UNIVERSIDADE FEDERAL DO ESPÍRITO SANTO
CENTRO DE CIÊNCIAS HUMANAS E NATURAIS
PROGRAMA DE PÓS GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS

SPATIAL AND TEMPORAL PATTERNS OF MEIOFAUNAL DIVERSITY IN COASTAL ECOSYSTEMS OF ESPÍRITO SANTO, BRAZIL

Gabriel Carvalho Coppo

Vitória

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Orientador: Angelo Fraga Bernardino

Tese submetida ao Programa de Pós Graduação em Ciências Biológicas (Biologia Animal) da Universidade Federal do Espírito Santo como requisito para obtenção do grau de Doutor em Ciências Biológicas (Biologia Animal).

Vitória

2023

AGRADECIMENTOS

Agradeço ao meu orientador, Dr. Angelo Fraga Bernardino, por todo o apoio, conhecimento passado, confiança depositada em mim, conversas, conselhos e oportunidades oferecidas para meu crescimento profissional e pessoal.

Aos demais professores integrantes do corpo docente do Programa de Pós-Graduação em Biologia Animal (Ciências Biológicas), por todo o conhecimento passado durante as disciplinas.

À Universidade Federal do Espírito Santo (UFES), pela estrutura disponibilizada e incentivo a realização da pesquisa e desenvolvimento científico.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), pela concessão da bolsa durante o período do Doutorado.

Aos meus pais, André Coppo e Tereza Coppo, pelo respeito as minhas decisões, e pelo apoio desde a graduação, mestrado e agora no doutorado. À Romy Schneider, por todo apoio desde sempre, pelos conselhos e pelas longas conversas sobre ciência, sobre o futuro e a vida. À Eduarda Coutinho, por todo o carinho, apoio e companheirismo durante este período.

A todos os membros da equipe do Grupo de Ecologia Bêntica pelo apoio, críticas, ensinamentos, companheirismo, que fizeram parte deste período de alguma maneira. Em especial à Drª Ana Carolina Mazzuco, Carla Frechiani, Daniela Gaurisas, Fabricio Gabriel, Luiz Eduardo Gomes e Patricia Stelzer, pela amizade e por todos os momentos vividos dentro e fora do laboratório. Sem esquecer também dos alunos de graduação que fazem parte do grupo de pesquisa, por todo o companheirismo e alegria no laboratório, em especial Ana Lara Flores, André Vassoler e Fernanda Alves.

Ao Dr. Fabiano Pais, por dispor de seu tempo mesmo durante o período de pandemia, para me ensinar bioinformática à distância, e por toda a contribuição e ajuda na realização de análises dos dados e redação de capítulos que compõem essa Tese.

Ao Dr. Kenneth M. Halanych, pelos ensinamentos, pelo carinho e recepção excepcionais em seu grupo de pesquisa na University of North Carolina Wilmington (UNCW). Também aos outros membros do grupo de pesquisa, Caitlin Redak, Candace Grimes e Kyle Donnelly, por todo o apoio e ajuda em todas as necessidades, além das conversas, trocas de conhecimento e momentos de diversão. As demais amizades que Wilmington e

a UNCW me trouxeram, em especial à Katy Boot, que juntamente com os outros, me fizeram sentir em casa, mudaram um pouco da minha visão da ciência e plantaram em mim a vontade de retornar.

Ao Dr. Sergio Netto, pelas conversas científicas que contribuíram para o meu desenvolvimento profissional, além da contribuição das análises de biopolímeros nas amostras de sedimento e redação de capítulos que compõem essa Tese.

Aos membros da banca de qualificação, Drª Elisandra Chiquito, Dr. Jean-Christophe Joyeux, e Dr. Sergio Netto, pela disponibilidade, ensinamentos e pelas contribuições para realização desta Tese, além da discussão sobre ciência, animais da meiofauna e análises de DNA.

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Table S2 Abundance of meiofaunal reads and diversity metrics (Phylogenetic Diversity and Shannon Diversity) obtained after metabarcoding sequencing from sediment samples collected at Gramuté beach, in SE Brazil, during all seasons. Data presented as mean ± standard deviation. Significant differences ($p<0.05$) are presented in bold. 128

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RESUMO

O termo meiofauna aplica-se a metazoários bentônicos com tamanhos variando entre 50µm e 500µm. Esses animais são onipresentes em ecossistemas de água doce e marinhos em diferentes profundidades. A meiofauna desempenha um papel ecológico fundamental nos sedimentos marinhos, atuando em diversos processos ecológicos e biogeoquímicos, ligando diferentes níveis tróficos, e são amplamente utilizados como bioindicadores para avaliação de impacto ambiental. Devido ao seu pequeno tamanho e alta diversidade, a identificação da meiofauna requer extenso trabalho e tempo de especialistas em diferentes filos. O uso de abordagens baseadas em DNA tem sido proposto como alternativa ao estudo da meiofauna a partir de amostras ambientais (Metabarcoding). Esta tese busca investigar, utilizando metabarcoding, padrões espaciais e temporais da diversidade da meiofauna em diferentes ecossistemas costeiros no sudeste do Brasil. O Capítulo 1 apresenta uma introdução geral ao tema central desta Tese, destacando a importância deste tipo de estudo e apresentando os objetivos dos capítulos seguintes. No Capítulo 2, foi observado um processo de transição na composição e diversidade da meiofauna no estuário do Rio Doce em diferentes momentos após um desastre com rejeitos de mineração. No Capítulo 3, os dados obtidos sugeriram maior diversidade filogenética e diferenças na assembléia bentônica associada a bancos de rodolitos de alta densidade. O Capítulo 4 demonstra a influência da sazonalidade e de fatores espaciais e ecológicos na diversidade, abundância e composição da meiofauna, além de indicar a existência de um filtro ecológico entre praias arenosas e poças de maré. O Capítulo 5 apresenta as conclusões gerais obtidas com esta Tese, e apresenta possíveis pesquisas futuras que podem se beneficiar dos resultados aqui apresentados.

Palavras-chave: Bentos; Rio Doce; Rodolitos; Poçase maré; Praia

ABSTRACT

The term meiofauna applies to benthic metazoans with sizes ranging between 50µm and 500µm.These animals are ubiquitous in freshwater and marine ecosystems at low or high depths.Meiofauna plays a key ecological role in marine sediments, acting in several ecological and biogeochemical processes, linking marine trophic levels, and are broadly used as bioindicators to assess environmental impacts. Due to its small size and high diversity, meiofauna identification requires extensive work and time of specialists on different phyla. The use of DNA-based approaches has been proposed as an alternative to the study of the meiofauna from environmental samples (Metabarcoding). This thesis seeks to investigate, using metabarcoding, spatial and temporal patterns of meiofauna diversity in different coastal ecosystems in southeastern Brazil. Chapter 1 presents a general introduction to the central theme of this Thesis, highlighting the importance of this type of study and presenting the objectives of subsequent chapters. In Chapter 2, a transition process was observed in the composition and diversity of meiofauna in the Rio Doce estuary at different times after a mining tailings disaster. In Chapter 3 the data obtained suggested greater phylogenetic diversity and differences in the benthic assemblage composition associated with high-density rhodolith beds. Chapter 4 demonstrates the influence of seasonality and spatial and ecological factors on the diversity, abundance, and composition of meiofauna, in additionto indicating the existence of an ecological filter between sandy beaches and tide pools. Chapter 5 presents the general conclusions obtained from this Thesis, and presenting possible future research that may benefit fromthe results presented here.

Keywords: Benthos; Rio Doce; Rhodoliths; Tide pools; Beach

CHAPTER 1

INTRODUCTION

Meiofauna, a group of small benthic invertebrate metazoans (50 – 500 μm), is considered a key component of marine benthic systems at all depths (Higgins and Tiel, 1988; Hakenkamp and Palmer, 2000; Giere, 2009). Only Gastrotricha, Gnathostomulida, Kinorhyncha, Loricifera, and Tardigrada are exclusively meiofauna (Higgins and Tiel, 1988; Giere, 2009), but other benthic invertebrates larger than 1000 μm may be considered meiofaunal organisms if they spend part of their life living as interstitial organisms (temporary meiofauna), where they also play important roles in marine sediments (McIntyre, 1969; Hakenkamp and Palmer, 2000). These organisms participate in the biomineralization of organic matter, decomposing debris, and acting in nutrient cycling (Coull,1999; Kenedy and Jacoby, 1999; Danovaro et al., 2000; Schratzberger and Ingels, 2018).Additionally, they are the most diverse component of the marine biota and the phylogenetically most diverse fauna on Earth (Schratzberger and Ingels, 2018).

Understanding species distribution and dispersal is crucial in a world facing environmental changes (e.g., global warming, pollution) and with an ever-increasing number of endangered species. Meiofaunal organisms have a ubiquitous occurrence, being encountered in all aquatic environments and climatic zones (Giere, 2009). In general, the spatial distribution of meiofaunal organisms is patchy and variable, and mainly related to physical and chemical parameters from sedimentary and hydrographic factors, such as grain size, watercontent, salinity, permeability, and oxygen concentration (Hulings and Gray, 1976; Giere, 2009). At small-scales (few centimeters to meters) it is also influenced by biotic and ecological factors, such as reproductive, potential predation, and other trophic interactions(Blanchard, 1991). Swan and Palmer (2000) suggested that the most important factors for meiofauna small-scale patterns are predation, food quantity and quality, competition, dispersal, localflow dynamics and sediment characteristics, such as grain size (Fricke and Flemming, 1983). The interdependence of environmental variables, like organic matter content and oxygen depletion, is also determinant in small-scales, demonstrating how closely biotic and abiotic factors are linked (Rundle et al., 2000; Giere et al., 2009).

Reviewing the tropical meiofauna, Alongi (1990a) demonstrated a large geographical and biotic variations, showing a great range of habitats for meiofauna including carbonate sands on beaches and shelf regions of carbonate sand, estuarine muds, mangroves, and enclosed lagoons. Meiofaunal density may vary from several hundreds toseveral thousand specimens per 10 cm^2 (McIntyre, 1968; Vanhove, 1993).

Temporal fluctuations contribute to meiofauna variability on abundance and diversity, seasonal changes are less pronounced in tropical areas, but most part of meiofaunal organisms present some seasonality, being more abundant during the warmest periods of the year (Coull and Giere, 1988; Arlt, 1993).

Grain size composition is an important factor that drives meiofaunal composition. Usually, in tropical areas, nematodes are the most abundant and diverse group, but in coarser sediments harpacticoids may prevail. On the other hand, a low- abundance of annelids may be explained by the presence of turbellarians, which are theirmain predator and are abundant in tropical ecosystems (Giere, 2009). Also, the abundanceand biomass of benthic assemblages are distinctly associated with spatial and temporal patterns of salinity variability, sediment content, and organic matter content (Gilberto et al., 2004; Mariano and Barros, 2015). In tropical Brazilian beaches, harpacticoids and nematodes are usually the most abundant taxa, representing around 35 and 30%, respectively (Giere, 2009). Depending on the grain size, meiofauna may occur in differentdepths into the sediment along the intertidal region. For instance, in sandy environments meiofaunal organismscan penetrate into the sediments from a few centimeters to meters (depending on the type of beach system); conversely, in muddy estuarine sediments, organisms are restricted to the first centimeters of the sediment (Venekey and Santos, 2017).

The Eastern Brazilian Marine Ecoregion, located within the Tropical Southwestern Atlantic province, extends along 1200 Km of coastline with latitudinal changes in mean rainfall and temperature (Spalding et al., 2007). The region is characterized by thepresence of lateritic reefs as well as great abundance of macroalgae and rhodolith beds (Mazzuco et al, 2020). In this region, sea surface temperature is 26 °C on average, varyingslightly between seasons, with higher temperatures during autumn and lower in winter. However, in the last two decades this region has experienced significative warming (+1 °C) (Bernardino et al., 2016; Mazzuco et al, 2020). Climate change effects, such as higher temperatures and lower rainfall are predicted to significantly impact benthic assemblages in Eastern ecoregion (Bernardino et al., 2018). This marine ecosystem is influenced by E-NE winds, strong internal tidal currents, and E-SE wave swells, with occurrence of cold fronts periodically influencing on the water column and wave action along the coast (Pereira et al.,2005; Pianca et al., 2010). Stronger swells are observed during summer and winter,and lower during fall (Mazzuco et al., 2020).

Natural stressors have always existed in marine ecosystems, but the frequency of

extreme environmental challenges is increasing and represents a planetary threat (Ingels et al., 2023). Environmental impacts can cause stress or even death of organisms, and an environment can be considered under extreme conditions when one or more physical or chemical factors are near limits of what is known to be tolerable by most life forms (e.g., extremely high or low temperatures, pressure, oxygen or salt concentration, or toxic compounds) (Zeppilli et al., 2018). High temperatures can be destructive for molecules and cells, interrupting body functions, although, several meiofaunal species have been reported to survive in hot springs at temperatures around 40 °C or even higher (Abebe et al., 2001; Zeppilli and Danovaro, 2009). Commonly, the exposureto chemical stressors may impact meiofaunal species in different ways, from reduction ofmovements over inactivity to toxicity, which can cause the death of organisms (Kaminsky, 2003). Nevertheless, nematodes are known to be resistant to Arsenic and other heavy metals at high concentrations. Arsenic, when in high concentrations in the environment, is known to be lethal for most metazoans, but *Auanema* sp. (freshwater nematode species) can survivein concentrations up to 500-times the human lethal dose, due to expression of the gene dbt that confer arsenic resistance (Shih et al., 2019). Many nematodes are not sensitive to moderate or high concentrations of heavy metals in marine or freshwater systems. For example, the genera *Monhystera* and *Theristus* that are dominant in estuaries contaminated by heavy metals, and are considered indicators for polluted sediments (Gyedu-Abadio et al., 1999).

Accurately identification of meiofaunal organisms by traditional morphological protocols is a difficult task as its labor-intensive, due to their microscopical size and enormous diversity of taxa, and requirestaxonomic specialists in different taxonomic groups, (Wang et al., 2023). Consequently, meiofaunal organisms are usuallyneglected in many biodiversity assessments, and researches tend to focus on few specifictaxonomic groups (e.g., benthic macrofauna and nematodes) (Urban-Malinga et al., 2005; Xuan et al., 2007; Neto et al., 2021), which limits our ability to better understand meiofaunal diversity and distribution patternsat local and global scales. In recent years, there has been a considerable advance in DNA-based methods to assess the biodiversity patterns of small-sized organisms, including meiofauna (Tang et al., 2012; Faria et al., 2018; Fais et al., 2020; Wang et al., 2023). Recent studies have successfully assessed, by environmental DNA metabarcoding (eDNA), meiofaunal diversity in different marine ecosystems, including estuaries (Bernardinoet al., 2019; Clarke et al., 2020), continental shelf (Bakker et al., 2019; MacNeil et al., 2022), and coastal sediments (Aylagas et al., 2018; Jeunen et

al., 2018). eDNA metabarcoding enable the bulk identification of multiple species using an environmental sample by simultaneously amplifying individual DNA barcodes (e.g., 18S and COI amplicons sequencing), also allowingthe identification of individuals that are too small or degraded (Steyaert et al., 2020). Furthermore,it does not require the taxonomic expertise that morphological methods do and provide great power to explore community responses to environmental stressors or changes in an accurate and effective manner (Lalliaset al., 2014; Leasi et al., 2018). Metabarcoding approaches can complement and expand traditional methodologies to assess biodiversity, and transform our ability to identify andassess the life on Earth, moving forward our current knowledge on marine biodiversity (Fonseca et al., 2010). Recent studies have demonstrated that metabarcoding is a promising alternative for acquiring biological/ecological data and broadly monitoring marine ecosystems (Wang et al., 2023). Metabarcoding approaches and molecular data can be used to assess diversity metrics, such as Faith`s Phylogenetic Diversity is calculated as the sum of branch lengths between root and tips, in a phylogenetic tree, for a community (Faith, 1992). This metric is a biodiversity measure based on evolutionary relationships between species, integrating information about phylogenetic positions as a result of evolutionary processes (e.g., speciation, radiation) (Erwin, 1991), and has been suggested to be relevant to environmental conservation, once it is related to ecological processes (e.g., extinction, biotic invasion), ecosystem functioning, and ecosystem services (Purvis et al., 2000; Winter et al., 2009; Faith et al., 2010; Srivastava et al., 2012).

This Thesis aimed to investigate, using eDNA metabarcoding, the spatial and seasonal patterns of meiofaunal diversity of coastal environments in SE Brazil (Brazilian Eastern Marine Ecoregion), evaluating how meiofaunal assemblages and diversity are influenced by anthropogenic impacts, habitat structural complexity, seasonality, and environmental parameters (meteo-oceanographic and sedimentary) in a changing world. To achieve this, the following chapters are presented as three original research articles.In chapter 2, we investigate and compare meiofaunal assemblages on the Rio Doce estuary 1.7and 2.8 years (2017 and 2018, respectively) after the initial contamination by mine tailings that occurred after the Fundão Dam rupture in November 2015 (Coppo et al., 2023). Chapter 3 evaluates the role of rhodoith beds as a diversity hotspot for benthic invertebrates in a Marine Protected Area, testing the influence of habitat structural complexity (rhodolith beds density) on meiofaunal assemblage composition and phylogenetic diversity (Coppo et al., in review). Chapter 4 tests whether the phylogenetic

diversity of meiofaunal assemblages changes seasonally on a sandy beach, and whetherthis diversity and assemblage composition is associated to seascape coverage in the studyarea. Additionally, chapter 4 investigates the existence of an ecological filter on assemblage composition and phylogenetic diversity between sand beach and insidetidepools.

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CHAPTER 2

TRANSITION OF AN ESTUARINE BENTHIC MEIOFAUNA ASSEMBLAGE 1.7 AND 2.8 YEARS AFTER A MINING DISASTER

Artigo publicado na edição especial *Advancing the Environmental DNA and RNA Toolkit for Aquatic Ecosystem Monitoring and Management* do periódico *PeerJ*.

Coppo G, Pais FS, Ferreira TO, Halanych KM, Donnelly K, Mazzuco AC, Bernardino AF. 2023.Transition of an estuarine benthic meiofauna assemblage 1.7 and 2.8 years after a mining disaster. PeerJ 11:e14992. (http://doi.org/10.7717/peerj.14992)

ABSTRACT

Estuaries are transitional coastal ecosystems and often threatened by multiple sources of human pollution. In 2015, mining tailings from an upstream dam failure caused massive metal contamination that impacted benthic assemblages on the Brazilian Rio Doce estuary. In this study, we investigate and compare meiofaunal assemblages using eDNA metabarcoding 1.7 years (2017) and 2.8 years (2018) after the initial contamination by mine tailings in order to evaluate the continued impact of sediment mine tailing contaminants on the structure of benthic assemblages after the disaster. The community was dominated by Arthropoda and Nematoda 1.7 yr after the impacts (42 and 29% of meiofaunal sequence reads, respectively) but after 2.8 years Arthropoda (64.8% of meiofaunal sequence reads) and Rotifera (11.8%) were the most common taxa. This continued impact on meiofaunal assemblage revealed a lower phylogenetic diversity (7.8-fold) in 2018, despite overall decrease in metal concentration (Al, Ba, Cr, As, Fe, Zn, Mn, Pb, Cd, Co) in sediments. Our data suggests that differences in benthic assemblages and loss of diversity may be influenced by contaminants in sediments of this estuary, and indicate that broad eDNA assessments are greatly useful to understand the full range of biodiversity changes in dynamic estuarine ecosystems.

Keywords: Environmental DNA, Pollution, Environmental Impact, Benthos, Rio Doce, Estuary, Meiofauna

INTRODUCTION

Estuaries are considered dynamic and transitional coastal ecosystems with a high variability in environmental conditions. Most of them are highly productive habitats and acting as nurseries for a great diversity of organisms. For this reason, estuaries are considered one of the most valuable ecosystems in the world, providing important ecological services (Costanza et al., 1997; McLeod et al., 2011; Pendleton et al., 2012; Janakiraman et al., 2017; Lana & Bernardino, 2018). Estuarine environments are naturally stressed and variable habitats due to their plasticity of physic-chemical processes that vary in short spatio-temporal scales (e.g., changes in salinity and tide) (Mulik, Sukumaran & Srinivas, 2020). Nonetheless, during the last century, the contamination of estuarine ecosystems has become a worldwide problem (Irabien et al., 2008)due to acute and chronic impacts generated by contamination and pollution, which in turns has changedthe composition of animal assemblages closely associated with the sedimentary matrix(Alves et al., 2013; Alves et al., 2015; Varzim et al., 2019).

Meiobenthos, or meiofauna, are sediment associated organisms between 50 and 500 µm (Higgins & Thiel, 1988; Meyer, 1990). Invertebrates larger than 1,000 µm may be included in the meiofauna if they spend part of their life as interstitial organisms (McIntyre, 1969; Hakenkamp & Palmer, 2000). Meiofauna undertake important ecological roles in estuarine ecosystems, through the biomineralization of organic matter and enhancing nutrient regeneration, linking trophic levels of the food web (Coull, 1999; Kennedy & Jacoby, 1999). Their high sensitivity to anthropogenic inputs make them excellent proxies for estuarine pollution (Coull, 1999), and bioindicators for the management of coastal environments (Ward & Jacoby, 1992). However, environmental changes in estuaries, caused by human activities, can strongly impact meiofauna community structure and functioning (Kennedy & Jacoby, 1999; Elliott & Quintino, 2007), often leading to functional and long-term ecological changes (Gomes et al., 2017). Salinity, organic matter content and sediment grain size, for example, are strongly related to the spatial distribution of meiofaunal organisms (Austen & Warwick, 1989; Coull, 1999; Rutledge & Fleeger, 1993; Walters & Bell, 1994; Gomes & Bernardino, 2020).

Due to the difficulty and labor requirements of accurately identifying meiofaunal organisms by traditional morphological protocols, these organisms are usually neglected in many biodiversity assessments. However, in recent years there havebeen considerable advances in applying DNA-based methods using metabarcoding

techniques to disentangle biodiversity patterns of microorganisms (Baird & Hajibabaei, 2012; Taberlet et al., 2012), including meiofauna (Tang, Li & Yan, 2012; Faria et al., 2018; Fais et al., 2020). Recent studies have successfully assessed, by environmental DNA (eDNA) metabarcoding, metazoan biodiversity in different marine ecosystems, such as estuaries (Bernardino et al., 2019; Clark et al., 2020), continental shelf (Bakker et al., 2019; MacNeil et al., 2022), and coastal sediments (Aylagas et al., 2018; Jeunen et al., 2018). This approach has proven to be useful in assessing the compositional data from meiofauna samples, thus becoming a powerful tool to overcome the limitation for meiofaunal morphological identification (Valentini, Pompanom & Taberlet, 2009; Medinger et al., 2010; Gielings et al., 2021).

The use of eDNA to measure and monitor marine and estuarine biodiversity is gaining popularity (Creer et al., 2010; Bik et al., 2012; Brannock & Halanych, 2015; Brannock et al., 2016; Mäechler et al., 2019; Ruppert, Kline & Rahman, 2019; Berry et al., 2020; Clark et al., 2020; Naro-Maciel et al., 2022). Recent metabarcoding studies using eDNA extracted from sediment (Avó et al., 2017; Lanzén et al., 2017; Faria et al., 2018; Nascimento et al., 2018; Bernardino et al., 2019; Fais et al., 2020; Castro et al., 2021; Pawlowski et al., 2022) demonstrated its usefulness to assess marine biodiversity. For the most part of biodiversity, eDNA metabarcoding can be more efficient than traditional morphological-based taxonomy, enable the bulk identification of multiple species in an environmental sample by simultaneously amplifying individual DNA barcodes, which can allow the identification of specimens that are small, cryptic or too degraded for morphological identification (Steyaert et al., 2020). In addition, it can be an effective technique for determining the quality and recovery of ecosystems following anthropogenic disasters, such as metal contamination after a rupture on a mining dam (Chariton et al., 2015; Cordier et al., 2017; Di Battista et al., 2020; Martínez et al., 2020; He et al., 2021; Leasi, Sevigny & Hassett, 2021).

In November 2015 a large mine tailing dam ruptured in SE Brazil,releasing nearly 50 million m^3 of iron ore tailings into the Rio Doce watershed. The minetailings load was carried over 600 Km downstream reaching the Rio Doce estuary and theAtlantic Ocean, where it severely impacted estuarine and coastal ecosystems nearby (Carmo et al., 2017; Queiroz et al., 2018; Bernardino et al., 2019; Magris et al., 2019; Gabriel et al., 2020a). The tailings, mainly composed of iron oxyhydroxides, were associated to different potentially toxic elements including Mn, Cr, Pb, Hg, As, La, and

Sc, which were 24 times higher for Mn (and more than 200 times higher for other metals, such as Zn and Cu) than before the incident (Queiroz et al., 2018; Queiroz et al., 2021). The first impacts of the tailings deposition in the estuary included loss of several macrofaunal benthic organisms (Gomes et al., 2017), contamination of aquatic organisms (Gabriel et al., 2020a; Queiroz et al., 2021) and changes in sediment bioturbation and biogeochemistry (Barcellos et al., 2021; Queiroz et al., 2021; Barcellos et al., 2022). The mine tailings impacted the benthic macrofauna diversity, composition and trophic groups (e.g., loss of surface-dwelling taxa), and these impacts were still observed on macrofauna even after almost four years (Gomes et al., 2017; Gabriel et al., 2020b).

eDNA metabarcoding identified effects of this disaster in the meiofaunal assemblages in the Rio Doce estuary in August 2017, 1.7 years after the tailings spill (Bernardino et al., 2019). At the time, high levels of Fe contamination were detected in the estuary sediment, suggesting that meiofaunal assemblages were partially influenced by environmental filtering from toxicity of highly contaminated sediments, since this metal concentrations acted as significant predictors of changes in dominant meiofaunal taxa (e.g., nematodes, copepods, ostracods and flatworms) (Bernardino et al., 2019). The Fe concentrations significantly increased by two times two days after the impact (Gomes et al., 2017), and in August 2017 continued to be 2–20 times higher compared to preserved (Piraquê-Acu-Mirim estuary) ¸ or polluted estuaries, such as the Vitória Bay, located in a metropolitan and industrial area approximately 100 km to the south of the Rio Doce estuary (Hadlichet al., 2018). As the time passes and the contamination impacts in Rio Doce are reduced,it is expected that these biological communities will exhibit some degree of recovery, which should be detected by long-term monitoring and biodiversity assessments.

Given the highly dynamic nature of the estuarine ecosystems, and the prediction that levels of contaminants in sediments will decrease with time (see Gabriel et al., 2021), we re-evaluated the Rio Doce meiofaunal assemblages 2.8 years (2018) after the initial impact. Our aim was to evaluate the continued impacts on meiofaunal assemblages in response to sediment contamination by metals, through biodiversity assessment and multivariate association. We hypothesized that meiofaunal composition and diversity would be affected by metal concentrations in the impacted estuarine sediments, leading to ecological recovery, and that higher phylogenetic diversity would occur with a reduction on the contaminant levels.

MATERIAL AND METHODS

Sampling sites and sampling procedures

The Rio Doce estuary (19◦ 380 to 19◦ 450 S, 39◦ 450 to 39◦ 550W; Fig. 1) is located in SE Brazil. This region has a tropical climate and two well-defined seasons, dry winters (April to September) and wet summers (October to March), with a monthly average rainfallof 145 mm (Alvares et al., 2013; Bernardino et al., 2015; Bissoli & Bernardino, 2018). The estuary is characterized by low salinity levels (0.05–8 ppt) and temperatures between
23.1 and 30.5 ◦C (Gomes et al., 2017; Bernardino et al., 2018; Lana & Bernardino, 2018; Gabriel et al., 2021).

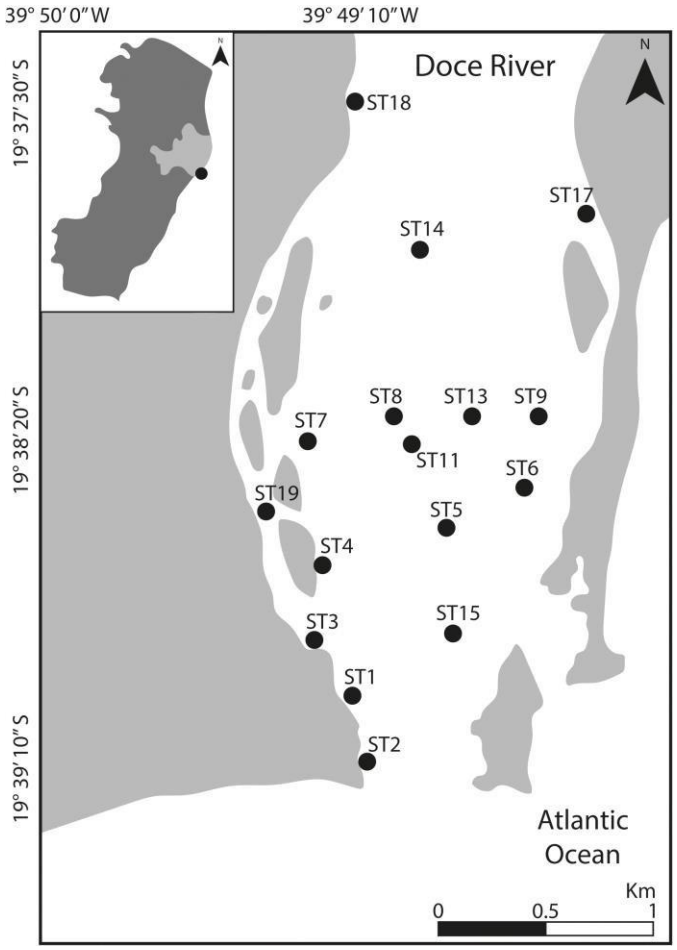

Figure 1 Location of the study area. Map indicating the sampling stations at Rio Doce estuary, on the SE Brazilian coast, in August 2018.

Sampling was carried out in August 2018 at 16 sampling sites distributed throughout the lower portion of the Rio Doce estuary, covering about five km from its mouth (Fig. 1). At each site, we collected two sediment samples (top five cm) using

sterile, DNA-free corers, which were immediately frozen in liquid nitrogen. Additional samples were obtained for determination of grain size, total organic matter and trace metal quantification. All sediment samples were stored in a freezer at −20 ◦C upon arrival at the laboratory until further analysis. Additionally, water temperature and salinity were measured at each site. Field sampling was approved by SISBIO-IBAMA (sampling license N 24700-1), and data were collected as previously described in Bernardino et al. (2019).

Grain size was determined according to Suguio (1973) by sieving and pipetting, and total organic matter (TOM) quantified gravimetrically by the weight loss method after combustion (500 ◦C for 3 h). Metal concentration in sediment samples was evaluated from two independent replicates. For the total trace metal contents, approximately1 g of the freeze-dried samples was digested by a tri-acid mixture (nine mL of HNO3 + three mL of HF 1 mol/L + five mL of H3BO3 5%; USEPA, 1996) in a microwave oven digestion system. Vessels containing the samples were shaken and heated at 110 ◦C for 4 h. Posteriorly, samples were diluted to 40 mL in deionized water. We determined the concentrations of trace metals (Al, Ba, Cr, As, Fe, Zn, Mn, Pb, Cd, Co) using aliquots of 0.1mL on an ICP-OES spectrometer (iCAP 6200; Thermo Scientific, Waltham, MA, USA; see Queiroz et al., 2018) in triplicate. Standard solutions were prepared from dilution of certified standards and certified reference materials (NIST SRM 2709a), and used for comparison to measured and certified values. Sedimentary and metals concentrations analysis were realized as previously described in Gabriel et al. (2020a).

DNA extraction and sequencing

Prior to DNA extraction, we elutriated the sediment samples using 45 mm sieves, following the protocol established by Brannock & Halanych (2015), using 950 mL of filtered seawater in a 1L flask, inverting the flask and decanted the liquid over the sieve after the flask was let to sit. After repeating this procedure 10 times, the sediment retained on the sieve was transferred to a sterile 50 mL falcon tube, and spun down using an EppendorfCentrifuge 5430 at room temperature for 3 min at 1,342g, and was aliquoted to 20 mL.The sample was mixed using a sterile pipette, and two separate one mL subsamples were aliquoted, transferred to separate sterile 1.5 mL tubes, and then stored at -20°C for DNA extraction. All glassware and materials used during the elutriation process were cleaned,sterilized, and autoclaved between samples. After elutriation, we extracted DNA from thesediment samples using the PowerSoil DNA Isolation® kit (Qiagen) following

the manufacturer's instructions. We verified DNA integrity on a 1% agarose gel and purity (260/230 and 260/280 ratios) using a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). We determined DNA concentration using a Qubit® 4 Fluorometer (Life Technologies-Invitrogen, Carlsbad, CA, USA), and samples were sent to ©NGS Genomic Solutions (Piracicaba, SP, Brazil) for metabarcoding sequencing and construction of the amplicon libraries by HiSeq Illumina platform (2x250 bp paired-end). TheV9 hypervariable region of the 18S SSU rRNA gene was amplified using primers Illumina_Euk_1391f forward primer (GTACACACCGCCCGTC) and Illumina_EukBr reverse primer (TGATCCTTCTGCAGGTTCACCTAC) (Medlin et al., 1988; Lane,1991; Amaral-Zettler et al., 2008; Stoeck et al., 2010).

Bioinformatic pipelines

We used QIIME2 2021.2 to process and analyze all demultiplexed raw paired-end reads (Bolyen et al., 2018). Fastq files were first imported as QIIME2 artifacts, and reads were denoised via DADA2(Callahan et al., 2016) with the DADA2 denoise-paired plugin, setting the p-trunc parameter to 220 to remove low-quality bases, and the p-trim set to 10 to remove primersequences.

The taxonomic composition of the amplicon sequence variants (ASV), generated after running the DADA2 plugin, were assigned using the machine learning Python library scikit-learn (Pedregosa et al., 2011). The feature-classifier plugin was used to generate the classification results by a pre-trained Naïve Bayes classifier trained on Silva 132 database clustered at 99% similarity (Quast et al., 2013), and the taxonomic profiles of each sample were visualized using the taxa-barplot plugin. Due to the difference on the sequencing depth, we normalized the datasets from both years to allow analysis and comparison with homogenous sampling depth. We used the 2018 dataset minimum sampling depth (2,282 reads) and resampled each station to the same depth. These filtered/subsampled datasets were used to calculate all diversity metrics.

We reanalyzed all sequences from the 2017 studyby Bernardino et al. (2019) following this pipeline to guarantee that both datasets (2017 and 2018 assessments) were treated and analyzed using the same techniques and procedures, and to guarantee we were doing a more accurate comparison. Phylogenetic trees were built for each dataset using QIIME2 with the align- to-tree-mafft-fasttree pipeline from the q2-phylogeny plugin. After that, we calculated Faith's phylogenetic diversity (PD) using the diversity core-metrics- phylogenetic pipeline, based on these phylogenetic trees. The PD is obtained summing the branch lengths on a phylogenetic tree, where longer branches correspond to

longer evolutionary times and more distinct taxonomic groups (Faith, 1992). Additionally, we plotted rarefaction curves for both datasets. Raw sequence data was deposited on NCBI (SRA: SRR21716030).

Statistical analysis

Only meiofaunal sequence reads were used for ecological and statistical analysis, and here we considered meiofaunal metazoans the five phyla that are exclusively meiofauna (Gnathostomulida, Kinorhyncha, Loricifera, Gastrotricha, and Tardigrada) and other metazoans that can be representative of meiofauna during any stage of life and play important role in the sediment (temporary meiofauna) (Higgins & Thiel, 1988; Giere, 2009). Normality of all environmental data were tested by ShapiroWilk test, and when necessary, data were log-transformed (log10 or log10(x + 1)). Differences in environmental variables, phylogenetic diversity, and the relative abundance of taxa between 2017 and 2018 assessments were assessed by Student's t -test (Student, 1908; Mann & Whitney, 1947). The differences on abundance between phyla were analyzed by a One-Way Analysis of Variance (ANOVA), followed by the Tukey post-hoc test for multiple comparisons (Tukey, 1949; Underwood, 1997). A Similarity Percentage Routine (SIMPER) was applied to analyze the contribution of each taxonomic group to the assemblage composition dissimilarity between the two datasets (Clarke, 1993). Linear regressions were performed to evaluate the relation between metals concentrations and phylogenetic diversity and, phyla relative abundances. A non-metrical multidimensional scaling (nMDS; Oksanen et al., 2022) ordination was performed with the meiofaunal assemblage composition in August 2017 and August 2018. A canonical analysis of principal coordinates (CAP; Anderson & Willis, 2003) was performed with theset of environmental variables that best explain the meiofaunal assemblage. Significant differences were defined when $p<0:05$. All graphical and analytical processes were performed in R environment (R Core Team, 2022).

RESULTS

Environmental conditions

In the 2018 assessment, the salinity in the estuary at the time of sampling was 0.14 ± 0.04, and the temperature ranged from 23.7°C to 26.3°C. Sediment grain size of sampled stations indicated a predominance of sand particles (minimum = 48.8% and maximum = 94.1%), and the total organic matter (TOM) varied between 1.5 and 11.8% (Table 1; Table S1). We found a significant decrease in concentration of all measured sediment trace metals compared to the 2017 assessment (p<0:05; Table 1; Table S1), except for arsenic which increased (p=0:536; Table 1). We measured an average sediment Fe concentration of 16,566 mg/kg. Associated metals, including As, Cr, and Cdstill showed concentrations above the limits allowed by the current legislation (5.9 mg/kg, 37.3 mg/kg, and 0.6 mg/kg, respectively).

Table 1 Environmental data from sedimentary samples. Sediment grain size, total organic matter (TOM), and metal concentrations (mg/Kg), as median, minimum and maximum, obtained from sampled station in Rio Doce estuary in August, 2017 and August, 2018. Significant differences ($p < 0.05$) are presented in bold. *Data from August, 2017 were obtained from Bernardino et al. (2019).

Variables	Year				
	2017*		2018		*p*
	Median	Min - Max	Median	Min – Max	
%Sand	87.8	11.8 – 96.2	85.5	48.8 – 94.1	0.532
TOM	3.20	1.50 – 16.8	4.00	1.50 – 11.8	0.646
Al	32,495	10,066 – 65,386	19,467	10,754 – 27,590	**<0.001**
As	2.84	<LQ – 53.1	4.29	0.15 – 12.6	0.536
Ba	238.7	33.3 – 688.4	68.3	26.1 – 177.3	**<0.001**
Cd	3.25	0.57 – 7.53	1.76	0.72 – 2.67	**<0.001**
Co	9.41	3.81 – 20.9	7.18	4.78 – 9.69	**0.004**
Cr	47.1	17.7 – 79.6	25.1	10.25 – 45.3	**<0.001**
Cu	8.83	2.31 – 16.1	4.05	0.64 – 6.65	**<0.001**
Fe	35,538.3	13,204.4 – 57,923.3	15,990.5	8,981.7 – 26,862.1	**<0.001**
Mn	551.8	148.4 – 1094.9	345.3	163.5 – 539.2	**<0.001**
Ni	14.5	7.17 – 28.6	10.1	6.27 – 15.0	**<0.001**
Pb	101.9	4.92 – 182.2	6.52	3.68 – 10.9	**<0.001**
Zn	35.4	15.3 – 85.9	27.4	14.6 – 46.1	**0.009**

Assemblage structure and phylogenetic diversity

We reanalyzed the data from the 2017 assessment and found a significantly higher number of meiofaunal sequence reads when compared to the 2018 assessment (2017 = 3,090,870 sequence reads; 2018 = 120,627 meiofaunal sequence reads; t = 11.147; p < 0.001; Table S3). In the 2017 dataset we identified 12 phyla, which is similar to the 10 phyla identified in the 2018 assessment, with the addition of Micrognathozoa, and Tardigrada. The most frequent phyla in the 2017 assemblages were Arthropoda (41.8%) and Nematoda (29.2%) (Fig.2A).

We detected a total of 162,330 sequences from the eDNA metabarcoding of Rio Doce estuarine sediments in 2018 (Table S2). After filtering the dataset to remove sequences that were not meiofaunal animals (e.g., bacteria, fungi, algae, protists), we obtained 120,627 sequence reads from ten phyla, most of them identified as Arthropoda (64.8% of sequence reads; Table 2) and Rotifera (11.8%; Table 2). The frequencies were significantly different between phyla (df = 9; F = 12.715; p < 0.001; Fig.2B; Table S3). The rarefaction curves suggest that the number of meiofaunal taxonomic groups were higher in 2017 than 2018 (Fig.3).

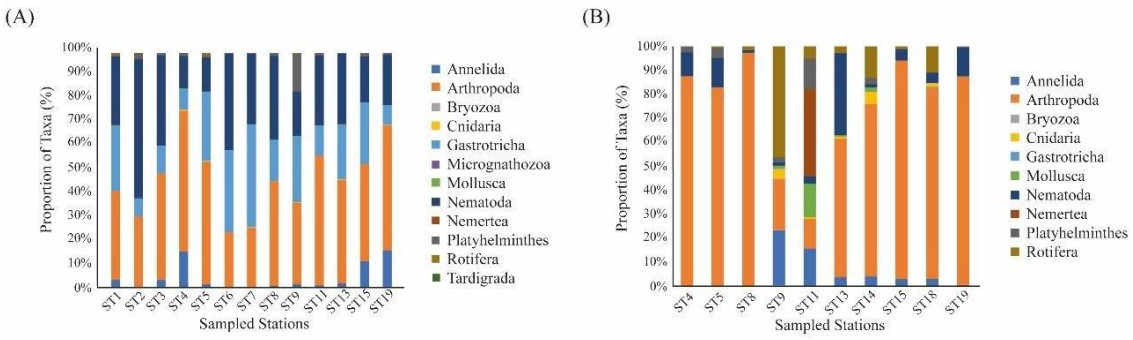

Figure 2 Frequency of identified taxa. Barplots showing **(A)** The proportion of identified Phylum at the Rio Doce estuary in the 2017, and **(B)** 2018 assessments, respectively.

Table 2 Frequency of meiofaunal sequences identified in the 2017 and 2018 assessments. Meiofauna assemblage composition and relative frequency of sequences of each amplicon sequence variants (ASVs) identified at Rio Doce estuary in 2017 and 2018 assessments.

Phylum	Class	Order	2017 Assessment	2018 Assessment
Annelida			-	5.79%
	Clitellata	Haplotaxida	0.21%	3.09%
		Rhynchobdellida	0.12%	-
	Polychaeta	Echiuroinea	0.00%	-
		Eunicida	1.97%	-
		Spionida	2.73%	0.13%
Arthropoda			0.20%	19.52%
	Arachnida	Acari	0.34%	0.09%
	Branchiopoda		-	0.25%
		Diplostraca	-	<0.01%
	Malacostraca	Eucarida	<0.01%	0.02%
	Maxillopoda		0.02%	7.07%
		Calanoida	0.01%	-
		Hexanauplia (Copepoda)	0.06%	-
		Cyclopoida	-	0.57%
		Harpacticoida	0.10%	0.13%
	Ostracoda	Halocyprida	-	0.02%
		Podocopida	33.68%	25.39%
Bryozoa	Gymnolaemata		0.03%	-
	Phylactolaemata	Plumatellida	-	0.08%
Cnidaria	Anthozoa	Actiniaria	<0.01%	-
		Zoantharia	0.01%	-
	Hydrozoa		0.01%	0.01%

		Anthoathecata	0.06%	0.02%
		Limnomedusae	0.01%	2.45%
	Myxozoa	Bivalvulida	0.01%	0.08%
Gastrotricha		Chaetonotida	23.11%	0.12%
Micrognathozoa			<0.01%	-
Mollusca			-	<0.01%
	Bivalvia		-	0.08%
		Myoida	0.04%	-
		Nuculoida	<0.01%	-
		Veneroida	0.01%	2.82%
	Gastropoda	Caenogastropoda	0.06%	-
		Heterobranchia	<0.01%	-
Nematoda	Chromadorea		0.08%	0.09%
		Aerolaimida	0.03%	-
		Chromadorida	0.02%	-
		Desmodorida	6.43%	0.23%
		Monhysterida	16.73%	4.52%
		Rhabditida	<0.01%	-
		Tylenchida	<0.01%	0.02%
	Enoplea	Dorylaimia	0.22%	1.39%
		Enoplida	6.72%	0.16%
		Triplonchida	0.38%	0.56%
Nemertea	Anopla	Heteronemertea	<0.01%	5.84%
	Enopla	Monostilifera	-	0.09%
Platyhelminthes			0.02%	0.62%
	Catenulida		0.01%	0.31%
	Monogenea	Monopisthocotylea	0.07%	0.10%
	Rhabditophora	Macrostomida	0.01%	1.97%
		Proseriata	0.91%	-
		Rhabdocoela	5.13%	0.47%
		Seriata	0.13%	0.03%
	Trematoda		-	0.18%
		Echinostomida	-	0.01%
Rotifera	Bdelloidea		<0.01%	14.90%
		Adinetida	-	0.05%
		Philodinida	-	0.09%
	Monogononta		<0.01%	0.60%
		Flosculariacea	0.01%	-
		Ploimida	0.23%	0.04%
Tardigrada	Eutardigrada	Parachela	0.03%	-

Further, we observed a significant decrease in phylogenetic diversity (PD) from 2017 to 2018. Meiofaunal assemblages in 2017 had a mean PD of 166.6 ± 35.1, while in 2018 meiofaunal PD was 21.3 ± 7.2; a significant decrease in PD of 7.8times in 2018 ($t = 23.320$, $df = 44$, $p < 0.001$). In addition, we observed the same pattern for Shannon diversity, with significant higher diversity in 2017 (2017 dataset = 5.46±0.48, and 2018 dataset = 4.75±0.79; $df = 21$; $t = 2.639$; $p = 0.015$).

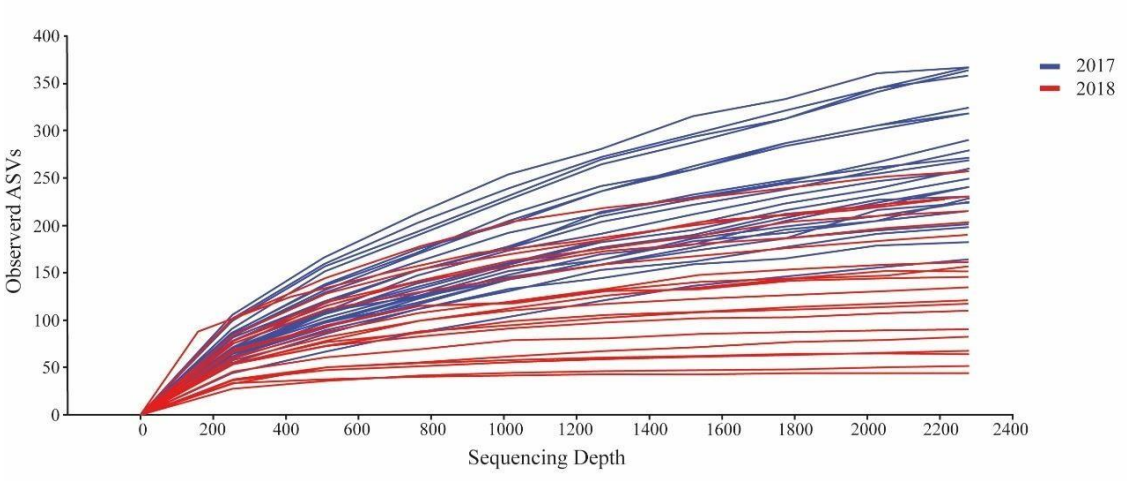

Figure 3 Rarefaction Curves. Rarefaction curves from the datasets from 2017 (blue) and 2018 (red).

Multivariate analysis revealed significant differences on the composition of meiofauna assemblages in the Rio Doce estuary between years (Fig.4). The phyla that most contributed to this difference are Nematoda (24%), Gastrotricha (23.3%), and Arthropoda (18.9%), which together contributed to 49.25% of the dissimilarity between the 2017and 2018 assemblages (Table 3).

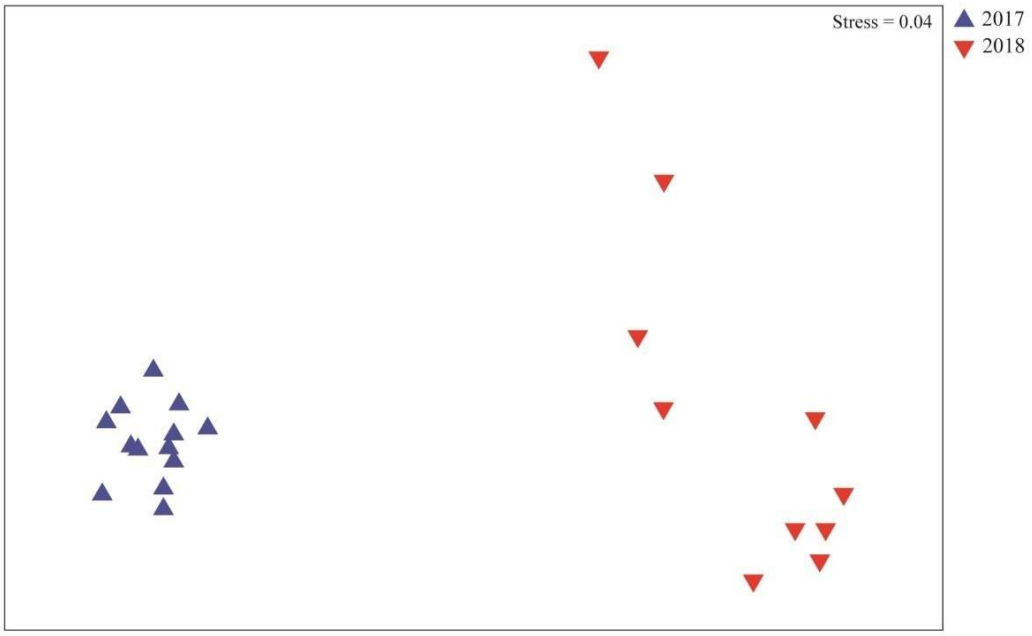

Figure 4 Meiofaunal community structure. Non-metric Multidimensional Scaling (nMDS) ordination based onmeiofaunal assemblage composition in August 2017 (blue triangles) and August 2018 (red triangles).

Table 3 SIMPER Results. Results from Similarity Percentage analysis (SIMPER) indicating the contribution of each Phylum to the dissimilarity between 2017 and 2018 assessments in the Rio Doce estuary. Av. Dissim. = Average Dissimilarity; Contrib. = Contribution.

Phyla	Av. Dissim.	Contrib. (%)	Cumulative (%)	2017 mean	2018 mean
Arthropoda	36.72	40	39.99	9.94e04	7.81e03
Nematoda	27.53	30	69.98	6.95e04	631

Gastrotricha	20.45	22.3	92.26	5.25e04	10.7
Annelida	4.09	4.46	96.72	1.14e04	816
Platyhelminthes	1.80	1.96	98.67	3.78e03	335
Rotifera	0.62	0.68	99.35	561	1.42e03
Nemertea	0.22	0.24	99.60	0.385	537
Mollusca	0.18	0.19	99.79	249	263
Cnidaria	0.14	0.15	99.94	233	231
Bryozoa	0.03	0.03	99.97	62	7.6
Tardigrada	0.02	0.03	100.00	56.8	0
Micrognathozoa	0.00	0.00	100.00	2	0

Association with metals and sediments

The results of assemblages' composition in 2018 displayed a negative relation betweenthe Al concentration and the relative abundance of Mollusca ASVs ($F = 4.964$; $R^2 = 0.209$; $p = 0.043$) and Platyhelminthes ASVs ($F = 4.408$; $R^2 = 0.185$; $p = 0.050$). Furthermore, we observed significant negative relation between the Zn concentration ($F = 14.31$; $R^2 = 0.412$; $p = 0.001$), Ni concentration ($F = 9.877$; $R^2 = 0.318$; $p = 0.006$), Pb concentration($F = 7.302$; $R^2 = 0.249$; $p = 0.015$), Co concentration ($F = 13.11$; $R^2 = 0.389$; $p = 0.002$) and phylogenetic diversity. Even other negative relationships were observed between phyla ASVs and metals concentrations, or between Faith's Phylogenetic Diversity and metals concentrations, they were not significative. The CAP analysis demonstrated that TOM, %Sand, Zn, Cu and Cd were the best set of variables to explain the distribution of meiofaunal assemblage in 2018, and this model significatively explained 66.66% of the distribution of the identified meiofaunal metazoans (Fig.5; $F = 2.378$; $p = 0.044$).

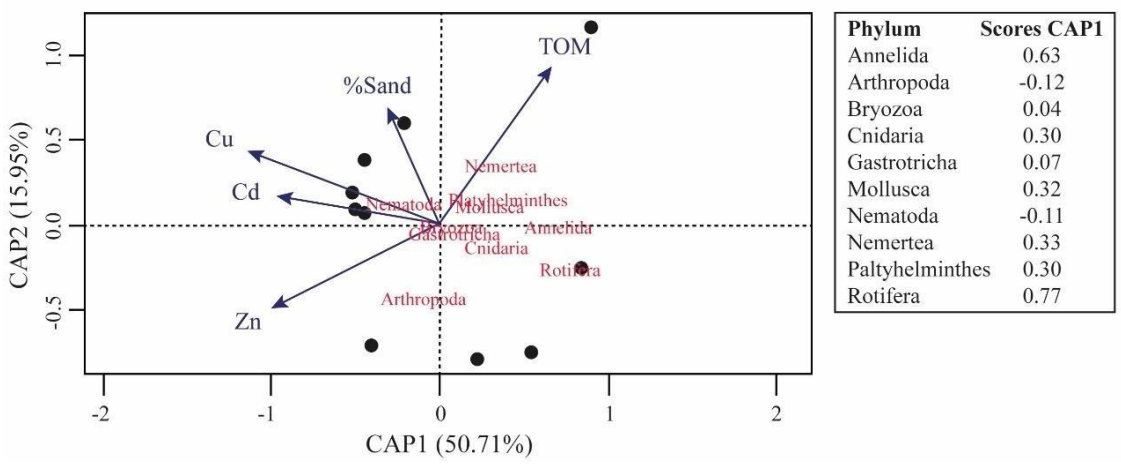

Figure 5 Canonical Analysis of Principal Coordinates Canonical Analysis of Principal Coordinates (CAP) ordination of samples according to multivariate distribution of the meiofaunal metazoans identified in the Rio Doce estuary in 2018.

Differences in the composition of assemblages, and in the phylogenetic diversity between 2017 and 2018 can also be observed on the respective phylogenetic trees. We can observe a phylogenetic tree more complex, diverse, and having longer branches in

2017 assessment(Fig.6A). In the phylogenetic tree from 2017 the branches are longer and more divided indifferent nodes, representing more diversity, especially in Nematoda, Gastrotricha and Platyhelminthes. In 2018 the meiofaunal assemblage changed, since the branches are shorter and less divided in different nodes. Is notable how Arthropoda and Rotifera became more representative phyla for the assemblage composition (Fig.6B).

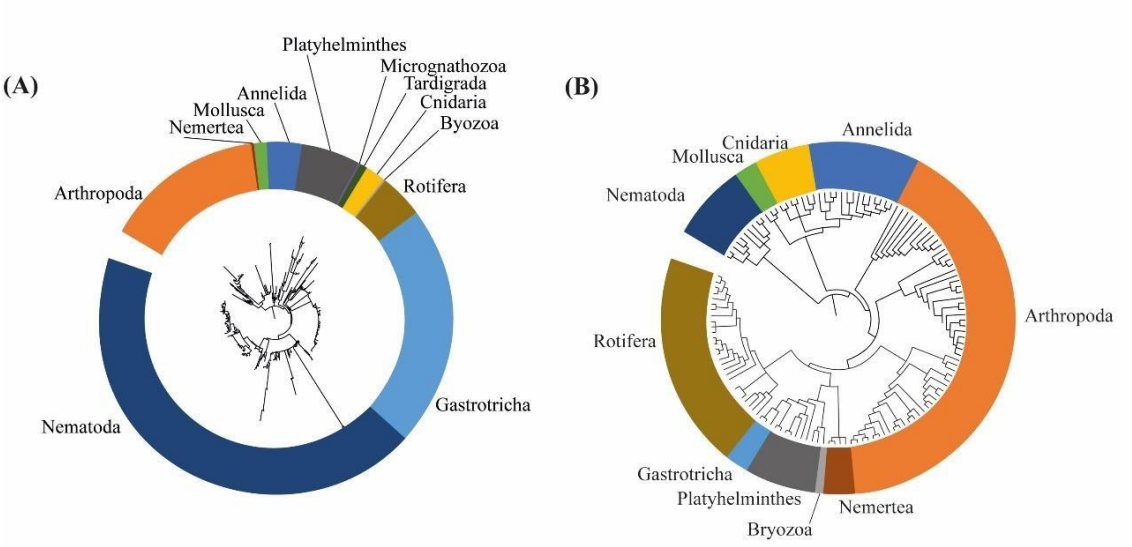

Figure 6 Phylogenetic trees. Phylogenetic trees based on the amplicon sequence variants (ASVs) identified from (A) 2017, and (B) 2018 assessments in the Rio Doce estuary.

DISCUSSION

eDNA metabarcoding of the Rio Doce estuary revealed a lower meiofaunal phylogenetic diversity 2.8 years after the mine tailing disaster, which is contrary to our initial hypothesis of a temporal increase of meiofaunal diversity along an expected decrease in sediment contamination. The temporal comparison of meiofauna assemblages showed significant changes in the composition and diversity (Fig.2; Fig.6) of meiofaunal organisms, which are markedly associated with the metal contamination in the sediments. Therefore, our results support that the meiofaunal assemblage in the Rio Doce estuary has changed substantially between 2017 and 2018, but with observed reductions in phylogenetic diversity, number of sequences, and changes in the relative abundance of each taxon.

Sediment metal concentrations decreased since the initial impacts were observed in the Rio Doce estuary, but concentrations are still well above pre-impact levels (Gomes et al., 2017; Gabriel et al., 2021). Estuaries are commonly considered ecosystems with low diversity, due to the highly dynamic hydrological conditions (Gray et al., 2002; Anila Kumary et al., 2008; Alves et al., 2013; Janakiraman et al., 2017; Hadlich et al., 2018). Nematodes and Arthropoda are common taxa in estuarine sediments (Coull, 1999; Dalto and Albuquerque, 2000), and at the Rio Doce they represented over 70% of the taxa sampled (Table 2; Fig.2). These taxa were key to differences observed between 2017 and 2018. In 2017, Nematoda was dominant in the same sampled stations representing 29.2% of sequences of meiofauna (Bernardino et al., 2019).

Copepods are known to be sensitive to pollution (Won et al., 2018), whereas nematodes are highly tolerant, with some species capable of detoxify absorbed or ingested metals by using metal-binding proteins (Montserrat et al., 2003; Ferraro et al., 2006). Millward and Grant (1995) applied toxicity tests on a nematode community from a severely contaminated estuary, and evidenced that nematodes are resistant to Cu. Thus, the higher dominance of nematodes in 2017 may be related to the higher levels of metals (Bernardino et al., 2019); and their decreased abundance in 2018 suggests a temporal succession of dominance; possibly related to a gradual decrease in pollution observed in the estuary (see Gabriel et al., 2021). This reduction on the relative abundance of nematodes (from 29.2% to 5.2% of total meiofaunal sequences), which are a potential indicator of contaminated sediments, may indicate an assemblage response to the reduction in the metal concentrations in the sediment, where other less tolerant taxa can compete with taxa that are more tolerant to toxicity.

The significant changes observed in meiofaunal assemblages supports the marked temporal changes in environmental conditions of the estuarine sediments. We additionally observed a stronger degree of dissimilarity in assemblages in 2018, which support high bottom heterogeneity and some recovery. The higher heterogeneity in sediment composition can be a source of species nestedness (or loss) in estuarine sediments (Menegotto et al., 2019), which could explain lower taxonomic diversity and higher dominance of Arthropods in 2018.

The distribution of metals (e.g., Cd, Cu and Zn) may help explain the distribution pattern of meiofaunal metazoans in 2018. McLeese et al. (1987) indicated Cd as non toxic at typical environmental concentrations. Some other studies on meiofauna suggest that

Cd does not affect species compositions (Austen and McEvoy, 1997; Austen and Somerfield, 1997). Trannum et al. (2004) did not observe negative effects from high concentrations of Cd on the recolonization of different benthic taxa. On the other hand, Wakkaf et al. (2020) observed Cd toxicity to meiobenthic nematodes. Copper, a common contaminant in bays and estuaries (Hadlich et al., 2018), and considered to be most toxic metal to many marine species (NAS, 1977), showed negative correlations with benthic recolonization rates in experiments realized by Olsgard (1999) and Trannum (2004). Although Zn is not considered toxic to marine organisms (Bryan and Langston, 1999), Gyedu-Abadio (2011) found influences of this metal on the structure of nematodes in two estuaries in South Africa.

Metal concentrations had a significant effect on meiofaunal assemblages after 2.8 years, in addition to sedimentary organic content and grain size. Organic matter contents in the sediment plays a key role, as a nutrient source in determining the distribution of benthic organisms (Paarsons et al., 1984; Neto et al., 2021). The distribution of some meiofaunalorganisms may be influenced by grain size, like crustaceans that are usually more abundant in coarse sediments (Tietjen, 1969; Hicks and Coull, 1983). Grain size determines structural and spatial conditions from the habitat, and indirectly influences the physical and chemical parameters of it (Giere, 2009). In fact, different studies suggest that abiotic factors, such as grain size and organic matter content, contribute to the patchy distribution of meiofaunal assemblages in a similar pattern observed in the present study (Nascimento et al., 2008; Alves et al., 2009; Faria et al., 2018; Fais et al., 2020).

We expected to detect a meiofaunal successional process towards assemblages with higher richness and diversity, when compared to the 2017 assessment, which would suggest a recovery process from chronic impacts of metal contamination. Our study showed that other factors can influence the rate at which biotic assemblages recover from environmental disasters. Our results suggest that the Rio Doce estuary was not yet on a recovery path after nearly 3 years from the initial impacts, as ecosystems are not considered recovered until a secondary succession returns the ecosystem to the pre-existing condition (Borja et al., 2010). In this sense, we would need continued long-term assessments to determine its a recovery trajectory (Latimer et al. 2003). The recovery of benthic communities can vary greatly from weeks (Danovaro et al., 1995) to decades(Jones and Schmitz, 2009; Borja et al., 2010; Aderhold et al., 2018), while some ecosystemsmay never be technically recovered and end up irreversibly in an alternative state (Borjaet al., 2010). Similarly, Fleeger et al. (2019) did not observe afull recovery of meiofaunal assemblages 6.5 years after an oil spill contamination. Our results corroborate those found by Gambi et al. (2020) that clearly detected the effect of long-term tailing discharge on benthic diversity after several decades from the end of themining. In our case, it is difficult or even impossible to determine the state of recovery the Rio Doce estuary since there were no baseline data or long-term studies of meiofaunalassemblages in this estuary.

The meiofaunal phylogenetic diversity from the Rio Doce estuary suggests losses of diversity in assemblage composition from 1.7 to 2.8 years after initial impacts. This may be a result or a response to the chronic effects of the metal concentrations following the disaster since, despite a significant decrease on metal concentrations, the contamination remains above reference values (Gabriel et al., 2021). These observed

differences in meiofauna assemblages may indicate changes in other biological components, and consequently in the whole estuarine ecosystem. The loss of some meiofauna phyla and the decrease in phylogenetic biodiversity may corroborates to thishypothesis.

CONCLUSION

In conclusion, we observed substantial differences on meiofaunal assemblage composition and diversity in the Rio Doce estuary from 1.7 to 2.8 years after a mine tailing disaster. Although sediment metal concentrations decreased with time, we observedfewer meiofauna taxa and lower phylogenetic diversity. Our results suggest that meiofaunal diversity are now influenced by total organic matter content and grain size, but the continuous contamination by trace metals including Cd, Cu and Zn seems to still affect assemblage diversity. On the other hand, the reduction on Nematoda relative abundance – a tolerant taxa to toxicity - may indicate a recovery of meiofaunal assemblages via competition with less tolerant taxa. Additionally, we reinforce that the use of eDNA approaches is an useful and cost-effective way to understand the dynamic ofestuarine ecosystems and temporal changes in biodiversity. The continued sampling and monitoring of the Rio Doce estuary would be of great importance to understand how this meiofaunal assemblage will respond during the successional process over time.

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SUPPLEMENTARY MATERIAL

Table S1 Student's t-test results. Results of Student's t-test comparing environmental variables between 2017 and 2018 sampling in the Rio Doce estuary.

Variable	df	t	p
TOM	21	0.629	0.646
%Sand	21	-0.984	0.532
Al	21	3.396	**<0.001**
As	21	0.689	0.536
Ba	21	2.956	**<0.001**
Cd	21	3.271	**<0.001**
Co	21	1.989	**0.004**
Cr	21	3.709	**<0.001**
Cu	21	3.443	**<0.001**
Fe	21	4.243	**<0.001**
Mn	21	2.327	**<0.001**
Ni	21	2.347	**<0.001**
Pb	21	5.870	**<0.001**
Zn	21	1.719	**0.009**
Phylogenetic Diversity	21	15.609	**<0.001**
Number of Sequences	21	11.147	**<0.001**

Table S2 Sampling stations and number of sequences. Number of sequences identified as meiofaunal metazoans in each sampled station in the 2018 assessment in the Rio Doce estuary.

Station	Number of Sequences
ST4	7,196
ST6	5,890
ST8	13,098
ST9	16,508
ST11	14,764
ST13	4,758
ST14	23,250
ST15	5,947
ST18	21,454
ST19	7,762

Table S3 ANOVA results for meiofaunal sequences. Results of ANOVA comparing the frequencies of meiofaunal representative sequences obtained in the 2018 assessment in the Rio Doce estuary.

Source of Variation	**df**	**F**	**p**
Between Groups	9	12.715	**<0.001**
Residual	90		
Total	99		

CHAPTER 3

ENVIRONMENTAL DNA SUPPORTS A HIGH PHYLOGENETIC DIVERSITY AND DISTINCT BENTHIC ASSEMBLAGES INRHODOLITH BEDS FROM SE BRAZIL

Artigo em revisão no periódico *Biodiversity and Conservation*

ABSTRACT

The global importance of rhodolith beds as a repository of biodiversity and carbon in the oceans has been highlighted due to rising exploratory interests worldwide and urgent need for better evaluation of their ecological value. Here we investigate the role of rhodolith beds as a biodiversity hotspot for benthic invertebrates in a marine protected area in SE Brazil, testing the influence of habitat structure on meiofaunal assemblage composition and phylogenetic diversity. Additionally, we evaluated congruence between taxonomic identification based on environmental DNA and traditional morphological approach, in detecting biological patterns. Our results revealed that sediments under high-density rhodolith beds have higher quantity and quality of organic matter as well as higher number of meiofaunal sequence reads when compared to areas of low-density rhodolith beds. Meiofaunal eDNA sequences in high-density rhodolith beds were mainly dominated by Crustacea and Mollusca, while Annelida and Crustacea were dominant in low-density rhodolith beds. Morphological survey identified a distinct community with lower relative dominance of Annelida and Crustacea, but higher Nematoda dominance in the sediment underneath both, high and low-density, rhodolith beds. Phylogenetic diversity based on eDNA sequences was not significantly different in low-density and high-density rhodolith beds. Our findings support the idea that rhodolith beds with distinct structure may hold similar diversity levels, but the environmental DNAmetabarcoding captures taxa that are not typically identified by morphological taxonomysurveys. Our results highlight the importance of the combined use of DNA-based and morphological approaches for a more detailed biodiversity survey of marine ecosystems.

Keywords: Habitat complexity; Phylogenetic diversity; Benthos; eDNA metabarcoding; Benthic diversity

INTRODUCTION

Rhodolith beds (RB) are important hotspots of marine life along continental shelves worldwide, particularly in the tropical South West Atlantic (Anderson et al. 2023), although also occurring abundantly from tropical to polar waters at depths ranging from 10 to 150m (Amado-Filho et al. 2012; Foster et al. 2013). The three-dimensional structure of rhodolith beds increases the structural complexity of the seafloor and may support greaterbiodiversity in these ecosystems (Steller et al. 2003; Berlandi et al. 2012; Cavalcanti et al. 2014; Teichert 2014; Riosmena-Rodríguez et al. 2017; Qui-Minet et al. 2018; Cerqueira Veras et al. 2020). These calcareous macroalgae are associated with biogeochemical processes and ecosystem services, such as calcium carbonate deposition, carbon sequestration, and maintenance of marine pH, that can affect nutrient availability (Schubert et al. 2019; Gabara 2020). Benthic diversity may be increased due to the seafloor structural complexity, which creates microhabitats and provides colonization areas for algae, invertebrates, and fish (Steller et al. 2003; Mazzuco et al. 2020; Neto et al. 2021; Stelzer et al. 2021), and nursery and feeding grounds for megafauna (Capitoli and Haimovichi 1993; Kamenos et al. 2004). Currently, trawling fishing and seabed mining are considered major threats to rhodolith bed habitats (Anderson et al. 2023), imposing an urgent need for biodiversity mapping and habitat evaluation.

It is recognized that the presence of rhodolith beds is important to maintain benthic diversity and abundance. Comparing seabed areas covered with rhodoliths and sand banks, Steller et al. (2003) observed higher richness and abundance of epibenthic, crypto- and infaunal species in high-density mesophotic rhodolith beds. These same beds also support higher abundance, biomass, and diversity of nematodes (Neto et al. 2021). In the nearshore, Rebecchi et al. (2022) observed five times higher density, 1.5-fold higher richness, and significantly higher biomass ofmeiofaunal assemblages on the sediment under rhodolith beds than a sandy beach. Additionally, Gabara et al. (2018) demonstrated that less disturbed rhodolith beds support more abundant, diverse, and stable benthic assemblages than less disturbed ones or sandbanks. Together, these studies support the idea that benthic biodiversity increases due to rhodolith formation.

One challenge when assessing benthic biodiversity in rhodolith beds in large areas is to perform precise and fast taxonomic identification of the meiofauna. Meiofauna are very small metazoans (invertebrates, between 50 – 500 μm) that are a key component of marine benthic diversity (Higgins and Thiel 1988; Hakenkamp and Palmer 2000). Apart frombeing the most diverse component of marine biota and the most phylogenetically diverse fauna on Earth (Schratzberger and Ingels 2018), these animals play important roles in marine benthic ecosystems, acting in debris decomposition, nutrient cycling, and energytransfer throughout the food web (Coull 1999; Danovaro 2000; Schratzberger and Ingels 2018). Meiofaunal specimens are commonly neglected on marine diversity assessments, due to the difficulty of accurately identifying them by traditional morphological protocols and due to the lack of specialist taxonomists (Curini-Galletti et al. 2012; Zeppilli et al. 2015). These challenges can compromise the estimative of richness and distribution patterns of marine meiofaunal species (Castro et al. 2021), and significantlyunderestimate ecosystem importance in marine biodiversity assessments

The identification of organisms is traditionally based on morphological features, but over the last two decades the use of DNA barcodes has been transforming the ability to assess life on the planet (Grant et al. 2021). Although environmental DNA (eDNA) metabarcoding use is increasing in monitoring studies, morphology-based identification remains the most common methodology (Steyaert et al. 2020). This is because it enables direct counting of individuals (which is a pitfall of eDNA methods). DNA metabarcoding, on the other hand, enables the bulk identification of multiple species in a sample, including the identification of specimens that are too small, cryptic or too degraded for morphology-based taxonomy (Elbrecht and Leese 2015; Steyaert et al. 2020). DNA metabarcoding and eDNA techniques are novel approaches to identify meiofaunal organisms and can complement and expand traditional methodologies to assess biodiversity (Fonseca et al. 2010). Recent metabarcoding studies have successfully characterized meiofaunal communities in coastal environments (Bernardino et al. 2019; Castro et al. 2021; Leasi et al. 2021), and consequently transforming the study of marine biology, as well as our ability to identify and assess the life on Earth (Berry et al. 2020;Mächler et al. 2019; Ruppert et al. 2019).

Fast and accurate identification of meiofaunal biodiversity associated with rhodolith beds is crucial to fully understand their spatial and temporal patterns thus providing meaningful information for conservation planning in areas with multiple stressors (Nelson 2009; Bernardino and Sumida 2017). In this study, we evaluated the meiofauna assemblage response to seafloor complexity (i.e., rhodolith bed density) in the SE continental shelf of Brazil. Furthermore, we compared traditional morphology-based taxonomy and eDNA metabarcoding approaches, particularly on their ability of detecting biological and ecological patterns. Based on the importance of these calcareous macroalgae beds to the structural complexity of the seafloor, this study tested the following hypotheses: i) the density of rhodolith beds positively influences the meiofaunal diversity in the underlying sediment, and ii) eDNA metabarcoding assessment detects higher meiofauna diversity than traditional morphological approach.

METHODS

Study area and sampling

The sampled rhodolith beds are located within the Costa das Algas Marine Protected Area, in the Eastern Marine Ecoregion of Brazil (Fig. 1). This tropical region is characterized by rainy summers and dry winters (Bernardino et al. 2015), and influencedby tropical waters, warm, blooms, high nutrients, and other pelagic seascapes (see Mazzuco and Bernardino 2022) of the Brazilian Current. Sea surface temperatures ranging from 21°C to 27°C and salinity between 34.6 and 36 ppt (Quintana et al. 2015; Mazzuco et al. 2019;2020). The continental shelf is predominantly composed by rodolith beds, which extend down to the shelf break at depths over 80 m (Amado-Filho et al. 2007).

Benthic sampling was performed by SCUBA diving during the summer of 2019 (January). Sample stations were divided in two categories according to the abundance of rhodolith nodules: high-density (H1, H2, and H3; Fig. 1) and low-density (L1 and L2; Fig. 1), using preliminary images. At high-density sites, the seafloor was fully covered by rhodoliths (204±18.7 nodules.m²) while at the low-density sites rhodolith nodules were separated by sediment patches (61±27.1 nodules.m²). Three random 50x50 cm quadrats were placed over the seafloor in each sampling station, where three sediment replicates were manually sampled using sterile DNA-free corers for eDNA-based identification. Additional samples were also collected for sediment analysis (grain size, total organic matter, carbonate content, and sedimentary organic biopolymers). All the samples were preserved at -20°C until analysis. Abiotic metadata (temperature, salinity, and depth) were obtained during sampling using a SonTek ® CastAway CTD. Field sampling was approved by SISBIO-IBAMA (sampling license N 24700-1).

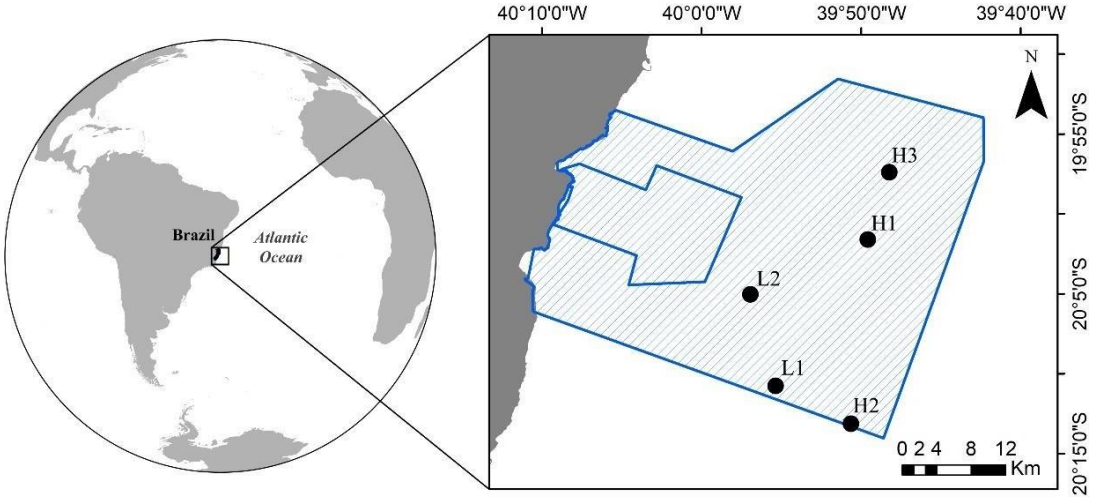

Fig. 1 Location of sampling sites with high (H1, H2 and H3) and low-density rhodolith beds (L1 and L2) on the SE Brazilian coast, within the Marine Protected Area Costa das Algas (polygon area).

Sediment analysis

The samples for granulometric analysis and carbonate content were thawed and dried at 60ºC for 48 hours. Grain size analysis was performed by sieving, following Suguio (1973). The dry sediment was macerated and sieved in mesh openings

from -1.5 Φ to 4 Φ (with 1Φ intervals) in a sieve shaker. The carbonate contents of sediment were determined by muffle combustion at 550° C for 4 h with an additional hour at 800° C.

The analysis of sedimentary organic biopolymers (proteins, carbohydrates, and lipids) followed Danovaro (2010)'s protocol. Total protein (PRT) was conducted after extraction with NaOH 0.5 M and determined according to Hartree (1972) modified by Rice (1982) to compensate for phenol interference. Total carbohydrates (CHO) were analyzed following Gerchacov and Hatcher (1972). Total lipids (LIP) were extracted from 1 g of homogenized sediment lyophilized by ultrasonication in 10 ml of chloroform: methanol (2:0 1 v/v) and analyzed following Marsh and Weinstein (1966). Sample blanks were performed for each analysis with pre-combusted sediments at 450 and 480° C for 4 h. The concentrations of PRT, CHO and LIP were expressed as bovine serum albumin, glucose, and tripalmitin equivalents, respectively. All analyzes were performed in triplicate. We converted the concentrations of PRT, CHO and LIP to carbon equivalents using a conversion factor of 0.49, 0.40, and 0.75, respectively (Fabiano and Danovaro, 1994). The sum of the protein, lipid, and carbohydrate carbon equivalents was reported as biopolymeric carbon (BPC) and used as a reliable estimate of the labile fraction of organic carbon (Fabiano et al. 1995). Additionally, we used protein-to-carbohydrate (PRT: CHO) and carbohydrate-to-lipid (CHO: LIP) ratios to assess the state of biochemical degradation processes (Galois et al. 2000).

DNA extraction and sequencing

DNA extraction and sequencing follow the steps described in Fig.2. Preceding DNA extraction, we elutriated the sediment samples using a 45 µm sieve, and aliquoted them to 20 mL in Falcon tubes. After that 1mL aliquots were separated into sterile 1.5mL tubes and stored them at -20°C (Brannock and Halanych 2015). All glassware was sterilized between samples to avoid cross-contamination. After elutriation, we extracted DNA from the sediment samples using the PowerSoil DNA® (Qiagen) kit following the manufacturer's instructions. DNA integrity, purity, and concentration were verified using 1% agarose gel, a NanoDrop One spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA), and a Qubit® 4 Fluorometer (Life Technologies-Invitrogen, Carlsbad, CA, USA), respectively. Metabarcoding sequencing and amplicon libraries were carried by ©NGS Genomic Solutions (Piracicaba, SP, Brazil) using the MiSeq Illumina platform (2 x 250 bp). We amplified the V9 hypervariable region of the 18S SSU rRNA gene using primers Illumina_Euk_1391 forward (GTACACACCGCCCGTC) and Illumina_EukBr reverse (TGATCCTTCTGCAGGTTCACCTAC) (Medlin et al. 1988; Amaral-Zettler et al. 2008; Stoeck et al. 2010), generating amplicons that could vary in size (mean 260±50 bp), once the reverse primer doesn't have its exact position conserved as the forward one.

Bioinformatic pipeline

The entire bioinformatic pipeline was run on an AMD Ryzen 1950x Crucial 64 GB (16x4) DDR4 2666MHz computer. QIIME2 2022.8 software was used to identify taxonomically the sequence reads with the demultiplexed raw paired-end reads (Bolyen et al. 2018). Firstly, we imported FastQC files as QIIME2 artifacts. Secondly, we denoised the paired-

end reads via DADA2 (Callahan et al. 2016) using the denoise-paired plugin, and removed low-quality bases and primer sequences.

Machine learning Python library scikit-learn was performed to assign the taxonomic composition of the amplicon sequence variants (ASV) generated after running the DADA2 plugin (Pedregosa et al. 2011). To identify taxonomically the sequences reads, we used a pre-trained Naïve Bayes classifier, trained on Silva 132 database (Quast et al. 2013) clustered at 99% similarity. Due to the difference in the number of sequence reads, we normalized datasets from both areas (high and low-density RBs) to allow analysis with homogeneous sampling depth. We used the low-density dataset minimum sampling depth (2,991 reads) and resampled each station to the same depth. These filtered datasets were used to run all analyses and calculate all diversity metrics. Additionally, we plotted rarefaction curves for both sampled areas (Supplementary Fig.S1).

Faith's Phylogenetic Diversity (PD) was calculated for each sample using the diversity core-metrics-phylogenetic pipeline, which is based on a phylogenetic tree generated previously using the *align-to-tree-mafft-fasttree* pipeline from the *q2-phylogeny* plugin in QIIME2. We calculated Shannon diversity as well, using the qiime diversity alpha pipeline and setting the p-metric parameter to "*shannon*". The raw sequence dataset was deposited in NCBI (SRR23510645), and it is also available in the Tropical and Subtropical Western South Atlantic Ocean Biogeographic Information System (WSAOBIS) (Coppo et al. 2023), as well as the morphological dataset (Netoet al 2023).

We compared the eDNA-based dataset with traditional morphology-based identification from sediment samples collected at the same sampled stations during the same sampling time, obtained from Neto (2020). Additionally, we compared sequence reads identified as Nematoda to the meiofauna morphologically identified as Nematoda, once it is, in general, the most representative taxon on meiofaunal assemblages.

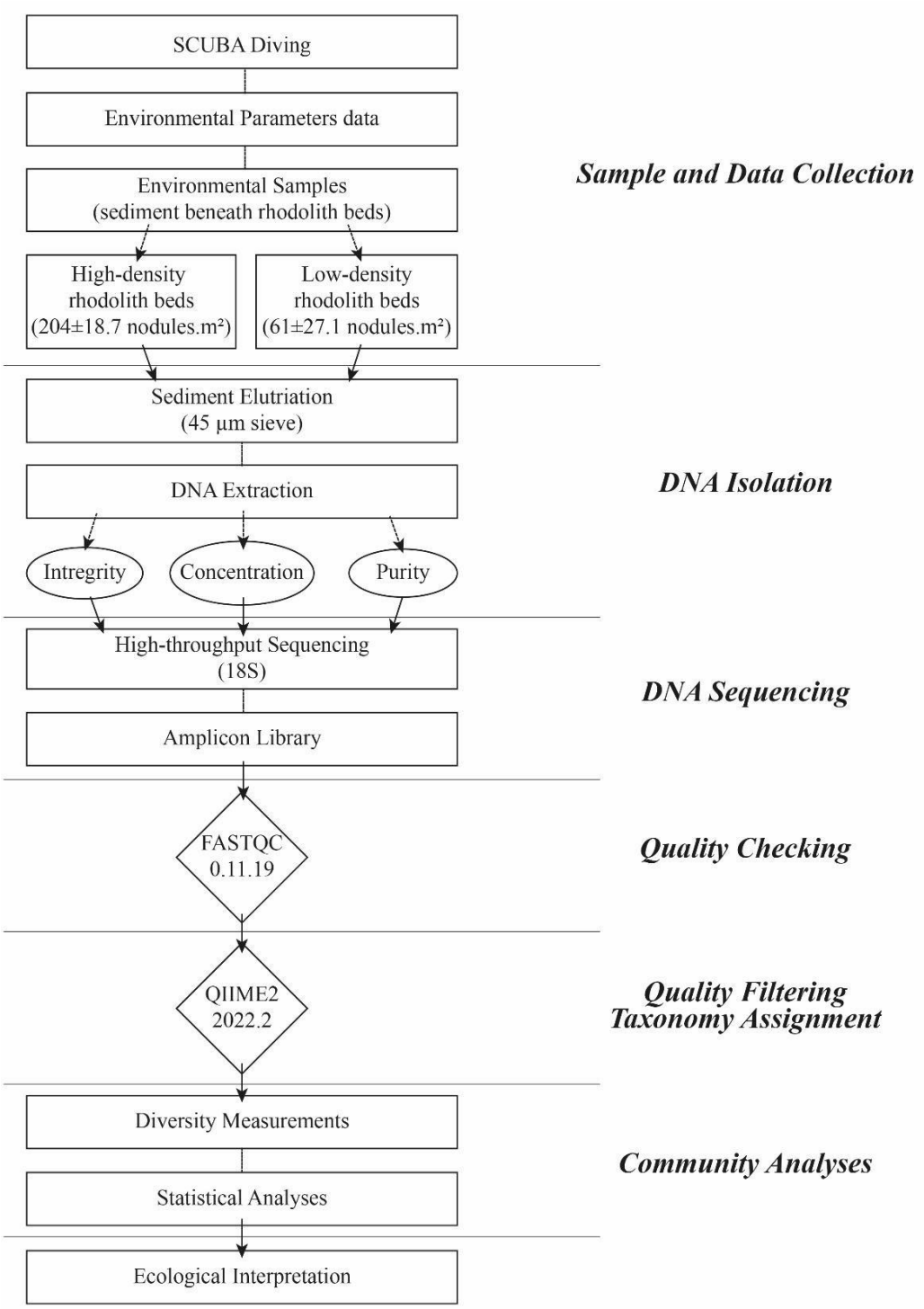

Fig. 2 Flowchart showing all steps in the methodology, from the samples and data collection until the ecological interpretation obtained from the bioinformatic pipeline and statistical analyses.

Statistical analysis

Differences in sediment parameters (granulometry, carbonate content, total organic matter, PRT, CHO, LIP, BPC concentration, PRT:CHO and CHO:LIP ratios), phylogenetic diversity, and the number of sequencereads between high and low-density rhodolith beds were analyzed by a Mann-Whitney Rank test (Mann and Whitney 1947). A Similarity Percentage Routine (SIMPER) was applied to define those taxa that contributed most to the dissimilarity between the habitats(Clarke 1993). Non-Metrical

Multidimensional Scaling (nMDS; Oksanen et al. 2022) ordinations was performed with the meiofaunal assemblages from high and low-density RBs. Additionally, we calculated multiple linear regressions between Shannon's diversity index andenvironmental variables that showed significant differences between high and low- density rhodolith beds. Analyses considered $\alpha = 0.05$. All graphical and analytical processes were performed in R environment (R Core Team 2022).

RESULTS

We observed significant differences in the density of rhodolith nodules (n/m²) between the high and low- density beds (H = 204±18.7 nodules/m², L = 61±27.1 nodules/m²; T = 21.000; p = 0.002; Table 1). Both areas were under similar depth range (H = 48.1±8.5 m, L = 43.6±5.5 m; T = 39.000; p = 0.308; Table 1). Sediments under the rhodolith nodules were overall composed of poorly selected medium to coarse sand. The sediment carbonate content was significantly higher (T = 29.000; p = 0.029) at high-density beds (3.7±1.6) than low-density beds (1.9±0.3). Sediment organic matter content (OM) was similar between both high and low-density beds(3.7±0.9 and 3.6±0.9, respectively; t = 0.824; p = 0.849; Table 1).

The sediments under high-density rhodolith beds had a higher concentration of proteins (p < 0.001), and lipids (p = 0.034) when compared to sediments under low-density beds (Table 1). We did not observe differences in sediment carbohydrate concentrations (p = 0.559), but the biopolymeric carbon (which represents the sum of protein, carbohydrate and lipids concentration) was significantly higher at the high-density RBs (p = 0.049; Table 1). The sediment organic matter in the RBs, independent of density, was mainly composed of carbohydrates, and lipids represented the smallest fraction. We observed differences in the ratio of protein/carbohydrates (PRT:CHO; p = 0.016), but not in carbohydrates/lipids (CHO:LIP, p = 0.087) between high and low-density RBs (Table 1).

Table 1 Environmental variables data, presented as mean ± standard deviation, obtained from sediment samples from high and low-density rhodolith beds. Significant differences (p<0.05) are presented in bold.

Environmental Variable	Rhodolith Bed Density		*p*
	High-Density	Low-Density	
Density of nodules (nodules/m²)	204±18.7	61±27.1	**0.002**
Depth	48.1±7.3	43.5±4.2	0.308
%Sand	75.6±11.2	85.5±6.6	0.074
%Gravel	22.6±11.4	12.8±6.6	0.082
%Silt	1.8±0.6	1.7±0.4	0.606
Carbonate	3.7±1.6	1.9±0.3	**0.018**
Organic Matter (OM)	3.7±0.9	3.6±0.9	0.849
Protein (PRT)	0.65±0.16	0.28±0.02	**<0.001**
Carbohydrate (CHO)	1.2±0.7	1.0±0.2	0.559
Lipids (LIP)	0.22±0.06	0.11±0.10	**0.034**

Biopolymeric Carbon (BPC)	2.0±0.7	1.4±0.3	**0.049**
PRT:CHO	0.87±0.80	0.29±0.06	**0.016**
CHO:LIP	6.2±4.9	34.9±41.2	0.087

A total of 118732 meiofaunal sequence reads were identified in the dataset, 12308±5333.2 (92% of sequence reads in the entire dataset) of them from the high-density RBs, and 1599±976.4 (8% of sequence reads in the entire dataset) were obtained from the low-density RBs (Mann-Whitney U Statistic = 16.000; T = 37.000; p = 0.216; Table 1). The number of sequence reads on the high-density RB varied from 442 to 49137, which was 8 to 12 fold the number of sequences on low-density RB (207 to 3460).

We also observed differences in assemblage composition between rhodolith beds with distinct nodule density. High-density beds were typically dominated by Arthropoda (62% of sequence reads; Fig.3A; Supplementary Fig.S2) and Mollusca (29% of sequence reads; Fig.3A; Supplementary Fig.S2), while low-density beds were mostly represented by Annelida (40% of sequence reads; Fig.3A; Supplementary Fig.S2) and Arthropoda (27% of sequence reads; Fig.3A; Supplementary Fig.S2). Sequence reads from Echinodermata and Nemertea were only identified in low-density beds, and sequence reads from Entoprocta were only identified in high-density beds (Fig.3A; Supplementary Fig.S2; Supplementary Table S1). Crustaceans (Eucarida) sequence reads were the most abundant in high-density beds (60.2%), while in low-density beds the most abundant sequence reads were from Copepods (Calanoida) (Supplementary Fig.S2; Supplementary Table S1). Additionally, the DNA-based identification detected a broader range of meiofaunal taxa when compared to traditional morphology-based taxonomy, which only detected Nematoda, Arthropoda (Copepoda), and Annelida (Polychaeta) (Fig.3B; Supplementary Table S2). Overall, on the morphological assessment,Nematodes were the most abundant identified taxa (62% of individuals), representing 56% at high-density and 71% at low-density rhodolith beds. Copepoda and Polychaeta represented 22% each in high-density beds, and 29% and 0%, respectively, in low-density beds (Fig.3B; Supplementary Table S3).

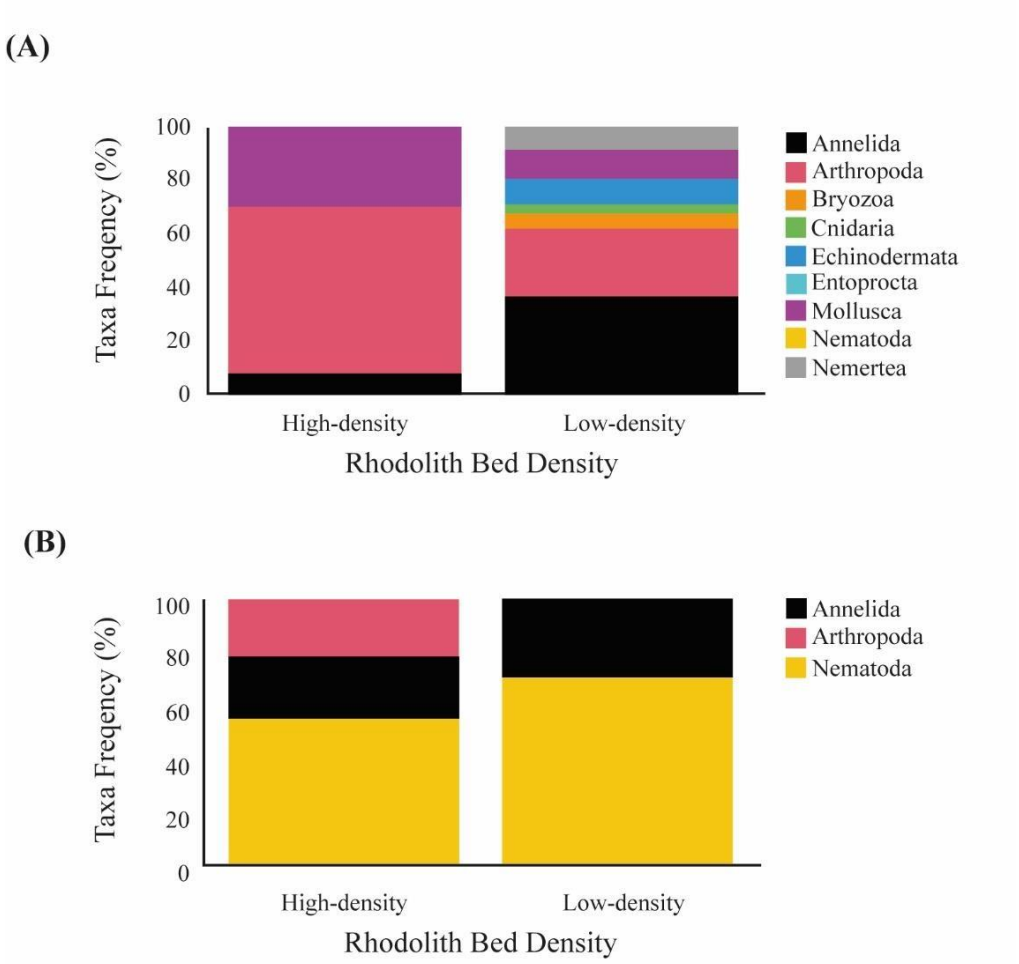

Fig. 3 Mean relative abundance of meiofaunal assemblage composition detected by (A) environmental DNA metabarcoding, and (B) by traditional morphological identification, in sediment samples collected underneath high and low-density rhodolith beds in SE Brazil.

Further, we observed a significant difference in Shannon diversity between high and low-density beds. Meiofaunal assemblage at high-density beds had a mean diversity of 3.2±1.4, in which was significantly lower than that at low-density RB (4.6±0.8), representing a significant difference of 1.4 times ($t = -2.303$; $df = 13$; $p = 0.038$; Table2). We observed a high variance in meiofaunal diversity within each rhodolith bed, whichwas more pronounced in high-density beds (1.99 to 5.43) when compared to the low- density beds (3.19 to 5.36). Similarly, higher phylogenetic diversity (PD) was observed at low-density RB (23.3±8.5), when compared to high-density RB (17.3±4.6; $t = -1.768$; $df = 13$; $p = 0.100$; Table 2).

Table 2 Abundance of meiofaunal reads and diversity metrics (Phylogenetic Diversity and Shannon Diversity) obtained after eDNA metabarcoding sequencing from sediment samples collected underneath high and low-density rhodolith beds in SE Brazil. Data presented as mean ± standard deviation. Significant differences ($p<0.05$) are presented in bold.

Variable	Rhodolith Bed Density		*p*
	High-Density	Low-Density	
Abundance of Reads	12308±5333.2	1599±976.4	0.316
Phylogenetic Diversity	17.3±4.6	23.3±8.5	0.100
Shannon Diversity	3.2±1.4	4.6±0.8	**0.038**

Meiofaunal assemblages identified from high and low-density beds showed different patterns in their composition (Fig.4). Multivariate analysis revealed that Arthropoda (Crustacea) (36.4%) and Mollusca (21.5%) were the phyla that most contributed to the dissimilarity between the assemblages. Meanwhile, Nematoda – traditionally a major representant of meiofauna – was not a crucial to the dissimilarity between high and low-density rhodolith beds assemblages, contributing 2.70% to the dissimilarity between both habitats. Although Echinodermata, Nemertea andEntoprocta were only found in one of both rhodolith densities, they did not have a majorcontribution to dissimilarity (5.31%, 4.71%, and 1.53%, respectively) due to their low relative abundance in our samples (Table 3; Supplementary Table S1).

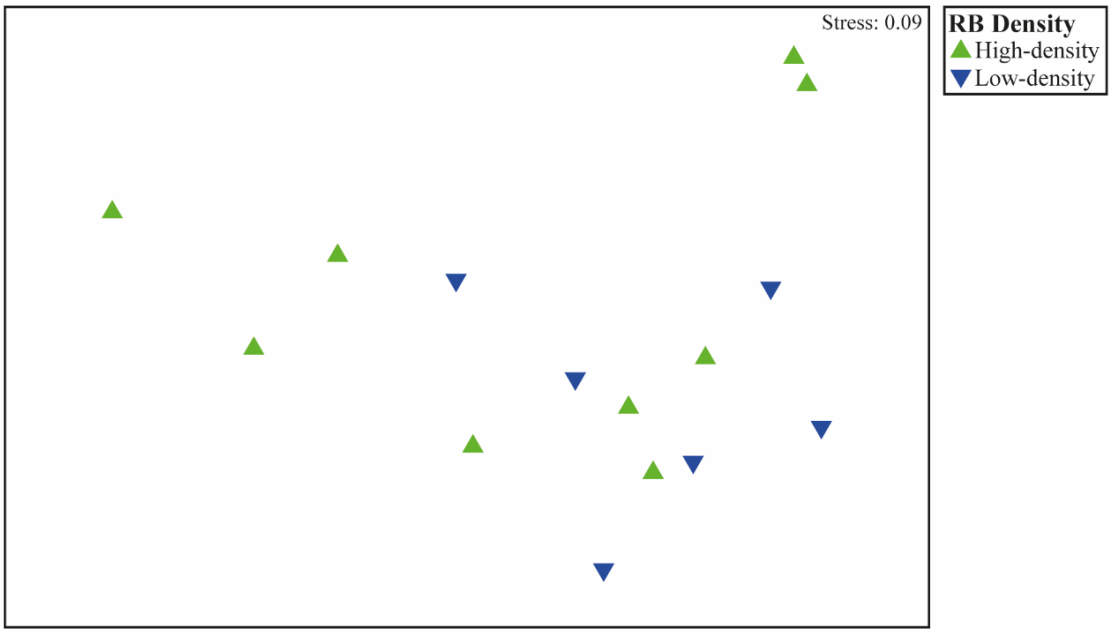

Fig. 4 Non-metric Multidimensional Scaling (nMDS) ordination based on meiofaunal assemblage composition (as relative abundance) at Phylum level, in high-density(green triangles) and low-density (blue triangles) rhodolith beds.

Table 3 Results from Similarity Percentage analysis (SIMPER) indicating each taxon contribution to the similarity between high and low-density rhodolith beds. Av. Dissim. = Average Dissimilarity; Diss./SD = Dissimilarity/Standard Deviation; Contrib. = Contribution.

Taxon	Av. Dissim.	Diss./SD	Contrib. %	Cumulative %
Arthropoda	21.02	0.94	36.41	34.41
Mollusca	12.47	0.79	21.59	58.00
Annelida	8.53	1.37	14.78	72.78
Bryozoa	4.87	0.94	8.43	81.22
Echinodermata	3.07	0.52	5.31	86.53
Nemertea	2.72	0.52	4.71	91.23
Cnidaria	2.62	1.40	4.54	95.77
Nematoda	1.56	0.95	2.70	98.47
Entoprocta	0.88	0.68	1.53	100

Arthropoda, Mollusca and Annelida specimens were more abundant in high-density RBs samples. Additionally, we observed that these taxa were highly associated with coarse sediment (%Gravel), carbonate content (%Carbonate), and the sediment content of lipids and proteins. Low-density RBs, on the other hand, were characterized by fine sediment (%Sand) (Fig.5).

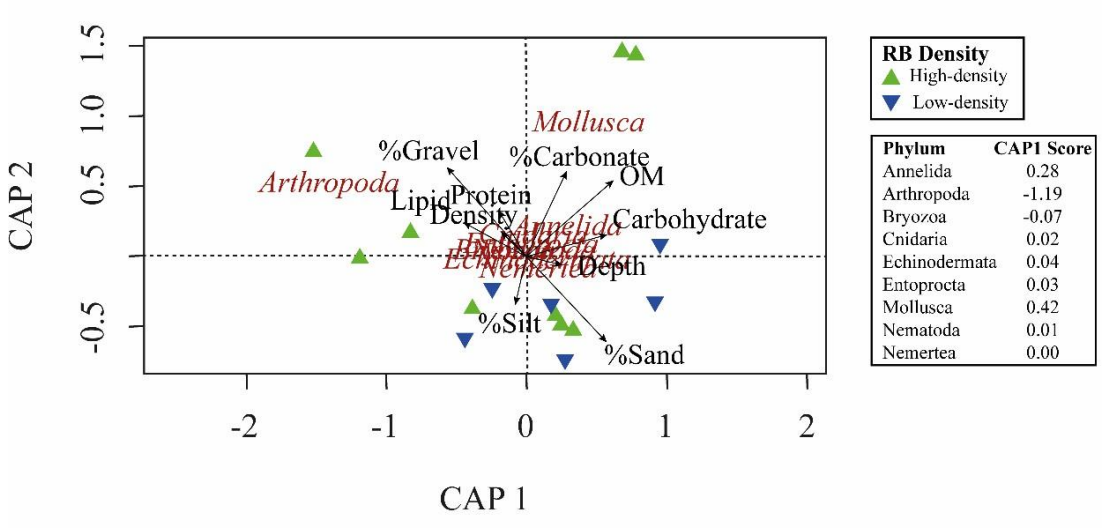

Fig. 5 Canonical Analysis of Principal Coordinates (CAP) of assemblage composition and environmental variables at high (green triangles) and low-density (blue triangles) rhodolith beds.

Variables that showed significant differences between high and low-density RBs (%Carbonate, PRT, LIP, BPC, and PRT:CHO) compose a model of variables likely to drive the diversity of meiofaunal assemblages on rhodolith beds ($R^2 = 0.638$; $F = 3.168$; $p = 0.063$). We observed negative relationships between protein content on sediment (PRT) and meiofaunal diversity ($p = 0.043$; Table 4), meanwhile biopolymeric carbon content (BPC), and protein/carbohydrate ratio (PRT:CHO) were positively correlated to meiofaunal diversity on RBs ($p = 0.026$ and $p = 0.035$, respectively; Table 4; Supplementary Table S3).

Table 4 Multiple linear regression statistical values from relation between Shannon's Index and environmental variables that shown significative differences between high and low-density rhodolith beds.

	Coefficient	Standard Error	t	*p*
Constant	4.753	1.551	3.064	**0.013**
Carbonate	-0.345	0.295	-1.170	0.272
PRT	-9.017	3.829	-2.355	**0.043**
LIP	-6.555	3.233	-2.028	0.073
BPC	2.766	1.037	2.667	**0.026**
PRT:CHO	2.575	1.041	2.475	**0.035**

The eDNA metabarcoding of Nematoda returned 121 sequence reads in the high-density RBs (85 % Chromadorea and 15% Enoplea), and 62 sequence reads in the low-density RB (100% Chromadorea). Meanwhile, the morphological assessment identified 414 nematodes specimens in the high-density RBs (88% Chromadorea and 12% Enoplea), and 64 specimens in low-density RBs (84% Chromadorea and 16% Enoplea). Based on the eDNA metabarcoding identification, the most refined taxonomic level reached was Order. eDNA metabarcoding only identified the Araeolaimida order (class Chromadorea). Furthermore, we were able to identify sequence reads classified as miscellaneous of Chromadorea and Enoplea. On the other hand, based on traditional morphological identification we could identify 49 genera, from 20 distinct families (Fig.6; Supplementary Table S2). Both in high and low-density RBs, the most abundant family was Desmodoridae, representing 22% and 24% of the assemblages, respectively (Supplementary Table S2).

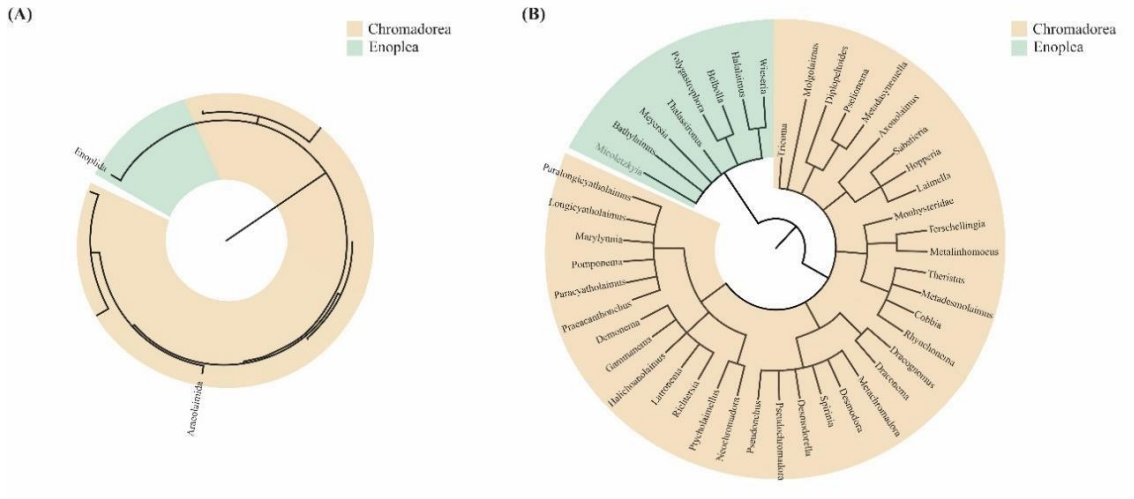

Fig. 6 Phylogenetic trees based on the detected Nematoda amplicon sequence variants (ASVs) identified by (A) environmental metabarcoding DNA (branches without identification were only identified at Class level), and (B) morphological identification, from samples of sediment underneath rhodolith beds in SE Brazil.

DISCUSSION

In this study, we observed assemblages that were 57.7% different between the high and low-density RBs sediments, and our findings did not support our hypothesis that thedensity of rhodoliths would act as a major driver to meiofaunal diversity. Here we foundthat rhodolith beds with distinct complexity (e.g., nodule density) may hold similar levelsof meiofaunal diversity, although the composition and dominance of taxa may slightly differ. Our eDNA assessment indicated that copepods and annelids are an important fraction of meiofaunal assemblages under rhodolith beds, which is in contrast with the high dominance of nematodes from the morphological assessment (Neto 2020).

We expected higher phylogenetic diversity on the high-density beds, since bottom complexity would provide more microhabitats for benthic organisms. These high complexity beds may also hold higher concentrations of proteins and lipids providing higher food quality for benthic organisms (Neto et al. 2021). However, our results based on the eDNA did not support this hypothesis, as we detected similar phylogenetic diversity between rhodolith beds. Although we observed a higher number of sequence reads in high-density rhodolith beds, the assemblage composition in those areas was highly dominated by copepods and mollusks which were underrepresented groups in morphology-based assessments (Neto et al. 2021).

Here we observed a high abundance of Crustacea, such as copepods, which may be associated with the higher microphytobenthos concentration as observed by Neto et al. (2021) and Todaro (2006), similarly, with a high algal input to the sedimentary organic matter(Neto 2020). The higher diversity observed on low-density rhodolith beds using eDNA assessment can be a result of the low dominance of nematodes, and not due to the presence of more taxonomic groups, as observed by Neto (2020) in the same sampled stations. This low dominance of Nematoda may be due to a higher predation pressure from other taxa at these sites (Grall et al. 2006). Stelzer et al. (2021) identified almost 30% of benthicmacrofauna as omnivores or carnivores in the same studied area, and the microhabitats generated by rhodolith beds may directly influence on competitive and predator-prey interactions (Scheffers et al. 2014).

Although we observed more sequences in high-density RB sediment samples, our findings showed that this habitat is dominated by Arthropoda (Crustacea), Mollusca and Annelida (99% of sequence reads). This dominance may contribute to a lower Shannon's diversity when compared to low-density RBs sediment samples. Meanwhile, we observed fewer sequence reads in low-density RBs sediment samples, but a more diverse assemblage, according to Shannon's Diversity Index (Table 2; Supplementary Table S1). Other studies observed similar meiofaunal assemblages' compositions in the sediment under rhodolith beds, mainly composed of nematodes, copepods and polychaetes (Neves and Costa 2022; Rebecchi et al. 2022).

Our study using eDNA metabarcoding returned more meiofaunal taxonomic groups when compared to Neto (2020) morphological approach (Copepoda, Nematoda, and Polychaeta), but we have detected similar ecological patterns comparinghigh and low-density bed, with higher Shannon diversity in low-density beds. The eDNA metabarcoding assessment detected a higher number of taxa when compared to

morphological identifications, a pattern that has been reported by other studies that have compared DNA-based and morphology-based approaches surveying of benthic biodiversity (Dell'Anno et al. 2015; Guardiola et al. 2016; Cahill et al. 2018). This occur due to howorganisms are detected using each methodology, or other errors associated to DNA-based methodologies (e.g., primer biases, PCR and sequencing errors). eDNA metabarcoding detectsorganisms by their genetic material present in sediment samples, including whole cells, extracellular DNA, and potentially entire organisms (Barnes and Turner 2016), yet morphological assessments relying on counting of entire individuals to detect its presence in the environment.

High and low-density RBs differed in their PRT and BPC contents, and on morphological-based assessments, the PRT:CHO ratio was previously related to high speciesdiversity (Neto et al. 2021) (Table 4). In our eDNA assessment, the PRT content showeda negative correlation to sequence diversity, while BPC content, and PRT:CHO had a positive relationship. Accordingly, this result indicates that the eDNA diversity and abundance may be positively influenced by high-quality OM, but the presence of strong competitors such Arthropoda (Crustacea) and Annelida, may suppress the overall meiofauna diversity.

Based on traditional morphological identification (and focusing on nematodes, which commonly is the main component of meiofaunal assemblages), Neto et al. (2021) obtained a more refined taxonomic identification. The result of this more refined taxonomy can be observed comparing the phylogenetic trees (Fig.6). The eDNA-based survey returned a tree with fewer nodes and shorter branches (that ends at Order level) when compared to the morphology-based identification that returned a tree ramified in longer branches (ending at Genus level). This higher refinement observed on the morphology-based taxonomy is explained by the effort of specialists in the identification of this phylum, and the lack of refined tropical nematode sequences on online databases. Other studies report the scarcity of reference sequences in databases asan issue, specially from tropical regions (Fais et al. 2020; Steyaert et al. 2020).

One of the concerns with eDNA metabarcoding approaches is the lack of DNA sequences broadly representing meiofauna in molecular databases. Most studies using this technique are in North Americaor Europe (Grant et al. 2021), so there is a lack of sequences on databases particularly from tropical species (Castro et al. 2021), which explains why we identified 183 sequences from nematodes, classified in two Class (Chromadorea and Enoplea), and twoOrder (Areolaimida and Enoplida), while Neto et al. (2021) identified a total of 49 genera(20 families) of nematodes using morphology-based taxonomical approach. Both approaches detected Chromadorea as the more abundant nematode class in both RBs density habitats,so we suggest that eDNA metabarcoding is capable of detecting similar overall biodiversity patterns to the traditional morphology-based identification, without significant loss of ecological information at higher taxonomic ranks. Another pitfall of eDNA metabarcoding is thelimited understanding of biodiversity in marine environments due to incomplete DNA- barcodes deposited in molecular databases and different methodological practices from research groups, which may result in marked differences in taxa identification between eDNA metabarcoding and traditional methods, as we observed here, and previously reported by other authors. (Cahill et al. 2018; Pawlowski et al. 2022; Keck et al. 2022; Willassen et al. 2022).

Some of the taxa recorded with eDNA may represent pelagic organisms or non-residential specimens, that could have been redistributed by mobile animals' activities or derived from the water column and settled on the sediment surface layer, as observed by Willassen et al. (2022). These sequence reads were not excluded since we were not able to determine the life stage of organisms, and some invertebrates larger than 1000 µm may be considered temporary meiofauna because they spend part of their life on benthic habitats (McIntyre 1969; Hakenkamp and Palmer 2000). The possible presence of exogenous eDNA requires caution on diversity and ecological interpretation frombenthic assemblages, once the sediment receives eDNA input from the water column, making it difficult to interpret whether sequence reads represent genuine localbenthic occurrences or exogenous eDNA from meroplanktonic life history stages or redistributed eDNA (Descôteaux et al. 2021; Willassen et al. 2022).

Since it is a novel technique, and in continuous development, eDNA metabarcoding will progressively become more accurate in estimating diversity as molecular databases improve their taxonomic coverage. As bioinformatic pipelines and high-throughput sequencing are in constant development, there is a need for more exploratory studies of poorly studied taxa such as marine benthic meiofauna (Steyaert et al. 2021). Most studies investigating the biology and ecology in RB beds aroundthe world have focused on the general biodiversity of macroalgae, macro and megafauna (Fredericq et al. 2014; Ling et al. 2015; Carvalho et al. 2020; Stelzer et al. 2021). Our study supports the idea that the combining morphology and eDNA-based approaches will increase the success of monitoring programs, and therefore should be carried outin association.

CONCLUSION

In conclusion, we found that high-density rhodolith beds support sediments with a higher abundance of meiofaunal sequences. However, this higher number of sequences returned assemblages with similar diversity in high and low-density rhodolith beds. Multivariate analysis revealed that meiofaunal community on rhodolith beds may be driven by the qualityof organic matter, and that ecological interactions may influence the composition of meiofaunal assemblages. Our results reveal the importance of integrating eDNA metabarcoding and morphological approaches to identify meiofaunal assemblages. The eDNA metabarcoding could detect similar spatial and diversity patterns of meiofaunal and nematofaunal assemblages as the traditional taxonomic assessments, without loss of ecological information at high taxonomic ranks, even with differences in the taxonomic refinement. It is important to combine morphological and metabarcoding approaches, and produce DNA barcodes for different markers and enrich molecular databases, especially in tropical seas with limited sampling and genomiclibraries, since the availability of sequences is a major factor to the success of these studies. The existence of monitoring programs to assess the diversity of rhodolith beds is crucialin order to understand the ecological importance of such ecosystems. Particularly, monitoring how these calcareous algae habitats and the associated benthic animals will respond to environmental impacts, including climate changeand rhodolith exploitation, is key.

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SUPPLEMENTARY MATERIAL

Table S1 Relative abundance of meiofaunal taxa identified based on environmental DNA metabarcoding from sediment samples collected underneath high and low-density rhodolith beds (RBs) in SE Brazil.

Phylum	Class	Order	High-density RB	Low-density RB
Annelida			<0.1%	-
	Polychaeta		2.0%	12.7%
		Eunicida	5.1%	1.3%
		Terebellida	0.3%	0.1%
		Phyllodocida	0.4%	13.3%
		Sabellida	0.1%	1.6%
		Scolecida	-	0.4%
		Spionida	0.1%	4.6%
Arthropoda (Crustacea)			1.0%	9.1%
	Malacostraca		-	0.5%
		Eucarida	60.2%	0.1%
		Peracarida	<0.1%	0.1%
	Copepoda	Calanoida	<0.1%	15.4%
		Harpacticoida	0.1%	1.2%
		Cyclopodia	<0.1%	-
	Ostracoda	Myodocopida	0.1%	1.0%
			0.1%	2.3%
Bryozoa	Gymnolaemata	Cheilostomatida	0.7%	7.1%
Cnidaria			<0.1%	-
	Anthozoa		0.4%	-
		Scleractina	<0.1%	-
	Hydrozoa		<0.1%	-
		Anthothecata	-	0.6%
	Scyphozoa	Coronatae	0.1%	0.4%
Echinodermata	Ophiuroidea		-	10.5%
Entoprocta			0.1%	-
Mollusca			<0.1%	1.0%
	Bivalvia		0.1%	6.1%
		Venerida	28.8%	2.7%
	Gastropoda		<0.1%	-
Nematoda	Chromadorea		0.1%	0.6%
		Araeolaimida	<0.1%	-
	Enoplea	Enoplida	<0.1%	-
Nemertea	Anopla	Heteronemertea	-	6.5%
	Hoplonemertea	Monostilifera	-	0.2%

Table S2 Relative abundance of meiofaunal taxa (identified based on traditional morphology-based taxonomy) from sediment samples collected underneath high and low-density rhodolith beds (RBs) in SE Brazil. Data obtained from Neto (2020).

Phylum	Class	Order	Family	Genus	High-density RB	Low-density RB
Annelida						
	Polychaeta				13.4%	-
Arthropoda (Crustacea)						
	Copepoda				13.7%	11.1%
Nematoda						
	Chromadorea					
		Araeolaimida				
			Axonolaimidae			
				Axonolaimus	3.0%	0.9%
			Comesomatidae			
				Hopperia	0.1%	-
				Laimella	-	0.3%
				Sabatieria	1.6%	0.4%
		Chromadorida				
			Chromadoridae			
				Neochromadora	2.0%	5.2%
				Ptycholaimellus	1.3%	0.4%
			Cyatholaimidae			
				Longicyatholaimus	0.1%	-
				Marylynnia	0.1%	0.5%
				Paracyatholaimus	0.1%	-
				Paralongicyatholaimus	-	0.1%
				Pomponema	0.8%	0.4%
				Praecanthonchus	0.1%	-
			Selachinematidae			
				Demonema	-	0.3%

		Gammanema	2.6%	2.4%
		Halichoanolaimus	0.4%	0.1%
		Latronema	0.1%	-
Desmodorida				
	Desmodoridae			
		Desmodora	3.7%	5.4%
		Desmodorella	0.5%	0.7%
		Metachromadora	1.0%	-
		Molgolaimus	0.7%	-
		Pseudochromadora	0.5%	-
		Pseudonchus	0.1%	-
		Spirinia	1.2%	-
	Draconematidae			
		Dracognomus	0.1%	-
		Draconema	-	0.8%
	Richtersiidae			
		Richtersia	2.9%	1.2%
Desmoscolecida				
	Desmoscolecidae			
		Tricoma	1.2%	2.0%
Monhysterida				
	Linhomoeidae			
		Didelta	0.1%	-
		Metalinhomoeus	0.1%	-
		Terschellingia	2.0%	2.5%
	Monhysteridae			
		Thalassomonhystera	0.1%	-
	Xyalidae			
		Cobbia	-	0.1%
		Metadesmolaimus	0.3%	-

			Rhynchonema	0.1%	0.1%
			Scaptrella	-	0.1%
			Theristus	0.7%	0.3%
	Plectida				
		Diplopeltoididae			
			Diplopeltoides	0.5%	-
		Ceramonematidae			
			Metadasynemella	0.1%	-
			Pselionema	0.4%	0.7%
			Pterygonema	0.1%	-
Enoplea					
	Enoplida				
		Enchelidiidae			
			Belbolla	-	0.3%
			Polygastrophora	0.1%	0.1%
		Ironidae			
			Thalassironus	0.1%	0.1%
		Oncholaimidae			
			Meyersia	0.9%	0.5%
		Oxystominidae			
			Halalaimus	3.8%	1.6%
			Wieseria	0.1%	-
		Phanodermatidae			
			Micoletzkyia	0.1%	-
		Tripyloididae			
			Bathylaimus	0.3%	-

Table S3 Complete results of multiple regression analysis between Shannon Diversity and significantly environmental variables (Carbonate, PRT, LIP, BPC, and PRT:CHO) obtained from sediment samples collected underneath rhodolith beds in SE Brazil.

	df	SS	MS	F	*p*
Regression	5	17.446	3.489	3.168	0.063
Residual	9	9.912	1.101		
Total	14	27.357	1.954		

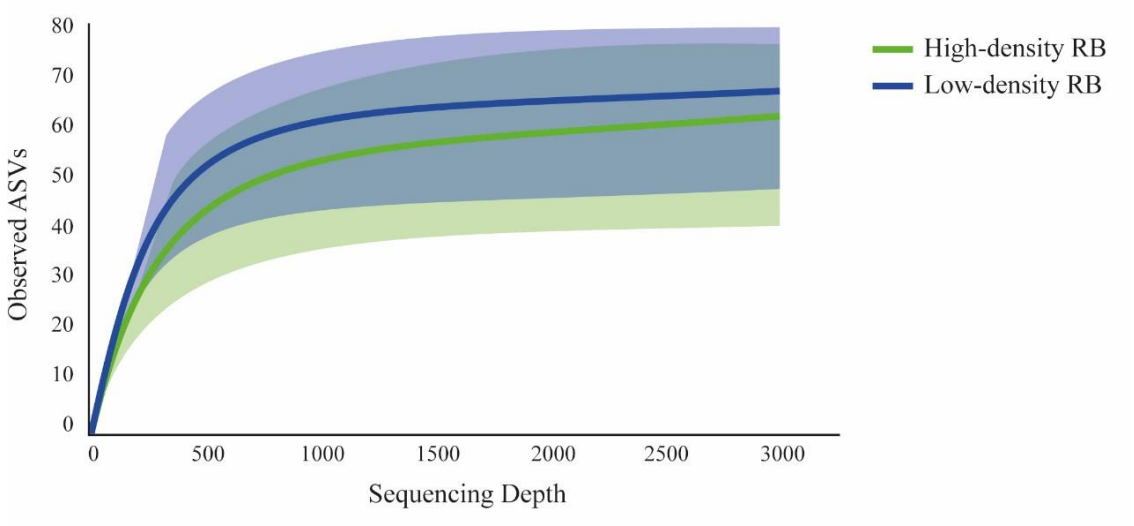


Fig. S1 Rarefaction curves from the high (green) and low-density rhodolith beds samples (blue). The shaded area represents the envelope for each rhodolith bed density dataset.

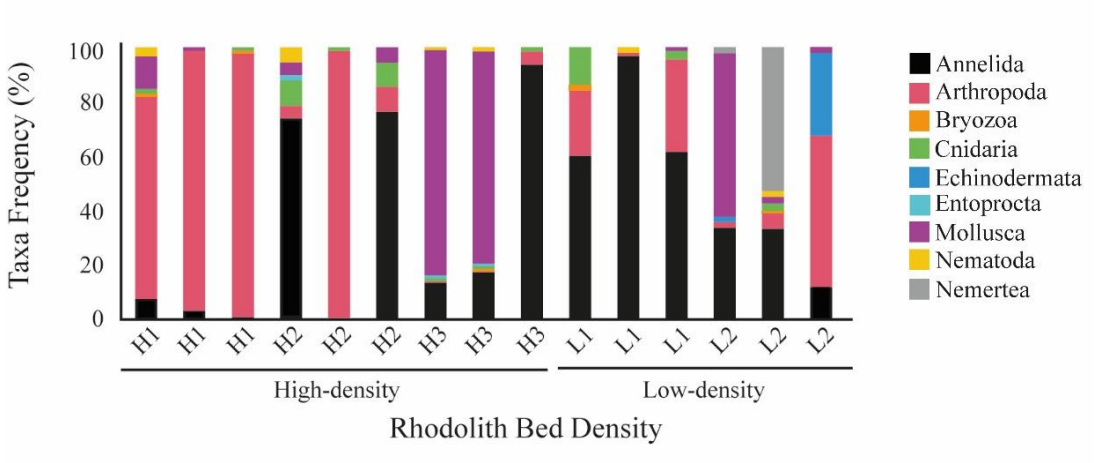


Fig. S2 Barplots showing the relative frequency of identified Phylum at high and low-density rhodolith beds persampled station.

CHAPTER 4

METABARCODING REVEALS ECOLOGICAL VARIABILITY BETWEENTIDE POOLS AND SAND BEACHES IN THE SW ATLANTIC

ABSTRACT

Understanding the diversity patterns of marine meiofauna is critical in a changing world. Here we investigate the seasonality of sand beach meiofaunal assemblages in response to costal oceanography dynamics based on the Seascapes remote sensing dataset. Additionally, we investigate whether tide pools located in close proximity to sand beaches may act as an ecological filter from sand beach meiofauna. We used metabarcoding from sediment samples to assess the meiofaunal assemblage compositionand diversity across ecosystems (sand beach x tide pools). We addressed the following hypothesis: (i) meiofaunal phylogenetic diversity and abundance of sequences is higher during warmer months in a sand beach; (ii) tide pools have different assemblage composition than sand beaches, with lower abundance of sequences and phylogenetic diversity. Our data support our initial hypotheses, revealing a higher abundance of reads,phylogenetic diversity, and Shannon diversity during warmer periods of the year. Meiofauna was dominated by Crustacea (46% of sequence reads), Annelida (28% of sequence reads) and Nematoda (12% of sequence reads), at sand beach characterized byhigh temperatures (> 25°C), high salinity (>31.5 ppt), and calm waters. Further, comparing sand beaches and tide pools, we observed differences on abundance of sequences, assemblage composition, and diversity, on local and regional scales, supporting our hypothesis of different meiofauna composition and lower diversity and abundance of reads at tide pools.

Keywords: tide pools; beach; meiofaunal assemblage; environmental DNA; phylogenetic diversity

INTRODUCTION

In benthic marine communities, spatial-temporal diversity patterns are mostly driven by substrate and oceanographic parameters (Blanchette et al., 2008; Griffiths et al., 2017; Mazzuco et al., 2019;2020). It is recognized that sediment grain size, coastal hydrodynamics, and food availability are typical drivers of meiofaunal communities (Giere, 2009). However, meiofaunal taxa may have specific adaptations and respond differently to environmental conditions, due to their differential ability of dispersion, locomotion, nutrition, development and reproduction (Curini-Galletti et al., 2012). Additionally, temperature can act as main driver on intertidal benthic communities, reducing their diversity, when it surpasses species' physiological limits (Vafeiadou et al., 2018; Starko et al., 2019; Mazzuco et al., 2020). In tropical humid regions, rainfall may additionally work as a major factor structuring meiofauna diversity in tropical sandy beaches (Gomes and Rosa-Filho, 2009; Venekey et al., 2014; Baia and Venekey, 2019).

Sandy beaches form an intricate ecosystem between marine and terrestrial environments, with a large diversity of organisms supporting important biogeochemical processes (Wu et al., 2018; Okamoto et al., 2022). Sandy beaches are influenced by global and local oceanographic processes, which in turn shape the community structure of these habitats. In addition, sandy beaches are under a range of anthropogenic impacts (including climate change) with signs of declining diversity in numerous areas worldwide(Bellwood et al., 2004; Aued et al., 2018). Understanding how marine diversity varies at local and regional scales contributes to the conservation ofthese ecosystems (Gaston et al., 2000; Underwood et al., 2000). Also, understandingwhich environmental factors are the main drivers of marine diversity and abundance, including spatio-temporal variations, is critical to establish a strong baseline that can be used in future comparisons.

The tidal effect along the intertidal zone may create isolated mesocosms of permanently immersed habitats, called tide pools (Mendonça et al., 2018). Tide pools are important habitats on the intertidal marine environment providing shelter and food resources to small animals (Vinagre et al., 2015; Dias et al., 2016). However, this habitat may have extreme environmental conditions (e.g., excessively high temperatures), potentially acting as an ecological trap as some organisms cannot leave the tidepools during the low-tide (Vinagre et al., 2018). Thus, it is possiblethat this mesocosm with different environmental conditions and pressures would hold benthic assemblages with distinct composition and lower diversity than nearby ecosystems suchas sand beaches.

Meiofauna is composed by organisms ranging from 42 to 500 µm, comprising at least 22 phyla, and often displaying high abundance and diversity in marine benthic systemas (Higgins andTiel, 1988; Giere, 2009) (McIntyre, 1969; Higgins & Thiel 1988; Hakenkamp and Palmer, 2000). These organisms play crucial ecological roles in the marine sediment, such as nutrient recycling, and transferring energy and matter into benthic and pelagic trophic food webs, linking different trophic levels (Coull, 1999; Giere, 2009). Due to its ecological importance, meiofaunal communities reflects the overall health of the marine benthos and are considered excellent bioindicators to monitoring marine environmental health, and testing general ecological hypotheses (Bonaglia et al., 2014).

Meiofaunal organisms may have a strong direct impact on benthic properties, modifying interactions between macrofaunal species and the environment (Zeppilli et al., 2015). In some shallow marine environments, such as tidal flats, meiofaunal secondary production may exceeds macrofaunal production (Warwick et al., 1979; Kuipers et al.,

1981), contributing up to 40% of the total benthic metabolism in sandy beaches (Fenchel, 1978). Previous studies have demonstrated that meiofaunal communities respond to warming in aquatic ecosystems (O'Gorman et al., 2012; Gingold et al., 2013), causing the mortality of dominant species in subtropical environments (Gingold et al., 2013), changes in biomass (Alsterberg et al., 2011), and altering body-size structure (Jochum et al., 2013).

In sandy beaches, the distribution and abundance of infaunal benthos are expected to respond to the swash climate and sediment characteristics (McLachlan et al., 1993). Wave action also play an important role on spatial variability (i.e., patchiness) of density and diversity due to the hydrodynamic stress (Covazzi et al., 2001). Along the intertidal zone of sandy beaches, temperature and salinity are highly variable and can also influence on the distribution and composition of organisms (Olafsson, 1991). The sand beach meiofauna is distributed mostly in the upper 2 (60-70% of individuals) to 7 centimeters (>95% of individuals) (Tietjen, 1969), but with seasonal variations (Coull and Bell, 1979).

Nematodes and copepods (Harpacticoida) are usually the most abundant meiofaunal taxa in sand beach sediments worldwide, although in some cases other taxa, may take over the first or second place (Hogue, 1978). Altaff et al. (2005) reported the meiofaunal assemblage, at Marina beach in India, to be composed by turbellarians, nematodes, polychaetes, oligochaetes, and harpacticoids. In a DNA-based study from intertidal sediments, Castro et al. (2021) found Annelida and Nematoda as the most frequent taxa using 18S high throughput sequencing. Albuquerque et al. (2007) found an assemblage composed by 12 taxa (Tardigrada, Nematoda, Copepoda, Turbellaria, Oligochaeta, Polychaeta, Halacaridae, Collembola, Ostracoda, Gastrotricha, Isopoda, and Cnidaria), dominated by tardigrads and nematodes (92% of total taxa) in a sandy beach in SE Brazil. Neves and Costa (2022) found meiofaunal assemblages dominated by Copepoda and Nematoda (69% of the total taxa) in SE Brazil. In tropical areas seasonal changes are less markedly defined, but meiofaunal organisms show some seasonality, with greater abundance during the warmest/rainy months (Coull, 1988; Albuquerque et al., 2007).

Predicting changes in diversity patterns from local to global scales is a research prime concern in a scenario of global environmental change, and it has been added to the protocols of diverse ocean observatories (Muller-Karger et al., 2017; Bax et al., 2019; Mazzuco et al., 2020). To predict how theseassemblages will respond in the future, firstly is necessary to understand the drivers of local-scale diversity patterns, and how organisms respond to environmental parameters and seasonality. Here, we aimed to assess meiofaunal diversity in a tropical sandy beachand tide pools to test whether or not (i) the phylogenetic diversity is influenced by seasonality; (ii) if the local diversity and assemblage composition would be related to regional (larger- scale influences) in marine seascapes; (iii) tide pools would host a subset of meiofaunal taxa from nearby sand beaches, thus revealing ecological filtering. We addressed the following hypotheses on this study: (i) meiofaunal phylogenetic diversity and abundance of sequences is higher during warmer months in Gramuté beach; (ii) tide pools have different assemblage composition than sand beaches, with lower abundance of sequences and phylogenetic diversity.

MATERIAL AND METHODS

Study area and sampling

The study was carried out at two sand beaches on the coast of Espirito Santo State thathave similar geomorphology, Gramuté and Rio Preto, both located within a marine protected area in the Eastern Brazilian Marine Ecoregion (Figure 1A). The sand beaches arelocated in a tropical region marked by dry winters and rainy summers (Bernardino et al.,2015), with sea surface temperatures ranging between 21°C and 27°C, and salinity ranging from
34.6 to 36 ppt (Quintana et al., 2015; Mazzuco et al., 2019;2020). This area has experienced significant warming on the last 40 years (Bernardino et al., 2015; Mazzuco et al., 2020).

The study was divided in two distinct sampling strategies. The first was atemporal assessment that lasted for one year (December 2019 to November 2020) in Gramuté beach. Sediment samples were collected in triplicate in three stations 20 metersdistant from each other (n = 9 sediment samples per month) in the subtidal zone, alwaysduring the low-tide (Figure 1B). Sediment samples were collected monthly during springtides over all seasons during the sampling period (Summer = December 2019 to February2020; Autumn = March 2020 to May 2020; Winter = June 2020 to August 2020; Spring
= September 2020 to November 2020).

The second sampling strategy consisted in testing the occurrence of ecological filtering between beach and tide pools within the marine protected area. To achieve that, we collected sediment samples monthly (n = 3 per season) during winter (June 2020 to August 2020) and spring (September 2020 to November 2020) at Gramuté beach and Rio Preto beach. The samples were collected in triplicate in three stations 20 meters distant from each other (n = 9 sediment samples per month) in the subtidal zone (spring tides), and inside three tide pools 20 meters distant from each other (n = 9 sediment samples per month).

All sediment samples were collected manually using sterile, DNA-free corers. Additionally, we collected samples for sediment analysis (grain size, total organic matter, carbonate content and sedimentary organic biopolymers) in both assessments, at Gramuté beach and Rio Preto beach, in the beach subtidal zone and inside tide pools. All samples were transported in thermic bags with ice, and stored at -20°C until analysis. Sea surface temperature and salinity were additionally measured *in situ*.

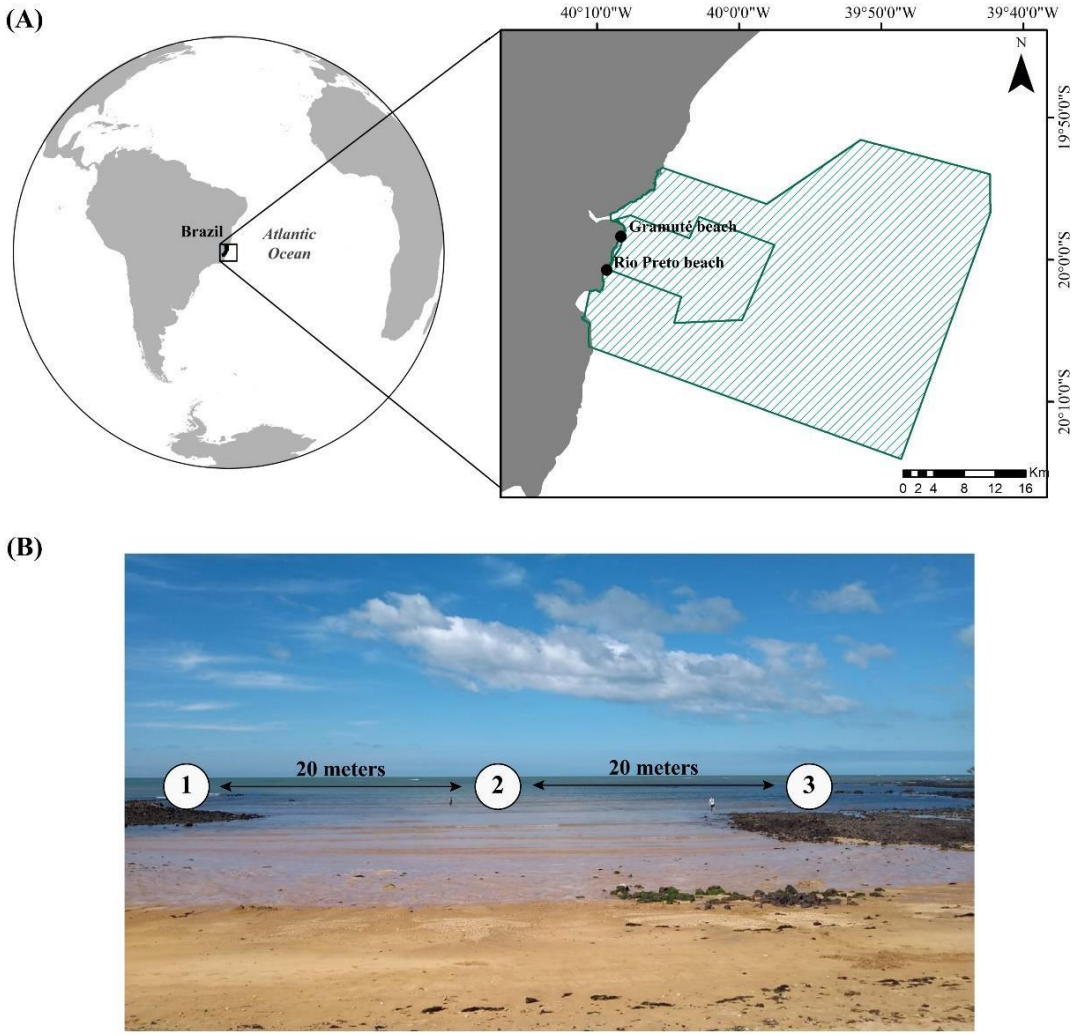


Figure 1 (A) Location of sampling sites at Gramuté and Rio Preto beach in the SE Brazilian coast, within the marine protected areas Refúgio da Vida Silvestre de Santa Cruz and Área de Proteção AmbientalCosta das Algas (polygon areas) **(B)** sampling design in Gramuté beach replicated at Rio Preto beach,on seasonal assessments and with the tide pools sampling.

Seascape Characterization

The use of satellite observation has improved the temporal monitoring of oceans for ecological and conservation applications, which is still poorly explored for coastal ecology purposes (Mazzuco and Bernardino, 2022). This approach can be used to support and to predict the loss of biodiversity in coastal ecosystems, providing crucial information to manage and mitigate global ocean changes with greater detail (Fagundes etal., 2020). The Marine Biodiversity Observation Network (MBON) Seascapes is obtained from satellite data measurements and modeled data that integrates multiple oceanic variables (sea surface temperature – SST, sea surface salinity – SSS, absolute dynamic topography – ADT, chromophoric dissolved organic material – CDOM, surface chlorophyll-a – CHLA, and normalized fluorescent line height – NFLH) to a categorization system of 33 water masses. This categorizationis designed to support marine observation networks with high resolution, and is considered a promising toolto assess and predict biological processes (Montes et al., 2020).

Oceanographic conditions were characterized through variation in MBON Seascape Pelagic Habitats Classification (Kavanaugh et al., 2014; 2016), as previously

described in Mazzuco and Bernardino (2022). This database is available on NOAA Coast and Ocean Watch Programs, at monthly frequency and along a 5Km² grid (Kavanaugh et al., 2014; 2016). We characterized the seascapes for the Área de Proteção Ambiental Costa das Algas (local scale ~ 30 km coastline, 465 Km², Longitude − 40.3º to − 39.8º, Latitude 20.3º to 19.8º) on a monthly basis during the study period (December 2019 – November 2020).

Sediment analysis

Sediment samples were dried at 60°C for 48 hours before all granulometric analysis. We macerated the dry sediment and sieved in mesh openings of -1.5 Φ to 4 Φ (with 1Φ intervals) in a sieve shaker. We determinate the carbonate contents of sediment by muffle combustion at 550° C for 4 h with an additional hour at 800° C, and we quantified total organic matter (TOM) gravimetrically by the weight loss after combustion (500 ◦C for 3 h) (Suguio, 1973).

To analyze sedimentary organic biopolymers (proteins, carbohydrates, and lipids), we followed the procedures of Danovaro (2010). Total protein analysis (PRT) was conducted after extraction with NaOH 0.5 M and determined according following Hartree (1972) modified by Rice (1982) to compensate for phenol interference. We analyzed total carbohydrates (CHO) according to Gerchacov and Hatcher (1972). Total lipids (LIP) were extracted from 1 g of homogenized sediment lyophilized by ultrasonication in 10 ml of chloroform: methanol (2:0 1 v/v) and analyzed following Marsh and Weinstein (1966). We carried out blanks for each analysis with pre-combusted sediments at 450 and 480° C for 4 h. The concentrations of PRT, CHO and LIP were expressed as bovine serum albumin, glucose and tripalmitin equivalents, respectively. We performed all analyzes in triplicate, and converted the concentrations of PRT, CHO and LIP to carbon equivalents using a conversion factor of 0.49, 0.40 and 0.75, respectively (Fabianoand Danovaro, 1994). We report the sum of protein, lipid and carbohydrate carbon equivalents as biopolymeric carbon (BPC) (Fabiano et al., 1995). Additionally, we used protein to carbohydrate (PRT: CHO) and carbohydrate to lipid (CHO: LIP) ratios to assessthe state of biochemical degradation processes (Galois et al., 2000).

DNA extraction and sequencing

Preceding DNA extraction, we elutriated the sediment samples on sieves using 45 µm mash, and aliquoted them to 20 mL in Falcon tubes, following Brannock and Halanych (2015) protocol. To elutriate sediment samples, we used 1L flask filled with 950 mL of filtered seawater. The flasks were let to sit before decanting the liquid over the sieve. This procedure was repeated ten times, and the sediment retained on the sieve was rinsedto Falcon tubes and then centrifuged at room temperature for 3 minutes at 1342 X g, usingan Eppendorf Centrifuge 5430. After that we separated 1mL aliquots on sterile 1.5mL tubes and stored them at -20°C (Brannock and Halanych, 2015) (Figure 2). All glasswarewere sterilized between samples to avoid cross contamination. Using the 1mL aliquots of elutriated sediment, we extracted the DNA using the PowerSoil DNA® (Qiagen) kit following the manufacturer's instructions. We verified the DNA integrity in 1% agarosegel, and the purity using NanoDrop One spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). DNA concentration was measured using a Qubit® 4 Fluorometer (Life Technologies-Invitrogen, Carlsbad, CA, USA). Metabarcoding

sequencing and amplicon libraries were carried out by ©NGS Genomic Solutions (Piracicaba, SP, Brazil) using the HiSeq Illumina platform (2 x 250 bp). We sequenced the V9 hypervariable region from 18S SSU rRNA gene using primers Euk_1391 forward (GTACACACCGCCCGTC) and EukBr reverse (TGATCCTTCTGCAGGTTCACCTAC) (Medlin et al., 1988; Amaral-Zettler et al., 2009; Stoeck et al., 2010).

Bioinformatic pipeline

We used QIIME2 2022.8 software to identify taxonomically the sequences with the demultiplexed raw paired-end reads (Bolyen et al., 2018). We imported FastQC files as QIIME2 artifacts, denoised them via DADA2 (Callahan et al., 2016) using the *denoise-paired* plugin, and removed low-quality bases and primer sequences.

We used the machine learning Python library *scikit-learn* to determine the taxonomic composition of the amplicon sequence variants (ASV) generated by the DADA2 plugin (Pedregosa et al., 2011). To identify taxonomically sequences, we used a pre-trained Naïve Bayes classifier, trained on Silva 132 database (Quast et al., 2013) clustered at 99% similarity. We normalized the datasets to allow analysis and comparisons with homogeneous sampling depth. We used the minimum sampling depth (1384 reads for seasonal assessment in Gramuté beach, and 363 reads for tide pools *vs.* sand beach assessment), and resampled each station to the same depth, then these filtered/subsampled datasets were used to calculate all diversity metrics. Additionally, we plot rarefaction curves.

Additionally, we calculated the Faith's Phylogenetic Diversity (PD) for each sample using the *diversity core-metrics-phylogenetic* pipeline, which is based on a phylogenetic tree generated by the *align-to-tree-mafft-fasttree* pipeline from the *q2-phylogeny* plugin in QIIME2. We calculated Shannon diversity as well, using the *qiime diversity alpha* pipeline by setting the *p-metric* parameter to "*shannon*". The raw sequences data are deposited in NCBI (SRR24675047).

Statistical analysis

Only meiofaunal sequences were used on statistical analysis, and here we considered temporary meiofaunal taxa (taxa that can be representative meiofauna at any stage of life, and play important role in the sediment) as meiofaunal organisms (Higgins and Tiel, 1988; Giere, 2009).

We performed Permutational Analysis of Variance (PERMANOVA) to compare environmental (temperature, salinity, carbonate content, grain size, total organic matter and its biopolymeric composition), seascape coverage, and meiofaunal data (diversity metrics, and abundance of sequence reads) between seasons (Summer, Autumn, Winter, and Spring) in Gramuté beach (Anderson et al., 2008). A canonical analysis of principal coordinates (CAP; Anderson & Willis, 2003) ordination plot was made with environmental variables (temperature, salinity, grain size, carbonate, organic matter, biopolymers) and the meiofaunal assemblage composition (square-root transformed).

PERMANOVA (Anderson et al., 2008) was also performed to compare meiofaunal assemblage composition under spatial (habitat - beach and tide pools; location - Gramuté and Rio Preto beach) and seasonal scales (season - spring and winter). We used a Student's *t*-test (Student, 1908; Mann and Whitney, 1947) to assess differences in environmental parameters, diversity metrics, and abundance of sequence reads, for each pair of factors. Additionally, a Similarity Percentage Routine (SIMPER) was applied to

analyze the contribution of each taxonomic group to the assemblage composition dissimilarity between sand beach and tide pools datasets (Clarke, 1993).

Significant differences were defined when $p<0.05$. All graphical and analytical processes were performed in R environment (R Core Team, 2022).

RESULTS

Seasonal variability at Gramuté sandy beach

Seasonal differences were observed over the year on environmental variables (temperature, salinity, grain size, carbonate, organic matter, biopolymers), which correspond to seasonal changes at Gramuté beach, SE Brazil (df = 3; Pseudo-F = 5.623; p = 0.001; Table 1). Sea surface temperature varied from 23.9 °C during winter to 28.0 °C in summer. The sediment is completely composed by sand, and its carbonate content range from 19% during autumn to 64% in spring. Total organic matter (TOM) concentration presented huge variance on Spring, with the lowest and highest values during the year (3.7% and 19.1%). The content of labile fraction of organic matter, which is represented by the biopolymeric carbon (BPC), had its highest peak during Winter (1562.6 mgC/g), and lower during Spring (241.1 mgC/g), with similar quality between all seasons.

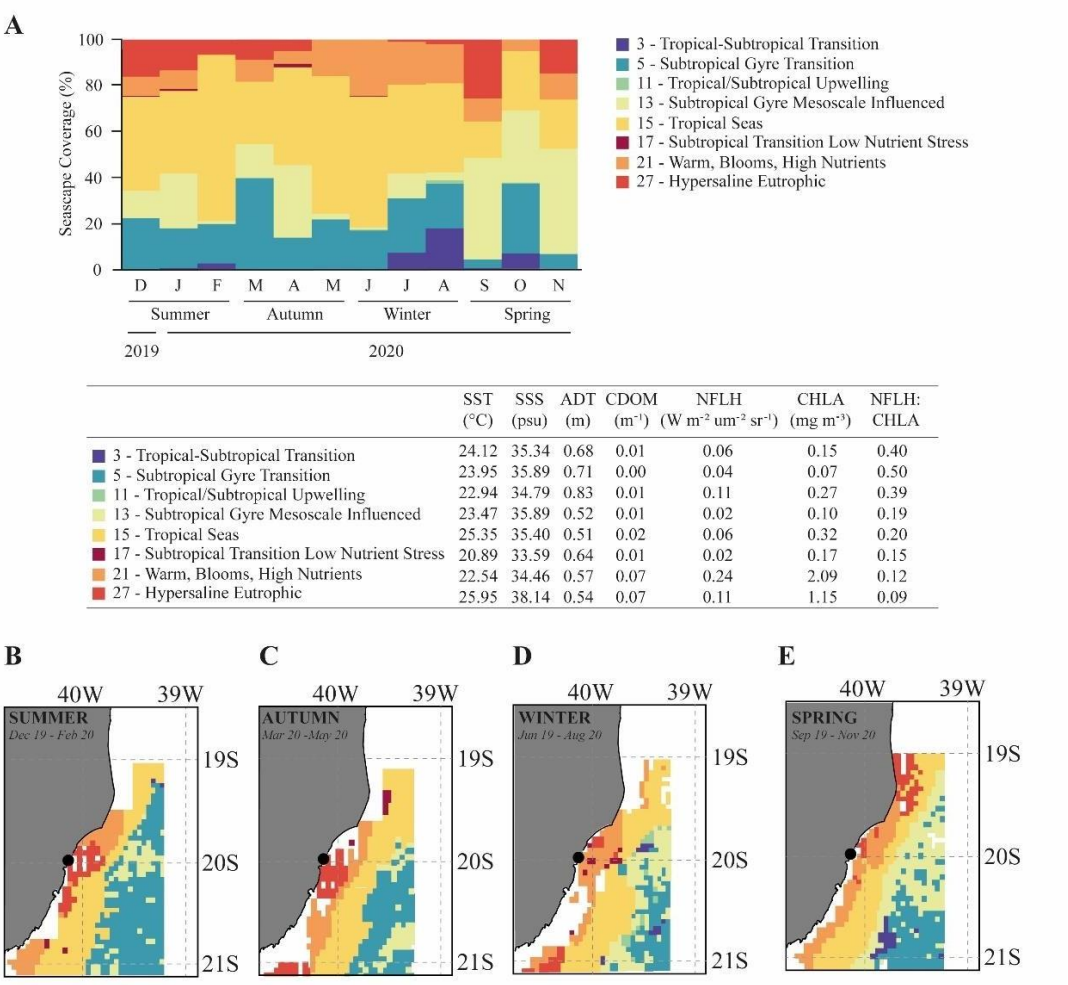
Table 1 Permutational Multivariate Analysis of Variance results from environmental data (temperature, salinity, grain size, carbonate, organic matter, biopolymers) collected in Gramuté beach, SE Brazil, during all seasons (summer, autumn, winter and spring). Significative results are considered when p<0.05, and are presented in bold. df = Degrees of Freedom; SS = Sum of Squares; MS = Mean of Squares.

Source	df	SS	MS	Pseudo-F	*p*
Season	3	182.48	60.826	5.623	**0.001**
Residual	32	343.52	10.704		
Total	35	525.00			

Overall, the Seascapes categories in this region are characterized by high sea surface temperature (SST > 20.9°C), high sea surface salinity (SSS> 33.6 psu) and calm waters (absolute dynamic topography - ADT ranging from 0.51 to 0.83m). The seascapes have wide ranges in dissolved organic matter (CDOM; 0.00 to 0.07 m^{-1}), chlorophyll-a concentration (CHLA; 0.07 to 2.09 $mg.m^{-3}$), and fluorescence (NFLH; 0.02 to 0.24 $W.m^{-2}.um^{-2}sr^{-1}$) (Figure 2). We observed changes in the frequency of seascapes in the studied area along the year (Table 2). Seascapes Tropical Seas (class 15 – 38.4%), Subtropical Gyre Transition (class 5 – 19.0%), Subtropical Gyre Mesoscale Influenced (class 13 – 18.3%), and Warm, Blooms, High Nutrients (class 21 – 12.4%) were the most frequent, with more than 80% of coverage during the study period (Figure 2). Summer (Dec – Feb), Autumn (Mar – May) and Winter (Jun – Aug) were dominated by the Seascape Tropical Seas (class 15), with 40.9%, 43.1% and 45.1% of coverage respectively (Figure 2). Although, Spring (Sep – Nov) was dominated by the seascape Subtropical Gyre Mesoscale Influenced (class13) with 42.7% of coverage (Figure 2).

Table 2 Permutational Multivariate Analysis of Variance results from MBON Seascapes coverage at local scale (~ 30 km coastline, 465 Km^2) at SE Brazil, during all seasons (summer, autumn, winter and spring). Significative results are considered when p<0.05, and are presented in bold. df = Degrees of Freedom; SS = Sum of Squares; MS = Mean of Squares.

Source	df	SS	MS	Pseudo-F	*p*
Season	3	120.12	40.041	8.014	**0.001**
Residual	32	159.88	4.996		
Total	35	280.00			



	SST (°C)	SSS (psu)	ADT (m)	CDOM (m^{-1})	NFLH (W m^{-2} um^{-2} sr^{-1})	CHLA (mg m^{-3})	NFLH: CHLA
3 - Tropical-Subtropical Transition	24.12	35.34	0.68	0.01	0.06	0.15	0.40
5 - Subtropical Gyre Transition	23.95	35.89	0.71	0.00	0.04	0.07	0.50
11 - Tropical/Subtropical Upwelling	22.94	34.79	0.83	0.01	0.11	0.27	0.39
13 - Subtropical Gyre Mesoscale Influenced	23.47	35.89	0.52	0.01	0.02	0.10	0.19
15 - Tropical Seas	25.35	35.40	0.51	0.02	0.06	0.32	0.20
17 - Subtropical Transition Low Nutrient Stress	20.89	33.59	0.64	0.01	0.02	0.17	0.15
21 - Warm, Blooms, High Nutrients	22.54	34.46	0.57	0.07	0.24	2.09	0.12
27 - Hypersaline Eutrophic	25.95	38.14	0.54	0.07	0.11	1.15	0.09

Figure 2 Monthly **(A)** and seasonal **(B – E)** variation in Seascapes coverage (%) between December 2019 to November 2020 in Gramuté beach, SE Brazil. Mean oceanographic values from oceanographic variables that identify each MBON Seascape water mass (class). SST - sea surface temperature, SSS - sea surface salinity, ADT - absolute dynamic topography, CDOM - chromophoric dissolved organic material, CHLA - chlorophyll-a, NFLH - normalized fluorescent line height.

We identified a total of 9,962 sequences from meiofaunal taxa in Gramuté beach. We observed significative differences in meiofaunal assemblage composition (df = 3; Pseudo-F = 2.353; p = 0.001; Table 3) and abundance of sequence reads between seasons (H = 12.884; df = 3; p = 0.005; Figure 3A) with lower abundance during spring (565 sequence reads) and higher on summer (3347 reads; Figure 3A). The abundance of sequence reads in winter (3160 reads; Figure 3A) was similar to the summer, and the number of sequence reads in autumn was similar to all other seasons (Figure 3A).

Table 3 Permutational Multivariate Analysis of Variance results from meiofaunal composition at Gramuté beach, SE Brazil, during all seasons (summer, autumn, winter and spring). Significative results are considered when p<0.05, and are presented in bold. df = Degrees of Freedom; SS = Sum of Squares; MS = Mean of Squares.

Source	df	SS	MS	Pseudo-F	*p*
Season	3	11842	3947.5	2.353	**0.001**
Residual	32	53682	1677.6		
Total	35	65526			

Overall, meiofaunal assemblage in Gramuté beach was mainly composed by Crustacea (46% of sequence reads), Annelida (28% of sequence reads) and Nematoda (12% of sequence reads). Crustacea and Annelida dominated the assemblage during summer (35% and 40% of reads, respectively), autumn (43% and 34% of reads, respectively), and spring (59% and 27% of reads). During winter, the most abundant taxa were Crustacea (57% of reads) and Nematoda (17% of reads) (Figure 3B). Nemertea were not detected duringautumn, Gastrotricha was not detected in spring, and Rotifera was not detected in neither.

Rarefaction curves suggest that the number of meiofaunal taxonomic groups detected during Spring was lower when compared to summer, autumn, and winter (Figure 4). The meiofaunal assemblage differ significantly between the sampled seasons in Gramuté beach (df = 3; Pseudo-F = 2.353; p = 0.001; Figure 5; Table S1). Dissimilarity levels ranged from 49.7% (between winter and summer) to 68.6% (between autumn and summer), and winter had distinct assemblage composition compared to the others sampled seasons. SIMPER analysis revealed that Annelida (ranging from 16.5% to 28.3%; Table S1, Crustacea (ranging from 21.8% to 26.7%; Table S1) and Nematoda (ranging from 13.9% to 21.8%; Table S1 were the taxa that most contributed to the differences among all seasons. Platyhelminthes contributed 15.4% to the total dissimilarity of 49.5% between autumn and spring (Table S1).

We observed significative differences on diversity patterns among seasons in Gramuté beach. Spring presented the lower phylogenetic diversity (9.23±1.88) when compared to autumn (11.88±1.82), summer (17.93±3.11) and winter (19.37±4.85) (F = 15.179; df = 3; p < 0.001; Figure 3C; Table S2). The same pattern was observed with Shannon diversity, spring had the lowest diversity (2.38±0.91) when compared to the other seasons (F = 14.930; df = 3; p < 0.001; Figure 3D; Table S2)

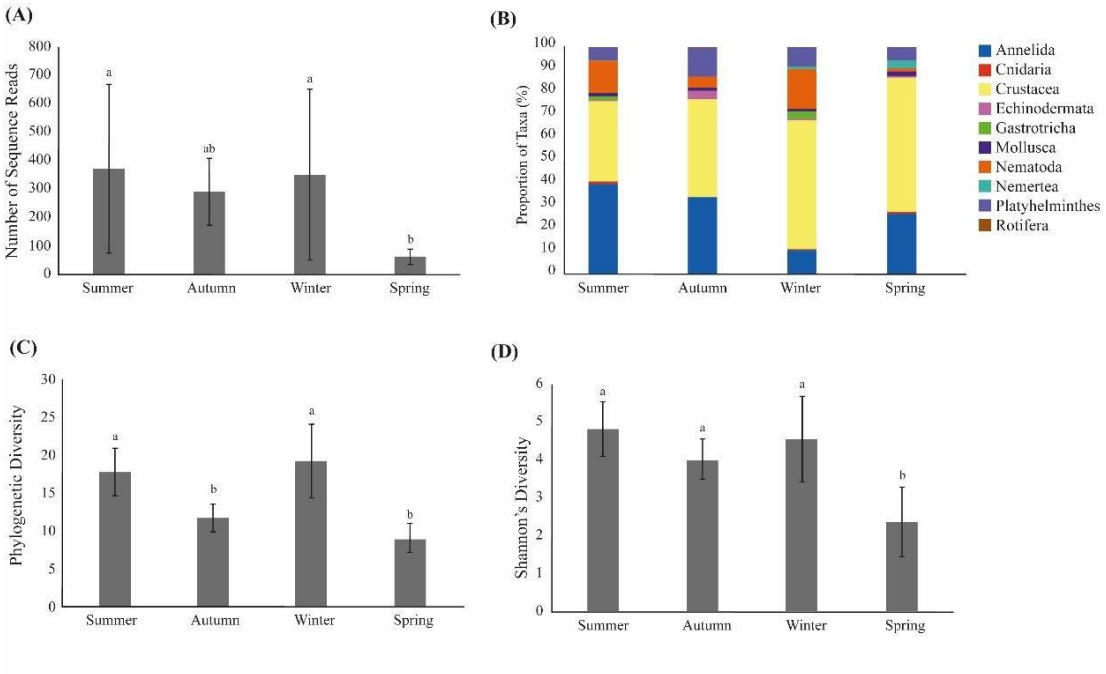


Figure 3 (A) Number of meiofaunal sequence reads (mean±SD) **(B)** Meiofaunal taxa proportion (%) **(C)** Faith's Phylogenetic Diversity (mean±SD) **(D)** Shannon's Diversity index (mean±SD), obtained after metabarcoding sediment samples from Gramuté beach, SE Brazil, in each season. Different letters represent significative statistical differences (p<0.05)

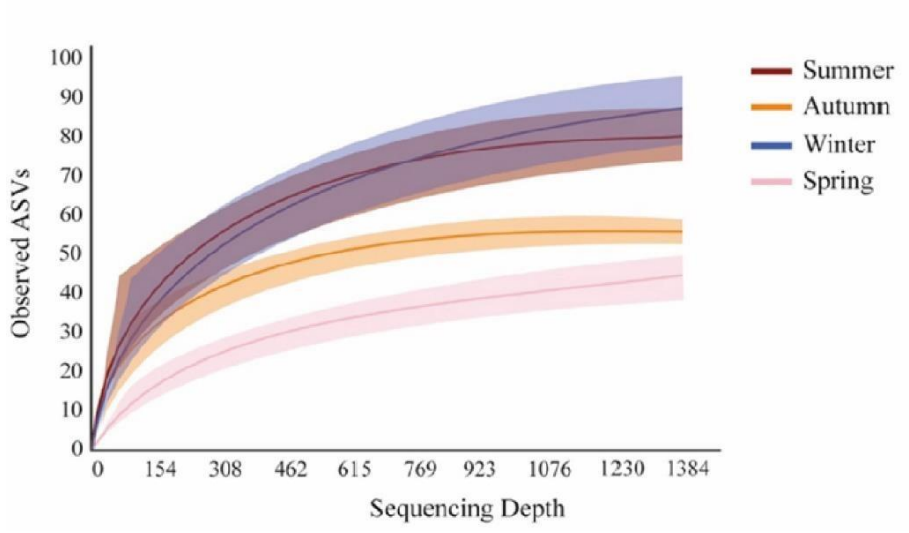


Figure 4 Rarefaction curves obtained from sediment samples metabarcoding collected at Gramuté beach, SE Brazil, during all seasons on a 1-year sampling. Solid lines represent a mean of observed ASVs at each sampling depth, and the shaded area represents the standard deviation.

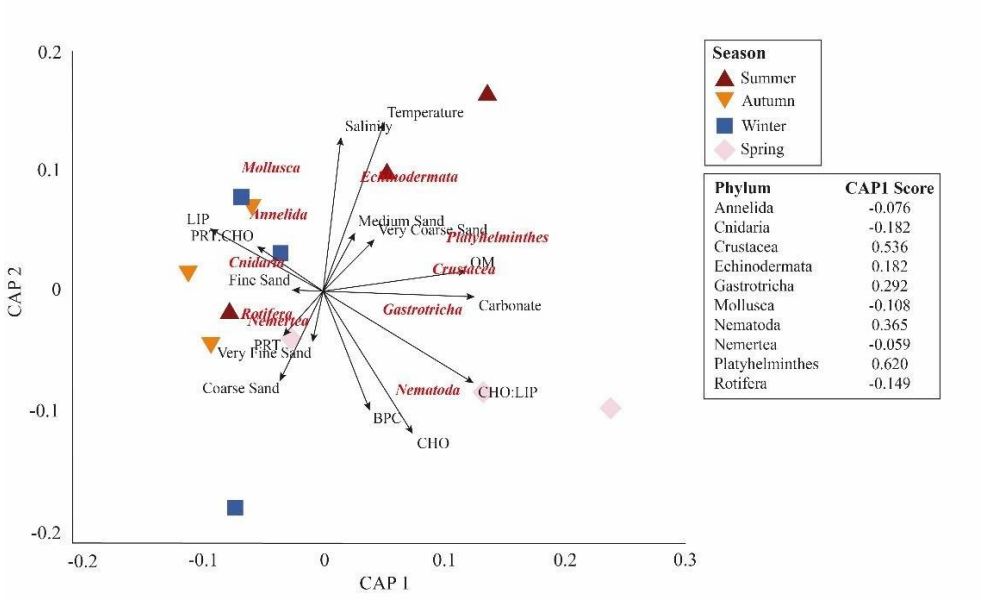


Figure 5 Canonical Analysis of Principal Coordinates (CAP) of assemblage composition and environmental variables (temperature, salinity, grain size, carbonate, organic matter, biopolymers) at Gramuté beach, SE Brazil, during all seasons.

Sand beaches vs. tide pools

Our second protocol to study ecological filtering in tide pools revealed marked spatial differences (location or regional scale) between the sites studied (Gramuté and Rio Preto beach; Pseudo-F = 17.607; df = 1; p = 0.001; Table 4). We observed higher temperature, sediment carbonate, TOM content, CHO and BPC contents, and CHO:LIP ratios at Gramuté beach (Table 5). Otherwise, Rio Preto beach presented higher (1.6-fold) sedimentary LIP content (t = -2.476; df = 22; p = 0.021; Table 5), and PRT:CHO ratio (t = -5.157; df = 22; p<0.001; Table 5).

At the scale of habitats (sand beach and tide pool) we also observed significant environmental differences (df = 1; Pseudo-F = 5.288; p = 0.001; Table 4). The sediment grain size was mainly composed of sand at both habitats (71.3% to 74.3%), but the water temperature was on average 1.8°C higher inside tide pools (T = 105.00; df = 22; p = 0.010; Table 5). We did not find statistical differences in the sediment total organic matter content, but LIP content was 1.87-times higher inside tide pools (t = -3.804; df = 22; p < 0.001; Table 5), as well as BPC content, that was 1.49-times higher (T = 114.00; df = 22; p = 0.040; Table 5). This suggests that tide pools and nearby sand beaches hold distinct sedimentary organic matter quantity and quality.

In addition, we observed seasonal (winter x spring) differences in water temperature and salinity (Pseudo-F = 3.856; df = 1; p = 0.007; Table 4). Spring had higher temperature (26.8±1.8 °C) in comparison to winter (25.5±1.4 °C) (p = 0.031; Table 4), that presented higher salinity (32.5±0.50 ppt) (p = 0.038; Table 5).

Table 4 Permutational Multivariate Analysis of Variance results from environmental data (temperature, salinity, grain size, carbonate, organic matter, biopolymers) comparisons between sand beach and inside tide pools (habitat), at Gramuté beach and Rio Preto beach (location), SE Brazil, during different seasons (spring and winter). Significative results are considered when p<0.05, and are presented in bold. df = Degrees of Freedom; SS = Sum of Squares; MS = Mean of Squares.

Source	df	SS	MS	Pseudo-F	*p*
Season	1	21.044	21.044	3.856	**0.007**
Habitat	1	28.863	28.863	5.288	**0.001**
Location	1	96.096	96.096	17.607	**0.001**
Season x Habitat	1	11.561	11.561	2.118	**0.044**
Season x Location	1	8.094	8.094	1.483	0.190
Habitat x Location	1	11.799	11.799	2.161	**0.048**
Season x Habitat x Location	1	11.217	11.217	2.055	0.060
Residual	16	87.325	5.458		
Total	23	276			

Table 5 Environmental variables (mean±SD) obtained from sediment samples from sand beach region and tide pools (habitat) at Gramuté beach and Rio Preto beach (location) during winter and spring (season). Statistical significative differences (p<0.05) are presented in bold.

Variable	Habitat		*p*
	Beach	Tide Pool	
Temperature (°C)	25.2±0.8	27.0±2.0	**0.010**
Salinity (ppt)	31.9±0.7	32.4±0.5	0.075
%Sand	71.3±31.9	74.3±28.3	0.399
%Gravel	28.7±9.2	25.7±8.2	0.399
Carbonate	0.32±0.20	0.50±0.31	0.112
TOM	5.5±5.3	7.6±6.6	0.583
PRT	92.1±22.3	102.9±26.6	0.295
CHO	1272.4±1114.5	1715.2±498.1	0.583
LIP	317.4±192.4	594.5±163.3	**<0.001**
BPC	792.1±413.9	1182.3±685.9	**0.040**
PRT:CHO	0.13±0.09	0.13±0.10	1.000
CHO:LIP	26.5±15.8	3.3±1.0	0.371
	Location		*p*
	Gramuté beach	Rio Preto beach	

Temperature (°C)	26.9±1.7	25.4±1.0	**0.029**
Salinity (ppt)	32.3±0.7	32.0±0.7	0.314
%Sand	95.4±2.6	50.2±24.3	**<0.001**
%Gravel	4.6±2.6	49.8±24.3	**<0.001**
Carbonate	0.62±0.14	0.20±0.19	**<0.001**
TOM	11.1±5.3	2.0±0.5	**<0.001**
PRT	92.3±23.4	102.7±26.7	0.313
CHO	2341.0±1228.9	646.6±500.2	**<0.001**
LIP	353.2±192.0	558.6±166.6	**0.021**
BPC	1246.5±519.2	727.9±214.8	**0.026**
PRT:CHO	0.06±0.04	0.20±0.08	**<0.001**
CHO:LIP	28.3±16.1	1.3±1.2	**<0.001**
	Season		*p*
	Winter	Spring	
Temperature (°C)	25.5±1.4	26.8±1.8	**0.031**
Salinity (ppt)	32.5±0.50	31.8±0.7	**0.038**
%Sand	83.0±23.1	62.7±32.7	0.093
%Gravel	17.1±6.7	37.3±32.7	0.093
Carbonate	0.43±0.28	0.38±0.28	0.436
TOM	6.9±6.0	6.3±6.2	0.707
PRT	95.4±68.2	100.2±26.0	0.596
CHO	434.0±180.0	1132.1±885.6	0.707
LIP	825.2±746.3	502.0±179.0	0.326
BPC	839.2±243.2	878.5±349.3	0.751
PRT:CHO	0.14±0.09	0.12±0.10	0.436
CHO:LIP	2.6±2.0	27.0±15.7	0.237

We observed significant differences in the meiofaunal assemblage composition between both locations (Gramuté and Rio Preto sand beaches; Pseudo-F = 2.24; df = 1; p = 0.008; Table 6). Gramuté beach had a higher number of sequence reads (104.61±34.75) when compared to Rio Preto (17.97±4.7; p<0.001; Table 7). At Gramuté beach, the meiofauna was dominated by Crustacea (57% of reads), Nematoda (15% of reads), and Annelida (13% of reads), but it also included Mollusca, Gastrotricha, Echinodermata, Cnidaria, Platyhelminthes, and Nemertea. At Rio Preto beach, however, we only detected specimens of Crustacea (57% of reads), Mollusca (32% of reads), and Nematoda (11% of reads). This difference on assemblage composition, resulted on a 2.31-times higher phylogenetic diversity at Gramuté beach (p<0.001; Table 7)

We observed significative differences on assemblage composition and read count between habitats (sand beach and tide pool) (Pseudo-F = 3.15; df = 1;p = 0.001; Table 6), with higher (7.2-fold) abundance of reads in sand beaches(113.33±18.89) when compared to nearby tide pools (15.78±8.22) (p = 0.001; Figure 6A; Table 7). The sand beach meiofauna was dominated by Crustacea (55.4%), Nematoda (14.3%), and Annelida (12.3%), meanwhile, tidepools were dominated by Crustacea (82.5%) and Nematoda (16.1%), with Annelida representing only 1.4% of readsat this habitat. We identified reads from Mollusca, Gastrotricha, Echinodermata, Cnidaria, Platyhelminthes, and Nemertea only at sand beach (Figure 6B).

SIMPER analysis revealed that tide pools and sand beach meiofaunal assemblages are on average 91% dissimilar. These differences are mostly due to the abundance of Crustacea (47.6%), Nematoda (16.1%), Annelida (15.2%), and Mollusca (10.1%), that were significantly more abundant in sand beaches (Table 8). The rarefaction curves suggest that at tide pools meiofaunal composition represents a fraction of the entire diversityof taxa found at sand beaches (Figure 7). Overall, we did not observe significant differences in the meiofaunal phylogenetic diversity (T = 1323.00; p = 0.175; Figure 6C) or Shannon diversity between tide pools and sand beaches (T = 1339.00; p = 0.783; Figure 6D). However, we observed local-scale differences between tide pools and sand beaches in Gramuté beach, which had higher phylogenetic diversity (14.1±6.1) at the sand beach when compared to the nearby tide pools (8.4±3.3; T = 369.00; p = 0.002).

Table 6 Permutational Multivariate Analysis of Variance results from meiofaunal data comparisons between sand beach and inside tide pools (habitat), in Gramuté beach and Rio Preto beach (location), SE Brazil, during different seasons (spring and winter). Significative results are considered when p<0.05, and are presented in bold. df = Degrees of Freedom; SS = Sum of Squares; MS = Mean of Squares.

Source	df	SS	MS	Pseudo-F	*p*
Season	1	5390.4	5390.4	1.361	0.106
Habitat	1	12490.0	12490.0	3.153	**0.001**
Location	1	8862.1	8862.1	2.237	**0.008**
Season x Habitat	1	6297.0	6297.0	1.590	**0.041**
Season x Location	1	6294.9	6294.9	1.589	0.051
Habitat x Location	1	12405.0	12405.0	3.132	**0.001**
Season x Habitat x Location	1	4512.8	4512.8	1.139	0.229
Residual	64	2.535e05	3961		
Total	71	3.098e05			

Table 7 Abundance of reads, phylogenetic diversity, and Shannon diversity data (mean±SD) obtained from sediment samples from sand beach and tide pools (habitat) at Gramuté beach and Rio Preto beach (location) during winter and spring (season). Statistical significative differences (p<0.05) are presented in bold.

Variable	Habitat		*p*
	Beach	Tide Pool	
Abundance of Reads	113.33±34.24	8.11±3.04	**0.001**
Phylogenetic Diversity	8.96±6.44	6.55±3.08	0.237
Shannon Diversity	3.50±1.12	3.53±0.71	0.783
	Location		*p*
	Gramuté beach	Rio Preto beach	
Abundance of Reads	104.61±34.75	17.97±4.70	**<0.001**
Phylogenetic Diversity	10.88±5.65	4.72±1.20	**<0.001**
Shannon Diversity	3.59±1.18	3.45±0.59	0.714
	Season		*p*
	Winter	Spring	
Abundance of Reads	99.61±34.46	21.83±8.12	**0.029**
Phylogenetic Diversity	8.97±6.75	6.32±2.06	0.750
Shannon Diversity	3.81±0.98	3.22±0.79	**0.007**

Table 8 Results from Similarity Percentage analysis (SIMPER) indicating each taxon contribution to the similarity between sand beach and tide pools. Av. Dissim. = Average Dissimilarity; Diss./SD = Dissimilarity/Standard Deviation; Contrib. = Contribution.

Taxon	Av. Diss.	Diss./SD	Contribution (%)	Cum.Contrib. (%)
Crustacea	43.34	1.27	47.6	47.6
Nematoda	14.64	0.58	16.1	63.7
Annelida	13.82	0.88	15.2	78.9
Mollusca	9.16	0.40	10.1	88.9
Platyhelminthes	3.89	0.35	4.3	93.2
Nemertea	2.65	0.45	2.9	96.1
Gastrotricha	1.87	0.40	2.0	98.1
Cnidaria	0.86	0.39	0.9	99.1
Echinodermata	0.81	0.31	0.9	100.0

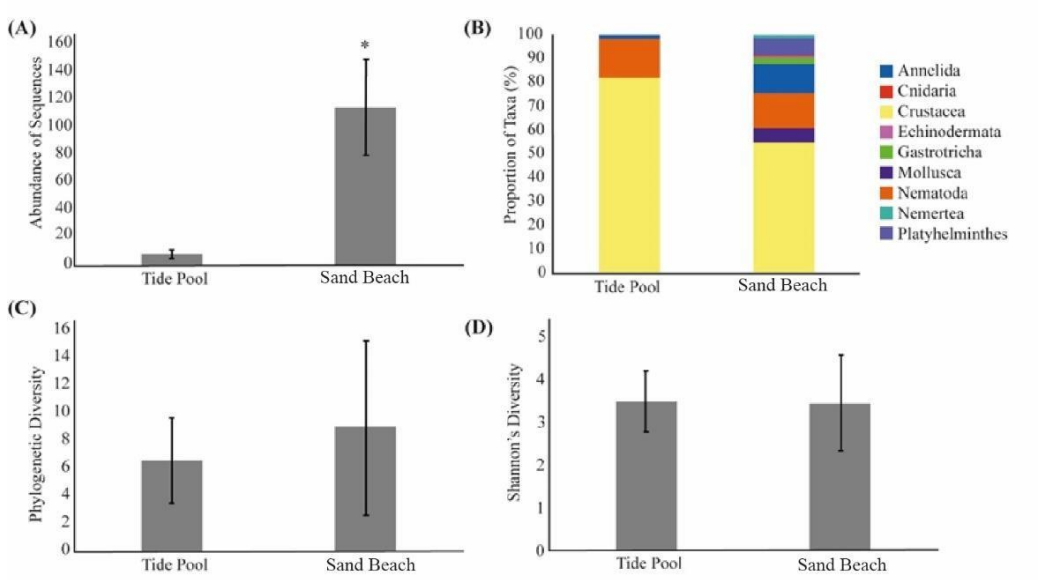


Figure 6 (A) Number of meiofaunal sequence reads (mean±SD) **(B)** Meiofaunal taxa proportion (%) **(C)** faith's Phylogenetic Diversity (mean±SD) **(D)** Shannon's Diversity index (mean±SD), obtained after metabarcoding of sediment samples collected inside tide pools and in the subtidal zone at Gramuté beach and Rio Preto beach, SE Brazil. The presence of an asterisk (*) indicate significative statistical differences ($p<0.05$).

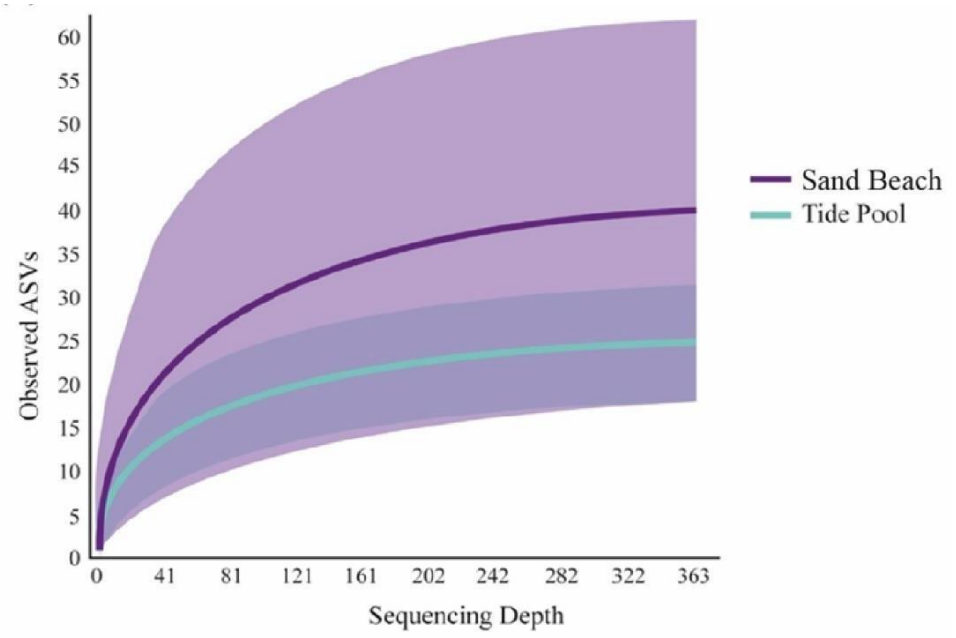


Figure 7 Rarefaction curves obtained from sediment samples metabarcoding collected at sand beaches and inside tide pools at SE Brazil. Solid lines represent a mean of observed ASVs at each sampling depth, and the shaded area represents the standard deviation.

DISCUSSION

Seasonal patterns of sandy beach meiofauna

Our findings suggest a marked seasonality of the meiofaunal assemblage composition, abundance of reads, and diversity at Gramuté beach. Higher phylogenetic diversity was observed during summer and winter, as well as higher abundance of sequences and Shannon diversity. We observed 5.9-times more sequences during summer when compared to spring, and the dissimilarities on assemblages between seasons reflected in differences of 1.9 and 2-times in phylogenetic diversity and Shannon's diversity, respectively. The observed seasonal differences at Gramuté beach reveal that meiofaunal assemblages in this tropical region support marked oscillations inthe coastal oceanography, recruitment, and possibly productivity regimes (Mazzuco et al., 2015; 2019; Mazzuco and Bernardino, 2022).

Neves and Costa (2022) recovered copepods and nematodes as dominant taxa during 1-year sampling (between 2013 and 2014) at the same beach, with annelids representing only 14% of the total individuals. Meanwhile, annelids were recoveredas a dominant taxon (28 % of sequence reads) in the present study, thus suggesting that eDNA approachesare either more efficient methods in detecting soft bodied species, such as polychaetes; or that we are partially measuring diversity (DNA sequences) from nearby coastal habitats (Derycke et al., 2021). Furthermore, we detected a broader range of meiofaunal diversity, identifying additional four phyla (Echinodermata, Gastrotricha, Nemertea, and Rotifera) not previously reported by Neves and Costa (2022) in their morphological assessment. This supports previous observations that metabarcoding assessments are more sensitive to cryptic meiofaunal taxa than traditional morphological assessments, at the cost of a lower taxonomic refinement (Dell'Anno et al. 2015; Guardiola et al. 2016; Cahill et al. 2018).

The seascapes characterization from our study suggests similar seasonal patterns for this tropical region as previously described by Mazzuco and Bernardino (2022). In this region we observed the presence of tropical water masses associated to higher abundance and diversity of meiofaunal taxa, which are followed by the intrusion of subtropical watermasses rich in nutrients. This peaks of abundance and diversity may be related to larval recruitment, even though some meiofaunal taxa does not have a larval phase (e.g., nematodes). This data highlight the relation between temperature and food supply to benthic abundance and diversity, and support our initial hypothesis of meiofaunal phylogenetic diversity and abundance of sequences associated to warmer months at Gramuté beach.

Sand beaches vs. tide pools

Our results revealed marked spatial differences on the composition and abundance of reads between locations (Gramuté beach and Rio Preto beach), habitat (sand beaches and tide pools), and their interaction; which suggests that ecological filtering between tide pools and sand beaches may not be a ubiquitous pattern. This is supported by our observed local differences on meiofaunal phylogenetic diversity. Environmental variables (temperature, salinity, grain size, carbonate, organic matter content, and biopolymers) showed differences on spatial and seasonal scales with quantity and quality of organic matter varying spatially.

We observed that tide pools had higher temperatures, and higher sediment lipid and biopolimeric carbon concentrations. The biopolymeric carbon represents the labile fraction of sedimentary organic matter that is available for benthic consumers (Fabiano et al., 1995; Bianchelli et al., 2008). This suggests that inside tide pools, there is a higher content of labile organic matter available for benthic consumers. However, also LIP (lower quality organic matter) represents a higher fraction of BPC inside tide pools (25%), when compared to sand beach (19%) (Table 2). This higher content of lower-quality organic matter, associated to the higher temperature inside the tide pools may explain the lower abundance of reads and diversity in this habitat. Meiofaunal assemblages aremainly drive by the quantity and quality of the sedimentary organic matter (Gabara et al.,2020; Neto et al., 2021). Additionally, the 1.8°C warmer environment inside the tide poolsmay exceed the tolerable limits of some taxa, what may explain the 13.9-fold difference on abundance of reads and low meiofauna richness detected inside tide pools (Annelida, Crustacea, and Nematoda). Changes in temperature can alter meiofaunal activity patterns, survival, growth, and reproductive rates (Giere, 2008). Wieser and Schiemer (1977) demonstrated that the meiofauna is more influenced by the maximum temperature experienced in the habitat, than the range of temperature, and although some species can adapt to warmer temperatures, their fitness is reduced and mortality risk increases (Schratzberger and Somerfield, 2020).

Changes in temperature may also influence on other parameters, such as oxygen concentration, ocean productivity, food web dynamics (Hoegh-Guldberg and Bruno, 2010), and on ecological interactions among taxa, since some nematodes speciesdecrease in density when exposed to warmer temperatures (Mevenkamp et al., 2018; Vafeiadou et al., 2018), but few tolerant opportunistic species may increase (Lee et al., 2017). Here we observed crustaceans as the dominant taxa both in sand beaches (>55% of reads) and tide pools (>82% of reads), and only nematodes (16%)and annelids (1%) were detected inside the tide pools, in addition to crustaceans. This represents adifference of 12-times in the abundance of annelids (12% in sand beaches). Kuhnert et al. (2010) observed on a manipulation experiment at tidal flats, that the abundance of harpacticoids copepods increased in sediments where burrowing polychaetes were excluded.

Tide pools and their associated meiofaunal assemblages may serve as an experimental laboratory to future climatic conditions. Global average temperature has increased approximately 0.2°C per decade over the last 30 years (Schratzberger and Somerfield, 2020), and locally in the Eastern Brazilian Marine Ecoregion, higher temperatures and lower rainfall are expected as a consequence of climate change (Bernardino et al., 2018). Meiofaunal species that persist in space and time facing environmental changes, will be tolerant to variations in their habitat, and abundances will change as function of alterations in the environment and in local biological interactions (Schratzberger et al., 2009). This possible loss of meiofaunalabundance and diversity may impact many attributes of benthic and pelagic ecosystems, such as energetic balance between ecological compartments of marineecosystems (Warwick, 1989).

CONCLUSION

In conclusion, we observed seasonal influence on meiofaunal diversity and abundance of reads at Gramuté beach, which is characterized by high temperatures, high salinity, and calm water masses. The abundance of sequences and diversity are higher during summer months and lower in spring, due to variation on water masses intrusion. Our tide pool *vs.* sand beach assessment revealed markedly spatial differences in regional and local scale on meiofaunal assemblage composition and abundance of reads, with a predominance of lower-quality organic matter content inside tide pools. We observed environmental differences between habitats (sand beach and tide pools) and locations (Gramuté and Rio Preto beach), which indicates that each beach is a unique environment. Local-scale influences produced a 1.68-times lower phylogenetic diversity inside tide pools at Gramuté beach, but this pattern was not observed at Rio Preto beach. Tide pools may represent an ecological filter for meiofauna in marine ecosystems, but is affected by local-scale environmental factors. These meiofaunal changes observed in habitat with warmer conditions and low-quality organic matter, may simulate a possible future scenario of a marine environment facing climate changes. We highlight the necessity of long-term monitoring programs to continue understanding how marine benthic organism will respond to future warmer environmental scenarios.

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SUPPLEMENTARY MATERIAL

Table S1 Results from Similarity Percentage analysis (SIMPER) indicating each taxon contribution to the dissimilarity between seasons (pair-wise comparisons) in Gramuté beach, SE Brazil. Av. Dissim. = Average Dissimilarity; Diss./SD = Dissimilarity/Standard Deviation; Contrib. = Contribution.

Taxon	Seasonal Comparison			
	Autumn vs Spring (Av. Diss. = 49.45%)			
	Av. Diss.	Diss./SD	Contribution (%)	Cum.Contrib. (%)
Crustacea	13.19	1.56	26.66	26.66
Annelida	11.12	1.08	22.49	49.15
Platyhelminthes	7.61	1.09	15.39	64.54
Nematoda	7.53	1.40	15.24	79.78
Gastrotricha	2.79	1.01	5.65	85.43
Mollusca	2.79	1.19	5.63	91.06
Echinodermata	2.55	0.82	5.16	96.23
Cnidaria	0.84	0.35	1.69	97.92
Rotifera	0.59	0.34	1.18	99.11
Nemertea	0.44	0.35	0.89	100.00
	Autumn vs Summer (Av.Diss. = 49.71%)			
	Av. Diss.	Diss./SD	Contribution (%)	Cum.Contrib. (%)
Annelida	11.28	1.13	22.70	22.70
Crustacea	10.82	1.28	21.76	44.46
Nematoda	7.59	1.09	15.27	59.73
Platyhelminthes	7.01	1.09	14.09	73.82
Gastrotricha	3.69	1.16	7.42	81.24
Cnidaria	2.56	0.61	5.14	86.38
Mollusca	2.44	1.19	4.90	91.28
Nemertea	2.09	0.71	4.21	95.49
Echinodermata	1.27	0.75	2.55	98.04
Rotifera	0.97	0.35	1.96	100.00
	Spring vs Summer (Av.Diss. = 56.00%)			
	Av. Diss.	Diss./SD	Contribution (%)	Cum.Contrib. (%)
Crustacea	14.01	1.29	25.02	25.02
Annelida	11.39	1.26	20.35	45.36
Nematoda	8.99	0.84	14.88	60.24
Platyhelminthes	7.48	0.66	13.36	73.60
Gastrotricha	3.67	0.87	6.55	80.15
Mollusca	2.56	1.15	4.56	84.71
Nemertea	2.37	0.65	4.24	88.95
Echinodermata	2.31	0.67	4.12	93.07
Cnidaria	2.26	0.50	4.04	97.10
Rotifera	1.62	0.49	2.90	100.00
	Autumn vs Winter (Av.Diss. = 66.70%)			
	Av. Diss.	Diss./SD	Contribution (%)	Cum.Contrib. (%)
Annelida	17.12	0.91	25.67	25.67
Crustacea	16.77	1.69	25.14	50.81
Nematoda	12.93	1.76	19.38	70.19
Platyhelminthes	7.84	1.34	11.75	81.94
Mollusca	3.55	1.13	5.32	87.26

Gastrotricha	3.50	1.00	5.24	92.51
Nemertea	1.79	0.60	2.68	95.19
Echinodermata	1.66	0.72	2.49	97.68
Cnidaria	1.55	0.54	2.32	100.00
	Spring vs Winter (Av.Diss = 62.91%)			
	Av. Diss.	Diss./SD	Contribution (%)	Cum.Contrib. (%)
Annelida	17.78	0.97	28.26	28.26
Crustacea	16.36	1.14	26.01	54.27
Nematoda	8.75	1.28	13.91	68.18
Platyhelminthes	8.35	0.65	13.28	81.45
Mollusca	4.14	1.08	6.57	88.03
Echinodermata	3.22	0.70	5.12	93.15
Nemertea	1.84	0.50	2.93	96.07
Rotifera	1.02	0.34	1.62	97.69
Cnidaria	0.78	0.50	1.25	98.94
Gastrotricha	0.67	0.34	1.06	100.00
	Summer vs Winter (Av.Diss = 68.62%)			
	Av. Diss.	Diss./SD	Contribution (%)	Cum.Contrib. (%)
Crustacea	18.32	1.24	26.69	26.69
Nematoda	14.98	0.90	21.83	48.52
Annelida	11.35	1.14	16.54	65.06
Platyhelminthes	6.71	0.55	9.78	74.84
Gastrotricha	4.75	0.85	6.92	81.76
Nemertea	3.80	0.78	5.54	87.31
Mollusca	3.34	1.02	4.86	92.17
Cnidaria	3.09	0.59	4.50	96.67
Rotifera	1.29	0.35	1.89	98.55
Echinodermata	0.99	0.49	1.45	100.00

Table S2 Abundance of meiofaunal reads and diversity metrics (Phylogenetic Diversity and Shannon Diversity) obtained after metabarcoding sequencing from sediment samples collected at Gramuté beach, in SE Brazil, during all seasons. Data presented as mean ± standard deviation. Significant differences ($p<0.05$) are presented in bold.

Variable	Season				*p*
	Summer	Autumn	Winter	Spring	
Abundance of Reads	371.9±296.6	291.1±97.0	351.1±299.5	62.8±27.1	**0.005**
Phylogenetic Diversity	17.9±3.1	11.9±1.8	19.4±4.9	9.2±1.9	**<0.001**
Shannon Diversity	4.8±0.7	4.0±0.5	4.6±1.1	2.4±0.9	**<0.001**

CHAPTER 5

CONCLUSION

The data acquired and presented in this document demonstrate the effectiveness of metabarcoding techniques to assess meiofaunal assemblages under different spatial and temporal scales, under different environmental conditions. It also showed how metabarcoding approaches can be used to elucidate changes in meiofaunal assemblages, including phylogenetic diversity, in response to environmental impacts over time; habitat complexity; seasonality;and habitats that may present extreme environmental conditions.

The obtained data revealed an unexpected decrease of meiofaunal phylogenetic diversity and sequence reads abundance after 2.8 years of the Fundão dam rupture disaster, which led to the release of an enormous volume of mine tailings in the Rio Doce basin. Comparingthis result to previous data on the same sampling sites, we detected substantial changes on the meiofaunal assemblage in the Rio Doce estuary. Even observing a decrease on sediment metals concentrations, the meiofaunal assemblage is still under the influence of trace metals (e.g., Cd, Cu, and Zn), associated to organic matter content and grain size. Although, differences on abundance of toxicity tolerant taxa (Nematoda) indicate some recovery of meiofaunal assemblage (Chapter 2).

Meiofaunal assemblages may differ significantly between habitats with different substrate complexity (rhodolith beds density). However, higher RB density not necessarily corresponds to higher meiofaunal diversity, even the composition and dominance of taxa differ slightly. The results revealed that meiofaunal diversity and abundance may be influenced by quantity and quality of labile organic matter, but it can also be influenced by ecological interactions among taxa, such as the presence of strong competitors. Additionally, metabarcoding may detect a broader range and similar diversity patterns of meiofaunal taxa in comparison to traditional morphology-based taxonomy, but with lower taxonomic resolution (Chapter 3).

Meiofaunal diversity and assemblage composition varies seasonally in tropical beaches, with higher phylogenetic diversity and abundance of reads during rainy season. The meiofaunal assemblage composition and abundance may differ significantly in habitats with potentially more extreme environmental conditions, such as tide pools, which may be influenced by local factors, like quantity and quality of organic matter, and temperature,that makes each beach a unique environment. Observed changes in tide pools assemblages may alert to future scenarios of environmental conditions impacted by climate change (Chapter 4).

In this Thesis assessed meiofaunal assemblages in different coastal environments in SE Brazil (Brazilian Eastern Marine Ecoregion), using metabarcoding approaches as the main methodology. Our findings support the use of metabarcoding approaches to investigate benthic diversity patterns in marine environments. Nevertheless, it is important to point out that applying an integrative approach (traditional morphological identification and metabarcoding) is especially important at tropical regions due tolow representation of taxa in molecular databases, once the availability of representative DNA sequences is still a major factor limiting the full potential of metabarcoding studies, and therefore should be taken into account. It is also necessary to highlight the importance of maintaining long-term studies to create historical series for marine meiofauna communities, so that spatio-temporaldata is available for comparisons in case of future environmental impacts. Furthermore, baseline studies such as this one will help to better understand how marine benthic assemblages will respond to different scenarios in a

changing world.