School of Molecular and Life Sciences

Characterising biodiversity patterns associated with marine oil and gas infrastructure using environmental DNA

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This thesis is presented for the Degree of

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—— DECLARATION ——

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgement has been made. This thesis contains no material which has been accepted for any other degree or diploma in any university.

Signed: Jason Bradley Alexander

Date: 25th April 2023

—— ABSTRACT ——

Marine artificial reefs are being strategically designed and deployed to fulfill a particular economic or ecological purpose, such as aid natural resource management, mitigate anthropogenic pressures, and promote tourism related industries (e.g., recreational fishing and SCUBA). Marine infrastructure, such as oil and gas platforms, can develop extensive biotic communities and, when decommissioned, are being considered for conversion to artificial reefs. Information on the spatial distribution of biota on infrastructure can help inform decommissioning options and environmental impact assessment. Environmental DNA metabarcoding (hereafter 'eDNA') is a molecular tool increasingly deployed in marine surveys. It can be utilised to enhance species detections and may sample broader assemblages than possible with methods relying on visual morphological characterisation. There are a small number of studies that sample using eDNA methods at oil and gas infrastructure. Consequently, their efficacy remains untested in comparison to conventional methods.

My research provides important steps in optimising eDNA metabarcoding for marine assemblage censusing on infrastructure and oil and gas platforms, with the primary aims of improving reliability, reproducibility, and streamlining the sampling process. To achieve this, I explore the impact that eDNA collection method has on eDNA output (Chapter 2), which is then expanded to investigate the synergies and nuances of eDNA results in comparison with conventional (ROV) sampling at oil and gas platforms in the Gulf of Thailand (Chapter 3). I document and assess spatial changes of eukaryotic diversity at the same oil and gas platforms and assess possible decommissioning options (Chapter 4), and the efficacy of eDNA sampling methods to track temporal changes at marine infrastructure in a high tidal flow environment (Chapter 5).

Environmental DNA studies have predominantly focused on the collection and isolation of DNA using a single collection method, commonly comprising filtered water samples, the results of which are assumed to represent the entire adjacent community and substrates. I test this assumption in Chapter 2, and apply seven methods of eDNA collection across two substrates (infrastructure epibenthos and the adjacent water column) to investigate the synergies in detection between method,

substrate and depth. Sampling was undertaken on the pylons of a decommissioned jetty in Perth, Western Australia, which had well-established biotic communities. Sequencing results were obtained using a broad metazoan assay, and indicated that there was very little overlap in community composition detected both between substrates and also methods targeting the same substrate. This surprising result has flow on implications for reproducibility in eDNA studies. Interestingly, methods that collected bulk organic material had reduced diversity, an important research outcome for bio-foul sampling on marine infrastructure.

Chapter 3 maintains the methodological focus and applies it to a 'real-world' scenario, comparing ROV surveys of fish to eDNA water samples at seven oil and gas platforms as well as five comparative sediment locations, from the Gulf of Thailand (GoT). The GoT is a relatively shallow, yet diverse tropical system which contains extensive oil and gas infrastructure. Exploring fish and elasmobranch communities, this research showed that eDNA collection methods detected much higher diversity, however there were surprisingly little overlap in taxa detection, indicating a complementary sampling approach of conventional and eDNA sampling may provide more robust community censusing tool.

To further explore the scalability and the potential for eDNA sampling to inform decommissioning options and assess environmental impact (Chapter 4), an additional four assays were used to broaden the existing data focus from Chapter 3 to capture across the eukaryotic tree of life. These six assays were applied to water, bio-foul and sediment samples. Using this data, I was able to evaluate the ability of eDNA to detect broad and fine scale spatial changes in location and depth using both taxonomic and taxonomy independent (using amplicon sequence variants, or ASVs) analysis. A comparison of the data showed similar trends indicating that, while taxonomic databases are deficient for the highly diverse GoT, taxonomic resolution was capable of detecting community shifts. From this data I make conclusions about the hypothetical impacts from decommissioning options to detected spatial composition.

Lastly, having assessed the ability of eDNA to elucidate spatial nuance in Chapters 2-4, I explore spatial and temporal differences in fish, elasmobranch and chordate communities from water samples at a newly installed artificial reef in the Exmouth

Gulf, in the northwest of Western Australia. Samples were collected over a 27-month period spanning pre- and post-installation, and while limited trends were detected at the artificial reef, this result was mirrored in some other habitat types sampled as comparison. This result may be reflective of the presence of transient species at the artificial reef, or potentially the result of DNA movement caused by mesotidal influence. However, the two assays detected a broad range of chordate taxa, including temporal detections in line with known annual migration patterns, such as the humpback whale (*Megaptera novaeangliae*). This temporal eDNA pattern indicates promise as a broader detection tool and, with optimisation, able to be enhanced to track biotic changes at newly installed artificial reefs.

Collectively, this research encompasses the eDNA profiling of approximately 434 samples targeting the water column, 216 samples targeting the epibenthos, 52 sediment samples, and additional comparative morphological identification samples. Combined with the application of nine assays, approximately 232 million raw metabarcoding reads were generated and analysed. My research is a valuable step in tailoring eDNA metabarcoding to studies of marine infrastructure. The sampling methods described within these studies are able to be modified and used by scientific and non-scientific (such as industry personnel) alike, thus improving eDNA metabarcoding accessibility across the board.

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We all know the old adage that "*it takes a village to raise a PhD student*" (-did I get that right?) and as much as I would like to think that I have been a stoic, self-sufficient mountain amid the chaos, in truth I have had a ridiculous amount of support from a lot of sources. My supervisory team have been front-and-center as founts of knowledge, ideas, and the occasional calming influence when I've fixated on, or headed into a downward spiral over the smallest details. Prof. Euan Harvey, Dr. Michael Marnane, Assoc. Prof. Zoe Richards, Prof. Michael Bunce and Dr. Olly Berry, thank you all for the invaluable help, guidance and perspective that you have provided over the past years, it has truly been appreciated. Even though after a draft paper ~~shredding~~ review, that appreciation may not have shone through. Also, my appreciation to Dr. Ben Saunders for providing support as the chair of my PhD supervisory panel. While not a supervisor, a huge thanks also to Dr. Travis Elsdon, for the statistics support and brainstorming.

A special thanks to the Trace and Environmental DNA (TrEnD) lab rats- or to those that once identified as such, as many have since finished studies and moved onto greener, research-oriented pastures. In particular, Georgia Nester, who has always been a great sounding board for research and life in general, and Tina Berry, without whom I probably would still be caffeine-free, but much poorer mentally without the early morning brain dumps and laboratory-methods chats. However, the TrEnD community is large and full of amazingly intelligent and diverse people, without which my PhD experience wouldn't have been half as fun. So many thanks to Katrina West, Jackie Jones, Matt Heydenrych, David Juszkiewicz, Kristen Fernandes, Mieke van der Heyde, Matt Power, Arne Adam, Alishum Ali, Josh Kestel, Miwa Takahashi, Sophie Preston, Josh Newton, Matt Campbell, Austin Guthrie and Frederik Seersholm for fun office chats and debates. As well as Sherralee Lukehurst, Nicole White, Georgia Peverley and Mahsa Mousavi-Derazmahalleh for laboratory and bioinformatic expertise. Straying from the TrEnD lab to the murky depths (-of a few doors down the hall) of the Fish Ecology Laboratory, I would like to thank Laura Fullwood, Damon Driessen, Karl Schramm, Jack Parker and Rowan Kleindienst for the field logistics/sampling/fish-identification/*insert-other-annoying-request* support over the last few years, and otherwise for just being great people. I also need to

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On the random pieces of art throughout this thesis

It's not until you need interesting and artistic, marine themed imagery for chapter title pages, and lack the artistic flair to create it yourself, that you realise just how many talented people you know. I decided to use various forms of artwork in this thesis to provide welcome visual relief for weary eyes. I would like to gratefully acknowledge the talented Penny Brooshooft (Eclette), Chris Cole (Tidal Scales) who uses Gyotaku art (one of which can be seen below), Mark Penhale who dabbles in X-ray art, and Justine Shailes, who provided these amazing visual pieces for use in this thesis.

Coral Trout
Gyotaku is a Japanese art form that dates back to the mid 1800s. Fishermen would use this technique as a method to record their outstanding captures to help tell their stories when they returned to shore. (Text adapted from website)
Image provided and displayed here with consent from Chris Cole of Tidal Scales (https://tidalscales.com.au/)

—— ACKNOWLEDGEMENT OF COUNTRY ——

Just as Australia has a wealth of idyllic natural places and a staggering diversity of flora and fauna to marvel at, it also has an amazing cultural diversity that has been here for many thousands of years prior to the arrival of European settlers. I have been fortunate enough to witness firsthand the deep connection, care and respect that the traditional owners have for the land, during what seems like a previous life working in zoology and environmental science. This amazing country is cared for by many nations, and I am extremely grateful for the privilege that I have had in being able to see some of these incredible locations, some of which during fieldwork for this PhD.

This research was predominantly completed on the lands of the Whadjuk Noongar people, which is the home of Curtin University, the Trace and Environmental DNA (TrEnD) laboratory, and also the location of field sampling for one of the studies. The remaining (Australia-based) fieldwork was completed in the land of the Jinigudera peoples, the traditional owners and custodians of the lands and waters around Exmouth Cape. I would like to express my gratitude to their Elders past and present for their care and stewardship of the land, and for the privilege of being able to conduct research in such amazing places. I would like to personally acknowledge that sovereignty was never ceded and therefore these regions, like all of Australia, always was and always will be Aboriginal land.

-Curtin University-

We acknowledge that Curtin University works across hundreds of traditional lands and custodial groups in Australia, and with First Nations people around the globe. We wish to pay our deepest respects to their ancestors and members of their communities, past, present, and to their emerging leaders. Our passion and commitment to work with all Australians and peoples from across the world including our First Nations peoples are at the core of the work we do, reflective of our institutions' values and commitment to our role as leaders in the Reconciliation space in Australia.

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—— STATEMENT OF CONTRIBUTIONS ——

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Alexander J.B., Marnane M.J., McDonald J.I., Lukehurst S.S., Elsdon T.S., Simpson T.J., Hinz S., Bunce M., Harvey E.S. (2023) Comparing environmental DNA collection methods for sampling community composition on marine infrastructure. *Estuarine, Coastal and Shelf Science*. https://doi.org/10.1016/j.ecss.2023.108283.

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Chapter 3:

Alexander J.B., Marnane M.J., Elsdon T.S., Bunce M., Songploy S., Sitaworawet P., Harvey E.S. (2022) Complementary molecular and visual sampling of fish on oil and gas platforms provides superior biodiversity characterisation. *Marine Environmental Research*. https://doi.org/10.1016/j.marenvres.2022.105692

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Chapter 4:

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Chapter 5:

Alexander J.B., Berry T.E., Richards Z.T., Harvey E.S. (2023) Temporal eDNA sampling reveals a high diversity, and high assemblage turnover rate, of chordates surrounding a newly installed integrated artificial reef. *[in preparation]*

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—— ABBREVIATIONS ——

ΔRn	Delta RN / Normalised Reporter value
ANOVA	Analysis of variance
ASV	Amplicon Sequence Variant
BLAST	Basic Local Alignment Search Tool
BP	base pairs
BRUV	Baited remote underwater video
CAP	Canonical analysis of principal coordinates
CO1	Mitochondrial cytochrome c oxidase 1
CSIRO	Commonwealth Scientific and Industrial Research Organisation
C_T	Cycle fluorescence threshold value
DNA	Deoxyribonucleic acid
DPIRD	Department of Primary Industry and Regional Development
eCells	Whole cells extracted from environmental samples
eDNA	Environmental DNA
EIA	Environmental impact assessment
GoT	Gulf of Thailand
IMS	Introduced of invasive marine species
INDVAL	Indicator values analysis
iPhD	Industry PhD programme
ITS2	Internal transcribed spacer 2
IUCN	International Union for Conservation of Nature
LCA	Lowest common ancestor
NCBI	National Center for Biotechnology Information
MDS	Multidimensional scaling
MMS	Man-made structures
mOTU	Molecular OTU
mtDNA	Mitochondrial DNA
NGS	Next generation sequencing
NRM	Natural Resource Management
OP	Off-platform
OTU	Operational Taxonomic Unit
PCO	Principal coordinates analysis
PCR	Polymerase Chain Reaction
PERMANOVA	Permuted analysis of variance

PF	Platform
qPCR	Quantitative PCR
RO	Reverse osmosis
ROV	Remote operated video
rRNA	Ribosomal RNA
RTP	Australian Government research Training Program Scholarship
RtR	Rigs-to-Reefs
SCUBA	Self-contained underwater breathing apparatus
SIMPER	Similarities percentage analysis
TMS	Tether management system
TrEnD	Trace and Environmental DNA Laboratory
UAV	Autonomous underwater vehicle
WA	Western Australia
WoRMS	World Register of Marine Species
zOTU	Zero-radius OTU

Chapter 1

Introduction

X-ray image of a mixture of whole and broken shells including nautilus shells. Image provided and displayed with consent from Mark Penhale.

1.1 Fantastic reefs and why we need them

Coral reefs are biologically diverse habitats with between 600,000 – 950,000 species inhabiting them (Reaka-Kudla, 1997). While coral reefs occupy approximately 0.1% of the world's surface, they are an important habitat that provide many ecosystem services and functions (Birkeland, 2015; Brandl et al., 2019) including coastal erosion protection and the sequestration of CO_2. They also have some of the highest gross productivity in terms of primary production (Birkeland, 2015; Hoegh-Guldberg et al., 2019). Humanity has depended on reef systems for thousands of years as a valuable source of food (Hodgson, 1999). Recently our use of coral reefs has expanded to include commercial fishing, including for the aquarium trade, as well as commercial and recreational tourism (such as recreational fishing and SCUBA diving). In recent decades the exploration of reefs for pharmaceutical compounds has exploded with over 200 potentially useful substances extracted from coral holobionts alone (van de Water et al., 2022). Currently, it's estimated that, considering these varied uses, coral reefs have an annual worth of $375 billion (van de Water et al., 2022). However, globally, coral reef ecosystems have been in decline due to the accumulation and compounding of anthropogenic stressors (Hoegh-Guldberg, 1999; Hughes, 2003; Bellwood et al., 2004; Hoegh-Guldberg et al., 2007).

The decline of coral reef systems and the overall health of the ocean, has been well documented (Sebens, 1994; Hughes, 2003; Pandolfi et al., 2011; Heery et al., 2017). Causes of this decline include the over exploitation of fish and coral stocks, an increased runoff into fringing coral reefs (e.g., freshwater, domestic chemicals, and sediment), and the movement, introduction or population growth of corallivores, such as the voracious crown of thorns starfish (Sebens, 1994; Hughes, 2003; Brander et al., 2007; Birkeland, 2015). The current state of global reefs is dire, with acidification reducing calcification of coral systems (Erez et al., 2011), as well as marine heat waves increasing in intensity and frequency (Skirving et al., 2019). These heat waves can result in corals ejecting their endosymbiotic autotrophs (*Symbiodinium* sp.), which can provide up to 100% of their daily energy requirements (Schoepf et al., 2013), resulting in the corals appearing white or 'bleached' (Hoegh-Guldberg, 1999). If these environmental stressors are prolonged then coral can die from this energy deficit. A recent series of consecutive marine heat waves, from 2014 – 2017, have been

documented with approximately 75% of coral reefs globally being subjected to bleaching temperatures, which in some areas, had up to 98% mortality rate of bleached coral (Eakin et al., 2019). Projections for reefs include the collapse of base coral diversity and the trophic systems they support or, at best, the reduction in coral diversity and a shift in community assemblage and species distributions (Adam et al., 2021). The flow on from these mass coral mortality events can lead to the collapse of trophic interactions and changes in functional species diversity, such as altering fish, benthic and planktonic communities (Bellwood et al., 2004, 2014). Over time this can lead to impacts on global coral reef fisheries (Cinner et al., 2009), reduction in habitat diversity and complexity (Pratchett et al., 2014), and in the long-term, this can cause a reduction of accretion of $CaCO_3$ resulting in less coastal protection and increased coastal erosion (Reguero et al., 2018; Hoegh-Guldberg et al., 2019).

Ongoing research is investigating aspects of promoting reef health and reclaiming diversity lost through past bleaching events, through the seeding of new coral from nursery stock (Howlett et al., 2021; Strudwick et al., 2022), or by promoting colonisation of heat tolerant *symbiodinium* relationships with corals (Caruso et al., 2021). Another avenue being explored is the reduction/dilution of pressures on existing natural reefs, or the provision of additional habitat in optimal locations, through the deployment of artificial habitats, or reefs (Higgins et al., 2022).

1.2 The background and rationale behind artificial reefs

Humans have used strategically placed structures in the marine environment for centuries, initially for fishing purposes as fish attracting and aggregating devices (Riggio et al., 2000; Baine, 2001). These form some of the first known artificial reefs. However, given the long history of human dependence on marine habitats, the ocean is scattered with man-made structures (hereafter 'MMS') that have the potential to recruit considerable biotic diversity (Rilov and Benayahu, 2000; Walker et al., 2007). These can refer to relictual structures such as shipwrecks and defunct jetties (Hylkema et al., 2021), or operational infrastructure such as wind turbines, piers, jetties and oil and gas infrastructure (e.g., pipelines and platforms; Lemasson et al., 2021; Elrick-Barr et al., 2022). There are many definitions of what constitutes an artificial reef,

however for the purposes of this thesis, I use the definition outlined in Seaman and Jenson (2000), stating that:

"*An artificial reef is one or more objects of natural or human origin deployed purposefully on the seafloor to influence physical, biological, or socioeconomic processes related to living marine resources*."

These intentionally deployed structures are becoming increasingly utilised globally to promote reef habitats in areas which were previously unsuited to high productivity reefs (Florisson et al., 2020). These purpose-designed artificial reefs are deployed to provide connection between natural habitats, provide protected refugia and provide habitats for recreational fishing and tourism activities such as SCUBA diving (Becker et al., 2018; Tynyakov et al., 2017; Lima et al., 2019; Vivier et al., 2021). Historically, artificial reefs have been created from an extensive list of material, including ash, tyres, vehicle bodies or rock/rubble, to name a few (Ramm et al., 2021). There has been a recent focus on the design and deployment of artificial reefs to optimise the outcomes for a particular purpose, such as the promotion of fish diversity. This has led to an increase in specialised 3D printed structures from limestone, cement and geopolymers (Baine, 2001; Ly et al., 2021). In this way, artificial reefs are being used to provide alternative habitat, optimally placed and designed to promote the diversity and abundance of recreationally important fishing species, or to provide economic benefits through tourism accessibility (Becker et al., 2018). Artificial reefs are also being deployed to increase commercial fishing yields, to promote snorkelling and SCUBA tourism (Stolk et al., 2007), to promote the conservation of biodiversity and promote local water quality enhancement (Seaman and Jensen, 2000; Becker et al., 2018).

These artificial reefs function to provide a vertical relief, which has been shown to attract fish species (Rilov and Benayahu, 1998, 2000), and the presence of internal cavities to provide habitat for fish of all life history stages (Kellison and Sedberry, 1998; Wilhelmsson et al., 2006; Blount et al., 2021). This vertical substrate also promotes the growth of benthic fauna (such as sponges and corals), autotrophs (sessile algae), while also providing refugia for invertebrate fauna (Layman and Allgeier, 2020; Neely et al., 2021; Higgins et al., 2022).

Existing MMS in the marine environment, and in particular the subsea components of oil and gas platform jackets (hereafter referred to as 'platforms') have a finite operational lifespan, often in the dozens of years, and can during that time recruit extensive biotic communities (Sammarco et al., 2004; Coolen et al., 2020; Madgett et al., 2023). After this operational lifespan, platforms are typically decommissioned, which involves the capping of wells, the removal of pipelines and, finally, the complete removal of the platform from the marine environment (Elrick-Barr et al., 2022). In recent decades, there has been an increasing research emphasis on exploring the conversion of MMS to artificial reefs as an alternative decommissioning option, a concept commonly referred to as Rigs-to-Reefs (RtR; Reggio, 1987).

1.3 From Rigs to Reefs

While the first planned RtR conversion was completed in 1979 (Macreadie et al., 2011), the merits of such conversions from MMS to artificial reefs have been widely debated (Baine, 2002; Salcido, 2005; Jagerroos and Krause, 2016). Key considerations outlined in the debate are the risk of leakage and contamination from structures, local maritime navigation safety, and the potential for facilitating species distribution expansion (Ounanian et al., 2020). The spread of introduced or invasive species, which have been documented to occur on some oil and gas platforms, is particularly concerning (Page et al., 2006; Braga et al., 2021), and consequently the conversion or removal of infrastructure (during transport) can facilitate the introduction of these taxa into new areas (Macreadie et al., 2011; Schläppy et al., 2021). There is also a view that these structures may merely serve to aggregate biota of various life history stages (adult, larval or juvenile), rather than enhance local biotic productivity (Macreadie et al., 2011; Jagerroos and Krause, 2016), a view that is relevant for artificial reefs and RtR conversions alike. However, this conversion process can have clear ecological benefits (Henrion et al., 2015) with some studies documenting a higher diversity at platforms than adjacent natural reef systems, mostly relating to fish diversity and abundance (Torquato et al., 2017; Harvey et al., 2021, Madgett et al., 2023). These structures can also provide habitat for complex depth stratified, benthic communities (Gallaway and Lewbel, 1982). Economically, this conversion process can also be beneficial by comparison to the complete removal of structures, which is forecast to cost £17 billion pound up till 2025 in the UK alone, or $210 billion in the United States

up to 2040 (van Elden et al., 2019). However, there are limited studies assessing the benefits of RtR conversions, and those that do are predominantly focused in the Gulf of Mexico, which has approximately 420 reefed platforms (Ajemian et al., 2015), or the North Sea (Picken et al., 2000).

The RtR framework outlines several strategic scenarios for the conversion of decommissioned platforms, which can be tailored to suit local natural resource management (NRM) (Figure 1.1). These scenarios include leave in-situ (unaltered), removal of the top section to facilitate safe shipping channels, with the top section either entirely removed from the marine environment (hereafter 'partial removal') or placed adjacent to the structure on the benthos (hereafter 'topping'). Alternatively, structures can be dissected at the base and either toppled in-situ, or removed and deployed at an optimal location to create an artificial reef (or tow and topple; Figure 1.1) (Macreadie et al., 2011; Henrion et al., 2015; Fowler et al., 2018; Sommer et al., 2019). While these options for decommissioning platforms are gaining acceptance with regulators, with an estimated 12,000 platforms globally, it is unlikely these will all be required, or suitable, for RtR conversion (van Elden et al., 2019). Determining the suitability of oil and gas platforms for conversion requires, in addition to other non-biological considerations, comprehensive assessment to determine the impacts to extant and surrounding biotic communities. This process can identify platforms that have extant populations of target taxa and therefore identify those that could potentially facilitate the expansion of invasive species (Page et al., 2006; Sammarco et al., 2010; McLean et al., 2022). Similarly, this assessment can inform the potential for impact to populations of conservation significant present (Robinson et al., 2013; Friedlander et al., 2014).

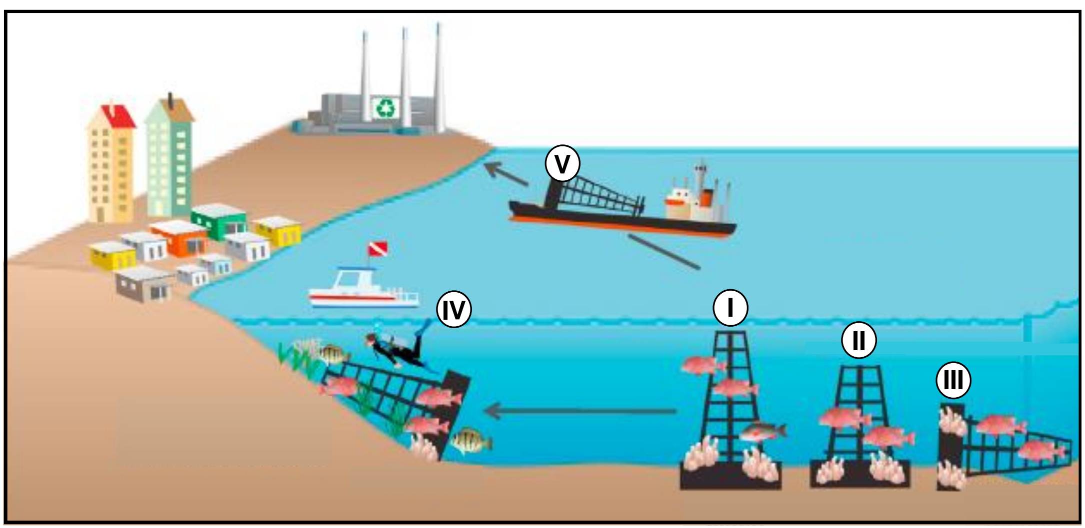

Figure 1.1: Decommissioning options commonly referred to in the RtR debate for oil and gas platforms, which typically occurs after the removal of topsides and equipment that has been in contact with hydrocarbons, such as piping and valves.
Decommissioning options include Leave *in-situ* (unaltered; I), partial dismantle to facilitate safe passage of shipping with the top section removed entirely or placed adjacent on the benthos, known as 'topping' (II), topple *in-situ* (III), cut and tow the platform for deployment as an artificial reef in an area selected for ecological or economic gain (IV), or the complete removal of all associated infrastructure from the marine environment (V), Figure adapted and reproduced with consent from Sommer et al. (2019).

Current methods of censusing biota associated with oil and gas infrastructure have involved the morphological identification of taxa, such as photographic sampling (Page et al., 2006), the analysis of video footage from ROV (Harvey et al., 2021), SCUBA underwater visual counts (Consoli et al., 2013), or the morphological analysis of scraped or suctioned epibenthic fauna (Page et al., 2007). These methods target specific taxa, generally fish, coral or invasive species (Torquato et al., 2017; Braga et al., 2021; Bull et al., 2023), and as such are unable to provide a broad tree-of-life or ecosystem approach to monitoring.

1.4 Monitoring diversity using environmental DNA (eDNA) metabarcoding

Environmental DNA (hereafter 'eDNA'), which is defined as any "*DNA that can be extracted from environmental samples (such as soil, water or air), without first isolating any target organisms*" (Taberlet et al., 2012). This DNA can be deposited in the environment through biological processes such as shedding, defecation, release of mucous, trophic level interactions (such as predation), or can comprise the collection of whole organisms, such as plankton or bacteria, within environmental samples

(Barnes and Turner, 2016; Compson et al., 2020). While eDNA has been isolated for decades for the purposes of exploring bacterial communities (Ogram et al., 1987), or monitoring phytoplankton (referred to as particulate DNA; Boehme et al., 1993), these early studies comprised of detections of single taxa that were isolated and identified based on the amplification of a known, taxonomically diagnostic region, or barcode region. Since the development of metabarcoding technologies, the prevalence and applications of eDNA in the literature have increased yearly (Jarman et al., 2018; Takahashi et al., 2023).

The emergence of eDNA metabarcoding, which uses next generation sequencing (NGS) technologies to facilitate the production of millions of amplicon sequence reads in parallel (Reuter et al., 2015), has resulted in a slew of applications for environmental monitoring (Deiner et al., 2017). In the marine environment, eDNA metabarcoding proof-of-concept studies first occurred in 2012 to document fish (Thomsen et al., 2012) and marine mammals (Foote et al., 2012). Since then, literature has grown yearly with applications for target species detections, such as introduced species and distribution tracking (Ardura et al., 2015b; Holman et al., 2019; Bowers et al., 2021), the detection of morphologically cryptic or rare taxa (Nester et al., 2020; Xia et al., 2021), diet analysis (Berry et al., 2017), ballast water analysis (Ardura et al., 2015a) and the detection of ecologically or economically important species (Bracken et al., 2019). The development of broad-target or "universal" metabarcoding assays (Leray et al., 2013; Pochon et al., 2013), has facilitated greater breadth of taxonomic detections from every sample, allowing for broad biomonitoring across the tree-of-life (Stat et al., 2017), or more broadly within target groups (Miya et al., 2015; Alexander et al., 2019; West et al., 2021). As such, eDNA methods provide a cost efficient, non-invasive, and highly sensitive method of detection with the potential for fine scale spatial (Jeunen et al., 2020), temporal (Berry et al., 2019) and taxonomic scalability (depending on completeness of reference databases). In eukaryotic biomonitoring, eDNA is proving to be a beneficial tool, either as a complement to conventional methods, such as those that rely on the characterisation of morphological characteristics (Alexander et al., 2019; Pereira et al., 2021), or as a stand-alone method.

Despite the well documented success of marine eDNA metabarcoding, a number of limitations are also acknowledged in the scientific literature. These include the risk for

field and laboratory contamination and the potential of false positives and negatives (Murray et al., 2015). The stochasticity of eDNA detections is also documented, resulting from the heterogeneous distribution of DNA in the marine environment (Jensen et al., 2022). While these can be, in part, mitigated by the implementation of field and laboratory controls, they cannot be completely eliminated. Additionally, and this is particularly relevant surveys for fish and elasmobranch, eDNA methods are currently unable to provide robust, quantitative population abundance or biomass data (Fonseca, 2018), which are commonly used and accepted metrics (Zintzen et al., 2012; Schobernd et al., 2014; Schramm et al., 2021). Environmental DNA study design can also be problematic as results are highly influenced by a range of factors, such as substrate selection, sampling method selection, local environmental conditions (e.g., temperature, pH, tidal influences, amongst many), season, assay selection, and selected quality filters, making the reproducibility of results challenging (Barnes et al., 2014; Murray et al., 2015; Antich et al., 2021).

Relevant to my research, eDNA methods have been under-utilised at MMS, artificial reefs and RtR conversions (Table 1.1) and therefore validation is required to understand the impact of environmental influences on DNA distribution and longevity at these structures. With optimisation at oil and gas platforms, in particular, eDNA metabarcoding methods can be utilised for decision making and impact assessment prior to decommissioning or RtR conversion. The simplification of field sampling methods, such as the reduction of in-situ secondary handling of samples (to reduce contamination) may aid in the uptake of eDNA as a mainstream industry and managerial tool.

Table 1.1: Studies identified applying eDNA metabarcoding approaches to MMS and artificial reefs.

Study	Location	Structure type	Substrate sampled	Target taxa (Barcode region)	Summary of major findings
Laroche et al., 2017	New Zealand	Floating Production Storage and Offloading (FPSO) platform	Sediment (transects)	Bacteria (16S rRNA), Eukaryota (18S rRNA)	Read abundance information of bacteria eRNA did not reveal any impact from the oil production activity. eDNA and RNA should be used for metabarcode monitoring.
Laroche et al., 2018	New Zealand	Floating Production Storage and Offloading (FPSO) platform	Sediment (transects)	Bacteria (16S rRNA), Eukaryota (18S rRNA), Foraminifera (18S rRNA)	Bacteria that degrade hydrocarbons were detected at impacted sites. Indicator taxa were specific to site conditions.
Cordier et al., 2019	Adriatic Sea	Platforms	Water and Sediment	Universal (CO1), Chordata (16S rRNA), Meiofauna, planktonic Eukaryota, benthic foraminifera (18S rRNA)	Assemblage changes along the distance gradient was obtained with the universal assay (18S). Some impact of platform activities on benthic and pelagic communities at very close distance closest to platforms (< 50 m).
Klunder et al., 2018	North Sea	Platforms	Sediment (transects)	Eukaryota (18S rRNA)	Number of benthic fauna families found eDNA metabarcoding on average three times higher than for the morphological approach. Differences more pronounced between transects rather than distance from platform.
Lanzén et al., 2021	North Sea	Platforms	Sediment	Universal (CO1), Eukaryota (18S rRNA)	Metabarcoding can complement to the current morpho-taxonomic approaches. Alpha diversity and community structure of both datasets correlated strongly with a physicochemical pressure index
Mauffrey et al., 2021	North Sea	Platforms	Sediment	Universal (CO1), Meiofauna (18S rRNA)	The metabarcoding and morphological data significantly changed with distance from platforms.

Study	Location	Structure type	Substrate sampled	Target taxa (Barcode region)	Summary of major findings
					Impact of the operational discharges was also detected
Mercaldo-Allen et al., 202	USA	Aquaculture Cage	Water	Fish (12S rRNA)	eDNA metabarcoding detected 42 fish species at the farm and reef. Six species were detected using both methods. Complementary approaches.
Sato et al., 2021	Japan	Artificial Reef	Water	Fish (12S rRNA)	High quantities of fish eDNAs at the ARs than at surrounding stations. eDNA and echo sounder had positive correlation between concentration and echo intensity.
Inoue et al., 2022	Japan	Artificial reef	Water	Fish (12S rRNA)	These results indicate that eDNA distribution is in follows fish distribution, (minimal movement by water). eDNA distribution reflects the spatio-temporal distribution of fish and also the degradation and transport of eDNA.
Krolow et al., 2022	Gulf of Mexico	Artificial Reef / Oil and gas infrastructure	Water	Fish (12S rRNA)	A higher species richness at artificial reefs versus paired control sites. Differences were detected between seasons, reef materials, and reef positions
Levy et al., 2023	Israel	Artificial Reef	Water and epibenthic scrape	Metazoa (CO1)	Tile tops had higher diversity and richness, however higher abundance of organisms on tile bottom.

1.5 Environmental DNA metabarcoding workflow

Given the scope for variability and adaptability within the metabarcoding workflow, as well as the detailed workflow outlined in subsequent chapters, this section is kept to a brief overview to provide context to terminologies and methods used throughout this research. Careful consideration is required when undertaking eDNA studies as the choices made at each processing step of the workflow can influence results (Figure 1.2; A-G), and therefore decisions should be made in relation to the study aims (Murray et al., 2015; Deiner et al., 2018; Mathieu et al., 2020; van der Loos and Nijland, 2020). Marine eDNA sampling has previously focused on the collection of water samples, which then require filtrations through a membrane of known pore size, a process that, depending on the volume and turbidity, can take considerable time (Takahashi et al., 2023). Alternatively, scraping of epibenthic (or bio-foul) colonisers, or sediment samples can be used to target different biotic assemblages (Figure 1.2-A). Collected samples, such as filtered water membranes or scraped organic material, are frozen or placed in stabilising agent to maintain DNA integrity until extraction, which generally occurs in a clean laboratory setting (Figure 1.2-B). DNA extraction is dependent on the type of sample collected and the laboratory workflow, typically samples that collect bulk material (scrapes and sediment) are homogenised thoroughly to mitigates bias in subsampling (Hestetun et al., 2021; Pawlowski et al., 2022). Assays, comprising one or more (if multiplexed) forward and reverse primer combinations, are applied to sample extracts and bind to conserved (or evolutionarily stable; Kocher et al., 1989) regions adjacent to the target barcode. These barcode regions, and primer binding sites, are then amplified using Polymerase Chain Reaction (PCR; Figure 1.2-C), resulting in the production of bulk synthetic copies (or amplicons) of the diversity present within that barcode region for each sample. In PCR, individual samples are combined with unique identifiers, which allow amplicons to be bioinformatically tracked back to the correct sample, later in the workflow. Based on the cycle threshold (CT) values, melt curves and delta-Rn (ΔRn) output of the PCR, samples are combined in equi-molar concentrations in a sequencing ‘library’ and sequenced using a NGS sequencer (Figure 1.2-D), such as the commonly utilised Illuminer Miseq (van der Loos and Nijland, 2020).

Raw sequencing files are taken and bioinformatically deconvoluted and demultiplexed (Figure 1.2-E), which sorts amplicons by sample and removes primers/sequence identifiers (Mousavi-Derazmahalleh et al., 2021). Quality filtering parameters are then bioinformatically applied to amplicons, though programmes such as DADA2 (Callahan et al., 2016) or OBITools (Boyer et al., 2016), which tracks and removes the likelihood of erroneous sequence reads based on stringency of filtering parameters, and produces a matrix of unique sequences by sample. Unique sequences, otherwise known as amplicon sequence variants ('ASVs') or zero-radius operational taxonomic units ('zOTU'), are then aligned with taxonomically identified reference material in a database (Figure 1.2-F), with the publicly available National Center for Biological Information's GenBank Nucleotide Database (or 'GenBank') a readily available choice in marine studies (Ardura, 2019). These taxonomically assigned sequences can then feed into research specific analysis (Figure 1.2-G). Alternately these unique sequences can be analysed as ASVs or bioinformatically clustered based on percentage similarity (molecular operational taxonomic units, or 'mOTUs'), according to available literature, as analysis independent of taxonomy, which can be preferred in studies where there is little supporting taxonomic framework (such as bacteria, viruses or diatoms; Apothéloz-Perret-Gentil et al., 2017; He et al., 2019).

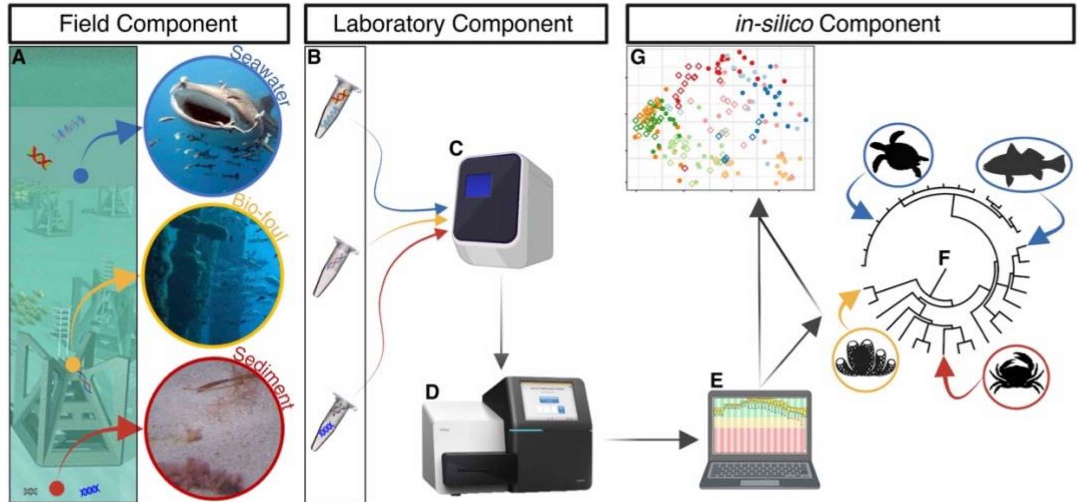

Figure 1.2: Schematic of the marine eDNA metabarcoding workflow starting with collection of environmental samples through to analysis of diversity.
The collection of eDNA sample collection commonly includes substrates such as water collection, bio-foul or sediment (A). DNA is extracted from environmental samples within a dedicated clean laboratory (B) and explored, amplified and combined with MID-tags using PCR (C) before being combined into sequencing libraries in equi-molar concentrations and then sequenced (D). Raw sequences are bioinformatically demultiplexed and quality-filtered (E) with the resulting ASVs/zOTUs aligned with a reference dataset to provide taxonomic resolution (F), or clustered based on known percentage differences in the target barcode region. This output can then be used in biodiversity analysis (G). Figure created with BioRender.com

1.6 Filling the Gap - Research question and thesis overview

The emergence of eDNA metabarcoding provides unique opportunities, driven by the requirement for a cost-effective sampling method for whole ecosystem monitoring (Berry et al., 2021). Applications in marine research have been well documented, and there is an ongoing transition from proof-of-concept studies to validation studies, commonly with an emphasis on comparison with conventionally used methods (Hajibabaei, 2022). However, the transition from a novel research approach through to an applied toolkit available to industry, with outcomes that are accepted in regulation (Bunce and Freeth, 2022) has lagged. Areas involving targeted species detections from eDNA, such as fisheries and biosecurity, have been adopted more readily (Ardura et al., 2015a; Bowers et al., 2021; Jerde, 2021). However, broad industry survey requirements, such as in baseline surveys, environmental impact assessments, as well as government approvals and compliance monitoring, rely predominantly on conventional methods (Figure 1.3). This is relevant to oil and gas infrastructure which is inherently difficult to sample already (due to depth profiles), and in particular, active platforms where access logistics can be challenging (health,

safety and security protocols). Furthermore, broad taxonomic censusing of platforms is difficult as few eDNA collection methods have been applied at these structures. As such the optimal methods, or combination of methods, are unknown. The optimisation of eDNA metabarcoding at oil and gas infrastructure is required to facilitate detections of spatial and temporal diversity changes, which can then be used to inform decommissioning and its impacts to biota. Furthermore, innovation in sampling is required to facilitate the uptake of field sampling by non-eDNA professionals (such as industry personnel). This includes research into eliminating the time consuming field processing of samples (such as the time-consuming filtration of water), thereby reducing the requirement for field laboratory spaces (a source of field contamination) and reducing the secondary handling of samples *in-situ*, which consequently can further reduce contamination risks.

The overarching question behind this research, therefore, is "Can eDNA metabarcoding be optimised for broad diversity monitoring at oil and gas infrastructure?" (Figure 1.3), with aspects of this question addressed across four data collection chapters (Chapters 2 – 5). These chapters have been written for publication, and are currently either in-preparation, in-review, or published.

In Chapter 2, I explore seven methods of eDNA sampling, targeting two different substrates. The aims of this chapter are, through the comparison of novel and existing sampling methods, to explore the detection capabilities and overlap of detections inherent to each method and substrate and the ability of each method to detect fine-scale depth distribution patterns. This chapter has been published in peer-reviewed journal *Estuarine, Coastal and Shelf Science* (Alexander et al., 2023).

Chapter 3 involves the field application of eDNA sampling methods at seven oil and gas platforms as well as five comparative sediment locations, within the Gulf of Thailand (GoT) to census fish assemblages. Comparing ROV transects to historical eDNA water samples, I explore the ability of eDNA methods to be used as a stand-alone technique in documenting the vertical (fine-scale spatial) changes in fish assemblages, as well as broad-scale spatial differentiation between platforms and non-platform locations. Chapter 3 has been published in peer-reviewed journal *Marine Environmental Research* (Alexander et al., 2022).

In Chapter 4, I apply a multi-assay and multi-substrate approach to holistically census eight platforms in the GoT, and explore the potential for taxonomic scalability across the eukaryotic tree-of-life using eDNA metabarcoding. I use this combined dataset to explore spatial patterns, determine if taxonomic resolution is sufficient to inform diversity trends, and make predictions about the hypothetical outcomes for biodiversity under a range of decommissioning scenarios (Figure 1.1). This chapter is been submitted for review with *Science of the Total Environment*.

Lastly, in Chapter 5, I explore the ability of eDNA sampling methods to document spatio-temporal changes in chordate communities that occur over a two-year period at an integrated artificial reef, which incorporates repurposed oil and gas infrastructure. In this study I explore temporal detections at the artificial reef and adjacent habitats, to understand the range of chordate species inhabiting the reef with wider regional context. In particular, I explore the timeframes over which the artificial reef might provide value as a habitat for recreationally important fish species, as it was deployed for this purpose.

The discussion (Chapter 6) provides a synthesis of findings from all data collection chapters, outlines the limitations inherent within this research, integrates the works in this thesis within the wider literature, and identifies remaining knowledge gaps and future research potential and applications for eDNA at oil and gas infrastructure.

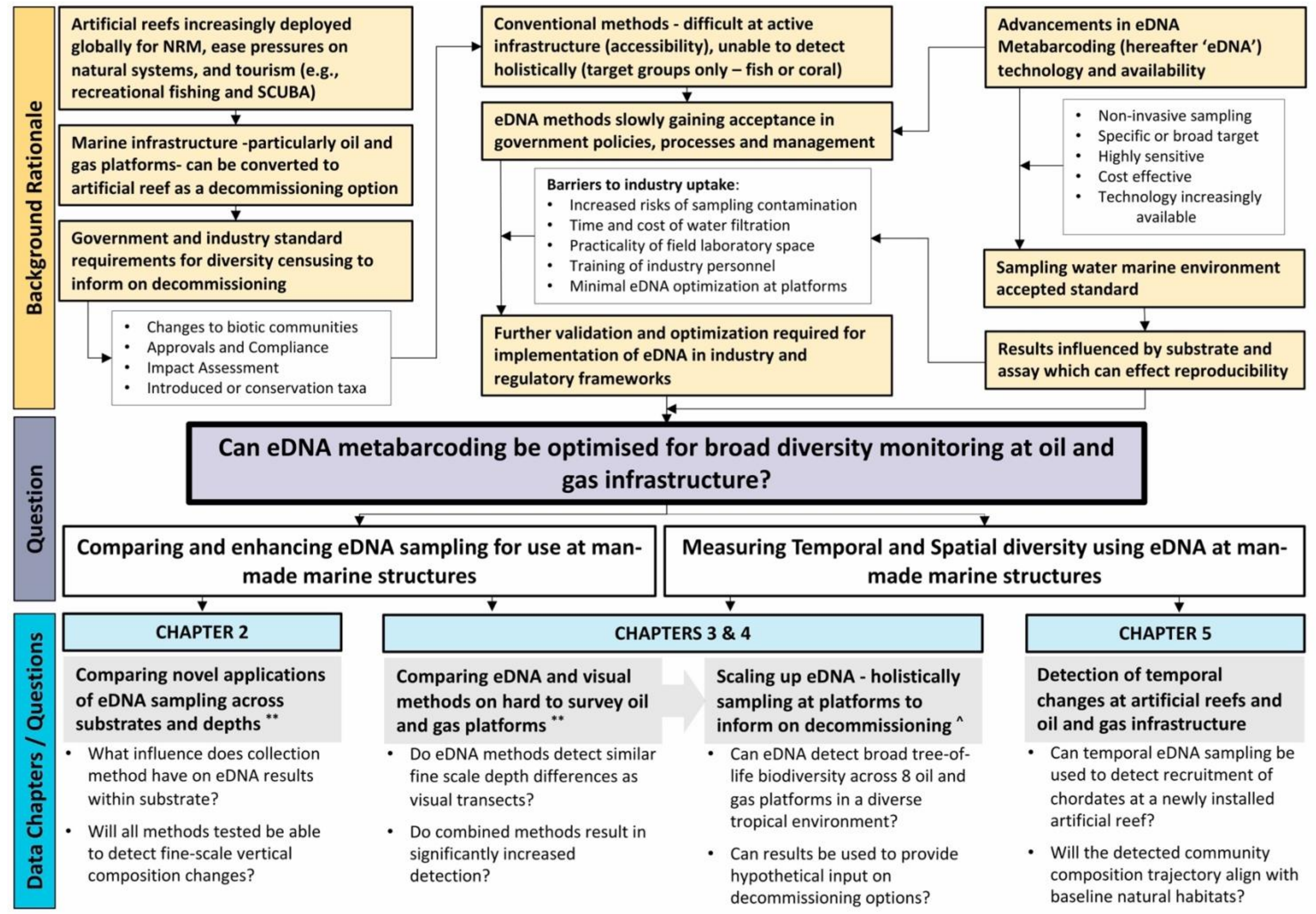

Figure 1.3: Flow diagram outlining the conceptual flow, themes and rationale found within this thesis.
(**chapter published, ^ chapter submitted for review)

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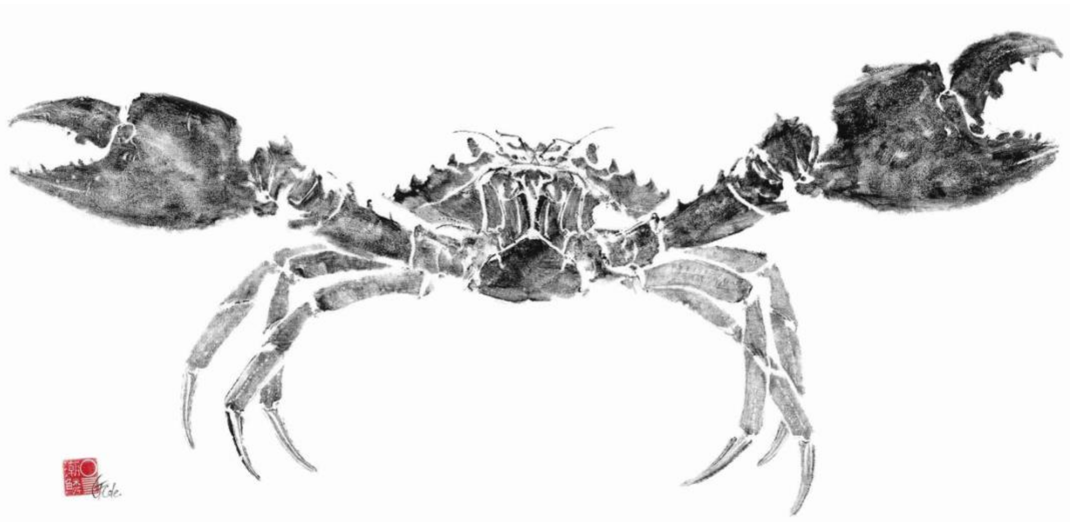
Chapter 2

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



Mudcrab Defense
Traditional methods of Gyotaku involves use Sumi ink (derived from soot) and Japanese paper made by hand using fibres from local plants (washi). Ink is applied directly onto the surface of the fish, or in this case crab, covering one full side and all of the features. Once coated, the sheet of washi is pressed against the fish. Every part must be applied to the paper to transfer the ink and create the effect. The washi is then removed to reveal an imprint of the animal showing its true size and features. (Text adapted from website)
Image provided and reproduced here with consent from Chris Cole of Tidal Scales (tidalscales.com.au)

2.1 Preface

This chapter consists of a published manuscript titled '**Comparing environmental DNA collection methods for sampling community composition on marine infrastructure**' which has been published in *Estuarine and Coastal Shelf Science*. The content in this chapter is the same as the published manuscript, with minor editorial changes to accommodate the thesis format.

The use of Environmental DNA metabarcoding is increasing, particularly in marine environments. There is a gap in the literature comparing and validating eDNA collection methods for different substrates. This study explores and compares the eDNA diversity collected by seven methods from two substrates at a decommissioned jetty. This chapter demonstrates the importance of informed study design when planning eDNA studies.

Alexander, J. B., Marnane, M. J., Elsdon, T. S., Bunce, M., Songploy, S., Sitaworawet, P., et al. (2022). Complementary molecular and visual sampling of fish on oil and gas platforms provides superior biodiversity characterisation. *Marine Environmental Research*, 105692. doi: 10.1016/j.marenvres.2022.105692.

2.1.1 Acknowledgements

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I would like to acknowledge the support and contributions of the co-authors of this chapter, Michael Marnane, Justin McDonald, Sherralee Lukehurst, Travis Elsdon, Tiffany Simpson, Shawn Hinz, Michael Bunce and Euan Harvey. A breakdown of author and co-author contributions is provided in Appendix I.

2.1.2 Permits

The field sampling for this study was completed under the Department of Primary Industries and Regional Development exemption number 3545v2.

2.1.3 Data accessibility

The unfiltered demultiplexed metabarcoding data from this chapter is available for download from Zenodo data repository (https://doi.org/10.5281/zenodo.7142167). Images and quadrat photos used in the morphological analysis as well as supporting presence/absence, site by family taxonomy matrices is available from: https://drive.google.com/drive/folders/1KDkQFjmOj6e7Eb27_jsYiVqsFsYFs0zE?usp=sharing

2.2 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.

2.3 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 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; van der 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 are 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.

2.4 Methods

2.4.1 Study area

The decommissioned Kwinana Bulk Jetty (hereafter KBJ) is located approximately 29.7 km south of Perth, Western Australia (Figure 2.1). 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 (Figure 2.1). All sampling occurred between the 20th and 23rd of October 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.

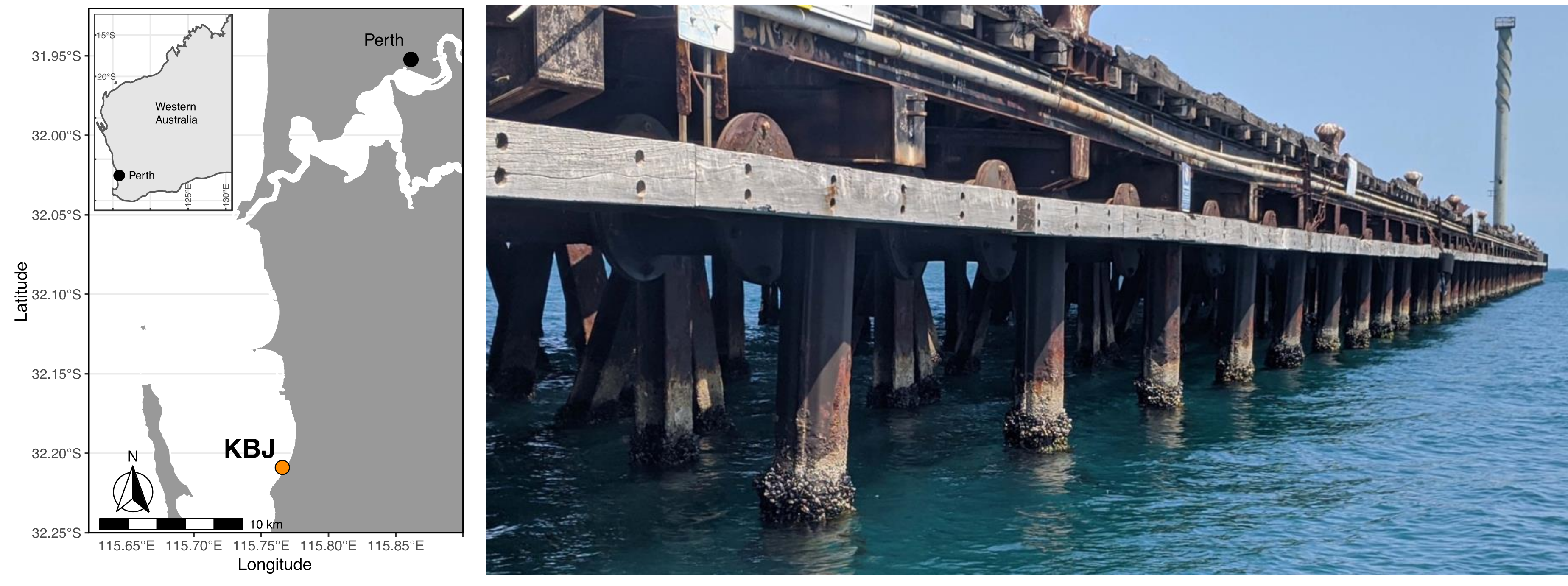

Figure 2.1: Locality (left) of the KBJ in relation to the Perth central business district, Western Australia (left inset).
Sample collection included eight methods of data collection on the north-facing surface of 15 pylons (right), at 0 m and 8 m depth below water surface between the 20th and 23rd of October, 2020.

2.4.2 Field sampling

2.4.2.1 Visual/morphological sample collection (quadrats)

Thirty 10 x 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 minutes 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 S2.1). 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.

2.4.2.2 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 minutes, where equipment was then air-dried before being exposed to UV radiation for a further 15 minutes. 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.

2.4.2.3 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 S2.2). These units, which are primarily produced as vapor collection substrates, were sterilised using an industrial autoclave set to 121°C for 30 minutes prior to sampling and placed into compressed (for ease during sampling) Ziplock bags and exposed to UV light for a minimum of 15

minutes. 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 (Supplementary S2.2). This sampling method was completed concurrently with the plankton tows (see below for sampling details).

2.4.2.4 Plankton tows

Plankton nets were custom made from 50 μm nylon mesh. The tapered nets (Supplementary S2.2) 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).

2.4.2.5 Quadrat swabs

Swab samples were collected using a 17 x 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.

2.4.2.6 ROV scrapes

ROV scrapes were completed using a standard SRV-8 ROV from RJE Oceanbotics (California, USA), attached by a 300 m tether (Supplementary S2.2). 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.

2.4.2.7 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; Supplementary S2.2). The surfaces are cleaned using a series of replaceable brushes to loosen bio-foul material, this was then vacuumed through a nylon 34 x 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-hour period.

2.4.2.8 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.

2.4.3 Laboratory processing

2.4.3.1 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 2.1, were

sterilised in 10% bleach solution and UV radiation, both for a minimum of 15 minutes. DNA digestion 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 sterilization 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.

Table 2.1: 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		900 / 100	All samples digested in rotation at 56°C
	• PM100 Planetary Ball Mill (Retsch; Germany); • stainless steel grinding jar; seven 20 mm stainless steel balls.	• 250 rpm; • 1 minute reversing intervals; • 4 to 12 minutes total.		
	B - 120 mg of centrifuged lysate digested			
Keel Crab and ROV scrape	A - Sample lysed		900 / 100	
	• TissueLyser (Qiagen; Netherlands)	• 30 Hz setting in 30 second intervals; • 90 to 180 seconds		
	B - 120 mg of centrifuged lysate digested			
Swab	A - Half swab dissected per sample and digested		540 / 60	
Water	A - Samples filtered.		540 / 60	
	• Pall microbiology pump; • 47 mm, 0.22 µm polyethersulfone filters (Pall Life Sciences; USA)	-		
	B - Half filter dissected per sample and digested.			
PUF tow	A - PUFs loaded into sterile syringe and flushed with 400 mL milli-Q water.		540 / 60	
	• 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)	-		
Plankton tow	A - nets flushed with milli-Q to concentrate eDNA.		540 / 60	
	B - last 3 cm removed and digested			

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).

2.4.3.2 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 minutes, 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.

2.4.4 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 Center 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 7,500 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.

2.4.5 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 (PCO) and Constrained Analysis of Principal Coordinates (CAP; 9,999 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 9,999 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 variation in community composition plateau (Anderson and Santana-Garcon, 2015).

2.5 Results

2.5.1 PCR, sequencing and bioinformatic statistics

Extract concentration varied considerably between the methods (Figure 2.2) and depths within method (Supplementary S2.3), 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 CT values showed that the PUF tow samples had earlier amplification in the exploratory PCR (Figure 2.2), 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 (Figure 2.2; Supplementary S2.3).

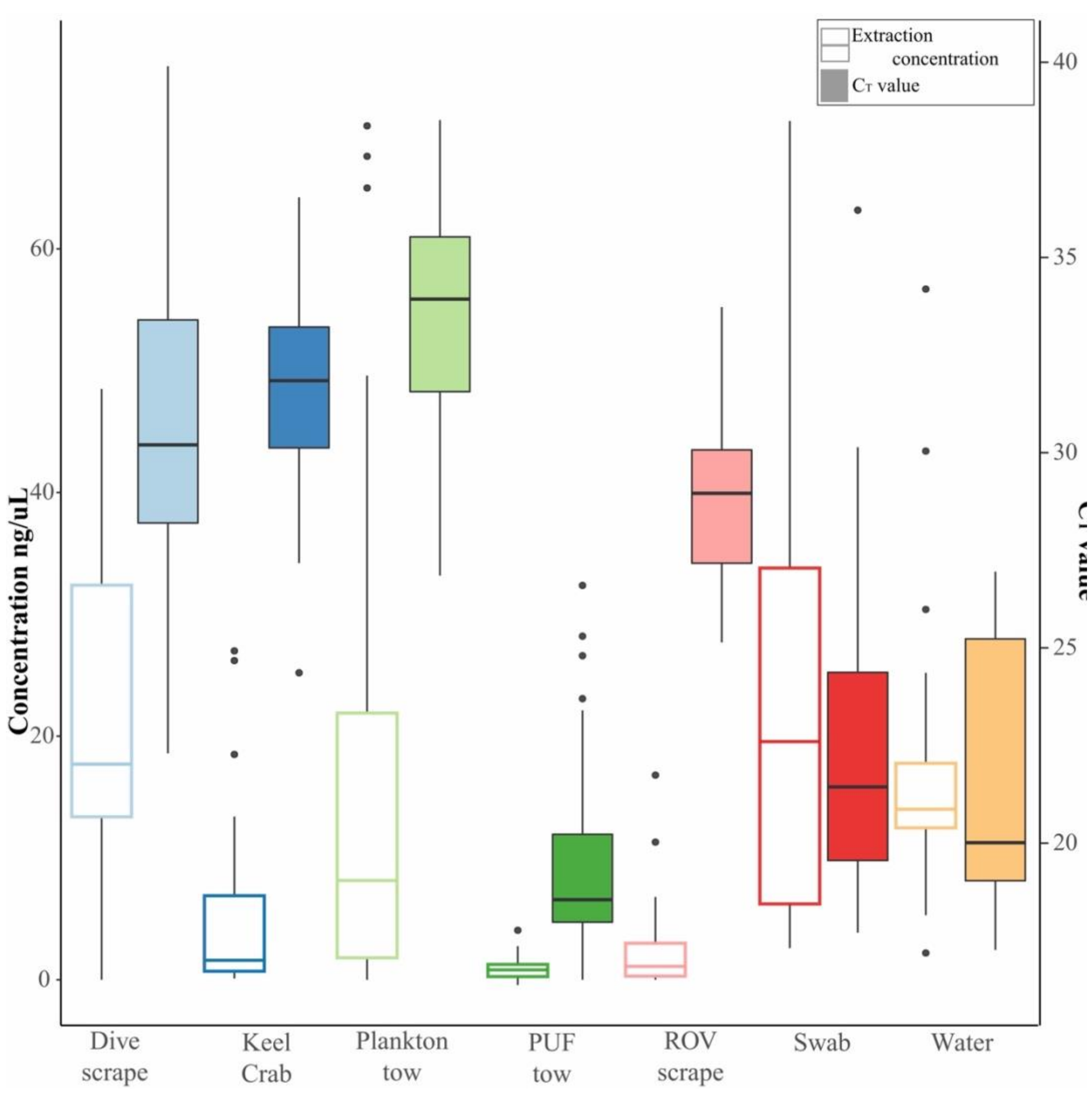

Figure 2.2: Summary of eDNA collected using each sampling method by concentration (ng/µL) with outliers trimmed above 80 ng/µL (swab only), and the corrected C_T values per method of untagged eDNA as an indicator of how much DNA product was available for use by the 18S sequencing assay.

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 ± 6,648.95), however ranged by method from 31,827 (SE ± 2,492; water), to 232,065 (SE ± 18,855; Keel Crab) (Supplementary S2.4 and S1.5). ASVs per method ranged between 681 (dive scrape) total detected and 2,183 (PUF tow), however 81% of the reads were unable to be assigned to family taxonomic level (Supplementary S2.6). Following the subsampling, 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.

2.5.2 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; Figure 2.3) 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.

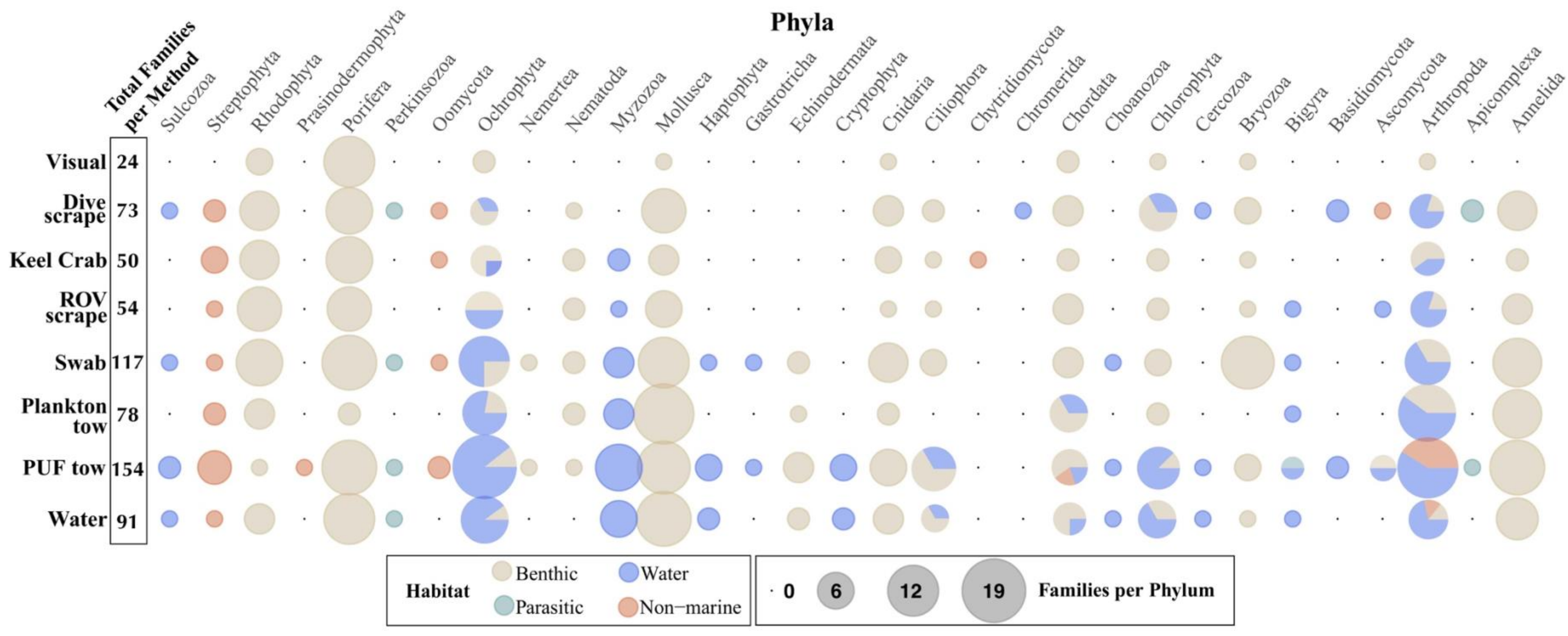

Figure 2.3: **Total number of families detected per phyla using the 18S universal assay across all eDNA sample collection methods, as well as the visual method used, and proportion of habitat types detected.**

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 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=9,697). 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=7,724), and between 0 m and 8 m depth with the ROV method (t=1.17, P(perm)=0.119, Unique Perms=9,853) (Supplementary S2.7 to S1.9). These results are corroborated by constrained and unconstrained ordination (Figure 2.4). The leave-one-out allocation analysis (Supplementary S2.10) 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 (Supplementary S2.10). 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

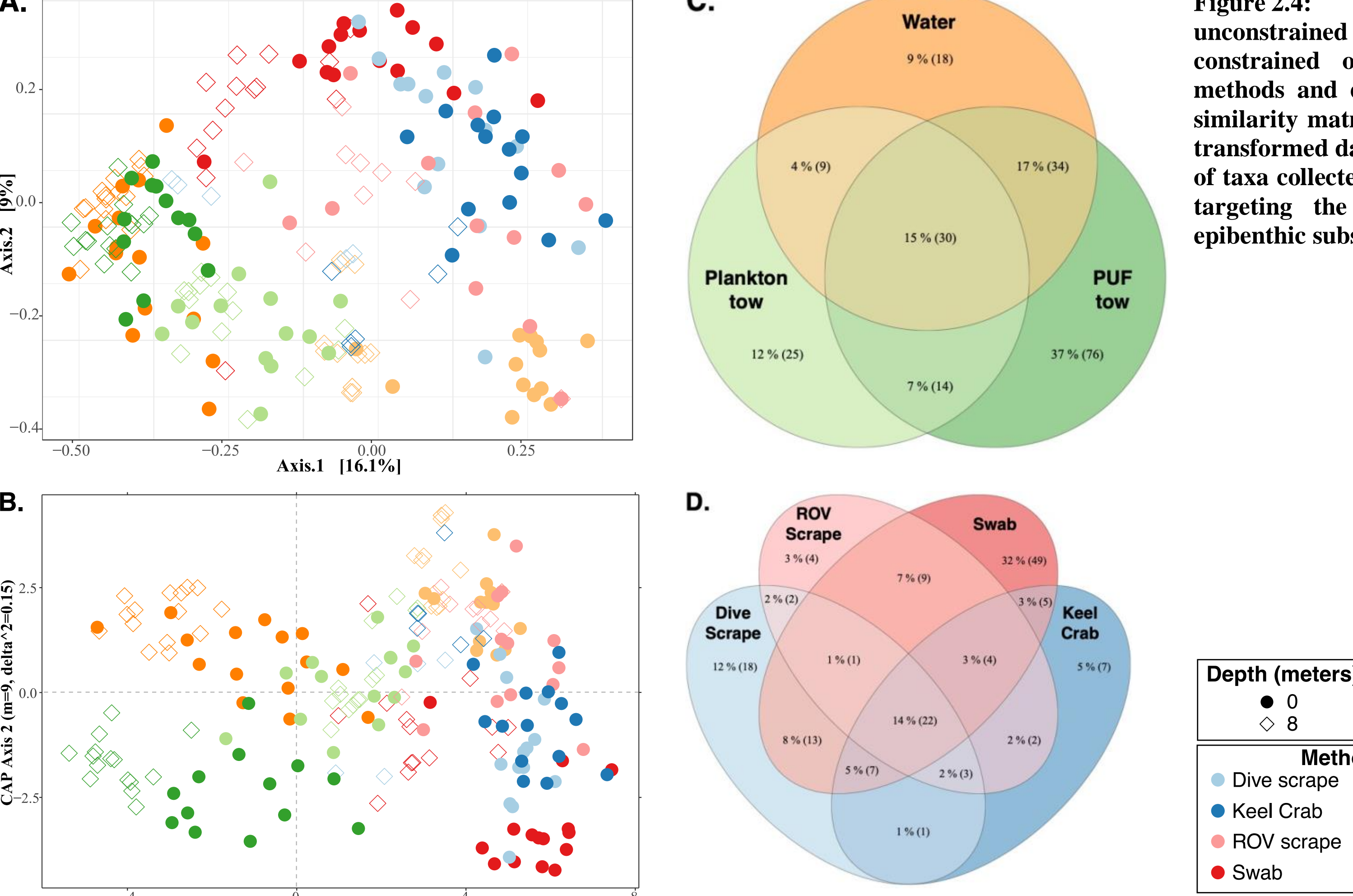

Figure 2.4: Comparison of the unconstrained ordination (PCoA; A) and constrained ordination (CAP; B) for methods and depth based on a Jaccard similarity matrix of the presence/absence transformed data, as well as a comparison of taxa collected detected by each method targeting the water column (C) and epibenthic substrates (D).

The characterisation of methods and depths were completed using a similarity percentage (SIMPER) analysis (Supplementary S2.11), 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 (Figure 2.5). 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 – 25 samples and therefore additional sampling will not greatly increase community composition (Figure 2.5).

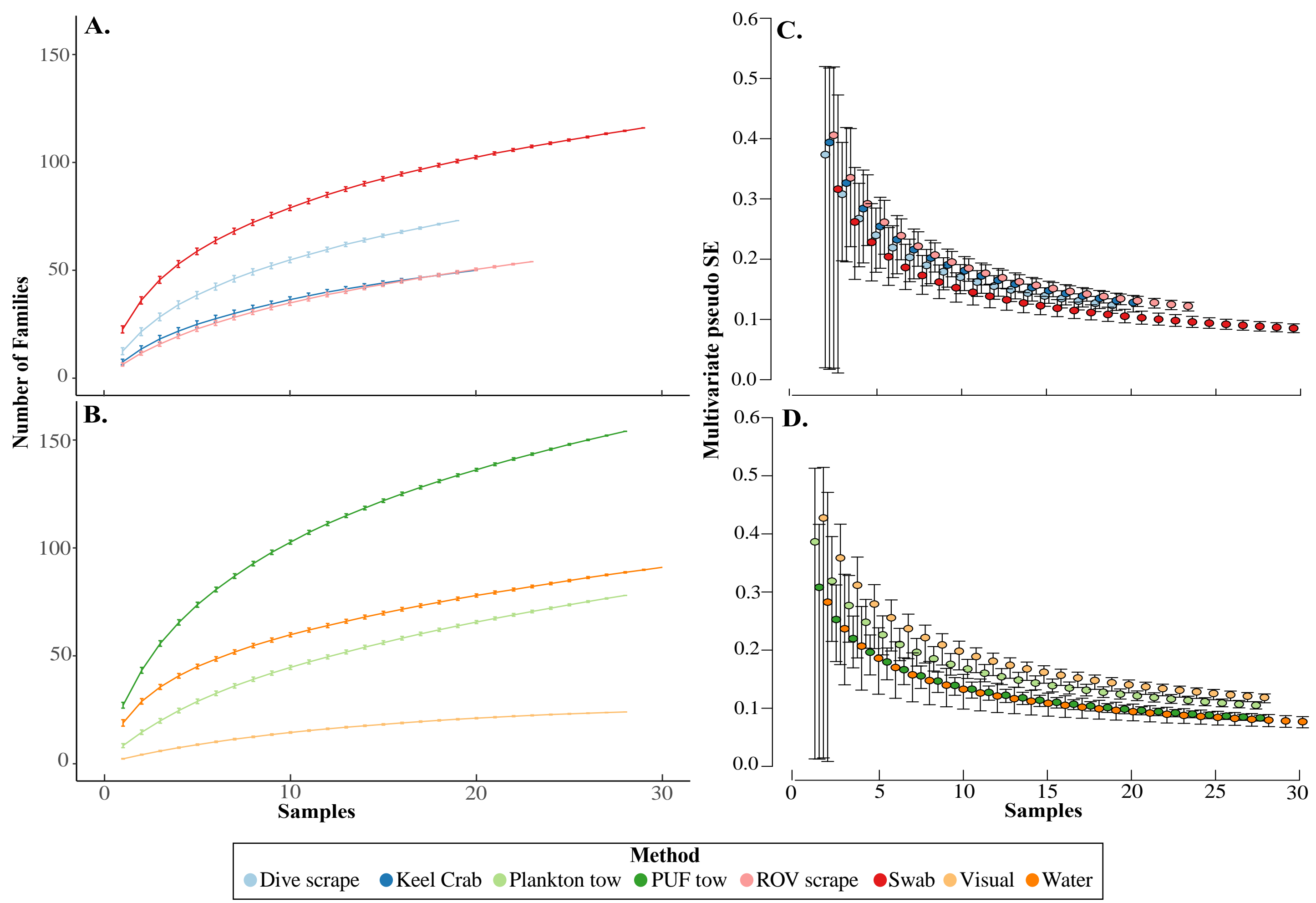

Figure 2.5: Family level observed accumulation curves for each sampling method (A-epibenthic sampling methods; and B-water column and visual) and the corresponding result of dissimilarity-based multivariate analysis displaying the mean multivariate pseudo standard error (right, C and D) based on Jaccard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

2.6 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 1,000 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 utilising 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.

2.7 References

Every reasonable effort has been made to acknowledge the owners of copyright material. I would be pleased to hear from any copyright owner who has been omitted or incorrectly acknowledged.

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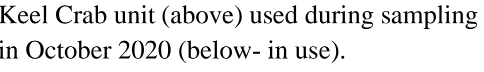
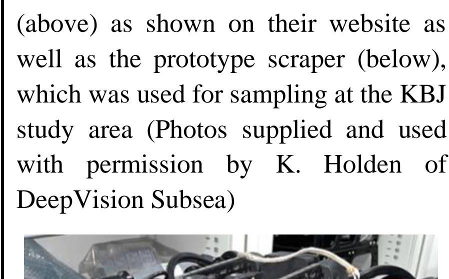
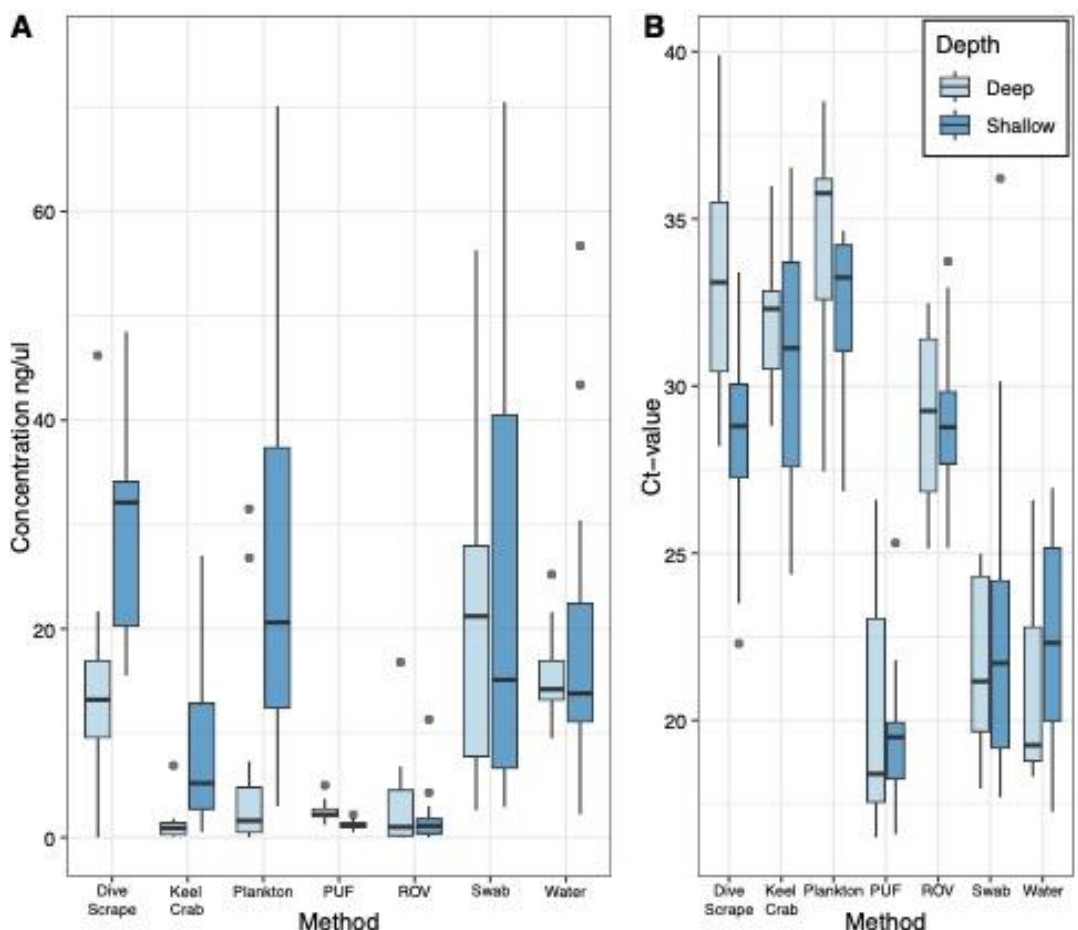
2.8 Supplementary information

Supplementary S2.1: Link to photographs of quadrat plates used in visual identification.

https://drive.google.com/drive/folders/1KDkQFjmOj6e7Eb27_jsYiVqsFsYFs0zE?usp=sharing

Polyurethane foam (PUF) sampling tow funnel with PUF units indicating where, within the unit, they would be positioned during sampling and a single PUF unit. Each unit is 76 mm long x 38 mm diameter (Tisch Environmental; USA).	Plankton sample tow funnel with plankton net attached.	Keel Crab unit (above) used during sampling in October 2020 (below- in use). 	SRV-8 ROV from RJE Oceanbotics (above) as shown on their website as well as the prototype scraper (below), which was used for sampling at the KBJ study area (Photos supplied and used with permission by K. Holden of DeepVision Subsea) 

Supplementary S2.2: Specialised equipment used in eDNA collection methods at the Kwinana Bulk Jetty study area, comprising (left to right): PUF tow sampler; Plankton tow sampler; the Keel Crab unit; and the ROV (above) and prototype scraper (below).
Photos only included of sampling methods requiring specialised equipment.



Supplementary S2.3: Summary of eDNA concentrations (ng/µl; A.) by sampling method by at both shallow and deep sampling depths as well as the spread of corrected CT values per method and depth of untagged eDNA (B.) as an indicator of how much DNA product was available for use by the 18S sequencing assay.

Supplementary S2.4: Link to raw, demultiplexed sequence data (stored on Zenodo data repository).

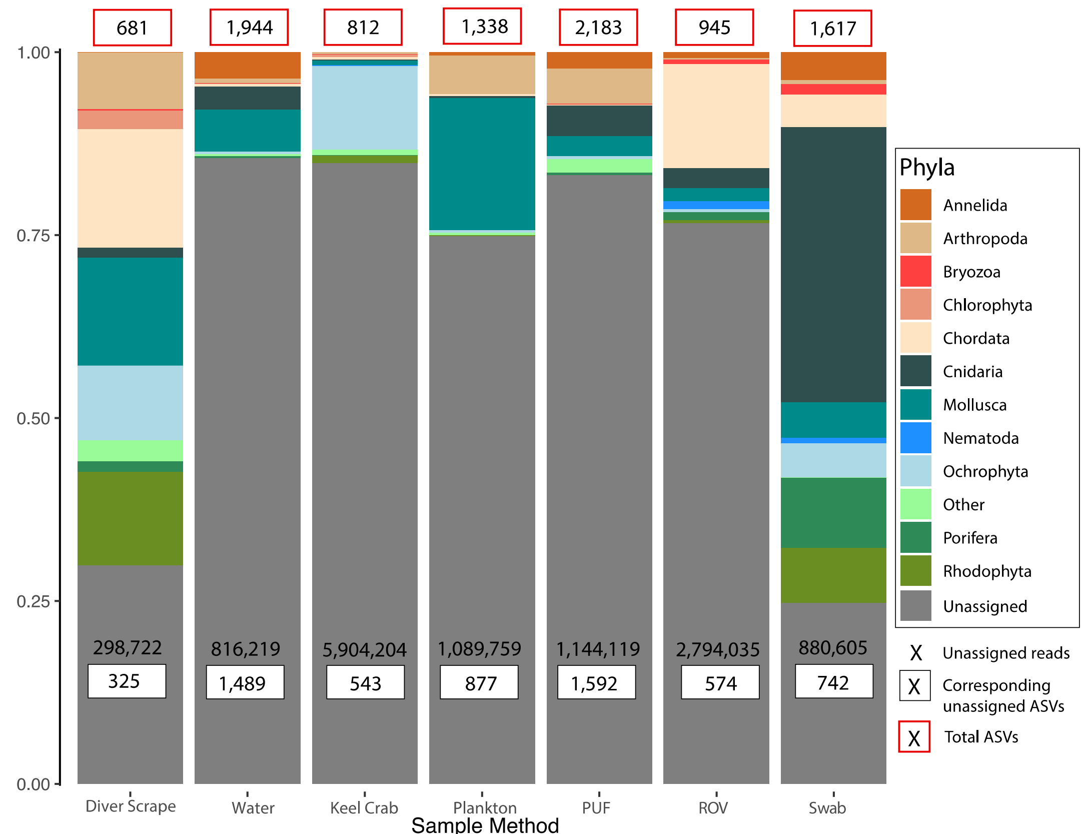
DOI:	https://doi.org/10.5281/zenodo.7142167

Supplementary S2.5: Table of mean sequencing results for quality filtering, denoising, read merging and chimera removal for each method of eDNA collection.

Mean final reads as well unique ASVs were accounted for after subsampling, data vetting and amalgamation of the dataset at the family level using the 'taxglom' function in R. The table includes the number of samples removed prior to analysis due to lack of samples identifiable taxonomically to the family level.

	Raw Reads	Filtered	Denoised forward	Denoised Reverse	Merged	Chimera removal	Number unique ASVs	Final reads post LCA, manual vetting and control removal	Final merged ASVs	# Samples removed
Water										
Mean	248,865	221,632	219,940	219,831	205,419	164,150	500	31,827	20	0
SE	11,248	11,996	11,925	11,949	12,628	14,193	31	2,492	2	
Plankton tow										
Mean	225,409	198,855	197,818	197,695	192,345	150,587	278	48,553	8	3
SE	18,757	20,098	20,078	20,069	19,783	16,420	16	4,689	1	
ROV scrape										
Mean	227,999	199,841	198,173	198,191	188,888	175,099	157	121,612	5	7
SE	19,891	20,140	20,171	20,183	19,777	19,398	13	18,565	1	
Keel crab										
Mean	313,570	291,721	291,130	291,186	283,143	254,801	214	232,065	5	10
SE	19,731	20,543	20,511	20,521	19,898	18,641	15	18,855	1	
Swab										
Mean	290,136	276,705	269,829	269,335	247,455	208,606	206	118,738	22	1
SE	31,096	24,162	23,637	23,513	23,476	23,432	16	15,801	2	
Dive scrape										
Mean	372,084	327,679	326,452	326,853	305,657	270,400	63	33,333	8	11
SE	31,459	29,129	29,047	29,045	25,534	23,094	9	10,232	2	
PUF tow										
Mean	219,999	169,317	163,157	165,043	144,896	112,463	544	45,861	25	2
SE	17,375	17,022	16,318	16,566	14,909	12,269	28	5,897	2	

Supplementary S2.6: Proportional representation of ASVs assigned at the phyla taxonomic level after quality filtering showing the proportion of reads unassigned, and the number of unassigned reads and corresponding ASVs.



Supplementary S2.7: PERMANOVA results of the presence-absence transformed data on Jaccard resemblance matrix with an estimated percentage breakdown of component variation (%V) attributed to the different levels.

	Family				
	DF	**Pseudo-F**	**P(perm)**	**Perm.**	**%V**
Method	7	10.3	<0.001	9627	23.3
Depth	1	8.1	<0.001	9852	4.7
Method × Depth	7	3.2	<0.001	9629	10.8
Residual	189				61.2

Supplementary S2.8: Pairwise test for levels within the factor 'Method' in the term 'Method x Depth'. Non-significant results are designated by **.

Groups	Depth = 0			Depth = 8		
	t	P(perm)	Perm.	t	P(perm)	Perm.
Visual X Water	3.54	<0.001	9902	4.03	<0.001	9893
Visual X Swab	3.94	<0.001	9884	3.01	<0.001	9866
Visual X Dive scrape	2.75	<0.001	9891	1.91	<0.001	5889
Visual X Plankton tow	3.12	<0.001	9885	2.46	<0.001	9892
Visual X PUF tow	3.70	<0.001	9859	3.66	<0.001	9871
Visual X ROV Scrape	2.21	<0.001	9891	1.81	<0.001	9858
Visual X Keel Crab	2.88	<0.001	9897	1.22	0.081 **	7724
Water X Swab	3.06	<0.001	9878	3.35	<0.001	9854
Water X Dive scrape	2.65	<0.001	9886	2.56	<0.001	7296
Water X Plankton tow	2.10	<0.001	9889	3.58	<0.001	9878
Water X PUF tow	2.03	<0.001	9871	3.08	<0.001	9838
Water X ROV Scrape	2.48	<0.001	9866	3.24	<0.001	9878
Water X Keel Crab	2.90	<0.001	9892	3.49	<0.001	9039
Swab X Dive scrape	2.00	<0.001	9874	1.53	<0.001	6668
Swab X Plankton tow	3.02	<0.001	9864	2.35	<0.001	9875
Swab X PUF tow	2.76	<0.001	9836	2.83	<0.001	9838
Swab X ROV Scrape	2.40	<0.001	9901	1.87	<0.001	9847
Swab X Keel Crab	2.43	<0.001	9881	2.38	<0.001	8684
Dive scrape X Plankton tow	2.47	<0.001	9892	1.44	0.007	6629
Dive scrape X PUF tow	2.77	<0.001	9847	2.10	<0.001	7303
Dive scrape X ROV Scrape	1.52	<0.001	9894	1.40	0.004	2760
Dive scrape X Keel Crab	1.39	0.005	9881	1.62	<0.001	461
Plankton tow X PUF tow	2.45	<0.001	9873	2.93	<0.001	9852
Plankton tow X ROV Scrape	2.15	<0.001	9871	1.95	<0.001	9874
Plankton tow X Keel Crab	2.61	<0.001	9904	1.89	<0.001	8699
PUF tow X ROV Scrape	2.71	<0.001	9839	2.92	<0.001	9809
PUF tow X Keel Crab	2.99	<0.001	9847	2.97	<0.001	9022
ROV Scrape X Keel Crab	1.31	0.015	9876	1.37	0.004	5710

Supplementary S2.9: Pairwise test for levels within the factor 'Depth' in the term 'Method x Depth'. Non-significant results are designated by **.

Groups	Depth groups 0 X 8 m		
	t	P(perm)	Perm.
Visual	2.32	<0.001	9783
Water	2.24	<0.001	9897
Swab	2.22	<0.001	9887
Dive Scrape	1.61	<0.001	6691
Plankton tow	1.81	<0.001	9870
PUF tow	2.11	<0.001	9878
ROV	1.17	0.119 **	9853
Keel Crab	2.00	<0.001	8691

Supplementary S2.10: Leave-one-out allocation of observations to groups.

Color shading indicates the correct classification (green) of the leave-one-out allocation, mis-classification between depth of the same method (yellow), different methods targeting the same substrate (such as epibenthic to epibenthic; light orange: and water column to water column; dark orange) and mis-classification between methods targeting different substrates (such as epibenthic to water column; red).

		Method																Total	% Correct
		Visual		Water		Swab		Dive Scrape		Plankton tow		PUF tow		ROV scrape		Keel Crab scrape			
		0	8	0	8	0	8	0	8	0	8	0	8	0	8	0	8		
Visual	**0**	13	1															14	93
	8	1	10														3	14	71
Water	**0**			9	5					1								15	60
	8				15													15	100
Swab	**0**			1		12								1		1		15	80
	8					1	12			1								14	86
Dive Scrape	**0**	1				2		8						1		1	1	14	57
	8			1					2					1	1			5	40
Plankton tow	**0**									12	2							14	86
	8									1	10				1		2	14	71
PUF tow	**0**								1			12						13	92
	8												15					15	100
ROV scrape	**0**	3						1			1			5	1	2		13	38
	8	1	1				3							1	3		1	10	30
Keel Crab	**0**							2						2		9	1	14	64
	8		2											1			3	6	50
																Total correct		**150/205 (73.2%)**	
																Mis-classification error		**26.8%**	

Supplementary S2.11: Results of the similarity percentage (SIMPER) analysis completed on presence/absence transformed data with a Jaccard similarity on Method and Depth.

	Family	Average Abundance	Average Similarity	Sim/SD	Contribution (%)	Cumulative Contribution (%)
Visual	Overall - Average similarity: 14.45					
	Dictyotaceae	0.46	8.84	0.49	61.19	61.19
	0 m Depth - Average similarity: 37.29					
	Dictyotaceae	0.86	29.05	1.50	77.90	77.90
	8 m Depth - Average similarity: 10.01					
	Dysideidae	0.29	4.1	0.25	41	41
	Sycettidae	0.21	1.43	0.18	14.28	55.28
Water	Overall - Average similarity: 43.18					
	Euterpinidae	0.87	4.97	1.15	11.51	11.51
	Pyuridae	0.87	4.4	1.24	10.18	21.68
	Euterpinidae	0.87	4.97	1.15	11.51	11.51
	Pyuridae	0.87	4.4	1.24	10.18	21.68
	Thalassiosiraceae	0.8	3.05	1.21	7.06	28.75
	Styelidae	0.8	2.99	1.24	6.92	35.66
	Chattonellaceae	0.77	2.7	1.11	6.26	41.93
	Rhizosoleniaceae	0.73	2.37	1.03	5.48	47.41
	Chalinidae	0.73	2.37	1.03	5.48	52.9
	0 m - Average similarity: 35.63					
	Euterpinidae	0.93	8.88	1.49	24.92	24.92
	Pyuridae	0.87	6.48	1.21	18.18	43.1
	Solenidae	0.6	3.05	0.64	8.57	51.67
	8 m - Average similarity: 60.67					
	Styelidae	1	4.06	12.04	6.69	6.69
	Pectinidae	1	4.06	12.04	6.69	13.38
	Rhizosoleniaceae	1	4.06	12.04	6.69	20.07
	Thalassiosiraceae	1	4.06	12.04	6.69	26.76
	Chattonellaceae	1	4.06	12.04	6.69	33.46
	Chalinidae	1	4.06	12.04	6.69	40.15
	Mycalidae	0.93	3.51	2.47	5.79	45.93
	Dysideidae	0.93	3.49	2.48	5.75	51.69
Swab	Overall - Average similarity: 36.565					
	Scrupariidae	0.83	3.14	1.32	8.6	8.6
	Syllidae	0.76	2.39	1.09	6.55	15.15
	Mycalidae	0.69	2.29	0.89	6.25	21.4
	Mytilidae	0.72	2.1	0.99	5.75	27.15
	Euterpinidae	0.66	1.93	0.76	5.29	32.43
	Lepraliellidae	0.66	1.84	0.8	5.04	37.47
	Grantiidae	0.66	1.72	0.82	4.7	42.17
	Chromadoridae	0.59	1.64	0.69	4.49	46.65
	Styelidae	0.55	1.33	0.62	3.64	50.29
	0 m - Average similarity: 48.4					
	Scrupariidae	0.93	4.14	2.03	8.56	8.56
	Dictyotaceae	0.93	4.05	2.11	8.37	16.92
	Corynidae	0.87	3.36	1.53	6.93	23.86
	Mytilidae	0.87	3.26	1.58	6.73	30.59
	Lepraliellidae	0.8	2.86	1.2	5.91	36.5

	Family	Average Abundance	Average Similarity	Sim/SD	Contribution (%)	Cumulative Contribution (%)
	Ulvaceae	0.8	2.65	1.25	5.47	41.97
	Styelidae	0.73	2.51	0.97	5.2	47.17
	Laodiceidae	0.73	2.27	1	4.69	51.85
	8 m - Average similarity: 36.84					
	Syllidae	0.86	3.06	1.48	8.31	8.31
	Mycalidae	0.79	2.74	1.16	7.42	15.74
	Euterpinidae	0.71	2.45	0.85	6.64	22.38
	Grantiidae	0.79	2.4	1.18	6.52	28.9
	Pectinidae	0.71	2.32	0.85	6.29	35.19
	Chromadoridae	0.71	2.24	0.97	6.08	41.28
	Scrupariidae	0.71	2.17	0.94	5.88	47.16
	Cirratulidae	0.64	1.74	0.76	4.73	51.89
Dive scrape	Overall - Average similarity: 25.49					
	Dictyotaceae	0.68	4.03	0.74	15.81	15.81
	Styelidae	0.58	2.61	0.61	10.23	26.04
	Pyuridae	0.58	2.45	0.57	9.63	35.66
	Rhodomelaceae	0.53	2.33	0.54	9.13	44.79
	Mytilidae	0.58	2.15	0.65	8.43	53.22
	0 m - Average similarity: 33.57					
	Dictyotaceae	0.93	7.57	1.43	22.56	22.56
	Erythrotrichiaceae	0.71	3.54	0.89	10.55	33.1
	Mytilidae	0.71	3.44	0.91	10.24	43.34
	Styelidae	0.64	3.25	0.71	9.67	53.01
	8 m - Average similarity: 20.61					
	Pyuridae	0.8	5.17	1	25.07	25.07
	Euterpinidae	0.6	1.98	0.61	9.6	34.67
	Lepraliellidae	0.6	1.98	0.61	9.6	44.28
	Chlorellalesincertaesedis	0.6	1.98	0.61	9.6	53.88
Plankton tow	Overall - Average similarity: 22.7					
	Euterpinidae	0.93	11.62	1.44	51.18	51.18
	0 m - Average similarity: 28.77					
	Euterpinidae	1	14.98	2.59	52.06	52.06
	8 m - Average similarity: 26.31					
	Euterpinidae	0.86	8.41	1.07	31.97	31.97
	Spionidae	0.64	3.35	0.75	12.74	44.71
	Solenidae	0.64	3.2	0.77	12.17	56.88
PUF tow	Overall - Average similarity: 38.52					
	Euterpinidae	1	3.87	4.46	10.05	10.05
	Solenidae	0.93	3.35	2.1	8.71	18.76
	Telonemidaincertaesedis	0.89	3.02	1.75	7.83	26.59
	Pyuridae	0.79	2.3	1.19	5.97	32.56
	Naviculaceae	0.79	2.15	1.22	5.59	38.15
	Thalassiosiraceae	0.75	1.97	1.09	5.12	43.26
	Rhizosoleniaceae	0.68	1.62	0.88	4.21	47.48
	Chattonellaceae	0.68	1.57	0.89	4.08	51.56
	0 m - Average similarity: 39.06					
	Euterpinidae	1	4.57	4	11.69	11.69
	Solenidae	1	4.57	4	11.69	23.38

	Family	Average Abundance	Average Similarity	Sim/SD	Contribution (%)	Cumulative Contribution (%)
	Telonemidaincertaesedis	0.85	3.17	1.38	8.13	31.51
	Obeliidae	0.85	2.87	1.47	7.35	38.85
	Poaceae	0.77	2.7	1.08	6.9	45.75
	Pyuridae	0.69	2.03	0.87	5.2	50.95
	8 m - Average similarity: 48.35					
	Euterpinidae	1	3.36	8.13	6.95	6.95
	Amoebophryaceae	1	3.36	8.13	6.95	13.9
	Naviculaceae	1	3.36	8.13	6.95	20.85
	Chattonellaceae	1	3.36	8.13	6.95	27.8
	Thalassiosiraceae	0.93	2.92	2.4	6.04	33.83
	Telonemidaincertaesedis	0.93	2.88	2.4	5.96	39.8
	Pyuridae	0.87	2.5	1.64	5.18	44.97
	Solenidae	0.87	2.48	1.64	5.12	50.1
ROV scrape	Overall - Average similarity: 18.76					
	Ceramiaceae	0.57	4.6	0.61	24.5	24.5
	Dictyotaceae	0.35	2.76	0.29	14.73	39.23
	Dasyaceae	0.43	2.59	0.45	13.83	53.06
	0 m - Average similarity: 21.74					
	Dictyotaceae	0.54	5.43	0.55	24.98	24.98
	Dasyaceae	0.54	3.99	0.59	18.33	43.31
	Ceramiaceae	0.46	2.79	0.47	12.82	56.13
	8 m - Average similarity: 17.12					
	Ceramiaceae	0.7	6.95	0.8	40.59	40.59
	Styelidae	0.5	3.04	0.5	17.76	58.36
Keel crab	Overall- Average similarity: 21.27					
	Rhodomelaceae	0.65	5.04	0.65	23.68	23.68
	Dictyotaceae	0.6	3.37	0.68	15.83	39.51
	Spyridiaceae	0.45	2.18	0.43	10.25	49.76
	Ceramiaceae	0.45	2.13	0.43	10.02	59.78
	0 m - Average similarity: 35.57					
	Rhodomelaceae	0.86	7.64	1.25	21.49	21.49
	Dictyotaceae	0.86	7.03	1.38	19.76	41.24
	Erythrotrichiaceae	0.64	3.82	0.74	10.73	51.98
	8 m - Average similarity: 4.44					
	Cucurbitaceae	0.33	4.44	0.26	100	100

Chapter 3

Complementary molecular and visual sampling of fish on oil and gas platforms provide superior biodiversity characterisation

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Shell Fish
"*On my first visit to the new museum, I bought the book Rare Treasures from the library of the Natural History Museum. The book came with a series of reproduction natural history prints, including the print which I have used as the background for this work - Louis Renard's (1678-1746) depiction of white-barred triggerfish, clown triggerfish and a crayfish. I chose to depict one of my own shell collections in an unnatural blue hue to complement the vintage print.*" (Text provided by P. Brooshooft)
Image provided and displayed here with consent from Penny Brooshooft of Eclette (eclette.com.au)

3.1 Preface

This chapter was published in *Marine Environmental Research* and consists of a manuscript titled '**Complementary molecular and visual sampling of fish on oil and gas platforms provide superior biodiversity characterisation**'.

As an emerging biomonitoring method, there is a need to compare and contrast data collected from eDNA metabarcoding methods to that collected from conventional methods. In this chapter, I analyse data collected concurrently from eDNA water samples ROV surveys targeting fish and elasmobranchs at seven platforms and five off-platform (sediment) sites. As oil and gas infrastructure can provide unique deep-sea hybrid zones between reef-associated and pelagic fish species, a robust sampling method, or methods, are required to comprehensively document the fish and elasmobranchs using these habitats. The outcomes of this chapter inform fisheries and conservation related monitoring and provide insights into the value of vertical infrastructure as deep-sea refugia for species.

Within this chapter it was noted that a diversity of reef-associated fish species were detected from the off-platform locations, which was hypothesised to result from detections of pelagic larval life-history stages. Since the publication of this manuscript in 2022, I received additional information about the density of oil and gas platform infrastructure within the Gulf of Thailand, particularly surrounding the sampled platforms, which is included in Chapter 4, and discussed in Chapter 6. This density in infrastructure warrants mention here as it could potentially influence results, and may have implications in the detected results at sediment sites.

Alexander, J. B., Marnane, M., McDonald, J. I., Lukehurst, S. S., Elsdon, T. S., Simpson, Tiffany J. S., et al. (2023). Comparing environmental DNA collection methods for sampling community composition on marine infrastructure. *Estuarine, Coastal and Shelf Science*, 108283. doi: 10.1016/j.ecss.2023.108283.

3.1.1 Acknowledgements

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from Mermaid Subsea Services. I would also like to thank Dr Rose Lines, Adam Koziol and Matt Heydenreich for assistance with processing lab samples and Laura Fullwood and Damon Driessen, from the Curtin Fish Ecology Laboratory for assisting with processing of video samples. I would like to thank Chevron for in kind contributions, such as field logistics and access, during sampling and funding to provide for laboratory processing. 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, and from the CSIRO Environomics Future Science Platform through the iPhD program.

I would like to acknowledge the support and contributions of the co-authors of this chapter, Michael Marnane, Travis Elsdon, Michael Bunce, Se Songploy, Paweena Sitaworawet and Euan Harvey. A breakdown of author and co-author contributions is provided in Appendix I.

3.1.2 Permits and ethics

While no permits were required for the taking of samples in the Gulf of Thailand, these samples were imported into Perth, Australia under non-prohibited goods permit number 0001530842. The ROV component of this study, targeting fish, was reviewed and approved by the Curtin University Animal Ethics Committee (ARE_2018_20).

3.1.3 Data accessibility

Raw sequencing output from this chapter can be found on Zenodo data repository (https://doi.org/10.5281/zenodo.5757217). Family and genus level presence/absence matrices are available from:
https://drive.google.com/drive/folders/1iumylFU1AYqtX9dKj-zJ33ktAECh0-qO?usp=share_link

3.2 Abstract

Offshore oil and gas platforms have the potential to provide complex refugia for fish and benthic colonisers. We compare two methods of biodiversity assessment for fish and elasmobranchs at seven decommissioned oil and gas platforms as well as five sediment sites, located 5 km from platforms, in the Gulf of Thailand. Using surveys

from stereo-video ROV transects, and data from Environmental DNA (eDNA) water-column samples, we detected fish and elasmobranch taxa from 39 families and 66 genera across both platform and sediment sites with eDNA, compared with 18 families and 29 genera by stereo-ROV with platforms yielding significantly greater species richness. This study demonstrates that the combination of stereo-video ROV and eDNA provide effective, non-extractive and complementary methods to enhance data capture. This approach sets new benchmarks for evaluating fish assemblages surrounding platforms and will enhance measurements of biota to inform decisions on the fate of oil/gas infrastructure

3.3 Introduction

Quantifying fish species diversity and distribution can be challenging, particularly in deep-water habitats (Zintzen et al., 2012, 2017), areas of reduced visibility (Jalal et al., 2020) and operating environments which are considered hazardous (such as ports, oil and gas infrastructure, and other areas of restricted access). In recent years, the use of Autonomous Underwater Vehicles (UAVs; Seiler et al., 2012), Remotely Operated Vehicles (ROVs; Schramm et al., 2020a, 2020b, Harvey et al. 2021) and Baited Remote Underwater stereo-Videos (BRUVs; Langlois et al., 2020) have been used as an alternative to underwater visual census by SCUBA divers. These methods can be used to collect data on the presence and abundance of fish, and biomass if data are collected using calibrated stereo-videos systems (Shortis and Harvey, 1998; Harvey et al., 2010). It is acknowledged that these visual methods have potential biases, which may cause them to not detect species that are small or visually cryptic (Robertson and Smith-Vaniz, 2008; Stat et al., 2019). Similarly, some fish taxa have been documented actively avoiding SCUBA divers (Watson and Harvey, 2007; Lindfield et al., 2014) and ROVs due to thruster noise, bubbles and lights (Stoner et al., 2008). Biases associated with baited camera techniques have also been raised, particularly the over-estimation of species richness by attracting species to a bait from other habitats (Schramm et al., 2020a). A multi-faceted sampling approach to biodiversity assessment can be used to help understand and mitigate individual method bias and increase detections (Harvey et al., 2012; Stat et al., 2019; Cole et al., 2021; Piggott et al., 2021; Valdivia-Carrillo et al., 2021).

Environmental DNA metabarcoding (hereafter 'eDNA') has been demonstrated to be a valuable tool for sampling diversity in the marine environment, and in particular for the detection of rare and cryptic taxa (Pearman et al., 2016; Nester et al., 2020), invasive species (Ardura et al., 2015b, 2015a) and in broad biodiversity assessments (Lindeque et al., 2013; West et al., 2020, 2021). It has also been demonstrated to be a complementary sampling tool for traditional methods to bolster species census (Alexander et al., 2019; Stat et al., 2019; Cole et al., 2021). Although metabarcoding has been successfully used to document fish or invertebrate communities around oil and gas infrastructure in the North Sea (Mauffrey et al., 2020), Adriatic Sea (Cordier et al., 2019) and New Zealand (Laroche et al., 2017), its efficacy for sampling more diverse, tropical assemblages associated with oil and gas infrastructure has not been explored. Additionally, data collected from eDNA methods have not been compared to visual methods, such as ROV for assessing assemblages associated with these vertical structures.

Oil and gas platforms (hereafter referred to as 'platforms') often have a functioning lifespan of decades, and can provide highly productive, deep-sea refugia for fish and benthic colonisers (Consoli et al., 2013; Robinson et al., 2013; Claisse et al., 2014; Friedlander et al., 2014; Sammarco et al., 2014; Kolian et al., 2017; Todd et al., 2020), often aided by enforced exclusion zones during the platform operational life (van Elden et al., 2019). Due to their high vertical relief, platforms, can be ecologically important interaction zones for reef and pelagic species (Torquato et al., 2017; Harvey et al., 2021), as well as important nursery habitat for fishery species (Love et al., 2019).

Depending on the jurisdiction, decommissioning strategies considered for offshore platforms may include full removal from the marine environment, leave-in-situ (topple or partially dismantle) or relocate structures to a reefing site (Techera and Chandler, 2015; Jagerroos and Krause, 2016; Fam et al., 2018; Bull and Love, 2019). Given the ecological and anthropogenic (fisheries) potential of these platforms (Page et al., 2006; Pajuelo et al., 2016; Creed et al., 2017), robust methods of documenting community assemblages are required to predict changes that may occur under different decommissioning strategies and scenarios. The vertical nature of platforms makes standard underwater fish census methods difficult, as most platforms are too deep for SCUBA based methods. Documenting assemblages associated with platforms has

mostly relied on the use of imagery from industry Remotely Operated Vehicles (ROVs) (McLean et al., 2017; Todd et al., 2020), and more recently small scientific ROVs (Schramm et al., 2020a). However, there have been limited surveys documenting fish community assemblages associated with platforms in the Gulf of Thailand (GoT) (Harvey et al., 2021).

The GoT is a relatively flat oceanic basin with a benthic substrate of predominantly silt/clay and maximum depth of approximately 84 m (Wattayakorn, 2012). This tropical gulf is bordered by Thailand, Cambodia and Vietnam, covers approximately 320,000 km^2 in sea area and contains approximately 75 km^2 of predominantly coastal coral reef systems (Cheevaporn and Menasveta, 2003; Yeemin et al., 2013). As a result of this extensive coral reef system, the GoT has a highly diverse fish assemblage, with an estimated 425 reef-associated fish species to occur (Satapoomin, 2000; Scaps, 2006). Overall, including species not solely associated with reef habitats (such as demersal and pelagic species), 631 species may utilise the Gulf (Froese and Pauly, 2021), including species of conservation significance such as the pointed sawfish (*Anoxypristis cuspidata*) and the Spadenose shark (*Scoliodon laticaudus*). Additionally, this diverse fish assemblage is the basis of an economically important commercial fishery which captures threadfin bream (genus *Nemipterus*), Indo-Pacific mackerel (*Rastrelliger brachysoma*), and anchovies (family Engraulidae), amongst others. The rapid expanse of these fisheries has led to fishing resource management issues and over harvesting (Ahmed et al., 2007).

This overexploitation of fish stocks, as well as increased sedimentation, eutrophication, and other anthropogenic pressures are impacting coastal reef systems (Cheevaporn and Menasveta, 2003; Sutthacheep et al., 2013; Kulanujaree et al., 2020). Given these pressures and the predominantly silt/clay habitat in GoT, the presence of more than 400 oil and gas platforms installed in the GoT (Thailand Ministry of Energy, 2021) may provide a deep-sea refugia facilitating recruitment and colonisation of natural coastal reefs. ROV sampling of a subset of oil and gas platforms in the GoT demonstrated that a diverse fish community with a very high abundance and biomass is associated with these structures (Harvey et al., 2021).

This study aims to compare and contrast visual biodiversity assessments of fish on and off platforms in the GOT collected using a stereo-video system (mounted on an ROV) to eDNA metabarcoding of water samples from the same locations at the same time. The primary objective is to understand the range of species detected using eDNA and the stereo-video method to inform the strengths and weaknesses of each method with the aim of developing more robust assessments of fish taxa. In addition, we were interested in understanding how each method performed in censusing fish communities associated with established platforms compared surrounding soft sediment habitats in the GoT. We also explore whether eDNA has the sensitivity to detect the depth stratification in fish assemblages associated with oil and gas platforms documented by Harvey et al. (2021).

3.4 Methods

3.4.1 Study area

The study area is located approximately 133 km east of the island of Koh Samui (Figure 3.1) in the GoT. Seven platforms, installed between 5 and 23 years prior to sampling, and five soft-sediment habitat sites (hereafter sediment sites) were sampled between 2 and 12 March 2018. These sediment sites are all located a minimum of 5 km from platforms, pipelines and other permanent man-made structures and were selected because they provided a similar benthic habitat to the platform locations prior to construction and operation. These sites are also representative of what the fish assemblage will likely revert to if platforms are fully removed. We acknowledge that it is possible some larger and more transient species might be moving between Platform and sediment sites. All sites are located between 133 and 163 km from the closest natural reef systems at a depth between 61 m and 73 m.

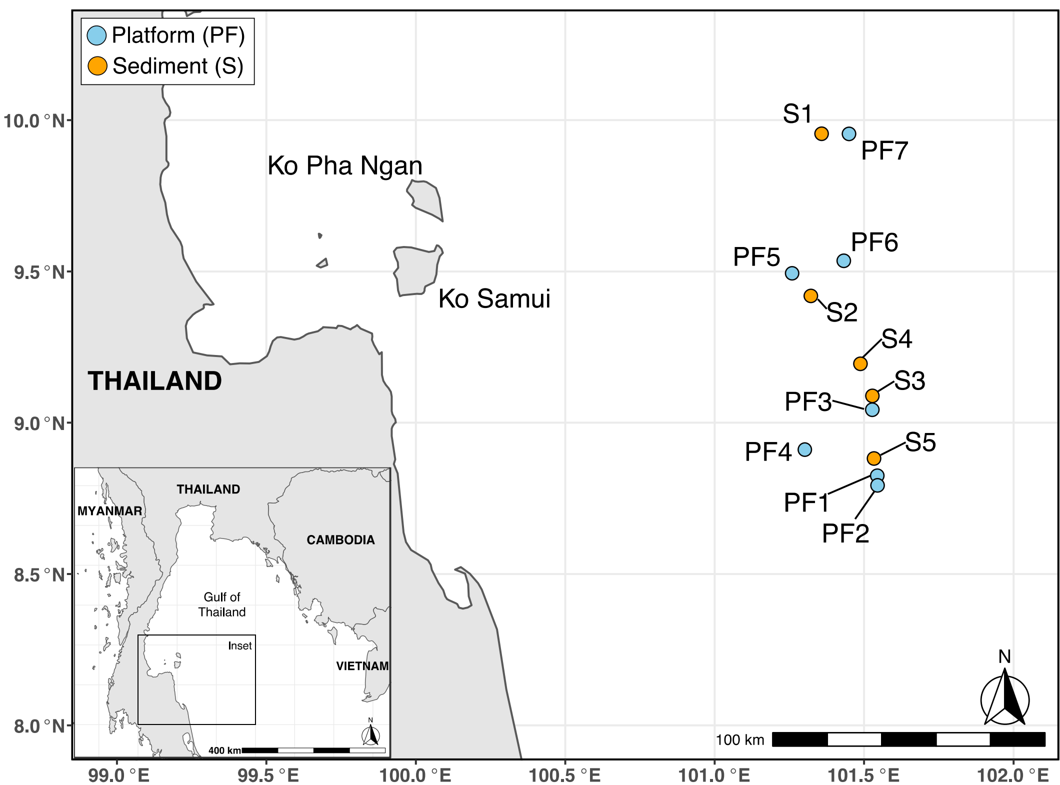

Figure 3.1: Locations of platform and sediment sites sampled in the GoT in March 2018. Sampling at each site comprised stereo-video ROV depth gradients for abundance, as well as four replicate 1 L eDNA water samples collected at 0 m, 30 m and 50 m depths, subsampled from 1.7 L Niskin water collector.

3.4.2 Field sampling

3.4.2.1 Fish stereo-video ROV sampling

The ROV data used in this study is a subset of the data collected by Harvey et al. (2021) looking at data points analogous with the eDNA seawater collection depths. We chose to use an observation class (SAAB Seaeye Tiger) ROV over a larger model to minimise operational noise (Stoner et al., 2008). The ROV was fitted with a calibrated underwater stereo camera system that was optimised to count and measure fish to a maximum distance of 7 m from the unit (Harvey et al., 2021). As artificial lights have been shown to alter the behaviour of some species of fish (Ryer et al., 2009), no lights were used during sampling and all work was completed during daylight hours. A tether management system (TMS) containing the ROV was lowered into the water to a depth of about 15 m. At the platform the ROV exited the TMS to collect samples. At the sediment sites the ROV remained inside the TMS and recorded video from there. While sampling with the ROV was completed at 7 specific depth zones, for the current study consistency in depths was maintained with eDNA

sampling depths (from 0 m, 30 m and 50 m depth from sea surface) by only using stereo-video ROV data subset from 5-7 m, 30-35 m and 50-55 m. All images were analysed and counts and identifications were completed using EventMeasure Software.

3.4.2.2 Seawater eDNA collection

Seawater samples were collected using a 1.7 L Niskin bottle that was sterilised in a 10% sodium hypochlorite solution (bleach) between samples and rinsed *in-situ* using seawater from the site to be sampled. At platform sites the Niskin bottle was deployed off a vessel, immediately adjacent to the platforms (within <50 m). Specific depths were targeted by lowering the open-ended Niskin bottle on a rope marked in 1 m increments to the desired depth and then deploying a weighted messenger to close the caps on the Niskin bottle. Upon retrieval, 1 L of collected seawater was subsampled, transferred to a sterile bottle and refrigerated until filtered. Twelve 1 L seawater samples were collected at each platform. Samples were taken adjacent to each leg of the platform at depths of 0 m, 30 m and 50 m, with four replicates collected at each depth, and one replicate collected at each platform leg at each depth. Sampling at sediment sites (S1-5) was completed to spatially mimic replicates taken at platforms, with four replicates taken per depth.

All 144 water samples were filtered within 4 hrs of collection using 47 mm, 0.22 μm polyethersulfone membranes, which were selected as samples were anticipated to have a low turbidity and subsequently high filtering rates. Filtering was completed with two peristaltic Sentino pumps (Pall Life Sciences, USA) in a temporary field laboratory on the research vessel, which was cleaned thoroughly with a 10% bleach solution. Likewise, all filtration equipment that came into contact with samples was sterilised in a 10% bleach solution between filtrations for a minimum of 15 mins, and rinsed with Reverse Osmosis (RO) water, to minimise risk of cross contamination. Control samples (1 L) of bleach solution and RO water were also filtered onto 0.22 μm polyethersulfone membranes between each site to test for contamination in the filtering and sterilisation workflows. After filtration, membranes were stored in uniquely labelled Ziplock bags at -20 °C and transported on dry ice to

the laboratory in Perth, Western Australia (imported under non-prohibited goods permit number 0001530842).

3.4.3 Laboratory processing

3.4.3.1 DNA extraction

All eDNA extraction, amplification and sequencing was completed using dedicated, clean laboratories. Filter membranes were thawed on ice, divided in half, with remaining samples returned to storage in -20 °C freezers as backup and archiving purposes. The second half was dissected into small pieces and incubated overnight at 56 °C in a solution of Proteinase K (60 µl) and Qiagen Buffer ATL (540 µl). DNA was then extracted from the filter membrane using the QIAgen DNeasy Blood and Tissue kit (Qiagen, Netherlands) protocol on the QIACube platform (Stat et al., 2017; Koziol et al., 2018), which produced 100 µl of DNA extract. To aid in detection of potential cross contamination, extraction controls were collected with each batch. Extracted DNA was stored at -20 °C.

3.4.3.2 PCR amplification and Indexing

Two metabarcoding assays were selected to amplify fish and elasmobranch biodiversity at sites in the GoT, targeting the 16S ribosomal and mitochondrial COI barcode regions (Table 3.1). Assay COIelasmobranchii consists of two forward primers, FishF1-degenerate and FishF2-degenerate, which were multiplexed with reverse primer Shark COI-MINIR-degenerate to broaden fish and, specifically, Elasmobranchii (shark and ray) detections.

Table 3.1: Assays and primers applied to water samples filtered from the Gulf of Thailand water samples.

	Primer	Primer Sequence (5'-3') (reference)	Amplicon Length	Annealing Temp. (°C)
16Sfish	16SF/D *	GACCCTATGGAGCTTTAGAC (Berry et al., 2017)	178 - 228	54
	16S2R-degen.	CGCTGTTATCCCTADRGTAACT (Deagle et al., 2007)		
COI-elasmobranchii	FishF1-degen. *	ACCAACCACAAAGANATNGGCAC (West et al., 2020)	110 - 241	52
	FishF2-degen. *	TCNACNAATCATAAAGATATCGGCAC (Fields et al., 2015)		
	Shark COI-MINIR-degen.	GATTATTACNAAAGCNTGGGC (West et al., 2020)		

* indicates forward primers

The PCR were completed on a StepOnePlus Instrument (Applied Biosystems), with a reaction volume of 25 µl, with 2 µl of eDNA extract per reaction. The PCR master mix consisted of 2.5 mM/L $MgCl_2$ (Applied Biosystems, USA), 10x PCR Gold buffer (Applied Biosystems), 0.25 mM/L 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 1 U AmpliTaq Gold DNA polymerase (Applied Biosystems). PCR conditions were as follows: an initial denaturation stage of 95 °C for 5 min, followed by 45 (COIelasmobranchii) or 50 (16Sfish) cycles of 95 °C for 30 s, then followed by assay specific annealing temperature (Table 3.1) for 30 s, 72 °C for 45 s, finishing with a final extension stage at 72 °C for 10 min. This PCR step was performed on both neat and 1/10 dilutions of each sample, with the optimal reaction selected based on C_T values, amplification and melt-curves. Each samples optimal dilution was then progressed through the indexing PCR outlined below.

Samples for both metabarcoding assays were indexed using a double indexing PCR protocol where samples were amplified using primers that incorporated a unique combination of indexes for each sample, so that each unique combination can be traced back to a sample and site (Sickel et al., 2015; Alexander et al., 2019). Reactions were set up on a Qiagility instrument (QIAgen), with amplification completed in duplicate using 2 µl of eDNA extract per duplicate. The same master mix and PCR conditions

were used, as outlined above, with the exception of 50 cycles employed for both assays. Negative extraction and PCR controls were included to control for contamination.

3.4.3.3 Library Preparation and Sequencing

Indexed duplicates were combined if the resulting amplification curve and melt plot were similar, otherwise 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 nine samples included in each minipool. Minipools were quantified using the Qubit 4.0 Fluorometer (Invitrogen) and amplicon lengths visualised using the Qiaxcel (QIAgen) before being blended into a single library based on equimolar values. Libraries were size selected between 180 to 450 bp using a Pippin Prep instrument (Sage Sciences, USA) to remove amplicons outside the target range, such as primer dimer artifacts. Following quantification (Qubit), the libraries were diluted to 2 nM for sequencing, which was completed using an Illumina MiSeq 300 cycle V2 kit with custom sequencing primers and a Q-score threshold of Q30.

3.4.4 Bioinformatics and Analysis

Bioinformatic analysis and taxonomic assignments were completed remotely using a high-performance cluster supercomputer (Pawsey Supercomputing Centre, Perth, WA, Australia). Raw sequence files were obtained directly from the MiSeq platform and deconvoluted using the OBITools (v1.2.9; Boyer et al., 2016) program for single-end sequencing. Batch sorting into unique MID-tag identifiers was completed, omitting short (<50 bp) sequences and those without an exact primer and tag match. MID-tags and primers were then removed leaving just the target amplicons for quality filtering. Sequence quality was visualised by sample using the FastQC package (v0.11.4; Andrews, 2010) to validate sequence length for quality filtering. Quality filtering of sequence reads were completed using the DADA2 package (Callahan et al., 2016) on R (v2.6.3; R Core Team, 2020) and Rstudio (v1.2.5042; RStudio Team, 2020), with expected errors at 0.5, no ambiguous bases (maxN=0), truncQ of two and amplicon length set between 100 and 250 (multiplexed elasmobranch primers) and 100 to 260 (16sFish). Remaining sequences were then dereplicated and converted into

Amplicon Sequence Variants (ASVs) prior to the removal of Chimeras using the "removeBimeraDenovo" function in DADA2.

Quality filtered ASVs were queried using the Basic Local Alignment Search Tool (v2.7.1; Altschul et al., 1990), with reference material from Genbank (open-source nucleotide reference database within the National Center for Biotechnology Information (NCBI)). BLAST parameters were applied as follows; maximum of 10 target sequences returned, query coverage set to 100%, e-value of 1e-3 and 90% minimum percent identity. Returned taxonomic designations were then collapsed to Lowest Common Ancestor (LCA) using the Python script within the eDNAflow automated workflow (Mousavi-Derazmahalleh et al., 2021) with a percent filtering threshold of 97%, coverage of 100% and insignificant difference threshold of two. All LCA ASVs were then manually vetted back against initial blast results and verified using the publicly available online database FishBase (Froese and Pauly, 2020). Finally, reads were filtered by relative abundance with a 0.5% threshold using the R packages Phyloseq and Vegan. Any contaminant ASVs from site specific bleach and RO water controls, as well as laboratory extract and negative template controls, were removed from the dataset using the R package microDecon (v1.0.2; McKnight et al., 2019).

3.4.5 Multivariate analysis

Statistical analysis and visualisation of data was completed using the Primer7 software (Clarke and Gorley, 2015) with the PERMANOVA + add on (Anderson et al., 2008) and Rstudio. As many ASVs were unable to be assigned to species or genus level, comparative statistical analyses were completed at the family and genera taxonomic level. Data from each depth were pooled per site and transformed to presence/absence data, and a resemblance matrix created using a Jaccard diversity index with the inclusion of a dummy variable (n=1). PERMANOVA (Anderson, 2001) was used to compare collection methods and depths, with both treated as fixed factors, the analyses were done using unrestricted permutation of raw data and 9,999 permutations. Data were visualised using Multidimensional Scaling (MDS) plots on bootstrapped data in Primer7. A similarity percentage (SIMPER) analysis was used to determine relative contribution of species at depth for each collection method.

3.5 Results

3.5.1 Sequence Results

DNA was successfully extracted and sequenced from all 144 seawater samples. Sequencing resulted in 12,144,222 and 15,290,811 raw reads from 16sFish and CO1Elasmo assays respectively (Supplementary S3.1). After demultiplexing, sequencing depth ranged from 13,338 ± 1,168 (mean ± SE, CO1Elasmo) to 56,667 ± 3,642 (16sFish), with both assays detecting greater sequence yields in samples from platforms (16,111 ± 1,512 for CO1Elasmo and 59,312 ± 3,586 for 16sFish) compared with sediment sites (11,501 ± 1,792 for CO1Elasmo and 49,444 ± 5,124 for 16sFish). Quality filtering, denoising, chimera removal and processing sequences through the DADA2 pipeline resulted in 520 unique Amplicon Sequence Variants (ASVs) from 16sFish and 2,629 from multiplexed CO1Elasmo assay, including target and non-target taxa (Supplementary S3.2).

Contamination is an ever-present risk in the eDNA metabarcoding workflow, and as such we detected non-target taxa including *Homo sapiens* (Human), *Gallus gallus* (red junglefowl) and *Hirundo rustica* (barn swallow) in high read abundance at many samples including controls. This DNA likely originated from waste at platforms and vessels. Additionally, in control samples we detected 855 fish and elasmobranch sequences from bleach water, RO water and laboratory controls (35 control samples) compared to 3,822,494 sequences detected in the 1 L eDNA water samples (representing 0.022% of target groups sequence abundance). The bulk of these (741 sequences) came from the sediment site S4 bleach water control, within which amplified *Encrasicholina punctifer* (buccaneer anchovy, Engraulidae), Carangidae sp., *Sardinella* sp. (Clupeidae) and *Dussumieria elopsoides* (slender rainbow sardine, Dussumieriidae) were detected. These baitfish taxa were widely detected in this study, especially *E. punctifer* that was ubiquitous at every site. These detections in control samples are likely to represent lab contamination, rather than a lack of efficacy of the bleach solution used to sterilise between samples, given both assays are shown to amplify *E. punctifer,* yet fish sequences were only detected using 16S fish assay. The MicroDecon algorithm was used to remove contamination from samples (Supplementary S3.3).

3.5.2 eDNA Diversity Detection

The 16Sfish assay detected greater diversity and comprised 66 taxa, of which 60 were assigned to species, from 55 genera and 36 families, compared to CO1elasmobranchii, which detected 23 taxa equating to 20 species and genera from nine families (Supplementary S3.4). Three families, eight genera and nine species were only detected in CO1elasmobranchii whereas 30 families, 42 genera and 49 species detected only with 16Sfish. Six families were detected with both assays. These were Blenniidae, Carangidae, Engraulidae, Mullidae, Platycephalidae and Scombridae. The combined genetic assays detected 80 fish and elasmobranch taxa across both platform and sediment sites. This comprised two classes, 24 orders, 39 families and 66 taxa identified to species-level and a further 14 assigned at a genus level due to limitation in the reference database and/or uncertainty in the underpinning taxonomic framework. Of these, two were from the class Elasmobranchii, being *Mobula japonica* (spinetail mobula, Mobulidae) and *Telatrygon zugei* (pale-edged stingray, Dasyatidae). The buccaneer anchovy was the most commonly and widest distributed taxon detected using eDNA metabarcoding, located at every site and from 61% of replicates with 3,094,700 positive reads post quality filtering. Three species not endemic to the GoT were also detected in low abundance from the study, sea trout (*Salmo trutta*, Salmonidae), Nile tilapia (*Oreochromis niloticus*, Cichlidae), and the silverside (*Argentina australiae*, Argentinidae).

Five taxa were detected at all platforms, including four reef-associated: *Decapterus maruadsi* (Japanese scad, Carangidae), *E. punctifer (*buccaneer anchovy, Engraulidae), *Selar crumenophthalmus* (bigeye scad, Carangidae), an undetermined species from the genus *Pterocaesio* (fusilier, Caesionidae), and a pelagic *Rastrelliger kanagurta* (Indian mackerel, Scombridae). Three species were detected at all sediment sites, including the Japanese scad, buccaneer anchovy and big-eyed scad.

Seventeen families were detected only in platform eDNA samples, while two families (Dactylopteridae and Synanceiidae) were only detected at sediment eDNA samples. Depth trends at sediment and platform sites were similar, with diversity highest at shallow depths, from which 16 families were detected in sediment and 30 from platform sites. Mid-depth samples returned the least diversity with 10 families at sediment, and 19 from platforms. Overall samples were dominated by the families

Engraulidae (*E. punctifer)* and Carangidae (*S. crumenophthalmus*) at both treatments, and a SIMPER analysis identified contributions of 45% and 14% at platforms, and 56% and 19% at sediment sites (Supplementary S3.5).

3.5.3 Stereo-video ROV Diversity Detection

As with eDNA, greater numbers of species were detected at platform sites than sediment sites using the stereo-video ROV. Only three species were detected at sediment sites; Atherinidae sp. (deep), *Echeneis naucrates* (mid: live sharksucker; Echineidae) and *Decapterus* sp. (shallow: Carangidae). Fish diversity sampled by stereo-video ROV at platforms varied with depth, with intermediate depths (30-35 m) having greater diversity (25 taxa) compared to shallow (5-7 m, 15 taxa) and deep (50-55 m 21 taxa) regions of the jackets. *Neopomacentrus cyanomos* (regal demoiselle; Pomacentridae) was most abundant at mid and deep depths, while *Thalassoma lunare* (moon wrasse; Labridae) was detected most frequently at shallow depths. These species were identified as key fish and elasmobranch community contributors using a SIMPER analysis, contributing 57% and 30% respectively (Supplementary S3.5).

3.5.4 Method Comparison

eDNA sampling detected a greater number of taxa at all depths at both platforms and sediment locations (Figure 3.2) than stereo-video ROV. Over all treatments and depths, eDNA detected 39 families and 66 genera, compared to 18 families and 29 genera detected by stereo-video ROV (Supplementary S3.4). Ten of the families were detected using both sampling methods, including Atherinidae (silversides), Belonidae (needlefishes), Caesionidae (fusiliers), Carangidae (jacks and pompanos), Ephippidae (spadefish, batfishes and scats), Labridae (wrasses), Pomacentridae (damselfishes), Scombridae (mackerels), Serranidae (groupers) and Siganidae (rabbitfishes).

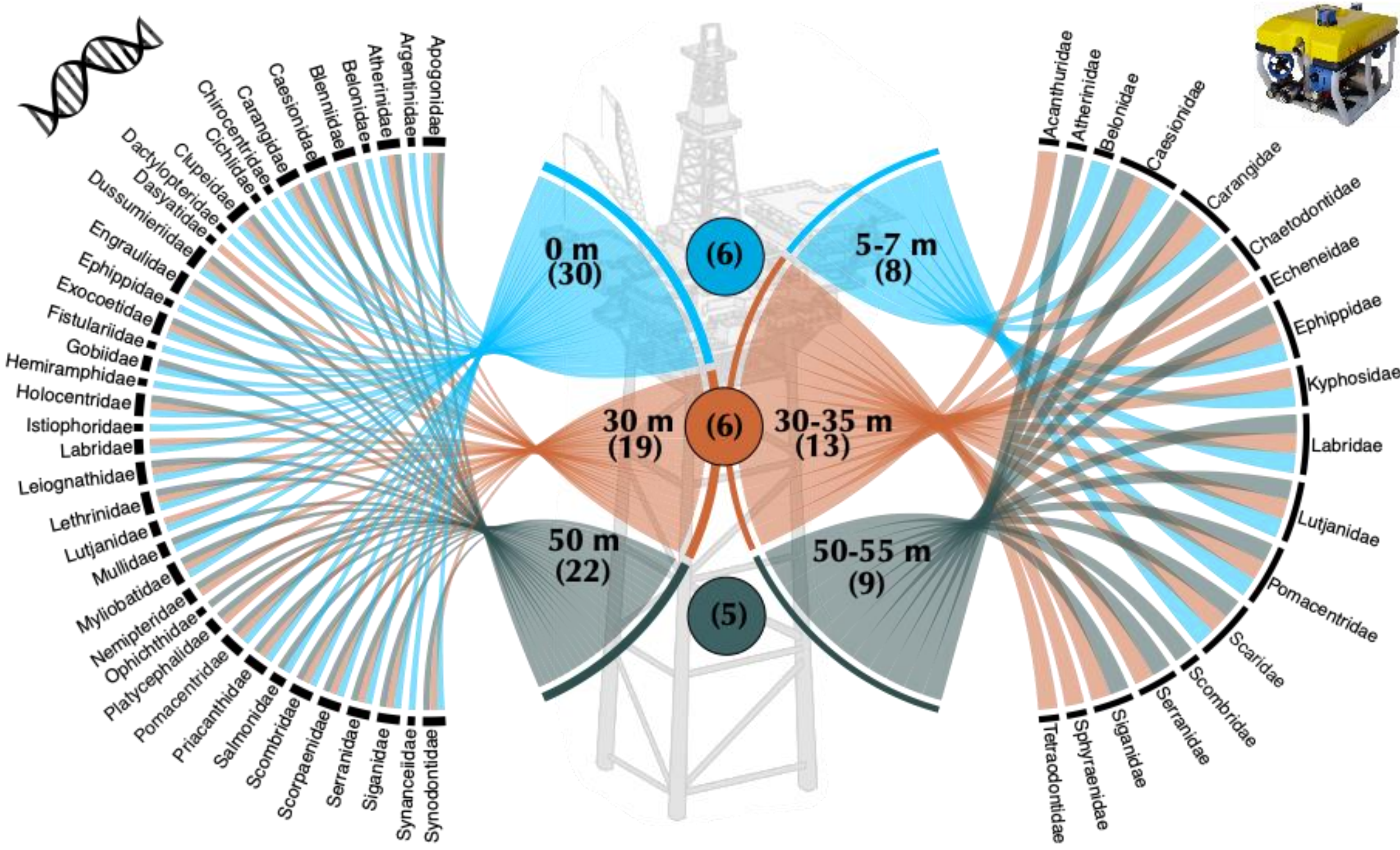

Figure 3.2: Detections of presence/absence transformed, family level taxa by depth using eDNA metabarcoding (left) and stereo-video ROV methods (right) on oil and gas infrastructure in the GoT.

The total number of families detected for each sampling method is indicated in brackets at each depth and method, with center circles indicating the number of families shared by both methods for shallow, middle and deep depths

The species compositions differed significantly at platforms and sediment sites, with eDNA detecting 35 families and 62 genera, with a mean detection of 22.42 ± 3.47 (mean ± SE) genera per platform site, compared to sediment sites that detected 20 families and 35 genera, with a mean of 13.4 ± 1.54 (mean ± SE) genera per sediment site. Stereo-video ROV detected 14 families and 25 genera (9.28 ± 0.94 per site, means ± SE) at platforms, and three families and genera (0.6 ± 0.4 per site, means ± SE) at sediment sites. Six families were detected at platforms only using stereo-video ROV, whereas 27 families were detected only in eDNA.

Depth sampling stratification showed that eDNA detections were highest at shallow depths at platforms (family 10.14 ± 1.58; genus 15.71 ± 2.36, means ± SE), and with stereo-video ROV at mid depths at platforms (family 6.14 ± 0.34; genus 7.71 ± 0.64, means ± SE). eDNA detections were lowest at mid depths at sediment sites (family 4.2 ± 0.86; genus 6 ± 1.22, means ± SE), while stereo-video ROV detections were lowest at all sediment depths with only one family and genus detected at each depth (Figure 3.3). At platforms, the families Carangidae, Lutjanidae and Pomacentridae were common across both methods and all depths.

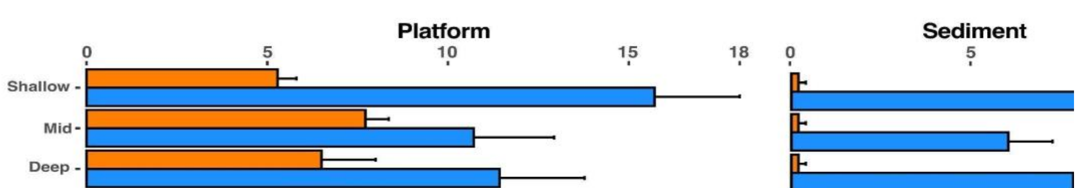

Figure 3.3: Mean number of genera (±SE) detected per depth at each treatment level (platform and sediment sites) for both stereo-video ROV (orange) and eDNA metabarcoding (blue) methods.
Family taxonomic level displayed similar trends (Supplementary S3.6)

Analyses of both genus and family level data showed significant differences with detection method (eDNA / stereo-video ROV), treatment (platform / sediment), depth (shallow/moderate/deep) and interactions of these (PERMANOVA; see Table 3.2). Pair-wise tests for the terms Method × Treatment × Depth within the factor Method were all significant, however pair-wise test indicated that eDNA results at platforms and sediment sites were similar at deep (30 m) depth (t=1.412, P(perm)=0.066, Unique Perms=9,949). Additionally, within the factor of Depth, Mid and Deep results at both platform (t=0.540, P(perm)=0.927, Unique Perms=9,943) and sediment (t=1.198,

P(perm)=0.209, Unique Perms=9,947) sites with the eDNA method were statistically insignificant, as well as shallow and mid at sediment (t=1.411, P(perm)=0.076, Unique Perms=9,945). Variance components indicate that variation is mostly unaccounted for (between 39.6 and 44.1 %). Method accounts for the highest variation (26.9 %) for genera, the interaction of Method × Treatment account for 12.1 % (Table 3.2).

Table 3.2: PERMANOVA results on Bray-Curtis similarities based on presence-absence transformed resemblance data.
%V represents percentage variation attributed to different levels.

	Family					Genus				
	DF	**Pseudo-F**	**P(perm)**	**Perm.**	**%V**	**DF**	**Pseudo-F**	**P(perm)**	**Perm.**	**%V**
Method	1	161.61	<0.001	9,942	26.9	1	146.59	<0.001	9,941	26.9
Treatment	1	73.8	<0.001	9,953	13.3	1	57.47	<0.001	9,942	11.7
Depth	2	4.26	<0.001	9,933	0.9	2	3.82	<0.001	9,907	0.8
Method × Treatment	1	27.25	<0.001	9,955	14.7	1	18.85	<0.001	9,950	12.1
Method × Depth	2	4.03	<0.001	9,933	1.6	2	4.13	<0.001	9,917	1.6
Treatment × Depth	2	2.33	0.018	9,957	1.0	2	2.91	0.001	9,906	1.0
Method × Treatment × Depth	2	2.85	0.004	9,936	2.0	2	2.11	0.007	9,922	1.7
Residual					39.6					44.1

Data visualization for both family and genus level supported PERMANOVA analyses and pair-wise tests, and showed clear differences between detections by methods (Figure 3.4). Taxonomic richness did not vary significantly using the eDNA detection methods, with similar trends at both family and genus levels.

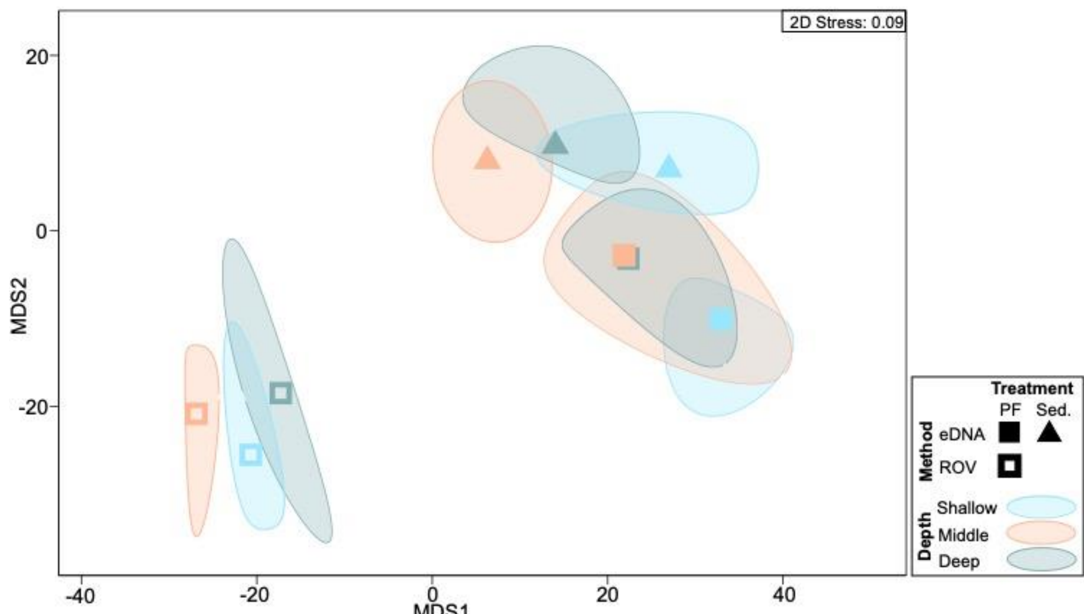

Figure 3.4: Bootstrapped MDS plot showing detection centroids and 95% confidence intervals for genus depth data by collection method, derived from a presence and absence transformed, Jaccard resemblance matrix.
Due to only single detections at each depth and therefore a lack of definable confidence interval, stereo-video ROV sediment samples were not included. Family taxonomic level displayed similar results (Supplementary S3.7).

The methods combined detected a total of 44 families and 82 genera for the study area, which included 41 and 22 families, 69 and 36 genera, from platform and sediment sites respectively. At the genus taxonomic level, this combined methods approach represented a detection increase of 14% for eDNA and 64% for stereo-video ROV at platforms; as well as a 6% and 92% increase for eDNA and stereo-video ROV respectively at sediment locations.

3.6 Discussion

A comparison of the data collected by both techniques shows the benefits of using both visual and eDNA sampling methods concurrently. When pooled, 82 genera were detected from both platforms and sediment areas, which is more than either individual technique. Compared to stereo-video ROV observations, eDNA detected greater numbers of taxa across all treatments and depths. However, the assemblage composition varied greatly between the techniques. For example, the elasmobranch species (*Mobular japonica* and *Telatrygon zugei*) were only detected by eDNA methods at two platform sites and not by stereo-video ROV. Although the stereo-video ROV and eDNA methods used in this study have previously been independently used

to characterise aspects of fish community assemblages on oil and gas infrastructure (Harvey et al., 2021), to our knowledge they have not been used in a complementary sampling of these structures.

Species which were smaller, less abundant, shier, or not typically associated with the vertical structure, such as the buccaneer anchovy (*Encrasicholina punctifer*), Smith's cardinalfish (*Jaydia smithi*) and the orange-fin ponyfish (*Photopectoralis bindus*) were only detected with eDNA. This may be partly due to small species being difficult to correctly identify from the video imagery (Holmes et al., 2013), or shy species being deterred by the noise or vibrations associated with ROV sampling (Stoner et al., 2008). eDNA also detected large, less abundant species, such as the indo-pacific sailfish (*Istiophorus platypterus*) and blue marlin (*Makaira nigricans*), both of which are pelagic, oceanic species and, therefore, less likely to enter the stereo-video ROV field of view. However, the stereo-video ROV not only detected larger bodied species, such as *Scarus ghobban,* or blue-barred parrotfish (Scaridae), but also detected the small, highly abundant (Harvey et al., 2021), reef-associated regal demoiselle (*Neopomacentrus cyanomos*) at every site.

Biases have been suggested for ROV sampling of fish including avoidance and attraction associated with lights on the ROV or noise from the thrusters and electronics (Stoner et al., 2008; Ryer et al., 2009; Schramm et al., 2020b). Although this was recognised during the study and mitigated to some degree by choosing a smaller, observation-class ROV over a larger, work-class ROV it is difficult to quantify the effect of the ROV noise and physical presence on fish behaviour. One possible implication of ROV avoidance is the incomplete characterisation of fish within any given habitats.

Species detections at sediment sites were greater using eDNA compared to stereo-video ROV, however, many of these positive detections were of families and species that are typically associated with reef habitat, such as Serranidae (groupers), Lutjanidae (snappers), Holocentridae (squirrelfish) and Lethrinidae (emperors) (Supplementary S3.4). Some of these species are known to forage widely over different substrates at night, such as snappers and emperors (Mueller et al., 1994; Heidmann et al., 2021), and eDNA detections may correspond to the recent physical

presence of these species in soft sediment habitat. However, some reef-associated species detected using eDNA in soft sediment habitat are not typically known to forage far from hard structures, such as groupers. For these species, detections may have been the result of DNA material drifting with currents into soft sediment habitat, transport through trophic interactions of carnivorous species, or alternatively, may have been the result of collection of DNA material from pelagic larval life stages in open water (Golani and Ben-Tuvia, 1985; Emel'yanova and Pavlov, 2020; Takahashi et al., 2020). eDNA has a far greater potential to detect fish species from pelagic egg or larval life-history phases, that would otherwise be missed by stereo-video ROV methods. If accurate, this detection of egg and larval stages has the potential to skew community assemblage detections, and therefore should be taken into consideration in future studies.

In order to minimise the likelihood of detecting DNA material from fish communities associated with oil and gas structures in soft sediment habitat, sampling of this habitat was undertaken a minimum of 5 km from any oil and gas infrastructure (or any known hard substrate). This distance was based on studies that demonstrated that DNA can display relatively high site fidelity in the marine environment (O'Donnell et al., 2017; Alexander et al., 2019; Jeunen et al., 2019). However, DNA persistence in marine environments has been reported to exhibit high variability, with a half-life of up to 51 hrs, depending on DNA fragment length and environmental factors, such as temperature (Collins et al., 2018; Jo et al., 2019; Murakami et al., 2019). Therefore, we are unable to eliminate the potential for DNA movement with currents from platforms to soft sediment sites. The presence of spatial and depth fidelity in the detected fish assemblages suggests that eDNA does not spread uniformly across large ocean areas in the GoT, however the spatial extent of DNA from platforms may potentially be mapped using a series of eDNA collection transects radiating out from platforms. Completed with sufficient replication, this could help determine if DNA from some species persists in the marine environment, or if detections correlate with those species known to forage over larger areas. The detection of species known to be strictly reef-associated at further points may indicate the detection of immature life-history phases. However, a study like this would need to be completed with current movement, seasonality, weather events, sampling time and tides in consideration, as these can influence the movement of DNA within the marine environment.

eDNA detected multiple species of interest that extended the current understanding of fish within GoT, and in doing so, the method shows its value in rapidly enumerating species presence in regions less studied. However, eDNA is also a highly sensitive method of detection and prone to field and laboratory contamination, leading to false detections such as false positives and negatives (Furlan et al., 2020; Martel et al., 2021). While there are risks of detection errors in all methods of ecological sampling, in eDNA sampling these can arise both from field sampling and PCR amplification within laboratory analysis. eDNA detections are a proxy for species detections, therefore the rate of DNA shedding, movement within the environment, and environmental conditions and degradation can also impact detection and detection error rate (Hinz et al., 2022). Stringent checks and controls throughout sampling (control collections), and through each step of the laboratory processes, such as inhibition control, exploratory sample PCR completed on every sample, and negative template, positive controls as well as and bioinformatic workflows, as applied in this study, can help mitigate these false detections (Murray et al., 2015; Alberdi et al., 2018).

In the present study, three species were detected that are not endemic to the region. These detections included the sea trout (*Salmo trutta*, Salmonidae), endemic to European and Mediterranean coastal waters, which had 1,020 sequence reads, and Nile tilapia (*Oreochromis niloticus*, Cichlidae), a freshwater or brackish water specialist endemic to the African continent, had 12,661 sequence reads. Detection of these species was considered to be an artifact of contamination by food waste disposal from vessels and/or accommodation. Interestingly, both species are cultured in Thailand for human consumption. Additionally, the silverside (*Argentina australiae*, Argentinidae), endemic to coastal southern Australia, was detected from two sites, though in very low read abundance (six sequence reads total). Given the very low sequence abundance, this detection is unlikely to represent a geographical distribution extension and more likely to represent a mis-identification in sequencing due to similar genetic makeup of a local species and potentially limited database resolution. All other families detected within the GoT, both with eDNA and ROV methods, are known to occur in the locality (Froese and Pauly, 2021).

Although eDNA has gained popularity as a method of cost-effective biodiversity assessment, there are a number of limitations to the methods commonly outlined in scientific literature, the most prevalent being a paucity of reference sequence material in publicly available databases, which can limit taxonomic resolution and designation (Pompanon et al., 2012). For example, of the estimated 630 fish and elasmobranch species known to occur within the GoT (Froese and Pauly, 2021), approximately 67% and 59% have corresponding material for the 16S and CO1 barcode regions respectively, with a combined 75% coverage, based on cross references of GenBanks open access Nucleotide database. Additionally, a limitation of eDNA methods when compared to stereo-video ROV, is the current inability to convert read abundance to information on species count, abundance or biomass data (Fonseca, 2018; Schramm et al., 2020b). These limitations are being increasingly addressed in literature (Lacoursière-Roussel et al., 2016b, 2016a; Di Muri et al., 2020; Rourke et al., 2021), however at this stage remain unresolved, and thus the description of species is largely limited to their presence or absence in a particular location and only where reference sequence material is available to confirm a positive identification. In addition, this study raises the possibility that DNA signal from larval life-history stages of some species may have given rise to confounded interpretations of habitat and biodiversity studies, in that results may be skewed to overly represent the pelagic larval community of an area.

In the context of quantifying fish communities associated with oil and gas platforms relative and surrounding habitat, this study indicates that a combination of stereo-video ROV and eDNA is a powerful two-pronged approach to describing fish communities across space, depth and likely time. Platforms are known to have horizontal fields (or halo effects) of influence that extend well beyond the immediate vicinity of structures, where they attract or hold fish due to the presence of the structure itself (Love and York, 2006), as well as vertical zonation in species presence (Torquato et al., 2017). Quantifying these horizontal and vertical fields can be challenging with visual methods due to limitations in light at depth and visibility with water clarity, that inhibit the ability to see species at distances away from the structures (Stanley, 1996; Rooker et al., 1997). Using eDNA as a complementary method provides a more comprehensive assessment of fish assemblages associated with platforms. While the focus of this study was exclusively fish, it is relevant that eDNA, as a method, is capable of profiling

assemblages of a much wider diversity of taxa (Stat et al., 2017), including species of relevance to decommissioning such as conservation significant or invasive species.

With an increasing amount of oil and gas infrastructure to be decommissioned, there is a need for robust assessments of fish assemblages associated with oil and gas structures. Having a greater understanding of the habitat value that offshore structures provides to fish assemblages, and how these assemblages are distributed in, around and adjacent to vertical structures, provides valuable information to inform the predicted outcome of decommissioning strategies (Macreadie et al., 2011; Sommer et al., 2019) including ongoing monitoring. The strength of the current study lies in the combination of the different survey techniques. Together, imagery from cameras and detections from eDNA provide a more comprehensive assessment of fish assemblages associated with platforms than each method individually. We propose that fish surveys on oil and gas infrastructure may be optimised by using eDNA and ROV sampling techniques both during operations and prior to decommissioning to inform management decisions. With an estimated 7,500 oil and gas platforms set to be decommissioned in the coming decades (Parente et al., 2006), and the need for robust censusing of diversity on these structures to inform decommissioning management, stereo-video ROV and eDNA provide non-extractive complementary methods of sampling while circumventing logistical constraints of sampling around oil and gas infrastructure, as well other subsea structures.

3.7 References

Every reasonable effort has been made to acknowledge the owners of copyright material. I would be pleased to hear from any copyright owner who has been omitted or incorrectly acknowledged.

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3.8 Supplementary information

Supplementary S3.1: Link to raw sequencing output

DOI:	https://doi.org/10.5281/zenodo.5757217

Supplementary S3.2: Link to summary table of amplicon read abundance throughout the bioinformatic process on eDNA samples for both 16Sfish and COIelasmobanchii Assays for samples collected at platform and sediment location in the Gulf of Thailand.

https://drive.google.com/drive/folders/1iumylFU1AYqtX9dKj-zJ33ktAECh0-qO?usp=share_link

Supplementary S3.3: ASVs and their taxonomic assignments removed entirely using the R package microDecon for both 16SFish and COIElasmobranchii assays on eDNA samples.

Location	ASV Number	Taxonomy (Family_Genus_Species) where available
16S Fish		
PF2	ASV_1	Engraulidae_Encrasicholina_*Encrasicholina punctifer*
PF4	ASV_39	Engraulidae_Encrasicholina_*Encrasicholina punctifer*
	ASV_55	Engraulidae_Encrasicholina_*Encrasicholina punctifer*
PF5	ASV_1	Engraulidae_Encrasicholina_*Encrasicholina punctifer*
	ASV_17	Siganidae_Siganus_dropped
PF6	ASV_1	Engraulidae_Encrasicholina_*Encrasicholina punctifer*
	ASV_4	Carangidae_Selar_*Selar crumenophthalmus*
	ASV_6	Clupeidae_Sardinella_dropped
	ASV_87	Belonidae_Tylosurus_dropped
S1	ASV_1	Engraulidae_Encrasicholina_*Encrasicholina punctifer*
	ASV_102	Leiognathidae_Photopectoralis_dropped
	ASV_125	Carangidae_dropped_dropped
	ASV_131	Carangidae_dropped_dropped
	ASV_159	Leiognathidae_Photopectoralis_dropped
	ASV_167	Carangidae_dropped_dropped
	ASV_170	Carangidae_dropped_dropped
	ASV_172	Engraulidae_Encrasicholina_*Encrasicholina punctifer*
	ASV_175	Carangidae_dropped_dropped
	ASV_176	Carangidae_dropped_dropped
S2	ASV_1	Engraulidae_Encrasicholina_*Encrasicholina punctifer*
	ASV_9	Priacanthidae_Priacanthus_*Priacanthus tayenus*
S3	ASV_1	Engraulidae_Encrasicholina_*Encrasicholina punctifer*
S4	ASV_1	Engraulidae_Encrasicholina_*Encrasicholina punctifer*
	ASV_167	Carangidae_dropped_dropped
	ASV_175	Carangidae_dropped_dropped
	ASV_2	Carangidae_dropped_dropped
	ASV_227	Clupeidae_Sardinella_dropped
	ASV_44	Scombridae_Rastrelliger_dropped
	ASV_6	Clupeidae_Sardinella_dropped
	ASV_69	Scombridae_Rastrelliger_*Rastrelliger kanagurta*
S5	ASV_1	Engraulidae_Encrasicholina_*Encrasicholina punctifer*
	ASV_36	Carangidae_Selar_*Selar crumenophthalmus*
COI Elasmobranchii		
PF2	ASV_1	Hominidae_Homo_*Homo sapiens*
	ASV_142	Hominidae_Homo_*Homo sapiens*
	ASV_147	Hominidae_Homo_*Homo sapiens*
	ASV_150	Hominidae_Homo_*Homo sapiens*
	ASV_237	Hominidae_Homo_*Homo sapiens*

Location	ASV Number	Taxonomy (Family_Genus_Species) where available
	ASV_299	Hominidae_Homo_*Homo sapiens*
	ASV_341	Hominidae_Homo_*Homo sapiens*
PF3	ASV_1	Hominidae_Homo_*Homo sapiens*
	ASV_13	Hominidae_dropped_dropped
	ASV_142	Hominidae_Homo_*Homo sapiens*
	ASV_147	Hominidae_Homo_*Homo sapiens*
	ASV_150	Hominidae_Homo_*Homo sapiens*
	ASV_237	Hominidae_Homo_*Homo sapiens*
	ASV_299	Hominidae_Homo_*Homo sapiens*
PF4	ASV_1	Hominidae_Homo_*Homo sapiens*
	ASV_142	Hominidae_Homo_Homo sapiens
	ASV_147	Hominidae_Homo_Homo sapiens
	ASV_150	Hominidae_Homo_Homo sapiens
	ASV_237	Hominidae_Homo_Homo sapiens
	ASV_299	Hominidae_Homo_Homo sapiens
PF5	ASV_1	Hominidae_Homo_Homo sapiens
	ASV_6	dropped_dropped_dropped
	ASV_142	Hominidae_Homo_*Homo sapiens*
	ASV_147	Hominidae_Homo_*Homo sapiens*
	ASV_150	Hominidae_Homo_*Homo sapiens*
	ASV_237	Hominidae_Homo_*Homo sapiens*
	ASV_299	Hominidae_Homo_*Homo sapiens*
	ASV_341	Hominidae_Homo_*Homo sapiens*
PF6	ASV_1	Hominidae_Homo_*Homo sapiens*
	ASV_5	Carangidae_Selar_*Selar crumenophthalmus*
	ASV_142	Hominidae_Homo_*Homo sapiens*
	ASV_147	Hominidae_Homo_*Homo sapiens*
	ASV_150	Hominidae_Homo_*Homo sapiens*
	ASV_237	Hominidae_Homo_*Homo sapiens*
	ASV_341	Hominidae_Homo_*Homo sapiens*
PF7	ASV_1	Hominidae_Homo_*Homo sapiens*
	ASV_13	Hominidae_dropped_dropped
	ASV_25	Carangidae_Atule_*Atule mate*
	ASV_150	Hominidae_Homo_*Homo sapiens*
S3	ASV_1	Hominidae_Homo_*Homo sapiens*
	ASV_5	Carangidae_Selar_*Selar crumenophthalmus*
S4	ASV_1	Hominidae_Homo_*Homo sapiens*
S5	ASV_32	Engraulidae_Encrasicholina_*Encrasicholina punctifer*

Supplementary S3.4: Link to table of detections from the Gulf of Thailand for both ROV and eDNA samples at Platform and Sediment locations, showing typical known environment (Reef-associated, Pelagic or Demersal) of taxa able to be identified to species.

https://drive.google.com/drive/folders/1iumylFU1AYqtX9dKj-zJ33ktAECh0-qO?usp=share_link

Supplementary S3.5: Results of SIMPER analysis on presence/absence transformed data with a Bray-Curtis similarity on treatment (Platform or Sediment) and method (eDNA and ROV) in the Gulf of Thailand.

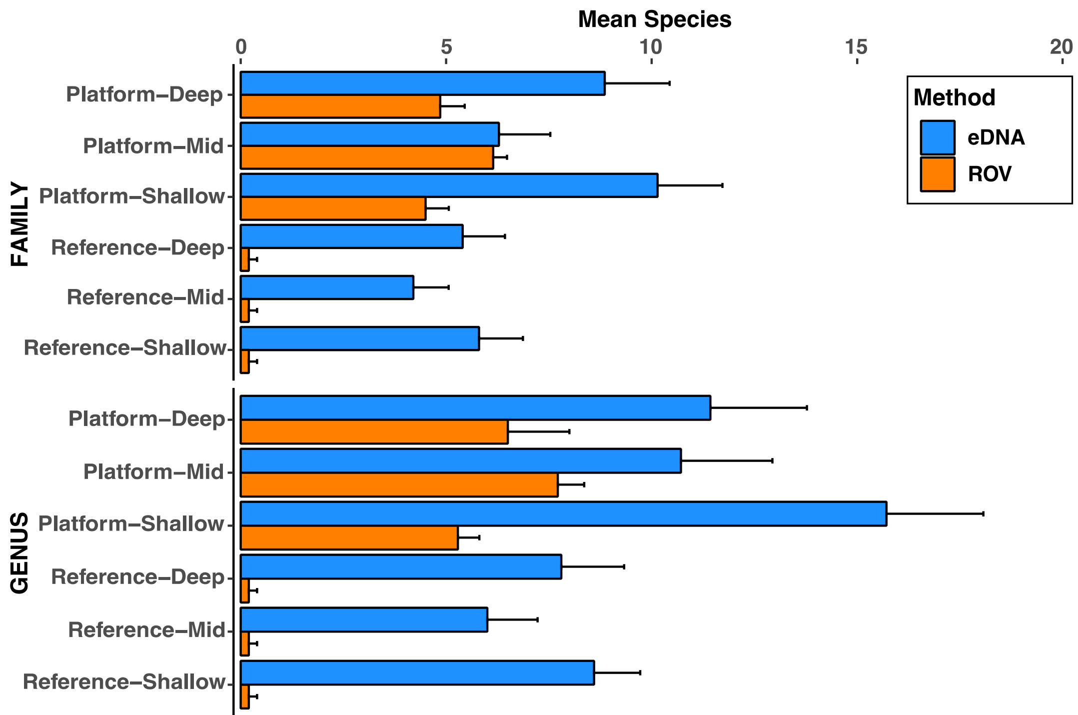
Species	Average Abundance	Average Similarity	Sim/SD	Contribution (%)	Cumulative Contribution (%)
Platform x eDNA Average similarity: 23.56					
Encrasicholina punctifer	0.77	10.72	0.92	45.5	45.5
Selar crumenophthalmus	0.48	3.21	0.5	13.64	59.14
Decapterus maruadsi	0.42	2.47	0.41	10.5	69.64
Rastrelliger kanagurta	0.3	1.23	0.29	5.2	74.85
Sediment x eDNA Average similarity: 23.06					
Encrasicholina punctifer	0.6	12.85	0.64	55.72	55.72
Selar crumenophthalmus	0.4	4.44	0.38	19.23	74.95
Platform x ROV Average similarity: 25.84					
Neopomacentrus cyanomos	0.64	14.85	0.75	57.46	57.46
Thalassoma lunare	0.48	7.75	0.53	30	87.46
Sediment x ROV Average similarity: -					
-	-	-	-	-	-

Species	Average Abundance		Average Dissimilarity	Dissimilarity /SD	Contribution (%)	Cumulative Contribution (%)
Platform_eDNA & Sediment_eDNA Average dissimilarity = 78.56	PF. eDNA	Sed. eDNA				
Encrasicholina punctifer	0.77	0.6	8.32	0.62	10.59	10.59
Selar crumenophthalmus	0.48	0.4	7.11	0.72	9.05	19.64
Decapterus maruadsi	0.42	0.27	6.89	0.61	8.77	28.41
Rastrelliger kanagurta	0.3	0.2	4.82	0.63	6.13	34.54
dropped (Auxis)	0.21	0.07	3.83	0.43	4.88	39.42

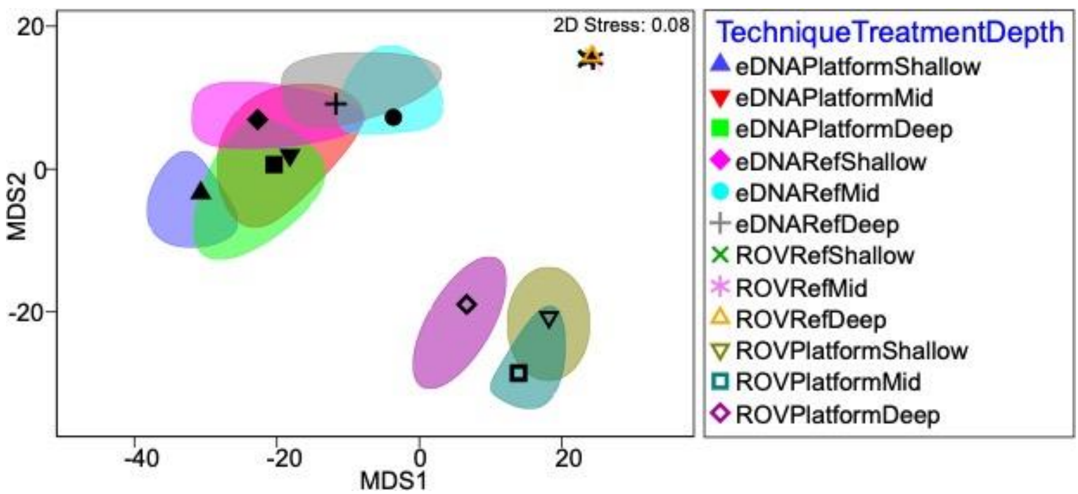
Species	Average Abundance		Average Dissimilarity	Dissimilarity /SD	Contribution (%)	Cumulative Contribution (%)
dropped (Pterocaesio)	0.21	0.15	3.68	0.52	4.69	44.11
Priacanthus tayenus	0.19	0.1	3.57	0.46	4.54	48.65
Sardinella gibbosa	0.19	0.13	3.18	0.48	4.05	52.7
Decapterus russelli	0.24	0.08	2.99	0.55	3.8	56.5
Euthynnus affinis	0.21	0.05	2.88	0.46	3.67	60.17
Photopectoralis bindus	0.21	0.05	2.45	0.49	3.11	63.28
dropped (Caranx)	0.19	0.02	1.93	0.47	2.45	65.73
Atherinomorus lacunosus	0.17	0	1.63	0.42	2.07	67.8
Selaroides leptolepis	0.12	0.02	1.32	0.33	1.68	69.48
Lethrinus lentjan	0.05	0.05	1.03	0.27	1.31	70.8
Platform_eDNA & Sediment_ROV Average dissimilarity = 100.00						
	PF. eDNA	Sed. ROV				
Encrasicholina punctifer	0.77	0	18.97	0.91	18.97	18.97
Selar crumenophthalmus	0.48	0	8.03	0.81	8.03	27
Decapterus maruadsi	0.42	0	8.03	0.55	8.03	35.03
dropped (Auxis)	0.21	0	7.08	0.35	7.08	42.11
Rastrelliger kanagurta	0.3	0	4.99	0.56	4.99	47.1
Priacanthus tayenus	0.19	0	4.88	0.35	4.88	51.98
Euthynnus affinis	0.21	0	4.4	0.35	4.4	56.38
Decapterus russelli	0.24	0	3.28	0.53	3.28	59.67
dropped (Pterocaesio)	0.21	0	3.15	0.43	3.15	62.82
Photopectoralis bindus	0.21	0	2.96	0.49	2.96	65.78
Sardinella gibbosa	0.19	0	2.73	0.45	2.73	68.51
dropped (Caranx)	0.19	0	2.56	0.46	2.56	71.07
Sediment_eDNA & Sediment_ROV Average dissimilarity = 100.00						
	Sed. eDNA	Sed. ROV				
Encrasicholina punctifer	0.6	0	26.42	0.9	26.42	26.42
Decapterus maruadsi	0.27	0	14.45	0.51	14.45	40.86
Selar crumenophthalmus	0.4	0	14.13	0.63	14.13	55
Rastrelliger kanagurta	0.2	0	6.45	0.45	6.45	61.44

Species	Average Abundance		Average Dissimilarity	Dissimilarity /SD	Contribution (%)	Cumulative Contribution (%)
dropped (Pterocaesio)	0.15	0	5.45	0.39	5.45	66.89
Sardinella gibbosa	0.13	0	4.36	0.3	4.36	71.25
Platform_eDNA & Platform_ROV Average dissimilarity = 98.60						
	PF. eDNA	PF. ROV				
Encrasicholina punctifer	0.77	0	11.08	0.99	11.24	11.24
Neopomacentrus cyanomos	0.11	0.64	9.69	0.82	9.83	21.07
Thalassoma lunare	0.02	0.48	7.09	0.73	7.19	28.26
Selar crumenophthalmus	0.48	0	5.49	0.81	5.56	33.83
Decapterus maruadsi	0.42	0	5.04	0.62	5.12	38.94
dropped (Auxis)	0.21	0	3.58	0.35	3.63	42.58
Rastrelliger kanagurta	0.3	0	3.41	0.57	3.46	46.03
Caranx sexfasciatus	0	0.19	2.83	0.36	2.87	48.9
Priacanthus tayenus	0.19	0	2.8	0.37	2.84	51.74
Euthynnus affinis	0.21	0	2.68	0.39	2.72	54.46
Decapterus russelli	0.24	0	2.38	0.52	2.41	56.87
Abudefduf vaigiensis	0	0.17	2.31	0.39	2.34	59.21
dropped (Pterocaesio)	0.21	0	2.18	0.45	2.21	61.43
Photopectoralis bindus	0.21	0	2.14	0.48	2.17	63.59
Sardinella gibbosa	0.19	0	1.95	0.45	1.98	65.57
dropped (Caranx)	0.19	0	1.87	0.45	1.89	67.47
Atherinomorus lacunosus	0.17	0	1.68	0.42	1.7	69.17
Lutjanus argentimaculatus	0	0.11	1.62	0.28	1.64	70.81
Sediment_eDNA & Platform_ROV Average dissimilarity = 99.99						
	Sed. eDNA	PF. ROV				
Encrasicholina punctifer	0.6	0	13.43	0.84	13.43	13.43
Neopomacentrus cyanomos	0	0.64	13.08	1.04	13.08	26.5
Thalassoma lunare	0	0.48	9.33	0.84	9.33	35.84
Selar crumenophthalmus	0.4	0	7.71	0.62	7.71	43.54

Species	Average Abundance		Average Dissimilarity	Dissimilarity /SD	Contribution (%)	Cumulative Contribution (%)
Decapterus maruadsi	0.27	0	6.83	0.47	6.83	50.38
Caranx sexfasciatus	0	0.19	3.71	0.41	3.71	54.09
Rastrelliger kanagurta	0.2	0	3.7	0.43	3.7	57.79
dropped (Pterocaesio)	0.15	0	3.03	0.37	3.03	60.82
Abudefduf vaigiensis	0	0.17	3.01	0.43	3.01	63.84
Sardinella gibbosa	0.13	0	2.39	0.3	2.39	66.22
Lutjanus argentimaculatus	0	0.11	2.12	0.31	2.12	68.35
Priacanthus tayenus	0.1	0	1.92	0.29	1.93	70.27
Sediment_ROV & Platform_ROV Average dissimilarity = 100.00						
	Sed. ROV	PF. ROV				
Neopomacentrus cyanomos	0	0.64	30.02	1.15	30.02	30.02
Thalassoma lunare	0	0.48	19.83	0.95	19.83	49.85
Caranx sexfasciatus	0	0.19	8.69	0.42	8.69	58.54
Abudefduf vaigiensis	0	0.17	5.92	0.47	5.92	64.46
Lutjanus argentimaculatus	0	0.11	4.72	0.32	4.72	69.18
Lutjanus lutjanus	0	0.09	3.69	0.32	3.69	72.87



Supplementary S3.6: Breakdown of mean (±SE) Family and Genera detected per depth at each treatment level (platform and sediment sites) for both ROV (orange) and eDNA metabarcoding (blue) methods.



Supplementary S3.7: Bootstrapped MDS plot showing detection centroids and 95% confidence intervals for family depth data by collection method, derived from a presence and absence transformed, Bray-Curtis resemblance matrix.

Chapter 4

Using environmental DNA to better inform decision making around decommissioning alternatives for offshore oil and gas infrastructure

Rockpools
This piece "*reflects the tiny worlds that exist in the tidal rockpools of the Kimberley. These smaller species of reef fish are often trapped in small rockpools through the lower tides and patiently drift among the seaweed waiting for the tide to return*." (text from website)
Image provided and reproduced here with consent from Chris Cole of Tidal Scales (tidalscales.com.au)

4.1 Preface

This chapter, based on the manuscript titled '**Using environmental DNA to better inform decision making around decommissioning alternatives for offshore oil and gas infrastructure**' has been submitted to *Science of the Total Environment* for review. The content within this chapter is the same as the submitted manuscript, with minor changes due to thesis formatting.

The quantity of oil and gas infrastructure in the marine environment necessitates a robust method of ecosystem monitoring which can also identify target populations and inform decommissioning options. With platforms being increasingly considered for conversion to artificial reefs, or opted to be left in the marine environment, a greater breadth of taxonomic and community resolution is required to inform these decisions. This chapter explores the scaling up of eDNA studies using available samples to extend the breadth of taxonomy detected to inform hypothetical decommissioning options of platforms in the Gulf of Thailand.

4.1.1 Acknowledgements

I would like to gratefully acknowledge the field and logistical support provided by and the crew of The Resolution, as well as to Donnie Cameron and the ROV crews from Mermaid Subsea Services. I would like to thank Tina Berry and Georgia Peverley from eDNA Frontiers for their expertise in processing samples through the eDNA workflow. I would like to acknowledge valuable support and funding from Chevron, who facilitated field eDNA collection and funded laboratory processing. 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, and from the CSIRO Environomics Future Science Platform through the iPhD program.

I would like to acknowledge the support and contributions of the co-authors of this chapter, Michael Marnane, Travis Elsdon, Michael Bunce, Paweena Sitaworawet, Se Songplow, Sarin Chaiyakul and Euan Harvey. A breakdown of author and co-author contributions is provided in Appendix I.

4.1.2 Permits

While no permits were required for the taking of samples in the Gulf of Thailand, these samples were imported into Perth, Australia under non-prohibited goods permit number 0001530842.

4.1.3 Data accessibility

Raw sequencing output from this chapter can be found on Zenodo data repository (https://doi.org/10.5281/zenodo.7787789). Presence/absence matrices are available from:
https://drive.google.com/drive/folders/1eP3y_YYSnQzNqssg91QB5ZurDfv3ga6h?usp=share_link

4.2 Abstract

Artificial reefs are being utilised globally to aid in natural resource management, conservation, restoration or the creation of unique marine habitats. There is discussion around the optimal construction materials and designs for artificial reefs, the influences these have on biological communities, and the resulting ecological and social benefits. This discussion also includes the ecological value of repurposed marine infrastructure, such as decommissioned oil and gas platforms. Platforms often have an operational life spanning multiple decades, over which time they can develop extensive and unique community assemblages. The creation of artificial reefs by repurposing oil and gas platforms can have ecological, economic and sociological merit. However, with more than 12,000 platforms requiring decommissioning globally, there is the need for holistic assessment of biological communities associated with these platforms to inform the potential outcomes of different decommissioning options. We use environmental DNA metabarcoding (eDNA) of water, bio-foul and sediment samples to census broad eukaryotic diversity at eight platforms in the Gulf of Thailand (GoT) and five nearby soft sediment habitat locations. We sampled three target depths at sites (shallow, mid, deep) and detected 430 taxa at platforms, with higher diversity in shallow (near-surface) samples (313 taxa), compared to mid (30 m collection depth; 261 taxa) and deep (50 m; 273 taxa). Three percent of taxa were shared among all depths at platforms with distinct assembles at each depth. Introduced species are an ongoing risk for platforms however the eDNA detected no known

introduced species. While the eDNA data provide broad taxon coverage and significant assemblage patterns, ongoing sampling innovation, assay design and local reference material require still require development to obtain the maximum benefit of the technique. This study highlights the versatility and scalability of eDNA metabarcoding to holistically census marine infrastructure and inform the management and potential conservation of extant communities

4.3 Introduction

Oil and gas platform jackets (hereafter termed 'platforms'), can provide habitat and refugia to a broad range of biotic diversity (Harvey et al., 2021; Kolian et al., 2017; Todd et al., 2020, 2018) and can also act as surrogate marine protected areas due to the enforcement of exclusion zones surrounding most operational structures (Alexander et al., 2022; Jagerroos and Krause, 2016). Once platform infrastructure has reached the end of operational life, it is typically decommissioned, which by accepted international guidelines and standards, has required the complete removal of all infrastructure (Techera and Chandler, 2015; Watson et al., 2023) resulting in the loss of the naturally accrued biotic diversity (Chandler et al., 2017; Fowler et al., 2014; Macreadie et al., 2011). With an increasing number of artificial reefs being installed globally for the purposes of fisheries enhancement, conservation and habitat restoration, the repurposing of existing marine infrastructure is starting to be viewed as a viable alternative to the construction and installation of purpose-built artificial reefs (Elrick-Barr et al., 2022). The operational lifespan of marine oil and gas platforms can be decades with the infrastructure developing unique and significant biotic assemblages (Harvey et al., 2021; Rezek et al., 2018; Schutter et al., 2019; Torquato et al., 2017). Depending on the decommissioning strategy, these structures can provide ecological and socio-economic benefits either immediately, or with a reduced timeframe than purpose deployed artificial reefs (Marnane et al., 2022). Given this diversity, the removal of infrastructure from the marine environment may work against the principles of environmental management that aims to protect biological diversity (Fowler et al., 2014).

Alternatives to the complete removal of oil and gas infrastructure are being considered and legislated in some jurisdictions on the condition that environmental and shipping safety concerns can be addressed (Fowler et al., 2018; Osmundsen and Tveterås, 2003;

Techera and Chandler, 2015). Within the Rigs-to-Reefs (hereafter RtR) framework, complete removal is still a viable option, and can still preferred in some scenarios. However, alternatives to this include the conversion of the infrastructure to permanent biotic refugia by toppling (laying the framework on its side), partial removal (cutting off the top section to facilitate safe shipping) of the structure *in-situ* in the original location, or the moving of the structure to a location where they are repurposed as an artificial reef, for example, in shallower water (Fowler et al., 2014; Macreadie et al., 2011). In some cases, this can provide a socially acceptable alternative location to promote tourism (recreational fishing and SCUBA diving; Sommer et al., 2019) to natural reefs with positive economic outcomes (Elrick-Barr et al., 2022). However, literature on reefing processes and RtR conversions has focused on platforms within the Gulf of Mexico (with ~2,900 active platforms; Sammarco et al., 2014) or the North Sea (Sommer et al., 2019).

Decommissioning decisions require information on the environmental and socioeconomic risks, weighed against the benefits of each option. With an estimated 12,000 global offshore platforms requiring decommissioning (van Elden et al., 2019), it's unlikely that these structures would all be suitable, or required, for RtR conversion. Information on the biotic diversity, an assessment of the presence and implications of non-endemic or introduced marine species (IMS), and the presence of species of conservation significance can all inform the predicted outcomes of different decommissioning options. Biodiversity assessment methods used on oil and gas platforms have predominantly focused on identifying target groups, and have mostly focused on fish (Harvey et al., 2021; Love et al., 2020; Torquato et al., 2017) and invasive marine species (Braga et al., 2021; Page et al., 2006). Methods used have relied predominantly on morphological methods for identifying taxa, such as photographic sampling (Page et al., 2006), the analysis of video footage from ROV (Harvey et al., 2021), SCUBA underwater visual counts (Consoli et al., 2013), or the morphological analysis of scraped or suctioned epibenthic fauna (Page et al., 2007).

Marine eDNA Metabarcoding (hereafter 'eDNA') shows promise as an alternative to holistically census and document platform biomes. eDNA has been shown to be an effective tool in broad-scale diversity detection (Stat et al., 2017; Takahashi et al., 2023; West et al., 2021), censusing cryptic and low abundance taxa, including invasive

marine species (Ardura et al., 2015; Bowers et al., 2021). It has been employed effectively as a stand-alone, or complementary method to traditional monitoring (Closek et al., 2019; Pearman et al., 2021). eDNA has been explored as a molecular approach for broadening survey focus, including sampling of oil and gas infrastructure (Alexander et al., 2022; Cordier et al., 2019; Laroche et al., 2017). This can be achieved by applying metabarcoding assays that amplify specific barcoding regions in a wide range of taxa. Metabarcoding data are then cross referenced to databases to provide taxonomic resolution. Broader sampling of biota can also be attained by incorporating different sampling methods and target substrates (Alexander et al., 2023; Koziol et al., 2018) such as sediment, the water column, and epibenthic surfaces or bio-foul.

The Gulf of Thailand (GoT) is a diverse, relatively shallow (approximately 80 m maximum), tropical gulf bordering Thailand, Cambodia and Vietnam (Wattayakorn, 2006). The gulf is dominated by silt and clay substrate, but there is approximately 75 km^2 of coastal coral reef systems (Cheevaporn and Menasveta, 2003; Wattayakorn, 2012). Due to the diversity in habitat, the GoT has important ecological and fisheries value (Ahmed et al., 2007; Cheevaporn and Menasveta, 2003). However, it also has an extensive history of oil and gas extraction, with around 450 fixed installations currently in place (Thailand Department of Mineral Fuels, 2022; Tularak et al., 2007). Some of the infrastructure within the GoT is reaching the end of operational life, and will require decommissioning. Currently, there is no legal requirement for the censusing of existing communities occupying oil and gas infrastructure in Thailand (Fam et al., 2018). However, data on the presence or absence of species of interest, such as conservation significant species (such as rare, threatened or endangered), introduced species, or species of economic importance, such as fished species, can provide valuable information to predict the biodiversity outcomes of a range of decommissioning alternatives.

This study used eDNA methods to holistically assess the biotic composition of eight oil and gas platforms in the Gulf of Thailand, as well as natural benthic habitats, which provide a contrast for the diversity that might have existed prior to the installation of the platforms. Utilising a suite of assays, substrates and depth profiles, we holistically explore the biotic diversity, with the specific aims of; (a) evaluating the ability of

eDNA methods to differentiate broad and fine scale spatial changes in location and depth; (b) exploring the effectiveness of eDNA detections using the existing taxonomic frameworks, as well as independent of taxonomy, using amplicon sequence variants (ASVs) to investigate if current frameworks (both local species taxonomy and reference databases) are sufficient to inform biodiversity trends; and (c) assessing what taxa are driving community characteristics. We then explore and apply these results to determine if the level of resolution provided within this study is sufficient to inform the possible effects of different decommissioning strategies.

4.4 Methods

4.4.1 Study area

Eight platforms and five off-platform (OP) sites were sampled in March, 2018. Platforms were located approximately 133 km east of Koh Samui in the Gulf of Thailand within a field of oil and gas infrastructure (Figure 4.1). OP sampling sites were located a minimum of 5 km from any subsea infrastructure and were included in the sampling design to provide baseline eDNA detections for the GoT as a comparison to platforms. The eight platforms included seven four-legged structures and one three-legged structure. These platforms had been installed between 5 and 23 years prior to sampling and were selected as they were targeted for upcoming decommissioning (Supplementary S4.1). OP locations were selected and sampled as they represent the habitat composition prior to the installation of the platforms, but also theoretically, what the biotic composition will return to post decommissioning and the removal of the structures. All sample locations were between 133 and 163 km from the closest natural reef systems, and ranged between 61 m and 73 m deep.

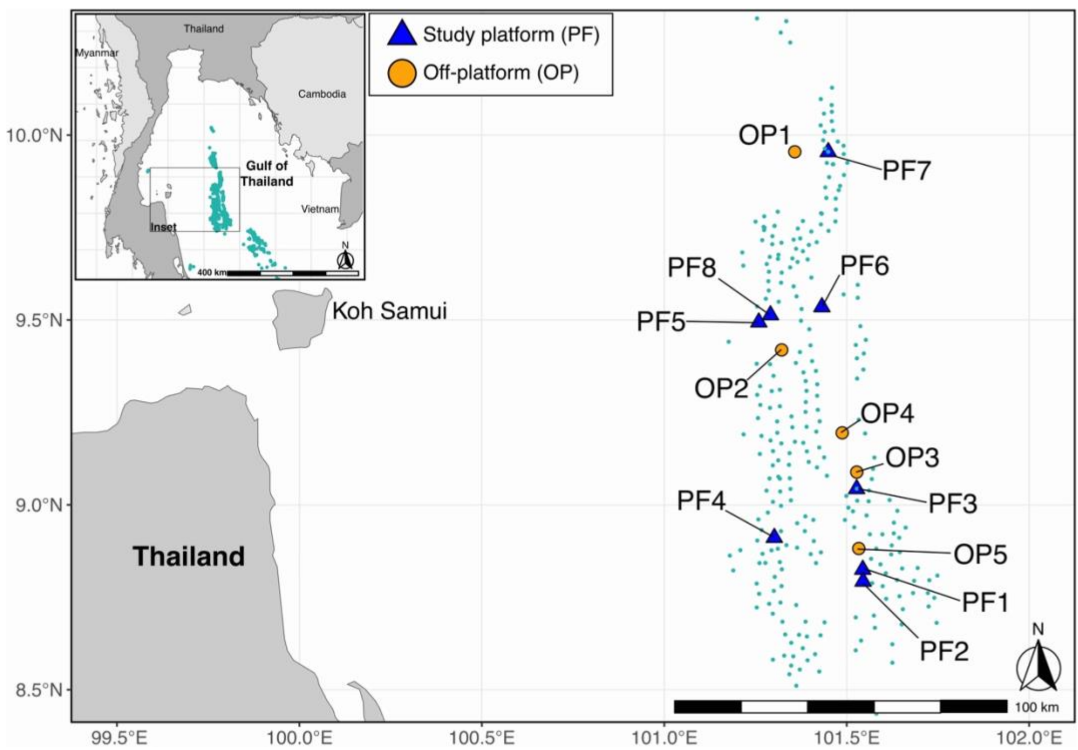


Figure 4.1: Location of study area within the Gulf of Thailand (inset), as well as platforms (PF) and off-platform (OP) sites in relation to the closest land mass (Koh Samui) and the mainland Thailand coast.

A combination of water, bio-foul and sediment were collected at both PF and OP sites. Teal-coloured points represent other oil and gas platforms not sampled in the current study.

4.4.2 Field sample collection

4.4.2.1 Water samples

A total of 156 water samples were collected at three depths, 0 m (surface), 30 m and 50 m below the surface, using a sterilised (10% bleach solution) 1.7 L Niskin bottle, with three to four replicates taken at each depth (depending on the number of legs of the platform). Water samples were collected adjacent to corners of each platform and, at OP sites, to spatially mimic platform site replication. At each site the Niskin was opened, set and rinsed with surface water to remove excess bleach, and lowered to the required sampling depth. The Niskin was then closed remotely using a weighted 'messenger' before being retrieved to the vessel. On the vessel, the water sample was subsampled into bleached, pre-labelled bottles that were rinsed with reverse osmosis (RO) water to remove bleach. These samples were then refrigerated (4 °C) until filtration, which occurred on the research vessel within 4 hours of collection. Filtration was completed using peristaltic Sentino pumps (Pall Life Sciences, USA) through 0.22 μm polyethersulfone membranes. All filtration equipment was sterilised in a 10% bleach solution between filtrations for a minimum of 15 minutes, and rinsed with RO

water, to minimise risk of cross contamination between samples. One litre control samples were collected and filtered for both the RO and bleach solution between each site to test efficacy of decontamination.

4.4.2.2 Bio-foul samples

Ninety-six bio-foul samples were collected from each platform leg at a depth of 10 m, 30 m and 50 m using a medium sized, a work class remotely operated vehicle or ROV (Quasar 125hp; SMD, UK) that was operated from the vessel. Samples were collected using a prototype aluminium scraper attached to a forward-facing manipulator arm of the ROV, which was scraped against the infrastructure to collect small fragments of the bio-foul. Each sample was transferred into individual mesh bags on a collection tray before collecting the next sample. While not logistically feasible to sterilise the prototype scraper between each sample on a platform, the scrapers and bags were sterilised prior to sampling and between ROV dives (with two dives completed at each platform) and platforms. Bio-foul samples were homogenised using an Omni TH (OMNI International, USA) and approximately 30-50 ml of the homogenized sample was placed in a 50 ml falcon tube.

4.4.2.3 Sediment samples

Sediment samples were collected using a small (3.5 L) Ekman grab within 50 m of each platform leg. The sterilised Eckman grab's jaws were opened and the grab was lowered from the vessel to the seafloor and triggered via a messenger to close. Upon retrieval to the vessel, the top doors of the Ekman grab were opened and sediment was sampled from approximately 5 locations among the top few millimetres of sediment within the Ekman grab using a sterilised and rinsed spoon, resulting in approximately 30-50 g of sediment in total at each sample. A total of 52 sediment samples were collected, which were placed in 50 ml falcon tubes. Between samples, the Ekman grab and subsampling equipment were thoroughly cleaned and sterilised using a 10% bleach solution for a minimum of 15 minutes, and rinsed with RO water prior to re-deployment. OP sites were sampled to spatially mimic sampling at platforms.

After collection, all eDNA samples were stored in uniquely labelled Ziplock bags at -20 °C until transport back to the laboratory. Samples were transported on dry ice under a non-prohibited goods permit (number 0001530842).

4.4.3 Laboratory processing

4.4.3.1 DNA digestion and extraction

Environmental DNA samples were extracted in dedicated clean laboratories. All equipment used during the digestion and extraction processes were soaked for a minimum of 15 minutes in a 10% bleach solution prior to use, and irradiated for 15 minutes using a UV oven, and all equipment re-bleached between samples. To determine sterilisation efficacy and detect potential cross-contamination, digestion, extraction and non-template controls were taken with each batch.

DNA digestion for both water and bio-foul samples followed the DNeasy Blood and Tissue (Qiagen; Netherlands) protocol. Water filters were defrosted, dissected in half, with half returned to storage in -20 °C as backup and for archiving purposes. The remaining half was further dissected and incubated overnight (minimum of 12 hrs) in a solution 540:60 µl ratio of ATL buffer and Proteinase K. Bio-foul samples were homogenised and tissue lysed using a TissueLyser II (Qiagen; Netherlands) in 30 second intervals for 90 to 180 seconds (sample dependant), on a 30 Hz setting. Following homogenisation, approximately 140 mg of sample was combined in a solution of 1260:140 µl ratio of ATL buffer and Proteinase K and digested overnight. After digestion, all water and bio-foul digests were extracted using a custom eDNA protocol on a QIACube platform (Qiagen; Netherlands). Sediment samples were homogenised with a TissueLyser II (settings: 20 Hz for 120 seconds in 30 second intervals) and then extracted manually, containing approximately 250 mg of sample, using a DNeasy Powersoil extraction (Qiagen; Netherlands) protocol. All extraction resulted in approximately 100 µl of extract in AE buffer.

4.4.3.2 Tagged amplification and sequencing

Assays were selected from scientific literature to provide a broad coverage of biotic diversity, which could be analysed as taxonomy dependent and independent (using amplicon sequence variants, or ASVs). A broad "universal" assay was selected and

applied to all collected substrates, while more specialised assays targeting hard coral, fish, elasmobranchs (sharks and rays), molluscs and crustaceans were applied to bio-foul and/or water samples (Table 1). To mitigate paucity in reference material, assays were selected targeting varied barcode regions, including the mitochondrial CO1 region, mitochondrial 16S rRNA, and the nuclear ribosomal ITS2 region. Two of the assays were multiplexed comprising PCR reactions with either two forward (elasmobranch assay; West et al., 2021) or reverse (hard coral, or Scleractinia) PCR primers (Table 4.1).

Assigned multiplex identifier tags, each consisting of 6 to 8 bp assigned in unique combination, were used to allow sequences to be bioinformatically assigned back to a sample. Initially, samples were explored via (untagged) PCR using neat and 1/10 dilutions to determine the optimal DNA input to progress with identifier tags (Murray et al., 2015). Both exploratory and final PCR reactions were completed on on a StepOnePlus Instrument (Applied Biosystems) with an initial denaturation stage of 95 °C for 5 minutes; followed by 45 (exploratory PCR) or 50 (final tagged PCR) cycles of; 95 °C for 30 s, followed by 30 s of the assay specific annealing temperature; 72 °C for 45 s; and a final extension stage at 72 °C for 10 min. The PCR master mix comprised a total 25 µl of 2.5 mM MgCl2 (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), with tagged PCR completed in duplicate.

The indexed duplicates were combined if the amplification curves, melt plots and ΔRn values were similar, otherwise minipools were formed with only the optimal reaction. Minipools were blended based on equi-molar ratios of the amplification ΔRn values with no more than seven 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 using a Pippen Prep instrument (Sage Sciences, USA) to exclude erroneous amplicons. Sequencing was completed on a

Miseq platform (Illumina, USA), with custom sequencing primers, using 500-cycle V2 (paired-end) and 300 cycle V2 (single-end) kits, using a Q-score threshold of Q30.

Table 4.1: PCR primers applied to water filters, bio foul and sediment eDNA collections from platform and sediment sites in the Gulf of Thailand. Assays were selected to detect broad eukaryotic diversity.

Assay Name (Target taxa)	**PCR Primers**	**Reference**	**Target Barcode**	**Sequence (5'-3')**	**Annealing Temp (°C)**	**Substrate**		
						Water	**Bio-foul**	**Sediment**
CO1 universal (broad target)	mlCOIintF *	(Leray et al., 2013)	CO1	GGWACWGGWTGAACWGTWTAYCCYCC	46	Y	Y	Y
	jgHCO2198_R	(Geller et al., 2013)		TANACYTCNGGRTGNCCRAARAAYCA				
Coral^ (Scleractinia)	SCLER5.8SForw *	(Brian et al., 2019)	ITS2	GARTCTTTGAACGCAAATGGC	55	Y	Y	
	SCLER28SRev			GCTTATTAATATGCTTAAATTCAGCG				
	CoralAcro_874Rev	(Alexander et al., 2019)		TCGCCGTTACTGAGGGAATC				
Fish (fish)	16SF *	(Berry et al., 2017)	16S	GACCCTATGGAGCTTTAGAC	54	Y		
	16S2R-degen.	(Deagle et al., 2007)		CGCTGTTATCCCTADRGTAACT				
Elasmobranch^ (sharks and rays)	FishF1-degen *	(West et al., 2020)	CO1	ACCAACCACAAAGANATNGGCAC	52	Y		
	FishF2-degen *	(Fields et al., 2015)		TCNACNAATCATAAAGATATCGGCAC				
	Shark COI-MINIR-degen	(West et al., 2020)		GATTATTACNAAAGCNTGGGC				
Crustacea (Crustacea)	Crust16S_F(short) *	(Berry et al., 2017)	16S	GGGACGATAAGACCCTATA	51		Y	
	Crust16S_R(short)			ATTACGCTGTTATCCCTAAAG				
Mollusca (molluscs)	Limacina_F *	(Berry et al., 2019)	CO1	TAATTGGNGGVTTTGGRAAYTG	52		Y	Y[&]
	Limacina_R			GTTCAHCCTRAYCCTRCNCC				

* indicates forward primer

^ multiplexed assay with either two forward or reverse primers

[&] substrate/assay combination failed to amplify in PCR and was not proceeded through sequencing

4.4.4 Bioinformatics and analysis

Raw sequence files were downloaded directly from the online Illumina Sequence Hub. Where feasible, raw sequence files from the same assay and substrate type were concatenated and processed through the bioinformatic workflow as one file to avoid the replication of ASVs within datasets. The demultiplexing and deconvolution of both paired and single-end sequence files were processed in 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), and efficacy verified using the cutadapt package (v3.7; Martin, 2011). Quality filtering (maxN=0, truncQ=2, maxEE=2 and a minimum amplicon length of 50 bp) was completed using the dada2 (v1.8.0; Callahan et al., 2016) pipeline in R, which was then subsequently used to merge paired-end reads, then identify and remove chimeric sequences. The resulting ASVs were queried against publicly available reference material from the National Center for Biological Information's (NCBIs) GenBank Nucleotide Database, which was accessed in May 2022. A 100% coverage and was required, an e-value of 1e-3 and 90% minimum percent identity in order to return a maximum of 10 taxonomic assignments. Species-level taxonomic assignments required a minimum 98% identity match and were taxonomy assigned based on the lowest common ancestor (LCA) using the Python script within the eDNAFlow automated workflow (Mousavi-Derazmahalleh et al., 2021). Taxonomic assignments were manually vetted back against the initial blast results, known distributions and against publicly available databases, World Register of Marine Species (WoRMS; accessed August 2022; WoRMS Editorial Board, 2022) and FishBase (accessed September 2022; Froese and Pauly, 2022). Results from field or laboratory controls were removed manually across that assay or sequence dataset, as indicated. Finally, reads were filtered by relative abundance with a 0.1% threshold using the R packages Phyloseq (v1.28.0; McMurdie and Holmes, 2013) and Vegan (v2.5.7; Oksanen et al., 2020), and data merged to form one dataset using the "merge_phyloseq" function in Phyloseq. All resulting samples with no reads were removed. Sampling effectiveness was explored using the BiodiversityR (Kindt and Coe, 2005) package within Rstudio. This analysis was completed at the ASV level within each substrate for all assays to determine if asymptote had been reached or to extrapolate what sampling effort was required.

4.4.5 Data exploration and multivariate analysis

All analyses were completed on presence/absence transformed data, as read abundance is not analogous with individual abundance and can be impacted by environmental conditions such as trophic interactions, season, and water movement. The overall differences in assemblage composition were explored at the ASVs level to capture data from taxa that were missing from the GenBank reference database. This taxonomy independent approach was explored using the Primer 7 software plus PERMANOVA+ add on (Anderson et al., 2008; Clarke and Gorley, 2015) with each method, water, bio foul and sediment, analysed independently. A fixed, two-factor PERMANOVA analysis was completed on Location (Platforms and Off-platforms) and Depth factors (0 m, 30 m and 50 m) on water samples, with pair-wise analyses completed on both Location and Depth. Fixed design PERMANOVA analyses were also completed on Depth (10 m, 30 m and 50 m) for bio foul samples and Location for sediment samples. All PERMANOVA analyses were completed on Jaccard similarity matrices with 9,999 permutations. ASV detection composition was further visualised by the above factors using non-metric MDS plots, which were bootstrapped to reduce variability within factors. Indicator species were explored for the above factors using the packages labdsv (v2.0.1; David W. Roberts, 2019) and indicspecies (v1.7.12; De Cacares and Jansen, 2016) completed on Rstudio. The dataset was then collapsed at the species taxonomic level for taxonomic dependant analysis and the same PERMANOVA analysis re-run to determine if database resolution for the region impacts analysis. With focus on the diversity at platforms only, an analysis of similarity percentages was completed on combined depth profiles, shallow, mid, deep and sediment, which was completed using the SIMPER function on the Primer 7 software, on Jaccard similarity matrix. A phylogenetic tree was produced using the taxonomy from NCBI through the "phyloT" website (https://phylot.biobyte.de/).

4.5 Results

4.5.1 Sequencing results and metrics

A total of 143,569,001 raw reads were returned from sequencing across all assays and methods employed (Supplementary S4.2 and

Supplementary S4.3). On completion of demultiplexing, quality filtering, the merging of paired-end sequences, and the removal of chimeric sequences, mean reads per sample were 74,062 (± SE 4,010), however varied by assay. The Crustacea and Mollusca assays (bio-foul samples) had had the highest mean read abundance, comprising (106,026 ± 4,579 and 120,985

± 3,112 respectively), with the Elasmobranch assay the lowest read abundance per sample (39,124 ± 2,113). Mean quality filtered reads per substrate using the CO1 universal assay, the only assay applied to samples of all three substrata, ranged from 51,997 (± 1,886; sediment) to 79,207 (± 3,466; water;
Supplementary S4.3).

Contamination from field and laboratory workflows were removed from subsequent analysis, however contamination ASVs varied by assay. ASVs that were identified as contamination and removed included non-target taxa such as bacterial and unassigned eukaryotes (from CO1 universal, Mollusca, Elasmobranch and Coral assays). Additionally, target ASVs that were identified from field and laboratory controls belonging to the species *Ostorhinchus semilineatus* (half-lined cardinal, phyla Chordata; 1 ASV, 19,199 reads, 0.6% of quality filtered reads), the anchovy family Engraulidae (phyla Chordata; 26 ASVs, 101 reads, <0.1%) were removed (fish assay), *Petroscirtes* sp. (phyla Chordata; 1 ASV, 12 reads, <0.1%; Elasmobranch assa) and *Urostyla grandis* (phyla Ciliophora; 8 ASVs, 29,994 reads, 0.8%; Coral assay). In addition, ASVs aligning to species that were used as positive controls in laboratory workflow, namely *Menippe mercenaria* (phyla Arthropoda; 2 ASVs, 109 reads; CO1 universal), *Homarus americanus* (phyla Arthropoda; 1 ASV, 2 reads; Crustacea), and *Plesiastrea versipora* (phyla Cnidaria; 1 ASV, 23 reads; Coral assay) were detected in some laboratory controls. Lastly ASVs that aligned to known non-marine species were omitted, which included the species *Gallus gallus* (chicken), *Homo sapiens* (human), the genus *Ovis* (likely cow), where DNA likely resulted from waste due to proximity to vessels and platforms.

Analysis of sampling effort by assay determined that Mollusca (bio-foul), CO1 universal and Coral (water and bio-foul) were sufficient to detect 90% of the ASV diversity (Supplementary S4.4), however for remaining assays additional sampling would have resulted in increased ASV diversity. Similarly, the success of each assay to assign ASVs to taxonomy was highly variable, which is reflective of the resolution of reference databases for the GoT, which is an understudied region. Combined, 25% of ASVs were assigned to species taxonomic level however ranged from 12% (Coral) to 67% (Crustacea; Supplementary S4.5). The coral assay was unable to assign 69% of ASVs past kingdom.

4.5.2 Location comparison

Overall, 462 taxa were identified overall with 236 resolved to species-level, 146 at genera and a further 46 at family, and the remaining 34 taxa aligning at a higher taxonomic level. A total of 430 (of which 216 were species-level) taxa were detected at platforms and 161 (87 species) at OP locations. The PERMANOVA showed significance across all factors (Location, Depth and Location x Depth) in a (substrate) combined, and within bio-foul and sediment substrates (Supplementary S4.6). The factor Location X Depth within the water substrate was non-significant (Pseudo-F=0.9, P (perm) = 0.734, Unique Perms = 9,830). Pairwise analysis on combined species-level data showed similarities in assemblages at both locations between shallow and mid, and mid and deep, however within the individual substrates, pairwise tests were significant (Supplementary S 4.7). Taxonomic diversity varied across substrates at each location (platform and OP). A higher mean diversity per sample was detected at platforms compared to OP locations in both water samples (platforms: 25.4 ± SE 0.6- total taxa: 214; OP: 23.4 ± 0.6, total taxa: 155), as well as sediment samples (platforms: 3.4 ± 0.4- total taxa: 33; and OP: 2.2 ± 0.2- total taxa: 14). At platforms, bio-foul had a mean of 26.4 (± 0.7- total taxa: 250) taxa per sample.

4.5.3 Platform diversity

Higher species diversity was detected in shallow samples at platforms (313 taxa), compared to deep (273) and mid (261; Figure 4.2). Three percent of taxa were shared among all depths at platforms, whereas 36% of species-level taxa were common to shallow, mid and deep samples. Overall diversity comprised seven kingdoms and 33 phyla (Figure 4.2).

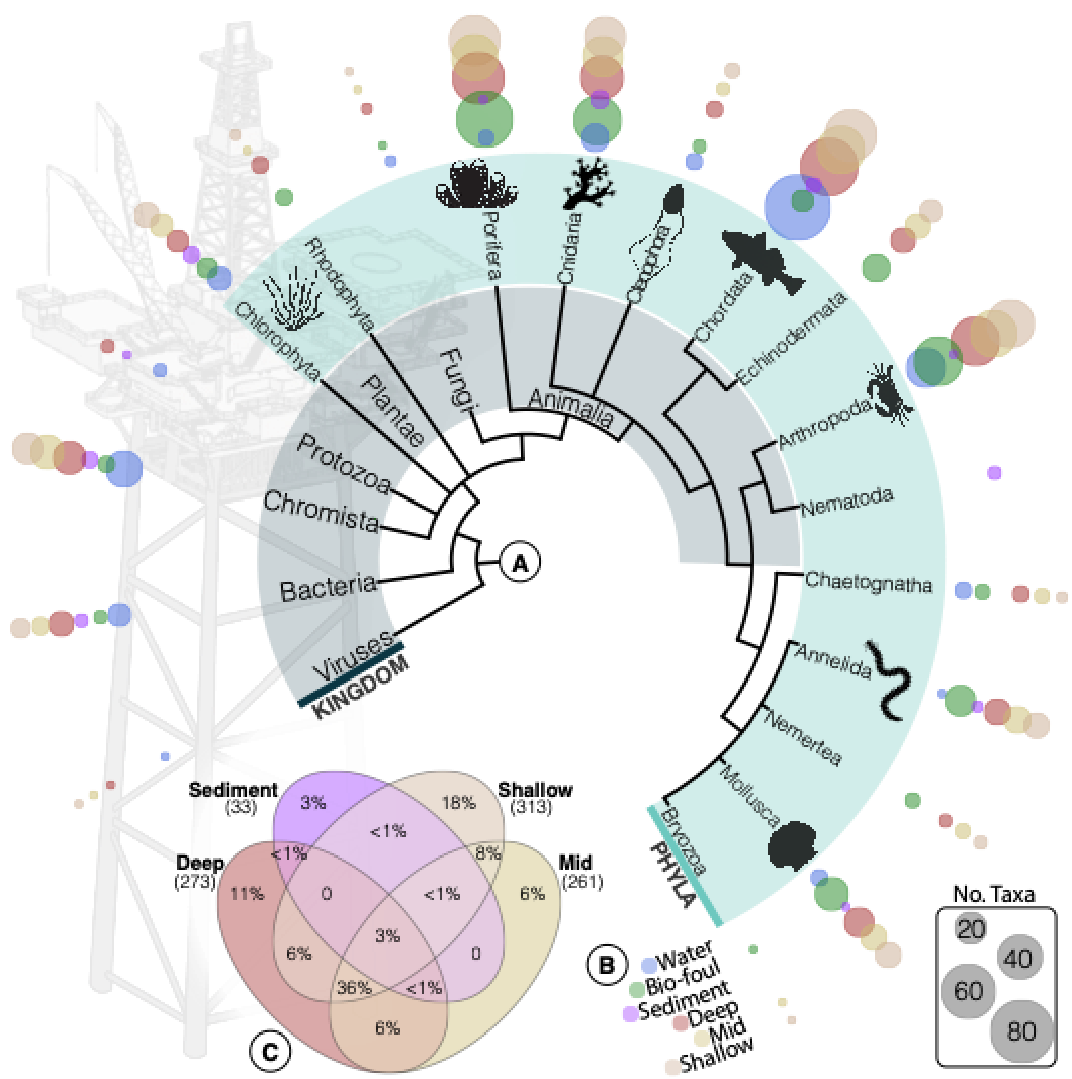


Figure 4.2: Biotic kingdoms detected from eight platforms within the Gulf of Thailand, with further phyla level breakdown of detected Plantae and Animalia (A).
Circles indicate the number of species-level taxa detected per group from each substrate and also each depth (combined water and bio-foul) sampled (shallow, mid and deep; B), and Venn diagram showing the percentage of taxa common to all depths at platforms utilised in this study (C).

Shallow bio-foul and water samples were dominated by zooplanktonic copepods (contributing 10%), phytoplankton (Chromista and Chlorophyta contributing 22%), and benthic species (contributing 12%; see Supplementary S4.8), with shallow samples displaying and average percent similarity of 24%. Planktonic species increased in percentage dominance with depth (mid 36%, deep 39%) whereas benthic fauna dominance decreased (mid 11%, deep 8%). INDVAL analyses completed on depths revealed shallow samples were characterised by 28 taxa, 26 from the phyla Animalia including reef associated fish species *Atherinomorus lacunosus* (wide-banded hardyhead silverside), *Scomberoides tol* (needlescaled queenfish) and *Selar*

crumenophthalmus (Bigeye scad), as well as brittle stars (Phyla Echinodermata; *Ophiactis savignyi* and *Ophiactis modesta*), amphipods (Arthropoda; *Elasmopus nkjaf* and *Stenothoe nhatrangensis*). Mid samples were characterised more by benthic diversity with five of the six taxa identified, including polychaete worms (Annelida; *Phyllochaetopterus ramosus* and *Phyllochaetopterus* sp.), sponges (Porifera; *Crambe crambe* and *Tedania* sp.) and soft coral (Alcyonacea; *Dendronephthya* sp.). Twenty-one taxa characterised deeper samples, which were predominantly benthic, comprising sponges, *Ophlitaspongia papilla*, oysters (Mollusca *Hyotissa hyotis*), soft coral (*Carijoa riisei*), ascidians (Chordata; *Ascidia ahodori*) and polychaetes (Annelida; *Dipolydora armata*), however also planktonic jellyfish (Phyla Cnidaria; *Nausithoe punctata*) and copepods (Arthropoda; *Clausocalanus minor*). Sediment samples were dominated by meiofauna, *Terschellingia longicaudata* and *Terschellingia* sp. (Nematoda) and protozoan, *Cunea thuwala* (Discosea).

4.5.4 ASV assemblage composition

By restricting eDNA data to groups that have good representation on reference databases, it is possible that patterns and insights are lost. Accordingly, taxonomy independent analysis on all samples (platform and OP sites) revealed a total of 3,112 ASVs detected from all substrates, including 2,261 from water samples (mean per sample 145 ± SE 3), 883 from bio-foul (56 ± 2), and 62 ASVs from sediment (4 ± 3). ASV diversity was higher at platforms in both water (151 ± 4) and sediment (4 ± 0.5) samples, in relation to OP samples which comprised 136 (± 4) and 3 (± 0.4) respectively. Assemblage composition at platforms differed with depth for both water and bio-foul samples, with mid depth having a higher mean ASV diversity (61 ± 4) than deep (54 ± 4) and shallow (49 ± 4), compared to depth profile in water samples which had the highest diversity in shallow samples, deep, and then mid, which was a trend mirrored in OP depth profiles (Figure 4.3).

PERMANOVA analysis showed that the detected assemblages of ASVs were non-significant across the factors Location X Depth (Pseudo-F=1.123, P (perm) = 0.105, Unique Perms = 9,708) within the water dataset, with factors Location and Depth both significant. Fixed factor PERMANOVA for sediment (Location only) and bio-foul (Depth only) were significant (Supplementary S4.9).

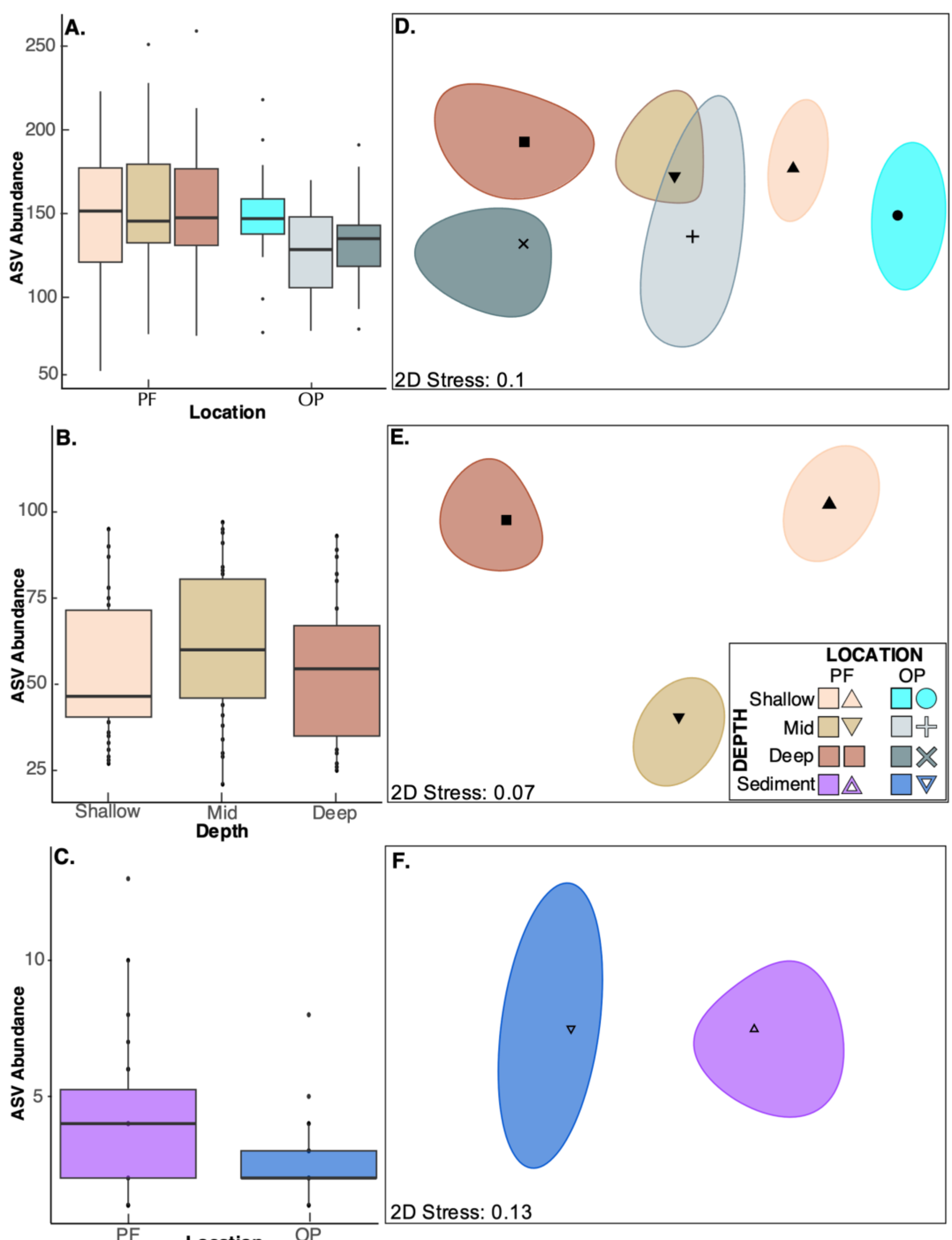


Figure 4.3: Observed alpha diversity for all assays applied to the target substrates including water (A.) from different locations (platform and OP) and depths; bio-foul (B.) at platform depths; and sediment (C.) at both locations, and bootstrapped nMDS plots (D-F) for the corresponding substrates showing 95% confidence intervals and centroids.
Data based on 9,999 permutations of a presence/absence transformed Jaccard resemblance matrix.

The ASVs that characterised a location or depth within each substrate were characterised by indicator species analysis. Although most indicator ASVs were unassigned at the species-level, some species were identified driving differences in

substrate detections (Table 4.2). Platform water samples were characterised by fish and zooplankton species (all within the genera *Calocalanus*), whereas deeper samples were characterised by phytoplanktonic groups, with 13 ASVs aligning to the algal species *Micromonas commoda*, and a further 19 ASV aligning to four species within Chromista (Table 4.2). Bio-foul samples were characterised by known fouling and epibenthic associated fauna such as amphipods (*Elasmopus nkjaf* and *Stenothoe nhatrangensis*), brittlestars (genus *Ophiactis*), annelids. Filter feeders, such as Sponges (Phyla Porifera), oyster (*Hyotissa hyotis*) and three ASVs aligning to soft coral (*Carijoa riisei),* dominated mid and deep bio-foul sample characterisation. while no indicator species, or ASVs, were identified from sediment OP sites. However, a phytoplanktonic chromist (*Pelagomonas calceolata*) characterised sediment samples associated with platforms, as well as the only meiofaunic nematode species detected, *Terschellingia longicaudata*. In OP water samples, INDVAL analysis identified fewer significant species, however a greater number of indicators unable to be aligned below Kingdom or Phyla at each depth. Similar to platforms, shallow and mid depths at OP sites were dominated by zooplanktonic copepods, in addition a sponge species, *Tethya seychellensis*, was also identified. Similarly, phytoplankton (Chromista and the Plantae phyla Chlorophyta) were characteristic of deeper OP samples

Table 4.2: Indicator species identified from Platforms and OP sites in the GoT showing ASVs aligning to species, significantly characterising water (location and depth), bio-foul (depth only) and sediment (location).

	Depth	Kingdom	Phyla	Species	No. Sig. ASVs	Sig. P-value (range)
Water						
PF	Shallow	Animalia	Arthropoda	*Calocalanus pavo*	-	0.01
		Animalia	Arthropoda	*Calocalanus plumulosus*	-	0.02
		Animalia	Chordata	*Atherinomorus lacunosus*	-	0.0
		Animalia	Chordata	*Oxyporhamphus micropterus*	-	0.03
		Animalia	Chordata	*Upeneus guttatus*	-	0.04
	Mid	Animalia	Chordata	*Euthynnus affinis*	-	0.01
	Deep	Animalia	Arthropoda	*Clausocalanus minor*	-	0.04
		Animalia	Chordata	*Ascidia ahodori*	2	0.01-0.03
		Animalia	Cnidaria	*Antipathes curvata*	-	0.03
		Animalia	Cnidaria	*Nausithoe punctata*	-	0.04
		Chromista	Haptophyta	*Phaeocystis globosa*	4	0.0-0.04
		Chromista	Ochrophyta	*Pelagomonas calceolata*	6	0.0-0.03

	Depth	Kingdom	Phyla	Species	No. Sig. ASVs	Sig. P-value (range)
		Chromista	Ochrophyta	*Pseudo nitzschia cuspidata*	-	0.0
		Chromista	Radiozoa	*Dictyocoryne truncatum*	8	0.0-0.03
		Plantae	Chlorophyta	*Chloropicon laureae*	-	0.02
		Plantae	Chlorophyta	*Micromonas commoda*	13	0.0-0.03
OP	Shallow	Animalia	Arthropoda	*Calocalanus plumulosus*	-	0.04
		Animalia	Chordata	*Selar crumenophthalmus*	-	0.03
		Animalia	Porifera	*Tethya seychellensis*	-	0.05
	Mid	Animalia	Arthropoda	*Farranula gibbula*	-	0.02
		Chromista	Radiozoa	*Dictyocoryne truncatum*	-	0.04
	Deep	Chromista	Haptophyta	*Phaeocystis globosa*	-	0.0
		Plantae	Chlorophyta	*Chloropicon roscoffensis*	2	0.0-0.01
		Plantae	Chlorophyta	*Micromonas commoda*	-	0.0
		Plantae	Chlorophyta	*Pseudoscourfieldia marina*	-	0.01
		Bacteria	Proteobacteria	*Vibrio fluvialis*	-	0.05
Bio-foul						
PF	Shallow	Animalia	Annelida	*Lumbrineris perkinsi*	-	0.02
		Animalia	Arthropoda	*Elasmopus nkjaf*	-	0.0
		Animalia	Arthropoda	*Stenothoe nhatrangensis*	-	0.0
		Animalia	Echinodermata	*Ophiactis modesta*	-	0.0
		Animalia	Echinodermata	*Ophiactis savignyi*	-	0.05
		Chromista	Ochrophyta	*Pelagomonas calceolata*	-	0.05
	Mid	Animalia	Annelida	*Lumbrineris perkinsi*	-	0.03
		Animalia	Porifera	*Crambe crambe*	-	0.0
	Deep	Animalia	Cnidaria	*Carijoa riisei*	3	0.00
		Animalia	Mollusca	*Hyotissa hyotis*	-	0.0
		Animalia	Porifera	*Chelonaplysilla erecta*	-	0.05
		Animalia	Porifera	*Ophlitaspongia papilla*	-	0.0
Sediment						
PF	-	Animalia	Nematoda	*Terschellingia longicaudata*	-	0.03
		Chromista	Ochrophyta	*Pelagomonas calceolata*	-	0.02
OP	-	-	-	-	-	-

4.6 Discussion

Using eDNA metabarcoding, we documented a broad eukaryotic diversity at oil and gas platforms in the highly diverse Gulf of Thailand, and revealed differences in assemblages among substrates, depths and locations. We demonstrate that platforms had developed complex biotic communities associated with the vertical relief of the infrastructure, a result reflected in both taxonomic and taxonomy-independent

analysis. This study also shows the taxonomic scalability of eDNA methods over conventional sampling, which often target specific taxa or assemblage components. In the complex, tropical community of the GoT, multiple assemblage components can be investigated through eDNA sampling with careful assay selection and analysis. This holistic, multi-substrate, multi-assay approach can be applied beyond oil and gas platforms to other marine infrastructure or natural habitat surveys. In particular, the ecosystem level data generated from this eDNA study can provide additional data to inform managers and regulators about the possible outcomes of different decommissioning options.

In marine eDNA research, the dominant collection method to date has comprised replicate water samples of varying volumes (Takahashi et al., 2023), which has been assumed to provide an overview of the adjacent substrates. From this research, we conclude that no single substrate was able to holistically document the entire detected diversity on or off platforms, a result corroborated by existing marine literature (Alexander et al., 2023; Koziol et al., 2018). The distinct assemblages associated with the various substrates have important implications for future monitoring surveys using eDNA methods at marine infrastructure, where the selection of substrate should be considered with an *a priori* knowledge of the primary study objectives.

Current Thailand legislation allows for a case-by-case assessment of decommissioning options using comparative assessment tools, such as the Best Practicable Environmental Option (BPEO; O'Riordan, 1989). While there are non-biological considerations involved, such as the potential for residual contaminants, physicochemical and geochemical parameters, we focus here on an ecological diversity perspective. The decommissioning of platforms typically involves the removal of all equipment that has contacted hydrocarbons (e.g., risers, valves and topsides), which is then followed by the cutting and decommissioning of the jackets (platform legs) (Bull and Kendall, 1994; Jagerroos and Krause, 2016). Platforms are then either completely removed, toppled in place, partially removed, or moved to an alternate location and repurposed as an artificial reef (Macreadie et al., 2011).

In the present study, the patterns of biotic diversity in relation to depth and the differences between biotic diversity associated with platforms compared to OP

locations can help to inform the outcomes of decommissioning options. While aspects of these impacts have been addressed at infrastructure elsewhere, such as exploring the coral or fish communities on reefed and standing infrastructure (Ajemian et al., 2015; Stunz and Coffey, 2020), or fish biomass and impacts to shell mounds under a partial dismantle scenario in California (Claisse et al., 2015), few studies have examined impacts to biotic diversity by following the fate of communities from before to after decommissioning. Consequently, there is little published information on survival rates of benthic and sedentary colonisers during the decommissioning process, particularly those taxa susceptible to photic and depth changes, such as corals and algae, which may affect subsequent colonisation of the biotic community.

The predicted outcomes for communities under each decommissioning scenario are summarised in Table 4.3, with specific examples for the taxa detected in the present study. Assuming that the OP sites represent a background diversity for open water and soft sediment habitats in the central GoT, the full removal of the infrastructure could see the local loss of up to 141 identified species that were only detected at platforms, or the potential loss of 36 shallow-water species (Supplementary S4.10) under a partial removal scenario (top section removed from marine environment). Inversely, under the leave *in-situ* scenario, all taxa would be retained at all platform depths with only minimal disturbance when removing associated equipment (e.g. topside structure, valves and risers; Sommer et al., 2019). Additionally, sediment assemblages are likely impacted from nutrient filtration as a result of the biotic community inhabiting the vertical structure above (Bomkamp et al., 2004). When platforms are removed, the likely reduction in nutrient input and complete removal of physical structures is predicted to result in sediment assemblages adjacent to platforms becoming similar to those in OP sediment assemblages over time.

Under the topple or top and leave in place scenarios, it is predicted that there would be a shift in community composition from shallow water benthic colonisers, such as autotrophs, to deeper adapted taxa, such as Porifera, as indicated by distinct assemblages detected at different depths in the present study. The impact of towing structures to a reefing location on assemblages is likely to depend on tow method (wet or dry), transit time, speed of tow (Marnane et al., 2022), as well as the morphology of biota. For example, colonial and encrusting species (such as some ascidians and

sponges) have documented increased survivorship at higher (vessel) transport speeds compared to softer bodied or branching benthic species (Coutts et al., 2010), which may have implications for towing transit success. For those species surviving the towing process, once at the reefing location their survival is likely to depend on reefing depth, with distinct assemblages detected at different depths in the present study indicating the potential for demise and transition to a different suite of species over time if the platforms are reefed at depths shallower or deeper than *in-situ*.

Table 4.3: The mechanisms of decommissioning expected to impact extant biological communities under five decommissioning options, including full removal, partial removal, Topple/top *in-situ*, Tow and topple, and leave *in-situ*, and the implications for the diversity detected during this study.
The grey indicates original platform position and black indicates the moved position of the platform under the decommissioning option. Figures recreated from original design in Fowler et al. (2018).

Decommissioning option	Generalised biotic impact of decommissioning alternative (from literature and present study)	Specific biotic impact of decommissioning alternative in the GoT (predicted from present study)
Full removal (of platform from marine environment)	• Immediate local loss of diversity associated with platform • Decline in diversity of soft sediment-associated biota under and surrounding platforms due to reduced nutrient filtration (Bomkamp et al., 2004) • Potential triggered spawning of some benthic colonisers, with potential implications for introduced species, if present (Donelan et al., 2022), and release of organic material during cutting removal and cleaning	• Local loss or dispersal of at least 141 identified species that were detected at platforms but not OP sites • Potential decline in soft sediment inhabiting species detected adjacent to platforms, such as the polychaete, *Timarete ceciliae*, and nematode, *Terschellingia longicaudata* • Potential vibration triggered spawning of benthic colonisers, such as ascidia (*Ascidia ahodori*) or Bryozoa (*Parasmittina* sp.) during cutting and removal
Partial Removal (top section removed from the marine environment)	• Immediate local loss of diversity associated with shallow sections of platforms • Potential change in soft sediment-associated assemblages under and surrounding platforms due to reduced nutrient filtration from top section of platform • Potential triggered spawning of some benthic colonisers, with potential implications for introduced species, if present, and release of organic material during cutting removal and cleaning	• Local loss of shallow water benthic species such as bivalves (*Barbatia trapezina*, *Isognomon legumen*, and *Pinna attenuata*), sponges (*Tethya wilhelma*, *Oscarella viridis*, *Crella cyathophora*) and algae (*Dictyota humifusa*), as well loss or dispersal of amphipods (*Podocerus jinbe*) and other arthropods (*Galathea* sp.) • Potential vibration induced spawning of benthic colonisers, such as ascidia (*Ascidia ahodori*) or Bryozoa (*Parasmittina* sp.) during cutting and removal
Toppled or topped *in-situ*	• Gradual loss of diversity associated with shallow section of platform due to changes in depth of toppled or topped platform • Gradual loss or re-orientation of attached biota due to changes in orientation of platform (toppled platform) • Gradual increase in diversity of deeper dwelling species	• Gradual loss or dispersal of a potential 36 identified species that were only detected in shallow sections of platform due to change in depth, including species adapted to wave surge zone, such as mussels (*Barbatia trapezina*) or encrusting sponges (*Crella cyathophora*)

Decommissioning option	Generalised biotic impact of decommissioning alternative (from literature and present study)	Specific biotic impact of decommissioning alternative in the GoT (predicted from present study)
or	• Potential triggered spawning of some benthic colonisers, with potential implications for introduced species, if present, and release of organic material during cutting removal and cleaning • Potential change and redistribution of sediment-associated assemblages under and surrounding platforms and extended under the toppled or topped structure	• Increase in deeper dwelling species, such as coral *Carijoa* sp. (Cnidaria), or *Igernella* sp. (Porifera), which were detected predominantly in deep samples
Tow and topple (creation of deployed artificial reef)	• Loss of some/all attached biota during towing, influenced by local regulations (requirement for cleaning), tow method (wet or dry), tow duration, and body morphology (Coutts et al., 2010) • Loss of fish and other motile species during towing (unless towed slowly; Marnane et al., 2022) • Gradual loss or re-orientation of attached biota due to change in orientation of toppled platform • Change in community composition associated with platform to deeper or shallower community depending on depth of toppling compared to *in-situ* depth • Decline in diversity of soft sediment-associated biota at site where platform was removed due to reduced nutrient filtration or loss of hard habitat • Potential triggered spawning of some benthic colonisers, with potential implications for introduced species, if present, and release of organic material during cutting removal and cleaning	• Maintenance of some reef-associated fish species if towed slowly (Marnane et al., 2022), including Moon wrasse (*Thalassoma lunare*), Goldband fusilier (*Pterocaesio chrysozona*) or Streaked spinefoot (*Siganus javus*). Likely reduction of juveniles from reef associated species unable to follow • Decline of soft sediment inhabiting species such as polychaete, *Timarete ceciliae*, and nematode, *Terschellingia longicaudata*

Decommissioning option	Generalised biotic impact of decommissioning alternative (from literature and present study)	Specific biotic impact of decommissioning alternative in the GoT (predicted from present study)
Leave *in-situ*	• No changes to existing diversity associated with platforms • No changes in diversity of soft sediment communities under and adjacent to platforms • No potential triggered spawning of benthic colonisers, or release of organic material	• Preservation of at least 141 identified species that were associated with platforms but not OP sites, including detected conservation significant species (*Stylophora pistillata*)

The value in conducting broad assemblage censusing pre-decommissioning is, in part, to understand the presence or absence of key taxa such as conservation significant or introduced species, both of which can be present in low abundance and biomass, which then may help inform decisions regarding decommissioning options. eDNA methods are highly sensitive and easily tailored to finding low abundance taxa (Nester et al., 2022). However, the use of single broad metabarcoding assays (such as the CO1 universal assay used in this study) may not be ideal for informing on the presence of low abundant searches (Wilcox et al., 2013; Xia et al., 2021). This can be mitigated by the use of narrow focus assays (target species or group specific), by increasing site replication, adopting a multi-assay approach, and incorporating *in-silico* analysis of target taxa. In this study, two conservation significant species were detected, of a potential 400 occurring within the broader GoT (IUCN red list database accessed in December 2022): the smooth cauliflower coral (*Stylophora pistillata*; family Pocilloporidae; Near-threatened) and the pelagic Indo-Pacific sailfish (*Istiophorus platypterus*; family Istiophoridae; Vulnerable) species. However, the sailfish species, detected from multiple replicates at one site, is likely only loosely to be associated with platform habitats. None of the eight regionally documented introduced marine species from Thailand were detected in this study. These included sponges (*Tetilla japonica*), arthropods (*Penaeus vannamei* and *Leucothoe spinicarpa*), tunicates (*Clavelina cyclus* and *Ecteinascidia thurstoni*), and mollusc species (*Mytilopsis adamsi*, *Mytilopsis sallei* and *Mytella strigata*) (Chavanich, 2010; Sanpanich and Wells, 2019). However, reference material for these species is limited, with three of the eight species entirely unrepresented, and *in-silico* analysis (allowing for two primer mismatches) indicating that only *M. strigata* had the potential to be amplified with the primers used in this study, yet was not detected. Also, no congeneric taxa for this introduced species were detected. However, this list may not reflect earlier introductions and cryptogenic species, and therefore not reflect the true number of extant non-native species present (Chavanich, 2010). While the primary aim of this study was to characterise broad diversity at the platforms, this finding highlights the importance, and implications, of assay selection, in targeted taxonomy searches.

While successful in the broad characterisation diversity using both taxonomic and taxonomy independent analysis at oil and gas platforms in the GoT, efficiencies in the selection of assay and substrate were evident in our results. The coral assay, for

example, was unable to assign almost 70% of ASVs past kingdom, with only 12% assigned to species, the lowest rate of all the assays used here. Given the estimated 292 identified scleractinian species occurring within the GoT (IUCN redlist database accessed in December 2022), the primary detection target of the coral assay (Alexander et al., 2019), this result could indicate a lack of hard corals inhabiting the structures, although four species were successfully detected (including 3 species of the genera *Tubastraea*). Alternatively, this could result from a lack of reference material from the ITS2 barcode region. Similarly, while the CO1 universal assay detected broad assemblages from water and bio-foul substrates, sediment substrate yielded few meiofauna species and significantly less ASVs by comparison. Detected sediment fauna comprised single annelid and nematode species and the remaining detections largely unassigned or green algae (genus *Chloropicon*). Informing management decisions currently relies heavily on taxonomic resolution (Kelly et al., 2014; Nielsen et al., 2023), especially for the detection of target taxa. Therefore, for future studies on platforms within the central GoT, we would not recommend the use of the coral assay, or the combination of the sediment substrate with broad CO1 universal assay, for informing on taxonomic resolution.

Given the developing field of marine eDNA, and in particular its use for censusing marine epibenthic assemblages, the optimisation of sampling and laboratory methods is ongoing, and likely dependent on location, environmental conditions and survey objectives. A number of recommendations can be drawn from this study, and existing literature, for future surveys on oil and gas platforms, which may be applicable to wider marine epibenthic sampling. During study design, the availability of assays and completeness of reference databases should be considered (through *in-silico* analysis; Bylemans et al., 2018) for target or local dominant taxa, which can determine potential for taxonomic analysis of results, or analysis of assemblages independent of taxonomic frameworks. Sampling substrates and methods target different biotic assemblages, and therefore careful consideration should be given to the objectives of the research study, with recent studies indicating increased diversity detections utilising methods that collect minimal bulk material (Alexander et al., 2023). Increasing vertical sampling replication may increase detected diversity and provide finer spatial nuance in informing on decommissioning, such as informing on optimal structure dissection. Finally, the application of an appropriate universal barcode assay, such as the CO1

universal assay used here, to all substrates may provide initial broad results and inform further assay direction required or on unrepresented target groups, if necessary for the study objectives.

The application of eDNA for the assessment of biotic diversity associated with marine infrastructure is relatively new, and particularly so in the Gulf of Thailand. eDNA metabarcoding can provide a very valuable, non-destructive tool for holistically censusing benthic, sedimentary and planktonic organisms, either as a complementary of stand-alone method broadly across the tree of life. Importantly the ability to store the digital data and the DNA extracts provide a powerful way to revisit sites and samples to build up temporal and spatial datasets. With appropriate collection of samples, selection of assays and analysis, eDNA censusing has considerable potential to aid in determining the decommissioning course of action. For example, exploring how biota respond to reefing or towing options may help shape future decisions. Likewise, these methods can be further tailored to detected known IMS. Finally, ongoing eDNA surveys of the GoT sites could provide valuable sentinel data on how oceans are responding to a range of anthropogenic pressures.

4.7 References

Every reasonable effort has been made to acknowledge the owners of copyright material. I would be pleased to hear from any copyright owner who has been omitted or incorrectly acknowledged.

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4.8 Supplementary information

Supplementary S4.1: Details of platform (PF) and off-platform (OP) sites selected for sampling within the Gulf of Thailand, including type, depth and age.
Sites were sampled for a combination of water, bio-foul and sediment.

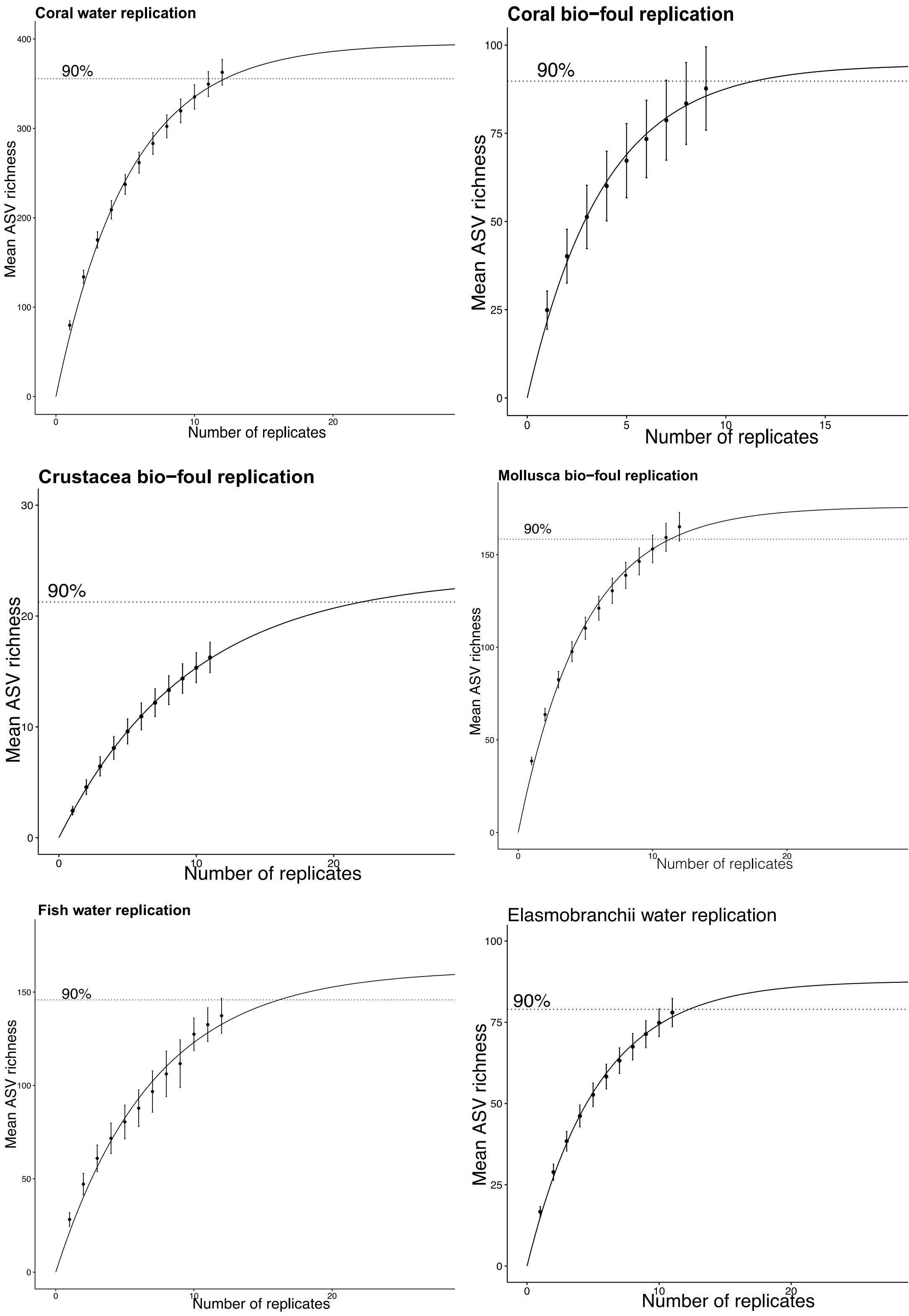
Site	Platform type	Depth to benthos (meters)	Installation date	Platform age at sampling (years)
PF1	Wellhead (4-Pile)	69.8	16-Jul-05	13
PF2	Wellhead (4-Pile)	65.9	12-Apr-06	12
PF3	Wellhead (4-Pile)	69.7	4-Mar-95	23
PF4	Wellhead (4-Pile)	61.1	5-May-13	5
PF5	Wellhead (4-Pile)	68.3	22-Sep-10	8
PF6	Wellhead (4-Pile)	73.5	28-Sep-12	6
PF7	Wellhead (Tripod)	73.8	9-Jan-00	18
PF8	Wellhead (4-Pile)	69.8	9-Jul-10	8
OP1	NA	72.9	NA	NA
OP2		69.7		
OP3		69.8		
OP4		70.1		
OP5		69.9		

Supplementary S4.2: Link to demultiplexed raw sequence data and sample index information.

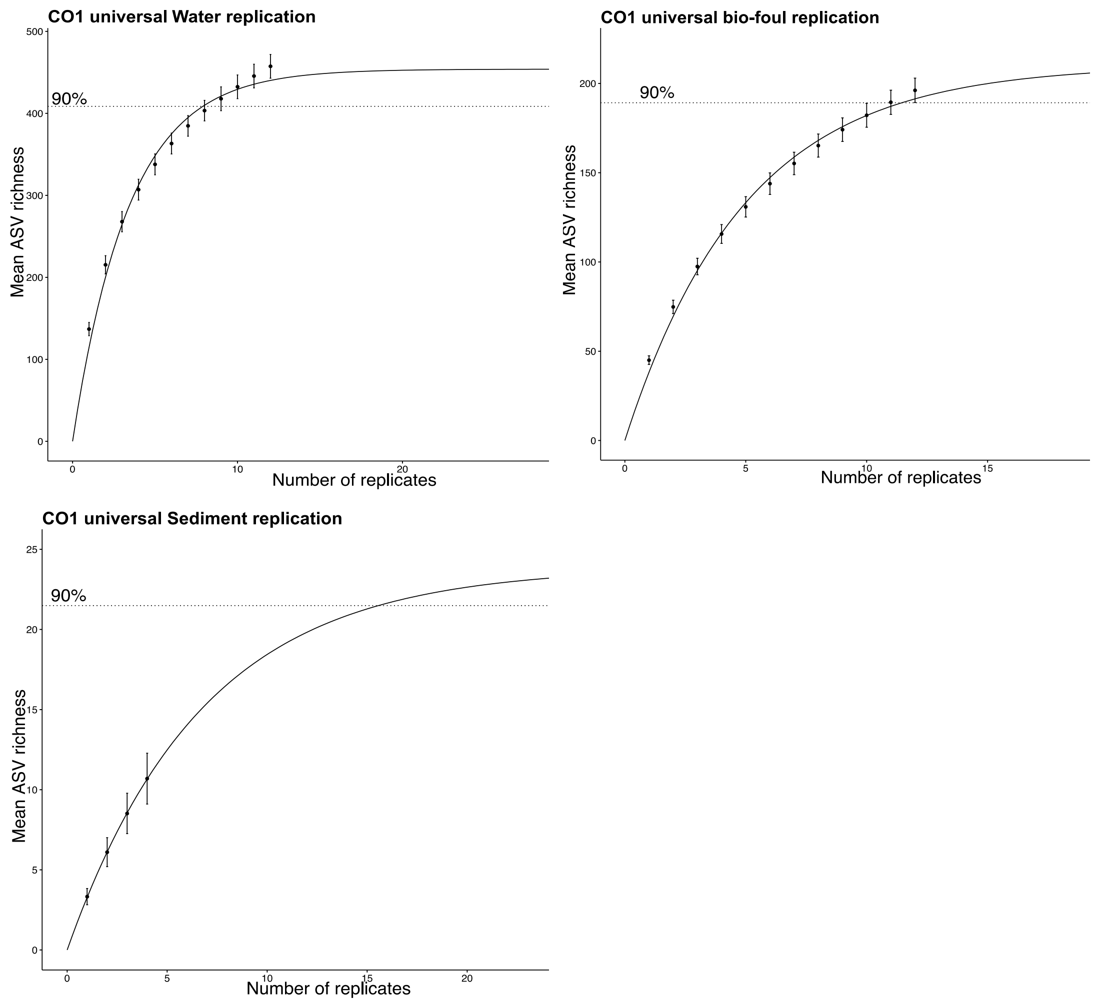
DOI: https://doi.org/10.5281/zenodo.7787789

Supplementary S4.3: Table of mean sequencing results for quality filtering, denoising, read merging and chimera removal for each assay.

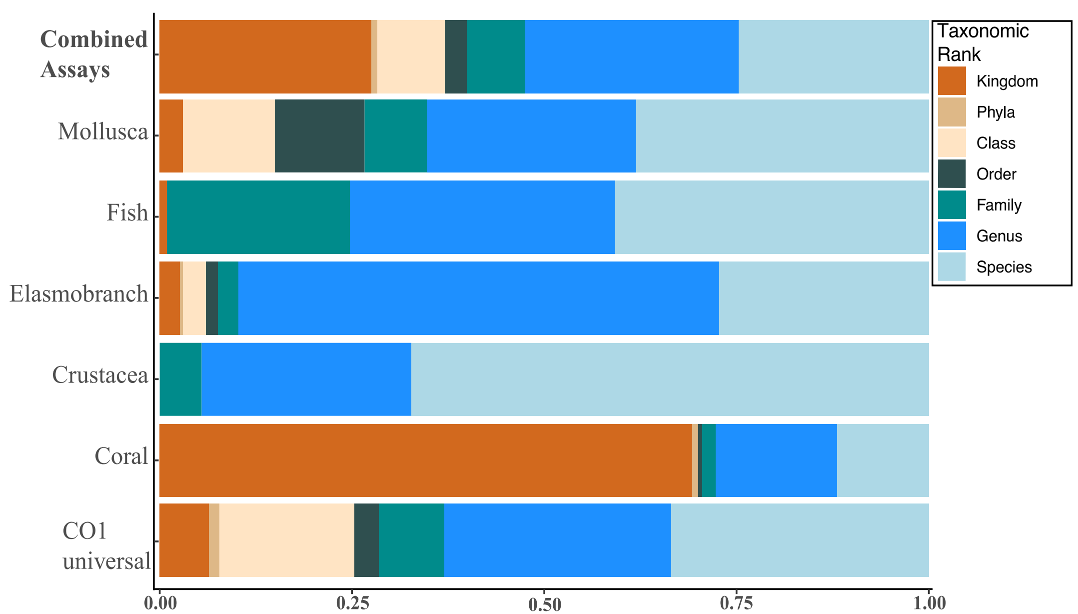
Total Raw Reads	Mean per sample						
	Mean Raw Reads	**Filtered**	**Denoised forward**	**Denoised Reverse**	**Merged**	**Chimera removal**	**ASVs**
CO1 universal							
45,301,455	97,632 (±5,097)	76,338 (±2,247)	76,055 (±2,239)	76,037 (±2,239)	73,281 (±2,157)	68,344 (±1,986)	94 (± 4)
Coral							
40,019,222	84,108 (2,842)	60,626 (±10,089)	60,580 (10,083)	60,572 (±10,079)	56,527 (±9,609)	55,004 (±9,269)	59 (±3)
Fish							
12,144,222	63,673 (±2,958)	55,284 (±3,047)	55,239 (±3,043)	-	-	54,676 (±3,001)	31 (±2)
Elasmobranchs							
15,290,811	42,393 (±2,144)	40,324 (±2,186)	40,018 (±2,174)	-	-	39,125 (±2,113)	19 (±1)
Crustacea							
15,351,796	106,188 (±4,606)	101,527 (±4,901)	106,335 (±4,601)	-	-	106,026 (±4,579)	2 (±0.2)
Mollusca							
15,461,495	125,229 (±3,409)	122,765 (±3,168)	122,554 (±3,164)	-	-	120,985 (±3,112)	39 (±1)



Coral water replication
90%
Mean ASV richness
Number of replicates
Coral bio−foul replication
90%
Mean ASV richness
Number of replicates
Crustacea bio−foul replication
90%
Mean ASV richness
Number of replicates
Mollusca bio−foul replication
90%
Mean ASV richness
Number of replicates
Fish water replication
90%
Mean ASV richness
Number of replicates
Elasmobranchii water replication
90%
Mean ASV richness
Number of replicates



Supplementary S4.4: Accumulation curves showing replication efficacy of each assay for the target substrates (water samples, bio-foul and sediment) to detect and extrapolated 90% of the ASV diversity present at each site.



Supplementary S4.5: Proportional taxonomic rank assignment for assays used on platform and OP sites in the Gulf of Thailand

Supplementary S4.6: PERMANOVA results on taxonomy collapsed at the species-level. PERMANOVA completed on presence-absence transformed Jaccard resemblance matrix. Non-significant results are designated by **

	ASV			
	DF	**Pseudo-F**	**P(perm)**	**Perm.**
Combined (Location and Depth)				
Location	1	12.2	<0.001	9876
Depth	3	15.5	<0.001	9851
Location × Depth	3	2.2	<0.001	9824
Residual	290			
Water (Location and Depth)				
Location	1	1.7	0.002	9876
Depth	2	3.7	<0.001	9822
Location × Depth	2	0.9	0.734 **	9830
Residual	150			
Bio-Foul (Depth)				
Depth	2	3.3	<0.001	9771
Residual	93			
Sediment (Location only)				
Location	1	2.5	0.008	9932
Residual	44			

Supplementary S 4.7: PERMANOVA pairwise test taxonomic dependent analysis (collapsed at species) for levels within the factor 'Depth' in the term 'Location' for combined substrates, and stand-alone water and bio-foul substrates.
Note that sediment is included as a depth factor for combined analysis as no depth profiling exists within the sediment collections.

Depth	**Location = Platforms**			**Location = OP**		
	t	**P(perm)**	**Perm.**	**t**	**P(perm)**	**Perm.**
Combined						
Shallow x Mid	1.3	0.08 **	9,885	1.2	0.0647 **	9,882
Shallow x Deep	1.9	<0.001	9,880	1.8	<0.001	9,898
Shallow x Sediment	4.2	<0.001	9,896	5.1	<0.001	9,928
Mid x Deep	1.3	0.0573 **	9,884	1.2	0.0459 **	9,872
Mid x Sediment	4.2	<0.001	9,900	5.3	<0.001	9,919
Deep x Sediment	4.2	<0.001	9,902	5.5	<0.001	9,921
Water						
Shallow x Mid	1.5	<0.001	9,873	1.2	0.0623 **	9,871
Shallow x Deep	2.1	<0.001	9,875	1.8	<0.001	9,897
Mid x Deep	1.4	0.002	9,878	1.2	0.0448	9,889
Bio-foul						
Shallow x Mid	1.6	<0.001	9,844			
Shallow x Deep	2.2	<0.001	9,858		N/A	
Mid x Deep	1.6	<0.001	9,855			

Supplementary S4.8: **Results of the SIMPER analysis completed on presence/absence transformed data with a Jaccard similarity on four depths, Shallow, Mid, Deep and Sediment.**

Assignment at species-level	Average abundance	Average similarity	Sim/SD	Contribution %	
				Individual	Cumulative
Group – Shallow (Average similarity: 23.6 %)					
Unassigned	0.78	2.26	1.2	9.55	9.55
Unassigned	0.55	1.14	0.63	4.82	14.37
Farranula gibbula	0.56	1.13	0.66	4.8	19.17
Unassigned	0.55	1.13	0.63	4.79	23.96
Unassigned	0.5	0.98	0.56	4.14	28.1
Unassigned	0.52	0.95	0.59	4.04	32.14
Unassigned	0.47	0.86	0.52	3.64	35.78
Unassigned	0.47	0.85	0.52	3.6	39.37
Chloropicon roscoffensis	0.48	0.79	0.54	3.33	42.7
Pelagomonas calceolata	0.48	0.77	0.54	3.27	45.97
Unassigned	0.44	0.74	0.47	3.14	49.11
Phaeocystis globosa	0.44	0.71	0.48	2.99	52.1
Unassigned	0.45	0.7	0.49	2.97	55.07
Unassigned	0.44	0.64	0.47	2.71	57.79
Lumbrineris perkinsi	0.41	0.55	0.43	2.33	60.12
Unassigned	0.41	0.55	0.43	2.32	62.44
Unassigned	0.38	0.51	0.39	2.17	64.61
Encrasicholina punctifer	0.34	0.44	0.36	1.86	66.47
Pseudoscourfieldia marina	0.34	0.42	0.36	1.79	68.26
Selar crumenophthalmus	0.33	0.4	0.34	1.7	69.97
Ophiactis savignyi	0.34	0.38	0.35	1.62	71.58
Group – Mid (Average similarity: 25.5 %)					
Unassigned	0.77	2.35	1.14	9.25	9.25
Farranula gibbula	0.66	1.73	0.84	6.8	16.05
Chloropicon roscoffensis	0.58	1.28	0.69	5.04	21.08
Unassigned	0.53	1.12	0.61	4.41	25.5
Unassigned	0.5	1.02	0.56	4.01	29.51
Unassigned	0.5	1	0.56	3.93	33.44
Unassigned	0.48	0.95	0.54	3.74	37.18
Phaeocystis globosa	0.48	0.94	0.54	3.7	40.88
Unassigned	0.48	0.93	0.54	3.66	44.54
Unassigned	0.47	0.88	0.52	3.45	47.99
Pseudoscourfieldia marina	0.45	0.83	0.49	3.25	51.25
Pelagomonas calceolata	0.47	0.79	0.52	3.11	54.35
Unassigned	0.44	0.77	0.47	3.03	57.38
Unassigned	0.44	0.77	0.47	3.01	60.39
Unassigned	0.42	0.71	0.45	2.79	63.18
Unassigned	0.41	0.61	0.43	2.39	65.58

Assignment at species-level	Average abundance	Average similarity	Sim/SD	Contribution %	
				Individual	Cumulative
Unassigned	0.36	0.47	0.38	1.85	67.43
Unassigned	0.34	0.45	0.35	1.78	69.2
Lumbrineris perkinsi	0.33	0.42	0.34	1.65	70.85
Group – Deep (Average similarity: 26.4 %)					
Unassigned	0.81	2.7	1.33	10.24	10.24
Pelagomonas calceolata	0.78	2.46	1.2	9.32	19.56
Chloropicon roscoffensis	0.66	1.66	0.85	6.29	25.85
Farranula gibbula	0.59	1.36	0.72	5.17	31.02
Unassigned	0.5	1.01	0.56	3.82	34.84
Phaeocystis globosa	0.5	1.01	0.56	3.82	38.66
Unassigned	0.48	0.95	0.53	3.59	42.25
Micromonas commoda	0.48	0.94	0.54	3.58	45.83
Unassigned	0.48	0.93	0.54	3.54	49.37
Unassigned	0.48	0.92	0.54	3.5	52.87
Unassigned	0.47	0.87	0.52	3.31	56.18
Unassigned	0.45	0.82	0.5	3.09	59.27
Unassigned	0.45	0.82	0.5	3.09	62.36
Pseudoscourfieldia marina	0.42	0.7	0.46	2.67	65.03
Carijoa riisei	0.39	0.59	0.41	2.22	67.25
Hyotissa hyotis	0.36	0.52	0.37	1.96	69.21
Unassigned	0.33	0.43	0.34	1.64	70.86
Group – Sediment (Average similarity: 28.6 %)					
Unassigned	0.75	17.89	0.93	62.52	62.52
Chloropicon roscoffensis	0.39	4.33	0.38	15.14	77.67

Supplementary S4.9: PERMANOVA results of the presence-absence transformed Amplicon Sequence Variance (ASV) data on Jaccard resemblance matrix.
Non-significant results are designated by **

	ASV			
	DF	**Pseudo-F**	**P(perm)**	**Perm.**
Water (Location and Depth)				
Location	1	1.7	<0.001	9742
Depth	2	4.3	<0.001	9737
Location × Depth	2	1.1	0.105 **	9708
Residual	150			
Bio-Foul (Depth)				
Depth	2	2.5	<0.001	9773
Residual	93			
Sediment (Location only)				
Location	1	1.9	0.007	9892
Residual	44			

Supplementary S4.10: Link to lists of species-level resolved taxa detected at each location depth at platforms and off-platform locations.

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Chapter 5

Temporal eDNA sampling reveals a high diversity, and high assemblage turnover rate, of chordates surrounding a newly installed integrated artificial reef

Collage of photos from the Exmouth Gulf and Cape. Images provided and displayed with consent from Justine Shailes and Euan Harvey

5.1 Preface

This data collection chapter is currently in preparation for submission, with the manuscript titled '**Temporal eDNA sampling reveals a high diversity, and high assemblage turnover rate, of chordates surrounding a newly installed integrated artificial reef**'.

This research investigates the use of eDNA metabarcoding to track temporal changes in chordate composition at a deployed artificial reef in the north-west of Western Australia. This reef, which comprises a mix of cleaned and repurposed oil and gas infrastructure, as well as designed concrete structures, was deployed to support local recreational fishing, which is economically important to the adjacent town, Exmouth. The ability to detect temporal changes at marine infrastructure can be beneficial in tracking the recruitment of populations of recreational fishing value. However, such studies can be broadened to monitor seasonal, annual, lunar or tidal cycles across an ecosystem. This chapter examines chordate diversity prior to the installation of the reef and for approximately two years post installation.

The original conceived idea with this study was to explore not only chordate data, but determine what effect that artificial reef deployment has on plankton, in particular copepod, diversity. A significant amount of time was spent trying to deconvolute the arthropod dataset from the CO1acartia assay, which was selected for this study as it detects both arthropods and chordates. The disjoint between copepod taxonomy and molecular resolution, led to this chapter focussing on just the chordates.

5.1.1 Acknowledgements

I would like to gratefully acknowledge the aid of the Fish Ecology laboratory for logistical support during field sampling, particularly Laura Fullwood, Damon Dreissen, Logan Hellmrinch, Sam Russell, Rowan Kleindienst, Chynna Cahill and John Totterdale. I would also like to acknowledge statistical brainstorming and support from Katrina West at CSIRO. 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 and from the CSIRO Environomics Future Science Platform through the iPhD program.

I would like to acknowledge the support and contributions of the co-authors of this chapter, Tina Berry, Zoe Richards and Euan Harvey. A breakdown of author and co-author contributions is provided in Appendix I.

5.1.2 Data accessibility

The demultiplexed sequence and sample data is available from:
https://drive.google.com/drive/folders/16mC0G1DgifOlAbfLo93JNcqLjDGGrbGv?usp=share_link

5.2 Abstract

Artificial reefs are being increasingly deployed in Australia to support the economically important recreational fishing industry. One such reef, King Reef, was installed in 2018 in the Exmouth Gulf, Western Australia, and is a mix of four structure types, including repurposed and cleaned oil and gas infrastructure. Deployed with the aim of providing habitat for recreationally important fish species, we assess the temporal changes in chordate diversity at the reef using eDNA metabarcoding (eDNA) over 27 months. Water eDNA samples were collected at seven sites across four habitats, comprising the artificial reef, sand, natural reef, and an area dominated by benthic filter feeders (sponge habitat), with the collections starting prior to installation of the artificial reef. Using two metabarcoding assays, a broad diversity of chordate diversity were detected across the Exmouth Gulf, including 161 species of fish, 22 sharks and rays, 2 species of turtles and four marine mammals. King Reef had the lowest chordate richness per phase of all the habitats. However, all habitats demonstrated a high turnover in the assemblage composition. We were unable to detect consistent changes in chordate composition attributable to the presence of the artificial reef, or temporal synergies of chordate composition to either the natural reef, sponge or sand habitats. This lack of temporal trends could be an accurate depiction of the transient nature of the species in the Exmouth Gulf. Alternatively, it could be a result of environmental factors (tidal or current movement). We show that temporal eDNA metabarcoding can enhance biodiversity detection and that, with further optimisation, shows promise for tracking community changes and recruitment at artificial reefs.

5.3 Introduction

There are approximately 150 artificial reefs in Australia (Florisson and Tropiano, 2017). Historically, the term "artificial reef" has referred to sunken vessels or abandoned marine infrastructure (Hylkema et al., 2021). However, in recent years, artificial reefs are increasingly being constructed to fulfill a particular economic or ecological purpose (Dafforn et al., 2015; Tynyakov et al., 2017; Lee et al., 2018; Ramm et al., 2021). These purpose-built artificial reefs are being deployed to restore and connect natural habitats, provide refugia in more protected areas and to provide optimally located habitats for recreational fishing and tourism activities such as SCUBA diving (Becker et al., 2017, 2018; Tynyakov et al., 2017; Lima et al., 2019; Vivier et al., 2021). Over the last 70 years, artificial reef construction has been trialled using a range of materials, including ash, tires, metal and ceramic (Ramm et al., 2021). However, there is an increasing focus on whether repurposing marine infrastructure into artificial reefs is a good use of this material (Sherman, 2002; Bull and Love, 2019; Lima et al., 2019). The repurposing of marine infrastructure can be economically beneficial compared to the construction or 3D printing (Ly et al., 2021) of artificial reefs (Elrick-Barr et al., 2022) and, depending on the original location and cleaning requirement, can provide an extant benthic diversity that may result in quicker recruitment of fish species (Marnane et al., 2022). However, the deployment of artificial reefs, and the use of repurposed marine infrastructure can promote marine connectivity and potentially facilitate distribution expansions, including invasive or introduced taxa (Macreadie et al., 2011; Henry et al., 2018; Castro et al., 2021). Within Western Australia (WA), there have been seven purpose-designed artificial reefs deployed to provide additional habitat for use by recreational fishers. The northernmost of these artificial reefs is located in the Exmouth Gulf (north-west WA), and uses both repurposed marine infrastructure to provide vertical habitat for fish species as well as designed concrete structures (Florisson et al., 2020).

The Exmouth Gulf is an economically and ecologically important area. Covering approximately 2,614 km^2, with a mean depth of 11.9 m, the gulf is predominantly a gradual sloped soft-benthic system, with mean tidal range of 1.8 m (Brunskill et al., 2001; Bathmann et al., 2021). The land mass bordering the gulf comprises the Cape Range peninsula to the west, which contains the Cape Range National Park and the

Exmouth township (21°56’S, 114°09’E), and arid plains to the east (McCook et al., 1995). The Ningaloo State Marine Park and World Heritage Area partially borders the northern boundary of the gulf, which extends around the point of the peninsular and encompasses one of the largest fringing reefs in the world (Smallwood et al., 2011). The Exmouth Gulf is also recognised as having valuable mangrove and seagrass habitat, which provide important nursery habitat to recreationally and important fish species (McCook et al., 1995; Loneragan et al., 2013; Bathmann et al., 2021). The gulf provides a sanctuary for a number of marine mammals, such as dugongs (*Dugong dugon*), three species of dolphin (Sprogis and Parra, 2022), and acts a nursery stopover for the annual migration of the humpback whales (*Megaptera novaeanglea*) (Preen et al., 1997; Bejder et al., 2019). In addition, over 63 species of cartilaginous fish (sharks and rays, class Chondrichthyes), approximately 790 teleost (class Actinopterygii) fish, six species of turtle and 15 species of sea-snake are known from the area (Sutton and Shaw, 2021). As a result of this diversity, the Exmouth town (population 3,074) is economically reliant on both the gulf and Ningaloo Marine Park as attractions for tourism, with approximately 218,000 visitors annually (as of 2018/2019), predominantly for eco-tourism and recreational fishing.

Recreational fishing contributes approximately $2.4 billion AUD to the WA annual economy (Ryan et al., 2021). The Exmouth Gulf is highly regarded as a recreational fishing destination for both pelagic (e.g., Spanish mackerel, *Scomberomorus commerson*), and demersal (e.g., spangled emperor, *Lethrinus nebulosus*) sport fishing species (Mitchell et al., 2018). Increases in recreational fishing can lead to increased ecological pressures on local target fish stocks, and within the wider environment (Brownscombe et al., 2019). To provide additional accessible habitat for recreational fishing tourism within proximity to the Exmouth boat ramps, the Exmouth Integrated Artificial Reef (hereafter referred to as "King Reef") was installed in 2018 (Florisson et al., 2020). At the time of installation, this artificial reef was the largest in the southern hemisphere and the first within Australia to integrate modified, repurposed oil and gas infrastructure. This repurposed infrastructure consisted of six large mid-depth buoyancy modules from a nearby gas production field and was complemented with 49 purpose made concrete structures (55 structures overall; Florisson et al., 2020). Placed over approximately 472 m^2 of seafloor in the Exmouth Gulf, King Reef comprises four different structure types; Fish Towers (modified buoyancy modules),

Pyramids, Apollo clusters, and Abitats (Figure 5.1). This diversity in structure type and depth profile, which ranges from 1-10 m off the seafloor, is designed to promote recruitment and the attraction of new individuals to the reef (Rilov and Benayahu, 2000). These structures were also designed to assist in post-recruitment survival of target species at different life history stages in order to promote overall production within the habitat (Gallaway et al., 2009; Arney et al., 2017; Florisson et al., 2020). However, assessing the success of these structures to attract, recruit and maintain populations of recreationally important fish species can be challenging.

Conventional monitoring for fish on marine infrastructure uses baited remote underwater videos (BRUVs), diver operated videos (DOVs) or unmanned or remotely operated vehicles (ROVs) to count, identify and measure the lengths of fish (Love et al., 2020; Rofallski et al., 2020; Schramm et al., 2020a, 2020b). These methods can be logistically difficult in low visibility and turbid conditions, which are common within the Exmouth Gulf due to oceanic swells and tidal movement re-suspending sediment (Dee et al., 2020). Research at marine infrastructure has shown that these methods detect only a part of the assemblage (Alexander et al., 2022) and they have the potential to skew result towards particular trophic levels, such as carnivorous or omnivorous species due to the use of baits and attractants (BRUVs; Schramm et al., 2020b). These methods can also elicit variable avoidance behaviour by fish, depending on SCUBA method and location (Lindfield et al., 2014; Gray et al., 2016), as well as light and ROV avoidance behaviours (Stoner et al., 2008; Ryer et al., 2009; Prato et al., 2017; Hellmrich et al., 2023).

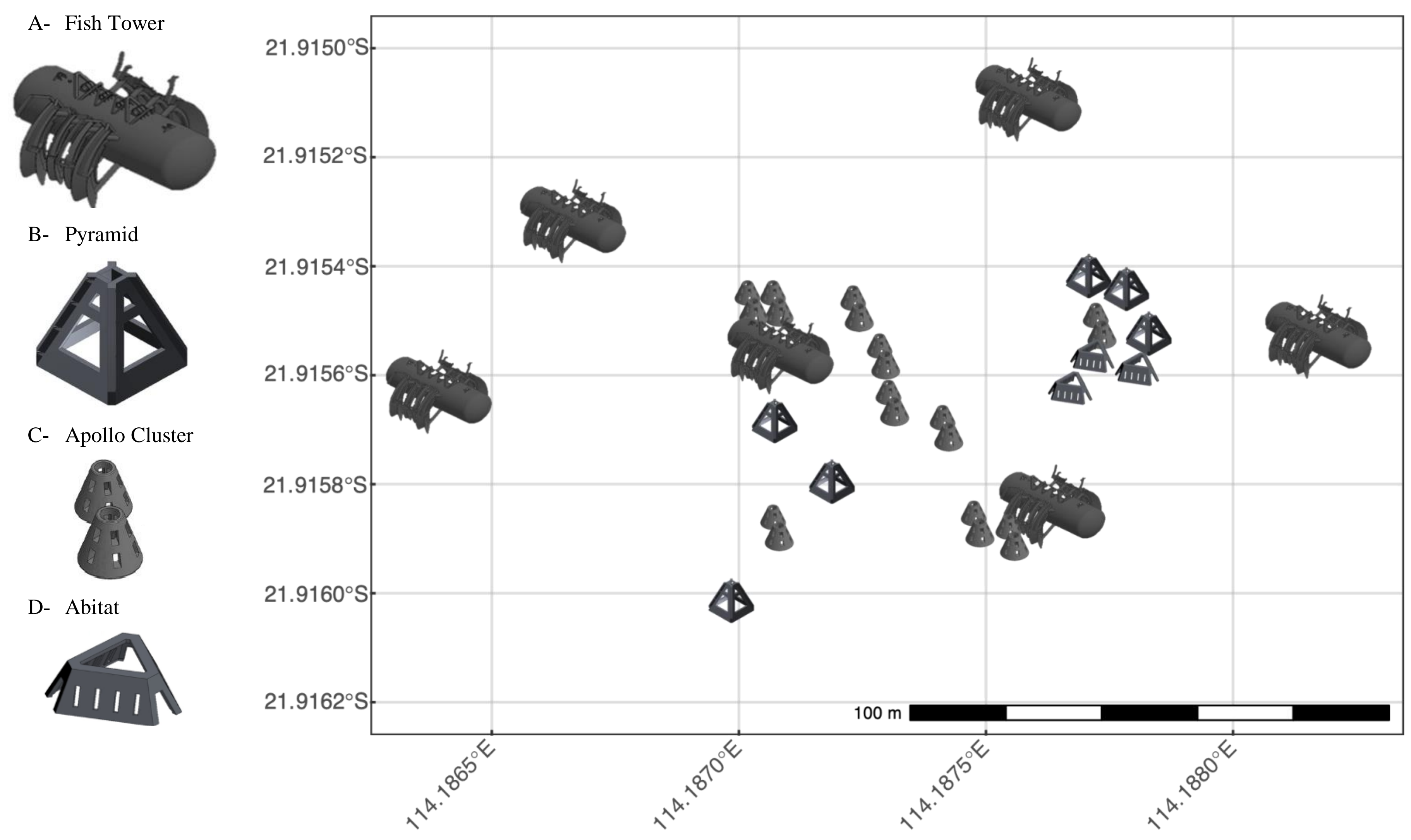

Figure 5.1: Schematic of the spatial distribution of King Reef, which was installed in the Exmouth Gulf in August, 2018. The reef was installed over approximately 472 m2, and comprised four structure types: Fish towers (A; re-purposed, modified oil and gas buoyancy modules), Pyramids (B), Apollo clusters (C); and Abitats (D).

Environmental DNA metabarcoding (hereafter "eDNA") is a non-invasive, and highly sensitive method for auditing marine biodiversity. It is increasingly being used as stand-alone method, or in tandem with conventional methods to enhance the breadth of diversity that is detected in complex and dynamic marine environments (Azevedo et al., 2020; Alexander et al., 2022). eDNA has marine applications in the detection of marine introduced species (Couton et al., 2019; Pearman et al., 2021), rare and cryptic species (Nester et al., 2020, 2022), broad-scale monitoring surveys (Stat et al., 2017; West et al., 2021b) and the detection of temporal patterns in biotic communities (Berry et al., 2019). For censusing and monitoring fish assemblages, eDNA has been demonstrated to have an equal or higher species richness detection capability when compared to conventional, morphological identification methods (Valentini et al., 2016; Zhang et al., 2020; Alexander et al., 2022). As a relatively new method of marine diversity detection, the frameworks supporting eDNA studies (e.g., reference material, and assay availability) and the impact of environmental conditions (tidal movement and longevity of eDNA), require more research and optimisation (Richards et al., 2022). While fish diversity has been explored using eDNA at both artificial reefs (Inoue et al., 2022; Levy et al., 2023) and marine infrastructure (Alexander et al. 2022; Borrell et al., 2017; Mauffrey et al., 2021), these have focussed on single-timepoint sampling. No research explores the temporal patterns recorded by eDNA from the perspective of the colonisation and succession of newly installed, integrated artificial reefs.

This study used eDNA techniques to explore the spatio-temporal diversity of chordates at King Reef and nearby natural habitats in the Exmouth Gulf, with particular focus on fish (class Actinopterygii), elasmobranchs (class Chondrichthyes) and marine mammals. Two metabarcoding assays were applied to water samples collected over a two-year period with the aims of (a) detecting a broad chordate diversity within the gulf; (b) exploring the temporal trends at King Reef with context to surrounding habitats; and (c) examining temporal detections of recreationally important fish species at King Reef as a proxy for determining the success of the artificial reef. We hypothesised that (1) the chordate diversity detected at the artificial reef would rapidly increase post-installation before reaching a plateau; (2) the composition of the fish and community composition would start to homogenise with the natural reef habitats located south of King Reef within the two years of sampling. Our primary objective in

this study is to explore and understand the range of species inhabiting King Reef within the context of the wider Exmouth Gulf and determine the success of the structure for supporting fish populations of recreational fishing importance.

5.4 Methods

5.4.1 Study area

King Reef is located approximately 6.6 km north-east of the town Exmouth in the Exmouth Gulf (Figure **5**.**2**) and was deployed within a flat, homogenous, sandy bed at approximately 17 m depth. The reef is approximately 15 km north of the nearest natural reef systems and approximately 5 km south of the Ningaloo Marine Park boundary, behind which lies an extensive habitat for benthic filter feeders, which is locally referred to as the sponge garden (Heyward et al., 2010).

Field sampling was completed in the Exmouth Gulf over six phases between June 2018 and September 2020 (27-month period), and comprised the repeat sampling four different habitat types the artificial reef (King Reef- KR), sand (SA), natural reefs (Camplin and Cooper Shoals- NR) and sponge habitat (SP; Figure **5**.**2**). Each phase included sampling of seven sites, two per each habitat type and one within the artificial reef (Supplementary S5.1). The exception to this was phase 1, where no sampling was completed within the sponge habitat (located within the Ningaloo State Marine Park), but was incorporated into the study for phase 2. Sampling mobilisations were timed to coincide with the neap tides to improve sampling logistics and reduce influence of tidal movement within the gulf.

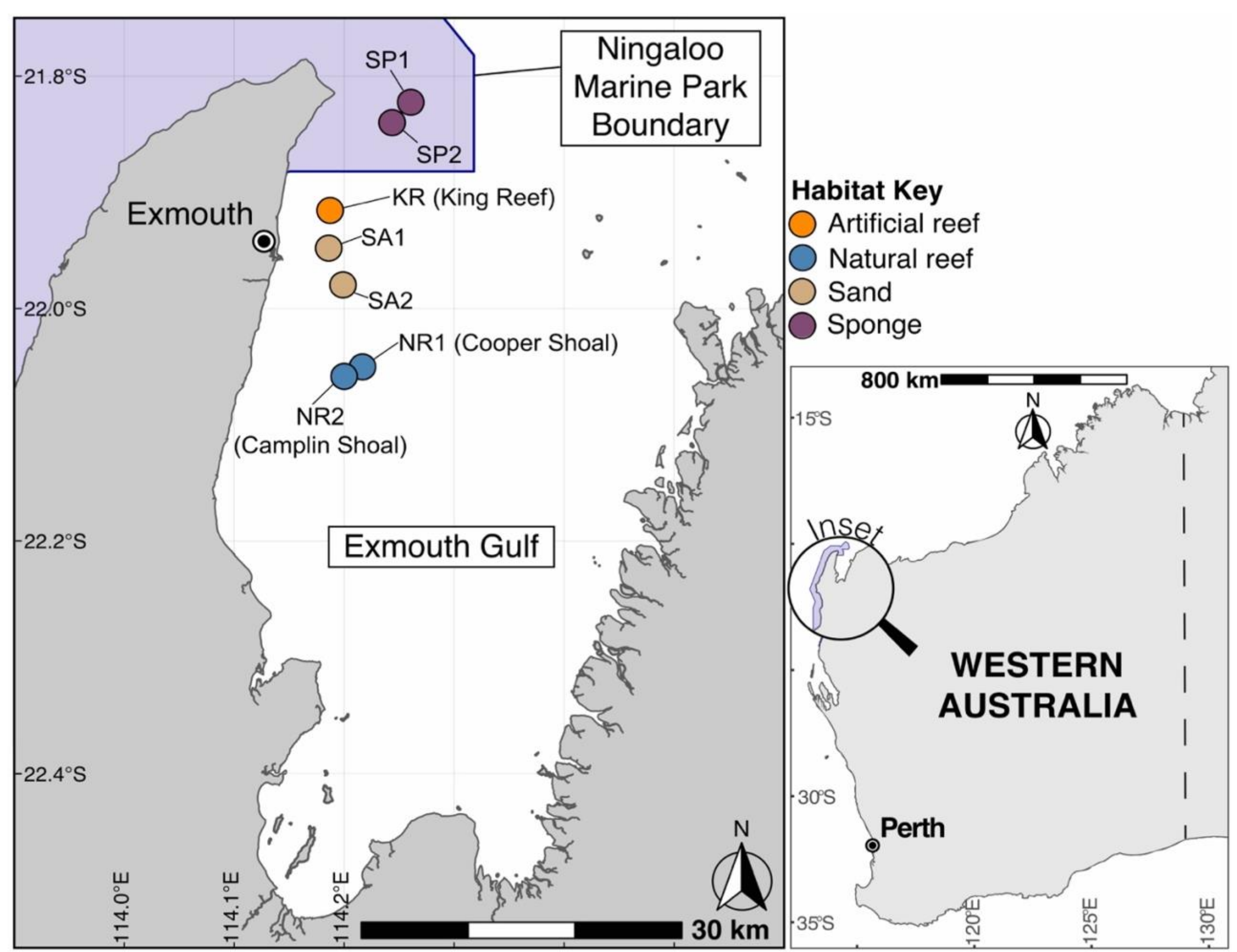

Figure 5.2: Exmouth Gulf study area location in relation to Perth, Western Australia (right), and the Exmouth Gulf (left), including sampling locations and habitat types within the gulf and the Ningaloo State Marine Park (blue shading; right). Habitat types comprise the Artificial Reef site (KR- King Reef), Natural Reef sites (NR), Sand sites (SA) and Sponge sites (SP).

5.4.2 Seawater eDNA collection

Using a boat depth sounder, a 1.7 L sterile Niskin water sampler was deployed to collect water from approximately 1 m above the benthos. On reaching the desired depth, a weighted messenger was released from the vessel via rope to trigger the closure of the Niskin water sampler, which was then brought to the surface. On the vessel, 1 L of water was subsampled into a sterile, labelled bottle, then placed on ice and refrigerated until filtration, which occurred within 6 hrs of collection. All sterilisation of eDNA field equipment was completed using a 10% sodium hypochlorite (bleach) solution soaked for a minimum of 15 minutes between sites. Nitrile gloves were worn during sampling and changed between sites and as necessary. A total of five 1 L seawater samples were collected at each site during every phase, with 35 water samples collected in total, with the exception of Phase 1 (see above). Two hundred seawater samples were collected overall across the six phases. At King Reef, effort was made to deploy the Niskin water sampler and collect from adjacent to the fish tower structures, where possible.

Filtration of collected water samples was completed in a cleaned, temporary field-laboratory using two peristaltic Sentino pump (Pall Life Sciences, USA), with water samples filtered onto 47 mm, 0.22 μm polyethersulfone membrane. All filtration equipment was soaked in a minimum 10% bleach solution for 15 minutes prior to use and between samples and rinsed using additional site water, which was collected for this purpose. Control samples of bleach solutions were taken and filtered daily to detect potential cross contamination. Sample and control filter papers were stored in uniquely labelled ziplock bags at -20 ^{o}C for transport back to laboratories in Perth.

5.4.3 Laboratory processing

5.4.3.1 <u>DNA extraction</u>

In dedicated clean laboratories, filtered membranes were thawed and divided, with half returned to storage in -20 °C freezers as backup and archiving purposes. The second filter half was dissected into small pieces and incubated overnight at 56 °C in a solution of Proteinase K (60 µl) and Qiagen Buffer ATL (540 µl). DNA was then extracted from the filter membrane using the QIAgen DNeasy Blood and Tissue kit (Qiagen, Netherlands) protocol on the QIACube platform (Koziol et al., 2018; Stat et al., 2017),

producing approximately 100 µl of eDNA extract. Digestion and extraction controls were collected with each processed batch to expose potential laboratory contamination. Extracted DNA was stored at -20 °C.

5.4.3.2 Primer selection

Two broad metabarcoding assays were used in this study, first (16Schordata) to target marine chordates, specifically fish (class Actinopterygii), sharks and rays (class Elasmobranchii), and secondly an assay with a broad metabarcoding focus, CO1acartia, which was originally designed to detect plankton and arthropods (Table 5.1). The plankton assay, CO1acartia (called Copepod3 in Berry et al., 2019), has also been demonstrated to detect more broadly across the eukaryotic tree-of-life, including fish, sharks and rays, which was used in this study to bolster detected diversity and, in combination, mitigate and reduce shortfalls in publicly available reference material at either the 16S or CO1 barcoding region. These assays were selected after *in-silico* testing of species lists from historic BRUV data showed this combination would successfully detect a larger portion of species.

Table 5.1: PCR assays and primer combination information applied to seawater samples from six collection timepoints in the Exmouth Gulf.
These combinations were selected to complement marine vertebrate detection targeting two mitochondrial barcode regions, the 16S and CO1 regions.

	Primer	Primer Sequence (5'-3') (Reference)	Amplicon Length	Target Region	Annealing Temp. (°C)
16Schordata	16Schordata_F*	GACGAGAAGACCCTRTGG (This study)	180-250	16S	52
	16Schordata_R	GCTGTTATCCCTRGGGTARC (This study)			
CO1acartia	acartia_F*	GGRGAYGATCARRTYTATAAYGT (Berry et al., 2019)	105	CO1	50
	acartia_R	TYATWCGWGGAAAHGCYATRTC (Berry et al., 2019)			

* Indicates forward primer

5.4.3.3 PCR amplification and indexing

PCR amplification of the target region were completed on a StepOnePlus Instrument (Applied Biosystems), with a reaction volume of 25 µl, and 4 µl of eDNA added extract per reaction. The PCR master mix consisted of 2.5 mM/L MgCl2 (Applied

Biosystems, USA), 10x PCR Gold buffer (Applied Biosystems), 0.25 mM/L 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 1 U AmpliTaq Gold DNA polymerase (Applied Biosystems). PCR conditions were as follows: an initial denaturation stage of 95 °C for 5 min, followed by 45 cycles of 95 °C for 30 s, then followed by assay specific annealing temperature (Table 5.1) for 30 s, 72 °C for 45 s, finishing with a final extension stage at 72 °C for 10 min. This initial, exploratory PCR step was completed on both neat and 1/10 dilutions of each sample, with the optimal reaction selected based on C_T values, amplification and melt-curves. The optimal dilution was then progressed through the indexing PCR outlined below using fresh eDNA extract or dilution.

Samples were assigned a unique forward and reverse 6-8 bp multiplex identifier tags (MID-tags). The optimal dilution for each sample was combined with unique MID-tags combinations and amplified via PCR, with reactions set up on a Qiagility instrument (QIAgen). This MID-tag PCR step was completed using the same master mix and PCR conditions outlined above, with the exception of 50 cycles for both assays to account for amplification of longer amplicons. Sequenced laboratory controls included negative template, digest and extract controls. Additionally, a synthetic positive control was included for each assay, which was selected *in silico* from available sequences on NCBIs publicly available GenBank Nucleotide Database (hereafter "GenBank"). These included *Menippe mercenaria* (Florida Stone Crab, voucher-MT797632; CO1 region) and *Xyrichtys novacula* (Pearly Razorfish, voucher-MN794015; 16S region).

5.4.3.4 Library preparation and sequencing

MID-tagged duplicate samples were combined if the resulting amplification curve and melt plot were similar, otherwise minipools were progressed using only the optimal replicate. Minipools were blended based on equi-molar ratios of the amplification ΔRn values and no more than eight samples were included in each minipool. Minipools were quantified using the Qubit 4.0 Fluorometer (Invitrogen) and amplicon lengths visualised using the Qiaxcel (QIAgen) before being blended into a single library based

on equimolar values. Libraries were size selected using a Pippen Prep instrument (Sage Sciences, USA) to 180 to 400 bp (CO1acartia), and 200 to 600 bp (16Schordata), based on visualised product and expected amplicon length, to remove amplicons outside the target range such as primer dimer artifacts. Following quantification (Qubit), libraries were diluted to 2 nM for sequencing, which was completed using a Illumina MiSeq 300 cycle V2 kit for single-end sequencing (CO1acartia) and 500-cycle V2 chemistry for paired-end sequencing (16Schordata) with custom sequencing primers.

5.4.4 Demultiplexing and bioinformatic Analysis

Demultiplexing and deconvolution of raw sequence data were completed in R (v3.6.3; R Core Team, 2020) through Rstudio (v1.2.5042; RStudio Team, 2020) using the package Insect (v1.4.0.9000; Wilkinson et al., 2018) for both paired and single-end data, with an additional verification step using the ShortRead (v1.44.3; Morgan et al., 2009) and Cutadapt (v3.7; Martin, 2011) packages. Demultiplexed data were quality filtered and error rates mapped using the DADA2 package (v1.8.0; Callahan et al., 2016) with the following parameters: maxN=0, truncQ=2, maxEE=2, and minimum amplicon length of 50 bp. Data was denoised and, for paired-end data (assay 16Schordata), chimeras removed prior to merging the forward and reverse complement sequences. The resultant Amplicon Sequence Variants (ASVs) were then transferred to an SGI of the Pawsey Supercomputing Centre (Kensington, Australia) and queried using the Basic Local Alignment Search Tool (BLAST) (v2.7.1; Altschul et al., 1990). The database was queried in March 2022 against reference sequences from GenBank, which was completed under the following parameters: maximum of 10 target sequences returned, query coverage=100%, e-value of 1e-3 and 95% minimum percent identity. Taxonomic designations were then collapsed to Lowest Common Ancestor (LCA) using the Python script within the eDNAflow automated workflow (Mousavi-Derazmahalleh et al., 2021). Results were manually vetted against the blast results and their taxonomy verified against the open access World Register of Marine Species database (WoRMS; accessed Jan 2022; WoRMS Editorial Board, 2021), or online database FishBase (Froese and Pauly, 2020). After this, using the R packages Vegan and Phyloseq, a 0.05% relative abundance threshold was applied to both assays, removing ASV read counts from samples that make up less than 0.5% of

the total for that ASV. Non-eukaryotic, non-marine, and ASVs unable to be assigned to the taxonomic family were manually removed, as were positive ASV detections from field and laboratory controls and, conservatively, ASVs belonging to the South American pilchard (*Sardinops sagax*), as this species is sold locally as baitfish. The detection of non-endemic species in broad eDNA studies is not uncommon, which may result from the misidentification of congeneric species within the database, or species with limited resolution within the selected barcode region (Jerde et al., 2021). Within this study, for a species to be classified as having a low likelihood to be a false positive, or represent a true range-extension, all locally (Exmouth Gulf) documented congeneric taxa must have coverage within GenBank, with in-silico analysis demonstrating binding of all congeneric taxa. Additionally, the likelihood of a species detection being a false positive was determined to be high if (a) there were limited reference material for the detected barcode region for the species in question; (b) the species was represented by a single ASV detected in low abundance read (<20 reads) within single replicates; and (d) comprised less than a 2 nucleotide base differences between the species amplicon and nearest taxonomic match (criterion similar to those outlined in West et al., 2020).

5.4.5 Analysis and multivariate Statistics

As read abundance does not directly correlate to population abundance, data were transformed to presence and absence prior to analysis using the package metagMisc (v0.0.4; Mikryukov, 2022). Data were then merged to the species taxonomic level using the "tax_glom" function in the R package phyloseq (v1.28.0; McMurdie and Holmes, 2013) and the phyla Chordata subsampled using the "subset_taxa" function within the same package. Cladograms were produced using the taxonomy from NCBI through the "phyloT" website (https://phylot.biobyte.de/). Species distributions and habitat preferences (which were binned into three categories: reef-associated, pelagic or demersal) were verified using data obtained from FishBase (Froese and Pauly, 2022) and Fishes of Australia (Bray and Gomon, 2022). Sampling effort was explored by phase and habitat using the BiodiversityR (Kindt and Coe, 2005) and phyloseq packages within Rstudio, to determine alpha diversity measures, test efficacy of current sampling in relation to target taxa and explore sampling effort requirements for different habitat types. The community composition of each habitat was visualised

and temporal changes explored using both site and habitat by sampling phase using the Primer7 software (Clarke and Gorley, 2015). The PERMANOVA + add on (Anderson et al., 2008) to the Primer7 software was utilised for permutational analysis of variance multivariate analysis using the factor Phase within each habitat type. Both similarity percentage (SIMPER; Primer7) and indicator species analyses (v1.7.12; De Cacares and Jansen, 2016; Rstudio) were undertaken to explore species composition and turnover and key taxa at King Reef and habitats over the six sampling timepoints. Each fish species (class Actinopterygii) was assigned a recreational fishing value of high, medium or low based on output from FishBase and Fishes of Australia database searches. These were assigned based on the following: High – If one or both database lists recreational or gamefish amongst human uses; Medium – recreational fishing not specifically mentioned however listed as baitfish; or Low – no recreational fishing uses cited. Finally, temporal functional diversity changes, and specifically trophic level functional diversity in fish and elasmobranchs (Ladds et al., 2018), were explored at King Reef to reveal trends in trophic level occurrences. Species were assigned a trophic level indicator (Froese and Pauly, 2022) and were binned into Low (indicator <2.9; comprising partly or predominantly herbivorous species), Medium (3-3.4), High (3.5-3.9), and Very High (4>; predominantly carnivorous species).

5.5 Results

5.5.1 Sequencing results

The combined assays generated 40,446,316 raw sequence reads; 24,296,605 from 16Schordata and 16,149,711 from CO1acartia (Supplementary S5.2). All seawater samples successfully amplified for both assays. After quality filtering, denoising and chimera removal the mean number of sequences per sample was 71,761 (± SE 3,340) for the 16Schordata, and 55,477 (± 2,244) for CO1acartia (Supplementary S5.3), which equated to 178 (± 8) and 168 (± 5) ASVs per sample. Contamination is an ever-present risk in eDNA field sampling and laboratory workflows. An ASV belonging to the planktonic copepod *Paracalanus indicus* (total 49 reads) was detected from CO1acartia field controls and manually removed across that time sequence. Likewise, the CO1acartia assay amplified 5 arthropod ASVs belonging to the planktonic genera *Paracalanus* (3 ASVs; total 37 reads) and *Bestiolina* (2 ASVs; total 6 reads) from laboratory workflow controls, extraction and non-template. The 16Schordata assay

amplified no target vertebrate taxa from field or laboratory controls, however contaminant detections of the synthetic positive control (*X. novacula*; 2 reads), as well as *Homo sapiens* (human) and bacterial ASVs were removed prior to analysis.

5.5.2 Exmouth Gulf chordate diversity

After completion of quality filtration steps, assigning taxonomy and relative abundance filtering, removing control ASVs and merging assay datasets, 1,692 ASVs were identified which comprised 6 kingdoms, 31 phyla, 53 classes, 121 orders and 204 families. In total, 668 ASVs were assigned to species-level, representing 274 unique species. Thirty-nine percent of ASVs were able to be assigned to species-level, with almost 50% of those not assigned belonging to non-target kingdoms such as Bacteria, Archaea or Chromista (Supplementary S5.4).

The selected assays successfully amplified broadly within Chordata (Figure 5.3). Five taxonomic classes were detected, 40 orders, 81 families, 153 genera and 195 species. Accumulation curves on habitat replicates indicate that the sampling effort required to detect a minimum 90% of species varies by habitat with King Reef requiring 8.3-8.5 replicates, to 18.2-18.4 replicates at sponge sites (Supplementary S5.5).

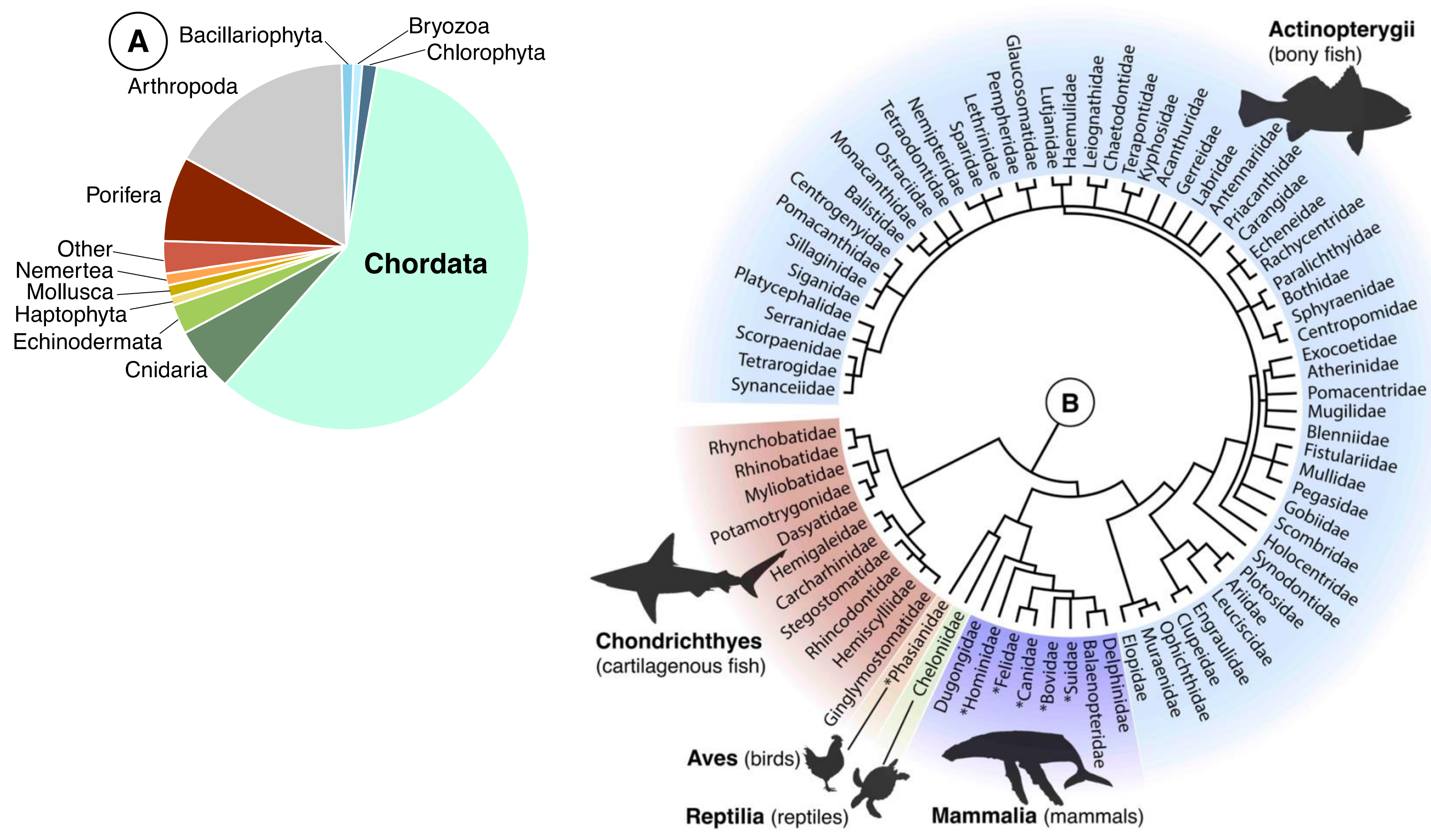

Figure 5.3: Proportional breakdown of taxa detected per phyla using the combined 16Schordata and CO1acartia assays at all sites and habitat types in the Exmouth Gulf (A), with the category "Other" comprising phyla with 2 or less taxa detections (Chaetognatha, Ciliophora, Cryptophyta, Ochrophyta, Prasinodermophyta, Rhodophyta, and Streptophyta). (B) represents a combined family-level dendrogram of the Chordata (fish, sharks, rays and mammals).
* Denotes non-marine fauna that were removed prior to multivariate analysis.

The two metabarcoding assays detected two species of sea turtle (loggerhead sea turtle, *Caretta caretta*; green sea turtle, *Chelonia mydas*), four marine mammals (humpback whale, *M. novaeangliae;* pantropical spotted dolphin, *Stenella attenuate*; Indo-Pacific bottlenose dolphin, *Tursiops aduncus*; dugong, *Dugong dugon*), in addition to the target 22 identified species of cartilaginous fish (class Chondrichthyes) and 161 species of bony fish (class Actinopterygii). 16Schordata identified 97 species of bony fish, 30 were identified only with CO1acartia, and 24 species using both assays (Supplementary S5.6). Seven of the 161 identified species of Actinopterygii detected had not been recorded from the Exmouth Gulf previously (Table 5.2), rather have known distributions in the wider Indo-Pacific, eastern Australian coast or the Pacific Ocean. Of these species, only *Brachaluteres ulvarum,* the Japanese inflator filefish, which had no congeneric species occurring within the Exmouth Gulf, was identified as a potential true range extension under the criteria applied (Table 5.2; see methods). Terrestrial chordate taxa detected and omitted from multivariate analysis included the genera *Gallus gallus* (domestic chicken) and *Felis* (likely domestic cat), and species *Bos taurus* (Cow), *Ovis aries* (Sheep), *Sus scrofa* (Pig), *Canis lupus familiaris* (Dog) and *Homo sapiens* (Human), which were detected across both assays.

Fish and elasmobranchs detected comprised demersal (14%), pelagic (10%) and reef-associated (55%) species (Bray and Gomon, 2022; Froese and Pauly, 2022), with the remaining taxa unable to be assigned a preferred habitat.

A search of the IUCN red list (accessed February 2023, IUCN, 2022; Supplementary S5.6), with bounding co-ordinates of the Exmouth Gulf, revealed 165 chordate species detected in this study were listed. Six species were classed as data deficient, 135 as being of least concern (97% of which were fish), six were near threatened, nine listed as vulnerable, seven endangered and two critically endangered, which included the bottlenose wedgefish (*Rhynchobatus australiae*) and the scalloped hammerhead (*Sphyrna lewini*; both class Chondrichthyes). Of the latter three categories, 73% of taxa were of the class Chondrichthyes. Additionally, Australian legislation recognises seven migratory species under federal legislation, dugong, humpback whale, pantropical spotted dolphin, and the Indo-Pacific bottlenose dolphin, as well as green turtles, loggerhead turtle and whale shark (the latter three are also listed as vulnerable; accessed February 2023; DBCA, 2022).

Table 5.2: List of target chordate species detected during this study which are previously unrecorded from the Exmouth Gulf.
The likelihood of these species to constitute a range extension is determined by the availability of broader congeneric reference material from the Exmouth Gulf, site and replicate detections.

Species name / Common name	**Known distribution**	**ASVs detected**	**No. Replicates**	**Total Reads detected**	**Congeneric taxa represented in GenBank**	**Nearest species nucleotide difference**	**likelihood for false positive**
Brachaluteres ulvarum / Japanese inflator filefish	Indo-Pacific. North and east Australia	2	1	7,492	N/A	14	Low
Carangoides bajad / Orange-spotted trevally	Indo-Pacific. East Australia.	3	2	696	No	3	High
Chaetodon guentheri / Crochet butterflyfish	Indo-Pacific	1	5	59	No	6	High
Gymnothorax sagmacephalus **-**	Indo-Pacific	1	1	2	No	29	High
Plectorhinchus chrysotaenia Yellow-striped sweetlips	Indo-Pacific	2	2	638	No	3	High
Pomacentrus wardi / Ward's damsel	Indo-Pacific. North Australia	2	5	6,197	No	1	High
Scorpaenodes xyris / Rainbow scorpionfish	East Australia. Pacific	1	4	2,073	No	31	High

5.5.3 King Reef chordate diversity

Forty-six chordate species were detected from King Reef in all six phases of sampling. This comprised 41 species of fish, 18 of which were characterised as being of high value for recreational fishing (Supplementary S5.6). Additionally, two sharks were detected, the graceful shark (*Carcharhinus amblyrhynchoides*), and sandbar shark (*Carcharhinus plumbeus*), as well the blue-spotted stingray (*Neotrygon kuhlii*) the bottlenose wedgefish (*R. australiae*). The humpback whale (*M. novaeangliae*) and Indo-Pacific bottlenose dolphin (*T. aduncus*) were also detected in phases 3-6. The detected chordate assemblage was classified as demersal (11 species), pelagic (7 species), and reef-associated (27 species, Supplementary S5.6).

5.5.4 Temporal diversity shifts in chordate community

The diversity of chordate taxa was highly variable and dependent on habitat and sampling time (Figure 5.4). Sponge habitat sites, in particular, ranged from 26 taxa detected in August 2018 (Phase 3) and increased to 78 taxa in the subsequent sampling in March 2019 (Phase 4). Natural reef systems were also variable with the final phase of sampling recording the lowest diversity for the habitat with 30 chordate taxa. The artificial reef habitat had the lowest total chordate diversity during every phase of sampling, including prior to installation of the artificial reef when the habitat was analogous to Sand habitat. However, mean taxa detection increased at the artificial reef marginally after the installation of the reef from 4.4 taxa (SE±0.4) to 6.3 (±0.5) with total chordate diversity detected equal or higher in post installation phases. An analysis of variance on presence absence transformed chordate using the factors before and after installation showed no significant difference ($F_{1,28}$=2.197, P=0.149). Chordate presence by phase was also non-significant ($F_{5, 1,536}$=0.352, P=0.881).

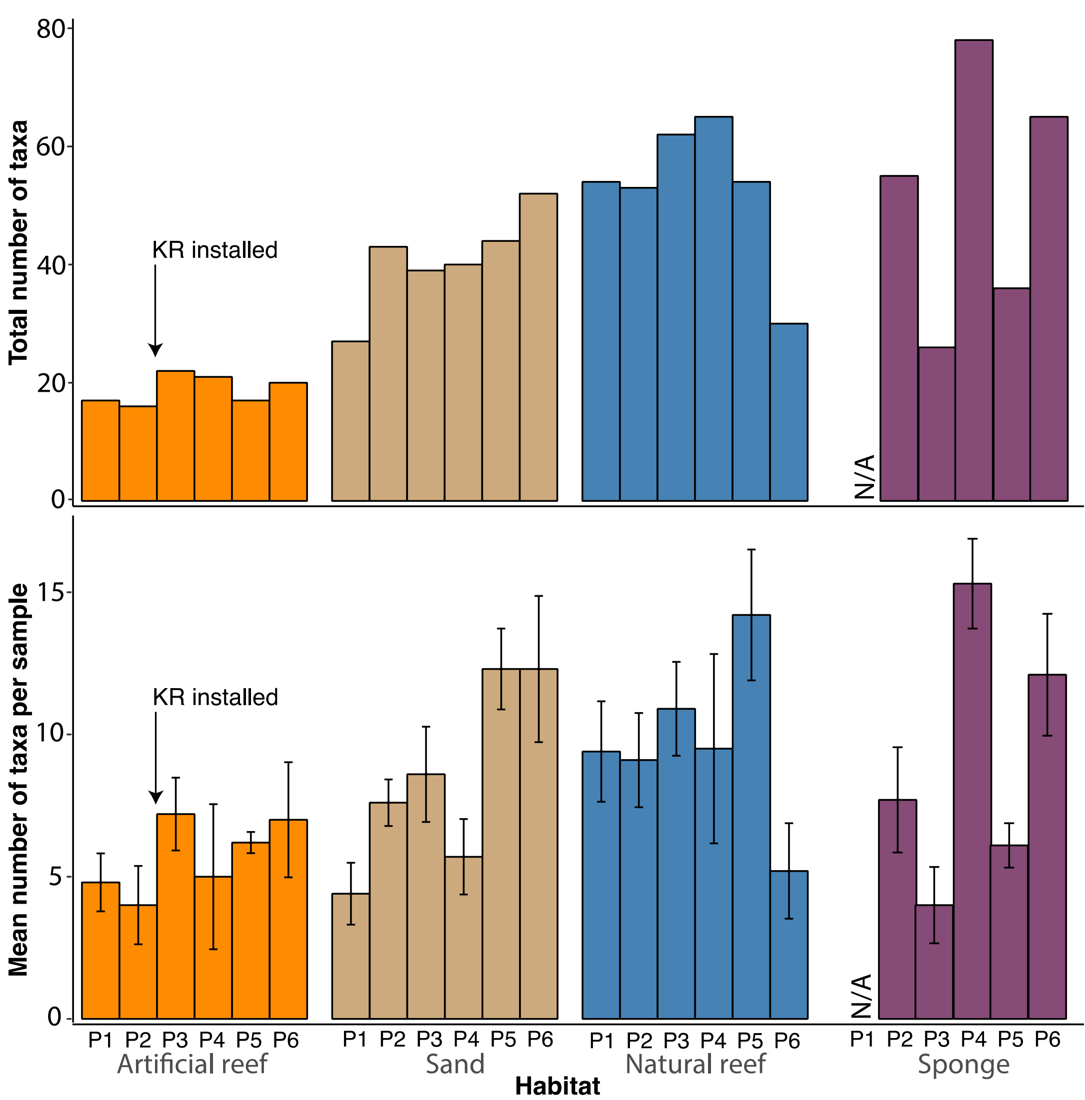

Figure 5.4: The total number of marine chordates taxa (above) and the mean number of taxa detected per replicate at each habitat (including standard error bars; below) sampled between June 2018 and September 2020.

Arrow indicates the point where the artificial reef was installed in August 2018, prior to the third sampling timepoint.

The permutational analysis of variance showed significance based on a two-factor fixed design of Phase and habitat, for both Phase (MS=8,991, Pseudo-F=2.73, P(perm)=<0.001) and Habitat (MS=11,366, Pseudo-F=3.45, P(perm)=<0.001) (Supplementary S5.7), with pair-wise analysis on the same design showing inconsistent compositional similarities between all habitats (Table 5.3). For example, in the first two phases, NR habitat demonstrated a significant difference from SA habitat, however AR habitat, prior to the installation of the King Reef, was unable to be differentiated in pair-wise analysis to SA. All habitats were significantly different in phases 3 and 4 (Table 5.3). Pairwise comparisons of Phase within Habitat for King Reef showed that 10 of the 15 tests were not significant (Supplementary S5.8). Of

those significant pair-wise tests, four were significant between chordate composition between phases pre- and post- installation of the reef, comprising Phases1/5, Phases1/6, Phases2/3 and Phases2/5, as well as Phases3/5.

Table 5.3: Pairwise test results of PERMANOVA, showing compositional similarity of the factor Habitat within Phase for all six phases of sampling at Exmouth Gulf.
Bold number indicated P(perm) and below signifies t-statistic for the pairwise test. Non-significant results are shaded in orange. Habitats indicated as follows; AR- Artificial Reef, NR- Natural Reef, SA- Sand and SP- Sponge. Results based on 9,999 permutations of a presence absence transformed Jaccard resemblance matrix.

Pairwise Test (Habitat)	Phase					
	1	2	3	4	5	6
AR / NR	**0.511** 0.975	**0.109** 1.159	**0.011** 1.251	**0.011** 1.478	**0.567** 0.947	**0.339** 1.049
AR / SA	**0.601** 0.947	**0.091** 1.158	**0.041** 1.272	**<0.001** 1.674	**0.617** 0.946	**0.406** 1.011
AR / SP	**N/A**	**0.548** 0.978	**0.014** 1.404	**0.004** 1.358	**<0.001** 1.685	**0.015** 1.279
NR / SA	**0.013** 1.344	**0.002** 1.354	**<0.001** 1.544	**0.022** 1.281	**0.21** 1.077	**0.021** 1.401
NR / SP	**N/A**	**0.005** 1.329	**<0.001** 1.655	**<0.001** 1.796	**<0.001** 1.767	**0.003** 1.483
SA / SP	**N/A**	**<0.001** 1.394	**<0.001** 1.880	**<0.001** 2.001	**<0.001** 1.841	**0.031** 1.247

This temporal variability in detected chordate composition across all habitats is reflected in PCO of centroids across phases (Supplementary S5.9), showing the detected differences in sponge habitat, and the inability to consistently differentiate between the remaining three habitat types, sand, natural reef and the artificial reef, both results are corroborated by bootstrapped nMDS results (Figure 5.5; A-F). The trajectory of the AR habitat shows no clear trajectory after the installation of the reef (Figure 5.5; G).

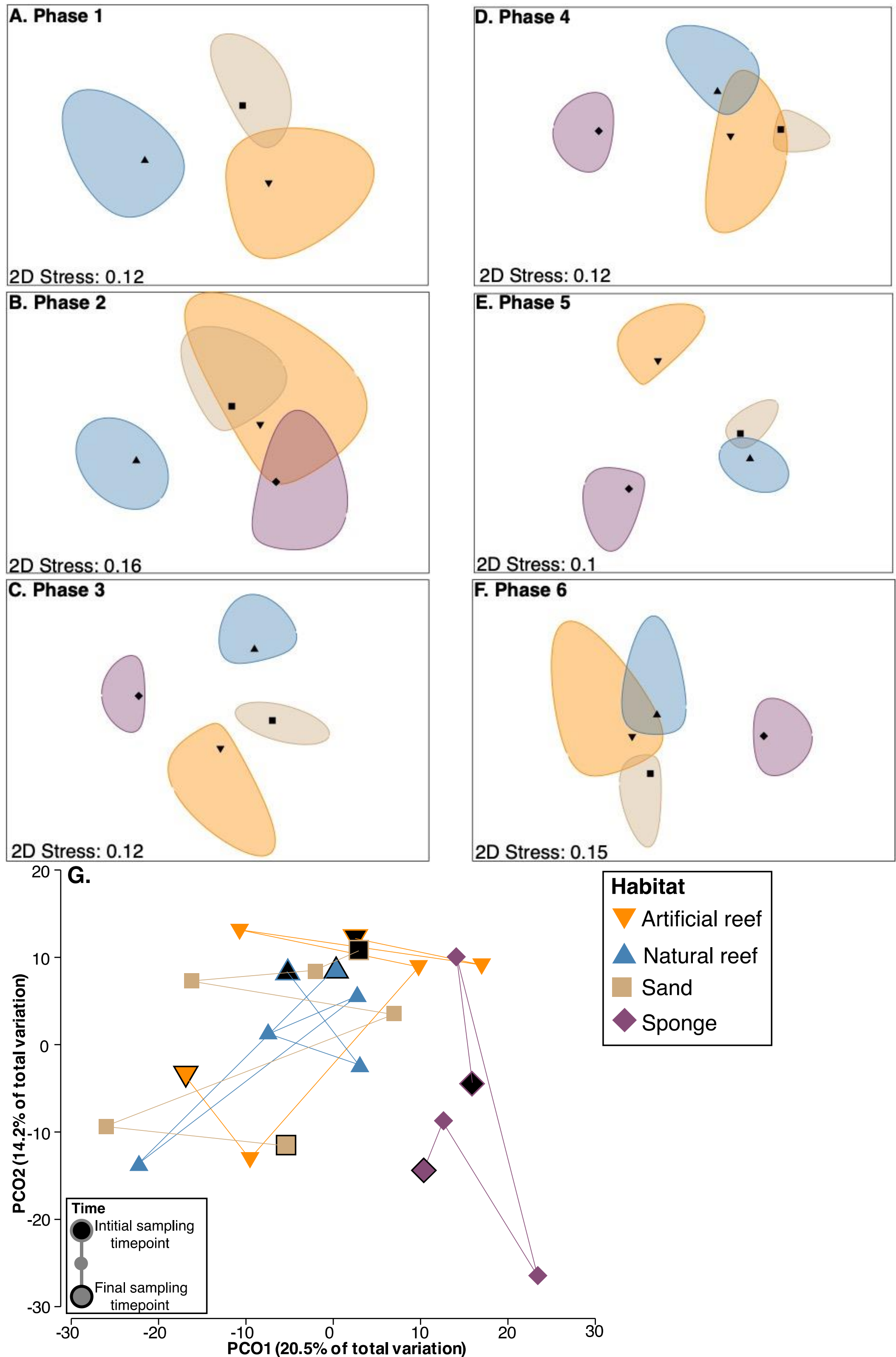

Figure 5.5: A – F: Temporal bootstrapped (based on 9,999 permutations), non-metric MDS plots, with each panel representing detected chordate assemblage per phase; and G: a Principal coordinates analysis (PCO) showing Chordata centroid movement by phase for all habitats by sampling phase.

Analyses output are based on Jaccard similarity matrices of presence and absence transformed data from amalgamated 16Schordata and CO1acartia output. Initial sampling phase designated by black fill in the respective habitat, whereas the final sampling effort is indicated by black outline.

As no indicator species were identified from the AR habitat (Supplementary S5.10), the top contributing chordate taxa in each phase at King Reef were identified by SIMPER analysis and ranked by their percentage (%) contribution (Table 5.4). All species identified from phases 1 and 2 (pre-installation) were reef-associated species, indicating some movement throughout the sandy benthic substrate prior to the installation of the reef, including the purple tuskfish (*Choerodon cephalotes*), four-lined terapon (*Pelates quadrilineatus*) from Phase 1, and the blue-barred parrotfish (*Scarus ghobban*). Immediately on installation of the King Reef (Phase 3) 19.2% of the community composition was contributed by the schooling pelagic species, the Australian spotted herring (*Herklotsichthys lippa*). Likewise in sampling phase 5, the greatest contributor to fish community composition was the pelagic goldstripe sardinella (*Sardinella gibbosa*), also schooling species (Whitehead et al., 1985). The remaining species were all reef-associated however, other than species identified from prior to the reef installation, the purple tuskfish, which contributed 25% and 18% in phases 4 and 5, and the four-lined terapon which detected 14% in phases 3 and six, there were no compositional crossover in dominant contributor's post King Reef installation. This trend of inconsistent detection is reinforced when exploring detection rates of the nine most commonly recorded fish species within replicates at each habitat and phase (Supplementary S5.11).

Table 5.4: Species identified by SIMPER analysis as characterising community assemblages at the AR habitat prior to the installation of King Reef, and at four sampling phases after installation.

Preferred habitat assigned based on FishBase, with genera level taxa conservatively not assigned.

	Taxa	Assigned Habitat	Average abundance	Average similarity	Sim/SD	Contribution (%)	Cumulative Contribution (%)
Pre-Installation	**Phase 1** - Average similarity: 15.99						
	Pelates sp.	-	0.6	6.75	0.6	42.18	42.18
	Choerodon cephalotes	Reef	0.4	2.5	0.32	15.63	57.82
	Pelates quadrilineatus	Reef	0.4	2.22	0.32	13.9	71.71
	Phase 2 - Average similarity: 6.29						
	Sardinella sp.	-	0.4	2	0.32	31.82	31.82
	Scarus ghobban	Reef	0.4	1.43	0.32	22.73	54.55
	Amblygaster sp.	-	0.4	1.43	0.32	22.73	77.27
Post Installation	**Phase 3** - Average similarity: 25.37						
	Pelates sp.	-	0.8	7.64	1.14	30.11	30.11
	Herklotsichthys lippa	Pelagic	0.6	4.87	0.59	19.2	49.31
	Sardinella sp.	-	0.6	3.5	0.62	13.78	63.1
	Pelates quadrilineatus	Reef	0.6	3.5	0.62	13.78	76.88
	Phase 4 - Average similarity: 3.81						
	Selaroides leptolepis	Reef	0.4	0.95	0.32	25	25
	Choerodon cephalotes	Reef	0.4	0.95	0.32	25	50
	Lutjanus carponotatus	Reef	0.4	0.95	0.32	25	75
	Phase 5 - Average similarity: 31.52						
	Sardinella gibbosa	Pelagic	0.8	10.94	1.16	34.71	34.71
	Choerodon cephalotes	Reef	0.6	5.64	0.62	17.88	52.6
	Sillago sp.	-	0.6	5.64	0.62	17.88	70.48
	Phase 6 - Average similarity: 25.33						
	Sillago sp.	-	0.8	9.27	1.02	36.58	36.58
	Pelates quadrilineatus	Reef	0.6	3.43	0.61	13.55	50.13
	Pelates sp.	-	0.6	3.43	0.61	13.55	63.68
	Pelates sexlineatus	Reef	0.4	2.22	0.32	8.77	72.46

5.5.5 King Reef temporal detections of recreationally important fish species

Of the 48 fish species that were determined to have high recreational fishing value, 18 were detected at King Reef, however 12 were singleton records from a single replicate within one phase. The purple tuskfish (*C.cephalotes*) was most commonly recorded at King Reef, however, as indicated by the SIMPER analysis, this was collected prior to the installation of the reef also (Figure 5.6). Only two species classified as high recreational fishing importance were recorded only after the installation of King Reef,

including Spanish flag snapper (*Lutjanus carponotatus*) and the yellowtail flathead (*Platycephalus westraliae*), of which both were detected across two sampling phases, post-installation. Similarly, few temporal trends were observed with the trophic functional diversity data (Supplementary S5.12).

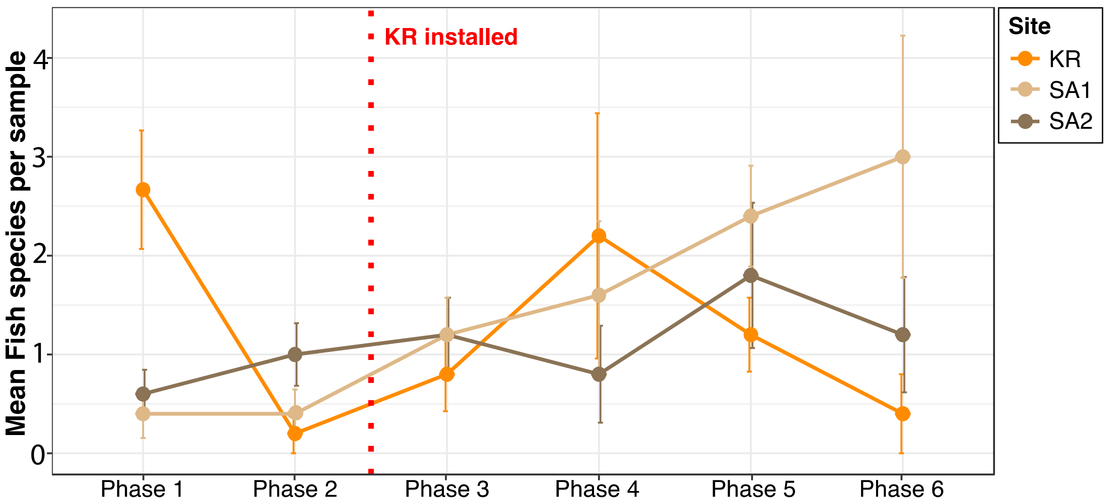

Figure 5.6: Mean abundance per replicate (including standard error bars) of recreationally important fish species for King Reef, in comparison to sand habitat sites. Dashed line indicates the approximate timing of the installation of King Reef, prior to Phase 3 sampling.

5.6 Discussion

In this study we applied two metabarcoding assays to water samples taken over a two-year period at the Exmouth Gulf to determine the efficacy of eDNA metabarcoding in tracking marine chordates and, in particular, fish, sharks and rays. We successfully detected a broad array of chordates known to occur within the area, and temporal detections coinciding with known migration patterns (*M. novaeangliae*), as well as some species previously not recorded from the Exmouth Gulf, such as the pantropical spotted dolphin (*S. attenuate*; Sprogis and Parra, 2022). While successful in detecting overall diversity, a primary aim of this study was to examine and document trends and explore the temporal relationship of species fidelity to the artificial reef. We also aimed to investigate the trajectory of the assemblage composition at the adjacent natural habitat types over two years post installation. We were unable to detect consistent changes in chordate composition attributable to the presence of the artificial reef, or temporal synergies of chordate composition to either the natural reef, sponge or sand habitats. This result was surprising, as there are documented examples of customised

artificial reefs providing habitat to a significantly greater richness (Higgins et al., 2022), and over time compositional dissimilarities between artificial reef and natural habitats become less significant (Komyakova et al., 2019). However, results on some studies are mixed (Walker and Schlacher, 2014), and indicate, over longer periods than were sampled in this study, that assemblages are unlikely to mimic natural habitat species composition (Becker et al., 2017). However, these temporal studies were completed using conventional methods of fish assemblage composition, such as stereo-BRUVs (Becker et al., 2017), rather than eDNA metabarcoding methods.

Historical literature has cited the movement of water through currents and water as a potential influence on eDNA results, through the transportation of genetic material (Goldberg et al., 2016; Andruszkiewicz et al., 2019). The mesotidal movement is a dominant hydrodynamic process which, along with wind, drives most water movement within the Exmouth Gulf (Brunskill et al., 2001). We have attempted to mitigate this potential impact by sampling through neap tides when water movement is lowest. However, however we are unable rule out the impact of water movement in the outcome of our findings within the Exmouth Gulf. Recent literature has indicated that water samples show relative site fidelity and that eDNA signal is not a significantly impacted by tidal movement (Kelly et al., 2018; Jeunen et al., 2019; Larson et al., 2022). These studies have predominantly been completed in cold water habitats and under varying tidal conditions. Seasonality was detected, particularly in the presence of species known in inhabit the gulf periodically, such as migratory species. Seasonal trends based on community assemblage, however, were more difficult to discern due to a high compositional turn-over in almost all habitats. While seasonality is likely to have an impact on results, the observed temporal trends at both habitat and site levels were inconsistent.

Additional factors potentially confounding eDNA results is the transient nature of these fish species. On closer examination of species identified from the SIMPER analysis (Table 5.4) as species contributing to and characterising species assemblage, the Labridae species *C. cephalotes*, while classified as reef-associated in FishBase, is also documented to occur at a range of habitats, such as seagrass (Fairclough et al., 2008), with most *Choerodon* known to occur in WA over a range of habitats from

(Fairclough, 2005). *P. quadrilineatus* and *S. ghobban* are known to inhabit sand, rubble and seagrass habitats (Jennings et al., 1996; Burfeind et al., 2009).

After the installation of the artificial reef, there was no period allocated for recruitment and colonisation of King Reef, and while a previous study identified 28 fish species using BRUVs (Florisson et al., 2020), compared to 22 detected in eDNA sampling immediately post installation (Phase 3), it is unclear what the impacts of the potential removal of target recreational fishing species during this time may have on long-term species recruitment. While this is a potential factor which we include here for scientific rigour, given the temporal turnover of chordate species at other habitats such as sponge and, predominantly, sand, we find it unlikely that this would have unduly impacted sampling results at the artificial reef.

A range of measures can potentially be used to optimise eDNA metabarcoding collection methods in the sub-tropical Exmouth Gulf. While sampling was completed on the artificial reef to target the fish towers, increased sampling effort at each tower per phase may have increases eDNA yield. Increasing consistency of temporal sampling (Berry et al., 2023), in addition to increasing sampling effort, may help elucidate seasonal effects, as well as collecting environmental data. Increasing the sampling time beyond two years may reveal patterns in recruitment to the artificial reef. Additionally, literature is emerging that sampling method can be an important factor in determining eDNA results (Alexander et al., 2023), in particular, methods that collect large amounts of organic material can potentially be influenced and swamped by the collection of single source organic material. Alternative sampling methods, such as plankton, passive or active sampling using a foam matrix, or a combination thereof, may yield greater diversity and potential greater trends in recruitment to King Reef than this study. Additionally, the selection of assay, and by-proxy available reference data sets, plays a highly important role in rigorousness of eDNA sampling. Actinopterygii diversity in Australian coastal waters is well documented.

While the primary aim of this study was to determine the compositional alignment of taxa from King Reef in relation to nearby natural habitats, the reef was installed to provide value to the recreational fishing community. Therefore, the overall success of

the artificial reef can be defined by the proportion of species targeted by recreational fishers. Of the three species detected across multiple replicates after the installation of the reef, the purple tuskfish (*C.cephalotes*), Spanish flag snapper (*L.carponotatus*) and the yellowtail flathead (*P.westraliae*), all were detected in more sample replicates at all other habitat types. With the data currently available it is not possible to determine if the presence of these species indicates recruitment or species transient detections. With the remaining species that were identified as having high recreational fishing value being represented by single records at King Reef, there is insufficient evidence to indicate positive recruitment and therefore we are unable to say if the reef habitat is providing any net benefit for recreational fishing.

While this study was successful in detecting a broad taxonomic diversity within the Phyla Chordata, there were also some notable exclusions from our eDNA results. These included the highly varied diversity (approximately 15 species) of sea snake which inhabit the gulf (Sprogis and Parra, 2022), of which the olive sea-snake (*Aipysurus laevis*) were noted to be abundant during the spring sampling events (September). Despite this, no sea-snake species were detected. Likewise, two species of sea turtle (*C. caretta* and *C. mydas*) were detected by the 16Schordata assay from three water replicates from the sponge habitat, however these were commonly observed during sampling at all locations and therefore this limited detection is notable. For the sea snake species (*A. laevis*), in-silico analysis revealed an inability of the 16Schordate assay to bind, accounting for lack of detection, and as such optimisation of assay. However recent literature has suggested that non-avian reptiles may shed DNA at a reduced rate in comparison to other marine fauna due to the presence of keratinised scales (Adams et al., 2019; West et al., 2021a), making detections of marine reptiles difficult even under optimal assay design.

Despite the limitations outlined above, broad chordate diversity was detected from sampling at the Exmouth Gulf, including a number of species of conservation significance. This result indicates that eDNA methods can successfully be used to detect diversity within the tidal gulf setting and, in time, can be optimised to temporally detect recruitment at artificial reefs. These optimisations may be through study design modifications, such as an increased sampling time frame post-installation, an increase in site replication, or maintaining more frequent and rigid

sampling time (consistency in sampling months) which would better help determine seasonal effects, which was not logistically feasible during this study. Alternatively, these optimisations may be through the assay development to fill known holes in taxonomic detectability (such as sea-snake), the use of alternative sampling methods targeting the water column, which may improve detections (Alexander et al., 2023), or even improving reference availability for local species. The anecdotal evidence of known species caught for recreational fishing purposes via recreational fishing surveys, would also provide valuable insight into recruitment.

King Reef supports a diverse chordate assemblage with compositional data showing considerable turn-over between sampling timepoints. In this study we show that eDNA metabarcoding can provide excellent chordate biodiversity detection capabilities and shows promise as a method for tracking temporal movement of species and potential for tracking recruitment are artificial reefs. While more exploration is required with focus on the study design of temporal eDNA metabarcoding studies to provide robust results for tracking recruitment trends, our results of this study have wider implications for eDNA detection at marine infrastructure. Given the difficulties associated with censusing diversity these marine structures, future research will benefit from the optimisation and enhancement of these temporal eDNA methods as either a complementary or stand-alone detection technique.

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5.8 Supplementary information

Supplementary S5.1: Sampling timepoints at each habitat prior and post King Reef installation.

Six sampling phases were completed starting in June 2018, with the final sampling timepoint in September 2020. A total of 200 seawater samples were collected

Sampling Phase	Sampling Dates	Samples Collected
Phase 1	June 2018	25
Phase 2	July 2018	35
- King Reef Structures Installed -		
Phase 3	August 2018	35
Phase 4	May 2019	35
Phase 5	September 2019	35
Phase 6	September 2020	35
	Total	**200**

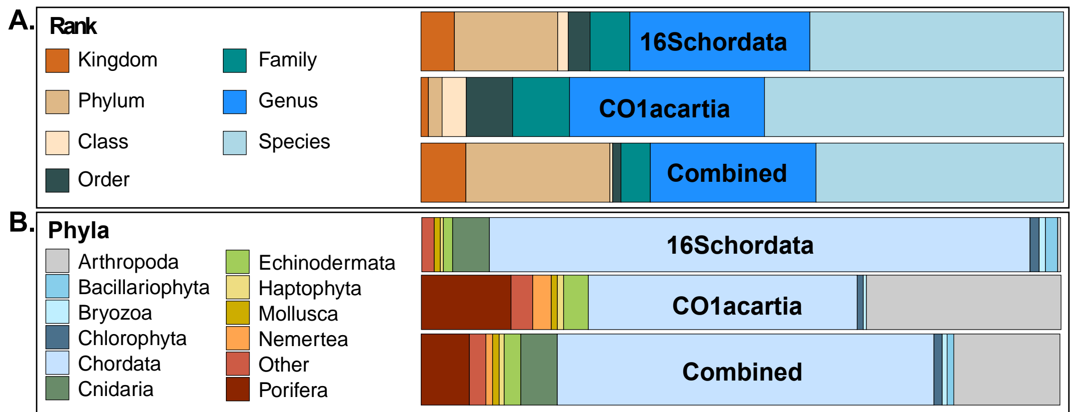
Supplementary S5.2: Link to unfiltered demultiplexed output files and sample information from both assays used in this chapter.

https://drive.google.com/drive/folders/16mC0G1DgifOlAbfLo93JNcqLjDGGrbGv?usp=share_link

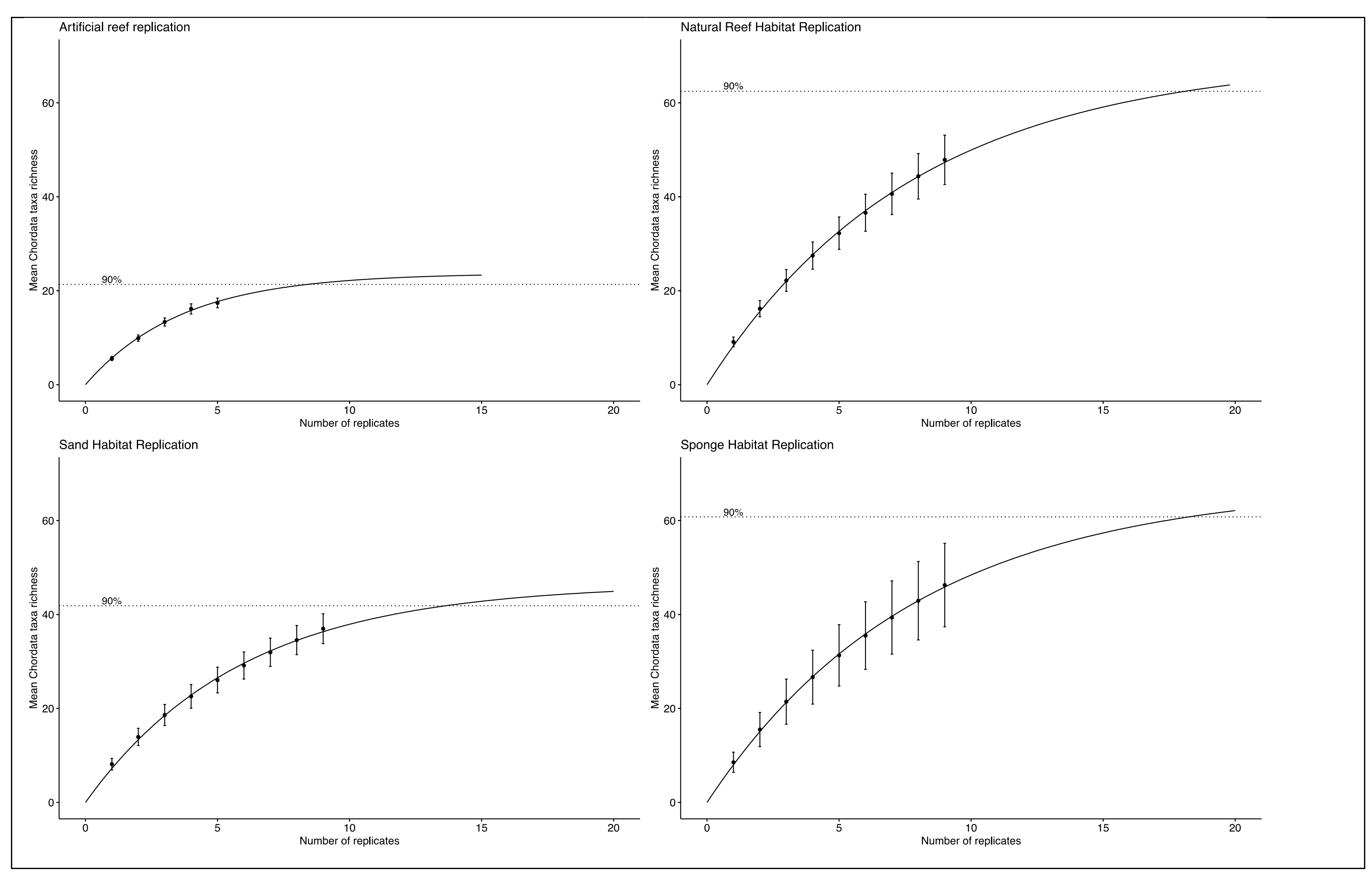
Supplementary S5.3: Summary of mean amplicon read abundance (and standard error) throughout the bioinformatic process on eDNA samples for both 16Schordata and COIacartia assays for samples collected at all habitat types and phases in the Exmouth Gulf.

Location	Mean reads past filter	Mean reads past denoise		Mean reads past chimera removal	Percent Remaining
		Forward*	Reverse*		
16Schordata (total reads – 24,296,605)					
Samples	75,298 ± 3,347	74,989 ± 3,337	75,015 ± 3,337	71,761 ± 3,340	95 ± 0
Controls	10,407 ± 7,170	10,388 ± 7,168	10,258 ± 7,170	9,795 ± 6,834	79 ± 6
Positive Control	151,019	149,891 /	150,353	148,922	99
CO1acartia (total read abundance – 16,149,711)					
Samples	60,284 ± 2,233	56,907 ± 2,310		55,477 ± 2,244	92 ± 1
Controls	53 ± 14	50 ± 13		38 ± 10	72 ± 3
Positive Control	265,452	26,5431		26,5430	100

* Paired-end sequencing only



Supplementary S5.4: Bar plots indicating the proportional breakdown of ASV taxonomic assignment for both PCR assays (A.) and the phyla level, proportional breakdown of number of taxa detections for 16Schordata and CO1acartia, and combined resolution.



Supplementary S5.5: Accumulation curves showing replication efficacy of current sampling to detect a minimum 90% of the extrapolated chordate taxa present at the Artificial Reef, Natural Reef, Sand and Sponge habitat types

Supplementary S5.6: Link to list of identified chordate species detected from the Exmouth Gulf over six sampling phases.

https://drive.google.com/drive/folders/16mC0G1DgifOlAbfLo93JNcqLjDGGrbGv?usp=share_link

Supplementary S5.7: PERMANOVA results of the presence-absence transformed data on Jaccard resemblance matrix with an estimated percentage breakdown of component variation (%V) attributed to the different levels.

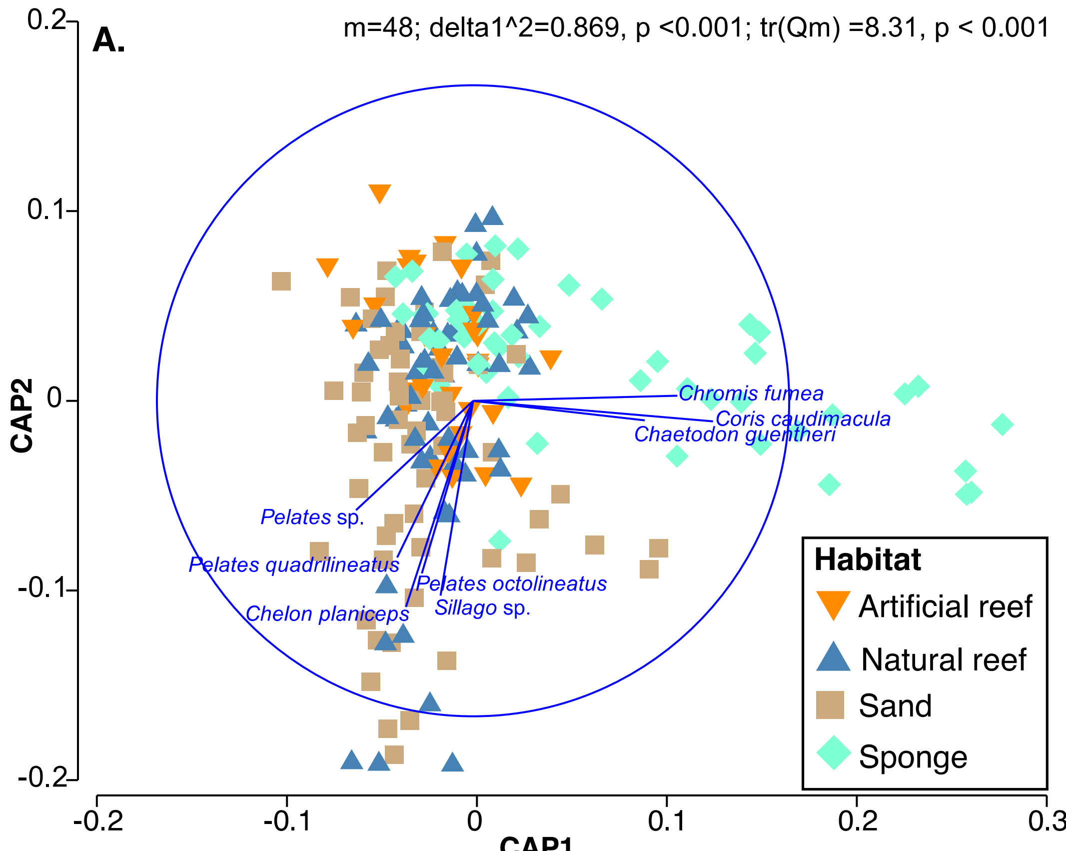
Results based on 9,999 permutations of a presence absence transformed Jaccard resemblance matrix.

	DF	Pseudo-F	P(perm)	Permutations	%V
Phase	5	2.7296	<0.001	9,735	4.9
Habitat	3	3.4504	<0.001	9,783	4.3
Phase × Habitat	14	1.631	<0.001	9,535	6.3
Residual	177				84.5

Supplementary S5.8: Pairwise test results of PERMANOVA, showing compositional similarity of the factor Phase within the habitat 'Artificial Reef' for all phases of sampling at Exmouth Gulf.

Results based on 9,999 permutations of a presence absence transformed Jaccard resemblance matrix.

Groups	t	P(perm)	Permutations
Phase1, Phase2	1.1171	0.1098	91
Phase1, Phase3	1.0042	0.4625	126
Phase1, Phase5	1.3888	0.022	126
Phase1, Phase4	1.0423	0.3086	126
Phase1, Phase6	1.2433	0.0315	126
Phase2, Phase3	1.2796	0.0413	91
Phase2, Phase5	1.4543	0.0079	91
Phase2, Phase4	1.0679	0.2681	56
Phase2, Phase6	1.34	0.0507	91
Phase3, Phase5	1.443	0.0083	126
Phase3, Phase4	1.2296	0.1031	126
Phase3, Phase6	1.1757	0.1445	126
Phase5, Phase4	1.3474	0.0544	126
Phase5, Phase6	1.1534	0.1994	126
Phase4, Phase6	1.2286	0.1305	126

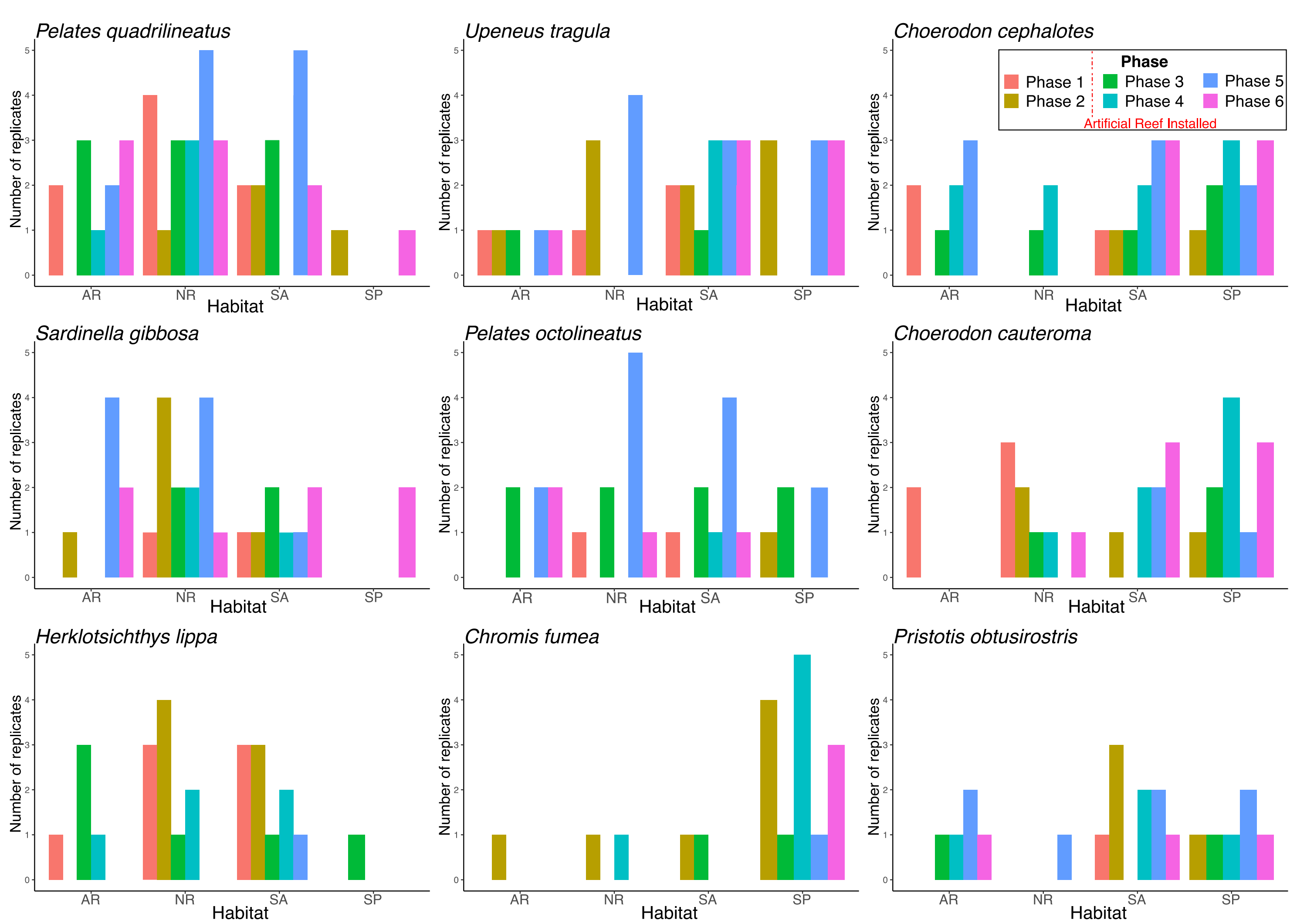


Supplementary S5.9: Canonical analysis of principal coordinates (CAP) plot on Chordata assemblage detected at each habitat type during all phases of sampling, both prior to and post installation of the artificial reef, and overlaid with a 0.5 pearsons correlation of focal species

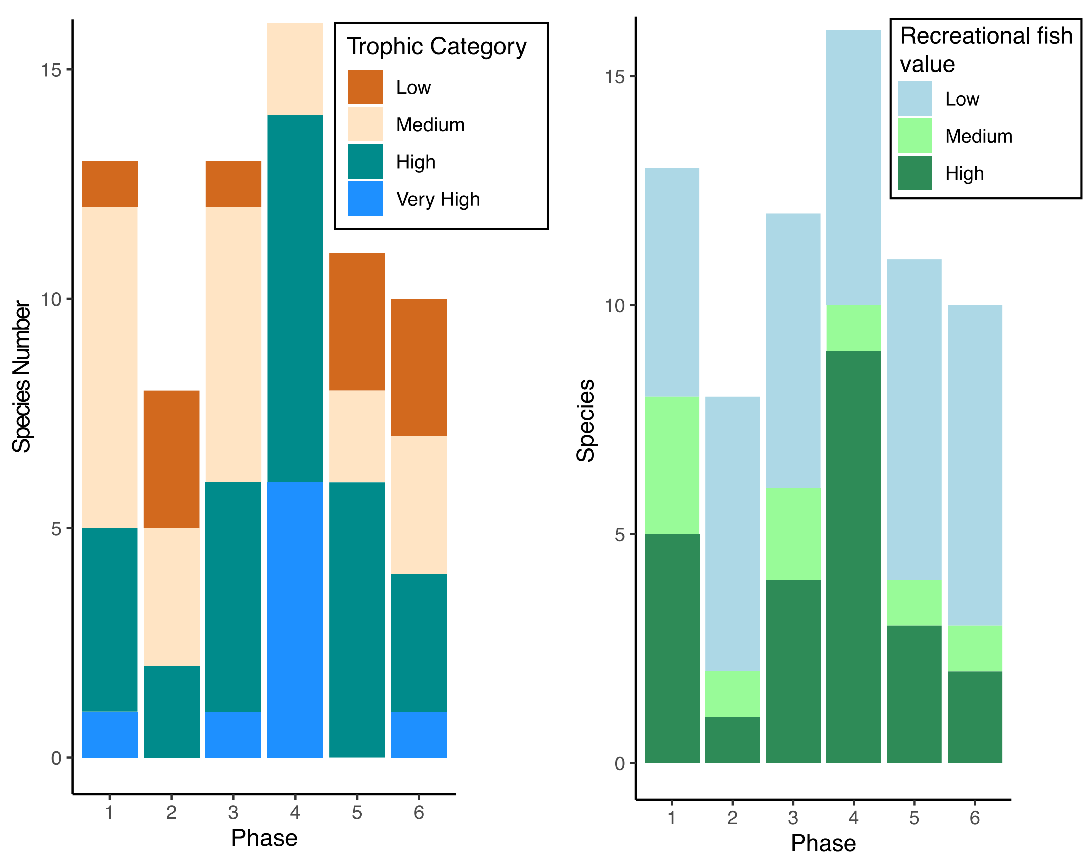
Supplementary S5.10: Indicator species identified from sampling locations in the Exmouth Gulf over all phases of sampling.

(Only significant indicator species displayed, with significance codes as follows- 0 '***', 0.001 '**', 0.01 '*').

Phase	Family	Species	Stat	P-value	Sig
NR1					
1	Hemigaleidae	*Hemipristis elongata*	0.632	0.0205	*
	Myliobatidae	*Aetobatus ocellatus*	0.6	0.0031	**
3	Scombridae	*Acanthocybium solandri*	0.632	0.0208	*
5	Synodontidae	*Saurida micropectoralis*	0.632	0.0222	*
NR2					
3	Paralichthyidae	*Pseudorhombus spinosus*	0.671	0.0013	**
5	Carcharhinidae	*Carcharhinus sorrah*	0.596	0.0003	***
	Mugilidae	*Chelon planiceps*	0.488	0.0009	***
	Sillaginidae	*Sillago maculata*	0.447	0.0067	**
	Gerreidae	*Gerres.* sp.	0.424	0.0358	*
	Terapontidae	*Pelates octolineatus*	0.368	0.0059	**
	Delphinidae	Unassigned	0.344	0.05	*
SA1					
3	Sillaginidae	*Sillago.schomburgkii*	0.516	0.0023	**
5	Terapontidae	sp.	0.577	0.0002	***
	Platycephalidae	*Platycephalus westraliae*	0.422	0.0096	**
6	Sillaginidae	sp.	0.496	0.0026	**
SA2					
3	Labridae	sp.	0.405	0.0451	*
5	Monacanthidae	*Paramonacanthus choirocephalus*	0.447	0.025	*
	Monacanthidae	*Paramonacanthus* sp.	0.447	0.0219	*
	Sillaginidae	*Sillago burrus*	0.365	0.0082	**
6	Carangidae	*Caranx bucculentus*	0.548	0.0066	**
SP1					
4	Scorpaenidae	*Scorpaenodes xyris*	0.671	0.001	***
	Labridae	*Coris caudimacula*	0.62	0.0001	***
	Nemipteridae	*Pentapodus* sp.	0.434	0.0088	**
	Haemulidae	*Diagramma labiosum*	0.424	0.0334	*
6	Blenniidae	*Blenniella chrysospilos*	0.632	0.0197	*
	Synodontidae	*Trachinocephalus myops*	0.632	0.0201	*
	Clupeidae	*Amblygaster sirm*	0.548	0.0054	**
SP2					
4	Potamotrygonidae	*Taeniura meyeni*	0.73	0.0003	***
	Pomacentridae	*Chromis fumea*	0.43	0.001	***
	Labridae	*Choerodon vitta*	0.405	0.0497	*
6	Labridae	*Coris pictoides*	0.632	0.0203	*
	Labridae	*Halichoeres nebulosus*	0.6	0.0038	**



Supplementary S5.11: Nine top contributing species of fish species detected at Exmouth Gulf with replicate detection by habitat and phase.

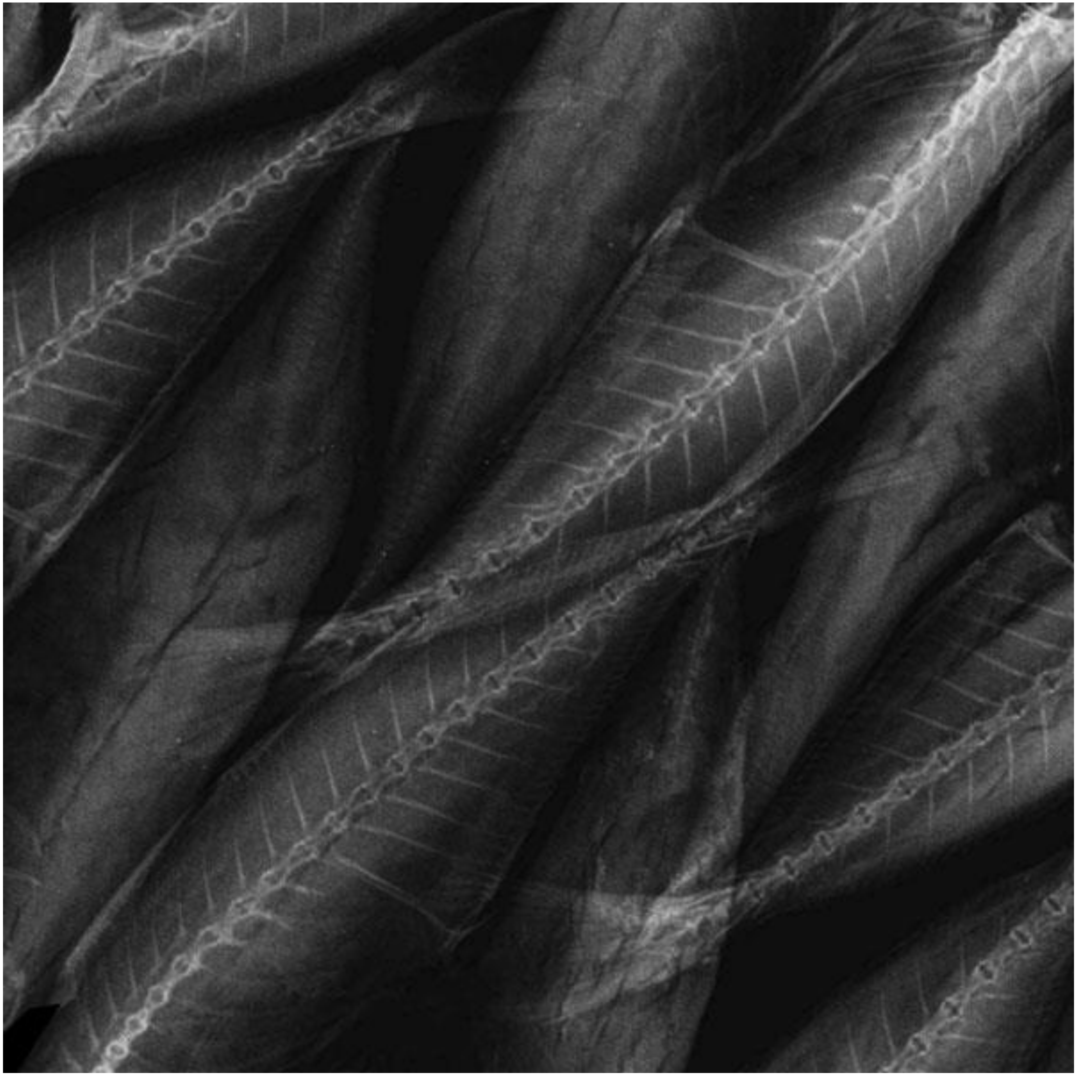


Supplementary S5.12: Breakdown of species detections of function trophic categories (left) and species of recreational fishing value (right).

Species typically within Low trophic categories comprise predominant herbivores, whereas Very High trophic categories are typically carnivores (see Methods).

Chapter 6

General discussion



X-ray composite of fish.
Image provided and displayed with consent from Mark Penhale.

6.1 Overview

The holistic monitoring of biological communities is challenging. Accordingly, there is an urgent need for robust data to inform baseline assessments, monitoring programmes, and regulatory management. Marine infrastructure, particularly oil and gas platforms, have the potential to provide biodiversity hotspots in otherwise low diversity areas (Friedlander et al., 2014). Being able to document marine assemblages in these complex ecosystems can inform decisions regarding decommissioning options. However, the development of a robust, scalable toolkit is needed that can be adopted by industry personnel and accepted in different jurisdictions or management agencies (Bunce and Freeth, 2022). Conventional methods of assessing biodiversity, while accurate for a subset of the taxa, lack the potential to be scaled up for ecosystem monitoring and censusing across multiple trophic levels. While environmental DNA (eDNA) methods are scalable and customisable (Leray et al., 2013; Stat et al., 2017; DiBattista et al., 2019), they can be challenging at marine infrastructure due to sampling logistics and field processing time and requirements. Additionally, there is a paucity of published literature focussing on this infrastructure (see Table 1.1) and as such there is a need for eDNA methodological validation. eDNA methods have the potential to be developed into an easily implementable sampling toolkit, which through sampling innovation and validation, can mitigate these restrictions. It is towards this goal that the research within this thesis makes significant progress.

This final chapter synthesises and distils the findings of the four research chapters (Chapters 2 – 5; Figure 6.1), and while the repetition of knowledge is kept to a minimum, the contextual overlap in these sections necessitates some duplication. Taking into consideration the limitations identified within these case studies, I discuss critical next steps in furthering this research and in the creation of a functional eDNA toolkit. I then address ‘bigger-picture’ steps in research direction, and discuss what this continued research can provide in terms of confidence in eDNA results at marine oil and gas infrastructure, but with implications for wider marine monitoring. Finally, I broadly discuss the requirements and path towards regulatory acceptance and the importance of data accessibility, which I see as critical hurdles to overcome in the uptake of eDNA as a mainstream biomonitoring tool at marine infrastructure, however with wider applicability for overall marine monitoring (Figure 6.1).

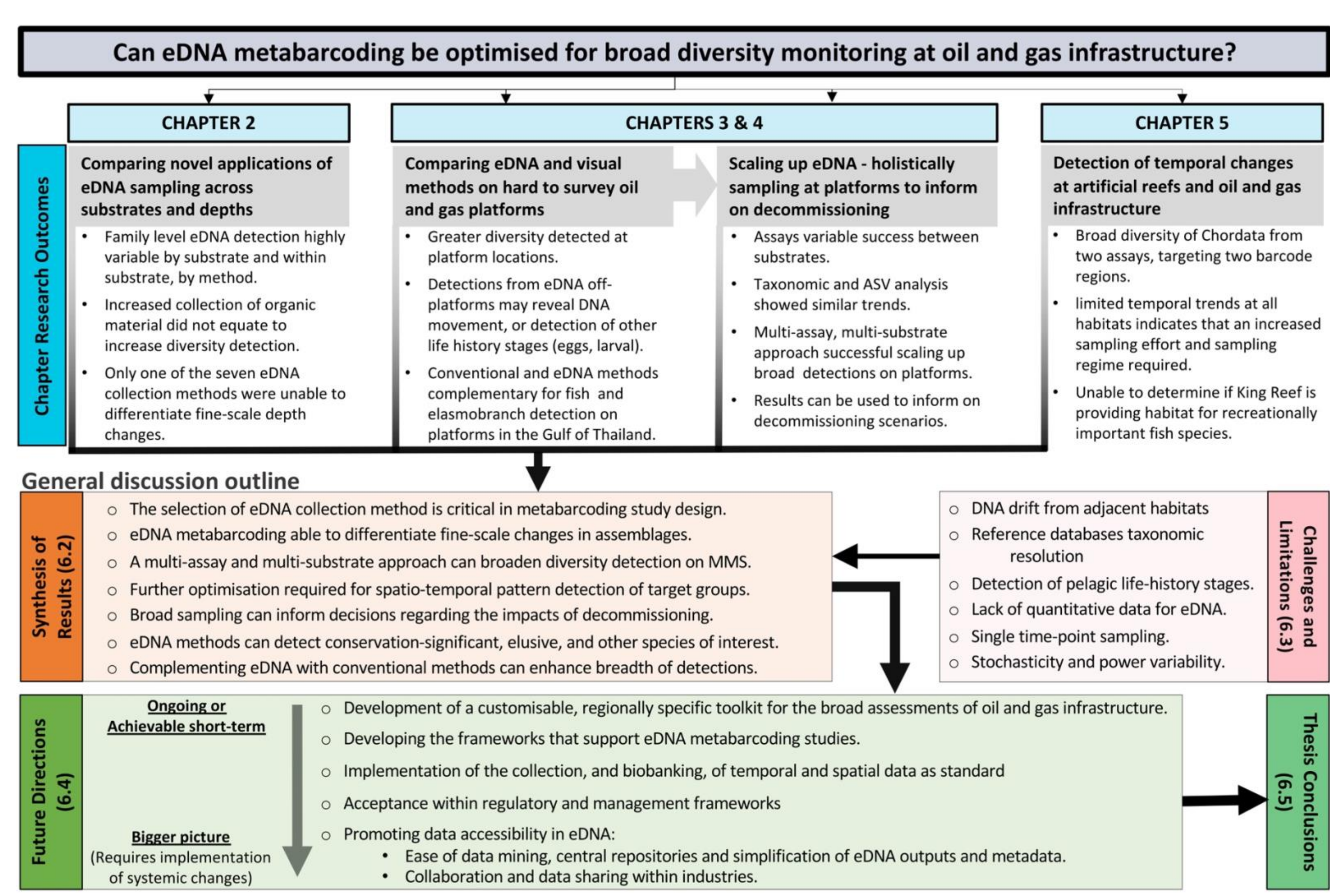


Figure 6.1: **Conceptual flow diagram summarising outcomes of each chapter, an overall synthesis and potential future directions from this research.**

6.2 A synthesis of data chapter results

6.2.1 eDNA collection method is critical in metabarcoding study design

The selection of marine substrate has been documented to impact eDNA detections (Koziol et al., 2018; Antich et al., 2021), a result also corroborated by Chapters 2 and 4 in this research. However, I demonstrate that the selection of sampling method utilised within a substrate is also critically important to eDNA metabarcoding results. Chapter 2 showed that, despite concurrent sampling of the water column, the Polyurethane Foam (PUF) tow method and plankton tow shared only 23% of taxa. This finding was similar for the epibenthic methods that consecutively sampled the same quadrat (diver scrapes and epibenthic swabs), where only 30% of taxa were overlapping. In terms of broad taxa detection, the PUF tow and the epibenthic swab methods yielded 77% and 79% of the total taxa detected within those substrates respectively, despite recovering only trace amounts of eDNA comparatively to the other methods targeting the same substrate. For epibenthic sampling, differences were primarily driven by the collection of bulk organic material in scrape methods, which may disproportionately collect and amplify genomic material from dominant taxa, missing rare taxa. Benthic sampling is conventionally completed by scraping the benthos or epibenthos directly (Rivera et al., 2021; Rallis et al., 2022) or by the scraping of settlement plates that are specifically deployed for monitoring (Levy et al., 2023). However, based on the results of this research, a survey approach that excludes the bulk removal of organic material, such as a swab method, may provide more accuracy and a greater range of detection.

Many of the published eDNA validation studies focus on comparisons with conventional, morphological methods (Ruppert et al., 2019; Takahashi et al., 2023), which are seen as a minimum threshold for acceptable detectability. However, the outcomes of this research provide an important evaluation of eDNA collection on marine infrastructure, demonstrating the variability inherent in each method. This outcome has critical implications for the careful selection of sampling methods when planning eDNA research or diversity monitoring on and adjacent to oil and gas platforms. Further research is required to investigate and validate these methods under different environmental conditions, and to innovate practical methods of sample collection at areas with limited access, such as offshore infrastructure.

6.2.2 eDNA metabarcoding can differentiate fine-scale changes in assemblages

The ability of a selected sampling method to differentiate fine-scale spatial changes is paramount in diversity monitoring across ecosystems and habitats, such as through photic zones or areas of variable wave movement on marine vertical infrastructure. All of the data collection chapters demonstrated the ability of eDNA methods to detect fine-scale spatial patterns in assemblages, ranging from zonation within an 8 m depth profile (evident in seven of the eight methods; Chapter 2) to broader habitat differentiation over approximately 10 km^2 (Chapter 5). Chapters 3 and 4 of the study revealed distinct depth stratification patterns over an approximately 75 m depth range, using water and bio-foul eDNA samples in the Gulf of Thailand (GoT). The placement of platforms can cause localised alterations in water flow (e.g., currents and eddies; Heery et al., 2017; Lawrence and Fernandes, 2022), so the observed fidelity of detection in water samples was notable and demonstrates the localized nature of eDNA signals. However, the impact to eDNA results from the density of adjacent oil and gas infrastructure, which in some cases were located <1 km from sampled platforms, needs further exploration (see Section 6.3).

At a broader scale, all sampling methods were successful at detecting compositional differences between assemblages associated with marine infrastructure and those associated with soft sediment habitat in the GoT (Chapters 3 and 4), which is a finding that is partly reflected in the Chapter 5 chordate detection results. While temporal data patterns from the Exmouth Gulf chordate analysis were more challenging, this habitat differentiation was reflected in the overall analysis. eDNA detections from the sponge habitat, in particular, showed consistent differences to eDNA detections in habitats located outside the Ningaloo Marine Park boundary. This ability to detect nuanced changes in compositional data, demonstrates the applicability of eDNA methods to provide information on spatial diversity trends. This outcome has critical implications for the assessment of impacts or to inform decisions regarding decommissioning alternatives.

6.2.3 A multi-assay and multi-substrate approach can broaden diversity detection on man-made structures (MMS)

The broad detection of biodiversity using a single assay are limited by reference material for a single barcode region, are more prone to assay amplification bias, and potential sequencing artefacts (Stat et al., 2017; van der Loos and Nijland, 2020). Chapters 3 – 5 indicate that a multi-assay metabarcoding approach provides increased diversity detection over single assay approaches. This efficacy can be seen in Chapter 4, where the epibenthic scrape results using the CO1 universal assay detected approximately 56% of the total 166 identified species, and the Mollusca, Crustacea and Coral assays each respectively contributing a further 31%, 10% and 3% of the diversity. This was also evident in Chapter 5, where chordate species detections would have been reduced by approximately 17.5% using a single assay (16Schordata) approach. Both of these studies showed that a combination of assays that amplify different barcode regions can potentially mitigate the paucity of reference material available for each region, which is also corroborated in eDNA literature (Alexander et al., 2019; van der Loos and Nijland, 2020; Ip et al., 2021; Ficetola and Taberlet, 2023).

Similarly, the sampling of multiple substrates is shown in this research to be beneficial in increasing the biotic assemblage sampled. This result is supported in Chapter 2, where novel PUF and swab methods, which yielded the highest diversity in their respective substrates, detected 62% and 47% individually, yet combined detected 81% of the overall diversity at the location. In Chapter 4, no single substrate was able to document the entire detected assemblage, further emphasising that multiple substrates target different biotic assemblages.

The implications that multiple assays and substrates are required for whole ecosystem monitoring will have repercussions for the increased cost of metabarcoding research and monitoring. A balance must therefore be found between robust sampling to inform the required study questions, and the cost of the research, which should be considered during study design. This is discussed further in the future directions (Section 6.4).

6.2.4 Further optimisation required for spatio-temporal detection of target groups

An important result of this body of research, particularly Chapter 5, is the lack of detected temporal trends from the deployed artificial reef in Exmouth Gulf. This underscores the importance of carefully considered study design, inclusive of logistics and environmental variables, prior to implementation. The primary purpose behind the deployment of this reef was to provide habitat for recreationally important fish species. However, the lack of clear trends in the data, for the artificial reef as well as in the surrounding habitats, combined with the high turnover of chordate composition, indicates that a modified sampling regime (e.g., increase site replication and sampling timepoints, and more consistency of sampling in the same months) may have helped elucidate temporal trends. Currently however, eDNA methods were unable to address any of the temporal study objectives for this chapter. Some studies have indicated that at least nine samples are required for detection of seasonal trends (Berry et al., 2023), however optimisation and validation would be required to determine if this is applicable for the Exmouth Gulf area. The potential use of complementary approaches, such as recreational fisher surveys or other morphological methodologies (e.g., BRUV/ROV), may have helped to reveal temporal trends. Alternatively, expanding the scope to broad diversity detections (beyond the phylum Chordata) may show finer community trends at the artificial reef. The data in this chapter demonstrate that experimental design, in terms of where, when and how often to sample, is still a challenge in eDNA studies.

6.2.5 Broad sampling can inform decisions regarding the impacts of decommissioning

The potential to customise eDNA metabarcoding workflows to suit study requirements and local environmental conditions is almost limitless. Using a combination of three substrates and six assays at oil and gas platforms in the GoT (Chapter 4), I was able to demonstrate utility in informing the outcome of a range of decommissioning scenarios as outlined in the RtR literature. The ability of eDNA to be able to holistically census taxonomic diversity and detect fine-scale spatial differentiation for the purposes of informing on decommissioning and management, underpins the entire purpose of this thesis. This study presents a pivotal first step for demonstrating the efficacy of integrating eDNA approaches for this purpose.

6.2.6 eDNA methods detect conservation-significant and elusive species, as well as other species of interest

In chapters 3 – 5, a total of 26 IUCN red listed species were detected, with a further seven of those species listed as protected within local and Federal Australian government legislation (Chapter 5). This result, while not the primary aim of this research, shows applicability of even broad assays to detect the presence of significant species. These species comprised elusive chordates, such as dugongs (*Dugong dugon*) and whales on known migration routes (Humpback whale; *Megaptera novaeanglea*), as well as hard coral species (*Stylophora pistillata*), and 22 species of IUCN red listed sharks and rays (Elasmobranchii).

The ability to detect species of interest is also seen in Chapter 2 where, while not the focus of the study, a parasitic species of commercial importance, *Perkinsus olseni* (Family Perkinsidae), was detected using three eDNA methods. This species is a highly transmissive parasitic protist that is known to impact heavily on abalone and mussel fisheries (Goggin and Lester, 1995). The diagnosis of this parasitic infection usually involves histology and electron microscopy, immunology, or, more recently, the sampling of infected tissue via PCR amplification combined with a species-specific assay (Abollo et al., 2006; Choi and Park, 2010). Therefore, the detection of this species using a broad assay in both epibenthic and water samples, demonstrates applicability of metabarcoding to inform fisheries health management and potentially early diagnosis of some pathogen outbreaks. These detections can also be enhanced via assay optimisation (target-specific assay applied to eDNA samples), or multiplexing of target-specific assay (for multiple target detections).

The detection of these species from MMS highlights metabarcoding applicability in the detection of rare and elusive taxa for conservation or economic purposes, with wider implications for introduced and invasive species. While the use of targeted or species-specific assays can increase detectability of rare, cryptic or species with a low biomass or shedding rate, my research shows the utility of broad eDNA methods to identify rare or taxa of interest. However, caution should be used in interpreting the absence of target species based on broad assay detections alone, as these can result

from preferential primer binding and PCR amplification bias of less abundant genomic material. Depending on the study questions, the use of a universal assay may provide an initial 'sweep' of results and inform further targeted assay application, if expected taxa remain undetected.

6.2.7 Complementing eDNA with conventional methods can enhance breadth of detections.

In Chapter 3, eDNA methods were demonstrated to complement conventional morphological methods to increase the documented diversity of fish and elasmobranch species at oil and gas platforms. This result is reflected, and well documented, in literature where eDNA and conventional methods have detected different components of the biotic assemblages (Kelly et al., 2017; Suter et al., 2021; Suarez-Bregua et al., 2022), and as such a combination of these methods can be used to mitigate the limits of detection (or lack of reference sequences in databases) of either method in isolation. However, the detectability and metrics collected vary by method, and as such combining datasets can be challenging, which is discussed in Section 6.3.

6.3 Study challenges and limitations

The research in this thesis demonstrates the applicability of marine eDNA metabarcoding as a tool for assessing broad diversity at MMS and artificial reefs. However, a number of limitations have been identified during the process of this research. While some of these are inherent within the eDNA metabarcoding workflow, and are well documented in literature, some are specific to this research or environments sampled. I touch on these identified limitations and challenges below, in consideration of the results outlined within this thesis (see Figure 6.1).

6.3.1 DNA drift from adjacent habitats

DNA movement and decay rates have long been queried in marine eDNA studies, as these can potentially account for false detections within results. Increasingly, research is finding that eDNA shows surprising location fidelity (Stat et al., 2018; Jeunen et al., 2019), a result mirrored within Chapter 2, where fine-scale (8 m) depth stratification was able to be elucidated. However, in Chapters 3 and 4, while vertical stratification was shown, the proximity of a substantial number of oil and gas infrastructure (see

Figure 4.1) in addition to those sampled, necessitates the inclusion as a limitation. The potential detection risk of genomic material from these platforms was not able to be eliminated, both at sampled platforms and off-platform locations. Literature identifies variation within DNA decay rates in open water, ranging from between 10 hours (in the Adriatic Sea; Dell'Anno, 2005) to up to 20 days (West Antarctic Peninsula; Cowart et al., 2018), and is highly dependent on environmental factors (Collins et al., 2018). While the impacts of eDNA dilution away their genetic source is suggested in literature (Stat et al., 2018), within a dense oilfield like the central GoT the concentration of platform infrastructure may result in erroneous eDNA detections from cumulative dilutions.

6.3.2 Reference databases taxonomic resolution

Taxonomic biodiversity assessment using eDNA methods are only as reliable as the quality and availability of the reference material for the target locality (Keck et al., 2023). Some groups, such as fish, are well studied and characterised in terms of taxonomic and database resolution, at least for the commonly utilised barcode regions (e.g., CO1, 16S and 12S), however many groups are less resolved. This is particularly true of benthic and epibenthic species, such as sponges, coral, and calcifying invertebrate species where many taxa are entirely unrepresented in publicly available databases, and much of the taxonomy is genetically unresolved or unknown (Nichols et al., 2022). This lack of species-level resolution was evident in Chapter 2 where, using a broad 18S assay with the analysis kept at a family level, a mean 66% of ASVs were taxonomically unassigned for the trialled methods. Likewise, in Chapter 4, where the resolution of unique sequences to species-level taxonomy ranged from 12% – 67%, with the remaining portion identified to a higher taxonomic level, or completely unassigned. While taxonomic analysis demonstrated similar trends to ASV (taxonomy independent) analysis in this study (Chapter 4), the lack of resolution limits the interpretability of the dataset, and particularly so if unresolved taxa are of special interest, such as conservation-significant or invasive species. The lack of reference material can often result in researchers using taxonomy-free approaches (e.g., ASVs), however for metazoan or eukaryotic research, often the preferred state would be to assign accepted taxonomy to each (meta)barcode.

6.3.3 Detection of pelagic life-history stages

In Chapter 3, a high diversity of reef-associated fish species were detected from off-platforms sites. While possible that these detections could result from DNA movement, as discussed in Section 6.3.1, this finding was attributed to the detection or collection of eggs or larval stage fish taxa given many marine biota, particularly fish, display pelagic life-history phases. As eDNA is a highly sensitive method of detection, this has the potential to skew ecological results by placing detections in a habitat from which species are not usually associated. This detection of varied life history stages has been documented in literature (Leduc et al., 2019; Garcia-Vazquez et al., 2021; Collins et al., 2022).

6.3.4 Lack of quantitative data for eDNA

A significant, general limitation in eDNA studies is the inability of eDNA methods to provide quantitative estimates, such as abundance and biomass, which are metrics currently used in fisheries management and monitoring (Cullis-Suzuki and Pauly, 2010). The shedding rates of species are variable and eDNA longevity is highly dependent on environmental conditions (Section 6.2), with trophic interactions also contributing to eDNA concentrations (Sassoubre et al., 2016). To confound this further, preferential primer binding, PCR amplification bias, and the preferential amplification of sequences can add complexity to determining abundance estimates (Fonseca, 2018; Nichols et al., 2018). Given these constraints, all data analysis undertaken was conservatively used data converted to presence/absence. The field of eDNA quantification is receiving attention (Sassoubre et al., 2016; Adams et al., 2019b; Rourke et al., 2023), with some studies exploring general read abundance trends as a proxy for community population and biomass trends, which have been successful in controlled tank experiments (Rourke et al., 2021; Jo and Yamanaka, 2022; Karlsson et al., 2022). Another emerging avenue of research is the extraction of intact cells only from eDNA samples and amplification of whole cells, or eCells, for use in population studies (Liu et al., 2012). However, it should be noted that these studies are centred on fish populations, with very little research exploring epibenthic coloniser populations or biomass.

6.3.5 Single time-point sampling

Single time point sampling, using any method, will only provide a snapshot of the biotic community present at the time of sampling. This is true of three of the chapters within this thesis, Chapters 2 – 4. The data used to inform decommissioning in Chapter 4 is based on a single time point. While it should be noted that this does not diminish the relevance of the results, seasonality in marine eDNA metabarcoding studies has also been documented (de Souza et al., 2016; Berry et al., 2019, 2023) and therefore additional sampling timepoints would likely provide greater breadth of taxa to inform decommissioning. This is particularly relevant for species that have seasonal variability in DNA shedding rates (often associated with reproduction; Collins et al., 2022; Ip et al., 2022). In some freshwater studies, as there are limited marine examples, this can include introduced and endangered species (Erickson et al., 2017; Troth et al., 2021). Additionally, seasonal variables (such as UV and temperature) can influence the breakdown and longevity of DNA in the environment, therefore affecting seasonal detectability (Wilcox et al., 2016).

6.3.6 Stochasticity and power variability

eDNA is able to provide good representation of biotic communities with a relatively small number of samples. However, the collection of eDNA samples is known to have stochasticity, as DNA (whole cells or fragments) are not homogeneously distributed throughout the environment (Smart et al., 2016; Bockrath et al., 2022), and due to seasonal and shedding effects (Sections 6.3.4 and 6.3.6). Increased site replication and temporal replication can help mitigate these stochastic effects (Adams et al., 2019a), however this increase in sampling effort can potentially increase research costs. This is particularly relevant if applying a multi-assay or multi-substrate study design, as exemplified in Chapter 4. Additionally, the sampling effort required for holistic or robust censusing varies by collection method (Chapter 2) and assay used (Chapter 4). This limitation is included here, not as an impact on the research outlined in this thesis, but as a potential challenge to holistic monitoring overall, including future efforts at oil and gas infrastructure.

6.4 Future directions

The eDNA metabarcoding field is expanding rapidly with new literature, seemingly, published on a daily basis. Even in the brief time-frame of this PhD tenure, there have been significant improvements in eDNA methodologies and their validation (Hajibabaei, 2022), as well as metabarcoding workflows and bioinformatic pipeline enhancements (Mousavi-Derazmahalleh et al., 2021; Zhu et al., 2023). In the marine and aquatic environment, this has resulted in new trialled methods (Thomas et al., 2019; Yamahara et al., 2019), greater detections, and a plethora of new assays and primer combinations (Takahashi et al., 2023). While this research represents important steps in the optimisation of eDNA metabarcoding at marine infrastructure, it is not a final product (if such a thing were ever to exist in science). The optimisation of eDNA metabarcoding methods and the broad censusing of marine infrastructure has been the focus of this research, however there is endless potential for the customisation of future focus trajectories. In this section, I outline five broad directions that I consider important as a logical progression from this research, some of which are centered in scientific validation and development (achievable short-term), and others critical to the promotion of eDNA as a mainstream application in the ecological monitoring tool belt (long-term goals; Figure 6.1). This path, I believe, will result in applied, robust eDNA metabarcoding as a standardised and mainstream tool within industry, as well as widely accepted and supported within local legislation.

6.4.1 Development of a customisable, regionally specific toolkit for the broad assessments of oil and gas infrastructure

The logical next step in this research is the development of a regional toolkit for the broad assessment of diversity on oil and gas platforms, initially for the GoT but customisable to other regional marine oil and gas hubs (e.g., Gulf of Mexico and North Sea). This development would naturally require studies into applying different methods of sampling (e.g., PUF or SWAB combinations) for communities at existing oil and gas infrastructure, the trial of different matrices as collection media, as well as mechanical means of deploying these sampling methods by ROV while minimising cross contamination. The elimination of water filtration could greatly increase the appeal of this toolkit to industries, as this can reduce field contamination, speed up sample collection, potentially promote increased sampling effort or redundancy

collection, and reduce the requirement for in-situ laboratory space. The development of a suite of regional taxa-specific assays would be beneficial to reduce redundancy in detections, with additional assays able to be added as needed based on targeted requirements. The integration and application of multiplexed primer combinations in PCR amplification, while challenging in terms of development, validation and bioinformatics (Ficetola and Taberlet, 2023), can provide higher resolution with fewer reactions, thereby reducing laboratory related time and costs.

The development, validation and implementation of such a toolkit could provide a standardised approach to monitoring, in terms of detection and statistical rugosity, and help inform industry on management and decommissioning options. The overall implementation of this toolkit should be developed with input from industry and government stakeholders, where the data will be applied and analysed.

6.4.2 Developing the frameworks that support eDNA metabarcoding studies

A common theme in many broad metabarcoding studies is the lack of resolution or a paucity in reference databases. While undoubtedly one of the most documented limitations, other linked and supporting frameworks also require consistent and ongoing enhancement. This can include the underpinning taxonomy on which reference databases are founded, given that many groups rely on taxonomy completed many decades ago, which often contains outdated genetic characterisation. Additionally, the ongoing maintenance and quality assurance of databases, which is particularly relevant and problematic in open-source platforms such as GenBank and BOLD (Jin et al., 2020). While some marine taxonomic groups are well characterised and represented, such as fish species, many groups are less studied, such as sponges (phyla Porifera; Yang et al., 2017) or ascidians (class Ascidiacea; Paz and Rinkevich, 2021). It should be noted however, that even well represented groups often only have representation in select barcode regions (Nester et al., 2020). This requirement for the future maintenance, enhancement and development of databases and underpinning taxonomy is ongoing. Already, there are initiatives underway with goals to provide barcode reference material for entire regions (such as the Australian National Biodiversity DNA Library; https://research.csiro.au/dnalibrary/). There is also ongoing research into obtaining reference sequences from historical, taxonomically

identified museum collections, which depending on accessibility of these data systems, may greatly boost the resolution of marine metabarcoding studies. The creation of a unique sequence, or molecular OTU, database for marine studies may help provide regional collection context in taxonomy independent analyses. This, when paired with the appropriate metadata, would allow for the retrospective species-level alignment as reference material and taxonomy is improved.

6.4.3 Implementation of the collection, and biobanking, of temporal and spatial data as standard

The implementation of standardised temporal and spatial sampling upon installation, as well as throughout the operational life, of oil and gas infrastructure can provide valuable information on primary and secondary colonisation, and compositional diversity turnover thereafter. Many invasive species are highly opportunistic, and it's likely that many of these taxa colonise early in operational life. Given the availability of unoccupied benthic substrate, early detection is crucial to prevent settlement and facilitate potential eradication (Willan et al., 2000). Of course, the lack of identifiable trends at marine infrastructure present in Chapter 5 indicates that further validation is required for tracking recruitment at marine infrastructure. This temporal approach can also be used to track community changes post-toppling or reefing in another location, which was the original concept for this PhD research (however was re-focused due to travel restrictions prevalent in 2020). The eight platforms utilised in Chapters 3 and 4 were reefed together to form an inshore artificial reef in late 2020. The temporal data collection prior to removal of the structures, in transit and on reefing could also have been used to validate predictions in Chapter 4, as well as track compositional changes from vertical offshore habitat through to shallow inshore reefs.

Additionally, the implementation of biobanking or long-term storage of eDNA samples (Jarman et al., 2018) can provide a valuable resource for the validation of results, querying new assays, or in future studies, management and research. This could be particularly useful in long-term monitoring programmes where, in a field such as metabarcoding, sequencing technology and protocols can change rapidly. Bio-banked samples would also allow the re-analysis and comparability of new and prior sampling episodes.

6.4.4 Acceptance within regulatory and management frameworks

The acceptance and utilisation of eDNA methods in baseline, impact assessment and compliance monitoring vary by jurisdiction and are not yet widely accepted (Hinz et al., 2022). Although it should be noted that limited eDNA applications have been adopted or planned in some jurisdictions, such as Canada, USA and Finland (Abbott et al., 2021; Norros, et al., 2022; Laschever et al., 2023). In part, this is due to the viewpoint that eDNA is still an emerging tool requiring validation. Currently there is a lack of regulatory input into eDNA monitoring requirements, and therefore the emphasis of eDNA research does not align with the applied and legislative requirement of regulators. Collaboration among research experts, regulatory bodies, and industry stakeholders can result in the development of eDNA guidance statements and regional standardization of protocols for consistent eDNA sampling and analysis. These guidelines have already been produced in some countries (e.g., The eDNA Society, 2019; Pawlowski et al., 2020; De Brauwer et al., 2023), and can offer valuable insights into minimum sampling requirements, bioinformatic thresholds, and quality assurance measures, providing a framework for robust and standardized eDNA research practices. While such documents would require periodic review to incorporate changing technologies, these collaborations can result in a more consistent application of eDNA methods which may increase regulatory acceptance. Eventually this uptake could support more holistic ecosystem or region-wide 'state of environment' reporting.

6.4.5 Promoting data accessibility in eDNA

Environmental DNA metabarcoding outcomes are enhanced by the availability of supporting data frameworks. This of course includes taxonomic and reference databases, but can also include contextual results and metadata from similar regional studies. The potential for enhancing metabarcoding studies via the integration of regionally relevant data, either through querying previous datasets, or access to lodged, pre-collected samples, is immense. To provide context to the potential availability of data, this research alone produced approximately 232 million raw sequencing reads from 702 samples, almost all of which will be unused post-publication. While research outputs and raw or demultiplexed sequence data are often uploaded to online

repositories upon publication, such as Zenodo (as used in this research) or Dryad Digital Repository, this is usually completed with a view of providing reproducibility of results and assessment of scientific rigor within the publication. Often these datasets are beyond the ability of regulatory, industry and non-research personnel to interpret, and the quality and interpretability of this data is highly variable (Berry et al., 2021).

The implementation of open-source data storage has the potential to be enormously advantageous in eDNA studies and monitoring, and should as a minimum include raw sequence files, sample indexes and appropriate field and laboratory metadata. The long-term storage of extracts and redundancy samples is more complicated, but would likely need to fall to regional regulation for implementation, through the lodgement of samples similar to the vouchering of specimens with local museums. Another beneficial avenue to explore would be the production of software to mine, collate and amalgamate data from a variety of existing databases. The output of this software would, by necessity, need to be customisable to feed into research goals, as well as formats accessible to industry and governmental grey literature (such as taxonomic outputs, rather than ASVs).

Secondary to this point of data availability, is the promotion of data sharing and collaboration between institutions. Industry organisations are often very cautious, or risk averse, with the sharing of data particularly with competitors, which would need to be overcome. The benefit of industry collaboration would be immense, given these organisations often face similar environmental barriers. Often industries report environmental conditions solely in the context of their own impacts or infrastructure, however this collaboration or increased availability of data (either sequencing or existing datasets) can allow for a more holistic approach of cumulative regional impacts and context.

6.5 Thesis conclusion

The ability to holistically census marine biotic communities is challenging, however is required to inform management decisions, regulation and conservation efforts. All survey methodologies have documented limitations which must be navigated, however conventional morphology-based survey methods can only target specific groups and are unable to be scaled up for ecosystem-wide diversity monitoring. While eDNA

metabarcoding also has known limitations, for the broad assemblage censusing at marine infrastructure, eDNA methods show the greatest promise as a scalable tool that can play an integral role in informing decisions regarding decommissioning and management options. However, an understanding of the strengths and weaknesses of eDNA methods is required in order to have confidence in, and to base important management decisions upon this data. The overarching question driving this research was, "Can eDNA metabarcoding be optimised for broad diversity monitoring at oil and gas infrastructure?". Throughout the thesis, I compared existing methods of monitoring fish assemblages with eDNA metabarcoding. I successfully identified novel methods of sampling eDNA on infrastructure that outperform conventional marine water sampling methods and epibenthic scrapes methods, which can potentially be applied at oil and gas infrastructure to increase the breadth of detections. Following this, I demonstrated the taxonomic scalability and potential of eDNA to detect across the tree-of-life in a multi-assay and multi-substrate approach in order to provide impact context to varied decommissioning options. Lastly, I detected highly diverse chordate assemblages associated with temporal sampling in, and surrounding, a deployed artificial reef, while demonstrating that further optimisation is required for temporal trend detection in some marine environments. These valuable lessons, when contextualised with the identified limitations, led to the identification of future research and focus trajectories.

This thesis demonstrates that DNA metabarcoding has enormous potential to be scaled up for ecosystem-wide monitoring, and provides a significant advancement in our understanding of the opportunities and limitations of eDNA methods for censusing assemblages on marine infrastructure. Additionally, I provide here key considerations to move these methods from a validation phase for research scientists, to an applied and holistically informative tool for industry and regulatory bodies. The applications for eDNA metabarcoding, including the eDNA toolkit, will continue to develop in the years ahead. This pathway, I believe, will lead to improved biodiversity conservation through informed decision-making in the marine environment, and particularly surrounding marine oil and gas infrastructure.

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Appendices

Appendix I – Co-author contribution statement

This thesis contains work that has been published and prepared for publication.

Chapter 2: Published in the journal Estuarine, Coastal and Shelf Science

Student Contribution statement: To Whom It May Concern, I, Jason Alexander, contributed to conceptualisation of this study, collected and processed samples, completed bioinformatics, analysed the data, and wrote and edited the manuscript:

Alexander J.B., Marnane M.J., McDonald J.I., Lukehurst S.S., Elsdon T.S., Simpson T.J., Hinz S., Bunce M., Harvey E.S. (2023) Comparing environmental DNA collection methods for sampling community composition on marine infrastructure. *Estuarine, Coastal and Shelf Science*. https://doi.org/10.1016/j.ecss.2023.108283.

Student
signature: Date: 01/03/2023

Chapter 3: Published in the journal Marine Environmental Research

Student Contribution statement: To Whom It May Concern, I, Jason Alexander, contributed to conceptualisation of this study, processed samples, completed all bioinformatics, analysed the data, and wrote and edited the manuscript:

Alexander J.B., Marnane M.J., Elsdon T.S., Bunce M., Songploy S., Sitaworawet P., Harvey E.S. (2022) Complementary molecular and visual sampling of fish on oil and gas platforms provides superior biodiversity characterisation. *Marine Environmental Research*. https://doi.org/10.1016/j.marenvres.2022.105692

Student
signature: Date: 01/03/2023

Chapter 4: Manuscript submitted to Science of the Total Environment

Student Contribution statement: To Whom It May Concern, I, Jason Alexander, contributed to conceptualisation of this study, partially processed samples, completed all bioinformatics, analysed the data, and wrote and edited the manuscript:

Alexander J.B., Marnane M.J., Elsdon T.S., Bunce M., Songploy S., Sitaworawet P., Harvey E.S. (2023) Using environmental DNA to inform decommissioning decisions for offshore oil and gas infrastructure. Submitted for review at *Science of the Total Environment.*

Student signature: Date: 01/03/2023

Chapter 5: Manuscript in preparation

Student Contribution statement: To Whom It May Concern, I, Jason Alexander, contributed to conceptualisation of this study, processed samples, completed all bioinformatics, analysed the data, and wrote and edited the manuscript:

Alexander J.B., Berry T.E., Richards Z.T., Harvey E.S. (2023) Temporal eDNA sampling reveals a high diversity, and high assemblage turnover rate, of chordates surrounding a newly installed integrated artificial reef. *[in preparation]*

Student signature: Date: 01/03/2023

Co-author signatures and dates affirming student contribution for data collection (thesis chapters 2-5) chapters and publications as outline above. Green boxes represent the corresponding chapter/s that co-authors contributed to.

Co-author Name (in order of appearance)	**Co-authored chapter**				**Signature** I, as a co-author, endorse that this level of contribution by the candidate as stated above, for the manuscripts that I am involved in, is appropriate	**Date**
	2	**3**	**4**	**5**		
Michael J. Marnane	■	■	■			13/04/2023
Justin I. McDonald	■					01/03/2023
Sherralee S. Lukehurst	■					01/03/2023
Travis S. Elsdon	■	■	■			01/03/2023
Tiffany J. Simpson	■					03/03/2023

Co-author Name (in order of appearance)	**Co-authored chapter**				**Signature** I, as a co-author, endorse that this level of contribution by the candidate as stated above, for the manuscripts that I am involved in, is appropriate	**Date**
	2	**3**	**4**	**5**		
Shawn Hinz						01/03/2023
Michael Bunce						10/03/2023
Euan S. Harvey						01/03/2023
Se Songploy						01/03/2023
Paweena Sitaworawet						14/04/2023
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Co-author Name (in order of appearance)	Co-authored chapter				Signature I, as a co-author, endorse that this level of contribution by the candidate as stated above, for the manuscripts that I am involved in, is appropriate	Date
	2	3	4	5		
Tina E. Berry						10/03/2023
Zoe T. Richards						22/04/23

7.1 Appendix II – Other Publications

Alexander, J. B., Bunce, M., White, N., Wilkinson, S. P., Adam, A. A. S., Berry, T., et al. (2019). Development of a multi-assay approach for monitoring coral diversity using eDNA metabarcoding. Coral Reefs. doi: 10.1007/s00338-019-01875-9.

Dugal, L., Thomas, L., Wilkinson, S. P., Richards, Z. T., Alexander, J. B., Adam, A. A. S., et al. (2022). Coral monitoring in northwest Australia with environmental DNA metabarcoding using a curated reference database for optimized detection. Environmental DNA 4, 63–76. doi: 10.1002/edn3.199.

White, N. E., Guzik, M. T., Austin, A. D., Moore, G. I., Humphreys, W. F., Alexander, J., et al. (2020). Detection of the rare Australian endemic blind cave eel (*Ophisternon candidum*) with environmental DNA: implications for threatened species management in subterranean environments. Hydrobiologia 847, 3201–3211. doi: 10.1007/s10750-020-04304-z.