High-throughput sequencing of Diatoms using V4 region of 18S rRNA gene in Bayug Island, Iligan City, Philippines

DARSHEL ESTER P. ESTOR[1], SHARON ROSE TABUGO[1,2,♥]

[1]Department of Biological Sciences, Mindanao State University-Iligan Institute of Technology, Iligan City, 9200, Philippines.
Tel./fax.: +63-917-1100599, ♥email: sharonrose.tabugo@g.msuiit.edu.ph
[2]Molecular Systematics and Conservation Genomics Laboratory, Center for Biodiversity Studies and Conservation, Premier Research Institute of Science and Mathematics, Mindanao State University-Iligan Institute of Technology. Iligan City, 9200 Philippines

Abstract. *Estor DEP, Tabugo SR. 2023. High-throughput sequencing of Diatoms using V4 region of 18S rRNA gene in Bayug Island, Iligan City, Philippines. Biodiversitas 24: 6343-6350.* Diatoms are the main contributors to aquatic primary production and are the most dominant phytoplankton. Their adaptability and growth in diverse natural resources enable them to thrive in various climates and geographical areas, making them suitable for monitoring and biotechnology applications. This study aimed to analyze the diatom composition in two study sites: a mangrove area and the estuary in Bayug Island. The analysis was conducted using metabarcoding of the V4 region of the 18S rRNA gene, amplified and sequenced on the Illumina MiSeq platform. Four amplicon libraries were generated, representing the diatom composition at the two study sites. Data processing was performed using the Parallel-Meta-Suite software. Following quality control and processing, 20,433 amplicon sequence variants (ASVs) were obtained. Among these, the genera *Navicula, Thalassiosira, Chaetoceros, Guinardia, Amphora,* and *Minidiscus* were the most abundant ASVs. The assessment of diatom alpha diversity using the Shannon index indicated a higher diversity in the estuary site. Furthermore, the analysis done within the Parallel Meta Suite suggested that the presence of these specific diatom species can be linked to salinity levels, and may indicate environmental disturbances such as water pollution. The presence of these diatoms has the potential to be utilized in ecological treatments, disease prevention, biotechnology, and as nutrient indicators for other marine organisms. Physico-chemical parameters were recorded in the estuary area (S1), with the average water pH measuring around 7.6, salinity at approximately 36.6 ppt, and water temperature at 26.7°C. In contrast, the measurements in the mangrove area (S2) revealed an average water temperature of 30°C, salinity of around 35 ppt, and a water pH of 7.7. This research will serve as baseline data for diatom community in the area and a pioneering work on using NGS and eDNA for diatom study.

Keywords: Abundance, diatoms, diversity, monitoring

INTRODUCTION

Mangrove estuaries are the freshwater links between rivers and seas, and are characterized by strong tidal currents, fluctuating water levels, varying salinity, and sediment concentrations (Ward et al. 2016). However, since the 1990s, rapid economic development, particularly in coastal industrial zones, has led to significant environmental degradation, resulting in the alarming decline in the richness and abundance of marine species (Meon and Phuoc 2014; Thomsen et al. 2015; Carraro et al. 2020) this, in turn, poses a risk to the equilibrium and welfare of various underwater ecosystems and the global economic structure (Depledge et al. 2017).

Consequently, many countries are now taking steps to assess and mitigate these escalating environmental impacts. Diatoms, a group of unicellular photosynthetic organisms, have emerged as valuable bioindicators for monitoring ecological health due to their sensitivity to physicochemical stressors and wide distribution, resulting in high species diversity (Vasselon et al. 2017). Diatoms are the main contributors to aquatic primary production and are the most dominant phytoplankton (Wu et al. 2014). Their adaptability to various climates and geographical areas makes them suitable for diverse applications (Jin and Agustí 2018).

Diatoms, which represent a branch of heterokonts resulting from the secondary endosymbiosis of a red alga, exhibit remarkable diversity, with estimates of species ranging from 20,000 to 2 million. They are encased in a unique silica-based structure called a frustule, characterized by intricately designed valves with tiny pores that optimize sunlight capture. Notably, diatoms possess golden chloroplasts due to the carotenoid pigment fucoxanthin. Beyond their physical characteristics, diatoms can also be categorized based on their habitat preferences (Oliveira and Bhattacharyan 2000). Planktonic diatoms exist in a free-floating state, epiphytic diatoms attach themselves to other organisms, and benthic diatoms are typically found near the bottom of aquatic environments (Oliveira and Bhattacharyan 2000).

Traditional environmental quality monitoring often relies on morphological taxonomy, which is time-consuming and demands a high level of taxonomic expertise (Carter et al. 2017; Mauvisseau et al. 2019; Vasselon et al. 2019). However, molecular taxonomy, facilitated by DNA metabarcoding, offers a fresh approach to environmental monitoring (Keck et al. 2017) and can improve the assessment of a marine environment (Dafform et al. 2015; Goldberg et al. 2015). This method involves the efficient identification

of species by amplifying a small segment of DNA extracted from environmental samples, such as soil, water, or sediment. DNA metabarcoding has proven effective in evaluating changes in community structure (Chariton et al. 2015; Keely et al. 2018), early detection of invasive species, and accurately assessing marine benthic and planktonic (Zaiko et al. 2015; Chain et al. 2015; Holman et al. 2019; Westfall et al. 2020; Perez-Burillo et al. 2022; Pawlowski et al. 2022).

Specifically, eDNA metabarcoding employs advanced next-generation sequencing (NGS) technology, enabling the simultaneous identification of numerous species (Klymus et al. 2017; Ruppert et al. 2019; Miya et al. 2020). This method amplifies a brief segment of eDNA from the desired organism using universal polymerase chain reaction (PCR) primers. Adapters and index sequences are added to both ends of the amplified fragments (amplicon) (Valentini et al. 2016; Miya et el. 2020). This efficient and sensitive approach has found wide application in various aquatic research areas, including freshwater environments (Evans et al. 2017; Nakagawa et al. 2018; Bylemans et al. 2019; McColl-Gausden et al. 2021), estuaries (Lobo et al. 2017; Zou et al. 2020), and marine ecosystem (Yamamoto et al. 2017; Holman et al. 2019; Nester et al. 2020).

However, it's important to note that eDNA metabarcoding is not a one-size-fits-all solution. Numerous challenges, including concerns about primer design, specificity, DNA extraction, PCR techniques, and the variability of aquatic environments, need to be addressed (Beng and Corlett 2020; Pawloksi et al. 2021; Foseca et al. 2023). Despite these challenges, eDNA offers potential solutions to significant ecological and conservation issues. In this context, the current research employs diatom eDNA as bioindicators to evaluate the ecological well-being of a reforested area on Bayug Island, Iligan City, Philippines. This pioneering work utilizes NGS and eDNA to establish baseline data for diatoms in the area, contributing to emerging diatom community studies and the marine conservation field.
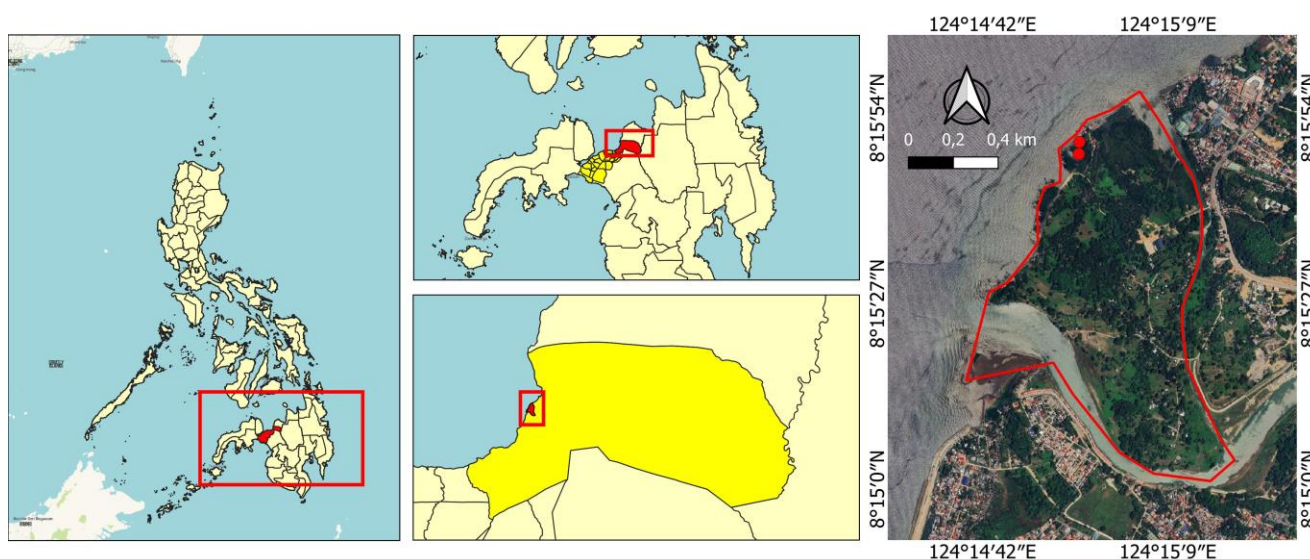
MATERIALS AND METHODS

Sampling site

The study area was situated on Bayug Island, within Iligan City, Philippines. Bayug Island is a sitio within Barangay Hinaplanon positioned 3.7 km; northeast of the Poblacion. Recognized as one of the reforested mangrove areas within Iligan Bay, it is located approximately 8°15'30" North and 124°14'56" East (Figure 1) (Almarez et al. 2014).

Diatoms eDNA collection

The study area is strongly influenced by riverine water and saline water from Iligan Bay. Two sites were established in the area, estuary (S1) and mangrove areas (S2). There were two replicates per site (S1R1, S1R2, S2R1, S2R2). Per replicate samples were processed in triplicates (3L each) and then pooled to constitute the 9L per site/replicate. A combined 36 liters of water (9L per site/replicate), was gathered for both locations, stored in containers, disinfected with bleach and thoroughly rinsed with distilled water before use. Physico-chemical parameters (temperature, pH, salinity) were carried out in situ using its respective laboratory apparatus. Seawater samples were filtered onsite using a 0.22 µm pore-size filter membrane in a Buchner funnel. Filter membranes were then stored in a capped, sterile container, placed in a portable cooler and immediately brought back to the Molecular Systematics and Conservation Genomics Laboratory, Center for Biodiversity Studies and Conservation (CBSC), Premier Research Institute of Science and Mathematics (PRISM), MSU-IIT, for eDNA extraction.

Figure 1. Map of the Philippines showing the reforested study site, Bayug Island, Philippines

DNA extraction, amplification, miseq sequencing and data processing

Before eDNA extraction, filtered seawater samples onsite were centrifuged at 13,000 rpm for 30 min, and the supernatant was removed and used as a starter. Samples were extracted using HiPurA Water Purification Kit (HiMedia) following the manufacturer's protocol. Extracted eDNA was evaluated using gel electrophoresis in Certified Molecular Biology Agarose gel (BIO-RAD) in 1 x TBE buffer using Cleaver Scientific electrophoresis system (MSMINIONE) before sending to Macrogen, South Korea for Metagenome Custom Amplicon Sequencing.

The V4 region of the 18S locus was amplified using the primer set of D512 forward: ATTCCAGCTCCAATAGCG and D978 reverse: GACTACGATGGTATCTAATC (Zimmerman et al. 2011). The PCR application started with an initial denaturation at 94°C for 2 minutes followed by five cycles consisting of denaturation at 94°C for 45s, annealing at 52°C for 45s, respectively, and elongation at 72°C for 1 min. This was followed by 35 cycles in which the annealing temperature was lowered to 52°C and a final elongation at 72°C for 10 minutes. Fragment length was done at 390 to 410 bp on MiSeq.
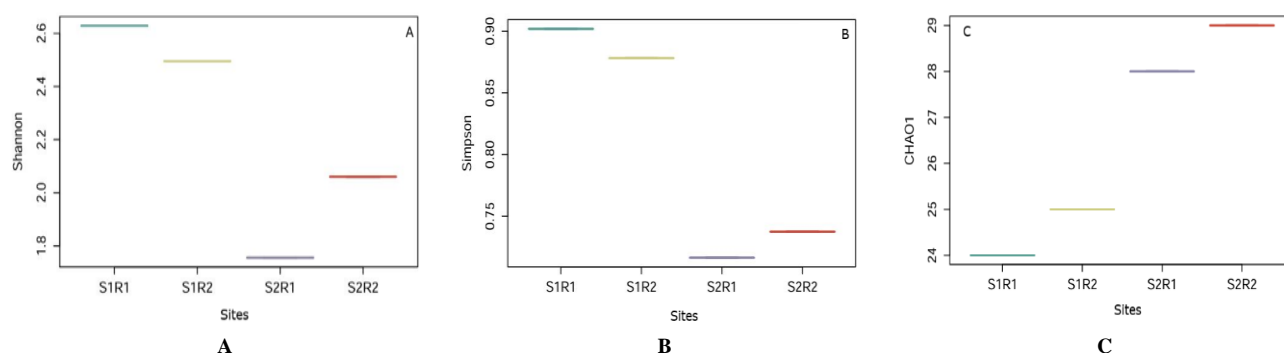
Pair-end reads were combined using the Flash Length Adjustment of Short Reads (FLASH) tool, and any incorrect or unreliable reads were subsequently eliminated (Magoč and Salzberg 2011). Ilumina MiSeq paired-end reads were processed using Parallel-Meta Suite (PMS) pipeline version 3.7 available from (https://github.com/qdu-bioinfo/parallel-meta-suite). Moreover, the pipeline encompasses a broad range of features, including data processing, statistical analysis, and visualization through a user-friendly graph-based interface (GUI), representing a notable leap forward in usability. It also allows the visualization of biodiversity indices (Shannon, Simpson, Chao1) providing insights into species relative richness and evenness. Simpson's index highlights the relative abundance of species, whereas the Shannon index concentrates on the species richness within a community. Concurrently, Chao1 is a nonparametric method for estimating the species count in a given community (Chen et al. 2022).
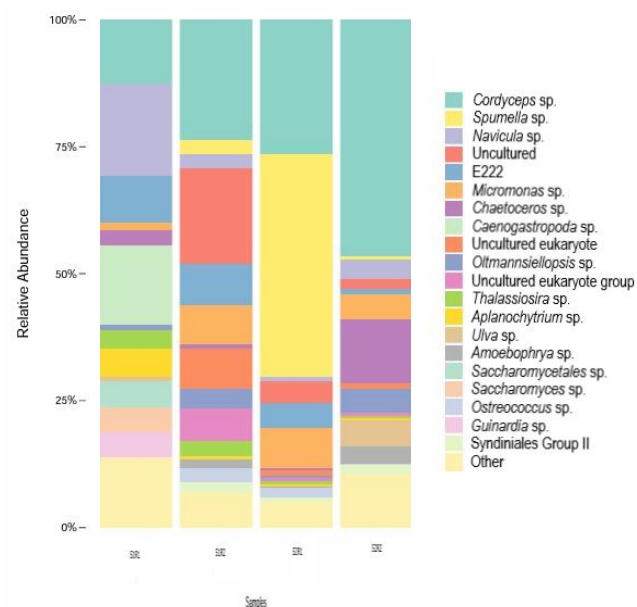
RESULTS AND DISCUSSION

Due to its exceptional biodiversity, the Philippines is acknowledged as one of the world's megadiverse nations (Venturina et al. 2020); thus, it faces a great ecological crisis from the destruction of many of its ecological systems (Lynette and Lava-Arreola 2020). Therefore, biomonitoring should consistently incorporate preservation and responsible utilization. From the entire set of high-quality reads obtained through metabarcoding, 20, 433 Amplicon Sequence Variants (ASVs) were produced, encompassing 116 families and 151 genera. These results were derived from the V4 region of the 18S rRNA gene within the two study areas. ASV's richness was used to calculate the alpha diversity of the samples. This has been consistent with species diversity indices (Shannon, Simpson, Chao1) (Figures 2A, 2B, 2C), indicating that Site 1 has the most species with a value of 2.6 within the study area. Among the genera obtained after taxonomic assignment *Navicula* species are the dominating diatoms across Bayug Island, Iligan City, Philippines (Figure 3). *Navicula* sp. are halotolerant species (Rincón et al. 2022) and are reactant in sensing chemicals (Kannaih et al. 2022). This can indicate that the water quality in the area is very polluted. Due to the untreated discharge of pesticides, factories and houses which is alarming to the aquatic ecosystem.

Based on the eDNA metabarcoding approach conducted at two sampling sites, a total of six genera were identified as the dominant species in the study area. These genera are *Navicula, Chaetoceros, Guinardia, Amphora, Minidiscus,* and *Thalassiosira.* These species exhibited the highest individual counts at site S1, as outlined in Table 1.

Figure 2. Biodiversity Indices of diatoms eDNA from the water samples of two sampling sites in Bayug Island, Iligan, Philippines. A. Shannon Index, B. Simpson Index, C. Chao1 index. All indices were analyzed and visually represented from the PMS pipeline (https://github.com/qdu-bioinfo/parallel-meta-suite)

Figure 3. Relative Abundance of diatoms species from water samples gathered within the two sampling sites in Bayug Island, Iligan, Philippines

The distribution of diatom taxa within the study area varies along the estuarine gradient, a phenomenon often influenced by changes in the ratios of riverine nutrients (Udalov et al. 2021; Nunes et al. 2022). The genera like *Navicula* and *Thalassiosira* have emerged as intriguing occurrences that demonstrate the complex interactions among environmental elements. These genera have demonstrated a strong association in total nitrogen level and other organic nutrients. They are equipped to exploit high-nutrient conditions, enabling them to grow rapidly and dominate the diatom community (Shen et al. 2018). This competitive advantage can lead to shifts in the balance of species within the ecosystem (Trabert et al. 2020; El-Sheek et al. 2023) which could have consequences on water quality, and lead to profound changes in the composition and overall health of the studied area. These alterations could set in motion a cascade of ecological effects, altering the composition and structure of various populations of organisms that rely on this ecosystem for their habitat and sustenance (Parfait et al. 2023). The prevalence of these diatom genera underscores the significance of site-specific ecological factors, impacting the composition of these vital microorganisms.

Moreover, the presence of these organic pollution indicators raises concerns about deteriorating water quality in certain regions. High nutrient levels can lead to eutrophication, where excessive algal growth consumes oxygen and degrades water quality, posing a threat to aquatic life and human activities (Rashid and Prakash 2022; Kamboj et al. 2022; Hassan et al. 2023). In the context of a recent study by Cui et al (2023), the negative effects observed in certain species within the genus *Chaetoceros* further highlight the delicate balance of aquatic ecosystems. These *Chaetoceros* species, driven by strong seasonal preferences have been associated with harmful algal blooms, which can disrupt food webs, impact water quality, and even pose a risk to public health (Daniels et al. 2015). *C. indicus* is composed of small diatoms that are not easily observed but their interaction in the trophic is important as their implication can affect the carbon cycling and organic matter export in an area (Daniels et al. 2015).

Apparently, they also hold qualities that enable them to become raw material for pharmaceutical, nanotechnology, and energy sources, leading to sustainable economy. Diatoms represent a significant reservoir of valuable resources in the form of fucoxanthin and polyunsaturated fatty acids, holding immense potential for nutraceuticals and biofuels (Sharma et al. 2021). Additionally, their secondary metabolites offer promising prospects for exploring and developing distinctive natural compounds. Among diatom species, *Skeletonema* sp. displayed the highest protein level, secondary metabolites, and total antioxidant activity (Bhattachariya et al. 2020). Note, that some species are considered in the field of medical context, and species of *Thalassiosira* are gaining a body of literature. They are found to produce compounds in natural habitats that can inhibit the production of other marine organisms like copepods (Matas et al. 2023). Also, it possessed a therapeutic effect that benefits health care (Sane et al. 2021; Sabia et al. 2018).

Meanwhile, genus *Navicula* species are found to be a good source of bioactive compounds (Nieri et al. 2023), and also possess high antioxidant properties (Fimbres-Olivarria et al. 2018), as well as carotenoids and phenolics which are important for food and nutrition industries, along with *Chaetoceros* species (Kuppusamy et al. 2017). In the study conducted by Joseph et al. (2016), *Navicula* species were found to possess the most potent strains for biodiesel production, followed by *Thalassiosira* species. The biomass production of diatom is essential in the food and pharmaceutical industries.

Table 1. List of genera that have abundant Amplicon Sequence Variants (ASVs) found in Bayug Island, Iligan City, Philippines

Genus	Species name	S1 (estuary area)	S2 (mangrove area)
Navicula	*Navicula* sp.	8828	2043
Chaetoceros	*Chaetoceros* sp.	1738	-
Guinardia	*Guinardia deliculata*	2083	5418
Amphora	*Amphora* sp.	328	-
Minidiscus	*Minidiscus trioculatus*	308	-
Thalassiosira	*Thalassiosira* sp.	2693	487
		15978	7948

On the other hand, salinity stress is an important factor that affects the production of metabolites. Many other species are directly used as feed for other organisms, like shrimp, during their larval stage (Xia et al. 2013). They also produced derived chemical substances that combat against various chronic disease; and acute bacterial and viral diseases such as HIV and Alzheimer's (Verma 2013; Zupo et al. 2014; Fimbres-Olivarria et al. 2018). Furthermore, the vast array of diatom sizes, with variations in frustule shapes among different species, offers an opportunity to select a specific diatom species that precisely matches specific requirements. This opens the door to the development of customized three-dimensional nanocomposites (Mishra et al. 2017). It is worth observing as well that the most investigations on biotechnological application of diatom metabolites are based on a few species such as *Thalassiosira* sp., *Chaetoceros* sp., *Skeletonema* sp. and *Navicula* sp. (Meyer et al. 2018). The advantages of microorganisms like diatoms, which are both complex and crucial in industry, have become increasingly apparent and have recently astounded the scientific community due to their industrial potential. Diatoms possess the ability to thrive in challenging environments, and their distinct pore structures and well-defined cell walls make them excellent candidates for producing a wide range of industrial products. The potential for utilizing diatom cells in industrial applications can be promising alongside advancements in microscopy, metabarcoding, analytical techniques, and genetic tools. Moreover, there has been a noticeable shift in the approach of both industry and academia toward the use of genetic tools, leading to a precise characterization of various molecular components of diatoms. Consequently, it is now feasible to carry out primary culturing, harvesting, and subsequent downstream processing of diatom cultures cost-effectively (Mishra et al. 2017; Saxena et al. 2020; Sharma et al. 2021).

The results underscore the efficacy of using environmental DNA (eDNA) to detect a wide array of diatom species, revealing the significance of bioindicators in assessing the water quality of the sampling site. However, to ensure effective marine monitoring, it is imperative to conduct these assessments regularly to document and track the stability and/or improvement of ecosystem health (Edgar et al. 2014). Ecological communities change over time, so this new method could help explain the present-day patterns and place a-conservation goals in a historical context by examining the ecological dynamics of population, communities, and ecosystems over hundreds to thousands of years (Bálint et al. 2018).

Environmental DNA (eDNA) analysis has gained popularity as a precise and cost-effective approach for managing species and aquatic ecosystems. Nonetheless, it presents certain challenges; eDNA begins to break down as soon as it is released into the environment, and its persistence can vary from days to months. Its longevity in aquatic environments is influenced by temperature, hydrological conditions, pH, salinity, UV radiation, substrate type, turbidity, and water chemistry (Strickler et al. 2015).

Additionally, the primers' function is critical to for avoiding amplifying non-targeted groups within environmental samples (Deiner et al. 2017). To address this, employing a range of primer pairs that target different genes, such as 12S, 16S, 18S, Cytochrome c oxidase subunit 1 (COI), and Cytb is advisable. This approach could enhance taxonomic sensitivity and mitigate potential biases associated with specific primers (Xiong et al. 2022).

Estuaries are renowned as the most productive ecosystems globally, receiving nutrients from terrestrial, marine, and anthropogenic sources (Cloern et al. 2014). Alterations in the estuarine water's physico-chemical conditions impact the phytoplankton communities because of the abbreviated life span of these organisms (Bergström et al. 2020). The recurring and cyclic seasonal fluctuation in physico-chemical factors (temperature, salinity, and nutrient levels), along with hydrological changes like tides, exert a significant impact on estuarine ecosystems, which subsequently have a direct or indirect effect on the composition and arrangement of phytoplankton communities (Valenzuela-Sanchez et al. 2021). Physico-chemical parameters were recorded in the estuary area (S1), with the average water pH measuring around 7.6, salinity at approximately 36.6 ppt, and water temperature at 26.7°C. In contrast, the measurements in the mangrove area (S2) revealed an average water temperature of 30°C, salinity around 35 ppt, and a water pH of 7.7. The dynamic nature of diatom species distribution corresponds to their adaptability to each area's nutrient cycling and environmental conditions such as salinity, water temperature and water pH.

Numerous studies have highlighted the adaptability of diatoms in mangrove and estuary gradients, showcasing their ability to modify their morphology in response to varying physico-chemical variables (Desrosiers et al. 2013; Carey and Fulweiler 2014; Nodine and Gaiser 2014; Hardikar et al. 2017). For instance, Tilli et al. (2022) noted that phytoplankton species like *Navicula* sp. exhibited optimal growth conditions within a temperature range of 21°C to 32°C. In the finding of Flanjak et al. (2022), the authors highlighted that *Chaetoceros* species can grow in different temperatures, but their optimal growth can be observed at 30°C leading to cell growth and faster reproduction. Species that preferred higher salinity levels, such as *Amphora, Guinardia* and *Navicula*, thrived within the salinity range of 35 ppt to 109 ppt (Ishika et al. 2018; Santos et al. 2022). On the other hand, *Minidiscus* species are found to be a small nano-sized diatom that potentially lack bloom-forming population, but, an important implication for carbon cycling and organic matter export in area in which potentially limited by physicochemical factors (Daniels et al. 2015).

Salinity has been identified as a key factor influencing shifts in plankton community structure, particularly diatoms, in many mangrove areas (Saifullah et al. 2014; Balqis et al. 2019; Halaludin et al. 2020), significantly impact their diversity (Hilaludin et al. 2020). Nursuhayati et al. (2013) have strongly suggested that diatoms are predominant in estuarine environments with high salinity levels. Furthermore, nutrients in mangrove areas tend to fluctuate in response to seasonal changes, including water column mixing and temperature fluctuations (Matos et al. 2022). The high diatom productivity in mangroves and estuaries can be

attributed to naturally available nutrients' supporting ecological balance and diversity (Rahamad et al. 2013; Saifullah et al. 2013). Moreover, species of *Guinardia* tend to grow more during monsoon weather, as they were significantly associated with water characteristics (Sahu et al. 2012). These research findings align with our observations, highlighting that diatoms swiftly respond to environmental variations in diverse gradients in temperature, nutrient availability, light levels, and water chemistry, which can vary significantly in aquatic ecosystems.

The most frequently identified amplicon sequence variants were associated with the genera *Navicula, Thalassiosira, Chaetoceros, Guinardia, Amphora,* and *Minidiscus,* which dominated the estuary area. These genera exhibit a strong correlation with physico-chemical changes such as temperature, salinity and nutrient levels, making them potential bioindicators of water quality. The study's findings suggest that the area's organic pollution may be attributed to several factors, including agricultural activities, the discharge of municipal and industrial waste, and the presence of urban clusters. These factors highlight the ecological pressure on the study area and emphasize the need for sustainable practices, regulatory measures, and conservation efforts to safeguard the health and balance of this critical ecosystem. Furthermore, understanding the relationship between dominant genera and environmental stressors can provide a foundation for informed decision-making and the development of targeted conservation strategies in the face of escalating environmental challenges. Additionally, some of these diatom species exhibit potential therapeutic effect which are beneficial to healthcare system. Environmental DNA (eDNA) can be collected in the field within a week, enabling surveys to be conducted during brief periods of favorable weather; this is important because rapid data collection is crucial for timely and responsive monitoring. Consequently, eDNA has the potential to significantly enhance current monitoring efforts by expanding geographical coverage, allowing temporal flexibility, and enabling assessments at different depths. This provides resource managers with crucial insights into biomonitoring responses.

However, the challenge lies in bridging the gap between traditional biomonitoring techniques that have been in use for many decades and emerging molecular methods. Overcoming challenges and capitalizing on the advantages of eDNA requires concerted efforts in standardization, education, cost analysis, regulatory acceptance, and public engagement. This connection is vital for maintaining seamless continuity in assessing water quality. Thus, this study contributes to the promising future of eDNA in water management.

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