CHARACTERIZING FISH SPECIES DIVERSITY IN THE UTHUKELA MPA USING ENVIRONMENTAL DNA METABARCODING

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PREFACE

The research contained in this dissertation was completed by the candidate based in the discipline of Marine Biology, School of Life Sciences, in the College of Agriculture, Engineering, and Science, University of KwaZulu-Natal, Westville Campus, South Africa, under the supervision of Dr A Macdonald. This Research was financially supported by the National Research Foundation and the WILD TRUST.

The research content presented in this dissertation has not previously been submitted for a degree in this or any other university, it is the original work of the candidate except where the work of others is acknowledged in the text.

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I……Dr Angus Macdonald…….as the candidate's Supervisor I agree to the submission of this thesis.

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DECLARATION 1 - PLAGIARISM

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ABSTRACT

The uThukela MPA, located on the east coast of South Africa in central part of the Natal Bight region is one of the 22 MPAs declared by the minister of environmental health as part of Operation Phakisa in 2018. The uThukela MPA is a significant area of larval retention used by many local fish species as a spawning and nursery site. Its environmental and biological factors are largely influenced by the oceanographic features such as the Agulhas current, Durban eddy and Richards Bay upwelling cell, and the uThukela River. eDNA is evolving as an effective tool for diversity monitoring. Living organisms leave their genetic material in their environment through the shedding of their hair, scales, skin, blood, and other biological secretions, resulting in a combination of genetic material in the environment known as environmental DNA (eDNA). eDNA makes it possible to detect different species from a single environmental sample through a process known as eDNA metabarcoding. This study aims to use environmental DNA metabarcoding to assess the spatial, temporal, and environmental variation in fish species diversity within the uThukela MPA and surrounding none-protected areas outside the MPA, to add to the growing baseline knowledge of fish species diversity and community composition in the uThukela MPA. This was achieved by assessing spatiotemporal variability in fish diversity and environmental variables at different spatial scales within uThukela MPA and in the non-protected areas outside the MPA. Water samples were collected from various depths in 12 and 14 reefs inside and outside the uThukela MPA winter of 2012 (June) and 2023 (July) respectively. DNA was extracted from the filtered water samples, amplified using the CO1 mitochondrial gene primers, and then later used to determine the species diversity of the fish community. eDNA detected spatial and temporal variation in alpha and beta diversity in the uThukela MPA at different spatial scales. Environmental variables showed various patterns at different spatial scales. The results indicated hydrodynamic and oceanographic factors are crucial in establishing and maintaining spatial variability and ecological connectivity in the uThukela MPA.

TABLE CONTENTS

LIST OF FIGURES

Figure **Page**

LIST OF TABLES

Table **Page**

CHAPTER 1: INTRODUCTION

1.1 Background

Marine ecosystems are one of the most biodiverse and productive ecosystems on earth, providing important goods and services such as food, medicine, climate regulation, recreational and educational opportunities (Goodwin et al., 2017). Despite their importance, these ecosystems are continually degraded by human activities such as over-exploitation, pollution, and climate change disturbances resulting in a significant decline in marine biodiversity (Thomsen and Willerslev, 2015). As a result, marine protected areas (MPAs) have emerged as a highly effective scientific tool for mitigating these impacts by promoting marine biodiversity conservation through the provision of refugia where marine life can recover and thrive (O'Leary et al., 2017; Harris and Lombard, 2018; Gjerde, 2007; Roberts et al., 2017). Understanding community composition and biodiversity of important taxonomic groups such as fish is crucial for establishing effective conservation policies (Capurso et al., 2023), and it also provides an opportunity to assess whether the management practices are appropriate, or they need to be adjusted (Gold et al., 2021). Environmental DNA is a promising tool with the potential to meet such biodiversity estimation objectives due to its non-invasive nature and relatively accurate species identification ability (Rishan et al., 2023).

Given the importance of marine ecosystems, several attempts have been made to establish conservation policies for recovering biodiversity and important ecosystems through MPAs (Capurso et al., 2023). MPAs are spatially delimited onshore and offshore areas of the ocean that are formally protected by a set of laws from specific anthropogenic activities (Humphreys and Clark, 2020; Kriegel et al., 2021). They have different goals, sizes, management approaches, and restrictions, resulting in different levels of protection (e Costa et al., 2016; Kriel et al., 2021). They range from no-take zones which restrict any extraction activities (e.g., fishing), to fisheries management zones that aim to sustain commercial and recreational activities such as commercial fishing, diving, etc (Day et al., 2012; Kriel et al., 2021). Some MPAs are designed to protect specific important species (e.g., endangered and vulnerable species) and habitats (e.g., breeding and nursery areas) (Day et al., 2012; Krieg et al., 2021) and to help recover damaged ecosystems and collapsed fisheries stocks (Lester et al., 2009). MPAs also help to improve the resilience of the ecosystem to both climate and overfishing events (Edga et al., 2007). Most importantly, MPAs do not only reserve natural systems and resources, but they directly meet a variety of human needs such as recreation, research, employment, and education (Kenchington, 1990). However, declaring an MPA does not automatically mean it will be successful in achieving its goals (Morris et al., 2023). For an MPA to effectively recover biodiversity, it must be adequately policed, appropriately managed (Fidler et al., 2022), and monitored regularly (Edga et al., 2014).

Effective MPA management and monitoring relies on the extensive collection and analysis of key species biodiversity data (McGeady et al., 2023). Much of marine biodiversity data collection have long relied on conventional survey methods such as visual surveys, trawl surveys, netting, and acoustic surveys (English et al., 2024). While these methods have been successfully used worldwide, they come with several limitations (Rishan et al., 2023). Trawl surveys are known for their destructive nature and are not very ideal for use in conservation research (Schadewell and Adams, 2021). Visually surveys are limited to species that are easily observable and those that are found in easily accessible areas (Polanco Fernández et al., 2021). Netting can be labour intensive, environmentally destructive, and may fail to capture low abundant species (Ratcliffe et al., 2020). In addition, conventional survey methods may fail to capture rare and cryptic species (Rishan et al., 2023). These limitations lead to incomplete assessment of biodiversity, especially in very complex and diverse environments such as those found in MPAs.

Nevertheless, molecular tools can now be used to provide new insight into species diversity and distribution as well as for monitoring biodiversity (Goodwin et al., 2017). Environmental DNA (eDNA) metabarcoding has evolved as a non-destructive method for assessing the biodiversity within MPAs (Rishan et al., 2023). eDNA metabarcoding uses high-throughput sequencing to detect a variety of organisms from a single environmental sample (Ruppert et al., 2019). It allows for a comprehensive assessment of species diversity and community composition of a given geographical area at a given time (Miya, 2022). With eDNA metabarcoding, a variety of taxa from abundant and easily accessible to rare and cryptic species can be detected over a very short period and at a lower cost (Ruppert et al., 2019). eDNA metabarcoding has therefore emerged as a world wild non-invasive tool for monitoring marine biodiversity in MPAs (Suarez-Bregua et al., 2022).

This technological advancement in biodiversity monitoring aligns with the growing realization of the benefits of MPAs, which has resulted in a global increase in MPA expansion (McGeady et al., 2023). The 2010 Convention on Biological Diversity (CBD) to which South Africa is one of the signatories, adopted the strategic plan for Biodiversity which aimed to increase the conservation, value, recovery, and sustainable use of biodiversity, thereby sustaining a healthy planet and maintaining ecosystem services for the benefit of all people (Bacon et al., 2019). The strategic plan for biodiversity committed to by South Africa under target 11 was to expand its MPAs by at least 10% by 2020 (Strategy Expansion, 2016). Despite falling behind most parts of the world, South Africa has made significant progress towards achieving its conservation goal (Adams and Kowalski, 2021). Prior to 2019, only 0.4% of the ocean was formally protected in South Africa (Harris et al., 2014). In May 2019 the minister of environmental health gazetted a network of 22 new MPAs, putting South Africa's MPAs at 5.4% (57 736 km^2) protection. One of the areas identified for protection is the KwaZulu-Natal (KZN) Bight, also known as "the Natal

Bight". The Natal Bight is a shallow unique region of larval retention, and recruitment, and a general nursery area for many fish species (Fennessy et al., 2016). Located in the centre of the KwaZulu-Natal Bight between Durban and Richards Bay is the uThukela MPA (Green et al., 2022), one of the three MPAs that were declared in the KwaZulu-Natal Bight. The uThukela MPA is an onshore and offshore MPA (Fielding, 2021) that covers 5666 km^2 of area which includes soft sediments (mosaic mud), gravel beds (Sink et al., 2012), reefs and submarine canyon habitats (Green et al., 2022). It is strongly influenced by the nutrient-poor warm Agulhas current which flows southwest along the shelf edge, bringing nutrient-poor water from the tropics (north) (Lutjeharms, 2006). The Agulhas current drives the overall cyclonic circulation on the Natal Bight (Guastella and Roberts, 2016). Despite the poor nutrient contribution from the Agulhas current, the uThukela MPA receives nutrients from other sources such as the nutrient-rich freshwater input and sediments from the uThukela River (Fennessy et al., 2016), upwelling at the Cape St. Lucia upwelling cell, Richards Bay upwelling cell and from the Durban eddy (Guastella and Roberts, 2016). These oceanographic features not only drive ecosystem productivity but also other ecosystem processes such as larvae dispersal and seasonal migrations (Roberts et al., 2010; Govender et al., 2023)

Despite the ecological value and uniqueness of the Natal Bight, it is threatened by anthropogenic activities from the developed area around the pots Durban and Richards Bay. The Thukela MPA was declared in October 2018 to protect the habitats and spawning grounds for different marine species found in this area, protecting overexploited species such as marine mammals (e.g., *Sousa plumbea*), sea-turtles (e.g., *Caretta caretta*) and several fisheries species (Green et al., 2022). Protected fisheries species include, seventy-four seabream (*Polysteganus undulosus*), geelbek (*Attractancies aequidens*), square tail kob (*Argyromus thorpei*), dusky kob (*Argyrosomus japonicus*), white edge rockcod (*Epinephelus albomarginatus*), Garrick (*Lichia amia*), black musselcracker (*Cymatoceps nasutus*) and hammerhead sharks (*Sphyrna spp*.) ensuring that their populations have sanctuaries in which to recover biomass and ensure long term food supply and employment security to the local coastal community (Fielding, 2021). In order to ensure that the Thukela MPA is effective in conserving natural resources and recovering biodiversity, it must be adequately policed and appropriately managed (Fidler et al., 2022).

1.2 Study rationale

While MPAs have the potential to overcome the biodiversity loss crisis, MPA management requires adequate data on biodiversity, environmental and ecological dynamics that shape the ecosystems (Júnior et al., 2016). Additionally, for an MPA to be effective in achieving its target conservation goals, its management zones must have some level of ecological connectivity to promote the recovery of populations from natural disturbances (Sale et al., 2021). Ecological connectivity is very crucial for ecosystem processes such as larvae dispersal and seasonal

migrations (Lima et al., 2021). Considering the rate at which marine biodiversity is deteriorating worldwide, the non-invasive nature of eDNA and its ability to capture a large amount of biodiversity data within a relatively short period makes it appropriate for biodiversity assessment in MPAs.

The uThukela MPA is now at its full implementation phase, however, not enough data about the biological features of the area is available and many of the available research data are fragmented (Fennessy et al., 2016). It is therefore necessary to add to the existing baseline knowledge to inform the management plan of the uThukela MPA and ensure the conservation of its resources. Baseline knowledge is essential as a reference against which success or effectiveness of MPA management can be measured at least 5 years after declaration of the MPA. Repeat fish biodiversity surveys inside and outside of the MPA using eDNA metabarcoding can be used as an experiment to assess fish biodiversity and ecological connectivity across all the MPA management zones. This study is part of the SmartZone project nested under the African Coelacanth Ecosystem Program.

1.3 Aims and Objectives

The aim of this study is to use environmental DNA metabarcoding to assess the spatial, temporal, and environmental variation in fish species diversity within the uThukela MPA and surrounding none protected areas outside the MPA, to add to the baseline knowledge of fish species diversity and distribution patterns in the MPA.

The objectives include:

(i) To assess spatiotemporal variability in environmental variables at different spatial scales in the uThukela MPA.

(ii) To assess spatiotemporal variability in alpha and beta diversity in the Thukela MPA at different spatial scales.

(iii) To compare the diversity detection success of different eDNA sampling efforts

(iv) To assess a combined effect of environmental variables on the fish species composition and distribution.

(v) To determine if uThukela MPA has source or sink population of the local fish communities.

(vi) To assess ecological connectivity between the MPA's management zones

(vii) To provide future recommendations for monitoring and conservation strategies based on the Results.

The objectives were archived by answering the following research questions:

(i) Is there a spatial and temporal variation in environmental variables between depths, management zones, inside and outside the MPA and between 2021 and 2023?

(ii) How does species richness, community composition and overall diversity vary between depths, management zones, inside and outside the MPA.

(iii) Are the different management zones ecologically connected through larvae dispersal.

(iv) How has fish species diversity changed between 2021 and 2023?

(v) Is there an effect of environmental variables on the composition and distribution and of fish species?

(vi) Does sampling more replicates in a selected sites increase diversity detected by eDNA?

(vii) How effective is the uThukela MPA in conserving fish biodiversity relative to the adjacent unprotected areas outside the MPA based on the eDNA metabarcoding results?

CHAPTER 2: LITERATURE REVIEW

2.1 Environmental DNA

Living organisms regularly shed their genetic material into their environment in the form of hair and skin cells, urine, faeces, the deteriorating remains of dead organisms, gametes (Bakker 2018), blood, and other biological secretions (Bohmann et al., 2014). This results to a mixture of genetic material known as environmental DNA (eDNA) which can be found in any environmental sample including water, soil, sediments, and ice (Rees et al., 2014). When an organism's genetic material becomes integrated into the environment, its original form can be altered by biological, chemical and physical processes such as microbial activities, photochemical oxidation, and mechanical breakdown of biological material respectively (Mauvisseau et al., 2022). The processes that are responsible for eDNA degradation are all influenced by environmental factors such as ultraviolet (UV) radiation, pH, and temperature (Barnes et al., 2014). Consequently, DNA in an environmental sample can exist as either degraded extracellular DNA or intact cellular DNA (Bakker, 2018). Macro-organismal and microbial DNA exist in different forms in the environmental samples because macro-organismal DNA only exists as part of the organism (free degraded DNA or cellular remains) whereas micro-organismal DNA may be present as the whole organism (whole cell) (Thomsen and Willerslev, 2015). The majority of metazoan eDNA exists as mitochondrial DNA (mtDNA) because mtDNA is protected from endonuclease degradation which rapidly degrades nuclear DNA in all cells during apoptosis (Murgia et al., 1992). In addition, mtDNA is more abundant compared to nuclear DNA copies per cell thus making mtDNA abundant in environmental samples (Wilcox et al., 2013). For this reason, mtDNA is mostly targeted in eDNA studies (Wilcox et al., 2013). However, which gene target is selected also depends on the purpose of the study.

eDNA can be collected and isolated from different environmental samples such as water, sediments, ice, etc, and used to investigate different ecological concepts (Thomsen and Willerslev, 2015). eDNA can be applied either in a species-specific approach, known as eDNA metabarcoding or in a multi species approach known as eDNA metabarcoding which both helps to detected species presence without prior knowledge of species presence in an environment (Valentini et al., 2016). The eDNA barcoding approach is not very different from DNA metabarcoding which uses the 650 bp sequence of the mitochondrial cytochrome oxidase subunit 1 (CO1) gene to identify vertebrates and invertebrates (Díaz-Ferguson et al., 2014**).** The two methods only differ in that, eDNA can use fragments that are as small as 100 base pairs (bp) or less and uses other genes besides CO1 (Díaz-Ferguson et al., 2014). As a result, the eDNA detection has been referred to as a "mini barcode" detection (Hajibabaei et al., 2007; Baird & Hajibabaei, 2012).

2.2 History of eDNA

The recovery and sequencing of eDNA have been developed quite substantially since its initial use (Biggs et al., 2015) as a method for aquatic ecological surveys (Ficetola et al., 2008). As a result, there has been an increase in interest in its use (Harper et al., 2019), including its application as a tool for both biodiversity assessment and target species detection (Handelsman, 2005). Microbiologists initially used the eDNA approach to assess the diversity of microbial communities from marine sediments in the mid 1980's, and they were the first to use the term eDNA (Willerslev et al., 2003). Following that, eDNA techniques were used in the 1990s, in phytoplankton, bacterial, and virus biomass monitoring studies (Thomsen and Willerslev, 2015). During that time, eDNA classification was determined by particle size (Paul et al., 1996), with aggregates of DNA larger than 0.2 μm linked with cells such as microbial DNA referred to as Particulate DNA or P-DNA, while DNA aggregates smaller than 0.2 μm were referred to as dissolved DNA or D-DNA (Paul et al., 1996).

eDNA has been recently developed to explain the identity of macro-organisms in aquatic environments (Ferris, 2020). Before that, eDNA was first applied to macro-organisms to detect extinct and extant mammals, birds, and plants in terrestrial sediments analysis in the 2000s (Poinar and Cooper, 2000). This resulted in another similar study in ancient cave sediments, ice cores, and ancient and contemporary sediments across different taxa, habitats, and climates (Willerslev et al., 2004, Yoccoz at al., 2012, Thomsen et al., 2012). Thereafter, Ficetola et al., (2008) used the eDNA approach to identify the presence of an invasive American frog species known as the American bullfrog (*Rana catesbeiana*) in more than 2500 France wetlands (Thomsen et al., 2012). eDNA techniques were later used to investigate the high-profile invasion of *Hypophthalmichthys molitrix* and *H. nobilis* (bigheaded carps) into Midwest western waters in the United States of America (USA) (Turner et al., 2014). In the marine environment, the eDNA techniques were first applied by Foote et al., (2012) and Thomsen et al., (2012) to do genetic monitoring of marine mammals and marine fish diversity (Bakker 2018). It was later used to detect the presence of sharks and rays (Weltz et al., 2017). In the past 5 years, eDNA technology has been increasingly used as a tool to monitor fish biodiversity, with at least 120 published studies that assessed fish community composition of several aquatic environments, including freshwater, marine (Leese et al., 2018; McElroy et al., 2020), and deep-sea habitats (McClenaghan et al., 2020). Other studies focused on modeling biotic (vectors, system volume, sample volume), and abiotic factors (factors eDNA dynamics, stream flow, discharge, and particle size) that influence eDNA persistence and detection (Piaggio et al., 2013). Before eDNA techniques can be used in a study, it is important to establish the dynamics of eDNA to determine the extent to which it can be applied.

2.3 eDNA dynamics in the marine environment

2.3.1 DNA shedding

Before eDNA can even be detected, it has to shed from an organism into the environment. Temporal variation of the eDNA concentration of any organism may be related to seasonal variations in DNA shedding rates, which are influenced by various factors such as changing weather conditions, hydrodynamic processes, and the productivity in their habitat (Troth et al., 2021). These factors can alter animal activities and life cycle patterns (Troth et al., 2021). Changes in animal behaviors strongly influence the rate at which DNA is released into the environment therefore affecting the amount of detectable eDNA in a given time and space across seasons (Wacker et al., 2019). For example, eDNA shedding rate was reported to increase during signal crayfish's egg reproduction season (Dunn et al., 2017). There was also a correlation between freshwater pearl mussel eDNA and the reproduction and spawning events in field population (Wacker et al., 2019). Sampling could therefore be restricted to spawning seasons as an approach to account for such effects (Buxton et al., 2018; Rees et al., 2017). However, there is a need for a more detailed knowledge of the relationship between seasonal activity patterns and DNA shedding rates before seasonal field sampling season restrictions could be implemented (Seymour et al., 2020). Furthermore, eDNA shedding rate is also strongly dependent on the density (Díaz-Ferguson & Moyer, 2014), body size and physiology of the species, which directly determines the amount of DNA shed to the environment (Ramirez-Amaro et al., 2022). All these factors depend on the taxonomic groups to which the target organism belongs.

2.3.2 eDNA persistence

After DNA is released into the environment, it becomes exposed to processes that can cause it to persist in the environment for some time until it degrades to undetectable concentrations, be absorbed in organic and inorganic particles, or transported to other location (Díaz-Ferguson and Moyer, 2014). The duration of eDNA fragments in the environment after their source has been removed from the system is known as eDNA persistence (Dejean et al., 2011). eDNA persistence is directly dependent on the nature of the environment (e.g., sediment, water column, fresh water, saline water etc.) (Turner et al., 2015), the concentration of DNA and Abiotic (temperature, UV radiation pH etc.) and biotic factors (microbial activities) present at a certain place at a given time (Colmenares et al., 2023). Experimental studies that investigated the persistence of eDNA in different environments have shown that the amount eDNA detectable in a given time and space decreases with time after the source has been removed from the environment (Dejean et al., 2011). The amount of eDNA detectable at a given time and place decreases faster in the water column than in sediments (Turner et al., 2015). This difference is linked to differences in eDNA concentration, with high DNA concentration resulting in longer persistence (Barnes et al., 2014).

Sediments receive the high eDNA concentration from faeces which are the major source of macrofaunal organisms' DNA because they can contain high DNA concentration and are excreted in large quantities which sink down to the substrate (Saba & Steinberg, 2012). DNA Persistence also varies between fresh water and marine systems, with faster degradation rates in marine systems than in freshwater (Thomsen et al., 2012; Sassoubre et al., 2016). However, Collins et al., (2018) found that longer DNA persistence in marine water was only found in studies that used unnatural water sources and where temperature was lower in fresh water. The higher salinity and pH and more stable temperatures in sea water promote DNA preservation and it has been found to correspond with the lowest degradation rates (Tsuji et al., 2017; Collins et al., 2018).

eDNA from different taxonomic groups and life history stages have different persistence values (Thomsen et al., 2012). eDNA persistence was estimated for different aquatic taxonomic groups, with freshwater fish ranging from 15 to 30 days (Ficetola et al., 2008; Goldberg et al., 2011), 15 to 30 days for amphibians (Ficetola et al. 2008; Goldberg et al. 2011) 0.9 to 7 days for marine mammals (Foote et al., 2012), 21 days for mud snails (Goldberg et al., 2013), and 14 days for reptiles (Piaggio et al., 2013). eDNA persistence in marine water is not yet understood and still requires extensive investigation (Andruszkiewicz et al., 2017).

After DNA has been shed into the environment, degradation is one of the most important factors in determining how long DNA will remain detectable (Nagarajan et al., 2022). As a result, eDNA persistence in the environment depends on the changes in degradation rates, which further influences the species detection and quantification (Goldberg et al., 2018), thus affecting the reliability of the data (Barnes et al., 2014)**.** Different habitats, species and environmental conditions are reported to have different DNA degradation rates (Collins et al., 2018). This difference is strongly influenced by changes in environmental factors such as temperature and UV radiation (Goldberg et al., 2018), bacterial and fungal concentration (Dejean et al., 2011), pH (Seymour et al., 2018) and salinity (Barnes et al., 2014). Temperature has been reported to have the strongest effect on DNA persistence (Caza-Allard, 2022). Higher temperatures directly decrease DNA persistence through DNA denaturation and indirectly by increasing microbial metabolism and enzyme kinetics (Barnes et al., 2014; Goldberg et al., 2018; Chipuriro et al., 2022). The molecular structure of DNA has long been known to be destabilized by higher temperatures through the breaking of hydrogen bonds between nitrogenous bases (Nagarajan et al., 2022). However, eDNA's molecular structure is only denatured when the temperature rises above 50°C (Nagarajan et al., 2022). Robson et al., (2016) reported that increasing the temperature to 35°C did not change the Invasive Mozambique Tilapias' eDNA degradation rates. Conversely, Qian et al., (2022) showed that DNA degradation of Chinese white shrimp *Fenneropenaeus chinensis* accelerated with temperature increase from 10 °C, 20 °C and 25 °C in laboratory experiments. Additionally, Moyer et al., (2014) demonstrated a decrease in DNA

persistence by 1.67 times for every 1.02 °C increase in temperature. Extracellular nucleases and microbes have been used to explain the increase DNA degradation rates at temperatures less than 50°C (Collins et al., 2018; Jo et al., 2019; Lamb et al., 2022) because DNA remains stable at temperatures less than 50°C (Lamb et al., 2022). Microorganisms such as bacteria release enzymes into the environment that break down extracellular molecules, causing the release of DNase which degrades the DNA (Torti et al., 2015). This is supported by experimental studies that found that DNA degrades faster in untreated water and slower in sterile water (Barnes et al., 2014; Lance et al., 2017).

Additionally, eDNA has been found to be very stable in low oxygen conditions whilst decaying rapidly by hydrolysis in oxygen-saturated conditions (Bozinovic & Pörtner 2015). Conversely, slow DNA degradation has been reported in waters with low a temperature, low solar ultraviolet B radiation a high pH (Pilliod et al. 2014; Strickler et al. 2015)**.** In a study comparing degradation rates in different seasons, eDNA degradation was found to be around 1.1 times faster in winter than in summer, though the results were statistically insignificant (Collins et al., 2018). The different responses of DNA degradation observed by different studies at different temperatures clearly suggest that it is a complex phenomenon, especially in the presence of other environmental factors.

Like temperature, high UV radiation has the same degradation effect on DNA, therefore decreasing its detectability (Barnes et al., 2014). UV-B can directly damage the structure of DNA resulting in the inhibition of DNA persistence (Andruszkiewicz et al., 2017). pH also affects DNA degradation, higher pH is associated with slow DNA degradation and vice versa (Strickler et al., 2015; Seymour et al., 2018) likely due to acidic conditions (Bruce et al., 2021). DNA has a longer persistence in alkaline water than in acidic water because of hydrolysis below pH 7.5 (Seymour et., al., 2018). Bochove et al., (2020), found no significant effect of pH on DNA degradation in artificial environments. pH affects DNA degradation significantly when it interacts with other environmental factors such as enzymes and microbial activity (Lance et al., 2017).

The spatial distribution patterns of eDNA can be strongly heterogeneous within different sites (Moyer et al., 2014). Habitat preferences of the target taxa, hydrological dynamics (Deiner and Altermatt, 2014; Jane et al., 2015), and the level of stratification in aquatic environments (Moyer et al., 2014) are some of the potential drivers of heterogeneous eDNA concentrations (Collins et al., 2018). Therefore, eDNA is strongly influenced by spatial heterogeneity in the natural marine environment, with the terrestrially influenced inshore environment having 1.6 times faster eDNA degradation than ocean-influenced offshore environments (Collins et al., 2018). Experimental estimates of differences in eDNA removal rates under typical marine water column conditions (inshore/offshore, winter/summer) made by Collins et al., (2018) showed that eDNA removal

occurred more quickly in inshore artificial conditions, suggesting that the natural offshore marine waters experience slower degradation rates**.** False negatives and positives may occur if such heterogeneity is not taken into consideration, particularly when rare and endangered species are targeted (Moyer et al., 2014).

Finally, eDNA transport is another crucial factor that contributes to eDNA degradation, especially in the ocean where long-distance transport is likely to occur (Thomsen et al., 2012a). eDNA can be transported to distances far from their production source location through water movement (Hinz et al., 2022). eDNA behaves like a small particle, hence the distance it travels is determined by sedimentation processes (Pont et al., 2018). In the marine environment, particle transport is also affected ocean the mixture of interacting currents (Harrison et al., 2019). Unfortunately, the reactivity dynamics of eDNA in the process of transportation is not a well-studied topic in marine systems (Hinz et al., 2022).

2.4 eDNA in conservation and ecology

2.4.1 Invasive species

Currently, eDNA is used in various fields of study, including biological and environmental studies to detect different taxonomic groups from various types of research fields such as fecal pollution tracking (Caldwell et al., 2011), forensics (van Oorschot et al., 2010), paleogenetic (Pedersen et al., 2015), and environmental biosafety (Nielsen et al., 2007). In ecology, the detection of invasive species is one of the areas that have successfully used eDNA techniques (Collins et al., 2018). The use of eDNA to detect invasive species is in fact one of the first applications of eDNA on macro-organisms. eDNA has been used as an early warning system to detect invasive (Jerde et al., 2011) and pathogenic species (Aintablian et al., 1998) when they are still low in density and have not yet established in the new environment (Collins et al., 2018). Sampling water from the transition areas such as ship ballast water, aquaculture transits, and then applying eDNA techniques to detect invasive species prevents invasive species from establishing in new environments (Collins et al., 2018). The use of eDNA to detect invasive species from transoceanic ship's ballast water has been proposed by Egan et al., (2013). Similarly, eDNA techniques been applied on ballast tank sediments and successfully captured dormant stages of benthic invertebrates (Darling and Tepolt, 2008; Harvey et al. 2009; Briski et al. 2011). The US Fish and Wildlife Services used eDNA techniques in the Mid-west USA to monitor the invasive Asian Carp instead of the labor-intensive and expensive traditional survey tools such as electrofishing and/or manual netting (Jerde et al., 2011). These studies demonstrate eDNA monitoring techniques' ability to detect the presence of invasive species, but they also demonstrate eDNA's usefulness in capturing hidden stages of invasive that are not easily observable (Xiong et al., 2022).

2.4.2 Rare, cryptic, and endangered species

eDNA techniques have also been used in detecting and monitoring rare, cryptic, and endangered species (Barnes and Turner, 2016) which can be very challenging and often require huge amounts of time and effort (Qu and Stewart, 2019)**.** Rare, cryptic, and endangered species are not easy to monitor, and legal restrictions may prevent regular handling of such organisms using more conventional techniques like trapping (Thomsen et al., 2012). Furthermore, conventional methods, which often involve repeated sampling can be expensive and may damage the target organism or its habitat (Beng and Corlett, 2020). The efficiency of the eDNA techniques in detecting rare, cryptic, and endangered species has been evaluated by several studies and it has been demonstrated to have a higher probability of accurately detecting the presence of a target species compared to conventional survey tools (Deiner et al., 2017). For example, in Indiana and Missouri, USA eDNA assays were successfully used to detect an amphibian of conservation concern known as the eastern hellbender (*Cryptobranchus a. alleganiensis*) (Olson et al., 2012). In the UK, the threatened great crested newt *Triturus cristatus's* presence was validated using eDNA assays (Rees et al., 2014a). eDNA techniques were also reported to outperform traditional methods in surveying endangered fish in Denmark (Sigsgaard et al., 2015).

2.4.3 Fisheries management

Due to its non-invasive nature being cost effective and more efficient in detecting biodiversity, eDNA metabarcoding has also been widely used in fisheries management (Ruppert et al., 2019). The eDNA techniques have been applied in monitoring fish composition, community structure, and spatial and temporal changes of fish communities in various aquatic ecosystems including lakes, rivers, ponds, streams, bays, and estuaries (Xiong et al., 2022). Pont et al., (2018) successfully described longitudinal fish assemble patterns in a large river (Rhone River) using eDNA metabarcoding. Hanfling et al., (2016), used eDNA metabarcoding in large lakes to provide accurate qualitative and quantitative data on fish communities by comparing it with the available comprehensive long-term gill-net data set in the UK (Xiong et al., 2022). Lastly, Stoeckle et al., (2017) showed that eDNA detected a temporal and spatial distribution of marine fish, similar to those recorded by conventional methods, highlighting the capability of eDNA in detecting spatiotemporal dynamics of fish communities. The population attributes such as abundance, distribution, and biomass can be measured by eDNA detection and quantification (Rees et al., 2014). Biomass is one of the fundamental biological parameters and yet can be difficult to measure in aquatic systems by traditional tools such as electrofishing and hydroacoustic surveys (Díaz-Ferguson, 2014). Biomass estimates provide information that contributes to the conservation of rare and endangered species and also plays a role in the management of population size (Begon, Townsend and Harper, 2005). In freshwater ecosystems, eDNA concentration has been used as a measure of distribution and biomass (Takahara et al.,

2012). Takahara et al., (2012) found a positive correlation between the known carp biomass per liter and copies of eDNA of a water sample taken from a pond containing common carp. eDNA was also used in a lagoon system to estimate common carp biomass (Rees et al., 2014). It was concluded that seasonal changes in eDNA concentration can be used to estimate species biomass in natural environments (Rees et al., 2014). In marine environments, changes in bacterial and phytoplankton communities that occur during blooming events in coastal waters have been correlated with eDNA concentration (Bailiff and Karl, 1991). Furthermore, population distribution of aquatic organisms such as fishes (Mahon et al., 2012), reptiles, (Piaggio et al., 2013) and amphibians have been correlated with their DNA concentration in the water sample (Ficetola et al., 2008; Goldberg et al., 2011). Additionally, several studies have demonstrated a positive correlation between eDNA read abundance and fish biomass (Evans et al., 2016; Thomsen et al., 2016). In contrast, studies like Kelly et al., (2014) and Knudsen et al., (2019), have contradicted that relationship. The complexity of eDNA ecology in natural systems makes the relative abundance estimation challenging, therefore, further research is required to determine the accuracy of predicting the relative biomass of species from eDNA read abundance (Ushio et al., 2018).

2.4.4 Diversity assessments

eDNA's ability to identify multiple species from a single sample makes it a quicker method for producing biodiversity estimates that are taxonomically comprehensive without relying on taxonomic expertise (Ji et al., 2013). eDNA metabarcoding has been successfully used to assess species diversity in various aquatic systems including rivers, estuaries, coastal areas, and oceans. Several fish biodiversity studies have used eDNA to monitor fish communities (Evans et al., 2016; Shaw et al., 2016; Stat et al., 2019). Li et al., (2022) recently used eDNA to assess the diversity and distribution of pelagic fish in the central Pacific Ocean. In a study that compared eDNA metabarcoding results with data from conventional visual methods, it was found that there is a correlation between the results from the two approaches (Port et al., 2016). Zou et al., (2020) compared results from eDNA metabarcoding with bottom trawling and found that eDNA captured more species than bottom trawling in a coastal wetland of the Pearl River Estuary. The use of eDNA metabarcoding to assess and monitor biodiversity is now one of the frontline methods available in the toolbox of ecologists and conservation scientists (Zou et al., 2020; Yamamoto et al., 2017).

2.5 eDNA metabarcoding versus conventional survey methods

For reliable biodiversity assessment, eDNA must be compared to the conventional methods such as visual surveys, trawl surveys, netting, and acoustic surveys. There is a growing number of publications in scientific literature that provides evidence that eDNA is more efficient in

characterizing community composition and diversity than conventional survey tools (e.g., Fujii et al., 2019; Handley et al., 2019; Li et al., 2019). Several studies successfully detect over 93% of marine fish species using eDNA both in aquariums and reef ecosystems (Thomsen et al., 2012, Kelly et al., 2014, Port et al., 2015, Miya et al., 2015, Valentini et al., 2016). Furthermore, eDNA was able to capture 50% more species than conventional tools, demonstrating the ability to detect species that conventional methods missed (e.g., Erdozaina et al., 2019; Fujii et al., 2019; Knudsen et al., 2019; Andruszkiewicz et al., 2017; Port et al., 2016; Yamamoto et al., 2017). In Greenland waters, eDNA was reported to have successfully detected 100% of species that were captured in trawl surveys (Thomsen et al., 2016). eDNA has clearly demonstrated a high sensitivity in capturing species composition, however, it does not always outperform traditional methods. Some studies have reported overlapping differences after comparing eDNA and visual methods on marine communities (Thomsen et al., 2012; Kelly et al. 2014; Ushio et al. 2018; Closek et al. 2019). Another study comparing eDNA metabarcoding and underwater visual census in two Cambodian tropical reefs found that eDNA captured the same number of species in one of the two sites however, eDNA also captured a wider phylogenetic diversity and smaller species (Fernández et al., 2021). In addition, eDNA also failed to detect the nurse shark in an area where it was known to be present (Bakker et al., 2017). As a result, traditional methods may be effective in capturing some species however, eDNA is able to reach species that traditional methods fail to capture. This therefore makes it necessary to use an approach that combines conventional tools with eDNA (Bohmann et al., 2014).

eDNA is also able to detect rare, cryptic, threatened, and endangered species that are often not very easy to detect by traditional survey tools (Willis, 2001; Port et al., 2016). For example, Yamamoto et al., (2017) found that some species detected by eDNA were not easy to detect by underwater visual. DNA has also been reported by several studies (Boothroyd et al., 2016; Simpfendorfer et al., 2016; Weltz et al., 2017; Lafferty et al., 2018) to effectively capture various threatened and endangered species in the aquatic environment over a large-scale sampling and these species only occurred rarely in the bay and mostly occurred as pelagic larvae.

Other than better detection capability, eDNA has other advantages over conventional methods (Thomsen et al., 2012). eDNA has the ability to capture a wide variety of marine vertebrate taxa over a short period (Thomsen et al., 2012). Camera traps, trawl grabs, and other physical methods are limited by time in that they are able to detect a small portion of the organisms that are in the environment at a given time, whereas with a single sample of eDNA broader assessments can be provided with less capacity and effort (Thomsen et al., 2016). For example, after comparing eDNA metabarcoding and traditional sampling tools of fish species in Maizuru Bay, Japan, Yamamoto et al., (2017) found that it took six hours of eDNA sampling to detect more than half of the species found in 14 years of sampling using underwater visual sampling.

Furthermore, eDNA is a very useful tool for monitoring biodiversity in marine areas with restricted access such as protected areas (Gold et al., 2021) and deep-sea habitats (Carugati et al., 2015). eDNA is particularly appropriate for monitoring biodiversity in MPA because it is not destructive to the individual being studied and its habitat (Thomsen et al., 2012; Schnell et al., 2012) as opposed to the commonly used traditional methods such as trawl surveys (Danielsen et al., 2005). Deep sea habitats are logistically very difficult and very costly to access with traditional fishing gear thus making it hard to monitor biodiversity in such areas (Carugati et al., 2015).

Lastly, eDNA sampling methods are not just sensitive in detecting species, quicker, and non-destructive, but they have a relatively simple sampling effort (Gold et al., 2021). Sampling eDNA in the marine environment requires 3 L of water or less and a simple filtering technique that does not require complicated training, even in remote areas (Miya et al., 2016). Whereas traditional survey methods rely on experts in species identification based on morphological features, which might result in misidentification if expertise is not available (Deiner et al., 2017).

Despite all these advantages, eDNA has its own limitations. It can detect a broader range of taxa compared to visual surveys however, it cannot detect a subset of fish species observed by visual methods, resulting from different community patterns across sites (Kelly et al., 2017). eDNA is also limited by an incomplete species detection due to PCR bias and an incomplete reference database which can be resolved by using a multiple marker and primer approach and an improved referenced database (Pikitch, 2018).

2.6 eDNA workflow

2.6.1 Sample collection

Collecting eDNA samples from different environments differs for each environment, purpose of the study, and target species and their potential abundance (Shu et al., 2020). In aquatic environments, water samples are usually collected by scooping water into a bottle and then use a microfiber filter to filter the water sample to concentrate the DNA (Yamamoto et al., 2016). Sampling depth is very crucial in determining successful species detection (Lu Shu et al., 2020). The depth from which samples are collected can range from surface, middle, and bottom, depending on the type of water body being sampled, and the target organism (Schumer et al., 2019). In streams, sampling is usually done on surface waters because of its well-mixed water with high turbulence (Strickland and Roberts, 2019). Lakes on the other hand are highly stratified, and it is recommended to take a sample that has water from the surface, middle, and bottom to reduce sampling bias for diversity assessment studies (Klobucar et al., 2017). In the marine environment, it is still not clear how hydrological and oceanographic processes influence the transportation and distribution of eDNA, therefore, there is a need for further research on how

sampling strategies in the marine environment can be improved to capture accurate biodiversity patterns (Bruce et al., 2021). Sampling depth in near-shore marine systems can be determined by the season in which the samples will be taken because some species move to shallow waters in summer for mating and move back to deep waters in winter (Bruce et al., 2021). Some species generally prefer deep water in summer and shallow water in winter (Bruce et al., 2021). Moreover, like lakes, the ocean water column can be vertically stratified due to haloclines or thermoclines, thus resulting in the uneven distribution of eDNA (Bruce et al., 2021). Therefore, it has been suggested that in tropical ocean ecosystems such as coral reefs, samples must be sampled from both the surface and along the depth gradient of the water column (Stauffer et al., 2021). Jeunen et al., (2020) also recommended that marine systems should be sampled at several depths for the whole community to be captured.

2.6.2 Replication

In order to capture the whole community diversity, the sampling strategy must be representative of the study area, regardless of the physical sampling method used (Creer et al., 2016). Therefore, it is essential to include enough replicates, because the number of replicates chosen at all stages from sampling to PCR and library preparation are all known to affect the taxonomic composition detection (Ficetola et al., 2015). A varying number of samples have been used in different studies. For example, Sigsgaard et al., (2015) used 3 × 15 ml water samples per site in a study to monitor endangered fish species in Jutland, Denmark in Europe. West et al., (2020) used 6 × 1 L water samples per site to detect coral reef fishes in the lagoons around the Cocos Islands in the eastern Indian Ocean. Stauffer et al., (2021) recently suggested that enough species richness could be reflected by at least 25 replicates of samples in the tropical oceans. Another factor that is very important in the sampling step of eDNA is the sampling volume. Sample volume can to a certain extent affect the biodiversity estimates from eDNA samples in sediments (Nascimento et al., 2018). Different water volumes have been used in studies when collecting eDNA from the water column (Deiner et al., 2015). Volumes of water samples that have been recorded in different studies range from 15ml to 45L (Schumer et al., 2019; Dressler et al., 2020; Fraija-Fernández et al., 2020; Sigsgaard et al., 2020; Brys et al., 2021). Hunter et al., (2019) compared the number of eDNA copies obtained from 200 ml of water filtered through one filter and 800 ml through four filters and found that 200 ml yielded 1.4 copies/µl and 800 ml yielded 6.39 copies/µl. A recommended volume of water samples is 2L to 3L per sampling station in natural environments (Deiner et al., 2015). The available literature shows that eDNA sample replicates and water volume are dependent on the nature and location of the study site and the purpose of the study.

2.6.3 Sample filtration

eDNA from water samples must be concentrated before it can be extracted because in its dilute state, it is not ideal for in situ detection (Nagarajan et al., 2022). Filtering the water is a common method used to concentrate the DNA, however, some studies have used precipitation and centrifugation (Eichmiller et al., 2015). Precipitation was used in the early studies of eDNA (Ficetola et al., 2008). However, filtration is more effective in capturing aquatic eDNA (Tsuji et al., 2019). Filtration can handle from 250 mL to 45 L of water whereas centrifugation and precipitation can handle only up to 1.5 ml of water (Doi et al., 2017). Furthermore, filtration has been shown by many studies (Deiner et al., 2015, Spens et al., 2017) to outperform precipitation even when equal volumes of water were used. Most fish eDNA capture studies have widely used the filtration method (Lu Shu et al., 2020). Yingchun Xing, (2022) found that filtration has been used in more than 90% of 371 studies on the application of eDNA in fish ecology**.** When compared with other methods, the filtration method has been shown to be more effective in producing a high DNA concentration compared to other methods (Eichmiller et al., 2015)

Filtration is done in the field or laboratory immediately or within 24 hours after sampling to minimize DNA degradation (Nagarajan et al., 2022). Various membrane filters with different pore sizes ranging from 0.22 to 3 μm have been used to capture eDNA from water samples (Wang et al., 2021). 0.45 and 0.7 μm membrane filters are the most commonly used filters with about 23% and 16% of fish eDNA studies who have used these (Wang et al., 2021**)**. Fish eDNA is better captured by a small pore size (<1 μm) membrane filter (Shu et al., 2020). Turner et al., (2014**)** suggested that the most common size of fish particles in environmental samples is 1–10 μm. Therefore, it is important to select a pore size according to particle size to ensure a successful fish DNA capture. However, large pore size (Schabacker et al., 2020) filters have been successfully used to capture eDNA (Bruce et al., 2021). In a situation where a filter with a small pore size is too small that it gets clogged, a larger pore size (>1 μm) filter can be used (Shu et al., 2020). Filter membranes do not only have different pore sizes but are made of different materials (Turner et al., 2014). Some of the most common filter membrane materials include, glass fiber (GF) filters, cellulose nitrate filters (CN), polyether sulfone (PF), and mixed cellulose acetate and nitrate (MCE) filters (Shu et al., 2020) and these membrane filters have the ability to adsorb DNA and proteins on their surface which improves the eDNA collection efficiency (Tsuji et al., 2019). The filter type to be used is determined by water turbidity and fish density during sampling time (Li et al., 2018). The GF filter is recommended for fresh water, and the MCE filter performs better in turbid water bodies with high species density (Eichmiller et al., 2015; Muha et al., 2019). Shu et al., (2020) recommended the 0.7 μm glass fiber filters. Kumar et al., (2019) on the other hand concluded that cellulose-based filters are the best performing filters compared to others. The appropriate filter choice between the GF and cellulose based filter is still an ongoing debate (Shu

et al., 2020). Cellulose nitrate filter yielded higher DNA copies than other filter types in experimental aquariums of bluegill sunfish *Lepomis macrochirus*, (Renshaw et al. 2015). This is supported by findings of the experimental aquarium of oriental weather loach *Misgurnus anguillicaudatus* (Hinlo et al., 2017). In field studies, mixed cellulose acetate and nitrate (MCE) filters yielded higher detection rates than glass filters for northern pike *Esox lucius* in lakes (Sepulveda et al. 2018). Lacoursiere-Roussel et al., (2016) on the other hand found that GF captured more brook charr *Salvelinus fontinalis* than the MCE filter. Because different water bodies and target species produce different results, a pre-experiment is required to determine a method that is the best for a particular study (Shu et al., 2020). However, GF filters were recommended for fish eDNA studies as this method has been used successfully to capture fish eDNA water samples obtained from various water bodies such as aquariums, and lentic or lotic systems (Shu et al., 2020).

The filtration processes involve pumping water through a filter with a certain pore size to collect the DNA on the surface of the filter (Xing, 2022). After filtration, samples can be stored until extraction (Wang et al., 2021). The desired storage method is one that minimizes DNA degradation and maximizes extraction efficiency (Xing, 2022). Some of the common storage methods that have been used in recent studies include, freezing the sample or keeping it in liquid nitrogen and keeping the filter in a fixed buffer such as ethanol or lysis buffer (Sugiura et al., 2021). Freezing is the commonly used preservation method appropriate for DNA that has been concentrated by centrifugation (Sigsgaard et al., 2015). However, most field environments do not make it possible to freeze samples, leaving preservation buffers as an alternative (Renshaw et al., 2015). Filtered samples are well suited for being preserved in a buffer or ethanol (Bagley et al., 2019).

2.6.4 DNA extraction

DNA extraction is the next step after sample collection. To minimize contamination risk, eDNA extraction must be conducted in an eDNA-dedicated environment with appropriate anti-contamination measures (Goldberg et al., 2016). DNA extraction is defined as “the process by which DNA is released from intact cells and organelles” (Eichmiller et al., 2015). eDNA can be extracted using liquid phase separation methods which include phenol-chloroform-isoamyl alcohol, the traditional extraction method also known as PCI (Deiner et al., 2015), cetyl trimethylammonium bromide also known as CTAB, salt DNA extraction protocols and several commercial DNA extraction kits (Shu et al., 2020). The liquid phase extraction with phenol-chloroform-isoamyl protocol is very effective and produces high DNA yields (Deiner et al., 2015). However, most researchers avoid it because it has significant health and safety concerns, thus leaving commercial kits as an alternative (Bruce et al., 2021). The most commonly used commercial DNA extraction kits for eDNA studies is the Qiagen DNeasy Blood and Tissue Kit,

followed by the PowerWater DNA Isolation Kit from MoBio (Shu et al., 2020). Kumar et al., 2020 and Tsuji et al., (2019) compared different commercial DNA extraction Kits for eDNA extraction and found eDNeasy Blood and Tissue Kit to be the best because it is less costly, easy to use, and less toxic than other kits. However, Eichmiller et al., (2016) discouraged the use of DNeasy blood and tissue kits for quantification because of its PCR inhibition. The PowerWater kit, on the other hand, is more effective than the DNeasy blood and tissue kit in that it produces better DNA yields and includes an inhibitor removal step (Yingchun Xing, 2022). However, it costs almost 4 times the cost of the DNeasy blood and tissue kit (Yingchun Xing, 2022). Additionally, Eichmiller et al., (2015) reported that the Biomedicals kit was more effective in detecting common carp (*Cyprinus carpio*) after comparing it with five other kits (PowerSoil, PowerWater, Biomedicals soil, DNeasy, and Stool Mini). As a result, the extraction kit choice involves a trade-off between better PCR without inhibitions and a better cost.

2.6.5 Genetic marker and primer choice and PCR

After eDNA extraction, individual or multiple species can be amplified using group-specific primers (e.g. fish, vertebrate, or invertebrates) through a process known as DNA metabarcoding (Taberlet et al., 2012). Before PCR, appropriate genetic markers and primers have to be chosen according to the target species, population, or community of interest (Wang et al., 2021). The common genetic markers that have been used are mitochondrial and nuclear genes (Shu et al., 2020). Due to its ability to describe biodiversity better and evaluate degraded DNA, mitochondrial genes have been used as the gold standard in eDNA studies (Rees et al., 2014). Ribosomal genes on the other hand can detect a large proportion of target species and have high primer specificity compared to protein-coding genes because they have many universally conserved sequences (Collins et al., 2019)**.** However, ribosomal markers are shorter, hence they have limited taxonomic resolution, and they may have detection bias (Hanfling et al., 2016). Early studies of eDNA metabarcoding mostly used the cytochrome b gene as the genetic marker to target fish (Minamoto et al., 2012**;** Thomsen et al., 2012b), as a result, the public databases such National Centre for Biotechnology Information, NCBI; the Barcode of Life Data System (BOLD) are very rich in extensive reference for CO1 (Kumar et al., 2022). However, its conservative nature makes the design of taxon-specific primers complicated (Clarke et al., 2014) because CO1 metabarcoding primers have a high degree of base degeneracy (Leray et al., 2013) which allows for a wide breadth of taxa to be amplified (Elbrecht and Leese, 2017). Moreover, a huge percentage of sequence reads produced by CO1 metabarcoding primers are non-target sequence reads (Hajibabaei et al., 2019). As a result, CO1 is no longer the most commonly used marker for eDNA metabarcoding (Girish et al., 2022) but many researchers now prefer to use 12S rRNA

(Hänfling et al., 2016; Port et al., 2016; Yamamoto et al., 2017; Ushio et al., 2018) and 16S rRNA (Shaw et al., 2016; Berry et al., 2017; Jeunen et al., 2018; Stat et al., 2018)

Choosing a marker for species diversity detection can be difficult because the performance of eDNA metabarcoding primers is different for different geographic regions (Shu et al., 2021). It is therefore necessary to test primers before commencing with a field survey (Shu at al., 2021). Furthermore, different primers may be biased to certain taxonomic groups therefore, to overcome primer bias resulting from using a single genetic marker and to increase the probability of species detection, many studies have employed the multiple genetic markers approach (Evans et al., 2017; Hanfling et al., 2016; Olds et al., 2016; Shaw et al., 2016; Shu et al., 2020). Universal primers are specifically designed to target diverse taxa through metabarcoding whereas specific primers target a single species (Shu et al., 2020). Various universal fish primers have been designed for 12S rDNA, Cytb, 16S rDNA, 18S rDNA, and COI (Wang et al., 2021). Several studies that target fish, have compared different eDNA metabarcoding primer sets (Bylemans et al., 2018, Collins et al., 2019, Evan et al., 2017, Shaw et al., 2017). In a recent study that evaluated the efficacy of 22 published metabarcoding primer sets covering different gene regions using in silico and in vitro analysis on freshwater fish in China, it was found that 12S rRNA primers outperformed 16S rRNA primers and CO1 primers (Zhang et al., 2020). Three 12S primer sets (MiFish_12S, Riaz_12S and Valentini_12S) one 16S primer set (Berry_16S), and one 18s primer set (MacDonald_18S) were compared, and Riaz_12S detected more species, followed by Berry-16S (Girish et al., 2022).

2.6.6 Bioinformatics

Following PCR, processing sequence data is another crucial step in eDNA metabarcoding, as biodiversity estimation relies on the stringency of bioinformatics (Evans et al., 2017; Xiong et al., 2022). Next gen sequencing generally yields thousands or millions of raw DNA sequence reads from each sample that often has random errors which are introduced during the amplification and sequencing process (Valdés, 2017). The raw reads must therefore be processed to produce reliable sequences that can be used to answer the main research question (Galhardo et al., 2018). Raw data processing in fish metabarcoding includes, sequence merging and trimming, quality filtering settings removing short and low-quality reads, removing chimeras and singletons, and sequence dereplication (Xiong et al., 2022). All these steps can be carried out by either denoising or clustering (Porter and Hajibabaei, 2020; Brandt et al., 2021). These procedures are different in that, denoising involves detecting sequences that contain errors and merging them with the correct original sequence to produce amplicon sequence variants (ASVs), whereas clustering involves combining sequences into meaningful biological entities known as operational taxonomic units (OTUs) without taking into consideration if they have errors or not

(Brandt et al., 2021). OTUs are generated based on a predefined similarity threshold ranging from 97% to 99% making them representative of the same species (Parada et al., 2016). ASVs on the other hand are generated based on sequence variance presence and are considered to be a more fined-grained taxonomic characterisation (Chiarello et al., 2022).

Whether OTUs or ASVs are more appropriate for metabarcoding data analysis is still a controversial topic. Some researchers argue that ASVs should be the standard because they have high resolution and can be reused across studies, while others believe that different genetic markers such as COI require clustering in order to conform to the biological species concept (Antich et al., 2021; Gielings et al., 2021). Antich et al., (2021) suggested that using clustering is beneficial for long-term biomonitoring studies since it reduces the data set, eases pooling, or makes it easy to compare studies. However, with COI the downfall of using denoising only is that a lot of intraspecific diversity is lost (Zizka et al., 2020; Antich et al., 2021). Some authors suggested that Denoising should be done within molecular operational taxonomic units (MOTUs) after clustering so that the right context of sequence variation and abundance skew will be produced (Antich et al., 2021). (Laroche et al., 2020; Brandt et al., 2019) Performed denoising first and then clustering and refining steps. Choosing between these two approaches is therefore dependant on the type of study, marker and bioinformatics tools available.

The standardization of bioinformatic in a pipeline is a more preferred choice of carrying out bioinformatics because it produces results that are of good quality and that are reproducible, however, different studies may require to use of a combination of bioinformatics tools (Xiong et al., 2022). There are several Denoising and clustering algorithms available to generate OTUs or ASVs. The most common ones include UNOISE (Edgar, 2016), Swarn (Mahé et al., 2014), Deblur (Amir et al., 2017), and DADA 2 (Callahan et al., 2016). Mbareche et al., (2020) suggested that these clustering algorithms must be combined with other tools because they can only filter PCR and sequencing errors but cannot remove inter-sample contamination, chimera, and tag-jump which can be performed by other tools such as QIIME2; Mothur, PERMA and SLIM. Each bioinformatics tool has its own pro and cons; therefore, a pipeline should be chosen based on its ability to fulfil the studies goal (Hakimzadeh et al., 2023).

2.6.7 Reference database

After raw Data filtration the generated OTUS or ASVs must be queried on a public data base in order to translate the sequences into biological species for further analysis (Schenekar et al., 2020). The most commonly used databases are National Center for biotechnology Information (NCBI) GenBank and Barcode of Life Data System (BOLD) (Collins et al., 2021). NCBI has been historically used to search nucleotide sequences and to store data from different sources such as GenBank, RefSeq and PDF databases (Agarwala et al. 2018; Galhardo et al., 2018). Later,

more sequence data bases were developed, including those that include more sources for sequence matching such as BOLD (Ratnasingham and Hebert, 2007; Galhardo et al., 2018**).** BOLD stores mostly DNA sequences of the COI gene and molecular morphological and distributional data for eukaryotes (Galhardo et al., 2018). OTUS or ASVs can be queried directly from these data bases through online tools such as BLAST (Collins et al., 2021). The completeness and accuracy of the reference data base directly affects the success of metabarcoding (Boyer et al., 2016, Machida et al., 2017; Gold et al., 2021) Unfortunately, despite the global effort to enrich the public reference data base, species sequences available in the public database is far from complete due to the enormous diversity worldwide, with about 32 000 known fish species in the aquatic environment worldwide (Nelson et al., 2016; Miya, 2022).

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CHAPTER 3: METHODS

3.1 Ethical clearance and sampling permit

Ethical clearance was not required for this study. eDNA sampling was conducted under the Oceans and Coastal Research and Fisheries Research and Development permit (permit number: RES2023-35), issued under section 83 of the Marine Living Resources Act 1998 by the South African Department of Forestry, Fisheries and the Environment (DFFE). All sampling followed the rules and guidelines set by the DFFE, with minimal impact on the environment.

3.2 Study site

This study was conducted inside the uThukela MPA (29°16.5′S 31°46.5′E), located between Durban and Richards Bay, and on selected unprotected areas outside the MPA (Figure 1). Sampling was carried out in the winter of 2021 (June) and 2023 (June-July), because of favourable sampling conditions (calmer water) in the sampling area around this time. In 2021, sampling was set to focus on thirteen reef stations, spanning 47-100 m in depth, with nine reefs inside (Dunford Hats, Pats Paride, Lens Ledge, Amatikulu HPM, Carpnters and Tongaat Pinnacles, Zini Hard Edge and Ballito Birthday Sundowners) and three reefs outside the uThukela MPA (Rosie Snowball, Umdloti Kob, Umdloti steep) (Table 3.1). However, Grumpy Reef could not be sampled due to unfavourable sampling conditions. The sampled reefs were selected to represent the MPA's four management zones which are, Controlled Bottom North, Controlled Bottom South, Controlled Pelagic, Restricted zone, and outside the MPA (Table 3.1). Each management zone was represented by at least 3 reefs. In 2023, the sampling sites were refined by adding one more reef (Zinkwazi HPM) to get a more accurate representation of the MPA.

Table 3.1: Sampling stations' coordinates and management zones represented by reefs inside and outside the uThukela MPA in 2021 and 20223.

			2021			2023		
Reef name	Depth (m)	Management Zone	Latitude (S)	Longitude (E)	Date	Latitude (S)	Longitude (E)	Date
Rosie Snowball	67	Outside the MPA	29°81.00	31°18.600	10/06/2021	29°48.990	31°11.160	04/07/2023
Umdloti Kob	50	Outside the MPA	29°73.5106	31°18.9521	11/06/2021	29°43.781	31°11.160	26/06/2023
Umdloti Steep	53	Outside the MPA	29°71.9497	31°21.1000	11/06/2022	29°42.282	31°12.720	25/06/2023
Dunford Hats	54	Controlled Bottom North	29°00.8185	32°13.1296	12/06/2023	29°00.414	32°07.873	01/07/2023
Lens Ledge	47	Controlled Bottom North	29°20.1931	31°94.3288	14/06/2026	29°12.397	31°56.267	02/07/2023
Amatikulu HPM	54	Controlled Bottom North	29°30.5000	31°82.7000	15/06/2029	29°18.090	31°49.319	03/07/2023
Pats Paradise	52	Controlled Pelagic	29°15.5993	32°02.5066	13/06/2024	29°09.787	31°01.823	01/07/2023
Zini Hard Edge	100	Controlled Pelagic	29°10.993	32°03.836	13/06/2025	29°10.814	32°03.691	01/07/2023
Grumpy Reef	48	Restricted				29°15.479	31°39.904	03/07/2023
Carpenters	52	Restricted	29°31.0900	31°83.5045	14/06/2027	29°18.591	31°57.334	02/07/2023
Sundowners	62	Restricted	29°39.8150	31°83.5045	15/06/2028	29°23.458	31°50.577	02/07/2023
Ballito Birthday	54	Controlled Bottom South	29°49.0432	31°39.1789	16/06/2030	29°29.523	31°23.882	30/06/2023
Tongaat Pinnacles	56	Controlled Bottom South	29°52.1000	31°35.8000	16/06/2031	29°31.234	31°21.772	27/06/2023
Zinkwazi HPM		Controlled Bottom South				29°23.625	31°33.032	30/06/2023

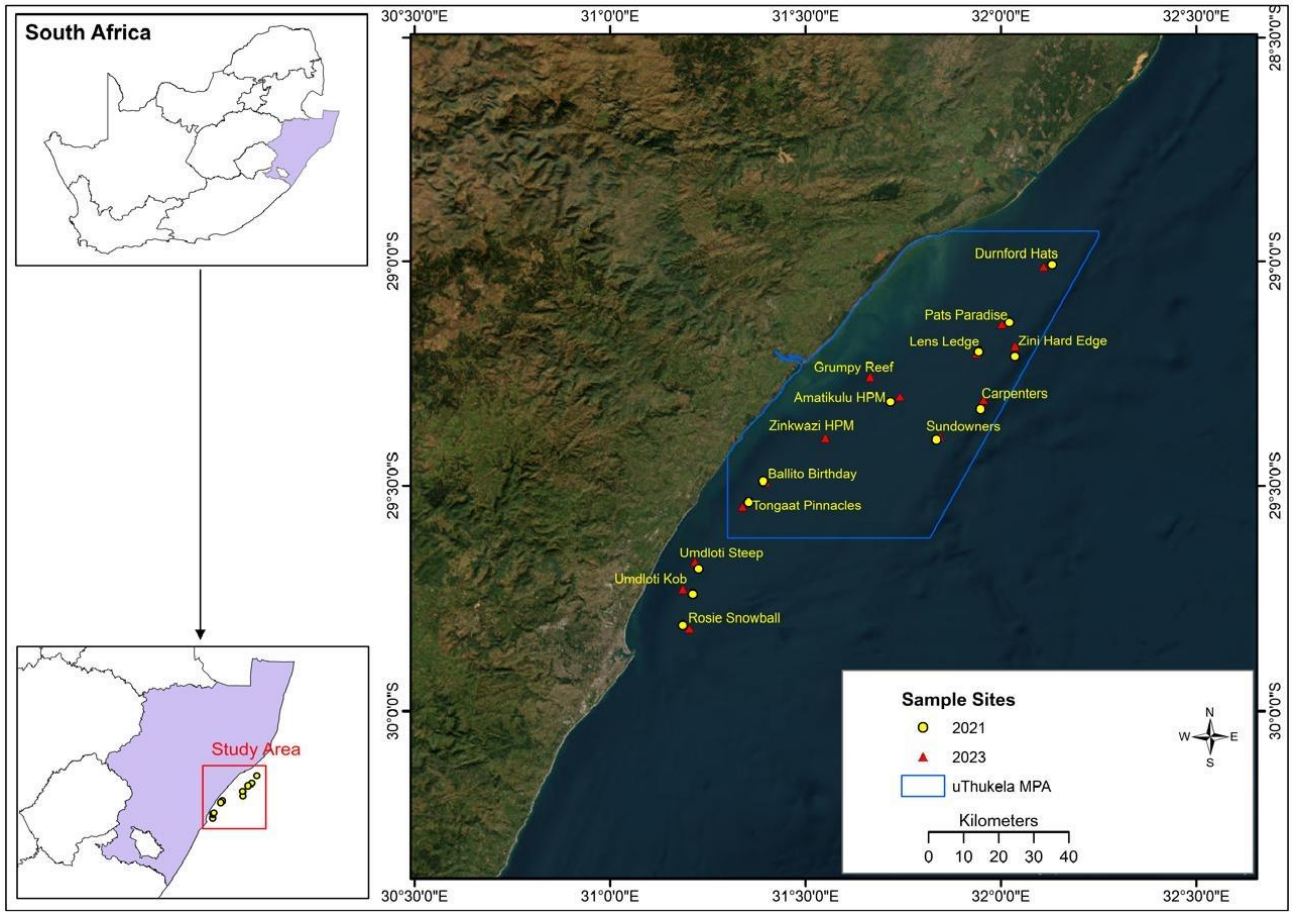


Figure 3.1: The location map of the fourteen sampling stations inside the uThukela MPA (Dunford Hats, Pats Paradise, Lens Ledge, Amatikulu HPM, Carpenters and Sundowners) and on the selected unprotected areas adjacent to the uThukela banks MPA (Rosie Snowball, Umdloti Kob, and Umdloti Steep) in KwaZulu-Natal on the east coast of South Africa in 2021 and 2023.

3.3 Field sampling

Water samples were collected from the Angra Pequena Research Vessel at twelve reef stations in 2021 and fourteen reef stations in 2023 located within the uThukela MPA and outside the MPA (figure 1). Outside the MPA representing the fished zone represented by 3 reefs with no restrictions at all. Sampling was done over seven days, from the 10th to the 16th of June 2021 and from the 25th to the 4th of July 2023. At each sampling station, seawater was collected from three distinct depths: 5 m 10 m, 15 m, 20 m, 25 m, 30 m, 35 m, 40 m, and 50 m above the sea floor to represent the whole depth gradient in 2021 (Stauffer et al., 2021). In 2023, the sampling protocol was refined to only include 5m, 10m, and 40m only representing the top, middle, and bottom layers of the water column to check how a reduced sampling effort of three replicates compares with sampling every 5m in 2021 and ultimately determine the efficient sampling effort. Six sterile 10-litre Niskin bottles mounted on a rosette sampler were used to collect water samples, with each bottle programmed to collect water at a specific depth. Prior to use, Niskin bottles were sterilized with 10% bleach and 70% ethanol using the 250 ml wash bottles and then flushed with filtered water to prevent cross-contamination between samples. A CTD device, also mounted on a rosette sampler, was used to measure the salinity, temperature, dissolved oxygen, and turbidity of the water at each sampled depth. The chlorophyll *a* data had some errors and was excluded completely from the environmental data. The collected seawater was transferred to 2-liter plastic bottles that had also been sterilized and flushed. The SeatermAF © V2 version 2.1.4 was used to program the Niskin bottles and the CTD device and to download the environmental data generated by the CTD device. The water samples were then filtered through a sterile analytical vacuum flask fitted with 0.4 µm pore-size hydrophilic immediately after collection, to minimize eDNA degradation. A vacuum pump was attached to the glass filtration assembly to facilitate filtration. A total of 6 litres per station representing 2 litres per depth and filter was filtered from the environmental samples. After filtration, sterile forceps were used to remove filters from funnels and to place them in sterile 5 ml screw-cap tubes with 70% ethanol. Samples were labelled with the year in which they were collected, reference number from the CTD device, and station from which they were taken from. Finally, tubes containing the filters were stored at room temperature for one year in 2021 and two weeks in 2023 until DNA extraction could be performed. One sterile and unused filter paper was used to represent a field blank per site. Each field blank was stored in the same conditions as the samples.

3.4 DNA extraction

All DNA extractions were conducted in the molecular biology laboratory designated for eDNA for the duration of the study. All the equipment and surfaces to be used were sprayed and wiped with 70% ethanol and 10% bleach prior to each extraction session to reduce the risk of contamination. DNA was extracted from filters following the standard protocol of the Qiagen DNeasy® Blood & Tissue kit following the manufacturer's protocol with minor modifications (QIAGEN, Hilden, Germany). The stored filter membranes, each representing 2 litres of sampled water, were cut into small pieces using

sterile scissors and placed into 1.5ml centrifuge tubes. DNA was extracted from the small pieces of filter paper following the manufacturer's protocol, with final illusion repeated twice for optimal DNA yield.

3.5 PCR amplification

Fish specific primers, Leray-XT/f 5'GGWACWRGWTGRACWITITAYCCYCC 3' and Lera.y-XT/r 5'TAIACYTCIGGRTGICCRAARAAYCA 3' (Wangensteen et al. 2017) targeting a region of the mitochondrial COI gene were used. PCR amplification followed a nested PCR approach, with the first PCR performed using the above-mentioned primer set and second PCR amplification was performed using the same primers but modified to include illumina Nextera® overhangs attached (Forward overhang: 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG 3', Reverse overhang: 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG). Each sample was amplified in a 25µl reaction, with each PCR reaction containing, 1 µl of forward and reverse primer, 0.16ul of 0.1 mg/mL Bovine Serum Albumin (BSA), 12.5 µl One Taq® 2X master mix with standard buffer, supplied by the New England BioLab®, 3.34 µl of ultra-pure water (New England BioLab®), and 2 µl of 10ng/ul genomic DNA. PCR conditions were as follows: 95°C for 10min of initial denaturation followed by 35 cycles at 95°C for 1min, 45°C for 1min, 72°C for 1min, and a final extension at 72°C for 5 min. Each PCR run included one negative control and one extraction blank sample. The second PCR followed the first PCR reaction with the following PCR conditions: 95°C for 5 min of initial denaturation followed by 45 cycles at 95°C for 30s, 45°C for 45 min, 72°C for 1min, and a final extension at 72°C for 10 min.

3.6 Sequencing

To prepare PRC products for sequencing, 10µl of each replicate for each sample including field blanks were pooled and sent for sequencing where they were first quantified on a Qubit 4.0 instrument (Life Technologies) and were purified using the AmpureXP purification beads (Beckman Coulter, High Wycombe, UK). High throughput sequencing was performed on an Illumina Miseq™ platform at the KZN Research Innovation and Sequencing Platform (KRISP).

3.7 Bioinformatics analysis

Raw sequences were demultiplexed using DRAGEN v3.10.12 to assign each sequence to its original sample based on the unique index assigned to sequences of each sample during PCR. Only sequences that matched the primer and sequence adaptor at a 100% level were kept for further processing.

The subsequent sequence filtering was carried out on the DADA2 pipeline, which removes primers, adaptors, singletons, and chimeras (Callahan et al., 2016) in R Studio v139. DADA2 also filters for errors, dereplicates sequences, removes sequences containing ambiguous bases, and denoises the data (Callahan et al., 2016). Filtering was based on a maximum allowable error of 0.1, and a minimum length of 150 bp for paired sequences. Sequence quality was also checked on DADA2, retaining only good quality reads, and then clustering them into Amplicon sequence variants (ASVs) based on the exact

sequence match (Callahan et al., 2016). ASVs' ability to distinguish sequences differing by one nucleotide makes them a preferred option (Mbareche et al., 2020).

3.8 Taxonomic identification

ASVs were identified to species level using custom reference library/database of fish species that have been identified in South Africa, both endemic and not endemic (both marine and freshwater species) downloaded from BOLD. Species assignment was done on DADA2 after sequences were clustered into ASVs.

3.9 Data analysis

3.9.1 Environmental variability

To test if environmental variables varied significantly between groups (depths, reefs, management zones, inside and outside the MPA and years), a Kruskal-Walli's rank sum test was performed between groups on R studio v139. The normality test which confirms if the environmental variables' data is normally distributed was conducted using the Shapiro-Wilk Test, and the assumption was satisfied ($P>0.05$).

3.9.1 Diversity analysis

3.9.1.1 Alpha diversity

All alpha and beta diversity analyses were carried out based on ASVs, using phyloseq v1.28.0 (McMurdie and Holmes, 2013) and Vegan v2.6-4 (Wickham, 2006) respectively on R Studio v139. Alpha diversity, which measures diversity within a specific group or sample, was quantified and compared at various spatial and temporal scales (between depths, reefs, management zones, inside and outside the MPA, and between 2021 and 2023) by estimating the Observe, Abundance-based Coverage Estimator (ACE), Shannon (Keylock, 2005) Fisher and Simpsons indices of diversity (Keylock, 2005). The Observed and ACE reflect species richness, with the ACE accounting for the presence of rare species (Kim et al., 2017), while the Shannon and Fisher indices consider the overall diversity. The Simpsons index places greater weight on species evenness, giving insight into species distribution (Schloss and Handelsman, 2006). In the current study, the observed, and ACE were used to infer species richness, while the Fisher and Shannon were used to infer the overall diversity. The Simpsons index was used to infer species evenness. The species abundance used to quantify the alpha diversity indexes was estimated from the number of sequences counted for each ASV. Read count is treated as a proxy for abundance because of the correlation between read counts and the number of individuals per sample (Patuzzi et al., 2019). Boxplots were used to visualize differences in alpha diversity between different groupings of the data set.

3.9.1.2 Beta diversity

Beta diversity, which estimates how much community composition varies across groups, was estimated by calculating the Bray-Curtis Dissimilarity Index which measures community dissimilarity by testing for differences in community similarity variances on the index scores (Bray & Curtis, 1957), using adonis2 in vegan v2.6-4 (Oksanen et al., 2013). The Bray-Curtis Dissimilarity Index is bounded between 0 and 1, where 0 means the two samples have the same taxonomic composition and 1 means they do not share any taxa. Before performing beta diversity analysis, the ASV data was transformed using the Hellinger transformation to account for the sparsity of ASV data caused by many zeros in the abundance table. The distance-based redundancy analysis (db-RDA) and the permutation test based on the ANOVA were used to test if the differences observed between groups were of biological importance. ASV data was tested for the assumption of normality using the Shapiro-Wilk Test, and the assumption was not satisfied. It is important to note that the PERMONVA test is the most popular and preferred method for comparing groups in ecological and metabarcoding studies, however, it could not be used in the current study because the assumption of homogeneity of variances in the ASV data was not satisfied. Differences in Beta diversity between groups were visualized on the non-metric multidimensional scaling (NMDS) plot by checking the clustering of samples between groups. The NMDS plot clusters samples that are more similar in community composition together and samples that are different further apart (Mbareche et al., 2020). To visualize overlaps in community composition between groups based on ASVs, a Venn diagram was constructed using the Venndiagram package (version 1.7.3) in R studio v139.

3.10 Taxonomic composition and relative abundance

Species composition and relative abundance were visualized on stacked bar plots to show how many species were dominant in each reef and sample and how abundant was each species detected per reef and sample.

3.11 Validity

The effect of environmental variables as individuals and as pairs on read length per sample before sequence filtering was tested using linear regression and a pairwise test respectively, after the Shapiro-Wilk Test satisfied the assumption of normality on the environmental and read length data. This was done to exclude the possible preferential degradation of eDNA template that happen as a result of environmental factors such that there are fewer intact fragments of the expected amplicon size for efficient amplification. The effect of environmental variables on community composition was tested using the db-RDA and ANOVA-based permutation test.

3.12 Sampling effort

A species rarefaction curve was constructed using the vegan package v2.6-4 on R studiov139 to determine if the sequencing depth and sample size were sufficient to fully capture the whole community diversity.

CHAPTER 4: RESULTS

4.1 Sequencing results

The high throughput sequencing yielded a total of 33 498 351 raw paired end reads from 75 samples across two sampling years, 2021 and 2023. After quality filtering, trimming, and removing chimeric sequences on the DADA2 pipeline (Callahan et al., 2016), 8 207 984 high-quality reads were retained. Sequence clustering generated 81 072 ASVs for downstream analysis.

4.2 Taxonomic identification

Taxonomic identification using DADA2 and a custom fish reference library downloaded from BOLD, identified only, 15 Orders, 17 Families, 20 Genus and 20 species at 95% Similarity threshold (Table 4.1). *Sardinella gibbose* was the most represented species with, followed by *Pseudomyxus capensis* with (Figure 4.17). 18 of the identified species have been previously documented in South Africa and other regions, while there was only 1 species endemic to South Africa (*Pseudomyxus capensis*).

Table 4.1: Taxonomic composition of identified AVSs across all sampling sites and years.

Taxonomic Level	Counts
Orders	10
Families	13
Genera	17
Species	20

4.3 Environmental variables

4.3.1 Spatial variability

The Kruskal-Walli's rank sum test found that in 2021, on a small scale, between depths, there was a significant variation in all environmental variables ($p < 0.05$) except for salinity ($H = 10.727$, df = 6, $p = 0.097$) (Table 4.2a). In 2023 a significant difference was observed only in dissolved oxygen ($H = 8.946$, df = 2, $p = 0.011$) between depths (Table 4.2a). Temperature ($H = 25.791$, df = 11, $p = 0.007$) and turbidity ($H = 27.169$, df = 11, $p = 0.004$) showed a significant difference between reefs, however, there was no significant in dissolved Oxygen and salinity ($H = 8.549$, df = 11, $p = 0.663$) (Table 4.2a). In 2023, all environmental variables showed a significant difference between reefs ($p < 0.05$) (Table 4.2a). In 2021, environmental variables between management zones showed a similar pattern as that of reefs in 2021, with no significant difference between dissolved oxygen ($H = 1.883$, df = 5, $p = 0.865$) and salinity ($H = 6.603$, df = 5, $p = 0.252$), while temperature ($H = 18.252$, df = 5, $p = 0.002$) and turbidity ($H = 13.869$, df = 5, $p = 0.016$) varied significantly (Table 4.2a). In 2023, only salinity had a significant difference between management zones ($H = 7.966$, df = 4, $p = 0.093$) (Table 4.2a). On a larger spatial scale between inside and outside, there was a significant difference in temperature ($H = 4.168$, df = 1, $p = 0.04$) and turbidity ($H = 5.337$, df = 1, $p = 0.021$) and no significant difference between in salinity ($H = 0.209$, df = 1, $p = 0.647$) and dissolved oxygen ($H = 0.488$, df = 1, $p = 0.485$) in 2021 (Table 4.2a). A similar pattern was found in 2023, with temperature ($H = 16.523$, df = 1, $p = 4.807e-05$) and turbidity ($H = 8.219$, df = 1, $p = 0.004$) having a significant difference between the inside and the outside of the MPA (Table 4.2a).

4.3.2 Temporal variation in environmental variables

Environmental variables showed no significant difference in dissolved Oxygen ($H = 0.18401$, df = 1, $p = 0.6679$) and turbidity ($H = 1.952$, df = 1, $p = 0.162$), while temperature ($H = 8.401$, df = 1, $p = 0.00375$) and salinity ($H= 5.270$, df = 1, $p = 0.022$) showed a significant difference between 2021 and 2023 (Table 4.2b).

4.3.3 Effect of environmental variables on read length

Environmental variables (temperature, salinity, turbidity, and dissolved oxygen) had no significant effect on read length both as individuals (Table 4.2a), pairs (Table 4.2b), confirmed by the linear regression and pairwise test ($p > 0.05$), indicating that differences in read length cannot be attributed to environmental variables alone. This is also evident on the linear regression plot, showing a weak correlation between environmental variables and read length (Figure 4.2).

Table 4.2: Summary table of the Kruskal-Wallis rank sum test for differences in environmental variables (Temperature, Salinity, Turbidity and dissolved Oxygen) between (a) depth, reefs, management zones, and inside and outside the MPA, and (b) years in uThukela MPA and selected none protected areas outside the MPA in 2021 and 2023.

(a)

		Depths			**Reefs**			**Management Zones**			**Inside and outside the MPA**		
Year	**Environmental variables**	***H***	**df**	***p***	***H***	**df**	***p***	***H***	**df**	***p***	***H***	**df**	***p***
2021	Temperature (°C)	25.791	11	0.007	18.252	5	0.003	18.252	1	0.003	4.168	1	0.041
	Turbidity (TNU)	27.169	11	0.004	13.869	5	0.016	13.869	1	0.016	5.337	1	0.021
	Salinity (ppt)	17.606	11	0.091	6.603	5	0.252	0.2519	1	0.252	0.209	1	0.648
	Dissolved Oxygen (mg/L)	8.5499	11	0.663	1.883	5	0.865	0.8651	1	0.865	0.488	1	0.485
2023	Temperature (°C)	28.051	13	0.009	19.275	4	0.001	19.275	1	0.001	16.523	1	4.81E-05
	Turbidity (TNU)	32.881	13	0.002	12.866	4	0.012	12.866	1	0.012	8.219	1	0.004
	Salinity (ppt)	29.933	13	0.005	7.966	4	0.093	7.9664	1	0.093	0.059	1	0.808
	Dissolved Oxygen (mg/L)	22.523	13	0.048	13.176	4	0.010	13.176	1	0.011	1.807	1	0.179

(b)

Environmental variables	***H***	**df**	***p***
Temperature (°C)	8.401	1	0.003
Turbidity (TNU)	1.952	1	0.162
Salinity (ppt)	5.270	1	0.021
Dissolved Oxygen (mg/L)	0.184	1	0.668

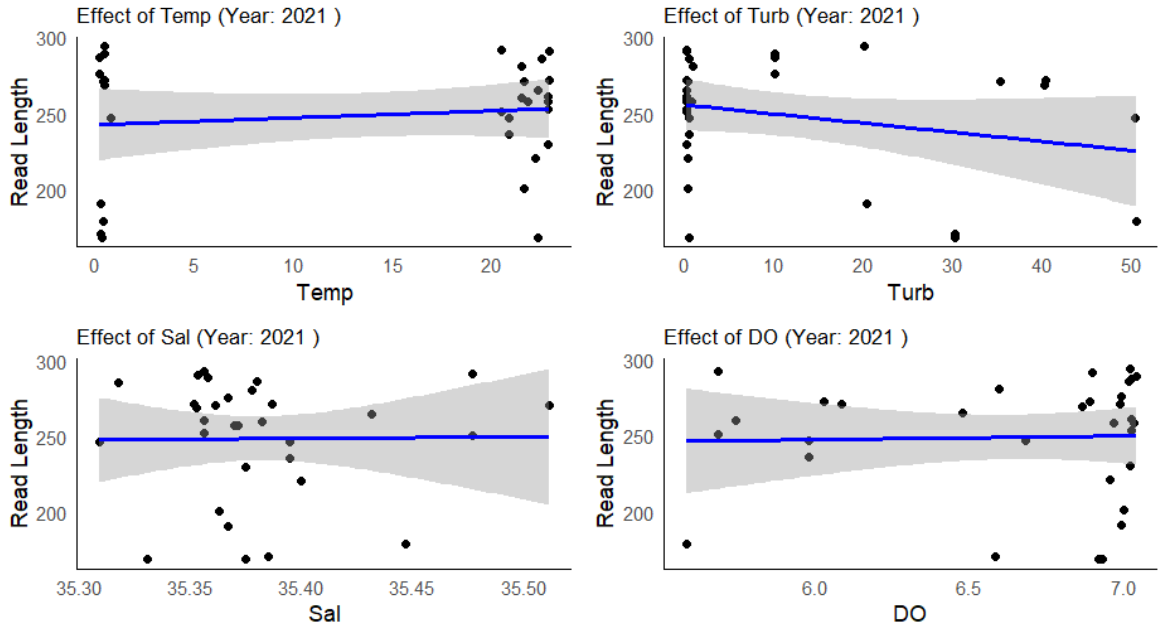
Table 4.3: Summary table for a (a) Linear regression analysis and a (b) pairwise comparison test for the effect of environmental variables of read length.

(a)

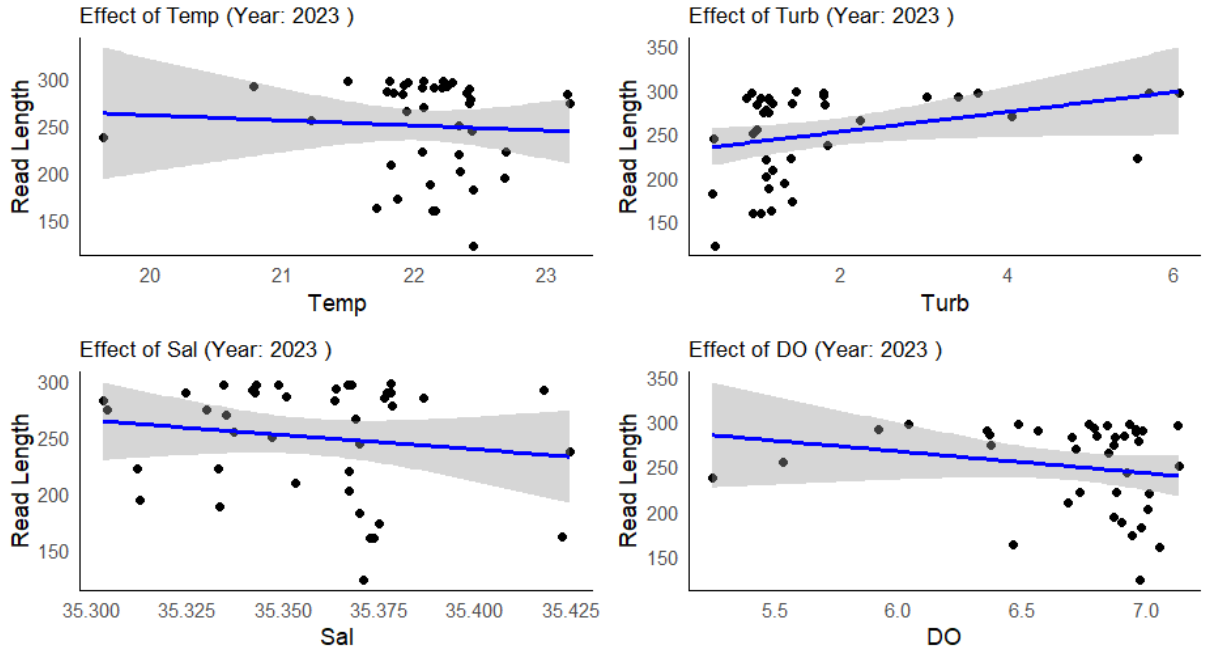
Year	**Environmental variables**	**F**	**df**	**R^2**	***p***
2021	Temperature (°C)	0.469	29	0.016	0.499
	Turbidity (TNU)	2.072	29	0.067	0.161
	Salinity (ppt)	0.004	29	0.000	0.948
	Dissolved Oxygen (mg/L)	0.028	29	0.000	0.868
2023	Temperature (°C)	0.156	38	0.004	0.695
	Turbidity (TNU)	4.243	38	0.100	0.046
	Salinity (ppt)	0.849	38	0.039	0.363
	Dissolved Oxygen (mg/L)	1.581	38	0.004	0.216

(b)

Year	Environmental variables	F	df	R^2	*p*
2021	Temperature (°C) and Turbidity (TNU)	1.462	28	0.094	0.249
	Temperature (°C) and Salinity (ppt)	0.231	28	0.016	0.795
	Temperature (°C) and Dissolved Oxygen (mg/L)	0.239	28	0.017	0.789
	Turbidity (TNU) and Salinity (ppt)	1.018	28	0.068	0.374
	Turbidity (TNU) and Dissolved Oxygen (mg/L)	1.008	28	0.067	0.378
	Salinity (ppt) and Dissolved Oxygen (mg/L)	0.048	28	0.003	0.953
2023	Temperature (°C) and Turbidity (TNU)	2.069	37	0.100	0.141
	Temperature (°C) and Salinity (ppt)	1.264	37	0.064	0.294
	Temperature (°C) and Dissolved Oxygen (mg/L)	2.486	37	0.066	0.097
	Turbidity (TNU) and Salinity (ppt)	2.486	37	0.119	0.097
	Turbidity (TNU) and Dissolved Oxygen (mg/L)	2.624	37	0.124	0.085
	Salinity (ppt) and Dissolved Oxygen (mg/L)	1.760	37	0.087	0.186



(a)



(b)

Figure 4.1: Scatter plot with correlation line using Spearman's Rank Correlation, showing the relationship between environmental variables (temperature, turbidity, salinity, and dissolved oxygen)

and average read length in 2021 (a) and 2023 (b) samples across all depths and reefs within uThukela MPA and outside the MPA.

4.4 Diversity Analysis

4.4.1 Alpha diversity

The results show that there was no significant variation in species richness (Figure 4.2a and b) and diversity (Figure 4.2c and e) between the three sampled depths across all richness and diversity indices (Observed, ACE, Shannon, Fisher) in 2021 and 2023. In 2021, species richness and diversity were highest at 5m (richness: Observed = 54, ACE = 56; diversity: Shannon = 3.43, Fisher = 14.6) and lowest at 40m (richness: Observed = 47.5 ASVs, ACE = 48; diversity: Shannon = 3.56, Fisher = 12.8) (Figure 4.2a, b c, and e). In 2023, there was a slight increase in diversity and species richness, at 10m and 40m and a decrease at 5m, with the highest diversity and species richness recorded at 10m (richness: Observed = 65, ACE = 66; diversity: Shannon = 3.77, Fisher = 15.8) and the lowest at 5m (richness: Observed = 46, ACE = 48.9; diversity: Shannon = 3.50, Fisher = 12.4) (Figure 4.2a, b c, and e).

The alpha diversity indices showed a noticeable spatial variation in species richness (Figure 4.3a and b) and diversity (Figure 4.3c and e) between reefs in 2021 and 2023. In 2021, diversity was high in reefs like Rosie Snowball, Ballito Birthday Reef, and Tongaat Pinnacles across all diversity indices, particularly Rosie Snowball, having the highest diversity (richness: Observed = 181 ASVs, ACE = 181; diversity: Shannon = 3.0, Fisher = 9.26) (Figure 4.3a, b c, and e). In contrast, the lowest species richness and diversity was recorded in Dunford Hats (Observed = 32.5 ASVs, ACE = 32.8; diversity: Shannon = 3.4, Fisher = 9.26) (Figure 4.3a, b c, and e). There was shift in species richness between 2021 and 2023, with reefs like Ballito Birthday, Carpenters, Danford Hats, Lens Ledge, Umdloti steep and Zini Hard edge showing an increase in species richness while reefs like Amatikulu HPM, Tongaat Pinnacles, Rosie snowball, Umdloti Kob, Zinkwazi HPM, and Sundowner showing a decrease in species richness and diversity (Figure 4.3a and b). The highest species richness and diversity in 2023 was recorded in Ballito Birthday (richness: Observed = 95, ACE = 97.2; diversity: Shannon = 4.1, Fisher = 24.9) and the lowest in Sundowners (richness: Observed = 36.5 ASVs, ACE = 39.1; diversity Shannon = 3.1, Fisher = 7.60) (Figure 4.3a, b c, and e).

Species richness (Figure 4.4a and b) diversity (Figure 4.4c and e) also varied between management zones in 2021 and 2023. The Controlled Bottom South zone had the highest diversity (richness: Observed = 69, ACE = 70.9; diversity: Shannon = 3.84, and Fisher = 15.6) in 2021 (Figure 4.4a, b c and e). In contrast, the Restricted zone had the lowest species richness and diversity (richness: Observed = 41.8, ACE = 43.6; diversity: Shannon = 3.21, Fisher = 9.97) in 2021 (Figure 4.4a, b c and e). In 2023,

species richness and diversity increased in all management zones, except outside the MPA. The Controlled Bottom South zone remained the zone with the highest diversity even in 2023 (richness: Observed = 85, ACE = 85; diversity: Shannon = 3.99, Fisher = 15.9), while the lowest was recorded in Controlled Pelagic zone (richness: Observed = 43.5, ACE = 43.9, diversity Shannon = 3.51, Fisher = 11.8) (Figure 4.4a, b, c, and e).

At a larger spatial scale, between inside and outside the MPA, species richness Figure 4.5a and b) and diversity (Figure 4.5c and e) was higher inside (richness: Observed = 52, ACE = 53.8; diversity: Shannon = 3.40 and Fisher = 12.1) than outside the MPA in 2021 (richness: Observed = 46, ACE = 47, diversity: Shannon = 3.62 and Fisher = 15.5) (Figure 4.5a, b, c, and e). In 2023, There was an increase in Species richness and diversity increased both, inside (richness: Observed = 59, ACE = 60.4, diversity: Shannon = 3.75 and Fisher = 14.9), and outside the MPA (richness: Observed = 49, ACE = 58, diversity: Shannon = 3.53 and Fisher = 14.6) (Figure 4.5a, b, c and e).

Species richness (Figure 4.6a and b), and diversity (Figure 4.6c and e) also varied on a temporal scale between 2021 and 2023) with the highest diversity recorded in 2023 (richness: Observed = 58, ACE = 60; diversity: Shannon = 3.73, Fisher = 14.7) and the lowest in 2021 (richness: observed = 50, ACE = 52.2, diversity: Shannon = 3.45, fisher = 12.9) (Figure 4.5a, b, c and e).

The Simpson index of diversity, which accounts for species evenness was relatively high at all spatial scales (depth, reef, management zones, inside and outside the MPA), ranging from 0.889 to 0.988, with no significant variation between depth in 2021 and 2023, indicating that species were evenly distributed in all reefs (Figure 4.2d, 4.3d, 4.4d,4.5d and 4.6d).

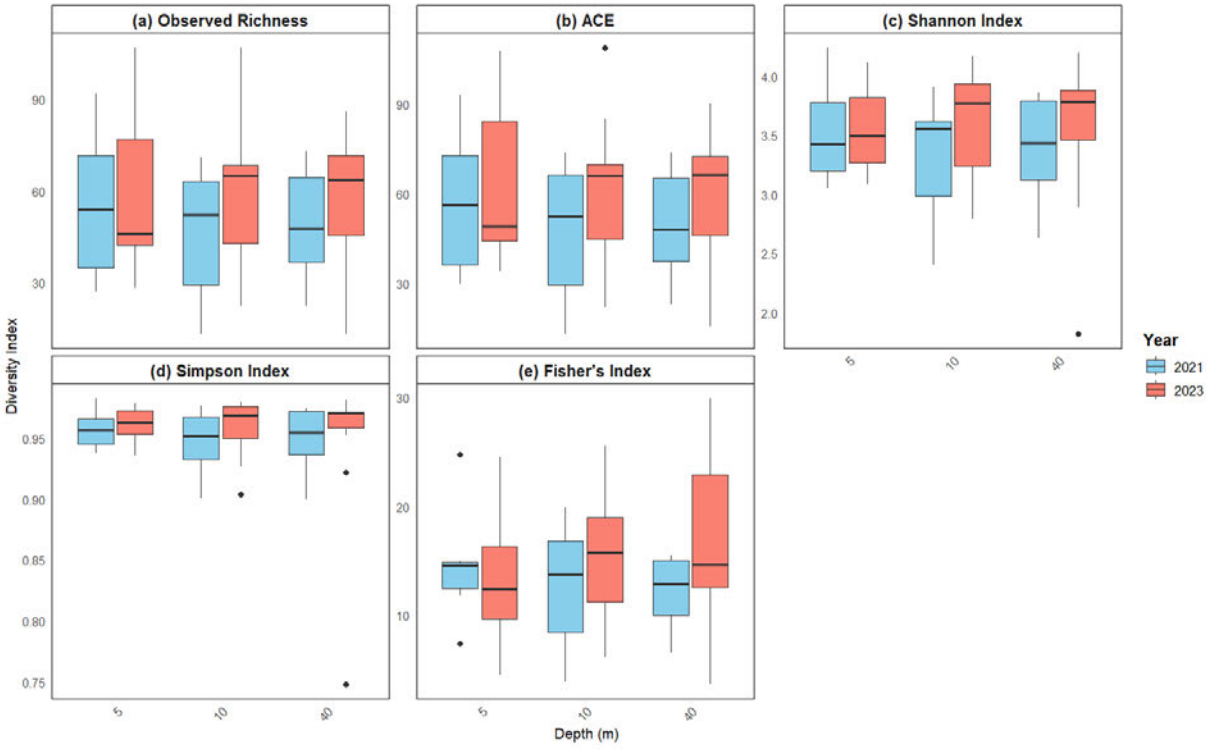


Figure 4.2: Alpha diversity indices, showing differences in species richness, diversity, and evenness between the three depths (5m, 10m and 40m above the sea floor) across 13 reefs in 2021 and 14 reefs in 2023 within uThukela MPA and in the adjacent unprotected areas outside the MPA.

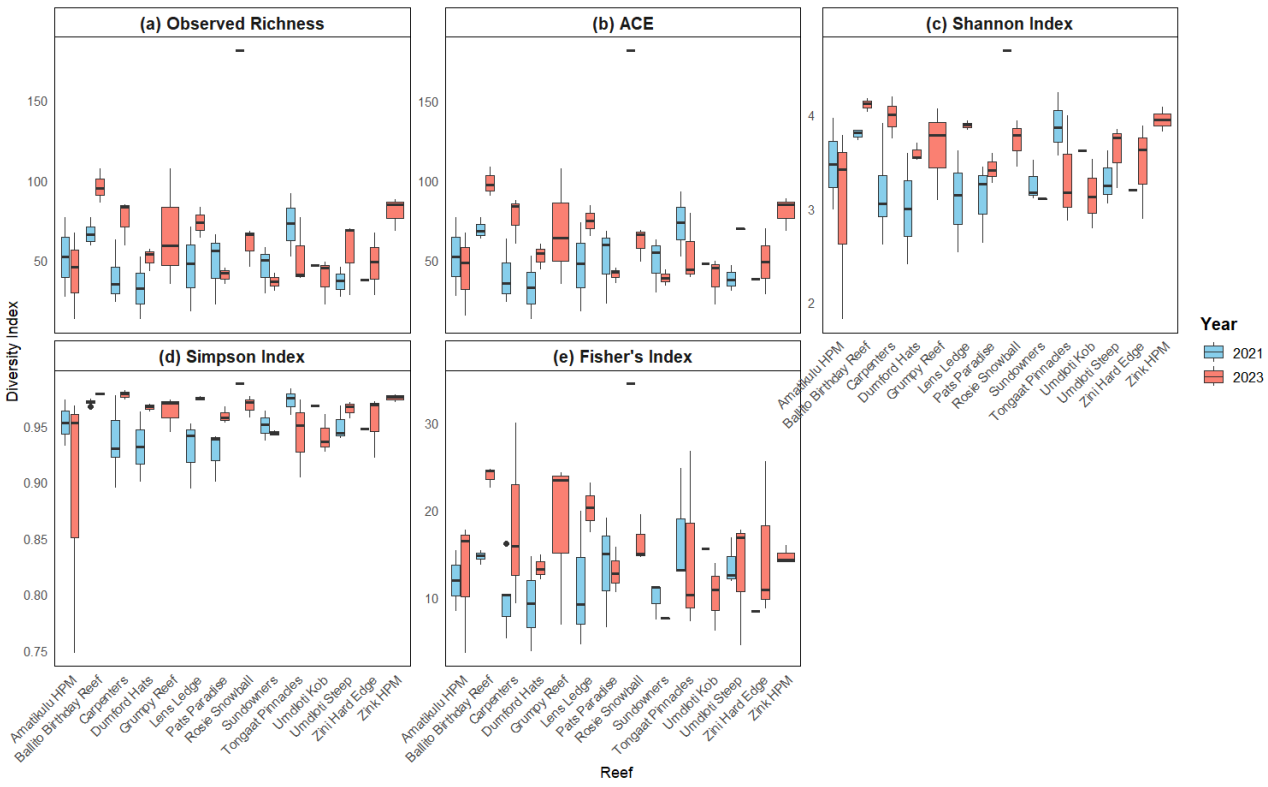


Figure 4.3: Alpha diversity indices, showing differences in diversity between 14 reefs across three depths in 2021 and 2023 in uThukela MPA and in the adjacent unprotected areas outside the MPA.

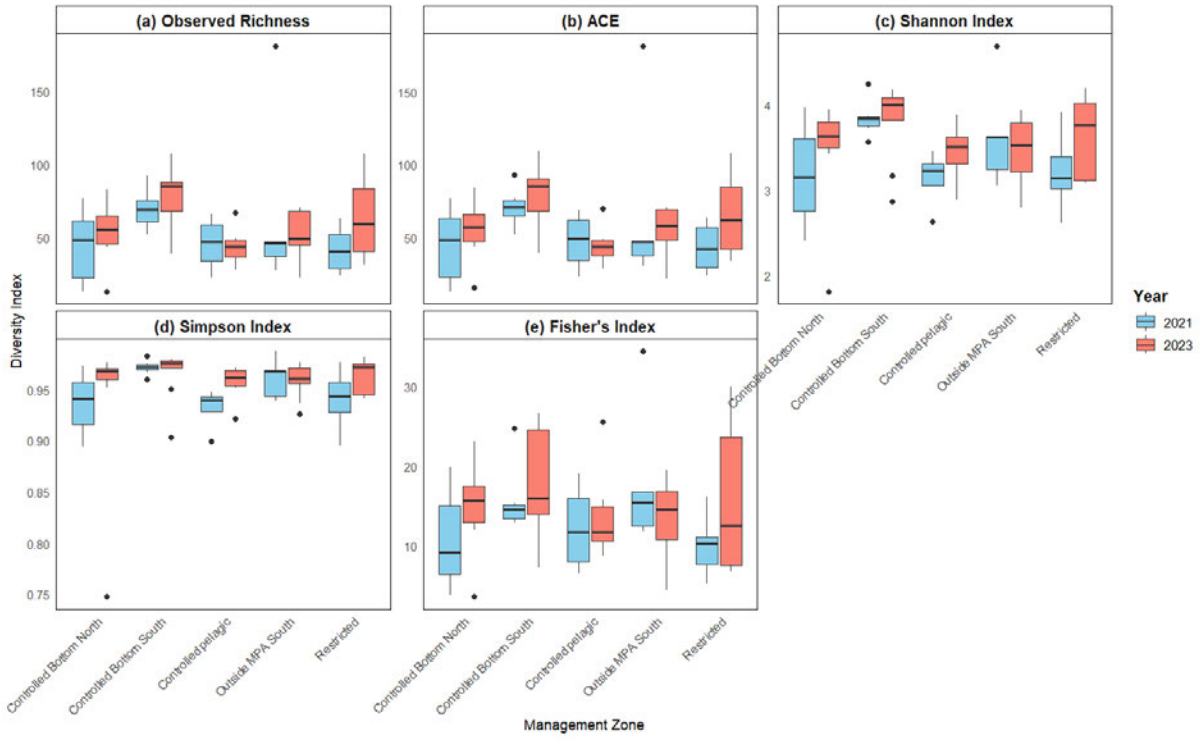


Figure 4.4: Alpha diversity indices, showing differences in diversity between five management zones across 13 reefs in 2021 and 14 reefs in 2023 depths in 2021 within uThukela MPA and in the adjacent unprotected areas outside the MPA.

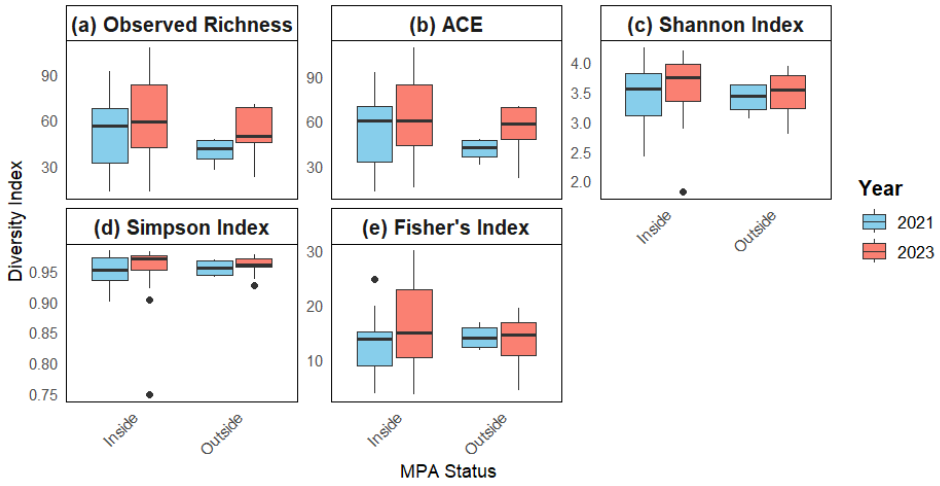


Figure 4.5: Alpha diversity indices showing differences in diversity between five management zones across 13 reefs in 2021 and 14 reefs in 2023 within uThukela MPA and in the adjacent unprotected areas outside the MPA.

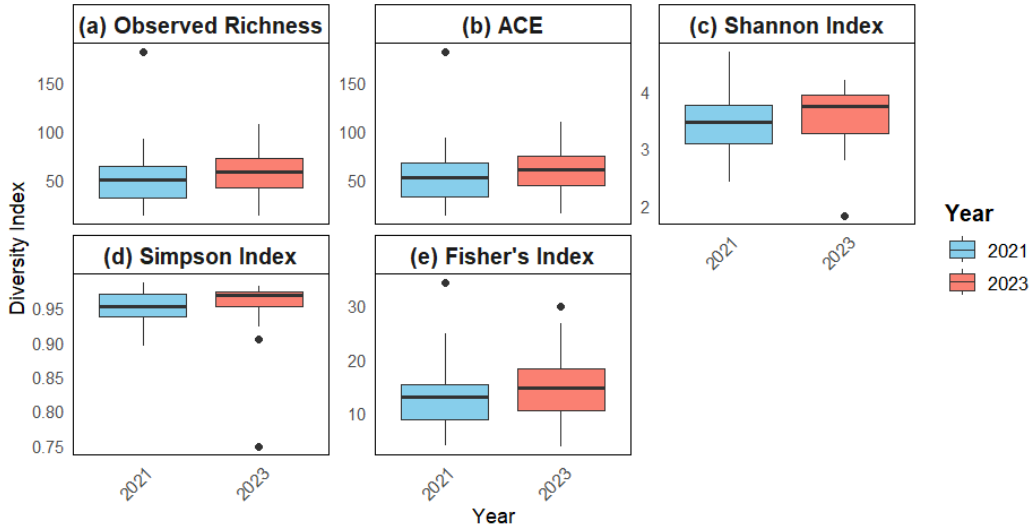


Figure 4.6: Alpha diversity indices showing differences in diversity across all sampled reefs and depths between 2021 and 2023 in the uThukela MPA and on the adjacent areas outside the MPA.

4.4.2 Beta diversity

There was a spatial variation in community composition associated with different depths across all reefs, with the highest community composition recorded at 10m, both in 2021 (168 unique ASVs) (Figure 4.7a) and 2023 (303 unique ASVs) (Figure 4.7b). The lowest community composition was

recorded at 5m above the sea floor in 2021 (112 unique ASVs) (Figure 4.7a) and 40m in 2023 (185 ASVs) (Figure 4.7b). There was minimal overlap between different pairs of depth, with communities from 10m and 40m having the highest number of shared ASVs (24 shared ASVs) and 10m and 5m and 5m and 40 having the lowest number of shared ASVs (7 shared ASVs) in 2021 (Figure 4.7a). In contrast, in 2023, the highest number of shared ASVs was between 10m and 5m (19 shared ASVs), and the lowest was between 5m and 40m (Figure 4.7b). Substantial overlap was observed between all three depths, indicating a high level of similarity in the community composition between the three depths in 2021 (67 shared ASVs) (Figure 4.7a). and 2023 (182 shared ASVs) (Figure 4.7b). The db-RDA test confirmed that the difference in community composition between different depths is not statistically significant in 2021 ($F = 0.6194$, $P = 0.981$) and 2023 ($F = 0.6847$, $P = 0.834$), indicating that the depth factor cannot be used to explain the variation in community composition or species assemblage in the water column across all sampled reefs both in 2021 and 2023. The NMDS plot shows a similar pattern, with no clear depth-related differences in community composition, as indicated by a poor clustering sampling according to depth in 2021 (Figure 4.7a) and 2023 (Figure 4.7b).

The db-RDA test shows that there was a significant difference in community composition between reefs across all depths, both in 2021 ($F = 1.4031$, $P = 0.013$) and 2023 ($F = 1.7598$, $P= 0.002$), indicating the differences between reefs have a significant effect on the community composition of reefs. The NMDS analysis based on the Bray Curtis dissimilarity distance, revealed a notable difference in clustering of samples at different reefs in 2021 (Figure 4.11a) and 2023 (Figure 4.11b)

The NMDS plot also shows a poor clustering of samples according to depth and reefs both in 2021 (Figure 4.9a) and 2023 (Figure 4.9b) indicating a lack of community structuring by depth and site. Samples from different depths and reefs show a range of community compositions across all depths and reefs. The db-RDA test confirmed that the combined effect of depth and reef was not statistically significant in 2021 ($F = 1.0293$, $P = 0.415$) (Figure 4.13b) and in 2023 ($F = 1.0862$, $P = 0.295$) (Figure 4.13b).

In 2021, the highest community composition was recorded in the restricted zone (204 unique ASVs) and the lowest in the controlled pelagic zone (69 unique ASVs) (Figure 4.9a). In 2023, the controlled bottom south zone (183 unique ASVs) had the highest community composition, although the controlled pelagic (109 unique ASVS) still had the lowest community composition (Figure 4.9b). There was a minimal overlap between all management zones, with only 39 shared ASV in 2021 (Figure 4.9a). and 57 shared ASVs in 2023 (Figure 4.9b). The difference observed in community composition between the MPA's management zones was confirmed statistically significant both in 2021 ($F = 1.3857$, $P = 0.019$) and 2023 ($F = 1.4417$, $P= 0.045$). This pattern can also be seen on the NMDS plot, which shows

some level of variation between samples from different management zones in 2021 (Figure 4.14) and 2023 (Figure 4.14b)

Community composition was consistently higher inside than outside the MPA in 2021 and 2023, with 609 unique ASVs inside the MPA in 2021 (Figure 4.9a) and 629 unique ASVs in 2023 (figure 4.9b). There was high overlap between communities inside the MPA and outside the MPA both in 2021 (87 shared ASVs) and 2023 (107 shared ASVs). The variation in community composition between inside and outside the MPA was not statistically significant either in 2021 ($F = 0.8638$, $P = 0.633$) or 2023 ($F = 1.1111$, $P = 0.293$), indicating that the observed variation in community composition inside and outside the MPA cannot be attributed to the MPA status only. The high similarity in community composition between inside and outside the MPA in 2021 (Figure 4.15a) and in 2023 (Figure 4.15b) is also evident on the NMDS plot, with many samples clustering closer to each other, indicating relatively similar communities.

There was significant variation in the overall community composition in the uThukela MPA and non-protected areas outside the MPA between 2021 and 2023 ($F = 1.7593$, $P = 0.02$). Community composition was higher in 2023 (731 unique ASVs) than in 2021 (722 unique ASVs) (figure 4.11). Despite the variation recorded, the overlap between 2021 and 2023 was notably high, with 151 ASVs common in both years (figure 4.11). Although the variation was significant, the NMDS plot also shows a strong overlap between samples from 2021 and 2023, indicating some level of community similarity (Figure 4.16).

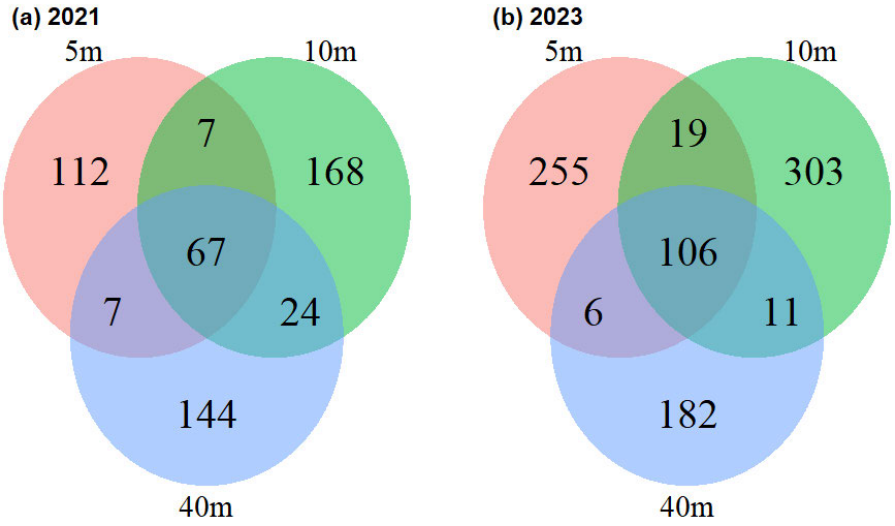


Figure 4.7: Venn diagram showing a total number of unique and shared ASVs obtained from three sampled depths (5m, 10m, and 40m above the sea floor) in 2021(a) and 2023 (b) across 15 reefs within the uThukela MPA and adjacent non-protected areas outside the MPA.

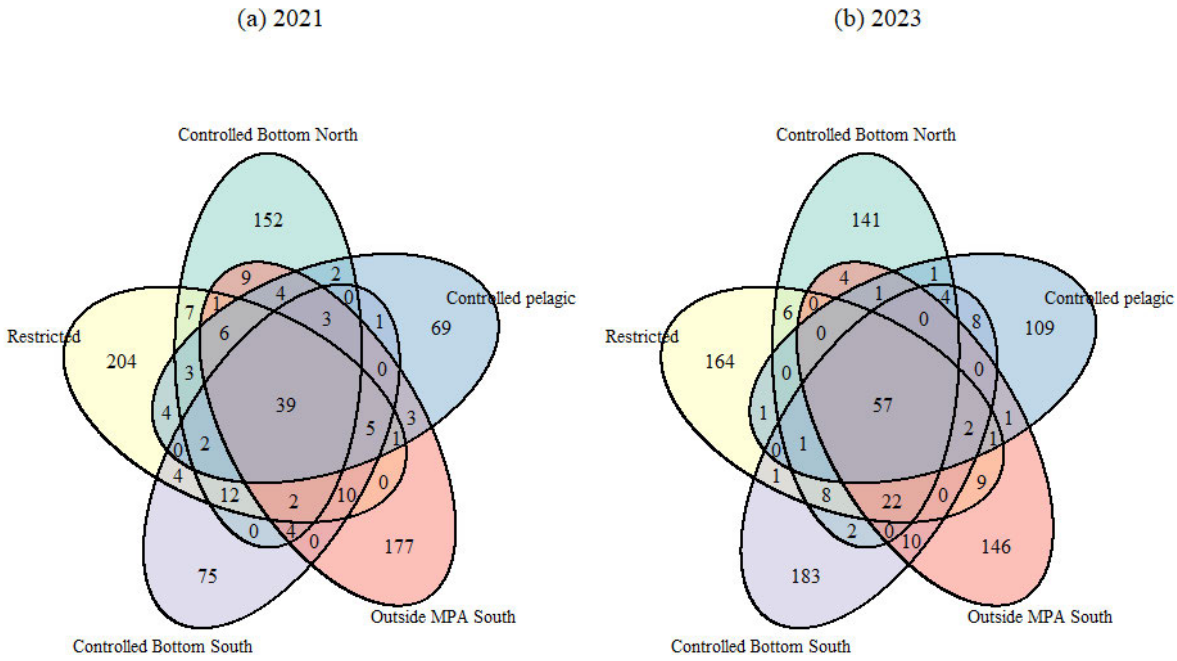


Figure 4.8: Venn diagram showing the total number of unique and shared ASVs between the uThukela MPA's management zones and the adjacent none protected areas outside the MPA 2021 (a) and 2023 (b) across fourteen reefs and three depths.

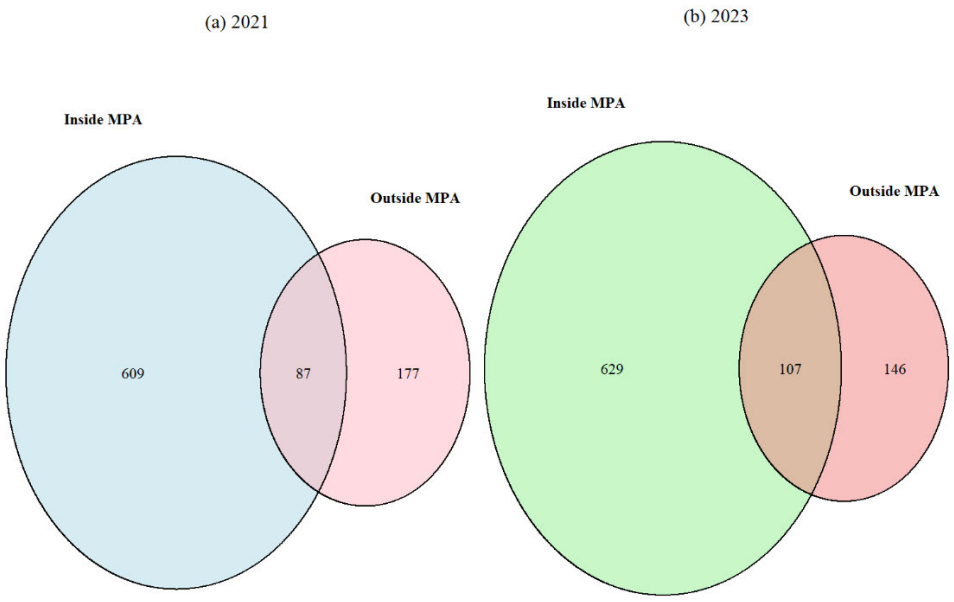


Figure 4.9: Venn diagram showing the total number of unique and shared ASVs between the area inside the uThukela MPA and the adjacent non-protected area outside the MPA 2021 (a) and 2023 (b) across fourteen reefs and three depths.

Figure 4.10: Venn diagram showing the total number of unique and shared ASVs between 2021 and 2023 across three depths and fourteen reefs with the uThukela MPA and adjacent none protected areas outside the MPA.

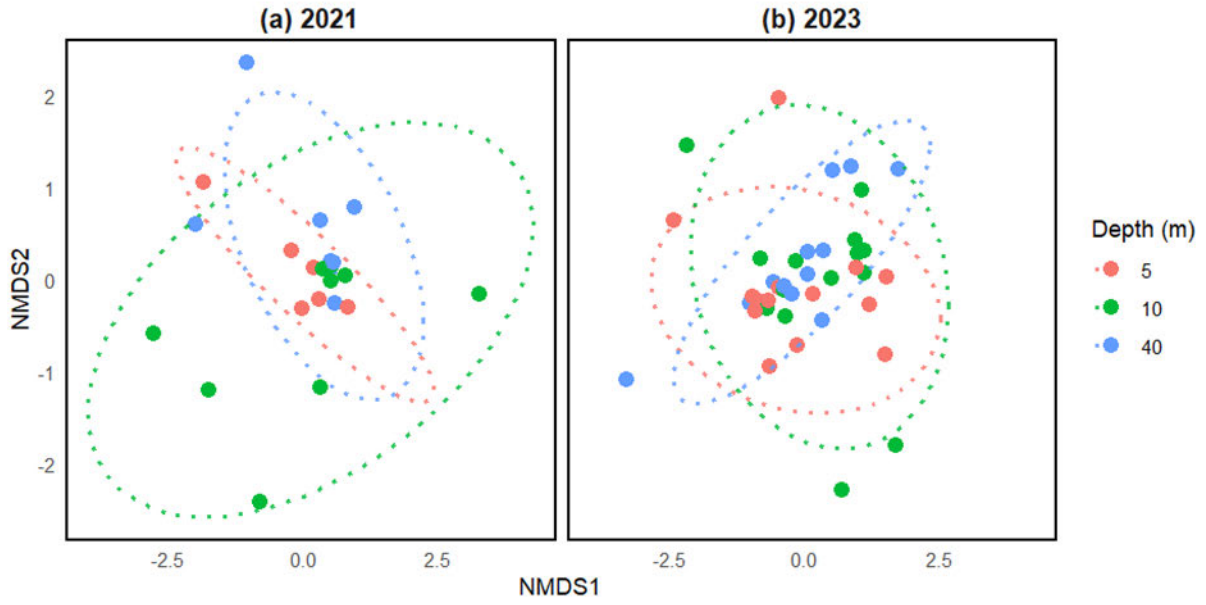


Figure 4.11: NMDS based on the Bray Curtis dissimilarity between depths (5m, 10m, and 40m) across 14 reefs within the uThukela MPA and on the adjacent areas outside the MPA in 2021 (a) and 2023 (b).

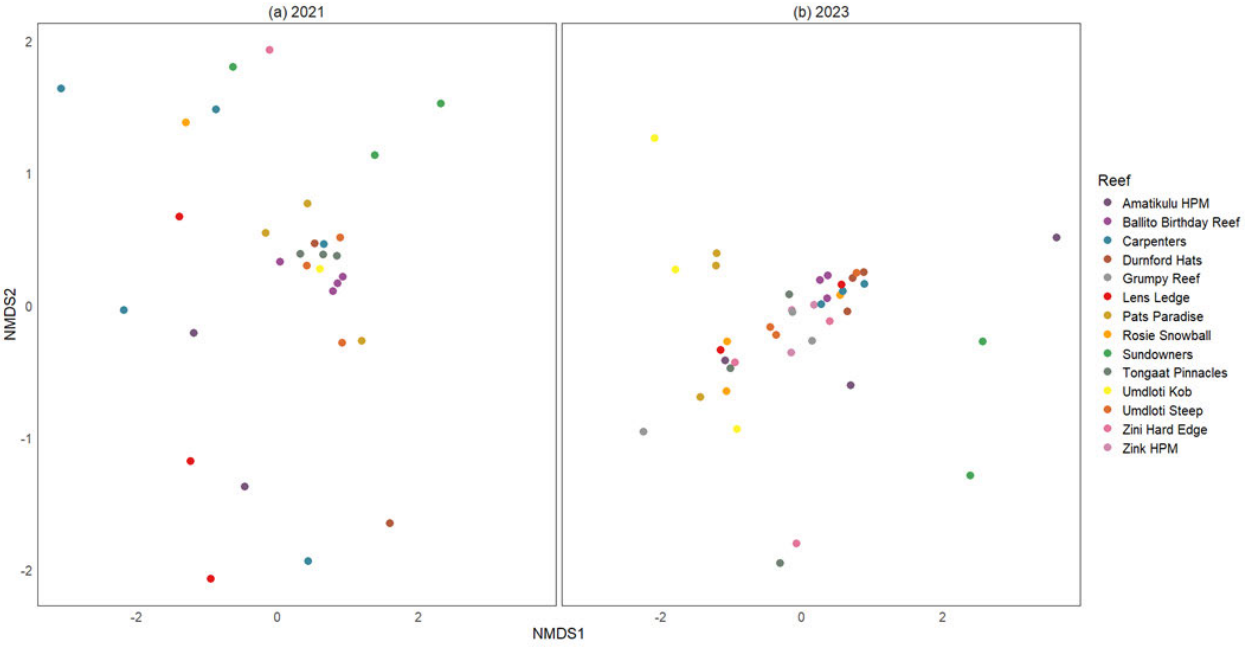


Figure 4.12: NMDS based on the Bray Curtis dissimilarity showing differences in community composition between 14 reefs across 3 depths within the uThukela MPA and on the adjacent areas outside the MPA in 2021 (a) and 2023 (b).

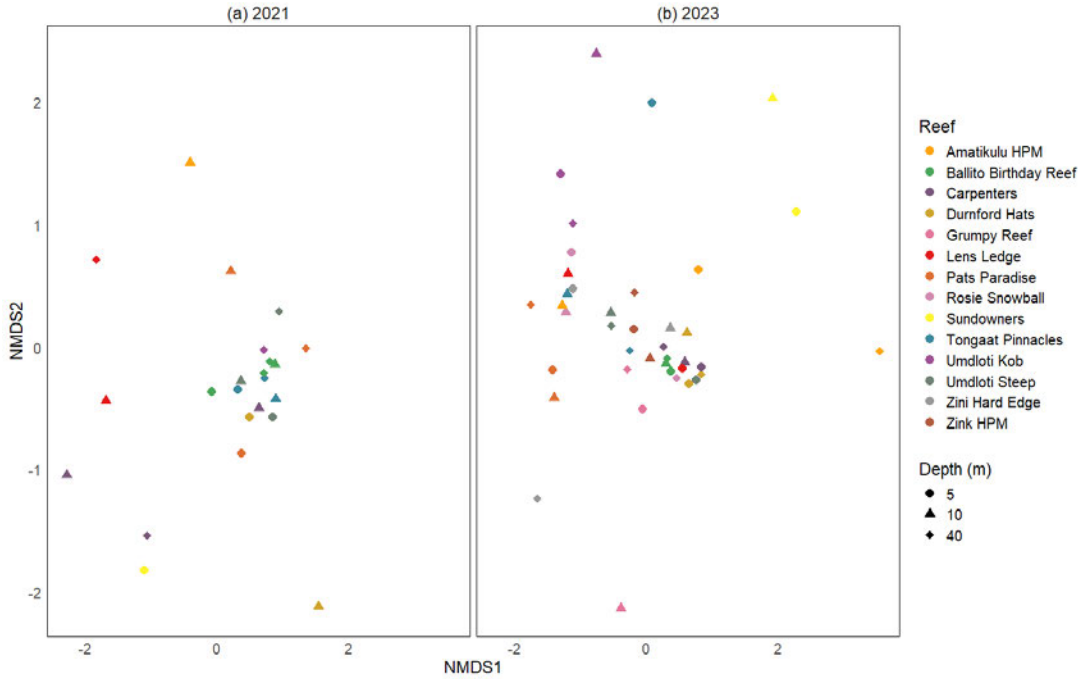


Figure 4.13: NMDS based on the Bray Curtis dissimilarity showing differences in community composition between fourteen reefs and three depths within the uThukela MPA and on the adjacent areas outside the MPA in 2021 (a) and 2023 (b).

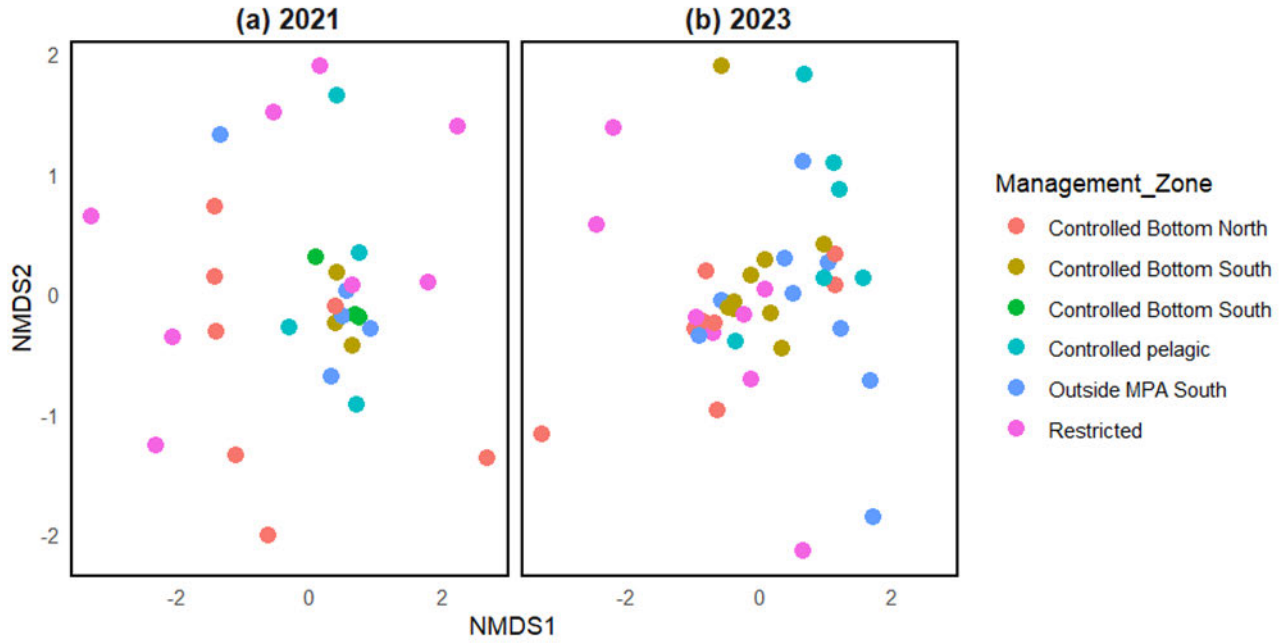


Figure 4.14: NMDS based on the Bray Curtis dissimilarity showing differences in community composition between six management zones across fourteen reefs and three depths within the uThukela MPA and on the adjacent areas outside the MPA in 2021 (a) and 2023 (b).

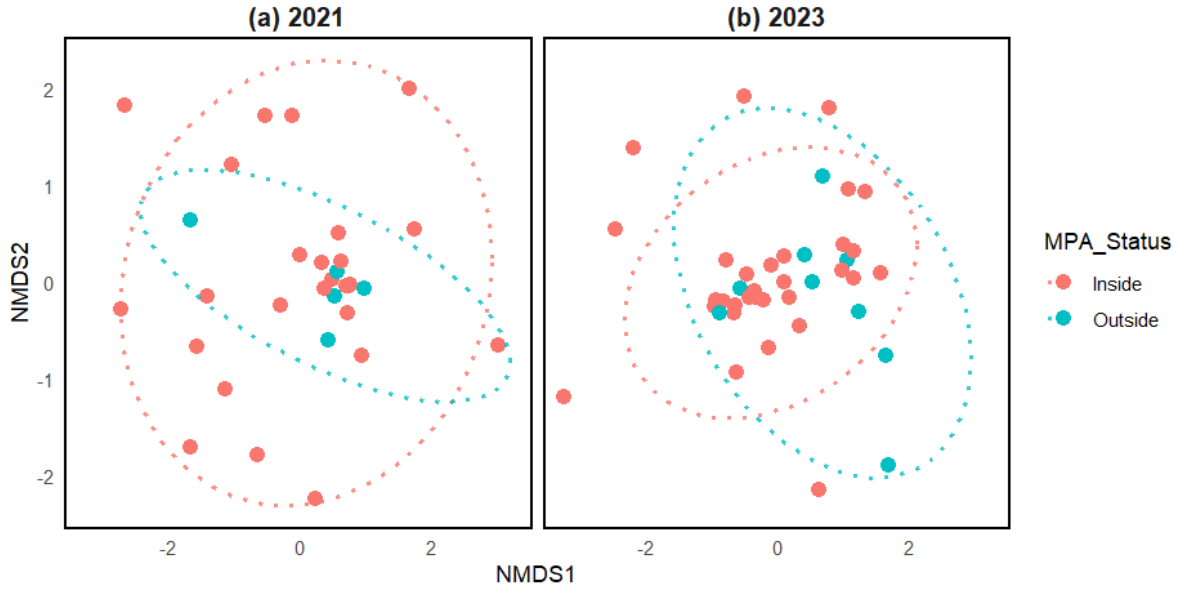


Figure 4.15: NMDS based on the Bray Curtis dissimilarity showing differences in community composition between the protected area inside the uThukela MPA and the adjacent area outside of the MPA adjacent areas outside the MPA in 2021 (a) and 2023 (b).

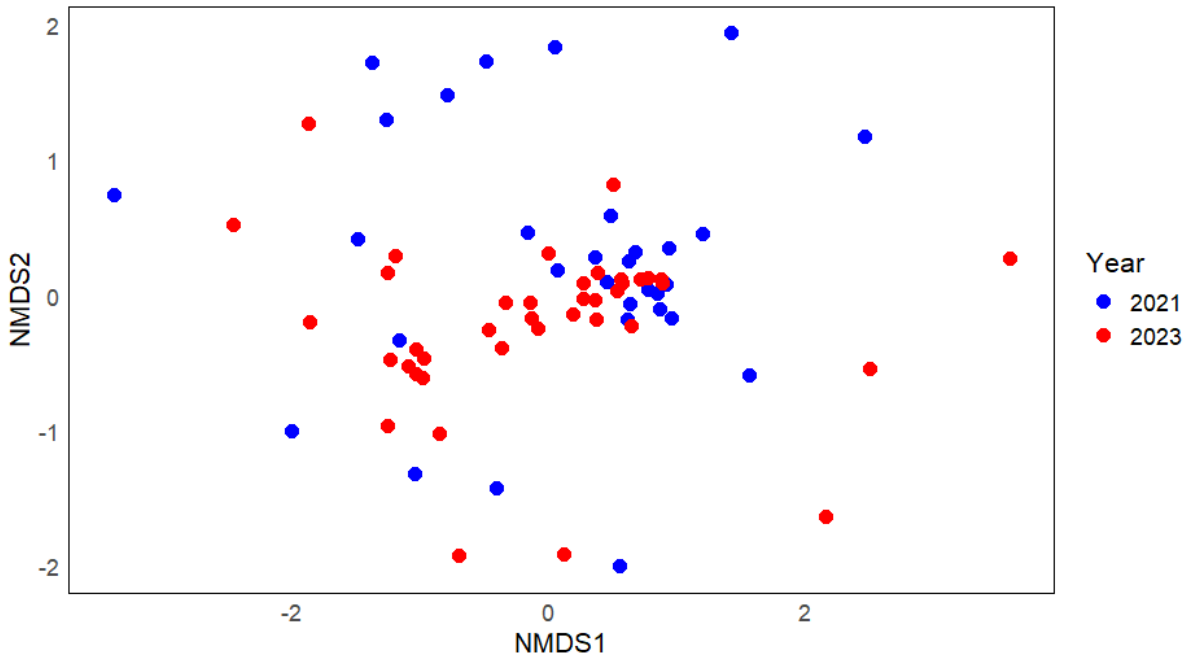


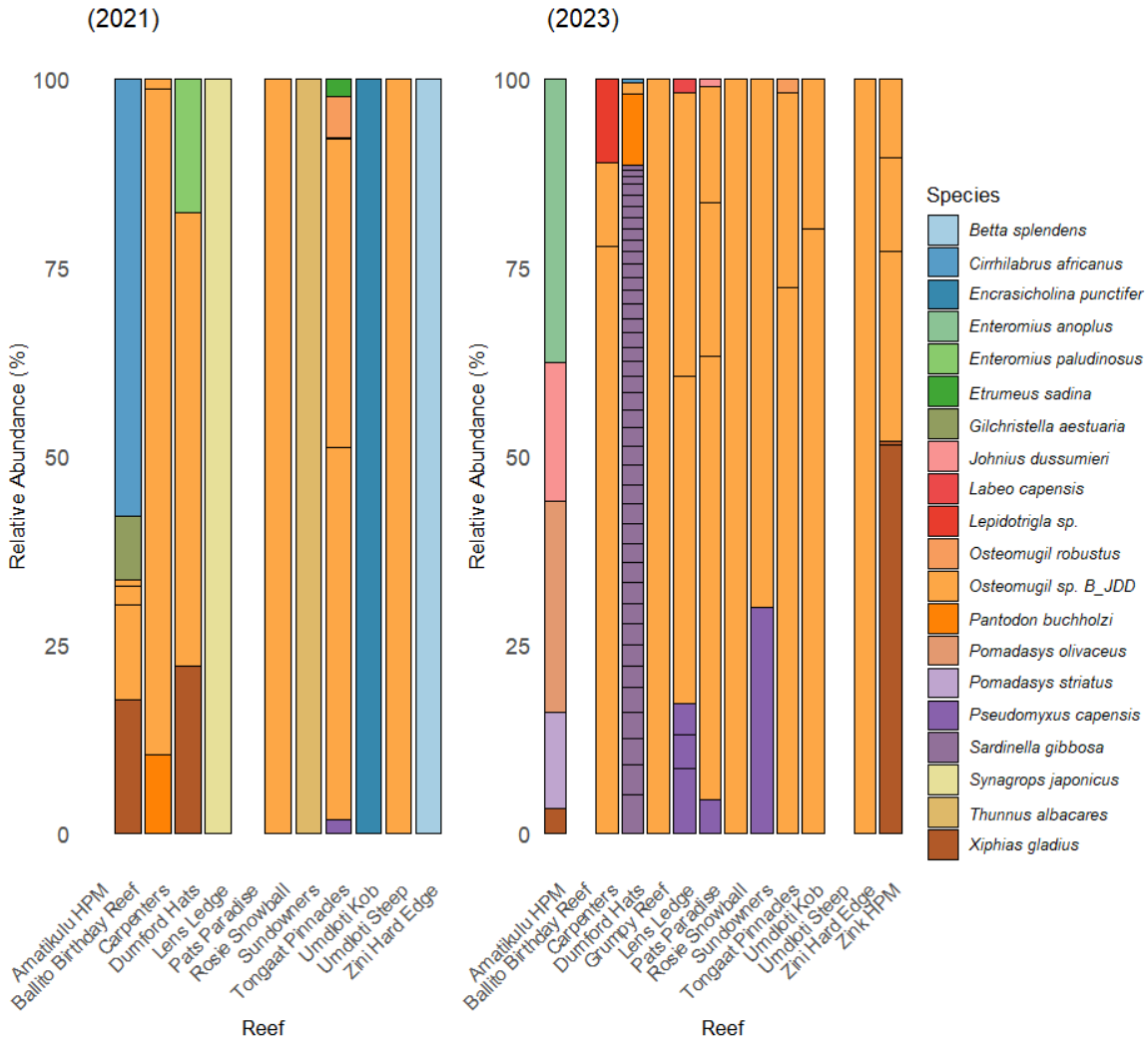
Figure 16: NMDS based on the Bray Curtis dissimilarity showing differences in community composition between 2021 and 2023 in the uThukela MPA and the adjacent area outside of the MPA adjacent areas outside the MPA,

4.7 Taxonomic composition and relative abundance

The stacked bar plot showed that, amongst the identified species majority of reefs were largely dominated by *Osteomugil sp.* both in 2021 and 2023 (Figure 4.17a). In 2021, *Osteomugil sp.* was detected in 6 reefs, while in 2023 it was detected in 11 reefs (Figure 4.17a). The least Dominant species was *Pseudomyxus capensis* in 2021 (Figure 4.17). and *Cirrhilabrus africanus* in 2023 (Figure 4.17a). In 2021, Amatikulu HPM had the highest number of identified species (4 species), while reefs like Dunford Hats, Pats Paradise, Rosie Snowball, uMdloti Kob, uMdloti Steep and Zini Haeds Edge were dominated by one species only (Figure 4.17a). In 2023, Amatikule HPM still had the highest number of identified species (5 species) and Grumpy reef, Rosie Snowball, uMdloti Kob, were dominated by one species (Figure 4.17a).

Within sample species composition and relative abundance showed that majority of the samples were dominated by one species, while fewer samples have more than one species making their total relative abundance (Figure 4.17b). The highest number of identified species detected in a sample was 3 species both in 2021 and 2023 (Figure 4.17b). Identified species that were detected in 2021 samples only, include *Encrasicholina punctifer, Thunnus albacares, Etrumeus sadina, Synagrops japonicus,* and *Enteromius paludinosus. Sardinella gibbose, Labeo capensis, Lepidotrigla sp, Johnius dussumieri, Pomadasys olivaceus, Enteromius anoplus,* and *Pomadasys striatus* detected in 2023 samples only. In 2021 only Pats Paradise had no identified species amongst its ASVs, while in 2023 it was Ballito Birthday and Umdloti steep (Figure 4.17b). In total, 12 species were identified in 2021, and 14 species were identified in 2023 (Figure 4.17b).

(a)


(2021)
(2023)
Relative Abundance (%)
100
75
50
25
0
Amatikulu HPM
Ballito Birthday Reef
Carpenters
Dumford Hats
Grumpy Reef
Lens Ledge
Pats Paradise
Rosie Snowball
Sundowners
Tongaat Pinnacles
Umdloti Kob
Umdloti Steep
Zini Hard Edge
Zink HPM
Reef
Species
Betta splendens
Cirrhilabrus africanus
Encrasicholina punctifer
Enteromius anoplus
Enteromius paludinosus
Etrumeus sadina
Gilchristella aestuaria
Johnius dussumieri
Labeo capensis
Lepidotrigla sp.
Osteomugil robustus
Osteomugil sp. B_JDD
Pantodon buchholzi
Pomadasys olivaceus
Pomadasys striatus
Pseudomyxus capensis
Sardinella gibbosa
Synagrops japonicus
Thunnus albacares
Xiphias gladius

(b)

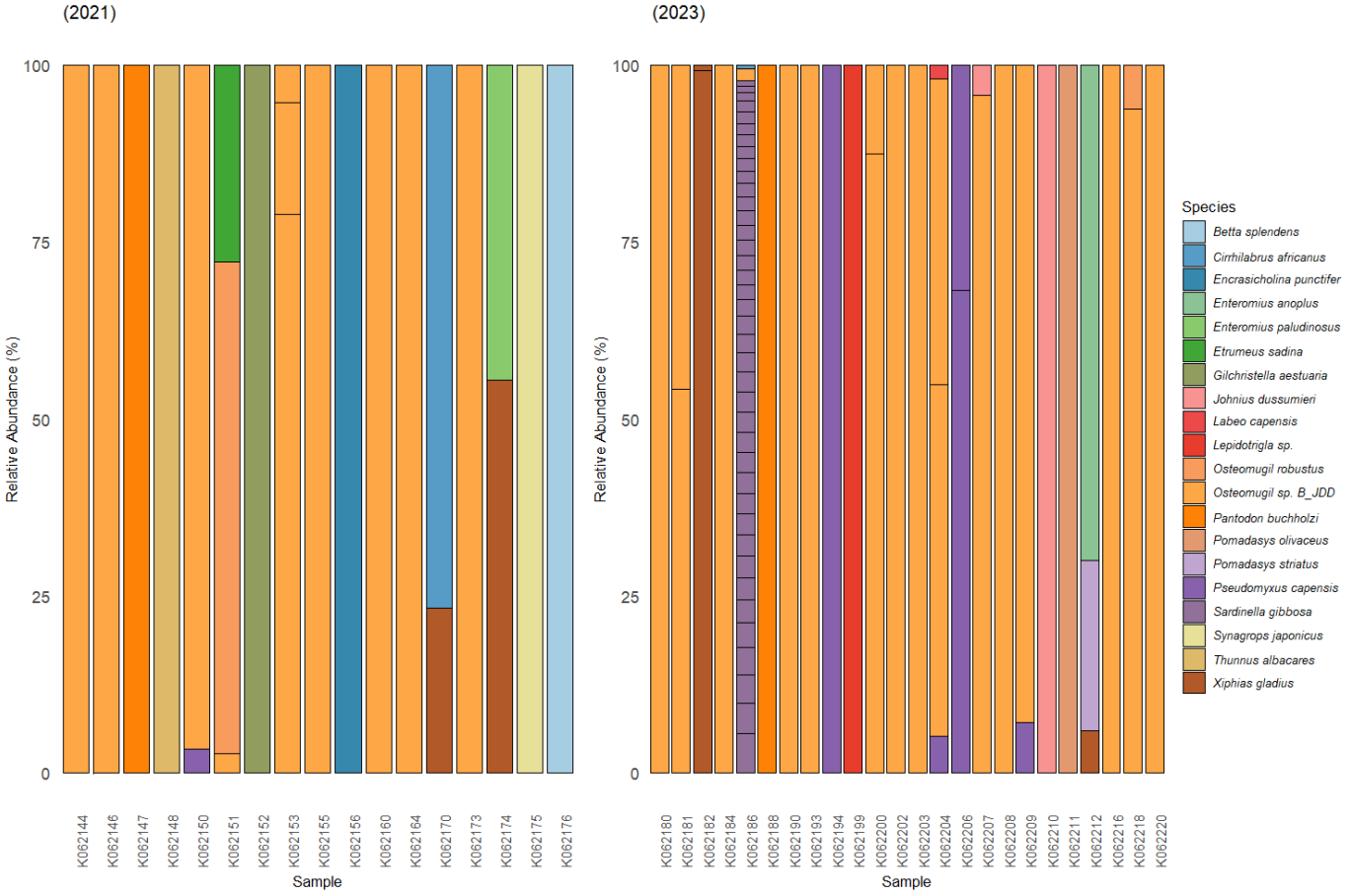


Figure 4.17: Species composition and relative abundance (%) of identified species per (a) sampled site/reefs and (b) sample in the uThukela MPA and adjacent none-protected areas in 2021 and 2023.

4.5 Effect of environmental variables

The environmental variables did not have a significant effect on community composition in 2021 (F = 0.7912, P = 0.929), however, in 2023 there was a significant environmental (F = 1.4004 p = 0.038). The db-RDA plot shows that dissolved Oxygen appears to have the strongest effect on community composition, followed by temperature and salinity in 2021 (Figure 4.18a). In 2023 community composition was strongly affected by temperature and turbidity (Figure 4.18b).

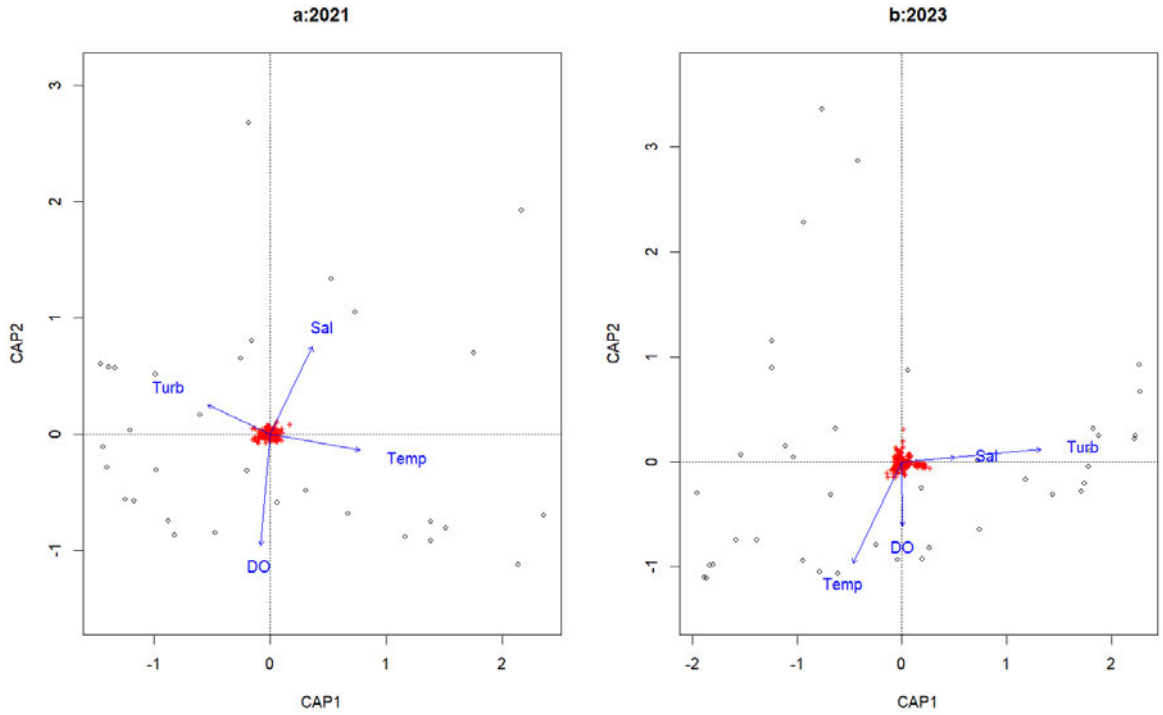


Figure 4.18: Distance based Redundancy Analysis (RDA) plots showing the relationship between environmental variables and community composition of each sampled reef within and outside the uThukela MPA in (a) 2021 and (b) 2023.

4.6 Sampling and sequencing effort

The species rarefaction curve shows that all reefs did not reach the equilibrium, indicating sample size used in each reef was not enough to capture the whole community diversity both in 2021 (Figure 4.20a) and 2023 (Figure 4.20b), despite the higher sampling effort used in 2021. In Contrast, the sequencing depth curve, shows that in most reefs a plateau was reached both in 2021 (Figure 4.19a) and 2023 (Figure 4.19a). The highest species richness was captured at a lower sequencing depth (<2000) (Figure 4.19).

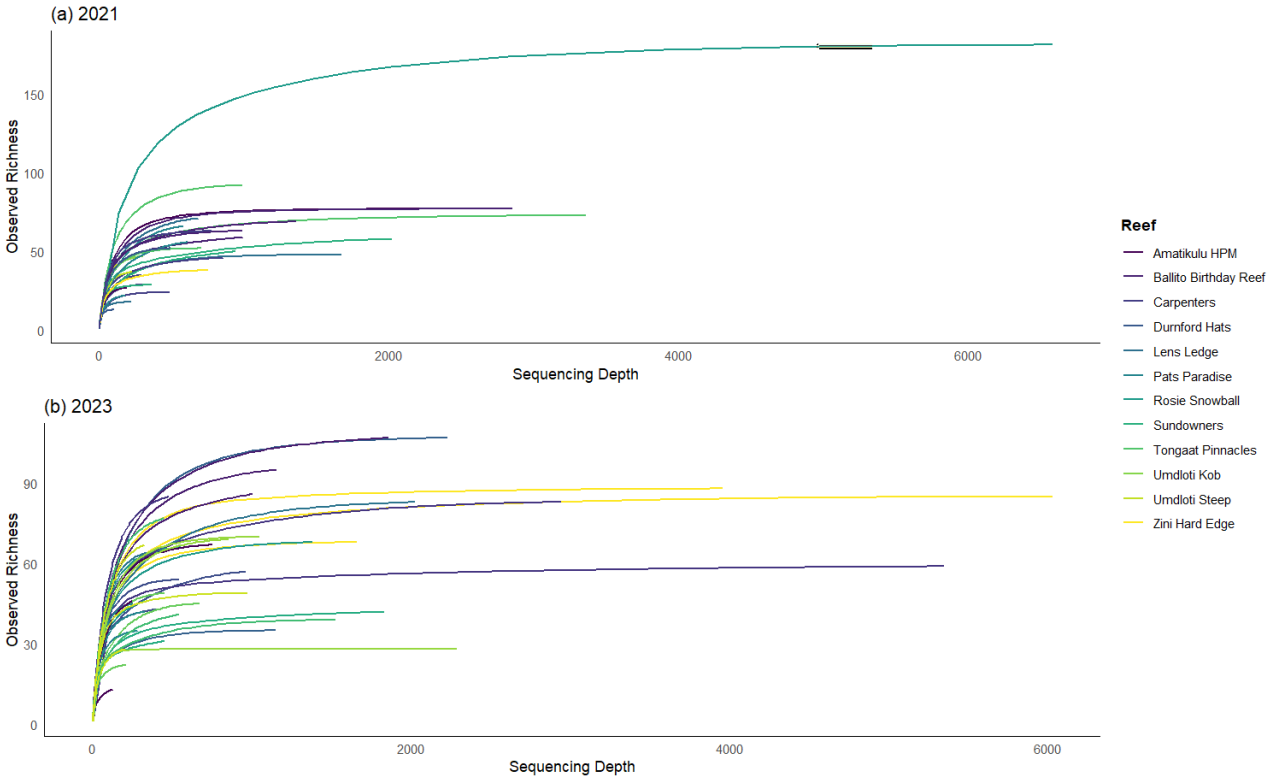


Figure 4.19: Species rarefaction curve, showing species richness as a function of sequencing depth of all samples from all sampled reefs in the uThukela MPA and on the unprotected reefs outside the MPA 2021(a) and 2023 (b).

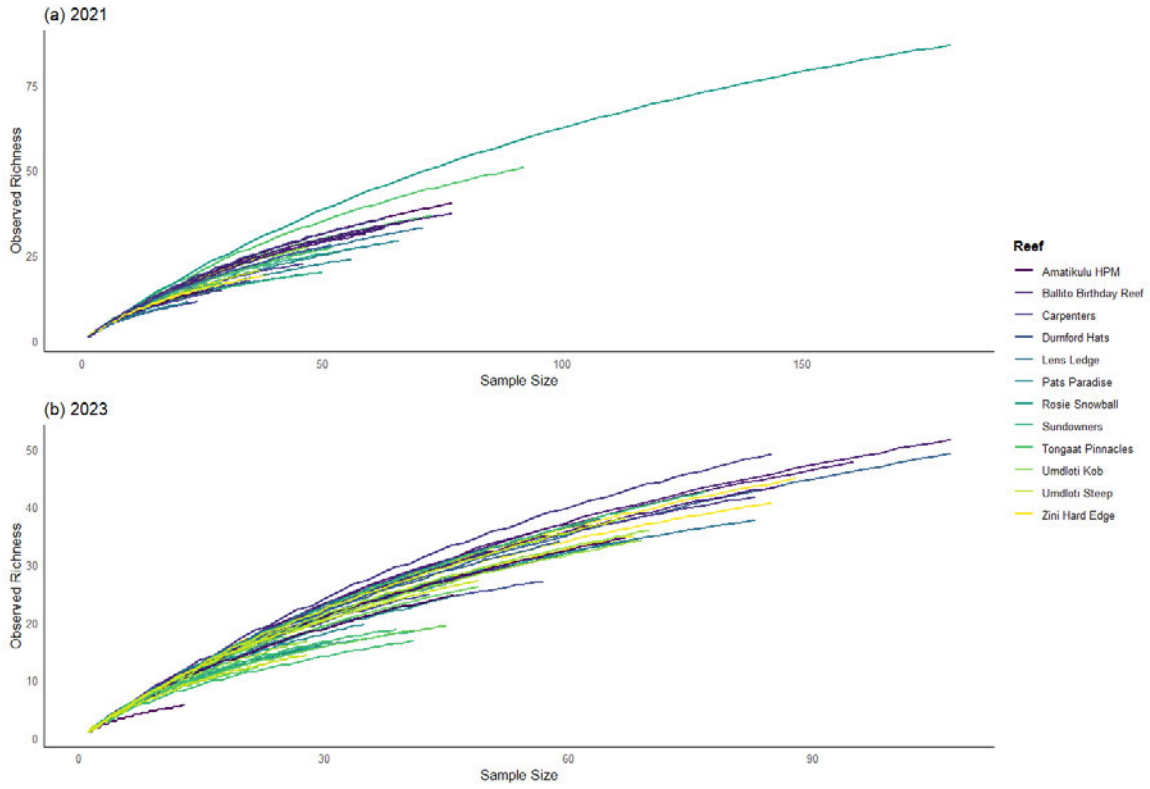


Figure 4.20: Species rarefaction curve of species richness as a function of the sample size in all sampled reefs in the uThukela MPA and on the unprotected reefs outside the MPA 2021(a) and 2023 (b).

CHAPTER 5: DISCUSSION

5.1 General

Biodiversity monitoring is a crucial step in MPA management as it tracks changes over time to check the impact of the conservation measures. Effective MPA monitoring relies on non-invasive and sensitive biodiversity survey tools and adequate baseline data against which the effect of protection can be measured. eDNA's ability to capture a huge amount of biodiversity data with relatively less sampling effort makes it appropriate not only as a tool for adding to the existing biodiversity data but also for tracking changes in biodiversity over time. This study uses eDNA metabarcoding to investigate the spatial and temporal variability in fish species diversity across different spatial scales in the uThukela MPA. This study does not only add to the existing baseline biodiversity knowledge, but it also gives insight into the dynamics of the eDNA signal in a dynamic marine environment. We compared alpha and beta diversity at different spatial scales in 2021 and 2023 using the mitochondrial COI gene marker. eDNA successfully detected horizontal spatial and temporal variation in species composition, richness, and overall diversity in the uThukela MPA. MPA's management strategies are guided by spatial variation, which influences biodiversity patterns (Wood et al., 2008). Therefore, to provide a complete picture of how biodiversity is changing in response to protection a multi-scale spatial approach to species richness and diversity quantification is needed (Blowes et al., 2020).

5.2 Environmental variables

5.2.1 Between depths

Environmental variables such as temperature, salinity, dissolved oxygen, etc. all play a crucial role in driving biodiversity patterns in marine ecosystems by influencing species distribution, community structure, and ecosystem function (Roland et al., 2012). Environmental variables also affect the degradation rates of eDNA in the water (Corinaldesi et al., 2008). Therefore, it is important to understand environmental variable dynamics in relation to eDNA and biodiversity patterns. In this study, environmental variables showed different trends at different spatial scales in both study years (Table 4.2). In 2021, all environmental variables except for salinity showed a significant variation between all sampled depths (Table 4.2a), indicating the presence of a strong vertical stratification across the depth gradient. The observed variability aligns with the global trends which show that environmental variables vary across the depth gradient, particularly with a decrease from surface to the boom waters (Costello et al., 2018). In 2023, all variables showed no significant variation between depths (Table 4.2a). This observation is not surprising, because upwelling-induced short-term fluctuations in environmental variables such as temperature and salinity have been previously

documented in the Natal Bight region (Pearce, 1978). In addition, the water in the Natal Bight region is known for its well-mixed water column (Lutjeharms et al., 2000). The changes from a stratified to a well-mixed water column in 2023, can be attributed to the changes in the sampling strategy of reduced replicates used in 2023. Costello et al., (2018) identified the importance of sampling at a fine spatial scale in shallower waters, in order to capture the increase in variability in environmental variables across the depth gradient. Alternatively, the reduced stratification observed in 2023 could suggest that the oceanographic processes that influence hydrodynamic processes such as upwelling-induced mixing across the depth gradient in the uThukela MPA vary over time. These results highlight the dynamic nature of the environmental conditions across the depth gradient in the uThukela MPA.

5.2.2 Between reefs and management zones

The horizontal trends showed that temperature and turbidity varied significantly between reefs, while dissolved Oxygen and salinity did not. In 2023, all environmental variables showed a significant variation between reefs (Table 4.2a). The environmental heterogeneity recorded in 2023 indicates that oceanographic and hydrological factors such as currents and upwelling zones influence reef's environmental conditions differently. This may be influenced by the bathymetry, a given reefs' proximity to oceanographic features, and terrestrial influences such as freshwater and sediment input from rivers (Leis and Goldman, 1983). For example, areas with muddy substrate tend to be very turbid during upwelling events, and high turbidity reduces sunlight penetration which then results in reduced primary production and therefore low dissolved oxygen levels (Kromkamp and Peene, 1995). Reefs that are close to the uThukela Estuary such as Grumpy Reef, Amatikulu HPM and Zonkwazi likely have high turbidity compared to the other reefs due to high levels of fine sediments input from the uThukela River discharge. The environmental variables trend between management zones in 2021 was consistent with that of reefs in 2021 (Table 4.2a), with environmental factors in 2021 showing no significant variation between management zones (Table 4.2a).

5.2.3 Between the protected and the non-protected area

It is important to determine if the environmental conditions vary between the protected area inside the MPA and the non-protected area outside the MPA, to distinguish the influence of the interaction between environmental factors and anthropogenic effects and that of environmental factors independently on species diversity and community composition. The pattern observed between inside and outside the MPA in both years was similar as that of reefs and management zones in 2021, with a significant difference in temperature and turbidity and no significant difference in dissolved oxygen and salinity (Table 4.2a). The consistency observed at different spatial scales shows that temperature tends to change with turbidity, suggesting that there might be a relationship between the two variables.

High turbidity has been reported to limit the depth at which sunlight heats the water (Löptien et al., 2011). Since the marine environment depends on sunlight as its primary source of heat, changes in turbidity influence water temperature.

5.2.4 Between years

There was no significant difference in environmental variables except temperature and salinity in the entire sampled area between 2021 and 2023 (Table 4.2b). The lack of variation at a larger temporal scale compared to the fine temporal scale highlights the importance of zonation in the MPA because environmental variability has important implications for the distribution of species in the MPA (Habtemariam and Fang, 2016).

5.3 Alpha diversity

5.3.1 between depths

Alpha diversity indices showed no significant variation in species richness and diversity between all depths in 2021 and 2023 (Figure 4.2a, b c, and e). The lack of variation in species richness and evenness between depths suggests that diversity is relatively uniform across the depth gradient in uThukela MPA, potentially because of the lack of vertical environmental stratification in the water column. A previous study also found that eDNA did not detect any vertical patterns in the overall species richness and evenness in fish communities in the North Sea (Dukan et al., 2024). In contrast, other studies have successfully detected a clear depth-related pattern in species richness and evenness (Priede et al., 2010; Pereira et al., 2018; Campbel et al., 2011). Moreover, a decrease in fish species richness associated with depth has been documented in several areas, including, the Tsitsikama National Park Marine protected area (Heyns-Veale et al., 2016), and the Bermuda archipelagos (Stefanoudis et al., 2019). However, these studies were conducted in the deep sea where environmental conditions are known to be harsh with a low food supply (Spence & Tingley, 2020). The upwelling and eddies dynamics along the Benguela current system are responsible mixing of the water column and nutrient supply in the Natal Bight region (Gibbons et al., 1999), therefore, they can be used to explain the uniform distribution of eDNA across depth gradients.

5.3.2 Between reefs and management zones

eDNA detected a spatial variation in alpha diversity between reefs, both in 2021 and 2023 (Figure 4.3). In 2021, Rosie Snowball showed the highest species richness and diversity, while the lowest species richness and diversity was in Dunford hats, whereas in 2023, the highest species richness was recorded in Ballito Birthday and the lowest in Sundowners (Figure 4.3a, b, c, and e). These results indicate that,

even though all the sampled sites are reef ecosystems, they possess some level of variation in their structural complexity and ecological factors. Badenhorst, (2021) reported similar findings of spatial heterogeneity in macrofaunal communities in the uThukela MPA. Similar studies recorded a patchy distribution of fish larvae communities in the Durban shelf area (Beckley and van Ballegooyen, 1992; Collocott, 2016). Productivity is one of the primary drivers of species richness and diversity in marine ecosystem (Fang et al., 2023). The oceanographic features such as currents, upwelling cells and eddies, and the uThukela Estuary are all primary sources of nutrients that drive productivity in this region, thus influencing species richness and diversity in this region (Scharler et al., 2016b; Meyer et al., 2002). The spatial variation in nutrient concentration driven by the dynamic oceanographic processes and terrestrial influence in the uThukela MPA is therefore an important factor in explaining observed variation in species richness and diversity. For example, some of the sampled reefs are more influenced by the uThukela Estuary, while others are influenced by the oceanographic features such as the nutrient-poor Agulhas current, the Durban eddy, the Richards Bay and St. Lucia upwelling cell, all of which influence the nutrient concentration of different reefs to varying extents (Meyer et al., 2002). The zooplankton biomass and distributions off Durban and Richards Bay are known to be influenced more by the upwelling than the Thukela River (Pretorius et al., 2016). Furthermore, Meyer et al., (2002) reported the upwelling cell at St Lucia to be the main nutrient source in the Natal Bight, providing the northern parts of the bight with ideal conditions for phytoplankton blooms. A Southwards decrease in nutrient concentration has been reported on the Natal Bight (Meyer et al., 2002). In addition to contributing nutrients, the uThukela estuary also influences the uThukela MPA with freshwater input and sediments deposition which shape the reefs' physical and environmental conditions, also contributing to habitat heterogeneity.

There was a shift in diversity in 2023, with reefs like Ballito Birthdays, Carpenters, Dunford hats, Lens Ledge, Rosie Snowball, Umdloti Steep, and Zini Hard Edge showing an increase in species richness, while the rest of the reefs, showed a decrease in species richness and diversity (Figure 4.3a, b c, and e). This is not rare in fish communities because they often exhibit varying levels of spatial and temporal changes in their richness and abundance in different parts of their habitat (Claudet et al., 2006). Therefore, a substantial proportion of changes in fish diversity are usually explained by their habitat structure (García-Charton et al., 2000, Ferraris et al., 2005). For example, the substrate of reefs located off the uThukela estuary such as Grumpy Reef, Amatikul HPM, and Zinkwazi HPM is known to be dominated by fine-grained sediments (mud) that come directly from the uThukela River (Green and MacKay, 2016, MacKay et al., 2016). Therefore, it is likely that these reefs favour species that thrive in turbid waters. The limitation associated with the sampling such as the inability to sample all reefs at

the same time of the day could also explain the observed variation between reefs. Jensen et al., (2022) showed that sampling at different times of the day can affect the number of species detected, with the dusk hours (19.00–21.00 h) showing a higher number of species detected compared to the later hours due to high activity during dusk hours. As a result, the time at which eDNA samples are taken can have serious implications on biodiversity interpretation.

Management zones also exhibited a difference in species richness and diversity in 2021 and 2023 (Figure 4.4a, b c, and e). The species richness patterns detected in 2021 differed from that of 2023 (Figure 4.4a, b c, and e). Species richness was comparatively higher in the Controlled Bottom South zone than all other management zones both in 2021 and 2023 while the lowest was recorded in the Restricted zone in 2021 and in the controlled pelagic zone in 2023 (Figure 4.4a, b c, and e). These results suggest that different management zones have a different response to varying levels of anthropogenic influences. The restricted zone was expected to have the highest species richness since most anthropogenic activities are restricted. The low species richness and diversity in the restricted zone and its proximity to the uThukela estuary support the observation that the uThukela River is not the greatest contributor of nutrients in the uThukela MPA (Meyer et al., 2002). Fish larvae display a migratory behaviour of moving towards the water with preferable conditions (Collocot, 2016). This is crucial to fish assemblage patterns, especially in the context of eDNA since eDNA acts like a footprint showing recent species presence. Hydrographic fronts (Sabatés et al., 2007) and local differences in environmental factors such as temperature, salinity, and chlorophyll *a* play a crucial role in influencing these patterns (Espinosa-Fuentes and Flore-Coto, 2004).

Spatial variability in community composition in uThukela MPA may be partly driven by the influence the Agulhas current, which interacts with the shelf, resulting in the formation of eddies and upwelling (Lamont et al., 2016). Upwelling brings nutrient-rich waters from the deep to surface water, thus promoting primary productivity which then supports high diversity in those areas (Lamont et al., 2016). Localized conservation measures, fishing restrictions, and habitat restoration also have an effect on the biodiversity patterns observed between management zones (Day et al., 2012). However, the impact of protection on marine diversity is not very clear due to the dynamic ecological process (Pettersen et al., 2022).

5.3.3 Between inside and outside the MPA

It is generally expected for biodiversity to be higher inside than outside the MPA (Coetzee et al., 2014). Similarly, in this study, species richness and diversity were consistently higher inside than outside the MPA in 2021 and 2023 (Figure 4.5 a, b c, and e). However, in 2023, there was an increase in species

richness both inside and outside the MPA (Figure 4.5 a, b c, and e). A similar pattern was reported by Blowes et al., 2020, where diversity and species richness were higher in protected than fished areas in the Mediterranean protected areas. Similar observations were made by Andriyuuono et al., (2019), where eDNA detected those urbanized areas had notably low species richness compared to nonurbanized areas. A high diversity and species richness inside the MPA does not only indicate a thriving population but could also suggest that the uThukela MPA does, in fact, serve as nurseries and spawning ground because it has been reported that a high number of juveniles tend to shed higher quantities of eDNA compared to their adult counterparts (Maruyama et al., 2014, Takeuchi et al., 2019), which in this particular case would be the adult populations outside the MPA. The observed differences between protected and unprotected areas reflect the effect of natural variability independently and a combined effect of natural variables and anthropogenic influences on species richness and diversity. As a result, these findings indicate that reduced anthropogenic pressures such as fishing and habitat destruction in the study area have a significant effect on the diversity of the local fish communities in the uThukela MPA. eDNA's ability to detect differences in fish diversity inside and outside the MPA showcases its potential as a tool for monitoring biodiversity in MPAs.

5.3.4 Temporal variability

Temporal monitoring of fish communities is essential for conservation purposes (Balint et al., 2018). There was temporal variation in the overall diversity detected between 2021 and 2023 across all sampled reefs in uThukela MPA and on the adjacent unprotected reefs outside the MPA (Figure 4.6 a, b c, and e). In 2023 a higher species richness and diversity than in 2021 was recorded, despite the high sampling effort of 2021 (Figure 4.6 a, b c, and e). Ecological communities do not only vary in space, but they also show temporal variation (Sales et al., 2021). Temporal replication may also increase the probability of capturing species that were previously missed, thus providing a more complete impression of species richness and community composition. Temporal changes in species diversity are very common in the marine environment due to temporal changes in environmental factors and biodiversity patterns (Lekve et al., 2002; Malcolm et al., 2007; Pettersen et al., 2022).

5.3 Species evenness

Simpon's index showed that species evenness was constantly high at all spatial scales (Figure 4.2d, 4.3d, 4.4, 4.5d, and 4.6d). This indicates that species were evenly distributed at all spatial scales. These findings are consistent with the findings of Collocott, (2016) who showed that all fish larvae taxa identified using plankton nests, were evenly distributed in the Natal Bight. In aquatic ecosystems,

species evenness is dependent on habitat variation, with greater habitat variation contributing to a high species evenness (Carvajal et al., 2015).

5.4 Beta diversity

5.4.1 Between depth

Fish community composition may differ considerably across different depths, depending on life history stages, behavioral patterns, and physiological tolerance to environmental gradients, thus requiring fish compositional response to MPA to be measured across depths (Rocha et al., 2018). In the current study, there was no significant difference in community composition between depths both in 2021 and 2023. Even though the lack of variability was consistent in both years, there was a higher community composition recorded at all depths in 2023 (Figure 4.7b) than in 2021 (Figure 4.7a). The observed lack of depth-related variation is supported by previous studies that found that eDNA did not capture any vertical structuring of fish communities across the depth gradient (Dukan et al., 2024; Westgaard et al., 2024). In contrast, studies like Portella et al., (2023); Zintzen et al., (2012); Pereira et al., (2018), did find depth to be a strong factor influencing community structuring in marine ecosystems. However, traditional sampling tools such as bottom long line, visual scuba surveys and baited underwater stereo video were used in these studies. Therefore, the contrasting results between eDNA and traditional survey methods suggest that the dynamic nature of eDNA in the marine environment may have potentially influenced the observed lack of variation in community composition across the depth gradient. Intracellular DNA behaves like a particle in water body (URycki et al., 2024), whereas dissolved eDNA behaves like other dissolved substances in a water body (Mauvisseau et al., 2022). Just like other particles, intracellular eDNA is subjected to water processes like sinking, resuspension, and mixing (Andruszkiewicz et al., 2019). Dissolved eDNA on the other hand is subjected to transport processes such like diffusion and advection which are also influenced by sinking to a lesser extent (URycki et al., 2024).

Vertical eDNA redistribution is mostly dependent on the level of stratification in the water column (Canals et al., 2021). In a well-mixed water column, depth-related patterns in community composition can be masked by the redistribution of eDNA particles throughout the water column (DiBattista et al., 2019). Several eDNA studies detected a strong eDNA vertical heterogeneity across the water column in waters with strong vertical stratification (Yamamoto et al., 20216; Andruszkiewicz et al., 2017; Jeunen et al., 2020). In a well-mixed turbulent water column Jeunen et al., (2020) found less distinct eDNA signal across depth. The observed differences in the eDNA signal from different studies indicate

that the eDNA signal is strongly influenced by the local oceanographic and hydrodynamic process that drives mixing in the water column. The hydrological and oceanographic processes that influence eDNA redistribution in the water column also affect environmental variables that shape species assemblages in the water column (Fang et al., 2023). The environmental variables discussed earlier characterized the water column in uThukela MPA as mixed or weakly stratified, which supports the heterogenous eDNA signal observed. As a result, the observed pattern detected by eDNA may in fact reflect the actual species composition in the uThukela MPA.

One of the most compelling aspects of beta diversity is its ability to show similarities between different communities by the amount of overlapping ASVs. eDNA detected a strong overlap between the three depths in both years (Figure 4.7a). However, there was an increase in the number of shared ASV from 67 shared ASVs in 2021 (Figure 4.7a) to 106 shared ASVs in 2023 (Figure 4.7b). A pairwise comparison showed that the depths that shared the highest number of ASVs were 10 m and 40 m and then at 5m and 10 m and 5 m and 40 m above the sea floor in 2021 (Figure 4.7a). In 2023, communities that were most similar, were from 10 m and 5 m, followed by 40 m and 10 m, and lastly 5 m and 40 m above the sea floor (Figure 4.7b). The strong overlap in species composition between all depths showed that depth is not an important factor in structuring fish communities in uThukela MPA. The strong overlap in community composition observed can be attributed to vertical diel migration (DVM) behaviour exhibited by some fish species. If a species exhibits vertical migration behaviour, its DNA signal will be homogenous throughout the depth range (Westgaard et al., 2024). Gjosaeter et al., (2017) recorded the vertical migration of fish and zooplankton in the water column over a 24-hour cycle in Arctic waters. Hydrological processes such as mixing by wind or the fast flow of freshwater discharge from the river, may have also influenced the observed overlaps. Jeunen et al., (2020) observed a strong overlap in eDNA signal related to the occurrence of the hydrological processes. The local hydrodynamic and oceanographic factors such as the Agulhas current, the Durban Eddy, and the Richards Bay upwelling cell all play a significant role in maintaining vertical ecological connectivity in the MPA by maintaining a well-mixed water column (Waters, 2023).

The vertical structuring of species across depth is a function of environmental variability which varies with different geographic locations, and hydrodynamic and oceanographic conditions (Fang et al., 2023). As a result, the observed lack of variability across the depth gradient in uThukela MPA can be explained by both the dynamic nature of hydrological and oceanographic conditions that influence environmental variability in the uThukela MPA and the dynamic nature of eDNA. It is difficult to distinguish between the effects of eDNA dynamics and the actual patterns displayed by the communities.

5.4.2 Between reefs and management zones

Marine communities in marine ecosystems are also subjected to horizontal variation (Jeunen et al., 2020. Ideally, an MPA must be designed in a way that ensures that its management zones are ecologically connected through larval dispersal and seasonal migrations to ensure the persistence and recovery of biodiversity (Balbar and Metaxas, 2019). The common way to track ecological connectivity is through larval distribution (Vazquez et al., 2021). However, larvae are not very easy to track due to their complex behaviour, small size, and nature of their habitat (Leis, 2006; North et al., 2008). Among other tools, eDNA can be used as a tool to track larvae distribution (Ratcliffe et al., 2021). uThukela MPA is known to be a region of larval retention because it provides a suitable habitat for spawning of many fish species (Hutchings et al., 2002), therefore, some of the eDNA sampled from this region is assumed to originate from larval stages, and local fish communities. At a small spatial scale, there was a significant variation in community composition between reefs and management zones in 2021 and 2023. This is also evident on the NMDS plot, showing a weak clustering of samples from the same reefs in 2021 (Figure 4.12b) and 2023 (Figure 4.12b). The interaction between depth and reef had no significant effect on community composition in 2021 and 2023, indicating that the variation in community composition between reefs can be explained by the structural and environmental differences between reefs. These results are consistent with the findings of Collocott, (2016), who found that fish larvae assemblages in the Natal Bight vary at fine spatial scale. Several other studies have also found a species-specific horizontal variation in larvae distribution at a small spatial scale in nearshore communities (Cowen, 2002; Swearer et al., 2002; Irisson et al., 2004). The horizontal distribution of larvae is dependent on the current path, flow rate, and other oceanographic features (Fang et al., 2023). Muhling (2006), observed that, distinct zooplankton assemblages were related to their proximity to the river mouth and upwelling cells.

At a larger spatial scale, there was a significant difference in community composition between uThukela MPA's management zones, with the Restricted Zones having the most diverse community composition, while the Controlled Pelagic zones had the least diverse community composition in 2021 (Figure 4.8a). These findings demonstrated the success of eDNA in capturing differences in protection levels of different management zones related to site selection in MPA designation. The Restricted zone is expected to have the highest community composition due to its high productivity and lower anthropogenic influence. The observed spatial variability may be attributed to oceanographic factors which may serve as barriers to larvae dispersal within uThukela MPA. These Results also suggest that the horizontal transport of eDNA is limited by distance. Canals et al., (2021); Monuki et al., (2021); Allan et al., (2021) noted that horizontal transport of eDNA in the Marine environment is limited to 10-

20m from their point of origin. A similar study indicated that on a small geographic scale, eDNA transfer from one habitat to another has been found to be negligible (Jeunen et al., 2020).

While the significant difference between management zones was consistent over time, there was a shift in community composition pattern in 2023, with the highest community composition recorded in the Controlled Bottom South zone, while the lowest was recorded in the Controlled Pelagic zone (Figure 4.8b). The Controlled Bottom North zone, Restricted zone, and outside the MPA zones showed a decline in diversity of community composition in 2023, with the highest decline recorded in the Restricted zone (Figure 4.8a). The sharp decline in the Restricted zone likely indicates a high sensitivity to environmental change which may have resulted from temporal changes in the uThukela River flow dynamics since the uThukela estuary is part of the Restricted zone. This highlights the importance of connectivity between coastal and offshore ecosystems in marine protected areas. Sampey et al., (2004) identified spawning patterns of adult fish as the major reason for temporal changes in fish composition using light traps. The NMDS plot, shows that some level of overlap between a number of samples and also high dissimilarity between some samples from different management zones in 2021 (Figure 4.14a) and in 2023 (Figure 4.14b). The horizontal distribution of fish assemblages, especially at the larvae stage, are shaped by oceanographic processes such as upwelling, flow direction (Franco-Gordo et al., 2001), advection currents (Hsieh et al., 2005) which all drive the differences in environmental factors and larval dispersal patterns (Cowen and Sponaugle, 1997). Therefore, the observed differences in the community composition patterns detected in 2021 and 2023 suggest possible temporal changes in local hydrological and oceanographic factors that influence the environmental conditions of community structuring in uThukela MPA.

There were minimal overlaps recorded between all management zones, which confirms the differentiation of habitats between management zones (Figure 4.8). Previous studies showed that beta diversity patterns are driven by spatial variability, even in ecologically connected ecosystems (Shaw et al., 2016). Therefore, the observed minimal overlaps and the significant variation in community composition between management zones is might not necessarily be an indication of weak ecological connectivity between uThukela MPA's management zones, however, it does raise a concern and necessitate further research into horizontal ecological connectivity. It should be noted that ecological connectivity is not only measured by the overlap in species composition, but there are much more complicated models used to predict connectivity between separated but similar habitats (Thorrold et al., 2002; Bryan-Brown et al., 2017; Burgess et al. 2014).

5.4.3 Between the protected and unprotected area

Another critical aspect of MPA monitoring is investigating if the MPA is ideally designed such that important nurseries inside the MPA are ecologically connected to the harvested populations outside the MPA through larvae dispersion and spill over effect to ensure that the protection benefits go beyond the MPA boundaries (Forcada et al., 2009). At a much broader scale, between inside and outside the MPA, there was no significant variation found in 2021 and 2023. However, there was an increase in diversity inside the MPA and a decrease outside the MPA in 2021 (Figure 4.9b) while 2021 showed a decrease (Figure 4.9b). Even though some samples from inside and outside the MPA show greater dissimilarity, the majority are closer together, indicating high community similarity both in 2021 (Figure 4.15a) and 2023 (Figure 4.15b). These findings are supported by Puspasari et al., (2023) who found that the catch composition of the fishery resources showed no significant variation in reef fish composition inside the Pieh Marine Protected Area and outside the MPA in Indonesia. In contrast, Varnes & Olsen, (2023) found a significant variation in catch composition between protected areas and unprotected areas in coastal Skagerrak in southern Norway. This difference detected in Norway's marine protected areas was after 10 years of protection, whereas it has only been 2 years since the uThukela MPA was declared. The lack of variation between communities inside and outside the MPA might be an indication of strong ecological connectivity between the presumed source populations inside the MPA and sink populations in the harvested populations outside the MPA. Hydrodynamic connectivity has been reported to play a significant role in maintaining fish populations in coral reef ecosystems (Holstein and Paris, 2014). There was a strong overlap between communities inside and outside the MPA in 2021 (Figure 4.9a) and 2023 (Figure 4.10b), the highest overlap was in 2023. The strong overlap further supports strong ecological connectivity between protected and non-protected areas.

5.4.4 Temporal variability

The overall community composition detected by eDNA in the whole study site was higher in 2023 than in 2021 despite the high number of replicates per site in 2021 (Figure 4.10). This indicates that sampling more sites rather than more replicates at one site is more effective in representing the whole community. Furthermore, the high number of detected species in 2023 highlights the importance of temporal replication to capture species that were not captured in the previous year.

5.4.5 Species composition and relative abundance

The stacked bar plot showed that, the majority of reefs were dominated by *Osteomugil sp. B_JDD* in both years (Figure 4.17a). *Pseudomyxus capensis* and *Cirrhilabrus africanus* were the least dominant species in 2021 and 2023 respectively (Figure 4.17a). In 2021 reefs like Dunford Hats, Pats Paradise, Rosie Snowball, uMdloti Kob, uMdloti Steep, and Zini Hard Edge were dominated by one species only,

while fewer reefs had more than one species, particularly Amatikulu HPM having the highest number of species both in 2021 (Figure 4.17a). 2023 showed a similar pattern with Amatikulu having the highest number species identified and uMdloti kob and Rosie Snowball consistently having one identified species. Species detection success and relative abundance are subjected to several methodological and analytical constraints that have not yet been overcome in eDNA metabarcoding studies (Figure 4.17a). Firstly, the bias associated with PCR has been identified as one of the potential sources of distortion of the relative abundance in amplified DNA from environmental samples by allowing the DNA of certain species to binding more successfully to the primers than others (Tsuji et al., 2022). PCR bias may result to the overestimation of certain favoured species and underestimation of others (Moinard et al., 2023). Despite the increase in the number of studies focusing on PCR dynamics in the context of metabarcoding, there has not been any standard PCR protocol for eliminating potential primer bias (Clarke et al., 2014; Pompanon et al., 2012; Moinard et al., 2023). Secondly, some aspects of bioinformatics processing such as sequence filtering threshold, error correction and taxonomic assignment can contribute to the underrepresentation or even complete exclusion of some species through stringent filtering criteria (Eckert et al., 2018). In the current study the strict filtering thresholds were applied to increase the integrity of the data. Lastly, the incompleteness of the public reference database, against which retrieved sequences can be matched in order to identify them to different taxonomic levels, is a major shortfall limiting the achievement of the taxonomic richness of eDNA metabarcoding data worldwide (Liu and Zhang, 2022).

eDNA metabarcoding is particularly interesting because of its ability to detect multiple species from a single sample. Within sample species composition showed that the majority of samples were dominated by one species, both in 2021 and 2023 (Figure 4.17b). The highest number of species detected in one sample was three species, both in 2021 and 2023 (Figure 4.17b). Species such as *Encrasicholina punctifer, Thunnus albacares, Etrumeus sadina, Synagrops japonicus,* and *Enteromius paludinosus*. were detected in 2021 samples only, while *Sardinella gibbose, Labeo capensis, Lepidotrigla sp, Johnius dussumieri, Pomadasys olivaceus, Enteromius anoplus, and Pomadasys striatus* were detected exclusively in 2023 (Figure 4.17b). Many of these species are known to commonly occur in this region, for example, Connell, (2007) recorded *Encrasicholina punctifer* in the Natal Bight region in fish eggs and larvae surveys. *Pomadasys olivaceus* has been documented as common in the Natal bight region (Fennessy et al., 2016). Some of the species identified are freshwater species. This is not surprising due to the influence of uThukela river which brings in freshwater and eDNA of residence species of the uThukela MPA. Some of the identified species, particularly those that have not been previously documented in the Natal Bight such as *Betta splendens* are likely misidentification that closely resemble

a resident species. For example, *Labeo capensis* may be a misidentification of *Labeo Rubromaculatus* a resident species of uThukela River, also known as Tugela Labeo. Overall, there were more identified species in 2023 than in 2021 (Figure 4.17b). The variation in species detected in 2021 and 2023 highlights the importance of temporal replication to chances of capturing species that were not previously captured, especially since more identified species were found in 2023 than 2021 sample (Figure 4.17b). While many species were not identified due to the incomplete reference database, the identification of commonly occurring species in the study site is an indication of the reliability of eDNA metabarcoding in capturing species composition and relative abundance.

5.6 Validity and reliability

eDNA in the open ocean is thought to be present in very small amounts (Hansen et al., 2018). As a result, eDNA analysis in the open ocean requires sufficient sampling that is representative of the sampling site in order to avoid underestimating species richness which is prone to false negative errors (Kawakami et al., 2023). The species rarefaction curve showed that all reefs did not reach a plateau, indicating that the sampling effort was not enough to capture the whole community diversity across the uThukela MPA, both in 2021 (Figure 4.19a) and 2023 (Figure 4.19b). These findings also show that the higher sampling effort used in 2021 did not result in higher maximum species richness. This is common in eDNA metabarcoding studies (Oosthuizen, 2021; Gold et al., 2021) since a standard sampling size has not been established. Despite not reaching the maximum number of species, the 2023 sampling effort consistently outperformed the 2023 indicating that sampling more sites in the study area is effective for capturing the whole community compared to sampling fewer sites and many replicates per site. Biomonitoring assessments rely on maximized spatial and temporal sampling (Czachur et al., 2022). Developing a standard protocol that can be used globally is still very challenging because marine ecosystems differ significantly on a local, regional, and global scale (Kawakami et al., 2023). The standard sampling effort is still a debated matter in eDNA metabarcoding studies in the ocean.

The db-RDA showed that environmental variables did not have a significant effect on community composition in 2021, however, in 2023 there was a significant effect. The db-RDA plot shows that dissolved Oxygen and salinity mostly influenced community composition in 2021 while temperature and turbidity most influenced community composition in 2023. This observation and the significant difference recorded in 2023 suggest that turbidity and temperature are more important in shaping species assemblages than salinity and dissolved Oxygen. This supports the observation made earlier where turbidity and temperature tended to change together. In addition, read length is a very critical

parameter in eDNA metabarcoding studies due to its sensitivity in discriminating between closely related species (Fonseca, 2018; Gaonkar and Campbell, 2024), reducing false positive and taxonomic artefact (Ramírez-Amaro et al., 2022). eDNA fragment size is a reflection of the quality of the extracted and sequenced DNA. The current study showed no significant effect of environmental variables on read length indicates that, indicating that the variation in fragment size between samples cannot to attributed solely to environmental factors.

5.7 MPA Management implications

The findings of this study not only contribute to the growing body knowledge on species diversity patterns across space and time but also gives insight into ecological dynamics that drive community composition, species richness, and diversity at different spatial scales. The observed variability across space and time highlights the role that habitat complexity, oceanographic factors, and environmental factors have in shaping biodiversity across similar habitats separated by distance. Differences in diversity over time highlight the dynamic nature of protected reef ecosystems, reflecting interannual changes in the diversity and community composition driven by natural processes such as spawning and recruitment and local oceanographic conditions such as currents, upwelling, and eddies. Comparing protected and unprotected areas helped to distinguish the combined effect of environmental and anthropogenetic influences and the effect of environmental factors independently. These results support the existing theoretical framework on the role of ecological connectivity, habitat complexity, and environmental gradients in structuring biodiversity distribution in complex marine ecosystems. In addition, this study highlights the success of eDNA in capturing spatial heterogeneity at different spatial scales, thus showcasing the potential of eDNA as a biomonitoring tool in marine protected areas.

The findings of this study clearly imply that any conservation approach taken for managing the uThukela MPA should take into account the homogeneity observed across the depth gradient. The lack of depth-related variation in species composition, richness, and diversity illustrates the need for maintaining the environmental coherence that is established by oceanographic and hydrological processes. Protecting these dynamics will increase the resilience of fish communities to climate change-induced environmental change over time. Horizontal variability provides crucial information on the areas of high diversity that should be prioritized for protection, and areas of low diversity that reflect habitat degradation and require restoration. As a result, this study can contribute to policy design by informing the design and management of MPAs. In addition, this study contributes to the goals of the national biodiversity frameworks, particularly the sustainable use and conservation of marine resources.

5.8 limitations

5.8.1 Potential PCR bias

While it is evident in this study and many previous studies that eDNA is undoubtedly a powerful tool for monitoring species diversity in marine ecosystems, it has its limitations that may be common throughout eDNA studies or that may be specific to a study. One of the potential limitations of this study is the common possibility of false negatives associated with PCR amplification. During PCR some species may be more successful in binding to the primers than other species, therefore resulting to false negatives of species that were in fact present in the sample (DiBattista et al., 2017). In this study, limitation was overcome adopting a nested PRC approach which first amplified the target gene marker and then later added the illumina adaptors which are particularly sensitive (Skelton et al., 2023)

5.8.2 Sampling time

The second limitation of this study is that water samples could not be collected at the same time of the day due to limited resources. Jensen et al., (2022) showed that there was a difference in the number of species detected at different times of the day, with the dusk hours (19.00–21.00) showing a higher number of species detected compared to the midday and night due to high activity during dusk hours. Therefore, sampling at different times of the day is a potential confounding factor in this study.

Sampling in the ocean can be very challenging, especially with eDNA's sensitivity to contamination. Some research vessels are small and unstable in the ocean therefore increasing the chances of contamination despite prevention measures adopted. Contamination is not only limited to field sampling, but it can also be from sampling to DNA extraction to PCR. Contamination can potentially introduce false positives (Goldberg et al., 2016), thus reducing the reliability of the results. This is very important, especially in monitoring studies as monitoring data directly inform conservation strategies. Therefore, both field blank and PCR blank are crucial to detect any contamination. No contamination was detected in this study.

5.8.3 The incomplete reference database

Lastly, the incomplete reference library/database is one of the most important limitations of eDNA. Some samples may be rich with species and may even have rare and cryptic species that were not previously known to exist in the sampling location, however if those species are not present in the refence data base they will not be identified to species level.

All these factors may result in a failure to detect some species, and the detection of species that are actually not present. However, eDNA's application for monitoring programs is still at its developmental

stage, therefore there is still room for improvement. Moreover, despite all these challenges the data provided by eDNA proved to be very informative in understanding biodiversity patterns needed for achieving the conservation goals in the uThukela MPA. therefore, this study advocates for eDNA as suitable method to complement traditional fish surveys and not as a replacement.

CHAPTER 6: CONCLUSION AND RECOMMENDATIONS

The main goal of marine protected areas is to recover biodiversity, and ecosystem function and promote resilience to climate change. For the uThukela MPA to achieve its goals it must ensure that important habitats of high diversity are adequately protected, degraded habitats are restored, and changes are regularly monitored to track the progress. All this relies on the availability of adequate knowledge of spatiotemporal patterns of biodiversity from the protected area. It was therefore necessary to add on the existing baseline biodiversity knowledge against which progress can be measured. This study

characterizes spatial and temporal variability of fish diversity in the uThukela MPA. The findings of this study show that fish diversity in the uThukela MPA is characterized by significant horizontal variability between reefs and management zones, suggesting structural and environmental heterogeneity, influenced by the oceanographic and hydrodynamic process. Despite the observed patterns between the MPA's management zones, the higher species richness and diversity detected inside than outside the MPA, indicate the effectiveness of the uThukela MPA's designation in conserving biodiversity. Temporal variation was also observed, reflecting internal variability in the oceanographic processes. In addition, the success of eDNA in capturing spatial variability at different spatial scales showcases its potential as a biodiversity assessment tool in dynamic marine ecosystems. While it is evident in this study and many others that eDNA is a sensitive, cost-effective, non-invasive method appropriate for biodiversity monitoring in marine protected areas, it has its limitations. The most reported limitation of eDNA also observed in this study is an incomplete reference library/database for species identification. A high risk of contamination is also one of eDNA's common limitations which affects the interpretation of the results because it introduces false positives.

This study showed that sampling many sites and few replicates is more effective in detecting a high number of species and diversity than sampling many replicates in fewer sites. Therefore, a sampling design that will ensure the whole community representation should aim for many sampling sites. One of the confounding factors in this study was sampling at different times of the day, which potentially introduces sampling bias. Sampling at different sites must therefore take place around the same time of the day unless the study aims to compare species detection at different hours of the day. The dynamic nature of the marine environment creates uncertainties in eDNA results interpretation, necessitating the integration of eDNA with conventional biodiversity survey tools. Furthermore, despite the influence of eDNA transport from one habitat to another being negligible, integrating eDNA sampling with the measurements of hydrodynamic processes taking place at the time of sampling would greatly improve the reliability of eDNA results. In conclusion, this study provides crucial spatiotemporal patterns of fish diversity in uThukela MPA, highlighting the need for conservation strategies that consider variability at different spatial scales. Taking advantage of molecular tools such as eDNA can enhance biodiversity assessment by providing bulk biodiversity data in a short period of time, therefore supporting sustainable management of marine protected areas.

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APPENDICES

Appendix A: Summary table of sample data

Sample	Depth_m	Reef	MPA_Status	Management_Zone	DO	Sal	Temp	Turb	Read_Length	Year
K062140	40	Lens Ledge	Inside	Controlled Bottom North	5.7454	35.3824	21.5341	0.214	260	2021
K062141	25	Amatikulu HPM	Inside	Controlled Bottom North	7.0217	35.3749	22.835	0.2262	230	2021
K062142	25	Carpenters	Inside	Restricted	6.4786	35.4317	22.3058	0.1926	265	2021
K062143	10	Lens Ledge	Inside	Controlled Bottom North	6.8971	35.3532	22.9057	0.2353	291	2021
K062144	40	Carpenters	Inside	Restricted	5.6875	35.477	20.5002	0.2567	292	2021
K062145	30	Sundowners	Inside	Restricted	6.5844	35.3853	0.2659	30.244	171	2021
K062146	10	Carpenters	Inside	Restricted	7.0243	35.3565	22.8341	0.2109	253	2021
K062147	35	Carpenters	Inside	Restricted	5.6875	35.477	20.5002	0.2567	251	2021
K062148	50	Sundowners	Inside	Restricted	5.5834	35.4468	0.4062	50.437	179	2021
K062150	5	Tongaat Pinnacles	Inside	Controlled Bottom South	6.9911	35.3669	0.2445	10.111	276	2021
K062151	40	Tongaat Pinnacles	Inside	Controlled Bottom South	6.0297	35.387	0.4581	40.359	272	2021
K062152	40	Ballito Birthday Reef	Inside	Controlled Bottom South	5.9807	35.395	20.8899	0.5344	236	2021
K062153	10	Ballito Birthday Reef	Inside	Controlled Bottom South	6.9872	35.3615	21.6213	0.3818	271	2021
K062154	10	Umdloti Steep	Outside	Outside MPA South	7.0202	35.3565	0.4947	20.132	294	2021

K06 2155	30	Rosie Snowball	Outside	Outside MPA South	6.919	35.3749	0.3452	30.202	169	2021
K06 2156	40	Umdloti Kob	Outside	Outside MPA South	6.0867	35.5116	0.4184	35.313	271	2021
K06 2159	10	Pats Paradise	Inside	Controlled pelagic	6.9656	35.3718	21.8035	0.8091	258	2021
K06 2160	40	Umdloti Steep	Outside	Outside MPA South	6.8655	35.3528	0.4642	40.251	269	2021
K06 2162	5	Pats Paradise	Inside	Controlled pelagic	6.9548	35.4001	22.2308	0.3269	221	2021
K06 2164	10	Tongaat Pinnacles	Inside	Controlled Bottom South	6.9915	35.3673	0.275	20.362	191	2021
K06 2166	5	Ballito Birthday Reef	Inside	Controlled Bottom South	6.9998	35.3629	21.6127	0.3788	201	2021
K06 2167	10	Amatikulu HPM	Inside	Controlled Bottom North	7.0322	35.3697	22.8514	0.2079	258	2021
K06 2168	5	Sundowners	Inside	Restricted	7.0241	35.38	0.2537	10.128	287	2021
K06 2170	40	Ballito Birthday Reef	Inside	Controlled Bottom South	5.9807	35.395	20.8899	0.5344	247	2021
K06 2171	5	Umdloti Steep	Outside	Outside MPA South	7.039	35.3581	0.5039	10.081	289	2021
K06 2172	40	Pats Paradise	Inside	Controlled pelagic	6.5945	35.3781	21.4844	0.9525	281	2021
K06 2173	5	Durnford Hats	Inside	Controlled Bottom North	7.0182	35.3179	22.5095	0.4703	286	2021
K06 2174	10	Durnford Hats	Inside	Controlled Bottom North	6.9306	35.3306	22.3637	0.5161	169	2021
K06 2175	25	Lens Ledge	Inside	Controlled Bottom North	6.892	35.352	22.9014	0.2323	272	2021

K06 2176	50	Zini Hard Edge	Inside	Controlled pelagic	6.68 02	35.3 094	0.80 91	50.3 55	247	20 21
K06 2179	10	Carpenters	Inside	Restricted	7.02 43	35.3 565	22.8 341	0.21 09	261	20 21
K06 2180	10	Zink HPM	Inside	Controlled Bottom South	7.05 48	35.3 726	22.1 374	1.04 71	160	20 23
K06 2181	5	Zink HPM	Inside	Controlled Bottom South	7.05 19	35.3 737	22.1 605	0.95 25	160	20 23
K06 2182	40	Zink HPM	Inside	Controlled Bottom South	6.46 43	35.4 226	21.7 185	1.16 61	162	20 23
K06 2183	10	Grumpy Reef	Inside	Restricted	6.86 72	35.3 126	22.6 911	1.32 79	194	20 23
K06 2184	5	Grumpy Reef	Inside	Restricted	6.88 02	35.3 118	22.6 943	1.41 03	222	20 23
K06 2185	40	Grumpy Reef	Inside	Restricted	6.03 83	35.3 668	21.4 967	3.64 12	297	20 23
K06 2186	10	Durnford Hats	Inside	Controlled Bottom North	6.76 68	35.3 343	22.0 715	6.05 21	297	20 23
K06 2187	40	Durnford Hats	Inside	Controlled Bottom North	6.71 85	35.3 352	22.0 732	4.05 31	270	20 23
K06 2188	5	Durnford Hats	Inside	Controlled Bottom North	6.73 04	35.3 332	22.0 668	5.55 77	222	20 23
K06 2190	5	Rosie Snowball	Outsid e	Outside MPA South	6.86 93	35.3 043	23.1 815	1.07 15	274	20 23
K06 2191	10	Rosie Snowball	Outsid e	Outside MPA South	6.87 72	35.3 032	23.1 647	1.00 44	283	20 23
K06 2192	40	Rosie Snowball	Outsid e	Outside MPA South	6.37 19	35.3 3	22.4 158	1.13 87	274	20 23
K06 2193	5	Sundowner s	Inside	Restricted	6.90 34	35.3 333	22.1 183	1.14 78	188	20 23
K06 2194	10	Sundowner s	Inside	Restricted	6.68 35	35.3 533	21.8 226	1.19 06	209	20 23

K06 2195	5	Ballito Birthday Reef	Inside	Controlled Bottom South	6.847	35.3689	21.944	2.2343	266	2023
K06 2196	10	Ballito Birthday Reef	Inside	Controlled Bottom South	6.8424	35.3679	21.9509	5.695	296	2023
K06 2197	40	Ballito Birthday Reef	Inside	Controlled Bottom South	6.4829	35.3782	21.811	1.4774	298	2023
K06 2198	5	Carpenters	Inside	Restricted	6.9411	35.3751	21.8737	1.4286	173	2023
K06 2199	10	Carpenters	Inside	Restricted	6.7999	35.3866	21.8406	1.4194	285	2023
K06 2200	40	Carpenters	Inside	Restricted	5.9185	35.418	20.7877	3.4184	292	2023
K06 2201	5	Umdloti Kob	Outside	Outside MPA South	6.9693	35.3785	22.4246	1.1143	278	2023
K06 2202	10	Umdloti Kob	Outside	Outside MPA South	6.9579	35.3779	22.4219	1.1448	289	2023
K06 2203	40	Umdloti Kob	Outside	Outside MPA South	6.9133	35.3762	22.3971	1.1814	285	2023
K06 2204	5	Lens Ledge	Inside	Controlled Bottom North	6.6992	35.3633	21.9161	1.8101	283	2023
K06 2206	10	Lens Ledge	Inside	Controlled Bottom North	6.7906	35.3639	21.9216	1.807	293	2023
K06 2207	40	Pats Paradise	Inside	Controlled pelagic	6.3671	35.3509	21.7905	1.1661	286	2023
K06 2208	5	Pats Paradise	Inside	Controlled pelagic	6.9603	35.3418	22.2551	3.0369	292	2023
K06 2209	10	Pats Paradise	Inside	Controlled pelagic	6.9323	35.3428	22.2225	1.807	297	2023
K06 2210	40	Amatikulu HPM	Inside	Controlled Bottom North	5.2479	35.4247	19.65	1.8467	237	2023

K06 2211	5	Amatikulu HPM	Inside	Controlled Bottom North	6.35 67	35.3 247	22.0 614	1.04 41	290	20 23
K06 2212	10	Amatikulu HPM	Inside	Controlled Bottom North	5.53 46	35.3 372	21.2 2	0.99 52	255	20 23
K06 2213	40	Umdloti Steep	Outsid e	Outside MPA South	6.91 96	35.3 697	22.4 354	0.48 56	244	20 23
K06 2214	5	Umdloti Steep	Outsid e	Outside MPA South	6.97 73	35.3 708	22.4 517	0.49 17	123	20 23
K06 2215	10	Umdloti Steep	Outsid e	Outside MPA South	6.97 91	35.3 698	22.4 452	0.46 42	182	20 23
K06 2216	5	Tongaat Pinnacles	Inside	Controlled Bottom South	7.01 14	35.3 673	22.3 398	1.10 51	220	20 23
K06 2217	10	Tongaat Pinnacles	Inside	Controlled Bottom South	7.00 76	35.3 672	22.3 448	1.11 73	202	20 23
K06 2218	40	Tongaat Pinnacles	Inside	Controlled Bottom South	6.56 11	35.3 771	22.1 552	1.14 78	290	20 23
K06 2219	5	Zini Hard Edge	Inside	Controlled pelagic	7.13 08	35.3 469	22.3 353	0.94 64	250	20 23
K06 2220	10	Zini Hard Edge	Inside	Controlled pelagic	7.12 59	35.3 486	22.2 951	0.93 73	296	20 23
K06 2221	40	Zini Hard Edge	Inside	Controlled pelagic	6.98 61	35.3 426	22.2 11	0.88 23	290	20 23