Biogeography, biodiversity, and barcodes: accounting for seagrass associated biodiversity along the South African coastline

Courtney Pierrette Fagg

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Supervised by: Prof Sophie von der Heyden

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Declaration

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Abstract

Environmental DNA metabarcoding is gaining momentum for global biodiversity studies. Many studies have demonstrated the ability of metabarcoding to characterize communities for biodiversity surveys, biogeographical analyses, and impact assessments. However, few studies have been performed along the complex coastline of South Africa, which is influenced by two current systems that shape three highly diverse biogeographic regions (the subtropical and warm temperate regions on the east coast, and cool temperate region on the west coast); especially in highly variable estuarine systems. Given the important ecological and economic services that estuaries, their seagrasses and their associated biodiversity provide, it is important that metabarcoding is assessed as a tool for biodiversity studies in South African estuaries. Yet there are still many unknowns surrounding metabarcoding in estuaries, such as the effect of sampling substrate choice on the communities returned, and the ability of metabarcoding to capture biogeographical variation. As such, this study aimed to investigate metabarcoding using a partial fragment of the mitochondrial cytochrome oxidase 1 (CO1) gene in six estuaries containing the seagrass, *Zostera capensis,* along the coastline of South Africa, with two estuaries per biogeographic region. Broadly, this project compared the multicellular, eukaryotic communities returned by different sampling substrates (water and three different sediment samples, including intertidal vegetated and unvegetated sediments, and subtidal vegetated sediment). There were some differences in community composition from water and sediment samples, likely due to the different communities harboured by the environments. It was expected that sediment substrates from different environments (intertidal and subtidal, and vegetated and unvegetated seagrass beds) would also capture different communities, yet there was no difference between them, which may likely reflect patchiness and insufficient sampling replication. This study further aimed to explore whether eDNA metabarcoding would capture the biogeographical signals associated with the South African coastline. Indeed, this approach was sufficient to detect variation in communities along the coastline to delineate biogeographic regions. The communities returned by regions were significantly different, with the strongest biogeographic differentiation between the east and west coast sites. Furthermore, species richness was found to be greatest in the warm temperate region as has been shown by previous studies. Finally, a redundancy analysis was used to explore the environmental variables that could explain the variation in communities between regions, which showed that nitrate, mean sea surface temperate and water quality were the three most powerful explanatory variables. In all, this study showed that selection of sampling substrate is critical for determining community structuring, and that overall metabarcoding is a useful tool to provide insights into biogeographical structuring of communities.

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All glory be to God, the creator of this universe and all its wonders, for the blessing of science and reason; that we may study and understand His creation and, through it, Him.

"For since the creation of the world His invisible attributes, His eternal power and divine nature, have been clearly seen, being understood through what has been made, so that they are without excuse."

- Romans 1:20

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Sampling substrates:

W= Water

IV= Intertidal vegetated sediment

SV= Subtidal vegetated sediment

IU= Intertidal unvegetated

Sampling sites:

Ol= Olifants

Be= Berg

Br= Breede

Sw= Swartkops

Mn= Mngazana

Um= Umhlatuze

Environmental variables:

SSTrange= Sea surface temperature range

SSTmean= Sea surface temperature mean

PIC= Particulate inorganic carbon

TSM= Total suspended matter

Introduction

Estuaries: valuable ecosystems under threat

Globally, coastal biodiversity encompassing marine and estuarine systems is under increasing pressure from environmental change due to anthropogenic influences (Wernberg *et al*., 2011; Brown *et al*., 2018; Madricardo *et al*., 2019; Bertocci *et al*., 2019). Given that ~65% of the human population lives along the coastal boundary and estuaries exist at the terrestrial-marine interface, the stressors faced by estuaries are complex and varied, making them one of the world's most threatened habitats (Halpern *et al*., 2015; Kaselowski and Adams, 2013; Whitfield and Harrison, 2021; Lima *et al*., 2019). This is problematic as estuaries are "critical transition zones", ranking among the most biologically productive ecosystems in the world (Gillanders *et al*., 2011; Kaselowski and Adams, 2013; Elliot *et al*., 2019; Jones *et al*., 2021). The rich biodiversity found in estuaries plays an important role in ecosystem services such as carbon sequestration, waste treatment, water purification and coastal protection (Turpie and Clark, 2007; Barbier *et al*., 2011; Kennedy *et al*., 2012; Ruiz-Frau *et al*., 2017; Mangan *et al*., 2020). Estuaries are also valuable resources for commercial and subsistence fisheries, as they provide critical breeding and feeding habitats, as well as nurseries, for economically important fish and invertebrate species (Harris *et al*., 2016; Turpie and Clark, 2007; Unsworth *et al*., 2019; Hallett *et al*., 2018).

Estuaries in South Africa provide a range of services through contribution to livelihoods, marine fisheries, amelioration of climate change damages, recreation, tourism, property values and a sense of wellbeing (Cooper *et al*., 2003; Crafford *et al*., 2013; van Niekerk *et al*., 2018). Although they comprise less than 2% of South Africa's territory, these highly productive ecosystems contribute ~R4.2 billion per annum to the South African economy (van Niekerk *et al*., 2018). Yet multiple anthropogenic pressures, such as alteration to freshwater flow, destructive land use and development, unsustainable fishing/bait collection, pollution, and invasive alien species have negatively impacted many estuaries along the South African coastline (van Niekerk *et al*., 2018; Whitfield *et al*., 2018; Adams *et al*., 2020; Whitfield and Harrison, 2021). Due to these impacts, 86% of South African estuaries are threatened with half of these being highly threatened (van Niekerk *et al*., 2018). In general, regional estuaries are under-protected (van Niekerk *et al*., 2018; Whitfield *et al*., 2019), to the detriment of important coastal ecosystem engineers, seagrass (Phair *et al*., 2020).

Seagrasses as ecosystem engineers

Seagrasses are classified as a foundation species (Hughes *et al*., 2009), as their physical characteristics provide critical habitats and biodiversity niches, particularly for juvenile stages of fishes and invertebrates (Constanza *et al*., 2014; Hughes *et al*., 2009). Seagrasses provide a direct link between coastal and offshore marine systems as nurseries for neighbouring fisheries (such as walleye pollock) whose early life-history stages require seagrass habitats (Unsworth *et al*., 2019). Seagrasses act as keystone species (Phair *et al*., 2020) and fundamental ecosystem engineers (Denny, 2021) through filtering nutrients (Worm *et al*., 2006), controlling erosion (Christianen *et al*., 2013), binding sediments (which can be many thousands of years old; Arnaud-Haond *et al*., 2012)

and providing coastal protection services (Christianen *et al*., 2013; Ondiviela *et al*., 2014). Although seagrasses only cover ~0.1% of the ocean space, they contribute to 10-18% of the entire oceanic carbon burial (Greiner *et al*., 2013); providing an important service as blue carbon stores (Kennedy *et al*., 2012; Gullström *et al*., 2018; de los Santos *et al*., 2020). However, despite their obvious ecological benefits in terms of both biodiversity and ecosystem services, seagrasses globally are poorly protected (Waycott *et al*., 2009; Phair *et al*., 2019). The global loss of seagrasses is estimated at ~7% per annum since 1990 (Waycott *et al*., 2009; Unsworth *et al*., 2019), with cascading consequences on the biodiversity they support and their ecosystem services (Barbier *et al*., 2011; McKenzie *et al*., 2020; Moksnes *et al*., 2021).

South African seagrasses

South Africa has both temperate and tropical species of seagrass from the genera *Halodule, Halophila, Ruppia, Syringodium, Thalassodendron* and *Zostera* (Green *et al*., 2003). This study will focus on the Cape dwarf-eelgrass, *Zostera capensis* (Figure 1), which is the dominant seagrass in South Africa, occurring in sheltered estuaries along the ~3000 km coastline (Adams, 2016). Due to its protected meristems, strong root system and flexible leaves, *Z. capensis* can grow in subtidal areas where there are strong tidal currents while also being able to withstand periods of exposure and desiccation (Adams, 2016), although many populations also have an intertidal ecotype (Barnes, 2020). The subtidal and intertidal ecotypes are known to have different light requirements, morphologies and to tolerate stress in different ways (Mokumo pers. Comm; Yuill, pers. Comm). *Zostera capensis* is found predominantly in permanently open estuaries but can occur in estuaries that close periodically to the sea (Adams, 2016), although this strongly depends on salinity levels remaining higher than about 15%. This seagrass has a wide distribution and is found in 62/300 estuaries from the Olifants estuary on the west coast to Kosi Bay on the east coast (Figure 4), yet the distribution is patchy due to a lack of suitable habitat (Short *et al*., 2010). *Zostera capensis* occupies less than 2000km of the South African coastline and is listed as “Vulnerable” in the IUCN Red Data Species List (IUCN, 2010). In fact, *Z. capensis* has been lost from several KwaZulu-Natal estuaries, as well as from parts of the Knysna estuary, due to the disturbance from development, changes in sediment load from the catchment, changes in salinity and run-off and an increase in turbidity (Adams, 2016). A decline in seagrass will be universally detrimental to dependent species, at both lower and higher trophic levels, given the interdependency of seagrasses and their associated biodiversity (Hughes *et al*., 2009). Thus, it is widely acknowledged that management strategies need to shift from species-by-species to an ecosystem-based approach.

Figure 1: Images of *Zostera capensis* seagrass beds.

Macrobenthic invertebrates and ecosystem functioning

It is broadly acknowledged that biodiversity contributes to ecosystem stability, resilience, function and important ecosystem services (Schultze and Mooney, 2012; Clarke *et al*., 2017; Thompson *et al*., 2018; Manning *et al*., 2019). The concept of Biodiversity and Ecosystem Functioning (BEF) emerged in the 1990′s (Naeem *et al*., 1994, Tilman *et al*., 1996) and describes the combined effects of all natural processes that sustain an ecosystem (Lam-Gordillo *et al*., 2020). Marine macrobenthic communities are important providers of ecosystem functioning (Lam-Gordillo *et al*., 2020) due to their functional traits. Functional traits are the components of an organisms' phenotype that determine its effect on ecosystem functioning (Weiss and Ray, 2019). Functional diversity (FD) is thus the most relevant measure for BEF, as it describes the value and range of functional traits according to species' taxonomic, physiological, and morphological characteristics (Wright *et al*., 2006).

Benthic ecosystems play an important role in the storage and cycling of organic matter and nutrients, through animal activities such as bioturbation, bioirrigation, grazing and assembling structures that bind sediment (e.g., burrows, tubes; Canuel *et al*., 2007; Rossi *et al*., 2017; Moraes *et al*., 2018). Animals may also influence sediment environmental conditions by controlling the availability of oxygen and other electron acceptors, reworking the sediment, and removing metabolites (Aller 1982; Lee 1992; Aller and Aller 1998). This demonstrates the impact biodiversity can have on ecosystem functioning, and as such, important ecosystem services. Indeed, a large body of research shows that greater biodiversity increases the number of expressed biological traits which have a greater effect on ecosystem functioning, compared to less diverse communities with poor functional expression (Hillebrand and Matthiessen, 2009; Reiss *et al*., 2009; Snelgrove *et al*., 2014).

Considering the growing evidence that total biodiversity promotes healthy ecosystem functions (Snelgrove, 1997; Loreau *et al*., 2002; Sandifer *et al*., 2015) and that sustaining biodiversity represents a practical framework for ecosystem-based management (Sandifer *et al*., 2015; Diaz *et al*., 2015), there is a need for more comprehensive approaches to monitoring marine biota in the face of

increasing anthropogenic pressures. Plans to maintain and preserve functioning estuarine ecosystems should fundamentally acknowledge the importance of estuarine biodiversity which, however, is generally poorly catalogued and understood (Fagg *et al*., 2021; Singh *et al*., 2021).

Biomonitoring of macrobenthic invertebrates

Accounting for all aspects of biodiversity in the sea still poses a profound challenge to marine scientists, especially when considering small-bodied, cryptic species (Andujar *et al*., 2017). A fundamental tool to provide data to support management of the marine environment is biomonitoring (Jones *et al*., 1996). Biomonitoring infers the state of an ecosystem from samples collected and identified using the best available taxonomic knowledge (Hajibabaei *et al*., 2016), where ecological conditions and status are determined by comparative analyses of pre-identified tolerant or sensitive taxa (Bioindicator species; Wiederholm, 1980; Hajibabaei *et al*., 2016). Macrobenthic invertebrates are important and integral parts of aquatic ecosystems, often forming the basis of food webs and as such play an important bioindicator role (Branch and Branch, 2018). Globally, macrobenthic invertebrate surveys have been used for the assessment of the ecological tatus of aquatic ecosystems (Lambshead *et al*., 1983; Macfarlane *et al*., 2001; Rosenberg *et al*., 2004; Muniz *et al*., 2005; Tweedley *et al*., 2012). However, few studies in South Africa have explored macrobenthic invertebrate diversity in seagrass beds, although research by Barnes (2013; 2019; 2020) suggests diverse assemblages.

Certainly, within the South African context, routine assessments of macrobenthic invertebrates have used mainly morphology-based approaches for species identification (Van Rensburg *et al*., 2020; Barnes, 2019; David *et al*., 2021) which proves time-consuming, as species are often small and difficult to identify, and requires taxonomic expertise (Bouchet *et al*., 2002; Lobo *et al*., 2017). This has limited our ability to investigate diversity patterns beyond a few major groups, most often macroinvertebrates and fishes (Tittensor *et al*., 2010).

Molecular tools for species identification

Molecular DNA-based species analysis methodologies, such as DNA barcoding, have become essential tools for the study of biodiversity (Bourlat *et al*., 2013; Stat *et al*., 2017; von der Heyden, 2017; Günther *et al*., 2018). Broadly speaking, genetic approaches to taxon diagnosis exploit the diversity among DNA sequences to identify organisms (Kurtzman 1994; Wilson 1995). These sequences can be viewed as genetic ‘barcodes’ (Hebert *et al*., 2003a), acting as a tag for rapid and accurate species identification at unprecedented scales and precision (Herbert *et al*., 2005; Hobern *et al*., 2020). The effectiveness of barcoding has been explored through projects such as the Barcode of Life Database (BOLD; www.barcodeoflife.org) which supports the generation and application of DNA barcode data. Such databases also provide crucial references for metabarcoding and environmental DNA studies (Weigand *et al*., 2019). However, the marine database is >70% incomplete (Weigand *et al*., 2019), which remains a challenge for DNA based biomonitoring (Elbrecht *et al*., 2017;

FraijaFernández *et al*., 2020; Leese *et al*., 2018) because gaps in reference libraries results in low resolution taxonomic matches, generating mislabelled sequences (Cristescu, 2014; Leese *et al*., 2018; Mohrbeck *et al*., 2015; Weigand *et al*., 2019). This is also problematic in South Africa, where the barcode database for invertebrate species, is underpopulated (Fagg *et al*. 2021).

In addition, although DNA barcoding has hugely contributed to delineating cryptic species where morphological taxonomy was unable to resolve species status (Bickford *et al*., 2003), it is not an efficient methodology for providing insights into the structure and composition of natural communities given that barcoding relies on non-degraded DNA from a single, tangible specimen and thus cannot infer the presence of a species that was not captured during sampling (Schenk *et al*., 2020). Furthermore, the technology used for DNA barcoding (namely, Sanger sequencing; Sanger *et al*.,1977) can only produce a single sequence per sequencing run, which can be limiting for large-scale studies of biodiversity (Shokralla *et al*., 2014). As such, many genomics centres have altered their approaches, incorporating more efficient technology, such as next-generation sequencing (NGS; reviewed in Shokralla *et al*., 2012), to sequence millions of DNA fragments in parallel (Shokralla *et al*., 2014).

Environmental DNA metabarcoding for community insights

A molecular tool for species detection through NGS is environmental DNA (eDNA) barcoding (or metabarcoding). As displayed in Figure 2, by collecting environmental samples containing DNA shed by organisms present in aquatic or terrestrial environments and comparing sequences to reference databases, it becomes possible to identify species and characterize communities, without *a priori* knowledge of their presence in the substrate (Taberlet *et al*., 2012; Rees *et al*., 2014; Foote *et al*., 2012; Davy *et al*., 2015; Valentini *et al*., 2015; Lamb *et al*., 2019). Environmental DNA methods are significantly less invasive and demonstrated higher detection capability, lower monitoring effort, and cost-effectiveness compared to traditional, morphology-based methods (Darling and Mahon 2011; Dejean *et al*. 2012; Valentini *et al*., 2015). Although technology exists to gather vast amounts of diversity data, such as Autonomous Reef Monitoring Structures (ARMS; Zimmerman and Martin, 2004), they may take years to gather sufficient data. On the other hand, eDNA metabarcoding offers a snapshot of a community which takes weeks to months to process (Ruppert *et al*., 2019). However, metabarcoding is currently hindered by incomplete reference databases (Weigand *et al*., 2019), which can restrict high resolution taxonomic assignment and thus limiting inferences that can be made about the composition of communities (Tapolczai *et al*., 2019). It is therefore important to compare diversity estimates provided by metabarcoding and traditional methods to determine the ability of metabarcoding to characterize the present community (Watts *et al*., 2019).

Environmental DNA metabarcoding within South Africa

In South Africa, metabarcoding has been used in marine studies to explore biogeographical structuring and connectivity of communities for fish (Czachur *et al*., 2021), invertebrates (Nielsen,

2021), zooplankton (Singh *et al*., 2021), metazoans, micro-eukaryotes and prokaryotes (Holman *et al*., 2021) along the environmental gradient of the South African coastline. The South African coastline is one of the most unique and dynamic in the world, influenced by two major current systems (namely, the warm Agulhas and cool Benguela; Griffiths *et al*., 2010) that shape three major, highly diverse, coastal biogeographic regions (namely, the subtropical, warm temperate and cool temperate regions). These regions are broadly defined by a change in sea surface temperature and primary productivity that shape diverse marine communities (Griffiths *et al*., 2010) and evolutionary trajectories (Teske *et al*., 2011; Wright *et al* 2015; Nielsen *et al*. 2021). The diverse biogeography of the South African coastline thus provides a unique insight into the effects of environmental variation on species diversity, as physicochemical changes can have varying physiological demands on the marine life that occupy these systems (Whitfield, 1999), especially in highly variable systems such as estuaries (Day *et al*., 1981). However, few studies have focused on estuaries within southern Africa, nor explored biogeographical patterns for estuaries along the environmental gradient of the South African coastline.

Metabarcoding as a tool in estuarine studies

Globally, metabarcoding has proven useful in estuarine studies for biodiversity monitoring (Ahn *et al*., 2020; Borrell *et al*., 2018; Zhang *et al*., 2019; Mártinez-Marqués, 2015) and ecosystem health assessments (Chariton *et al*., 2015; Clark *et al*., 2020). In South Africa, most estuarine studies have used metabarcoding to determine diversity estimates for benthic diatom (Nunes *et al*., 2019; Nunes *et al*., 2021) and bacterial communities (Matcher *et al*., 2021), which have been limited to single estuaries (St Lucia, Nunes *et al*., 2019; Sundays, Matcher *et al*., 2021). No studies have investigated the multicellular, eukaryote community, encompassing macrobenthos, in estuarine systems along the coastline of South Africa. Metabarcoding in estuaries is considered particularly challenging due to high turbidity, which clogs filters, and elevated levels of PCR inhibitors (Sanches & Schreier, 2020). Coupled with the transport of eDNA from upstream and tidal movements, the interpretation of results is more complicated for estuaries (Hallam *et al*., 2021). This may explain why so few metabarcoding studies have been performed on South Africa's estuaries relative to other marine systems (Czachur *et al*., 2021; Holman *et al*., 2020; Holman *et al*., 2021; Singh *et al*., 2021), which has resulted in little understanding of both the dynamics and potential of metabarcoding for South African, estuarine studies.

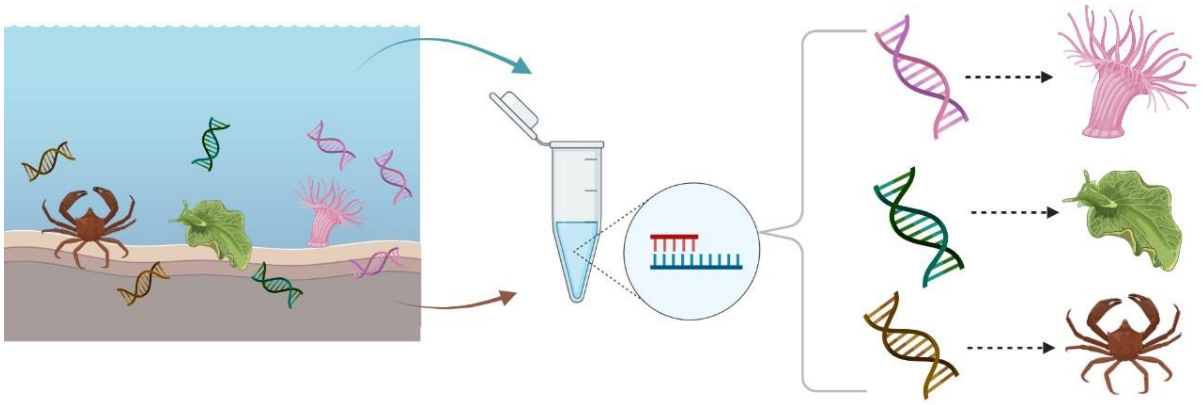

Figure 2: Diagram to illustrate the principle of metabarcoding

Metabarcoding sampling substrates affects the communities returned

The four commonly sampled substrates for metabarcoding include surface water, sediment, settlement plates and plankton tows (Koziol *et al*., 2019). A meta-analysis of the eukaryotic metabarcoding studies up to 2017 found that ~96% used a single biological substrate (primarily water), while only ~4% investigated two or more substrates (Koziol *et al*., 2019), despite studies that revealed that the biological substrate sampled for eDNA analysis is a critical factor influencing the biotic composition characterized from the marine environment (Koziol *et al*., 2019; Buxton *et al*., 2017; Sakata *et al*., 2020), In addition, most studies comparing different sample types have focused primarily on fishes (Sales *et al*., 2019; Sakata *et al*., 2021) with few studies investigating other groups, such as invertebrates.

There are still many unknowns on the origin and fate of eDNA, including the distance it travels and length of time it persists (Gleason *et al*., 2020; Ely *et al*., 2021), yet studies have investigated eDNA concentrations for different sampling substrates. For example, Turner *et al*. (2015) found that eDNA was more concentrated in sediments than water. Sakata *et al*. (2020) concluded that this is likely due to the different decay rates of eDNA in sediment and water, as sediment eDNA is more protected from degradation than aqueous eDNA. In addition, aqueous eDNA diffuses rapidly and is carried by currents, thus reflecting a wider spatial scale, while sediment eDNA progressively accumulates and is protected from degradation, thus reflecting a longer timescale (Sakata *et al*., 2020). The variation on biota obtained from different sampling substrates would thus be qualitatively different (Sakata *et al*., 2020). Indeed, studies have recovered different communities (but with some overlap) and identified different species when analysing water and sediment samples (Holman *et al*., 2019; Sakata *et al*., 2020). In general, however, studies have found that benthic species may be more effectively detected from sediment samples and pelagic species from those taken in the water column (Koziol *et al*., 2019; Shaw *et al*., 2016; Antich *et al*., 2020). Gleason *et al*. (2020) recommends comparing how targeted groups are represented by total sequence reads to determine which method best captures the community of interest.

The detection and absorption of eDNA is further affected by changes in sediment characteristics (Harrison *et al*., 2019; Buxton *et al*., 2018; Sakata *et al*., 2020). The binding capacity of eDNA is dependent on the sediment type as well as measures of pH, dissolved salts, moisture, temperature, and the presence of cell fragments and biofilms (Levy-Booth *et al*., 2007; Sirois and Buckley, 2009; Nielsen *et al*., 2007; Cai *et al*., 2009; Morrissey *et al*., 2015; Pietramellara *et al*., 2007; Montanaro *et al*., 2011). Several studies have shown that seagrasses affect nutrient composition and microbial processes in the sediment, as well as increase sedimentation and decrease resuspension (Short, 1983; Caffrey and Kemp, 1990; Short *et al*., 1993; Pedersen *et al*., 1997; Risgaard-Petersen *et al*., 1998; Hansen *et al*., 2000). This, coupled with the finding that seagrass beds and neighbouring unvegetated sand in estuaries harbour different macrofaunal communities (Barnes, 2017), implies that eDNA metabarcoding of vegetated beds and adjacent, unvegetated sediment should return different communities. In addition, intertidal and subtidal environments also have different sedimentological components (Fernandes *et al*., 2019) and species assemblages (Barnes *et al*., 2020), which would likely affect the results of metabarcoding.

Rationale for this thesis and thesis layout

Environmental DNA metabarcoding is a useful tool to determine the presence of a species in an environment or characterising natural communities without *a priori* knowledge of which species may be present (Lamb *et al*., 2019). However, eDNA metabarcoding research is currently hindered by incomplete reference databases (Weigand *et al*., 2019), which prevents species-level matches and gives low-resolution results (Tapolczai *et al*., 2019). A foundational step to improve the efficacy of metabarcoding is to augment the DNA barcode database for high-resolution sequence matches (Weigand *et al*., 2019). This study therefore aimed to sequence the mitochondrial cytochrome c oxidase subunit 1 (CO1) region of macrobenthic invertebrates from South African estuaries to upload to BOLD (Appendix 1). Furthermore, little is known of the dynamics of eDNA, especially in the South African estuarine context, yet studies suggest that different sampling substrates return different communities (Koziol *et al*., 2019; Antich *et al*., 2020).

Given the valuable ecological and economic services that South Africa's estuaries, their seagrass, and their associated biodiversity provide (van Niekerk *et al*., 2018; Manning *et al*., 2019), the applicability of eDNA metabarcoding in estuaries needs to be investigated to provide a baseline for future eDNA metabarcoding studies, as this method could be a less invasive option with a higher detection capability, lower monitoring effort, and cost-effectiveness compared to traditional, morphology-based methods (Darling and Mahon 2011; Dejean *et al*. 2012; Valentini *et al*., 2015). This study therefore aimed to investigate the multicellular eukaryotic communities returned by eDNA metabarcoding, encompassing invertebrates associated with seagrass meadows, sampled along the South African environmental gradient with various sampling substrates. Specifically, I wanted to investigate to what degree communities were shared between different sampling substrates, namely water and sediment substrates, including sediments from vegetated, unvegetated, intertidal and subtidal environments. I further aimed to explore how communities were structured along the biogeographical gradient of the southern African coastline and to determine which sampling

substrates captured the most variation between biogeographic regions. In addition, I aimed to determine the environmental drivers that best explained the variation in communities returned by regions. The thesis is structured into the following chapters:

Chapter 1: Comparing metabarcoding sampling substrates

Chapter 2: Investigating South Africa's coastal biogeographical patterns using metabarcoding

Chapter 3: Conclusions

Chapter 1: Comparing metabarcoding sampling substrates

Introduction

Metabarcoding for insights into marine communities

Although DNA barcoding has hugely contributed to delineating cryptic species where morphological taxonomy was unable to resolve species status (Bickford *et al*., 2003), it is not an efficient methodology for providing insights into the structure and composition of natural communities given that barcoding relies on non-degraded DNA from a single, tangible specimen and thus cannot infer the presence of a species that was not captured during sampling (Piper *et al*., 2019).

An alternative tool for species detection is through metabarcoding of environmental DNA (eDNA). By collecting environmental samples containing DNA shed by organisms present in aquatic or terrestrial environments, it becomes possible to identify species and characterize communities, without *a priori* knowledge of their presence in the substrate (Taberlet *et al*., 2012; Rees *et al*., 2014; Foote *et al*., 2012; Davy *et al*., 2015; Valentini *et al*., 2015). Two main approaches using eDNA have been proposed: eDNA barcoding (a species-specific approach), which aims at detecting a single species in the environment, and eDNA metabarcoding (or multi-species approach), which simultaneously identifies several taxa from an environmental sample; extending the analysis to a community of individuals (Taberlet *et al*., 2012; Aylagas *et al*., 2014). For example, multiple studies have found that fish biodiversity surveys using eDNA metabarcoding returned similar, sometimes larger, communities to traditional survey methods (i.e., visual or capture surveys, Fujii *et al*., 2019; Yamamoto *et al*., 2016; Yamamoto *et al*., 2017; Hanfling *et al*., 2016; Shaw *et al*., 2016). Various studies have employed metabarcoding to assess species composition in communities of aquatic and terrestrial arthropods (Beng *et al*., 2016; Elbrecht, Vamos, Meissner, Aroviita, and Leese, 2017; Ji *et al*., 2013), vertebrates (Sato *et al*., 2017), diatoms (Vasselon *et al*., 2017) and fungi (Aas *et al*., 2017; Bellemain *et al*., 2012; Tedersoo *et al*., 2018). Such metabarcoding analyses consistently revealed more species than morphological approaches while being more time and cost efficient (Brandon-Mong *et al*., 2015; Elbrecht, Peinert, & Leese, 2017; Elbrecht, Vamos *et al*., 2017; Hebert *et al*., 2018; Ji *et al*., 2013; Shokralla *et al*., 2015; Vivien, Lejzerowicz, & Pawlowski, 2016; Yu *et al*., 2012).

Choice of sampling substrate affects downstream results: water compared to sediment

The biological substrate sampled for eDNA analysis is a critical factor influencing the biotic composition characterized from the environment (Buxton *et al*., 2017; Koziol *et al*., 2018; Holman *et al*., 2019). The four commonly sampled substrates for marine environments include surface water, sediment, settlement plates and plankton tows (Koziol *et al*., 2018). Water is the most often used, although it appears that no single substrate can capture the broad spectrum of taxa. This is likely because of organismal traits, including history traits and habitat preference, will likely influence the detection of organisms' DNA in a given substrate (Koziol *et al*., 2018; Buxton *et al*., 2017). For example, Crane *et al*. (2021) found that during most of their life cycle, the European green crab (*Carcinus maenas*) shed low levels of eDNA- demonstrating the importance of considering life stage and sampling methodology when using eDNA to monitor biodiversity. Indeed, Koziol *et al*. (2018) found that taxonomic composition varied significantly depending on the substrate sampled, suggesting that the "suitability (and bias) of an eDNA substrate will depend on the focal taxa". Furthermore, sampling location may affect results, as eDNA metabarcoding has demonstrated the ability to distinguish vertical structuring of aquatic communities in the water column (Jeunen *et al*., 2020). As such, there is a need to take into consideration both oceanographic (such as water column stratification) and biological processes (such as vertical community structuring) when designing sampling strategies for marine eDNA metabarcoding surveys (Jeunen *et al*., 2020).

In addition, there are many unknowns on the origin and fate of eDNA, including the distance it travels and length of time it persists in an environment (Gleason *et al*., 2020). Most studies comparing the basic properties of sample types have been performed for fish eDNA (e.g., Turner *et al*., 2015), and suggest that eDNA is more concentrated in sediments than water. This is likely due to the different decay rates of eDNA in sediment and water (Sakata *et al*., 2020). As displayed in Figure 3, aqueous eDNA is degraded rapidly by water state, temperature, sunlight (UV) and pH (Andruszkiewicz *et al*., 2017; Eichmiller *et al*., 2016; Strickler *et al*., 2015; Tsuji *et al*., 2017), while sediment eDNA is more protected from degradation by binding to sediment molecules and through the presence of biofilms (Levy-Booth *et al*., 2007; Pietramellara *et al*., 2009; Shogren *et al*., 2017). Indeed, sediment may harbour 8-1800 times more eDNA (Turner *et al*., 2015) and return more OTUs (Holman *et al*., 2019) compared to water samples in freshwater and saltwater ecosystems.

Furthermore, sediment and water may reflect different scales due to differences in transport and decay rate (Sakata *et al*., 2020; Holman *et al*., 2019). Aqueous eDNA diffuses rapidly and is likely carried by currents, thus reflecting a wider spatial scale (Sakata *et al*., 2020; Ely *et al*., 2021). Eventually, aqueous eDNA precipitates through vertical transport and may be incorporated into sediments, the rate at which depends on particle size (Buxton *et al*., 2018; Sakata *et al*., 2020). Sediment eDNA progressively accumulates and is protected from degradation, thus reflecting a longer timescale (Sakata *et al*., 2020). Indeed, sedimentary eDNA may persist for a short time or for thousands of years, depending on the sediment characteristics (Buxton *et al*., 2018; Balint *et al*., 2018; Nelson-Chorney *et al*., 2019). As such, aqueous eDNA may provide a current, yet spatially broad, reflection of a community (Ely *et al*., 2021), while sediment eDNA may provide more historical information (Nelson-Chorney *et al*., 2019). As such it is not surprising that studies have recovered

different communities when analysing water and sediment samples (Holman *et al*., 2019; Sakata *et al*., 2020). In general, benthic species may be more effectively detected from sediment samples and pelagic species from those taken in the water column (Koziol *et al*., 2018; Shaw *et al*., 2016), thus it is expected that sediment and water substrates would capture different communities. To determine which sampling substrate best captures the community of interest, Gleason *et al*. (2020) recommend comparing how groups are represented by sampling substrates.

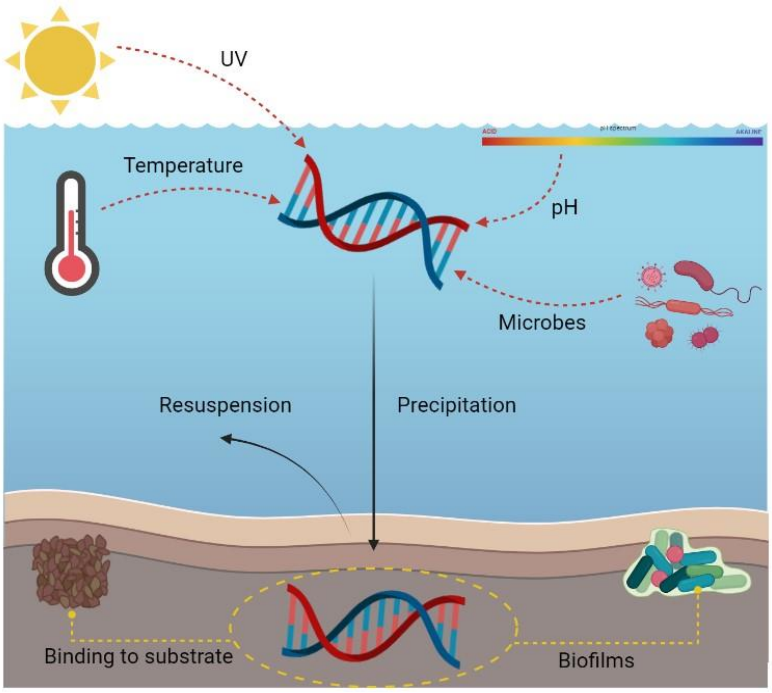

Figure 3: A diagram to illustrate the variables affecting the DNA degradation rate in water and sediment.

Vegetated compared to unvegetated seagrass beds

The detection and absorption of eDNA is further affected by changes in sediment characteristics (Harrison *et al*., 2019; Buxton *et al*., 2018; Sakata *et al*., 2020). The binding capacity of eDNA is dependent on the sediment type as well as measures of pH, dissolved salts, moisture, temperature, and the presence of cell fragments and biofilms (Levy-Booth *et al*., 2007; Sirois and Buckley, 2009; Nielsen *et al*., 2007; Cai *et al*., 2009; Morrissey *et al*., 2015; Pietramellara *et al*., 2007; Montanaro *et al*., 2011). Estuaries have been shown to possess different sedimentological components (Valgamaa *et al*., 2008), which not only affect the DNA binding capacity of sediment, but also the associated faunal assemblages (Thrush *et al*., 2003; Anderson, 2008; Pratt *et al*., 2014).

Several studies have shown that seagrasses affect nutrient composition (Short, 1983; Caffrey and Kemp, 1990; Short *et al*., 1993; Risgaard-Petersen *et al*., 1998) and microbial processes in the sediment (Pedersen *et al*., 1997; Hansen *et al*., 2000), as well as increase sedimentation and decrease resuspension (de Boer *et al*., 2007). In addition, vegetative cover has been shown to affect animal assemblages (Park *et al*., 2019; Jankowska *et al*., 2019), as seagrass provide a shelter and substrate for numerous benthic species, as well as a direct food source for grazing invertebrates (Gartner *et al*., 2013). For example, a study by Jankowska *et al*. (2019) found that the presence of seagrass meadows increased ecological stability in the faunal assemblage through a larger range of

food sources used by consumers. As such, it is expected that vegetated sediment would harbour a greater species richness, as well as a greater DNA binding capacity, and would thus capture a broader community than unvegetated sediment. I therefore expect vegetated and unvegetated sediment substrates to capture different communities.

Intertidal compared to subtidal environments

Intertidal and subtidal environments also have different sedimentological components (Fernandes *et al*., 2019) and species assemblages (Barnes *et al*., 2020), which would likely affect the results of metabarcoding. Indeed, Fernandes *et al* (2019) found that subtidal sediments were usually anoxic and presented higher amounts of clay and silt than intertidal sediments, which may affect eDNA detection as clay substrates have lower detection capability (Buxton *et al*., 2018). Furthermore, clay reduces the abundance of key species in estuaries (Pratt *et al*., 2014), which may explain why intertidal zones display greater species diversity than subtidal zones (Barnes and Claassens, 2020). As such, since intertidal beds have been shown to display both greater detection capabilities and greater species diversity, it is expected that intertidal sediment would capture a broader community than subtidal sediment. I therefore expect intertidal and subtidal sediment substrates to capture different communities.

Aims and hypotheses

In South Africa, no eDNA metabarcoding studies have been carried out in estuaries. As such, there is no knowledge of how faunal communities may differ between seagrass and unvegetated environments, or whether their communities differ between intertidal and subtidal sediments. There have also been no studies examining potential differences between water and sediment substrates within the context of metabarcoding. However, such information is crucial to develop eDNA metabarcoding in the region, specifically determining optimal sampling strategies. Therefore, the aim of chapter was to compare the communities returned by different metabarcoding sampling substrates, namely water and sediment substrates- including intertidal vegetated, intertidal unvegetated and subtidal vegetated sediments.

Aim 1: Compare the communities returned by water and sediment substrates.

H0: Community composition will differ between sediment and water substrates, with little overlap.

Aim 2: Compare the communities returned by sediments from vegetated and unvegetated environments.

H0: Vegetated and unvegetated sediments will capture different communities.

Aim 3: Compare the communities returned by sediments from intertidal and subtidal environments.

H0: Intertidal and subtidal sediments will capture different communities.

Materials and methods

Study sites

Sampling was performed in the late winter/early spring season of 2020 (August- October) in six estuaries harbouring *Zostera capensis:* the Umhlatuze, Mngazana, Swartkops, Breede, Berg and Olifants estuaries (Figure 4). Importantly, these estuaries are representative of overall diversity of estuarine sites in South Africa and cover the entire biogeographical gradient of the coastline (van Niekerk *et al*., 2020). Furthermore, these six estuaries are included in the top 50 South African estuaries in terms of conservation importance, where the conservation importance is calculated on the basis of weighted size, habitat, zonal type rarity and biodiversity importance scores (Turpie *et al*., 2002).

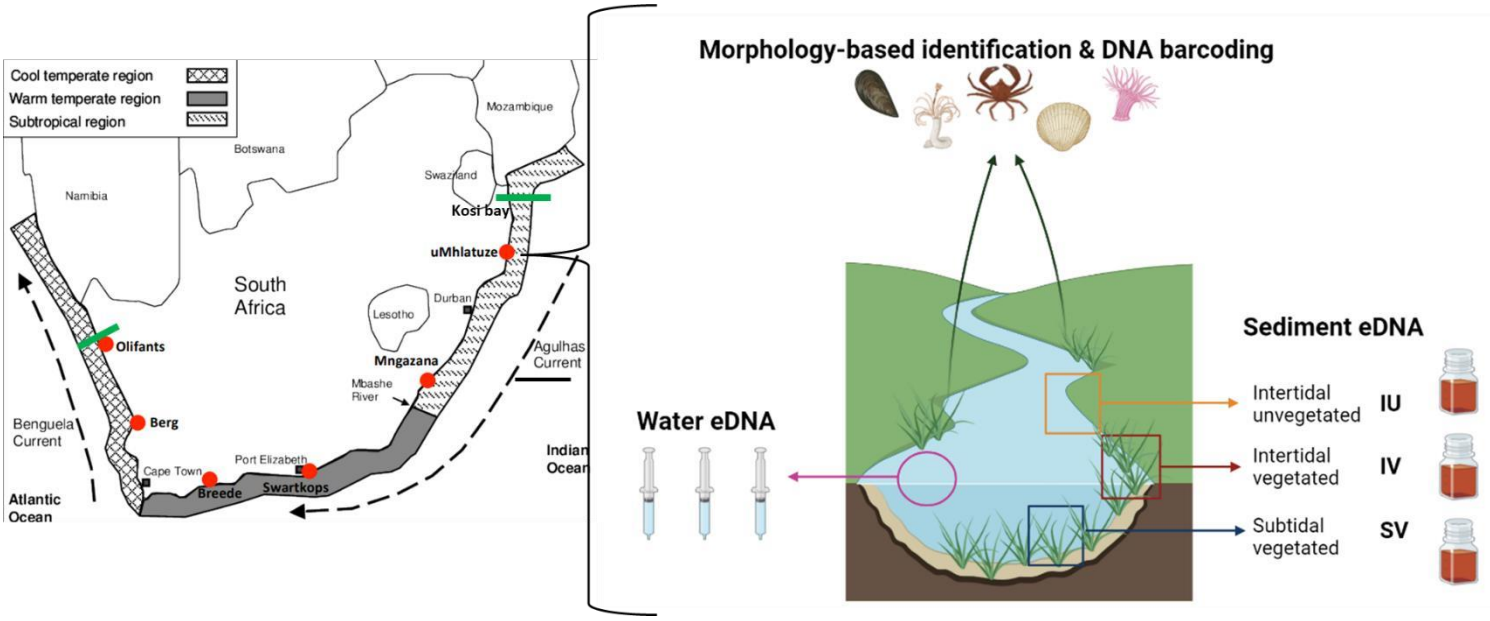

Figure 4: Map and sampling design. Red dots indicate sampling sites and green lines indicate the South African range of *Zostera capensis* on the map. The dominant currents, as well as major biogeographic breaks, are indicated.

Sampling design

Figure 4 provides an overview of the sampling design. Samples for metabarcoding were collected from both water and sediment substrates (henceforth referred to as sampling substrates) from the same site within each estuary under permit number RES2020/32. Sampling sites consisted of one point within the estuary, in a large seagrass bed (~1km long) near the mouth of the estuary (where salinity is highest), as both seagrass cover (Attrill *et al*., 2000; Lee *et al*., 2001; Jelbart *et al*., 2006)

and salinity (Awad *et al*., 2002; Harrison and Whitfield, 2006; Adams *et al*., 2016) positively influence species richness and abundance.

All equipment, containers and gloves were sterilised with a 10% bleach solution prior to entering the field and all equipment was sterilised using the 10% bleach between collecting samples. Three Sterivex filters (Sterivex—GP capsule filter pore size 0.22 µm; Millipore Sigma, Darmstadt, Germany) were used for collecting and transporting DNA based on prior eDNA extraction efficiency studies (Spens *et al*., 2017) and following best practice in the von der Heyden lab. Water samples were collected five metres apart along the edge of the seagrass bed from the surface of the water column. Water around the seagrass bed and upstream from the researcher was repeatedly collected and pushed through the filter using a 50ml syringe until the filter displayed resistance, which varied across sites depending on the turbidity. For each Sterivex filter, 150ml to 500ml of water was sampled. The Sterivex filter samples were airdried with the 50ml syringe and filled with 2ml of ATL buffer (Qiagen) to preserve the DNA captured in the filter. Sterivex filters were capped, sealed with parafilm, placed into sterile, labelled plastic bags and stored at room temperature until DNA extractions could be completed.

Sediment samples were collected from three points within a site, namely intertidal vegetated (IV), intertidal unvegetated (IU) and subtidal vegetated (SV) beds (see Figure 4), which were spaced one to three metre apart. These sampling environments were chosen as they have been shown to both harbour diverse faunal assemblages (Barnes and Claassens, 2020; Park *et al*., 2019; Jankowska *et al*., 2019) and present varying sedimentological properties which may affect DNA adhesion and preservation (Valgamaa *et al*., 2008; Fernandes *et al*., 2019; Buxton *et al*., 2018). For each, 45ml of sediment was collected from the sediment surface with a spatula and placed into a container, labelled, sealed with parafilm and set on ice until return to the laboratory. Subtidal sediments were collected upstream from the researcher, however multiple attempts were required to collect 45ml of sediment as sediment often washed away. Sediment samples were kept frozen at -25°C until DNA extractions could be completed.

eDNA extraction, amplification, and sequencing

A brief overview of the metabarcoding procedure is depicted in Figure 5. Environmental DNA extractions were performed in a sterile laboratory (all equipment and bench space wiped with 10% bleach solution as well as exposed to UV for ~30 minutes prior to usage), separate from main laboratory facilities. Sediment eDNA was extracted using the Qiagen DNeasy PowerSoil kit (Pearman *et al*., 2020), according to the manufacturer's instructions. Samples were extracted in triplicate and pooled. Extracted DNA was stored at -25°C. Water eDNA was extracted from the filters using the DNeasy Blood & Tissue Kit (Qiagen,) according to a modified protocol (Czachur, pers. Comm), and stored at −25°C. The three water samples for each site were pooled. For each extraction procedure, a blank was included. The DNA concentration of the samples and blanks was tested for with a Broad Range Qubit Assay to ensure adequate DNA concentration of samples (>1ng/µl) and no contamination.

Extracted eDNA samples were sent for PCR amplification and sequencing at the Advanced Identification Methods Lab (AIM Lab) in Berlin, Germany. Amplification of the CO1-5P target region and preparation of the MiSeq libraries was performed with a 2-step PCR. A 313 bp long mini-barcode region was amplified by PCR (Leray *et al*., 2013; Morinière *et al*., 2016), using forward and reverse HTS Leray primers, with complementary sites for the Illumina sequencing tails in triplicate. The second PCR reaction used index primers with unique i5 and i7 inline tags and sequencing tails for amplification of indexed amplicons. Equimolar amplicon pools were made and size selected using preparative gel electrophoresis. MagSi-NGSprep Plus beads (Steinbrenner Laborsysteme GmbH, Wiesenbach, Germany) were used to purify the pooled DNA. A bioanalyzer (High Sensitivity DNA Kit, Agilent Technologies) was used to check the bp distribution and concentration of the amplicons before the creation of the final library. High-throughput sequencing (HTS) was performed on an Illumina MiSeq using v2 (2*250 bp, 500 cycles, maximum of 20mio reads) chemistry (Illumina).

Bioinformatic analyses

Bioinformatic analyses were performed by AIM Lab and following this protocol; raw FASTQ files from Illumina were processed using the VSEARCH suite v2.9.1 (Rognes, Flouri, Nichols, Quince, & Mahé, 2016) and Cutadapt v1.18 (Martin, 2011). Forward and reverse reads for each sample were merged with the VSEARCH program “fastq_mergepairs” and a minimum overlap of 10 bp, presenting sequences of ~313 bp. Forward and reverse primers were removed using Cutadapt. To discard sequences for which primers were not consistently detected at a minimum 90% identity, the “discard_untrimmed” option was used. Quality filtering with the “fastq_filter” in VSEARCH was used to keep sequences with zero expected errors (“fastq_maxee”). Sequences were dereplicated with “derep_fulllength,” at the sample level, and then concatenated into one FASTA file, which was dereplicated. The VSEARCH program “uchime_denovo” was used to filter out chimeric sequences from the FASTA file. The remaining sequences were clustered into OTUs at 97% identity with a greedy centroid-based clustering program, “cluster_size”. OTUs were blasted against databases (BOLD, NCBI and RDP) in February 2021, including taxonomy and BIN information, with Geneious (v.10.2.5—Biomatters, Auckland—New Zealand), and following the methods of Morinière *et al*. (2016). The resulting csv file which included the OTU ID, BOLD Process ID, BIN, Hit%-ID value (percentage of overlap similarity- i.e., identical basepairs- of an OTU query sequence with its closest match in the database), length of the top BLAST hit sequence, phylum, class, order, family, genus, and species information for each detected OTU was exported from Geneious and combined with the OTU Table generated by the bioinformatic pipeline. The results were filtered by Hit-%-ID value and total read numbers per OTU. Entries with identifications below 97% and total read numbers below 0.01% of the summed reads per sample were removed from the analysis. OTUs were then assigned to the respective BIN. Additionally, the API provided by BOLD was used to retrieve BIN species and BIN countries for every OTU, and the Hit-%-IDs were aggregated over OTUs that found a hit in the same BIN and shown in the corresponding column as % range. To validate the BOLD BLAST results, a separate BLAST search was carried out in Geneious (using the same parameters) against a local copy of the NCBI nucleotide database downloaded from (ftp://ftp.ncbi.nlm.nih.gov/blast

/db/). Interactive Krona charts were produced from the taxonomic information using KronaTools v1.3 (Ondov, Bergman, & Phillippy, 2011). Species identification was based on high-throughput sequencing (HTS) data grouped to genetic clusters (OTUs), blasted, and assigned to barcode index numbers ("BINs": Ratnasingham & Hebert, 2013) which are a good proxy for species numbers (Hausmann *et al*., 2013; Ratnasingham & Hebert, 2013).

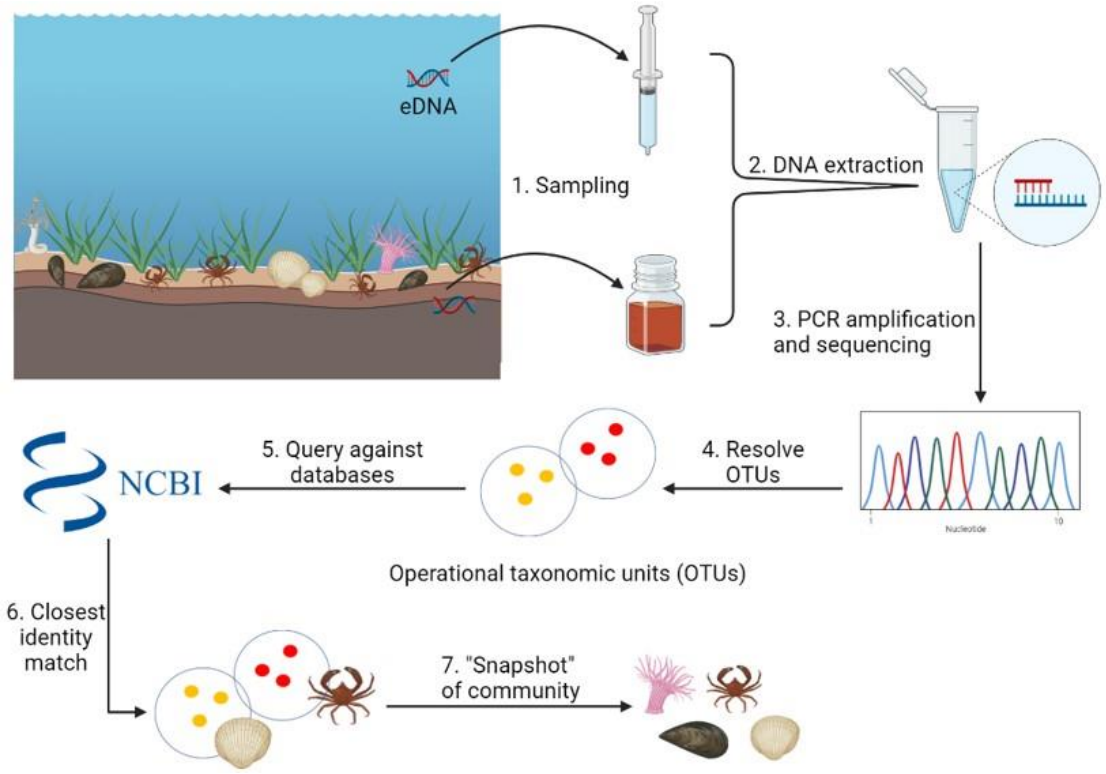

Figure 5: Overview of the metabarcoding workflow

Statistics

All data manipulation and statistical analyses were performed using RStudio (Allaire, 2012) and PAST v4.03 (Hammer *et al*., 2001) and data visualisation was performed with Excel (Raubenheimer, 2017), DisplayR (Displayr | Analysis and Reporting Software for Survey Data; Chasapi *et al*., 2020) and PAST v4.03 (Hammer *et al*., 2001). Only OTUs for multicellular, marine eukaryotes were retained, and a read threshold of 0.01% of total reads (28 reads) was applied (Buchner *et al*., 2021). The NCBI reference database was used for OTU identity given it is one of the largest and most important sources of biological data (Winter, 2017; Pirovano *et al*., 2017; Buchmann and Holmes, 2019).

Calculating OTU counts

Total OTU counts were calculated for both site and sampling substrates. To investigate the degree of taxonomic resolution that metabarcoding returned, the number of OTUs with the lowest classification

at taxonomic levels was calculated. The total number of OTUs returned for taxonomic levels was also calculated.

Comparing the total number of OTUs by sampling substrate

The number of OTUs were used as an indication of community size, given that OTUs are a proxy for species numbers (Hausmann *et al*., 2013; Ratnasingham & Hebert, 2013). To compare the size of communities returned across sites by sampling substrates, Chi-squared comparisons of the number of OTUs returned by substrates were performed in RStudio with expected probabilities of $\frac{1}{2}, \frac{1}{2}$. Boxplots of the number of OTUs returned by substrates were constructed in Excel, and Venn diagrams to illustrate the size and overlap of communities were made in DisplayR.

Comparing the communities returned by substrates

To explore community composition, the number of OTUs, and percentage of total OTUs, returned by phyla for sampling substrates and sites was visualised with stacked barplots in Excel. To identify the dominant phyla returned by substrates, pie charts of the total number of OTUs for phyla were created per substrate in Excel.

To visualise the similarity between communities captured by substrates across sites, samples were grouped by sites and a Principal Coordinates Analysis (PCoA) scatter plot (1st and 2nd axis) of OTUs was generated in PAST v4.03, with convex hulls around each site's samples. A transformation exponent of c=2 and Jaccard distances for the similarity index were used. Jaccard distances were chosen as OTU data was binary (presence/absence) and this ecological index has been shown to be appropriate for metabarcoding and biogeographical studies (Salazar, 2018). The plot used an eigenvalue scale.

To further visualise the similarity between communities returned by substrates, samples were grouped by substrates and a non-metric multidimensional scaling plot (nmMDS plot) of Jaccard distances was created in PAST v4.03.
To determine whether substrates returned different communities, A 2-way PERMANOVA with Jaccard distances as the similarity index and 999 permutations, was performed in
PAST v4.03 by grouping samples according to substrate and biogeographic region. A 2-way PERMANOVA was used to assess differences in multivariate centroids and dispersion between substrates (Holman *et al*., 2021), and to determine an interaction between substrates and regions. Pairwise p-values were calculated in PAST v4.03 to investigate the individual comparisons between regions. To elucidate the OTUs contributing to the dissimilarity between substrates, a SIMPER analysis (Gibert and Escarguel, 2019) was performed with all groups pooled and using the Bray-Curtis distance measure (Mumby, 2001). The OTUs with the greatest contributing percentage were included in a correspondence analysis scatter plot (Abdi and Bera, 2014) to display the OTUs at phylum level contributing to 25% of the dissimilarity between substrates.

Results

Metabarcoding returned a total of 288225 reads for 3533 OTUs. Figure 6 displays the species list received from AIM lab as a KRONA chart (interactive version: Krona - FP_MR_ITS). After filtering for Eukaryotic, multicellular marine OTUs, and applying a read threshold of 0.01% of total reads (28 reads), 11340 reads for 242 OTUs were retained. The number of OTUs returned by both sites and substrates is displayed in Table 1. The range of OTUs returned by substrates at sampling sites is [11; 74]. In total, water returned the fewest OTUs, while subtidal vegetated returned the most OTUs. Table 2 shows the number of OTUs with lowest classification at taxonomic levels for the sampling substrates. Almost half of all OTUs were classified only to the Kingdom or Phylum level (111 out of 242 OTUs).

Table 1: The number of OTUs returned by sites and substrates. W = water, IV = intertidal vegetated, SV= subtidal vegetated, IU= Intertidal unvegetated

Sites		**Substrate**			**Total**
	W	IV	SV	IU	
Umhlatuze	17	26	31	11	55
Mngazana	40	29	20	29	76
Swartkops	25	36	17	49	67
Breede	44	58	74	69	105
Berg	24	32	41	34	68
Olifants	39	31	33	36	66
Total	126	144	161	156	**242**

Table 2: The number of OTUs with lowest classification at taxonomic levels (Kingdom, Phylum, Class, Order, Family, Genus, Species) for sampling substrates, and total number of OTUs at taxonomic levels. Abbreviations as per Table 1.

Substrate	**Taxonomic level**							**Total**
	K	P	C	O	F	G	S	
W	39	12	19	22	28	1	5	126
IV	54	18	23	18	23	4	4	144
SV	61	14	27	21	29	2	7	161
IU	61	16	27	19	26	3	4	156
Total OTUs with lowest classification	88	23	37	36	47	3	8	
Total OTUs returned	242	154	131	94	58	11	8	

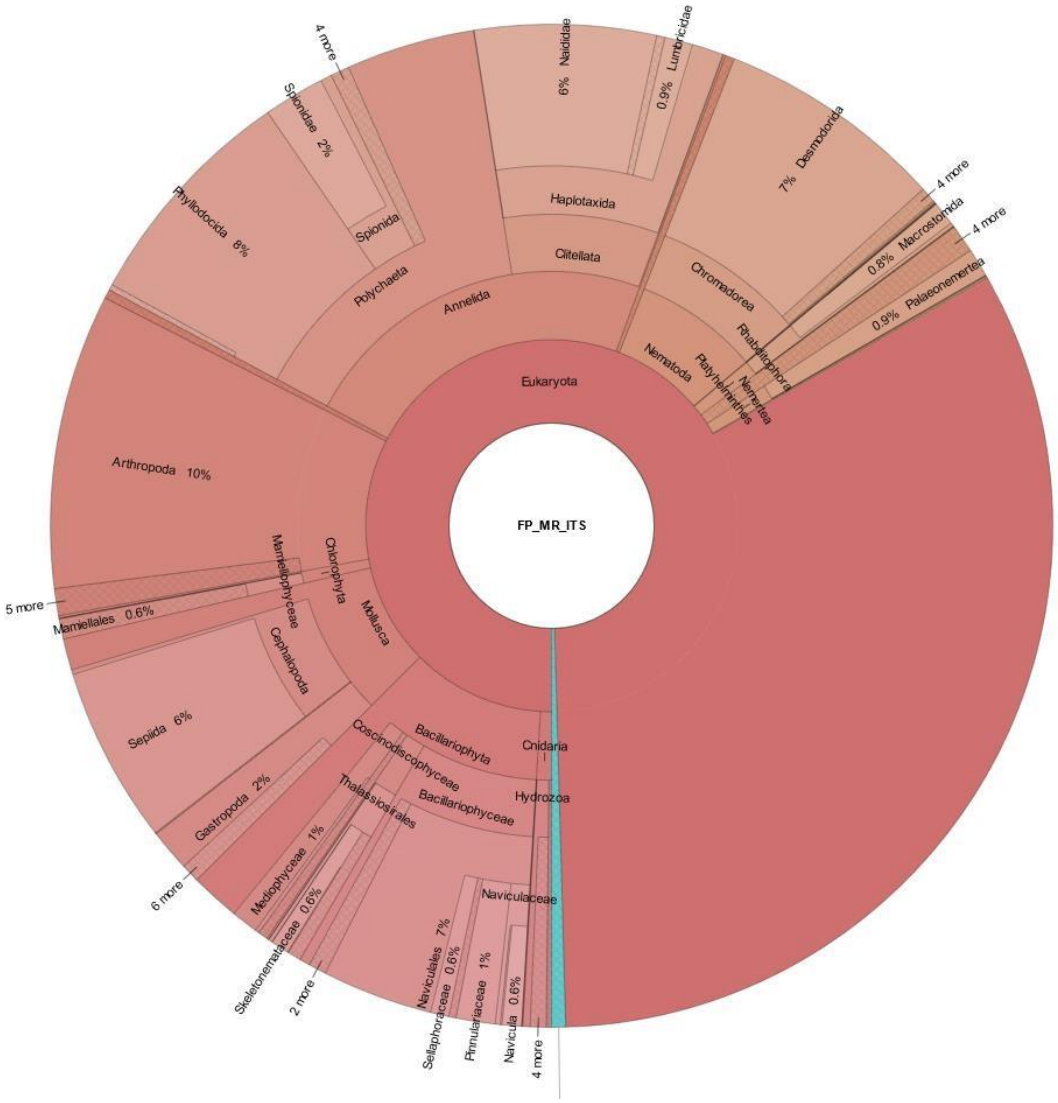

Figure 6: KRONA chart to display the composition of total results (3533 OTUs) returned by AIM lab.

Comparing the size of the communities returned by sampling substrates

Metabarcoding sampling substrates returned varying OTU numbers across sites (Figures 7 and 8). The size of communities returned by sampling substrates at sites were compared with Chi-squared tests using the data in Table 1, which showed some significant differences across the comparisons (8 out of 36 comparisons were significant, Table 3). Most differences were between water and the sediment substrates (namely, intertidal unvegetated and subtidal vegetated). Water and subtidal vegetated returned significantly different community sizes for most sites, yet the trend was inconsistent. Due to the few significant differences and the inconsistency in results, it cannot be concluded that there was a trend of different community sizes between any sampling substrates.

Broadly, sediment samples were more similar to each other, and there was little overlap with water, yet there was some variation as to the degree of overlap depending on sampling site (Figure 8). Overall, a large proportion of OTUs appear to be unique to either the sediment or water substrates, indicating unique communities.

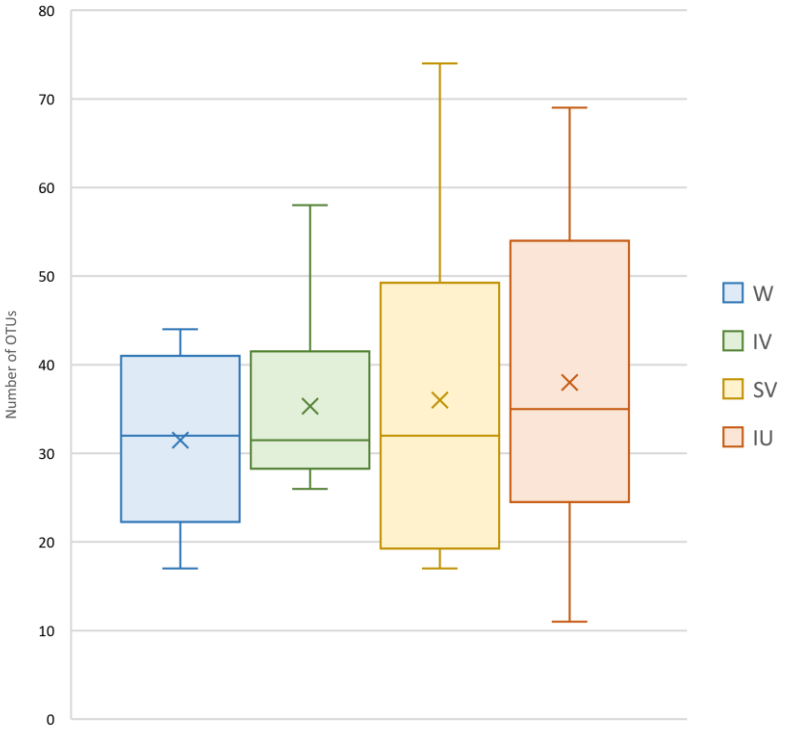

Figure 7: Boxplot of the number of OTUs returned across six sites by sampling substrates.

Table 3: The results of Chi-squared tests on the number of OTUs returned by sampling substrates at sites. Where the difference is significant (p< 0.05), the relationship is shown.

Water compared to sediments						
Sites	**W vs IV**		**W vs IU**		**W vs SV**	
Berg	X-squared = 1.143, df = 1, p= 0.285		X-squared = 1.724, df = 1, p= 0.189		X-squared = 4.446, df = 1, p= 0.035	SV > W
Olifants	X-squared = 0.914, df = 1, p= 0.339		X-squared = 0.120, df = 1, p= 0.729		X-squared = 0.5, df = 1, p= 0.480	
Swartkops	X-squared = 1.984, df = 1, p= 0.159		X-squared = 7.784, df = 1, p= 0.005	IU > W	X-squared = 1.524, df = 1, p= 0.217	
Breede	X-squared = 1.921, df = 1, p= 0.166		X-squared = 5.531, df = 1, p= 0.0187	IU > W	X-squared = 7.627, df = 1, p= 0.006	SV > W
Umhlatuze	X-squared = 1.884, df = 1, p= 0.170		X-squared = 1.286, df = 1, p= 0.257		X-squared = 4.083, df = 1, p= 0.043	SV > W
Mngazana	X-squared = 1.754, df = 1, p= 0.185		X-squared = 1.754, df = 1, p = 0.185		X-squared = 6.667, df = 1, p= 0.010	W > SV
Sediment comparisons						
Sites	**IV vs SV**		**IV vs IU**		**SV vs IU**	
Berg	X-squared = 1.120, df = 1, p= 0.292		X-squared = 0.061, df = 1, p= 0.806		X-squared = 0.653, df = 1, p= 0.419	
Olifants	X-squared = 0.063, df = 1, p= 0.803		X-squared = 0.373, df = 1, p= 0.541		X-squared = 0.130, df = 1, p= 0.718	
Swartkops	X-squared = 6.811, df = 1, p= 0.009	IV > SV	X-squared = 1.988, df = 1, p= 0.159		X-squared = 15.515, df = 1, p= 8.185e-05	
Breede	X-squared = 1.939, df = 1, p= 0.164		X-squared = 0.953, df = 1, p= 0.329		X-squared = 0.175, df = 1, p= 0.6759	
Umhlatuze	X-squared = 0.439, df = 1, p= 0.508		X-squared = 6.081, df = 1, p= 0.0137	IV > IU	X-squared = 9.524, df = 1, p= 0.002	
Mngazana	X-squared = 1.653, df = 1, p= 0.199		X-squared = 0, df = 1, p= 1		X-squared = 1.653, df = 1, p= 0.199	

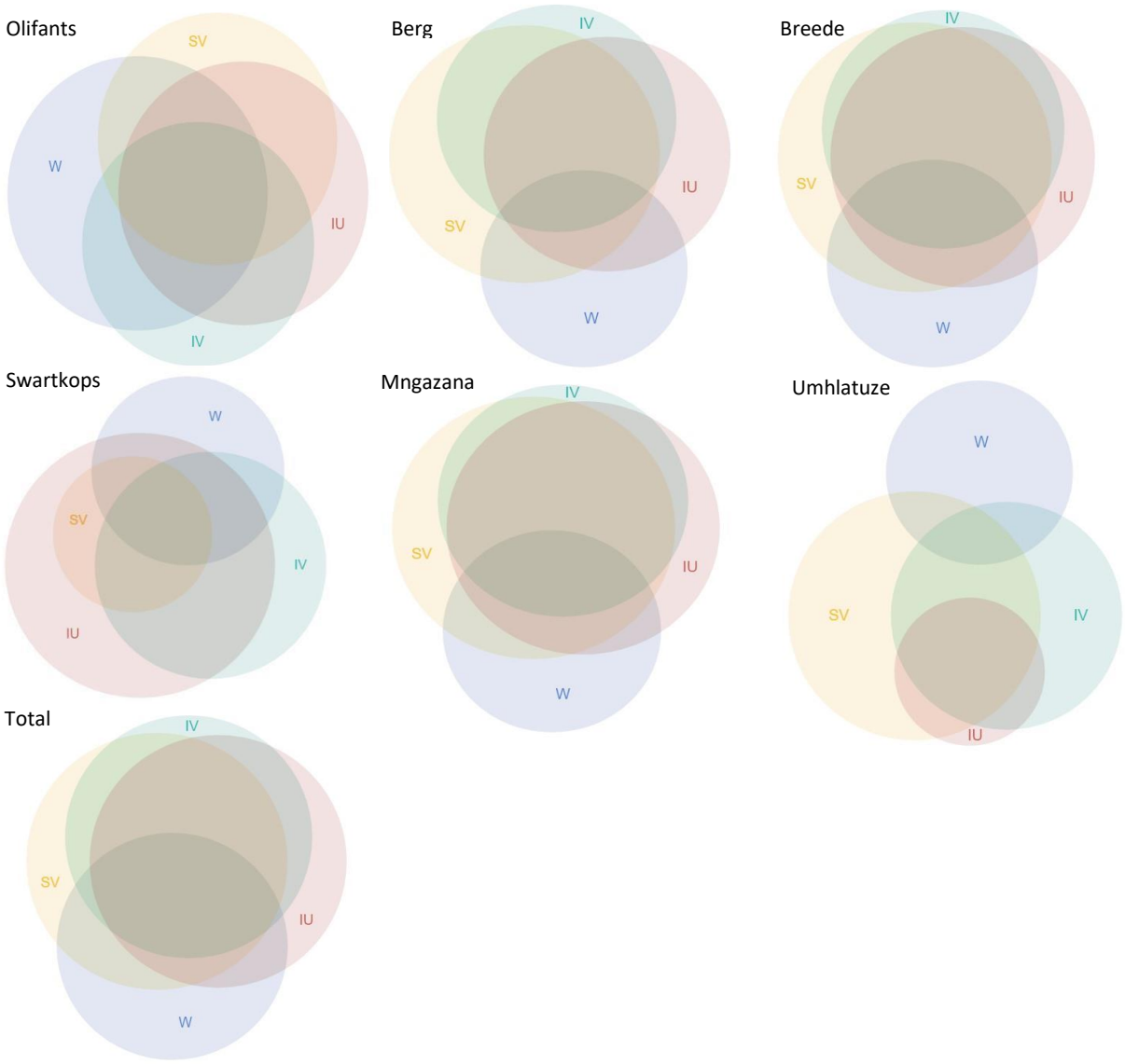

Figure 8: Venn diagrams of the number and overlap of OTUs returned for substrates at sites and in total.

Comparing communities from different substrates

The results demonstrate that sediment substrates from different environments (intertidal, subtidal, vegetated, unvegetated) captured similar communities, while sediment and water substrates captured unique proportions of communities, with some overlap. To investigate community composition returned by substrates, the proportions of OTUs returned for phyla by sampling substrates were visualised (Figures 9 and 10). Some phyla had similar proportions across substrates, while others were more represented in a particular substrate, yet generally the presence and abundance of OTUs for phyla varied across both sampling substrates and sites (see Figure 9). For example, Arthropoda returned the largest proportion of OTUs across substrates (25-27%; Figure 10), which remained consistent (consistent given the variance is 1% from the mean) across substrates. Mollusca also returned a consistent and relatively large proportion of OTUs across substrates (12-14%). Water had higher proportions of Ochrophyta and Cnidaria than sediment substrates and was the only substrate to return OTUs for Chordata. Meanwhile, sediment substrates captured higher, yet varying, proportions of OTUs for the “worms” (Annelida, Nematoda, Nemertea, and Platyhelminthes), than

water. Generally, sediment substrates captured similar proportions of phyla, with most variation between water and sediments.

The PCoA plot (Figure 11) shows the structuring of communities across substrates at sites. Generally, water appeared further removed from the sediment substrates, which showed grouping and thus community similarity. The distinction between the water and sediment samples was most pronounced for the Breede, Berg and Umhlatuze samples. Sediments showed the least distinction from the water sample for the Swartkops and Olifants estuaries.

The structuring of communities across substrates was further visualised with a nmMDS plot (Figure 12). The plot shows grouping of the sediment substrates, apart from the water substrate. The water samples for Berg, Breede, Mngazana and Umhlatuze present further apart from their corresponding sediment samples than the other sites, thus driving the dissimilarity in community composition between water and the sediment substrates. The water samples for Swartkops and Olifants showed the least distinction from the sediment samples.

The 2-way PERMANOVA showed no significant difference in the communities returned by substrates (PERMANOVA, $F= 0.968$, $p= 0.516$), and pairwise comparisons (Table 4) revealed no significant differences between substrates as the range of p-values was: [0.068; 0.984]. As such, sediment substrates from different environments (vegetated, unvegetated and sub and intertidal) were highly similar. While communities were not significantly different between water and sediment substrates, they still displayed a distinction in community structure at some sites.

To investigate the OTUs and taxa driving the dissimilarity between substrates, a SIMPER analysis was carried out (results are included in Table A3, Appendix 2). The OTUs contributing to 25% of the dissimilarity were included in a correspondence analysis scatter plot (Figure 13), where longer distance between substrates indicates dissimilarity. The plot therefore shows a large dissimilarity between the water substrate and the cluster of sediment substrates, thus capturing the pattern of community structure with only 25% dissimilarity. The OTUs that had the greatest contribution to the dissimilarity were mainly from the phyla Ochrophyta and Arthropoda, yet many OTUs were only at the Kingdom level and do not provide much insight. The scatter plot demonstrates, however, that these OTUs were the main contributors to the pattern of dissimilarity between the water and sediment substrates.

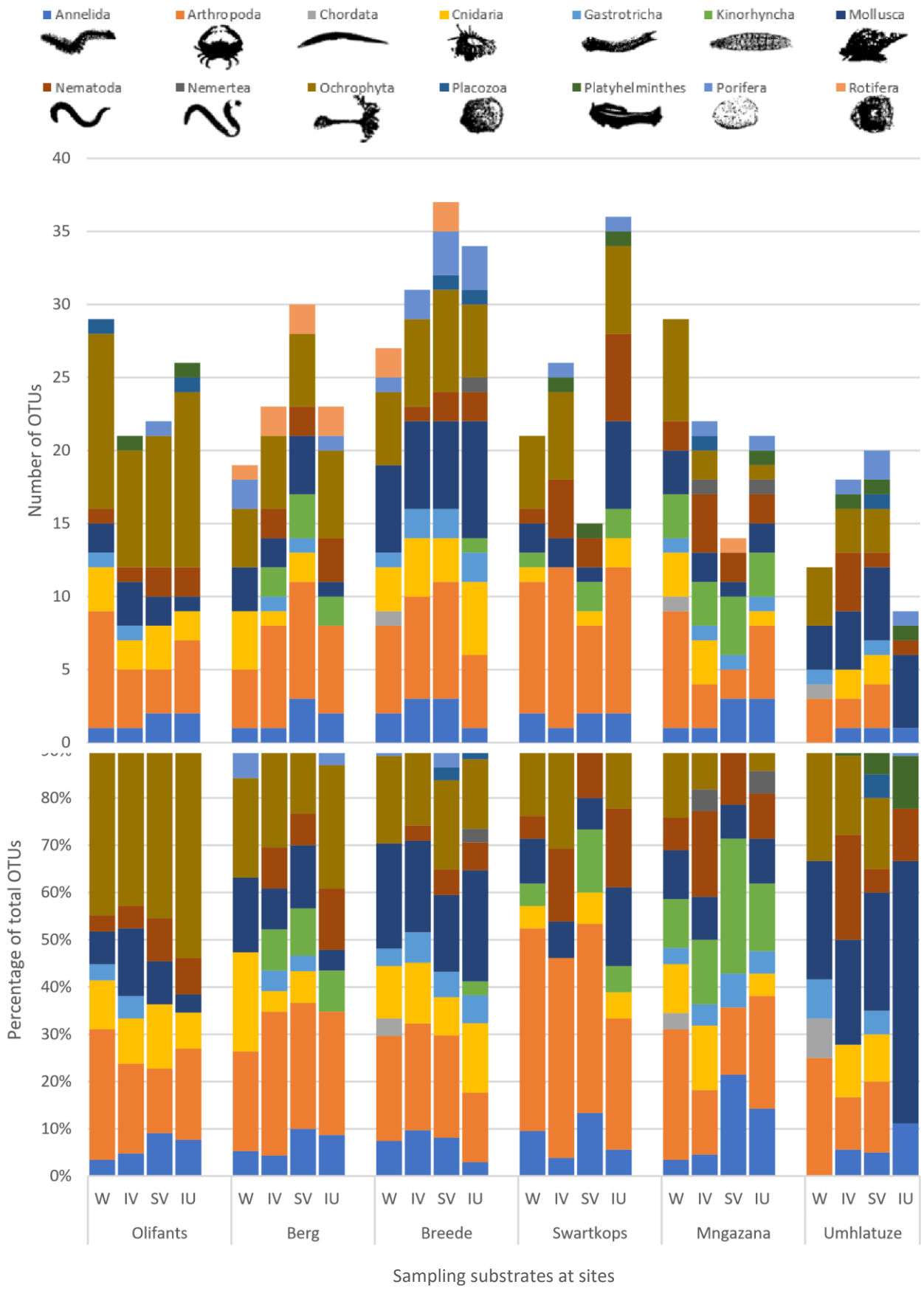

Figure 9: Stacked bar plots displaying the number of OTUs (top) and percentage of total OTUs (bottom) for phyla returned by sampling substrates at sites.

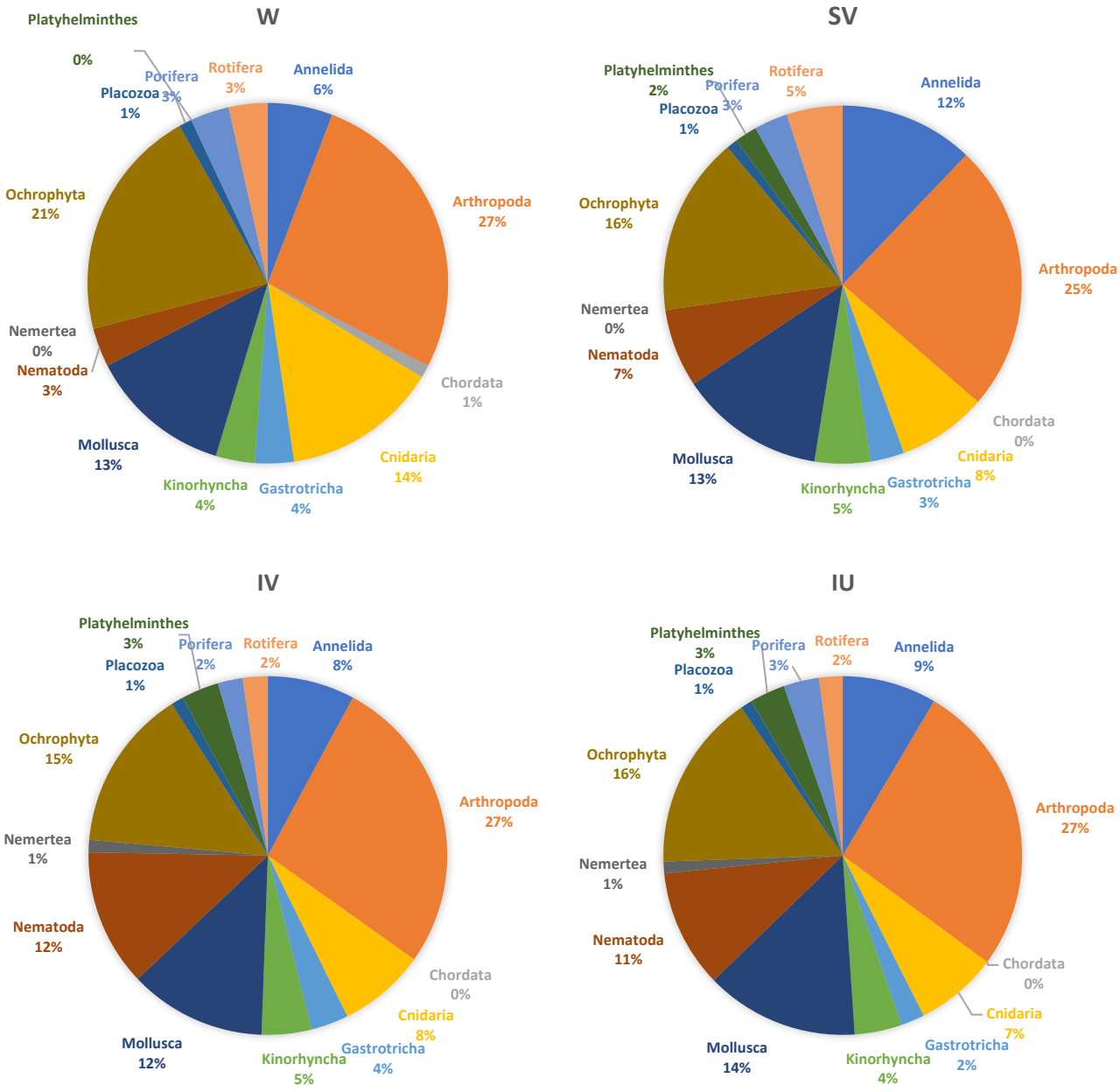

Figure 10: Pie charts of the proportions of total OTUs returned for phyla by sampling substrates.

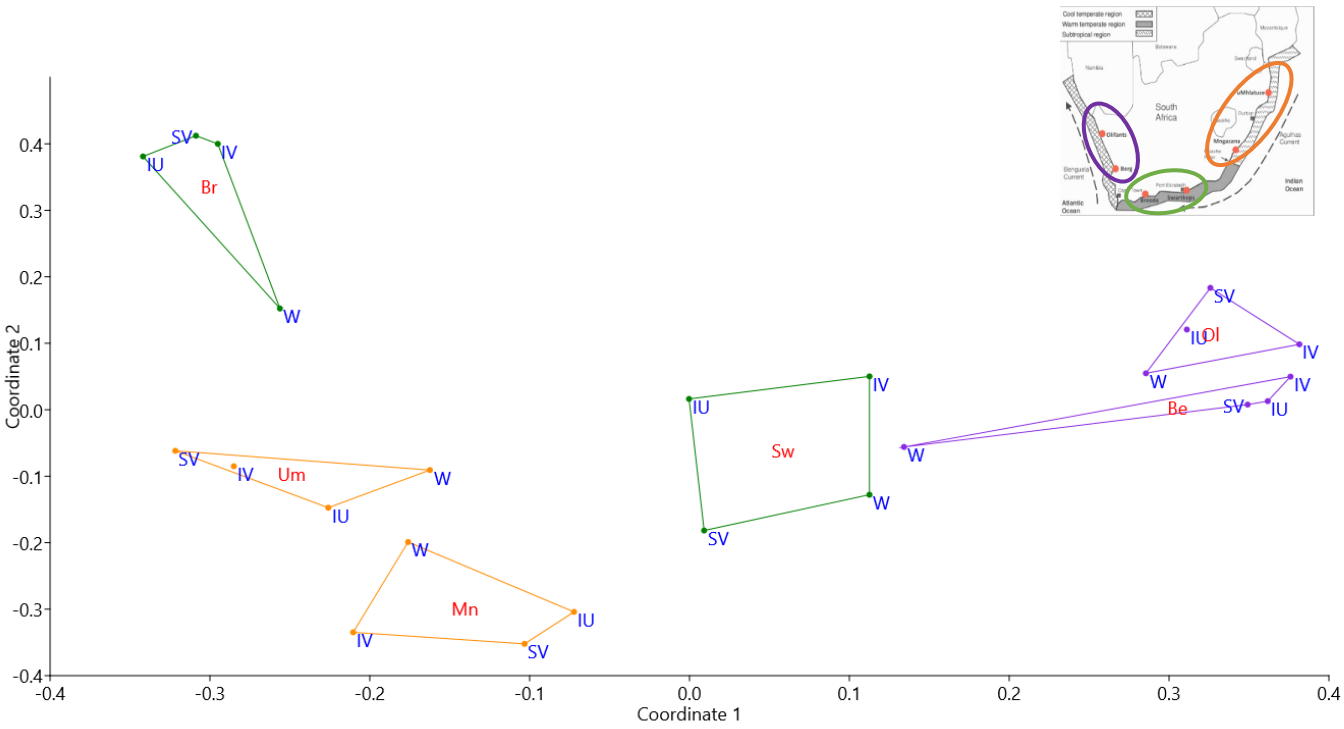

Figure 11: PCoA scatter plot (axes 1 and 2) of OTUs returned by sampling substrates at sites, with convex hulls around each site's samples. Transformation exponent c=2; similarity index= Jaccard distances; eigenvalue scale. Sites: Um= Umhlatuze, Mn= Mngazana, Sw= Swartkops, Br= Breede, Be= Berg, Ol= Olifants. Substrates: W= water, IV= intertidal vegetated, SV= subtidal vegetated, IU= intertidal unvegetated. Map indicates the colours of convex hulls which represent biogeographic regions: purple= cool temperate, green= warm temperate, orange= subtropical

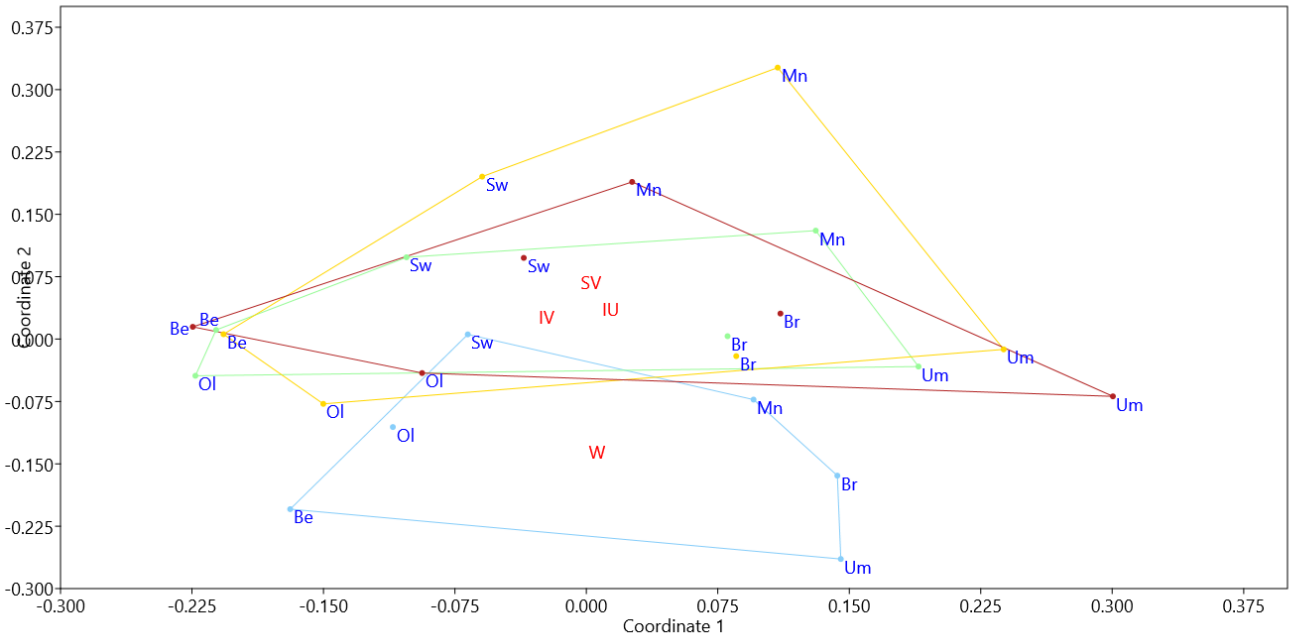

Figure 12: nmMDS plot (axes 1 and 2) of Jaccard distances of OTUs returned by sites, grouped by sampling substrate. Colours of convex hulls indicate sampling substrates: blue= water, red= intertidal unvegetated, yellow= subtidal vegetated, green= intertidal vegetated. Sites are abbreviated as per figure 11.

Table 4: The pairwise comparison (p-values) of a PERMANOVA on communities captured by sampling substrates. The p-values of water compared to sediment substrates are in blue, and sediment compared to sediment are in red.

	W	IV	SV	IU
W		0.087	0.200	0.068
IV	0.087		0.984	0.973
SV	0.200	0.984		0.980
IU	0.068	0.973	0.980	

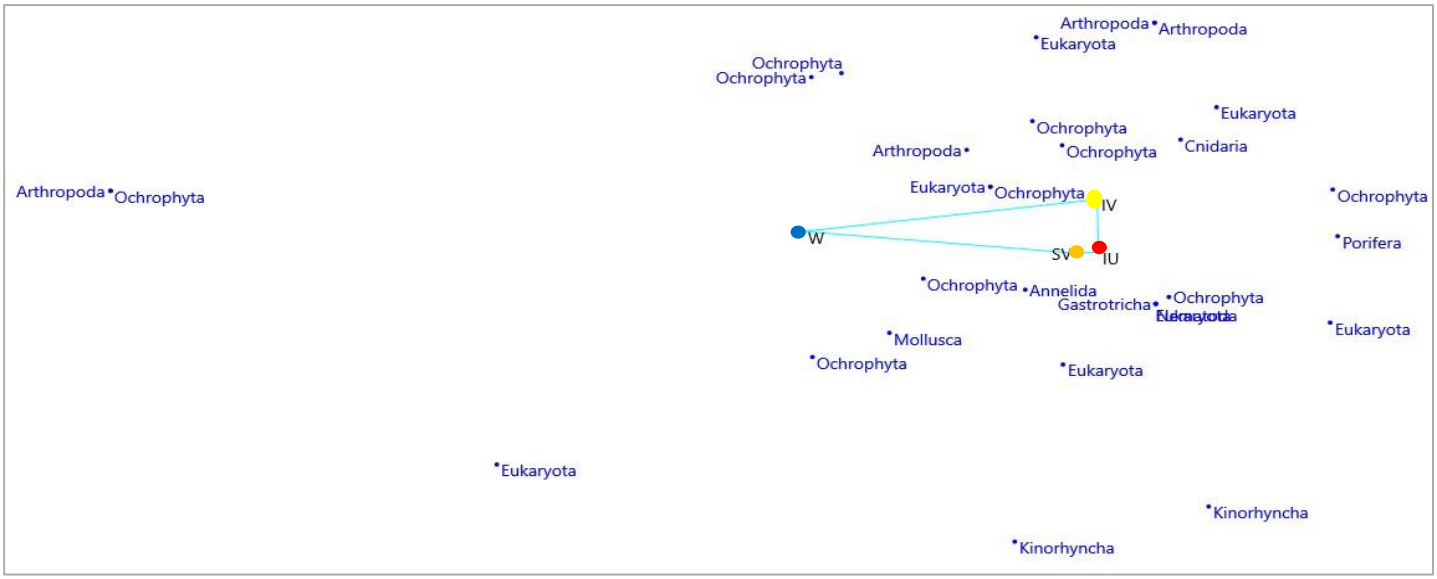

Figure 13: A correspondence analysis scatter plot of the OTUs contributing to 25% of the dissimilarity between substrates. OTUs are indicated at phylum level unless resolution was only to kingdom level.

Discussion

This study aimed to compare the communities returned by different metabarcoding sampling substrates, namely water and sediment, including sediment from vegetated and unvegetated, and intertidal and subtidal, environments. The trend of community size for sampling substrates was inconsistent across sites, and thus it cannot be concluded whether a particular substrate captured a broader community. Sediment substrates from different environments captured highly similar communities, with little variation between intertidal and subtidal, and vegetated and unvegetated, environments. On the other hand, water and sediment substrates had some overlap in community composition, y*et al*so captured distinct proportions of communities.

Comparing the communities returned by water and sediment substrates

The biological substrate sampled for eDNA analysis is a critical factor influencing the biotic composition characterized from the marine environment (Koziol *et al*., 2018; Buxton *et al*., 2017). Given that eDNA in sediment is generally better protected from decay and degradation than in water (Sakat *et al*., 2020; Buxton *et al*., 2018), it was expected that sediment substrates, especially intertidal

vegetated, would return more OTUs than water. However, this study found no difference in the number of OTUs, and thus the size of the communities, returned by substrates. This may be a factor of the patchy distribution of eDNA (see for example Bessey *et al*., 2020; Kumar *et al*., 2021; Fais *et al*., 2020) and as in this study only one sample was collected for each sediment substrate, it is likely that the sampling regime may not compensate enough for patchiness. Possibly, under-sampling and under-sequencing may not have captured the full range of eDNA harboured by the different habitats.

The relationship between substrates (i.e., amount of overlap) differed between sampling sites. As seen in the PCoA plot (Figure 11), water generally appeared further removed from the sediment substrates, which showed grouping and thus community similarity. The distinction between the water and sediment samples was most pronounced for the Breede, Berg and Umhlatuze samples. Indeed, the nmMDS plot (Figure 12) showed that these sites drove the dissimilarity in community composition between the water and sediment substrates. Meanwhile, the water samples for Swartkops and Olifants showed the least distinction from the sediment samples. The sampling regime did not change across sites, thus it is likely that the inconsistency in community structuring is due to under-sampling.

Generally, sediment and water substrates have shown to recover different species (but with some overlap) and different communities (Holman *et al*., 2019; Sakata *et al*., 2020), which was also the case for this study. Some studies have also shown that benthic species may be more effectively detected from sediment samples and pelagic species from those taken in the water column (Koziol *et al*., 2018; Shaw *et al*., 2016). Gleason *et al*. (2020) recommends comparing how targeted groups are represented by total OTUs to determine which method best captures the community of interest. Unfortunately, there was generally poor resolution of the taxonomic identification of OTUs, which made it impossible to test if specific groups (such as benthic versus pelagic species) that could explain the dissimilarity between water and sediment substrates. Not only is the lack of taxonomy an impediment in this regard, but for many species in South Africa there is a lack of understanding of even their basic life histories (see for example, Simon *et al*., 2021; Barnes and Daniels, 2019). As such, it is not only necessary to build additional barcode databases, but to better understand the basic ecology and biology of species inhabiting estuaries. Furthermore, not many OTUs were captured, which may be due to the stringent filtering approach (as only multicellular, eukaryotic OTUs with a read threshold above 28 were included) or may reflect a low total biodiversity at these sites.

Interestingly, some phyla had similar proportions of total OTUs across substrates, while others were more represented in a particular substrate, as seen in Figure 10. For example, Arthropoda and Mollusca returned large proportions of OTUs which remained consistent across substrates. Arthropoda and Mollusca are generally surface dwellers, moving at the interface of sediment and water (Branch and Branch, 2018), which may explain their prominence in both sediment and water substrates. Water had higher proportions of Ochrophyta and Cnidaria than sediment substrates and was the only substrate to return OTUs for Chordata. Studies have shown that in lotic systems, the transport of eDNA from its source is almost inevitable once it is released into the water (Nevers *et al*., 2020). In fact, eDNA in water can travel up to 50km (Nevers *et al*., 2020; Jane *et al*., 2015), although it is likely to persist at much smaller spatio-temporal scales (Tillotson *et al*., 2018; Bedwell and Goldberg, 2020; Monuki *et al*., 2021). It is likely that the water sample captured eDNA from a larger

spatial scale, which may explain why Ochrophyta, which was not noticed during sampling and usually thrives at the sublittoral rocky fringe (Bennion *et al*., 2019; Falace *et al*., 2018), was so prevalent in water. Meanwhile, sediment substrates captured higher, yet varying, proportions of OTUs for the "worms" (Annelida, Nematoda, Nemertea, and Platyhelminthes) than water, which has previously been reported in other metabarcoding studies (Holman *et al*., 2019). Worms burrow into sediment with little exposure to the water column (Branch and Branch, 2018), which likely explains their prominence in sediment. However, the varying proportions of phyla returned by water and sediment substrates demonstrates that water and sediment captured different communities, yet with some overlap for sediment surface dwellers, as water captured a broader spatial scale and sediment captured the sediment-dwelling taxa. These results are congruent with previous studies that have found water and sediment substrates to capture different communities, with some overlap (Turner *et al*., 2015; Holman *et al*., 2019; Sakata *et al*., 2020) and provide interesting insights into sampling designs for future eDNA studies (see conclusions chapter).

Comparing the communities returned by sediments from vegetated and unvegetated environments

The detection and absorption of eDNA is further affected by changes in sediment characteristics (Harrison *et al*., 2019; Buxton *et al*., 2018; Sakata *et al*., 2020), such as may be found between vegetated and unvegetated sediments. In addition, several studies have shown that seagrass meadows affect nutrient composition (Short, 1983; Caffrey and Kemp, 1990; Short *et al*., 1993; Risgaard-Petersen *et al*., 1998) and microbial processes in the sediment (Pedersen *et al*., 1997; Hansen *et al*., 2000), as well as increase sedimentation and decrease resuspension (de Boer *et al*., 2007), thus making sediments associated with seagrass dynamic role players in how they might capture and retain eDNA. In addition, vegetative cover has been shown to affect animal assemblages (Barnes and Barnes, 2012; Park *et al*., 2019; Jankowska *et al*., 2019), as seagrass provide a shelter and substrate for numerous benthic species, as well as a direct food source for grazing invertebrates (Gartner *et al*., 2013). As such, it was expected that vegetated sediment would harbour a greater species richness, as well as a greater DNA binding capacity, and would thus capture a broader community than unvegetated sediment. However, comparisons both in OTU number (Figure 7) and community composition (Figures 11 and 12) found no significant difference in the size or composition of the communities between sediments from vegetated and unvegetated environments across all estuaries. The similarity of communities returned by vegetated and unvegetated sediments differed across sites, with no concrete trend (Figure 11). Indeed, there was little variation in the proportions of phyla returned by the vegetated and unvegetated sediments (Figure 10). The nmMDS plot (Figure 12) and 2-way PERMANOVA confirmed that there was no significant difference in the communities returned by vegetated and unvegetated sediments. This similarity in the communities returned by different environments may be due to the sampling protocol, as sediments were sampled only one to three metres apart, which may not be sufficient to constitute a change in habitat and thus a change in community. Indeed, it may be that many of the metazoans recovered may move easily over such a spatial scale. More structured sampling across different spatial scales will be key to understanding the spatial scale of eDNA turnover.

Comparing the communities returned by sediments from intertidal and subtidal environments

Furthermore, intertidal and subtidal environments also have different sedimentological components (Fernandes *et al*., 2019) and species assemblages (Barnes *et al*., 2020), which would likely affect the results of metabarcoding. For example, Fernandes *et al.* (2019) found that subtidal sediments were usually anoxic and presented higher amounts of clay and silt than intertidal sediments, which may affect eDNA detection as clay substrates have lower detection capability (Buxton *et al*., 2018). Furthermore, clay reduces the abundance of key species in estuaries (Pratt *et al*., 2014), which may explain why intertidal zones display greater species diversity than subtidal zones (Barnes and Claassens, 2020). As such, since intertidal beds have been shown to display both greater detection capabilities and greater species diversity, it was expected that intertidal sediment would capture a broader community than subtidal sediment. As with the vegetated/unvegetated comparison, there was also little difference in the size (Figure 7) and composition (Figures 11 and 12) of communities returned by intertidal and subtidal sediment substrates. There was some variation in the phyla returned (Figure 10), as the subtidal sediment captured higher proportions of Rotifera and Annelida, while intertidal sediments captured more OTUs for Nemertea (that were not present in subtidal sediments) and captured more OTUs for Nematoda. However, the nmMDS plot (Figure 12) and 2-way PERMANOVA showed that statistically, there were no differences between sub- and intertidal communities.

The sampling protocol for this study might have played a large role in the similarities between sediment types, as sediments were sampled ~1 metre apart, which may not be sufficient to constitute a change in habitat and thus a change in community. Furthermore, studies have shown that both macrobenthic invertebrates (Barnes, 2019; Barnes, 2021) and eDNA (Bessey *et al*., 2020; Kumar *et al*., 2021; Fais *et al*., 2020) display patchiness in their distribution. This may result in false negatives, which should be avoided by well-structured geographic sampling regimes and larger numbers of environmental samples (Kaiser and Barnes, 2008; Bessey *et al*., 2020; Kumar *et al*., 2021), yet this study only collected sediment from a single source, which may not be sufficient to capture the full community harboured by the environments. However, despite some of the limitations of the study design, eDNA metabarcoding still captured a broad array of the estuarine community and provided novel insights into the utility of water and sediments in capturing natural communities.

Chapter 2: Investigating South Africa's coastal biogeographical patterns using metabarcoding

Introduction

Biogeography of the South African coastline

The South African coastline is one of the most unique and dynamic in the world, lying at the boundary of two major current systems, the Agulhas and Benguela (Griffiths *et al*. 2010). The east coast of South Africa is influenced by the warm, south-flowing Agulhas Current of the Indian Ocean, with average sea surface temperatures of 19-22 °C (Harrison, 2004; Lutjeharms *et al*., 2000), while the west coast is influenced by the cold, north-flowing Benguela Current of the Atlantic Ocean, with average sea surface temperatures of 13-15 °C (Tinley, 1985; Shannon, 1989). These contrasting current systems shape a highly diverse fauna and flora distributed along three biogeographic provinces: the cool-temperate west coast, the warm-temperate south coast, and the subtropical east coast (Stephenson and Stephenson, 1972; Awad *et al*. 2002; Figure 4). These regions are broadly defined by a change in sea surface temperature and primary productivity that shape diverse marine communities (Griffiths *et al*., 2010) and evolutionary trajectories (Teske *et al*., 2011; Wright *et al* 2015; Nielsen *et al*. 2021).

Furthermore, South Africa can be divided into several climate zones (Tyson 1986). The east coast is a subtropical humid zone that has a much higher rainfall (with a peak in summer) than the west coast, mainly due to moisture and heat that is transferred from the ocean to the atmosphere in the subtropical region (Cooper 2001, Hutchings *et al*. 2002, Taljaard *et al*. 2009). The southern coast of South Africa is a warm-temperate zone, with varying rainfall regimes (Heydorn & Tinley 1980, Cooper 2001), while the west coast is partitioned into the highly arid northern portion with erratic rainfall, and the Mediterranean-type southern portion with a predictable winter rainfall regime (James *et al*., 2013). This climatic variability results in variation in rainfall and river runoff patterns along the coastline (James *et al*., 2013). The diverse biogeography of the South African coastline thus provides a unique insight into the effects of environmental variation on species diversity, as physicochemical changes can have varying physiological demands on the marine life that occupy these systems (Whitfield, 1999), especially in highly variable systems such as estuaries (Day *et al*., 1981).

Biogeography of South African estuaries

Over 300 outlets intersect the South African coastline, ranging from relatively large, permanently open estuaries to small coastal streams (Harrison, 2004). Generally, there is a strong relationship between estuarine zoogeography and the physicochemical conditions along the South African coastline (Harrison, 2004). for example, Harrison (2004) found that estuarine temperatures follow the trend for marine coastal waters, "decreasing from the subtropical east coast, along the warm-temperate south coast and up the cool-temperate west coast". Yet as estuaries are formed where rivers meet the sea, they are affected by variations in both terrestrial and marine conditions (Day *et al*., 1981, Cooper, 2001). In fact, the conditions in South African estuaries are markedly different from those in the

adjacent marine inshore waters (James *et al*., 2013). These inshore waters typically experience turbulent wave action (McLachlan *et al*. 1981), while estuaries are more sheltered, calm and shallow (James *et al*., 2013). Furthermore, features such as climate, geomorphology, and tidal and fluvial patterns, play a major role in determining the chemical properties of South African estuaries (Day *et al*., 1981). As such, the physicochemical elements- such as temperature, salinity, pH, water current, nutrient levels, dissolved oxygen, and turbidity- are more variable in estuaries than in corresponding coastal and marine sites (Elliott *et al*., 2007; Whitfield 1999), resulting in regional variations in climate and oceanographic conditions (Harrison, 2004). In addition, estuaries experience varying levels of anthropogenic pressures such as flow modifications, urban and agricultural pollution, development and habitat loss and alien invasive vegetation (River Health Programme, Dallas, 2007; Van Deventer *et al*., 2019; Van Niekerk *et al*., 2019). The cumulation of varying physicochemical elements and anthropogenic pressures causes estuaries within each biogeographic region to possess distinctive physicochemical characteristics (Harrison, 2004), making it difficult to disentangle the factors driving diverse faunal assemblages.

Species richness along the biogeographical gradient

The effects of changing environmental conditions on the ecology of different habitats is driven ultimately by the underlying physiology and tolerances of organisms and their ability to cope with environmental fluctuations (Smyth *et al*., 2016). The strong gradient of environmental variation along the South African coastline shapes a unique assemblage of coastal biodiversity (Griffiths *et al*. 2010), with strong biogeographic and phylogeographic structure (Branch & Branch 2018; Griffiths *et al*. 2010; Teske *et al*., 2011). Species distribution ranges are largely governed by thermal tolerance (Vasconcelos *et al*.,2015), “with more species tolerant of warm temperatures” (Gaston, 2000). Indeed, temperature has been found as an explanatory variable structuring the genomic diversity of many marine species, such as invertebrates (Nielsen, 2021) and fishes (Teske *et al*., 2019; Golla *et al*., 2020; Czachur *et al*., 2021), and in many taxa there is a trend of increasing species richness (number of species in a community) along the gradient of increasing temperature from west to east coast (Awad *et al*., 2002; Griffiths *et al*. 2010).

Although no two estuaries are the same with respect to biotic or abiotic characteristics (Harrison and Whitfield, 2006), estuarine species generally demonstrate biogeographic patterns and high levels of regional endemicity (Awad *et al*., 2002; Harrison, 2004). Most estuarine studies have been focussed on fishes (Olisah and Adams, 2021), and those investigating biogeographic patterns have found a gradual decrease in taxonomic richness from east to west, attributed to a decreasing number of tropical marine species as the warm Agulhas Current moves offshore (Harrison, 2002; Harrison and Whitfield, 2005). Interestingly, tropical fish species have been found to have westwards movements into warm-temperate estuaries (James *et al*. 2013), suggesting that climatic changes, such as warming of the Agulhas Current, contribute to novel estuarine communities. However, in contrast to fishes, studies on invertebrates (Awad *et al*., 2002) and macrophytes (Adams *et al*., 2016), have demonstrated a greater species richness in the warm temperate region. Warm-temperate estuaries have elevated salinities and generally lower turbidity compared to both cool-temperate and

subtropical estuaries (Harrison and Whitfield, 2006), which are primary determinants influencing the biogeography of many estuarine species (Awad *et al*., 2002; Harrison and Whitfield, 2006; Adams *et al*., 2016). As such, the drivers of species patterns in the region are complex, particularly for estuarine communities that are governed by a dynamic array of environmental variation.

Metabarcoding as a tool to delineate biogeographic patterns and key stressors

Environmental DNA metabarcoding is gaining global momentum for biodiversity surveys (Santoferrana *et al*., 2018; West *et al*., 2021; Ritter *et al*., 2021; Bucklin *et al*., 2021) including in coastal South Africa (Czachur *et al*., 2021; Holman *et al*, 2021), as eDNA metabarcoding has been shown to reliably detect organisms across many different ecosystems (Deiner *et al*., 2017). Further, eDNA metabarcoding has been successfully applied to understand spatial patterns of biodiversity across multiple taxa (Holman *et al*., 2021; Czachur *et al*., 2021; West *et al*., 2021) and demonstrates a high level of sensitivity that is able to discern fine-scale patterns of biodiversity across complex oceanographic regions and elucidate biogeographic breaks in community structure (Pitz *et al*. 2020; West *et al*., 2021). In South Africa, metabarcoding of aqueous DNA has been used to assess the biogeographical patterns of fishes (Czachur *et al*., 2021) and metazoans, protists and bacteria (Holman *et al*., 2021) and zooplankton (Singh *et al*. 2021), to provide novel insights into species richness and biogeographical structuring. However, few studies have employed this technique to assess broad biogeographic patterns across multiple taxonomic groups (DiBattista *et al*., 2021), especially in the context of South African estuaries.

Furthermore, eDNA metabarcoding has been used to explore the main environmental variables structuring the communities of rivers, estuaries, and lakes along an environmental gradient, and to predict their pollution status with benthic eukaryote (Chariton *et al*., 2015), diatom (Pissaridou *et al*., 2021), microeukaryote (Al *et al*., 2021) and bacteria, Protista, and metazoan communities (Li *et al*., 2018). Generally, nutrients, turbidity and pH were the main driving stressors affecting community structure (Chariton *et al*., 2015; Pissaridou *et al*., 2021; Li *et al*., 2018), and variation in anthropogenically-driven environmental conditions shaped communities (Li *et al*., 2018; Al *et al*., 2021). Notably, similarities in the communities harboured by estuaries reflected their environmental condition (Chariton *et al*., 2015). In South Africa, few studies have investigated metabarcoding as a tool to explore the variables structuring communities or predict the main impacts of estuaries, despite the increase in anthropogenic impacts on estuaries (van Niekerk and Turpie, 2019). One study by Matcher *et al*. (2018) examined the influence of different human activities on the diversity and structure of bacterial communities in three warm-temperate estuaries. Both sediment and water substrates were sampled to compare the biogeographical structuring between the substrates. The study found that both substrates returned a similar biogeographical trend, yet the degree in overlap between sites' communities differed between substrates, and sediment exhibited a higher degree of species evenness (relative abundances of species within a community). Matcher *et al*. (2018) concluded that metabarcoding is a useful tool to examine community patterns and delineate anthropogenic impacts, at least for microbial communities.

Aims and hypotheses

The aim of chapter 2 was to assess eDNA metabarcoding as a tool to delineate biogeographical structuring and species richness patterns, and to investigate these patterns between multiple sampling substrates. Furthermore, this chapter aimed to explore environmental variables that explain the variation between estuaries (sites), to delineate the factors influencing community structure and species richness patterns.

Aim 1: Investigate biogeographical structuring of estuarine communities along the South African coastline with metabarcoding.

> H0: Sites within biogeographic regions will be more similar than sites between regions, displaying biogeographic structuring.
>
> H0: Biogeographical structuring will persist for individual mediums.

Aim 2: Explore the trend of species richness along the biogeographical gradient.

> H0: Species richness will be greatest in the warm temperate region, as warm-temperate estuaries have elevated salinities and lower turbidity than the subtropical or cool temperate estuaries.

Aim 3: Explore environmental variation as a driver of community composition and structure.

> H0: Sea surface temperature, pH, nutrients and turbidity will be the greatest explanatory variables of the variation in communities between sites.

Materials and methods

The OTU data from chapter 1 was used to investigate biogeographical patterning of sites. A community matrix consisting of presence/absence values (1/0) for all OTUs returned by sampling substrates (water and intertidal vegetated, intertidal unvegetated and subtidal vegetated sediments) across sites was built and utilised for downstream analyses.

Statistical analyses

Statistical analyses were performed in PAST v4.03 (Hammer *et al*., 2001), unless otherwise stated. Data visualisation was performed with Excel (Raubenheimer, 2017), DisplayR (Displayr | Analysis and Reporting Software for Survey Data; Chasapi *et al*., 2020) and PAST v4.03 (Hammer *et al*., 2001). Results were rounded up/down to three decimal places.

Investigating biogeographical structuring of communities with eDNA metabarcoding

To visualise the relationship between each of the six sites, samples were grouped by biogeographic region, with two estuaries per region, in an nmMDS plot of Jaccard distances. The individuality of samples was maintained, as they were classified by site and sampling substrate, and nested within

biogeographic regions as opposed to being treated as equal replicates. Jaccard distances were chosen as this ecological index has been shown to be appropriate for biogeographical studies (Salazar, 2018). A 2-way PERMANOVA was performed by grouping samples according to substrate and region to assess differences in multivariate centroids and dispersion between coastlines (Holman *et al*., 2021), and to determine an interaction between mediums and regions. The 2-way PERMANOVA used Jaccard distances as the similarity index and 999 permutations. PERMANOVA Pairwise p-values were calculated to investigate the individual comparisons between regions. To elucidate the OTUs contributing to the dissimilarity between biogeographic regions, a SIMPER analysis (Gibert and Escarguel, 2019) was performed with all groups pooled and using the Bray-Curtis distance measure (Mumby, 2001). The OTUs with the greatest contributing percentage were included in a correspondence analysis scatter plot (Abdi and Bera, 2014) to display the OTUs at phylum level contributing to 25% of the dissimilarity between biogeographic regions.

To investigate whether biogeographical structuring existed for different sampling substrates, nmMDS plots of Jaccard distances were created per substrate, with sites grouped by biogeographic region. Venn diagrams of sites' OTUs were made with DisplayR per substrate to visualise the overlap in communities between sites. To determine whether structuring was significant for each substrate, PERMANOVAs were performed with Jaccard distances as the similarity index and 999 permutations.

Exploring species richness along the biogeographical gradient

The number of OTUs returned by sites were used as indicators of species richness, as OTUs may act as a proxy for species in high-throughput sequencing (Macheriotou *et al*., 2018). To determine the trend of species richness across sites, the number of OTUs returned at sites by sampling substrates was tabulated and plotted in a line and points graph with Excel. To compare species richness across sites, the normality of data (number of OTUs returned at sites by sampling substrates) was first tested for with a Shapiro-Wilks test (Razali and Wah, 2011) with 999 Monte Carlo permutations. A Levene's tests was performed to determine homogeneity of variance from means and medians (Gastwirth *et al*., 2009). With conditions met, a One-way ANOVA (Girden, 1992) was performed with a Permutation (n=99999). For pairwise comparisons of sites, a Tukey's pairwise test was performed following Copenhaver-Holland (1988).

To visualise the species richness patterns of biogeographic regions, box and whisker plots were created in Excel for all substrates combined and for each substrate. To determine whether there was a significant difference in the species richness of biogeographic regions, sites were grouped for multiple comparisons tests for all substrates combined, and for each substrate. First, the normality and variance of data were tested for with Shapiro-Wilks tests (Razali and Wah, 2011) with 999 Monte Carlo permutations, and Levene's tests (Gastwirth *et al*., 2009), respectively. With conditions met, a One-way ANOVA (Girden, 1992) was performed with a Permutation (n=99999) for the water, subtidal vegetated and intertidal unvegetated substrates. A Kruskal-Wallis test of equal medians was performed for intertidal unvegetated. A Welch F test and post-hoc Games-Howell test (Games and

Howell, 1976) were performed in PAST v4.03 and RStudio (Allaire, 2012), respectively, for all mediums combined.

Exploring environmental variation as a driver of community structure

Environmental variables for sites were collected from GMED (GMED - Download Data Layers (auckland.ac.nz)), Planet OS (PlanetOS by Intertrust) and the literature (van Niekerk, 2018). The latter specifically focussed on estuarine-level data collected in South Africa, which was in contrast to the GMED and Planet OS data, which captures broader offshore and inshore environmental variation. 22 environmental variables (hereafter referred to as "variables") encompassing chemical composition, site characteristics and health condition were used (Table A6, Appendix 3). Chemical composition included the concentrations of chemical elements that have been shown to affect community composition (such as nitrate; Okyere, 2019; Duque *et al*., 2021). Site characteristics included physical measures, such as sea surface temperature, that interact with the physical tolerances of organisms to affect their range distributions and thus community structure (Blanchette *et al*. 2008). Measures of health condition provided a score out of 100 that assessed the biological and physical states of estuaries (a higher score indicates greater estuarine health, as determined by van Niekerk, 2018).

Variables were standardized with a Z-score: [(x-mean)/stdev] (Wu *et al*., 2001). To investigate multicollinearity between variables, Multivariate Multiple Linear Regressions (Brieman and Friedman, 1997) were performed with each variable acting as a dependent variable. Ecologically important variables (such as sea surface temperature; Moreno *et al*., 2021) were prioritized, and highly correlated variables (with R^2 values >0.7; Variyath and Brobbery, 2020) were removed. A Principal Components Analysis (Wold *et al*., 1987) of correlation was performed to visualise the relationship between variables.

A Redundancy Analysis (RDA; Van Den Wollenberg, 1977), was then performed with forward selection of variables to obtain the model that had the highest adjusted R^2 value (as described by Capblancq and Forester, 2021). An RDA scatter plot of Axes 1 and 2 was made with Scaling Type 2. Samples were grouped by sites, with convex hulls around groups that were coloured to indicate biogeographic regions. Scaling Type 2 was selected as it is explanatory variable focused (Aiello-Lammens and Silander, 2019). The significance of the model was tested with a permutation (n=999). A sunburst chart was created in Excel to display the proportion of variance that was partitioned between the constrained (canonical) and unconstrained (residual) axes. Partial RDAs were performed to isolate the effects of single explanatory variables by removing each variable from the model (Liu, 1997). The percentage variance that the variable explained was calculated as:

Full model (cumulative canonical % variance) – Partial model (cumulative canonical % variance).

A sunburst plot was created in Excel to compare the percentage variance explained by each variable.

Results

Investigating biogeographical structuring of communities through metabarcoding

Results strongly suggest that communities are structured biogeographically, with distinct communities in each of the biogeographic provinces. For example, the nmMDS plot (Figure 14) clearly shows a separation of sites along the biogeographic continuum, with some overlap between the east coast regions, the subtropical and warm temperate, and a clearer distinction between the east and west coast sites. The 2-way PERMANOVA confirmed that biogeographic regions captured significantly different communities (PERMANOVA, F= 4.206, p= 0.000) and pairwise comparisons (Table 5) were all significant. In addition, there was no interaction between medium and regions (PERMANOVA, F=0. 488, p=1).

To investigate the OTUs and taxa responsible for the dissimilarity between regions, a SIMPER analysis was performed (results are included in Table A5, Appendix 3). The OTUs with highest contributing percentages contributing to 25% of the dissimilarity were included in a correspondence analysis scatter plot (Figure 15). The latter showed that OTUs of the phyla Ochrophyta and Arthropoda were prominent contributors to the dissimilarity between regions, and tended to cluster around the cool temperate region, indicating their uniqueness to the region. A few OTUs from Cnidaria, Porifera and Kinorhyncha also contributed to the dissimilarity and clustered towards the subtropical region. However, some OTUs were only identified to the Kingdom level and were dispersed across regions.

The nmMDS plots and VENN diagrams for individual substrates (Figure 16) confirmed biogeographic patterning of OTUs across sites for each substrate, with the VENN diagrams offered better resolution of the overlap in communities returned by sites. For example, the intertidal vegetated substrate showed the clearest biogeographical structuring in both the nmMDS plot, and the VENN diagram. The other sampling substrates showed less pronounced biogeographical structuring in both their nmMDS plots and VENN diagrams, with some overlap of the orbitals of east coast sites for subtidal vegetated and intertidal unvegetated substrates, and the communities between biogeographically distant sites. Overall, all sampling mediums displayed biogeographical structuring, especially between east and west coast sites, but structuring was not significant for individual substrates.

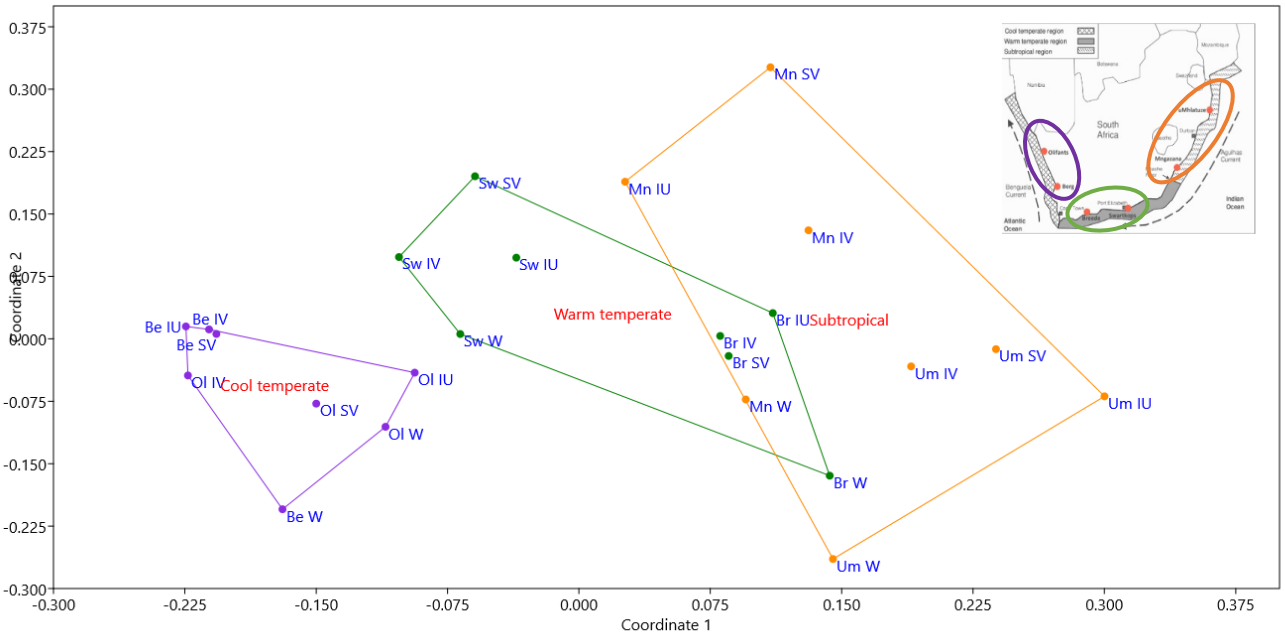

Figure 14: nmMDS plot of Jaccard distances of OTUs for sites, grouped by biogeographic regions. Map is included on the top right to show location of regions. Samples are indicated by site and substrate. Sites: Um= Umhlatuze, Mn= Mngazana, Sw= Swartkops, Br= Breede, Be= Berg, Ol= Olifants. Substrates: W= water, IV= intertidal vegetated, SV= subtidal vegetated, IU= Intertidal unvegetated.

Table 5: The pairwise comparison (p-values) of a PERMANOVA on communities captured by biogeographic regions.

	Cool temperate	Warm temperate	Subtropical
Cool temperate		0.0003	0.0002
Warm temperate	0.0003		0.0007
Subtropical	0.0002	0.0007	

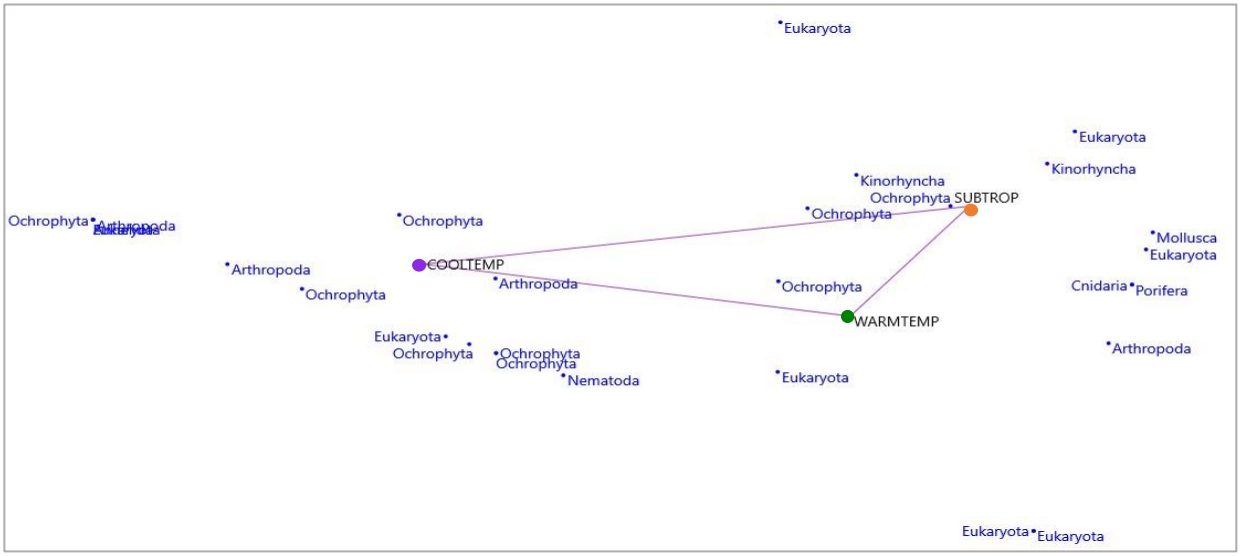

Figure 15: A correspondence analysis scatter plot of the OTUs contributing to 25% of the dissimilarity between biogeographic regions. OTUs are indicated at phylum level unless resolution was only to kingdom level.

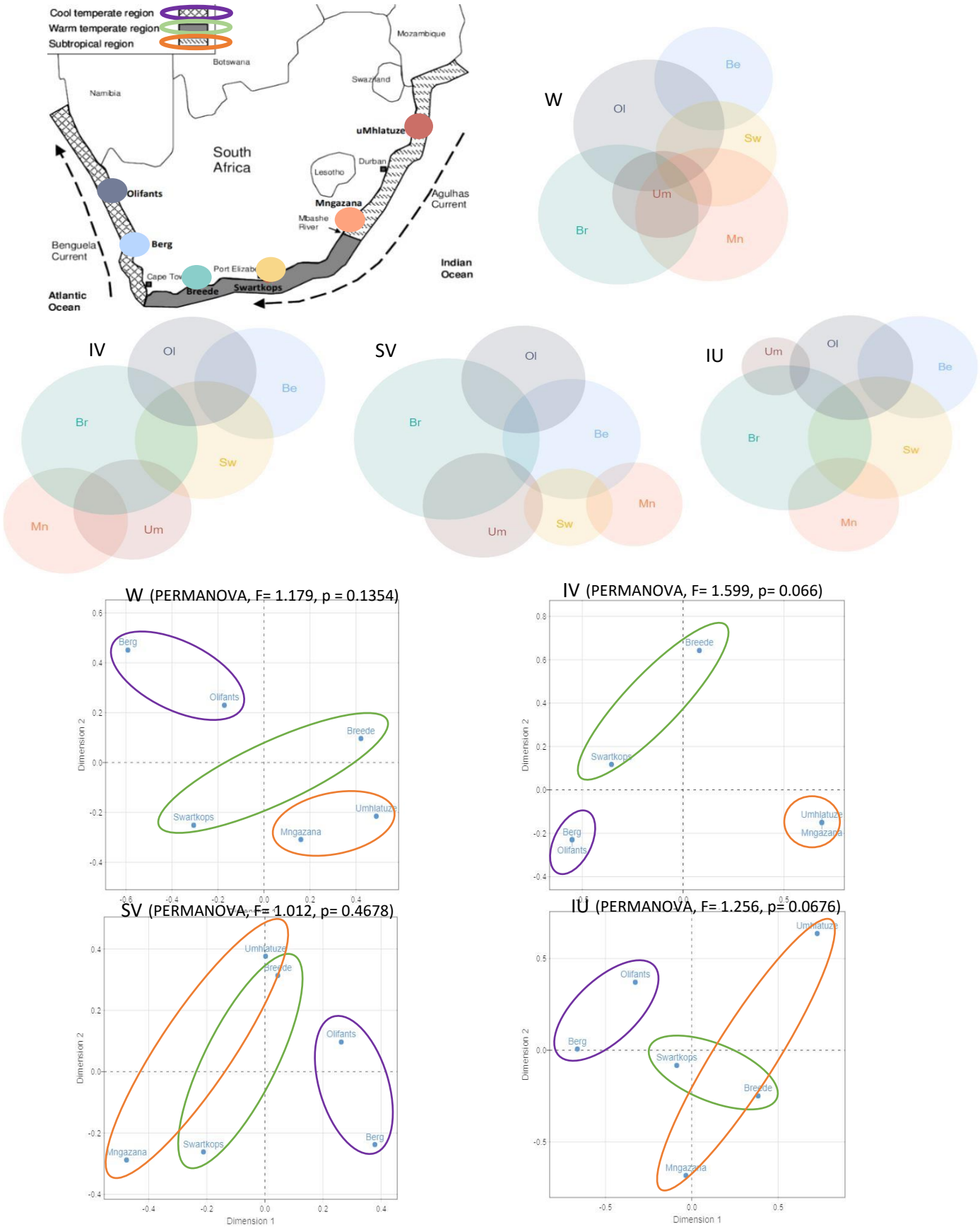

Figure 16: Venn diagrams of the number and overlap of OTUs (top) and nmMDS plots of Jaccard distances (bottom) of sites, per substrate. PERMANOVA results are indicated for each substrate. Sites are grouped by biogeographic region, as indicated by the map at the top.

Exploring species richness along the biogeographical gradient

Results show that metabarcoding captured a species richness trend that was similar across sampling substrates. The general trend of species richness (calculated as number of OTUs per site) increased from the west coast, cool temperate sites towards Breede, with a sharp decline at Swartkops, to the lowest species richness at Umhlatuze (Figure 17). Data was normally distributed and homoscedastic (Table A4, Appendix 3). The results of the ANOVA indicate a highly significant difference in the species richness across sites (F= 7.668, p= 0.001). The pairwise comparisons (Table 6) show that Breede returned significantly more OTUs than all other sites.

The general trend of species richness for biogeographic regions was warm temperate > cool temperate > subtropical (Figure 18), although much of the warm-temperate richness was driven by the Breede River estuary. The difference in species richness between biogeographic regions was only significant when substrates were combined (Table 7). Water captured little difference between regions, thus most of the variation was captured by the sediment substrates. The pairwise comparisons (Table 8) show that the warm temperate region returned more OTUs than the other regions, however the difference was not significant.

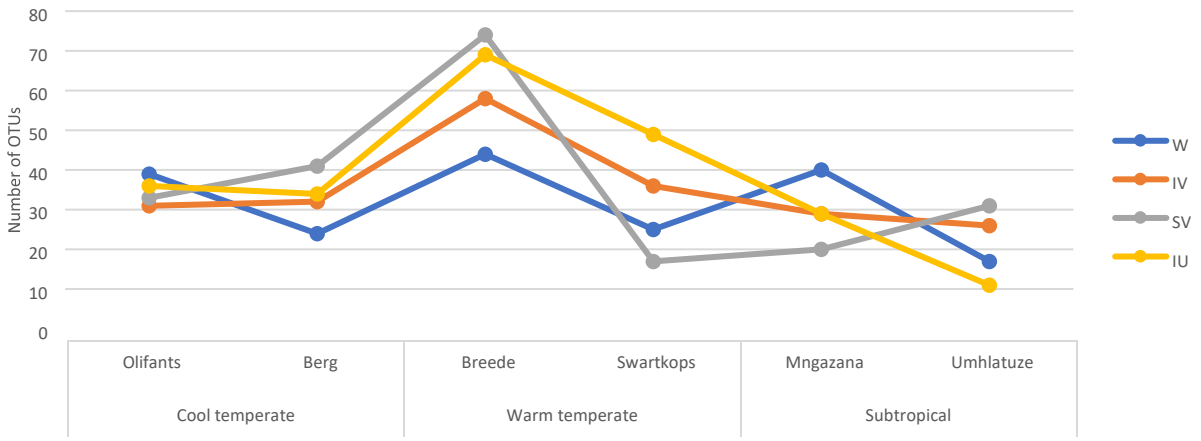

Figure 17: Line and points graph of the number of OTUs returned by sampling substrates across sites.

Table 6: The results of a posthoc Tukey test for the number of OTUs returned by sites through multiple sampling substrates. Tukey's Q values are below the diagonal, and p-values (same) are above. Significance values ($p<0.05$) are in **bold.**

	Olifants	**Berg**	**Breede**	**Swartkops**	**Mngazana**	**Umhlatuze**
Olifants		0,999	**0,014**	0,998	0,972	0,409
Berg	0,408		**0,007**	1	0,997	0,575
Breede	5,399	5,806		**0,005**	**0,003**	**0,000**
Swartkops	0,611	0,204	6,01		0,999	0,661
Mngazana	1,07	0,662	6,468	0,458		0,837
Umhlatuze	2,75	2,343	8,149	2,139	1,681	

Table 7: The results of multiple comparisons tests of the number of OTUs returned by biogeographic regions for all substrates combined and for individual substrates. Significant values (p<0.05) are in **bold**.

Medium	Test						
	Normality		Homogeneity of variance	Multiple comparisons test			
	W	pvalue	p-value	Test	Statistic	df	pvalue
Combined	0.924	0.071	**0,007**	Welch F	F= 4.366	11.89	**0.038**
W	0.898	0.363	N/A	ANOVA	F= 0.097	2	0.910
IV	0.763	**0.026**		KruskalWallis	H= 4.571	2	0.102
SV	0.861	0.192		ANOVA	F= 0.352	2	0.729
IU	0.971	0.898		ANOVA	F= 6.379	2	0.083

Table 8: The results of a posthoc Games-Howell test on the number of OTUs returned by biogeographic regions for all substrates combined. The estimated difference (X-Y) is below the diagonal, p-values (same) are above the diagonal.

	Cool temperate	Warm temperate	Subtropical
Cool temperate		0,253	0,104
Warm temperate	-12,8		0,055
Subtropical	8,38	21,1	

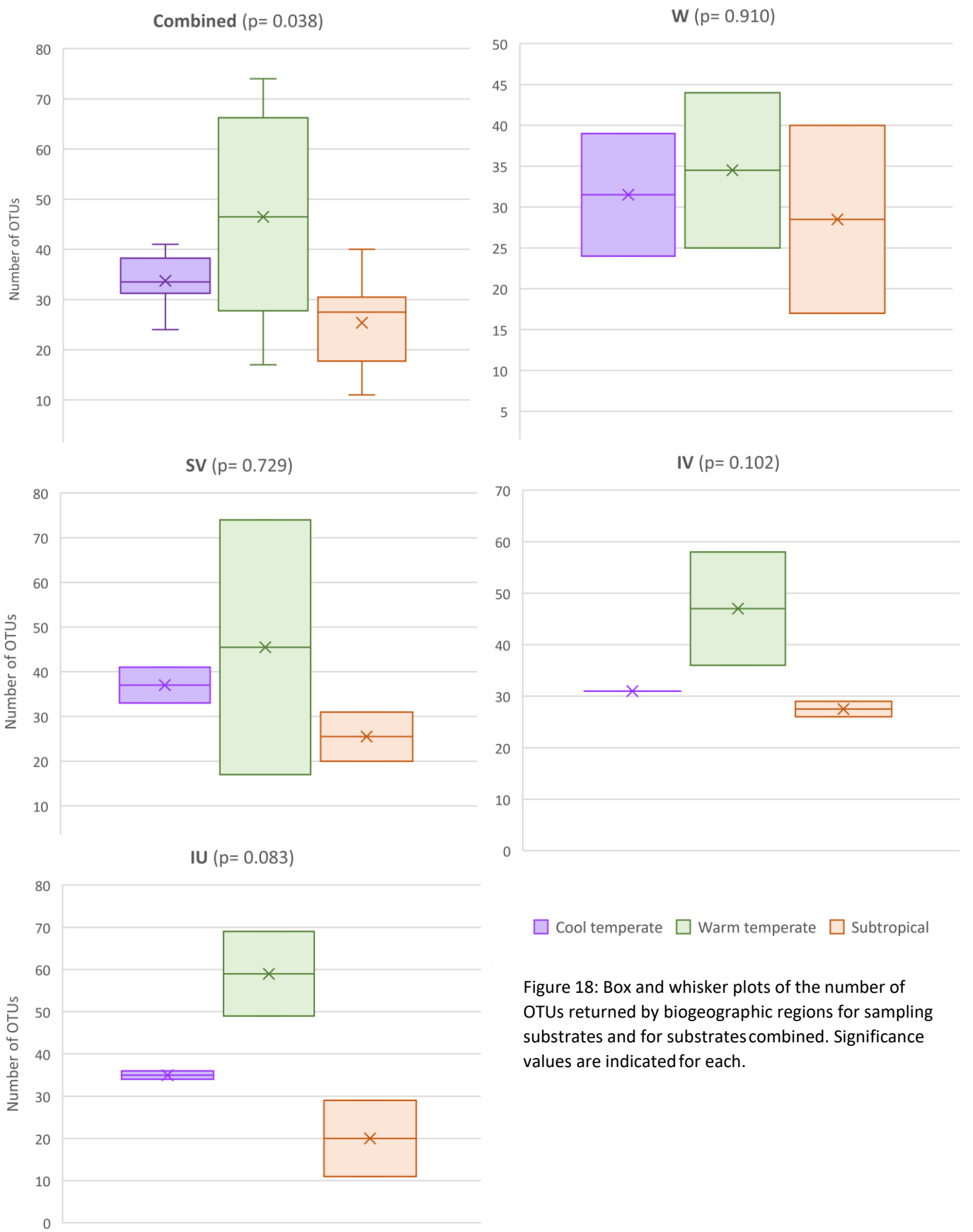

Figure 18: Box and whisker plots of the number of OTUs returned by biogeographic regions for sampling substrates and for substrates combined. Significance values are indicated for each.

Exploring environmental variation as a driver of community composition and structure

The Principal Components Analysis (PCA) scatter plot (Figure 19) displays the correlation between all 22 environmental variables. Most of the variation in the environmental variables was captured by components 1 and 2 (~77%), thus components 1 and 2 were used for the PCA.

After the multivariate multiple linear regression, 10 uncorrelated and ecologically important variables were retained (Table 9). Environmental variables included site characteristics, chemical composition, and habitat scores (variables and their sources are included in Table A6, Appendix 3). The results of the RDA showed that around 58% of the variation was explained by the canonical axes and therefore by the environmental variables (the full eigenvalue results are included in Table A7, Appendix 3). Figure 20 shows the partitioning of variation that is explained by the canonical and residual axes. Axes 1 and 2 cumulatively explained 46.66% of the variation and were thus used for the RDA scatter plot (Figure 21). The overall model was highly significant and explained 91.53% of the variation in the community matrix (R^2= 0.9153, R^2adj= 0.8501, F= 14.04, p= 0.001).

Scaling type 2 was used for the RDA as it is explanatory variable focused, thus the angles reflect a linear correlation (correlation= cosine(ɵ)) and right-angled projections of response variables (sites) onto explanatory variables (environmental variables) indicates their values. Longer lines mean the variable strongly drives the variation in the community matrix, and direction of lines indicates a positive or negative relationship with sites. From the RDA scatter plot, it appears that mean sea surface temperature (SST), nitrate concentration, surface current and water quality had the strongest impact as explanatory variables. Mean SST had a positive relationship with samples from the subtropical region, while nitrate had a positive relationship with cool temperate samples. Surface current was positively associated with the cool temperate samples and the Swartkops samples, while water current was positively associated with the Breede samples.

To further investigate the variation explained by the environmental variables, partial RDAs were performed. The percentage of canonical variance that each variable explained was plotted in a sunburst chart (Figure 22). Mean SST explained the most variation (21%), followed by nitrate concentration (19%) and water quality (19%). The full results of the partial RDAs are included in Table 8 (Appendix 3).

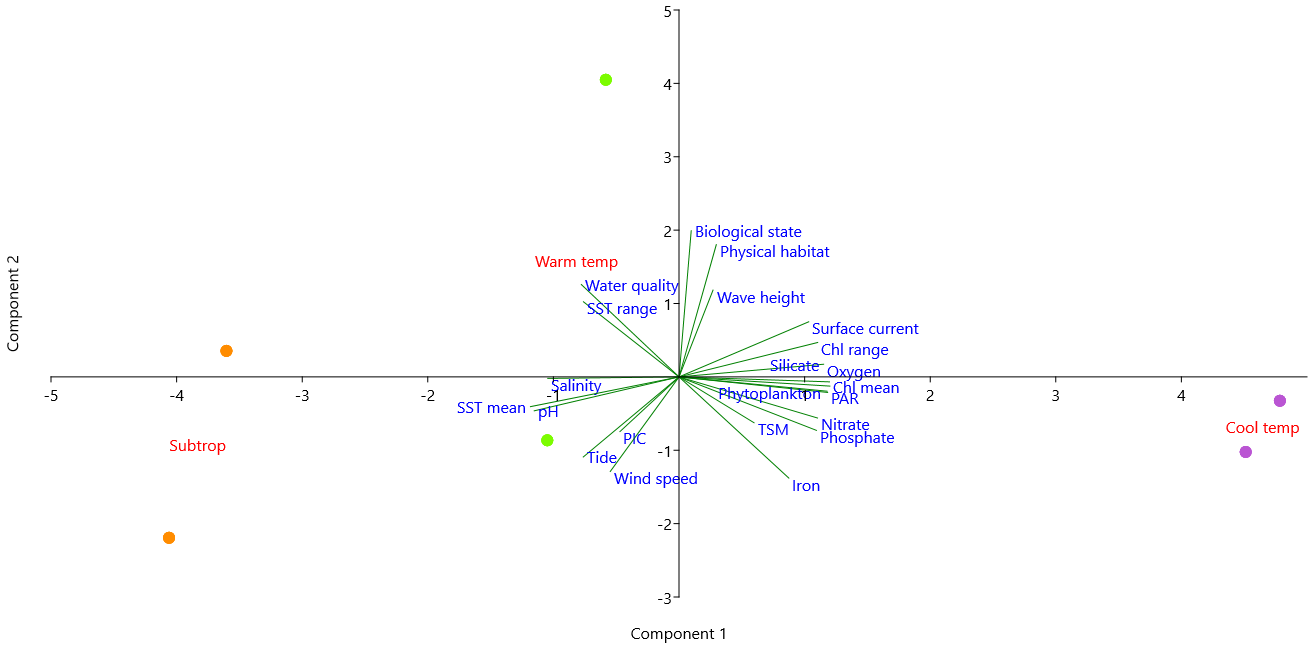

Figure 19: Principal Components Analysis scatter plot of the correlation of 22 environmental variables; components 1 and 2 displayed.

Table 9: The results of a multivariate multiple linear regression to show retained variables and closely correlated variables (R^2>0.7) that were removed. Abbreviations: SST= sea surface temperate, Chl= chlorophyll, PAR= photosynthetically active radiation, PIC= particulate inorganic carbon.

Retained variables	Correlated variables	R^2
Nitrate	Phosphate	0,858
	Chl mean	0,848
	PAR mean	0,887
	pH	0,742
	Silicate	0,734
	Iron	0,737
	Oxygen	0,883
Physical habitat	Biological state	0,864
	Wind speed	0,704
PIC	None	
Salinity	Chl mean	0,783
	Oxygen	0,713
SST mean	Silicate	0,945
	pH	0,951
	Chl range	0,944
	Oxygen	0,867
SST range	Phosphate	0,760
	Iron	0,725
Surface current	Chl range	0,954
Tide	None	
Water quality	Phytoplankton	0,941
Wave height	None	

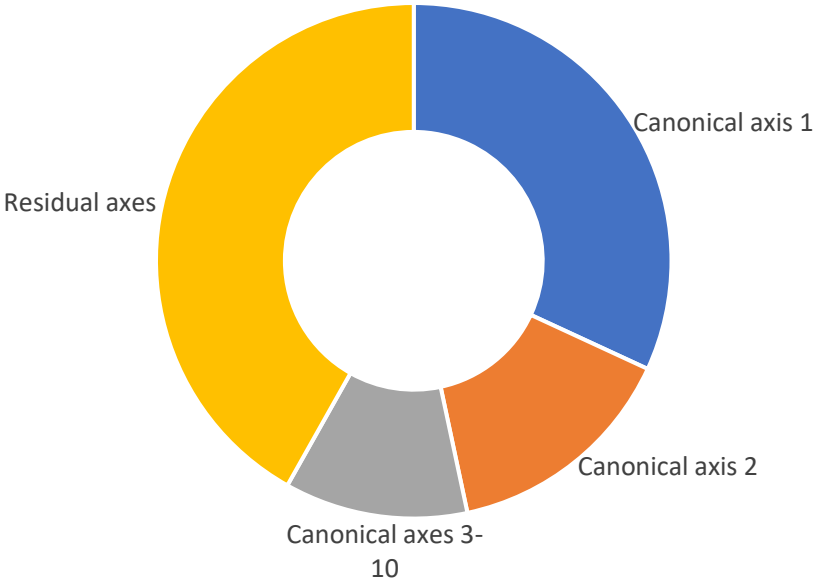

Figure 20: A sunburst plot to display the proportion of variance explained by the canonical and residual axes of the Redundancy Analysis.

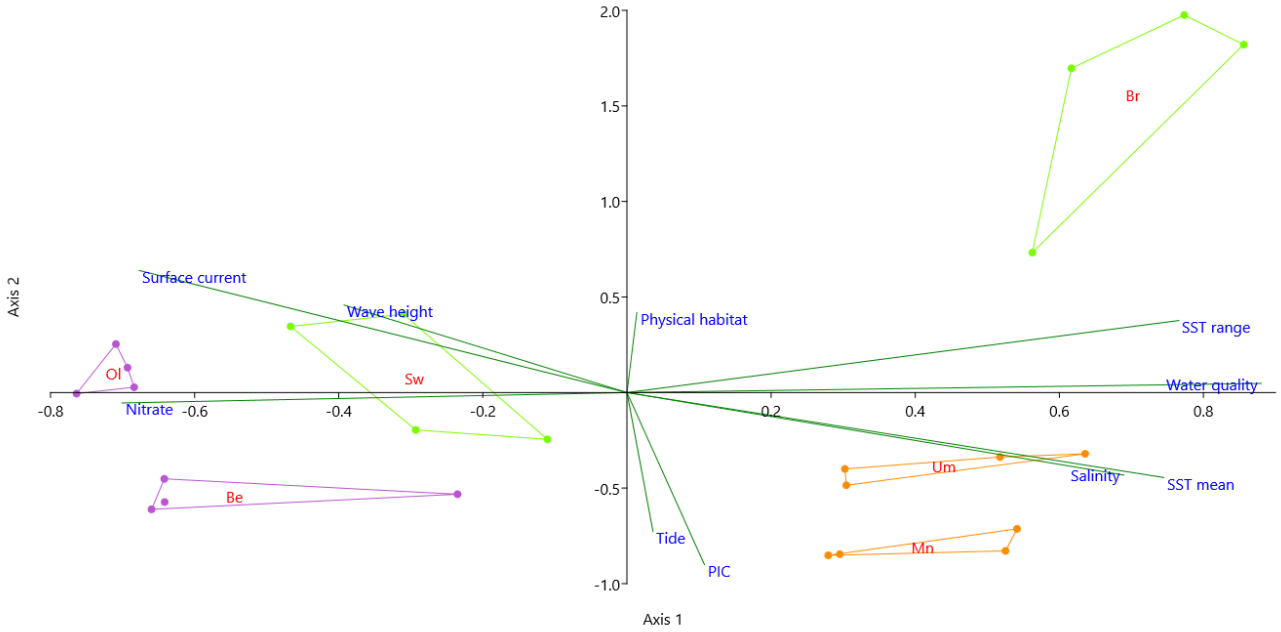

Figure 21: Redundancy Analysis scatter plot (Scaling Type 2) to display the relationship and impact of explanatory variables on the community matrix. Axes 1 and 2 are shown and samples are grouped by site, with convex hulls coloured by biogeographic region. Sites: Um= Umhlatuze, Mn= Mngazana, Sw= Swartkops, Br= Breede, Be= Berg, Ol= Olifants. Biogeographic regions: orange= subtropical, green= warm temperate, purple= cool temperate. Abbreviations for variables as per Table 9.

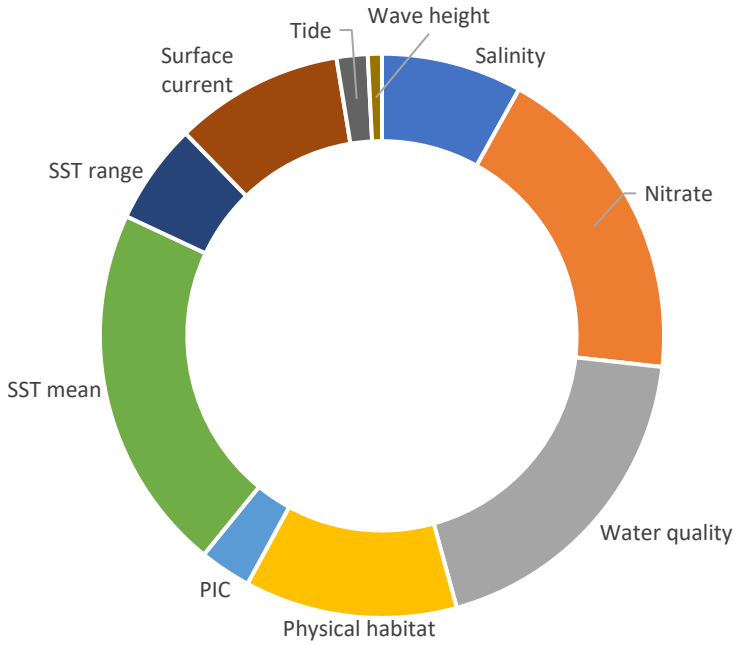

Figure 22: A sunburst plot to display the proportion of variance explained by environmental variables. Abbreviations for variables as per Table 9.

Discussion

This chapter utilised eDNA metabarcoding across six South African estuaries to not only test whether this could detect biogeographic patterns, but also to examine species richness and potential environmental drivers of community structuring for multicellular eukaryotes, encompassing meio- and macrofauna. My results suggest that metabarcoding was able to capture biogeographical structuring of faunal communities from six estuaries housing *Zostera capensis* seagrass and identified the biogeographical break between the east and west coast sites. Furthermore, species richness was greatest in the warm temperate region and least in the subtropical region, and mean sea surface temperate (SST), nitrate concentration and water quality had the greatest impact as explanatory variables of the variation in community structure between regions. In all, results had improved resolution and greater significance when multiple sampling substrates were combined as opposed to a single substrate, an important finding that will be valuable for future eDNA metabarcoding sampling strategies.

Investigating biogeographical structuring of metabarcoding communities

To date, few studies have employed eDNA metabarcoding to assess broad biogeographic patterns across multiple taxonomic groups (e.g., DiBattista *et al*., 2021), nor compared the results of multiple sampling substrates on recovering biogeographic patterns. The South African coastline, with its distinct biogeographic zones, is an idea place to test the power of eDNA for resolving species

distribution patterns. Promising results from coastal areas suggest that for fishes (Czachur *et al*. 2021), and metazoans, micro-eukaryotes and prokaryotes (Holman *et al*., 2021), eDNA metabarcoding is able to detect biogeographic patterns. However, those studies excluded estuaries, which are particularly interesting given that some of their biota is transient (i.e., marine species utilising estuaries as nursery/breeding places; Unsworth *et al*., 2019) and because of their generally quite dynamic environmental variability that can be significantly different to adjacent coastal areas (Harrison, 2004; James *et al*., 2013).

The results of this study showed that metabarcoding is a useful tool to investigate biogeographic patterns of estuaries, as it captured sufficient variation in communities to discern biogeographic regions, even based on just two estuaries per region. Notably, the 2-way PERMANOVA showed that sites within a biogeographic region are more similar than sites between regions, indicating three clear biogeographic regions (Figure 14). Differences in species composition between the biogeographic regions has long been recognized (Teske *et al*., 2007a), and likely reflect a combination of life history traits (including larval behaviour, Muller *et al*., 2012), the inability of poorly dispersing species to cross biogeographic barriers (Teske *et al*., 2006), and the adaptation of species to the environmental conditions characteristic of their marine biogeographic region (Teske *et al*., 2008, 2011 2011, 2019). Indeed, barriers formed by geographical distance and ecologically and physiologically different marine habitats may result in genetic divergence of species due to low levels of gene-flow (Teske *et al*., 2006), or in locally adapted communities (Teske *et al*., 2011). As such, many of the coastal species present in two or more regions are split into phylogroups whose distributions are limited to single regions (Ridgway *et al*. 1998; Evans *et al*. 2004; Teske *et al*. 2006, 2007a, b; Zardi *et al*. 2007). This seems particularly true for estuarine species, as estuaries possess highly variable environmental parameters (including water temperature, currents, salinity, and nutrient concentrations) that contribute to biogeographical structuring through dispersal barriers and local adaptations (Ridgway *et al*. 1998; Teske *et al*., 2006; Teske *et al*. 2008; Teske *et al*., 2011). Indeed, a study on three estuarine crustaceans (*Upogebia africana, Exosphaeroma hylecoetes*, and *Iphinoe truncate*) showed phylogeographic breaks that coincided with biogeographic boundaries, although the extent of structuring differed based on their modes of dispersal (Teske *et al*., 2006). In addition, the varying physicochemical characteristics of biogeographic regions has resulted in locally adapted communities and regional endemicity, as many coastal marine invertebrates are restricted to certain regions (Awad *et al*., 2002; Scott *et al*., 2012; Whitfield *et al*., 2016). It is likely that metabarcoding is detecting patterns of phylogeographic structuring and regional endemicity, yet due to the poor resolution of OTUs (as many were resolved only to Kingdom or Phylum level) it is difficult to fully clarify species distribution patterns. However, the SIMPER analysis and correspondence analysis scatter plot (Figure 15) indicated that OTUs of the phyla Ochrophyta, Arthropoda, Cnidaria, Porifera and Kinorhyncha were prominent contributors to the dissimilarity between regions.

Furthermore, metabarcoding identified a strong biogeographic break between the east and west coast communities, which were least similar (Awad *et al*. 2002; Teske *et al*., 2011). Biogeographic and phylogeographic breaks between cool-temperate and warm-temperate biota (Emmanual *et al*., 1992) often coincide with the confluence of the Agulhas and Benguela currents, as breaks have been reported near Cape Point (Teske *et al*., 2007b; von der Heyden *et al*., 2008) and Cape Agulhas (Evan

et al., 2004; Teske *et al*., 2007a). Indeed, Cape Point has shown to be the most pronounced transition zone between biogeographical regions, due to differences in sea surface temperature, current flow direction, nutrients, and upwelling cells between the eastern and western shorelines (Griffiths *et al*., 2010), which reduce gene flow and dispersal between adjacent populations (von der Heyden *et al*., 2011). The eastern coast is influenced by the southwards flowing, nutrient poor, ~24°C Agulhas Current (Wright *et al*., 2015), while the western coast is influenced by the northward flowing ~12°C Benguela Current (Griffiths *et al*., 2010), with several strong upwelling cells (Shannon, 1985; Laudien *et al*., 2003). Cold-water upwelling has shown to be a strong dispersal barrier for numerous marine species (Lessios *et al*., 2003; Olivares Banuelos *et al*., 2008), resulting in limited dispersal of marine organisms from the Indian Ocean on the east coast, to the Atlantic Ocean on the west coast (Rocha *et al*., 2005; Floeter *et al*., 2008). Indeed, the distinct physicochemical conditions of the warm- and cool-temperate regions, such as temperature, nutrients, currents, and upwelling cells, have contributed to biogeographical structuring of estuarine communities for fishes (Whitfield *et al*., 2021), molluscs (Evans *et al*., 2004), shrimps (Evans *et al*., 2004), and crustaceans (Teske *et al*., 2006).

In addition, my study found that biogeographical structuring between east and west coast sites persisted for the different sampling substrates, even though the VENN diagrams (Figure 16) showed that the patterning of OTUs across sites was highly variables between substrates. Matcher at al. (2018) also found that different sampling substrates (namely sediment and water) captured a similar biogeographical trend in three warm temperate estuaries, yet the degree in overlap between sites' communities differed between substrates, and sediment exhibited a higher degree of species evenness (relative abundances of species within a community). In this study, intertidal vegetated sediment captured the most biogeographic structuring. When multiple sampling substrates were combined, the biogeographic structure was more pronounced and significant, indicating that multiple sampling substrates are most effective in delineating biogeographical structuring- likely due to substrates capturing different communities (especially water and sediment, as demonstrated in Chapter 1) that when combined are more representative of the faunal community.

Exploring species richness along the biogeographical gradient

The effects of changing environmental conditions on the ecology of different habitats is driven ultimately by the underlying physiology and tolerances of organisms and their ability to cope with environmental fluctuations (Smyth *et al*., 2016). Species distribution ranges are largely governed by thermal tolerance (Belanger *et al*., 2012; Vasconcelos *et al*., 2015), "with more species tolerant of warm temperatures" (Gaston, 2000) and in coastal South Africa, temperature has been found as an explanatory variable structuring the genomic diversity of many marine species, such as invertebrates (Nielsen, 2021), an endemic seagrass (Phair *et al*. 2019) and fishes (Teske *et al*., 2019; Golla *et al*., 2020; Teske *et al*. 2021). For many taxa there is a trend of increasing species richness (number of species in a community) along the gradient of increasing temperature from west to east coast (Awad *et al*., 2002; Griffiths *et al*. 2021). However, in contrast to fishes, some studies of estuarine invertebrates suggest a higher species richness on the warm-temperate south coast (Awad *et al*.,

2002; Teske and Wooldridge, 2004; Griffiths *et al*., 2010), likely due to the affinity of macroinvertebrates to higher salinities and lower turbidities, which is characteristic of warm-temperate estuaries in South Africa (Teske and Wooldridge, 2004; Harrison and Whitfield, 2006). Yet the pattern of species richness is often group dependent, as species richness for some groups, including fishes, gastropods, bivalves, echinoderms, and brachyurans, increases eastward towards the subtropical region, whereas other taxa, such as polychaetes, isopods, and amphipods, display a greater species richness in the warm-temperate region (Griffiths *et al*., 2010).

One of the expectations of this study was that the east coast estuaries would return a higher species richness than the west coast estuaries, yet only Breede followed the expected trend (Figure 17). It was found that generally, species richness increased from the west coast, cool temperate sites towards Breede, with a sharp decline at Swartkops, to the lowest species richness at Umhlatuze, across all substrates sampled. Indeed, when estuaries were grouped into their biogeographic regions, the trend of species richness was warm-temperate > cool-temperate > subtropical (Figure 18). This is in stark contrast to, for example, Czachur *et al*. (2021) who showed that for fishes, species richness increased from the west to the east coast, and Griffiths *et al*. (2010) and Awad *et al*. (2002) who showed that across macroinvertebrate groups, species richness was greatest along the east coast. However, few studies have been performed for invertebrates, especially meiofauna, as these can be difficult to sample and classify due to morphological similarities (see, for example, Slenzka *et al*., 2013; Wiedhase *et al*., 2016; Williams *et al*., 2017; Sato-Okoshi *et al*., 2017; Kara *et al*., 2020; Malan *et al*., 2020). As such, the incongruency of this studies' findings with previous research may be due to a lack of research investigating species richness patterns of total biodiversity in South African estuaries, especially as no studies have used metabarcoding- which offers complementary information to morphological approaches (Cahill *et al*., 2017; Groendahl *et al*., 2017)- to assess these patterns. It is therefore possible that these findings represent novel patterns of species richness for estuarine invertebrates.

However, it is also possible that metabarcoding is detecting local scale impacts on the invertebrate communities of east coast estuaries, as the Umhlatuze, Mngazana and Swartkops estuaries returned the lowest species richness and are located near highly developed areas and ports (namely Richards Bay Harbour, Port St John's, and Port Elizabeth, respectively). It may be that human-related activities- such as disturbance from development, changes in sediment load from the catchment, and an increase in turbidity (Adams, 2016; Izegaegbe *et al*., 2020) are disrupting invertebrate communities in these east coast estuaries, as has been shown for the Umhlatuze (Izegaegbe *et al*., 2020), as well as some North American (Freeman *et al*., 2019) and southeast Australian estuaries (Fowles *et al*., 2018), yet the magnitude of impact is difficult to quantify (Dafforn *et al*., 2012; Izegaegbe *et al*., 2020).

An interesting finding of this study was that the Breede in the warm temperate region returned the greatest species richness (Figure 17), which may likely be due to elevated salinities (it is a large permanently open estuary) and lower turbidity (Harrison and Whitfield, 2006), which are primary determinants influencing the biogeography of many estuarine species (Awad *et al*., 2002; Harrison and Whitfield, 2006; Adams *et al*., 2016). However, very little is known about the drivers of species diversity in estuaries and "the relative roles of different local and regional processes in determining

community structure" (Alves *et al*., 2020), especially as historical data is often lacking (Izegaegbe *et al*., 2020).

Notably, the trend of species richness varied by substrate, with most of the variation captured by the sediment substrates (Figure 17). Only once substrates were combined was there a significant difference in the species richness of biogeographic regions (Figure 18), demonstrating again, as for the detection of biogeographic patterns, that a combination of sampling substrates, especially sediments, may capture clearer structuring of species richness across biogeographic regions.

Exploring environmental variables that explain the variation in communities between regions

Environmental DNA metabarcoding has been used to explore the variables driving community structure (Nielsen, 2021; Teske *et al*., 2019; Golla *et al*., 2020) and the main stressors of aquatic systems (Chariton *et al*., 2015; Pissaridou *et al*., 2021; Al *et al*., 2021). Generally, nutrients, turbidity and pH have been found as the main driving stressors affecting community structure (Chariton *et al*., 2015; Pissaridou *et al*., 2021; Li *et al*., 2018), and variation in anthropogenically-driven environmental conditions shaped communities (Li *et al*., 2018; Al *et al*., 2021).

To investigate environmental variables that could explain the variation in the community matrix (presence/absence values of OTUs across sites), a highly significant RDA model was created for this study that included nine environmental variables which showed that mean SST, nitrate concentration and water quality were the three strongest explanatory variables (Figure 21). Other studies have also found SST mean (Belanger *et al*., 2012; Moreno *et al*., 2021), nitrate concentration (Okyere, 2019; Duque *et al*., 2021), and water quality (Yuksek *et al*., 2006; Balushkina and Golubkov, 2018) to be strong explanatory variables of community variation in meio- and macrofaunal communities.

Sea surface temperature is the most widely explored environmental variable thought to be important for explaining marine coastal biogeographical structure for regional and global studies (see Valentine 1966, Roy *et al*. 1994, Blanchette *et al*. 2008) – which is well founded in early studies connecting thermal tolerances to species distributions (Hutchins 1947) and additionally by more recent studies of global (Kelley *et al*., 2011; Belanger *et al*. 2012; Schultz *et al*., 2016; Hewitt *et al*., 2016) and individual species distributions (Jones *et al*. 2010; Staveley *et al*., 2019; Moreno *et al*., 2021). As such, SST measures (such as SST mean, min, max and range) are the most cited variables thought to be responsible for the biogeographic structure of many coastal marine organisms (Fenberg *et al*., 2015).

Furthermore, Duque *et al*. (2021) found that high nitrate concentration (likely through agricultural runoff, which is a persistent threat to South African estuaries; NBA, van Niekerk and Turpie, 2019) was the main cause of low water quality, and the main anthropogenic factor impacting invertebrate communities. Indeed, while nitrate concentration was highest in the cool-temperate region, the score for water quality (a higher score indicates better quality) was lowest in the cool-temperate region (Figure 21). These variables did not have a strong correlation yet may both be representing the negative effects of increased runoff in semi-enclosed water bodies such as estuaries (Okyere, 2019),

which has shown to affect larval mortality rates (Camargo *et al*., 2005; Muir *et al*., 1991) and may thus be negatively impacting the communities associated with the cool temperate estuaries.

In all, the results of the RDA are congruent with previous research that has shown SST mean, nitrate concentration, and water quality to be strong explanatory variables of biogeographical structuring of coastal, and now estuarine, communities, but of course, this will require additional insights from other estuaries in South Africa.

Chapter 3: Conclusions

Environmental DNA metabarcoding is gaining momentum for global biodiversity studies, which have demonstrated the ability of metabarcoding to characterize communities for biodiversity surveys (Deiner *et al*., 2017; Taberlet *et al*., 2012; Rees *et al*., 2014; Foote *et al*., 2012; Davy *et al*., 2015; Valentini *et al*., 2015), biogeographical analyses (Holman *et al*., 2021; Czachur *et al*., 2021; West *et al*., 2021), and impact assessments (Chariton *et al*., 2015; Li *et al*., 2018; Pissaridou *et al*., 2021; Al *et al*., 2021). However, few studies have been performed along the complex coastline of South Africa, which is influenced by two current systems, the warm Agulhas and cool Benguela (Walker, 1990), that shape three highly diverse biogeographic regions (Stephenson and Stephenson, 1972; Griffiths *et al*. 2010). In fact, no regional studies have explored biogeographical patterns in highly variable estuarine systems, which possess varying physicochemical characteristics (Day *et al*., 1981) and are heavily impacted through anthropogenic pressures (van Niekerk and Turpie, 2019), resulting in regional variations in the conditions (Elliott *et al*., 2007; Whitfield 1999)- and thus associated biodiversity (Smyth *et al*., 2016). Given the important ecological and economic services that estuaries, their seagrass, and their associated biodiversity provide (van Niekerk and Turpie, 2019), it is important that metabarcoding be assessed as a tool for biodiversity studies in South African estuaries. Yet there were still many unknowns surrounding metabarcoding in estuaries (Gleason *et al*., 2020; Ely *et al*., 2021), such as the effect of sampling substrate choice on the communities (Sakata *et al*., 2020; Matcher *et al*., 2018), and the ability of metabarcoding to capture biogeographical variation (Matcher *et al*., 2018).

As such, this study investigated metabarcoding in six estuaries housing *Zostera capensis* seagrass along the coastline of South Africa, with two estuaries per biogeographic region and broadly aimed to compare the communities returned by different sampling substrates (water and sediment substrates). Results suggested a that water captured a significantly different community to all the sediment substrates. Indeed, water captured larger proportions of the phyla Ochrophyta, Cnidaria and Chordata, while sediment substrates returned larger, yet varying, proportions of the “worms” (Annelida, Nematoda, Nemertea, Platyhelminthes). Previous research has demonstrated that water and sediment substrates capture different communities (Holman *et al*., 2019; Sakata *et al*., 2020), likely due to the different faunal assemblages harboured by the environments (Koziol *et al*., 2018; Shaw *et al*., 2016), and the different information (spatial for water, historical for sediment) that they present (Nelson-Chorney *et al*., 2019; Ely *et al*., 2021). This demonstrates the importance of selecting metabarcoding substrates based on the target species/community, as well as the type of information (i.e., spatial, or historical) required. One key recommendation from this study would be that for future estuarine biodiversity studies, both water and sediment samples are included, to more fully capture the broad array of species diversity that call estuaries their home.

It was further expected that sediment substrates from different environments (namely intertidal and subtidal, and vegetated and unvegetated seagrass beds) would capture different communities, due to differences in sedimentological components- which affect eDNA preservation and resuspension (Harrison *et al*., 2019)- and species assemblages (Park *et al*., 2019; Barnes *et al*., 2020). Yet this study found no significant difference in the communities returned by sediment substrates, indeed

sediments presented highly similar communities. Since both eDNA (Bessey *et al*., 2020) and macrobenthic invertebrates (Barnes, 2019; Barnes, 2021) display patchiness in their distribution, and as only one sample was collected for each sediment substrate per site, it is possible that sampling did not capture the full variation in communities returned by different sediment substrates. In addition, samples were collected only one to three metres apart, which may not be sufficient to constitute a change in environment. Future sampling regimes to compare sediment substrates should include large transects of each sediment type to capture more distinct communities, and multiple replicates along the transects to compensate for patchiness. Furthermore, samples for this study were collected at only one point in time, which may not be sufficient to capture the broad array of biodiversity. As many estuaries in South Africa are important breeding and nursery grounds for fishes and invertebrates that might utilise estuaries differentially throughout the year, it is recommended to include temporal sampling to include such variation (Stoeckle *et al*., 2017; Salter, 2018; Zhang *et al*., 2019). Environmental DNA turnover can occur at the scale of hours to days (Collins *et al*., 2018; Salter, 2018; Beentjies *et al*., 2019; Seymour *et al*., 2021; Ely *et al*., 2021) and therefore including a longer timeframe is important for capturing the full array of estuarine biodiversity. Indeed, Beentjies *et al*. (2019) found that temporal replicates at one-week intervals captured as much dissimilarity as spatial replicates, which increased for longer timescales. Therefore, spatial replicates alone may not be enough to capture the full taxonomic diversity of marine systems, as communities may experience shifts in short timescales, highlighting the importance of including temporal replicates for metabarcoding studies of biodiversity (Beentjies *et al*., 2019).

In addition to revealing novel insights into substrate sampling, the results from my work demonstrate that metabarcoding captured sufficient variation in communities along the coastline to delineate biogeographic regions. Indeed, the communities returned by regions were significantly different, with a strong biogeographic break between the east and west coast sites, likely due to the influence of two different current systems and their physicochemical properties (Walker, 1990). A redundancy analysis was used to explore the environmental variables that could explain the variation in communities between regions, which showed that nitrate, mean sea surface temperate (SST) and water quality were the three most powerful explanatory variables, which is congruent with previous research (Nitrate: Okyere, 2019; Duque *et al*., 2021; SST: Belanger *et al*., 2012; Moreno *et al*., 2021; Water quality: Yuksek *et al*., 2006; Balushkina and Golubkov, 2018). Furthermore, species richness was found to be greatest in the warm temperate region, likely due to elevated salinities ad lower turbidity (Harrison and Whitfield, 2006), coupled with the warm Agulhas waters (Harrison, 2004), which are primary determinants influencing the biogeography of many estuarine species (Awad *et al*., 2002; Harrison and Whitfield, 2006; Griffiths *et al*., 2010; Adams *et al*., 2016). Biogeographical structuring and species richness patterns were significant only when multiple sampling substrates were included in the analyses, indicating again that a combination of sampling substrates (especially water and sediment substrates) captures a broader range of variation in communities and should be included for future biogeographical studies of estuaries.

An important aspect of molecular studies (including metabarcoding surveys) is primer choice (Leray and Knowlton, 2016, Hajibabaei *et al*., 2019). Multiple studies for biodiversity analyses have shown that results (such as species composition and richness) are sensitive to primer choice (Leray and

Knowlton, 2016; Hajibabaei *et al*., 2019; Holman *et al*., 2019; Castro *et al*., 2021), which can prove problematic for long-term biodiversity studies as amplicon data from different regions cannot be compared (Leray and Knowlton, 2016). This study used the Folmer primers targeting the mitochondrial cytochrome c oxidase subunit 1 (CO1) region for PCR amplification of eDNA (Folmer *et al*., 1994), which have been shown to "often fail or perform poorly, producing faint products despite attempts at optimization" (Geller *et al*., 2013). Mismatches with the target annealing position for many taxa makes the Folmer primers not truly 'universal' in applicability (Geller *et al*., 2013), which may have resulted in primer biases and skewed results. Future studies should make use of the degenerate primers (Geller *et al*., 2013) to target the CO1 region as they have been shown to be more universal and less erroneous than the Folmer primers (Geller *et al*., 2013; Miralles *et al*., 2019).

Furthermore, many studies have targeted hypervariable regions of the 18S rRNA gene (e.g., Fonseca *et al*., 2010; Pawlowski *et al*., 2011; Fonseca *et al*., 2014; Brannock *et al*., 2014; Guardiola *et al*., 2015; Chariton *et al*., 2015; Banerji *et al*., 2018; Minerovic *et al*., 2020; Egge *et al*., 2021), as this primer is highly versatile, yet "primer versatility comes at the price of taxonomic resolution" (Leray and Knowlton, 2016). Indeed, evidence is mounting that suggests that all 18S rRNA regions seriously underestimate diversity compared to CO1 (Tang *et al*., 2012; Leray and Knowlton, 2016; Holman *et al*., 2019). For example, Holman *et al*. (2019) found that the 18S variable regions do not provide sufficient taxonomic resolution for species-level censuses of macroinvertebrates and suggests a combination of broad-range primers (such as 18S) and hypervariable regions (such as the CO1 region). Another study by Tang *et al*. (2012), demonstrated that CO1 provided sufficient taxonomic resolution to identify cryptic lineages, which increased diversity estimates compared to 18S. Indeed, mitochondrial DNA (mtDNA) is particularly suitable for molecular discrimination of animals (Machida and Tsuda, 2010; Allio *et al*., 2017; Andujar *et al*., 2018), as the high substitution rate, maternal mode of inheritance and absence of recombination, presents low levels of mtDNA variation within a species, yet distinct genetic variation between species (Wilson *et al*., 1985; Tsauosis *et al*., 2005; Raupach *et al*.,2010). As such, this study targeted the mitochondrial CO1 gene for improved taxonomic resolution of marine animals. Although all multicellular, eukaryotic OTUs were retained, metabarcoding of the CO1 region captured more OTUs for macroinvertebrate phyla (such as Arthropoda and Mollusca) and only a few OTUs for Chordata in the water substrate. Further, despite the success of CO1 for discriminating animal species for barcoding, there are some drawbacks of using mitochondrial markers, such as co-amplification of nuclear mitochondrial pseudogenes (Song *et al*., 2008; Hazkani-Covo *et al*., 2010), incomplete lineage sorting (Petit and Excoffier, 2009; Kemppainen *et al*., 2009), and heteroplasmy (Hoeh *et al*., 1991). As such, it is widely acknowledged that a combination of nuclear and mitochondrial markers should be used as complementary measures to assess patterns of biodiversity to control for sequencing biases and method drawbacks (Leray and Knowlton, 2016; Hajibabaei *et al*., 2019; Holman *et al*., 2019; Castro *et al*., 2021). Therefore, future metabarcoding studies of South African estuaries assessing total biodiversity should include a combination of broad-range nuclear and hypervariable mitochondrial primers.

In addition, the taxonomic resolution of CO1 was poor, as nearly half of all OTUs were identified only to the Kingdom or Phylum level, and as such does not provide insight into species-level distributions

for biodiversity surveys. Indeed, many of the OTUs that were contributors to the dissimilarity between the communities returned by different sampling substrates, and biogeographic regions, were only identified to the Kingdom or Phylum level, which prevented finer-scale analyses of community structure. A foundational step to improve the efficacy of metabarcoding is to augment the DNA barcode database for high-resolution sequence matches (Weigand *et al*., 2019). A smaller part of this study sequenced the mitochondrial CO1 region of 18 macrobenthic invertebrates from 12 species to upload to BOLD (Appendix 1). The CO1 library was also included in the reference database for metabarcoding, which found little to no overlap in species-level returns between the CO1 sequences and the OTUs. In fact, only *Hydrobia knysnaensis* was captured by both morphology-based identification and metabarcoding. Four of the species that were sequenced are novel to BOLD, highlighting the general lack of barcode data for marine species (Fagg *et al*., 2021). Further to this, Singh *et al*. (2021) assessed the current state of DNA barcode databases for marine zooplankton in South Africa and revealed incomplete databases for all taxa examined. Barcode records were dominated by commercially important species (such as fish and decapod crustaceans) and by species occurring in accessible shoreline ranges (Singh *et al*., 2021). Considering the growing evidence that total biodiversity promotes healthy ecosystem functions (Snelgrove, 1997; Loreau *et al*., 2002; Sandifer *et al*., 2015), there is a need for more comprehensive approaches including all aspects of biodiversity, to monitor marine biota in the face of increasing anthropogenic pressures, and as such a need for complete reference databases for improved metabarcoding surveys.

Furthermore, the use of OTUs in this study may have contributed to the lack of taxonomic resolution. Operational taxonomic units are clusters of reads that differ by less than a fixed sequence dissimilarity threshold, usually 3% (Westcott and Schloss, 2015; Kopylova *et al*., 2016). It is widely acknowledged, though, that this is at best a bioinformatical approximation of a species (Schloss and Westcott, 2011; Tikhonov *et al*., 2015) and we are far from the “one OTU= one species” ideal (Forster *et al*., 2019). Callahan *et al*. (2017) presented an alternative approach to OTUs, namely amplicon sequence variants (ASVs), which infer the biological sequences in the sample before amplification and sequencing, thus avoiding possible amplification biases or sequencing errors. Furthermore, as ASVs can distinguish sequence variants differing by a single nucleotide. As such, ASVs have demonstrated better sensitivity and specificity than I methods and may better determine ecological patterns (Eren *et al*., 2013; Eren *et al*., 2015; Callahan *et al*., 2016a; Needham *et al*., 2017; Callahan *et al*., 2017). A further advantage of ASVs over OTUs is that they are “reusable across studies, reproducible in future data sets and not limited by incomplete reference databases” (Callahan *et al*., 2017). As such, future eDNA metabarcoding studies of estuarine biodiversity should include ASVs as the unit of marker-gene analysis and reporting.

Although eDNA metabarcoding has many advantages over traditional, morphology-based surveys for community studies, such as a higher detection capability coupled with a lower monitoring effort, the method can still prove costly compared to morphological identification of communities. Indeed, a cost-benefit analysis by Fernandez *et al*. (2018) found that the metabarcoding approach is more expensive than traditional methods for assessing macroinvertebrate communities but requires less sampling and identification efforts. In this study, it could be argued that the cost of eDNA metabarcoding may not be justifiable given the lack of taxonomic resolution and the limitations posed on sampling design (as

multiple sampling replicates for substrates at each site was too costly to include), yet this study still provided insights into biogeographical structuring that would take much longer to achieve with traditional methods- thus saving on time costs. However, to further address the costs associated with eDNA metabarcoding, technological improvements and a wider use of eDNA metabarcoding may decrease the associated sequencing costs (Fernandez *et al*., 2018). In addition, the sequencing process can be externalized to specialized companies, reducing the costs of implementing next-generation sequencing technology and the use of expensive platforms (Borrell *et al*., 2017). Furthermore, augmenting the DNA barcode database would increase taxonomic resolution (Weigand *et al*., 2019), thus justifying the costs for future eDNA metabarcoding studies.

In all, the results of this study highlight opportunities for future eDNA metabarcoding research of South African estuarine fauna. Firstly, while this study provided a baseline for eDNA metabarcoding sampling strategies by demonstrating that water and sediment substrates may capture unique proportions of communities, replication could not be included due to cost constraints, which may have resulted in under-sampling. As such, communities should be assessed at a finer scale with multiple sampling replicates from various points within a single estuary, such as the well-studied Knysna estuary (Barnes, 2019; van Rensburg, 2019; Barnes, 2021). Yet, to fully explore not just the patterns of community structure between substrates, but also the relationship with environmental conditions, environmental measurements of ecologically important variables (such as salinity, Harrison and Whitfield, 2005; sea surface temperature, Belanger *et al*., 2012; Moreno *et al*., 2021; and sediment characteristics, Rodil *et al*., 2021) should be taken with eDNA samples. In addition, morphological assessments of biodiversity within various substrates could also be compared with the results of eDNA metabarcoding to determine congruency in the communities returned (see for example, Pereira *et al*., 2021). Secondly, while this study demonstrated the ability of eDNA metabarcoding to delineate biogeographic regions, it would be ideal to explore more study sites with multiple sampling substrates (as this study demonstrated that a combination of sampling substrates captures greater biogeographical structuring), while also including more sampling replicates. However, such replication on a large scale would prove to be costly, highlighting the inherent trade-offs between sampling design and cost that must be considered for eDNA metabarcoding studies.

In conclusion, my study provides novel baseline information for metabarcoding surveys of biodiversity in South African estuaries, bringing to light novel findings, recommendations, and future directions of research. Overall, this study lays the foundation for biomonitoring through eDNA metabarcoding with a particular focus on seagrass ecosystems, to help support ongoing conservation and management efforts for this valuable ecosystem engineer.

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Appendix 1: Barcoding efforts for macrobenthic invertebrates in South Africa

Materials and methods

Sampling

Sampling was conducted at low tide in exposed intertidal seagrass beds in the Umhlathuze, Mngazana, Swartkops, Breede, Langebaan, Berg and Olifants estuaries under permits RES2020/23 and CRC/2021-2022/012--2021/V1 for Langebaan. Within each estuary, five 10x10m sites spanning the distribution were sampled randomly. This allowed for biodiversity capture throughout the distribution, thus accounting for local environmental variation (such as differences in salinity or temperature) which have been shown to shape benthic communities in other estuarine systems (Barnes, 2010; Vonk *et al*., 2010; Al-Wedaei *et al*., 2011). From each site, five core samples were randomly collected for single-species barcoding studies. Cores have a diameter of 11cm and depth of 10cm (current and previous research in Knysna shows that this sampling approach collects a diverse and representative invertebrate community). A total of 25 cores were collected for each of the six estuaries; calculated as 5 cores/site for 5 sites. The sediment from the core was size fractionated gently through stainless steel sieves of mesh size >730 µm and all sediment and invertebrates remaining on the sieve were placed into a clean, labelled jar (including site name) with fresh seawater and set on ice in a polystyrene box for processing later that day.

DNA barcoding: DNA extraction, amplification, and sequencing

Specimens were identified at species level through morphology-based identification (hereafter referred to as “morphology”) with reference to taxonomic keys (*Two Oceans* by Branch, 2017; *Guide to the marine isopods of southern Africa* by Kensley, 1978) and the under the guidance of taxonomist Professor Richard Barnes. Correct nomenclature was checked for on the World Register of Marine Species (WoRMS - World Register of Marine Species). Metadata, such as photographs and source locations, were recorded for each specimen. DNA extraction and polymerase chain reactions (PCR) were carried out at the Marine Genomics Lab at Stellenbosch University. DNA extraction was performed with a Macherey-Nagel NucleoSpin DNA extraction kit following the manufacturer’s protocol. For small-sized samples, the Qiagen QIAamp DNA Micro Kit was used. A partial fragment of the mtDNA-CO1 gene of ~700 bp was targeted, following Geller *et al*. (2013), with multiple primer combinations (Folmer *et al*., 1994; Geller *et al*., 2013 and followed Fagg *et al*. 2021). PCR products were visualised through gel electrophoresis on a 1% agarose gel stained with ethidium bromide and gel purified. The PCR products were bidirectionally sequenced on an ABI 3730xl DNA Analyser at the Central Analytical Facility at Stellenbosch University. All sequences were edited in Geneious 11 (Kearse *et al*. 2012) and EMBOSS transeq (EMBOSS Transeq < Sequence Translation Sites < EMBL-EBI) was used to check for pseudogenes. Sequences were compared to the BOLD database with the BLASTn function on Geneious 11 to ensure correct taxonomic identity. Species were searched for on BOLD to determine their presence on the reference database. All sequences and metadata will be uploaded to the Barcode of Life Database (BOLD).

Statistics

To compare the species identified through metabarcoding and morphology-based identification, VENN diagrams (Figure A2) were made to illustrate the overlap in species identified.

Results

A total of 164 macroinvertebrate specimens were collected from 30 species, with one to five specimens per species. Table A1 displays the catch and sequencing records for species at sites. After multiple sequencing attempts with various combinations of primers and PCR conditions, only 18 specimens from 12 species were successfully sequenced. Sequenced species were of the class Bivalvia (3 species), Gastropoda (3) and Malacostraca (6). As displayed in Table A2, when compared with the BOLD database through the BLASTn function, three sequences returned matching hits at family level, six at genus and nine at species level. At the time of searching (27 October 2021), eight species were present on BOLD, while three were represented at genus level and one at family level. As such, sequences for four new species (*Eumarcia paupercula, Fissurella mutabilis, Melita zeylanica* and *Atrina squamifera*) were added to BOLD (project code: HONS). Images for the newly sequenced species are included in Figure A1.The VENN diagrams (Figure A2) show little overlap in the species identified through metabarcoding and morphology-based identification, demonstrating that the methods captured different taxa at species level.

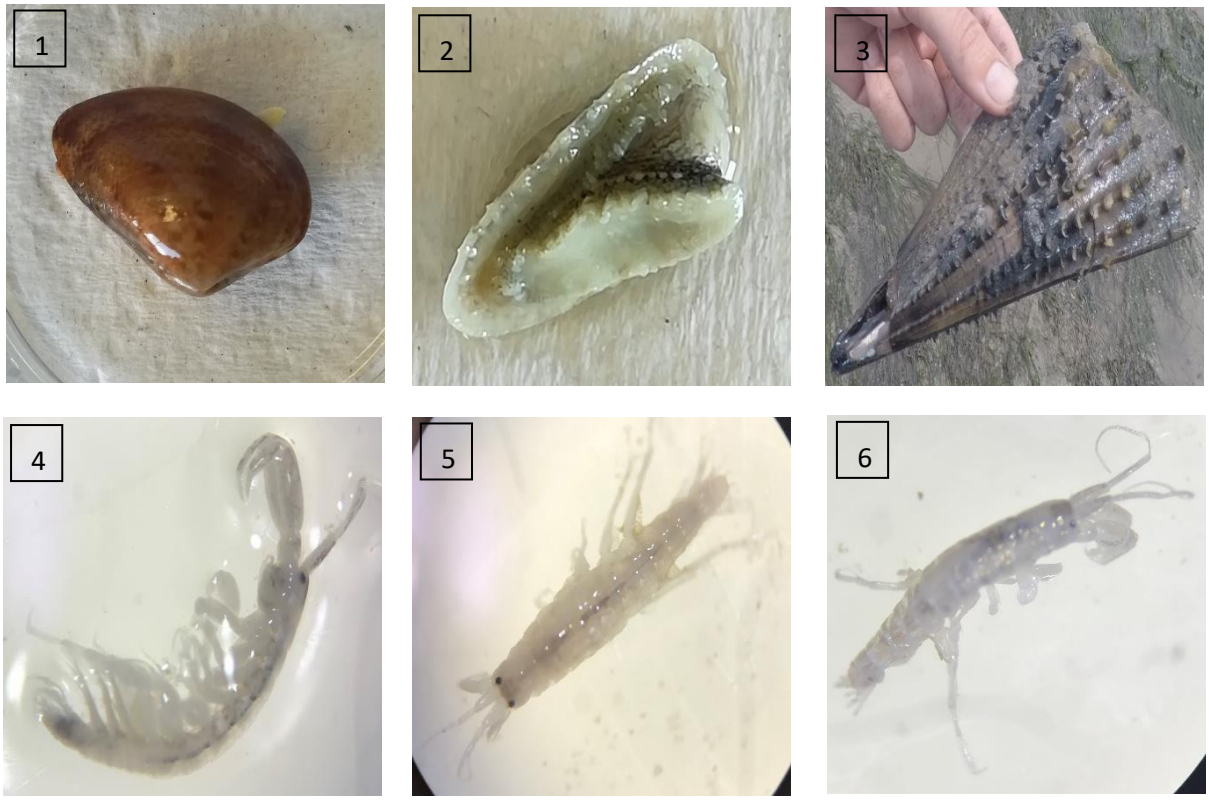

Figure A1: Specimen images of newly sequenced species. 1= H3 *Eumarcia paupercula*, 2= L30 *Fissurella mutabilis*, 3= S42 *Atrina squamifera,* 4= O16 *Melita zeylanica*, 5= O17 *Melita zeylanica*, 6= O18 *Melita zeylanica*.

Table A1: The number of specimens collected and sequenced for species at sites. The bottom row indicates the total number of specimens collected/ number of species per site, with the last cell indicating the total number of specimens sequenced/ number of species sequenced.

	Phylum	Class	Species	Site							Number of specimens collected for species	Number of specimens sequenced for species
				Ol	Be	La	Br	Sw	Mn	Um		
	Cnidaria	Anthozoa	*Bunodactis reynaudi*			*3*					3	
			Haloclava capensis			*5*					5	
	Crustacea	Malacostraca	*Cardisoma carnifex*					*2*			2	
			Danielella edwardsii				*3*	*1*	*5*		9	*2*
			Diogenes brevirostris			*5*	*3*	*5*			13	2
			Diogenes extricatus							*1*	1	
			Exosphaeroma truncatitelson	*2*	*5*			*1*			8	
			Hymenosoma orbiculare	*1*	*5*	*2*	*5*	*3*			16	1
			Kraussillichirus kraussi					*3*			3	
			Melita zeylanica	*5*	*5*						10	3
			Mesopodopsis africana					*3*			3	
			Paridotea ungulata	*1*							1	1
			Upogebia africana					*1*			1	1
			Upogebia capensis		*2*						2	
	Echinodermata	Asteroidea	*Parvulastra exigua*					*1*			1	
		Holothuroidea	*Holothuria nobilis*			*5*					5	
	Mollusca	Bivalvia	*Atrina squamifera*					*1*			1	1
			Bulla ampulla					*1*			1	
			Crepidula porcellana			*5*					5	
			Dosinia hepatica				*5*			*5*	10	1
			Eumarcia paupercula							*1*	1	1
			Salmacoma litoralis					*5*	*5*		10	
			Solen cylindraceus					*1*		*2*	3	
			Venerupis corrugata			*2*					2	
		Gastropoda	*Fissurella mutabilis*			*5*					5	1
			Haminoea alfredensis					*6*			6	2
			Hydrobia knysnaensis	*5*	*5*						10	2
			Littorina saxatilis						*2*		2	
			Nassarius kraussianus				*5*	*5*	*5*	*5*	20	
			Tricolia capensis	*5*							5	
Total	**4 phyla**	**6 classes**	**30 species**	**19/ 6**	**22/ 5**	**32/ 8**	**21/ 5**	**39/ 15**	**17/ 4**	**14/ 5**	**164 specimens**	**18/ 12**

Table A2: The BLASTn and BOLD search results for specimen sequences.

Specimen label	Sampling location	Species	Species present on BOLD	BLASTn top match	% Pairwise identity
H3 *E. paupercula*	Umhlatuze	*Eumarcia paupercula* *	No, only family	*Tapes dorsatus* isolate DZJ0414 cytochrome oxidase subunit I (CO1) gene, partial cds; mitochondrial	78.1
H9 *D. hepatica*	Umhlatuze	*Dosinia hepatica*	Yes	*Dosinia corrugata* voucher Cheng200805DL cytochrome c oxidase subunit I (CO1) gene, partial cds; mitochondrial	84.3
L15 *U. africana*	Langebaan	*Upogebia africana*	Yes	*Upogebia africana* isolate Uafr30 cytochrome oxidase subunit I gene, partial cds; mitochondrial	99.5
L17 *H. orbiculare*	Umhlatuze	*Hymenosoma orbiculare*	Yes	*Hymenosoma orbiculare* isolate HNam2 cytochrome oxidase subunit I (CO1) gene, partial cds; mitochondrial	92.3
L30 *F. mutabilis*	Langebaan	*Fissurella mutabilis* *	No, only genus	*Fissurella natalensis* voucher MCZ:Mala:378455_2 cytochrome c oxidase subunit 1 (CO1) gene, partial cds; mitochondrial	85.9
O1 *H. knysnaensis*	Olifants	*Hydrobia knysnaensis*	Yes	*Hydrobia knysnaensis* isolate 2345 cytochrome c oxidase subunit I (CO1) gene, partial cds; mitochondrial	85.5
O16 *M. zeylanica*	Olifants	*Melita zeylanica* *	No, only genus	*Melita shimizui* 651 mitochondrial CO1 gene for cytochrome c oxidase I, partial cds	78.3
O17 *M. zeylanica*	Olifants	*Melita zeylanica* *	No, only genus	*Melita shimizui* 651 mitochondrial CO1 gene for cytochrome c oxidase I, partial cds	79.7
O18 *M. zeylanica*	Olifants	*Melita zeylanica* *	No, only genus	*Melita shimizui* 651 mitochondrial CO1 gene for cytochrome c oxidase I, partial cds	78.6
O19 *P. ungulata*	Olifants	*Paridotea ungulata*	Yes	*Paridotea ungulata* cytochrome oxidase subunit I (CO1) gene, partial cds; mitochondrial gene for mitochondrial product	98.9
O5 *H. knysnaensis*	Olifants	*Hydrobia knysnaensis*	Yes	*Hydrobia knysnaensis* isolate 2345 cytochrome c oxidase subunit I (CO1) gene, partial cds; mitochondrial	99.4
S15 *D. edwardsii*	Swartkops	*Danielella edwardsii*	Yes	*Tubuca rosea* mitochondrion, complete genome	84.7
S2 *D. brevirostris*	Swartkops	*Diogenes brevirostris*	Yes	*Diogenes brevirostris* voucher MB-A066756 cytochrome oxidase subunit 1 (CO1) gene, partial cds; mitochondrial	94.2
S3 *D. brevirostris*	Swartkops	*Diogenes brevirostris*	Yes	*Diogenes brevirostris* voucher MB-A066755 cytochrome oxidase subunit 1 (CO1) gene, partial cds; mitochondrial	97.1

S37 *H. alfredensis*	Swartkops	*Haminoea alfredensis*	Yes	*Haminoea alfredensis* isolate 174 cytochrome oxidase subunit I (CO1) gene, partial cds; mitochondrial	99
S4 *H. alfredensis*	Swartkops	*Haminoea alfredensis*	Yes	*Haminoea alfredensis* isolate 174 cytochrome oxidase subunit I (CO1) gene, partial cds; mitochondrial	99.7
S42 *A. squamifera*	Swartkops	*Atrina squamifera* *	No, only genus	*Atrina fragilis* voucher Malaga1 cytochrome c oxidase subunit I (CO1) gene, partial cds; mitochondrial	96.6
Z9 *D. edwardsii*	Mngazana	*Danielita edwardsii*	Yes	*Tubuca rosea* mitochondrion, complete genome	85.4

* newly sequenced species

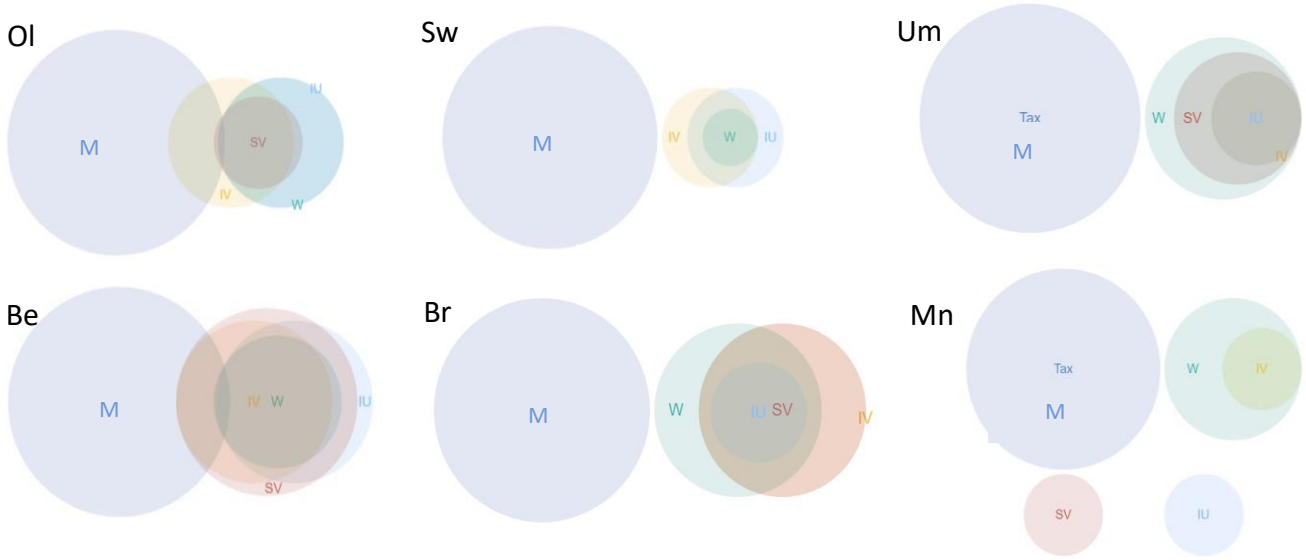

Figure A2 Venn diagrams of the species identified through morphology-based identification (M) and metabarcoding mediums (W, IV, IU, SV) at each site.

Appendix 2: Additional information for Chapter 1

Table A3: Results of a SIMPER analysis on the OTUs contributing to the dissimilarity between sampling substrates. Results are displayed up to a cumulative 25%.

OTU	Av. dissim	Contrib. %	Cumulative %	Mean W	Mean IV	Mean SV	Mean IU	OTU ID at taxonomic levels						
								K	P	C	O	F	G	S
OTU_3184;size=43	0,7977	0,9817	0,9817	1	0	0	0	Eukaryota	Arthropoda					
OTU_138;size=1362	0,7946	0,9779	1,96	0,667	0,333	0,333	0,5	Eukaryota	Mollusca	Gastropoda	Littorinimorpha	Assimineidae	*Assiminea*	*Assiminea capensis*
OTU_123;size=813	0,791	0,9735	2,933	0,167	0,333	0,5	0,667	Eukaryota	Kinorhyncha	Cyclorhagida		Echinoderidae		
OTU_2;size=20677	0,7854	0,9665	3,9	0,333	0,667	0,667	0,667	Eukaryota	Nematoda	Chromadorea	Desmodorida			
OTU_448;size=105	0,7823	0,9627	4,862	0,5	0,667	0,333	0,5	Eukaryota	Ochrophyta	Phaeophyceae				
OTU_5;size=2778	0,7696	0,9471	5,809	0,5	0,5	0,333	0,333	Eukaryota	Arthropoda	Branchiopoda	Diplostraca			
OTU_33;size=1498	0,7674	0,9444	6,754	0,667	0,5	0,333	0,167	Eukaryota	Ochrophyta	Phaeophyceae	Ectocarpales	Chordariaceae	*Myrionema*	*Myrionema balticum*
OTU_192;size=355	0,7669	0,9438	7,698	0	0,667	0,5	0,5	Eukaryota	Ochrophyta	Phaeophyceae	Laminariales	Laminariaceae		
OTU_166;size=464	0,7524	0,926	8,624	0,167	0,667	0,333	0,5	Eukaryota						
OTU_1242;size=86	0,7295	0,8978	9,521	0,333	0,333	0,333	0,5	Eukaryota						
OTU_1897;size=203	0,7272	0,8949	10,42	0,333	0,167	0,5	0,333	Eukaryota	Kinorhyncha	Cyclorhagida		Echinoderidae		
OTU_414;size=159	0,726	0,8935	11,31	0,333	0,5	0,333	0,333	Eukaryota	Ochrophyta	Phaeophyceae	Fucales			
OTU_263;size=232	0,6987	0,8599	12,17	0,167	0,5	0,333	0,333	Eukaryota	Cnidaria	Hydrozoa	Leptothecata	Obeliidae		
OTU_171;size=321	0,6949	0,8552	13,02	0,5	0,333	0,333	0,333	Eukaryota	Ochrophyta	Phaeophyceae	Fucales			
OTU_167;size=412	0,692	0,8516	13,88	0,333	0,5	0,167	0,333	Eukaryota						
OTU_28;size=2245	0,6834	0,841	14,72	0,333	0,333	0,333	0,333	Eukaryota	Annelida	Polychaeta	Capitellida	Capitellidae	*Capitella*	
OTU_69;size=6664	0,6773	0,8335	15,55	0,167	0,5	0,333	0,167	Eukaryota	Arthropoda					
OTU_1001;size=55	0,6622	0,815	16,37	0	0,5	0,333	0,5	Eukaryota	Porifera	Demospongiae				
OTU_1013;size=81	0,6583	0,8101	17,18	0,5	0,167	0,167	0,333	Eukaryota	Ochrophyta	Phaeophyceae	Ectocarpales	Chordariaceae		
OTU_169;size=331	0,6532	0,8038	17,98	0,833	0	0	0	Eukaryota	Ochrophyta	Phaeophyceae	Ectocarpales			
OTU_380;size=107	0,6523	0,8028	18,78	0,333	0,333	0,167	0,333	Eukaryota						
OTU_114;size=441	0,6515	0,8018	19,58	0,5	0,333	0,167	0,167	Eukaryota	Ochrophyta	Phaeophyceae				
OTU_1425;size=49	0,6456	0,7946	20,38	0,667	0	0,333	0	Eukaryota						
OTU_932;size=47	0,6441	0,7927	21,17	0,167	0,333	0,167	0,5	Eukaryota	Ochrophyta	Phaeophyceae	Fucales			
OTU_149;size=428	0,6297	0,7749	21,95	0,333	0,333	0,167	0,333	Eukaryota	Ochrophyta	Phaeophyceae	Dictyotales			
OTU_4762;size=288	0,6251	0,7693	22,72	0,167	0,5	0,333	0,167	Eukaryota	Arthropoda	Insecta				
OTU_57;size=1476	0,6206	0,7637	23,48	0,167	0,333	0,333	0,333	Eukaryota						
OTU_1004;size=84	0,5978	0,7357	24,22	0,167	0,333	0,333	0,333	Eukaryota	Gastrotricha		Chaetonotida	Chaetonotidae		
OTU_409;size=445	0,5919	0,7285	24,94	0	0,333	0,333	0,333	Eukaryota						

Appendix 3: Additional information for Chapter 2

Table A4: The results of a Shapiro-Wilks test for normality and Levene's Test for homoscedasticity of OTU data

	Olifans	Berg	Breede	Swartkops	Mngazana	Umhlatuze
N	4	4	4	4	4	4
Shapiro-Wilk W	0,9787	0,9839	0,9494	0,9806	0,94	0,965
p(normal)	0,8941	0,9245	0,7124	0,9053	0,6541	0,8104
Levene´s test, from means	p (same):	0,1688				
Levene´s test, from medians	p (same):	0,1976				

Table A5: Results of a SIMPER analysis on the OTUs contributing to the dissimilarity between biogeographic regions. Results are displayed up to a cumulative 25%.

OTU	Av. dissim	Contrib. %	Cumulative %	Mean Cool temp	Mean Warm temp	Mean Subtrop	OTU ID at taxonomic levels						
							K	P	C	O	F	G	S
OTU_2;size=20677	1.048	1.2	1.2	0.875	0.875	0	Eukaryota	Nematoda	Chromadorea	Desmodorida			
OTU_28;size=2245	1.025	1.173	2.373	1	0	0	Eukaryota	Annelida	Polychaeta	Capitellida	Capitellidae	*Capitella*	
OTU_414;size=159	0.935	1.071	3.444	0.875	0.25	0	Eukaryota	Ochrophyta	Phaeophyceae	Fucales			
OTU_138;size=1362	0.9046	1.036	4.48	0	0.75	0.625	Eukaryota	Mollusca	Gastropoda	Littorinimorpha	Assimineidae	*Assimine a*	*Assiminea capensis*
OTU_5;size=2778	0.8882	1.017	5.497	0.75	0.375	0.125	Eukaryota	Arthropoda	Branchiopoda	Diplostraca			
OTU_192;size=355	0.873	0.9998	6.497	0.75	0.5	0	Eukaryota	Ochrophyta	Phaeophyceae	Laminariales	Laminariaceae		
OTU_166;size=464	0.8041	0.9209	7.418	0.375	0.75	0.125	Eukaryota						
OTU_167;size=412	0.8009	0.9172	8.335	0.625	0.375	0	Eukaryota						
OTU_263;size=232	0.776	0.8887	9.224	0	0.625	0.375	Eukaryota	Cnidaria	Hydrozoa	Leptothecata	Obeliidae		
OTU_4762;size=288	0.7757	0.8883	10.11	0.75	0.125	0	Eukaryota	Arthropoda	Insecta				
OTU_1242;size=86	0.7709	0.8829	11	0.125	0.375	0.625	Eukaryota						
OTU_69;size=6664	0.7663	0.8776	11.87	0	0.625	0.25	Eukaryota	Arthropoda					
OTU_409;size=445	0.7561	0.8659	12.74	0.75	0	0	Eukaryota						
OTU_448;size=105	0.7541	0.8636	13.6	0.5	0.5	0.5	Eukaryota	Ochrophyta	Phaeophyceae				
OTU_123;size=813	0.7469	0.8554	14.46	0.375	0.375	0.5	Eukaryota	Kinorhyncha	Cyclorhagida		Echinoderidae		
OTU_1758;size=53	0.7446	0.8528	15.31	0.75	0	0	Eukaryota	Ochrophyta	Phaeophyceae				
OTU_15;size=8767	0.7425	0.8503	16.16	0.75	0	0	Eukaryota	Arthropoda	Insecta				
OTU_1897;size=203	0.7419	0.8497	17.01	0.125	0.375	E	Eukaryota	Kinorhyncha	Cyclorhagida		Echinoderidae		
OTU_33;size=1498	0.7397	0.8471	17.86	0.25	0.5	0.5	Eukaryota	Ochrophyta	Phaeophyceae	Ectocarpales	Chordariaceae	*Myrionema*	*Myrionema balticum*
OTU_114;size=441	0.7224	0.8274	18.68	0.625	0.125	0.125	Eukaryota	Ochrophyta	Phaeophyceae				

OTU_932;size=47	0.7213	0.826	19.51	0.5	0.375	0	Eukaryota	Ochrophyta	Phaeophyceae	Fucales			
OTU_149;size=428	0.7165	0.8205	20.33	0.5	0.375	0	Eukaryota	Ochrophyta	Phaeophyceae	Dictyotales			
OTU_1001;size=55	0.6881	0.7881	21.12	0	0.625	0.375	Eukaryota	Porifera	Demospongiae				
OTU_423;size=119	0.6845	0.7839	21.9	0	0.75	0	Eukaryota						
OTU_171;size=321	0.6841	0.7835	22.69	0.375	0.5	0.25	Eukaryota	Ochrophyta	Phaeophyceae	Fucales			
OTU_380;size=107	0.6838	0.7831	23.47	0.375	0	0.5	Eukaryota						
OTU_456;size=108	0.67	0.7673	24.24	0	0.75	0	Eukaryota						
OTU_57;size=1476	0.6543	0.7493	24.99	0	0.5	0.375	Eukaryota						

Table A6: Environmental variables included in the biogeographical analysis of community variance

Variable type	Environmental variable	Units	Source
Habitat score	Biological state	Score out of 100	Van Niekerk (2018) Table A1, Appendix A
	Hydrology	Score out of 100	Van Niekerk (2018) Table A1, Appendix A
	Physical habitat	Score out of 100	Van Niekerk (2018) Table A1, Appendix A
	Water quality	Score out of 100	Van Niekerk (2018) Table A1, Appendix A
Site characteristics	Particulate Inorganic Carbon (PIC) mean	mg.m-3	GMED - Download Data Layers (auckland.ac.nz)
	pH	-	GMED - Download Data Layers (auckland.ac.nz)
	Photosynthetically active radiation (PAR) mean	Einstein/m²/day	GMED - Download Data Layers (auckland.ac.nz)
	Salinity	psu	Planet OS - HYCOM (Hybrid Coordinate Ocean Model) global ocean forecast (Global Ocean Forecasting System (GOFS) 3.1 output on the GLBy0.08)
	Sea surface temperature mean (SSTmean)	°C	GMED - Download Data Layers (auckland.ac.nz)
	Sea surface temperature range (SSTrange)	°C	GMED - Download Data Layers (auckland.ac.nz)
	Surface current (surcurrent)	m/s	GMED - Download Data Layers (auckland.ac.nz)
	Tide average	m	GMED - Download Data Layers (auckland.ac.nz)
	Total Suspended Matter (TSM) mean	g.m-3	GMED - Download Data Layers (auckland.ac.nz)
	Waveheight	m	GMED - Download Data Layers (auckland.ac.nz)
	Windspeed	m/s	GMED - Download Data Layers (auckland.ac.nz)
Chemical composition and nutrients	Chlorophyll-a (Chla) mean	mg/m³	GMED - Download Data Layers (auckland.ac.nz)
	Chlorophyll-a (Chla) range	mg/m³	GMED - Download Data Layers (auckland.ac.nz)
	Iron	mmol/m^3	Planet OS - Global Ocean Biogeochemical Analysis and Forecast
	Nitrate	µmol/l	GMED - Download Data Layers (auckland.ac.nz)
	Phosphate	mmol/m^3	Planet OS - Global Ocean Biogeochemical Analysis and Forecast
	Silicate	µmol/l	GMED - Download Data Layers (auckland.ac.nz)

Table A7: Eigenvalue results of the Redundancy Analysis for canonical and residual axes.

Axis	Eigenvalue	%	Cumulative %	XY corr. (R)
Canonical				
1	14.249	31.89	31.89	0.9395
2	6.5992	14.77	46.66	0.9001
3	2.6693	5.974	52.63	0.9473
4	2.0319	4.547	57.18	0.9569
5	0.43987	0.9844	58.16	0.9436
6	1.388E-30	3.106E-30	58.16	1.336E-14
7	2.8238E-31	6.319E-31	58.16	5.03E-15
8	2.3798E-31	5.326E-31	58.16	4.807E-15
9	1.2978E-31	2.904E-31	58.16	1.676E-15
10	1.0813E-31	2.42E-31	58.16	3.827E-15
Residual				
11	3.9065	8.742	66.9	
12	2.5185	5.636	72.54	
13	1.3746	3.076	75.62	
14	1.1716	2.622	78.24	
15	1.0384	2.324	80.56	
16	0.93609	2.095	82.66	
17	0.87329	1.954	84.61	
18	0.81855	1.832	86.44	
19	0.77536	1.735	88.18	
20	0.62602	1.401	89.58	
21	0.58846	1.317	90.9	
22	0.54977	1.23	92.13	
23	0.52549	1.176	93.3	
24	0.47711	1.068	94.37	

25	0.47265	1.058	95.43	
26	0.43105	0.9647	96.39	
27	0.38016	0.8508	97.24	
28	0.36491	0.8166	98.06	
29	0.33952	0.7598	98.82	
30	0.28168	0.6304	99.45	
31	0.16856	0.3772	99.83	
32	0.075133	0.1681	100	
33	0.001554	0.003478	100	

Table A8: Results of partial redundancy analyses for each explanatory variable.

Environmental variable excluded from RDA	Cumulative canonical percentage variance		
	Full model	Partial model	Difference: Full model - Partial model
Salinity	58,16	52,54	5,62
Nitrate	58,16	45,16	13
Water quality	58,16	44,97	13,19
Physical habitat	58,16	49,68	8,48
PIC	58,16	56,1	2,06
SST mean	58,16	43,49	14,67
SST range	58,16	54,14	4,02
Surface current	58,16	51,43	6,73
Tide	58,16	59,4	-1,24
Wave height	58,16	57,6	0,56