks did directly rank with the concentration of salmon DNA ($3,096, 2,941, 1,817$, and $1,405$ reads for samples of $1.0, 0.1, 0.01$, and $0.001$ ng/$\mu$L, respectively; Figure 2b). Habitat did not affect the number of salmon reads (ANOVA, $F_{2,18} = 1.641$, $p = .221$), but the extraction method did affect the number of salmon reads (ANOVA, $F_{2,18} = 5.057$, $p = .018$; Figure 2b), with the combination protocol having fewer salmon reads than the MO BIO protocol. There was no interaction between the two fixed factors (ANOVA, $F_{4,18} = 0.504$, $p = .733$).

4 | DISCUSSION

Using eDNA to monitor the presence of animals is an emerging technique that can enhance our understanding of the distribution of organisms, particularly when using DNA in marine sediments, that contain a fingerprint of animals in the ecosystems integrated over

![FIGURE 1](image.png) The amount of salmon extracellular DNA extracted from sediment (MO BIO protocol) after being treated to remove existing DNA and adding 5 µl of salmon DNA. There were no differences in variances (Levene’s Test, $p = .133$) or means (ANOVA, $p = .529$) among the sediment collected from different habitats. Boxplots show the median with the upper and lower quartiles, while the whiskers extend to the extreme data point but no more than 1.5 times the respective quartile. The mean is indicated by the circle.
FIGURE 2  The number of reads (a) and reads after rarefaction (b) of salmon sequenced from DNA that was extracted using 3 different protocols from sediment from three different habitats. Boxplots show the median with the upper and lower quartiles, while the whiskers extend to the extreme data point but no more than 1.5 times the respective quartile. The mean is indicated by the circle. Extraction and PCR blanks are shown, as well as positive blanks with four different concentrations of salmon DNA (0.001, 0.01, 0.1, and 1.0 ng/µl) as shown by size of asterisks.

humic substances present in organic-rich sediment (Buxton et al., 2017). We found that there was no difference in the number of salmon reads between the Lever extraction and the other two methods, but that the MO BIO extraction had more salmon reads than the combination extraction. In the study that developed the extraction protocol, the Lever extraction had greater DNA yields than the MO BIO when tested on different sediment types, which was attributed to high PO₄ concentrations and making the pH > 9 during initial protocol steps (Lever et al., 2015). In this study, the reduced reads in the combination protocol likely resulted from the samples being over-processed and losing DNA with the additional steps resulting in a reduced DNA yield as opposed to the extra processing resulting in more purified DNA.

Our experiments were designed to build upon previous experiments that compared DNA extracted from sediments with naturally occurring DNA which varies among and within types of sediment. Our findings are based on equal DNA additions to determine the effect of sediment type and extraction on DNA yield. Although this gives us more insight into how sediment type affects DNA extraction, it may differ from naturally occurring eDNA. We simulated DNA addition to the sediment by adding salmon sperm DNA to sediment and mixing it, but this may differ from how DNA is added and adsorbed to the sediment in nature. For example, the exogenous DNA spike may not have been absorbed to the sediment, perhaps because it was added at too high of a concentration, resulting in equal amounts of extracted DNA. Future studies could add a known amount of exogenous DNA to sediment in natural conditions. However, such a study would not be able to isolate the effect of sediment from habitat-specific DNA degradation. A second consideration with regard to comparing eDNA in different habitats is that in most metabarcoding experiments, equimolar samples are used for sequencing and the number of reads per sample is standardized (rarefaction), which likely minimize potential eDNA artifacts resulting from differences among habitats. However, eDNA studies do have a detection limit, such as a minimum number of reads at which a taxa is determined to be present and not just type II error. Thus, determining how habitat-specific characteristics affect DNA extraction and subsequent metabarcoding results become more important as species become less abundant. We suggest future studies add sequentially lower concentrations of the exogenous DNA spike to sediment, in addition to testing different habitats to improve our understanding of whether sediment types may affect eDNA detection.

Detecting organisms and assessing diversity within different habitats, as well as measuring species overlap among habitats, is a key aspect of ecology. Yet, the majority of survey techniques do not have perfect detection. Findings from sampling methods can find different taxa assemblages among habitats because of survey artifacts and not actual differences in the communities. For example, visual surveys quantified the greatest lobster abundance on structurally complex habitat while traps measured the greatest lobster abundance on structure-less substrate (Geraldi, Wahle, & Dunnington, 2009). In addition, some fish species were not consistently detected when comparing surveys by visual census, traps, and video camera, which may impart be from biases associated with habitat characteristics (Bacheler et al., 2017). There is a clear need to develop and test survey techniques that have consistent species detections among different locations regardless of abiotic and biotic factors. This is particularly pertinent in complex marine habitats, such as coral reefs, mangrove forests, and seagrass meadows, which support high biodiversity including many cryptic species that take shelter within the structure. Our findings suggest that eDNA offers a new tool that could improve ecologists’ ability to quantify and compare biodiversity among marine habitats.

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CONFLICT OF INTEREST

None declared.

DATA AVAILABILITY STATEMENT

Sequences from this study have been deposited at NCBI's SRA under project no. PRJNA579138 (https://www.ncbi.nlm.nih.gov/sra/PRJNA579138).

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