Estuarine, Coastal and Shelf Science 283 (2023) 108283

Contents lists available at ScienceDirect

Estuarine, Coastal and Shelf Science

journal homepage: www.elsevier.com/locate/ecss

Comparing environmental DNA collection methods for sampling community composition on marine infrastructure

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ARTICLE INFO

Keywords:
Epibenthic sampling
eDNA sampling
Diversity detection
Biofoul
Water sampling

ABSTRACT

Broad scale monitoring of marine diversity is challenging, with many techniques limited to sampling only a small portion of the actual diversity present. For this reason, molecular methods, such as environmental DNA (eDNA) metabarcoding, are becoming increasingly popular, especially in locations that are logistically difficult to sample (for example, ports, offshore platforms and other restricted marine infrastructure). eDNA studies in marine environment have predominantly focused on the collection and isolation of DNA from water. Recent literature suggests this approach may not be effective for detecting taxa from adjacent epibenthic substrates. In this study we compare a visual, morphological approach utilizing three eDNA sampling methods targeting the water column and four methods targeting the epibenthic substrate: three methods scraping and one swabbing the epibenthos. Sampling was completed at two depths on and adjacent to a decommissioned jetty, with all methods detecting significant community compositions. Only 2.8% of family-level taxonomic detections were found across all eDNA sampling methods, and all but one scraping method were able to detect fine scale community shifts associated with depth. The epibenthic sampling methods ranged from 50 to 117 families detected, with those methods that collected bulk DNA material (all scraping methods) detecting considerably lower diversity. The methods targeting the water column detected between 78 and 154 families, with the polyurethane foam (PUF) tow method detecting the highest number of families, indicating that the physical matrix may be better at retaining traces of DNA within the water column. While further validation is required, this study provides a base toolkit for the broad characterisation of vertical diversity at both natural and man-made marine structures such as oil and gas platforms. Additionally, these highly varied results demonstrate the importance of appropriate substrate selection to sample for a given study objective and indicates that multiple sampling methods may be required to holistically characterise diversity across a chosen environment using eDNA.

1. Introduction

Environmental DNA samples are environmental substrate collections from which the genomic DNA of numerous biotic taxa can be extracted and sequenced using next generation sequencing technology to identify taxa within a target community at the time of sampling (Taberlet et al., 2012; Thomsen and Willerslev, 2015; Forsström and Vasemägi, 2016). Within the marine environment, environmental DNA (hereafter eDNA) metabarcoding has proven effective for monitoring diversity broadly across target groups, or entire tree of life detections (Stat et al., 2019; West et al., 2021), the detection of invasive marine species (Borrell et al., 2017; Cowart et al., 2018; Xia et al., 2018), as well as cryptic or naturally rare species that are not detected using visual survey methods (Nester et al., 2020; Bonfil et al., 2021; Xia et al., 2021). This molecular approach, as either as a stand-alone or complementary to visual surveys (Alexander et al., 2019, 2022), has advantages over visual marine

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https://doi.org/10.1016/j.ecss.2023.108283
Received 3 November 2022; Received in revised form 1 February 2023; Accepted 25 February 2023
Available online 28 February 2023
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surveys (such as SCUBA, diver operated video (DOV) or remotely operated video (ROV) transects, and baited remote underwater video (BRUV) sampling) in that there is a reduced requirement for taxonomic expertise that is often limiting and expensive, especially when the study focus is broad and would require numerous taxonomic experts (Goldstein and DeSalle, 2010; Loos and Nijland, 2020). This non-invasive technique removes the logistical limitations such as the need for specialised field equipment and safety protocols inherent in SCUBA based methods, is less constrained by weather (Gold et al., 2021), and sampling and processing is comparatively cost and labour effective (Pereira et al., 2021).

The default standard in marine eDNA sampling has been filtered water replicates from the water column (Stat et al., 2017), which have relied on proximity to detect diversity from adjacent surfaces (Alexander et al., 2019; Gold et al., 2021). Some studies have focused on sediment (Pawlowski et al., 2022) and benthic substrates by deploying settlement plates (Nichols et al., 2022). However, as with all biodiversity sampling methods, eDNA metabarcoding has documented biases and limitations (Pompanon et al., 2012; Fonseca, 2018; Jo et al., 2019). The sampling method, substrate and assay selection can all drastically influence eDNA results (Wegleitner et al., 2015; Koziol et al., 2018; Sakata et al., 2020), meaning that an *a priori* knowledge of the target assemblage, habitat, and depth should drive experimental design. Recent studies have demonstrated that single collection methods are not accurately depicting diversity from all substrates, such as benthic or epibenthic surfaces (Koziol et al., 2018; Antich et al., 2021; West et al., 2022) and that epibenthic studies show a higher portion of taxa that are underrepresented in the water column (Gaither et al., 2022). Therefore, in studies where the target taxa is epibenthic, such as invasive marine species that are primarily benthic (Glasby et al., 2007), or in broad tree of life censusing studies, low volume water filtration of the adjacent water column may not be ideal.

Additionally, eDNA metabarcoding has a high sensitivity to low abundance DNA (Beng and Corlett, 2020). This can promote detection of valid yet low-abundance species, however it can also result in a higher risk of contamination throughout the field and laboratory workflows. With focus on sample collection and processing, filtering water eDNA samples is often completed *in-situ* in temporary field laboratories under sub-optimal conditions compared to dedicated clean laboratories, increasing the risk of cross and erroneous DNA contamination. Contamination can be partially mitigated by the use of controls and by employing stringent filtering parameters during the bioinformatic workflow (Murray et al., 2015). However, it can be difficult to determine the efficacy of removing all contamination, such as aerosolised DNA contamination, which may not present in controls. The time required to filter water samples can also vary considerably depending on the water quality (such as turbidity, salinity, and the level of organic compounds present) and can be limiting in studies where the sampling timeframe is short, such as when hiring vessels or equipment or sampling near operational infrastructure.

There is a need for further development of these techniques and the robust testing and comparison of field based eDNA collection methods that can not only better target species, particularly in habitats that are logistically difficult to access, but also to reduce the potential for contamination by removing *in-situ* processing steps. To date, eDNA comparison studies have focused on comparing morphological methods to eDNA methods that target single substrates in a proof-of-concept approach. However, few studies have rigorously explored side-by-side comparisons of eDNA methods targeting the same substrates to determine the optimal field methodologies to maximise results.

This study explores and compares seven methods of eDNA collection at a single location, three methods targeting the water column (filtered water, plankton net tow and polyurethane foam (PUF) tow), and four targeting established vertical epibenthic communities on marine infrastructure (ROV scrape, manual dive scrape, Keel Crab scrape, and epibenthic swab). In addition, a method of visual, morphological, or epibenthic taxa identification is utilised as a baseline. The methods were conceptualised and designed to utilise readily available equipment and technology and, where possible, reduce unnecessary field sampling steps and to have the potential for further validation and streamlining to reduce field-based contamination. This study aims to explore the broader, family level detection capabilities inherent in each collection method and determine the impact that substrate and method has on sampling efficiency and results. The primary objective in this study is to determine if all methods targeting each substrate return a comparable diversity, while developing an efficient method for detecting established epibenthic diversity. Finally, we explore the ability of each method to determine the fine scale vertical spatial differentiation, as this is an important consideration when selecting a sampling method.

2. Methods

2.1. Study area

The decommissioned Kwinana Bulk Jetty (hereafter KBJ) is located approximately 29.7 km south of Perth, Western Australia (Fig. 1). Constructed in 1956, it is now a popular location for recreational SCUBA diving due to the extensive recruitment of flora, fauna, and a diverse fish community present around the pylons, which was the primary reason for the selection of this site in the current study. Secondly, this site was selected due to the close proximity to dedicated clean laboratories at Curtin University for sample processing. The sampling of 15 consecutive pylons was completed from a boat starting approximately 40 m from shore, along the north-facing side of the jetty to minimise light variability effects (Fig. 1). All sampling occurred between the 20th and October 23, 2020. Two depths were sampled on each pylon (0 m, or surface, and 8 m) to ascertain whether the methods could detect visually obvious differences in the assemblage composition. All manual sampling was completed by snorkelling (surface) and SCUBA (8 m) methods.

2.2. Field sampling

2.2.1. Visual/morphological sample collection (quadrats)

Thirty 10 × 10 cm photo quadrats were produced using stainless steel wire and temporarily affixed to each pylon using elastic straps. Each quadrat was used just once to limit the movement of eDNA between sampling points. Quadrats were pre-labelled and bulk sterilised in ultra violet (UV) for 15 min and stored in a Ziplock bag until sampling. These quadrats also formed the base collection point for quadrat swabs and dive scrapes. Quadrat photos were analysed manually by a local benthic expertise (see supplementary Section S1 for photographs). Only specimens able to be definitively assigned to family level were retained for analysis, so as to align with eDNA sampling methods analysis. Photographs were taken using an Olympus TG-6 camera in an underwater housing.

2.2.2. Water samples

A total of 30 1 L water samples were collected, which included 15 at surface and 15 at 8 m, and were collected prior to any SCUBA sampling. Surface water samples were collected by submerging the 1 L water bottle adjacent to the target pylon, while 8 m water samples were collected using a 1.7 L Niskin water sampler and subsampled into sterile 1 L jars on the surface.

Unless stated otherwise, the sterilisation of eDNA field collection equipment was completed using a 10% bleach solution for 15 min, where equipment was then air-dried before being exposed to UV radiation for a further 15 min. *In-situ* field sterilisation of equipment between samples was completed using only the 10% bleach. All Ziplock bags and falcon tubes used were pre-labelled with unique identifier codes to reduce errors during sampling.

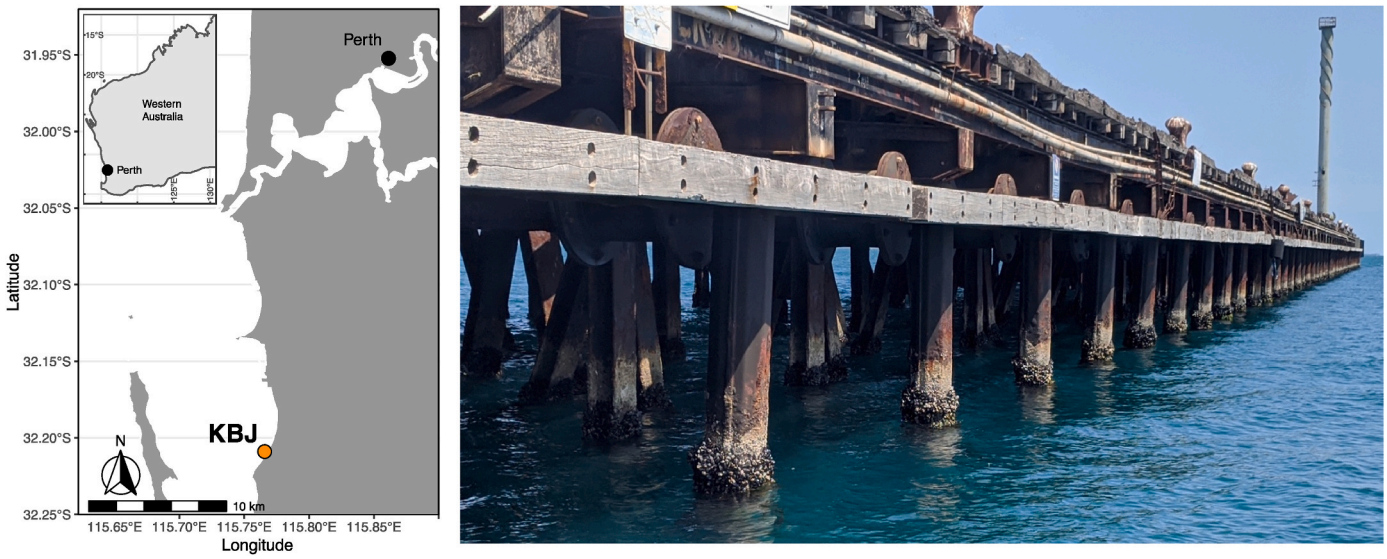

Fig. 1. Locality (left) of the KBJ in relation to the Perth central business district, Western Australia (Left inset). Sample collection included eight methods of data collection on the north-facing surface of 15 pylons (right), at 0 m and 8 m depth below water surface between the 20th and 23rd of October 2020.

2.2.3. PUF tows

Each PUF tow was completed using two positively charged cylindrical polyurethane foam units at each sampling site that were approximately 76 mm long x 38 mm in diameter (Tisch Environmental; USA; supplementary section S2). These units, which are primarily produced as vapor collection substrates, were sterilised using an industrial autoclave set to 121 °C for 30 min prior to sampling and placed into compressed (for ease during sampling) Ziplock bags and exposed to UV light for a minimum of 15 min. To sample, both PUFs were placed within a sampling unit comprising of an open funnel aperture, with a mouth diameter of 146 mm, narrowing to 35 mm where the PUFs are placed behind a course grate (approximately 3 mm grid size) to stop large particulates but not impede water flow (see supplementary section S2). This sampling method was completed concurrently with the plankton tows (see below for sampling details).

2.2.4. Plankton tows

Plankton nets were custom made from 50 μm nylon mesh. The tapered nets (see supplementary section S2) were 385 mm long and designed to fit over a 120 mm custom steel tow funnel. The nets were sterilised inside 50 mL falcon tubes, which were then filled with Milli-Q water to provide negative buoyancy while diving.

The plankton and PUF tows were completed by manually swimming the custom frame around the target pylon with a transect length approximately 5 m. For safety logistics while diving, and to reduce contamination variables, PUF and plankton nets for 8 m samples were changed in-situ while diving. For both methods, the sampling media (PUF and net) were placed back in the original vessel (50 mL falcon tube or Ziplock bag).

2.2.5. Quadrat swabs

Swab samples were collected using a 17 × 12.5 cm section of nylon material. Prior to sampling, the material was sterilised and sealed in Ziplock bags. In-situ, swab samples were removed from the Ziplock bag and wiped vigorously against the substrate within the quadrat against the pylon before being replaced back in the sealed Ziplock for storage.

2.2.6. ROV scrapes

ROV scrapes were completed using a standard SRV-8 ROV from RJE Oceanbotics (California, USA), attached by a 300 m tether (see supplementary section S2). Samples were collected using a prototype cylindrical, serrated scraper on the forward ROV arm with an aperture of 30 mm. A sterile nylon mesh catch bag was attached to the base of the cylindrical scraper using a cable tie. During sampling, the ROV was operated from the boat, with the scraper serrations of the scraper used to remove biological material, with the concept that material would release and be collected by the nylon mesh bag. After each scrape, samples were returned to the boat where the nylon bag was removed and placed back into the Ziplock bag. These scrapers were sterilised between samples.

2.2.7. Keel Crab scrape

A Keel Crab underwater drone (Keel Crab; Italy) was repurposed to collect eDNA samples from both sampling depths at each pylon. These drones are primarily designed for cleaning and maintenance of boat hulls and suction to the surface using a vacuum (https://keelcrab.com; see supplementary section S2). The surfaces are cleaned using a series of replaceable brushes to loosen biofoul material, this was then vacuumed through a nylon 34 × 34 cm mesh bag affixed to a metal frame using Velcro and elastic. This mesh bag acted to collect all large organic and particulate matter, which in this study was collected as our eDNA sample.

Sampling was completed using a standard Keel Crab unit, with a 50 m cable, standard 180 μm nylon collection bag, and hard medium nylon brushes. Prior to sampling, collection bags were sterilised and stored in individual Ziplock bags. All Keel Crab replaceable brushes were sterilised between collections. Sampling was completed by boat adjacent to each pylon over a 5 h period.

2.2.8. Dive scrape

Dive scrapes were collected within each quadrat using a sterilised 5 cm wide, sterilised, metal paint scraper. Collected material was scraped directly into large Ziplock bags.

All of the eDNA samples taken across all methods were stored on ice and, on return to dedicated clean laboratories, at −20 °C until further processing and extraction.

2.3. DNA digestion and extraction

Sample processing, digestion and extraction protocols were completed in a dedicated clean lab wearing nitrile gloves to help prevent cross contamination. All equipment used in processing of samples and pre-digestion steps, as outlined in Table 1, were sterilised in 10% bleach solution and UV radiation, both for a minimum of 15 min. DNA digestion

Table 1
Summary of the pre-digestion steps completed, equipment used and equipment settings for each eDNA collection method, including sample digest conditions. After digestions, the workflow for each method was the same.

Method	Pre-digestion steps		ATL/ Proteinase K vol (μL)	Digestion
	• Equipment (where relevant)	• Setting		
Dive Scrape	A - Sample lysed • PM100 Planetary Ball Mill (Retsch; Germany); •stainless steel grinding jar; seven 20 mm stainless steel balls. B - 120 mg of centrifuged lysate digested	• 250 rpm; • 1 min reversing intervals; • 4–12 min total.	900/100	All samples digested in rotation at 56 °C
Keel Crab and ROV scrape	A - Sample lysed • TissueLyser (Qiagen; Netherlands) B - 120 mg of centrifuged lysate digested	• 30 Hz setting in 30 s intervals; • 90–180 s	900/100	
Swab	A - Half swab dissected per sample and digested		540/60	
Water	A - Samples filtered. • Pall microbiology pump; • 47 mm, 0.22 μm polyethersulfone filters (Pall Life Sciences; USA) B - Half filter dissected per sample and digested.	–	540/60	
PUF tow	A - PUFs loaded into sterile syringe and flushed with 400 mL milli-Q water. • 140 mL monoject syringe B – flushed water filtered as per water sample. • Pall microbiology pump; • 47 mm, 0.22 μm polyethersulfone filters (Pall Life Sciences; USA)	–	540/60	
Plankton tow	A - nets flushed with milli-Q to concentrate eDNA. B - last 3 cm removed and digested		540/60	

followed the DNeasy Blood and Tissue (Qiagen; Netherlands) protocol, with minor modifications based on collection method and amount of organic material collected. Control samples were collected to determine the efficacy of the sterilisation process on sampling methods, as well as the digestion and extraction controls. Where possible, additional eDNA sample, such as half of filter or additional lysate material, was returned to storage at −20 °C as a contingency.

After digestion, the supernatant for each sample (minimum of 400 μL) was loaded into a QIAcube (Qiagen; Netherlands) unit for automated extraction, following a customised eDNA extract protocol resulting in approximately 100 μL of DNA extract. After extraction, the concentration of DNA extracts were verified using a NanoDrop One Spectrophotometer (Thermofisher, USA).

2.3.1. Amplification and sequencing

An assay targeting the conserved 18S rRNA barcode region was selected as it is able to detect broadly across the marine tree of life to a higher taxonomic level, such as order or family (Stat et al., 2017; DiBattista et al., 2020). As we were not focused on species level resolution for the purposes of this study, all analyses were completed at the family taxonomic level. The assay comprised of a single forward primer (18S_uni_1F; 5′ - GCCAGTAGTCATATGCTTGTCT - 3′) and reverse (18S_uni_400R: 5′ - GCCTGCTGCCTTCCTT - 3′) combination, with an annealing temperature of 52 °C (Pochon et al., 2013).

An exploratory PCR was used to determine the concentration required for optimal DNA amplification. The PCR master mix consisted of 2.5 mM $MgCl_2$ (Applied Biosystems; USA), 10x PCR Gold buffer (Applied Biosystems), 0.25 mM dNTPs (Astral Scientific; Australia), 0.4 mg/mL bovine serum albumin (Fisher Biotec; Australia), 0.4 μmol/L forward and reverse primers, 0.6 μL of a 1:10,000 solution of SYBR Green dye (Life Technologies; USA), and AmpliTaq Gold DNA polymerase (Applied Biosystems). The PCR reaction volumes were 25 μL and were performed on a StepOne Plus Instrument (Applied Biosystems) under the following PCR conditions: an initial denaturation stage of 95 °C for 5 min, followed by 45 cycles of: 95 °C for 30 s, followed by 52 °C (assay annealing temperature) for 30 s, 72 °C for 45 s, finishing with a final extension stage at 72 °C for 10 min. This initial PCR was completed on neat, 1/10 and 1/100 dilutions across all samples, with the optimal dilution selected for each sample selected based on the amplification curve, C_T value and melt-curve.

Based on results from the initial PCR outlined above, using fresh sample extract from the optimal dilutions, samples were assigned a unique 6–8 bp multiplex identifier tag, which allows the amplicon to bioinformatically traced back to the correct sample and site. The DNA and tags were amplified in duplicate using PCR, with reactions set up on a Qiagility instrument (Qiagen; Netherlands) using the same master mix to 25 μL and PCR conditions as outlined above, with the exception of 50 cycles to compensate for the longer amplification product. Negative extraction and PCR controls were included to ensure validity of results.

The indexed duplicates were combined if the amplification curves and melt plots were similar, otherwise the least optimal was discarded, and the minipools were progressed using only the optimal replicate. Minipools were blended based on equi-molar ratios of the amplification ΔRn values with no more than 10 samples included in each minipool. All minipools were quantified (Qubit 4.0 Fluorometer; Invitrogen) and amplicon peaks visualised (Qiaxcel; QIAgen) before being blended into a single library based on equimolar values. This library was then size selected to between to 250 to 600 base pairs (Pippen Prep; Sage Sciences, USA) to reduce primer dimer and erroneous amplicons. Sequencing was completed using two 500-cycle V2 chemistry for paired-end sequencing on a Miseq platform (Illumina, USA) following the manufacturer's protocol using a Q-score threshold of Q30.

2.3.2. Bioinformatics

Raw sequence data were downloaded from the online Illumina Sequence Hub and transferred to Zeus, an SGI of the Pawsey Supercomputing Centre (Kensington, Australia), for bioinformatic processing. Demultiplexing and deconvolution of raw sequences was completed through R (v3.6.3; R Core Team, 2020) on Rstudio (v1.2.5042; RStudio Team, 2020) using the package Insect (v1.4.0.9000; Wilkinson et al., 2018). Demultiplexed data were quality filtered prior to merging of paired-end reads and chimera removal, which were completed using the DADA2 package (v1.8.0; Callahan et al., 2016). The resulting Amplicon Sequence Variants (ASVs) were then queried against the National Centre for Biological Information's (NCBIs) publicly available GenBank Nucleotide Database (accessed in December 2021). Amplicon sequence variants (ASVs) resulting from dereplication were blasted against NCBI's GenBank nucleotide database, which required 100% query coverage and a minimum percent identity of 95% to successfully return a BLAST result. Taxonomy was assigned based on the lowest common ancestor (LCA) using the Python script within the eDNAFlow automated workflow (Mousavi-Derazmahalleh et al., 2021) with a percent filtering threshold of 98%, coverage of 100% and insignificant difference threshold of two percent. All dropped taxonomic assignments were manually vetted back against initial blast results and their taxonomy verified against the open access World Register of Marine Species database (WoRMS; accessed Jan 2022; WoRMS Editorial Board, 2021). Any positive results from field and laboratory controls were removed by

manually removing positive ASVs across the method or sequence dataset, as indicated, prior to taxonomy assignment. To reduce bias and normalise across multiple sequencing runs, all samples were rarefied and subsampled to 7500 sequences based on asymptote of visualised rarefaction curves using the "rarefy_even_depth" function of the R package vegan (v2.5.7; Oksanen et al., 2020). ASVs were merged to the family taxonomic level using the "tax_glom" function in the R package phyloseq (v1.28.0; McMurdie and Holmes, 2013) and ASVs that were unable to be assigned to family level were removed. Finally, samples were removed that had no reads at the family level taxa.

2.3.3. Data exploration, statistics, and multivariate analysis

The amount of eDNA being utilised in each reaction across each method was visualised by plotting the C_T values of the exploratory PCR with diluted samples corrected for on the basis that a 10-fold dilution equates to a −3.33 shift in C_T value under optimum PCR efficiency (Liu et al., 2021). As read abundance does not directly correlate to population abundance, and environmental factors (temperature, season, light) and trophic interactions can influence localised eDNA collection, after sequencing, data were transformed to presence and absence prior to analysis. A dominant habitat type was assigned to each family using available data from the WoRMS database, Encyclopedia of Life (EOL; Accessed Feb 2022; Parr et al., 2014) and the Atlas of Living Australia (ALA; Accessed Feb 2022; Atlas Of Living Australia, 2019), while acknowledging that families have habitat dependant life-history stages, such as planktonic larval stages in sponges. The data were explored using the R packages vegan and phyloseq, and community assemblage visualised via Principal Coordinates Analysis (PCoA) and Constrained Analysis of Principal Coordinates (CAP; 9999 permutations), with a leave-one-out analysis that was completed using the 'plot_ordination' and 'CAPdiscrim' functions within the same packages. Differences in the assemblage composition were explored using the PERMANOVA + add on for Primer 7 (Anderson et al., 2008; Clarke and Gorley, 2015). The analysis used the PERMANOVA routine with a two factor design with technique (8 levels) and depth (2 levels), both being fixed, on a Jaccard similarity matrix with 9999 permutations. Pair-wise analyses were completed exploring Method factor level within depth, and Depth factor levels within Method. Permuted multivariate precision was calculated to visualise the level of replication required for the detected assemblage variation to reach asymptote (Anderson and Santana-Garcon, 2015).

3. Results

3.1. PCR, sequencing and bioinformatic statistics

Extract concentration varied considerably between the methods (Fig. 2) and depths within method (see supplementary Section S3), with the swab having the highest mean (27.7 ng/μL) and PUF tow method having the lowest at 1.8 ng/μL, which also had the most consistent concentrations of all the methods, ranging from 0.5 to 5 ng/μL. Interestingly, the corrected amplification C_T values showed that the PUF tow samples had earlier amplification in the exploratory PCR (Fig. 2), indicating that more eDNA was available within the samples be utilised by the assay. DNA was successfully extracted and sequenced from all 210 samples. Sequencing yielded a total of 27, 405, 374 raw reads, with dive scrape detecting higher mean raw read counts per method, and PUF and plankton tows the least number of raw reads per method, prior to bioinformatic and quality filtering steps (Fig. 2; supplementary Section S4).

Overall, 20, 249, 700 reads passed bioinformatics and quality filtering processes prior to the merging and removal of unassigned ASVs, equating to a mean reads per sample of 90,284 (SE ± 6648.95), however ranged by method from 31,827 (SE ± 2492; water), to 232,065 (SE ± 18,855; Keel Crab) (see supplementary Section S5). ASVs per method ranged between 681 (dive scrape) total detected and 2183 (PUF tow), however 81% of the reads were unable to be assigned to family taxonomic level (see supplementary Section S6). Following the subsampling,

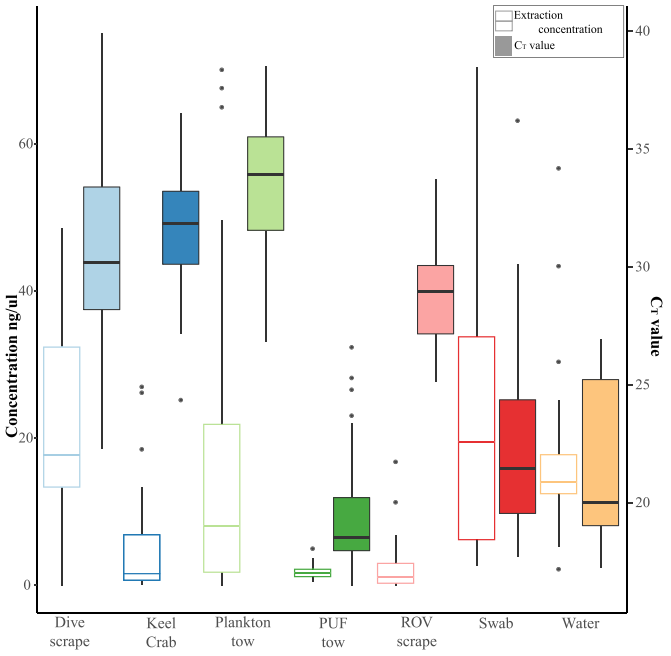

Fig. 2. Summary of eDNA collected using each sampling method by concentration (ng/μL) with outliers trimmed above 80 ng/μL (swab only), and the corrected C_T values per method of untagged eDNA as an indicator of how much DNA product was available for use by the 18S sequencing assay.

removal of unassigned reads and merging ASVs step, a total of 249 taxa were assigned to family taxonomic level, with a mean family richness per sample ranging from 5.13 (SE ± 1.1; Keel Crab scrape) to 26.51 (SE ± 1.4; PUF tow). Upon completion of all quality filtering and subsampling steps, 34 eDNA samples had insufficient reads and were removed from subsequent analysis. Samples were removed from all methods except water, with dive scrape (n = 11), Keel Crab scrape (n = 10) and ROV scrape (n = 7) the methods with the greatest number removed. Plankton, PUF and swab methods had the least samples removed after quality filtering with 3 samples, 2 and 1 sample, respectively.

3.2. Diversity detection and methods comparison

Sequencing identified 249 families from seven methods of eDNA collection, which represented 31 eukaryotic phyla. Visual identification from quadrat photographs increased overall detected diversity to 257 families. Overall, methods targeting epibenthic substrates ranged from 24 families (visual method) to 117 families (swab; Fig. 3) and 78 (plankton) to 154 (PUF) for methods targeting the water column. The phylum Porifera was the most commonly detected, with a mean 10.4 families detected per method (SE ± 1.4), and the phyla Chromerida, Chytridiomycota and Prasinodermophyta least detected, with single family detections within a single method. Four families (1.5% of total) were detected in all sampling methods eDNA and visual, including Ulvaceae (phylum Chlorophyta), Styelidae (Chordata), Mytilidae (Mollusca) and Mycalidae (Porifera). Seven families (2.8% of total) were detected using all eDNA sampling methods, but not represented in visual samples.

When assigned to habitat type (benthic, water column, parasitic and non-marine species) detections were dominated by benthic families in all collection method, and the only habitat detected with visual methods. Twenty-three families detected were classified as non-marine and included detections of Streptophyta (terrestrial plants), Arthropoda (insects and terrestrial mites), Chordata (aves), Oomycota and Ascomycota (terrestrial and freshwater fungi). Non-marine taxa were

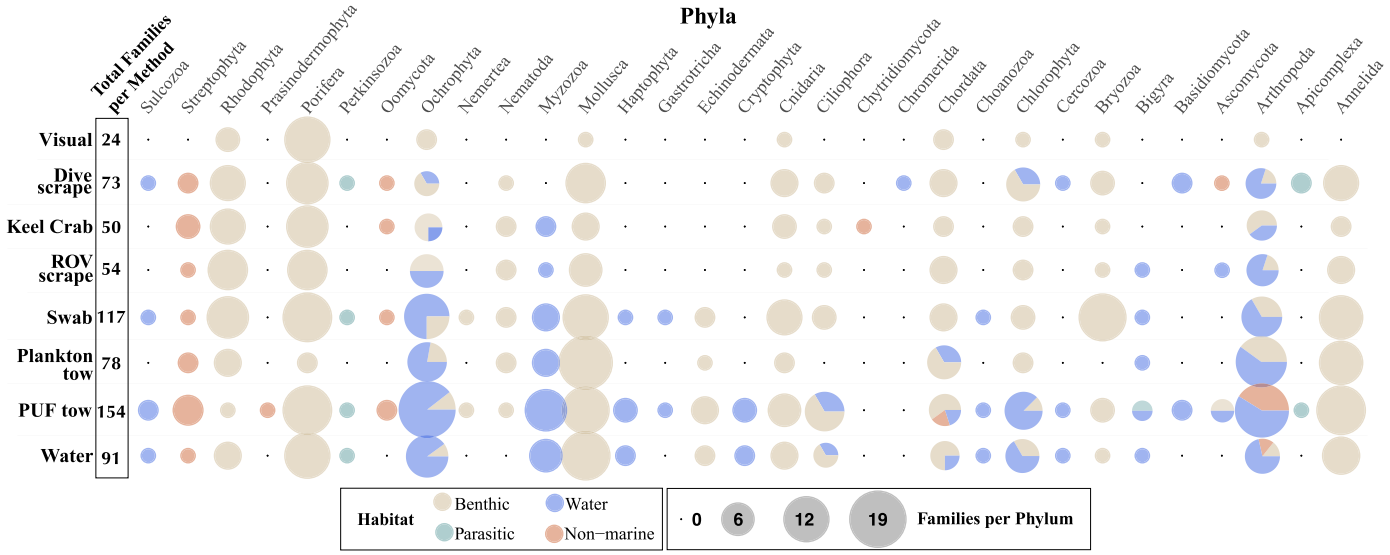

Fig. 3. Total number of families detected per phyla using the 18S universal assay across all eDNA sample collection methods, as well as the visual method used, and proportion of habitat types detected.

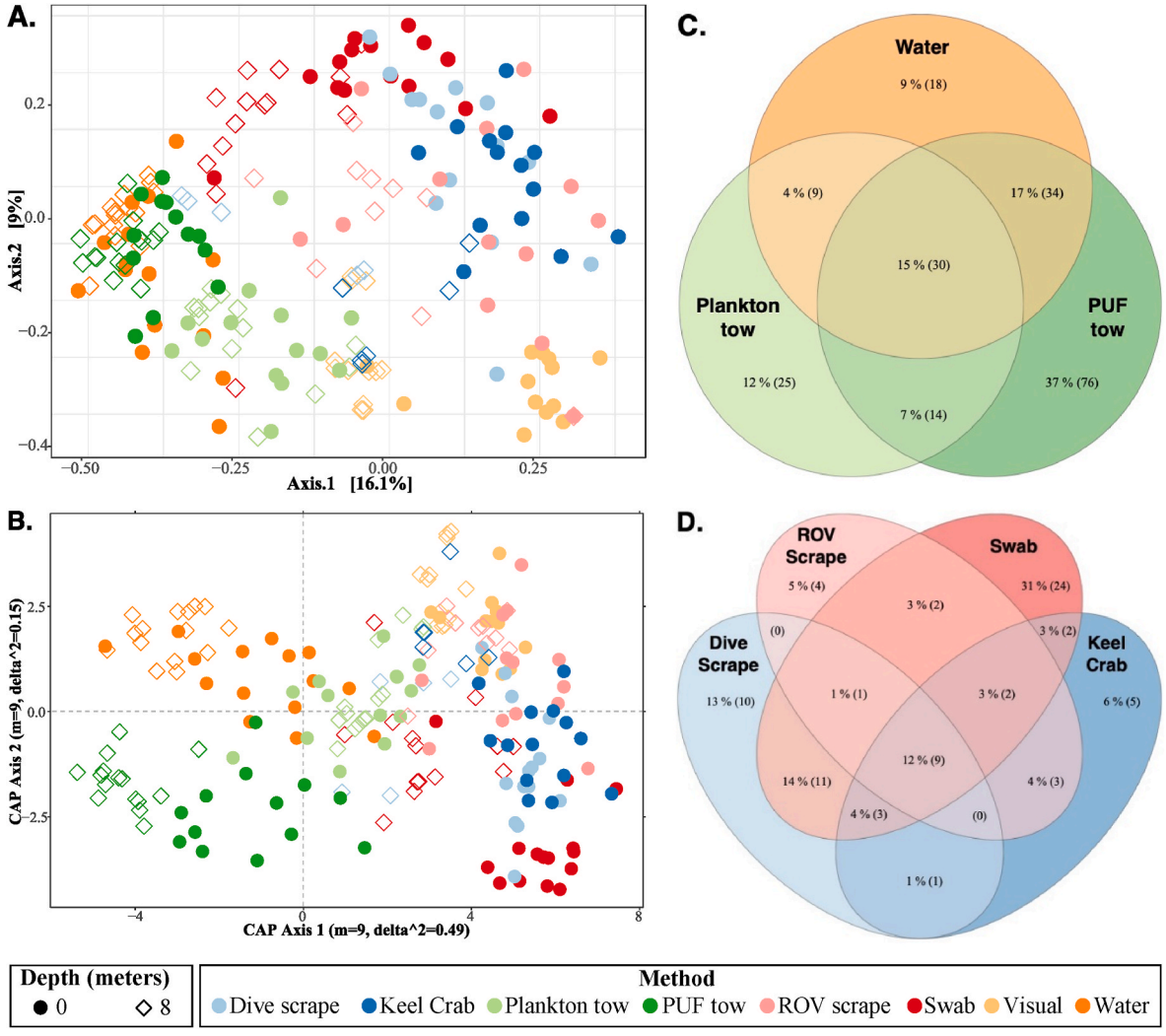

Fig. 4. Comparison of the unconstrained ordination (PCoA; A.) and constrained ordination (CAP; B.) for methods and depth based on a Jaccard similarity matrix of the presence/absence transformed data, as well as a comparison of taxa collected detected by each method targeting the water column (C.) and epibenthic substrates (D.).

detected across all of the eDNA collection methods, with all of the methods detecting less than five families, except the PUF tows that detected 16 non-marine families. Five parasitic families were detected from the phyla Perkinsozoa, Bigyra, and Apicomplexa.

The PERMANOVA analysis compared the overall detected community composition of sampling methods showed significant differences in the assemblage sampled across methods (Pseudo-F = 9.680, P(perm) < 0.001, Unique Perms = 9697). Pairwise analyses showed significance differences across all results, except between methods Visual X Keel Crab at 8 m depth (t = 1.22, P(perm) = 0.081, Unique Perms = 7724), and between 0 m and 8 m depth with the ROV method (t = 1.17, P(perm) = 0.119, Unique Perms = 9853) (see Supplementary Tables S7–S9). These results are corroborated by constrained and unconstrained ordination (Fig. 4). The leave-one-out allocation analysis (see Supplementary Table S10) had an overall 27% chance of being mis-assigned, over all methods and depths, with epibenthic targeted sampling methods, ROV, Keel Crab and Dive scrape had a lower average (46% correct SE 5.23) correct leave one out allocations at both depths compared to Swab and water column sampling methods (84% correct SE 4.88). Mis-classification occurred between depths within the same method, with water sampling having highest mis-classification within method (n = 5). Between methods targeting the same substrate, epibenthic method mis-classifications made up 97% of the 35 mis-classifications (see Supplementary Table S10). Mis-classifications between methods targeting different substrates were lower (n = 6), with three plankton methods at 8 m mis-classified as ROV and Keel Crab scrapes of the same depth.

The characterisation of methods and depths were completed using a similarity percentage (SIMPER) analysis (see Supplementary Table S11), which showed that the harpacticoid copepod family Euterpinidae (phyla Arthropoda) was dominant in all methods targeting the water column, and contributed 10% (PUF samples), 12% (water) and 51% (plankton) of the biotic detections for those methods. The dive scrape and visual methods were dominated by the brown algae family Dictyotaceae (phyla Ochrophyta), which contributed 16% and 61%, respectively. The remaining methods were dominated by the families Scrupariidae (phyla Bryozoa; swab samples; 9%), Ceramiaceae (phyla Rhodophyta; ROV samples; 24%), and Rhodomelaceae (phyla Rhodophyta, Keel Crab samples; 24%). The average similarity of epibenthic sampling methods at 0 m depth ranged between 21.7% (ROV scrape method) and 48.4% (Swab), and 4.4% (Keel Crab) and 36.8% (Swab) at the 8 m sampling depths. Water column sampling methods ranged from 28.8% (Plankton tow) to 39.1% (PUF tow) at 8 m depth, and 26.3% (Plankton tow) and 60.7% (Water) at 8 m depth, with the family Euterpinidae being the dominant family in all of the methods and depths, with the exception of water at 8 m (Styelidae; phyla Chordata).

Accumulation curves and multivariate precision analysis showed that only the visual method accumulation curve came close to reaching asymptote. This result indicated that additional sampling may not yield additional family level detections with further visual samples (Fig. 5). The seven eDNA sampling methods did not reach asymptote, suggesting that additional sampling would increase family level abundance. Estimates of total family diversity for water eDNA sampling methods range from 125/94 (Plankton tow) and 222/177 (PUF tow) using Chao2/Bootstrap estimators, and epibenthic eDNA methods from 72/59 (Keel Crab) and 161/132 taxonomic families (Swab method). However multivariate precision analysis pseudoSE indicates that permuted assemblage variability between replicates levels off between 20 and 25 samples and therefore additional sampling will not greatly increase community composition (Fig. 5).

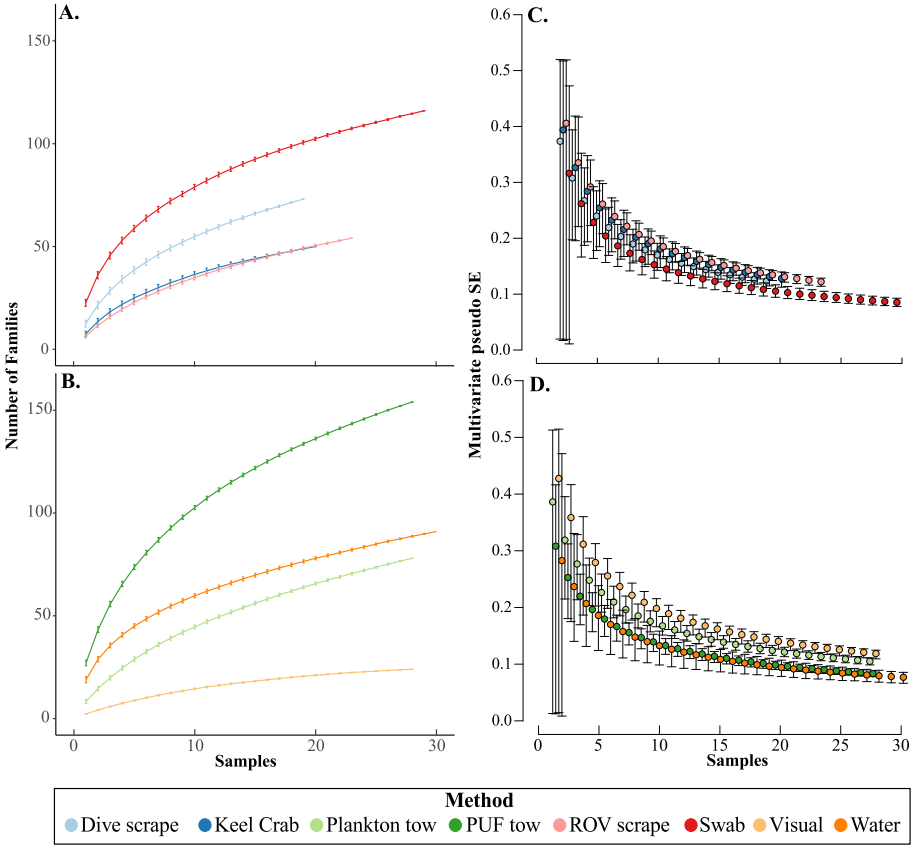

Fig. 5. Family level observed accumulation curves for each sampling method (A-epibenthic sampling methods; and B-water column and visual) and the corresponding result of dissimilarity-based multivariate analysis displaying the mean multivariate pseudo standard error (right) based on Jaccard dissimilarities with 2.5 and 97.5 percentiles as error bars (resample N = 10,000).

4. Discussion

Our results showed some compositional crossover between collection methods, particularly among those sampling the same substrate. It is the differences in detection between methods, however, that is arguably more important. In particular for methods that were concurrently sampled and targeted the same substrate, such as Plankton and PUF tows, which highlights that sampling method used can greatly affect the detected community composition. In methods targeting the epibenthos, it was noted that those that incorporated large amounts of organic material (such as dive, Keel Crab and ROV scrape methods) were potentially impacted by the disproportionate representation of single source DNA material from dominant taxa. This resulted in a reduced overall diversity (i.e. swamping of assays by dominate taxa). This finding was consistent with some studies that compared bulk-sample metabarcoding and water eDNA metabarcoding (Macher et al., 2018). These collection methods may also introduce increased PCR inhibitors into the samples, such as the collection of calcium from bivalve shells and other naturally occurring organic compounds, that can disrupt PCR amplification (McKee et al., 2015). Of the four epibenthic sample methods trialled in this study, only the swab method relied on the detection of trace benthic DNA (over the collection of bulk eDNA), which detected the highest family level diversity for the substrate.

With the exception of the ROV scrapes, all of the methods detected changes in the assemblage composition with depth. These significant differences between depth, method and substrate indicate that environmental DNA is not homogeneous throughout the environment. Within the water column, eDNA movement can be limited vertically by physicochemical stratification, such as salinity and temperature gradients, which has been documented over short (16 m) and large (over 1000 m) vertical spatial scales (Jeunen et al., 2020; Canals et al., 2021), however this has not been demonstrated using epibenthic eDNA samples.

This fine-scale depth differentiation has important practical implications, in particular for epibenthic substrates, when targeting subsea infrastructure, such as oil and gas platforms. With sufficient replication, the epibenthic methods utilised in this study, such as the epibenthic swab method, can translate to larger marine structures, and be used to provide robust vertical profiling. This has practical implications for informing on the presence of conservation significant and invasive marine species, as well as general epibenthic assemblage composition, which are important considerations in decommissioning and predicting outcomes for these epibenthic communities under the commonly accepted decommissioning strategies (e.g. removal, toppling, topping or reefing structures (Macreadie et al., 2011)).

Consistent with previous method comparison studies, our study highlights the risks of relying on single collection method in broad diversity eDNA studies, as this may skew results towards the substrate and method used (Koziol et al., 2018; Rivera et al., 2021; Alexander et al., 2022). Multiple sampling methods may be required across multiple substrates to adequately describe diversity depending on the study focus. In addition, this method-dependant community detection has implications for general ecological monitoring using eDNA methods, as there is a lack of robust reproducibility unless employing a similar collection technique and primer combination. Although this is more a general issue, it can have implications for the broader uptake of eDNA sampling for temporal or repeat monitoring, management and impact assessments, and the adoption of eDNA methods within governmental and conservation frameworks (Ruppert et al., 2019).

Although the technology is established to analyse eDNA, further development to determine optimal substrates and methods is required to have high confidence in study outputs. Marine eDNA method comparisons have predominantly focused on contrasting and comparing traditional visual (or traditionally accepted) methods with either water (Stat et al., 2018; Alexander et al., 2019, 2022; Ip et al., 2021; Lee et al., 2022) or sediment eDNA sampling (Pawlowski et al., 2022) to gauge the overall method efficacy. Other studies have compared technical aspects within those methods (such as filter types, pore sizes, filtered volume or environmental conditions; Deiner et al., 2018; Kumar et al., 2022) on eDNA metabarcoding output. Recently, sampling methods have been trialled to include the use of plankton nets to successfully monitor ballast water (Ardura et al., 2015; Zaiko et al., 2015) and the monitoring of bulk plankton diversity through the use of a continuous plankton recorder (Deagle et al., 2017; Govindarajan et al., 2021; Suter et al., 2021). Similarly, the use of ROV technology to collect eDNA samples is not new (Harvey et al., 2016), however has predominantly focused on targeted collection of water and deep-sea sediments and cores, requiring the use of large, industrial research ROV units (Laroche et al., 2020; McLean et al., 2020). Epibenthic eDNA and visual comparison studies have relied on the time consuming deployment and collection of colonisation substrates, such as settlement tiles and autonomous reef monitoring structures, that are scraped, homogenised, and processed (Harper et al., 2021; Nichols et al., 2022). While this method can detect a higher diversity of encrusting and calcifying epibenthic organisms, considerable time is required for deploying and retrieving such tools and for the colonisation of target taxa (Gaither et al., 2022; Nichols et al., 2022).

We successfully trialled and compared novel methods of sampling both epibenthic and water substrates. However, the novel field collection methods used in this study (PUF tow, swabs, ROV and Keel Crab scrapes), have only been trialled under a narrow range of environmental conditions and, as such, require further validation to determine efficacy under broader conditions (Cristescu and Hebert, 2018). This validation includes the exploration of the physical mechanisms behind eDNA capture, maximising sampling efficiencies and minimising contamination risks. Of the methods trialled here, this is particularly interesting for the PUF tow method, where the sample extracts yielded consistently low total genomic DNA concentrations compared to other methods examined, but inversely yielded the highest detected ASV and eukaryotic diversity. We hypothesise this interaction may be a result of the sampling method which omits large pieces (over 3 mm) of organic material (e.g. free-floating algae) due to the design of the PUF sampler. This combined with a pre-digestion step of compressing and flushing the PUFs prior to filtration, where the PUF matrix may retain much of the remaining multicellular and smaller organic material (less than 3 mm, such as plankton) allowing mostly cells and DNA to pass through to filtration. Conversely, other methods such as water sampling, may be collecting and extracting whole planktonic organisms, resulting in higher DNA concentrations, but reduced diversity at both the ASV and family level. In addition, the impact of the positive charge inherent in the PUF material should be considered, as this may result in organic material and DNA adhering to the filter matrix. While this has not been explored in active filtration or sampling methods, in some passive sampling trials, completed using different material, has found this effect to be negligible (Bessey et al., 2021) with an increase in available surface area more important in eDNA capture in passive sampling (Bessey et al., 2022).

These methods have the potential to provide researchers and environmental managers with alternative approaches that are capable of detecting a broad diversity of taxa in the marine environment, as well as considerations for how combinations of sampling methods and substrates targeted can increase the comprehensiveness of detection. Applications for these methods range from diversity characterisation and censusing across a chosen environment, to a more nuanced spatial and depth detection or greater sampling specificity when targeting substrate dependent taxa. This research has wider implications in promoting eDNA based surveys outside of the scientific community by reducing the reliance on scientific personnel, eliminating water filtration time and limitations, and therefore the reduction of associated costs, as well as the utilizing sampling media which are readily available. In addition, these methods can be automated using available ROV technology to reduce occupational health and safety requirements associated with SCUBA

methods, and to target more logistically challenging locations to provide a more comprehensive approach. Moving forward with these methods, priorities should be to explore the eDNA capture method, as well as similar PUF and swab matrices to streamline collection and laboratory workflows and further minimise the contamination potential. With further development, these methods have the potential to be a staple resource in the arsenal for research, industry, and government for exploring and managing marine environments.

Funding

This project was funded by Chevron through a research grant to Curtin University from the Western Australian Energy Research Alliance (AES 17-P2TD-151-A1). JBA received industry scholarship funding from Commonwealth Scientific and Industrial Research Organisation (CSIRO) and Chevron.

Permits and ethics

All field sampling was completed under the Department of Primary Industries and Regional Development exemption number 3545v2. No ethics were required for the taking and processing of eDNA samples.

CRediT authorship contribution statement

Jason B. Alexander: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Michael J. Marnane:** Writing – review & editing, Conceptualization. **Justin I. McDonald:** Writing – review & editing, Formal analysis, Conceptualization. **Sherralee S. Lukehurst:** Writing – review & editing, Data curation. **Travis S. Elsdon:** Writing – review & editing, Formal analysis, Conceptualization. **Tiffany Simpson:** Writing – review & editing, Conceptualization. **Shawn Hinz:** Writing – review & editing. **Michael Bunce:** Writing – review & editing, Formal analysis, Conceptualization. **Euan S. Harvey:** Writing – review & editing, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Jason B Alexander reports financial support, article publishing charges, equipment, drugs, or supplies, and writing assistance were provided by Chevron Energy Technology Co. Jason B Alexander reports financial support was provided by Commonwealth Scientific and Industrial Research Organisation. Euan S Harvey reports a relationship with Chevron Energy Technology Co that includes: funding grants. Michael Bunce reports a relationship with Chevron Energy Technology Co that includes: funding grants. Travis Elsdon reports a relationship with Chevron Energy Technology Co that includes: employment. Michael Marnane reports a relationship with Chevron Energy Technology Co that includes: employment. Shawn Hinz reports a relationship with Chevron Energy Technology Co that includes: consulting or advisory. Sherralee S Lukehurst reports a relationship with BHP Group Ltd that includes: funding grants.

Data availability

Data will be made available on request.

Acknowledgments

We gratefully acknowledge the logistical and safety support provided by the Fremantle Port Authority, under whose jurisdiction the KBJ study area fell within. We would like to thank Kevin Holden at DeepVision Subsea and Tony Scarangella at Andro Maritime Service Australia, for their assistance in field sampling. We would like to thank Rowan Kleindienst, Laura Fullwood and Damon Driessen, from Curtin University, for assisting with the field sampling and Matthew Hewitt from Department of Primary Industry and Resource Development (DPIRD) for assistance with equipment used for bulk sample processing. SL acknowledges the support from the BHP-Curtin alliance within the framework of the "eDNA for Global Environment Studies (eDGES)" programme. This work was supported by resources provided by the Pawsey Supercomputing Research Centre with funding from the Australian Government and the Government of Western Australia.

Appendix ASupplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecss.2023.108283.

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