School of Molecular and Life Sciences

Faculty of Science and Engineering

Monitoring blue biodiversity: examining diversity patterns in coastal and marine environments using environmental DNA

Georgia May Nester

0000-0001-9721-4512

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Doctor of Philosophy

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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.

Date: 2nd June 2023

Dedication

Dedicated to my Grampy

- Donald James Miller -

04-10-1931 – 04-05-2023

I love the sea, I love the navy

Bugger me, I must be crazy

Abstract

Coastal and marine ecosystems are facing unprecedented levels of disturbance and biodiversity loss globally. Baseline knowledge of diversity and richness patterns is often lacking or inadequate for many ecosystems and species, impeding targeted conservation efforts. Environmental DNA (eDNA) techniques are increasingly used in this context, advancing the current scope of coastal and marine ecosystem monitoring and expanding biodiversity knowledge. The technique characterises DNA shed by organisms into their immediate environment to infer species presence and assemblage composition. My thesis explores the applications of eDNA in coastal and marine ecosystems for monitoring small-bodied cryptic taxa (Chapters 2 and 3), and biodiversity within severely underrepresented (Southern Ocean) and uncharacterised (deep-sea) ecosystems (Chapters 4 and 5). Overall, this body of research seeks to develop and advance coastal and marine eDNA techniques to improve knowledge about biodiversity.

To assess the viability of eDNA for monitoring small-bodied cryptic taxa, I targeted Syngnathidae taxa (pipefishes, seahorses, and seadragons) using eDNA metabarcoding (Chapter 2). Many Syngnathidae taxa are considered threatened, however, many more lack the data necessary to assess their population status and distribution. The primary objective of this study was to evaluate and optimise a set of eDNA metabarcoding assays capable of detecting Syngnathidae taxa in the context of other fish assemblages. Using two newly designed assays on water samples obtained from coastal waters of the Perth Metropolitan region, four different species of Syngnathidae were detected for the first time using eDNA. This result demonstrates the potential for eDNA approaches to be used for monitoring small-bodied cryptic taxa and discriminating community composition over small spatial scales.

To further examine the value of eDNA approaches for detecting cryptic small-bodied taxa, a targeted eDNA assay was developed and optimised for the critically

endangered estuarine pipefish (*Syngnathus watermeyeri*). In this study, I compared eDNA obtained from replicate water samples to conventional seine netting across the historical range of *S. watermeyeri* in the Eastern Cape of South Africa (Chapter 3). *S. watermeyeri* was successfully detected in two of the five estuaries surveyed using both survey methods, with the positive detection rate of eDNA being four times higher than that of seine netting. In addition, the occurrence of the pipefish was found to be highly dependent on the presence of eelgrass. In providing a more complete picture of the conservation status of this critically endangered species, this study facilitated the development of a long-term eDNA monitoring program and the identification of priority conservation areas.

To expand and validate eDNA approaches in a broader context, patterns in Animalia biodiversity across a latitudinal gradient in the Southern Ocean were explored (Chapter 4). eDNA metabarcoding was compared to a continuous plankton recorder (CPR) survey, conducted simultaneously along a transect from Hobart (Tasmania) to Davis Station (Antarctica). Little overlap was found in the species detected by the methodologies, highlighting the need for employing multiple monitoring methods to adequately capture the biodiversity of a region. Importantly, eDNA metabarcoding detected 16 non-native, pest, or invasive taxa, with several detected near the continent of Antarctica. This research establishes and validates an approach for large-scale biomonitoring in the open ocean. The findings demonstrate that a long-term eDNA biomonitoring program is urgently needed in the Antarctic region to provide an early warning signal of invasive species that may threaten the persistence of local fauna.

Lastly, to explore the biodiversity in uncharacterised deep-sea ecosystems, an eDNA metabarcoding survey was undertaken across the Cape Range submarine canyon system, Western Australia. The canyon is one of the largest in Western Australia, extending over 120 km and reaching approximately 4600 m in depth. This study was conducted in tandem with the first biological survey of the canyons, and eDNA (COI Leray and 16S Fish assays) collected from ~1700 litres of water revealed 109 putative undescribed species, new records (species or genus), or range extensions for Western

Australia (Chapter 5). Multivariate statistics indicate community composition varied between canyon sites for both assays, with strong patterns in diversity observed across a depth gradient for the COI Leray assay. No depth patterns were observed in diversity for the 16S Fish assay, likely due to biological characteristics (e.g., vertical migrations and body size) of the target taxa as well as sampling timing (e.g., potential spawning events). This research revealed that high-quality metabarcoding compositional data can be obtained from deep-sea ecosystems, and used to characterise the biodiversity within these heterogeneous but little-known habitats.

Collectively, my research contributes to the expansion and refinement of eDNA monitoring approaches for coastal and marine ecosystems. By genetically profiling over 500 water samples, and filtering ~3000 L across four study regions, I have demonstrated the viability of eDNA approaches for monitoring cryptic and threatened taxa. I have also demonstrated eDNA biomonitoring can deliver early warning signals for biological invasions in coastal and marine environments, and provide unprecedented insight into the diversity of marine life inhabiting deep-sea ecosystems. In a broad context, my research generated data and baselines for vulnerable taxa and ecosystems, at a time where careful management and monitoring is pivotal to conserving coastal and marine biodiversity.

Acknowledgements

The completion of this thesis has been one of the hardest things I have ever had to do, but any challenge has been greatly outweighed by the experiences I have had and the people I have shared them with. I feel very fortunate to have undertaken this PhD, I have met the most amazing people and explored places that I dreamed of visiting as a little girl — Antarctica, Africa, and the deep-sea. But I could not have done any of this without the support of my family, friends, and supervisors.

If I am acknowledging those who have supported me on this journey, I have to start with my family. To my parents, your unwavering love and support is what made me think I could take on my dreams. I have so much respect and adoration for you both. You helped me become a mermaid as a child, and an adult. I can truly say, I have the best parents in the world. James, you are my baby brother and you always made life fun. To my Granny, you are always so interested in what I do and make me feel heard and special. To my Grampy, Nanna and Poppy, I wish you were here to see me graduate but I know that you would be proud.

Candy, you have quite literally been there from the start. Our bond was instant, unbreakable, and eternal. We have been through so much together, but every time I knew you would be there without fail. I am so lucky to have a best friend like you.

My sweet Johan. You are my world. You cared for me during this time in a way that I will never forget. I could not have done this without you. *Ek is baie lief vir jou, engel.*

To my dear friends Nikki and Rory, you both supported me and helped me throughout this journey so much. You also very nearly destroyed my liver on several occasions. I appreciate you both very much.

My supervisors believed in me, supported me, and checked in on me. Euan, we have known each other for almost a decade now and I am so proud of the relationship we

have. You always had my back, always picked my corner, and I cannot tell you how much that meant to me. Your check-ins were always appreciated. Zoe, you did so much for my self-esteem. You want what is best for me and make me feel like I can do anything- which may be dangerous. Mike, your enthusiasm about literally anything to do with science is second to none. Your passion was contagious, you were a great lab lead, and I will always hold the TrEnD laboratory close to my heart. Thank you for taking me on, I will always be grateful.

The TrEnD lab, we have changed so much over the years but we are always an amazing group. I have met some of my best friends in the TrEnD lab and I feel very fortunate to have been a part of something so inspiring. Dr Katrina West, you were my mentor, my roommate, then my best friend. Jason Alexander, you may be the second most sarcastic and cynical person I know (second only to myself), but you made this PhD journey a lot brighter. Thank you for being there for me in many ways. Special thanks to Matthew Heydenrych and Sophie Preston for making sure I surfaced from the depths of my PhD every now and then. The rest of the past and present TrEnD lab rats and frontiers staff, I have found lifelong friends in you all. The whole Fish Ecology Laboratory has been an amazing support, but a special thanks to Karl Schramm for coffee and introducing me to red wine risotto.

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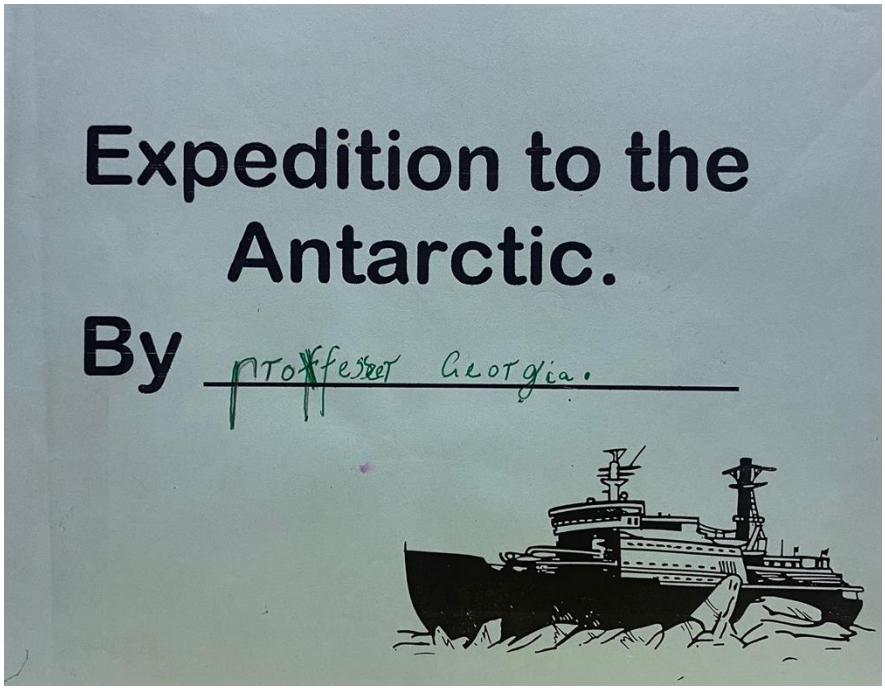
While not a 'proffesser', and my journey in science hasn't involved as many expeditions, overnight stays in igloos, or up-close interactions with seals, penguins, and killer whales as my 6-year-old self imagined, I am very proud of my progress so far.



Expedition to the Antarctic written by 'proffesser' Georgia Nester in June 2000.

Acknowledgement of Country

Personal

I have a deep appreciation and respect for Australian biodiversity and ecosystems as a whole. My trips to Nyinggulu (Ningaloo) as a child made me fall in love with the region, and inspired me to follow this path. In this appreciation, I am able to recognise that I live and work on stolen land. I would like to personally acknowledge the traditional custodians of the land upon which I conducted my research, the Whadjuk Noongar people and the (West) Thalanyji people. I recognise their continued connection to land and sea country, and the cultural and spiritual significance these regions have. I wish to pay my deepest respects to Elders past, present, and emerging, extending that respect to all First Nations people across the nation. I strive to live by the philosophy: "*Look after country, and country will look after you".*

Always was, always will be, Aboriginal land.

Institution

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

Chapter 2:

Nester, G. M., De Brauwer, M., Koziol, A., West, K. M., DiBattista, J. D., White, N. E., Power, M., Heydenrych, M. J., Harvey, E., & Bunce, M. (2020). Development and evaluation of fish eDNA metabarcoding assays facilitate the detection of cryptic seahorse taxa (family: Syngnathidae). *Environmental DNA*, 2(4), 614–626. https://doi.org/10.1002/edn3.93

This study was designed by G.M.N., M.D.B., M.B., and J.D.D.; samples were collected by G.M.N. and M.D.B, samples were processed and molecular research was conducted by G.M.N., A.K., M.P., N.W., and K.W.; the reference database was created by J.D.D., A.K., and M.H.; metabarcoding assays were designed by K.W., A.K., and G.M.N.; statistical analysis was conducted by G.M.N. with advice from E.H., M.D.B, and M.B.; and all authors contributed to writing this paper.

Chapter 3:

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Chapter 4:

Nester, G.M., Suter, L., Polanowski, A.M., Kitchener, J.A., Bunce, M., Deagle, B. eDNA metabarcoding reveals biodiversity patterns and facilitates early detection of invasive species in the Antarctic Southern Ocean *Manuscript in preparation*.

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This study was designed by G.M.N., Z.R., M.B., and N.W.; eDNA samples were collected, processed, and molecular research was conducted by G.N.; physical specimens were processed and identified by N.W., L.K., A.H., G.M., and Z.R.; G.N., conducted statistical analysis; G.N., and Z.R., contributed to the writing of this chapter.

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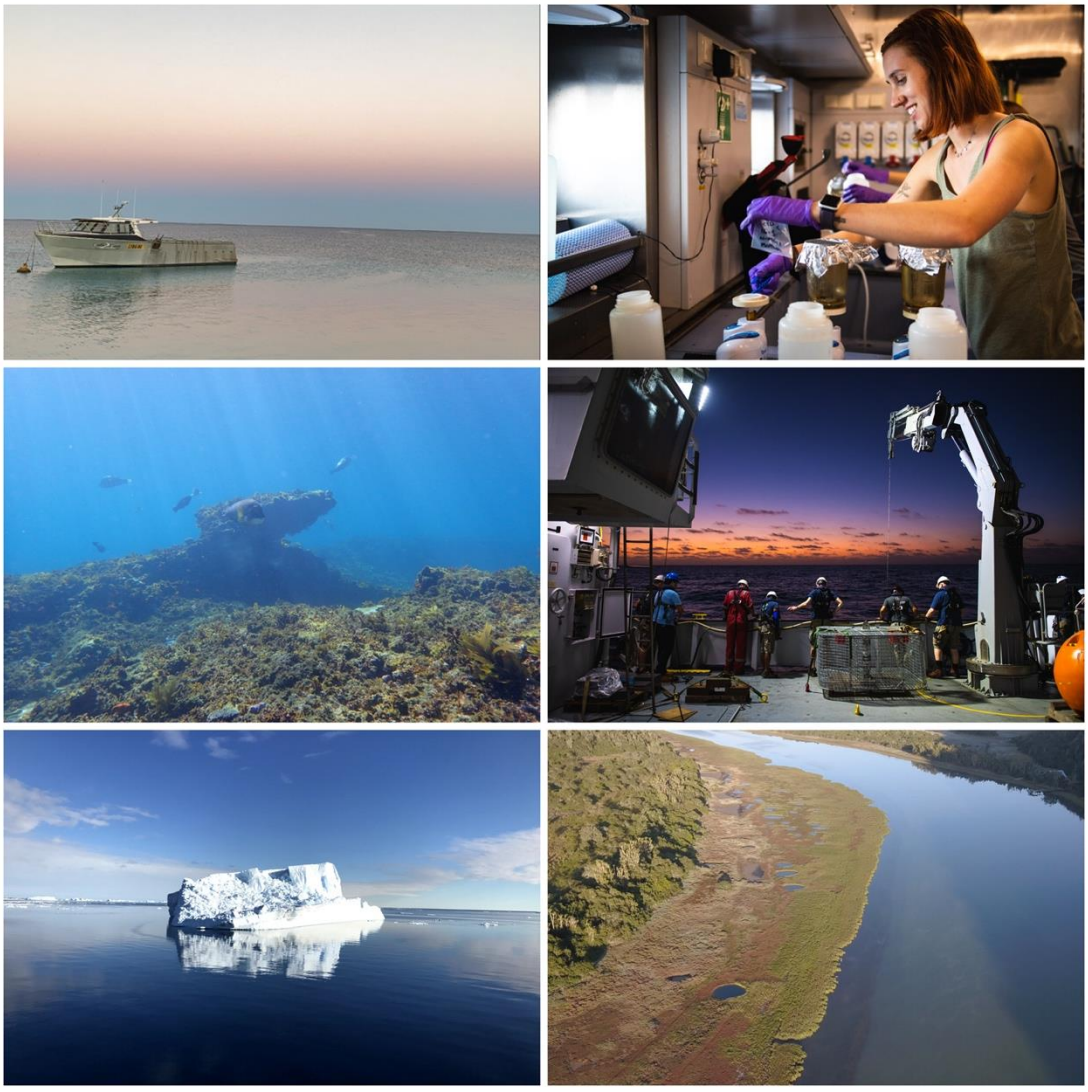
Acronyms

AAD	Australian Antarctic Division
AAZ	Antarctic zone
ACC	Antarctic Circumpolar Current
ALA	Atlas of Living Australia
ANOVA	Analysis of variance
BLAST	Basal Local Alignment Search Tool
BOLD	Barcode of Life Database
bp	Base-pair(s)
BRUV	Baited remote underwater video
CAP	Canonical analysis of principal coordinates
CATAMI	Collaborative and Automated Tools for Analysis of Marine Imagery
COI	Mitochondrial cytochrome c oxidase I
CPR	Continuous plankton recorder
Ct	Cycle threshold
CTD	Conductivity, temperature, depth rosette
CytB	Mitochondrial cytochrome b
dbRDA	Dissimilarity-based redundancy analysis
ddPCR	Digital-droplet PCR
DistLM	Distance-based linear model
DNA	Deoxyribonucleic acid
DPIRD	Department of Primary Industries and Regional Development
eDNA	Environmental DNA
eRNA	Environmental RNA
gDNA	Genomic DNA
GISD	Global Invasive Species Database
GRIIS	Global Register of Introduced and Invasive Species
IMS	Invasive marine species
INSDC	International Nucleotide Sequence Database Collaboration
LCA	Lowest common ancestor
LOD	Limit of detection

LOQ	Limit of quantification
MID	Multiple identifier
NBDL	National Biodiversity DNA Library
NCBI	National Center for Biotechnology Information
NGS	Next-generation sequencing
NMDS	Non-metric multidimensional scaling
OTU	Operational taxonomic unit
PA	Presence/absence
PCO	Principal coordinate analysis
PCR	Polymerase chain reaction
PERMANOVA	Permutational multivariate analysis of variance
PFZ	Polar frontal zone
qPCR	Quantitative PCR
RNA	Ribonucleic acid
ROV	Remotely operated underwater vehicle
rRNA	Ribosomal RNA
SACCF	Southern ACC Front
SACCZ	South of the ACC zone
SAF	Subantarctic front
SAZ	Subantarctic zone
SBDY	Southern boundary
SCUBA	Self-contained underwater breathing apparatus
SIMPER	Similarity percentage analyses
SSS	Sea-surface salinity
SST	Sea-surface temperature
STF	Subtropical front
STZ	Subtropical zone
TrEnD	Trace and Environmental DNA Laboratory
UVC	Underwater visual census
WAM	Western Australian Museum
WoRMS	World Register of Marine Species
ZOTU	Zero-radius operational taxonomic unit

Chapter 1

Introduction



1.1 Coastal and marine biodiversity under threat

Coastal and marine ecosystems cover more than 70% of the Earth's surface and encompass a wide range of highly productive and valuable habitats, including coral reefs, estuaries, mangroves, deep-sea, and open-ocean. These ecosystems support approximately 20% of the global biological diversity (Grosberg et al., 2012; Mora et al., 2011; Román-Palacios et al., 2022), with nearly 263, 000 species currently described (WoRMS Editorial Board, 2023 as of 18 April 2023) and an estimated one to two-thirds yet to be discovered (Appeltans et al., 2012). Coastal and marine ecosystems perform vital ecological functions such as climate regulation, carbon sequestration, nutrient cycling, habitat connectivity, and energy flow (Barbier, 2017; Bernhardt & Leslie, 2013; Cooley et al., 2022; Costanza et al., 2014). In addition to their intrinsic ecological value, the significance of these ecosystems is frequently linked to the socio-economic value of the ecosystem services they provide (Barbier, 2017; Barbier et al., 2011; Liquete et al., 2013; Mehvar et al., 2018). Ecosystem services, which encompass both direct (e.g., food provision, fisheries, aquaculture, tourism) and indirect benefits (e.g., climate and weather regulation, coastal protection, nutrient cycling, indigenous and spiritual values), play a fundamental role in sustaining livelihoods and promoting overall well-being by deriving benefits from nature (Georgian et al., 2022; Selig et al., 2019; Ward et al., 2022). However, as the human capacity to industrialize these ecosystems continues to expand (Jouffray et al., 2020), simultaneous escalation of threats posed by anthropogenic activities and climate change challenges the sustainability, resilience, and ability of these ecosystems to provide these critical services (Cavanagh et al., 2021; Cooley et al., 2022; Halpern et al., 2019; IPCC, 2023; Yi & Jackson, 2021).

Climate change impacts coastal and marine ecosystems globally through warming and acidification, sea-level rise, and the intensification of extreme weather events (Cooley et al., 2022; Gissi et al., 2021; IPCC, 2023; Poloczanska et al., 2016). At local to regional scales, climate change exacerbates the effects of non-climate anthropogenic impacts, such as habitat degradation, marine pollution, overfishing and overharvesting, nutrient enrichment, and the introduction of non-indigenous species (Cooley et al., 2022; Herbert-Read et al., 2022; IPCC, 2023). The combined

and synergistic effects of these stressors alter ecosystem structure, function and biodiversity beyond the range of natural fluctuations (Canadell & Jackson, 2021; Halpern et al., 2015). Changes have already been widely documented in coastal and marine ecosystems at various temporal and spatial scales, including range expansions or contractions (Booth et al., 2011; Sanford et al., 2019; Wernberg et al., 2016; Yamano et al., 2011), changes in community structure (Courtney et al., 2020; Edgar et al., 2023; Hughes et al., 2018; Ilarri et al., 2022), and instances of mass mortality in coral, kelp, mangrove, and seagrass habitats (Goldberg et al., 2020; Hughes et al., 2017; Krumhansl et al., 2016; Serrano et al., 2021). In spite of this, there is still a great deal of uncertainty regarding our ability to prevent further loss and improve ecosystem resistance (McClenachan et al., 2012; Thurstan et al., 2015).

Effective conservation actions and monitoring programs require baseline knowledge of historical and contemporary biodiversity patterns and their distributions (Borja, 2014; Edgar et al., 2023; Hooper et al., 2012). Biodiverse (and interconnected) ecosystems are more stable and resilient to disturbances (Georgian et al., 2022; Lefcheck et al., 2015). However, maintaining optimal or baseline levels of biodiversity requires knowledge of their original state prior to human impacts or disturbance (Thurstan, 2022). For many coastal and marine ecosystems, this knowledge is often missing or incomplete (Luypaert et al., 2020; Selig et al., 2014). Part of the disconnect between recognising human impacts on these ecosystems, and the timely implementation of mitigation and conservation strategies, can be attributed to the logistical challenges of underwater surveys (Bourlat et al., 2013; Elliott, 2011). Comprehensive biodiversity surveys are challenging in these environments due to their reduced accessibility and often vast expanse, the occurrence of rare and cryptic species, unresolved taxonomy, declining taxonomic expertise, and increased cost (Conde et al., 2019; Kindsvater et al., 2018; Richardson & Poloczanska, 2008; Scheele et al., 2018). Accordingly, long-term datasets that span multiple trophic levels, a crucial requirement for effective ecosystem-based monitoring (Harvey et al., 2020; Hughes et al., 2005; Magurran et al., 2010), are rare. Furthermore, our ability to directly observe ecological changes below the water's surface only began in recent decades, while our exploitation of their services spans centuries (Thurstan, 2022).

This disparity has resulted in a collective historical unawareness of past ecological changes across generations of scientists and managers, causing an intergenerational shift in the perception of how a natural or pristine ecosystem should look and function, popularly termed the 'shifting baseline syndrome' (McClenachan et al., 2018; Papworth et al., 2009; Pauly, 1995; Soga & Gaston, 2018).

The paucity of appropriate and comprehensive baselines in coastal and marine ecosystems poses a challenge in monitoring current biodiversity and anticipating how it might respond to future changes (Kopf et al., 2015; Rodrigues et al., 2019). Failure to acknowledge and incorporate shifting baselines into conservation and management efforts may result in underestimating biodiversity loss, thereby leading to the establishment of conservation targets based on already degraded baselines (Gonzalez et al., 2016; Mehrabi & Naidoo, 2022). This, in turn, can result in silent extinctions, the collapse of ecosystem services and an overall loss of biodiversity (Costello, 2015; Duarte et al., 2020; McCauley et al., 2015; Penn & Deutsch, 2022; Richards & Day, 2018; Worm et al., 2006). While shifting baseline syndrome can occur in any changing environment, it is especially prevalent in remote (and out of sight) coastal and marine ecosystems with limited historical records (Dayton et al., 1998; Plumeridge & Roberts, 2017; Thurstan et al., 2015). Mitigation and possible prevention of these impacts necessitates the rapid generation of ecosystem-wide baseline data for various habitats (Geist & Hawkins, 2016; Soga & Gaston, 2018; Ward et al., 2022), which can serve as the foundation for developing long-term monitoring datasets with sufficient power to detect ecological changes or disturbance.

Historically, through necessity, conventional approaches for surveying biodiversity in coastal and marine environments involved 'catch and look' techniques relying on morphological identification. With technological advances, this morphed into non-invasive methods such as baited remote underwater videos (BRUVs), underwater visual census (UVC) via belt transects or timed swims, remotely operated underwater vehicles (ROV), and acoustics (Harvey et al., 2007; Mallet & Pelletier, 2014; Murphy & Jenkins, 2010). Alternatively, invasive methods such as trawling, clove oil, euthanisation with rotenone, baited long-line, or electrofishing may also be utilised

(Ackerman & Bellwood, 2000; Danovaro et al., 2016; Hill et al., 2005; Hylton et al., 2017; Robertson & Smith-Vaniz, 2010). These approaches are capable of species-level identification and yield valuable data on life stages, population sizes, and biomass (Murphy & Jenkins, 2010; Radinger et al., 2019). However, these approaches may not completely capture the biodiversity within a sampling region or habitat, as they generally target a single taxonomic group, such as fish or corals, or a subset of these groups (Alexander et al., 2022; Bourlat et al., 2013; Richards, 2013). Furthermore, addressing the impacts of climate change and environmental degradation necessitates long-term datasets that encompass various ecological and spatial scales (Almpanidou et al., 2016; Hobday, 2011; Rilov et al., 2019; Trifonova et al., 2022). Yet, the time, cost, and taxonomic expertise typically associated with conventional surveys make it increasingly challenging to obtain such comprehensive data. As a result, many coastal and marine biodiversity studies are limited in duration and localized in scope (Ficetola et al., 2008), with most environments monitored at an ecosystem level using surrogate information (Richards et al., 2022). Environmental DNA (eDNA) approaches offer a new lens on biomonitoring, and represent a significant step towards whole ecosystem monitoring across multiple spatial and ecological scales. However, at the outset it should be stated that eDNA is no 'silver bullet', rather it is a powerful tool to be interwoven into other approaches.

1.2 Environmental DNA (eDNA)

The subsequent sections offer a concise introduction to environmental DNA (eDNA), encompassing both single and multiple target-taxa approaches. Additionally, a general workflow for conducting eDNA studies is presented, accompanied by an overview of each step involved. This section underscores critical considerations such as sampling volume, filtration methods, primer design, taxonomic assignment employing reference databases, as well as the significance of methodological standards and controls within eDNA research. However, it should be noted that the workflow overview does not encompass detailed discussions pertaining to sample substrate selection, PCR replication, library preparation, subsequent sequencing, bioinformatics pipelines, or data analyses (though see Bohmann et al., 2022; Koziol et al., 2019; Mathon et al., 2021; Ruppert et al., 2019; Taberlet et al., 2018).

1.2.1 A brief introduction

Environmental DNA (eDNA) refers to any genetic material that can be extracted from an environmental sample without the need for isolating the target taxon or taxa (Taberlet et al., 2018). This genetic material derives from both whole microscopic organisms (i.e., microbes) or extracellular DNA (i.e., shed cells or tissue, blood, mucous, faeces, etc.). Once DNA is shed into the environment, its preservation and availability vary greatly depending on a range of environmental conditions (Barnes et al., 2014). As such, eDNA can be extracted from a wide range of environments and substrates (e.g., water, sediment, snow, flowers/leaves, ice and sediment cores, air, etc.) to address fundamental questions about both ancient (Haile et al., 2009; Kjær et al., 2022; Sønstebø et al., 2010) and contemporary biodiversity (Franklin et al., 2019; Lynggaard et al., 2022; Newton et al., 2023; West et al., 2020). Since its conception and consolidation as a biomonitoring method, eDNA surveys in aquatic environments have significantly increased (Figure 1-1). This rapid global expansion has been accompanied by the invention of novel sampling and laboratory procedures, allowing eDNA surveys to be developed, tested, and applied in almost every type of aquatic ecosystem (Takahashi et al., 2023). However, the majority of these have targeted freshwater environments, with coastal and marine systems being comparatively underrepresented, accounting for less than a third of ecosystems where eDNA has been deployed (Figure 1-1).

Target genetic material is amplified from eDNA samples using polymerase chain reaction (PCR). Due to the degraded nature of DNA found in environmental samples, amplifications generally target short fragments of DNA less than 500 base-pairs (bp; Taberlet et al., 2018). Target amplification can be at the individual level using species-specific ‘targeted’ assays, or broader by amplifying entire or multiple taxa groups using taxonomically broad assays (eDNA metabarcoding). While these approaches use similar technologies to identify and assign taxa, they differ in their specific goals and environmental applications.

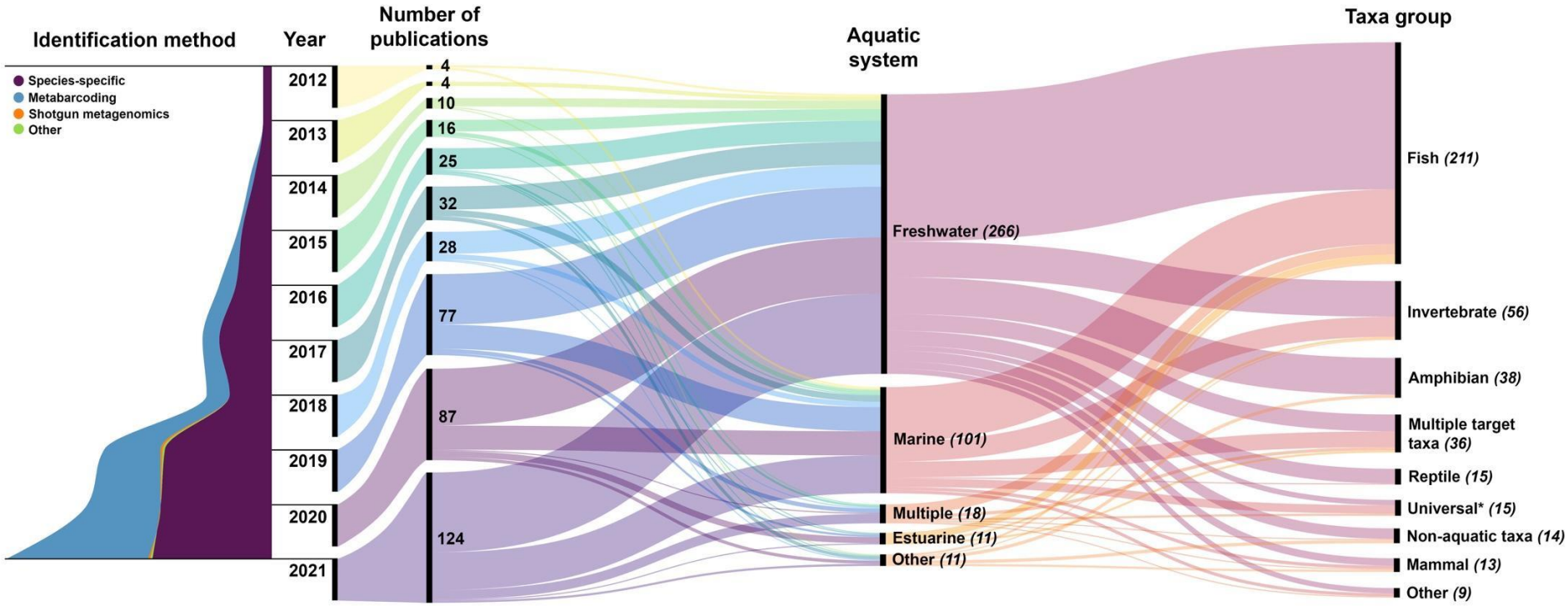


Figure 1-1: A sankey diagram showing trends in aquatic eDNA studies. Trends in eDNA studies conducted between 2012 and 2021. The number of publications, types of aquatic systems, and taxa groups that were dominant over the time period are indicated. On the left, trends in eDNA identification methods over the time period are shown. I created this figure as part of a review on the past 10 years of aquatic eDNA research (Takahashi et al., 2023), it is included in this thesis with permission from other co-authors and the journal *Science of the Total Environment*.

1.2.2 Species-specific detections

Species-specific eDNA surveys have been used in aquatic environments for over a decade to infer species presence without first isolating the target organism (Ficetola et al., 2008; Jerde et al., 2011). The approach was first applied in aquatic ecosystems (for macro-organisms) to successfully detect the invasive American bullfrog in natural wetland systems in France, demonstrating the efficacy of eDNA surveys for determining species presence and distributions (Ficetola et al., 2008). The findings of this study and the subsequent interest it generated, paved the way for eDNA biomonitoring, which, in turn, initiated exponential growth in aquatic eDNA research (Figure 1-1). Although later research continued to focus on invasive species detections (Dejean et al., 2012; Mahon et al., 2013), the heightened sensitivity and improved detection capabilities of the approach compared to conventional monitoring methods prompted investigations into identifying endangered and threatened species. Here, eDNA has confirmed species distributions (Laramie et al., 2015; Villacorta-Rath et al., 2022) and expanded known ranges (Gorički et al., 2017), tracked migration patterns (Halvorsen et al., 2020; Thalinger et al., 2019), and assessed population genetics (Adams et al., 2022; Baker et al., 2018).

1.2.3 Multi-taxa approaches (eDNA metabarcoding)

The use of taxonomically broad eDNA assays, known as metabarcoding, provides biodiversity and composition data for whole communities and ecosystems across the tree-of-life (Stat et al., 2017; Thomsen et al., 2012). Metabarcoding assays typically target specific groups, such as elasmobranchs or corals (Alexander et al., 2020; West et al., 2021), or they can have broader 'universal' targets, such as eukaryotes (Leray et al., 2013). As a result, assays need to be conserved enough to amplify multiple taxa within a target group, but variable enough to discriminate between species (or lower depending on marker resolution) within the target group (see section 1.3.3, Taberlet et al., 2018). The progression to multi-taxa detections was made possible through the introduction of high-throughput (or next-generation) sequencing platforms such as the 454 Life Sciences instrument in 2005. These platforms allow for parallel sequencing of multiple complex environmental samples, generating millions of sequencing reads for the simultaneous detection of multiple taxa (Shokralla et al.,

2012; Taberlet et al., 2018). The resulting data is then quality filtered and compared to reference databases, providing multiple taxonomic assignments (Taberlet et al., 2018).

eDNA metabarcoding surveys are commonly conducted in coastal and marine ecosystems to estimate species richness and diversity (Miya et al., 2015; Stat et al., 2017; Thomsen et al., 2012), and to enhance our understanding of species interactions and co-occurrences (Balasingham et al., 2017; Kačergytė et al., 2021). Beyond this, methodological advancements have made it possible to assess the quality and health of water bodies (David et al., 2021; Hajibabaei et al., 2011), and to evaluate the effects of anthropogenic activities and disturbances on biodiversity (DiBattista et al., 2020; Lanzén et al., 2021; Li et al., 2018; Pawlowski et al., 2014).

1.3 eDNA workflow and study considerations

Although the potential applications of eDNA for coastal and marine biodiversity monitoring are extensive, and the benefits of rapidly developing new eDNA methods are evident, there is a potential risk of losing or diminishing common methodological standards. These standards are essential in enabling effective cross-study comparisons and data sharing. However, several steps have been taken in this direction (Buxton et al., 2021; Goldberg et al., 2016; Minamoto, 2022; Takahashi et al., 2023), and the following sections provide a brief summary of key considerations for undertaking eDNA studies using both species-specific and metabarcoding approaches – this background underpins the core research themes within this thesis.

1.3.1 General workflow

eDNA workflows will somewhat differ between applications, environments, and laboratories, however, the basic workflow will usually include the following sequential steps: (i) environmental sampling; (ii) DNA extraction; (iii) PCR amplification of target taxa using species-specific or metabarcoding primers; (iv) library preparation and sequencing; (v) bioinformatics, taxonomic assignment and (vi) statistical analyses. The workflow and key considerations for each step are outlined in Figure 1-2, and discussed briefly in the following sections.

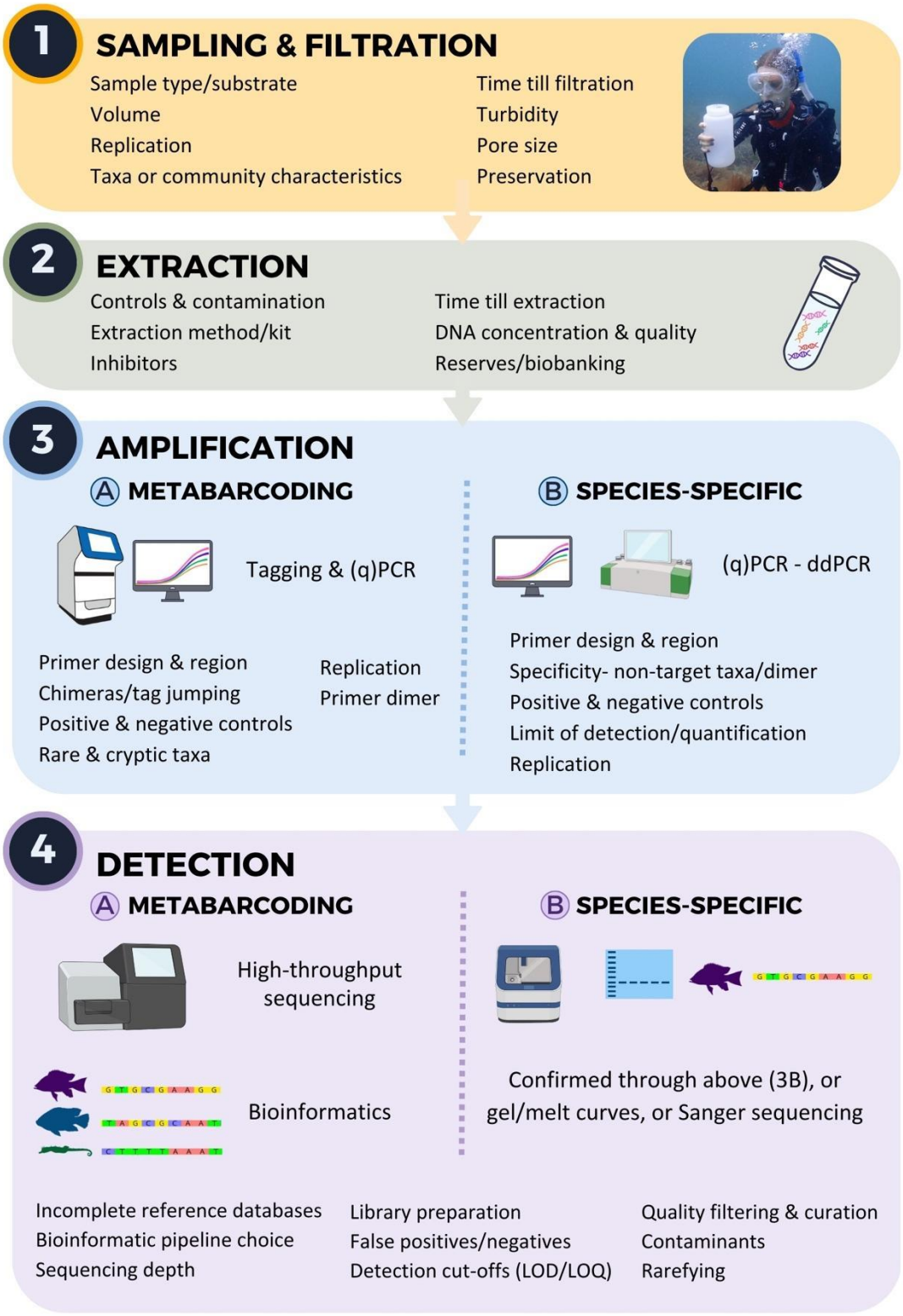


Figure 1-2: Basic eDNA workflow for both species-specific and metabarcoding approaches, with essential considerations for each step indicated.

1.3.2 Sampling, filtration, and extraction

eDNA surveys can be conducted in coastal and marine ecosystems using various substrates, however, as this thesis solely uses water samples, the following overview and recommendations pertain to water samples. While the volume of water samples used in eDNA surveys varies widely in the literature, ranging from millilitres (≤ 15 mL, (Dejean et al., 2012) to thousands of litres (Suter et al., 2021), the majority of current studies employ 1 L water samples (Takahashi et al., 2023). Likewise, the number of field replicates varies substantially in the literature, with the majority of studies currently implementing 1 – 3 biological field replicates (Takahashi et al., 2023). Logically, larger volumes of water and increased replication would improve detection rates, but there is a trade-off as sampling large volumes and quantities can be logistically challenging, particularly in remote locations (Villacorta-Rath et al., 2022). The choice of volume and replication is heavily dependent on target taxa, type of ecosystem, study aims, and risks associated with false negatives (Takahashi et al., 2023). For example, biosecurity studies targeting invasive species have higher risks associated with false negatives, and should therefore aim to increase the volume sampled and the number of biological replicates sampled. While there can be no general rule for coastal and marine eDNA surveys and a priori assessments are required, implementing larger volumes (> 1 L) with more than 3 biological field replicates per site will increase detection sensitivity (species-specific) and diversity detected (metabarcoding).

DNA continues to degrade once samples are collected (Barnes & Turner, 2016), so it is crucial that samples are appropriately preserved and filtered to accurately capture the genetic diversity at the time of collection. Filtering on site is preferred, but is often only possible for smaller water volumes or non-turbid environments (Harper et al., 2019), or requires equipment that may not be within budget constraints (e.g., Smith-Root eDNA Sampler; Thomas et al., 2018). The majority of studies will refrigerate or keep samples on ice until filtration is possible (Takahashi et al., 2023). However, DNA copy numbers have been shown to significantly decrease after 24 hours of collection regardless of storage method (Hinlo et al., 2017; Holman et al., 2022). Therefore,

samples should be filtered as soon as possible, ideally within 24 hours of collection. There are two primary methods for filtering water samples: active (Thomsen et al., 2012) and passive (Bessey et al., 2021; Kirtane et al., 2020; McQuillan & Robidart, 2017) filtration. The specific techniques employed can vary greatly, but the most widely used method is active filtration with a pumping mechanism through a membrane of various pore sizes (Takahashi et al., 2023). While a recent study has shown the methods achieve similar estimates of biodiversity (Bessey et al., 2021), the sampling effort was not equal, with 146 passive membranes deployed and only 9 x 1 L samples actively filtered. Therefore, active filtration is likely the most effective approach at present.

Pore size of filter membranes is study and site specific, with no consistent patterns evident throughout the literature (Takahashi et al., 2023). Generally, larger pore sizes (≥ 0.45 µm) are more appropriate for macro-organisms, larger water volumes, and more turbid environments (Kumar et al., 2021), while smaller pore sizes (≤ 0.22 µm) are better suited to microorganisms and smaller water volumes (Suter et al., 2021). Post-filtration membrane storage has not been comprehensively compared across all techniques or various ecosystems, but the most common methods are freezing (preferably at -80°C) or storing in lysis buffer (Renshaw et al., 2015). DNA can be extracted from filter membranes using a variety of methods, with silica binding chemistry (e.g., Qiagen kits) being the most common (Takahashi et al., 2023). Depending on substrate and DNA concentrations, commercial extraction kits are often modified e.g., different purification columns, adjusting volume of lysis solution and Proteinase K, altering digestion incubation time, etc.

1.3.3 Primer design and amplification

The design of species-specific primers for use in metabarcoding assays requires genetic information on the target taxa, as well as non-target closely related or co-occurring taxa (Alberdi et al., 2018). Accordingly, some of the most commonly targeted mitochondrial regions are, 12S, 16S, cytochrome b and cytochrome oxidase subunit 1 which have high coverage of a broad range of taxa in genetic databases (Tsuji et al., 2019). Primers should be designed in-silico with minimal mismatches to

target taxa (preferably none), whilst incorporating as many mismatches to non-target taxa as possible (Rees et al., 2014). Furthermore, primers can incorporate probes (TaqMan) to enhance sensitivity, but this requires a third region specific to the species of interest (Taberlet et al., 2018). DNA is then amplified through either PCR, qPCR, or digital-droplet PCR (ddPCR), although PCR is not recommended due to associated contamination risks (Tsuji et al., 2019). Both qPCR and ddPCR offer high specificity, sensitivity, and quantification ability, but ddPCR has a higher tolerance to PCR inhibitors and does not need a standard curve for absolute quantification (Nathan et al., 2014). However, the costs associated with ddPCR instruments and consumables have led to qPCR being the mainstream approach (Taberlet et al., 2018; Tsuji et al., 2019).

Designing metabarcoding primers also requires specificity to a particular group while excluding other taxa, but frequently requires the use of degenerate bases (ambiguous bases that allow multiple nucleotides at a particular position) to increase variability for the amplification of entire taxa groups (Elbrecht & Leese, 2016; Riaz et al., 2011). Metabarcoding primers commonly used in coastal and marine environments include MiFish-U (Miya et al., 2015), 12S-V5-a (Riaz et al., 2011), and Teleo (Valentini et al., 2016), with amplification typically achieved using qPCR. One of the key distinctions between species-specific and metabarcoding samples lies in the sequencing requirement of the latter. For metabarcoding samples, molecular identification (MID) tags are essential to differentiate and subsequently sequence each sample in a pool of samples (Bohmann et al., 2011; Schnell et al., 2015). As sequencing is often not the end-point for species-specific approaches (rather qPCR; see Figure 1-2), this is not an essential requirement. MID tags can be added to amplicons/each sample in three ways: *1*) two-step PCR using target taxa primers and sequencing adapter tails in the first round, and ligating sequencing adapters and MID tags in the second round; *2*) single-step PCR using target taxa primers, and subsequently ligating sequencing adapters and MID tags; and *3*) single-step PCR using fusion tagged primers with pre-ligated MID tags and sequencing adapters (Taberlet et al., 2018). The choice of MID tagging method largely depends on the sequencing platform and the available resources (Taberlet et al., 2018).

1.3.4 Taxonomic assignment and reference databases

In eDNA studies, the taxonomic identification of sequences typically involves comparing unknown eDNA sequences to reference genetic databases and assigning taxa based on percentage similarity. This can be done manually (e.g., using software like Geneious) or through the use of bioinformatic pipelines (e.g. eDNAflow; Mousavi-Derazmahalleh et al., 2021). The accuracy and confidence of these taxonomic assignments heavily relies on the completeness of reference databases for the chosen primer gene region and targeted taxon or taxa group (Deagle et al., 2014; Weigand et al., 2019). Therefore, when designing primers for species-specific or metabarcoding studies, it is crucial to consider the availability and quality of sequences in reference databases. This includes not only sequences for the target taxon or taxa groups, but also for non-target species that may co-occur or are closely related. By ensuring the comprehensiveness of reference databases, we can enhance the accuracy of taxonomic identification of eDNA sequences from samples, leading to more reliable and interpretable study results. Likewise, when assigning eDNA sequences to taxa, reference databases should be checked for potentially absent closely related or genetically similar species. Furthermore, assignments should always be queried against known distributions and habitat characteristics of the assigned taxa. This meticulous approach ensures that taxonomic assignments are based on robust data, and minimizes the risk of misidentification or erroneous conclusions in eDNA studies. Despite global initiatives (such as the international Barcode of Life, Weigand et al., 2019), existing reference databases are still incomplete, and certain gene fragments may be more suitable than others for metabarcoding work depending on the taxonomic group studied (Schenekar et al., 2020). However, efforts to build and expand global reference databases (e.g., International Nucleotide Sequence Database Collaboration (INSDC), Australia's National Biodiversity DNA Library (NBDL), or Germany's Mare-MAGE), coupled with the decreasing cost of sequencing, are anticipated to reduce the gap in reference databases and enhance the accuracy of taxonomic identifications in eDNA studies in the future (Taberlet et al., 2018).

1.4 Ecological and biogeographic research gaps

Many species and ecosystems lack established baselines, mainly due to the elusive nature of certain species, or the remote and hazardous nature of the ecosystems in question. However, eDNA studies have emerged as a promising approach to establish these baselines, as they enable non-invasive monitoring of small-bodied and cryptic taxa, and ecosystem-wide monitoring of hard-to-reach environments. Despite their potential, eDNA methodologies have not been extensively tested across all environments or taxa groups, and require further refinement and standardization. For example, the Southern Ocean, characterised by its harsh and challenging environmental conditions, has only been the subject of three ecosystem-wide eDNA monitoring studies, all of which were limited to a single location (Mariani et al., 2019) or relatively short transects (Cowart et al., 2018; Suter et al., 2021). Similarly, despite the deep-sea being widely recognized as both underexplored and threatened, there has been a notable lack of studies utilizing eDNA to investigate biodiversity in renowned biodiversity hotspots such as submarine canyons. Lastly, while eDNA is recognized for improving detection rates of rare and cryptic species, the number of studies that have identified Syngnathid taxa (pipefish, pipehorses, seahorses and seadragons), which are primarily data-deficient or vulnerable small-bodied cryptic taxa, remains scarce (Cole et al., 2022; Jensen et al., 2022), and no surveys have specifically targeted this group. Addressing these knowledge gaps and standardizing methodologies is critical to fully harnessing the potential of eDNA, hence further testing and refinement of eDNA approaches is the overall focus of the body of research presented in this thesis.

1.5 Thesis structure

This thesis addresses the overarching question: 'How can environmental DNA (eDNA) address ecological and biogeographic research gaps and generate baseline data for threatened ecosystems and species lacking this information?' The primary objective of my thesis is to establish baselines and explore how environmental DNA (eDNA) surveys can be applied to assess gradients of response, with a specific emphasis on 'small and scarce' and 'broad and unexplored' taxa and ecosystems (Figure 1-3). This central question and objective will be addressed in studies conducted across a

diverse range of coastal and marine ecosystems that vary greatly in scale, from small estuaries and rivers to submarine canyons and open-ocean transects. By generating data and baselines for vulnerable taxa and ecosystems, this thesis contributes to the critical need for careful management and monitoring of coastal and marine biodiversity in the current conservation context.

In Chapters 2 and 3 of my thesis, I focus on the development and refinement of eDNA methodologies for small-bodied cryptic taxa ('small and scarce'), particularly Syngnathidae taxa. In Chapter 2 of my thesis, I critically evaluate the ability of two commonly used fish metabarcoding assays (MiFish and 16S Fish) to detect Syngnathid taxa (seahorses, pipefish, seadragons and pipehorses) in Western Australia. I aim to determine whether these taxa are inadvertently omitted by current broad-spectrum fish assays. Additionally, I develop two new metabarcoding assays that are designed to detect Syngnathidae in environmental samples, in the context of other fish assemblages. To enhance the accuracy of taxonomic assignment across a wide range of bony fish taxa, I create a custom 16S rRNA fish database using specimens collected from the coastline of Western Australia. This research aims to contribute to the development of non-invasive methods for monitoring and managing cryptic Syngnathidae species, which may be crucial for their survival in the face of growing anthropogenic pressures and climate change. This chapter is published in the peer-reviewed journal *environmental DNA* (Nester et al., 2020) and is formatted accordingly.

In Chapter 3 of my thesis, I further explore the value of eDNA approaches for detecting cryptic small-bodied taxa, by developing and optimizing an eDNA protocol for the critically endangered estuarine pipefish (*Syngnathus watermeyeri*). Mapping the distribution of species in coastal and marine environments can be challenging, especially when dealing with low-abundance species like *S. watermeyeri*, which is estimated to have a population size of only 100 – 250 adult individuals. In this study, I developed a species-specific qPCR assay and compared eDNA obtained from replicate water samples to conventional seine netting conducted across the historical range of *S. watermeyeri* in the Eastern Cape of South Africa. The goal of this chapter

is to refine the eDNA protocol for critically endangered species in turbid environments, as well as to facilitate the development of a long-term monitoring program and identify priority conservation areas. This chapter is published in the peer-reviewed journal *environmental DNA* (Nester et al., 2023) and is formatted accordingly.

To expand and validate eDNA approaches in a broader context ('broad and unexplored'), patterns in Animalia biodiversity across a latitudinal gradient in the Southern Ocean are explored in Chapter 4. This chapter comprises one of the longest eDNA transects to date, spanning over 3000 nautical miles in a journey from Hobart (Tasmania) to Davis Station (Antarctica). eDNA metabarcoding was performed using a broad-spectrum COI assay (Leray et al., 2013), and the results are compared to a continuous plankton recorder (CPR) survey conducted simultaneously. The goal of this research was to establish and validate an approach for large-scale eDNA biomonitoring in the open ocean. To achieve this, I test two combinations of volume and pore sizes and compare the results to the CPR data. Through this comparison, I highlight the strengths and limitations of each approach, and emphasize the risks associated with solely relying on a singular monitoring approach for long-term biodiversity datasets. This chapter is formatted for publication in the peer-reviewed journal *Science of the Total Environment*.

Lastly, Chapter 5 explores the biodiversity in uncharacterised deep-sea ecosystems using eDNA. The Cape Range and Cloates Canyons are two large submarine canyons on the north-western margin of Australia, reaching depths of 4900 m and 4400 m respectively (Post et al., 2022). The canyons are recognised as key ecological features within the Gascoyne Marine Park (Falkner et al., 2009), but to date remain biologically unexplored. An eDNA metabarcoding survey was conducted across these canyons using the Leray COI and 16S Fish assays, in parallel with the first biological survey of the canyons. This allowed for comparisons between eDNA and morphological surveys, as well as the creation of a custom reference database to enhance the accuracy of taxonomic assignment. The goal of this chapter was to determine how informative eDNA can be in heterogenous but unexplored deep-sea ecosystems. This

chapter is formatted for publication in the peer-reviewed journal *Diversity and Distributions.*

The discussion (Chapter 6) summarises and critiques the findings from the data chapters, placing them into a broader context for ecological applications of eDNA and conservation management. This chapter also explores the future of coastal and marine eDNA research, providing insights into the development of the approach for biomonitoring. The data chapters within this thesis are written and formatted as stand-alone journal articles. As a result, there is repetition throughout, particularly in the methodologies sections.

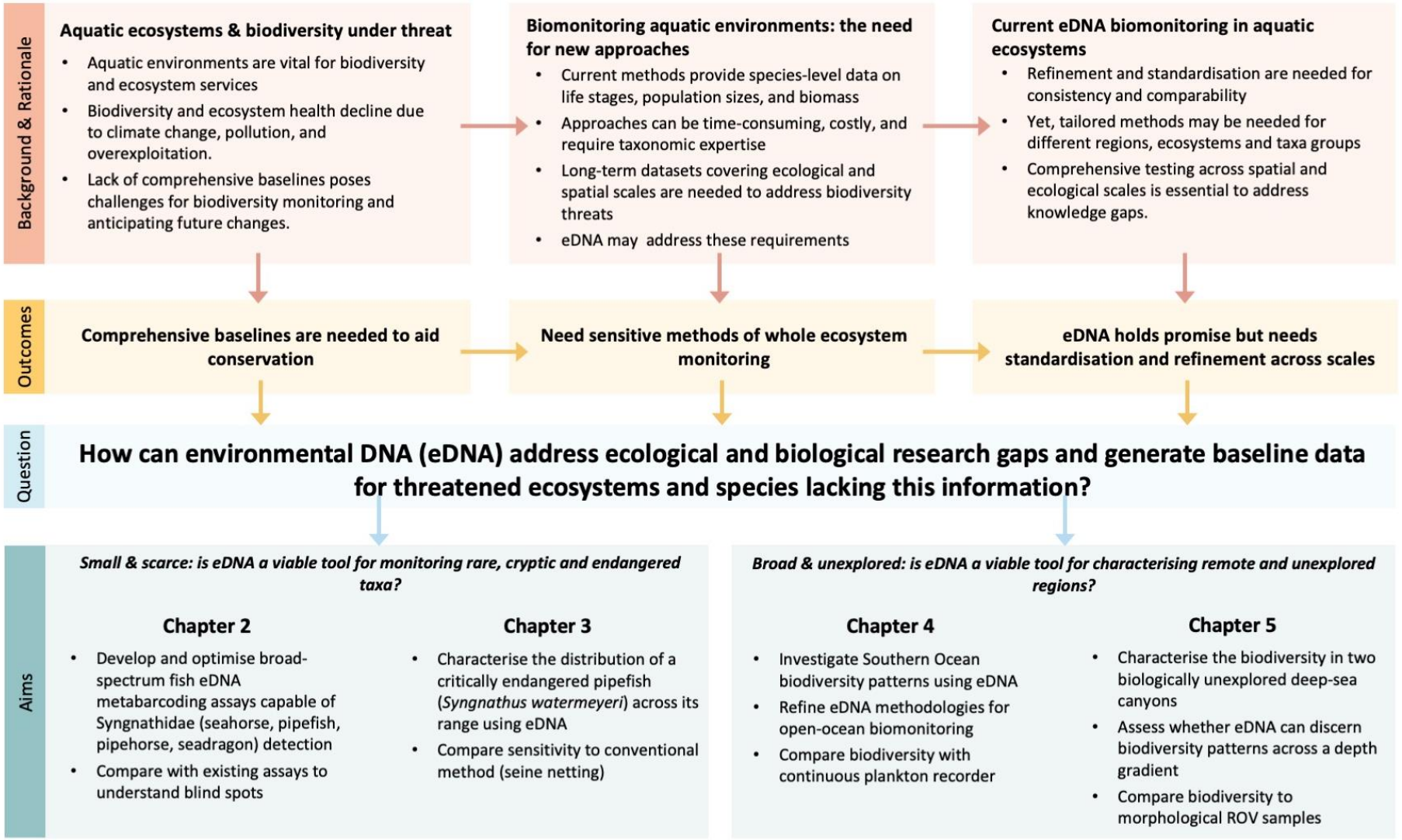


Figure 1-3: Thesis conceptual flow diagram outlining the background and rationale, key research question, and specific aims (chapters).

1.6 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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CHAPTER 2

Development and evaluation of fish eDNA metabarcoding assays facilitates the detection of cryptic seahorse taxa (family: Syngnathidae)

2.1 Prologue

Chapter 1 highlighted the strength of eDNA metabarcoding for characterizing species assemblages of whole ecological communities. The utility of eDNA metabarcoding is vast, and its ability to detect rare and threatened or invasive species in coastal and marine ecosystems has been well demonstrated (Bylemans et al., 2016; Klymus et al., 2017; Ma et al., 2016). However, at present, the majority of these investigations have focused on large charismatic fish species such as sawfish (Sani et al., 2021) and sharks (Bakker et al., 2017). Smaller, more cryptic fish species present a greater challenge due to their small relative biomass, and the high dynamism associated with most coastal and marine environments. At the time of writing, the reporting of Syngnathidae taxa via eDNA metabarcoding is rare (Thomsen et al., 2012), and to my knowledge, no one has reported the detection of a seahorse. The following chapter aims to generate a set of eDNA metabarcoding assays capable of detecting Syngnathidae taxa in the presence of other fish assemblages in the Perth metropolitan region (Western Australia).

The Western Australian (WA) coast is long and exposed, spanning more than 12 000 km (Phelps et al., 2018). Bound by the Indian Ocean, it is home to some of the world's most remarkable ecosystems and marine wildlife (Department of Biodiversity, Conservation and Attractions, n.d.). The coastal waters of the region are adorned with submerged limestone reefs, and local maritime history tells of the many vessels that met their demise in these perilous waters (Pearce, 1991). The WA coast has unique oceanographic conditions (Figure 2-1), with a combination of large-scale downwelling processes and small-scale seasonal coastal upwelling influencing the ecosystems and biodiversity present in the region (Garcia-Corral et al., 2021). The Leeuwin Current off WA is a unique anomaly among eastern boundary currents, being the only poleward-flowing current in the southern hemisphere (Cresswell & Golding, 1980). It differs from other eastern boundary currents by transporting warm, low-salinity water southward along the coast, leading to the absence of strong upwelling and nutrient-rich water in the region (Cresswell & Golding, 1980; Waite et al., 2007). This warm current facilitates the southward transport of planktonic larvae of many tropical species, enabling the unusual presence of corals and fish at these

temperate latitudes where they would not typically be found (Feng et al., 2009; Richards et al., 2016; Thomson & Frisch, 2010).

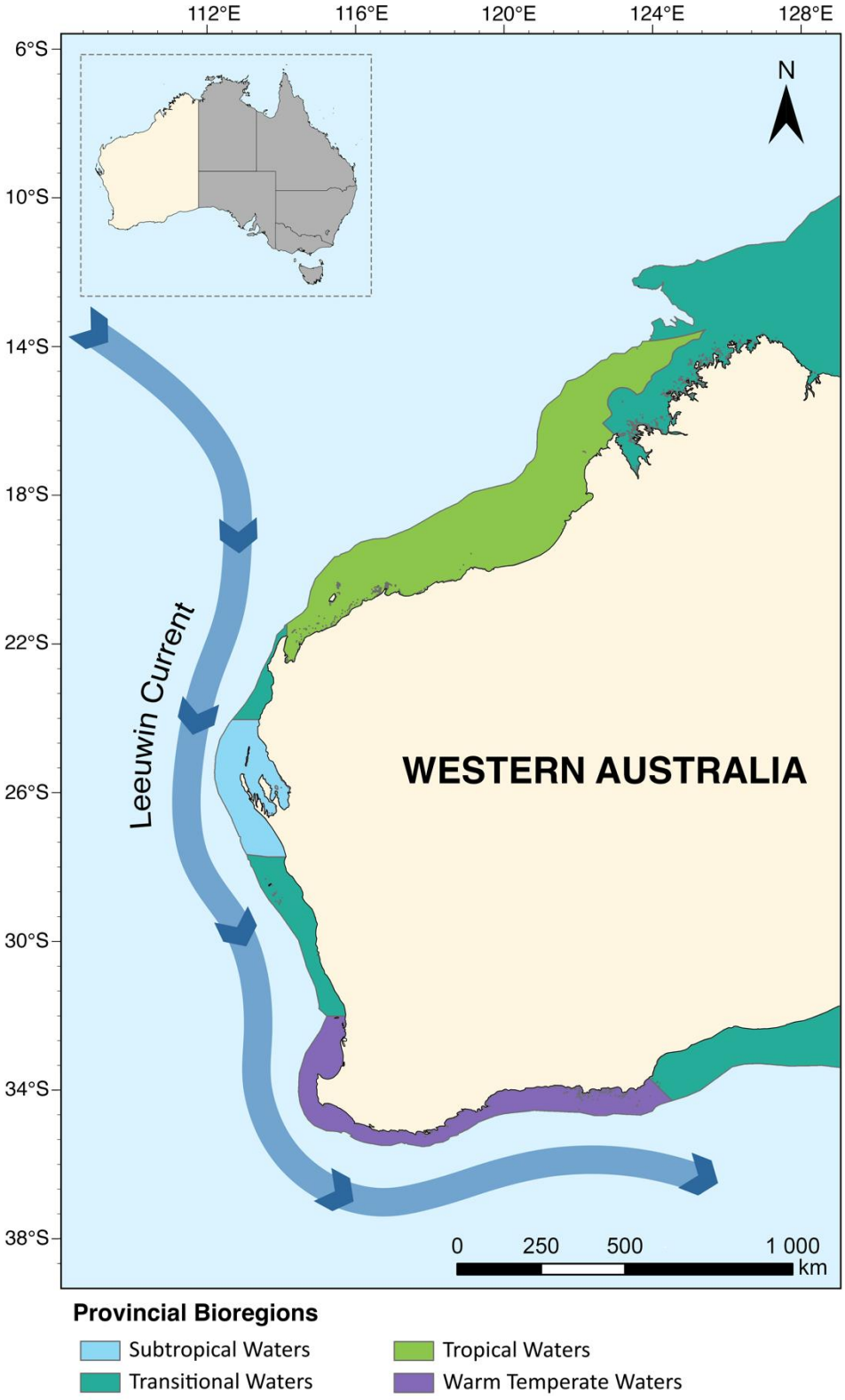

Figure 2-1: Provincial Bioregions for the Western Australian (WA) Coast.
Western Australian coast with the location of WA in relation to Australia indicated in yellow in the inset. The Leeuwin Current is represented by the blue line with arrows. Map data source: Department of the Environment, 1995.

The coastal and marine habitats of Western Australia exhibit high biodiversity and endemism, which can be attributed to the combined influences of the Leeuwin Current and the prolonged geographical isolation of Australia from other landmasses (Butler et al., 2010; Kendrick et al., 2009; Veevers, 1991). The Perth metropolitan region, situated in a transitional zone between subtropical and warm-temperate coastal and marine habitats, serves as a unique ecosystem (Richards et al., 2016). The region is known for its distinct biotic assemblages, supporting a variety of commercially and recreationally important species, including the Western rock lobster (*Panulirus cygnus*), and several prawn and snapper species. Moreover, it also supports several species of Syngnathidae (seahorses, pipefish, pipehorses, and seadragons), a flagship group for conservation and ecotourism. Australia is home to 128 species of Syngnathidae in 40 genera, 65 of which are found in Western Australian waters (Bray, 2019), including several iconic species such as the endemic Ruby (*Phyllopteryx dewysea*) and Leafy Seadragons (*Phyllopteryx eques*). The following chapter aims to develop an eDNA metabarcoding toolkit for the detection of Syngnathidae taxa in the context of other fish assemblages.

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2.1.2 Data accessibility

This chapter resulted in a manuscript that was published in the peer reviewed journal *environmental DNA* (Nester et al., 2020, DOI: https://doi.org/10.1002/edn3.93). As the first author, permission is automatically granted to reproduce this copyrighted material if it constitutes less than half of the total material in said publication. This chapter is a reproduction of the aforementioned manuscript.

The demultiplexed metabarcoding sequencing data generated from this study is available from https://doi.org/10.1002/edn3.93. The Western Australian 16S rRNA fish database sequences are available on NCBI under the accession MN473514 to MN473874.

2.2 Abstract

Environmental DNA (eDNA) metabarcoding methods have demonstrated their potential as non-invasive techniques for the monitoring and conservation of marine fishes, including rare and endangered taxa. However, the majority of these investigations have focused on large-bodied taxa such as sharks and sturgeons. In contrast, eDNA studies on small-bodied cryptic taxa are much less common. As a case in point, seahorses (members of the Syngnathidae family), have never been detected by eDNA, despite the fact that globally there are 14 species classified as "Threatened" by the IUCN. Here we critically evaluate the ability of two existing broad-spectrum fish metabarcoding assays (MiFish and 16S Fish) and explore the efficacy of two newly designed fish metabarcoding assays (16S_FishSyn_Short and 16S_FishSyn_Long) to detect Syngnathidae amidst a wide spectrum of fish species. Furthermore, a custom Western Australian 16S rRNA fish database was created to increase the likelihood of correct taxonomic assignments. With the newly designed assays, we detected four Syngnathidae species in a targeted eDNA survey of the Perth metropolitan area (Western Australia). These detections include the seahorse species *Hippocampus subelongatus* and *Hippocampus breviceps*, which represents the first time seahorse species have been detected using eDNA. The existing MiFish and 16S Fish assays did not detect any Syngnathidae. This evaluation of all four fish metabarcoding assays reinforces the view that every PCR assay has 'blind-spots'. In the context of complex environmental samples, no assay is universal and false negatives will occur due to a combination of PCR efficacy, primer binding, assay sensitivity, degeneracies in the primers, template competition and amplicon length. Taken together, these data indicate that eDNA methodologies, with ongoing optimisations, will become an integral part of monitoring small-bodied cryptic taxa such as seahorses, gobies and blennies and can assist in mapping species' distributions and prioritising conservation areas.

2.3 Introduction

Biodiversity loss is a major societal and economic concern (Cardinale et al., 2012), with increasing anthropogenic pressures and climate change resulting in a continuous decline of global biodiversity and ecosystem health (Butchart et al.,

2010). To detect changes in ecological communities, monitoring programs need to be sensitive and provide accurate data on species presence/absence and their distribution (Baker et al., 2016). This data is often difficult to obtain in aquatic environments using traditional methods due to the reduced accessibility or visibility of the environment, the occurrence of rare and cryptic species (Jerde, Mahon, Chadderton, & Lodge, 2011), limited taxonomic expertise (Hopkins & Freckleton, 2002) and funding limitations (Lundquist & Granek, 2005). Rare aquatic species are, by nature, difficult to monitor and study in their marine or estuarine environments (Pikitch, 2018). For these taxa, knowledge regarding species richness at a particular location, range distribution and population size is often incomplete or "data deficient" (Niemiller et al., 2017). Rapid advances in DNA sequencing technologies offer the opportunity to generate high quality biodiversity data with increased sensitivity (Bourlat et al., 2013; Thomsen et al., 2012), rapidly advancing the current scope of aquatic ecosystem monitoring (Bush et al., 2019).

One approach that is transforming the way aquatic ecosystems are monitored is through environmental DNA (eDNA, i.e., genetic material derived from whole microscopic organisms or shed from multicellular organisms (DiBattista et al., 2017; Taberlet, Coissac, Hajibabaei, & Rieseberg, 2012)), which can be obtained from environmental samples, such as water, sediment, soil etc and used to pinpoint species distributions. An extension of this approach, is eDNA metabarcoding, which specifically refers to the simultaneous detection of multiple species through the design and application of taxonomically broad PCR-based assays. When applied to environmental samples, it can characterise species assemblages of whole ecological communities (Deiner et al., 2017; Klitgaard Hansen, Bekkevold, Clausen, & Nielsen, 2018; Taberlet, Bonin, Zinger, & Coissac, 2018). The utility of eDNA is vast, with research demonstrating increased taxonomic resolution (Lim et al., 2016; Sigsgaard, Carl, Møller, & Thomsen, 2015; Valentini et al., 2016) and greater sensitivity in the detection of rare and invasive species (Dejean et al., 2012; Jerde et al., 2011; Piaggio et al., 2014) compared to traditional monitoring methods. Furthermore, by avoiding the need for visual observation, capture and direct sampling (Goldberg et al., 2016), eDNA surveys frequently overcome some of the cost, time, bias and at times invasive

nature associated with traditional monitoring methods (Jeunen et al., 2019; Thomsen & Willerslev, 2015).

eDNA metabarcoding surveys have been applied to a wide range of aquatic ecosystems including rivers (Bylemans, Gleeson, Hardy, & Furlan, 2018; Bylemans, Gleeson, Lintermans, et al., 2018); lakes (Fujii et al., 2019); open ocean (Truelove, Andruszkiewicz, & Block, 2019); coastal (Andruszkiewicz et al., 2017; Koziol et al., 2019) and reefs (DiBattista et al., 2017, West et al. 2020) primarily using broad-spectrum fish metabarcoding assays 16S Fish (Berry et al., 2017; Deagle et al., 2007) and MiFish-U (Miya et al., 2015), which respectively target the 16S and 12S rDNA regions of the mitochondrial genome. Despite the frequent use of 16S Fish and MiFish-U as broad-spectrum fish metabarcoding assays, there has been no critical comparison of the taxon range and sensitivities of these assays on a set of diverse environmental samples. These comparative studies are needed when planning an eDNA experimental design to understand which metabarcoding assays are most suitable. Furthermore, no studies using MiFish-U or 16S Fish have reported the detection of Syngnathidae taxa (pipefishes, seahorses and seadragons) as of present.

The Syngnathidae family is large, with almost 300 marine, brackish and freshwater species distributed globally (Wilson & Orr, 2011). Syngnathidae are extremely vulnerable to human impact and population declines due to life history traits such as low fecundity, restricted distributions, high rates of endemism and limited mobility (Foster & Vincent, 2004; Shokri, Gladstone, & Jelbart, 2009). Many Syngnathidae species are considered threatened (7.2%; IUCN, 2019) with population declines attributed to exploitation for the aquarium trade, Traditional Chinese Medicines (TCM), habitat degradation, and as bycatch in commercial trawl fisheries (Lourie, 2000; Luo, Qu, Li, Wang, & Lin, 2015; Martin-Smith & Vincent, 2006). However, many more species (over 30%) lack the data necessary to assess their extinction risk (IUCN, 2019). With the risk of a ‘silent extinction’ for many Syngnathidae species, the design of a non-invasive method for monitoring and managing these cryptic species may be critical to their survival.

False negatives (failure to detect a species when they are in fact present) are significant in the management of threatened species (Delaney & Leung, 2010; Ficetola et al., 2014; Furlan & Gleeson, 2017). For this reason, we aimed to determine if the Syngnathidae family are being inadvertently omitted by current broad-spectrum fish assays. Australia is home to 128 species of Syngnathidae in 40 genera, 65 of which are found in Western Australian waters (Bray, 2019). The Perth metropolitan area in Western Australia was chosen as our study site as it encompasses several habitat types, including brackish and salt water (Kendrick & Hyndes, 2003). To increase the likelihood of correct taxonomic assignment across a broad range of bony fish taxa, a custom 16S rRNA fish database was created using specimens collected along the coastline of Western Australia. The primary objective of this study was therefore to evaluate and optimise a set of broad-spectrum fish eDNA metabarcoding assays capable of detecting Syngnathidae in environmental samples in the context of other fish assemblages. In addition, we critically evaluate four fish metabarcoding assays across a set of diverse environmental samples, to better understand the strengths, weaknesses and limitations of each assay in isolation or when used in combination.

2.4 Methods

2.4.1 Western Australian 16S rRNA fish database curation

Curated databases for select gene regions enhance the utility and taxonomic range of current metabarcoding databases (Deiner et al., 2017). A custom 16S rRNA fish database was created using a combination of targeted sampling and subsamples provided and taxonomically identified by the West Australian Department of Primary Industries and Regional Development (DPIRD) for target and bycatch species (Table S 2-1). Tissue samples from 303 vouchered fish species were removed from storage buffer (20% salt-saturated DMSO or 95% ethanol) and dried before subsampling with a target weight of 20 mg. Extraction of DNA from the subsampled tissues was automated using the QIAcube extraction platform (Qiagen) and the DNeasy Blood and Tissue Kit (Qiagen) with modified protocols. DNA extracts were quantified using the NanoDrop™ 2000 spectrophotometer (Thermo Fisher, USA). Samples were

pooled (utilising DNA quantification results) for sequencing by taxonomic family, with no species from identical families within the same sequencing pool. This reduced the chances of taxonomic ambiguity between closely related species during bioinformatic analysis due to sequencing error. Metabarcoding and next-generation sequencing (NGS) was performed as below and a reference haplotype for each species was determined. These sequences were deposited into GenBank under the accession MN473514 to MN473874.

Primer design and validation

Several PCR metabarcoding assays were designed to target the 16S rRNA region of the mitochondrial genome of Syngnathidae species and species of fish typically detected using the 16S Fish assay. Publicly available (NCBI) Syngnathidae and other fish 16S reference sequences were aligned using Geneious v. 10.2.6 to identify short conserved regions capable of amplifying degraded DNA commonly encountered in environmental samples (Table S 2-2). Assays were designed based on guidelines specified in a previous study (Bustin & Huggett, 2017). In brief, the assays were free from secondary structures, had balanced GC content and similar annealing temperatures on forward and reverse primers.

To determine efficacy, these newly developed assays and MiFish-U and 16S Fish were tested in silico (Figure S 2-1) and in vitro through quantitative PCR (qPCR) of tissue and environmental samples. Reactions were performed using neat and a three point ten-fold dilution series of single-source H. subelongatus tissue (obtained from De Brauwer et al., 2019). The assays were further tested on aquarium water that held H. subelongatus, among other species, from the Aquarium of Western Australia to determine their ability to detect Syngnathidae taxa in controlled environmental samples. Tissue and filtered aquarium water were extracted and amplified as described below. Two optimal assay sets (termed 16S_FishSyn_Short and 16S_FishSyn_Long) were selected for further testing based on their reliability to amplify Syngnathidae mtDNA in tissue and aquarium samples. These assays were used, in conjunction with the 16S Fish and MiFish-U assays, throughout the remainder of the study (Table 2-1)

Table 2-1: Existing fish eDNA metabarcoding assays (MiFish-U and 16S Fish) and newly designed metabarcoding assays (16S_FishSyn_Short and 16S_FishSyn_Long) used throughout this study.

Assays	Gene region	Amplicon length (bp)	Primer sequence (5'>3')	GC content (%)	Annealing temperature (°C)	Reference
16S Fish forward	16S	~200	GACCCTATGGAGCTTTAGAC	45	54	Berry et al. 2017
16S Fish reverse			CGCTGTTATCCCTADRGTAACT	50		Deagle et al. 2007
MiFish-U forward	12S	~170	GTCGGTAAAACTCGTGCCAGC	57.1	60	Miya et al. 2015
MiFish-U reverse			CATAGTGGGGTATCTAATCCCAGTTTG	44.4		
16S_FishSyn_Short forward	16S	~80	GACGAGAAGACCCTGTGGAGC	61.9	55	This study
16S_FishSyn_Short reverse			CCGYGGTCGCCCCAAC	80		
16S_FishSyn_Long forward	16S	~200	GACGAGAAGACCCTDTGGAG	57.9	55	This study
16S_FishSyn_Long reverse			GRATTGCGNTGTTATCCCT	47.1		

2.4.2 Site description & sample collection

Sampling was conducted over a week period in May 2018, in Perth, Western Australia (Table S 2-3). The south-west region of Western Australia is known for its unique fish assemblages and high rates of endemism (Melville-Smit, Larsen, de Graaf, & Lawrence, 2010; Richards et al., 2016). The temperate, coastal waters of the Perth metropolitan region were chosen for this study as several Syngnathidae species inhabit this area; including *H. subelongatus*, *Stigmatopora argus* and *Filicampus tigris* (Kendrick & Hyndes, 2003). Samples were collected from five sites; (i) Bicton Baths, (ii) Blackwall Reach, (iii) Ammo Jetty, (iv) Rockingham Wreck Trail, (v) and Mt Henry Bridge (Figure 2-2). These specific sites were chosen as Syngnathidae presence was confirmed through recent recreational diver observations.

Five replicate surface (0 – 2 m deep) and bottom water (7 – 12 m deep) samples were collected via SCUBA and snorkelling at each site (n = 50) as a means of empirically evaluating the effects of depth selection on the detection rates of each assay. Water samples (1 L per replicate) were collected using sterile Nalgene bottles that were opened underwater at the sampling site and then immediately closed following sampling. Water samples were filtered using a Sentino peristaltic pump onto 47 mm filter membranes (Pall Life Sciences) within four hours of sampling to minimise DNA degradation. A 0.22 µm pore size was used for ocean sites (Ammo Jetty and Rockingham Wreck Trail) and 0.45 µm pore size for river sites due to increased turbidity in these environments (Bicton Baths, Blackwall Reach and Mt Henry). Filter membranes were frozen at -20°C until DNA extraction.

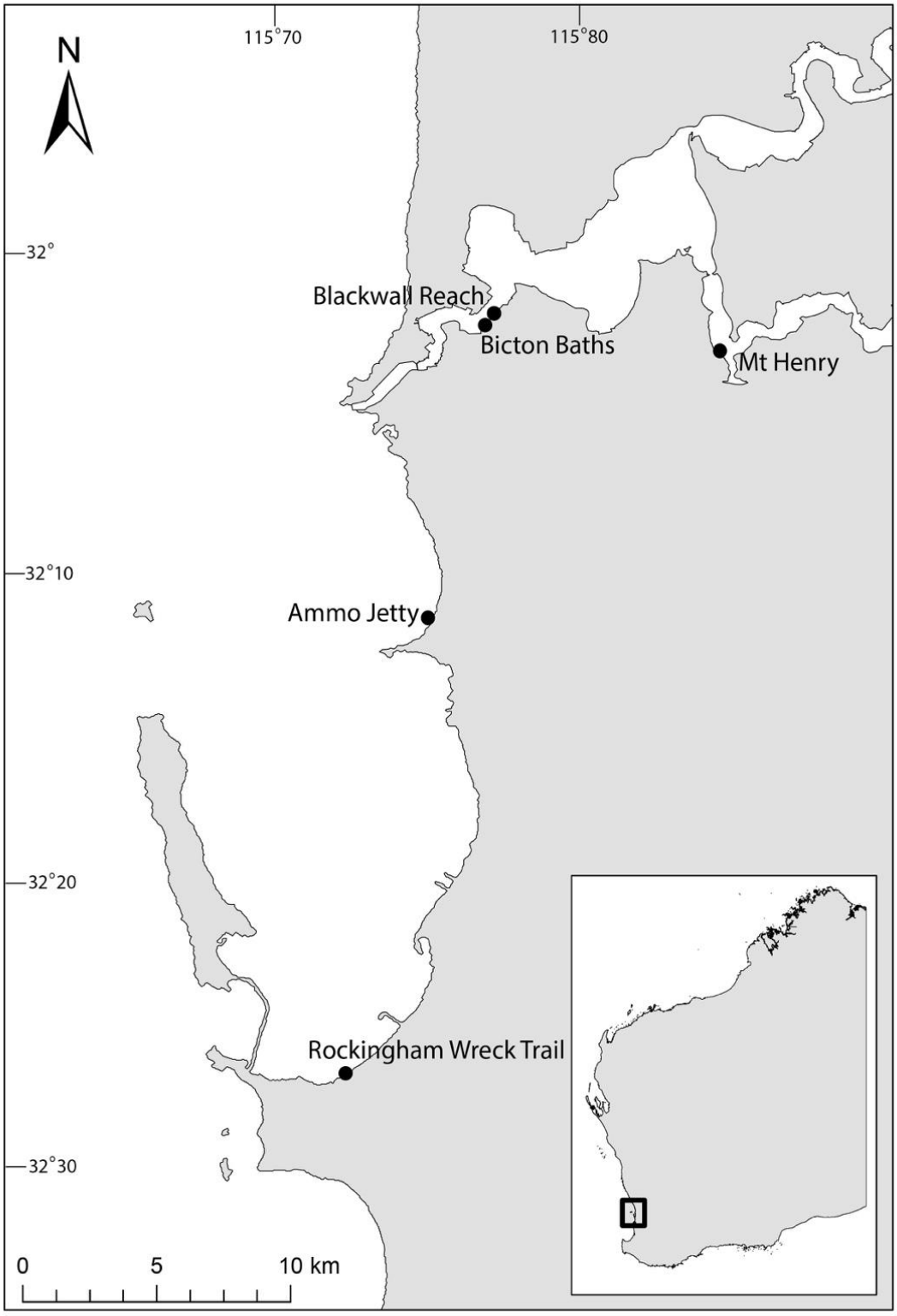

Figure 2-2: Map of the Perth metropolitan region study sites.
Perth metropolitan region with inset showing Western Australia (Perth metropolitan region indicated by the black square). Sampling sites used for this metabarcoding study are indicated by the black dots (Mt Henry, Bicton Baths, Blackwall Reach, Ammo Jetty and Rockingham Wreck Trail).

2.4.3 DNA extraction & metabarcoding

Water samples were extracted using a DNeasy Blood and Tissue kit (Qiagen) and a modified protocol (Stat et al., 2018). Extraction controls (i.e., no sample) were implemented for every site and extracted alongside the water samples. Metabarcoding was performed in duplicate on each DNA extract and control for each assay (Table 2-1). qPCR reactions (25 mL) consisted of the following: 2.5 mM/L $MgCl_2$ (Applied Biosystems, USA), 1× PCR Gold buffer (Applied Biosystems), 1 U AmpliTaq Gold DNA polymerase (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 primer (specified in Table 1), 0.6 µl of a 1:10,000 solution of SYBR Green dye (Life Technologies, USA) and 4 µl of template DNA. qPCR amplifications were performed on a StepOnePlus Real-Time PCR System (Applied Biosystems, Waltham, Massachusetts). To reduce the likelihood of index-tag switching and chimera production, multiple unique forward and reverse fusion tag combinations were added to the qPCR products, each consisting of an adapter sequence, gene specific primers, and a unique multiple identifier (MID). A 'no template' control was also included in each qPCR to detect any cross-contamination between samples (n = 16). Additionally, a positive control (Hippocampus subelongatus tissue) was used in duplicate for each primer set. Thermocycler conditions were as follows: 95°C for 5 m, 50 cycles of 95°C for 30 s, annealing temperature of 54°C (16S Fish), 55°C (16S_FishSyn_Short and 16S_FishSyn_Long) and 60°C (MiFish), completed by a 72°C elongation step. Extraction and negative controls showed no sign of amplification and were therefore excluded from downstream analyses.

Resulting amplicons were pooled in approximate equimolar ratios, size-selected using a Pippin Prep (Sage Science) and purified using a QIAquick PCR Purification Kit (Qiagen). The final library was quantified using a QIAxcel Advanced System (Qiagen) and a Qubit Fluorometric Quantitation machine (Thermo Fisher) and sequenced on an Illumina Miseq platform using a 300 cycle Miseq V2 Reagent Kit and custom

sequencing primers at Curtin University in Perth, Western Australia. Raw sequence data was deposited into GenBank under the accession SRX6841776.

2.4.4 Bioinformatics & taxonomic assignment

Sequences with 100% matches to Illumina sequencing adapters, index barcodes and template-specific primers were retained for downstream analysis using Geneious v. 10.2.6. Usearch v. 10 (Edgar, 2010) was used to quality filter and discard reads with error rates of 1%, short reads (< 50 bp) and chimeras. Resulting sequences were dereplicated into unique sequences and denoised into zero-radius operational taxonomic units (ZOTUs; denoised OTUs that aim to report correct biological sequences at a higher resolution than OTUs (Callahan, McMurdie, & Holmes, 2017; Edgar, 2016)). To remove erroneous ZOTUs caused by co-occurrence error, the LULU algorithm (Frøslev et al., 2017) was applied using R Studio v. 1.2.1335 (RStudio Team, 2015). ZOTUs were compared to a GenBank (NCBI) reference database and the custom Western Australian 16S fish database using the Basic Local Alignment Search Tool for nucleotides (BLASTn) on the Zeus system (Pawsey Supercomputing Centre, Western Australia).

ZOTUs with BLASTn parameters of E value above 10^{-5}, percentage identity below 94% and query coverage per subject below 99% were removed to decrease uncertainty surrounding ZOTU taxonomic assignment (Alberdi, Aizpurua, Gilbert, & Bohmann, 2018; Porter & Hajibabaei, 2018; Xiong & Zhan, 2018). Taxonomic identities of ZOTUs were assigned and visualised in MEGAN v6 (METaGenome ANalyzer; Huson et al., 2016) using the LCA (lowest common ancestor) parameters: min bit score 100.0 and reports restricted to the top 10% of matches. To be conservative, taxonomic assignments were further evaluated against knowledge of species distributions ("Fishes of Australia," 2019). The output from MEGAN v6 was exported to Geneious v. 10.2.6 to create the phylogram.

2.4.5 Statistical analysis

PRIMER v. 7 (Clarke & Gorley, 2015) was used to compare the effects of assay and site on taxonomic composition. Data was transformed into presence-absence format and a Jaccard resemblance matrix (Schaalje & Beus, 1997) was created. A permutational multivariate analysis of variance (PERMANOVA) with factors site (Bicton Baths, Blackwall Reach, Ammo Jetty, Rockingham Wreck Trail and Mt Henry) and assay (16S Fish, MiFish-U, 16S_FishSyn_Short and 16S_FishSyn_Long) was performed using the PERMANOVA+ add on in PRIMER v. 7 (Anderson, 2001; Anderson, 2005). All PERMANOVA tests were conducted using unrestricted permutation of raw data and 9999 permutations. In the presence of significant effects, pairwise comparisons using PERMANOVA were performed to determine where the significant differences occurred. To visualize patterns in the data, non-metric multidimensional scaling (nMDS) plots and canonical analysis of principal coordinates (CAP) plots were generated in PRIMER (Anderson, 2005) with the PERMANOVA+ add on. Using the vegan package in R v. 3.6.0 and RStudio v. 1.2.1335 (R Core Team, 2019; RStudio Team, 2015; Oksanen et al., 2018), rarefaction curves were generated to confirm adequate sequencing depth (Figure S 2-2).

2.5 Results

2.5.1 In silico and in vitro evaluation of fish metabarcoding assays

Existing fish metabarcoding assays (MiFish-U and 16S Fish) and newly developed fish metabarcoding assays (16S_FishSyn_Short and 16S_FishSyn_Long) were evaluated *in vitro* and *in silico* to infer their ability to detect Syngnathidae and more broadly fish taxa. The MiFish-U assay failed to amplify control seahorse tissue from *H. subelongatus,* whereas the 16S Fish assay returned a weak (but positive) amplification. Both existing assays detected the bulk of fish species present in the control aquarium samples, however neither detected *H. subelongatus*. *In silico* analyses, using reference 12S and 16S Syngnathidae sequences, revealed that the existing assays likely performed poorly due to primer mismatches (Figure S 2-1). 16S_FishSyn_Short and 16S_FishSyn_Long successfully amplified *H. subelongatus* from both the seahorse tissue and aquarium samples, with additional fish taxa

detected in the latter (Table S 2-4). To further evaluate the efficacy of these assays to detect fish, including Syngnathidae, a survey was conducted in the Perth metropolitan area.

2.5.2 Evaluation of fish metabarcoding assays based on seawater collected in the Perth metropolitan region

A total of 4.6 million metabarcoding reads were obtained across the four fish assays after quality filtering. All assays reached asymptote in the rarefaction analyses. Assay selection significantly altered the taxonomic composition and number of species detected (*Pseudo-$F_{(1-3)}$ = 33.865, p < 0.*001), with pairwise comparisons indicating all assays were significantly different from one another (*p < 0.001*). The differences in community structure between the assays is visualized through the CAP plot (Figure 2-3).

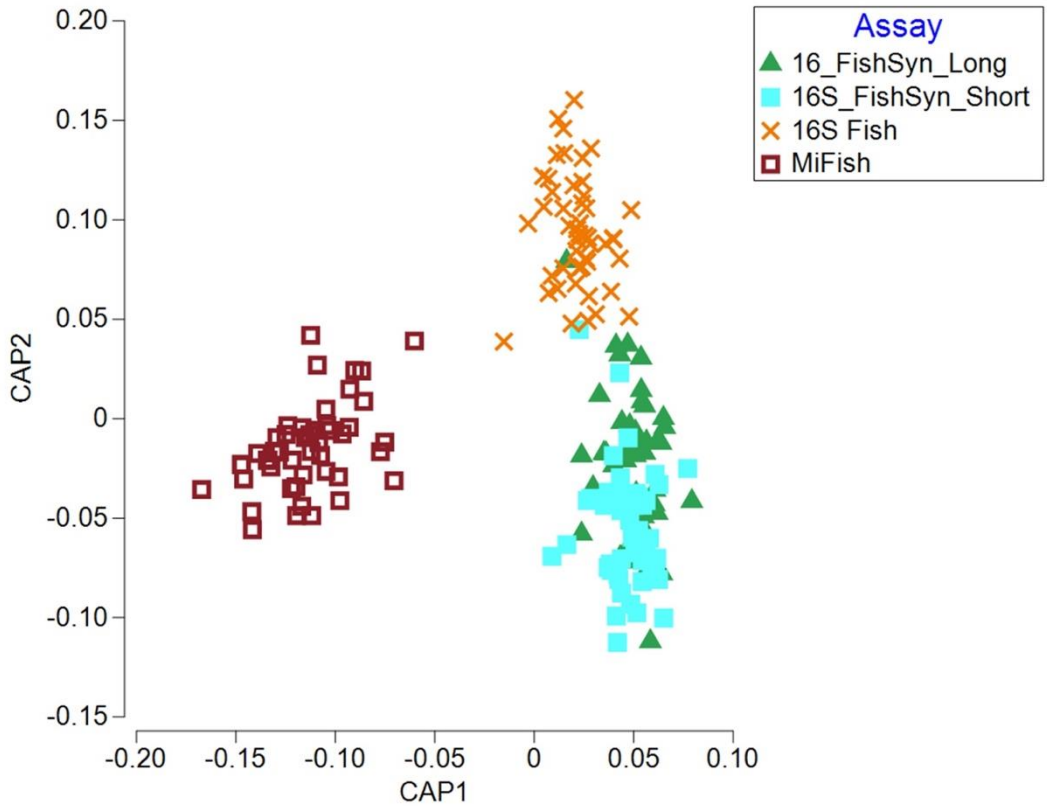

Figure 2-3: Canonical analysis of principal coordinates plot using a presence/absence transformed (Jaccard) data set of fish families detected (Perth, Western Australia).
Samples are classified by assay used with distance between samples representing similarity in biological assemblage.

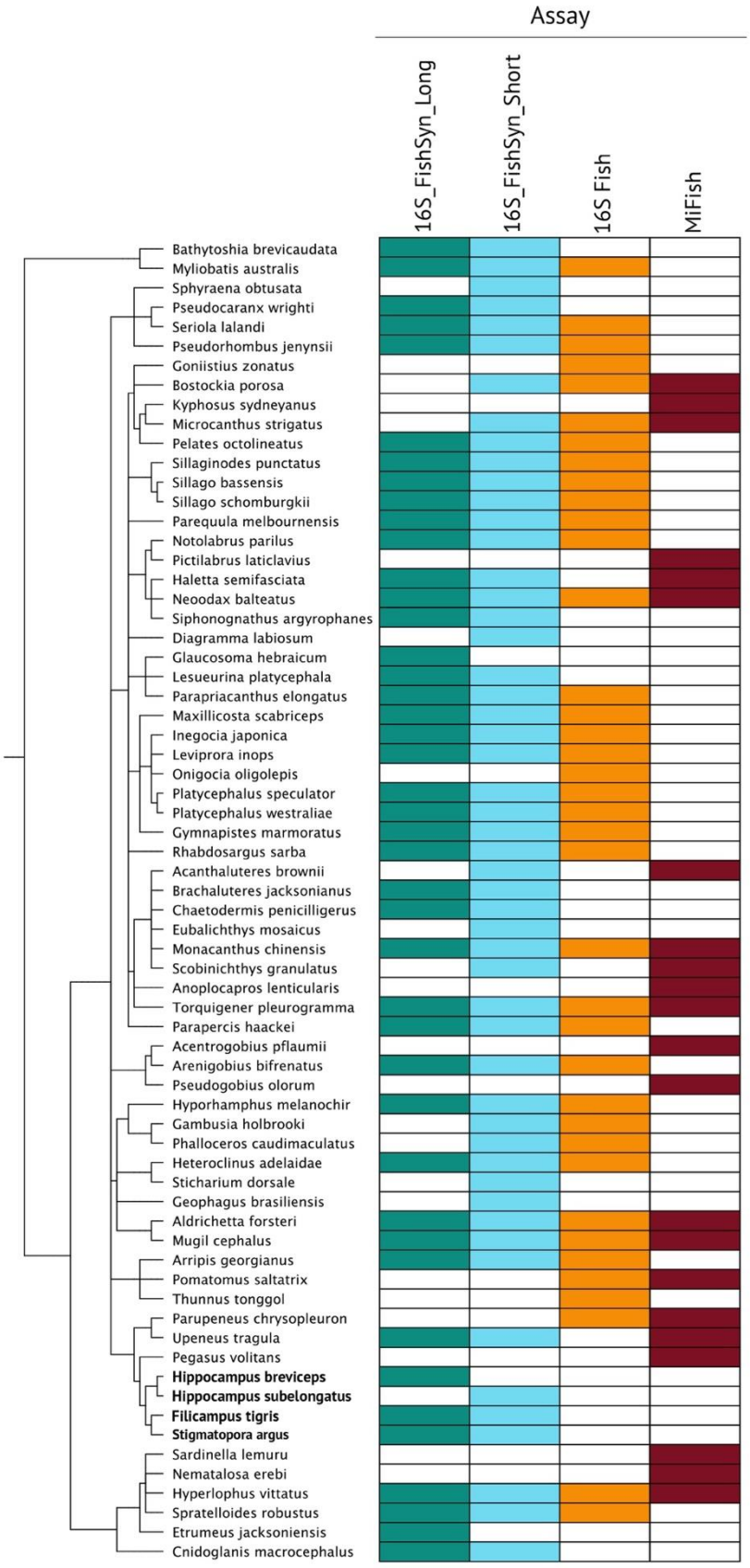

Figure 2-4: Taxonomic phylogram of total fish families detected across five locations in the Perth metropolitan region of Western Australia.

Taxonomic detections of each assay (16S_FishSyn_Long, 16S_FishSyn_Short, 16S Fish and MiFish-U) are indicated by the shading of each respective square. MEGAN v.6 and Geneious v.10.2.6 were used to create phylogenetic tree.

A total of 68 fish species were detected with the four applied assays. 16S_FishSyn_Short was the best-performing assay detecting 52 of 68 (76.5%) species, followed by 16S_FishSyn_Long with 43 of 68 (63.2%) species detections. In comparison, 16S Fish and MiFish detected 38 of 68 (55.9%) and 22 of 68 (32.4%) fish species, respectively. Relative to the best-performing assay (16S_FishSyn_Short), the combination of all four assays resulted in 23.5% more species-level detections. The taxonomic specificities and deficiencies of each assay are highlighted in Figure 2-4. Fish assemblages across each site displayed significant differences in the species that were detected (Figure 2-5; $Pseudo\text{-}F_{(1\text{-}4)} = 21.963, p < 0.001$). This difference was further validated through pairwise comparisons indicating all sites were significantly different from one another ($p < 0.001$).

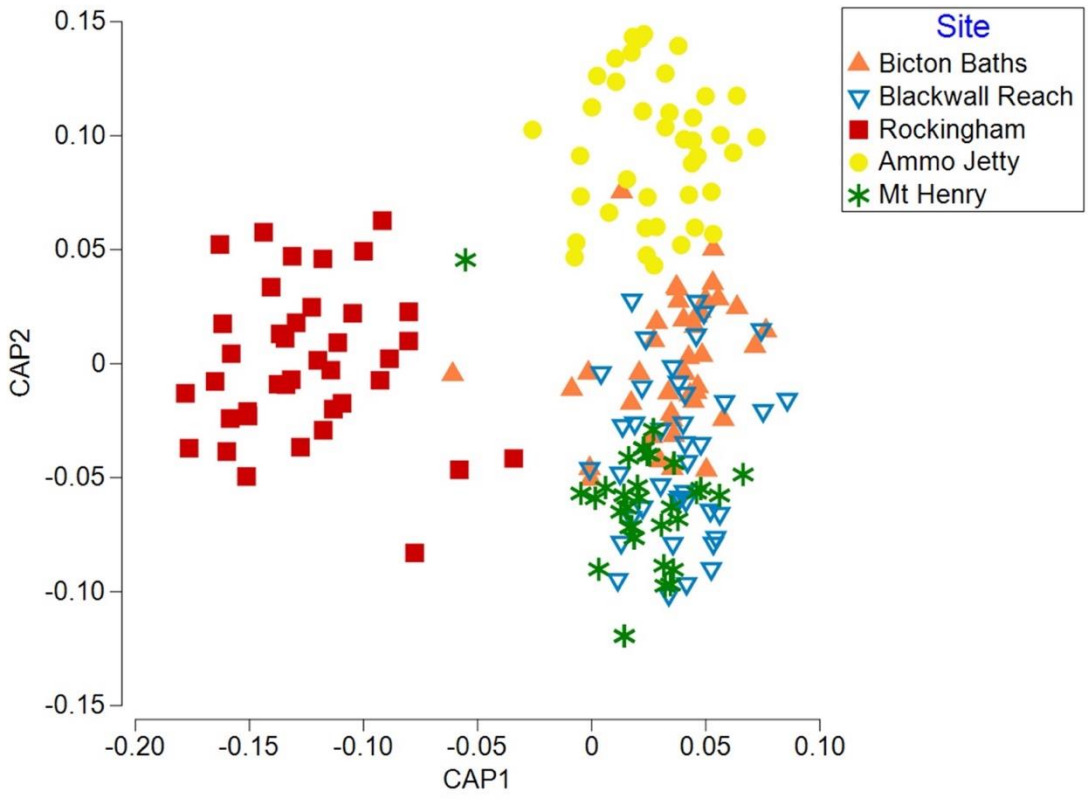

Figure 2-5: Canonical analysis of principal coordinates plot using a presence/absence transformed (Jaccard resemblance matrix) data set of fish species detected in this metabarcoding study (Perth, Western Australia).
Samples are classified by site (Bicton Baths, Blackwall Reach, Rockingham, Ammo Jetty and Mt Henry) with distance between samples representing similarity in biological assemblage. CAP plot was created using Primer 7 v. 7 using the PERMANOVA+ add on (Anderson, 2005).

Only two (16S_FishSyn_Short and 16S_FishSyn_Long) of the four applied assays successfully detected Syngnathidae species at the Perth metropolitan sites (Figure 2-6). A total of four species of Syngnathidae were detected with these newly developed metabarcoding assays: *Hippocampus subelongatus* (Rockingham Wreck Trail), *Hippocampus breviceps* (Bicton Baths), *Syngnathus argus* (Bicton Baths, Blackwall Reach and Ammo Jetty) and *Filicampus tigris* (Ammo Jetty). Both of the assays developed in this study successfully detected the two pipefish species, *Syngnathus argus* and *Filicampus tigris*. However, each assay detected a unique seahorse species with 16S_FishSyn_Short detecting *Hippocampus subelongatus* and 16S_FishSyn_Long detecting *Hippocampus breviceps.* Furthermore, the frequency of detection was low, with only 0.02% of the total quality filtered reads assigned to Syngnathidae.

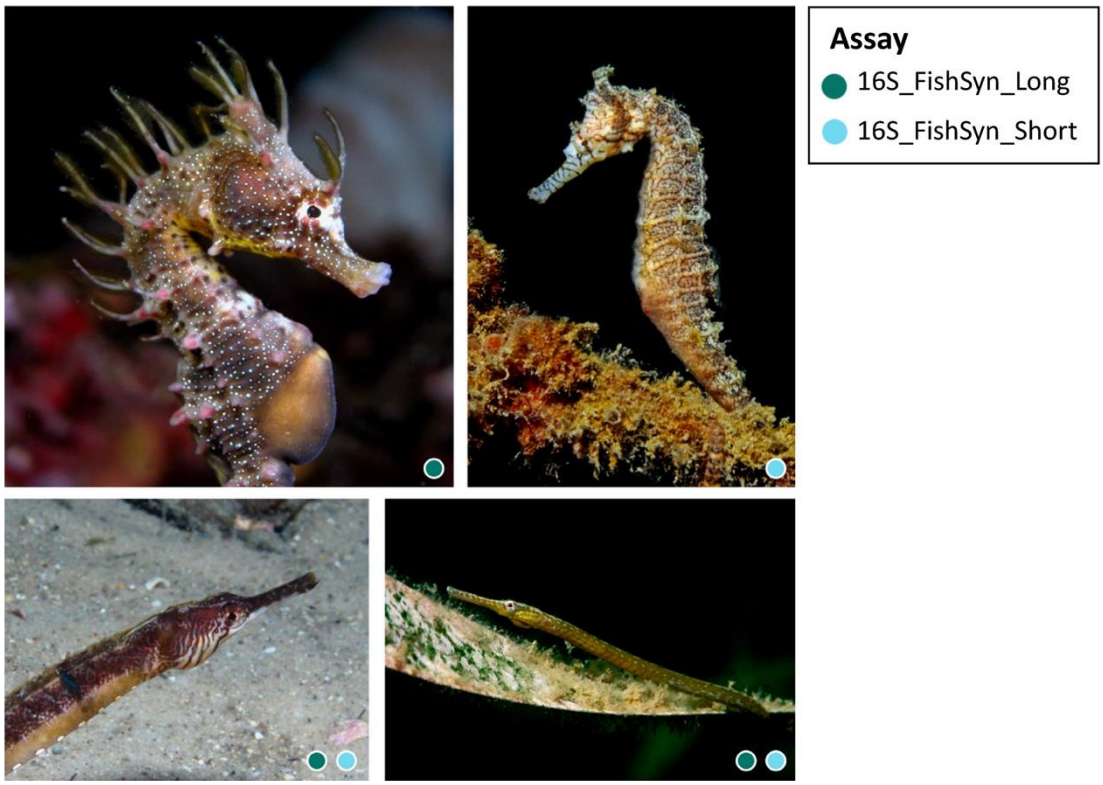

Figure 2-6: Total Syngnathidae taxa (N = 4) detected in this metabarcoding study across five locations in the Perth metropolitan region of Western Australia.
Clockwise from top left: *Hippocampus breviceps; Hippocampus subelongatus; Stigmatopora argus* and *Filicampus tigris*. Assay detections are indicated by the coloured dots detailed in the figure legend. Image credit: Dave Harasti-used with permission.

Through our empirical evaluation of the effects of depth selection on the detection rates of each assay, we detected more fish species per assay on average in bottom water (44.25 $\pm$ 0.95) than in surface water (43.25 $\pm$ 1.23; $p < 0.001$). Furthermore, bottom water detected more Syngnathidae species (n = 4) than surface water (n = 2), with seahorse species only detected in bottom water samples.

2.5.3 Performance of the Western Australian 16S rRNA fish database using seawater collected in the Perth metropolitan region

The use of the Western Australian 16S rRNA fish database provided an additional two species assignments for the 16S gene region; *Onigocia oligolepis* and *Goniistius gibbosus,* and one additional genus assignment; *Leidotrigla*. Moreover, the use of the custom database resulted in better resolution by resulting in more (N=3) species assignments for the 16S gene region. Of the 46 species that were not detected using the MiFish-U assay, 26 (56.5%) of these were due to mismatches and 20 (43.5%) were due to missing 12S rRNA sequences in the NCBI database. For the 16S Fish assay, 30 species were not detected with 28 (93.35%) of these due to mismatches and 2 (6.65%) due to missing 16S gene region sequences in the NCBI and custom fish database. The 16S_FishSyn_long assay failed to detect 25 species, of these 21 (84%) were due to mismatches and 4 (16%) were due to missing 16S gene region sequences. Of the 16 species not detected by the 16S_FishSyn_short assay, 12 (75%) of these were due to mismatches and 4 (25%) were due to missing 16S rRNA sequences.

2.6 Discussion

The metabarcoding assays MiFish-U (Miya et al., 2015) and 16S Fish (Berry et al., 2017; Deagle et al., 2007) are widely used throughout the eDNA literature as broad-spectrum fish metabarcoding assays as they target a taxonomically diverse range of fish species (DiBattista et al., 2017; Fujii et al., 2019; Miya et al., 2015; Stat et al., 2018). In this study, we demonstrated that these assays are inadvertently omitting Syngnathidae taxa in their detections due to primer binding factors. These assays may also be confounded by the low biomass of Syngnathidae relative to the other (more abundant) fish taxa. We subsequently developed two new fish metabarcoding assays

capable of Syngnathidae detection, and evaluated the taxon detections of these four assays in a varied set of environmental samples. While all aquatic environments differ in their composition, relative biomass and genetic background, we hope the taxonomic strengths and weaknesses discovered in these metabarcoding experiments might aid in assay selection and experimental design in future eDNA studies.

When considered holistically, there were clear differences in assay performance, with 16S_FishSyn_Short and 16S_FishSyn_long detecting 52 of 68 and 43 of 68 fish species respectively, while 16S Fish and MiFish detected 38 of 68 and 23 of 68 fish species respectively. The newly developed metabarcoding assays detected 18 fish species that 16S Fish and MiFish did not. Among these undetected species were the Western Australian dhufish (*Glaucosoma hebraicum*), the flathead sandfish (*Lesueurina platycephala*), the slender snake blenny (*Sticharium* dorsale) and the smooth stingray (*Bathytoshia brevicaudata*). However, for the 16S_FishSyn_Short assay, this increase in detection rate may be due to its shorter amplicon length. As eDNA is released into the environment the degradation process begins (Bista et al., 2017), with short fragments of DNA degrading slower than larger fragments (Deagle, Eveson, & Jarman, 2006). Therefore, there may be a greater probability of detection using shorter amplicon assays relative to longer ones. While there is an increase in detection rates, previous studies have shown that assays targeting longer DNA fragments will selectively detect newly released eDNA (Jo et al., 2017; Wu et al., 2019), providing a more contemporary insight to fish community assemblage.

While they did not detect the largest number of species, the MiFish-U and 16S Fish assays detected 12 species that 16S_FishSyn_Short and 16S_FishSyn_Long did not. Evidently, each assay used has its own 'blind spots' with overall taxonomic composition varying between assays. While primer mismatches (and base degeneracies) are common in PCR assays, the impact of these on taxa detections is difficult to evaluate in a set of environmental samples. By extrapolating the results, we have demonstrated that all fish metabarcoding assays will inadvertently omit a selection of the fish biota and that some groups (e.g. Syngnathidae) will be notable

false negatives. Traditionally, studies have used one universal metabarcoding assay to estimate fish biodiversity (DiBattista et al., 2017; Stat et al., 2018). However, our data reiterates the importance of a multigene approach with multiple metabarcoding assays (Stat et al., 2017) as each assay has advantages and disadvantages related to diversity and taxonomic resolution based on the availability of reference sequences (Berry et al., 2017; Deagle, Jarman, Coissac, Pompanon, & Taberlet, 2014). Furthermore, the use of multiple metabarcoding assays can provide higher confidence levels when there are multiple hits to the same organism (Stat et al., 2017).

With fish assemblages at each site displaying significant differences in the species detected, we demonstrated that eDNA surveys are ecologically informative over small spatial scales (~8.9 km average distance between sites). In accordance with other studies (West et al. 2020; Jeunen et al., 2019; Koziol et al., 2019; Murakami et al., 2019; O'Donnell et al., 2017; Stat et al., 2018), we suggest that eDNA signals dilute or degrade rapidly in aquatic ecosystems and are able to provide a contemporary snapshot of spatially distinct community assemblages. Furthermore, we identified a slight saltwater to freshwater gradient across the Perth metropolitan region and consistent with previous research, we suggest that eDNA signals are not impacted by localised oceanography (tides, currents and upwellings) as much as one might expect (Jeunen et al., 2019; O'Donnell et al., 2017).

The results of this study suggest that while the MiFish and 16S Fish assays might be capable of detecting the majority of teleost fish, they are unsuitable for detecting Syngnathidae species in complex multi-species environmental samples. Critical to the aims of this study, we successfully detected Syngnathidae species across a range of habitats in the Perth metropolitan region using the 16S_FishSyn_Short and 16S_FishSyn_Long assays. With Syngnathidae populations declining due to exploitation for the aquarium trade and habitat degradation (Luo et al., 2015; Martin-Smith & Vincent, 2006), eDNA provides a much-needed non-invasive method for monitoring threatened populations. Importantly, this study represents the first time a seahorse species has been detected using eDNA. Further optimisations (including

the development of a Syngnathidae specific assay) are clearly needed as each of our assays (16S_FishSyn_Short and 16S_FishSyn_Long) detected different seahorse species in the water samples. The heterogeneity of eDNA in the environment can introduce a stochastic effect when sequencing sample and PCR replicates in which less abundant or smaller biomass species may not be found in all replicates (Beentjes, Speksnijder, Schilthuizen, Hoogeveen, & Van Der Hoorn, 2019). While we took five sampling replicates, it has been suggested that as many as nine sample replicates are needed to obtain accurate biodiversity estimates from eDNA (Grey et al., 2018). This is further confounded by the fact that the relative biomass of seahorses to other fish biomass is likely extreme.

The frequency of detection for Syngnathidae species was low, less than 0.02% of the total reads. This indicates that the biomass of Syngnathidae DNA relative to other fish is low, possibly due to their: (i) low relative abundance, (ii) low overall biomass and/or (iii) low DNA shedding rates. It is possible that Syngnathidae species may not have been detected in some samples as their DNA concentrations were below the detection threshold of the newly designed metabarcoding assays. Furthermore, not all eDNA sampling material are ideal to detect all taxa and selection of eDNA material heavily influences assemblages derived from metabarcoding data (Koziol et al., 2019). In our empirical testing of bottom water versus surface water, Syngnathidae species were detected at a greater frequency in bottom water samples, with seahorse species only detected in bottom water. However, the low sample numbers are not sufficient to formally test this. We hypothesise that this difference could be due to the higher velocity associated with surface water (Cheng & Gartner, 2003) dispersing and degrading DNA faster than bottom water where seahorses reside. This finding may hold importance for rare and cryptic species like Syngnathidae as their small relative biomass makes them challenging to detect with generic fish assays (Pikitch, 2018).

DNA metabarcoding relies heavily on the availability of high-quality sequences correctly identified by taxonomic experts for accurate species assignments. Current metabarcoding databases are incomplete as not all taxa have barcodes available, this

is further exacerbated for understudied organisms. The use of Western Australian 16S rRNA fish database provided an additional two species and one genus assignments, and increased taxonomic resolution for the 16S gene region. As the number of publicly available sequences grows, the probability of incorrect taxonomic assignment is reduced and taxonomic resolution is improved (Andújar, Arribas, Yu, Vogler, & Emerson, 2018; Somervuo et al., 2017). Curated databases with accurate sequences are of paramount importance to the growth of eDNA metabarcoding as a biodiversity survey tool and will increase the functionality and applicability of metabarcoding data (Andújar et al., 2018).

The choice of barcoding region and assay can greatly affect species assignments and inferences on biodiversity (Cristescu, 2014; Zhang, Chain, Abbott, & Cristescu, 2018). Of the species that weren't detected for the 12S gene region using the MiFish-U assay, 43.5% of these were missing from the NCBI database. In comparison, of the species that weren't detected for the 16S gene region (using 16S Fish, 16S_FishSyn_Long and 16S_FishSyn_Short assays), an average of 15.9% of these were due to missing sequences from the NCBI and the Western Australian 16S rRNA fish database. This highlights the difference in taxonomic coverage and availability of fish sequences between the 12S and 16S mitochondrial gene region. The results of this study could have been affected by this difference through an underestimation of the number of species present, resulting in false negatives. While it is clear that these gaps in current databases will continue to impair the efficacy of eDNA metabarcoding for some time, the availability of reference sequences continues to grow and with it the likelihood of false negatives decreases. Our results emphasise the importance of making an informed choice on a suitable target gene region based on the availability of sequences for your target species.

2.7 Conclusion

Through the development of 16S_FishSyn_Short and 16S_FishSyn_Long, we have successfully developed two generic fish eDNA metabarcoding assays capable of detecting Syngnathidae species, as well as a wide range of other fish taxa, in the marine environment. Consistent with previous research, our findings reiterate that

no metabarcoding assay is ‘universal’ (Stat et al., 2017), and that taxa of conservation importance like Syngnathidae may be missed from eDNA surveys due to a lack of suitable assays. The Syngnathidae family is a flagship group for conservation due to their captivating nature and the iconic status of several species (De Brauwer & Burton, 2018). However, with over 30% of species listed as data deficient, the difficulties associated with undertaking robust conservation assessments on this group are evident. Given the wide spread conservation concerns for these taxa (Vincent, Foster, & Koldewey, 2011), further eDNA work will focus on optimising these Syngnathidae specific assays to develop an effective conservation toolkit for this family.

2.8 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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2.9 Supplementary information

Table S 2-1: Details of the species used to create the 16S Western Australian Fish Database.

Database was created using a combination of targeted sampling and subsamples provided and taxonomically identified by the West Australian Department of Primary Industries and Regional Development (DPIRD) for target and bycatch species. Species ID, name, location caught, and NCBI accession number have been provided.

Seq ID	Species name	Location caught	Accession number
>Seq1	*Abalistes stellatus*	[Australia: Cape Bossut, Kimberley, WA]	MN473514
>Seq2	*Abudefduf bengalensis*	[Australia: Lacepedes, Kimberley, WA]	MN473515
>Seq3	*Abudefduf septemfasciatus*	[Australia: Dampier Archipelago, WA]	MN473516
>Seq4	*Abudefduf septemfasciatus*	[Australia: Eaglehawk Bay, Dampier Archipelago, WA]	MN473517
>Seq5	*Abudefduf sexfasciatus*	[Australia: Coral Bay, WA]	MN473518
>Seq6	*Abudefduf sexfasciatus*	[Australia: Enderby Island North, Dampier Archipelago, WA]	MN473519
>Seq7	*Abudefduf vaigiensis*	[Australia: Coral Bay, WA]	MN473520
>Seq8	*Acanthistius serratus*	[Australia: WA]	MN473521
>Seq9	*Acanthistius serratus*	[Australia: WA]	MN473522
>Seq10	*Acanthocybium solandri*	[Australia: Scott Reef, WA]	MN473523
>Seq11	*Acanthurus grammoptilus*	[Australia: Malis Island, Dampier Archipelago, WA]	MN473524
>Seq12	*Acanthurus olivaceus*	[Australia: Christmas Island, WA]	MN473525
>Seq13	*Acanthurus tennentii*	[Australia: Christmas Island, WA]	MN473526
>Seq14	*Acanthurus tristis*	[Australia: Christmas Island, WA]	MN473527
>Seq15	*Alepes apercna*	[Australia: Lacepedes, Kimberley, WA]	MN473528
>Seq16	*Alepes apercna*	[Australia: Shark Bay or Exmouth Gulf, WA]	MN473529
>Seq17	*Alepes apercna*	[Australia: Shark Bay or Exmouth Gulf, WA]	MN473530
>Seq18	*Amphiprion clarkii*	[Australia: Cape Bossut, Kimberley, WA]	MN473531
>Seq19	*Amphiprion clarkii*	[Australia: Pring, Shark Bay, WA]	MN473532
>Seq20	*Anampses lennardi*	[Australia: Malis Island, Dampier Archipelago, WA]	MN473533
>Seq21	*Anodontostoma chacunda*	[Australia: Admiralty Gulf, North Kimberley, WA]	MN473534
>Seq22	*Anyperodon leucogrammicus*	[Australia: Scott Reef, WA]	MN473535
>Seq23	*Aphareus furca*	[Australia: Christmas Island, WA]	MN473536

>Seq24	*Aphareus rutilans*	[Australia: Scott Reef, WA]	MN473537
>Seq25	*Apistus carinatus*	[Australia: Admiralty Gulf, North Kimberley, WA]	MN473538
>Seq26	*Apogon apogonides*	[Australia: Cape Bossut, Kimberley, WA]	MN473539
>Seq27	*Apogonichthyoides nigripinnis*	[Australia: Cape Bossut, Kimberley, WA]	MN473540
>Seq28	*Aprion virescens*	[Australia: Scott Reef, WA]	MN473541
>Seq29	*Argyrosomus hololepidotus*	[Australia: The Prongs, Shark Bay, WA]	MN473542
>Seq30	*Arothron hispidus*	[Australia: Shark Bay or Exmouth Gulf, WA]	MN473543
>Seq31	*Arripis truttaceus*	[Australia: Bussleton, WA]	MN473544
>Seq32	*Bodianus frenchii*	[Australia: Mindarie, Perth, WA]	MN473545
>Seq33	*Bodianus vulpinus*	[Australia: Jurien Bay, WA]	MN473546
>Seq34	*Caesio caerulaurea*	[Australia: Shark Bay or Exmouth Gulf, WA]	MN473547
>Seq35	*Caesio cuning*	[Australia: Eaglehawk Bay, Dampier Archipelago, WA]	MN473548
>Seq36	*Caesio cuning*	[Australia: Malis Island, Dampier Archipelago, WA]	MN473549
>Seq37	*Carangoides cf. hedlandensis*	[Australia: Shark Bay or Exmouth Gulf, WA]	MN473550
>Seq38	*Carangoides chrysophrys*	[Australia: Admiralty Gulf, North Kimberley, WA]	MN473551
>Seq39	*Carangoides chrysophrys*	[Australia: Albany, WA]	MN473552
>Seq40	*Carangoides equula*	[Australia: Lacepedes, Kimberley, WA]	MN473553
>Seq41	*Carangoides fulvoguttatus*	[Australia: Cape Bossut, Kimberley, WA]	MN473554
>Seq42	*Carangoides gymnostethus*	[Australia: Gascoyne, WA]	MN473555
>Seq43	*Carangoides humerosus*	[Australia: Admiralty Gulf, North Kimberley, WA]	MN473556
>Seq44	*Carangoides malabaricus*	[Australia: Admiralty Gulf, North Kimberley, WA]	MN473557
>Seq45	*Carangoides talamparoides*	[Australia: Admiralty Gulf, North Kimberley, WA]	MN473558
>Seq46	*Caranx bucculentus*	[Australia: Admiralty Gulf, North Kimberley, WA]	MN473559
>Seq47	*Caranx bucculentus*	[Australia: Shark Bay or Exmouth Gulf, WA]	MN473560

>Seq48	*Caranx ignobilis*	[Australia: Broadmayoer Shoal, Kimberley, WA]	MN473561
>Seq49	*Centroberyx gerrardi*	[Australia: South Coast, WA]	MN473562
>Seq50	*Centroberyx australis*	[Australia: Canal Rocks, WA]	MN473563
>Seq51	*Centroberyx australis*	[Australia: Jurien Bay, WA]	MN473564
>Seq52	*Centroberyx gerrardi*	[Australia: Two Rocks 57BM, WA]	MN473565
>Seq53	*Cephalopholis argus*	[Australia: Christmas Island, WA]	MN473566
>Seq54	*Cephalopholis aurantia*	[Australia: Christmas Island, WA]	MN473567
>Seq55	*Cephalopholis boenak*	[Australia: Malis Island, Dampier Archipelago, WA]	MN473568
>Seq56	*Cephalopholis miniata*	[Australia: Christmas Island, WA]	MN473569
>Seq57	*Cephalopholis miniata*	[Australia: Christmas Island, WA]	MN473570
>Seq58	*Cephalopholis sexmaculata*	[Australia: Rowley Shoals, WA]	MN473571
>Seq59	*Cephalopholis sonnerati*	[Australia: Christmas Island, WA]	MN473572
>Seq60	*Chaetoderma penicilligerus*	[Australia: Lacepedes, Kimberley, WA]	MN473573
>Seq61	*Chaetodon assarius*	[Australia: Geographe Bay, WA]	MN473574
>Seq62	*Chaetodon aureofaciatus*	[Australia: Malis Island, Dampier Archipelago, WA]	MN473575
>Seq63	*Chaetodon mitratus*	[Australia: Christmas Island, WA]	MN473576
>Seq64	*Chaetodontoplus duboulayi*	[Australia: Cape Bossut, Kimberley, WA]	MN473577
>Seq65	*Chaetodontoplus duboulayi*	[Australia: Gregories, Shark Bay, WA]	MN473578
>Seq66	*Cheilinus chlorurus*	[Australia: WA]	MN473579
>Seq67	*Cheilinus undulatus*	[Australia: Cocos-Keeling, WA]	MN473580
>Seq68	*Cheilodactylus gibbosus*	[Australia: Geographe Bay, WA]	MN473581
>Seq69	*Chelidonichthys kumu*	[Australia: Eclipse Island, Albany, WA]	MN473582
>Seq70	*Chironemus maculosus*	[Australia: Esperance, WA]	MN473583
>Seq71	*Chlorurus microrhinos*	[Australia: Abrolhos 103AS, WA]	MN473584
>Seq72	*Chlorurus microrhinos*	[Australia: WA]	MN473585
>Seq73	*Chlorurus sordidus*	[Australia: Christmas Island, WA]	MN473586
>Seq74	*Chlorurus sp.*	[Australia: Cocos-Keeling, WA]	MN473587
>Seq75	*Choerodon cauteroma*	[Australia: Gascoyne, WA]	MN473588
>Seq76	*Choerodon cauteroma*	[Australia: Malis Island, Dampier Archipelago, WA]	MN473589
>Seq77	*Choerodon cephalotes*	[Australia: Exmouth Gulf, WA]	MN473590
>Seq78	*Choerodon cephalotes*	[Australia: Shark Bay or Exmouth Gulf, WA]	MN473591
>Seq79	*Choerodon cyanodus*	[Australia: Cattle Well, Shark Bay, WA]	MN473592

>Seq80	*Choerodon cyanodus*	[Australia: Malis Island, Dampier Archipelago, WA]	MN473593
>Seq81	*Choerodon jordani*	[Australia: Abrolhos, WA]	MN473594
>Seq82	*Choerodon schoenleinii*	[Australia: Lacepedes, Kimberley, WA]	MN473595
>Seq83	*Choerodon vitta*	[Australia: Cape Bossut, Kimberley, WA]	MN473596
>Seq84	*Choerodon vitta*	[Australia: Cape Bossut, Kimberley, WA]	MN473597
>Seq85	*Chromis klunzingeri*	[Australia: Geographe Bay, WA]	MN473598
>Seq86	*Chromis lineata*	[Australia: Christmas Island, WA]	MN473599
>Seq87	*Chromis nigrura*	[Australia: Christmas Island, WA]	MN473600
>Seq88	*Chrysophrys auratus*	[Australia: Mindarie, Perth, WA]	MN473601
>Seq89	*Cookeolus japonicus*	[Australia: Bremer Bay Canyon, WA]	MN473602
>Seq90	*Coris cf. pictoides*	[Australia: Malis Island, Dampier Archipelago, WA]	MN473603
>Seq91	*Coris pictoides*	[Australia: Cape Bossut, Kimberley, WA]	MN473604
>Seq92	*Dactyloptena cf. orientalis*	[Australia: Shark Bay or Exmouth Gulf, WA]	MN473605
>Seq93	*Dascyllus aruanus*	[Australia: Cocos-Keeling, WA]	MN473606
>Seq94	*Dascyllus trimaculatus*	[Australia: Christmas Island, WA]	MN473607
>Seq95	*Decapterus maruadsi*	[Australia: Carnac Island, Cockburn Sound, WA]	MN473608
>Seq96	*Decapterus muroadsi*	[Australia: Shark Bay or Exmouth Gulf, WA]	MN473609
>Seq97	*Diagramma pictum*	[Australia: Cape Bossut, Kimberley, WA]	MN473610
>Seq98	*Diploprion bifasciatum*	[Australia: Cape Bossut, Kimberley, WA]	MN473611
>Seq99	*Elates ransonnettii*	[Australia: Admiralty Gulf, North Kimberley, WA]	MN473612
>Seq100	*Engraulis australis*	[Australia: Pinnaroo Point, Perth, WA]	MN473613
>Seq101	*Enoplosus armatus*	[Australia: Mangles Bay, WA]	MN473614
>Seq102	*Epinephelus amblycephalus*	[Australia: Ashmore Reef, WA]	MN473615
>Seq103	*Epinephelus bilobatus*	[Australia: Enderby Island North, Dampier Archipelago, WA]	MN473616
>Seq104	*Epinephelus chlorostigma*	[Australia: Scott Reef, WA]	MN473617
>Seq105	*Epinephelus cyanopodus*	[Australia: Scott Reef, WA]	MN473618
>Seq106	*Epinephelus fasciatus*	[Australia: Enderby Island North, Dampier Archipelago, WA]	MN473619
>Seq107	*Epinephelus fuscoguttatus*	[Australia: Rowley Shoals, WA]	MN473620
>Seq108	*Epinephelus miliaris*	[Australia: Rowley Shoals, WA]	MN473621

>Seq109	*Epinephelus morrhua*	[Australia: Clerke Reef, Rowley Shoals, WA]	MN473622
>Seq110	*Epinephelus multinotatus*	[Australia: Cattle Well, Shark Bay, WA]	MN473623
>Seq111	*Epinephelus multinotatus*	[Australia: WA]	MN473624
>Seq112	*Epinephelus poecilonotus*	[Australia: Rowley Shoals, WA]	MN473625
>Seq113	*Epinephelus polyphekadion*	[Australia: Cocos-Keeling, WA]	MN473626
>Seq114	*Epinephelus quoyanus*	[Australia: Enderby Island North, Dampier Archipelago, WA]	MN473627
>Seq115	*Epinephelus radiatus*	[Australia: Rowley Shoals, WA]	MN473628
>Seq116	*Epinephelus retouti*	[Australia: Christmas Island, WA]	MN473629
>Seq117	*Epinephelus tauvina*	[Australia: Christmas Island, WA]	MN473630
>Seq118	*Equulites cf. leuciscus*	[Australia: Shark Bay or Exmouth Gulf, WA]	MN473631
>Seq119	*Equulites cf. moretoniensis*	[Australia: Shark Bay or Exmouth Gulf, WA]	MN473632
>Seq120	*Equulites sp.*	[Australia: Admiralty Gulf, North Kimberley, WA]	MN473633
>Seq121	*Erythrocles schlegelii*	[Australia: Exmouth Gulf, WA]	MN473634
>Seq122	*Etelis carbunculus*	[Australia: Imperieuse Reef, Rowley Shoals, WA]	MN473635
>Seq123	*Etelis radiosus*	[Australia: Exmouth, WA]	MN473636
>Seq124	*Etelis sp*	[Australia: Exmouth Gulf, WA]	MN473637
>Seq125	*Eubalichthys mosaicus*	[Australia: Geographe Bay, WA]	MN473638
>Seq126	*Feroxodon multistriatus*	[Australia: Lacepedes, Kimberley, WA]	MN473639
>Seq127	*Fistularia commersonii*	[Australia: Admiralty Gulf, North Kimberley, WA]	MN473640
>Seq128	*Gazza minuta*	[Australia: Lacepedes, Kimberley, WA]	MN473641
>Seq129	*Gephyroberyx darwini*	[Australia: Rockingham 56BI, WA]	MN473642
>Seq130	*Gerres longirostris*	[Australia: Cocos-Keeling, WA]	MN473643
>Seq131	*Gerres oyena*	[Australia: Admiralty Gulf, North Kimberley, WA]	MN473644
>Seq132	*Gerres oyena*	[Australia: Shark Bay or Exmouth Gulf, WA]	MN473645
>Seq133	*Glaucosoma buergeri*	[Australia: Gascoyne, WA]	MN473646
>Seq134	*Glaucosoma magnificum*	[Australia: Cape Bossut, Kimberley, WA]	MN473647
>Seq135	*Gnathanodon speciosus*	[Australia: Cape Bossut, Kimberley, WA]	MN473648
>Seq136	*Gnathanodon speciosus*	[Australia: Gascoyne, WA]	MN473649
>Seq137	*Gracila albomarginata*	[Australia: Christmas Island, WA]	MN473650

>Seq138	*Gymnapistes marmoratus*	[Australia: Mangles Bay, Perth, WA]	MN473651
>Seq139	*Gymnocranius grandoculis*	[Australia: Imperieuse Reef, Rowley Shoals, WA]	MN473652
>Seq140	*Gymnosarda unicolor*	[Australia: Christmas Island, WA]	MN473653
>Seq141	*Gymnothorax cribroris*	[Australia: Lacepedes, Kimberley, WA]	MN473654
>Seq142	*Gymnothorax thyrsoideus*	[Australia: Lacepedes, Kimberley, WA]	MN473655
>Seq143	*Halichoeres nigrescens*	[Australia: Enderby Island North, Dampier Archipelago, WA]	MN473656
>Seq144	*Hemigymnus melapterus*	[Australia: Malis Island, Dampier Archipelago, WA]	MN473657
>Seq145	*Herklotsichthys lippa*	[Australia: Admiralty Gulf, North Kimberley, WA]	MN473658
>Seq146	*Heteroclinus adelaidae*	[Australia: Mangles Bay, Perth, WA]	MN473659
>Seq147	*Hipposcarus cf. longiceps*	[Australia: Cocos-Keeling, WA]	MN473660
>Seq148	*Hoplostethus gigas*	[Australia: WA]	MN473661
>Seq149	*Hyporhamphus melanochir*	[Australia: Mangles Bay, Perth, WA]	MN473662
>Seq150	*Hyporhamphus quoyi*	[Australia: Shark Bay, WA]	MN473663
>Seq151	*Inegocia japonica*	[Australia: Lacepedes, Kimberley, WA]	MN473664
>Seq152	*Inegocia japonica*	[Australia: Lacepedes, Kimberley, WA]	MN473665
>Seq153	*Inegocia japonica*	[Australia: Shark Bay or Exmouth Gulf, WA]	MN473666
>Seq154	*Iniistius jacksonensis*	[Australia: Cape Bossut, Kimberley, WA]	MN473667
>Seq155	*Iniistius sp.*	[Australia: Christmas Island, WA]	MN473668
>Seq156	*Inimicus cf. sinensis*	[Australia: Shark Bay or Exmouth Gulf, WA]	MN473669
>Seq157	*Inimicus sinensis*	[Australia: Admiralty Gulf, North Kimberley, WA]	MN473670
>Seq158	*Istiompax indica*	[Australia: Ocean Beach, South Coast, WA]	MN473671
>Seq159	*Jaydia cf. ellioti*	[Australia: Shark Bay or Exmouth Gulf, WA]	MN473672
>Seq160	*Jaydia cf. poecilopterus*	[Australia: Shark Bay or Exmouth Gulf, WA]	MN473673
>Seq161	*Kathetostoma nigrofasciatum*	[Australia: Doubtful Bay, WA]	MN473674
>Seq162	*Katsuwonus pelamis*	[Australia: Gascoyne, WA]	MN473675
>Seq163	*Labroides dimidiatus*	[Australia: Gregories, Shark Bay, WA]	MN473676
>Seq164	*Lagocephalus lunaris*	[Australia: Cockburn Sound, WA]	MN473677

>Seq165	*Lagocephalus sceleratus*	[Australia: Cape Bossut, Kimberley, WA]	MN473678
>Seq166	*Lagocephalus sceleratus*	[Australia: WA]	MN473679
>Seq167	*Lates calcarifer*	[Australia: Whyndham, Kimberley, WA]	MN473680
>Seq168	*Lactoria cornuta*	[Australia: Lacepedes, Kimberley, WA]	MN473681
>Seq169	*Latropiscis purpurissatus*	[Australia: Albany, WA]	MN473682
>Seq170	*Latropiscis purpurissatus*	[Australia: WA]	MN473683
>Seq171	*Lepidotrigla papilio*	[Australia: Doubtful Bay, WA]	MN473684
>Seq172	*Leptoscarus vaigiensis*	[Australia: Coral Bay, WA]	MN473685
>Seq173	*Lethrinus erythracanthus*	[Australia: Scott Reef, WA]	MN473686
>Seq174	*Lethrinus genivittatus*	[Australia: Enderby Island North, Dampier Archipelago, WA]	MN473687
>Seq175	*Lethrinus laticaudis*	[Australia: Montgomery Reef, Kimberley, WA]	MN473688
>Seq176	*Lethrinus miniatus*	[Australia: Carnarvon, WA]	MN473689
>Seq177	*Lethrinus nebulosus*	[Australia: Enderby Island North, Dampier Archipelago, WA]	MN473690
>Seq178	*Lethrinus obsoletus*	[Australia: Imperieuse Reef, Rowley Shoals, WA]	MN473691
>Seq179	*Lethrinus punctulatus*	[Australia: Enderby Island North, Dampier Archipelago, WA]	MN473692
>Seq180	*Lethrinus rubrioperculatus*	[Australia: Christmas Island, WA]	MN473693
>Seq181	*Lethrinus variegatus*	[Australia: Enderby Island North, Dampier Archipelago, WA]	MN473694
>Seq182	*Lethrinus xanthochilus*	[Australia: Clerke Reef, Rowley Shoals, WA]	MN473695
>Seq183	*Lethrinus xanthochilus*	[Australia: Cocos-Keeling, WA]	MN473696
>Seq184	*Leviprora inops*	[Australia: Mindarie, Perth, WA]	MN473697
>Seq185	*Lutjanus argentimaculatus*	[Australia: Scott Reef, WA]	MN473698
>Seq186	*Lutjanus bitaeniatus*	[Australia: Biggie Island, Kimberley, WA]	MN473699
>Seq187	*Lutjanus bohar*	[Australia: Scott Reef, WA]	MN473700
>Seq188	*Lutjanus carponotatus*	[Australia: Malis Island, Dampier Archipelago, WA]	MN473701
>Seq189	*Lutjanus erythropterus*	[Australia: Dampier Archipelago,WA]	MN473702
>Seq190	*Lutjanus johnii*	[Australia: Hall Point, Kimberley, WA]	MN473703
>Seq191	*Lutjanus malabaricus*	[Australia: Admiralty Gulf, North Kimberley, WA]	MN473704
>Seq192	*Lutjanus malabaricus*	[Australia: WA]	MN473705
>Seq193	*Lutjanus rufolineatus*	[Australia: Scott Reef, WA]	MN473706

>Seq194	*Lutjanus russellii*	[Australia: Gascoyne, WA]	MN473707
>Seq195	*Lutjanus sebae*	[Australia: Pilbara, WA]	MN473708
>Seq196	*Melichthys niger*	[Australia: Cocos-Keeling, WA]	MN473709
>Seq197	*Meuschenia flavolineata*	[Australia: Geographe Bay, WA]	MN473710
>Seq198	*Meuschenia freycineti*	[Australia: Mangles Bay, WA]	MN473711
>Seq199	*Microcanthus strigatus*	[Australia: Cockburn Sound, WA]	MN473712
>Seq200	*Mobula japanica*	[Australia: Cheynes Beach, Albany, WA]	MN473713
>Seq201	*Mola mola*	[Australia: Bremer Bay, WA]	MN473714
>Seq202	*Monacanthus chinensis*	[Australia: Cockburn Sound, WA]	MN473715
>Seq203	*Mugil cephalus*	[Australia: Denham Bait Bin, Shark Bay, WA]	MN473716
>Seq204	*Mugil cephalus*	[Australia: Denham Bait Bin, Shark Bay, WA]	MN473717
>Seq205	*Mulloidichthys cf. vanicolensis*	[Australia: Shark Bay or Exmouth Gulf, WA]	MN473718
>Seq206	*Naso cf. tonganus*	[Australia: Shark Bay or Exmouth Gulf, WA]	MN473719
>Seq207	*Neoglyphidodon melas*	[Australia: Malis Island, Dampier Archipelago, WA]	MN473720
>Seq208	*Neopomacentrus filamentosus*	[Australia: Lacepedes, Kimberley, WA]	MN473721
>Seq209	*Neosebastes nigropunctatus*	[Australia: Capes to Capes, WA]	MN473722
>Seq210	*Neosebastes pandus*	[Australia: Capes to Capes, WA]	MN473723
>Seq211	*Neosebastes thetidis*	[Australia: Capes to Capes, WA]	MN473724
>Seq212	*Netuma thalassina*	[Australia: Admiralty North, Kimberley, WA]	MN473725
>Seq213	*Nuchequula gerreoides*	[Australia: Shark Bay or Exmouth Gulf, WA]	MN473726
>Seq214	*Onigocia oligolepis*	[Australia: Cape Bossut, Kimberley, WA]	MN473727
>Seq215	*Oplegnathus woodwardi*	[Australia: Jurien Bay, WA]	MN473728
>Seq216	*Opthalmolepis lineolata*	[Australia: Hopetown, WA]	MN473729
>Seq217	*Opthalmolepis lineolata*	[Australia: Rossiter Bay, WA]	MN473730
>Seq218	*Ostorhinchus aureus*	[Australia: Shark Bay or Exmouth Gulf, WA]	MN473731
>Seq219	*Ostorhinchus cf. apogonoides*	[Australia: Shark Bay or Exmouth Gulf, WA]	MN473732
>Seq220	*Ostracion cubicus*	[Australia: Malis Island, Dampier Archipelago, WA]	MN473733

>Seq221	*Paracaesio xanthura*	[Australia: Gascoyne, WA]	MN473734
>Seq222	*Parachaetodon ocellatus*	[Australia: Eaglehawk Bay, Dampier Archipelago, WA]	MN473735
>Seq223	*Parachaetodon ocellatus*	[Australia: Lacepedes, Kimberley, WA]	MN473736
>Seq224	*Paracirrhites arcatus*	[Australia: Christmas Island, WA]	MN473737
>Seq225	*Paracirrhites forsteri*	[Australia: Christmas Island, WA]	MN473738
>Seq226	*Paracirrhites hemistictus*	[Australia: Christmas Island, WA]	MN473739
>Seq227	*Paramonacanthus choirocephalus*	[Australia: Cape Bossut, Kimberley, WA]	MN473740
>Seq228	*Paramonacanthus choirocephalus*	[Australia: Cape Bossut, Kimberley, WA]	MN473741
>Seq229	*Paramonacanthus choirocephalus*	[Australia: Cape Bossut, Kimberley, WA]	MN473742
>Seq230	*Paramonacanthus choirocephalus*	[Australia: Shark Bay or Exmouth Gulf, WA]	MN473743
>Seq231	*Paramonacanthus filicauda*	[Australia: Admiralty Gulf, North Kimberley, WA]	MN473744
>Seq232	*Parapercis nebulosa*	[Australia: Cape Bossut, Kimberley, WA]	MN473745
>Seq233	*Parapercis nebulosa*	[Australia: Shark Bay or Exmouth Gulf, WA]	MN473746
>Seq234	*Parma mccullochi*	[Australia: Wanneroo Reef, WA]	MN473747
>Seq235	*Parma occidentalis*	[Australia: Wanneroo Reef, WA]	MN473748
>Seq236	*Parma victoriae*	[Australia: Geographe Bay, WA]	MN473749
>Seq237	*Parupeneus heptacanthus*	[Australia: Cape Bossut, Kimberley, WA]	MN473750
>Seq238	*Parupeneus indicus*	[Australia: Cape Bossut, Kimberley, WA]	MN473751
>Seq239	*Parupeneus indicus*	[Australia: Malis Island, Dampier Archipelago, WA]	MN473752
>Seq240	*Pelates octolineatus*	[Australia: WA]	MN473753
>Seq241	*Pempheris sp.*	[Australia: Cockburn Sound, WA]	MN473754
>Seq242	*Pempheris sp.*	[Australia: Gregories, Shark Bay, WA]	MN473755
>Seq243	*Petroscirtes breviceps*	[Australia: Coral Bay, WA]	MN473756
>Seq244	*Pictilabrus laticlavius*	[Australia: Geographe Bay, WA]	MN473757
>Seq245	*Plagiogeneion macrolepis*	[Australia: Rockingham, WA]	MN473758
>Seq246	*Plagiogeneion rubiginosum*	[Australia: WA]	MN473759
>Seq247	*Platax batavianus*	[Australia: Cape Bossut, Kimberley, WA]	MN473760
>Seq248	*Platycephalus australis*	[Australia: Admiralty Gulf, North Kimberley, WA]	MN473761
>Seq249	*Platycephalus chauliodous*	[Australia: WA]	MN473762

>Seq250	*Platycephalus conatus*	[Australia: Dampier Archipelago, WA]	MN473763
>Seq251	*Platycephalus conatus*	[Australia: Windy Harbour, WA]	MN473764
>Seq252	*Platycephalus endrachtensis*	[Australia: Lacepedes, Kimberley, WA]	MN473765
>Seq253	*Platycephalus longispinis*	[Australia: Doubtful Bay, WA]	MN473766
>Seq254	*Platycephalus speculator*	[Australia: WA]	MN473767
>Seq255	*Platycephalus westraliae*	[Australia: Cape Bossut, Kimberley, WA]	MN473768
>Seq256	*Platycephalus westraliae*	[Australia: Lacepedes, Kimberley, WA]	MN473769
>Seq257	*Plectorhinchus flavomaculatus*	[Australia: Gascoyne, WA]	MN473770
>Seq258	*Plectorhinchus flavomaculatus*	[Australia: Geographe Bay, WA]	MN473771
>Seq259	*Plectorhinchus gibbosus*	[Australia: Cape Bossut, Kimberley, WA]	MN473772
>Seq260	*Plectorhinchus polytaenia*	[Australia: Lacepedes, Kimberley, WA]	MN473773
>Seq261	*Plectorhynchus polytaenia*	[Australia: Eaglehawk Bay, Dampier Archipelago, WA]	MN473774
>Seq262	*Plectropomus areolatus*	[Australia: Cocos-Keeling, WA]	MN473775
>Seq263	*Plectropomus leopardus*	[Australia: Scott Reef, WA]	MN473776
>Seq264	*Plectropomus maculatus*	[Australia: Cattle Well, Shark Bay, WA]	MN473777
>Seq265	*Plectropomus maculatus*	[Australia: Eaglehawk Bay, Dampier Archipelago, WA]	MN473778
>Seq266	*Plectropomus oligacanthus*	[Australia: Scott Reef, WA]	MN473779
>Seq267	*Polydactylus cf. multiradiatus*	[Australia: Shark Bay or Exmouth Gulf, WA]	MN473780
>Seq268	*Polyprion cf. americanus*	[Australia: Lancelin, WA]	MN473781
>Seq269	*Polyprion oxygeneios*	[Australia: Cape Naturaliste, WA]	MN473782
>Seq270	*Pomadasys argyreus*	[Australia: Admiralty Gulf, North Kimberley, WA]	MN473783
>Seq271	*Pomadasys cf. maculatus*	[Australia: Shark Bay or Exmouth Gulf, WA]	MN473784
>Seq272	*Pomadasys kaakan*	[Australia: Admiralty North, Kimberley, WA]	MN473785
>Seq273	*Pomadasys maculatus*	[Australia: Admiralty Gulf, North Kimberley, WA]	MN473786
>Seq274	*Pomatomus saltatrix*	[Australia: WA]	MN473787
>Seq275	*Priacanthus macracanthus*	[Australia: Lacepedes, Kimberley, WA]	MN473788
>Seq276	*Priacanthus tayenus*	[Australia: Admiralty Gulf, North Kimberley, WA]	MN473789

>Seq277	*Priacanthus tayenus*	[Australia: Shark Bay or Exmouth Gulf, WA]	MN473790
>Seq278	*Prionobutis microps*	[Australia: Porosus Creek, North Kimberley, WA]	MN473791
>Seq279	*Pristipomoides auricilla*	[Australia: Clerke Reef, Rowley Shoals, WA]	MN473792
>Seq280	*Pristipomoides filamentosus*	[Australia: Kimberley, WA]	MN473793
>Seq281	*Pristipomoides multidens*	[Australia: Pilbara, WA]	MN473794
>Seq282	*Pristipomoides sieboldii*	[Australia: WA]	MN473795
>Seq283	*Pristipomoides typus*	[Australia: Pilbara, WA]	MN473796
>Seq284	*Pristipomoides zonatus*	[Australia: Scott Reef, WA]	MN473797
>Seq285	*Pristotis obtusirostris*	[Australia: Cape Bossut, Kimberley, WA]	MN473798
>Seq286	*Pristotis obtusirostris*	[Australia: Shark Bay or Exmouth Gulf, WA]	MN473799
>Seq287	*Protonibea diacanthus*	[Australia: Goodenough Bay, WA]	MN473800
>Seq288	*Psammoperca waigiensis*	[Australia: Eaglehawk Bay, Dampier Archipelago, WA]	MN473801
>Seq289	*Psettodes erumei*	[Australia: Admiralty Gulf, North Kimberley, WA]	MN473802
>Seq290	*Pseudorhombus argus*	[Australia: Cape Bossut, Kimberley, WA]	MN473803
>Seq291	*Pseudorhombus arsius*	[Australia: Cape Bossut, Kimberley, WA]	MN473804
>Seq292	*Pseudorhombus arsius*	[Australia: Shark Bay or Exmouth Gulf, WA]	MN473805
>Seq293	*Pseudorhombus cf. jenynsii*	[Australia: Shark Bay or Exmouth Gulf, WA]	MN473806
>Seq294	*Pseudorhombus jenynsii*	[Australia: Cape Bossut, Kimberley, WA]	MN473807
>Seq295	*Pseudorhombus spinosus*	[Australia: Cape Bossut, Kimberley, WA]	MN473808
>Seq296	*Ptereleotris microlepis*	[Australia: Cocos-Keeling, WA]	MN473809
>Seq297	*Pterocaesio digramma*	[Australia: Shark Bay or Exmouth Gulf, WA]	MN473810
>Seq298	*Pterois mombasae*	[Australia: Lacepedes, Kimberley, WA]	MN473811
>Seq299	*Rachycentron canadum*	[Australia: Cape Inscription, Shark Bay, WA]	MN473812
>Seq300	*Randallichthys filamentosus*	[Australia: Esperance, WA]	MN473813
>Seq301	*Rhynchostracion nasus*	[Australia: Shark Bay or Exmouth Gulf, WA]	MN473814
>Seq302	*Rogadius asper*	[Australia: Admiralty Gulf, North Kimberley, WA]	MN473815

>Seq303	*Saloptia powelli*	[Australia: Rowley Shoals, WA]	MN473816
>Seq304	*Sardinella brachysoma*	[Australia: Admiralty Gulf, North Kimberley, WA]	MN473817
>Seq305	*Sardinella gibbosa*	[Australia: Lacepedes, Kimberley, WA]	MN473818
>Seq306	*Saurida tumbil*	[Australia: Cape Bossut, Kimberley, WA]	MN473819
>Seq307	*Saurida undosquamis*	[Australia: Princess Royal Harbour, WA]	MN473820
>Seq308	*Scarus ghobban*	[Australia: Malis Island, Dampier Archipelago, WA]	MN473821
>Seq309	*Scarus prasiognathus*	[Australia: WA]	MN473822
>Seq310	*Scarus rivulatus*	[Australia: Malis Island, Dampier Archipelago, WA]	MN473823
>Seq311	*Scatophagus argus*	[Australia: Porosus Creek, North Kimberley, WA]	MN473824
>Seq312	*Scomberomorus commerson*	[Australia: Cattle Well, Shark Bay, WA]	MN473825
>Seq313	*Scomberomorus commerson*	[Australia: Perth, WA]	MN473826
>Seq314	*Scomberomorus queenslandicus*	[Australia: Emeriau Point, Kimberley, WA]	MN473827
>Seq315	*Selaroides cf. leptolepis*	[Australia: Shark Bay or Exmouth Gulf, WA]	MN473828
>Seq316	*Selaroides leptolepis*	[Australia: Admiralty Gulf, North Kimberley, WA]	MN473829
>Seq317	*Seriola dumerili*	[Australia: Gascoyne, WA]	MN473830
>Seq318	*Seriolina nigrofasciata*	[Australia: Admiralty Gulf, North Kimberley, WA]	MN473831
>Seq319	*Seriolina nigrofasciata*	[Australia: Lacepedes, Kimberley, WA]	MN473832
>Seq320	*Siganus canaliculatus*	[Australia: Cape Bossut, Kimberley, WA]	MN473833
>Seq321	*Siganus canaliculatus*	[Australia: Coral Bay, WA]	MN473834
>Seq322	*Siganus canaliculatus*	[Australia: Shark Bay, WA]	MN473835
>Seq323	*Siganus cf. margaritiferus*	[Australia: Coral Bay, WA]	MN473836
>Seq324	*Siganus virgatus*	[Australia: Malis Island, Dampier Archipelago, WA]	MN473837
>Seq325	*Sillago cf. sihama*	[Australia: Onslow, WA]	MN473838
>Seq326	*Sillago ingenua*	[Australia: Shark Bay, WA]	MN473839
>Seq327	*Sillago robusta*	[Australia: Perth, WA]	MN473840
>Seq328	*Sillago schomburgkii*	[Australia: Denham Bait Bin, Shark Bay, WA]	MN473841
>Seq329	*Sillago schomburgkii*	[Australia: Denham Bait Bin, Shark Bay, WA]	MN473842

>Seq330	*Sillago schomburgkii*	[Australia: South Coast, WA]	MN473843
>Seq331	*Sillago sp.*	[Australia: Admiralty Gulf, North Kimberley, WA]	MN473844
>Seq332	*Sphyraena pinguis*	[Australia: Gregories, Shark Bay, WA]	MN473845
>Seq333	*Stegastes cf. obreptus*	[Australia: Gregories, Shark Bay, WA]	MN473846
>Seq334	*Symphorus nemaptophorus*	[Australia: Enderby Island North, Dampier Archipelago, WA]	MN473847
>Seq335	*Terapon cf. puta*	[Australia: Shark Bay or Exmouth Gulf, WA]	MN473848
>Seq336	*Terapon cf. theraps*	[Australia: Shark Bay or Exmouth Gulf, WA]	MN473849
>Seq337	*Thalassoma cf. hardwicke*	[Australia: Gregories, Shark Bay, WA]	MN473850
>Seq338	*Thalassoma lunare*	[Australia: Coral Bay, WA]	MN473851
>Seq339	*Thalassoma lunare*	[Australia: Enderby Island North, Dampier Archipelago, WA]	MN473852
>Seq340	*Thunnus tonggol*	[Australia: Gascoyne, WA]	MN473853
>Seq341	*Thyrsitoides marleyi*	[Australia: Jurien Bay, WA]	MN473854
>Seq342	*Tilodon sexfasciatus*	[Australia: Cockburn Sound, WA]	MN473855
>Seq343	*Torquigener pallimaculatus*	[Australia: Cape Bossut, Kimberley, WA]	MN473856
>Seq344	*Torquigener pallimaculatus*	[Australia: Shark Bay or Exmouth Gulf, WA]	MN473857
>Seq345	*Torquigener whitleyi*	[Australia: Shark Bay or Exmouth Gulf, WA]	MN473858
>Seq346	*Trachinocephalus myops*	[Australia: Shark Bay or Exmouth Gulf, WA]	MN473859
>Seq347	*Trachinotus baillonii*	[Australia: Cocos-Keeling, WA]	MN473860
>Seq348	*Tragulichthys jaculiferus*	[Australia: Shark Bay or Exmouth Gulf, WA]	MN473861
>Seq349	*Upeneus asymmetricus*	[Australia: Cape Bossut, Kimberley, WA]	MN473862
>Seq350	*Upeneus asymmetricus*	[Australia: Mangles Bay, WA]	MN473863
>Seq351	*Upeneus asymmetricus*	[Australia: Shark Bay or Exmouth Gulf, WA]	MN473864
>Seq352	*Upeneus sulphureus*	[Australia: Shark Bay or Exmouth Gulf, WA]	MN473865
>Seq353	*Upeneus sundaicus*	[Australia: Admiralty Gulf, North Kimberley, WA]	MN473866
>Seq354	*Upeneus tragula*	[Australia: Enderby Island North, WA]	MN473867
>Seq355	*Variola albimarginata*	[Australia: Ashmore Reef, WA]	MN473868

>Seq356	*Variola louti*	[Australia: Cape Inscription, Shark Bay, WA]	MN473869
>Seq357	*Variola louti*	[Australia: Cocos-Keeling, WA]	MN473870
>Seq358	*Variola louti*	[Australia: Cocos-Keeling, WA]	MN473871
>Seq359	*Yongeichthys cf. nebulosus*	[Australia: Shark Bay or Exmouth Gulf, WA]	MN473872
>Seq360	*Zabidius novemaculeatus*	[Australia: Admiralty Gulf, North Kimberley, WA]	MN473873
>Seq361	*Zeus faber*	[Australia: Eclipse Island, WA]	MN473874

Table S 2-2: List of fish 16S reference sequences used in this study for metabarcoding assay development of 16S_FishSyn_Long and 16S_FishSyn_Short.

Sequences sourced from Western Australian 16S rRNA fish database (Genbank accession MN473514 to MN473874) and NCBI. Sequences were aligned in Geneious v. 10.2.6 for assay development.

Species	Sequence source	NCBI accession number
Abalistes stellatus	Custom 16s Fish Database	MN473514
Abudefduf bengalensis	Custom 16s Fish Database	MN473515
Abudefduf septemfasciatus	Custom 16s Fish Database	MN473516
Abudefduf septemfasciatus	Custom 16s Fish Database	MN473517
Abudefduf sexfasciatus	Custom 16s Fish Database	MN473518
Abudefduf sexfasciatus	Custom 16s Fish Database	MN473519
Abudefduf vaigiensis	Custom 16s Fish Database	MN473520
Acanthistius serratus	Custom 16s Fish Database	MN473521
Acanthistius serratus	Custom 16s Fish Database	MN473522
Acanthocybium solandri	Custom 16s Fish Database	MN473523
Acanthopagrus butcheri	NCBI	KX234643
Acanthurus grammoptilus	Custom 16s Fish Database	MN473524
Acanthurus olivaceus	Custom 16s Fish Database	MN473525
Acanthurus tennentii	Custom 16s Fish Database	MN473526
Acanthurus tristis	Custom 16s Fish Database	MN473527
Alepes apercna	Custom 16s Fish Database	MN473528
Alepes apercna	Custom 16s Fish Database	MN473529
Alepes apercna	Custom 16s Fish Database	MN473530
Amphiprion clarkii	Custom 16s Fish Database	MN473531
Amphiprion clarkii	Custom 16s Fish Database	MN473532
Anampses lennardi	Custom 16s Fish Database	MN473533
Anodontostoma chacunda	Custom 16s Fish Database	MN473534
Anyperodon leucogrammicus	Custom 16s Fish Database	MN473535
Aphareus furca	Custom 16s Fish Database	MN473536
Aphareus rutilans	Custom 16s Fish Database	MN473537
Apistus carinatus	Custom 16s Fish Database	MN473538
Apogon apogonides	Custom 16s Fish Database	MN473539
Apogonichthyoides nigripinnis	Custom 16s Fish Database	MN473540
Aprion virescens	Custom 16s Fish Database	MN473541
Argyrosomus hololepidotus	Custom 16s Fish Database	MN473542
Arothron hispidus	Custom 16s Fish Database	MN473543
Arripis georgianus	NCBI	DQ532841
Arripis truttaceus	Custom 16s Fish Database	MN473544
Bodianus frenchii	Custom 16s Fish Database	MN473545
Bodianus vulpinus	Custom 16s Fish Database	MN473546
Caesio caerulaurea	Custom 16s Fish Database	MN473547
Caesio cuning	Custom 16s Fish Database	MN473548

Caesio cuning	Custom 16s Fish Database	MN473549
Carangoides cf. hedlandensis	Custom 16s Fish Database	MN473550
Carangoides chrysophrys	Custom 16s Fish Database	MN473551
Carangoides chrysophrys	Custom 16s Fish Database	MN473552
Carangoides equula	Custom 16s Fish Database	MN473553
Carangoides fulvoguttatus	Custom 16s Fish Database	MN473554
Carangoides gymnostethus	Custom 16s Fish Database	MN473555
Carangoides humerosus	Custom 16s Fish Database	MN473556
Carangoides malabaricus	Custom 16s Fish Database	MN473557
Carangoides talamparoides	Custom 16s Fish Database	MN473558
Caranx bucculentus	Custom 16s Fish Database	MN473559
Caranx bucculentus	Custom 16s Fish Database	MN473560
Caranx ignobilis	Custom 16s Fish Database	MN473561
Centroberyx australis	Custom 16s Fish Database	MN473562
Centroberyx australis	Custom 16s Fish Database	MN473563
Centroberyx gerrardi	Custom 16s Fish Database	MN473564
Centroberyx gerrardi	Custom 16s Fish Database	MN473565
Cephalopholis argus	Custom 16s Fish Database	MN473566
Cephalopholis aurantia	Custom 16s Fish Database	MN473567
Cephalopholis boenak	Custom 16s Fish Database	MN473568
Cephalopholis miniata	Custom 16s Fish Database	MN473569
Cephalopholis miniata	Custom 16s Fish Database	MN473570
Cephalopholis sexmaculata	Custom 16s Fish Database	MN473571
Cephalopholis sonnerati	Custom 16s Fish Database	MN473572
Chaetoderma penicilligerus	Custom 16s Fish Database	MN473573
Chaetodon assarius	Custom 16s Fish Database	MN473574
Chaetodon aureofaciatus	Custom 16s Fish Database	MN473575
Chaetodon mitratus	Custom 16s Fish Database	MN473576
Chaetodontoplus duboulayi	Custom 16s Fish Database	MN473577
Chaetodontoplus duboulayi	Custom 16s Fish Database	MN473578
Cheilinus chlorurus	Custom 16s Fish Database	MN473579
Cheilinus undulatus	Custom 16s Fish Database	MN473580
Cheilodactylus gibbosus	Custom 16s Fish Database	MN473581
Chelidonichthys kumu	Custom 16s Fish Database	MN473582
Chironemus maculosus	Custom 16s Fish Database	MN473583
Chlorurus microrhinos	Custom 16s Fish Database	MN473584
Chlorurus microrhinos	Custom 16s Fish Database	MN473585
Chlorurus sordidus	Custom 16s Fish Database	MN473586
Chlorurus sp.	Custom 16s Fish Database	MN473587
Choerodon cauteroma	Custom 16s Fish Database	MN473588
Choerodon cauteroma	Custom 16s Fish Database	MN473589
Choerodon cephalotes	Custom 16s Fish Database	MN473590
Choerodon cephalotes	Custom 16s Fish Database	MN473591

Choerodon cyanodus	Custom 16s Fish Database	MN473592
Choerodon cyanodus	Custom 16s Fish Database	MN473593
Choerodon jordani	Custom 16s Fish Database	MN473594
Choerodon schoenleinii	Custom 16s Fish Database	MN473595
Choerodon vitta	Custom 16s Fish Database	MN473596
Choerodon vitta	Custom 16s Fish Database	MN473597
Choeroichthys sculptus	NCBI	KY065523
Chromis klunzingeri	Custom 16s Fish Database	MN473598
Chromis lineata	Custom 16s Fish Database	MN473599
Chromis nigrura	Custom 16s Fish Database	MN473600
Chrysophrys auratus	Custom 16s Fish Database	MN473601
Cookeolus japonicus	Custom 16s Fish Database	MN473602
Coris cf. pictoides	Custom 16s Fish Database	MN473603
Coris pictoides	Custom 16s Fish Database	MN473604
Dactyloptena cf. orientalis	Custom 16s Fish Database	MN473605
Dascyllus aruanus	Custom 16s Fish Database	MN473606
Dascyllus trimaculatus	Custom 16s Fish Database	MN473607
Decapterus maruadsi	Custom 16s Fish Database	MN473608
Decapterus muroadsi	Custom 16s Fish Database	MN473609
Diagramma pictum	Custom 16s Fish Database	MN473610
Diploprion bifasciatum	Custom 16s Fish Database	MN473611
Elates ransonnettii	Custom 16s Fish Database	MN473612
Engraulis australis	Custom 16s Fish Database	MN473613
Enoplosus armatus	Custom 16s Fish Database	MN473614
Epinephelus amblycephalus	Custom 16s Fish Database	MN473615
Epinephelus bilobatus	Custom 16s Fish Database	MN473616
Epinephelus chlorostigma	Custom 16s Fish Database	MN473617
Epinephelus cyanopodus	Custom 16s Fish Database	MN473618
Epinephelus fasciatus	Custom 16s Fish Database	MN473619
Epinephelus fuscoguttatus	Custom 16s Fish Database	MN473620
Epinephelus miliaris	Custom 16s Fish Database	MN473621
Epinephelus morrhua	Custom 16s Fish Database	MN473622
Epinephelus multinotatus	Custom 16s Fish Database	MN473623
Epinephelus multinotatus	Custom 16s Fish Database	MN473624
Epinephelus poecilonotus	Custom 16s Fish Database	MN473625
Epinephelus polyphekadion	Custom 16s Fish Database	MN473626
Epinephelus quoyanus	Custom 16s Fish Database	MN473627
Epinephelus radiatus	Custom 16s Fish Database	MN473628
Epinephelus retouti	Custom 16s Fish Database	MN473629
Epinephelus tauvina	Custom 16s Fish Database	MN473630
Equulites cf. leuciscus	Custom 16s Fish Database	MN473631
Equulites cf. moretoniensis	Custom 16s Fish Database	MN473632
Equulites sp.	Custom 16s Fish Database	MN473633

Erythrocles schlegelii	Custom 16s Fish Database	MN473634
Etelis carbunculus	Custom 16s Fish Database	MN473635
Etelis radiosus	Custom 16s Fish Database	MN473636
Etelis sp	Custom 16s Fish Database	MN473637
Eubalichthys mosaicus	Custom 16s Fish Database	MN473638
Feroxodon multistriatus	Custom 16s Fish Database	MN473639
Filicampus tigris	NCBI	KY065542
Fistularia commersonii	Custom 16s Fish Database	MN473640
Gazza minuta	Custom 16s Fish Database	MN473641
Gephyroberyx darwini	Custom 16s Fish Database	MN473642
Gerres longirostris	Custom 16s Fish Database	MN473643
Gerres oyena	Custom 16s Fish Database	MN473644
Gerres oyena	Custom 16s Fish Database	MN473645
Glaucosoma buergeri	Custom 16s Fish Database	MN473646
Glaucosoma magnificum	Custom 16s Fish Database	MN473647
Gnathanodon speciosus	Custom 16s Fish Database	MN473648
Gnathanodon speciosus	Custom 16s Fish Database	MN473649
Gracila albomarginata	Custom 16s Fish Database	MN473650
Gymnapistes marmoratus	Custom 16s Fish Database	MN473651
Gymnocranius grandoculis	Custom 16s Fish Database	MN473652
Gymnosarda unicolor	Custom 16s Fish Database	MN473653
Gymnothorax cribroris	Custom 16s Fish Database	MN473654
Gymnothorax thyrsoideus	Custom 16s Fish Database	MN473655
Halichoeres nigrescens	Custom 16s Fish Database	MN473656
Hemigymnus melapterus	Custom 16s Fish Database	MN473657
Herklotsichthys lippa	Custom 16s Fish Database	MN473658
Heteroclinus adelaidae	Custom 16s Fish Database	MN473659
Hippocampus barbiganti	NCBI	AY277286
Hippocampus barbouri	NCBI	AF354999
Hippocampus breviceps	NCBI	AY277287
Hippocampus camelopardalis	NCBI	AY277295
Hippocampus capensis	NCBI	AY277304
Hippocampus erectus	NCBI	AF355007
Hippocampus guttulatus	NCBI	AY277307
Hippocampus hippocampus	NCBI	AY277306
Hippocampus reidi	NCBI	AY277301
Hippocampus subelongatus	NCBI	AY277288
Hippocampus whitei	NCBI	AY277290
Hipposcarus cf. longiceps	Custom 16s Fish Database	MN473660
Hoplostethus gigas	Custom 16s Fish Database	MN473661
Hyporhamphus melanochir	Custom 16s Fish Database	MN473662
Hyporhamphus quoyi	Custom 16s Fish Database	MN473663
Inegocia japonica	Custom 16s Fish Database	MN473664

Inegocia japonica	Custom 16s Fish Database	MN473665
Inegocia japonica	Custom 16s Fish Database	MN473666
Iniistius jacksonensis	Custom 16s Fish Database	MN473667
Iniistius sp.	Custom 16s Fish Database	MN473668
Inimicus cf. sinensis	Custom 16s Fish Database	MN473669
Inimicus sinensis	Custom 16s Fish Database	MN473670
Istiompax indica	Custom 16s Fish Database	MN473671
Jaydia cf. ellioti	Custom 16s Fish Database	MN473672
Jaydia cf. poecilopterus	Custom 16s Fish Database	MN473673
Kathetostoma nigrofasciatum	Custom 16s Fish Database	MN473674
Katsuwonus pelamis	Custom 16s Fish Database	MN473675
Labroides dimidiatus	Custom 16s Fish Database	MN473676
Lactoria cornuta	Custom 16s Fish Database	MN473677
Lagocephalus lunaris	Custom 16s Fish Database	MN473678
Lagocephalus sceleratus	Custom 16s Fish Database	MN473679
Lagocephalus sceleratus	Custom 16s Fish Database	MN473680
Lates calcarifer	Custom 16s Fish Database	MN473681
Latropiscis purpurissatus	Custom 16s Fish Database	MN473682
Latropiscis purpurissatus	Custom 16s Fish Database	MN473683
Lepidotrigla papilio	Custom 16s Fish Database	MN473684
Leptoscarus vaigiensis	Custom 16s Fish Database	MN473685
Lethrinus erythracanthus	Custom 16s Fish Database	MN473686
Lethrinus genivittatus	Custom 16s Fish Database	MN473687
Lethrinus laticaudis	Custom 16s Fish Database	MN473688
Lethrinus miniatus	Custom 16s Fish Database	MN473689
Lethrinus nebulosus	Custom 16s Fish Database	MN473690
Lethrinus obsoletus	Custom 16s Fish Database	MN473691
Lethrinus punctulatus	Custom 16s Fish Database	MN473692
Lethrinus rubrioperculatus	Custom 16s Fish Database	MN473693
Lethrinus variegatus	Custom 16s Fish Database	MN473694
Lethrinus xanthochilus	Custom 16s Fish Database	MN473695
Lethrinus xanthochilus	Custom 16s Fish Database	MN473696
Leviprora inops	Custom 16s Fish Database	MN473697
Lutjanus argentimaculatus	Custom 16s Fish Database	MN473698
Lutjanus bitaeniatus	Custom 16s Fish Database	MN473699
Lutjanus bohar	Custom 16s Fish Database	MN473700
Lutjanus carponotatus	Custom 16s Fish Database	MN473701
Lutjanus erythropterus	Custom 16s Fish Database	MN473702
Lutjanus johnii	Custom 16s Fish Database	MN473703
Lutjanus malabaricus	Custom 16s Fish Database	MN473704
Lutjanus malabaricus	Custom 16s Fish Database	MN473705
Lutjanus rufolineatus	Custom 16s Fish Database	MN473706
Lutjanus russellii	Custom 16s Fish Database	MN473707

Lutjanus sebae	Custom 16s Fish Database	MN473708
Melichthys niger	Custom 16s Fish Database	MN473709
Meuschenia flavolineata	Custom 16s Fish Database	MN473710
Meuschenia freycineti	Custom 16s Fish Database	MN473711
Microcanthus strigatus	Custom 16s Fish Database	MN473712
Mobula japanica	Custom 16s Fish Database	MN473713
Mola mola	Custom 16s Fish Database	MN473714
Monacanthus chinensis	Custom 16s Fish Database	MN473715
Mugil cephalus	Custom 16s Fish Database	MN473716
Mugil cephalus	Custom 16s Fish Database	MN473717
Mulloidichthys cf. vanicolensis	Custom 16s Fish Database	MN473718
Naso cf. tonganus	Custom 16s Fish Database	MN473719
Neoglyphidodon melas	Custom 16s Fish Database	MN473720
Neopomacentrus filamentosus	Custom 16s Fish Database	MN473721
Neosebastes nigropunctatus	Custom 16s Fish Database	MN473722
Neosebastes pandus	Custom 16s Fish Database	MN473723
Neosebastes thetidis	Custom 16s Fish Database	MN473724
Netuma thalassina	Custom 16s Fish Database	MN473725
Nuchequula gerreoides	Custom 16s Fish Database	MN473726
Onigocia oligolepis	Custom 16s Fish Database	MN473727
Oplegnathus woodwardi	Custom 16s Fish Database	MN473728
Opthalmolepis lineolata	Custom 16s Fish Database	MN473729
Opthalmolepis lineolata	Custom 16s Fish Database	MN473730
Ostorhinchus aureus	Custom 16s Fish Database	MN473731
Ostorhinchus cf. apogonoides	Custom 16s Fish Database	MN473732
Ostracion cubicus	Custom 16s Fish Database	MN473733
Paracaesio xanthura	Custom 16s Fish Database	MN473734
Parachaetodon ocellatus	Custom 16s Fish Database	MN473735
Parachaetodon ocellatus	Custom 16s Fish Database	MN473736
Paracirrhites arcatus	Custom 16s Fish Database	MN473737
Paracirrhites forsteri	Custom 16s Fish Database	MN473738
Paracirrhites hemistictus	Custom 16s Fish Database	MN473739
Paramonacanthus choirocephalus	Custom 16s Fish Database	MN473740
Paramonacanthus choirocephalus	Custom 16s Fish Database	MN473741
Paramonacanthus choirocephalus	Custom 16s Fish Database	MN473742
Paramonacanthus choirocephalus	Custom 16s Fish Database	MN473743
Paramonacanthus filicauda	Custom 16s Fish Database	MN473744
Parapercis nebulosa	Custom 16s Fish Database	MN473745
Parapercis nebulosa	Custom 16s Fish Database	MN473746
Parma mccullochi	Custom 16s Fish Database	MN473747
Parma occidentalis	Custom 16s Fish Database	MN473748

Parma victoriae	Custom 16s Fish Database	MN473749
Parupeneus heptacanthus	Custom 16s Fish Database	MN473750
Parupeneus indicus	Custom 16s Fish Database	MN473751
Parupeneus indicus	Custom 16s Fish Database	MN473752
Pelates octolineatus	Custom 16s Fish Database	MN473753
Pempheris sp.	Custom 16s Fish Database	MN473754
Pempheris sp.	Custom 16s Fish Database	MN473755
Petroscirtes breviceps	Custom 16s Fish Database	MN473756
Phycodurus eques	NCBI	KY065585
Phyllopteryx taeniolatus	NCBI	AF355027
Pictilabrus laticlavius	Custom 16s Fish Database	MN473757
Plagiogeneion macrolepis	Custom 16s Fish Database	MN473758
Plagiogeneion rubiginosum	Custom 16s Fish Database	MN473759
Platax batavianus	Custom 16s Fish Database	MN473760
Platycephalus australis	Custom 16s Fish Database	MN473761
Platycephalus chauliodous	Custom 16s Fish Database	MN473762
Platycephalus conatus	Custom 16s Fish Database	MN473763
Platycephalus conatus	Custom 16s Fish Database	MN473764
Platycephalus endrachtensis	Custom 16s Fish Database	MN473765
Platycephalus longispinis	Custom 16s Fish Database	MN473766
Platycephalus speculator	Custom 16s Fish Database	MN473767
Platycephalus westraliae	Custom 16s Fish Database	MN473768
Platycephalus westraliae	Custom 16s Fish Database	MN473769
Plectorhinchus flavomaculatus	Custom 16s Fish Database	MN473770
Plectorhinchus flavomaculatus	Custom 16s Fish Database	MN473771
Plectorhinchus gibbosus	Custom 16s Fish Database	MN473772
Plectorhinchus polytaenia	Custom 16s Fish Database	MN473773
Plectorhynchus polytaenia	Custom 16s Fish Database	MN473774
Plectropomus areolatus	Custom 16s Fish Database	MN473775
Plectropomus leopardus	Custom 16s Fish Database	MN473776
Plectropomus maculatus	Custom 16s Fish Database	MN473777
Plectropomus maculatus	Custom 16s Fish Database	MN473778
Plectropomus oligacanthus	Custom 16s Fish Database	MN473779
Polydactylus cf. multiradiatus	Custom 16s Fish Database	MN473780
Polyprion cf. americanus	Custom 16s Fish Database	MN473781
Polyprion oxygeneios	Custom 16s Fish Database	MN473782
Pomadasys argyreus	Custom 16s Fish Database	MN473783
Pomadasys cf. maculatus	Custom 16s Fish Database	MN473784
Pomadasys kaakan	Custom 16s Fish Database	MN473785
Pomadasys maculatus	Custom 16s Fish Database	MN473786
Pomatomus saltatrix	Custom 16s Fish Database	MN473787
Priacanthus macracanthus	Custom 16s Fish Database	MN473788
Priacanthus tayenus	Custom 16s Fish Database	MN473789

Priacanthus tayenus	Custom 16s Fish Database	MN473790
Prionobutis microps	Custom 16s Fish Database	MN473791
Pristipomoides auricilla	Custom 16s Fish Database	MN473792
Pristipomoides filamentosus	Custom 16s Fish Database	MN473793
Pristipomoides multidens	Custom 16s Fish Database	MN473794
Pristipomoides sieboldii	Custom 16s Fish Database	MN473795
Pristipomoides typus	Custom 16s Fish Database	MN473796
Pristipomoides zonatus	Custom 16s Fish Database	MN473797
Pristotis obtusirostris	Custom 16s Fish Database	MN473798
Pristotis obtusirostris	Custom 16s Fish Database	MN473799
Protonibea diacanthus	Custom 16s Fish Database	MN473800
Psammoperca waigiensis	Custom 16s Fish Database	MN473801
Psettodes erumei	Custom 16s Fish Database	MN473802
Pseudorhombus argus	Custom 16s Fish Database	MN473803
Pseudorhombus arsius	Custom 16s Fish Database	MN473804
Pseudorhombus arsius	Custom 16s Fish Database	MN473805
Pseudorhombus cf. jenynsii	Custom 16s Fish Database	MN473806
Pseudorhombus jenynsii	Custom 16s Fish Database	MN473807
Pseudorhombus spinosus	Custom 16s Fish Database	MN473808
Ptereleotris microlepis	Custom 16s Fish Database	MN473809
Pterocaesio digramma	Custom 16s Fish Database	MN473810
Pterois mombasae	Custom 16s Fish Database	MN473811
Rachycentron canadum	Custom 16s Fish Database	MN473812
Randallichthys filamentosus	Custom 16s Fish Database	MN473813
Rhynchostracion nasus	Custom 16s Fish Database	MN473814
Rogadius asper	Custom 16s Fish Database	MN473815
Saloptia powelli	Custom 16s Fish Database	MN473816
Sardinella brachysoma	Custom 16s Fish Database	MN473817
Sardinella gibbosa	Custom 16s Fish Database	MN473818
Saurida tumbil	Custom 16s Fish Database	MN473819
Saurida undosquamis	Custom 16s Fish Database	MN473820
Scarus ghobban	Custom 16s Fish Database	MN473821
Scarus prasiognathus	Custom 16s Fish Database	MN473822
Scarus rivulatus	Custom 16s Fish Database	MN473823
Scatophagus argus	Custom 16s Fish Database	MN473824
Scomberomorus commerson	Custom 16s Fish Database	MN473825
Scomberomorus commerson	Custom 16s Fish Database	MN473826
Scomberomorus queenslandicus	Custom 16s Fish Database	MN473827
Selaroides cf. leptolepis	Custom 16s Fish Database	MN473828
Selaroides leptolepis	Custom 16s Fish Database	MN473829
Seriola dumerili	Custom 16s Fish Database	MN473830
Seriolina nigrofasciata	Custom 16s Fish Database	MN473831
Seriolina nigrofasciata	Custom 16s Fish Database	MN473832

Siganus canaliculatus	Custom 16s Fish Database	MN473833
Siganus canaliculatus	Custom 16s Fish Database	MN473834
Siganus canaliculatus	Custom 16s Fish Database	MN473835
Siganus cf. margaritiferus	Custom 16s Fish Database	MN473836
Siganus virgatus	Custom 16s Fish Database	MN473837
Sillago cf. sihama	Custom 16s Fish Database	MN473838
Sillago ingenua	Custom 16s Fish Database	MN473839
Sillago robusta	Custom 16s Fish Database	MN473840
Sillago schomburgkii	Custom 16s Fish Database	MN473841
Sillago schomburgkii	Custom 16s Fish Database	MN473842
Sillago schomburgkii	Custom 16s Fish Database	MN473843
Sillago sp.	Custom 16s Fish Database	MN473844
Sphyraena pinguis	Custom 16s Fish Database	MN473845
Stegastes cf. obreptus	Custom 16s Fish Database	MN473846
Stigmatopora argus	NCBI	AF355014
Symphorus nemaptophorus	Custom 16s Fish Database	MN473847
Syngnathus acus	NCBI	AF354991
Syngnathus temminckii	NCBI	JX228161
Syngnathus watermeyeri	NCBI	JX228163
Terapon cf. puta	Custom 16s Fish Database	MN473848
Terapon cf. theraps	Custom 16s Fish Database	MN473849
Thalassoma cf. hardwicke	Custom 16s Fish Database	MN473850
Thalassoma lunare	Custom 16s Fish Database	MN473851
Thalassoma lunare	Custom 16s Fish Database	MN473852
Thunnus tonggol	Custom 16s Fish Database	MN473853
Thyrsitoides marleyi	Custom 16s Fish Database	MN473854
Tilodon sexfasciatus	Custom 16s Fish Database	MN473855
Torquigener pallimaculatus	Custom 16s Fish Database	MN473856
Torquigener pallimaculatus	Custom 16s Fish Database	MN473857
Torquigener pleurogramma	NCBI	AY679674
Torquigener whitleyi	Custom 16s Fish Database	MN473858
Trachinocephalus myops	Custom 16s Fish Database	MN473859
Trachinotus baillonii	Custom 16s Fish Database	MN473860
Tragulichthys jaculiferus	Custom 16s Fish Database	MN473861
Upeneus asymmetricus	Custom 16s Fish Database	MN473862
Upeneus asymmetricus	Custom 16s Fish Database	MN473863
Upeneus asymmetricus	Custom 16s Fish Database	MN473864
Upeneus sulphureus	Custom 16s Fish Database	MN473865
Upeneus sundaicus	Custom 16s Fish Database	MN473866
Upeneus tragula	Custom 16s Fish Database	MN473867
Vanacampus margaritifer	NCBI	KY065603
Variola albimarginata	Custom 16s Fish Database	MN473868
Variola louti	Custom 16s Fish Database	MN473869

Variola louti	Custom 16s Fish Database	MN473870
Variola louti	Custom 16s Fish Database	MN473871
Yongeichthys cf. nebulosus	Custom 16s Fish Database	MN473872
Zabidius novemaculeatus	Custom 16s Fish Database	MN473873
Zeus faber	Custom 16s Fish Database	MN473874

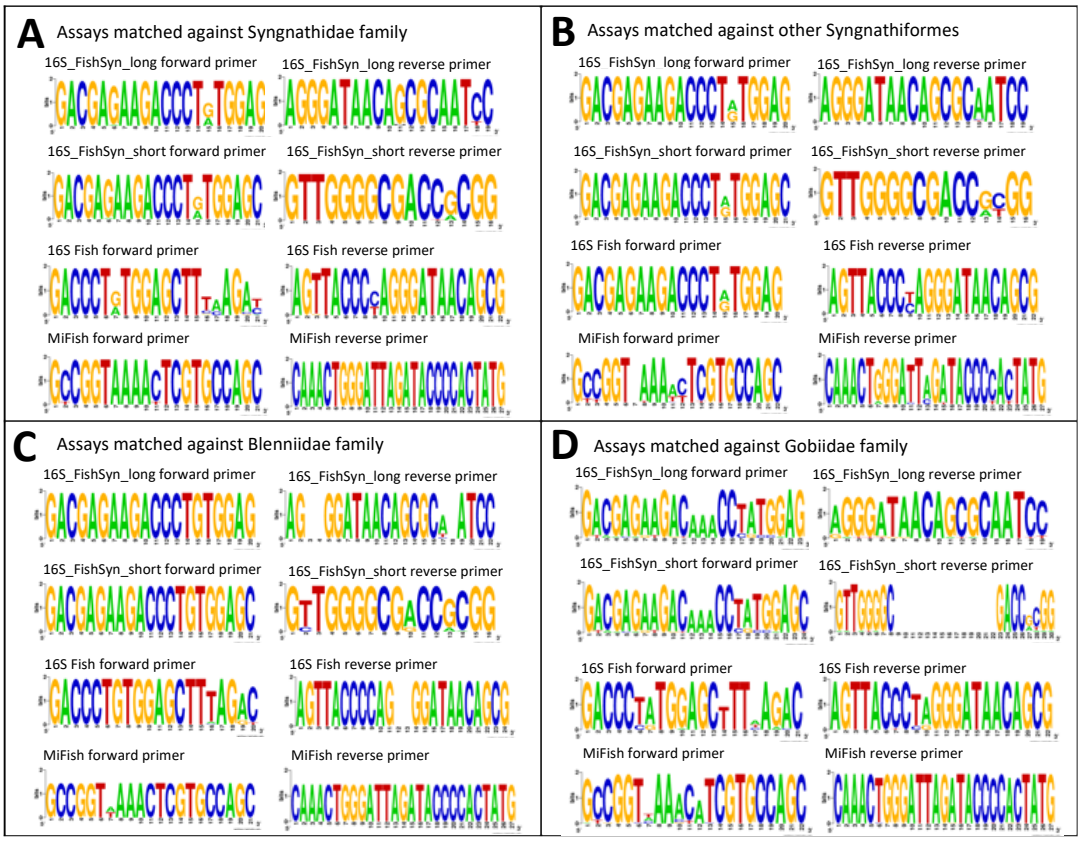

Figure S 2-1: Sequence logo plots for forward and reverse primers for the metabarcoding assays (16S_FishSyn_Long, 16S_FishSyn_short, 16S Fish and MiFish-U) used throughout this study.
Assays were matched against the Syngnathidae family (A), other Syngnathiformes not including Syngnathidae (B), the Blenniidae family (C), and the Gobiidae family (D). Figure was created using web logo https://weblogo.berkeley.edu/

Table S 2-3: Sampling details of the metabarcoding study including unique ID's, sample collectors, substrate type, depth, date and location details. All sampling was conducted over a week period in May 2018 via snorkelling and SCUBA, in Perth, Western Australia.

Sample ID	Collectors	Substrate	Depth (m)	Sampling country	State	Sampling Location	Latitude	Longitude	Sampling Date
F18_1111	GN & MDB	Bottom Water	6.2	Australia	Western Australia	Bicton Baths	-32.02.78	115.77.75	2-May-18
F18_1112	GN & MDB	Bottom Water	8.1	Australia	Western Australia	Bicton Baths	-32.02.77	115.77.76	2-May-18
F18_1113	GN & MDB	Bottom Water	7.6	Australia	Western Australia	Bicton Baths	-32.02.76	115.77.77	2-May-18
F18_1114	GN & MDB	Bottom Water	8	Australia	Western Australia	Bicton Baths	-32.02.75	115.77.78	2-May-18
F18_1115	GN & MDB	Bottom Water	9	Australia	Western Australia	Bicton Baths	-32.02.73	115.77.77	2-May-18
F18_1116	GN & MDB	Surface Water	<2	Australia	Western Australia	Bicton Baths	-32.02.78	115.77.76	2-May-18
F18_1117	GN & MDB	Surface Water	<2	Australia	Western Australia	Bicton Baths	-32.02.78	115.77.77	2-May-18
F18_1118	GN & MDB	Surface Water	<2	Australia	Western Australia	Bicton Baths	-32.02.76	115.77.78	2-May-18
F18_1119	GN & MDB	Surface Water	<2	Australia	Western Australia	Bicton Baths	-32.02.74	115.77.78	2-May-18
F18_1120	GN & MDB	Surface Water	<2	Australia	Western Australia	Bicton Baths	-32.02.74	115.77.80	2-May-18
F18_1121	GN & MDB	Bottom Water	8.3	Australia	Western Australia	Blackwall Reach	-32.01.99	115.78.37	2-May-18
F18_1122	GN & MDB	Bottom Water	7.5	Australia	Western Australia	Blackwall Reach	-32.01.98	115.78.39	2-May-18
F18_1123	GN & MDB	Bottom Water	8	Australia	Western Australia	Blackwall Reach	-32.01.96	115.78.41	2-May-18
F18_1124	GN & MDB	Bottom Water	9.1	Australia	Western Australia	Blackwall Reach	-32.01.96	115.78.42	2-May-18
F18_1125	GN & MDB	Bottom Water	7.4	Australia	Western Australia	Blackwall Reach	-32.01.94	115.78.43	2-May-18
F18_1126	GN & MDB	Surface Water	<2	Australia	Western Australia	Blackwall Reach	-32.02.00	115.78.37	2-May-18
F18_1127	GN & MDB	Surface Water	<2	Australia	Western Australia	Blackwall Reach	-32.01.99	115.78.38	2-May-18
F18_1128	GN & MDB	Surface Water	<2	Australia	Western Australia	Blackwall Reach	-32.01.98	115.78.40	2-May-18

F18_1129	GN & MDB	Surface Water	<2	Australia	Western Australia	Blackwall Reach	-32.01.97	115.78.41	2-May-18
F18_1130	GN & MDB	Surface Water	<2	Australia	Western Australia	Blackwall Reach	-32.01.97	115.78.42	2-May-18
F18_1131	GN & MDB	Surface Water	<2	Australia	Western Australia	Rockingham Wreck Trail	-32.27.27	115.73.04	3-May-18
F18_1132	GN & MDB	Surface Water	<2	Australia	Western Australia	Rockingham Wreck Trail	-32.27.25	115.73.07	3-May-18
F18_1133	GN & MDB	Surface Water	<2	Australia	Western Australia	Rockingham Wreck Trail	-32.27.24	115.73.11	3-May-18
F18_1134	GN & MDB	Surface Water	<2	Australia	Western Australia	Rockingham Wreck Trail	-32.27.22	115.73.12	3-May-18
F18_1135	GN & MDB	Surface Water	<2	Australia	Western Australia	Rockingham Wreck Trail	-32.27.21	115.73.14	3-May-18
F18_1136	GN & MDB	Bottom Water	11.2	Australia	Western Australia	Rockingham Wreck Trail	-32.27.25	115.73.03	3-May-18
F18_1137	GN & MDB	Bottom Water	9.7	Australia	Western Australia	Rockingham Wreck Trail	-32.27.21	115.73.07	3-May-18
F18_1138	GN & MDB	Bottom Water	10.1	Australia	Western Australia	Rockingham Wreck Trail	-32.27.18	115.73.11	3-May-18
F18_1139	GN & MDB	Bottom Water	11.9	Australia	Western Australia	Rockingham Wreck Trail	-32.27.16	115.73.15	3-May-18
F18_1140	GN & MDB	Bottom Water	10.5	Australia	Western Australia	Rockingham Wreck Trail	-32.27.13	115.73.15	3-May-18
F18_1141	GN & MDB	Surface Water	<2	Australia	Western Australia	Ammo Jetty	-32.12.42	115.75.88	3-May-18
F18_1142	GN & MDB	Surface Water	<2	Australia	Western Australia	Ammo Jetty	-32.12.40	115.75.87	3-May-18
F18_1143	GN & MDB	Surface Water	<2	Australia	Western Australia	Ammo Jetty	-32.12.41	115.75.85	3-May-18
F18_1144	GN & MDB	Surface Water	<2	Australia	Western Australia	Ammo Jetty	-32.12.39	115.75.83	3-May-18
F18_1145	GN & MDB	Surface Water	<2	Australia	Western Australia	Ammo Jetty	-32.12.40	115.75.80	3-May-18
F18_1146	GN & MDB	Bottom Water	6.5	Australia	Western Australia	Ammo Jetty	-32.12.41	115.75.86	3-May-18
F18_1147	GN & MDB	Bottom Water	6.8	Australia	Western Australia	Ammo Jetty	-32.12.40	115.75.84	3-May-18
F18_1148	GN & MDB	Bottom Water	7.5	Australia	Western Australia	Ammo Jetty	-32.12.40	115.75.82	3-May-18
F18_1149	GN & MDB	Bottom Water	8.6	Australia	Western Australia	Ammo Jetty	-32.12.39	115.75.81	3-May-18
F18_1150	GN & MDB	Bottom Water	8.9	Australia	Western Australia	Ammo Jetty	-32.12.39	115.75.79	3-May-18

F18_1151	GN & MDB	Surface Water	<2	Australia	Western Australia	Mt Henry	-32.03.60	115.85.64	8-May-18
F18_1152	GN & MDB	Surface Water	<2	Australia	Western Australia	Mt Henry	-32.03.60	115.85.66	8-May-18
F18_1153	GN & MDB	Surface Water	<2	Australia	Western Australia	Mt Henry	-32.03.60	115.85.68	8-May-18
F18_1154	GN & MDB	Surface Water	<2	Australia	Western Australia	Mt Henry	-32.03.59	115.85.70	8-May-18
F18_1155	GN & MDB	Surface Water	<2	Australia	Western Australia	Mt Henry	-32.03.58	115.85.72	8-May-18
F18_1156	GN & MDB	Bottom Water	4.4	Australia	Western Australia	Mt Henry	-32.03.59	115.85.68	8-May-18
F18_1157	GN & MDB	Bottom Water	4.8	Australia	Western Australia	Mt Henry	-32.03.58	115.85.70	8-May-18
F18_1158	GN & MDB	Bottom Water	5.6	Australia	Western Australia	Mt Henry	-32.03.57	115.85.70	8-May-18
F18_1159	GN & MDB	Bottom Water	5.2	Australia	Western Australia	Mt Henry	-32.03.56	115.85.73	8-May-18
F18_1160	GN & MDB	Bottom Water	4.7	Australia	Western Australia	Mt Henry	-32.03.54	115.85.71	8-May-18

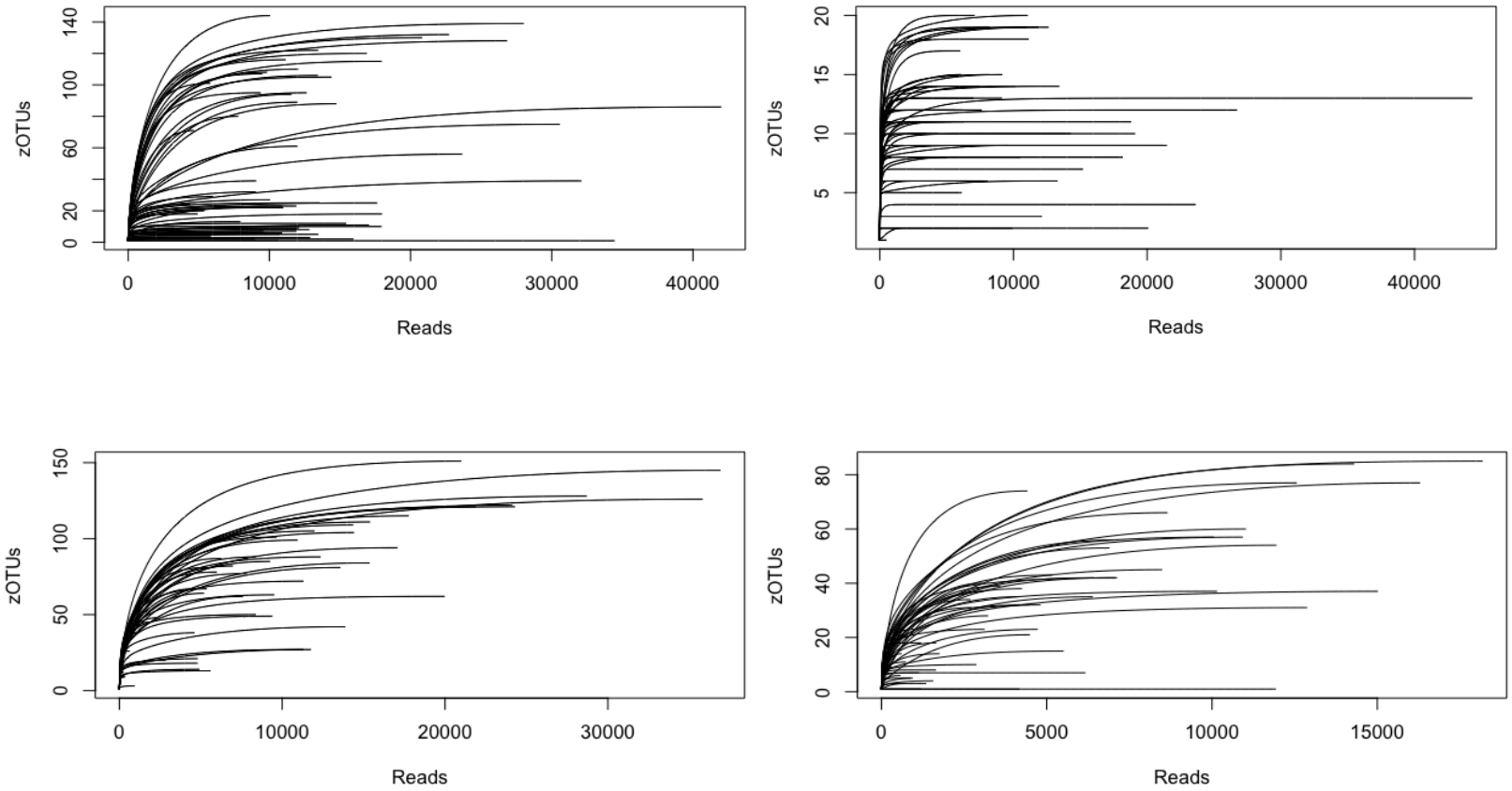

Figure S 2-2: ZOTU based rarefaction plots for each assay used in this study (16S Fish, MiFish, 16S_FishSyn_Long and 16S_FishSyn_Short).
Each assay used reaches asymptote, demonstrating that sequencing has been conducted with sufficient depth and eDNA samples and assays have achieved sufficient sequencing coverage.

Table S 2-4: Complete list of species detected in aquarium tank holding *Hippocampus subelongatus* from the Aquarium of Western Australia (AQWA) using fish metabarcoding assays 16S Fish, 16S_FishSyn_Long, 16S_FishSyn_Short, and MiFish.

Assignments with percentage identity above 98% were kept. Aquarium water is filtered and run on an interconnected circuit system, therefore there are other species DNA present in the aquarium system as reflected in the table below.

Order	Family	Species	Percent identity	16s Fish	16s_FishSyn_Long	16s_FishSyn_Short	MiFish
Perciformes	Sparidae	*Acanthopagrus australis*	100	0	12	24	0
Perciformes	Sparidae	*Acanthopagrus butcheri*	100	0	0	24	0
Perciformes	Sparidae	*Acanthopagrus butcheri*	99.55	0	12	0	0
Mugiliformes	Mugilidae	*Aldrichetta forsteri*	100	48640	17799	25257	19339
Mugiliformes	Mugilidae	*Aldrichetta forsteri*	98.718	341	117	19	0
Tetraodontiformes	Aracanidae	*Anoplocapros inermis*	98.225	0	0	0	976
Tetraodontiformes	Aracanidae	*Anoplocapros inermis*	98.507	3401	899	2530	0
Tetraodontiformes	Aracanidae	*Anoplocapros lenticularis*	98.81	0	0	0	976
Tetraodontiformes	Aracanidae	*Anoplocapros lenticularis*	98.507	3401	899	2530	0
Rhinopristiformes	Trygonorrhinidae	*Aptychotrema vincentiana*	100	150	41	882	0
Rhinopristiformes	Trygonorrhinidae	*Aptychotrema vincentiana*	99.5	883	256	0	0
Perciformes	Gobiidae	*Arenigobius bifrenatus*	99.497	4643	347	1584	0
Perciformes	Clinidae	*Cristiceps australis*	100	0	84	0	0
Perciformes	Clinidae	*Cristiceps australis*	99.552	0	84	0	0
Perciformes	Labridae	*Halichoeres brownfieldi*	100	9903	1276	1676	0
Syngnathiformes	Syngnathidae	*Hippocampus subelongatus*	100	0	3048	3398	0
Mugiliformes	Mugilidae	*Mugil cephalus*	99.415	0	0	0	829

Mugiliformes	Mugilidae	*Mugil cephalus*	100	21	35	3196	0
Perciformes	Pinguipedidae	*Parapercis haackei*	100	1386	511	1674	0
Perciformes	Blennidae	*Petroscirtes breviceps*	100	0	0	0	464
Perciformes	Blennidae	*Petroscirtes thepassii*	99.405	0	0	0	464
Perciformes	Blennidae	*Petroscirtes variabilis*	99.405	0	0	0	464

CHAPTER 3

Characterising the distribution of the critically endangered estuarine pipefish (*Syngnathus watermeyeri*) across its range using environmental DNA

3.1 Prologue

Chapter 2 explored the efficacy of eDNA metabarcoding for detecting Syngnathidae taxa in the context of other fish assemblages. This study demonstrated that eDNA approaches are poised to become an integral part of monitoring small-bodied cryptic taxa, and with ongoing optimisation, can assist in mapping species distributions and prioritising conservation areas. To further explore the potential of eDNA approaches for detecting cryptic small-bodied taxa, the following chapter develops a targeted eDNA toolkit for the detection of the critically endangered *Syngnathus watermeyeri* in estuary systems in South Africa.

South Africa's coastline spans approximately 3000 km, and is bound by the Atlantic Ocean in the west and the Indian Ocean in the east (Griffiths et al., 2010). The coastal region of South Africa encompasses four distinct biogeographical regions, namely the Cool Temperate, Warm Temperate, Subtropical, and Tropical (Turpie et al., 2000). Along this coastline lie an approximate 290 estuaries (van Niekerk et al., 2019). South African estuaries exhibit significant diversity in their physical and biological attributes, leading to the recognition of nine distinct ecosystem types (van Niekerk et al., 2019). These estuaries are characterized by their high variability, with rapid fluctuations in conditions such as salinity, temperature, turbidity, water currents, and dissolved oxygen concentrations occurring both spatially and temporally (Whitfield, 1994). These ecosystems are highly productive and support remarkable biodiversity, despite covering just 2% of the country's territory (van Niekerk et al., 2019). As a consequence, they are recognized as "super ecosystems" of significant ecological importance. However, South Africa's estuaries and the biodiversity they support are under threat, with more than 40% of estuary systems classified as degraded (van Niekerk et al., 2020, 2022; Whitfield, 1994).

Despite the presence of legislation and management tools, the conservation of estuaries in South Africa remains a significant and ongoing challenge. Approximately 65% of the country's estuarine area has undergone substantial alteration as a result of human activities, leading to adverse impacts on ecological processes and the diminished provision of ecosystem services by estuaries (van Niekerk et al., 2019).

South African estuaries face several major pressures, including altered flow regimes resulting from water abstraction and damming, pollution from wastewater discharge, stormwater inputs, and litter, overexploitation of living resources such as bait collection and fishing, and physical habitat alteration due to development and land use changes (van Niekerk et al., 2013). Moreover, the protection status of the country's estuaries is inadequate, with less than 1% of the estuarine area (representing less than 10% of estuarine ecosystem types) being effectively protected (van Niekerk et al., 2019). As a result, estuaries are now recognized as the most threatened ecological realm in South Africa (van Niekerk et al., 2019).

Estuaries are particularly important coastal systems for some syngnathids (Aylesworth et al., 2015; Claassens, Hodgson, et al., 2022; Masonjones et al., 2010; Whitfield et al., 2017). These unique and charismatic organisms are found in various estuarine habitats along the South African coastline. Estuaries serve as vital habitats for Syngnathid species, providing refuge against hydrodynamic forces and tidal regimes, acting as feeding and nursery grounds, and offering protection from predators (Claassens, de Villiers, et al., 2022; Shokri et al., 2009; Vincent et al., 1995). Despite their significance, knowledge gaps persist regarding the distribution and abundance of Syngnathid species within these estuarine systems. These gaps impede the effectiveness of conservation measures amidst mounting anthropogenic pressures and the loss of critical habitats.

The estuarine pipefish (*S. watermeyeri*) *is* endemic to South Africa, and is one of only two pipefish species in the world that is found in estuaries exclusively. The species is the only critically endangered member of the Syngnathid family, with an estimated 100-250 individuals remaining. Despite this, limited evidence on the development, implementation, or success of conservation actions exist (Claassens, de Villiers, et al., 2022; Stephenson et al., 2020). The following chapter implements a targeted eDNA survey across the historical range of *S. watermeyeri* to identify priority conservation areas, and provide a sensitive method of monitoring this imperilled species.

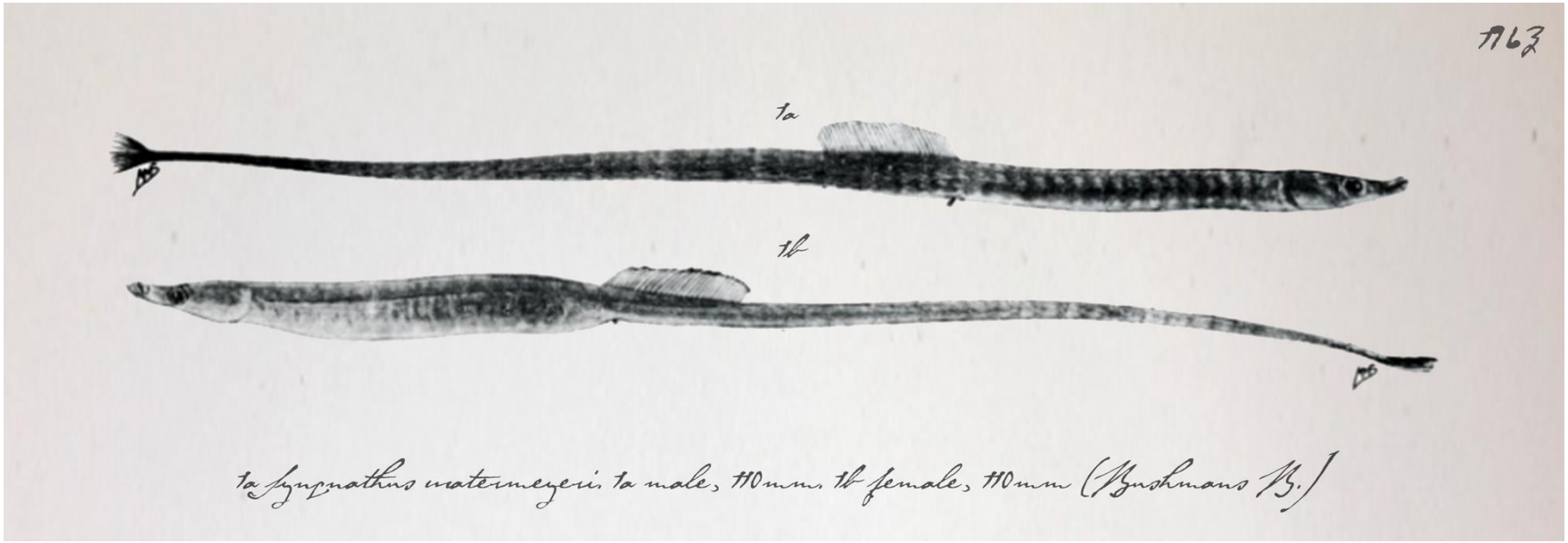

Figure 3-1: Male (3-1a) and female (3-1b) *Syngnathus watermeyeri* described by J.L.B. Smith and illustrated by M.M. Smith in 1963 in South Africa. Specimen 3-1b is a type specimen collected from the Bushmans River. Image is adapted from Smith, 1963.

3.1.1 Chapter acknowledgements

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For valuable field assistance, I thank Nina de Villiers. Lastly, I would like to acknowledge the Pawsey Supercomputing Centre (WA) for bioinformatic assistance. Cover photos for this chapter are the property of Jason Boswell, Nina de Villiers, and Louw Claassens, and have been included with permission.

3.1.2 Data accessibility

This chapter resulted in a manuscript that was published in the peer-reviewed journal *environmental DNA* (Nester et al., 2023, DOI: https://doi.org/10.1002/edn3.365). As first author, permission is automatically granted to reproduce this copyrighted material if it constitutes less than half of the total material in said publication. This chapter is a reproduction of the aforementioned manuscript. Raw sequencing data generated in this study has been uploaded to Zenodo and is publicly available under https://doi.org/10.5281/zenodo.5481837.

3.2 Abstract

The effective management of rare and threatened species, especially in areas where population sizes have diminished, relies on knowledge of their population size, threats, and distribution. Robust mapping of distribution presents a particular challenge in aquatic environments for cryptic species, especially those with low abundance. Environmental DNA (eDNA) approaches can offer improved detection rates of many rare and threatened species when compared with traditional sampling approaches. In this study, we developed and optimized a targeted eDNA assay for the critically endangered estuarine pipefish (*Syngnathus watermeyeri*). eDNA sampling and seine netting were undertaken at 39 sites across the historical range of *S. watermeyeri* in the Eastern Cape of South Africa in 2019. At each site, five water samples were collected for eDNA analysis (n = 195) along with three seine netting hauls (n = 117). Habitat and environmental data were collected at each location to explore what physical and biotic parameters might correlate with pipefish presence/absence. We successfully detected *S. watermeyeri* in two estuaries (Kariega and Bushmans) using both survey methods. Importantly, the positive detection rate of eDNA (66.7%) was four times that of seine netting (16.7%), highlighting the value of eDNA as a monitoring tool for rare and cryptic species. Null detections in the Kasouga, East Kleinemonde, and West Kleinemonde estuaries add to the growing body of evidence that the estuarine pipefish has been extirpated from these locations and is now only found in two estuarine systems. The occurrence of *S. watermeyeri* was found to be highly dependent on the cover of submerged macrophytes such as *Zostera capensis* (eelgrass). By providing a more complete picture of the conservation status of this critically endangered species, this work facilitates the development of a long-term monitoring program and the identification of priority conservation areas.

3.3 Introduction

Marine ecosystems are facing unprecedented levels of disturbance and biodiversity loss globally (Worm et al., 2006), with rare species often being the most vulnerable (Purvis et al., 2000). Species are generally considered rare if their abundances are low, geographical ranges are restricted, or they have restricted habitat tolerances (Gaston, 1994; Rabinowitz, 1981). Their scarcity and sparse distribution can increase the difficulties in sampling due to the time and specialized equipment that is often needed to avoid false negatives (Jones et al., 2002; Stoeckle et al., 2017). Furthermore, small population sizes often require the use of nondestructive or nonextractive sampling methodologies with minimal impacts on the species and the habitats and ecological communities that support them (De Brauwer et al., 2020; Pikitch, 2018). In complex aquatic habitats, these difficulties have led to inadvertent omissions of rare species in ecological studies or their overall underrepresentation (Chapman, 1999). Researchers have argued that rare species have a greater likelihood of extinction than more common species, due to either demographic stochasticity or increased sensitivity to habitat changes (Fagan et al., 2002; Laurance, 1991; Musick, 1999; Pimm et al., 1988).

The Syngnathidae family (pipefishes, pipehorses, seahorses, and seadragons) is a highly diverse group of fish with over 300 species distributed globally (Hamilton et al., 2017; Wilson & Orr, 2011). Syngnathids are known for their unusual morphology, remarkable crypsis, and specialized reproduction (Kendrick & Hyndes, 2005). Populations are extremely vulnerable to human impact and population declines due to life history traits such as low fecundity, restricted distributions, and limited mobility (Foster & Vincent, 2004; Jennions & Møller, 2003; Shokri et al., 2009). Many Syngnathidae species are considered threatened (up to 38%; Pollom et al., 2021) with population declines attributed to bycatch in commercial trawl fisheries, habitat degradation, use in traditional Chinese medicines, and exploitation for the aquarium trade (Lourie, 2000; Luo et al., 2015; Martin-Smith & Vincent, 2006; Vincent et al., 2011).

The estuarine pipefish (*Syngnathus watermeyeri*; Figure 3-2) is the only critically endangered member of the Syngnathidae family (Pollom, 2017). The total number of adult individuals remaining from the species is estimated to be between 100 and 250 (Pollom, 2017). The species is so imperiled that it was classified as extinct in 1994 (Whitfield & Bruton, 1996) before being rediscovered in 1996 (Cowley & Whitfield, 2001). *Syngnathus watermeyeri* is endemic to South African estuaries and has a restricted range in the Kariega and Bushmans estuaries, with historical populations in the East Kleinemonde, West Kleinemonde, and Kasouga estuaries now thought to be locally extinct (Cowley & Whitfield, 2001; Sheppard et al., 2011; Vorwerk et al., 2007; Whitfield et al., 2017; Whitfield & Bruton, 1996). However, to date, there is no species recovery or monitoring plan in place, and conservation sites have not been identified for the species. A complete understanding of the crisis facing *S. watermeyeri*, and the implementation of a recovery strategy, are currently limited by the difficulties of accurately determining their conservation status, distribution, and habitat requirements.

Historically, information on abundance and distribution of *S. watermeyeri* has primarily been collected through seine netting (Mwale et al., 2013; Ter Morshuizen & Whitfield, 1994; Vorwerk et al., 2008). This approach relies heavily on taxonomic expertise to distinguish between *S. watermeyeri* and its sister taxon *S. temminckii*, which is found in great abundance in the same habitats. Furthermore, seine netting can be logistically difficult given the species rarity, benthic nature, and association with dense seagrass habitats (Cowley & Whitfield, 2001; Vorwerk et al., 2007). Environmental DNA (eDNA) determines species presence by matching trace amounts of genetic material left in the environment to reference genetic databases (Ficetola et al., 2008). eDNA barcoding has been used for over a decade and its effectiveness in detecting rare and cryptic taxa, including Syngnathidae (Nester et al., 2020), in a range of marine environments is widely demonstrated (Nevers et al., 2018; Sakata et al., 2017; Sigsgaard et al., 2015). For the detection of rare and cryptic species, eDNA can be more sensitive and effective than traditional surveying methods (Dejean et al., 2012; Jerde et al., 2011; Piaggio et al., 2014) and has the added benefit of being a nondestructive and nonextractive sampling technique. The primary objective of this

study was to determine priority areas for the conservation of *S. watermeyeri* and compare this to historic distribution reports. The specific aims of the study were to: (i) assess the efficacy of eDNA in the detection of *S. watermeyeri*, (ii) compare the sensitivity of eDNA with that of seine netting to assist in the development of a long-term monitoring plan, and (iii) explore potential relationships between habitat or environmental variables and pipefish presence/absence.

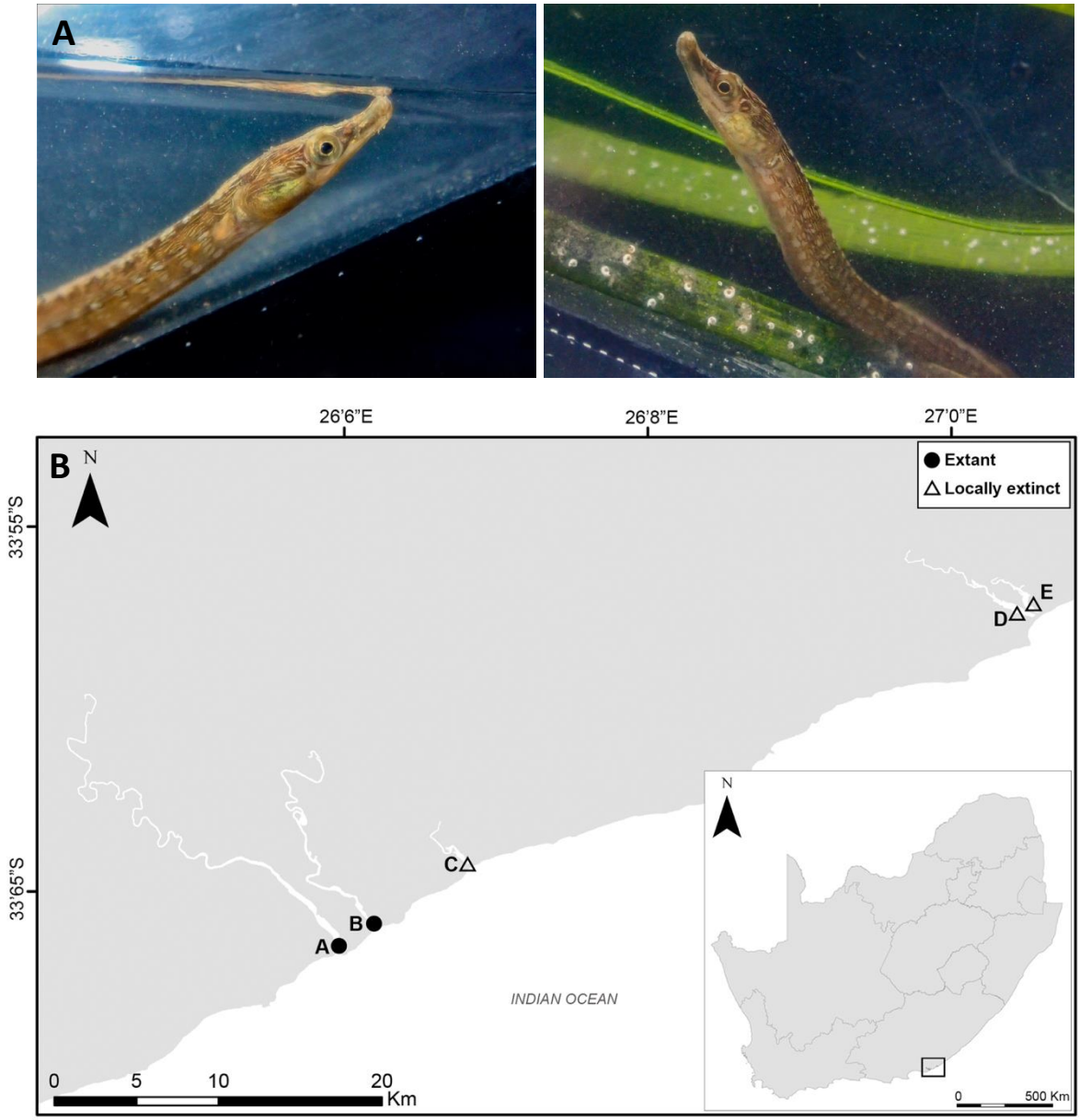

Figure 3-2: Photographs of *Syngnathus watermeyeri* and sampling locations.
(A) Syngnathus watermeyeri photographed (Louw Claassens) in the Bushmans estuary in October 2019. (B) Location of estuaries sampled (n = 5) within the historic range on the Eastern Cape of South Africa (insert). Extant populations (solid circles) - Bushmans (A) and Kariega (B) estuaries, and locally extinct populations (open triangles) - Kasouga (C), West Kleinemonde (D) and East Kleinemonde (E) indicated.

3.4 Methods

3.4.1 Assay development

3.4.1.1 Assay design

An eDNA assay for *S. watermeyeri* targeting a 140 base-pair (bp) region of the mtDNA cytochrome b (CytB) gene was developed using all publicly available sequences on NCBI (accession no. KY407926, KY407927, JX228139, and JX228140). To locate potential primer binding sites, sequences were aligned in Geneious (ver. 10.1.3 Biomatters Ltd) using MUSCLE with 10 iterations. The 140 bp region identified is a suitable length capable of amplifying degraded DNA isolated from environmental samples (Taberlet et al., 2018). The primer pair was designed to be free from secondary structures, and had balanced GC content and similar annealing temperatures for the forward and reverse (Table S 3-1). The *S. watermeyeri* sequences were then aligned (MUSCLE with 10 iterations) with all available sequences for the only other Syngnathidae species within its distribution (sister taxon *S. temminckii*), and 10 other nontarget co-occurring or closely related species. The assay was manually designed to preferentially amplify the target species and maximize the number of mismatches at the 3' end of the nontarget species (Table S 3-2). The Basal Local Alignment Search Tool (BLAST) was used to further validate the assay, ensuring a 100% match to the target species when blasted against the GenBank database. The following CytB assay, herein CytB_SW, was designed and used for the remainder of the study: CytB_SW_F 5' GCACCAATCTTGTCCAATGAATC 3' and CytB_SW_R 5' TCTTGTCAGCGTCCGAGTTC 3'.

3.4.1.2 Positive controls

To facilitate optimisation and specificity testing, genomic DNA (gDNA) was extracted (DNeasy Blood and Tissue kit, Qiagen) from the tissue of three specimens of *S. watermeyeri* (previously sampled by the South African Institute for Aquatic Biodiversity), and the total gDNA concentration was measured (Qubit Fluorometer, Thermo Fisher). A 10-fold serial dilution series ranging from 10−1 to 10−6 was established for each tissue-derived gDNA extract with starting concentrations ranging from 2.51 to 3.46 ng/µl. For specificity testing on potential co-occurring

species (*S. temminckii*), double-stranded gBlock fragments (Integrated DNA Technologies) were synthesized as tissue was not available (Table S 3-3). A double-stranded gBlock fragment of *S. watermeyeri* was also synthesized to determine the assay's limit of detection (LOD). The gBlock fragments for *S. watermeyeri* and two haplotypes of *S. temminckii* (starting concentrations: 1.25 × 1010, 2.29 × 1010, and 2.22 × 1010 copies/µl, respectively) were serially diluted ten-fold from 1 × 105 to 1 × 10−2 copies/µl. All qPCR plates included nontemplate controls and positive controls of undiluted *S. watermeyeri* gDNA.

3.4.1.3 Assay optimisation

Optimal assay conditions were determined by testing different annealing temperatures (°C), thermocycler conditions, and primer (nM) and magnesium chloride ($MgCl_2$) concentrations (mM) using various matrices. The conditions generating the lowest cycle threshold (Ct) value, strong singular peaks on melt curves, and a single visible band on agarose gel were considered to be optimal and implemented throughout the remainder of the study. Primer annealing temperatures calculated using Primer3 were 58.6°C and 60°C for the forward and reverse primer, respectively. However, the nearest neighbour annealing temperatures were calculated as 63.2°C and 65°C for the forward and reverse primer, respectively. To validate the optimal annealing temperature, a gradient qPCR was undertaken in duplicate from 58°C to 65°C (with 1°C increases) against each *S. watermeyeri* tissue-derived gDNA and gBlock fragment at a predicted 10,000 copies per reaction. Under a three-step cycling regime, three different sets of thermocycler conditions were simultaneously trialled across the qPCR gradient to enhance reaction specificity (Table 3-1).

Optimal primer and $MgCl_2$ concentrations were determined using various matrices with primer concentrations between 200 and 600 nM (100 nM increments) and $MgCl_2$ concentrations between 1.5 and 2.5 mM (0.5 mM increments). Combinations were assessed by qPCR against a dilution series of *S. watermeyeri* gDNA (in triplicate) using the optimized thermocycler conditions and annealing temperature (see 3.5.1).

All qPCR master mixes were prepared in a dedicated ultra-clean laboratory facility designed for trace DNA work at the TrEnD laboratory, Curtin University (Perth, Western Australia).

Table 3-1: Conditions of the three qPCR thermocycling protocols trialled for optimisation of the *Syngnathus watermeyeri* cytochrome b (CytB_SW) qPCR assay, with differences between the protocols highlighted in bold.

Protocol	Initial denaturation	Amplification (50 cycles)	Final extension
1	95°C for 5 min	95°C for **15 s** Annealing temp for 30 s 72°C for **20 s**	72°C for **1 min**
2	95°C for 5 min	95°C for **30 s** Annealing temp for 30 s 72°C for **30 s**	72°C for **5 min**
3	95°C for 5 min	95°C for **30 s** Annealing temp for 30 s 72°C for **45 s**	72°C for **10 min**

3.4.1.4 Assay specificity and sensitivity testing

Species-specificity was assessed by qPCR amplification of gBlock fragments of co-occurring sister taxon *S. temminckii*. The two CytB haplotypes chosen for specificity testing (accession no. KY407918 and KY407925) were the most genetically similar to *S. watermeyeri* (90.16% and 90.71%, respectively). For each haplotype, qPCR was performed on a dilution series (theoretical concentrations of 1×10^{-3} to 1×10^{5} copies/µl) below the predicted range of DNA concentrations found in environmental samples (Wilcox et al., 2013). qPCR was performed in duplicate using the predetermined optimal assay conditions (see *Assay optimisation and validation*, pg. 121). Melt curves were generated and amplicons were run on an agarose gel to confirm the expected size and assess potential nonspecific amplification (i.e., multiple bands) and primer dimer structures.

The ability of the CytB_SW assay to detect *S. watermeyeri* at low copy numbers was determined via a 10-fold serial dilution of the gBlock fragment from 5.17×10^{-7} ng/µl (theoretical 1000 copies/µl) to 5.17×10^{-13} ng/µl (theoretical 0.001 copy/µl). Each

dilution was run in ten qPCR replicates with 2 µl of the template using the specified optimal assay conditions (see *Assay optimisation and validation*, pg. 121). Data from the replicate curves were analyzed in RStudio (v 1.4.1106) to determine the LOD and LOQ of the assay (Klymus et al., 2020; Merkes et al., 2019).

3.4.2 Criteria for positive detection

For an eDNA sample to be considered a positive detection for *S. watermeyeri*, requirements were as follows: (1) at least one qPCR replicates generating a Ct value consistent with the assays LOD per field replicate; (2) absence of nonspecific bands and peaks on agarose gel and melt curve analysis; (3) no amplification in extraction or non-template (negative) controls and; (4) 100% sequence identity of the amplified fragment to *S. watermeyeri*.

3.4.3 Field collection

3.4.3.1 Site description

Sampling was conducted at five estuaries in the Eastern Cape of South Africa: Kariega, Bushmans, Kasouga, East Kleinemonde, and West Kleinemonde (Figure 3-2; Table S 3-4). These estuaries were chosen as two are known habitats for *S. watermeyeri* (Kariega and Bushmans; Vorwerk et al., 2007) and three have historical reports of populations (Kasouga, East Kleinemonde, and West Kleinemonde; Cowley & Whitfield, 2001; Whitfield & Bruton, 1996; Whitfield et al., 2017). These estuaries also provide a habitat for the most common pipefish found in South African estuaries, sister taxon *S. temminckii* (Mwale et al., 2014).

The Kariega and Bushmans estuaries are permanently open systems, 18 and 33 km long, respectively (Harrison, 2004; Vorwerk et al., 2008). Although originating from two different catchments, the mouths of these estuaries are only 2 km apart. These estuaries are marine-dominated due to freshwater deprivation, damming, and flow restrictions that have arisen from several impoundments (Hodgson, 1987; Ter Morshuizen & Whitfield, 1994; Vorwerk et al., 2008). As a result of the increased salinity in these estuaries, *Zostera capensis* (eelgrass) and *Codium* spp. (algae) are the

most dominant submerged macrophyte species (Adams & Talbot, 1992). By contrast, the remaining three estuaries are smaller temporarily open/closed systems dominated by the largest freshwater spiral ditchgrass *Ruppia cirrhosa* due to less marine influence and typically lower salinity levels (Cowley & Whitfield, 2001; Henninger et al., 2008; Tweddle, 2004; Whitfield et al., 2008).

3.4.3.2 Sample collection

This research was conducted under authorization received from the South African Department of Environmental Affairs (permit no. RES2019/37). Ethical authorization for the research was received from the Rhodes University Animal Research Ethics Committee (permit no. 2019–0853-921). Pipefish were handled ethically in accordance with the National Health and Medical Research Council of Australia.

A total of 195 water samples were collected for eDNA and 117 seine hauls were undertaken at 39 sites across the five estuaries over 3 weeks in October 2019 (Table S 3-4). The sampling effort was concentrated in known locations of the estuarine pipefish (Kariega and Bushmans) with 15 sites in each estuary. Three sites were sampled in each of the remaining smaller estuaries (Kasouga, East Kleinemonde, and West Kleinemonde). Sites were chosen randomly but separated by ~600 m to decrease the likelihood of encountering transported DNA (Jeunen et al., 2019) or a previously sampled pipefish, and to allow for sampling to be conducted within a feasible time frame. The exception was site 15 within the Bushmans estuary, which was in the upper reaches ~9 km from the nearest site due to boat access constraints. This was also the uppermost navigable limit of the Bushmans estuary, with suitable habitat and vegetation only found further downstream at site 14.

For eDNA analysis, five 1 liter water samples (n = 195) were collected at each of the 39 sites from the surface (0 – 1 m deep) using sterile Nalgene bottles. Water samples were collected prior to seine netting and kept on ice. Water samples were individually filtered within 3 h of collection using a Pall Sentino Microbiology peristaltic pump (Pall Corporation) onto 47 mm cellulose filter membranes. A 45 µm pore size was

used to capture sufficient DNA while decreasing filtration time and preventing clogging due to high turbidity and sediment load present in the samples (Kumar et al., 2022). Between the filtration of each replicate, all filtering equipment was soaked in 10% bleach for a minimum of 10 min and rinsed with desalinated and filtered water. One litre samples of the bleach and desalinated tap water were taken at the end of each filtering day to be included as filtration controls. These filtration controls aim to detect potential cross-contamination in water filtering between samples and sites. Water membranes were frozen in 540 µl of ATL lysis buffer (Qiagen), transported to a quarantine facility within the Trace & Environmental DNA (TrEnD) Laboratory (Perth, Western Australia), and stored at −20°C until further processing.

At each site, three replicate seine net sweeps (5 × 1 m) separated by 15 m were conducted. Sweeps ran parallel to shore and allowed for 15 m2 to be surveyed at each site. All pipefish found were placed in separate containers with water from the estuary and identified to species level by following the identification key in Mwale et al. (2014). Following this, pipefish were immediately released back to the capture site. At each sampling location, salinity, pH, and temperature (°C) were recorded using an OTT Hydrolab HL4 sonde. Additionally, habitat availability was assessed by determining vegetation type and percentage of vegetation cover determined visually using the area of the seine net (1 × 5 m). Owing to distinct differences in habitat type and a lack of cover, comparable habitat variables could not be recorded at Kasouga, East Kleinemonde, and West Kleinemonde.

3.4.4 eDNA sample processing

Water membranes stored in 540 µl of ATL lysis buffer were thawed and extracted using a DNeasy Blood and Tissue kit (Qiagen) with the following modifications: addition of 60 µl of Proteinase K during the cell lysis phase and incubated at 56°C for 12 h. Extraction controls were included for every site and extracted alongside the water samples to detect any laboratory or between-sample contamination. Extraction was completed using a QIAcube (Qiagen) DNA extraction system with DNA eluted in 100 µl of AE buffer. Extracts were assessed through qPCR for quality and to

optimize levels of DNA input with the following dilutions: neat, 1/5, 1/10, and 1/100. qPCR was performed with 4 µl of eDNA template (at optimized dilution) using the CytB_SW assay under the optimized reaction conditions (see *Assay optimisation and validation*, pg. 121). Each PCR plate included no-template, positive controls, and calibration standards (gBlock dilutions). Three qPCR replicates were implemented for each field replicate (n = 5) across the 39 sites.

To confirm assay specificity, all field replicates that amplified were sequenced. DNA extraction and no-template controls showed no sign of amplification. To facilitate sequencing, custom fusion-tagged primers were utilized consisting of an Illumina sequencing adapter, a unique multiple identifier (MID), and the CytB_SW primer sequence. Amplicon size was assessed prior to sequencing on an agarose gel and an automated capillary electrophoresis system (QIAxcel Advanced System; Qiagen), accounting for the size of the single-fusion MID-tag primers used. Amplicons were pooled in approximate equimolar ratios and purified using a QIAquick PCR Purification Kit (Qiagen). The resulting library was quantified using a QIAxcel Advanced System and a Qubit Fluorometer (Thermo Fisher). The library was sequenced on an Illumina Miseq platform using a 300-cycle V2 Nano reagent kit (Illumina) following the manufacturer's guidelines (raw sequencing data: https://doi.org/10.5281/zenodo.5481837).

3.4.5 Bioinformatics and taxonomic assignment

Bioinformatics and taxonomic assignments of sequencing data were performed using eDNAFlow, a fully automated workflow that processes eDNA data from raw sequences to curated and noncurated zero-radius operational taxonomic units (ZOTUs) and their abundance tables (Mousavi-Derazmahalleh et al., 2021). Sequences were quality filtered, demultiplexed, denoised, and erroneous sequences were removed using a combination of AdapterRemoval (Schubert et al., 2016), OBITools (Boyer et al., 2016), Usearch (Edgar, 2010), and LULU (Frøslev et al., 2017) via Zeus, an SGI Linux cluster based in the Pawsey Supercomputing Centre.

For taxonomic assignments, quality filtered ZOTUs were queried against the NCBI GenBank nucleotide database using a Lowest Common Ancestor (LCA) Python script within eDNAFlow (Mousavi-Derazmahalleh et al., 2021). The thresholds of percentage identity and query coverage were set above 70% and 80%, respectively. These bioinformatic and taxonomic thresholds and quality parameters were kept relaxed to determine the specificity of the primer and detect any potential nontarget species. Taxonomic identities of ZOTUs were distinguished by percentage similarity of the BLAST hit, 100% similarity was required for positive detection of *S. watermeyeri*.

3.4.6 Statistical analysis

Multivariate analyses were conducted using presence/absence (PA) data only due to current limitations in quantitative approaches to relative read abundance. Differences in PA between the estuaries were explored using Primer v.7 (Anderson, 2001) with the PERMANOVA+ (Anderson et al., 2008) add-on. Data were transformed into PA format, and a Jaccard resemblance matrix (Schaalje & Beus, 1997) was created. A permutational multivariate analysis of variance (PERMANOVA) was conducted using 9999 unrestricted permutations of raw data. In the presence of significant differences, pairwise comparisons were performed to determine where they occurred.

As *S. watermeyeri* was not detected with either method (eDNA or seine) in three of the five estuaries, further analyses were focused on estuaries with positive detections. A Wilcoxon rank sum test was conducted in RStudio (RStudio Team, 2015) to compare the positive detections of *S. watermeyeri* between the methodologies used. Data were then transformed using Primer into PA format and a Jaccard resemblance matrix (Schaalje & Beus, 1997) was created. A distance-based linear model (DistLM; with adjusted R^2 selection criterion, best selection procedure, and 9999 permutations) was conducted on the Jaccard resemblance matrix in Primer to test the relationship between *S. watermeyeri* presence and habitat or environmental variables (temperature, salinity, pH, and vegetation percentage cover). The fitted

values were then visualized in Primer using dissimilarity-based redundancy analysis (dbRDA) ordination.

3.5 Results

3.5.1 Assay optimisation and validation

We successfully developed, optimized, and validated a sensitive and species-specific assay targeting a 140 bp region of *S. watermeyeri* cytochrome b. Assay optimisation experiments resulted in a 25 µl qPCR assay containing 0.5 µM forward and reverse primer, 2 mM $MgCl_2$, 1× AmpliTaq Gold PCR buffer, 1 U AmpliTaq Gold DNA polymerase (Applied Biosystems), 0.25 mM dNTPs (Astral Scientific), 0.1 mg BSA (Fisher Biotec), 0.6 µl of 5X SYBR Green dye (Life Technologies), and 2 µl template (4 µl for eDNA samples), made to volume with ultrapure water (Life Technologies). A three-step cycling regime with thermocycler conditions of 95°C for 5 min, followed by 50 cycles of 95°C for 15 s, 61°C for 30 s, 72°C for 20 s, with a final extension of 72°C for 1 min provided the greatest qPCR reaction specificity.

The optimized assay uses 0.5 µM of forward and reverse primer in a 25 µl reaction with the above conditions and an annealing temperature of 61°C. Specificity testing resulted in no amplification from gBlock fragments of the nontarget species *S. temminckii*, where the gBlock of the target species successfully amplified in all 10 qPCR replicates from 5.17×10^{-5} ng/µl (10^5 copies/µl) to 5.17×10^{-9} ng/µl (10 copies/µl). Melt curve analysis confirmed the product melted at 82°C, with primer dimer not visible on melt curve plots or through an agarose gel. The modelled LOQ for the assay was determined to be 25 copies/µl, and the effective LOD using three qPCR replicates for each biological replicate was 0.82 copies/µl (Figure 3-3).

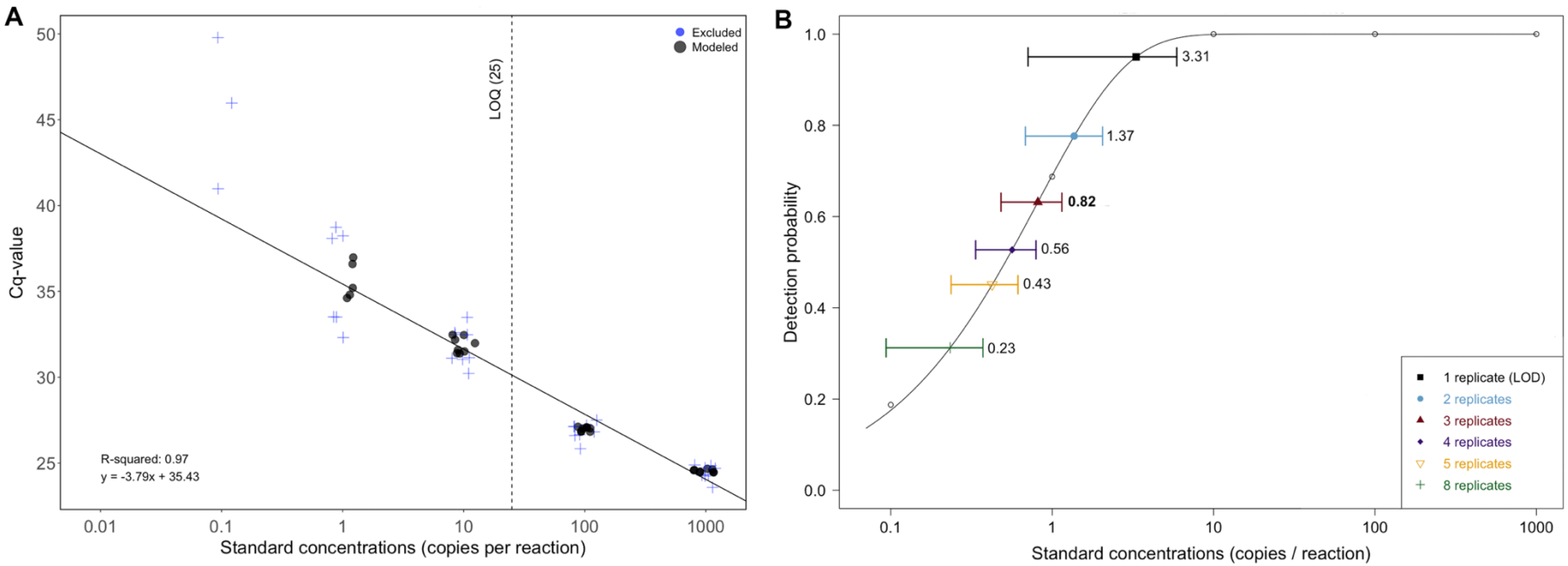

Figure 3-3: Limit of detection and limit of quantification plot for CytB_SW assay.

(**A**) Standard curve generated for the CytB_SW assay on a 10-fold serial dilution of a. *S. watermeyeri* cytochrome b gBlock fragment. Dilutions were run in 10 qPCR replicates with concentrations between 5.17 x 10^{-7} ng/µl (theoretical 1000 copies/µl) and 5.17 x 10^{-13} ng/µl (theoretical 0.001 copies/µl). The LOQ of the assay (25 copies/µl) is indicated by the dotted line. (**B**) the LOD for CytB_SW for differing amounts of qPCR replicates per field replicate, as determined by a two-parameter Weibull type II function. The effective LOD implemented in this study, 3 replicates, is indicated in bold text (0.82).

3.5.2 Field testing

The newly designed CytB_SW assay successfully discriminated between the two *Syngnathus* species in the region, with sequencing confirming that the amplified fragments consisted only of the intended target sequence and not sister taxon *S. temminckii* or other nontarget species (Table S 3-5). We did not detect *S. watermeyeri* in any of the negative fields, DNA extraction, or qPCR controls. The estuarine pipefish was detected in two of the five estuaries (Kariega and Bushmans, Figure 3-4). Using either eDNA and/or seine netting, it was detected at a total of 20 of the 39 (51.3%) sampled locations. More specifically, the pipefish was detected at nine sites in the Kariega estuary and 11 sites in the Bushmans estuary (Figure 3-4).

eDNA detected *S. watermeyeri* at a total of 20 of the 30 sites (66.7%) across the Kariega and Bushmans estuaries. In comparison, seine netting detected the pipefish at a total of five sites (16.6%) across the two estuaries. A Wilcoxon rank sum test (α=0.05) found that the number of sites with positive pipefish detections was significantly higher using eDNA methodologies than seine netting ($p < 0.001$). eDNA detected *S. watermeyeri* at 15 sites where no individuals were caught via seine netting (Table 3-2), equivalent to 75% of the total positive detections. Conversely, all positive detections using seine netting occurred at sites with positive eDNA detections. For all positive site detections, seine netting found pipefish in 1 of 3 replicates (33.3%). Detections were more variable within eDNA water replicates, with positive detections ranging from 1 of 5 replicates to all replicates (Figure 3-4).

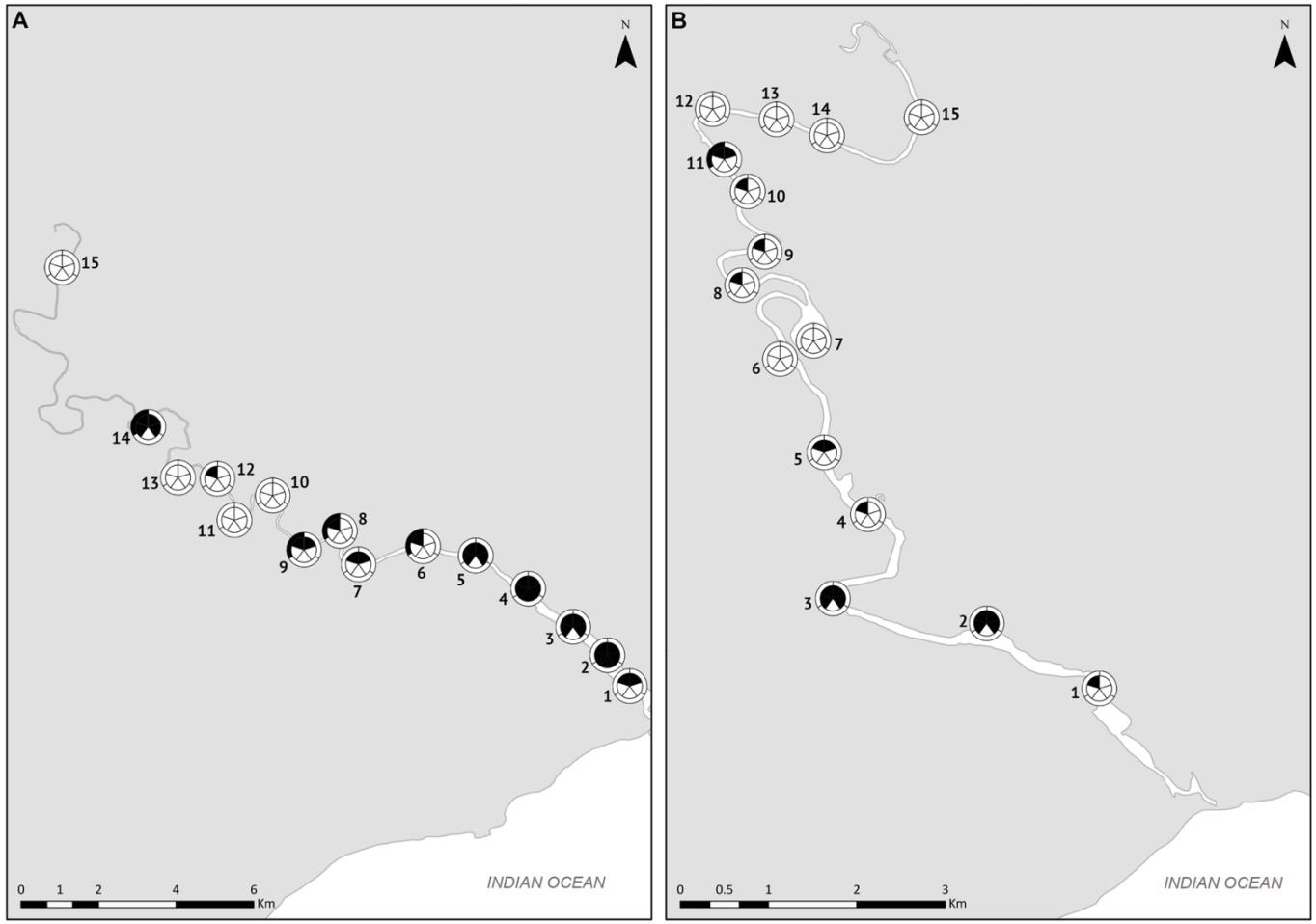

Figure 3-4: Survey sites in the Bushmans (A) and Kariega (B) estuaries in South Africa in October 2019.

A total of 15 sites were sampled in each estuary (n=30) with 5 x 1 L eDNA water samples and 3 seine net sweeps (5 x 1 m) collected at each site. The pie represents the number of eDNA water replicates taken, a shaded wedge indicates a positive eDNA hit for *S. watermeyeri* as confirmed through sequencing. The surrounding ring represents the number of seine net sweeps conducted with a shaded ring indicating a positive identification of *S. watermeyeri.*

Table 3-2: Comparison of detections and non-detections using eDNA methodologies and seine net sweeps for 30 sites across the Bushmans and Kariega estuaries in South Africa. Table shows the number of sites and the proportion of sites in parentheses.

	eDNA detection	eDNA non-detection
Seine detection	5 *(0.16)*	0 *(0)*
Seine non-detection	15 *(0.50)*	10 *(0.33)*

The five estuaries showed significant differences in PA composition (*Pseudo-*$F_{[4]} = 11.344$, $p < 0.001$), with pairwise comparisons confirming all sites, were significantly different from one another ($p < 0.05$). DistLM analysis revealed the measured environmental variables (salinity, temperature, pH, and percentage vegetation cover) explained 14.2% of the total variation in the PA of *S. watermeyeri* within the Kariega and Bushmans estuaries (Figure 3-5). Percentage cover was found to be the only significant variable ($p < 0.001$), explaining 7.6% of the total variation. Sites with positive pipefish detections had an average habitat coverage of 82.5% (±3.34%) compared with 48.2% (±6.31%) at sites with no pipefish detections. The Kariega and Bushmans estuaries were dominated by *Zostera capensis* (eelgrass) and *Codium* spp. (algae; Table 3-3). The remaining estuaries were comparatively barren or characterized by patchy *Ruppia cirrhosa* (spiral ditchgrass) habitats and dense filamentous green algae in the water column (Table 3-3).

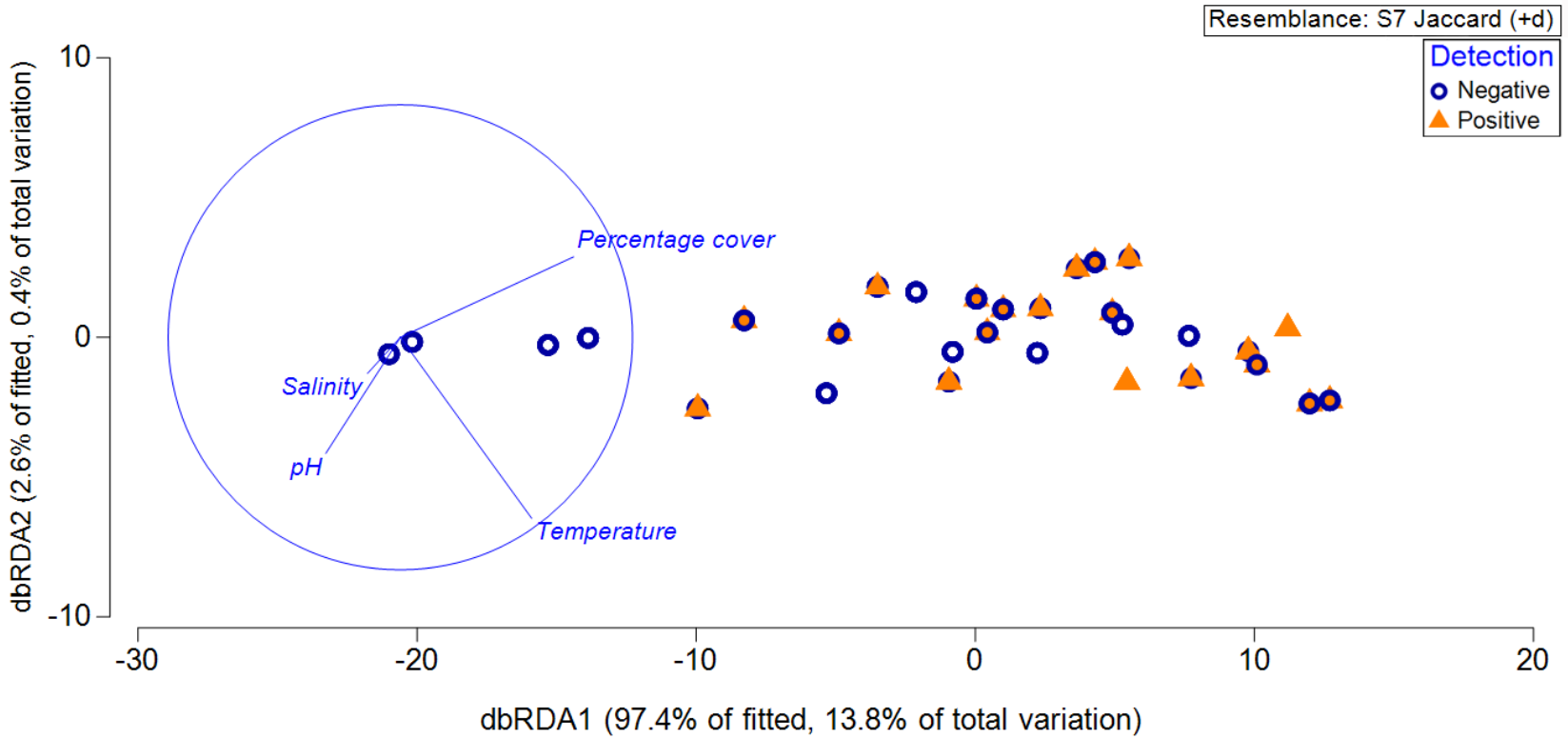

Figure 3-5: Distance-based redundancy analysis (dbRDA) plot for environmental variables influencing *S. watermeyeri* presence in the Kariega and Bushmans estuaries.

Distance-based redundancy analysis (dbRDA) plot illustrating the DistLM results for the effects of environmental variables on positive and negative detections of *S. watermeyeri* across the Kariega and Bushmans estuaries. Vector length is proportional to their contribution to the total variation explained by the model.

Table 3-3: Environmental and habitat variables (temperature (°C), salinity, pH, percentage cover and dominant vegetation) recorded at five estuaries (Kariega, Bushmans, Kasouga, East Kleinemonde and West Kleinemonde) in South Africa in October 2019. Percentage cover could not be recorded at the smaller estuaries (Kasouga, East Kleinemonde and West Kleinemonde).

Estuary	Environmental/habitat variables	Mean	Range
Kariega	*Temperature*	20.19 ± 0.10	19.10-21.07
	Salinity	35.33 ± 0.06	34.77-35.87
	pH	8.30 ± 0.01	8.25-8.42
	Percentage cover	80.67 ± 3.61	10-100
	Dominant vegetation	*Zostera capensis* and *Codium* spp.	
Bushmans	*Temperature*	22.65 ± 0.11	21.28-23.74
	Salinity	36.36 ± 0.06	35.71-37.14
	pH	8.27 ± 0.01	8.14-8.37
	Percentage cover	61.47 ± 4.63	0-100
	Dominant vegetation	*Zostera capensis* and *Codium* spp.	
Kasouga	*Temperature*	17.93 ± 0.04	17.81-18.06
	Salinity	32.55 ± 0.01	32.52-32.57
	pH	8.2 ± 0	8.19-8.20
	Dominant vegetation	Barren	
East Kleinemonde	*Temperature*	18.26 ± 0.03	18.17-18.35
	Salinity	32.48 ± 0.01	32.45-32.50
	pH	8.19 ± 0	8.19-8.19
	Dominant vegetation	*Ruppia cirrhosa* and filamentous green algae	
West Kleinemonde	*Temperature*	17.93 ± 0.04	17.81-18.69
	Salinity	32.55 ± 0.01	32.52-32.43
	pH	8.20 ± 0	8.19-8.20
	Dominant vegetation	*Ruppia cirrhosa* and filamentous green algae	

3.6 Discussion

This study demonstrates the efficacy of eDNA biomonitoring for detecting *S. watermeyeri* in water samples collected from estuarine habitats. This is consistent with the rapidly growing body of research validating eDNA as a powerful tool for detecting rare, threatened and small-bodied species in freshwater and marine ecosystems (Nester et al., 2020; Sakata et al., 2017; Schmelzle & Kinziger, 2016; Weltz et al., 2017). By confirming the species presence in the Kariega and Bushmans estuaries, this study adds to the increasing evidence that *S. watermeyeri* is restricted to these two estuaries and local extinctions have occurred in the Kasouga and the East and West Kleinemonde estuaries (Claassens et al., 2022; Cowley & Whitfield, 2001; Sheppard et al., 2011; Vorwerk et al., 2007; Whitfield et al., 2017; Whitfield & Bruton, 1996).

We developed, tested, and optimized a targeted assay for the detection of the critically endangered *S. watermeyeri*, capable of amplifying low concentrations of target DNA and excluding closely related, co-occurring species (*S. temminckii*). Designing highly specific and sensitive assays is particularly important for the detection of rare species as the target DNA copy number is likely to be very low (Wilcox et al., 2013). Although the developed assay has demonstrated specificity and increased sensitivity over seine netting, we recognize its limitations and advocate for the development of a probe to enhance sensitivity. The current use of this assay without a probe necessitates the use of agarose gel, melt point curves, and sequencing to confirm the presence of *S. watermeyeri*. Future use of the assay should employ Sanger sequencing over next-generation sequencing to reduce overall costs. However, the improved detection probability over seine netting demonstrates its ability to inform future conservation efforts and to be incorporated as a central component of future monitoring and recovery plans for this critically endangered species. Future monitoring efforts in known locations should include exhaustive eDNA and habitat surveys to better understand distribution patterns and facilitate regular monitoring of seasonal or temporal changes.

Surveying the known range of *S. watermeyeri* confirmed its presence in the Bushmans and Kariega estuaries, with no detections in the Kasouga, East, or West Kleinemonde estuaries. Vegetation cover (%) was found to be the most significant environmental variable contributing to pipefish presence or absence. Previous surveys in the Kariega and East Kleinemonde estuaries also found the presence and abundance of the species to be highly dependent on dense beds of submerged macrophytes like *Zostera capensis* (Vorwerk et al., 2007; Whitfield & Cowley, 2018). In conjunction with a recent study (Claassens et al., 2022), we identified *Codium* spp., which forms large free-floating beds among Z. capensis, as an important habitat supporting *S. watermeyeri* population. Studies have shown that increasing macrophyte and habitat complexity leads to larger overall invertebrate abundance and diversity in comparison to structurally simpler habitats (Gartner et al., 2013; Veiga et al., 2014). Therefore, *Codium* present throughout the Bushmans and Kariega estuaries may provide increased prey availability (small crustaceans) and refuge for the cryptic *S. watermeyeri* (Whitfield, 1995). We recommend vegetation mapping across estuaries within the region to identify potential locations to re-introduce this critically endangered species. The success and possible expansion of these reintroductions can be monitored with eDNA surveys.

The Kariega and Bushmans estuaries have been subject to long-term decreases in freshwater flows exacerbated by drought in recent years, resulting in reversed salinity gradients established after 2015 (Wasserman et al., 2020). Vorwerk et al. (2007) suggested that the re-emergence of *S. watermeyeri* in the Kariega estuary was related to the reinstatement of a salinity gradient; however, the presence of the species during this survey suggests that it may not be as sensitive to increased salinity as previously thought. Freshwater deprivation and the resulting saline conditions likely promoted the expansion of *Zostera* and *Codium* beds throughout the estuaries (Adams & Talbot, 1992) and, as a result, may have been beneficial for estuarine pipefish populations. However, if the deprivation of freshwater inflows and drought persists, there is a possibility that the mouths of these estuaries may close. Mouth closure can cause shifts in the state of macrophyte habitats (Riddin & Adams, 2010), which in turn would have profound implications for estuarine pipefish populations.

Pipefish populations in these estuaries are also threatened by potential floods, which can completely remove submerged macrophyte beds from estuaries (Talbot & Bate, 1987; Whitfield et al., 2008). Flooding, and the associated habitat loss, were presumed responsible for the local extinction of populations in the East and West Kleinemonde estuaries (Vorwerk et al., 2007; Whitfield et al., 2017). The expected increase in the frequency and severity of droughts and floods due to climate change necessitates a rigorous monitoring protocol for *S. watermeyeri* populations, toward which eDNA surveys can make a significant contribution.

Seine netting surveys were conducted bi-annually in the East Kleinemonde estuary by Whitfield et al. (2017) from 1994 to 2014. They found *S. watermeyeri* within this estuary from 1996 to 2003 when dense macrophyte beds were present along the length of the estuary (Whitfield et al., 2017). However, a major flooding event in 2003 resulted in the loss of these macrophyte beds and no subsequent detections of *S. watermeyeri* were reported. Our study supports the suggestion that *S. watermeyeri* populations did not recover from this flooding event and the recovery of the macrophytes in this system has not since led to the re-establishment of the population. Vorwerk et al. (2007) surveyed 60 sites within the Kariega estuary in 2006 and reported positive detections of *S. watermeyeri* at 11 of these sites. This study was replicated annually by Whitfield et al. (2017) from 2012 to 2015, with only one positive detection in 2013. Both studies had positive detections restricted to the middle and upper reaches of the estuary (Vorwerk et al., 2007; Whitfield et al., 2017). Using seine netting, we were only able to detect *S. watermeyeri* at one site in the upper reaches of the Kariega estuary. Comparatively, eDNA detections were spread throughout the entirety of this estuary. With previous studies confirming that eDNA signals degrade rapidly in aquatic systems and can be informative over small spatial scales (Jeunen et al., 2019; Murakami et al., 2019; Stat et al., 2018; West et al., 2020), we further emphasize the benefits of monitoring this cryptic species with eDNA and the difficulties associated with conventional surveying methods.

Our research directly compared detection rates of eDNA to seining methods for the critically endangered estuarine pipefish. We successfully detected *S. watermeyeri* at

all locations where the species was observed visually through the use of seine netting. The eDNA methodology had greater sensitivity than seine netting, is non-invasive, less destructive, and can detect DNA from a species irrespective of age or size. This emphasizes the benefits of using eDNA for a long-term monitoring plan of this cryptic threatened species. Our results demonstrate success detecting *S. watermeyeri* from 1 L field replicates; however, it is possible that increasing the volume of water filtered per sample may enhance detection rates and lower overall Ct values. The effects of extraction kit, master mix, and inhibitor removal steps (dilutions or PCR inhibitor removal spin-column kits) on estuarine water samples are inconsistent throughout the literature (Gaither et al., 2022; Kumar et al., 2022; Sanches & Schreier, 2020), with some studies observing high DNA yield through the use of the DNeasy Blood and Tissue kit, nitrocellulose filter membranes, and dilutions implemented in this study (McKee et al., 2015; Sanches & Schreier, 2020). However, the use of a PCR inhibitor removal spin-column kit (ZYMO) or a qPCR master mix designed to operate in the presence of high levels of inhibitors has been proven to reduce inhibitor levels and enhance sensitivity (Hunter et al., 2019; Kumar et al., 2022). The cost–benefit trade-offs between detection probability and financial investment must be considered in future studies.

This study does not attempt to update the population size estimates of this critically endangered species; however, doing so is critical for conservation planning and management purposes. Although eDNA showed a greater detection rate than seine netting, it did not provide abundance data and the extent to which eDNA can be used to measure species abundance remains largely uncertain (Deiner et al., 2017; Li et al., 2021). The ability to infer abundance from eDNA has been explored for several aquatic species using different methodologies (Doi et al., 2015; Hänfling et al., 2016; Lacoursière-Roussel et al., 2016; Pilliod et al., 2013; Takahara et al., 2012; Thomsen et al., 2016); however, its use is still in its infancy and relies on rigorous laboratory experimentation to determine shedding rates and the effect of abiotic factors (Wilcox et al., 2016; Yates et al., 2019). Further research is required before eDNA technologies can be used to accurately and reliably estimate population sizes.

3.7 Conclusion

The development of an eDNA-based toolkit for *S. watermeyeri* presents an exciting opportunity for developing a highly sensitive long-term monitoring plan and the possibility to implement broad-scale surveying to better understand its current distribution. Our results support the work of others in suggesting that localized extinctions of *S. watermeyeri* have occurred across its former range. More exhaustive eDNA surveys within these estuaries with increased sample numbers and more holistic metabarcoding will further identify where conservation efforts should be concentrated. Although the increased sensitivity of eDNA methodologies is evident in this study, presently it cannot entirely replace traditional or conventional methodologies that provide valuable population size and health estimates. With climate change increasing the frequency and severity of droughts and flooding, the apparent sensitivity of this critically endangered species to vegetation cover emphasizes the importance of catchment-level management plans that include eelgrass restoration to prevent further population declines and extinction. This study has provided information that will revolutionize current conservation efforts of the species and can be used to inform a species recovery plan for the critically endangered estuarine pipefish.

3.8 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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3.9 Supplementary information

Table S 3-1: General properties of the CytB_SW assay, an eDNA assay targeting the cytochrome b region of *Syngnathus watermeyeri*.

Data retrieved from Geneious Primer 3 and bioinformatics.org/sms2/pcr_primer_stats.html

General properties		
Primer name	SW_CytbB_F	SW_CytB_R
Primer sequence	GCACCAATCTTGTCCAATGAATC	GAACTCGGACGCTGACAAGA
Sequence length	23	20
Base counts	G=3; A=7; T=6; C=7; Other=0;	G=6; A=7; T=2; C=5; Other=0;
GC content (%)	43.48	55
Molecular weight (Daltons)	6967.6	6160.07
nmol/A260	4.57	4.95
micrograms/A260	31.87	20.51
Primer 3 basic Tm (degrees C)	58.6	60
Basic Tm (degrees C)	53	54
Salt adjusted Tm (degrees C)	48	49
Nearest neighbour Tm (degrees C)	63.22	64.95
PCR suitability tests (Pass / Warning)		
Single base runs	Pass	Pass
Dinucleotide base runs	Pass	Pass
Length	Pass	Pass
Percent GC	Pass	Pass
Tm (Nearest neighbour)	Warning: Tm is greater than 58;	Warning: Tm is greater than 58;
GC clamp	Pass	Pass
Self-annealing	Pass	Pass
Hairpin formation	Pass	Pass

Table S 3-2: List of non-target and exclusion species for specificity testing of the CytB_SW assay.

Species include the only other Syngnathid within its known distribution (sister taxa *S. temminckii*), and 10 non-target co-occurring or closely related species. The number of mismatches for the target and non-target species is provided.

				Number of mismatches		
Species	**Common name**	**Potential co-occurrence**	**In vitro testing**	**Forward primer**	**Reverse primer**	**Total**
Syngnathus watermeyeri	Estuarine pipefish	Yes	Yes	0	0	0
Syngnathus temminckii	Longsnout pipefish	Yes	Yes	4	2	6
Syngnathus acus	Greater pipefish	No	No	4	4	8
Syngnathus tenuirostris	Narrow-snouted pipefish	No	No	5	3	8
Syngnathus taenionotus	Darkflank pipefish	No	No	5	3	8
Syngnathus typhle	Broadnosed pipefish	No	No	6	3	9
Gilchristella aestuaria	Gilchrist's round herring	Yes	No	6	6	12
Oreochromis mossambicus	Mozambique tilapia	Yes	No	7	4	11
Lichia amia	Leerfish	Yes	No	5	9	14
Psammogobius knysnaensis	Knysna sandgoby	Yes	No	8	8	16
Pomatomus saltatrix	Bluefish	Yes	No	6	5	11
Glossogobius callidus	River goby	Yes	No	6	6	12

Table S 3-3: Details of the GBlocks (synthetic DNA) used for specificity and sensitivity testing of the CytB_SW assay.

Assay mismatches indicated in red where present.

Species	Accession no.	Similarity to *S. watermeyeri* gBlock	200 bp sequence (primers in black, mismatches in red)
Syngnathus watermeyeri (Estuarine pipefish)	Alignment of KY407926, KY407927, JX228139 & JXX228140 KY407918	N/A	CCTATATAG**GCACCAATCTTGTCCAATGAATC**TGAGG GGGATTCTCAGTTGACAATGCAACCCTCACACGATTTT TCGCCTTCCATTTCCTACTCCCGTTTATTGTTACCGCCA CCACACTTATCCACCTTTTATTCCTTCACGAAACAGGCT CCAATAACCCAGCAGGATT**GAACTCGGACGCTGACAA GA**TCTCTTTT
Syngnathus temminckii (Longsnout pipefish)		90%	CCTATGTCG**GCACCGATCTTGTCCAATGGGTT**TGAGG GGGGTTCTCAGTTGACAATGCAACCCTCACACGGTTTT TCGCCTTCCATTTCCTACTCCCGTTTATTGTCGCTGCCG CCACGGTTGTTCACCTTTTATTCCTTCACGAGACAGGC TCCAATAACCCAGCAGGGTT**AAACTCGGACGCTGACA AAA**TCTCTTTT
Syngnathus temminckii (Longsnout pipefish)	KY407925	90.50%	CCTATGTCG**GCACCGATCTTGTCCAATGGGTT**TGAGG GGGGTTCTCAGTTGACAATGCAACCCTCACACGGTTTT TCGCCTTCCATTTCCTACTCCCGTTTATTGTCGCTGCCG CCACGGTTGTCCACCTTTTATTCCTTCACGAGACAGGC TCCAATAACCCAGCAGGGTT**AAACTCGGACGCTGACA AAA**TCTCTTTT

Table S 3-4: Details of the sites sampled (estuary, site number, latitude, longitude and date the water sample was collected)

Estuary	Site	Latitude	Longitude	Date collected
Kariega	1	-33.671	26.673	4-Oct-19
Kariega	2	-33.665	26.663	4-Oct-19
Kariega	3	-33.661	26.647	4-Oct-19
Kariega	4	-33.652	26.650	5-Oct-19
Kariega	5	-33.645	26.646	5-Oct-19
Kariega	6	-33.636	26.641	5-Oct-19
Kariega	7	-33.636	26.643	5-Oct-19
Kariega	8	-33.629	26.637	5-Oct-19
Kariega	9	-33.617	26.636	5-Oct-19
Kariega	10	-33.629	26.642	6-Oct-19
Kariega	11	-33.626	26.640	6-Oct-19
Kariega	12	-33.620	26.637	6-Oct-19
Kariega	13	-33.612	26.634	4-Oct-19
Kariega	14	-33.614	26.645	4-Oct-19
Kariega	15	-33.612	26.655	4-Oct-19
Bushmans	1	-33.682	26.656	5-Oct-19
Bushmans	2	-33.679	26.654	5-Oct-19
Bushmans	3	-33.672	26.646	6-Oct-19
Bushmans	4	-33.662	26.633	6-Oct-19
Bushmans	5	-33.655	26.619	7-Oct-19
Bushmans	6	-33.651	26.609	7-Oct-19
Bushmans	7	-33.656	26.594	8-Oct-19
Bushmans	8	-33.651	26.590	10-Oct-19
Bushmans	9	-33.651	26.583	11-Oct-19
Bushmans	10	-33.641	26.575	10-Oct-19
Bushmans	11	-33.646	26.565	11-Oct-19
Bushmans	12	-33.637	26.562	12-Oct-19
Bushmans	13	-33.636	26.552	12-Oct-19
Bushmans	14	-33.624	26.546	13-Oct-19
Bushmans	15	-33.585	26.526	13-Oct-19
Kasouga	1	-33.652	26.733	15-Oct-19
Kasouga	2	-33.649	26.728	15-Oct-19
Kasouga	3	-33.642	26.714	15-Oct-19
East Kleinemonde	1	-33.539	27.047	16-Oct-19
East Kleinemonde	2	-33.534	27.040	16-Oct-19
East Kleinemonde	3	-33.533	27.042	16-Oct-19
West Kleinemonde	1	-33.540	27.044	16-Oct-19
West Kleinemonde	2	-33.538	27.039	16-Oct-19
West Kleinemonde	3	-33.538	27.036	16-Oct-19

Table S 3-5: Sites with positive detections of *S. watermeyeri* and the number of sequencing reads detected.

All positive detections required 100% percent identity match and query coverage of 100%.

Site	Reads
Kariega_1_5	14663
Kariega_2_1	12229
Kariega_2_2	16738
Kariega_2_3	15839
Kariega_2_5	12979
Kariega_3_1	9331
Kariega_3_2	4793
Kariega_3_4	1560
Kariega_3_5	16770
Kariega_4_3	12852
Kariega_5_2	13110
Kariega_5_4	194
Kariega_8_3	26785
Kariega_11_1	24975
Kariega_12_1	5921
Kariega_12_5	19444
Bushmans_1_2	12883
Bushmans_1_3	498
Bushmans_2_1	9984
Bushmans_2_2	21552
Bushmans_2_3	11485
Bushmans_2_4	13541
Bushmans_2_5	18164
Bushmans_3_1	21265
Bushmans_3_2	54191
Bushmans_3_4	25321
Bushmans_3_5	52794
Bushmans_4_1	17844
Bushmans_4_2	18549
Bushmans_4_3	25042
Bushmans_4_4	15736
Bushmans_4_5	25626
Bushmans_5_1	138724
Bushmans_5_2	7
Bushmans_5_3	17978
Bushmans_5_5	69
Bushmans_6_5	10198
Bushmans_7_2	20194
Bushmans_7_3	11035
Bushmans_8_2	12
Bushmans_9_4	2
Bushmans_9_5	1
Bushmans_12_4	2
Bushmans_14_2	1
Bushmans_14_3	3
Bushmans_14_4	6
Bushmans_14_5	3822
tissue	10569

CHAPTER 4

eDNA metabarcoding reveals biodiversity patterns and facilitates early detection of invasive species in the Antarctic Southern Ocean

4.1 Prologue

Chapters 2 and 3 have explored the applicability of eDNA for detecting 'small and scarce' Syngnathid taxa in coastal and marine ecosystems. These studies demonstrated the sensitivity of eDNA approaches for detecting these rare and cryptic taxa both in the context of other fish assemblages (Chapter 2), and in the presence of closely related sister taxon (Chapter 3). To explore the use of eDNA in 'broad and unexplored' ecosystems, this chapter employs eDNA metabarcoding to characterise biodiversity across the Southern Ocean.

The Southern Ocean, a vast body of water encircling Antarctica, is renowned as one of the most formidable oceans on our planet (McCann, 2019). The ocean came into existence between 40 to 20 million years ago, as tectonic forces gradually opened passages between the Antarctic, South American, and Australian continents (Antonello, 2017; Kennett, 1977). This geological process gave rise to the Antarctic Circumpolar Current and isolated Antarctica, allowing the ocean to circulate continuously around the continent (Fitzsimons, 2000; Kennett, 1977). While its northern limits have been the subject of international disagreement since 1928, the Southern Ocean was reinstated as the world's fifth ocean in 2000 (McCann, 2019).

The Southern Ocean was first systematically explored when the British *HMS Challenger* reached these latitudes in 1874 during its pioneering expedition (De Broyer et al., 2014; McCann, 2019). This ground-breaking voyage set the stage for subsequent scientific endeavours in the region, shedding light on the previously unexplored waters and laying the foundation for our understanding of the Southern Ocean's unique ecosystem (Figure 4-1). Exploration of this region became more frequent, and by the 1950s the number of described species was over 6 000, and was around 9 000 in 2010 (David & Saucède, 2015a). Our knowledge of Southern Ocean biodiversity has significantly increased since these pioneering voyages, leading to a better understanding of species ecology and ecosystem functioning (David & Saucède, 2015b).

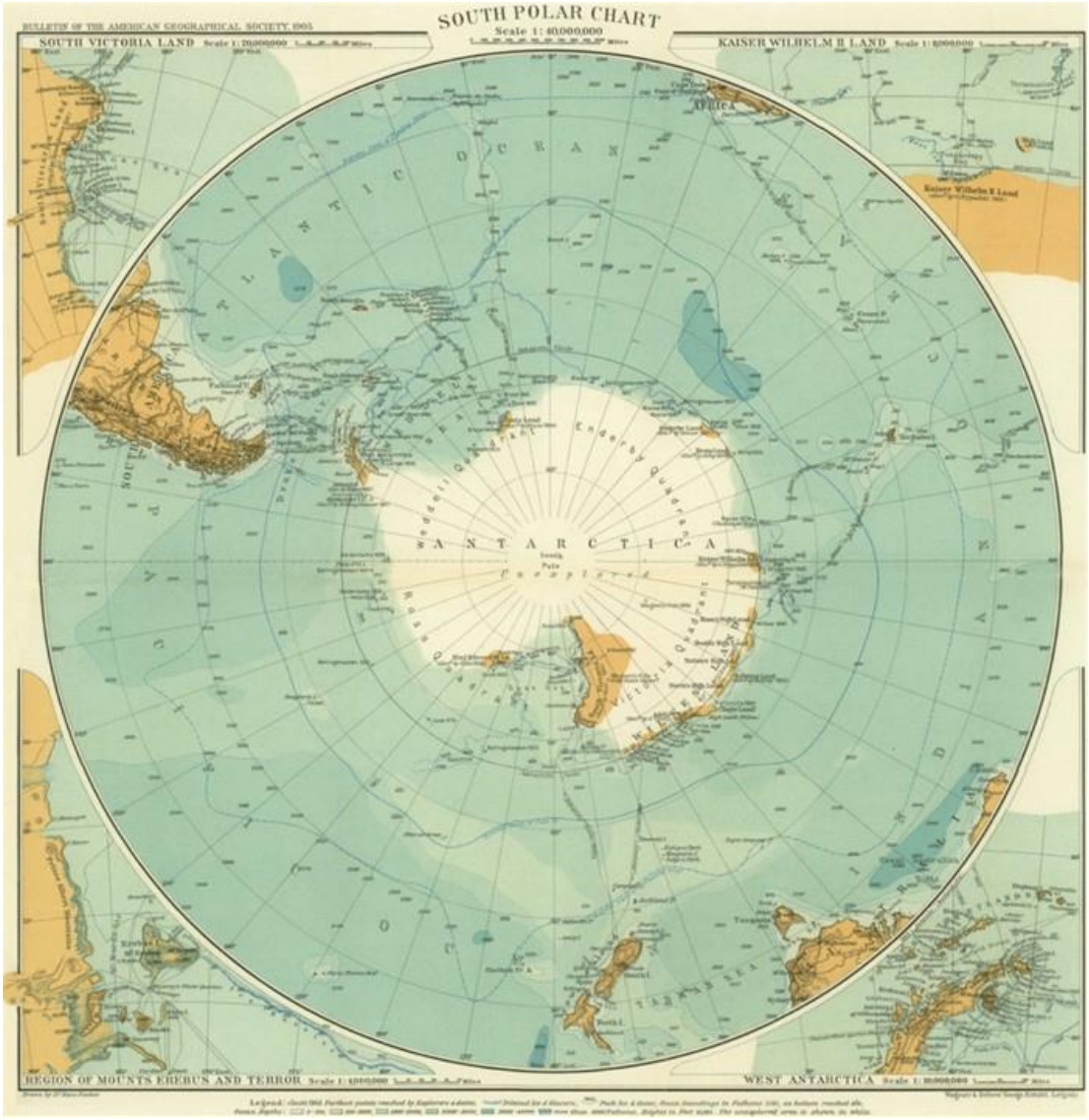

Figure 4-1: An early map of Antarctica.
Drawn by Dr. Hans Fischer for publication in the Bulletin of the American Geographical Society Vol. 37, No. 11 in 1905. Only the brown areas indicated on the map had been surveyed at the time, while the white areas were still completely unexplored. This period of time marked a resurgence in Southern Ocean exploration, with expeditions from multiple nations revolutionising our knowledge and perception of Antarctica and the surrounding polar regions ("The South Polar Chart," 1905).

Polar seas had long been considered low biodiversity regions due to their extreme environmental conditions being perceived as uninhabitable (David & Saucède, 2015b). The Southern Ocean, however, defies this notion, harbouring some of the healthiest marine habitats that remain on our planet (Brooks et al., 2022). This unique ecosystem supports a rich tapestry of species which provide remarkable wilderness, scientific, and ecological value (Brooks et al., 2022). The subtle molecular adaptation of the fauna in this region to surviving and thriving in this cold and harsh environment all point to a long evolutionary history of specialized adaptations (Clarke & Crame, 1989). Accordingly, much of the Southern Ocean's biodiversity is comprised of organisms that are endemic to this particular region (Rogers et al., 2012). Among the iconic features of the Southern Ocean is the remarkable abundance of apex predators, including seabirds (e.g., albatross, petrels) and mammals (e.g., whales, seals, orcas) which depend on the nutrient-rich waters (Bestley et al., 2020; Murphy et al., 2021). Certain species, like the emperor penguin (*Aptenodytes forsteri*), are exclusively adapted to solely inhabit this region, successfully navigating its highly seasonal conditions year-round (Bestley et al., 2020). Other populations, notably baleen whale species such as the humpback whale (*Megaptera novaeangliae*), undertake mass migrations to the region during spring and summer to take advantage of the high productivity during these periods (Bestley et al., 2020; Riekkola et al., 2018).

Despite being geographically distant from human populations, the Southern Ocean has been subject to significant human activity over the course of history (Antonello, 2017). The exploration of this region and scientific advancements opened the door to extensive commercial exploitation, especially as marine resources in the Northern Hemisphere became scarce (Antonello, 2017). Unfortunately, many of these early fisheries were marked by overexploitation, leading to the near extinction of Antarctic fur seals and the severe depletion of whale populations by the mid – late 19th century (Rogers et al., 2015). Prior to the implementation of a whaling moratorium in 1985, more than 2 million whales were taken from the Southern Ocean (Rocha et al., 2014). These exploitative practices had a profound impact on the delicate balance of the Southern Ocean ecosystem long before the effects and impacts of climate change

were understood (Rogers et al., 2020). Accordingly, the Southern Ocean stands as a testament to the awe-inspiring power and fragile interconnectedness of our planet's oceans. To safeguard the unique biota of this remarkable ocean, it is fundamental that we confront the challenges of monitoring the biodiversity within this remote and immense environment. The following chapter elucidates broad-scale biodiversity patterns across a 3000 nautical mile (~5500 km) Southern Ocean transect using eDNA, and attempts to refine eDNA methodologies for monitoring this invaluable ecosystem.

4.1.1 Chapter acknowledgements

I would like to acknowledge the support and contributions of the co-authors of this chapter: Leonie Suter, Bruce Deagle, Andrea Polanowski, and John Kitchener. A breakdown of author contributions can be found in Appendix 1: Copyright statements. I would like to acknowledge that this project was funded by the Australian Antarctic division. I would also like to thank the crew aboard the *RSV Aurora Australis* for CPR deployments.

4.1.2 Data accessibility

All data generated and R scripts created have been deposited in the following Zenodo repository https://doi.org/10.5281/zenodo.7982407. This includes raw sequencing data, morphology data, sampling information, and all outputs for analyses conducted on R.

4.2 Abstract

The Southern Ocean surrounding Antarctica harbours some of the most pristine marine environments remaining, but it is increasingly vulnerable to anthropogenic pressures, climate change, and non-native species invasions. Monitoring biotic responses requires temporal and spatial baselines - traditionally, this has been obtained by continuous plankton recorder (CPR) surveys. Here, we conduct one of the longest environmental DNA (eDNA) transects yet, spanning over 3000 nautical miles from Hobart (Australia) to Davis Station (Antarctica) aboard the *RSV Aurora Australis*. The efficacy of eDNA metabarcoding for long-term biomonitoring was evaluated by comparing two water volume and filter pore size combinations: LargeVF (12 L with 20 µm) and SmallVF (2 L with 0.45 µm). Employing a broad COI metabarcoding assay, we found LargeVF samples were better suited to open-ocean monitoring, detecting more target template and rare or low abundant species. Comparisons with simultaneously conducted CPR transect (N=4) revealed that eDNA detections were more diverse than CPR, with 7 (4 unique) and 4 (1 unique) phyla detections respectively. While both methods effectively delineated biodiversity patterns across the Southern Ocean, eDNA demonstrated an advantage by enabling surveys to be conducted in the presence of sea-ice. Accordingly, 16 species of concern were detected in Hobart or the Antarctic region (south of 60°S) using eDNA, which we attribute to hull biofouling, a recognized pathway for marine introductions into Antarctica. Given the vulnerability of Antarctic environments to potential introductions in a warming Southern Ocean, this work demonstrates the need for ongoing biosecurity vigilance. We propose the integration of eDNA metabarcoding to enhance long-term CPR surveys in the Southern Ocean, emphasising the urgency of its implementation. We anticipate the temporal and spatial interweaving of CPR, eDNA, and biophysical data will generate a more nuanced picture of the Southern Ocean and Antarctic ecosystems, with profound implications for their conservation and preservation.

4.3 Introduction

The Southern Ocean surrounding Antarctica harbours some of the largest, most pristine remaining marine environments (Brooks et al., 2022). Although the Southern Ocean covers approximately just 10% of the world's oceans, it plays a disproportionately important role through the multiple ecosystem services it provides (Grant et al., 2013). It is particularly important for climate regulation through storing significant amounts of heat and carbon dioxide (Chen et al., 2019; Cavanagh et al., 2021). The high biological productivity is valuable in oxygen production, fuelling marine food webs and supporting important fisheries (Grant et al., 2013; Murphy et al., 2021). It is also an important home to a unique and diverse array of marine biota, boasting high levels of endemism (Rogers et al., 2012). However, Southern Ocean and Antarctic environments, and the valuable services they provide, are rapidly changing in response to increased anthropogenic pressures and climate change (Chown et al. 2015; Johnston et al. 2022).

The Southern Ocean is comprised of several biogeographic zones formed by the oceanographic fronts (Constable et al., 2014), in particular the boundary of the strong Antarctic Circumpolar Current (ACC) which encircles Antarctica and creates a sharp gradient between cold polar water and warmer waters further north. Bathymetry and sea ice coverage also influence the biotic habitat within the Southern Ocean (Constable et al., 2003; Massom & Stammerjohn, 2010). The environmental heterogeneity between and within these zones results in a variety of complex ecosystems characterised by distinct ecological communities that vary in species richness and diversity (Griffiths, 2010). These communities are highly susceptible to change in response to multiple local and global drivers (Morley et al. 2020a; Grant et al. 2021), exacerbated by anthropogenic impacts including climate change and resource exploitation (Rogers et al. 2020; Brooks et al. 2022). Moving forward, monitoring temporal and spatial changes in biodiversity, species distributions, and community structure will be essential in making ecosystem-based management decisions to conserve the unique biota of the Southern Ocean (Brooks et al. 2016) and recognising the key role it plays in the dynamics of the oceans globally.

To date, biodiversity monitoring in the Southern Ocean has been inhibited by various logistical challenges (Griffiths, 2010), resulting in a paucity of comprehensive baseline data. Spatial coverage in biodiversity data has been limited by the remoteness of some parts of the Southern Ocean, which are rarely visited by scientific expeditions (Griffiths, 2010). Often scientific expeditions are limited to the summer months in the polar regions, as winter sea ice extent and short days make winter expeditions challenging, resulting in a temporal bias of biodiversity data sets (Howell et al., 2021). It is clear more spatiotemporally complete datasets are needed to provide holistic baselines and for comprehensively investigating biodiversity patterns and the underlying processes.

Continuous plankton recorder (CPR) surveys are routinely implemented to monitor Southern Ocean Animalia diversity, particularly plankton communities (Hunt & Hosie, 2003; McLeod et al., 2010). The CPR is a device towed behind a ship at approximately 10 m depth, collecting plankton samples onto a continuously winding silk (Hunt & Hosie, 2003; Reid et al., 2003). CPR surveys have been conducted annually in the Southern Ocean since 1991, and have covered roughly 285, 000 nautical miles (~527, 800 km) to date (Takahashi & Hosie, 2021). Providing some of the longest running biological datasets, its longevity owing partly to its robust and standardised design that allows it to be used opportunistically on any vessel with towing capabilities (Hunt & Hosie, 2003; Richardson et al., 2006). However, the method requires detailed taxonomic identification of large numbers of specimens (McLeod et al., 2010), a time-consuming process that can be difficult when specimens are damaged (Deagle et al., 2018). Furthermore, CPR surveys are designed to specifically target plankton communities and are therefore restricted in the taxonomic comprehensiveness of baseline data they can provide.

Environmental DNA (eDNA) biomonitoring presents a promising approach to complement and expand the current taxonomic scope of CPR surveys. eDNA metabarcoding describes biodiversity patterns through the amplification of trace amounts of DNA naturally shed into the environment by organisms. The technique typically targets DNA barcodes using broad-spectrum metabarcoding assays able to

profile a wide range of biota without the need for visual observation or identification of specimens. eDNA studies in various marine environments have demonstrated its ability to enrich temporal and spatial surveys by capturing biota across the tree of life (Berry et al., 2019, 2023; Minamoto et al., 2017). Notably, eDNA data can discriminate fine-scale spatial and habitat variation (Jeunen et al., 2019; West et al., 2021), and has demonstrated increased sensitivity to the presence of endangered or invasive species in comparison to conventional methods (Klymus et al., 2017; Nester et al., 2022). Early detection of non-native species is particularly important in the Antarctic region (south of 60°S) given the vulnerability of its unique biota to the negative impacts of non-native settlement, and is consistent with the key principles of the Protocol on Environmental Protection to the Antarctic Treaty (1991).

Here, using ship-based surveys we conduct one of the longest eDNA transects to date, spanning over 3000 nautical miles (~5500 km). The primary objective of the study was to investigate patterns in Animalia biodiversity in the Southern Ocean using CPR and eDNA metabarcoding. In an attempt to refine the eDNA methodologies and determine their viability for surveying open ocean Animalia communities, two combinations of water volume and filter pore sizes were tested (12 L with 20 µm, and 2 L with 0.45 µm). Smaller pore sizes can retain smaller particles and hence potentially more eDNA per water volume, but they can clog up relatively quickly, particularly in the presence of non-Animalia microorganisms (Suter et al., 2021; Turner et al., 2014), and hence only small water volumes can be filtered. Conversely, larger water volumes may contain more Animalia template, but require larger pore sizes for filtering. As well as exploring the logistics of collection we compared taxonomic resolution, diversity, and richness between these two approaches, and investigated whether these differences would impact ecological inferences. By making further comparisons to CPR surveys, we examine fine-scale patterns in Animalia biodiversity across the Southern Ocean, highlighting the strengths and potential 'blind spots' of both methodologies. Given the remoteness of the Southern Ocean and the difficulties associated with sampling the region, there is great potential to integrate water sampling into voyages to Antarctica and use this data to

inform decision-making, ocean-ecosystem models and conservation across the Southern Ocean.

4.4 Methods

4.4.1 Sampling

4.4.1.1 eDNA sampling

Samples (n = 138) were collected aboard the RSV Aurora Australis on a resupply voyage between Hobart, Tasmania (42°52'54.84"S 147°20'29.76"E) and Davis Station, Antarctica (66°26'14.28"S 77°28'24.6"E) in November 2019 (Figure 4-2; Table S 4-1). Two combinations of water volume and filter pore sizes were tested at each sampling location: 12 L with 20 µm, and 2 L with 0.45 µm, herein referred to as "LargeVF" and "SmallVF" respectively. Water samples were collected and filtered approximately every 4 hours (4.52 $\pm$ 0.23) via the ship's uncontaminated seawater line (4 $\pm$ 2 m depth). SmallVF water samples (2 L, n = 69) were filtered using a Sentino microbiology peristaltic pump (Pall Life Sciences) through 47 mm diameter, 0.45 µm pore size polyethersulfone filter membranes (Pall Life Sciences). Simultaneously, LargeVF water samples (12 L, n=69) were filtered using a Masterflex L/S console pump system (Cole-Parmer) through 25 mm diameter, 20 µm pore size nylon filter membranes (Merck). SmallVF filter membranes (47 mm diameter) were cut in half and immediately preserved at -80°C, with one half to be analysed and one to be stored as a reserve and a form of eDNA biobanking (Jarman et al., 2018). LargeVF filter membranes (25 mm diameter) were stored whole at -80°C. The LargeVF filter membranes were not cut in half due to the smaller diameter of this membrane. Filtration equipment was rinsed with a 10% bleach solution and freshwater from the laboratory in between every sample, and soaked for 15 minutes with 10% bleach every tenth sample. Field controls consisted of 500 mL samples (n = 10) of laboratory freshwater and the 10% bleach solution used for sterilisation.

4.4.1.2 CPR sampling

CPR sampling was conducted in parallel to the eDNA sampling using a Type II CPR (Mark V) and following standard Southern Ocean CPR methods (Hosie et al., 2003).

The CPR was fitted with 270 µm nylon mesh and towed 100 m behind the ship at approximately 10 m depth. Four CPR transects were completed between latitudes 47°8’12.84”S and 59°3’38.52”S, spanning a total of 1,858 nautical miles (~3400 km; Figure 4-2; Table S 4-2). The instrument was not used in the presence of sea-ice, limiting sample collection at the Southern part of the voyage. CPR silk spools were fixed in 10% buffered formalin for morphological identification.

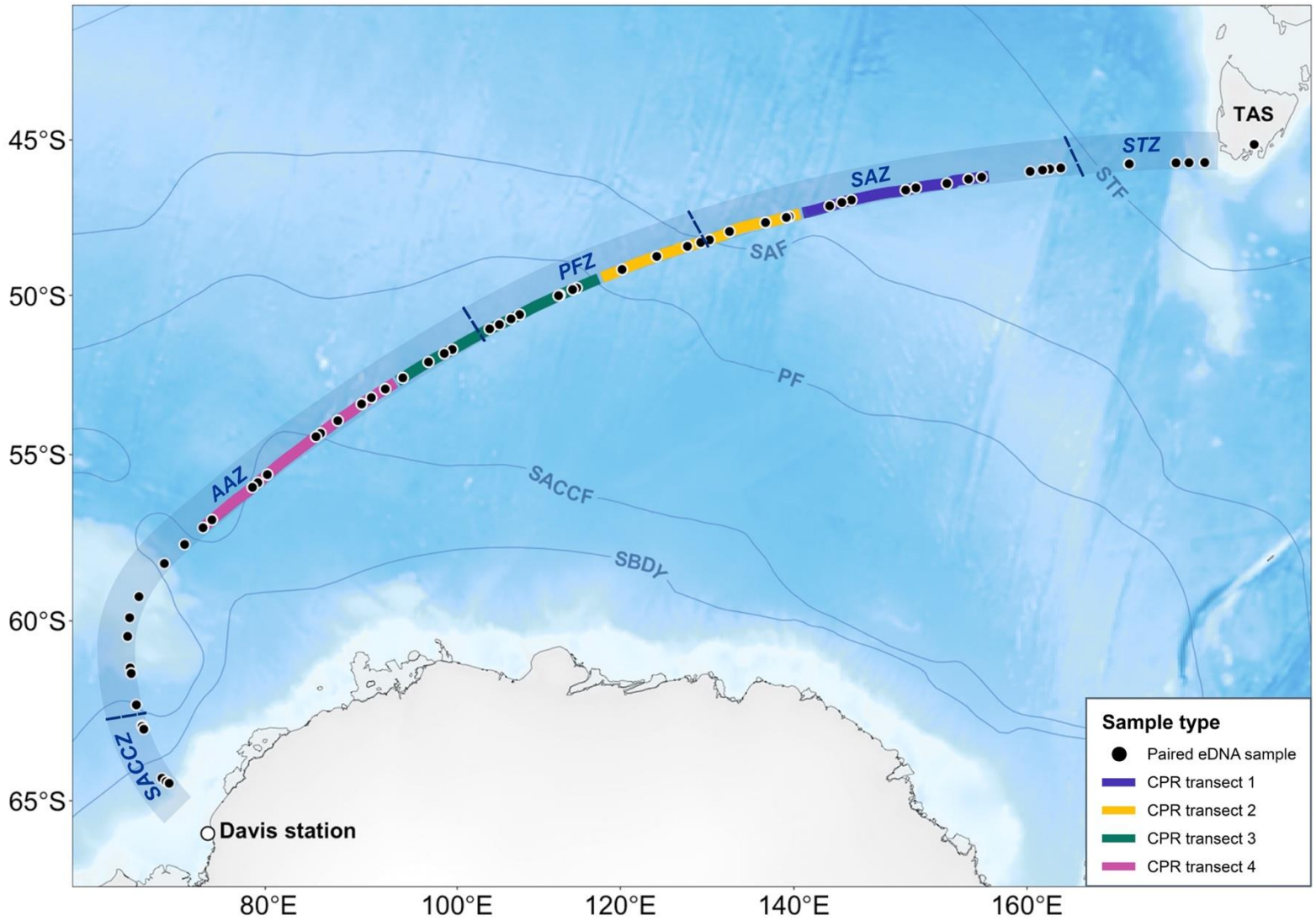

Figure 4-2: Map of the Southern Ocean sampling region. Sampling began at Hobart, Tasmania (TAS) and ended at Davis Station, Antarctica. Paired eDNA samples (LargeVF and SmallVF) are indicated by the black dots and CPR transects are differentiated sequentially by coloured lines. The Subtropical zone (STZ), Subantarctic zone (SAZ), Polar Frontal zone (PFZ), Antarctic zone (AAZ), and South of the ACC zone (SACCZ) are demarcated by dashed lines. Fronts are visualised here according to Orsi et al. (1995). Map created using "SOmap" R package (Maschette, et al., 2019).

4.4.2 eDNA methods

4.4.2.1 Laboratory processing

DNA was extracted from the filter membrane using a DNeasy Blood and Tissue Kit (Qiagen) in an automated QIAcube (Qiagen) DNA extraction system with the following modifications: 540 µl of ATL lysis buffer, 60 µl of Proteinase K, and a 3-hour digestion at 56°C. Extraction controls were processed in parallel with all samples to detect any laboratory or between sample contamination. Final DNA extracts were eluted in 100 µl of AE buffer.

DNA was amplified to target Animalia taxa using mitochondrial cytochrome c oxidase subunit I (COI) markers: m1COIintF 5' GGWACWGGWTGAACWGTWTAYCCYCC 3' (Leray et al., 2013) and jgHCO2198 5' TAIACYTCIAAYCAYAARGAYATTGG 3' (Geller et al., 2013), herein referred to as COI Leray. Samples were serially diluted (1/5, 1/10 and 1/100) to optimise DNA input levels for quantitative PCR (qPCR) and remove potential PCR inhibitors. No-template controls were included on each qPCR plate. Metabarcoding was performed through the use of fusion-tagged primers consisting of Illumina compatible sequencing adapters, a unique 6 – 8 bp multiples identifier tag (MID-tag), and the COI Leray primer. Each sample and control were processed in duplicate using the same MID tag, to reduce stochasticity for species with low amounts of template DNA. qPCR reactions (25 µl) consisted of the following concentrations: 2 mM $MgCl_2$, 1× AmpliTaq Gold PCR buffer, 1 U AmpliTaq Gold DNA polymerase (Applied Biosystems), 0.4 µM dNTPs (Astral Scientific), 0.1 mg BSA (Fisher Biotec), 0.6 µL of 5X SYBR Green dye (Life Technologies), 0.4 µM forward and reverse primer, 4 µL of eDNA template (at optimised dilution), made to volume with Ultrapure Distilled Water (Life Technologies).

qPCR amplifications were performed using a StepOnePlus Real-Time PCR System (Applied Biosystems) in a single-step process using an adjusted touchdown thermocycler protocol with conditions: 94°C for 10 min, 16 cycles of 95°C for 10 s, 62°C (-1°C per cycle) for 30 s, and 72°C for 45 s, followed by 25 cycles of 46°C for 30 s, with a final extension of 72°C for 5 minutes. qPCRs were prepared in dedicated

clean facilities within the TrEnD Laboratory (Curtin University). Amplicons were visualised on 1.5% agarose gels and duplicate reactions from the same eDNA template were combined and then pooled into a library at equimolar ratios. The resulting library was size selected using a Pippin Prep (Sage Science), purified using a Qiaquick PCR Purification Kit (Qiagen), and quantified using a Qubit 4.0 Fluorometer (Invitrogen) and a Qiaxcel Advanced System (Qiagen). The final library was diluted to 2 nM and sequenced on an Illumina MiSeq platform (Illumina) using a 500 cycle (2 x 250 bp) MiSeq V2 Reagent Kit for paired-end sequencing.

4.4.2.2 Bioinformatics and taxonomic assignments

Bioinformatics and taxonomic assignments of sequencing data were performed using eDNAFlow, a fully automated workflow that processes eDNA data from raw sequences to curated and non-curated zero-radius operational taxonomic units (ZOTUs) and their abundance tables (Mousavi-Derazmahalleh et al., 2021). Briefly, sequences were quality filtered, demultiplexed, denoised and erroneous sequences were removed using a combination of AdapterRemoval (Schubert et al., 2016), OBITools (Boyer et al., 2016), and Usearch (Edgar, 2010) via Zeus, an SGI Linux cluster based in the Pawsey Supercomputing Centre (Kensington, Western Australia). Resulting ZOTUs were queried against NCBI's GenBank nucleotide database using BLASTn. We used MEGAN v6 (Huson et al., 2016) to assign taxonomy with LCA parameters "min score": 300; "top percent": 5; "min support": 1; "min percent read to cover (query coverage)": 100. Taxonomic assignments of Animalia taxa were then manually curated and checked for additional entries using the BOLD identification engine (Ratnasingham & Hebert, 2007). Following the approach of Suter et al. (2020), species that hit at 100% within a ZOTU and could not be distinguished based on their COI sequence were merged, e.g., '*Calanus propinquus/similimus'*. All taxonomic assignments were further evaluated against knowledge of species distributions using the World Register of Marine Species (WoRMS Editorial Board, 2022) and Atlas of Living Australia (ALA – ala.org.au), and their status (invasive or pest) checked using the Global Invasive Species Database (GISD — iucngisd.org), the Global Register of

Introduced Species (GRIIS — griis.org), and the CABI Invasive Species Compendium (cabi.org/isc).

ZOTUs assigned to contaminants, non-Animalia, and non-marine Animalia were removed and excluded from further analysis. These detections included humans (*Homo sapiens*), dog (*Canis lupus*), and groups such as insects (class Insecta) and birds (class Aves). Potential cross-contaminant ZOTUs were identified as marine Animalia ZOTUs with reads present in either field, extraction, or PCR controls. ZOTUs with more than 0.5% of reads originating from controls were entirely removed (Table S 4-3 and Table S 4-4). Below this, the number of reads present in controls or the percentage of the ZOTU comprised of the control (whichever was higher) was removed from the samples. For example, a ZOTU assigned to *Clausocalanus brevipes* had 21,300 assigned reads of which 2 (0.009%) were present in a field control. We therefore removed either 2 reads or 0.009% from each sample, whichever resulted in a higher number of reads. We opted for this approach over removing ZOTUs entirely as we found the total portion of control reads (23 reads) from these target taxa ZOTUs (57,405 reads) to be just 0.04%. A total of 17 ZOTUs were entirely removed and 8 ZOTUs had reads removed. We believe our approach is conservative without removing indicator species or unnecessarily impacting the spatial trends of our data. Four eDNA samples (two LargeVF and two SmallVF) failed to sequence (less than 100 reads) and were removed from analysis, along with the corresponding or matching sample.

4.4.3 CPR morphological analysis

CPR silks (n = 368) from the four tows were processed at the Australian Antarctic Division following standard methods (Hosie et al., 2003). In brief, silks were cut into sections representing five nautical miles and zooplankton were identified and counted under a dissecting microscope. Zooplankton were identified to the lowest taxonomic level possible and developmental stages of euphausiids and copepods were recorded. Taxa within some broad lineages were not differentiated (e.g., Chaetognatha), and damaged or unidentifiable specimens were grouped at coarser

taxonomic levels where necessary (e.g., indeterminate Hyperiidea and indeterminate euphausiid furcilia).

4.4.4 Identification of Southern Ocean fronts and zones

The Southern Ocean is divided into biophysical zones by the numerous Antarctic Circumpolar Current (ACC) fronts, namely: the Subtropical Front (STF-S), the Subantarctic Front (SAF-N and SAF-S), the Polar Front (PF-N and PF-S), the Southern ACC Front (SACCF-N and SACCF-S), and the Southern Boundary (SBDY). Water mass properties are relatively homogenous within each of these zones (Orsi et al., 1995; Sokolov & Rintoul, 2002). From north to south, these are: **(1)** Subtropical zone (STZ) extending north of the STF-S, **(2)** the Subantarctic zone (SAZ) extending from the STF-S to the SAF-S, **(3)** Polar Frontal zone (PFZ) covering the SAF-S to the PF-S, **(4)** Antarctic zone (AAZ) spanning the PF-S to the SBDY, and **(5)** South of the ACC zone (SACCZ) covers the area below the SBDY (Figure 4-2). These fronts and the zones they delimit each have unique environmental characteristics and form distinct habitats that support unique biota (Bost et al., 2009). As such, we aimed to integrate detected Animalia biodiversity with the biophysical properties of Southern Ocean zones.

While the notion of a front as a water mass boundary is universally accepted (Chapman, 2014), the delineation of Southern Ocean fronts and their defining characteristics vary between studies (Chapman et al., 2020). We opted to define the fronts based on local criteria, in consideration with criteria adopted by other researchers and knowledge of where fronts are likely to be located over the Austral summer (November to March; Figure S 4-1 – 2). We used gradient thresholding of ship measured water properties found in the immediate geographic area (sea-surface temperature (SST, °C) and sea-surface salinity (SSS, psu)). While the specific locations of ACC fronts are more reliably and easily identified by deep oceanographic observations (Orsi et al., 1995), sharp changes in SST and SSS have been shown to correspond to the average front positions (Sokolov & Rintoul, 2002). Underway SST and SSS were continuously measured using a thermosalinograph (SeaBird SBE21) at

approximately 7 m depth. This approach reflects the localised dynamics of the study area, and our defining characteristics are unambiguous (Table 4-1).

Table 4-1: Sea surface temperature (SST °C) and sea surface salinity (SSS) characteristics used to define the fronts of the Southern Ocean and related references.

Front	SST	SSS	References
SAF - N	9	33.9	Lutjeharms & Valentine, 1984; Belkin & Gordon, 1996; Hunt & Hosie, 2003, 2005
SAF - S	6	33.75	
PF - N	4.5		Lutjeharms & Valentine, 1984; Sparrow et al., 1996; Holliday & Read, 1998; Hunt & Hosie, 2003
PF - S	2.5		
SACCF - N	2	33.7	Orsi et al., 1995; Sokolov & Rintoul, 2003; Hunt & Hosie, 2003, 2005
SACCF - S	1.8	33.73	
SB - ACC	0.5	33.8	Holliday & Read, 1998

4.4.5 Statistical analyses

Raw eDNA taxa were classified as 'Animalia', 'non-Animalia', or 'unclassified' post curation and contamination removal (see 4.1.2). Differences in overall read numbers and the proportion of Animalia taxa (for both reads and ZOTUs) between LargeVF and SmallVF samples was explored visually in R Studio (v4.2.1; R Studio Team, 2022). To test the differences observed, a paired Wilcoxon t-test was conducted between the eDNA volumes. As we wanted to formally test sequencing depth and the relationship between the eDNA methods, the proportion of assigned Animalia reads, and the number of species detected, samples were not rarefied. Rarefying the samples would have reduced the sequencing and diversity patterns we wanted to observe and test, leading to false inferences about choice of volume and filter size. To examine the taxonomic resolution of each volume (i.e., the ability to accurately distinguish taxa), the proportion of Animalia assignments matched to various

taxonomic levels (i.e., 'unclassified', order, family, genus, and species) was calculated.

Animalia community composition was assessed at species level across Southern Ocean zones for the eDNA samples. Species identified as hull fouling were removed from analysis. Alpha diversity was calculated for the LargeVF and SmallVF samples using Hill numbers at q = 0 (species richness), q = 1 (exponential Shannon's entropy index), and q = 2 (inverse Simpson's index) within each zone (Alberdi & Gilbert, 2019), and the differences assessed using either paired t-tests or Wilcoxon paired t-tests. Community composition was then visualised using non-metric multi-dimensional scaling (nMDS) and a Jaccard matrix of presence-absence data. Differences in Southern Ocean zone composition were tested using PRIMER v7 with the PERMANOVA+ add-on (Anderson et al., 2008). A permutational multivariate analysis of variance (PERMANOVA) was used on the Jaccard matrix to test community variation between methods (LargeVF and SmallVF) and between Southern Ocean zones (STZ, SAZ, PFZ, AAZ, and SACCZ). In the presence of significant differences, pairwise comparisons were performed to determine where they occurred. indicator species analysis was performed using the R package indicspecies (De Cáceres et al., 2016) to determine relationships and patterns between species detected and Southern Ocean zones. CPR community composition was individually assessed using the same analyses for presence-absence and abundance.

To allow for comparisons between morphological CPR and eDNA data using the aforementioned analyses, samples were subset to those paired by latitude and longitude and transformed to presence-absence matrices. eDNA data were subset to samples taken within the length of the CPR transect, and only CPR silks with coordinates matching eDNA samples were compared (see 4.1.2). This resulted in 41 sampling locations, each with a LargeVF, SmallVF, and CPR sample (n = 123). For eDNA and CPR comparisons, Hill numbers were only calculated at q = 0 to account for methodological differences. To visualise differences in community composition a principal coordinate analysis (PCO) was conducted.

An accompanying R markdown file including code for all analyses and figure generation, and accompanying data files is available via Zenodo (see 4.1.2). Results of Primer v7 outputs are provided in Table S 4-5 – Table S 4-13.

4.5 Results

4.5.1 Southern Ocean physical zonation

Changes in the physical characteristics of the Southern Ocean are used to characterise the fronts of the ACC. While these fronts can only be precisely identified at depths (Orsi et al., 1995), both SST and SSS have shown regions of steep physical change that correspond to the average position of the fronts (Sokolov & Rintoul, 2002). In our study, the southern branch of the STF (STF-S) was located at 46°S 140.4°E. The northern branch of the SAF (SAF-N) was located at 48.2°S 135.5°E and the southern branch (SAF-S) at 50.5°S 124.5°E. The northern PF (PF-N) was located at approximately 51.8°S 119.9°E and the southern branch (PF-S) at 54.7°S 108.5°E. The transect crossed the SACCF north (SACCF-N) at 57.9°S 99°E and 59.4°S and 87°E, however, the transect may have been continuously crossing the front between these coordinates. The southern branch of the SACCF (SACCF-S) was located at approximately 57.9°S and 83°E, and the SBDY at 63.1°S and 78°E. We identified the zones of the Southern Ocean using the observed physical characteristics of Southern Ocean fronts (Figures S1 and S2), and following boundaries previously used (Table 1). Average SST logically decreased from north to south, with 12.2°C in the STZ, 9.09°C in the SAZ, 3.67°C in the PFZ, 1.04°C in the AAZ, and -0.42°C in the SACCZ. Salinity was highest closer to the continents and lowest in the open ocean zones with average SSS of 34.9 in the STZ, 34.32 in the SAZ, 33.89 in the PFZ, 33.89 in the AAZ

4.5.2 eDNA Animalia comparison

4.5.2.1 eDNA sequencing statistics and depth

A total of 2.43 million sequencing reads were generated using the COI Leray metabarcoding assay post-quality, denoising and chimera filtering. These were assigned to 1273 ZOTUs, of which 808 were assigned to Animalia taxa. Using the LCA algorithm, 619 of the 808 Animalia ZOTUs were taxonomically resolved to species

level, representing 98.16% of the total Animalia reads. Two ZOTUs could not be resolved and were excluded from analysis, both were hits to '*zooplankton environmental sample*' on GenBank. While a reasonably large number of ZOTUs were assigned to Animalia taxa, this represented just 46.86% of the overall reads (1.14 million sequencing reads).

Overall sequencing read numbers were similar between the SmallVF samples (1.27 million reads) and the LargeVF samples (1.16 million reads). However, just 11.37% of reads within the SmallVF samples were assigned to Animalia taxa, compared to 85.66% of reads within the LargeVF samples (Figure S 4-3). SmallVF samples were largely dominated by subkingdoms Chlorophyta (47.49%), Harosa (19.89%), and Hacrobia (15.09%). A Wilcoxon signed rank test indicated differences in Animalia read numbers between the paired samples, with LargeVF median sample ranks (11,376 reads) significantly higher than SmallVF median sample ranks (696 reads; $V=2077$, $p < 0.001$). These differences are reflected spatially within the data, with the proportion of Animalia reads within SmallVF samples consistently lower than LargeVF samples across all Southern Ocean zones (Figure 4-3). This is particularly pronounced in the SAZ where overall reads were higher for SmallVF samples in comparison to LargeVF samples (529,515 and 204,424 respectively), but Animalia read proportion was considerably lower with 0.008% and 85.23% respectively. For LargeVF samples, Animalia read proportion relatively high across all zones (> 75%). The highest proportion for SmallVF samples was observed in the open ocean AAZ where mean species richness was highest, potentially reflecting the number of samples taken within this zone.

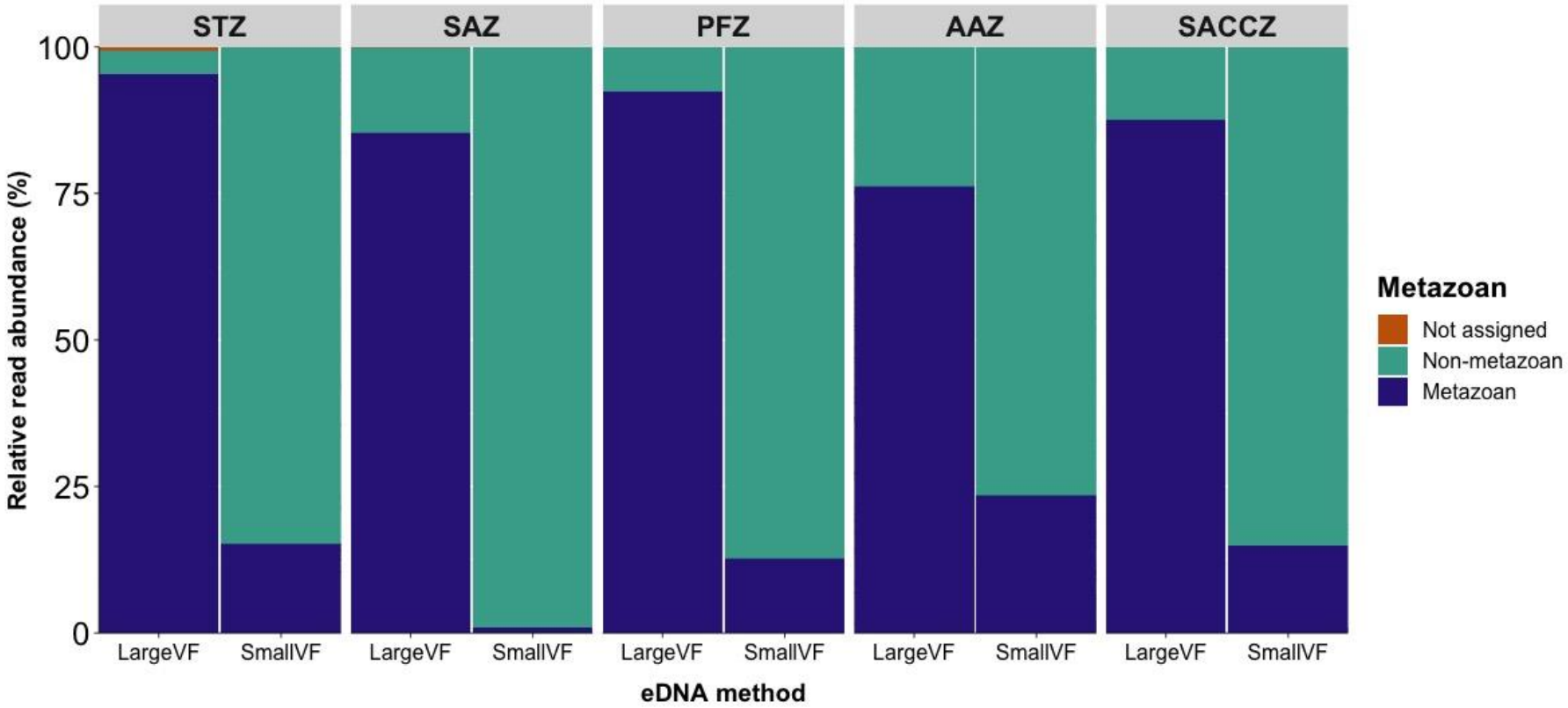

Figure 4-3: Proportion of reads classified as 'Animalia', 'non-Animalia', or 'not assigned' across LargeVF (12 L and 20 µm filter pore size) and SmallVF (2 L and 0.45 µm filter pore size) samples within Southern Ocean zones. eDNA samples (water) were amplified using the COI Leray metabarcoding assay.

4.5.2.2 Overall diversity using eDNA

A total of 68 marine Animalia species were detected using both eDNA sampling methods, representing 54 genera, 43 families and 24 orders. Predominant families were Calanidae (7 species), Euphausiidae (5 species), Clausocalanidae (5 species), and Myctophidae (5 species). Taxonomic resolution was comparable between the LargeVF and SmallVF samples, with over 76% of ZOTUs assigned to species level for both volumes (78.07% and 76.95% respectively). The number of species detected was higher in the LargeVF samples (65) than the SmallVF samples (54), with 14 species exclusively found in the LargeVF samples (Figure 4-4a). The majority of these 14 species were arthropods (8; Figure 4-4b). Mean species richness was significantly higher in the LargeVF samples (10.03 $\pm$ 0.44) compared to SmallVF samples (8.4 $\pm$ 0.44; $t = 4.825$, $df = 64$, $p < 0.001$). When comparing relative read abundance and detection frequency of species assignments, both eDNA volumes were dominated by Arthropoda (Figure S 4-4). SmallVF samples were dominated by *Ctenocalanus citer* for both read abundance (42.41%) and frequency of detection (53; see 4.1.2). Comparatively, LargeVF samples were dominated by *C. citer* in terms of read abundance (32.87%) and *Oithona similis* for frequency of detection (58; see 4.1.2).

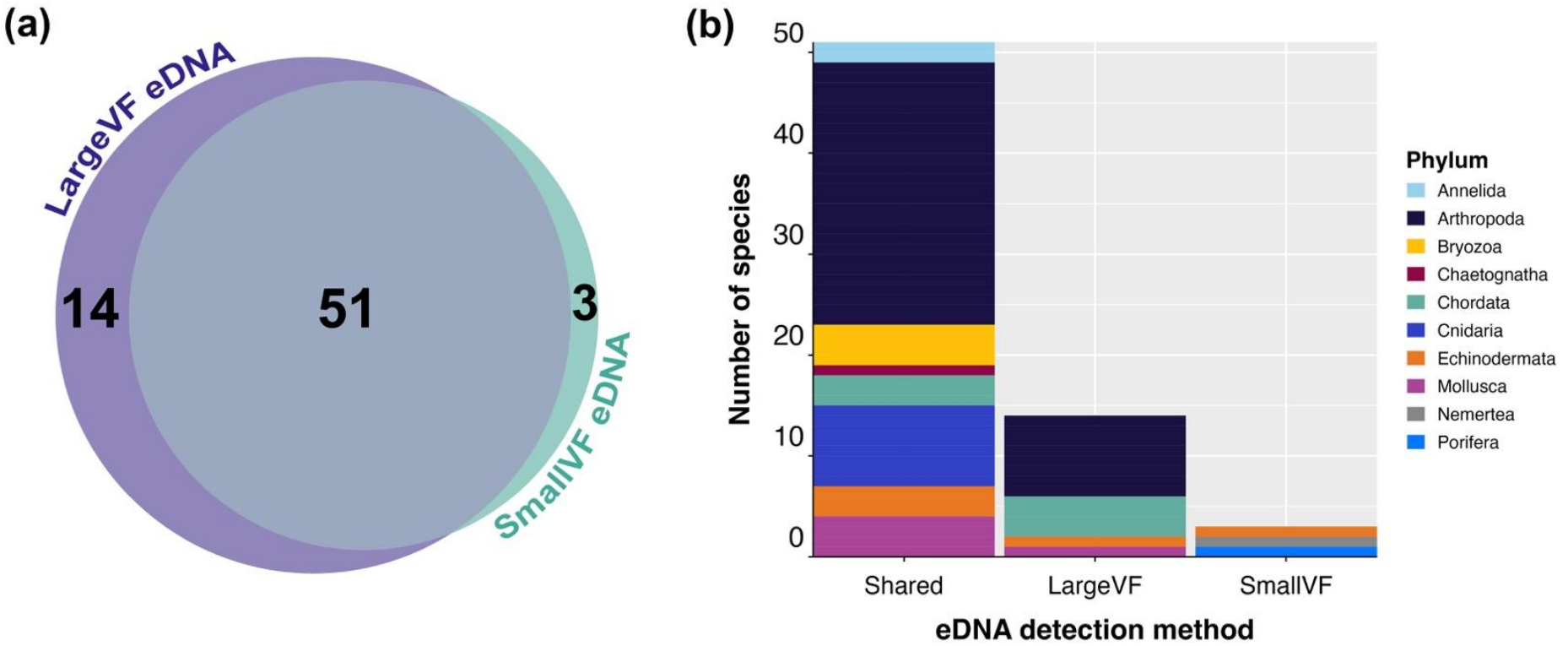

Figure 4-4: Unique and shared species detected by LargeVF (12 L and 20 µm filter) and SmallVF (2 L and 0.45 µm filter) eDNA metabarcoding in the Southern Ocean. (a) Venn diagram of the number of unique species detected by LargeVF (12 L and 20 µm filter) and SmallVF (2 L and 0.45 µm filter) eDNA metabarcoding, and the number of shared species detections using these approaches. (b) The number of unique species detections exclusive to and shared between LargeVF and SmallVF eDNA metabarcoding. All metabarcoding was performed using the COI Leray metabarcoding assay.

4.5.2.3 Spatial trends

Animalia diversity was compared across the Southern Ocean Zones for both eDNA appraoches (Figure 4-5). Overall, mean species richness (q = 0) was greatest in the AAZ (LargeVF: 9.75 $\pm$ 0.37; SmallVF: 7.88 $\pm$ 0.44) and the PFZ (LargeVF: 8.84 $\pm$ 0.47; SmallVF: 6.33 $\pm$ 0.33). Mean species richness was lowest in the SACCZ and the STZ for LargeVF (4.5 $\pm$ 0.72) and SmallVF (3.17 $\pm$ 1.38) samples respectively. Although following similar trends (Figure 4-5), mean species richness was significantly higher in LargeVF samples across all Southern Ocean zones with the exception of the SACCZ (Table S 4-14). Hill numbers became comparable between the volumes as they increased, with no significant differences at q = 1 or q = 2. The significant difference in species richness between LargeVF and SmallVF, and lack thereof for the abundance weighted diversity indices, suggests that LargeVF samples may be detecting more rare or low-abundant species, but a similar number of abundant species in comparison to SmallVF samples.

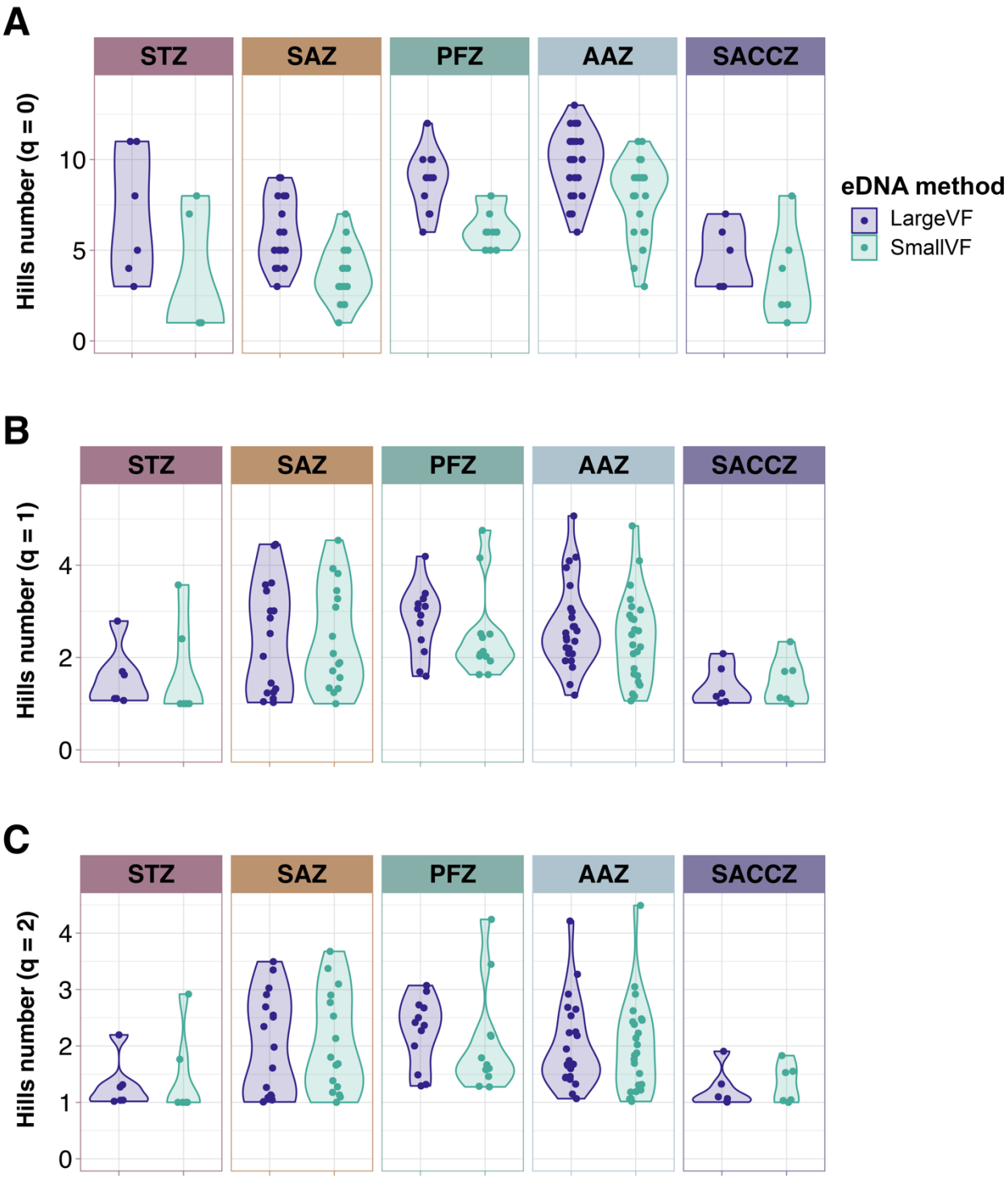

Figure 4-5: Comparison of Animalia species diversity detected using LargeVF (12 L and 20 µm filter) and SmallVF (2 L and 0.45 µm filter) eDNA methodologies across Southern Ocean zones. Hill numbers were calculated at q = 0 (species richness), q = 1 (Shannon diversity) and q = 2 (Simpson diversity) within each zone. Species abundance becomes more weighted with increasing Hill numbers.

PERMANOVA analysis of the presence-absence matrix confirmed community composition significantly differed between the eDNA survey methods ($t = 1.979$, $df = 1$, $p = 0.002$) and Southern Ocean zones ($F = 22.604$, $df = 4$, $p < 0.001$), with pairwise testing indicating all zones were distinct in composition (Table S 4-11). Taxonomic composition was dependent on both eDNA volume and zone, with a significant interaction ($F = 1.569$, $df = 4$, $p = 0.039$) and pairwise testing (Table S 4-9) further demonstrating that eDNA collection method can considerably alter observed spatial trends. However, no strong separations of taxonomic composition based on eDNA volume and filter size combination were evident in the nMDS (Figure 4-6) indicating that differences between the zones were greater than differences between sampling methods. Communities appeared to transition gradually along the South Ocean transect, with sites near zone boundaries sharing similar composition to those in the adjacent zone (Figure 4-6). Indicator species analyses identified copepod taxa *Calanoides acutus*, *C. citer*, *Calanus propinquus/similimus*, and *Metridia lucens* as contributing most to similarities between the zones (see 4.1.2)

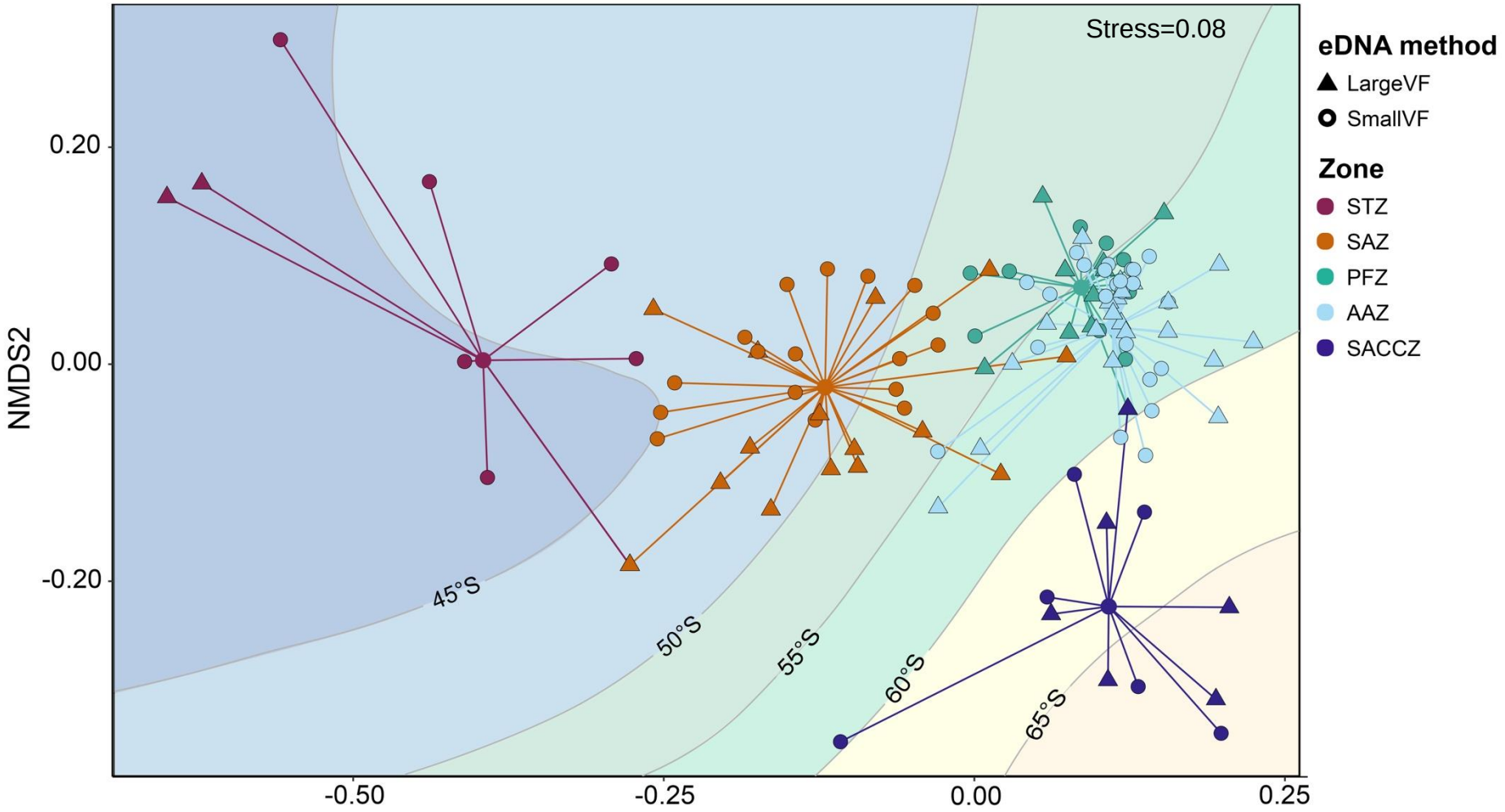

Figure 4-6: Non-metric multi-dimensional (nMDS) scaling plot of Animalia taxonomic composition across Southern Ocean zones using two eDNA methodologies: LargeVF (12 L and 20 µm filter) and SmallVF (2 L and 0.45 µm filter).

nMDS was conducted on a Jaccard resemblance matrix of presence-absence data, centroids are indicated by the junction of each zone. The relationship of the samples to latitude is overlaid.

4.5.2.4 Detection of species of concern via eDNA

We detected the presence of 16 species of concern in eDNA samples (Figure 4-7). These species are either classified as invasive to Tasmania, non-native to Tasmania, or native to Tasmania but detected close the continent of Antarctica (south of 60°S,Table S 4-15 – 16). These species comprised five phyla: Cnidaria (6, class Hydrozoa), Bryozoa (4, class Gymnolaemata), Arthropoda (2, classes Malacostraca and Thecostraca), Echinodermata (2, class Asteroidea), and Mollusca (2, class Bivalvia). The majority of species (87.5%) were detected at the beginning of the transect in the Derwent Estuary, Hobart (Tasmania). Detections along the transect were sparse in the open ocean region, with the exception of bryozoan species *Bougainvillia muscus*, *Bugulina flabellata*, and hydrozoan species *Coryne eximia*. At ~59°S detections were seen to increase for multiple species, coinciding with the onset of sea-ice. Total read numbers were relatively low (< 100) for half of the species detected (Table S 4-15), reflecting the use of a broad-spectrum Animalia assay.

Overall read numbers for species of concern were significantly higher in LargeVF samples than SmallVF samples ($V = 9135$, $p < 0.001$). While frequency of detection was higher in SmallVF samples than LargeVF samples, with 140 and 180 detections respectively. Despite this, the number of unique species detected was higher in LargeVF samples than SmallVF samples, with 16 (100%) and 14 (87.5%) species detected respectively. SmallVF samples failed to detect crab species *Metacarcinus novaezelandiae* and sea star *Patiriella regularis*. Both species were only detected in one sample taken at the start of the transect in the Port of Hobart. Reads (16,130) and Animalia read proportion (72.94%) were higher in this LargeVF sample in comparison to the paired SmallVF sample taken simultaneously (8229 and 56.98%, respectively).

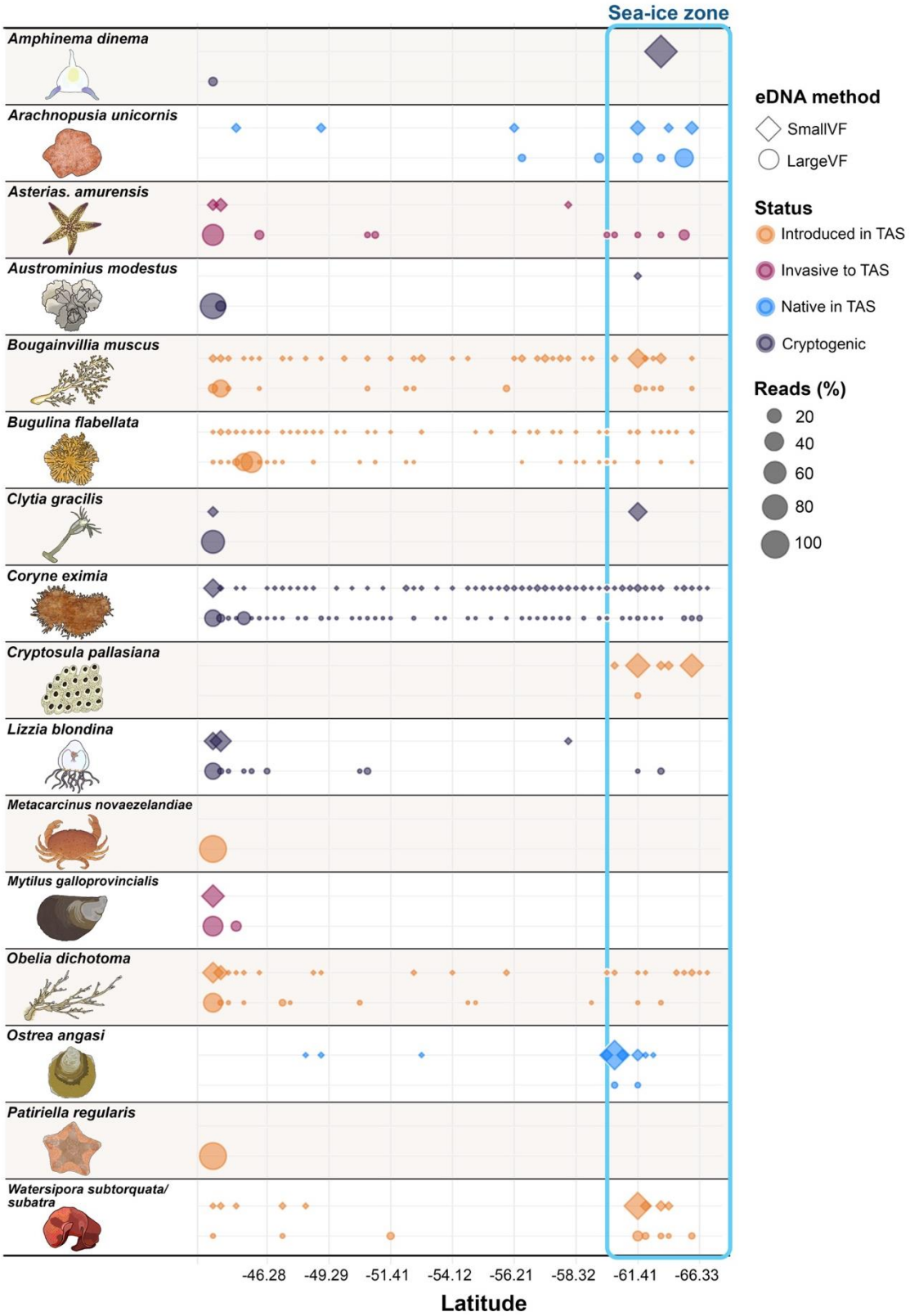

Figure 4-7: Read proportion of species of concern detected using eDNA methodologies, LargeVF (diamond) and SmallVF (circle). Reads are plotted on the x axis by latitude in the southerly direction of the transect (Hobart, Tasmania to Davis Station, Antarctica. Proportions are calculated by total reads per individual species, and not by the overall total reads, so that detection trends of less abundant reads are not overlooked due to the presence of more dominant/abundant reads. The status of each species is indicated by the different colours (see legend) as well as the onset of sea-ice (blue box).

4.5.3 Morphological CPR comparison

4.5.3.1 CPR overview

Morphological analysis of CPR tows (n = 368) identified 55,465 individual specimens, of which 48,282 (87.05%) were classified as Animalia taxa. Taxonomic assignments were predominantly resolved to species level (44), followed by genus (15), family (4), order (5), class (4), phylum (2), and other (1; chordate larvae). A total of 32 unique Animalia species (28,441 specimens) were detected across the CPR tows, representing 58.91% of the total specimens recorded. CPR species assignments were dominated by Arthropod taxa (99.17%), with 24 unique species and 28,205 individual specimens. Copepod taxa *O. similis* was the most dominant species observed in the CPR tows (66.06%).

4.5.3.2 Diversity comparison

Diversity was compared between paired CPR and eDNA samples i.e., eDNA water samples and CPR silks taken at the same coordinates. Taxonomic resolution was comparable between eDNA methods, with almost 80% of ZOTUs assigned to species level for both LargeVF (79.96%) and SmallVF (78.81%) method, while almost 60% of specimens detected across CPR silks (58.84%) were identified to species level. At the phylum level, eDNA detected 7 phyla, of which 4 were exclusive to the survey method (Bryozoa, Cnidaria, Echinodermata, and Mollusca). Comparatively, CPR detected 4 phyla of which Annelida was exclusive to the survey method (Figure 4-8b). Unique species detections were highest using LargeVF eDNA (35), followed by SmallVF (29), and CPR (18). We found little overlap between methodologies, with only 11 species shared between all methodologies (Figure 4-8a). Arthropod taxa were dominant across all methodologies and comprised majority of the species detections that were shared between methodologies (11, Figure 4-8b). Species exclusive to CPR comprised arthropod (3), annelid (2) and one tunicate species. eDNA exclusive detections were comprised of 6 different phyla, predominantly arthropod (5), cnidarian (4), and bryozoan (3) taxa (Figure 4-8b), and comprised 8 non-native, pest, or invasive species.

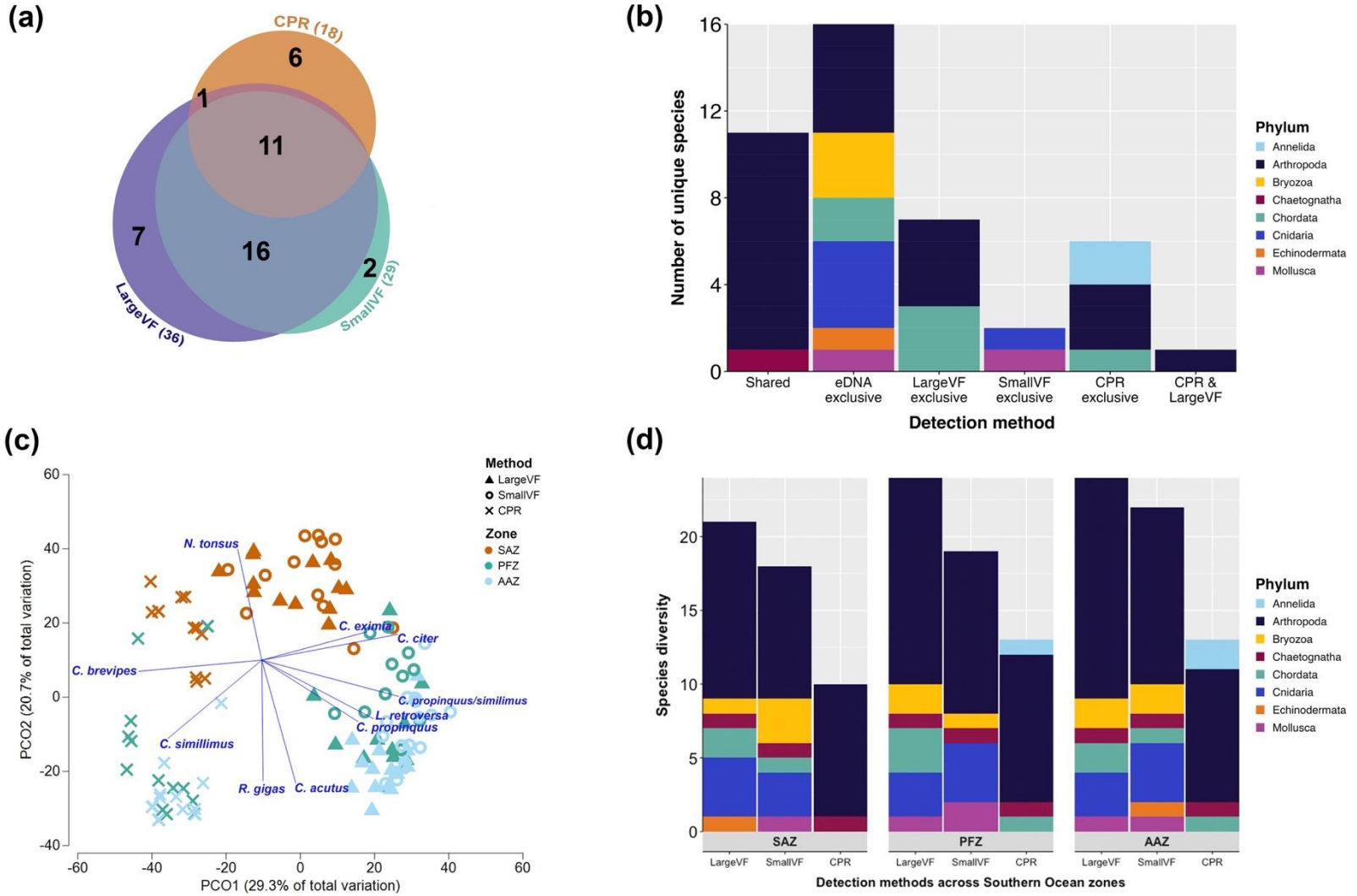

Figure 4-8: Animalia species composition detected by eDNA metabarcoding (LargeVF and SmallVF) using the Leray COI assay and CPR surveys in the Southern Ocean. (a) Proportional Euler diagram of unique and shared species between methods. (b) Number of unique and shared species per Animalia phyla for each method. (c) Principal coordinate analysis (PCO) of taxonomic composition detected by each method, conducted on a Jaccard resemblance matrix of presence-absence data. Species contributing most to the variation in composition are overlayed, with vector length proportional to the strength of the correlation (Pearson correlation > 0.5). (d) Species diversity per phyla, detected using three methods across Southern Ocean zones.

4.5.3.3 Spatial trends

When comparing CPR to eDNA methods, significant interactions were observed between Southern Ocean zones and sampling method in the presence-absence matrix (Pseudo-$F_{2\text{-}4}$ = 4.704, $p < 0.001$). Pairwise testing revealed that while all methods and zones significantly differed from one another, composition did not differ between LargeVF and SmallVF samples within the PFZ. These interactions are illustrated in the PCO (Figure 4-8c), with both sampling method (PCO1) and Southern Ocean zone (PCO2) having strong effects on community composition. Despite the effects of sampling method, patterns in composition were similar between methods, with a transition from SAZ to AAZ across all methods (Figure 4-8c). Overlapping between the PFZ and AAZ was seen in all methods, with indicator analyses revealing similarities were driven by *Rhincalanus gigas* and *C. propinquus/similimus* (eDNA) or *C. similimus* (CPR). Diversity and composition differed between the zones depending on the chosen survey method, with observed diversity highest in the PFZ and AAZ for CPR and LargeVF samples, and the AAZ for SmallVF samples (Figure 4-8d). While all zones were dominated by arthropod taxa across methods, each method comprised a unique assemblage (Figure 4-8d). Richness was highest on average in the AAZ for all methods, with hill numbers (q = 0) following similar trends across the methodologies (Figure S 4-5).

4.6 Discussion

4.6.1 eDNA metabarcoding baseline of the Southern Ocean

In light of mounting anthropogenic pressures and the escalating risk of non-native species invasions in the warming Southern Ocean, there is an urgent need to develop monitoring tools that can augment and enhance long-term CPR surveys in the region to capture biodiversity across the tree of life. This would enable the development of more diverse temporal and spatial baselines to monitor potential biotic responses to these increasing pressures. Therefore, this study aimed to refine eDNA approaches for Southern Ocean monitoring, and compare their efficacy to simultaneously conducted CPR surveys. While both methods effectively revealed biodiversity

patterns in the Southern Ocean, eDNA exhibited advantages by enabling surveys to be conducted in the presence of sea-ice. This not only expanded the breadth of taxa detected, but enabled us to provide early warning signals for potential biological invasions to the region, underscoring the benefits of incorporating eDNA into the monitoring toolkit for this vulnerable ecosystem. We emphasise that the integration of CPR, eDNA, and biophysical data in a temporal and spatial framework can provide a more comprehensive understanding of the Southern Ocean and Antarctic ecosystems to better inform future conservation efforts.

4.6.2 Long-term conservation impacts

The extreme nature of the Southern Ocean requires a range of techniques to monitor the effects of climate change and increasing anthropogenic pressures (Gutt et al., 2018; Howell et al., 2021). Multiple resupply voyages occurring over the Austral summer present an exciting opportunity to develop spatial and temporal baseline data for a broad range of Southern Ocean taxa. The simplicity of the sampling protocol could facilitate the beginning of a 'community science' program, as suggested by Howell et al. (2021), with non-specialists collecting samples annually on resupply voyages. While sampling quality and reproducibility will need to be carefully managed (Lukyanenko et al., 2016), the ability to generate large amounts of spatiotemporal data is invaluable, and the importance of developing taxonomically diverse baseline data cannot be understated.

To reduce the logistical challenges of eDNA sample collection and to increase the extent of potential survey areas, automated eDNA samplers have been designed and are being increasingly implemented in field surveys (Breier et al., 2020; Formel et al., 2021; Govindarajan et al., 2022; Hendricks et al., 2022; Herfort et al., 2016; Ribeiro et al., 2019). Automated eDNA sampling can be either active through the use of autonomous underwater vehicles (Truelove et al., 2022) or drones (Aucone et al., 2023), or passive by leaving filter membranes submerged for extended periods of time (Bessey et al., 2021). These automated approaches could allow samples to be taken at great depths and in hard-to-reach areas, such as under ice shelfs, with the

potential to dramatically increasing the survey area beyond any current biological survey method. Moreover, the automation of laboratory processing, bioinformatic pipelines and analyses is simultaneously addressing issues of reproducibility and standardisation and increasing the speed at which eDNA data can be generated (Mousavi-Derazmahalleh et al., 2021).

4.6.3 eDNA derived biodiversity patterns of the Southern Ocean

Using eDNA metabarcoding, we successfully identified 68 unique Animalia species from 10 different phyla across the Southern Ocean. Community composition between Southern Ocean zones displayed significant differences in the species detected, demonstrating that eDNA surveys can be informative over large scales and in open ocean systems. The composition detected by eDNA followed similar spatial patterns for both methodologies, and was largely congruent with previously established Southern Ocean diversity profiles (Hunt & Hosie, 2003; McLeod et al., 2010, 2010; Pinkerton et al., 2020; Takahashi & Hosie, 2021). Given the growing evidence suggesting eDNA signals degrade or dilute rapidly over small spatial scales (Alexander et al., 2020; Jeunen et al., 2019; Koziol et al., 2019; Nester et al., 2020; West et al., 2020), our study extends this phenomenon to the open ocean, revealing eDNA's utility in elucidating broad biodiversity patterns. However, our study did not explicitly investigate eDNA transport and this finding should be interpreted with caution.

In analysing biodiversity patterns across Southern Ocean zones, we removed potential hull-fouling organisms. Prior to this, clustering was observed between zones closest to Hobart (STZ and SAZ) and the zone closest to Antarctica (SACCZ). This was unlike previous diversity profiles of the Southern Ocean, and was unexpected given clear poleward changes in Southern Ocean community composition have previously been described (Cornils et al., 2018; Hunt & Hosie, 2005; Pakhomov & McQuaid, 1996; Pinkerton et al., 2020; Takahashi et al., 2010), and the ability of eDNA to detect spatially distinct assemblages is well documented (O'Donnell et al., 2017; Stat et al., 2017; West et al., 2021). This highlights the significant impact of hull-

fouling organisms on inferences of spatial patterns using eDNA data, emphasizing the importance of thorough data checking and the removal of potential hull-fouling species prior to analysis.

4.6.4 Refining eDNA methodologies for open-ocean monitoring

Applications of eDNA biomonitoring in open ocean environments are expanding (Canals et al., 2021; Shelton et al., 2022; Suter et al., 2021, 2023, p. 202; Takeuchi et al., 2019), and refinement of these methodologies is required to determine their viability for long-term monitoring. Contributing to this, we evaluated patterns in diversity and richness between two combinations of water volume and filter pore sizes, 12 L with 20 µm (LargeVF), and 2 L with 0.45 µm (SmallVF). We found that although the number of sequencing reads were comparable between eDNA methodologies, assigned Animalia reads were substantially lower in SmallVF samples. We attribute this to a combination of smaller pore size of the filter paper and smaller volume of water filtered: smaller pore sizes are more likely to retain large amounts of non-Animalia micro-organisms that can co-amplify with the Leray-COI assay(i.e., algae), while the small water volume likely contains overall less Animalia template than the larger water volume.

Interestingly, recent work using the same combinations of water volume and pore sizes found that larger water volumes and pore sizes were more likely to detect more fragmented eDNA than smaller volumes with smaller pore sizes (Suter et al., 2023). This suggests that short fragments of eDNA, potentially bound to organic matter (Nagler et al., 2022), can be retained by coarse or large filters, while non-Animalia microorganisms may be captured less frequently than with small pore-sized filters. Given the Leray-COI assay targets Animalia taxa, our results suggest that pore size and water volume can considerably impact assay performance and efficacy. As our study was conducted in the open-ocean with relatively low numbers of Animalia taxa (Suter et al., 2021), these findings may not be applicable to coastal environments with high numbers of diverse Animalia species. Additionally, these assignments are usually excluded from analyses or not reported in studies, particularly when

established assays are used. However, this information can only assist in refining eDNA metabarcoding as a monitoring tool that is effective across a wide variety of environments. We therefore support others in encouraging studies to report the assignment and proportion of non-target reads (Collins et al., 2019), particularly when established 'universal' assays are used.

Animalia taxonomic resolution within eDNA methodologies was comparable, with high proportions of assignments to species level for both methods. However, we found LargeVF samples produced more taxonomic assignments and detected more unique species along the transect than SmallVF samples. These unique species comprised a small portion of the total LargeVF reads, indicating that more rare and low abundant species were detected, a trend that was reflected in Hill number analyses. This is again most likely attributed to the large pore size increasing the proportion of Animalia template in LargeVF samples, and therefore increasing the likelihood of detecting rare or low abundant taxa. The recommendation of pore sizes varies in the literature, with a large number of studies suggesting the choice relies heavily on the characteristics of the sampling environment (Barnes et al., 2021; Budd et al., 2021; Cooper et al., 2022; Kumar et al., 2021). We recognise the difference between our pore sizes is large, and the use of different volumes introduces bias. Nevertheless, for open ocean environments, we suggest pore sizes larger than 0.45µm will maximise detected Animalia diversity and richness, and recommend further testing of large volumes (12 L or greater) across commonly used larger pore sizes (1.2 µm, 5 µm, 10 µm, and 20 µm) to further optimize eDNA sampling for these environments.

4.6.5 Detection of species of concern

We detected the presence of 16 species of concern classified as invasive to Tasmania, non-native to Tasmania, or native to Tasmania but detected close the continent of Antarctica (south of 60°S). Hull biofouling and ballast water constitute the two main anthropogenic pathways for marine species to Antarctica (Hughes & Ashton, 2017). Ballast water introductions into the region are considered a minimal threat as vessels

generally take on ballast water in the region following unloading of cargo (Lee & Chown, 2009; Lewis et al., 2003), and discharge of ballast water is prevented by the Antarctic Treaty (ATCM (Antarctic Treaty Consultative Meeting), 2006). Comparatively, hull biofouling has been described as the greatest potential pathway for marine introductions (Lee & Chown, 2009). Six of the species recorded have previously been recorded on the hull of the *RSV Aurora Australis* (Lewis et al., 2003, 2004, 2005), and the remaining are either known biofouling (GISD - iucngisd.org, GRIIS - griis.org) or possible biofouling organisms (hull fouling recorded at genus levels, (Lee & Chown, 2009; Lewis et al., 2004, 2005). We therefore believe the presence of these species as biofouling on the *RSV Aurora Australis* presents the most likely explanation for their detection and potential transport.

Detections of these species were relatively sparse in open ocean zones with the exception of two bryozoan (*B. muscus* and *B. flabellata*) and one hydrozoan species (*C. eximia*) that were detected almost consistently along the transect. While it is plausible that the frequency of these detections could be the result of large populations on the hull of the ship, we are not able to reject the possibility that these species are present in the ships uncontaminated seawater line due to their small size and colonial growth forms (ALA – ala.org.au). As sampling over the side of the vessel is largely impossible given the harsh conditions of the Southern Ocean and other methods (such as CTD drops) are not feasible aboard a resupply vessel, the potential growth of species within the uncontaminated seawater line must be assessed prior to future eDNA surveys.

An increase in detection frequency was observed at ~59°S for the remaining species of concern, coinciding with the onset of sea-ice. The abrasion of sea-ice on vessel hulls is thought to act as a natural hull cleaner, removing fouling organisms with the sheer force associated with pushing through sea-ice (Hughes & Ashton, 2017; Lee & Chown, 2009; Lewis et al., 2004). The sudden reappearance and increase in detections indicate that these biofouling species may have been scraped off the hull into the water column, potentially breaking apart encrusting or colonial organisms and increasing the likelihood of eDNA detection. Although abrasion by sea-ice is

considered a significant factor reducing the number of marine biofouling species from being introduced to the region (Hughes & Ashton, 2017), it cannot be relied upon to prevent introduction or settlement. Ships may not encounter sea-ice due to temporal variation in sea-ice extent and thickness (McCarthy et al., 2019), as well as an overall decline in response to climate change (Eayrs et al., 2021; Ludescher et al., 2019; Raphael & Handcock, 2022). Furthermore, particular areas of ships may not be subject to ice abrasion, highlighted by Lee and Chown (2007) who revealed the invasive mussel *Mytilus galloprovincialis* had survived transportation throughout the broader Antarctic region within two sea chests of the *SA Agulhas*.

Our study detected the Northern Pacific sea star (*Asterias amurensis*), a species previously identified as a high-risk potential invader to Antarctic ecosystems (L. J. Clarke et al., 2022; Holland et al., 2021) The Northern Pacific sea star is an aggressive global invader due its high fecundity (Agüera & Byrne, 2018; Ling et al., 2012) and broad thermal tolerance (Byrne et al., 2016), and the detrimental effects it has on native species and community composition are well documented (Hayes et al., 2005; Lowe et al., 2000). The species displays extensive phenotypic plasticity, is able to alter its spawning times to align with local conditions, and has an introduced range in cold climate locations including Canada and Alaska (Buttermore et al., 1994; Byrne et al., 1997, 2016; Ling et al., 2012). Crucial to our findings, recent environmental modelling indicated the Northern Pacific sea star could survive at all stations under current or future climate scenarios (Holland et al., 2021).

It is crucial to recognise that we cannot determine the source or developmental stage of eDNA detections, and we therefore cannot rule out the possibility that these detections represent transported DNA, larvae, or dead organisms. Additionally, while these species were not detected in any field or laboratory controls implemented, overall read numbers were low, most likely due to the use of a broad-spectrum Animalia assay. To increase the sensitivity of these detections and provide a comprehensive overview of their potential distribution, we recommend the implementation of a suite of species-specific assays targeting these species of concern. Interestingly, the number of potential pest, non-native or invasive species

detected was higher in LargeVF samples, despite the frequency of detection being greater in SmallVF samples. To explore the effect of pore size and water volume on the detection of these species, we recommend future work using the suite of assays across these combinations. The reporting of invasive marine species (IMS) in Antarctic regions is infrequent (Aronson et al., 2015; Avila et al., 2020, p. 2020; Cárdenas et al., 2020; Fraser et al., 2018; Hughes & Ashton, 2017; McCarthy et al., 2019; Tavares & De Melo, 2004; Thatje & Fuentes, 2003), and while no established IMS populations are currently reported, the rapid warming of the region may see an increase in the number and range of organisms capable of surviving (McCarthy et al., 2019). It is widely recognised that preventing IMS establishment is a more successful and cost-effective approach than eradication (Finnoff et al., 2007; Hanley & Roberts, 2019), a particularly difficult task in remote locations (Rout et al., 2011). Currently, there are no systematic IMS surveillance programs in the Antarctic region (Holland et al., 2021). The implementation of eDNA surveys presents a unique opportunity to monitor these otherwise hard to detect threats.

4.6.6 Comparison of eDNA and CPR for Southern Ocean monitoring

We successfully identified 87 unique species from 10 different phyla across the Southern Ocean using a combination of eDNA metabarcoding (LargeVF and SmallVF) and CPR transects. When comparing to paired CPR samples (silk segments) taken at the same coordinates, both eDNA methods detected more species across a wider range of phyla than CPR samples. eDNA survey methods detected seven phyla of which four were unique, while CPR detected four phyla of which Annelida was unique. Nonetheless, all survey methods detected numerous unique species, and the overlap of species detected between approaches was small. Unique CPR detections were primarily copepod species, which can be attributed to its targeted approach for surveying plankton taxa. However, widespread and abundant copepod taxa *Clausocalanus brevipes* and *Thysanoessa macrura* had noticeably fewer detections in eDNA samples. It is possible that these species were not detected as frequently with eDNA methodologies due to low shedding rates of invertebrate taxa with exoskeletons and the known difficulties associated with detecting these taxa groups

(Danziger & Frederich, 2022; Tréguier et al., 2014). Similarly, detection frequency may have been affected by diel vertical migrations undertaken in the water column (Hays, 2003). Similar eDNA studies have successfully reflected this diel migration in samples taken at the surface (Suter et al., 2021) and at depth (Feng et al., 2022). However, the surface samples in Suter at al. (2021) were taken at much shorter intervals (every 30 minutes) than in the present study, suggesting that sampling effort should be increased if the detection of these patterns is desired. Furthermore, we used a broad 'universal' assay with a large amplicon length and primer degeneracies to expand the diversity of taxa detected. In such assays, common reads with fewer mismatches will often dominate PCR amplification, and rare templates may be underrepresented, resulting in PCR biases. Additionally, the relatively long amplicon length of the Leray-COI assay may not be effective at detecting degraded DNA fragments. Targeted group-specific assays with relatively short amplicon lengths may provide a more contemporary insight into these assemblages through increased detection rates (Jo et al., 2017; Wu et al., 2019). However, this approach comes with an increase in both time and financial costs, and should be considered with the specific project aims in mind.

Southern Ocean CPR surveys are among the most temporally and spatially extensive biological datasets available, providing invaluable baseline data for plankton taxa in a rapidly changing environment. Our data highlights the familiar strengths and limitations of the approach. Its targeted design (e.g., small mouth opening) may inadvertently omit species detected with eDNA metabarcoding, or damage specimens beyond identification. Additionally, certain life stages and morphology types (e.g., gelatinous) present challenges for morphological identification with CPR, and may only be broadly identified (e.g., egg, larvae). In contrast, eDNA metabarcoding can identify taxa regardless of life stage or specimen quality, but relies on often incomplete reference databases for accurate species assignments. In addition, eDNA survey locations are more versatile than CPR surveys – eDNA surveys can be conducted into the sea-ice zone. In doing so, we not only expanded the diversity and richness detected, but were able to detect several non-native species of concern in the Antarctic region that got scraped off the hull in the sea-ice zone.

Surveys can also be expanded to include the whole water column (Canals et al., 2021; Feng et al., 2022), extending the current footprint and providing a method to detect these species of concern if settlement occurs i.e. at depth. Ultimately, each approach has its benefits, and the strengths of a combined genetic and conventional monitoring approach are well documented (Deagle et al., 2018; Nester et al., 2022; Stat et al., 2018; West et al., 2022). We advocate for the integration of an eDNA survey to be conducted in parallel with CPR surveys.

4.7 Conclusion

Monitoring the growing effects of anthropogenic pressures and climate change in the Southern Ocean requires a baseline understanding of the distribution and composition of its taxonomically diverse marine biota. Here we demonstrate the potential of eDNA metabarcoding as a long-term biomonitoring tool, and its ability to complement existing CPR surveys. Our evaluation of eDNA methodologies found larger volumes (12 L) with larger pore sizes (20 µm) were more suited to open ocean surveys as they detected greater proportions of Animalia template, and a higher number of rare or low abundant species. The detection of several non-native species using eDNA metabarcoding was a significant finding of the study, and further highlights the known weaknesses of monitoring remote environments using a singular approach. Given the vulnerability of Antarctic environments to the introduction of non-native species, future work should optimise a suite of targeted group or species-specific assays to enhance sensitivity and increase the likelihood of detecting these species in a timely manner. By combining these eDNA detections with CPR and ongoing collection of biophysical data, the number of species detected increased more than two-fold. We also found that although CPR surveys inadvertently omit several phyla, both methods detected high numbers of unique taxa. We advocate for the incorporation of eDNA metabarcoding to support long-term CPR surveys of the Southern Ocean, noting that the ability to overlay more metabarcoding data (across the tree-of-life) and biobank samples has not been explored here.

As the allocation of monitoring resources and establishment of potential mitigation strategies heavily relies on ecological interpretations of long-term datasets, the timely implementation of these surveys cannot be understated. With multiple annual resupply voyages across the Southern Ocean occurring over the Austral Summer, a unique opportunity is presented to monitor long-term spatial and temporal patterns in the region, expanding upon taxonomic diversity of the long-standing CPR surveys. We anticipate that such combined approaches will have profound implications for the conservation and preservation of the Southern Ocean and the Antarctic region.

4.8 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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4.9 Supplementary material

Table S 4-1: Sampling information for eDNA water samples collected aboard the RSV Aurora Australis on a voyage from Hobart (Tasmania) to Antarctica in October-November 2019

Sample Number	Water Volume	UTC Date	UTC Time
V1 001	2L	22/10/2019	1:07
V1 002	12L	23/10/2019	4:13
V1 003	2L	23/10/2019	
V1 004	2L	25/10/2019	6:20
V1 005	12L	25/10/2019	
V1 006	2L	25/10/2019	10:34
V1 007	12L	25/10/2019	
V1 008	2L	25/10/2019	20:08
V1 009	12L	25/10/2019	
V1 010	2L	26/10/2019	1:20
V1 011	12L	26/10/2019	
V1 012	2L	26/10/2019	5:23
V1 013	12L	26/10/2019	
V1 014	2L	26/10/2019	11:18
V1 015	12L	26/10/2019	
V1 016	2L	27/10/2019	0:14
V1 017	12L	27/10/2019	
V1 018	2L	27/10/2019	4:08
V1 019	12L	27/10/2019	
V1 020	2L	27/10/2019	8:08
V1 021	12L	27/10/2019	
V1 022	2L	27/10/2019	11:03
V1 023	12L	27/10/2019	
V1 024	2L	27/10/2019	19:35
V1 025	12L	27/10/2019	
V1 026	2L	27/10/2019	
V1 027	12L	27/10/2019	
B⃠1	2L	27/10/2019	23:50
B⃠2	12L	27/10/2019	
W⃠1	2L	27/10/2019	
W⃠2	12L	27/10/2019	
V1 028	2L	28/10/2019	5:04
V1 029	12L	28/10/2019	
V1 030	2L	28/10/2019	8:15

V1 031	12L	28/10/2019	
V1 032	2L	28/10/2019	12:16
V1 033	12L	28/10/2019	
V1 034	2L	28/10/2019	21:39
V1 035	12L	28/10/2019	
V1 036	2L	29/10/2019	0:30
V1 037	12L	29/10/2019	
V1 038	2L	29/10/2019	5:23
V1 039	12L	29/10/2019	
V1 040	2L	29/10/2019	8:31
V1 041	12L	29/10/2019	
V1 042	2L	29/10/2019	12:30
V1 043	12L	29/10/2019	
V1 044	2L	29/10/2019	21:57
V1 045	12L	29/10/2019	
V1 046	2L	30/10/2019	1:48
V1 047	12L	30/10/2019	
V1 048	2L	30/10/2019	6:19
V1 049	12L	30/10/2019	
V1 050	2L	30/10/2019	9:23
V1 051	12L	30/10/2019	
V1 052	2L	30/10/2019	13:31
V1 053	12L	30/10/2019	
V1 054	2L	30/10/2019	21:00
V1 055	12L	30/10/2019	
V1 056	2L	31/11/19	1:04
V1 057	12L	31/11/19	
V1 058	2L	31/11/19	5:09
V1 059	12L	31/11/19	
V1 060	2L	31/11/19	9:08
V1 061	12L	31/11/19	
V1 062	2L	31/11/19	12:55
V1 063	12L	31/11/19	
V1 064	2L	31/11/19	16:09
V1 065	12L	31/11/19	
V1 066	2L	31/11/19	21:01
V1 067	12L	31/11/19	
V1 068	2L	1/11/2019	1:02

V1 069	12L	1/11/2019	
V1 070	2L	1/11/2019	5:21
V1 071	12L	1/11/2019	
V1 072	2L	1/11/2019	8:11
V1 073	12L	1/11/2019	
V1 074	2L	1/11/2019	13:00
V1 075	12L	1/11/2019	
V1 076	2L	1/11/2019	16:17
V1 077	12L	1/11/2019	
V1 078	2L	1/11/2019	22:00
V1 079	12L	1/11/2019	
V1 080	2L	2/11/2019	1:18
V1 081	12L	2/11/2019	
V1 082	2L	2/11/2019	5:42
V1 083	12L	2/11/2019	
V1 084	2L	2/11/2019	10:31
V1 085	12L	2/11/2019	
V1 086	2L	2/11/2019	14:01
V1 087	12L	2/11/2019	
V1 088	2L	2/11/2019	17:20
V1 089	12L	2/11/2019	
V1 090	2L	2/11/2019	22:00
V1 091	12L	2/11/2019	
V1 092	2L	3/11/2019	1:52
V1 093	12L	3/11/2019	
V1 094	2L	3/11/2019	6:45
V1 095	12L	3/11/2019	
V1 096	2L	3/11/2019	9:15
V1 097	12L	3/11/2019	
V1 098	2L	3/11/2019	13:56
V1 099	12L	3/11/2019	
V1 100	2L	3/11/2019	16:59
V1 101	12L	3/11/2019	
V1 102	2L	3/11/2019	23:00
V1 103	12L	3/11/2019	
V1 104	2L	4/11/2019	3:06
V1 105	12L	4/11/2019	
V1 106	2L	4/11/2019	7:35

V1 107	12L	4/11/2019	
V1 108	2L	4/11/2019	10:22
V1 109	12L	4/11/2019	
V1 110	2L	4/11/2019	15:47
V1 111	12L	4/11/2019	
V1 112	2L	4/11/2019	22:51
V1 113	12L	4/11/2019	
V1 114	2L	5/11/2019	2:51
V1 115	12L	5/11/2019	
V1 116	2L	5/11/2019	7:00
V1 117	12L	5/11/2019	
V1 118	2L	5/11/2019	9:35
V1 119	12L	5/11/2019	
V1 120	2L	5/11/2019	15:36
V1 121	12L	5/11/2019	
V1 122	2L	5/11/2019	23.09
V1 123	12L	5/11/2019	
V1 124	2L	6/11/2019	2:55
V1 125	12L	6/11/2019	
V1 126	2L	6/11/2019	7:05
V1 127	12L	6/11/2019	
V1 128	2L	6/11/2019	11:12
V1 129	12L	6/11/2019	
V1 130	2L	6/11/2019	16:31
V1 131	12L	6/11/2019	
V1 132	2L	6/11/2019	23:08
V1 133	12L	6/11/2019	
V1 134	2L	7/11/2019	3:00
V1 135	12L	7/11/2019	
V1 136	2L	7/11/2019	10:15
V1 137	12L	7/11/2019	
B⃠3	2	7/11/2019	
B⃠4	12	7/11/2019	
W⃠3	2	7/11/2019	
W⃠4	12	7/11/2019	
V1 138	2	9/11/2019	1:32
V1 139	12	9/11/2019	

Table S 4-2: Sampling information for CPR samples (silk segments) collected aboard the *RSV Aurora Australis* on a voyage from Hobart (Tasmania) to Antarctica. Samples were collected across four tows/transects in October-November 2019.

The tow shaded grey was not analysed in this study as it is part of an ongoing IMOS study.

Ship Code	Season	Voyage	CPR Tow	CPR tow (this study)	Start Position		End Position		Start / End Dates		Distance (Nm)	Segments	Route
AA	2019/20	1	1	IMOS study	44° 17.985' S	145° 04.610' E	47° 08.050' S	136° 01.761' E	25/10/2019	27/10/2019	417	83	Hobart - Davis
AA	2019/20	1	2	1	47° 08.050' S	136° 01.761' E	49° 54.227' S	126° 51.832' E	27/10/2019	29/10/2019	403	81	Hobart - Davis
AA	2019/20	1	3	2	49° 54.734' S	126° 50.816' E	52° 51.104' S	115° 58.480' E	29/10/2019	31/10/2019	446	89	Hobart - Davis
AA	2019/20	1	4	3	52° 51.882' S	115° 54.735' E	56° 00.250' S	103° 13.366' E	31/10/2019	2/11/2019	494	96	Hobart - Davis
AA	2019/20	1	5	4	56° 00.673' S	103° 11.700' E	59° 09.105' S	088° 25.428' E	2/11/2019	4/11/2019	515	102	Hobart - Davis

Table S 4-3: Identification of potential contaminants and non-target Animalia ZOTUs for removal.

ZOTUs assigned to contaminants, non-Animalia, or non-marine Animalia taxa (indicated by 'C') were entirely removed. Potential cross-contaminant ZOTUs with more than 0.5% of reads originating from controls (indicated by '>1%') were also entirely removed. Below this, ZOTUs were kept (indicated in bold text). For these ZOTUs, the number of reads present in controls or the percentage of the ZOTU comprised of the control (whichever was higher) was removed from the samples.

In control	ZOTU	Phylum	Class	Order	Family	Genus	Species	Comment/ Action	Reason	% ID	Total Reads
No	Zotu3579	Arthropoda	Collembola	Entomobryomorpha	Isotomidae	*Isotomurus*	*Isotomurus maculatus*	Remove ZOTU	C	100	26
No	Zotu4546	Arthropoda	Collembola	Entomobryomorpha	Isotomidae	*Isotomurus*	*Isotomurus maculatus*	Remove ZOTU	C	99.7	20
No	Zotu2907	Arthropoda	Insecta	Diptera	Calliphoridae	*Calliphora*	*Calliphora*	Remove ZOTU	C	100	48
No	Zotu5331	Arthropoda	Insecta	Hymenoptera	Braconidae	*Diaeretiella*	*Diaeretiella rapae*	Remove ZOTU	C	100	11
No	Zotu2243	Arthropoda	Insecta	Lepidoptera	Erebidae	*Lithosiini*	*Lithosiini*	Remove ZOTU	C	98.7	73
No	Zotu7434	Arthropoda	Insecta	Lepidoptera	Limacodidae	*Doratifera*	*Doratifera oxleyi*	Remove ZOTU	C	98.7	4
No	Zotu1862	Chordata	Aves	Charadriiformes	Laridae	*Larus*	*Larus*	Remove ZOTU	C	100	104
No	Zotu5542	Chordata	Mammalia	Carnivora	Canidae	*Canis*	*Canis lupus familiaris*	Remove ZOTU	C	100	12
No	Zotu7124	Chordata	Mammalia	Carnivora	Canidae	*Canis*	*Canis lupus familiaris*	Remove ZOTU	C	100	6

Yes	Zotu3622	Chordata	Mammalia	Carnivora	Felidae	*Felis*	*Felis*	Remove ZOTU	C	99.7	32
Yes	Zotu5627	Chordata	Mammalia	Carnivora	Felidae	*Felis*	*Felis*	Remove ZOTU	C	99.4	19
No	Zotu3622	Chordata	Mammalia	Carnivora	Felidae	*Felis*	*Felis*	Remove ZOTU	C	99.7	32
No	Zotu5627	Chordata	Mammalia	Carnivora	Felidae	*Felis*	*Felis*	Remove ZOTU	C	99.4	19
Yes	Zotu233	Chordata	Mammalia	Primates	Hominidae	*Homo*	*Homo sapiens*	Remove ZOTU	C	100	4724
No	Zotu2720	Chordata	Mammalia	Primates	Hominidae	*Homo*	*Homo sapiens*	Remove ZOTU	C	100	100
No	Zotu1998	Chordata	Mammalia	Primates	Hominidae	*Homo*	*Homo sapiens*	Remove ZOTU	C	100	92
No	Zotu2162	Chordata	Mammalia	Primates	Hominidae	*Homo*	*Homo sapiens*	Remove ZOTU	C	99.7	82
No	Zotu2273	Chordata	Mammalia	Primates	Hominidae	*Homo*	*Homo sapiens*	Remove ZOTU	C	99.3	74
No	Zotu2685	Chordata	Mammalia	Primates	Hominidae	*Homo*	*Homo sapiens*	Remove ZOTU	C	100	62
No	Zotu2816	Chordata	Mammalia	Primates	Hominidae	*Homo*	*Homo sapiens*	Remove ZOTU	C	100	46
No	Zotu4107	Chordata	Mammalia	Primates	Hominidae	*Homo*	*Homo sapiens*	Remove ZOTU	C	100	20

No	Zotu7570	Chordata	Mammalia	Primates	Hominidae	*Homo*	*Homo sapiens*	Remove ZOTU	C	100	11
No	Zotu233	Chordata	Mammalia	Primates	Hominidae	*Homo*	*Homo sapiens*	Remove ZOTU	C	100	9475
No	Zotu1728	Chordata	Mammalia	Primates	Hominidae	*Homo*	*Homo sapiens*	Remove ZOTU	C	99.7	226
No	Zotu1981	Chordata	Mammalia	Primates	Hominidae	*Homo*	*Homo sapiens*	Remove ZOTU	C	100	154
No	Zotu2180	Chordata	Mammalia	Primates	Hominidae	*Homo*	*Homo sapiens*	Remove ZOTU	C	100	122
Yes	**Zotu131**	**Arthropoda**	**Copepoda**	**Calanoida**	**Calanidae**	***Neocalanus***	***Neocalanus tonsus***	**Keep ZOTU**	**<1%**	**98.7**	**9231**
Yes	**Zotu184**	**Arthropoda**	**Copepoda**	**Calanoida**	**Calanidae**	***Neocalanus***	***Neocalanus tonsus***	**Keep ZOTU**	**<1%**	**98.4**	**9468**
Yes	**Zotu198**	**Arthropoda**	**Copepoda**	**Calanoida**	**Calanidae**	***Neocalanus***	***Neocalanus tonsus***	**Keep ZOTU**	**<1%**	**98.4**	**8085**
Yes	**Zotu2291**	**Arthropoda**	**Copepoda**	**Calanoida**	**Calanidae**	***Neocalanus***	***Neocalanus tonsus***	**Keep ZOTU**	**<1%**	**98.1**	**193**
Yes	**Zotu70**	**Arthropoda**	**Copepoda**	**Calanoida**	**Clausocalanidae**	***Clausocalanus***	***Clausocalanus brevipes***	**Keep ZOTU**	**<1%**	**100**	**21300**
Yes	**Zotu387**	**Arthropoda**	**Copepoda**	**Calanoida**	**Clausocalanidae**	***Clausocalanus***	***Clausocalanus pergens***	**Keep ZOTU**	**<1%**	**100**	**1656**
Yes	**Zotu222**	**Arthropoda**	**Copepoda**	**Calanoida**	**Paracalanidae**	***Paracalanus***	***Paracalanus sp. B AC0013***	**Keep ZOTU**	**<1%**	**100**	**6518**

Yes	**Zotu720**	**Arthropoda**	**Copepoda**	**Calanoida**	**Paracalanidae**	***Paracalanus***	***Paracalanus sp. B AC0013***	**Keep ZOTU**	**<1%**	**99.7**	**954**
Yes	Zotu243	Arthropoda	Copepoda	Calanoida	Calanidae	*Neocalanus*	*Neocalanus tonsus*	Remove ZOTU	>1%	99	9
Yes	Zotu2902	Arthropoda	Copepoda	Calanoida	Calanidae	*Neocalanus*	*Neocalanus tonsus*	Remove ZOTU	>1%	98.1	1
Yes	Zotu3739	Arthropoda	Copepoda	Calanoida	Calanidae	*Neocalanus*	*Neocalanus tonsus*	Remove ZOTU	>1%	98.1	20
Yes	Zotu4405	Arthropoda	Copepoda	Calanoida	Calanidae	*Neocalanus*	*Neocalanus tonsus*	Remove ZOTU	>1%	98.1	1
Yes	Zotu493	Arthropoda	Copepoda	Calanoida	Calanidae	*Neocalanus*	*Neocalanus tonsus*	Remove ZOTU	>1%	98.1	2
Yes	Zotu1519	Arthropoda	Copepoda	Calanoida	Clausocalanidae	*Clausocalanus*	*Clausocalanus pergens*	Remove ZOTU	>1%	99	55
Yes	Zotu1428	Arthropoda	Copepoda	Calanoida	Paracalanidae	*Paracalanus*	*Paracalanus sp. F AC0013*	Remove ZOTU	>1%	99	2
Yes	Zotu1594	Arthropoda	Copepoda	Calanoida	Paracalanidae	*Paracalanus*	*Paracalanus sp. F AC0013*	Remove ZOTU	>1%	99.4	14
Yes	Zotu317	Arthropoda	Copepoda	Calanoida	Paracalanidae	*Paracalanus*	*Paracalanus sp. F AC0013*	Remove ZOTU	>1%	99.7	71
Yes	Zotu369	Arthropoda	Copepoda	Calanoida	Paracalanidae	*Paracalanus*	*Paracalanus sp. F AC0013*	Remove ZOTU	>1%	100	1180
Yes	Zotu2243	Arthropoda	Insecta	Lepidoptera	Erebidae	*Lithosiini*	*Lithosiini*	Remove ZOTU	>1%	98.7	73

Yes	Zotu716	Chaetognatha	Sagittoidea	Aphragmophora	Sagittidae	*Pseudosagitta*	*Pseudosagitta gazellae*	Remove ZOTU	>1%	100	787
Yes	Zotu3141	Nematoda	Chromadorea	Plectida	Plectidae	*Plectus*	*Plectus murrayi*	Remove ZOTU	>1%	92.3	38
Yes	Zotu8058	Nematoda	Chromadorea	Plectida	Plectidae	*Plectus*	*Plectus murrayi*	Remove ZOTU	>1%	92	8
Yes	Zotu1092	Rotifera	dropped	dropped	dropped	dropped	dropped	Remove ZOTU	>1%	90.5	18
Yes	Zotu2135	Rotifera	Eurotatoria	Ploima	Synchaetidae	*Synchaeta*	*Synchaeta pectinata*	Remove ZOTU	>1%	90.2	5
Yes	Zotu303	NA	NA	NA	NA	NA	Uncultured zooplankton	Remove ZOTU	>1%	99.7	29

Table S 4-4: ZOTUs identified as cross-contamination due to low number of target taxa reads present in controls (less than 0.05%). The number of reads present in controls or the percentage of the ZOTU comprised of the control (whichever was higher) was removed from the samples.

ZOTU	Phylum	Class	Order	Family	Genus	Species	% ID	Assignment	Reads in control	% of ZOTU in control	Total reads	
											Before	After
Zotu131	Arthropoda	Copepoda	Calanoida	Calanidae	*Neocalanus*	*Neocalanus tonsus*	98.7	Species	3	0.03	9231	9131
Zotu184	Arthropoda	Copepoda	Calanoida	Calanidae	*Neocalanus*	*Neocalanus tonsus*	98.4	Species	5	0.05	9468	9340
Zotu198	Arthropoda	Copepoda	Calanoida	Calanidae	*Neocalanus*	*Neocalanus tonsus*	98.4	Species	1	0.01	8085	8061
Zotu222	Arthropoda	Copepoda	Calanoida	Paracalanidae	*Paracalanus*	*Paracalanus sp. B AC0013*	100	Species	1	0.02	6518	6507
Zotu2291	Arthropoda	Copepoda	Calanoida	Calanidae	*Neocalanus*	*Neocalanus tonsus*	98.1	Species	1	0.52	193	176
Zotu387	Arthropoda	Copepoda	Calanoida	Clausocalanidae	*Clausocalanus*	*Clausocalanus pergens*	100	Species	5	0.3	1656	1606
Zotu70	Arthropoda	Copepoda	Calanoida	Clausocalanidae	*Clausocalanus*	*Clausocalanus brevipes*	100	Species	2	0.01	21300	21252
Zotu720	Arthropoda	Copepoda	Calanoida	Paracalanidae	*Paracalanus*	*Paracalanus sp. B AC0013*	99.7	Species	5	0.52	954	926

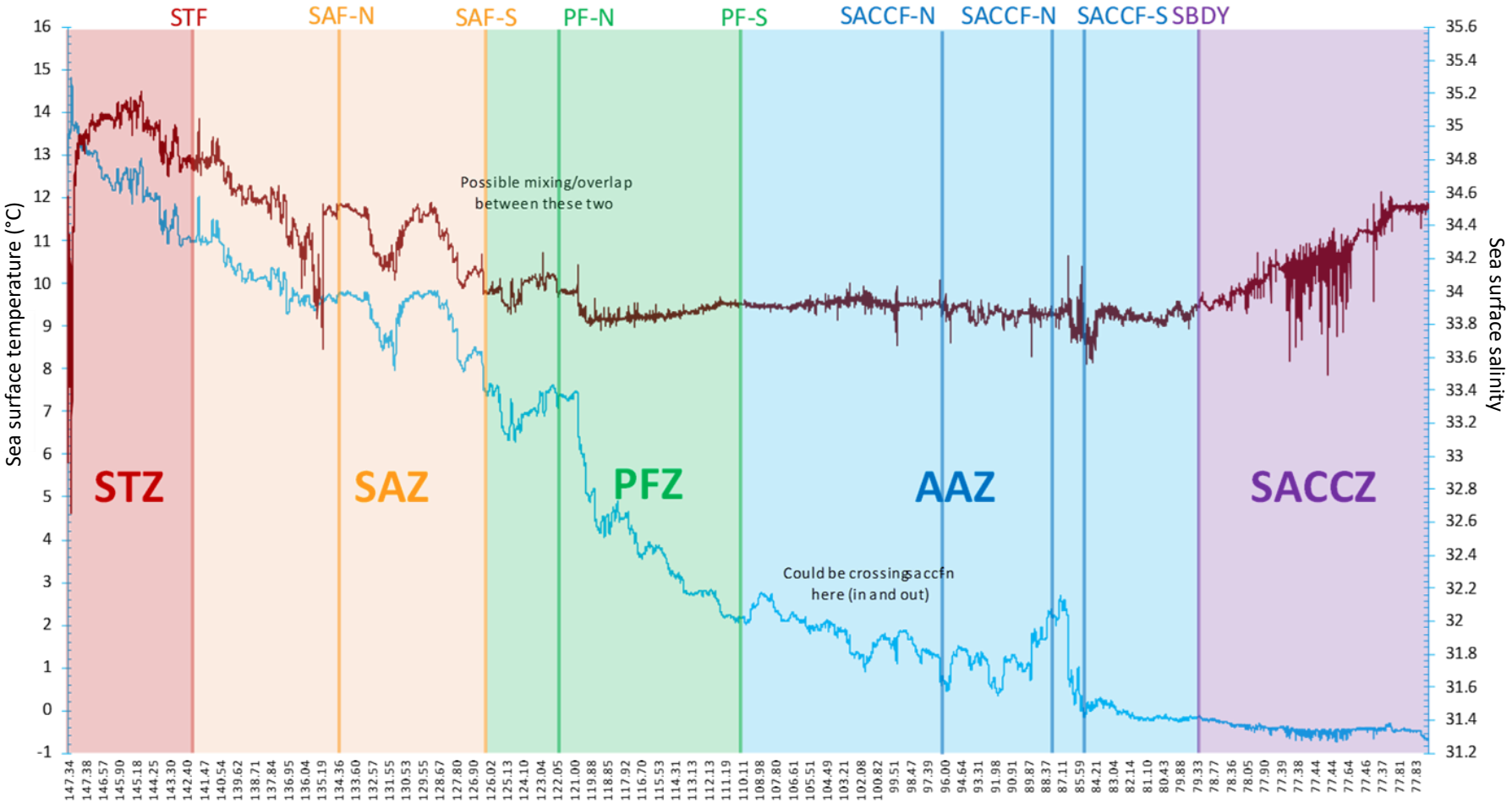


Figure S 4-1: Zones of the Southern Ocean plotted against latitude.

Zones were identified in this study using sea surface temperature (SST, °C) and sea surface salinity (SSS, psu) data obtained from a thermosalinograph (SeaBird SBE21) at approximately 7 m depth aboard the RV Aurora Australis. Sampling period was from October 22nd to November 9th, 2019, in the Austral Summer.

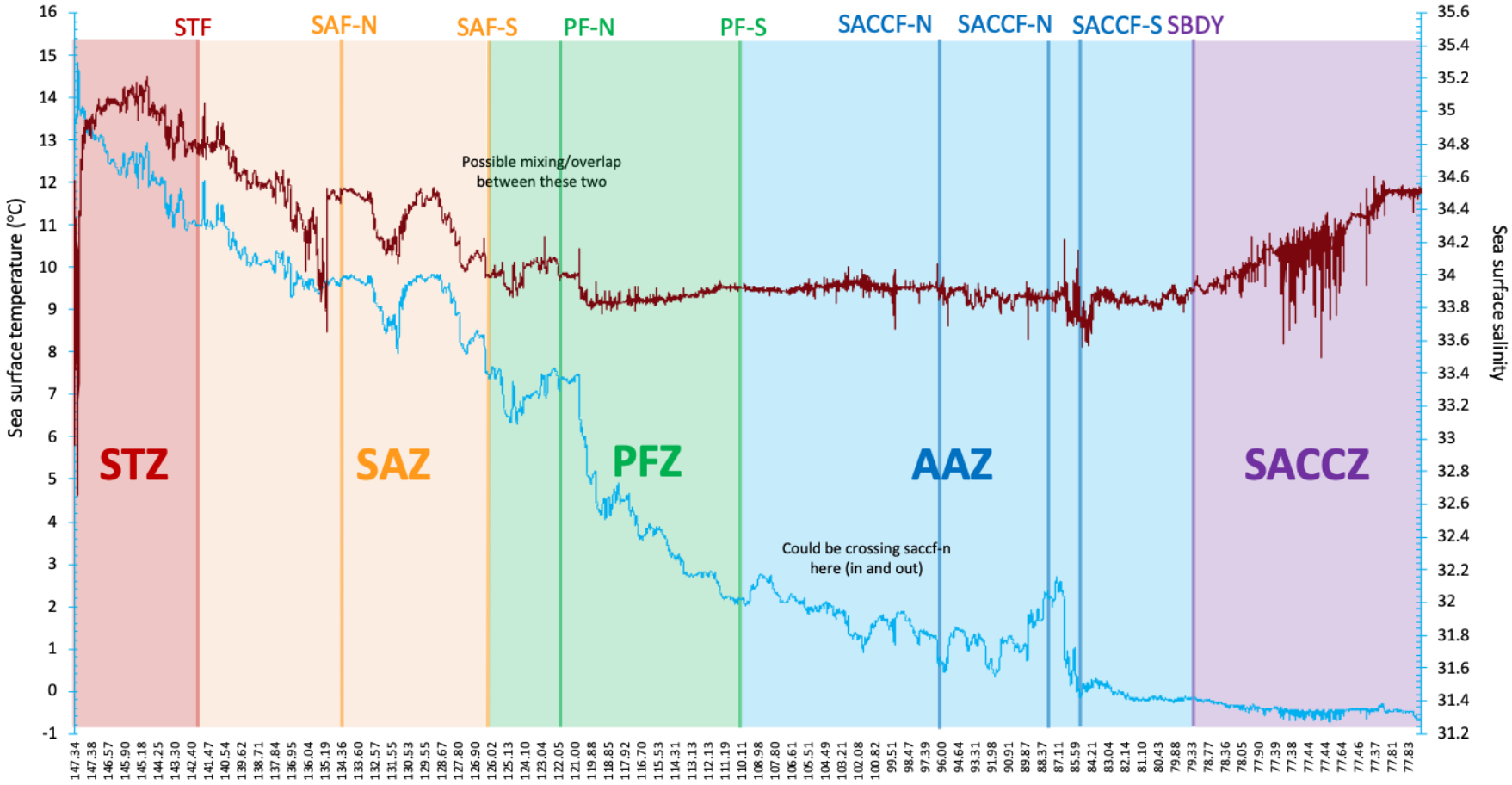


Figure S 4-2: Zones of the Southern Ocean plotted against longitude.
Zones were identified in this study using sea surface temperature (SST, °C) and sea surface salinity (SSS, psu) data obtained from a thermosalinograph (SeaBird SBE21) at approximately 7 m depth aboard the RV Aurora Australis. Sampling period was from October 22nd to November 9th, 2019, in the Austral Summer.

Table S 4-5: eDNA PERMANOVA - output comparing taxonomic composition between eDNA methodologies (SmallVF and LargeVF).

Analysis was conducted using Primer v7 with 9999 permutations on a Jaccard resemblance matrix of presence-absence data using fixed effects of Zone (Southern Ocean), Type (SmallVF and LargeVF), and Zone x Type.

Variable	df	SS	MS	Pseudo-F	P (perm)	Unique perms
Zone	4	1.17E+05	29336	17.049	0.0001	9876
Type	1	8546.5	8546.5	4.9668	0.0003	9941
Zone x Type	4	10042	2510.4	1.4589	0.0436	9858
Res	120	2.06E+05	1720.7			
Total	129	3.43E+05				

Table S 4-6: Pairwise eDNA PERMANOVA output for effects of interaction term - eDNA methodologies (Type) and Zone- on taxonomic composition.

Significant effects between eDNA methodologies within Southern Ocean zones were explored. Analysis was conducted using Primer v7 with 9999 permutations on a Jaccard resemblance matrix of presence-absence data.

Zone	Groups	t	P (perm)	Unique perms	Avg group similarity (%)
STZ	12L, 2L	1.4574	0.0816	119	18.997
SAZ	12L, 2L	1.3854	0.0636	9946	38.186
PFZ	12L, 2L	1.6134	0.0212	9719	55.748
AAZ	12L, 2L	1.3339	0.0961	9929	56.077
SACCZ	12L, 2L	1.8313	0.6628	462	27.094

Table S 4-7: Pairwise eDNA PERMANOVA - output for comparing taxonomic composition of eDNA methodologies (SmallVF and LargeVF).

Analysis was conducted using Primer v7 with 9999 permutations on a Jaccard resemblance matrix of presence-absence data.

Groups	t	P (perm)	Unique perms	Avg group similarity (%)
12L, 2L	2.2286	0.0002	9929	28.697

Table S 4-8: Pairwise eDNA PERMANOVA - output for Southern Ocean zones across both eDNA methodologies.

Analysis was conducted using Primer v7 with 9999 permutations on a Jaccard resemblance matrix of presence-absence data.

Groups	t	P (perm)	Unique perms	Avg group similarity (%)
STZ, SAZ	3.2757	0.0001	9933	15.378
STZ, PFZ	5.4423	0.0001	9946	2.6377
STZ, AAZ	6.2006	0.0001	9936	2.44
STZ, SACCZ	2.8991	0.0001	9904	1.5663
SAZ, PFZ	5.0086	0.0001	9936	26.61
SAZ, AAZ	6.7744	0.0001	9939	21.307
SAZ, SACCZ	3.4958	0.0001	9941	12.931
PFZ, AAZ	2.7456	0.0001	9937	52.473
PFZ, SACCZ	4.2192	0.0001	9933	16.085
AAZ, SACCZ	4.5632	0.0001	9940	18.161

Table S 4-9: PERMANOVA: CPR vs eDNA - output comparing taxonomic composition of eDNA methodologies (SmallVF and LargeVF) to CPR surveys across Southern Ocean zones.

Analysis was conducted using Primer v7 with 9999 permutations on a Jaccard resemblance matrix of presence-absence data using fixed effects of Zone (Southern Ocean), Type (SmallVF, LargeVF, and CPR), and Zone x Type.

Variable	df	SS	MS	Pseudo-F	P (perm)	Unique perms
Zone	2	79919	39959	31.651	0.0001	9920
Type	2	56034	28017	22.192	0.0001	9915
Zone x Type	4	23754	5938.6	4.7039	0.0001	9892
Res	114	1.44E+05	1262.5			
Total	122	3.08E+05				

Table S 4-10: Pairwise PERMANOVA: eDNA vs CPR - output for effects of interaction term of survey methodologies (Type: SmallVF, LargeVF, and CPR) and Zone (Southern Ocean) on taxonomic composition.

Significant effects between survey methodologies within Southern Ocean zones were explored. Analysis was conducted using Primer v7 with 9999 permutations on a Jaccard resemblance matrix of presence-absence data.

Zone	Groups	t	P (perm)	Unique perms	Avg group similarity (%)
SAZ	12L, 2L	1.6128	0.0058	9933	35.691
	12L, CPR	3.3681	0.0001	9918	33.657
	2L, CPR	3.7215	0.0001	9911	22.38
PFZ	12L, 2L	1.3928	0.0577	9880	49.481
	12L, CPR	4.3629	0.0001	9935	20.131
	2L, CPR	4.2494	0.0001	9932	15.282
AAZ	12L, 2L	1.9617	0.0002	9952	60.363
	12L, CPR	5.7094	0.0001	9945	29.075
	2L, CPR	5.6749	0.0001	9933	21.454

Table S 4-11: Pairwise eDNA PERMANOVA - output for Southern Ocean zones across both eDNA methodologies.

Analysis was conducted using Primer v7 with 9999 permutations on a Jaccard resemblance matrix of presence-absence data

Groups	t	P (perm)	Unique perms	Avg group similarity (%)
STZ, SAZ	3.2757	0.0001	9933	15.378
STZ, PFZ	5.4423	0.0001	9946	2.6377
STZ, AAZ	6.2006	0.0001	9936	2.44
STZ, SACCZ	2.8991	0.0001	9904	1.5663
SAZ, PFZ	5.0086	0.0001	9936	26.61
SAZ, AAZ	6.7744	0.0001	9939	21.307
SAZ, SACCZ	3.4958	0.0001	9941	12.931
PFZ, AAZ	2.7456	0.0001	9937	52.473
PFZ, SACCZ	4.2192	0.0001	9933	16.085
AAZ, SACCZ	4.5632	0.0001	9940	18.161

Table S 4-12: PERMANOVA: CPR vs eDNA - output comparing taxonomic composition of eDNA methodologies (SmallVF and LargeVF) to CPR surveys across Southern Ocean zones.

Analysis was conducted using Primer v7 with 9999 permutations on a Jaccard resemblance matrix of presence-absence data using fixed effects of Zone (Southern Ocean), Type (SmallVF, LargeVF, and CPR), and Zone x Type.

Variable	df	SS	MS	Pseudo-F	P (perm)	Unique perms
Zone	2	79919	39959	31.651	0.0001	9920
Type	2	56034	28017	22.192	0.0001	9915
Zone x Type	4	23754	5938.6	4.7039	0.0001	9892
Res	114	1.44E+05	1262.5			
Total	122	3.08E+05				

Table S 4-13: Pairwise PERMANOVA: eDNA vs CPR - output for effects of interaction term of survey methodologies (Type: SmallVF, LargeVF, and CPR) and Zone (Southern Ocean) on taxonomic composition.

Significant effects between survey methodologies within Southern Ocean zones were explored. Analysis was conducted using Primer v7 with 9999 permutations on a Jaccard resemblance matrix of presence-absence data.

Zone	Groups	t	P (perm)	Unique perms	Avg group similarity (%)
SAZ	12L, 2L	1.6128	0.0058	9933	35.691
	12L, CPR	3.3681	0.0001	9918	33.657
	2L, CPR	3.7215	0.0001	9911	22.38
PFZ	12L, 2L	1.3928	0.0577	9880	49.481
	12L, CPR	4.3629	0.0001	9935	20.131
	2L, CPR	4.2494	0.0001	9932	15.282
AAZ	12L, 2L	1.9617	0.0002	9952	60.363
	12L, CPR	5.7094	0.0001	9945	29.075
	2L, CPR	5.6749	0.0001	9933	21.454

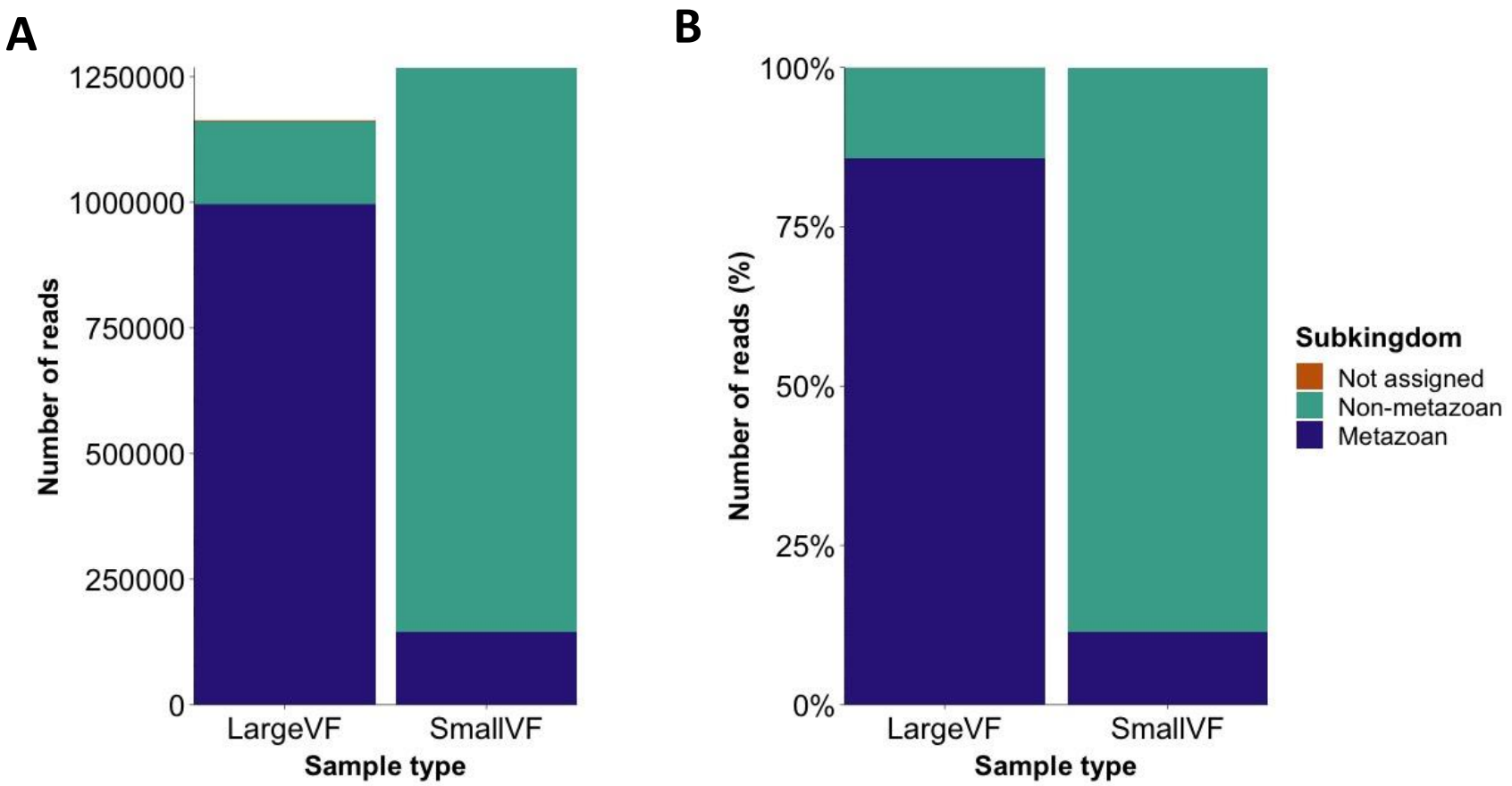


Figure S 4-3: eDNA subkingdom assignments.
Number of reads (A) and proportion of reads (B) assigned to Animalia, non-Animalia, and unclassified taxa between LargeVF and SmallVF eDNA samples using the Leray-CO1 assay

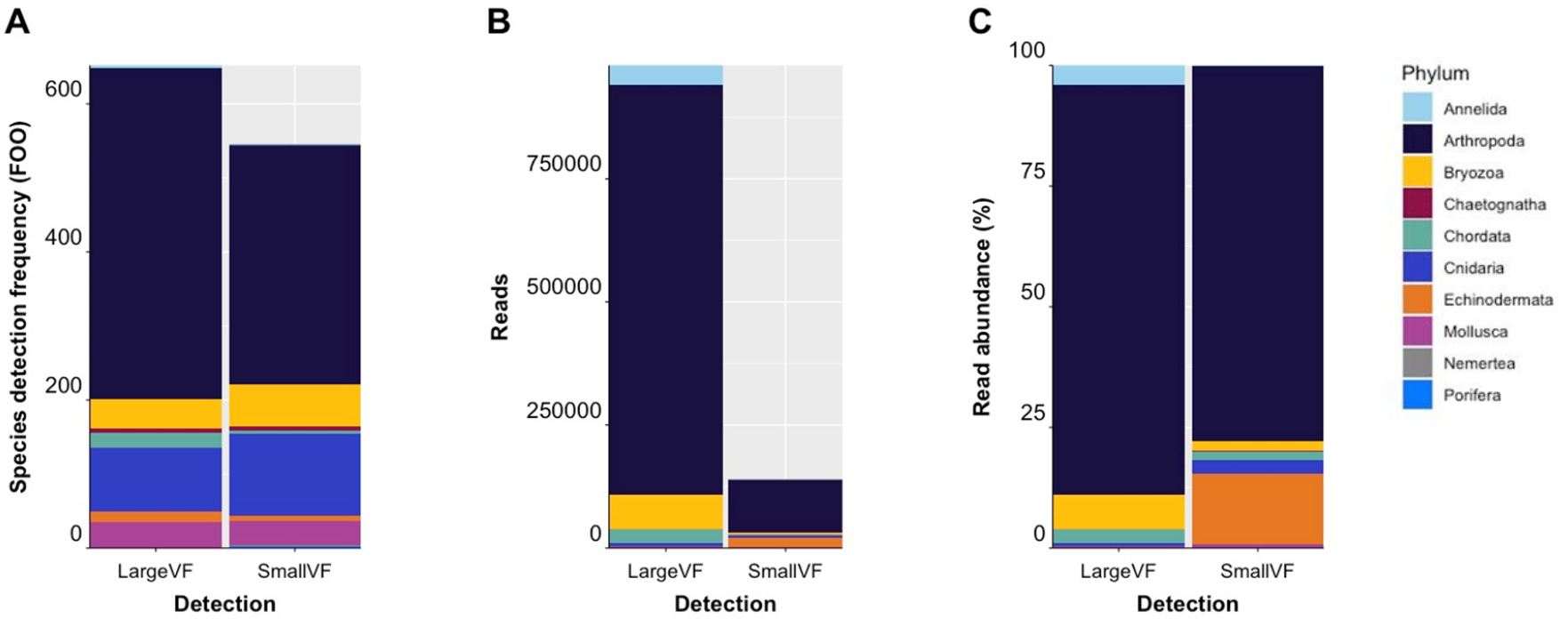


Figure S 4-4: eDNA methodology comparisons across Animalia phyla.
Detection frequency (frequency of occurrence, A), number of reads (B), and read abundance (%, C) of species level detections across different phyla in LargeVF and SmallVF eDNA samples. Samples of water were taken in the Southern Ocean in a transect from Hobart (Tasmania) to Davis Station (Antarctica) in October-November 2019, and were amplified using the Leray COI metabarcoding assay.

Table S 4-14: Statistical comparisons of differing Hills numbers between eDNA methodologies (LargeVF and SmallVF).

Paired t-tests or Wilcox tests were implemented depending on normality results. Statistical significance is indicated by '*'.

Test	Zone	Hills number	Group	Test statistic	p	sig
Wilcox	STZ	0	LargeVF & SmallVF	21	0.035	*
Wilcox	SAZ	0	LargeVF & SmallVF	105	0.011	*
Wilcox	PFZ	0	LargeVF & SmallVF	66	0.004	**
Wilcox	AAZ	0	LargeVF & SmallVF	236	<0.001	***
Wilcox	SACCZ	0	LargeVF & SmallVF	16	0.281	
t-test	STZ	1	LargeVF & SmallVF	10	1.000	
t-test	SAZ	1	LargeVF & SmallVF	64	0.860	
t-test	PFZ	1	LargeVF & SmallVF	52	0.339	
t-test	AAZ	1	LargeVF & SmallVF	189	0.277	
t-test	SACCZ	1	LargeVF & SmallVF	8	0.688	
t-test	STZ	2	LargeVF & SmallVF	10	1.000	
t-test	SAZ	2	LargeVF & SmallVF	61	0.744	
t-test	PFZ	2	LargeVF & SmallVF	51	0.380	
t-test	AAZ	2	LargeVF & SmallVF	156	0.877	
t-test	SACCZ	2	LargeVF & SmallVF	8	0.688	

Table S 4-15: Distribution & status information for putative detections of non-native, pest, or invasive species.

Invasive species lists checked were the CABI Invasive Species Compendium, Global Invasive Species Database (GISD), and the Global Register of Introduced and Invasive Species (GRIIS). Species were detected in the Southern Ocean outside of their ranges (or are known pest/invasive species) using eDNA methods (LargeVF and SmallVF). Water samples were amplified using the Leray COI metabarcoding assay.

Species	Common name	Phyla (Class)	Present in Tasmania/Australia	Listed as invasive	Listed as introduced to Aus	Reads	Hull-fouling organism	Native range	Comments	Resources
Metacarcinus novaezelandiae	Pie crust crab	Arthropoda (Malacostraca)	TAS	No	Yes	5	Yes	New Zealand		ALA, WoRMS and GRIIS
Austrominius modestus	Australasian barnacle	Arthropoda (Thecostraca)	TAS, VIC, NSW, SA	No	Unclear	121	Yes	Australia/New Zealand	Status debated as is native to NZ and mostly found in ports	ALA, WoRMS, CABI Invasive Species Compendium, GISD and GRIIS
Arachnopusia unicornis		Bryozoa (Gymnolaemata)	NSW and SA (ALA, GRIIS) Australia wide (WoRMS)	No	No	29		Australia/New Zealand		ALA, WoRMS and GRIIS
Bugulina flabellata		Bryozoa (Gymnolaemata)	TAS, VIC, NSW	No	Yes	72755	Yes	Northeastern Atlantic Ocean and the Mediterranean Sea	Introduced on WoRMS GRIIS	ALA, WoRMS and GRIIS

Cryptosula pallasiana	Bryozoa (Gymnolaemata)	WA, SA, VIC, TAS, NSW, QLD	No	Yes	69		North Atlantic, Mediterranean, and New Zealand (WoRMS)	TAS specimen collected off ship (CSIRO event ID 1157477)	ALA, WoRMS and GRIIS
Watersipora subtorquata/subatra	Bryozoa (Gymnolaemata)	Australia wide (subatra no registered occurrences)	No	Yes	154	Yes	Unclear	Subtorquata: Aus it is only considered pest Subatra: minimal literature but widely considered invasive	ALA, WoRMS, GISD and GRIIS
Amphinema dinema	Cnidaria (Hydrozoa)	Port Phillip Bay, VIC	No	Unclear	18		Circum-(sub)tropical	1 record collected/identified by Dr Jeanette E Watson (Museum VIC)	ALA and GRIIS
Bougainvillia muscus	Cnidaria (Hydrozoa)	TAS, VIC, NSW	No	Yes	1567	Yes	British Isles, North Sea, and Mediterranean Sea	Introduced on GRIIS Australia 2022	ALA, WoRMS and GRIIS

Clytia gracilis		Cnidaria (Hydrozoa)	NSW	No	Unclear	20	Yes	Atlantic Ocean, Mediterranean Sea, and North Sea	1 record collected/identified by Dr Jan E Watson (Aus Museum)	ALA, WoRMS and GRIIS
Coryne eximia		Cnidaria (Hydrozoa)	VIC, NSW, TAS (WoRMS only)	No	Unclear	5908		South-east Pacific Ocean, and Atlantic		ALA, WoRMS, CABI Invasive Species Compendium,and GRIIS
Lizzia blondina	British naked-eyed Medusæ	Cnidaria (Hydrozoa)	All Aus coastal waters (only on WORMS). None ALA	No	Unclear	287		Pacific Ocean and Atlantic Ocean	Hit on BOLD and NCBI- no other close hits	WoRMS, ALA, GRIIS
Obelia dichotoma	Sea thread hydroid	Cnidaria (Hydrozoa)	TAS, VIC, NSW, SA, QLD	No	Yes	1143	Yes	Pacific Ocean and Atlantic Ocean		ALA, WoRMS, CABI Invasive Species Compendium, and GRIIS
Asterias amurensis	North Pacific seastar	Echinodermata (Asteroidea)	TAS, VIC	Yes	Yes	78	Yes	North Pacific waters (Japan, Russia, north China, Korea)	On the Global Invasive Species Database's list of the 100 Worst Invasive Species	ALA, WoRMS, CABI Invasive Species Compendium, GISD and GRIIS

Patiriella regularis	New Zealand cushion star	Echinodermata (Asteroidea)	TAS	No	Yes	26		New Zealand	Concerns that it is outcompeting native seastar *Marginaster littoralis*	ALA, WoRMS and GRIIS
Mytilus galloprovincialis	Mediterranean mussel	Mollusca (Bivalvia)	TAS, WA, SA, VIC, NSW	Yes	Yes	22	Yes	Mediterranean, Black and Adriatic Seas		ALA, WoRMS, CABI Invasive Species Compendium, GISD and GRIIS
Ostrea angasi	Angasi oyster	Mollusca (Bivalvia)	TAS, WA, SA, VIC, NSW, QLD	No	No	314		Australia		ALA, WoRMS and GRIIS

Table S 4-16: Sample data for putative detections of non-native, pest, or invasive species.

Species were detected in the Southern Ocean outside of their ranges (or are known pest/invasive species) using eDNA methods (LargeVF and SmallVF). Water samples were amplified using the Leray COI metabarcoding assay.

Zone	Sample	Type	Latitude	Species	Status	Reads
STZ	V1_002	LargeVF	-42.882	*Metacarcinus novaezelandiae*	Introduced to Tasmania	5
STZ	V1_002	LargeVF	-42.882	*Mytilus galloprovincialis*	Invasive to Tasmania	11
STZ	V1_002	LargeVF	-42.882	*Obelia dichotoma*	Introduced to Tasmania	512
STZ	V1_002	LargeVF	-42.882	*Watersipora subtorquata/subatra*	Introduced to Tasmania	1
STZ	V1_002	LargeVF	-42.882	*Clytia gracilis*	Unclear	14
STZ	V1_002	LargeVF	-42.882	*Lizzia blondina*	Unclear	92
STZ	V1_002	LargeVF	-42.882	*Austrominius modestus*	Unclear	108
STZ	V1_002	LargeVF	-42.882	*Asterias amurensis*	Invasive to Tasmania	45
STZ	V1_002	LargeVF	-42.882	*Bougainvillia muscus*	Introduced to Tasmania	101
STZ	V1_002	LargeVF	-42.882	*Bugulina flabellata*	Introduced to Tasmania	96
STZ	V1_002	LargeVF	-42.882	*Patiriella regularis*	Introduced to Tasmania	26
STZ	V1_002	LargeVF	-42.882	*Coryne eximia*	Unclear	1936
STZ	V1_002	LargeVF	-42.882	*Amphinema dinema*	Unclear	1
STZ	V1_003	SmallVF	-42.882	*Mytilus galloprovincialis*	Invasive to Tasmania	9
STZ	V1_003	SmallVF	-42.882	*Bugulina flabellata*	Introduced to Tasmania	41
STZ	V1_003	SmallVF	-42.882	*Coryne eximia*	Unclear	1507
STZ	V1_003	SmallVF	-42.882	*Watersipora subtorquata/subatra*	Introduced to Tasmania	1
STZ	V1_003	SmallVF	-42.882	*Lizzia blondina*	Unclear	63
STZ	V1_003	SmallVF	-42.882	*Clytia gracilis*	Unclear	1
STZ	V1_003	SmallVF	-42.882	*Bougainvillia muscus*	Introduced to Tasmania	31
STZ	V1_003	SmallVF	-42.882	*Obelia dichotoma*	Introduced to Tasmania	377
STZ	V1_003	SmallVF	-42.882	*Asterias amurensis*	Invasive to Tasmania	5
STZ	V1_004	SmallVF	-42.882	*Coryne eximia*	Unclear	36
STZ	V1_004	SmallVF	-42.882	*Lizzia blondina*	Unclear	100
STZ	V1_004	SmallVF	-42.882	*Bugulina flabellata*	Introduced to Tasmania	638
STZ	V1_004	SmallVF	-42.882	*Watersipora subtorquata/subatra*	Introduced to Tasmania	2
STZ	V1_004	SmallVF	-42.882	*Obelia dichotoma*	Introduced to Tasmania	141
STZ	V1_004	SmallVF	-42.882	*Bougainvillia muscus*	Introduced to Tasmania	25
STZ	V1_004	SmallVF	-42.882	*Asterias amurensis*	Invasive to Tasmania	8
STZ	V1_005	LargeVF	-42.882	*Bougainvillia muscus*	Introduced to Tasmania	548
STZ	V1_005	LargeVF	-42.882	*Bugulina flabellata*	Introduced to Tasmania	111

STZ	V1_005	LargeVF	-42.882	*Obelia dichotoma*	Introduced to Tasmania	11
STZ	V1_005	LargeVF	-42.882	*Coryne eximia*	Unclear	270
STZ	V1_005	LargeVF	-42.882	*Austrominius modestus*	Unclear	11
STZ	V1_005	LargeVF	-42.882	*Lizzia blondina*	Unclear	5
STZ	V1_008	SmallVF	-44.13	*Bugulina flabellata*	Introduced to Tasmania	246
STZ	V1_008	SmallVF	-44.13	*Bougainvillia muscus*	Introduced to Tasmania	8
STZ	V1_008	SmallVF	-44.13	*Obelia dichotoma*	Introduced to Tasmania	2
STZ	V1_009	LargeVF	-44.13	*Bougainvillia muscus*	Introduced to Tasmania	9
STZ	V1_009	LargeVF	-44.13	*Bugulina flabellata*	Introduced to Tasmania	7
STZ	V1_009	LargeVF	-44.13	*Coryne eximia*	Unclear	15
STZ	V1_009	LargeVF	-44.13	*Obelia dichotoma*	Introduced to Tasmania	2
STZ	V1_009	LargeVF	-44.13	*Lizzia blondina*	Unclear	1
STZ	V1_010	SmallVF	-44.351	*Bugulina flabellata*	Introduced to Tasmania	82
STZ	V1_010	SmallVF	-44.351	*Watersipora subtorquata/subatra*	Introduced to Tasmania	1
STZ	V1_010	SmallVF	-44.351	*Arachnopusia unicornis*	Native in Tasmania	1
STZ	V1_010	SmallVF	-44.351	*Coryne eximia*	Unclear	1
STZ	V1_010	SmallVF	-44.351	*Obelia dichotoma*	Introduced to Tasmania	1
STZ	V1_011	LargeVF	-44.351	*Bugulina flabellata*	Introduced to Tasmania	2091
STZ	V1_011	LargeVF	-44.351	*Coryne eximia*	Unclear	3
STZ	V1_011	LargeVF	-44.351	*Mytilus galloprovincialis*	Invasive to Tasmania	2
STZ	V1_012	SmallVF	-44.531	*Bougainvillia muscus*	Introduced to Tasmania	1
STZ	V1_012	SmallVF	-44.531	*Bugulina flabellata*	Introduced to Tasmania	109
STZ	V1_012	SmallVF	-44.531	*Obelia dichotoma*	Introduced to Tasmania	2
STZ	V1_012	SmallVF	-44.531	*Coryne eximia*	Unclear	3
STZ	V1_013	LargeVF	-44.531	*Bugulina flabellata*	Introduced to Tasmania	26367
STZ	V1_013	LargeVF	-44.531	*Lizzia blondina*	Unclear	1
STZ	V1_013	LargeVF	-44.531	*Coryne eximia*	Unclear	1059
STZ	V1_013	LargeVF	-44.531	*Obelia dichotoma*	Introduced to Tasmania	1
STZ	V1_014	SmallVF	-45.17	*Bugulina flabellata*	Introduced to Tasmania	50
STZ	V1_014	SmallVF	-45.17	*Bougainvillia muscus*	Introduced to Tasmania	1
STZ	V1_015	LargeVF	-45.17	*Lizzia blondina*	Unclear	2
STZ	V1_015	LargeVF	-45.17	*Bugulina flabellata*	Introduced to Tasmania	41442
STZ	V1_015	LargeVF	-45.17	*Coryne eximia*	Unclear	2
SAZ	V1_016	SmallVF	-46.12	*Bugulina flabellata*	Introduced to Tasmania	63
SAZ	V1_016	SmallVF	-46.12	*Bougainvillia muscus*	Introduced to Tasmania	1
SAZ	V1_016	SmallVF	-46.12	*Obelia dichotoma*	Introduced to Tasmania	1
SAZ	V1_017	LargeVF	-46.12	*Bougainvillia muscus*	Introduced to Tasmania	2
SAZ	V1_017	LargeVF	-46.12	*Bugulina flabellata*	Introduced to Tasmania	54
SAZ	V1_017	LargeVF	-46.12	*Asterias amurensis*	Invasive to Tasmania	5
SAZ	V1_017	LargeVF	-46.12	*Coryne eximia*	Unclear	6

SAZ	V1_018	SmallVF	-46.278	*Bugulina flabellata*	Introduced to Tasmania	61
SAZ	V1_018	SmallVF	-46.278	*Coryne eximia*	Unclear	1
SAZ	V1_019	LargeVF	-46.278	*Coryne eximia*	Unclear	1
SAZ	V1_019	LargeVF	-46.278	*Lizzia blondina*	Unclear	4
SAZ	V1_019	LargeVF	-46.278	*Bugulina flabellata*	Introduced to Tasmania	22
SAZ	V1_020	SmallVF	-46.389	*Coryne eximia*	Unclear	5
SAZ	V1_021	LargeVF	-46.389	*Bugulina flabellata*	Introduced to Tasmania	45
SAZ	V1_021	LargeVF	-46.389	*Coryne eximia*	Unclear	1
SAZ	V1_022	SmallVF	-46.57	*Bougainvillia muscus*	Introduced to Tasmania	4
SAZ	V1_022	SmallVF	-46.57	*Watersipora subtorquata/subatra*	Introduced to Tasmania	2
SAZ	V1_022	SmallVF	-46.57	*Bugulina flabellata*	Introduced to Tasmania	46
SAZ	V1_022	SmallVF	-46.57	*Coryne eximia*	Unclear	6
SAZ	V1_023	LargeVF	-46.57	*Watersipora subtorquata/subatra*	Introduced to Tasmania	1
SAZ	V1_023	LargeVF	-46.57	*Bugulina flabellata*	Introduced to Tasmania	34
SAZ	V1_023	LargeVF	-46.57	*Coryne eximia*	Unclear	2
SAZ	V1_023	LargeVF	-46.57	*Obelia dichotoma*	Introduced to Tasmania	26
SAZ	V1_024	SmallVF	-47.261	*Bugulina flabellata*	Introduced to Tasmania	10
SAZ	V1_024	SmallVF	-47.261	*Coryne eximia*	Unclear	1
SAZ	V1_024	SmallVF	-47.261	*Bougainvillia muscus*	Introduced to Tasmania	1
SAZ	V1_025	LargeVF	-47.261	*Obelia dichotoma*	Introduced to Tasmania	1
SAZ	V1_026	SmallVF	-47.45	*Bugulina flabellata*	Introduced to Tasmania	46
SAZ	V1_026	SmallVF	-47.45	*Coryne eximia*	Unclear	1
SAZ	V1_027	LargeVF	-47.45	*Coryne eximia*	Unclear	1
SAZ	V1_028	SmallVF	-47.8	*Watersipora subtorquata/subatra*	Introduced to Tasmania	1
SAZ	V1_028	SmallVF	-47.8	*Ostrea angasi*	Native in Tasmania	1
SAZ	V1_028	SmallVF	-47.8	*Bugulina flabellata*	Introduced to Tasmania	37
SAZ	V1_028	SmallVF	-47.8	*Coryne eximia*	Unclear	3
SAZ	V1_028	SmallVF	-47.8	*Bougainvillia muscus*	Introduced to Tasmania	2
SAZ	V1_029	LargeVF	-47.8	*Coryne eximia*	Unclear	6
SAZ	V1_030	SmallVF	-48.22	*Obelia dichotoma*	Introduced to Tasmania	2
SAZ	V1_030	SmallVF	-48.22	*Bugulina flabellata*	Introduced to Tasmania	48
SAZ	V1_030	SmallVF	-48.22	*Coryne eximia*	Unclear	4
SAZ	V1_031	LargeVF	-48.22	*Bugulina flabellata*	Introduced to Tasmania	66
SAZ	V1_032	SmallVF	-48.381	*Arachnopusia unicornis*	Native in Tasmania	1
SAZ	V1_032	SmallVF	-48.381	*Bugulina flabellata*	Introduced to Tasmania	18
SAZ	V1_032	SmallVF	-48.381	*Ostrea angasi*	Native in Tasmania	2
SAZ	V1_032	SmallVF	-48.381	*Bougainvillia muscus*	Introduced to Tasmania	1
SAZ	V1_032	SmallVF	-48.381	*Obelia dichotoma*	Introduced to Tasmania	1
SAZ	V1_033	LargeVF	-48.381	*Coryne eximia*	Unclear	19
SAZ	V1_035	LargeVF	-49.138	*Coryne eximia*	Unclear	1

SAZ	V1_036	SmallVF	-49.288	*Coryne eximia*	Unclear	3
SAZ	V1_036	SmallVF	-49.288	*Bugulina flabellata*	Introduced to Tasmania	6
SAZ	V1_037	LargeVF	-49.288	*Coryne eximia*	Unclear	2
SAZ	V1_038	SmallVF	-49.49	*Bugulina flabellata*	Introduced to Tasmania	2
SAZ	V1_038	SmallVF	-49.49	*Bougainvillia muscus*	Introduced to Tasmania	7
SAZ	V1_040	SmallVF	-50.107	*Coryne eximia*	Unclear	1
SAZ	V1_041	LargeVF	-50.107	*Coryne eximia*	Unclear	2
SAZ	V1_043	LargeVF	-50.151	*Coryne eximia*	Unclear	1
SAZ	V1_043	LargeVF	-50.151	*Lizzia blondina*	Unclear	1
SAZ	V1_043	LargeVF	-50.151	*Obelia dichotoma*	Introduced to Tasmania	6
SAZ	V1_043	LargeVF	-50.151	*Bugulina flabellata*	Introduced to Tasmania	11
SAZ	V1_044	SmallVF	-50.44	*Coryne eximia*	Unclear	1
SAZ	V1_044	SmallVF	-50.44	*Bugulina flabellata*	Introduced to Tasmania	1
SAZ	V1_044	SmallVF	-50.44	*Bougainvillia muscus*	Introduced to Tasmania	6
SAZ	V1_045	LargeVF	-50.44	*Asterias amurensis*	Invasive to Tasmania	1
SAZ	V1_045	LargeVF	-50.44	*Coryne eximia*	Unclear	5
SAZ	V1_045	LargeVF	-50.44	*Bougainvillia muscus*	Introduced to Tasmania	8
SAZ	V1_045	LargeVF	-50.44	*Lizzia blondina*	Unclear	7
SAZ	V1_046	SmallVF	-50.918	*Bugulina flabellata*	Introduced to Tasmania	4
SAZ	V1_047	LargeVF	-50.918	*Bugulina flabellata*	Introduced to Tasmania	42
SAZ	V1_047	LargeVF	-50.918	*Asterias amurensis*	Invasive to Tasmania	2
SAZ	V1_047	LargeVF	-50.918	*Coryne eximia*	Unclear	2
SAZ	V1_048	SmallVF	-51.289	*Coryne eximia*	Unclear	1
SAZ	V1_049	LargeVF	-51.289	*Coryne eximia*	Unclear	3
PFZ	V1_050	SmallVF	-51.41	*Bugulina flabellata*	Introduced to Tasmania	2
PFZ	V1_050	SmallVF	-51.41	*Bougainvillia muscus*	Introduced to Tasmania	4
PFZ	V1_051	LargeVF	-51.41	*Coryne eximia*	Unclear	2
PFZ	V1_051	LargeVF	-51.41	*Watersipora subtorquata/subatra*	Introduced to Tasmania	5
PFZ	V1_054	SmallVF	-52.025	*Coryne eximia*	Unclear	43
PFZ	V1_055	LargeVF	-52.025	*Bugulina flabellata*	Introduced to Tasmania	14
PFZ	V1_055	LargeVF	-52.025	*Bougainvillia muscus*	Introduced to Tasmania	6
PFZ	V1_056	SmallVF	-52.549	*Obelia dichotoma*	Introduced to Tasmania	5
PFZ	V1_056	SmallVF	-52.549	*Bougainvillia muscus*	Introduced to Tasmania	7
PFZ	V1_056	SmallVF	-52.549	*Coryne eximia*	Unclear	2
PFZ	V1_057	LargeVF	-52.549	*Bugulina flabellata*	Introduced to Tasmania	13
PFZ	V1_057	LargeVF	-52.549	*Coryne eximia*	Unclear	11
PFZ	V1_057	LargeVF	-52.549	*Bougainvillia muscus*	Introduced to Tasmania	5
PFZ	V1_058	SmallVF	-53.476	*Ostrea angasi*	Native in Tasmania	1
PFZ	V1_058	SmallVF	-53.476	*Bugulina flabellata*	Introduced to Tasmania	73
PFZ	V1_058	SmallVF	-53.476	*Bougainvillia muscus*	Introduced to Tasmania	30
PFZ	V1_058	SmallVF	-53.476	*Coryne eximia*	Unclear	8
PFZ	V1_062	SmallVF	-53.287	*Coryne eximia*	Unclear	2

PFZ	V1_063	LargeVF	-53.287	*Coryne eximia*	Unclear	2
PFZ	V1_065	LargeVF	-53.497	*Coryne eximia*	Unclear	2
PFZ	V1_068	SmallVF	-54.12	*Coryne eximia*	Unclear	4
PFZ	V1_068	SmallVF	-54.12	*Bougainvillia muscus*	Introduced to Tasmania	1
PFZ	V1_068	SmallVF	-54.12	*Obelia dichotoma*	Introduced to Tasmania	2
PFZ	V1_072	SmallVF	-54.43	*Coryne eximia*	Unclear	6
PFZ	V1_072	SmallVF	-54.43	*Bougainvillia muscus*	Introduced to Tasmania	2
PFZ	V1_073	LargeVF	-54.43	*Coryne eximia*	Unclear	2
PFZ	V1_073	LargeVF	-54.43	*Obelia dichotoma*	Introduced to Tasmania	1
PFZ	V1_074	SmallVF	-54.56	*Bugulina flabellata*	Introduced to Tasmania	1
PFZ	V1_074	SmallVF	-54.56	*Coryne eximia*	Unclear	2
PFZ	V1_075	LargeVF	-54.56	*Obelia dichotoma*	Introduced to Tasmania	1
PFZ	V1_075	LargeVF	-54.56	*Coryne eximia*	Unclear	1
AAZ	V1_076	SmallVF	-55.161	*Coryne eximia*	Unclear	14
AAZ	V1_078	SmallVF	-55.27	*Coryne eximia*	Unclear	4
AAZ	V1_078	SmallVF	-55.27	*Bugulina flabellata*	Introduced to Tasmania	19
AAZ	V1_079	LargeVF	-55.27	*Coryne eximia*	Unclear	1
AAZ	V1_080	SmallVF	-55.499	*Coryne eximia*	Unclear	6
AAZ	V1_082	SmallVF	-55.922	*Coryne eximia*	Unclear	41
AAZ	V1_082	SmallVF	-55.922	*Obelia dichotoma*	Introduced to Tasmania	7
AAZ	V1_083	LargeVF	-55.922	*Coryne eximia*	Unclear	1
AAZ	V1_083	LargeVF	-55.922	*Bougainvillia muscus*	Introduced to Tasmania	32
AAZ	V1_084	SmallVF	-56.208	*Arachnopusia unicornis*	Native in Tasmania	1
AAZ	V1_084	SmallVF	-56.208	*Bougainvillia muscus*	Introduced to Tasmania	4
AAZ	V1_084	SmallVF	-56.208	*Bugulina flabellata*	Introduced to Tasmania	7
AAZ	V1_084	SmallVF	-56.208	*Coryne eximia*	Unclear	26
AAZ	V1_086	SmallVF	-56.417	*Bougainvillia muscus*	Introduced to Tasmania	24
AAZ	V1_086	SmallVF	-56.417	*Coryne eximia*	Unclear	16
AAZ	V1_087	LargeVF	-56.417	*Coryne eximia*	Unclear	3
AAZ	V1_087	LargeVF	-56.417	*Arachnopusia unicornis*	Native in Tasmania	1
AAZ	V1_087	LargeVF	-56.417	*Bugulina flabellata*	Introduced to Tasmania	1
AAZ	V1_088	SmallVF	-56.571	*Bugulina flabellata*	Introduced to Tasmania	11
AAZ	V1_088	SmallVF	-56.571	*Coryne eximia*	Unclear	14
AAZ	V1_089	LargeVF	-56.571	*Coryne eximia*	Unclear	1
AAZ	V1_090	SmallVF	-56.966	*Coryne eximia*	Unclear	64
AAZ	V1_090	SmallVF	-56.966	*Bougainvillia muscus*	Introduced to Tasmania	8
AAZ	V1_090	SmallVF	-56.966	*Bugulina flabellata*	Introduced to Tasmania	112
AAZ	V1_091	LargeVF	-56.966	*Coryne eximia*	Unclear	1
AAZ	V1_092	SmallVF	-57.26	*Coryne eximia*	Unclear	41
AAZ	V1_092	SmallVF	-57.26	*Bougainvillia muscus*	Introduced to Tasmania	35
AAZ	V1_093	LargeVF	-57.26	*Coryne eximia*	Unclear	5

AAZ	V1_094	SmallVF	-57.333	*Coryne eximia*	Unclear	12
AAZ	V1_094	SmallVF	-57.333	*Bougainvillia muscus*	Introduced to Tasmania	2
AAZ	V1_094	SmallVF	-57.333	*Bugulina flabellata*	Introduced to Tasmania	22
AAZ	V1_095	LargeVF	-57.333	*Coryne eximia*	Unclear	2
AAZ	V1_096	SmallVF	-58.401	*Coryne eximia*	Unclear	24
AAZ	V1_096	SmallVF	-58.401	*Bugulina flabellata*	Introduced to Tasmania	8
AAZ	V1_096	SmallVF	-58.401	*Bougainvillia muscus*	Introduced to Tasmania	17
AAZ	V1_097	LargeVF	-58.401	*Bugulina flabellata*	Introduced to Tasmania	3
AAZ	V1_097	LargeVF	-58.401	*Coryne eximia*	Unclear	3
AAZ	V1_098	SmallVF	-58.15	*Coryne eximia*	Unclear	27
AAZ	V1_098	SmallVF	-58.15	*Lizzia blondina*	Unclear	4
AAZ	V1_098	SmallVF	-58.15	*Bougainvillia muscus*	Introduced to Tasmania	6
AAZ	V1_098	SmallVF	-58.15	*Asterias amurensis*	Invasive to Tasmania	1
AAZ	V1_099	LargeVF	-58.15	*Coryne eximia*	Unclear	8
AAZ	V1_100	SmallVF	-58.316	*Coryne eximia*	Unclear	10
AAZ	V1_100	SmallVF	-58.316	*Bugulina flabellata*	Introduced to Tasmania	8
AAZ	V1_101	LargeVF	-58.316	*Bugulina flabellata*	Introduced to Tasmania	2
AAZ	V1_102	SmallVF	-58.411	*Bougainvillia muscus*	Introduced to Tasmania	1
AAZ	V1_102	SmallVF	-58.411	*Coryne eximia*	Unclear	27
AAZ	V1_103	LargeVF	-58.411	*Coryne eximia*	Unclear	7
AAZ	V1_103	LargeVF	-58.411	*Bugulina flabellata*	Introduced to Tasmania	12
AAZ	V1_104	SmallVF	-59	*Bougainvillia muscus*	Introduced to Tasmania	6
AAZ	V1_104	SmallVF	-59	*Coryne eximia*	Unclear	3
AAZ	V1_105	LargeVF	-59	*Obelia dichotoma*	Introduced to Tasmania	1
AAZ	V1_106	SmallVF	-59.141	*Coryne eximia*	Unclear	1
AAZ	V1_106	SmallVF	-59.141	*Bugulina flabellata*	Introduced to Tasmania	1
AAZ	V1_107	LargeVF	-59.141	*Arachnopusia unicornis*	Native in Tasmania	2
AAZ	V1_107	LargeVF	-59.141	*Coryne eximia*	Unclear	6
AAZ	V1_107	LargeVF	-59.141	*Bugulina flabellata*	Introduced to Tasmania	1
AAZ	V1_110	SmallVF	-59.43	*Ostrea angasi*	Native in Tasmania	20
AAZ	V1_110	SmallVF	-59.43	*Bugulina flabellata*	Introduced to Tasmania	16
AAZ	V1_110	SmallVF	-59.43	*Coryne eximia*	Unclear	20
AAZ	V1_110	SmallVF	-59.43	*Obelia dichotoma*	Introduced to Tasmania	2
AAZ	V1_111	LargeVF	-59.43	*Asterias amurensis*	Invasive to Tasmania	1
AAZ	V1_111	LargeVF	-59.43	*Bugulina flabellata*	Introduced to Tasmania	8
AAZ	V1_111	LargeVF	-59.43	*Coryne eximia*	Unclear	3
AAZ	V1_112	SmallVF	-59.748	*Cryptosula pallasiana*	Introduced to Tasmania	1
AAZ	V1_112	SmallVF	-59.748	*Ostrea angasi*	Native in Tasmania	235
AAZ	V1_112	SmallVF	-59.748	*Obelia dichotoma*	Introduced to Tasmania	4
AAZ	V1_112	SmallVF	-59.748	*Coryne eximia*	Unclear	4
AAZ	V1_112	SmallVF	-59.748	*Bougainvillia muscus*	Introduced to Tasmania	18

AAZ	V1_113	LargeVF	-59.748	*Asterias amurensis*	Invasive to Tasmania	1
AAZ	V1_113	LargeVF	-59.748	*Bugulina flabellata*	Introduced to Tasmania	2
AAZ	V1_113	LargeVF	-59.748	*Ostrea angasi*	Native in Tasmania	6
AAZ	V1_114	SmallVF	-60.39	*Coryne eximia*	Unclear	39
AAZ	V1_114	SmallVF	-60.39	*Ostrea angasi*	Native in Tasmania	19
AAZ	V1_115	LargeVF	-60.39	*Coryne eximia*	Unclear	6
AAZ	V1_116	SmallVF	-60.88	*Coryne eximia*	Unclear	37
AAZ	V1_116	SmallVF	-60.88	*Bugulina flabellata*	Introduced to Tasmania	39
AAZ	V1_117	LargeVF	-60.88	*Coryne eximia*	Unclear	2
AAZ	V1_118	SmallVF	-61.41	*Austrominius modestus*	Unclear	2
AAZ	V1_118	SmallVF	-61.41	*Arachnopusia unicornis*	Native in Tasmania	4
AAZ	V1_118	SmallVF	-61.41	*Ostrea angasi*	Native in Tasmania	23
AAZ	V1_118	SmallVF	-61.41	*Obelia dichotoma*	Introduced to Tasmania	1
AAZ	V1_118	SmallVF	-61.41	*Watersipora subtorquata/subatra*	Introduced to Tasmania	98
AAZ	V1_118	SmallVF	-61.41	*Coryne eximia*	Unclear	114
AAZ	V1_118	SmallVF	-61.41	*Bugulina flabellata*	Introduced to Tasmania	262
AAZ	V1_118	SmallVF	-61.41	*Cryptosula pallasiana*	Introduced to Tasmania	33
AAZ	V1_118	SmallVF	-61.41	*Clytia gracilis*	Unclear	5
AAZ	V1_118	SmallVF	-61.41	*Bougainvillia muscus*	Introduced to Tasmania	435
AAZ	V1_119	LargeVF	-61.41	*Arachnopusia unicornis*	Native in Tasmania	2
AAZ	V1_119	LargeVF	-61.41	*Watersipora subtorquata/subatra*	Introduced to Tasmania	13
AAZ	V1_119	LargeVF	-61.41	*Obelia dichotoma*	Introduced to Tasmania	2
AAZ	V1_119	LargeVF	-61.41	*Lizzia blondina*	Unclear	1
AAZ	V1_119	LargeVF	-61.41	*Coryne eximia*	Unclear	20
AAZ	V1_119	LargeVF	-61.41	*Ostrea angasi*	Native in Tasmania	4
AAZ	V1_119	LargeVF	-61.41	*Bougainvillia muscus*	Introduced to Tasmania	47
AAZ	V1_119	LargeVF	-61.41	*Asterias amurensis*	Invasive to Tasmania	1
AAZ	V1_119	LargeVF	-61.41	*Cryptosula pallasiana*	Introduced to Tasmania	1
AAZ	V1_119	LargeVF	-61.41	*Bugulina flabellata*	Introduced to Tasmania	52
AAZ	V1_120	SmallVF	-62.391	*Obelia dichotoma*	Introduced to Tasmania	1
AAZ	V1_120	SmallVF	-62.391	*Watersipora subtorquata/subatra*	Introduced to Tasmania	7
AAZ	V1_120	SmallVF	-62.391	*Coryne eximia*	Unclear	21
AAZ	V1_120	SmallVF	-62.391	*Ostrea angasi*	Native in Tasmania	2
AAZ	V1_120	SmallVF	-62.391	*Bougainvillia muscus*	Introduced to Tasmania	12
AAZ	V1_121	LargeVF	-62.391	*Watersipora subtorquata/subatra*	Introduced to Tasmania	4

AAZ	V1_121	LargeVF	-62.391	*Bougainvillia muscus*	Introduced to Tasmania	1
AAZ	V1_121	LargeVF	-62.391	*Coryne eximia*	Unclear	4
AAZ	V1_122	SmallVF	-62.57	*Bugulina flabellata*	Introduced to Tasmania	1
AAZ	V1_122	SmallVF	-62.57	*Ostrea angasi*	Native in Tasmania	1
AAZ	V1_122	SmallVF	-62.57	*Coryne eximia*	Unclear	16
AAZ	V1_122	SmallVF	-62.57	*Bougainvillia muscus*	Introduced to Tasmania	4
AAZ	V1_123	LargeVF	-62.57	*Coryne eximia*	Unclear	2
AAZ	V1_123	LargeVF	-62.57	*Bougainvillia muscus*	Introduced to Tasmania	3
AAZ	V1_124	SmallVF	-63.582	*Watersipora subtorquata/subatra*	Introduced to Tasmania	8
AAZ	V1_124	SmallVF	-63.582	*Cryptosula pallasiana*	Introduced to Tasmania	2
AAZ	V1_124	SmallVF	-63.582	*Bugulina flabellata*	Introduced to Tasmania	17
AAZ	V1_124	SmallVF	-63.582	*Bougainvillia muscus*	Introduced to Tasmania	82
AAZ	V1_124	SmallVF	-63.582	*Amphinema dinema*	Unclear	17
AAZ	V1_124	SmallVF	-63.582	*Coryne eximia*	Unclear	41
AAZ	V1_125	LargeVF	-63.582	*Watersipora subtorquata/subatra*	Introduced to Tasmania	2
AAZ	V1_125	LargeVF	-63.582	*Bougainvillia muscus*	Introduced to Tasmania	13
AAZ	V1_125	LargeVF	-63.582	*Bugulina flabellata*	Introduced to Tasmania	17
AAZ	V1_125	LargeVF	-63.582	*Coryne eximia*	Unclear	2
AAZ	V1_125	LargeVF	-63.582	*Arachnopusia unicornis*	Native in Tasmania	1
AAZ	V1_125	LargeVF	-63.582	*Obelia dichotoma*	Introduced to Tasmania	5
AAZ	V1_125	LargeVF	-63.582	*Lizzia blondina*	Unclear	6
AAZ	V1_125	LargeVF	-63.582	*Asterias amurensis*	Invasive to Tasmania	1
SACCZ	V1_126	SmallVF	-64.276	*Arachnopusia unicornis*	Native in Tasmania	1
SACCZ	V1_126	SmallVF	-64.276	*Cryptosula pallasiana*	Introduced to Tasmania	2
SACCZ	V1_126	SmallVF	-64.276	*Bugulina flabellata*	Introduced to Tasmania	7
SACCZ	V1_126	SmallVF	-64.276	*Watersipora subtorquata/subatra*	Introduced to Tasmania	4
SACCZ	V1_127	LargeVF	-64.276	*Watersipora subtorquata/subatra*	Introduced to Tasmania	1
SACCZ	V1_128	SmallVF	-64.313	*Obelia dichotoma*	Introduced to Tasmania	7
SACCZ	V1_128	SmallVF	-64.313	*Coryne eximia*	Unclear	5
SACCZ	V1_128	SmallVF	-64.313	*Bugulina flabellata*	Introduced to Tasmania	25
SACCZ	V1_130	SmallVF	-64.421	*Coryne eximia*	Unclear	37
SACCZ	V1_130	SmallVF	-64.421	*Obelia dichotoma*	Introduced to Tasmania	3
SACCZ	V1_131	LargeVF	-64.421	*Arachnopusia unicornis*	Native in Tasmania	12
SACCZ	V1_131	LargeVF	-64.421	*Coryne eximia*	Unclear	35
SACCZ	V1_131	LargeVF	-64.421	*Asterias amurensis*	Invasive to Tasmania	7

SACCZ	V1_132	SmallVF	-66.161	*Coryne eximia*	Unclear	12
SACCZ	V1_132	SmallVF	-66.161	*Arachnopusia unicornis*	Native in Tasmania	3
SACCZ	V1_132	SmallVF	-66.161	*Cryptosula pallasiana*	Introduced to Tasmania	30
SACCZ	V1_132	SmallVF	-66.161	*Obelia dichotoma*	Introduced to Tasmania	13
SACCZ	V1_132	SmallVF	-66.161	*Bougainvillia muscus*	Introduced to Tasmania	2
SACCZ	V1_132	SmallVF	-66.161	*Bugulina flabellata*	Introduced to Tasmania	98
SACCZ	V1_133	LargeVF	-66.161	*Watersipora subtorquata/subatra*	Introduced to Tasmania	3
SACCZ	V1_133	LargeVF	-66.161	*Coryne eximia*	Unclear	41
SACCZ	V1_133	LargeVF	-66.161	*Bougainvillia muscus*	Introduced to Tasmania	4
SACCZ	V1_133	LargeVF	-66.161	*Bugulina flabellata*	Introduced to Tasmania	5
SACCZ	V1_134	SmallVF	-66.33	*Coryne eximia*	Unclear	11
SACCZ	V1_134	SmallVF	-66.33	*Obelia dichotoma*	Introduced to Tasmania	1
SACCZ	V1_135	LargeVF	-66.33	*Coryne eximia*	Unclear	69
SACCZ	V1_138	SmallVF	-66.437	*Coryne eximia*	Unclear	1
SACCZ	V1_138	SmallVF	-66.437	*Obelia dichotoma*	Introduced to Tasmania	1

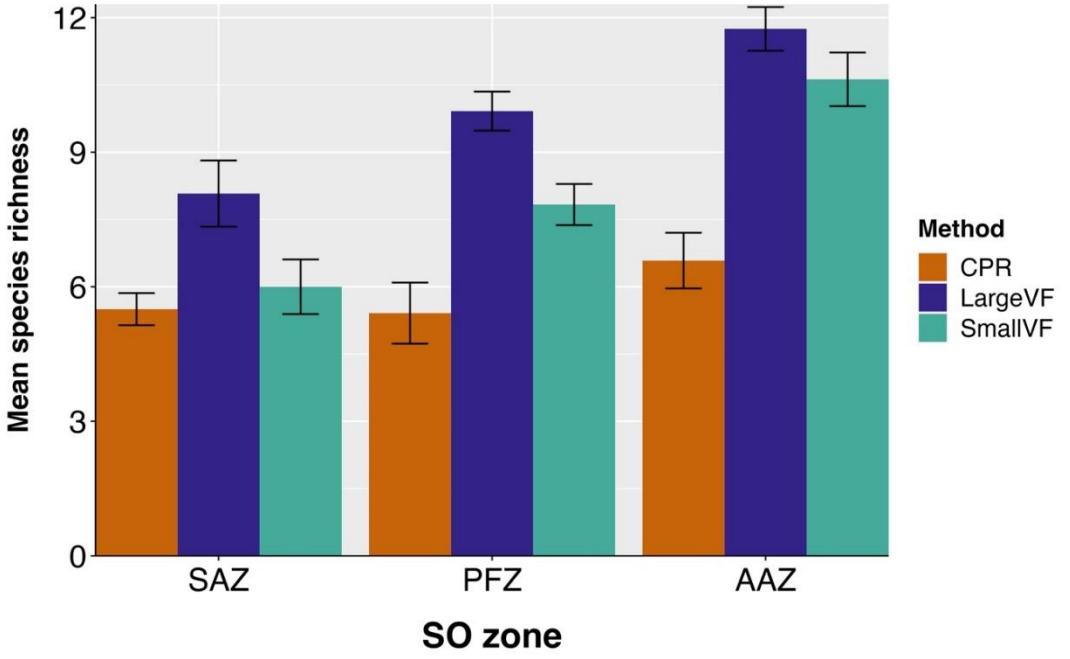
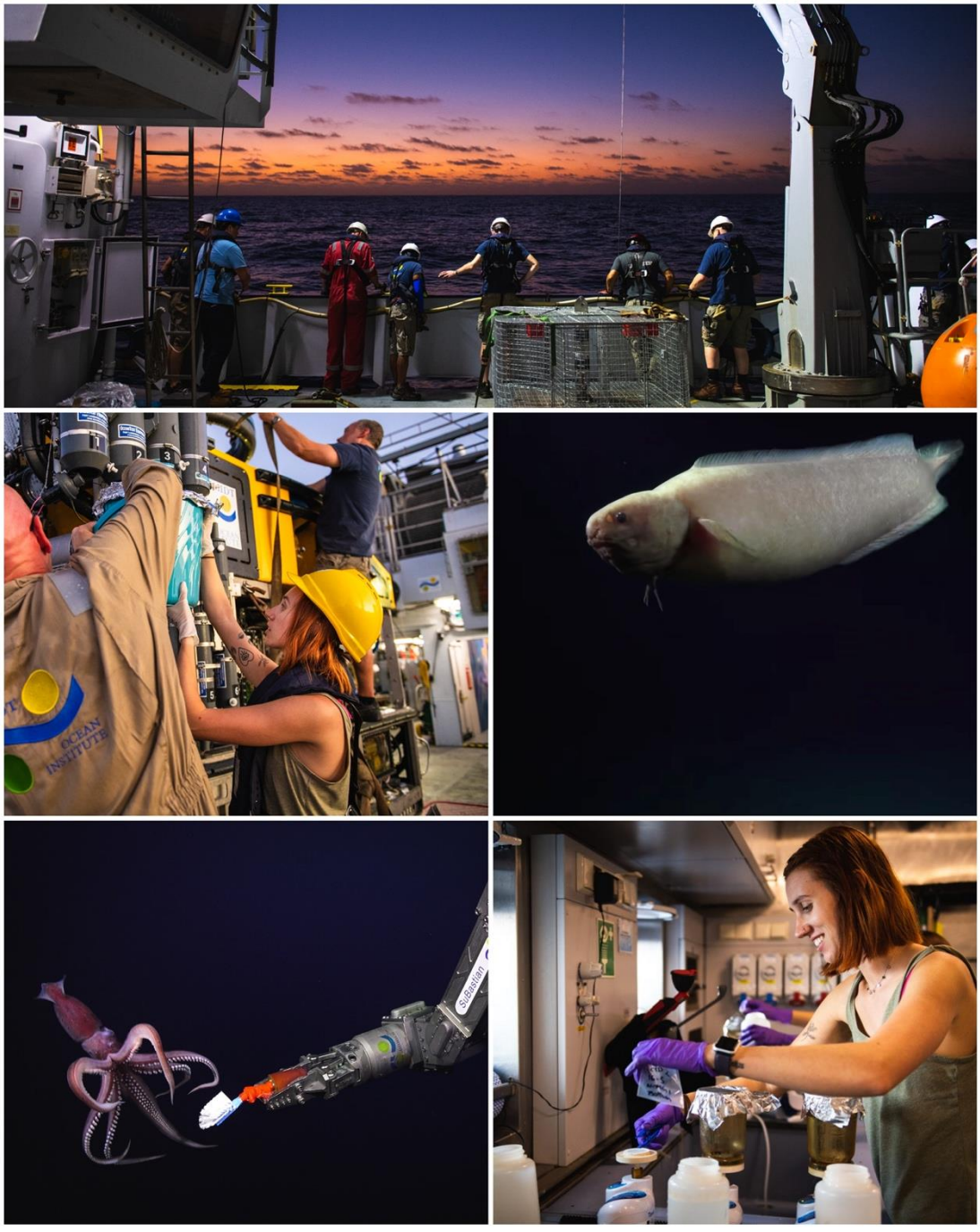


Figure S 4-5: Richness using eDNA and CPR.

Mean Animalia species richness (q=0) across Southern Ocean zones using eDNA metabarcoding (LargeVF and SmallVF) and CPR surveys. eDNA metabarcoding was performed using Leray COI assay.

CHAPTER 5

eDNA metabarcoding reveals submarine canyon biodiversity across depth gradient



5.1 Prologue

The ocean covers 71% of the surface of the earth and the deep-sea (herein depths below 200 m) comprises the vast majority of this area (Clark et al., 2016). Exploration of deep-sea habitats and the documentation of their fauna began in the late 19th century. The expedition of the HMS Beacon led by Prof. Edward Forbes in 1842 is considered the first biological deep-sea cruise (Ramirez-Llodra, 2020). The crew dredged to depths of 420 m, finding fewer species with increasing depth. This led to Forbes establishing the 'Azoic Theory', suggesting that no life existed in the depths of the ocean (Forbes, 1843). However, Sir Charles Thomson's transformative circumglobal expedition aboard the HMS Challenger three decades later shattered the perception of the deep-sea as desolate and barren (Ramirez-Llodra, 2020; Thomson, 1873). Thomson's findings unveiled a remarkably diverse and vibrant deep-sea fauna (Figure 5-1), characterized by elaborate and delicate organisms (Thomson, 1873).

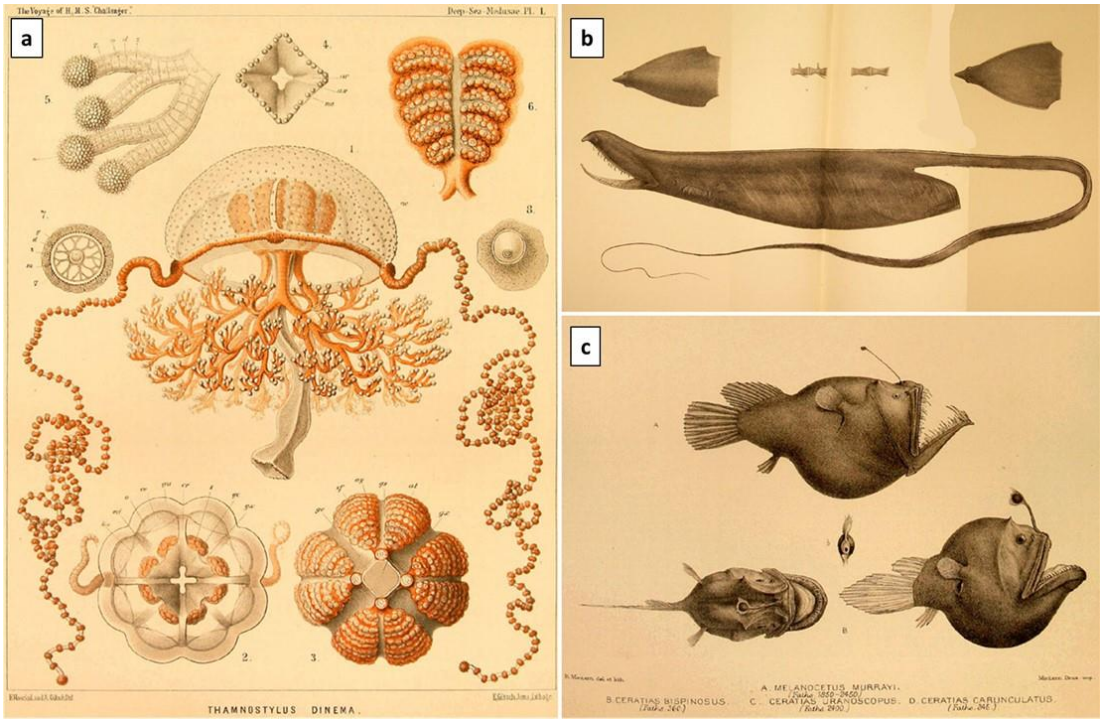


Figure 5-1: Deep-sea illustrations.

Illustrations of some of the many deep-sea creatures discovered between 1873 and 1876 during the historic *HMS Challenger* voyages, including (a) the medusae *Thamnostylus dinema*; (b) the gulper eel *Saccopharynx ampullaceus*; and (c) the bathypelagic anglerfish *Melanocetus murrayi* and *Ceratias bispinosus* (now *Diceratias bispinosus*; (Günther, 1887; Haeckel, 1880).

Over time, the exploration of the deep-sea expanded, driven by a desire to comprehend its intricate ecosystems. The advancement of deep-sea research is intrinsically linked to the progress in sampling and exploration techniques specifically designed for the challenges of observing the deep-sea *in situ* (Robison, 1999). In 1930, Beebe's Bathysphere emerged as the first deep-sea research submersible, enabling direct observations of the seabed in 1930 (Beebe, 1932, 1934). Subsequent decades witnessed rapid advancements in deep-sea technology, leading to the creation of innovative instruments like Remote Operated Vehicles (ROV) and Autonomous Underwater Vehicles (AUV) (Ramirez-Llodra, 2020). These monitoring tools facilitate in-situ observations, explorations, and experimental investigations, providing valuable insights into the composition, structure, and functioning of deep-sea ecosystems (Ramirez-Llodra, 2020; Ramirez-Llodra, Brandt, et al., 2010). As a result of these advancements, 22 new deep-sea habitats have been discovered in the last 170 years (Ramirez-Llodra, 2020), such as seamounts, hydrothermal vents, cold-seeps, and submarine canyons (Ramirez-Llodra, 2020, 2020). These habitats support an abundance of new and endemic species. Notably, a recent study in the Clarion-Clipperton Zone (4,000 – 6,000 m deep) revealed an estimated 5000 species previously unknown to science (Rabone et al., 2023), underscoring the vast potential for discovery within the depths of our ocean.

Yet, as our knowledge expands, so too does our awareness of the fragility of the deep-sea. While recent technological advancements have granted us access to the deepest reaches of the ocean, the global deployment of these instruments is hindered by the high costs of suitable vessels and limited infrastructure (Howell et al., 2021). Consequently, the deep-sea remains one of the least explored and understood environments on our planet (Paulus, 2021). This limited understanding of deep-sea ecosystems poses a significant challenge to developing effective management strategies to mitigate the mounting pressures of climate change (Hoegh-Guldberg & Bruno, 2010), deep-sea mining (Ramirez-Llodra et al., 2011), bottom trawling (Pusceddu et al., 2014), and overfishing (Clark, 2001). Addressing these threats requires a deeper understanding of the intricacies of deep-sea

ecosystems and the development of robust conservation measures that balance human activities with the preservation of these vulnerable habitats.

To improve our understanding (and thus our conservation abilities) of the deep-sea, innovative approaches are needed to explore and characterise biodiversity in the depths of our oceans. The following chapter uses eDNA to explore biodiversity patterns along depth gradients in two biologically uncharacterised submarine canyons off the coast of Western Australia. The chapter shows how eDNA can be used to generate valuable biological baseline datasets, reveal spatial biodiversity patterns, and generate records of putative undescribed species and potential range extensions, thereby providing a foundation for future conservation endeavours.

5.1.1 Chapter acknowledgements

I would like to acknowledge the support and contributions of the co-authors of the chapter Zoe Richards and Mike Bunce. For valuable specimen identification I would like to acknowledge Nerida Wilson, Lisa Kirkendale, Glenn Moore, Andrew Hosie, Jenelle Richie, and David Juskiewicz. For valuable field assistance I would like to thank Kaycee Handley. For operation of equipment and valuable field assistance I would like to thank Jason Rodriguez, Kaarel Rais, Paul 'Jimbo' Duncan, Russell Coffield, Corey Peyres, Ben Chiong, Kris Ingram, and Deborah Smith. I would like to thank all crew aboard the *RV Falkor* for field assistance. This chapter was funded by the Western Australian Museum, Schmidt Ocean Institute, a PADI Foundation Grant, and the Chevron Science Engagement Initiative of the Year (2020 Premier's Science Awards).

5.1.2 Data accessibility

All data generated and R scripts created have been deposited in the following Zenodo repository DOI: https://doi.org/10.5281/zenodo.7981207. This includes raw sequencing data, morphology data, sampling information, ZOTU tables for both metabarcoding assays, SIMPER results, and taxa of interest table.

5.2 Abstract

Submarine canyons are globally recognized as biodiversity hotspots, yet accessing and sampling deep-sea environments pose significant challenges for characterizing their biota. In this study, we conducted the first comprehensive biological survey of the Cape Range and Cloates submarine canyons in the East Indian Ocean using a combination of environmental DNA (eDNA) metabarcoding, remotely operated vehicle (ROV) underwater imagery, and specimen collection. Two metabarcoding assays (COI Leray and 16S Fish) were applied to 178 ten-litre water samples collected across 5 depths: surface, 200m 500m, 1000, and bottom (1750m – 4540 m). The application of these assays unveiled 226 species spanning 126 families, with each canyon detecting unique species. We identified 109 putative undescribed species, new records, or range extensions, including potential undescribed species of the monotypic ctenophore *Velamen* aff. *parallelum*, new records of the elusive giant squid Architeuthis dux, as well as several migratory mammal species like the deep-diving Pygmy Sperm whale (*Kogia breviceps*). Lastly, this study highlights the crucial role of metabarcoding assay choice in shaping observed spatial biodiversity patterns. The Leray COI assay revealed clear vertical stratification patterns, with differences between depths surpassing canyon-based differences for broader Animalia taxa. Conversely, the 16S Fish assay displayed greater canyon-based variations than depth-related differences. These assay disparities stemmed from the biological characteristics of the target taxa, and the dynamic nature of submarine canyons influencing vertical mixing. This underscores the complexity of interpreting eDNA-based spatial biodiversity patterns, emphasizing the need to consider target taxa, environmental factors, and ecological complexities in future investigations. Collectively, our findings highlight the efficacy of eDNA metabarcoding in exploring submarine canyon biodiversity and its broader potential for characterizing biodiversity in unexplored deep-sea habitats. The implementation of eDNA metabarcoding in deep-sea research shows promise as a powerful tool for enhancing conservation practices and addressing the growing pressures threatening these unique ecosystems.

5.3 Introduction

Submarine canyons are major geographical features of continental shelf margins (Harris et al., 2014), connecting relatively shallow productive shelves to deep-ocean basins (Puig et al., 2014). They act as conduits for the transportation of organic matter and macrophytes between the continental shelf and abyssal depths (Harris & Whiteway, 2011; Vetter & Dayton, 1999). The concentration of organic matter enhances local primary productivity in these canyons (De Leo et al., 2010; Vetter et al., 2010), providing key feeding, spawning, and recruitment grounds for both commercially important and charismatic megafauna species (Company et al., 2012; D'Onghia et al., 2015; Farrugio, 2012; Fernandez-Arcaya et al., 2013; Moors-Murphy, 2014; Yoklavich et al., 2000). The complex and abrupt topography of these canyons creates unique habitats that disrupt expected diversity patterns (Levin et al., 2010; Puig et al., 2014), leading to locally distinct assemblages, high rates of endemism, and potential speciation through physical isolation (Cunha et al., 2011; Gunton et al., 2015; Levin et al., 2001; Ramirez-Llodra, Company, et al., 2010; Schlacher et al., 2007). As a result, these environments are largely considered to be biodiversity hotspots (De Leo et al., 2010; Robertson et al., 2020; Vetter et al., 2010) that provide important refuge areas for a large number of rare and threatened species (Bianchelli et al., 2010; Fabri et al., 2014; Huvenne et al., 2011).

Although submarine canyons are widespread along continental margins, patterns in community composition and species richness have only been investigated in a relatively small subset of submarine canyons (De Leo & Puig, 2018; Matos et al., 2018). Many remain largely unexplored with many undescribed species (Matos et al., 2018) due to the inherent difficulties associated with accessing and sampling these environments (Ramirez-Llodra, Brandt, et al., 2010). The deep-sea is immense and remote, and comprehensive surveys of this environment are often expensive, and limited in time and space (Duhamet et al., 2023; Paulus, 2021; Ramirez-Llodra, Brandt, et al., 2010). Additionally, the majority of current baseline biodiversity data is obtained through benthic sampling techniques, with relatively limited attention given to pelagic biodiversity in these ecosystems (St. John et al., 2016; Webb et al., 2010), primarily due to the difficulties posed by the vast expanse of the deep pelagic

environment (Ramirez-Llodra, Brandt, et al., 2010). Despite these difficulties, these submarine canyons are increasingly threatened by direct and indirect anthropogenic pressures from fisheries, climate change, pollution, and extraction or mining (Daly et al., 2018; Fabri et al., 2014; Jamieson et al., 2019; Orejas et al., 2009; Trotter et al., 2019). As a result, these ecosystems may experience a silent erosion of deep benthic and pelagic biota, with many species driven to extinction before they are discovered.

Biological surveys of submarine canyons are logistically and financially constrained by the challenges of comprehensively sampling across broad spatial scales (over large distances and to great depths) in often remote locations (McClain & Barry, 2010; Moors-Murphy, 2014). While trawling has been a popular approach for surveying deep-sea ecosystems (Nóbrega et al., 2023; Orlov & Volvenko, 2022; Sánchez et al., 2008), it is not possible across areas of unsuitable substrates (Koslow et al., 2000) and should be avoided in unexplored regions given the substantial and often irreversible effects it can have on these vulnerable ecosystems (Good et al., 2022; Puig et al., 2012). For example, a study in the north-western Mediterranean Sea examined the effects of daily bottom trawling for deep-sea shrimp in a submarine canyon and found significant declines in organic matter content (up to 52%), slower carbon turnover (around 37%), decreased meiofauna abundance (80%), and lower biodiversity (50%) in trawled areas compared to untrawled areas (Pusceddu et al., 2014).

Visual surveys conducted using remotely operated underwater vehicles (ROV) are a non-invasive alternative that have frequently been used for biodiversity surveys in deep-sea environments (Ayma et al., 2016; de Mendonça & Metaxas, 2021; Dumke et al., 2018; Marsh et al., 2012), and provide a more targeted approach than ‘blind’ trawl or grab survey methods. However, the use of equipment like ROVs can easily introduce sampling variability and bias through sampling design (e.g., opportunistic video vs. imagery transects (Duffy et al., 2014)) and by affecting the behaviour of deep-sea fauna (Ayma et al., 2016; Lorance & Trenkel, 2006). Additionally, survey time is generally limited in deep-sea environments, and more cryptic species may not be detected in visual surveys (Sward et al., 2019). It is near impossible to confidently

identify organisms to species level from video transects, hence the development of surveys using unmanned vehicles driven by ship-based pilots with advanced specimen collection skills. The collection of specimens enables specimen identifications to be verified using morphological and molecular means and greatly increases confidence in biodiversity studies (Baco & Cairns, 2012; Bode et al., 2017).

The use of contemporary genetic techniques, such as environmental DNA (eDNA) metabarcoding, has become increasingly common in biomonitoring and is now at the forefront of biodiversity research (Takahashi et al., 2023). eDNA metabarcoding describes patterns in biodiversity through the amplification of trace amounts of DNA naturally shed into the environment by organisms, profiling a wide-range of biota without the need for visual observation. Surveys using eDNA have demonstrated increased sensitivity to the presence of rare and endangered species in comparison to conventional survey methods (Nester et al., 2023), expanding on total observed diversity. Importantly, it has been successfully used in deep-sea environments to describe communities across both spatial (Guardiola et al., 2016; Laroche et al., 2020; Lejzerowicz et al., 2014) and depth gradients (Canals et al., 2021; Feng et al., 2022), overcoming the difficulties associated with sampling these environments and expanding the breadth of biodiversity detected.

The Cape Range and Cloates Canyons are two large submarine canyons on the north-western margin of Australia, reaching depths of 4900 m and 4400 m respectively (Post et al., 2022). These canyons incise the continental shelf, connecting deep abyssal waters to shallow waters adjacent to the Ningaloo Reef (Post et al., 2022). The heads of these canyons are associated with localised areas of high productivity due to the interaction of upwelling nutrient-rich Antarctic Intermediate Water with the Leeuwin Current (Brewer et al., 2007; Lowe et al., 2012). The canyons are recognised as key ecological features within the Gascoyne Marine Park (Falkner et al., 2009), but to date remain biologically unexplored. The overall lack of understanding of these ecosystems prevents effective management of the canyons as part of the Gascoyne Marine Park network (Bond & Jamieson, 2022), and inhibits conservation actions in the face of increasing oil and gas initiatives.

Here, we conduct the first biological survey of the deep-sea Cape Range and Cloates Canyons in the East Indian Ocean using a combination of eDNA metabarcoding and ROV underwater imagery and specimen collection. As eDNA approaches rely heavily on comprehensive genetic databases, which are lacking for many remote or unexplored locations, we created a custom reference database using museum-vouchered specimens collected from the canyons via ROV. Our study uses eDNA metabarcoding to: *(1)* assess variation in diversity and community composition between the canyons and along a depth gradient (surface, 200 m, 500 m, 1000 m, 'bottom'); *(2)* uncover putative undescribed species records and range extensions; *(3)* compare diversity patterns and taxonomic resolution captured by eDNA and ROV sampling approaches carried out simultaneously. eDNA technologies are yet to be widely trialled in Australia's deep-sea marine environments, and as such, the potential for species discovery and new records is high. By characterising the biodiversity of the Cape Range and Cloates Canyons, we will generate important biological baseline information which can provide a foundation for future management and conservation efforts.

5.4 Methods

5.4.1 ROV imagery and specimen collection

Remote Operated Video (ROV) dives were completed across 16 sites in the Cape Range (n = 12) and Cloates (n = 4) Canyons in March – April 2020 (Figure 5-2). Poor weather reduced the number of sites visited in the Cloates Canyon. Imagery transects (quantitative and non-quantitative) and specimen collection were completed on board the *RV Falkor* using the ROV SuBastian. SuBastian is a custom-built work class ROV that conducts scientific work down to 4500 m (Schmidt Ocean Institute, 2019). It is equipped with a Sulis Subsea Z70 deep-sea science camera, with 4K optics and sensors for temperature, depth, conductivity and oxygen (Schmidt Ocean Institute, 2019). A robotic arm was used to collect biological specimens and place them into multi-chamber insulated bioboxes.

Sites were chosen using random points along a depth gradient, points far from the canyon wall were moved to the nearest such location in order to fully utilise the ROV's capability to traverse challenging canyon terrain. Twelve quantitative transects visual were surveyed in the Cape Range Canyon, with none undertaken in the Cloates Canyon due to poor weather delays. Quantitative transects were run for 500 m upslope, at an approximate 2 m above the seafloor or rock wall and a speed of 0.3 knots. Still images were acquired every 5 secs with additional frames added manually if required. Transect images were annotated using SQUIDLE+ (http://squidle.org/) and images acquired every 10-20 secs to ensure continuous imagery with minimal spatial overlap. Biota and substrates were characterised consistent with the national standard operating procedures established for marine imagery analysis (Carroll et al., 2018), using the CATAMI image classification scheme (Collaborative and Automated Tools for Analysis of Marine Imagery, (Althaus et al., 2015). Specimens collected were identified to the lowest taxonomic level by Western Australian Museum (WAM) taxonomists aboard the *RV Falkor* or at the WAM Collections and Research Centre (Welshpool, Western Australia).

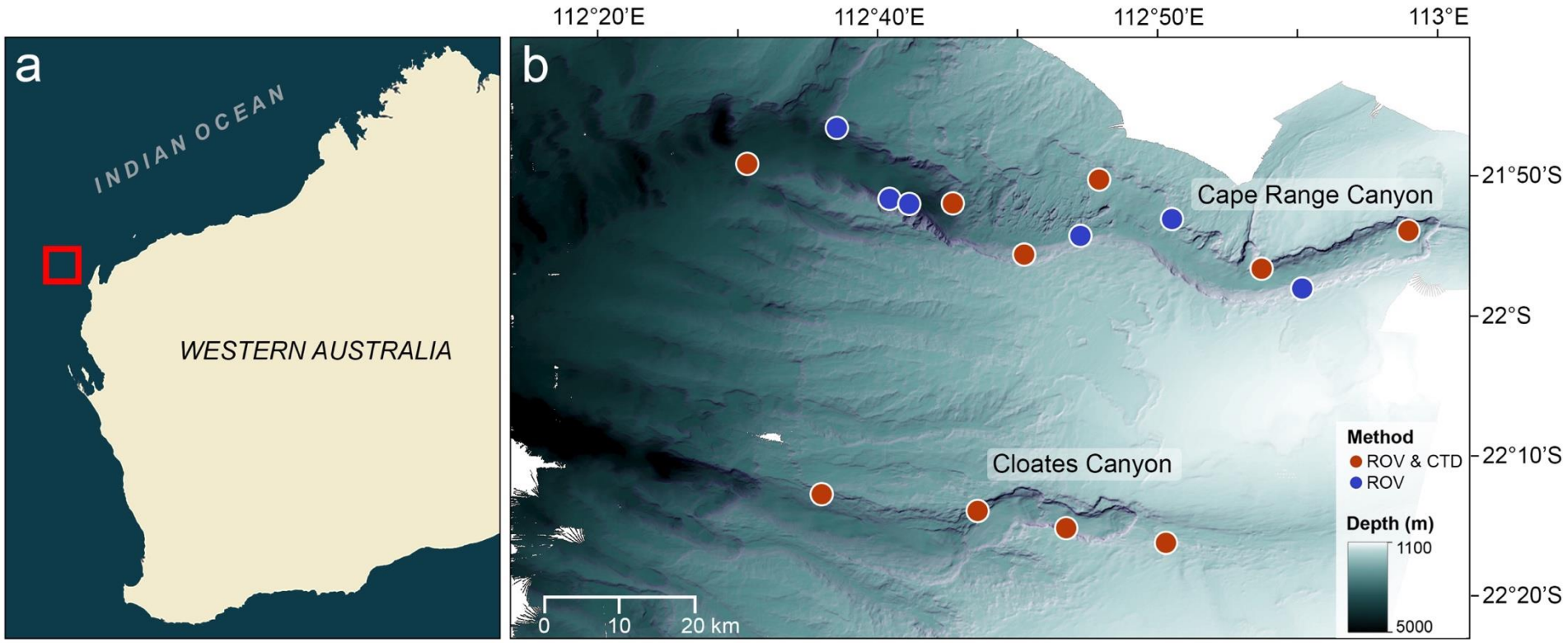


Figure 5-2: Cape Range and Cloates Canyon sampling sites (Western Australia). (a) Location of the Cape Range and Cloates submarine canyons (indicated by the red square) in the eastern Indian Ocean off the coast of Western Australia. (b) Sampling sites within the Cape Range (top, n = 12) and Cloates (bottom, n = 4) submarine canyons, with ROV and CTD (red dots), and ROV only (blue) sites indicated. All sites within the Cape Range Canyon had a visual transect conducted.

5.4.2 eDNA methodologies

5.4.2.1 Field sampling

Water samples (n = 178) were collected from 16 sites within the Cape Range and Cloates Canyons (Figure 5-2). Ten-litre water samples were collected in Niskin bottles using a Conductivity, Temperature, Depth rosette (CTD) and an ROV (SuBastian). All ROV samples were taken on the seafloor (1745 – 4510 m), with two 5 L samples taken at the beginning and end of a 500 m transect conducted at each site. The 5 L samples were combined to make one 10 L sample for the start and end of each transect. The volume was selected based on the CTD's Niskin bottle capacity, and the ROV Subastians configuration of four 5-litre Niskin bottles. CTD samples were taken at depths of surface (0 – 10 m), 200 m, 500 m, 1000 m, and seafloor (2020 – 4370 m), with 3 replicate 10 L water samples taken at each depth. Water samples were individually filtered within 3 h of collection using a Sentino microbiology peristaltic pump (Pall Life Sciences) through 47 mm diameter, 0.45 µm pore size polyethersulfone filter membranes (Pall Life Sciences). Pore size was selected as samples were anticipated to have low turbidity and subsequently high filtering rates. Filter papers were cut in half and immediately preserved at -80°C, with one half to be analysed and one to be stored as a reserve and a form of eDNA biobanking (Post et al., 2020; Post et al., 2022). Filtration equipment was soaked in a 10% bleach solution (changed daily) for a minimum of 10 min and rinsed with desalinated and filtered water in between each sample. One litre samples of the bleach solution and desalinated water were taken at the end of each filtering day to be included as filtration controls (n = 42). Filter membranes were transported to a quarantine facility within the Trace & Environmental DNA (TrEnD) Laboratory (Perth, Western Australia) for further processing.

5.4.2.2 DNA extraction and metabarcoding

DNA was extracted from half of the filter membrane using a DNeasy Blood and Tissue Kit (Qiagen) with the following modifications: 540 µl of ATL lysis buffer, 60 µl of Proteinase K, and a 12-hour digestion at 56°C. Extraction controls (i.e., no sample) were processed in parallel with all samples to detect any laboratory or between-

sample contamination. Extraction was completed using a QIAcube (Qiagen) DNA extraction system. Final DNA extracts were eluted in 100 µl of AE buffer and stored at -20°C. Samples were serially diluted (1/5 and 1/10) to optimise DNA input levels for quantitative PCR (qPCR) and remove potential PCR inhibitors.

DNA was amplified using the 16S Fish assay and the COI Leray assays (Table 5-1). The COI Leray assay broadly targets all Animalia taxa and was selected to maximize diversity detections. Comparatively, the 16S Fish assay has been optimized to specifically target bony fish. Negative controls were included on each qPCR plate to control for contamination. To reduce the likelihood of index-tag switching and chimera production, metabarcoding was performed through the use of fusion-tagged primers consisting of an Illumina-compatible adapter sequence, a unique multiple identifier (MID) tag, and a respective primer sequence for each assay. Each sample and control were processed in duplicate using the same MID tag, to reduce stochasticity for species with low amounts of template DNA. qPCR reactions (25 µl) consisted of the following: 0.5 µM forward and reverse primer (Table 5-1), 2 mM $MgCl_2$, 1× AmpliTaq Gold PCR buffer, 1 U AmpliTaq Gold DNA polymerase (Applied Biosystems), 0.25 mM dNTPs (Astral Scientific), 0.1 mg BSA (Fisher Biotec), 0.6 µl of 5X SYBR Green dye (Life Technologies), and 4 µl template DNA, made to volume with Ultrapure Distilled Water (Life Technologies). qPCR amplifications were performed on a StepOnePlus Real-Time PCR System (Applied Biosystems) under a three-step cycling regime. Thermocycler conditions were as follows: 95°C for 5 min, followed by 50 cycles of 95°C for 30 s, annealing temperature (Table 5-1) for 30 s, and 72°C for 45 s, completed by a 72°C elongation step for 10 min. All qPCR reactions were prepared in dedicated clean room facilities at the TrEnD Laboratory (Curtin University). Resulting amplicons were pooled at equimolar ratios, size selected using a Pippin Prep (Sage Science), and purified using a QIAquick PCR Purification Kit (Qiagen). The final libraries were quantified using a QIAxcel Advanced System (Qiagen) and a Qubit Fluorometer (Thermo Fisher). Libraries were sequenced on an Illumina MiSeq platform using either a 500-cycle MiSeq V2 Reagent Kit for paired-end sequencing (COI Leray), or 300-cycle MiSeq V2 Reagent Kit for single-end sequencing (16S Fish).

Table 5-1: PCR assay information for marine eDNA metabarcoding in the Cape Range and Cloates canyons.

The 16S Fish and COI Leray assay target the 16S and COI gene regions respectively. In the primer name, the 'F' refers to the forward primer and 'R' to the reverse primer.

PCR assay	Target taxa	Primer name	Sequence (5'–3')	Target length (bp)	Annealing temp (°C)	Reference
16S Fish	Bony Fish	16SF/D	GACCCTATGGAGCTTTAGAC	178–228	54	(Berry et al., 2017)
		16S2R-degenerate	CGCTGTTATCCCTADRGTAACT			(Deagle et al., 2007)
COI Leray	Animalia	m1CO1intF	GGWACWGGWTGAACWGTWTAYCCYCC	304-313	46	(Leray et al., 2013)
		jgHCO2198	TAIACYTCIAAYCAYAARGAYATTGG			(Geller et al., 2013)

5.4.2.3 Bioinformatics and taxonomic assignments

Bioinformatics and taxonomic assignments of sequencing data were performed using eDNAFlow, a fully automated workflow that processes eDNA data from raw sequences to curated and noncurated zero-radius operational taxonomic units (ZOTUs) and their abundance tables (Mousavi-Derazmahalleh et al., 2021). In brief, sequences were quality filtered, demultiplexed, denoised, and erroneous sequences removed using a combination of AdapterRemoval (Schubert et al., 2016), OBITools (Boyer et al., 2016), and Usearch (Edgar, 2010) via Zeus, an SGI Linux cluster based in the Pawsey Supercomputing Centre (Kensington, Western Australia). Resulting ZOTUs were queried against NCBI's GenBank nucleotide database (accessed September 2022) and a custom reference database of 175 WAM vouchered specimens obtained during the voyage (herein WAM custom database) using BLASTn. Thresholds for percentage identity and query coverage were set above 100% and 90% respectively. ZOTUs were assigned taxonomy using a lowest common ancestor python script within eDNAFlow (Mousavi-Derazmahalleh et al., 2021). Taxonomic assignments were then manually curated and checked for additional entries using the Barcode of Life Data (BOLD) system (Ratnasingham & Hebert, 2007). All taxonomic assignments were further evaluated against knowledge of species distributions using the World Register of Marine Species (WoRMS Editorial Board, 2022), Atlas of Living Australia (ALA – ala.org.au), and the WAM collections database. Final curated assignments and putative new records or range extensions were evaluated by taxonomic experts at the Western Australian Museum (see 5.1.2). To present putative new occurrence records we required taxa to be either species in a monospecific genera (with less than 96% identity to closest relative), congeneric taxa to be barcoded for the gene region of the respective assay, or tissue confirmation via WAM taxonomists.

ZOTUs assigned to contaminants, non-Animalia, and non-marine Animalia were removed and excluded from further analysis. These detections were primarily humans (*Homo sapiens*), Bacteria, and Chromista. Several species of fish (*Arripis georgianus*, *Katsuwonus pelamis*, *Thunnus alalunga*, *T. albacares* and *Mugil*

cephalus) were identified as potential contamination due to their use as bait in fish and crustacean traps throughout the voyage. To be conservative, ZOTUs assigned to these species were removed regardless of if the species are known to be present in the region.

5.4.2.4 Statistical analysis

Taxonomic datasets for Animalia (COI Leray) and fish (16S Fish) were analysed separately to account for potential assay biases. Overall differences in richness and diversity between canyons (Cape Range and Cloates) and depths (CTD samples only: surface, 200 m, 500 m, 1000 m, and bottom) were explored visually in R Studio (v4.2.1; R Studio Team, 2022). To examine taxonomic resolution of each assay (i.e., the ability to accurately distinguish taxa), the proportion of assignments matched to various taxonomic levels ('unclassified', order, family, genus, and species) was calculated. Differences in species richness between canyons and depths were assessed using ANOVAs. Differences in community composition were explored using PRIMERv7 with the PERMANOVA + add-on (Anderson et al., 2008). Data were transformed into presence-absence matrices and converted to a Jaccard matrix. A permutational multivariate analysis of variance (PERMANOVA) was used to test community variation between canyons and depths (CTD samples only). Pairwise comparisons were performed in the presence of significant differences to determine where composition differed. Community composition was visualised by principal coordinate analysis (PCO). Similarity percentage analyses (SIMPER) were also conducted in PRIMER v7 to identify taxa contributing most to pairwise dissimilarity between canyons and depths. An indicator analysis was performed using the R package indicspecies (De Cáceres et al., 2016) to distinguish relationships and patterns in the taxa detected between canyons and depths. Lastly, to examine potential depth stratification, each assay was analysed separately across the sampling depths.

Comparisons between eDNA metabarcoding and ROV transects could only be made at the phyla level due to the low taxonomic resolution of the ROV annotations. eDNA

samples were subset to include only those taken at sites where ROV transects were completed and the proportion of phyla detected at each transect was then compared between the methodologies. All R code used is available in R markdown format (see 5.1.2).

5.5 Results

5.5.1 Overview and specimens collected

The max surveyed depths were 4490 m in the Cape Range Canyon and 3375 m in the Cloates Canyon. Over 1000 biological specimens were collected during this expedition, and up to 30 putative undescribed species of marine animals across numerous phyla were discovered. Significant findings from this voyage include the collection of the first giant hydroids in Australian waters (> 1 m tall), and what may be the longest animal (a siphonophore) to have been observed in the world (~46 m). All faunal specimens have been registered into databases at the WA Museum where they will undergo additional taxonomic and genetic analysis. A total of 2570 seafloor images were annotated from 12 quantitative transects in Cape Range Canyon. Lastly, eDNA was extracted from 178 samples, comprising more than 1700 L of water from 16 sites.

5.5.2 eDNA sequencing statistics

A total of 10, 969, 297 sequencing reads were obtained using the COI Leray and 16S Fish metabarcoding assays. For the COI Leray assay 1689 ZOTUs were assigned, of which 695 (41.15%) were retained post-curation and contamination removal. For the 16S Fish assay, 493 ZOTUs were assigned, of which 458 (92.9%) were retained post-curation and contamination removal. The mean number of reads per sample was 5644 $\pm$ 423.12 and 46,068.13 $\pm$ 2233.95 for COI Leray and 16S Fish respectively. The low number of assigned reads produced by the COI Leray assay can be attributed to its high degeneracy, which is known to amplify widely beyond its target taxa Animalia (Collins et al., 2019; Deagle et al., 2014; Hintikka et al., 2022).

5.5.3 eDNA diversity and richness

The two metabarcoding assays collectively identified 226 species, representing 126 families in 56 orders, providing new baselines for 11 different phyla in the biologically unexplored Cape Range and Cloates Canyon. A total of 299 Animalia taxa were detected using the COI Leray assay, with majority resolved to species level (163), with the remainder resolved to genus (83), family (31), order (15) and class (7). Predominant phyla were Arthropoda (32 families), Cnidaria (31 families), Chordata (25 families), Mollusca (13 families), and Echinodermata (13 families; Figure 5-3). This trend was reflected in the number of assigned reads, with copepod (44.01%) and hydrozoan (40.6%) taxa comprising 84.61% of the total reads. The majority of species-level detections were associated with mesopelagic depths (including vertical migrating species, 36.42%), followed by epipelagic (20.99%), mesopelagic and bathypelagic (13.58%), bathypelagic (12.96%), deep-sea benthic (9.26), and bathypelagic and abyssopelagic (6.79%). The most frequently occurring species were siphonophore species *Lensia campanella* and *Rosacea* sp., detected 75 and 59 times respectively. The use of the WAM custom database resolved an additional 53 taxa from 79 ZOTUs, with 42 taxa (79.25%) resolved to species level.

Using the 16S Fish assay, 236 fish taxa were detected (class: Teleostei and Chondrichthyes), representing 58 fish families (Figure 5-4). Majority of these taxa were assigned at a species level (79), followed by genus (56), order (36), family (33) and class (32). Predominant fish families included lanternfish (Myctophidae; 8 genera), cusk-eels (Ophidiidae; 7 genera), and snake mackerels (Gempylidae; 5 genera). The majority of fish species detected were associated with mesopelagic depths (including vertical migrating species, 37.97%), followed by bathypelagic (20.25%), epipelagic (17.72%), mesopelagic and bathypelagic (15.19%), and bathypelagic and abyssopelagic (8.86%). *Seleniolycus* sp. and *Barathrites iris* were the most frequently detected fish species, with 73 and 72 respective detections. An additional 43 fish families were detected using the 16S Fish assay, with five fish families only detected using the COI Leray assay (Figure 5-4). The use of the WAM custom database resolved an additional 28 fish taxa from 42 ZOTUs, with 13 taxa (46.43%) resolved to species level.

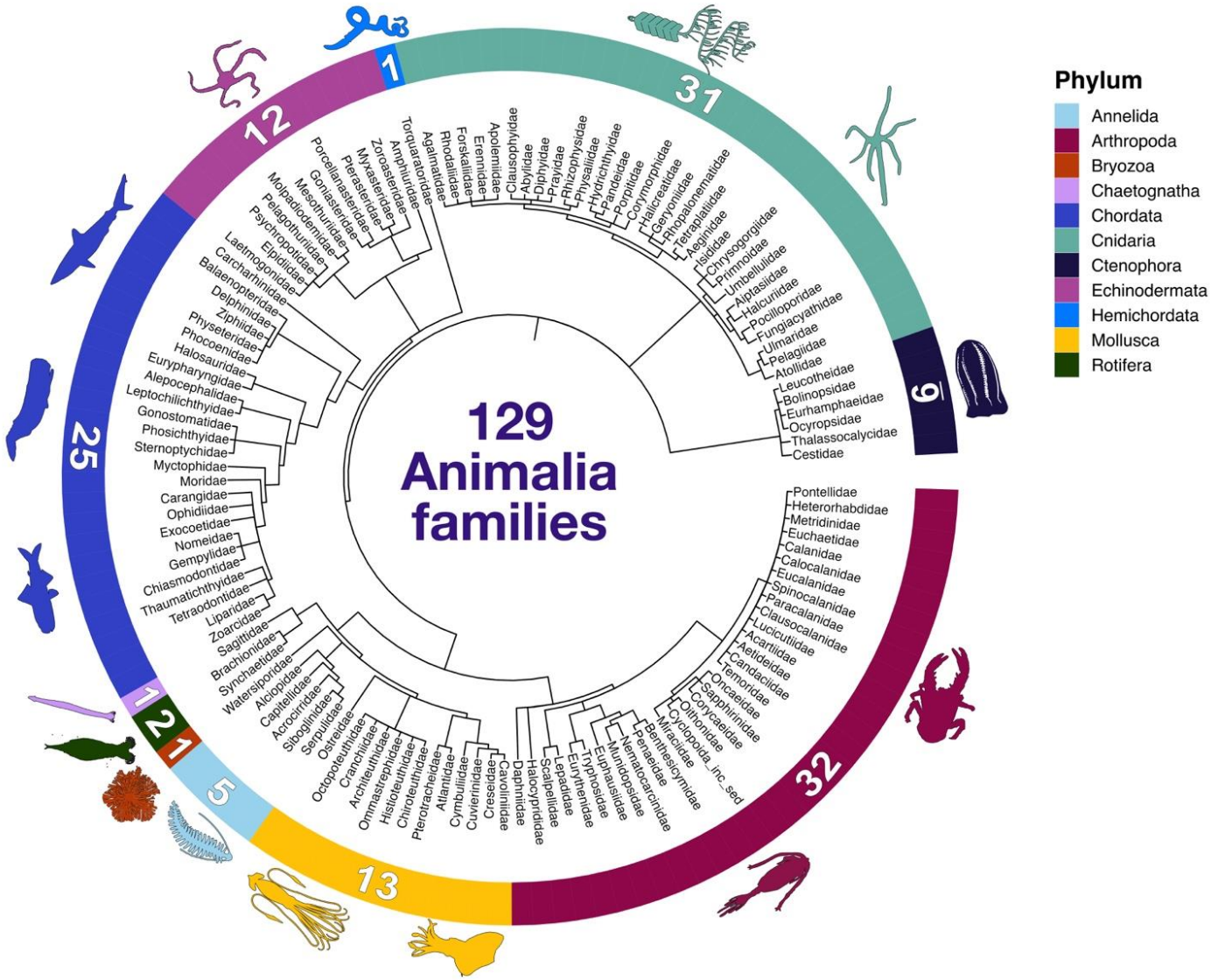


Figure 5-3: Family level taxonomic phylogram of Animalia taxa (n=129) detected in the Cape Range and Cloates canyons in the Indian Ocean. eDNA metabarcoding was performed using the COI Leray assay. Taxa silhouettes are coloured according to the phyla they represent.

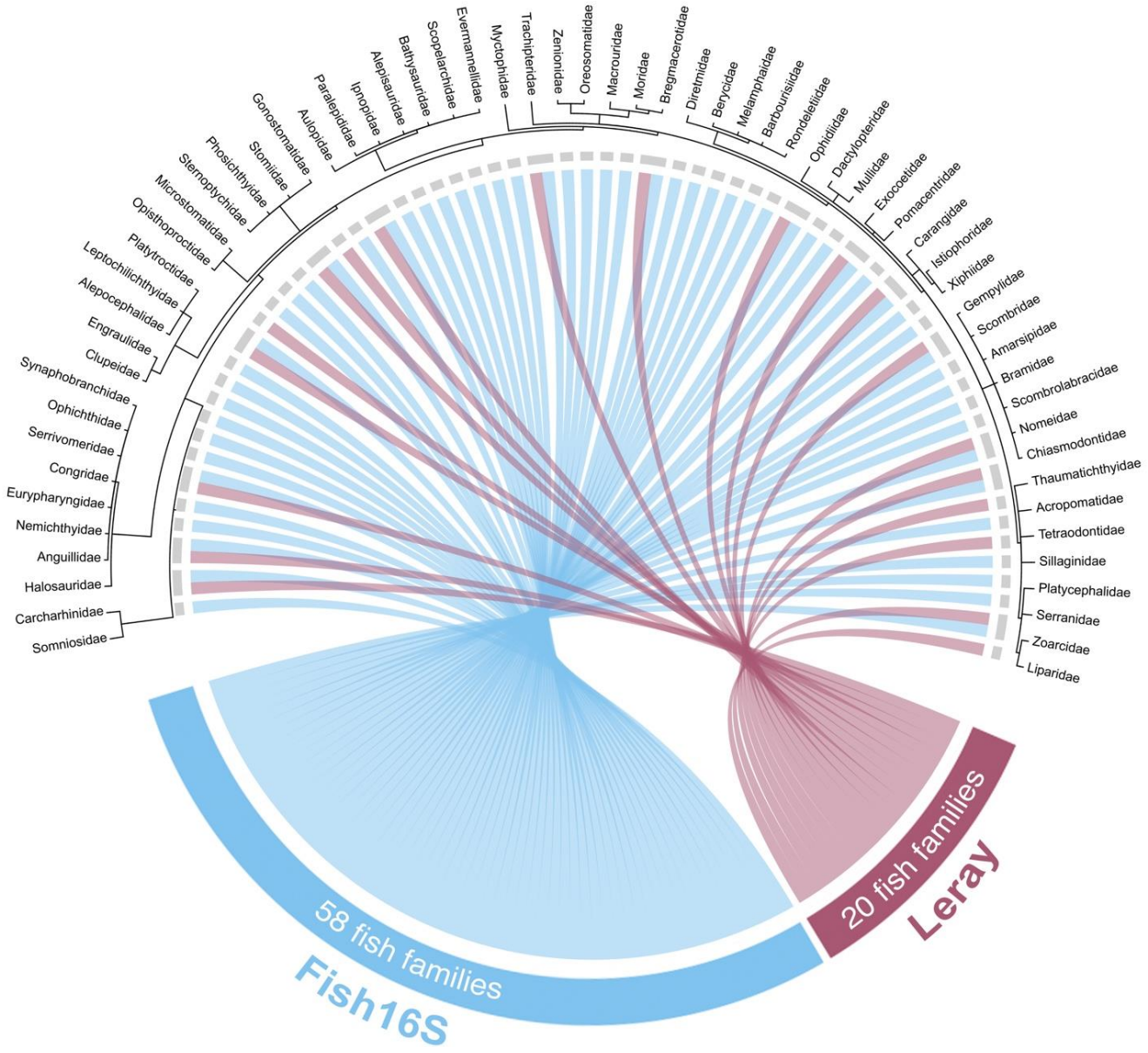


Figure 5-4: Family level taxonomic phylogram of bony fish (Class: Teleostei) and shark (class: Chondrichthyes) taxa detected via eDNA in the Cape Range and Cloates submarine canyons in the Indian Ocean. eDNA metabarcoding was performed using the 16S Fish (blue) and COI Leray (red) assays. The two metabarcoding assays yielded 78 fish and shark families in total.

A total of 109 taxa were identified as putative undescribed species, new records (genus or species), or range extensions (genus or species) across both assays (see 5.1.2). These detections were verified (WAM taxonomists) and a subset is presented in Figure 5-5 and Table 5-2. Of these taxa, 32.11% were putative undescribed species or records that blasted against WAM-verified tissue e.g., were visually confirmed. The remaining 67.89% were based on eDNA hits to the NCBI database.

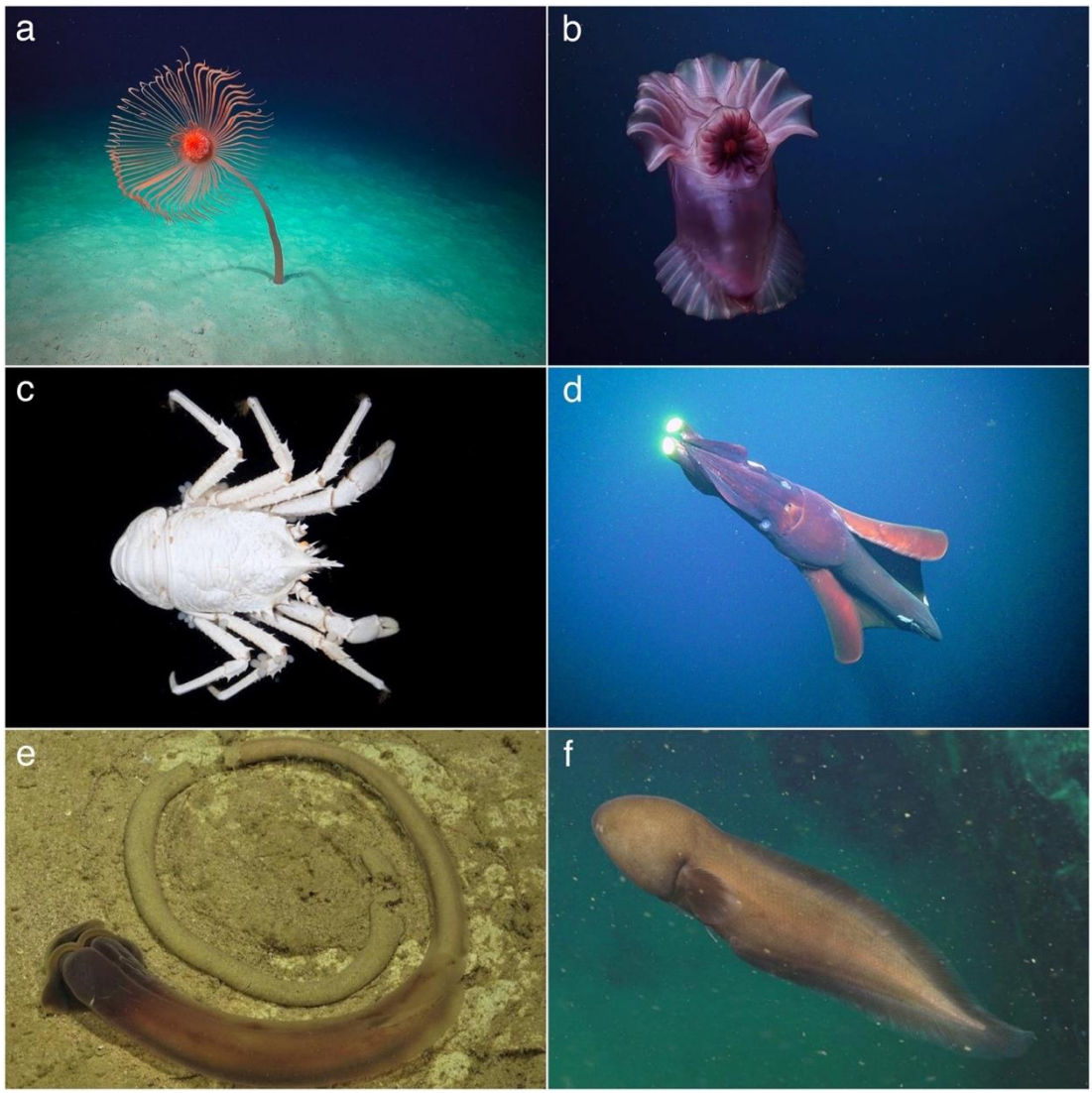


Figure 5-5: A subset of taxa of interest detected in the Cape Range and Cloates submarine canyons in the Indian Ocean (Western Australia). Taxa were detected using eDNA metabarcoding with the COI Leray and 16S Fish assays. Taxa include (a) giant hydroid *Branchiocerianthus* sp.; (b) deep-sea cucumber *Enypniastes* sp.; (c) squat lobster *Munidopsis* cf. subsquamosa; (d) Dana octopus squid *Taningia danae;* (e) acorn worm *Tergivelum* sp.*;* (f) faceless cusk eel *Typhlonus nasus.*

Table 5-2: A subset of taxa of interest detected in the Cape Range and Cloates submarine canyons (Indian Ocean- Western Australia) using eDNA.

Taxa were detected using the COI Leray and 16S Fish metabarcoding assays. Whether the taxa were assigned using the custom Western Australian Museum (WAM) database or NCBI is indicated under 'database'. Also indicated is whether they form a monospecific genus (under 'monospecific').

Phyla	Species	Common name	Database	Distribution	Comment	Mono-specific
Annelida	*Swima* sp. nov.		WAM	Northeast and West Pacific Ocean, Celebes Sea (Phillipines)	Possible undescribed species (sampled on board)	
	Teuthidodrilus sp. nov.	Squidworm	WAM	Celebes Sea (Phillipines)	Possible undescribed species (sampled on board)	Y
Arthropoda	*Eurythenes maldoror*		WAM	Weddell Sea, Argentinian Basin, North Atlantic and North Pacific	New Indian Ocean record	
	Munidopsis. cf. *subsquamosa*	Squat lobster	WAM	Northeast Pacific Northwest and Southwest Atlantic	New Indian Ocean record	
	Nigmatullinus acanthitelsonis		WAM	Atlantic	New record for Australia	
Chordata	*Acanthonus armatus*	Bony-eared assfish	WAM	Circumtropical	New record for WA	
	Amarsipus carlsbergi		NCBI	Indo-pacific	New East Indian Ocean record	Y
	Apagesoma australe		NCBI	Crozet Islands (Southern Ocean)	New genus record for Australia	
	Bassozetus sp.	Cusk eel	WAM	Circumglobal	Possible undescribed species (sampled on board)	
	Bathysaurus ferox	Deepsea lizardfish	NCBI	Circumglobal	Northerly range extension	
	Coryphaenoides armatus	Abyssal grenadier	NCBI	Circumglobal	New record for WA	
	Evermannella indica	Indian sabretooth	NCBI	Indo-pacific	New genus record for WA	
	Halosauropsis macrochir	Abyssal halosaur	NCBI	Circumglobal	Northerly range extension	

	Ipnops agassizii	Grideye spiderfish	WAM	Indo-pacific	New record for WA	
	Kogia breviceps	Pygmy Sperm Whale	NCBI	Circumglobal	Possible range extension- elusive species	
	Leucicorus sp.	Cusk-eel	WAM	Western Atlantic and Eastern Pacific	Possible undescribed species (sampled on board)	
	Pachycara sp.	Eelpout	WAM	Circumglobal	Possible undescribed species (sampled on board)	
	Penopus sp.	Cusk-eel	WAM	Western Atlantic and Western Pacific	Possible undescribed species (sampled on board)	
	Pseudorca crassidens	False killer whale	NCBI	Circumtropical	Evidence of independent resident coastal population	
	Rhadinesthes decimus	Slender Snaggletooth	NCBI	Circumglobal	New genus record for WA	Y
	Seleniolycus sp.	Eelpout	WAM	Southern Ocean (Antarctic)	Possible undescribed species (sampled on board)	
	Stemonidium hypomelas	Black serrivomerid eel	NCBI	Circumglobal	New record for WA	Y
	Typhlonus nasus	Faceless cusk	WAM	Indo-pacific	New record for WA	
	Zenion japonicum	Japanese dory	NCBI	Western Pacific	New genus record for WA/large range extension of species. No other species in genus (3) found in WA	
	Ziphius cavirostris	Cuvier's Beaked Whale	NCBI	Circumglobal	Northerly range extension	
Cnidaria	*Apolemia lanosa*		NCBI	Eastern Pacific	New genus record for WA	
	Athorybia rosacea		NCBI	Atlantic, Mediterranean Sea, and Pacific	New East Indian Ocean record	
	Branchiocerianthus sp.	Giant hydroid	WAM	Circumglobal	First giant hydroid in Australia	
	Erenna sp.		NCBI	Atlantic and Pacific	New genus record for WA	
	Halistemma rubrum		NCBI	Cosmopolitan	New record for WA	

	Hydrichthys boycei		NCBI	North Atlantic and North Pacific	New genus record for Australia and Indian Ocean	
	Liriope tetraphylla		NCBI	Circumglobal	Possible undescribed species	Y
	Marrus sp.		NCBI	Atlantic, Antarctic, Arctic, and East Pacific	New East Indian Ocean record	
	Pandea sp.		NCBI	Circumglobal	New genus record for WA	
	Rhizophysa eysenhardtii		NCBI	Atlantic and Meditteranean	New record for Indian ocean, possible undescribed species	
Ctenophora	*Eurhamphaea* sp.		NCBI	Tropical cosmopolitan	New genus record for WA	
	Velamen aff. Parallelum		NCBI	Circum(sub)tropical	Possible undescribed species	Y
Echinodermata	*Enypniastes eximia*	Deep-sea cucumber	WAM	Circumglobal	New record for north WA	Y
	Evoplosoma claguei	Deep-sea corallivore	NCBI	North-west Atlantic, and Pacific	New Indian Ocean record	
	Psychropotes sp.		WAM	North Pacific	New East Indian Ocean record	
Hermichordata	*Tergivelum* sp.	Acorn worm	WAM	North Atlantic and North-West Pacific	Possible undescribed species (sampled on board)	
Mollusca	*Architeuthis dux*	Giant squid	NCBI	Circumglobal	New record or range extension	
	Asperoteuthis acanthoderma	Thorny whiplash squid	WAM	Central Indo-Pacific	New East Indian Ocean record	
	Stigmatoteuthis hoylei	Flowervase jewel squid	WAM	Tropical Indian and Pacific	New record for WA	
	Taningia danae	Dana octopus squid	WAM	Cosmopolitan	Rare bioluminescent flashing squid	
	Teuthowenia megalops		WAM	North Atlantic	New Indian Ocean record	

5.5.4 eDNA derived patterns across canyons

The number of unique species detections was higher in the Cape Range Canyon (12 sites) than the Cloates Canyon (4 sites) for both the COI Leray (Figure 5-6a) and 16S Fish assay (Figure 5-6c). Despite the greater number of sites within the Cape Range Canyon, both the Cape Range and Cloates canyons had unique taxa detections. However, no significant differences in species richness were observed between the canyons for either COI Leray ($t = -0.346$, $df = 176$, $p = 0.73$) or 16S Fish assays ($t = -0.643$, $df = 174$, $p = 0.521$). Community composition differed significantly between the canyons for both the COI Leray ($F_{1,138} = 3.966$, $p < 0.001$) and 16S Fish assay ($F_{1,137} = 12.016$, $p < 0.001$). However, strong separations between canyon community composition were only visually evident in the fish (16S Fish) PCO (Figure 5-6d). No separations between canyon community composition were visually evident in the COI Leray PCO (Figure S 5-1), indicating that differences between depths were greater than differences between canyons for broader Animalia taxa.

SIMPER analysis identified prominent taxa contributing to the pairwise dissimilarity between the community composition of Cape Range and Cloates canyons using the COI Leray assay (Table S 5-1). This indicated a higher detection rate of *Rosacea* sp. (order: Siphonophorae), Warming's lantern fish *Ceratoscopelus warmingii* (order: Myctophiformes), and eelpout *Seleniolysus* sp. (order: Perciformes) within the Cape Range Canyon. Whilst the Cloates Canyon had more detections of *Athorybia* (order: Siphonophorae), *L. campanella* (order: Siphonophorae), *Leucothea* (phylum: Ctenophora), eelpout *Pachycara* sp. (order: Perciformes), and cusk eel *B. iris* (order: Ophidiiformes). These differences were reflected in the 16S Fish assay data, with higher detection rates of *Seleniolysus* sp. (order: Perciformes) in the Cape Range, and cusk eel *B. iris* (order: Ophidiiformes) and eelpout *Pachycara* sp. (order: Perciformes) in the Cloates Canyon (Table S 5-2).

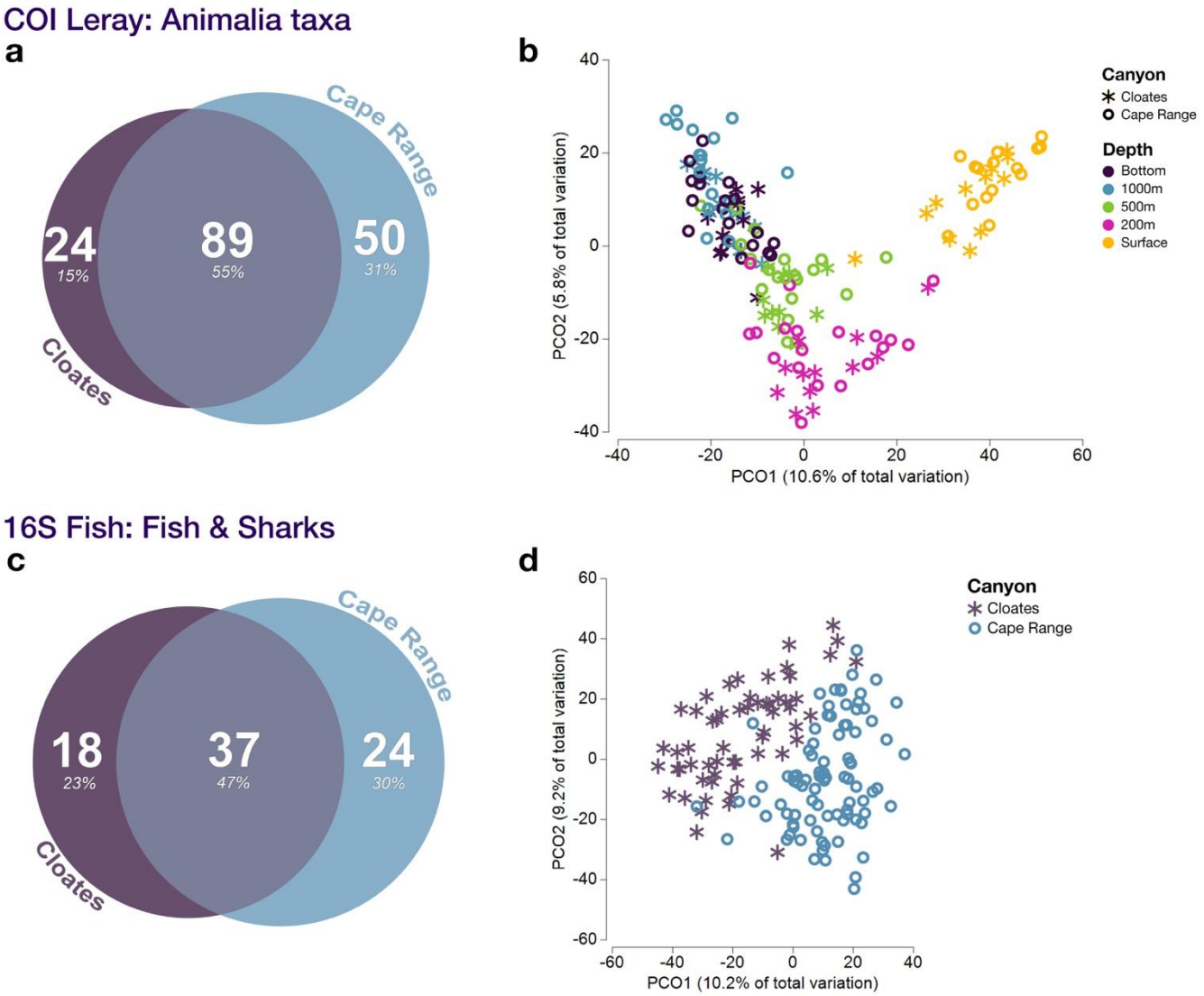


Figure 5-6: Venn diagrams and PCOs for the Cape Range and Cloates Canyons. (a) Venn diagram of the number of unique and shared Animalia species detected using the COI Leray assay in the Cape Range and Cloates canyons; (b) PCO of animalia taxonomic composition across 5 depths (surface, 200m, 500m, 1000m, and bottom) in the Cape Range and Cloates canyons; (c) Venn diagram of the number of unique and shared fish and shark species detected using the COI Leray assay in the Cape Range and Cloates canyons; (d) PCO of fish taxonomic composition across 5 depths (surface, 200m, 500m, 1000m, and bottom) in the Cape Range and Cloates canyons.

5.5.5 eDNA derived patterns across depth gradients

Along the CTD depth gradient, the number of unique species detected were highest in the bathypelagic bottom water samples at both Cape Range (56) and Cloates (49) using COI Leray (Figure S 5-2). Mean species richness was highest in bottom (15.5 $\pm$ 2.18) and surface (15.4 $\pm$ 0.25) water at Cape Range, and bottom water (17 $\pm$ 3.24) and 1000 (16.5 $\pm$ 4.41) for Cloates (Figure S 5-2). Comparatively, the number of unique fish species (16S Fish) were highest in the epipelagic samples at both Cape Range (200 m; 37) and Cloates Canyon (surface; 28 (Figure S 5-3)). Mean species richness followed the same trend with 12 $\pm$ 1.65 and 11.25 $\pm$ 1.12 species at the Cape Range (200 m) and Cloates (surface) respectively (Figure S 5-3). The reported differences in species richness between CTD depths were not significant for either assay (Table S 5-3,Table S 5-4, and Table S 5-5).

Within the Animalia dataset (COI Leray), we found both surface and 200 m samples to be dominated by Copepoda species, with 26 and 23 unique species detected respectively (Table 5-3). The 500 m and 1000 m samples were dominated by Teleostei species with 16 and 12 unique species detected, while bottom water samples were dominated by Teleostei and Hydrozoa, with 24 and 21 unique species detected respectively. Within the fish (16S Fish) dataset, species-level detections were more uniform. However, Ophiidiformes (order) were seen to dominate all depths (Table 5-4).

Table 5-3: Number of unique species detected per Animalia class at each sampling depth in the Cape Range and Cloates Canyons using the Leray COI eDNA metabarcoding assay.

		Depths				
Phylum	**Class**	**Surface**	**200**	**500**	**1000**	**Bottom**
Annelida	Polychaeta					5
Arthropoda	Copepoda	26	23	9	4	10
	Malacostraca	1	2	1	3	7
	Ostracoda		3	2	3	
	Thecostraca					2
Bryozoa	Gymnolaemata			1		1
Chaetognatha	Sagittoidea		1	1	1	
Chordata	Chondrichthyes					2
	Mammalia	1				8
	Teleostei	7	12	16	12	24
Cnidaria	Anthozoa	1	1			4
	Hydrozoa	9	10	11	11	21
	Scyphozoa		1	1		1
Ctenophora	Tentaculata			1		1
Echinodermata	Asteroidea			1	4	3
Holothuroidea	Hemichordata			1		1
	Enteropneusta			1		1
Mollusca	Cephalopoda	1	3	3	6	10
	Gastropoda	1	1			2
	Total	**47**	**57**	**49**	**44**	**103**

Table 5-4: Number of unique species detected per fish (class: Teleostei) and shark (class: Chondrichthyes) order at each sampling depth in the Cape Range and Cloates using the 16S Fish eDNA metabarcoding assay.

		Depth				
Class	**Order**	**Surface**	**200**	**500**	**1000**	**Bottom**
Chondrichthyes	Carcharhiniformes					1
Teleostei	Anguilliformes	1	1	2	1	1
	Argentiniformes				1	
	Aulopiformes	2	6	6	4	4
	Beloniformes	3	1		2	3
	Beryciformes		2	1		2
	Carangiformes	3				
	Gadiformes	1	1	1	1	2
	Istiophoriformes	2				1
	Lampriformes				1	1
	Myctophiformes	4	7	5	1	4
	Notacanthiformes	1	1	1	1	2
	Ophidiiformes	8	8	8	6	8
	Pempheriformes	1	1			
	Perciformes	3	2	2	2	2
	Scombriformes	5	7	2	8	5
	Stomiiformes	3	5	6	4	5
	Syngnathiformes	2				
	Trachichthyiformes			1	1	
	Zeiformes		1			1

Community composition significantly differed between CTD depths for both COI Leray ($F_{4,138}$ = 8.39, p < 0.001) and 16S Fish ($F_{4,137}$ = 4.817, p < 0.001), with pairwise testing indicating all depths were distinct in composition (Table S 5-6, Table S 5-7, Table S 5-8, and Table S 5-9). Taxonomic composition was seen to transition along the depth gradient for the COI Leray assay, with samples clustering into their respective depths in the PCO (Figure 5-6b). Overlapping between samples increased with depth. SIMPER analysis revealed differences between depths were largely driven by higher detection rates of copepod taxa (*Clausocalanus furcatus, Farranula gibbula, Calocalanus pavo*) in the epipelagic zone (surface and 200 m), and siphonophore taxa (genus *Apolemia*) in the mesopelagic, bathypelagic and abyssopelagic zones (500 m, 1000 m, bottom; see 5.1.2). Although community composition significantly differed for 16S Fish samples, no separations of depth were evident in the PCO (Figure S 5-4), indicating that differences between canyons were greater than differences between depths. Indicator analysis revealed similarities between depths were largely driven by lanternfish (order: Myctophiformes) and Stomiiformes (dragonfish, lightfish and hatchetfish), known to vertically migrate through the water column (see 5.1.2).

5.5.6 eDNA vertical stratification

Using the COI Leray assay, we observed vertical stratification of our eDNA samples along the water column relative to both depth and species. Epipelagic species (those living in the upper 200 m) represented 95.89% of the reads assigned to surface water samples (< 10 m), while bathypelagic (living 1000 – 4000 m) and benthic species represented 61.56% of the reads assigned to bottom water samples (> 1740 m). Bathypelagic and benthic species were found in higher read numbers below 1000 m; however, we did detect small proportions of bathypelagic eDNA in surface (16 reads) and 200 m (56 reads) samples.

These patterns were not reflected in the 16S Fish dataset, with minimal vertical stratification evident. Epipelagic species comprised the small proportion of the overall reads (1.87%), with species detections largely restricted to this zone. We

found bathypelagic species comprised 66.29% of the overall reads, and more than 60% of the assigned species reads at each sampling depth. This distribution of bathypelagic species eDNA throughout the water column contradicts the findings of the COI Leray dataset.

5.5.7 ROV transect comparison

Comparisons between eDNA (COI Leray) and ROV transects were done at the phyla level due to the low taxonomic resolution of ROV observations. Within the confinements of the transect, both methodologies detected 9 phyla. Phyla exclusive to eDNA and morphological ROV were Chaetognatha and Porifera respectively. No Porifera taxa were detected using eDNA, as the metabarcoding assay used (COI Leray) does not amplify or bind to Porifera taxa. Composition of eDNA detections within the transect were primarily Cnidaria (23.47%), Chordata (20.41%), and Arthopoda taxa (15.31%), while morphological ROV samples were dominated by Porifera (39.65%) and Echinodermata taxa (29.52%, Figure S 5-5).

5.6 Discussion

5.6.1 eDNA metabarcoding for monitoring submarine canyons

This study is the first to biologically characterise the Cape Range and Cloates Submarine Canyons off the coast of Western Australia, and demonstrates the efficacy of eDNA metabarcoding for characterizing biodiversity in unexplored deep-sea ecosystems. There is a pressing need to efficiently but comprehensively survey submarine canyons, given that distinct community assemblages and high rates of endemism they support are increasingly threatened by anthropogenic pressures and climate change. In this study, we address this data gap by using an eDNA metabarcoding approach to audit the diversity of marine life in two canyon systems and across a depth gradient within one canyon. The sequencing results are cross-checked with a locally curated genetic reference library built from expertly identified tissues that were collected by ROV during the expedition. The overall community detected by eDNA was then compared to records obtained visually on video transects. Despite being restricted to a single time-point, our survey highlights the

importance of non-destructive methods for assessing community composition and multitrophic diversity, provides a baseline for future monitoring efforts, and generates novel hypotheses related to the use of eDNA to discern potential undescribed species and range extensions. As all aquatic environments differ in composition, relative biomass, and genetic background, we hope the strengths and weaknesses of these experiments might aid future eDNA metabarcoding studies in deep-sea and unexplored aquatic environments.

5.6.2 Biodiversity of the Cape Range and Cloates Canyons

The biodiversity and community composition data obtained in this study provides new baselines for 11 different phyla in these biologically unexplored submarine canyons. Submarine canyons are widely recognised as biodiversity hotspots (De Leo et al., 2010; Robertson et al., 2020), their complex topography and enhanced productivity often results in distinct species assemblages and large aggregations of migratory species (Moors-Murphy, 2014). This survey revealed the Cape Range and Cloates Canyons support and provide habitat to a high diversity of taxa, with 226 species identified within 126 families using the COI Leray and 16S Fish assays.

The number of unique taxa species detections was higher in the Cape Range Canyon than in the Cloates Canyon for both assays, however, we attribute this to the increased number of sites in the Cape Range Canyon. Interestingly, despite the inequalities in sampling effort, each Canyon exhibited unique species detections and distinct community composition. Submarine canyons are complex habitats with specific hydrographic, sedimentological, and geochemical characteristics (De Leo & Puig, 2018). These habitats often exhibit fine-scale variations in biodiversity due to the high habitat heterogeneity present at the local scale (Ramirez-Llodra, Brandt, et al., 2010; Vetter et al., 2010). The differences between canyons likely relate to the larger size and depth of the Cape Range Canyon as opposed to the Cloates Canyon. Shallower bathymetry may influence the extend of upwelling and nutrient provision to pelagic zones, altering the transport of eDNA and thus the distribution and composition of species detected. Our findings indicate that the distribution and

composition of species within the Cape Range and Cloates canyons may be influenced by canyon-specific environmental conditions. This suggests the possibility of biologically distinct communities being supported by these canyons. However, to comprehensively explore these biodiversity patterns, future studies should employ equal sampling effort at finer spatial resolution, and investigation into canyon-specific environmental conditions. Exploring the potential interactions between environmental conditions and species distribution can shed light on the mechanisms driving the community composition patterns observed in these unique habitats.

5.6.3 Potential undescribed species and records

As no previous biological survey had been conducted in the Cape Range and Cloates submarine canyons, the potential for discovery and significant findings was high. Using eDNA metabarcoding, our study identified 109 taxa as putative undescribed species, new records (genus or species), or range extensions (genus or species) across both assays, highlighting how little is known about these largely inaccessible deep-sea ecosystems (Brandt et al., 2007; Costello & Chaudhary, 2017; Glover et al., 2018; Kaiser et al., 2022; Lambshead & Boucher, 2003).

Most putative undescribed species were confirmed through tissue sampling of specimens collected aboard the RV Falkor, such as the verified *Teuthidodrilus* sp. nov., a monotypic squidworm previously documented only in the Philippines (Osborn et al., 2011). However, a few potential undescribed species were suggested solely based on eDNA detections without specimen verification. For instance, *Velamen* aff. *parallelum* was proposed as a potential undescribed species within a family comprising two monotypic genera (*Velamen* and *Cestum*). Although the blast similarity percentage (96%) did not reach the threshold for species identification, the closest match was to *Cestum veneris* at 90%, leading to the inference that it is a potential undescribed species. Overall, the high number of putative undescribed species detected in the Cape Range and Cloates Canyons raises questions about the assumed cosmopolitan nature of deep-sea species. Moreover, studies have explored examined whether speciation and net diversification rates differ between the shallow

realm and the deep-sea (Miller et al., 2022; Rabosky et al., 2018), though limited representation in phylogenetic reconstructions hinders a comprehensive analysis for most deep-sea taxa (Lee et al., 2019). Although these putative eDNA reports were verified (WAM taxonomists), like visual survey approaches, they will require further verification by specimen collection. Nevertheless, the use of eDNA holds promise in guiding targeted survey efforts, not only for indicating potential undescribed species or confirming occurrence records, but also for aiding in the collection and recovery of unresolved taxa that may potentially represent undescribed species.

Our study revealed a substantial number of significant species that could potentially be new records or range extensions for Western Australia (WA), the Indian Ocean, or Australia. These include the first record of giant hydroid *Branchiocerianthus* (specimen verified) in Australia, new WA record of the bony-eared assfish *Acanthonus armatus*, and new WA records of the elusive giant squid *Architeuthis dux*. The high abundance of potential new records and range extensions in deep-sea environments may be attributed to the homogeneity of their cold-water conditions, facilitating species' range expansion over large distances while maintaining environmental similarity. However, deep-sea environments are recognized as both highly diverse and heterogeneous (Levin et al., 2010; McClain & Barry, 2010; Robertson et al., 2020), with studies demonstrating genetic differences among species within the same ecosystem over small and broad spatial scales (Quattrini et al., 2013; Schüller, 2011; Watanabe et al., 2021; Xu et al., 2021). For instance, a recent investigation of the Southern Ocean identified 27 undescribed species of *Doris kerguelenensis* (Order: Nudibranchia) from a sample of 1000 specimens (Maroni et al., 2022). Therefore, the prevalence of putative new records and extensions is more likely a reflection of the limited exploration and understanding of these environments.

We also detected the presence of several migratory mammal species including the deep-diving Pygmy Sperm whale (*Kogia breviceps*), and Cuvier's Beaked Whale (*Ziphius cavirostris*). The occurrence of these species correlates with the abundance of prey organisms such as copepods, squid, siphonophores, and fish, detected by

eDNA methodologies. Given the data-deficient status of the Pygmy Sperm whale (Kiszka & Braulik, 2020) and the potential range extension of Cuvier's Beaked whale, our findings demonstrate the utility of eDNA as a valuable tool for gaining insights into the ecology of these rarely observed taxa. Moreover, our study supports the notion of the Cape range and Cloates Canyons as biodiversity hotspots supporting large aggregations of predators and apex consumers (Sleeman et al., 2007). The detection of such diverse biota further emphasizes the ecological importance of preserving and safeguarding these unique and fragile deep-sea environments.

5.6.4 eDNA vertical stratification

The choice of metabarcoding assay clearly plays a significant role in influencing the observed vertical stratification patterns. The COI Leray assay revealed distinct vertical stratification patterns, correlating eDNA samples with depth and species, while the 16S Fish assay did not exhibit such patterns. Specifically, using the COI Leray assay, the eDNA signal of bathypelagic and benthic species showed progressive degradation as it ascended through the water column, while surface water samples were dominated by epipelagic species. However, these patterns were not evident in the 16S Fish dataset. The COI Leray assay detects a wider range of taxa, including more benthic species, which has likely impacted the observed differences in vertical stratification patterns. For example, benthic organisms inhabiting bathyal or abyssopelagic depths may exhibit limited vertical mobility, resulting in stratified distributions of eDNA throughout the water column that are better captured by universal assays such as Leray COI. Similarly, the higher mobility and biomass of fish may mean they are more likely to disperse their DNA extensively throughout the water column. Therefore, this discrepancy between assays is partially attributed to the biological characteristics of the target taxa.

Our findings contradict previous studies in deep-sea ecosystems (Canals et al., 2021), which demonstrated that eDNA of pelagic fish can be detected at the depth of release and deeper, but rarely shallower. Unlike Canals et al. (2021), our study focused on the distribution of total reads assigned to species throughout the water column,

rather than the relative abundance at specific depths. This approach was chosen to assess the proportional distribution of eDNA from deep-sea and epipelagic species, considering potential variations in read distribution across depths. When analyzing our fish data using a similar approach, we observed improved but still less pronounced vertical stratification patterns compared to Canals et al. (2021). These differences could be attributed to variations in sampling timing and location. Canals et al. conducted their sampling in the Bay of Biscay during both day and night, while our opportunistic study focused on submarine canyons, primarily sampling at night, which may have higher detection rates of vertical migrators. Furthermore, the unique ecological and physical characteristics of canyons, such as the concentration of diel vertical migrators (Genin, 2004; Santora et al., 2018), displacement of deep-water species to coastal zones (De Leo et al., 2010), topographically induced upwelling (Moors-Murphy, 2014; Sobarzo et al., 2001), and enhanced mixing through internal tides and wave generation (Kunze et al., 2002; Waterhouse et al., 2017), could contribute to the observed differences. Additionally, strong tidal currents near the continental shelf may lead to turbulence and well-mixed water (Dipper, 2022), potentially explaining the limited vertical stratification patterns in the fish data.

A major limitation of current eDNA metabarcoding studies, is the inability to distinguish different life stages of organisms, such as fish larvae or eggs. Despite growing interest and recent advances in this area, the discrimination of different life stages using eDNA necessitates a targeted species-specific assay that can effectively between intra-specific or haplotype differences (Bylemans et al., 2016; Hayer et al., 2020; Holmes et al., 2022; Marshall & Stepien, 2019; Takeuchi et al., 2019). Studies have suggested that deep-sea fish species reproduce near the sea floor, with fertilized eggs floating to shallower waters, and juveniles subsequently migrating down to typical adult depths (D'Onghia et al., 2015; Fernandez-Arcaya et al., 2013; Porcu et al., 2020). Accordingly, during spawning periods fish DNA will likely be ubiquitous throughout the water column, confounding any vertical spatial patterns observed. Therefore, interpreting vertical stratification patterns of fish must be done with caution, especially when excessive mixing throughout the water column is expected. Further research and consideration of various biological and ecological

factors, such as spawning period and water movement, are needed to accurately interpret and understand the vertical distribution patterns of fish using eDNA.

5.6.5 Comparisons between eDNA metabarcoding and ROV transects

Comparisons between eDNA metabarcoding and morphological ROV sampling were conducted at the phylum level, taking into consideration the limited taxonomic resolution obtained from the ROV observations. eDNA metabarcoding was therefore a superior approach for obtaining broad biodiversity data at a high taxonomic resolution. Although both approaches detected 9 phyla, the detections obtained through eDNA metabarcoding were more evenly distributed, whereas the morphological ROV sampling was dominated by Porifera. The absence of arrow worms (Chaetognatha) in the ROV observations could be attributed to the challenges associated with their relatively small size and elusive characteristics (fast-moving), which may have made them difficult to detect using ROV sampling methods. Comparatively, eDNA metabarcoding did not detect any sponge taxa (Porifera), likely due to the limitations of the chosen metabarcoding assays, COI Leray and 16S Fish, which do not effectively bind to or amplify sponge taxa. However, it should be noted that the study's objective was to capture a wide range of taxa at high taxonomic levels, which led to the utilization of broad Animalia and fish assays. Furthermore, one of the added benefits of eDNA metabarcoding is the ability to overlay additional metabarcoding data across the tree of life and biobank samples. Samples that are stored appropriately (biobanking) can be revisited as new assays are developed, and the metabarcoding data can be reanalysed as reference databases improve. This allows for retrospective analyses and expands the scope of biodiversity assessments over time, making eDNA metabarcoding a valuable tool for long-term biodiversity monitoring. This is especially crucial in remote or inaccessible ecosystems like submarine canyons where baseline data may be scarce, as it offers an opportunity to collect data for continuous monitoring and conservation initiatives.

5.6.6 Methodological advancements and future directions

The incomplete nature of reference databases poses a significant bottleneck in the taxonomic assignment of eDNA sequences. Our results, consistent with prior research (Dugal et al., 2022; West et al., 2022), demonstrate that the development of locally curated databases can greatly enhance taxonomic resolution. Through the utilization of a custom reference database of specimens obtained on the voyage, we were able to achieve an additional 15% of taxonomic assignment and resolution, underscoring the effectiveness of curated databases, particularly in hard-to-reach and unexplored environments. Our study also supports previous findings (West, Richards, et al., 2020; West, Stat, et al., 2020) in highlighting the potential of eDNA metabarcoding to elucidate putative undescribed species records, occurrences, and range extensions. While we acknowledge that not all researchers will have the resources to confirm putative detections with expert taxonomists, we believe that through meticulous checking of species distributions and thorough manual curation of taxonomic assignments, such detections could be validated (e.g., see West, Richards, et al. 2020). This underscores the importance of robust quality control measures, including manual curation and species distribution checks, in bioinformatic processes and taxonomic assignments to ensure accurate and reliable data, particularly when identifying potentially undescribed species records, occurrences, or range extensions.

The findings of our study revealed that the sequencing depth was inadvertently influenced by the metabarcoding assay used. The sequencing depth was found to be higher in the 16S Fish assay compared to the Leray assay, which is attributed to the presence of an extensive number of unassigned sequences in the Leray assay (75%). While this has been reported in existing literature (Atienza et al., 2020; Collins et al., 2019; Hintikka et al., 2022; Wangensteen et al., 2018), it is crucial to consider this issue in future studies, especially in challenging or unexplored environments, or when cost constraints may restrict the implementation of multiple assays. Furthermore, our research revealed significant differences in vertical stratification between the two assays employed. This finding underscores the limitations of making assumptions or inferences about eDNA behaviour based on a single group of taxa or a single assay,

further emphasizing the need for cautious consideration of assay and thorough validation of eDNA metabarcoding protocols in diverse environments or with different target taxa.

We acknowledge that our results may be influenced by sampling time, which could increase the presence of diel vertical migrators in our data. However, as our study aimed to rapidly characterize biodiversity in the target regions using multiple approaches, our sampling was opportunistic in nature. Nevertheless, we recommend that future deep-sea eDNA studies carefully consider sampling during specific times of day or night, or both, to account for diurnal migrating species. By doing so, a more comprehensive understanding of temporal dynamics and ecological patterns associated with eDNA in deep-sea environments can be achieved, while mitigating potential confounding factors and enhancing the robustness of the findings.

5.7 Conclusion

Our study employed eDNA metabarcoding (COI Leray and 16S Fish), ROV underwater imagery, and specimen collection to conduct the first comprehensive biological survey of the Cape Range and Cloates Canyons in the East Indian Ocean (Western Australia). Deep-sea ecosystems are notoriously under-surveyed due to logistical challenges in accessing and sampling these environments. However, the increasing anthropogenic pressures they face necessitate the rapid generation of baseline biodiversity data for effective conservation efforts. Through the application of eDNA metabarcoding, we were able to obtain high-resolution biodiversity and community composition data for 11 different phyla in these submarine canyons. Our findings demonstrate the applicability of eDNA metabarcoding in characterizing biodiversity across the entire tree-of-life, even in the presence of suboptimal reference databases for deep-sea environments.

Notably, we identified 109 taxa as putative undescribed species, new records (genus or species), or range extensions (genus or species) using both metabarcoding assays. This significant number of putative undescribed species and records emphasizes the significant knowledge gaps and unexplored nature of these regions. Furthermore, it

underscores the potential of eDNA to not only detect elusive taxa, but also establish novel records and potentially identify previously unknown species. While further validation through specimen collection is required for some of these putative reports, our results indicate that eDNA holds promise as a valuable tool for guiding targeted survey efforts to for aid in the collection and recovery of unresolved taxa that may potentially represent undescribed species.

In our investigation of vertical stratification patterns of biodiversity, we observed that the choice of metabarcoding assay significantly influenced interpretations and inferences. This was attributed to the biological characteristics of the target taxa of each assay, and the unique and specific features of the environment sampled. Therefore, understanding the intricate relationship between eDNA-derived biodiversity patterns and the underlying biological, ecological, and physical factors is imperative for accurately interpreting these patterns. This complexity highlights the importance of integrating habitat and environmental data in future studies to accurately interpret spatial biodiversity patterns using eDNA. Our study not only demonstrates the efficacy of implementing eDNA metabarcoding in submarine canyons, but also highlights the broader potential of this tool for exploring other uncharted deep-sea environments. The implementation of eDNA metabarcoding has the potential to serve as a powerful tool for enhancing our understanding of biodiversity in these ecosystems, thus enabling more effective conservation practices in response to escalating anthropogenic pressures.

5.8 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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5.9 Supplementary material

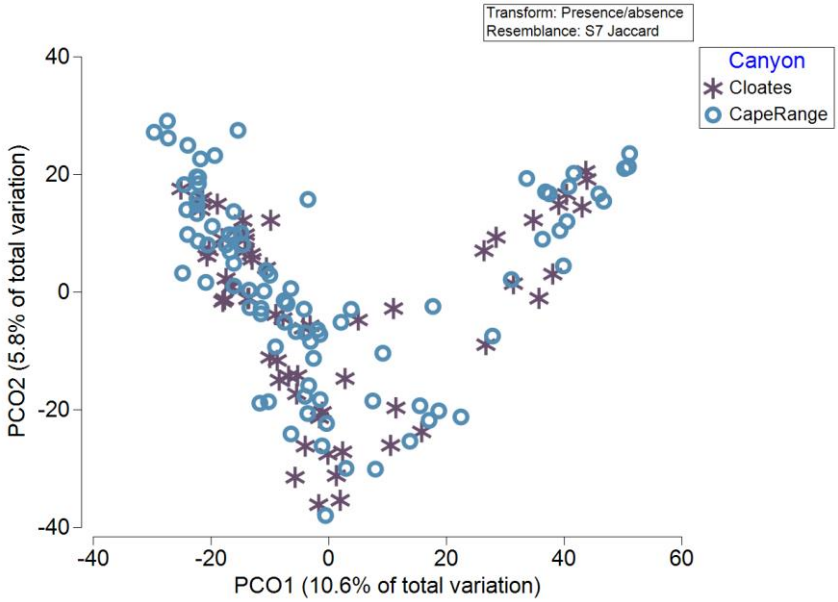


Figure S 5-1: PCO of Animalia taxonomic composition across 5 depths (surface, 200m, 500m, 1000m, and bottom) in the Cape Range and Cloates canyons.

eDNA metabarcoding was performed using the COI Leray assay.

Table S 5-1: Simper analysis for COI Leray metabarcoding assay of eDNA water samples collected at the Cape Range and Cloates submarine canyons. SIMPER compares dissimilarity between the canyons.

Groups Cloates & CapeRange	**Average dissimilarity = 77.99**					
	Group Cloates	**Group CapeRange**				
Species	**Av.Abund**	**Av.Abund**	**Av.Diss**	**Diss/SD**	**Contrib%**	**Cum.%**
Athorybia	0.4	0.2	2.71	0.77	3.48	3.48
Lensia campanella	0.37	0.31	2.43	0.77	3.11	6.59
Leucothea	0.38	0	1.96	0.7	2.52	9.11
Pachycara sp	0.35	0	1.95	0.64	2.5	11.61
Barathrites iris	0.35	0	1.86	0.65	2.38	13.99
Vinciguerria nimbaria	0.2	0.15	1.62	0.56	2.08	16.07
Chuniphyes multidentata	0.22	0.17	1.58	0.53	2.02	18.1
Calanoida	0.28	0.33	1.57	0.55	2.01	20.11
Apolemia sp	0.22	0.08	1.55	0.54	1.99	22.1
Asperoteuthis acanthoderma	0.18	0.13	1.4	0.52	1.79	23.89
Rosacea sp. 3 BO-2009	0.15	0.36	1.37	0.54	1.76	25.65
Chelophyes contorta	0.2	0.17	1.33	0.56	1.7	27.35
Eudoxoides spiralis	0.15	0.16	1.28	0.52	1.63	28.99
Apolemia	0.33	0.32	1.26	0.46	1.61	30.59
Ceratoscopelus warmingii	0.07	0.15	1.11	0.42	1.42	32.02
Seleniolycus sp	0.07	0.18	1.07	0.49	1.37	33.39
Eurhamphaea	0.18	0.06	1.05	0.46	1.34	34.73
Cyclopoida	0.08	0.17	0.93	0.4	1.19	35.92
Apolemiidae	0.05	0.16	0.9	0.42	1.16	37.08
Physalia physalis	0.13	0.23	0.9	0.44	1.16	38.24
Stigmatoteuthis hoylei	0.12	0.03	0.85	0.36	1.1	39.33
Diaphus	0.08	0.05	0.84	0.35	1.08	40.41
Eudoxoides mitra	0.1	0.08	0.8	0.42	1.03	41.44
Antimora rostrata	0.05	0.14	0.74	0.4	0.95	42.39
Rhopalonema	0.07	0.11	0.74	0.39	0.95	43.34
Isididae	0.12	0.11	0.7	0.41	0.9	44.24
Clausocalanus parapergens	0.02	0.08	0.66	0.3	0.85	45.09

Clausocalanus furcatus	0.25	0.23	0.64	0.35	0.82	45.91
Acartia negligens	0.1	0.18	0.64	0.38	0.82	46.73
Ctenocalanus vanus	0.07	0.09	0.62	0.33	0.8	47.53
Liriope	0.07	0.05	0.62	0.31	0.8	48.32
Pseudosagitta maxima	0.08	0.02	0.62	0.32	0.79	49.12
Pandeidae	0.05	0.06	0.62	0.3	0.79	49.91
Calocalanus plumulosus	0.1	0.15	0.61	0.33	0.79	50.69
Clausocalanus	0.07	0.09	0.61	0.37	0.78	51.47
Calocalanus pavo	0.13	0.19	0.57	0.34	0.73	52.2
Umbellula	0.07	0.08	0.56	0.34	0.71	52.91
Phosichthys argenteus	0.05	0.02	0.55	0.26	0.71	53.62
Colobonema	0.05	0.03	0.55	0.26	0.7	54.33
Rhizophysidae	0.03	0.06	0.53	0.3	0.69	55.01
Chrysogorgia	0.12	0.02	0.52	0.36	0.66	55.68
Enypniastes sp.	0.08	0.02	0.51	0.3	0.65	56.33
Proceroecia	0.07	0.02	0.48	0.29	0.62	56.94
Gennadas parvus	0.1	0.01	0.48	0.31	0.62	57.56
Subeucalanus mucronatus	0.07	0	0.47	0.27	0.6	58.16
Hydrichthys	0.02	0.06	0.45	0.25	0.57	58.73
Clausocalanus arcuicornis	0.07	0.01	0.44	0.28	0.56	59.29
Munidopsis. cf. subsquamosa	0.05	0.05	0.43	0.29	0.55	59.85
Corycaeidae	0.03	0.09	0.41	0.31	0.53	60.38
Paracalanus	0.07	0.1	0.4	0.31	0.51	60.89
Pleuromamma xiphias	0.05	0	0.39	0.23	0.5	61.4
Farranula gibbula	0.22	0.16	0.39	0.28	0.5	61.9
Benthodytes sibogae	0.07	0.05	0.39	0.31	0.5	62.4
Conchoecia	0.02	0.03	0.38	0.21	0.49	62.89
Psychropotes sp	0.07	0.05	0.38	0.31	0.49	63.38
Rhizophysa eysenhardtii	0.03	0.05	0.38	0.24	0.49	63.87
Mikroconchoecia sp. USNM IZ 1448470	0.02	0.05	0.38	0.24	0.49	64.36
Copilia mirabilis	0.1	0.02	0.38	0.3	0.48	64.84
Typhlonus nasus	0.05	0.03	0.37	0.28	0.47	65.31

Lilyopsis	0.05	0.02	0.35	0.25	0.45	65.77
Silax	0.1	0.08	0.35	0.33	0.45	66.22
Siphonophorae	0.02	0.1	0.35	0.33	0.45	66.67
Pelagia noctiluca	0.02	0.07	0.35	0.26	0.45	67.12
Lucicutia flavicornis	0.03	0.03	0.34	0.25	0.43	67.55
Euchaeta rimana	0.07	0.02	0.34	0.27	0.43	67.99
Eurythenes maldoror	0.08	0	0.34	0.29	0.43	68.42
Valenciennellus	0.02	0.03	0.34	0.22	0.43	68.85
Gastropoda	0.02	0.03	0.33	0.21	0.43	69.27
Gonostoma	0.03	0.02	0.33	0.23	0.43	69.7
Macrosetella gracilis	0.07	0.01	0.33	0.25	0.43	70.13

Table S 5-2: Simper analysis for 16S Fish metabarcoding assay of eDNA water samples collected at the Cape Range and Cloates submarine canyons. SIMPER compares dissimilarity between the canyons.

Groups Cloates & CapeRange	**Average dissimilarity = 74.59**					
	Group Cloates	**Group CapeRange**				
Species	**Av.Abund**	**Av.Abund**	**Av.Diss**	**Diss/SD**	**Contrib%**	**Cum.%**
Barathrites iris	0.81	0.15	4.05	1.32	5.44	5.44
Barathrites	0.68	0.01	3.73	1.19	5	10.44
Pachycara sp	0.58	0.02	3.05	1.04	4.08	14.52
Ophidiiformes	0.59	0.14	3.02	0.97	4.05	18.57
Seleniolycus sp	0.22	0.53	2.72	0.89	3.64	22.21
Exocoetus	0.61	0.63	2.58	0.8	3.46	25.67
Teleostei	0.71	0.59	2.41	0.79	3.23	28.9
Bassozetus sp2	0.27	0.28	2.16	0.73	2.9	31.8
Lampadena luminosa	0.25	0.23	1.99	0.69	2.67	34.47
Bathypterois sp.	0.19	0.26	1.95	0.67	2.61	37.08
Antimora rostrata	0.05	0.34	1.84	0.69	2.46	39.54
Stomiiformes	0.37	0.22	1.79	0.67	2.4	41.94
Acanthonus armatus	0.05	0.27	1.6	0.59	2.14	44.08
Apagesoma australe	0.14	0.16	1.33	0.51	1.78	45.87
Typhlonus nasus	0.14	0.18	1.32	0.57	1.77	47.64
Pachycara	0.25	0	1.28	0.55	1.71	49.35
Diaphus	0.49	0.49	1.27	0.53	1.7	51.05
Myctophiformes	0.41	0.26	1.21	0.52	1.62	52.67
Myctophidae	0.31	0.23	1.2	0.56	1.61	54.28
Ophidiidae	0.15	0.13	1.14	0.49	1.53	55.81
Leucicorus sp.	0.15	0.09	1.13	0.51	1.51	57.32
Penopus sp	0.2	0	1.12	0.46	1.5	58.82
Cyclothone pseudopallida	0.15	0.09	1.08	0.48	1.44	60.26
Bassozetus galatheae	0.07	0.15	0.95	0.46	1.27	61.53
Cheilopogon	0.12	0.14	0.85	0.4	1.14	62.67
Gempylus serpens	0.08	0.1	0.83	0.43	1.11	63.78
Sternoptyx diaphana	0.1	0.11	0.82	0.46	1.09	64.88
Omosudis lowii	0	0.15	0.8	0.41	1.07	65.95
Ipnops agassizii	0.03	0.11	0.77	0.37	1.03	66.98
Cubiceps	0.03	0.14	0.75	0.42	1	67.98
Cyclothone	0.07	0.1	0.73	0.39	0.98	68.96
Aulopiformes	0.12	0.05	0.72	0.43	0.97	69.93
Aulopidae	0.05	0.08	0.69	0.36	0.93	70.86

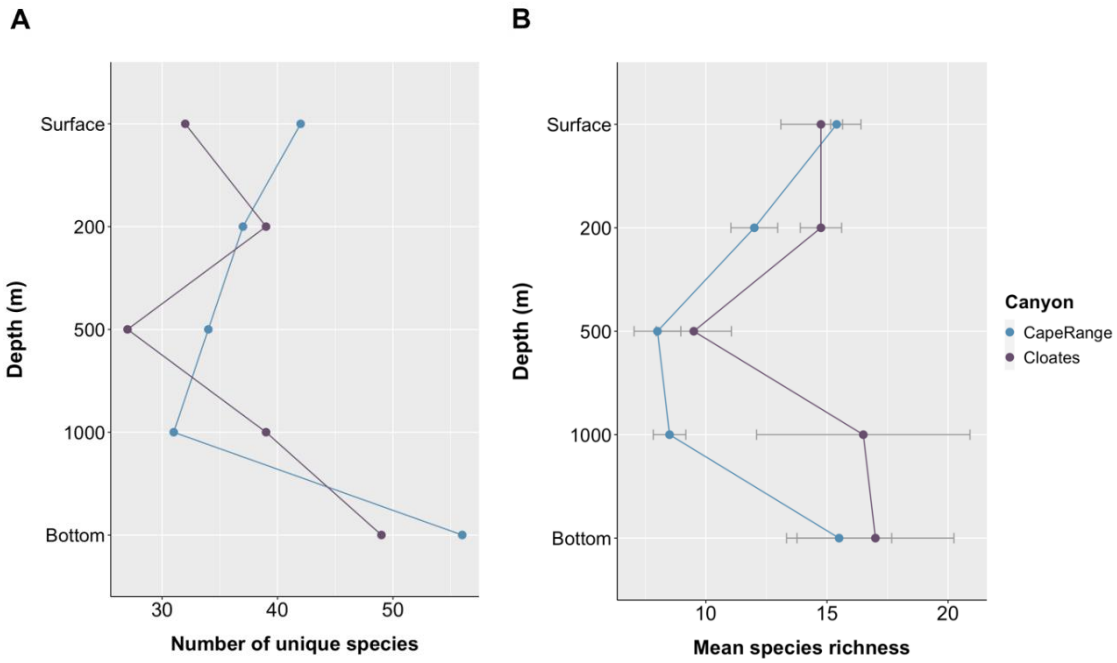


Figure S 5-2: Number of unique (A) species obtained and mean species richness (B) documented across different depths using COI Leray assay.

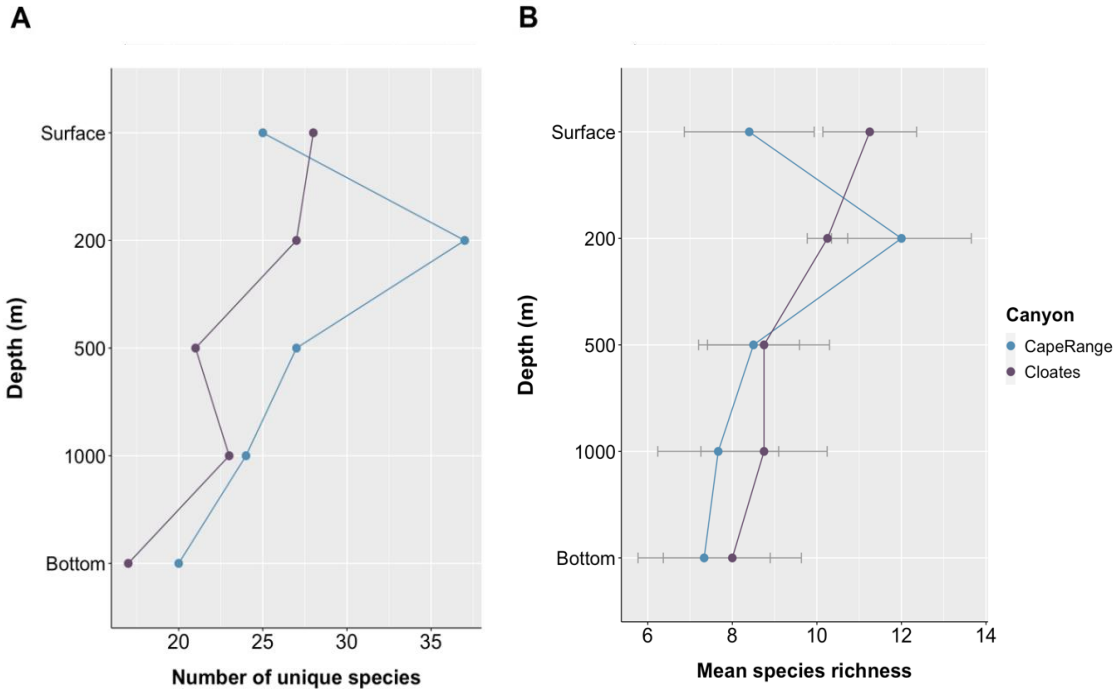


Figure S 5-3: Number of unique (A) species obtained and mean species richness (B) documented across different depths using 16S Fish assay.

Table S 5-3: ANOVA results for differences in mean richness between canyons (Cape Range and Cloates) and depths using COI Leray metabarcoding assay

	Df	Sum sq	Mean sq	F value	Pr(>F)
Canyon	1	88.95	88.955	5.4063	0.025359
Depth	4	341.35	85.337	5.1865	0.001898
Canyon:Depth	4	99.26	24.815	1.5082	0.218645
Residuals	39	641.7	16.454		

Table S 5-4: TUKEY results for differences in mean richness between canyon depths across the Cape Range and Cloates Canyons using COI Leray metabarcoding assay

Group	diff	lwr	upr	p adj
CapeRange:200-CapeRange:1000	3.50	-4.352	11.352	0.886
CapeRange:500-CapeRange:1000	-0.50	-8.352	7.352	1.000
CapeRange:Bottom-CapeRange:1000	7.00	-0.852	14.852	0.116
CapeRange:Surface-CapeRange:1000	6.90	-1.335	15.135	0.169
Cloates:200-Cloates:1000	-1.75	-11.366	7.866	1.000
Cloates:500-Cloates:1000	-7.00	-16.616	2.616	0.331
Cloates:Bottom-Cloates:1000	0.50	-9.116	10.116	1.000
Cloates:Surface-Cloates:1000	-1.75	-11.366	7.866	1.000
Cloates:200-CapeRange:200	2.75	-6.028	11.528	0.987
CapeRange:500-CapeRange:200	-4.00	-11.852	3.852	0.784
CapeRange:Bottom-CapeRange:200	3.50	-4.352	11.352	0.886
CapeRange:Surface-CapeRange:200	3.40	-4.835	11.635	0.925
Cloates:500-Cloates:200	-5.25	-14.866	4.366	0.713
Cloates:Bottom-Cloates:200	2.25	-7.366	11.866	0.998
Cloates:Surface-Cloates:200	0.00	-9.616	9.616	1.000
CapeRange:Bottom-CapeRange:500	7.50	-0.352	15.352	0.072
Cloates:Bottom-Cloates:500	7.50	-2.116	17.116	0.245
Cloates:Surface-Cloates:500	5.25	-4.366	14.866	0.713
CapeRange:Surface-CapeRange:Bottom	-0.10	-8.335	8.135	1.000
Cloates:Surface-Cloates:Bottom	-2.25	-11.866	7.366	0.998

Table S 5-5: ANOVA results for differences in mean richness between canyons (Cape Range and Cloates) and depths using 16S Fish metabarcoding assay

	Df	Sum sq	Mean sq	F value	Pr(>F)
Canyon	1	4.36	4.3597	0.4205	0.5205
Depth	4	85.82	21.4562	2.0694	0.1035
Canyon:Depth	4	25.37	6.3418	0.6116	0.6567
Residuals	39	404.37	10.3684		

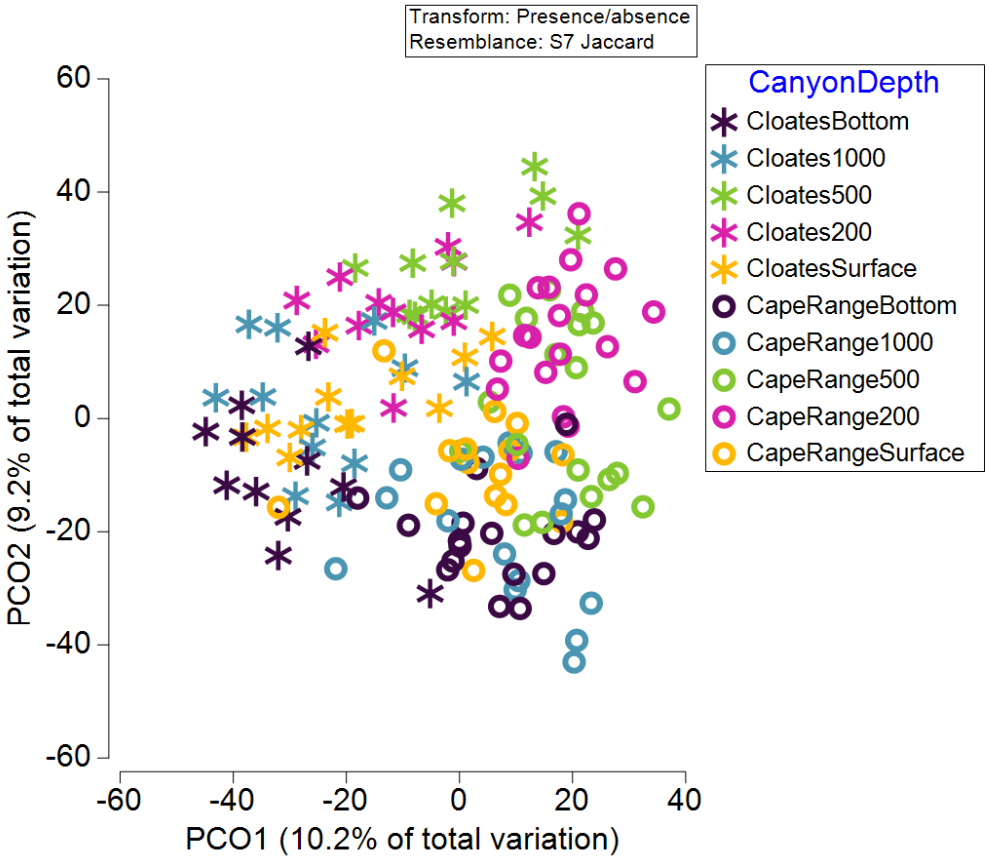


Figure S 5-4: PCO of fish (class: Teleostei and Chondrichthyes) taxonomic composition across 5 depths (surface, 200m, 500m, 1000m, and bottom) in the Cape Range and Cloates canyons.

eDNA metabarcoding was performed using the 16S Fish assay.

Table S 5-6: PERMANOVA results for community composition in the Cape Range and Cloates submarine canyons using the COI Leray metabarcoding assay.

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Canyon	1	14045	1.40E+04	3.9663	0.0001	9842
Depth	4	1.19E+05	29715	8.3918	0.0001	9712
CanyonxDepth	4	22211	5.55E+03	1.5681	0.0001	9722
Res	138	4.89E+05	3.54E+03			
Total	147	6.48E+05				

Table S 5-7: Pairwise results for community composition of COI Leray metabarcoding assay for tests between Canyons (Cape Range and Cloates) and depths.

Factor	Groups	t	P(perm)	Unique perms
Canyons	Cloates, CapeRange	1.9916	0.0001	9877
Depth	Bottom, 1000	2.2677	0.0001	9850
	Bottom, 500	2.0206	0.0001	9823
	Bottom, 200	2.8336	0.0001	9842
	Bottom, Surface	3.8416	0.0001	9883
	1000, 500	2.1735	0.0001	9851
	1000, 200	2.9972	0.0001	9865
	1000, Surface	4.0826	0.0001	9893
	500, 200	2.0934	0.0001	9853
	500, Surface	3.1803	0.0001	9855
	200, Surface	3.4396	0.0001	9879

Table S 5-8: PERMANOVA results for community composition in the Cape Range and Cloates submarine canyons using the 16S Fish metabarcoding assay.

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Canyon	1	38230	38230	11.877	0.0001	9892
Depth	4	58686	14672	4.5581	0.0001	9773
CanyonxDepth	4	18410	4602.4	1.4299	0.0023	9794
Res	166	5.34E+05	3218.8			
Total	175	6.55E+05				

Table S 5-9: Pairwise results for community composition of 16S Fish metabarcoding assay for tests between Canyons (Cape Range and Cloates) and depths.

Factor	Groups	t	P(perm)	Unique perms
Canyon	Cloates, CapeRange	3.4463	0.0001	9865
Depth	Bottom, 1000	1.3075	0.0435	9891
	Bottom, 500	2.3974	0.0001	9918
	Bottom, 200	1.785	0.0002	9881
	Bottom, Surface	1.5782	0.0013	9871
	1000, 500	2.176	0.0001	9852
	1000, 200	1.8159	0.0001	9841
	1000, Surface	1.6232	0.0001	9832
	500, 200	1.28	0.0339	9861
	500, Surface	2.161	0.0001	9882
	200, Surface	1.7033	0.0001	9859

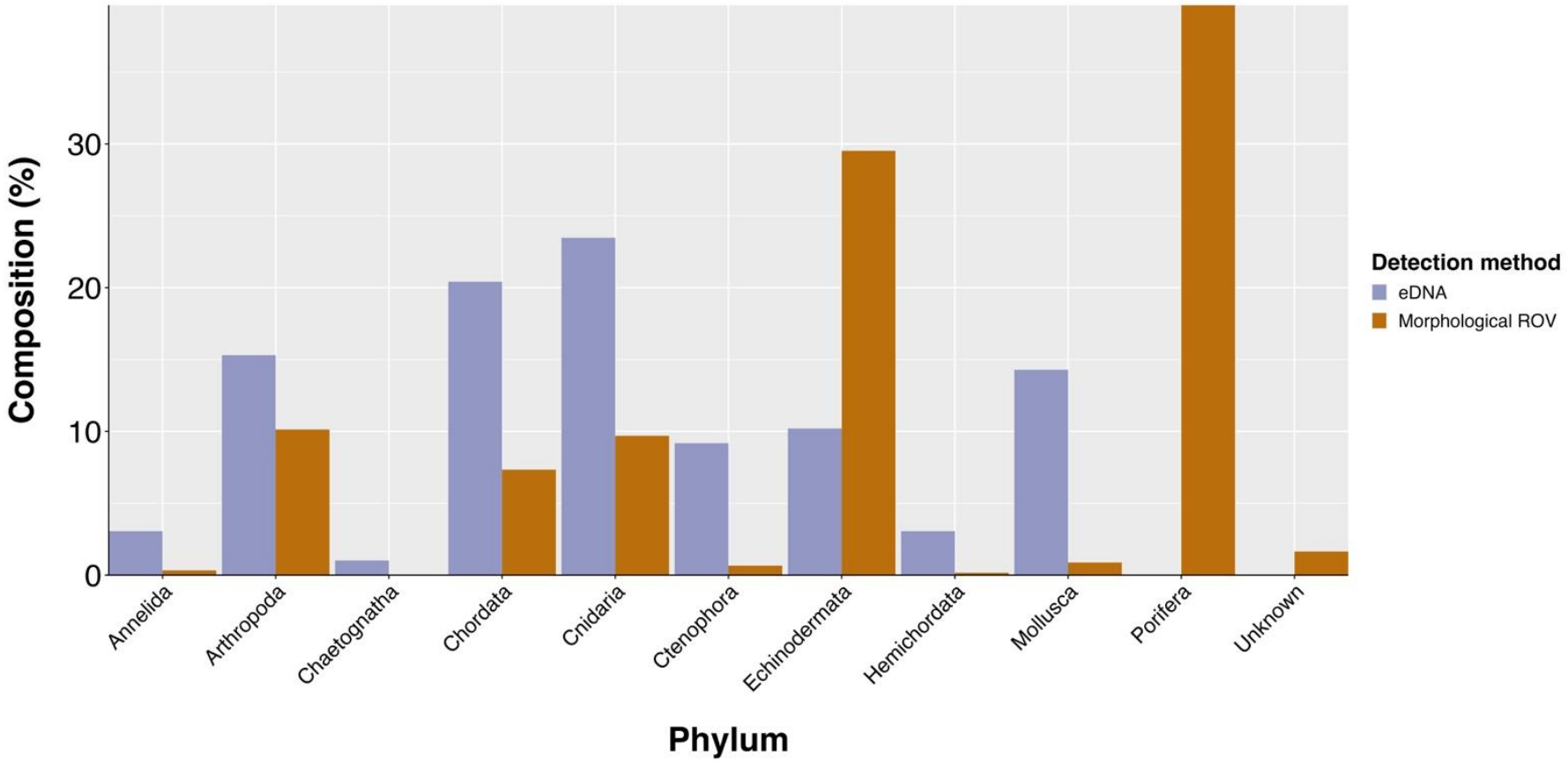
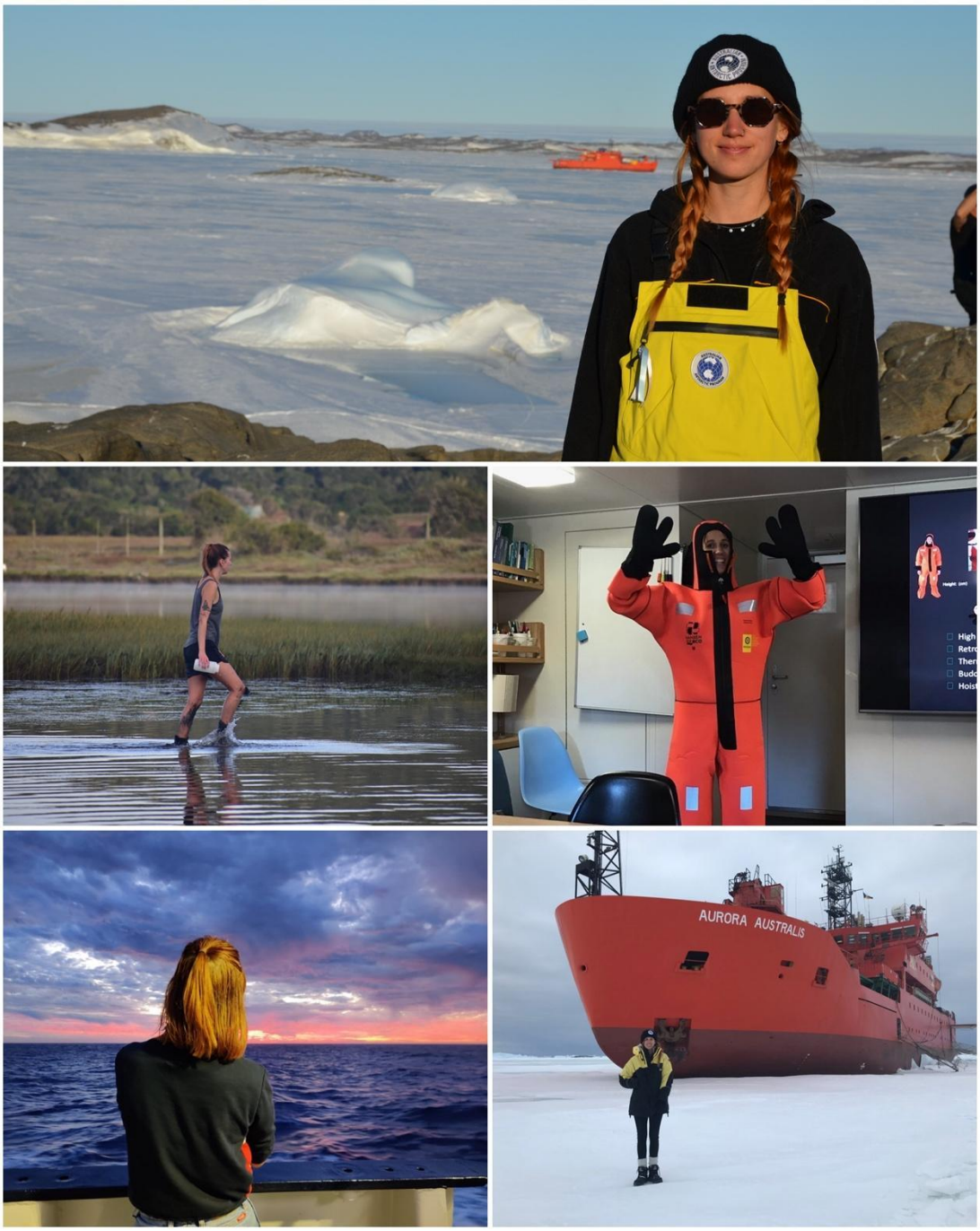


Figure S 5-5: Composition of eDNA and morphological ROV (video transects).
Composition on the y axis refers to relative abundance in terms of read numbers (eDNA) or abundance counts (ROV)

CHAPTER 6

Discussion



6.1 Thesis findings and significance

The research conducted in this thesis contributes to the expansion of eDNA applicability in coastal and marine ecosystems, with a focus on 'small and scarce' taxa, such as threatened Syngnathids, as well as 'broad and unexplored' ecosystems like the Southern Ocean and the deep-sea. Coastal and marine ecosystems exhibit considerable heterogeneity with respect to biodiversity, ecological process, and environmental conditions. Consequently, employing a uniform approach to eDNA surveys and analysis may inadequately address research questions and introduce biases. This research has emphasized the need for nuanced applications of eDNA methods tailored to these diverse ecosystems, shedding light on the intricacies and complexities inherent in each surveyed ecosystem. In doing so, this thesis adds to the growing body of literature (Eble et al., 2020; Reimer & Gösser, 2023; Richards et al., 2022; Takahashi et al., 2023) that seeks to interweave eDNA applications into the study of diverse coastal and marine environments.

This thesis discussion integrates the key concepts, findings, and limitations from each data chapter, offering an evaluation of the research's significance for eDNA studies and its broader implications in conservation applications (Figure 6-1). The chapter also discusses future research directions that have emerged as a result of this research, while providing insights into potential advancements in the overarching field of eDNA.

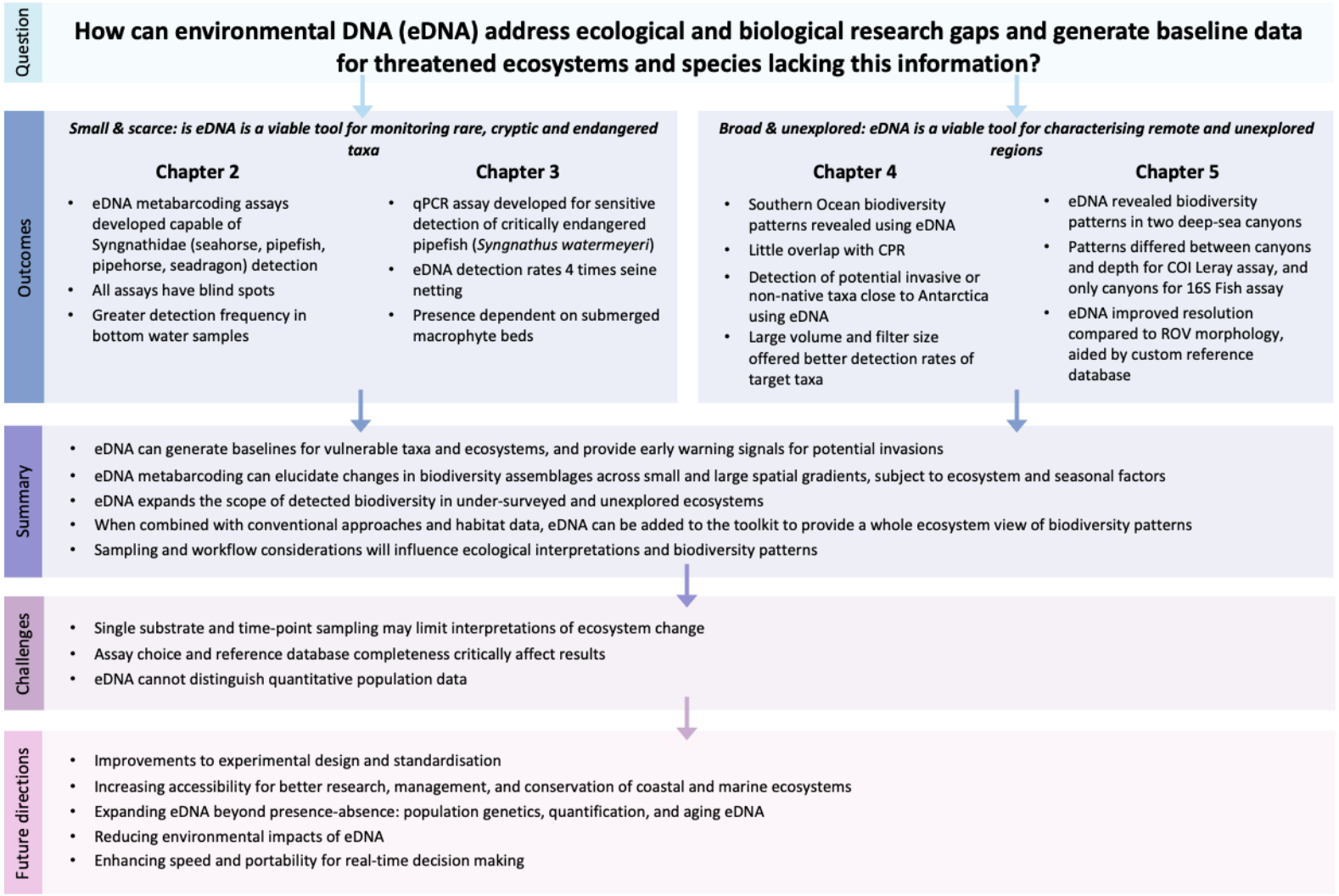


Figure 6-1: Conceptual flow diagram summarising the outcomes of each chapter, an overall synthesis of findings and challenges faced, and the future directions emerging from this research.

6.1.1 eDNA sensitively detects endangered, elusive, and potential invasive species

Throughout this research the application of eDNA surveys detected a range of endangered, elusive, or potentially invasive taxa. In Chapter 2, eDNA metabarcoding detected four Syngnathidae species, including the first detection of seahorse species (*Hippocampus subelongatus* and *H. breviceps*) using eDNA, illustrating the potential of this method for monitoring small-bodied cryptic taxa. This potential was further demonstrated in Chapter 3, where eDNA detected the critically endangered Estuarine pipefish (*Syngnathus watermeyeri*) in two of the five estuaries surveyed. The detection of the pipefish using eDNA was significant, considering its endangered status and the challenges posed by visual observations due to estuarine turbidity, and the pipefish's ability to camouflage in the submerged macrophyte beds it inhabits. Given detection rates were four times higher with eDNA versus seine netting, this non-invasive sampling approach yields demonstratable promise for monitoring the presence and distribution of threatened species.

Applying eDNA across a large spatial scale in the Southern Ocean (Chapter 4) enabled the detection of 16 species of concern (non-native, pest, or invasive taxa), with several detected near the continent of Antarctica. Proactive detection of non-native species is crucial in the Antarctic region (south of 60°S) given the susceptibility of its unique biota to the adverse effects of biological invasions (e.g., competition, disease, habitat alteration, increased predation, etc). While acknowledging the potential for these detections to represent transported DNA, larvae, or deceased organisms, this research provides compelling evidence of the imminent risk posed by invasive species. Consequently, there is an urgent need for further validation of these detections and a thorough assessment of the level of threat these taxa pose to Antarctic biosecurity. Future research should enhance sensitivity through species-specific or target-specific (genus or family level) assays, or through the multiplexing of these assays. Overall, these detections highlight the capacity of eDNA to provide early warning signals, a crucial aspect for mitigating biosecurity threats prior to catastrophic and potentially irreversible changes to Antarctic community composition and health.

In the deep-sea (Chapter 5), 109 taxa were identified as putative undescribed species, new records (genus or species), or range extensions (genus or species) using two eDNA metabarcoding assays. These detections include the first record of a giant hydroid (genus *Branchiocerianthus*) in Australia, new records of the elusive giant squid *Architeuthis dux*, and a putative undescribed species of squidworm (*Teuthidodrilus* sp. *nov*.) previously only documented in the Philippines (Osborn et al., 2011). This study highlights the potential of eDNA to not only detect elusive taxa, but establish new records and potentially identify undescribed species.

Overall, this thesis emphasises the versatility and power of eDNA as a tool for monitoring and conserving elusive or threatened taxa, and addressing biosecurity concerns. Its effective application across diverse and challenging environments highlights the benefits of incorporating eDNA into the toolkit to enhance our understanding and strengthen conservation efforts of threatened species and ecosystems.

6.1.2 eDNA metabarcoding elucidates spatial biodiversity patterns subject to ecosystem and seasonal factors

The efficacy of eDNA metabarcoding as a monitoring and management tool relies, in part, on its ability to discern and characterize fine-scale spatial biodiversity patterns. Consistent with existing literature, the data chapters presented in this thesis demonstrate how eDNA collected from surface water (0 – 10 m) can successfully identify biodiversity patterns at both small (Chapter 2) and broad spatial scales (Chapter 4). In Chapter 4, eDNA metabarcoding detected differences in community assemblages across the Southern Ocean attributed to oceanic fronts, and demonstrated the influence of hull-fouling organisms on observed biodiversity patterns. However, in Chapter 5, discerning these patterns across a large vertical gradient in the deep-sea (surface – ~5000m), posed significant challenges. Previous studies have documented vertical stratification of eDNA within the water column, but surveyed smaller depth ranges (Alexander et al., 2022; Canals et al., 2021) or

employed a single metabarcoding assay (Govindarajan et al., 2022). In the Cape Range and Cloates submarine canyons, vertical stratification patterns were observed when employing the universal Animalia Leray COI assay, however, the fish-specific assay 16S Fish failed to reveal vertical stratification patterns. Disparity between the two assays in the ability to reveal spatial patterns was unanticipated but has important implications for assay choice when designing studies across large depth gradients. This result was attributed to the biological characteristics (e.g., vertical migrations and body size) of the target taxa of each assay, as well as sampling timing (e.g., spawning events) which is also likely to affect vertical biodiversity patterns.

Lastly, the interpretation of vertical biodiversity patterns relies on the specific ecosystem under study. Submarine canyons for example, which are characterised by complex topography and hydrodynamics, can increase vertical mixing and act as conduits between shallow coastal areas and the deep-sea, thereby influencing the transport of eDNA and interpretation of biodiversity patterns. Understanding the intricate relationship between eDNA biodiversity patterns and the underlying biological, ecological, and physical factors is imperative for accurately interpreting eDNA-derived biodiversity data. Approached holistically, the findings presented in these data chapters underscore the complexity of interpreting spatial biodiversity patterns using eDNA and emphasize the importance of incorporating these factors into study design and execution.

6.1.3 eDNA expands the scope of detected biodiversity and provides valuable insights into under-surveyed areas

Open ocean and deep-sea ecosystems are logistically challenging to survey, and are therefore often understudied and uncharacterised. This frequently leads to suboptimal, or incomplete reference databases that greatly impedes the ability of eDNA metabarcoding to generate high-resolution assignments and robust assemblage data. In Chapters 4 and 5, eDNA metabarcoding was applied to samples collected from across the Southern Ocean and within submarine canyons respectively. In both chapters, the majority of the taxonomic assignments generated

were resolved to species level, followed by genus (Chapter 5) or family level (Chapter 4). Moreover, both chapters extended the scope of biodiversity captured by conventional approaches conducted simultaneously (see 6.1.4). This finding indicates that eDNA metabarcoding can reveal a wide array of taxonomic diversity at high resolutions in under-surveyed ecosystems, which is an important finding given the challenges associated with surveying these complex environments.

It is important to note that the high resolution achieved in these studies should not be misconstrued as an indication of the completeness of these reference databases (see 6.2.3). Further surveying and barcoding initiatives are needed to facilitate comprehensive eDNA metabarcoding surveys, particularly in the deep-sea. However, a benefit of applying eDNA in unexplored areas is its potential to guide conventional sampling efforts in regions with a high proportion of poorly resolved taxonomy. This approach can facilitate the inclusion of local, morphologically identified reference material, thereby enhancing the completeness of reference databases. Although the efficacy of eDNA metabarcoding relies on the availability of reference material, this research demonstrates that even with incomplete databases, eDNA methods provide a viable alternative and complement to conventional surveying methods in inaccessible ecosystems.

6.1.4 Incorporating eDNA into conventional monitoring practices broadens biodiversity detections and can greatly impact conservation efforts

Throughout this thesis, eDNA surveys were compared with conventional morphological survey methods such as seine netting (Chapter 3), continuous plankton recorder (CPR, Chapter 4), and remotely operated video specimen collection (Chapter 5). The results consistently demonstrated that eDNA surveys exhibited higher sensitivity and captured a wider range of biodiversity. However, it is important to acknowledge that a positive eDNA detection does not necessarily imply the presence of a viable organism, as the current capability of eDNA to provide robust metrics beyond the presence or absence of genetic material is limited. Although numerous studies exploring population metrics like abundance (Lacoursière-Roussel

et al., 2016; Luo et al., 2023), health (David et al., 2021; DiBattista et al., 2020), and sex (Nichols & Spong, 2017) using eDNA exist, the reliability of these metrics remains constrained due to the early stage of method development (see 6.3.3).

The outcomes of this research align with the prevailing consensus in the literature, supporting the integrated use of eDNA surveys alongside complementary conventional approaches. While this may seem general or clichéd, this research offers nuanced perspectives by providing specific insights into the environments where these approaches were employed. For example, in Chapter 5, a multi-marker metabarcoding survey in deep-sea submarine canyons discovered putative undescribed species or distribution records not detected during visual surveys undertaken with the ROV. This result emphasises the diverse nature of these ecosystems and the capacity of eDNA sampling methods to detect a broader spectrum of fauna in unexplored environments like the deep-sea. Similarly, in Chapter 4, eDNA metabarcoding successfully detected invasive species in the Antarctic region that were missed by concurrent CPR surveys. This information carries significant implications for conservation and can guide the development of long-term eDNA monitoring programs targeting potential invasive species. Lastly, in Chapter 3, eDNA exhibited increased sensitivity than seine netting in detecting the critically endangered estuarine pipefish, demonstrating its potential to guide conservation efforts and monitor the success of future reintroduction programs.

The collective findings from these data chapters underscore the significance of integrating eDNA into the monitoring toolkit, both as a complementary approach and as an effective initial screening tool. With its reduced field effort, eDNA proves invaluable for rapidly identifying taxa of interest in survey areas, including endangered or invasive species, or potential undescribed species or records. Therefore, eDNA can serve as an initial sweep or pilot study to inform decisions on appropriate sampling methods, strategies, resource allocation, and the necessity of specimen collection.

6.1.5 Workflows and protocols should be tailored for target taxa and ecosystem

Research in this thesis reinforces a growing body of literature that eDNA is not a 'one-size-fits-all' methodology, and that workflow components should be carefully considered and customised to address study-specific objectives and environmental conditions. For example, sampling was ship-based in the Southern Ocean (Chapter 4) and the deep-sea (Chapter 5), and accordingly larger volumes of water could be obtained, with the results of Chapter 4 suggesting that larger volumes of water increase the diversity of detected Animalia taxa. However, due to the SCUBA-based or manual collection involved in chapters 2 and 3, the use of larger sample volumes would have been impractical. Based on the specific findings in Chapters 2-5, the following broad recommendations are proposed:

(1) Enhance the detection sensitivity of low biomass taxa, such as Syngnathids, particularly in the presence of other fish assemblages, by collecting water samples closer to the target habitat (i.e., bottom water) to increase DNA capture (Chapter 2);

(2) Optimize assay and laboratory workflows (thermocycler conditions, assay concentration, temperature, etc.) to improve the detection of critically endangered or small-bodied taxa in turbid environments, and consider incorporating inhibition removal steps (e.g., Zymo inhibitor removal) to potentially increase sensitivity (Chapter 3);

(3) Increase sample volumes (> 12 L) and filter pore sizes (> 0.8 µm) for open-ocean surveys to expand the breadth of detected Animalia biodiversity, and carefully account for potential bias from hull biofouling species during ecological interpretations (Chapter 4);

(4) Account for the time of day and season in deep-sea sample collection, as these factors can impact results and patterns observed, and plan sample drops accordingly to minimize potential confounding effects (Chapter 5).

6.2 Exploring the limitations and emerging future directions

The data in this thesis significantly contributes to the advancement and refinement of eDNA monitoring approaches for detecting low-abundant, target taxa and establishing baselines for vulnerable coastal and marine ecosystems. However, it is important to acknowledge that this work represents a step in the journey towards

integrating eDNA into the marine monitoring toolkit. It is also worth noting the rapid pace of the eDNA field at this point in time - some of the literature published over the tenure of this thesis may have resulted in different approaches. While limitations were identified and explored within each of the data chapters, this section aims to discuss some of the future challenges and direction(s) that this research has highlighted or that have recently emerged in the eDNA literature.

6.2.1 Single substrate and single point-in-time sampling

The data chapters of this thesis examine biodiversity at a single time point. While this does not detract from the significance of the findings from each study, incorporating time series data would enhance the interpretation of ecological trends and strengthen confidence in the conclusions (Berry et al., 2019). Seasonal effects observed in eDNA studies of various organisms emphasize the importance of considering seasonal context when conducting these surveys in coastal and marine communities (see 6.1.5). Strategic alignment of eDNA studies with periods of heightened target organism activity or accounting for migration and spawning times increases the likelihood of detecting target taxa and improves the accuracy of inferences regarding biodiversity patterns. Revisiting the study sites within this thesis, particularly across different seasons, would therefore enrich the data and enhance the robustness of the studies.

Furthermore, water was the sole eDNA substrate sampled in this thesis. It is well-documented that taxonomic composition varies significantly depending on the sampled substrate, and using a single substrate likely underestimates the overall diversity of sampling localities (Brandt et al., 2021; Holman et al., 2019; Koziol et al., 2019). A recent study compared seven eDNA methods targeting epibenthic taxa using a universal 18S assay and found that only 2.8% of family-level detections were shared across all sampling methods (Alexander et al., 2023). As such, the data in this thesis may target a subset of the diversity that is present in the water column due to potential substrate biases. For future eDNA surveys of these ecosystems, it is recommended to incorporate multiple substrates where feasible, or to carefully

select substrate(s) that are best suited for sensitively detecting the target taxa and addressing the study objective.

6.2.2 eDNA assay design and optimisation

The assays developed in this thesis exhibit notable strengths for eDNA surveys, yet further enhancements are necessary to improve detection efficacy and accuracy for biomonitoring purposes. The 16S_FishSyn_Short and Long assays (Chapter 2) extend the detection capability to include cryptic Syngnathidae taxa within broader fish metabarcoding surveys. However, given the relatively low biomass of these taxa relative to other fish, the future development of a family-specific assay is recommended to decrease the risk of false negatives. Metabarcoding will always necessitate a trade-off between generating broad assemblage data and the sensitivity of detection. The CytB_SW assay (Chapter 3) sensitively detected estuarine pipefish across its range. However, as recent studies have suggested the population is highly inbred (Weiss et al., 2022), the development of a haplotype-specific assay may be useful for examining and monitoring genetic diversity.

6.2.3 Reference databases

The challenges of incompleteness and taxonomic bias in publicly available reference databases are widely acknowledged in eDNA literature (Jerde et al., 2021; Miya et al., 2015; Takahashi et al., 2023). Often databases demonstrate a bias towards well-studied, economically important, or charismatic taxa groups in easily accessible environments (Meiklejohn et al., 2019; Porter & Hajibabaei, 2018). However, even the well-represented taxa groups are often confined to one or two widely used barcode regions (e.g., 16S, 12S, and COI).

In this thesis, the comprehensive biodiversity documentation and sequence coverage observed in the easily accessible Perth metropolitan region (Chapter 2), compared to the hard-to-reach submarine canyons (Chapter 5), highlight the disparity caused by these biases. The implementation of custom reference databases in these regions resulted in the resolution of an additional two and 51 taxa in the Perth region and

the deep-sea, respectively. This discrepancy highlights the underrepresentation of hard-to-reach or under-surveyed ecosystems, such as the deep-sea, in public databases, emphasizing the importance of curating custom reference databases to improve taxonomic coverage and mitigate biases. Initiatives to generate robust reference databases are ongoing, relevant to this research is the National Biodiversity DNA Library for Australia. Once published there will be considerable value in updated taxonomic assignments from metabarcoding data, such as the data from this thesis. Ideally, datasets in the future might be 'live' and dynamically update (see 6.3.2).

6.2.4 Obtaining quantitative or population data through eDNA analysis

Perhaps the most widely acknowledged limitation of eDNA surveys is the current inability to provide quantitative estimates of population size or biomass (Takahashi et al., 2023), accordingly this thesis solely analysed presence-absence data. In eDNA metabarcoding, relative sequencing depth has been used as a proxy for population size and/or biomass (Jo et al., 2017; Lacoursière-Roussel et al., 2016). However, eDNA longevity and concentration in the environment are highly dependent on environmental conditions, seasonality, and variable shedding rates between species (Barnes et al., 2014; Collins et al., 2018, 2022). This is further complicated by PCR inhibition and amplification biases such as primer binding efficiencies and preferential target amplification (Fonseca, 2018; Nichols et al., 2018). While relative quantification is feasible for species-specific eDNA studies using qPCR or ddPCR (Doi et al., 2015; Takahara et al., 2012), absolute quantification is hindered by these challenges. However, the field of eDNA quantification is gaining momentum as new and promising methods emerge (Sigsgaard et al., 2020), aiming to propel eDNA data beyond presence-absence assessments (see 6.3.3).

6.3 Future avenues for eDNA biomonitoring

eDNA has become the fastest-growing biomonitoring tool, with improved methodologies developed, tested, and applied across diverse ecosystems spanning water, land, and air (Franklin et al., 2019; Lynggaard et al., 2022; West et al., 2020). These improvements have resulted in improved confidence in eDNA results, a

reduction in overall costs, and a plethora of new assays in the toolkit (Takahashi et al., 2023). At the outset of this thesis, the use of eDNA was still relatively novel and suggestions for its implementation in biosecurity or long-term conservation management strategies were often met with apprehension (Darling, 2019). Over the tenure of this thesis (2019-2023), however, eDNA monitoring programs are being widely embraced and integrated into management strategies by industry and government agencies (Petruniak et al., 2021), and a multitude of citizen science programs and initiatives exist (Agersnap et al., 2022). Undoubtedly, the momentum of eDNA biomonitoring is increasing, and with advancements in methods, standards, and reference databases, the future prospects are vast. While section 6.2 examined the limitations and emerging research directions of this thesis, this section explores broader areas of future research that I consider key to enhancing the efficacy and validity of eDNA biomonitoring.

6.3.1 Advancing experimental design and standardisation

The challenges of robust eDNA experimental design were evident in the diverse ecosystems and novel research questions explored in this thesis. Many of these ecosystems and questions had not been investigated using eDNA at the outset of this thesis. Consequently, determining the appropriate level of replication and spatial or temporal coverage *a priori* was difficult. Within each chapter, there were instances where experimental design could have been improved to mitigate uncertainties or improve statistical power that only became apparent during data analysis. Ideally, a larger number of samples would be collected and processed beyond the initial estimate, but practical constraints, such as budget and logistics, typically impede this. However, resampling to address these limitations post-data collection is often impracticable. One potential solution to address these challenges is the adoption of biobanking as a standard practice (Jarman et al., 2018), whereby samples are collected (perhaps in numbers in excess of what is thought appropriate), extracted, and stored appropriately for future use. This approach allows additional samples to be collected as backups or for potential future analysis, and grants researchers the ability to revisit stored samples when new research questions arise. As the field

progresses, however, more diverse habitats will be sampled using eDNA, leading to greater clarity and improved experimental design.

Similarly, standardising eDNA methodologies can advance the field by mitigating variation and bias in data collection, processing, and analysis, thereby improving the accuracy and validity of eDNA results (Goldberg et al., 2016; Minamoto et al., 2021). This can facilitate cross-study data comparisons and enable larger temporal and spatial scales (Takahashi et al., 2023). However, standardising eDNA methodologies for coastal and marine environments is challenging due to the diversity of ecosystems and the rapidly evolving nature of the field. This is exemplified by the lack of consensus for sampling these environments (Takahashi et al., 2023), and the emerging research demonstrating that different substrates are needed to capture different portions of biological assemblages (Alexander et al., 2023). In this thesis, diverse coastal and marine habitats were sampled, necessitating adjustments in sampling, workflow, and bioinformatic protocols to maximise detected biodiversity and increase sensitivity. Given these intricacies, best-practice guidelines should provide contextual information to guide appropriate experimental design that addresses differences in study aims, target organisms, environments and available materials and infrastructure.

6.3.2 Increasing accessibility and integrating diverse knowledge systems

Despite being considered a transformative tool for biodiversity monitoring, the potential of eDNA remains largely unrecognised due to a lack of data accessibility (Berry et al., 2021). While improving, it is fair to say that eDNA sequencing data is not readily available to practitioners, industry, government, or citizen scientists globally (Berry et al., 2021; Shea et al., 2023). Assays are scattered across research papers (though see Takahashi et al., 2023), and although sequencing data is accessible, it is often in formats unsuitable for general users and lacks integration into a single comprehensive database or archive (Berry et al., 2021). Furthermore, although the aforementioned concept of eDNA biobanking was proposed in 2018 (Jarman et al., 2018), the practical implementation necessary to enable effective utilization and

resource sharing by researchers is still pending. To give context to the issue, this thesis generated a substantial amount of data, with over 80 million raw sequencing reads obtained from more than 500 samples. While this data has been deposited in publicly available databases (see chapter data availability statements), researchers will need to refer to the published papers, this thesis itself, or perform targeted searches using relevant keywords to locate and access the data.

Encouraging progress has been made regionally, with countries like Australia expanding biodiversity occurrence databases such as the *Atlas of Living Australia* (ALA.org.au, 2023) and the *Global Biodiversity Information Facility* (GBIF.org, 2023) to include eDNA occurrence records. However, it is my opinion that the need to improve eDNA data accessibility is as pressing as the need to expand reference databases. A global database, though ambitious, could enable researchers to upload eDNA data, or it could be automatically mined from registered biodiversity databases. Uploaded data would be accompanied by reproducible metadata encompassing workflow (assay, laboratory, bioinformatic) and sampling and habitat details. Ideally, this database should also include specific research objectives and information on methods that were attempted but proved unsuccessful. A user-friendly platform enabling specific taxa or region searches and providing subsequent access to downloadable data, like the WilderLab platform (https://www.wilderlab.co.nz) or the ANEMONE DB (https://db.anemone.bio/), would undoubtedly appeal to non-experts and result in broader applications in biodiversity and health (see https://www.wilderlab.co.nz/tici). Similarly, access to study aims and protocols would be invaluable for researchers, preventing duplication of efforts and the waste of resources.

Addressing knowledge gaps and tackling biodiversity loss requires engaging and integrating diverse knowledge systems beyond the confinements of academia. Local and traditional knowledge from communities and indigenous peoples brings a unique understanding and perspective that enriches biodiversity data (Jessen et al., 2022; Robinson et al., 2021), and is key for the development and implementation of successful monitoring and restoration efforts (Higgs et al., 2014; Reyes-García et al.,

2019; Uprety et al., 2012). Likewise, citizen science initiatives prove invaluable for expanding spatial and temporal data and enabling long-term monitoring programs (Fraisl et al., 2022; van Strien et al., 2022). Only by recognizing the pressing need for researchers to embrace increased eDNA data accessibility and sharing, can harness the full potential of eDNA as a transformative tool for biodiversity monitoring.

6.3.3 Beyond presence-absence

Advancements in environmental genetics and eDNA technologies are expanding eDNA applications beyond presence-absence assessments towards the integration of population genetic information and more accurate abundance estimates. Although the use of eDNA in population genetics is still in its early stages, several studies have demonstrated its potential to gather population-level genetic data through species-specific (Adams et al., 2022; Baker et al., 2018; Parsons et al., 2018; Sigsgaard et al., 2017) and metabarcoding approaches (Elbrecht et al., 2018; Stat et al., 2017). However, the short target fragments in eDNA studies create difficulties in obtaining sufficient information to assess genetic variation (Hurst & Jiggins, 2005; Rubinoff et al., 2006). The development of longer molecular markers (Deiner et al., 2017), permitted by third-generation sequencing technologies like nanopore sequencing (Bleidorn, 2016), is anticipated to improve genetic coverage and overcome this limitation. At present, however, eDNA bioinformatic pipelines and classification tools predominantly rely on algorithms designed for short-read data, and are not equipped to deal with the high error rate of nanopore data (Ciuffreda et al., 2021).

In parallel, environmental RNA (eRNA) is being increasingly employed to capture biological diversity (Bowers et al., 2021; Pochon et al., 2017), examine targeted gene expression (Pochon et al., 2015; Zaiko et al., 2018), and monitor responses to environmental stressors (Adams et al., 2019). The rapid degradation rate of eRNA allows for differentiation between signals from living organisms and legacy signals (Marshall et al., 2021; Pochon et al., 2017); however, this necessitates specialized protocols for collection and storage (Pochon et al., 2017). Lastly, the extraction and amplification of single-cells from eDNA samples (eCells) is receiving considerable

interest (Adams et al., 2019; Mason et al., 2012). Targeting whole individual cells offers the opportunity to assess genetic variation at the individual level and obtain accurate abundance estimations (Adams et al., 2019). However, this approach faces significant challenges in complex environmental samples where DNA degradation is variable, and the efficiency of amplification can be impeded by cell aggregation. Current research primarily focuses on commercial fish species, and the application of eCells for biodiversity studies remains a distant prospect.

6.3.4 Enhancing speed and portability for effective management

Advancing the speed and portability of eDNA biomonitoring is crucial for real-time decision-making and effective management. Recent advancements in field methods, such as integrated backpack water sampling systems (Thomas et al., 2018), have shown promise for streamlining data collection and reducing sampling times. The recent emergence of subsurface automated samplers is particularly promising (Formel et al., 2021; Hendricks et al., 2022, 2023), especially for ecosystems that are difficult to access. These systems can be left *in situ* to collect samples autonomously, significantly reducing sampling time and effort. Current advances are even developing extraction and qPCR capabilities in these automated samplers (Hansen et al., 2020; Yamahara et al., 2019). Furthermore, advancements in portable eDNA sequencing platforms, like the MinION Oxford Nanopore sequencer, are leading to real-time in situ species identification. Moving forward, artificial intelligence (AI) will likely revolutionise the eDNA workflow. For example, AI could be used to guide autonomous vehicles for sample collection (Yamahara et al., 2019), optimise bioinformatic pipelines and taxonomic assignments (Mousavi-Derazmahalleh et al., 2021), and manage and handle large-scale databases. Such technological advances will significantly improve sampling capabilities and cut down processing times, thereby greatly expanding our ability to explore and unravel spatial and temporal patterns in marine biodiversity. This will be particularly significant for data-poor coastal and marine ecosystems, where our understanding of these systems in the face of growing pressures remains limited.

1.1.1 *Reducing environmental impact*

eDNA is widely recognised as a rapid and non-invasive monitoring tool, yet there is a need to enhance its sustainability to uphold the claim of being a 'non-invasive' approach. Recent advancements in the field have focused on improving efficiency and user-friendliness, such as the use of syringes equipped with enclosed filters, which not only facilitate easy sampling for professionals and non-experts but also minimize contamination risks. However, these syringes and plastic-enclosed filters are typically designed for single-use. Single-use consumables are preferred by eDNA researchers to avoid false-positive results caused by insufficient sterilization, or false-negative results due to residual bleach from sterilization methods affecting subsequent samples (Goldberg et al., 2016; Thomas et al., 2019). Nevertheless, to uphold the non-invasive claim of eDNA, steps should be taken towards developing more environmentally friendly methods that reduce both laboratory and field waste without compromising contamination risks. A good example of progress in this direction is the development of the Smith-Root self-preserving eDNA packs, which are predominantly biodegradable (Thomas et al., 2018). Additional efforts could be directed towards increased commitment to plastic recycling and the exploration of filtration systems powered by solar energy. While these suggestions represent only a fraction of the potential improvements, even small steps towards enhancing the environmental sustainability of eDNA surveys would further bolster the credibility of eDNA as a non-invasive monitoring tool.

6.4 Thesis conclusion

The need to effectively respond to escalating environmental impacts is driving the demand for more rapid and comprehensive methods to measure changes in biodiversity. Testament to this is the soaring expansion of eDNA technologies and applications in coastal and marine ecosystems. Despite their potential, eDNA methodologies have not been extensively tested across all environments or taxa groups, and more research is needed to explore how eDNA surveys can be applied to assess gradients of response in coastal and marine ecosystems. The overarching question of this thesis was: 'How can environmental DNA (eDNA) address ecological and biogeographic research gaps and generate baseline data for threatened

ecosystems and species lacking this information?' To address these research gaps, I assessed the efficacy of eDNA for detecting 'small and scarce' taxa, such as Syngnathids, as well as surveying 'broad and unexplored' ecosystems like the Southern Ocean and the deep-sea. The research conducted within this thesis demonstrates the viability of eDNA approaches for monitoring cryptic and threatened small-bodied taxa, and how these approaches can be effectively employed in long-term conservation initiatives. It showcases the capacity of eDNA to reveal spatial patterns in biodiversity across both small and large scales, and highlights how these patterns can be influenced by environmental factors and the presence of hull-fouling organisms. Additionally, the thesis demonstrates the capability of eDNA biomonitoring to serve as an early warning signal for biological invasions, enabling proactive management and mitigation strategies. Lastly, this thesis showcases the remarkable insights that eDNA can provide into the diversity of marine life in deep-sea ecosystems, uncovering previously unknown or understudied species and elucidating the complex ecological dynamics of these habitats. The contributions of this thesis extend to the expansion and refinement of eDNA methods in coastal and marine ecosystems, and underscore the importance of recognizing that eDNA is not a universally applicable methodology. Instead, careful consideration and customization of workflow components are essential to effectively address the specific objectives and environmental conditions of each study. In summary, this thesis emphasizes the versatility and power of eDNA as an invaluable tool that, when integrated into research and management toolkits, enhances our understanding and strengthens conservation efforts for threatened species and ecosystems. In an era where meticulous management and monitoring are crucial for preserving coastal and marine biodiversity, eDNA proves to be an invaluable asset.

6.5 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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Appendix 1: Copyright statements

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Chapter 2

To Whom It May Concern, I, Georgia Nester, contributed to the conceptualisation of the study, collected and processed the samples, analysed the data, and wrote and edited the resulting manuscript/chapter presented within this thesis:

Nester, G. M., De Brauwer, M., Koziol, A., West, K. M., DiBattista, J. D., White, N. E., Power, M., Heydenrych, M. J., Harvey, E., & Bunce, M. (2020). Development and evaluation of fish eDNA metabarcoding assays facilitate the detection of cryptic seahorse taxa (family: Syngnathidae). *Environmental DNA, 2(4)*, 614–626. https://doi.org/10.1002/edn3.93

Chapter 3

To Whom It May Concern, I, Georgia Nester, contributed to the conceptualisation of the study, collected and processed the samples, analysed the data, and wrote and edited the resulting manuscript/chapter presented within this thesis:

Nester, G. M., Heydenrych, M. J., Berry, T. E., Richards, Z., Wasserman, J., White, N. E., De Brauwer, M., Bunce, M., Takahashi, M., & Claassens, L. (2023). Characterizing the distribution of the critically endangered estuarine pipefish (*Syngnathus watermeyeri*) across its range using environmental DNA. Environmental DNA, 5(1), 132–145. https://doi.org/10.1002/edn3.365

Chapter 4

To Whom It May Concern, I, Georgia Nester, contributed to the conceptualisation of the study, collected and processed the samples, analysed the data, and wrote and edited the resulting manuscript/chapter presented within this thesis:

Nester, G.M., Suter, L., Polanowski, A.M., Kitchener, J.A., Deagle, B. eDNA metabarcoding reveals biodiversity patterns and facilitates early detection of invasive species in the Antarctic Southern Ocean. *Manuscript in Preparation.*

Chapter 5

To Whom It May Concern, I, Georgia Nester, contributed to the conceptualisation of the study, collected and processed the samples, analysed the data, and wrote and edited the resulting manuscript/chapter presented within this thesis:

Nester, G.M., Bunce, M., Richards, Z. eDNA metabarcoding reveals submarine canyon biodiversity across depth gradient. Manuscript in preparation..

Appendix 2: Research Outputs and Outreach

Research articles

Takahashi, M., Saccò, M., Kestel, J. H., **Nester, G. M.**, Campbell, M. A., Van Der Heyde, M., Heydenrych, M. J., Juszkiewicz, D. J., Nevill, P., Dawkins, K.L., Bessey, C., Fernandes , K., Miller, H., Power, M., Mousavi-Derazmahelleh, M., Newton, J. P., White, N. E., Richards, Z. T. & Allentoft, M. E. (2023). Aquatic environmental DNA: A review of the macro-organismal biomonitoring revolution. *Science of the Total Environment*, *873*, 162322.

Suter, L., Wotherspoon, S., Kawaguchi, S., King, R., MacDonald, A. J., **Nester, G. M.**, Polanowski, A. M., Raymond, B. & Deagle, B. E. (2023). Environmental DNA of Antarctic krill (*Euphausia superba*): Measuring DNA fragmentation adds a temporal aspect to quantitative surveys. *Environmental DNA*.

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Outreach

2023 *Characterising the distribution of a critically endangered pipefish in South Africa using eDNA* [oral presentation], eDNA conference 2023 (Hobart, TAS). Received oral presentation award.

2023 *Characterising biodiversity in two deep-sea canyons in Western Australia*. [lightning oral presentation], eDNA conference 2023 (Hobart, TAS).

2022 Led the Sequence our Seas program at Presbyterian Ladies' College

2022 DNA in the water shows South African scientists where to find a rare pipefish [article], The Conversation. https://theconversation.com/dna-in-the-water-shows-south-african-scientists-where-to-find-a-rare-pipefish-193229

2020 *An ocean of opportunity; New discoveries, experiences and friendships; and Sampling and Observing the deep sea* [blog posts], Schmidt Ocean Institute https://schmidtocean.org.

2020 Environmental DNA allows for the detection of cryptic seahorse species [blog post], Critter Research https://crittersresearch.com

2019 *eDNA in estuarine systems* [oral presentation], public outreach seminar (Kenton, South Africa).

2019 *Syngnathid eDNA research* [radio broadcast], Triple R (Australia).