

High-throughput 18S rRNA V4 region sequencing reveals diatom community structure in selected areas of Lanao del Norte, Philippines

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Abstract. Diatoms are considered the most characteristic phytoplankton, playing an important role in aquatic primary production. They are versatile and therefore capable of living in every type of climate and environment, making them ideal for monitoring and biotechnological applications. This study determined and compared diatom assemblages from four sites: an estuary, a mangrove, and two marine sanctuaries, through metabarcoding of the V4 region of the 18S rRNA gene, sequenced on the Illumina MiSeq platform. Seven amplicon libraries were generated, each corresponding to the diatom assemblage of a site. Results from Parallel-Meta-Suite software yielded 36,770 amplicon sequence variants (ASVs), corresponding to 80 families and 121 genera. The most abundant genera included *Chaetoceros*, *Melosira*, *Thalassiosira*, and *Navicula*. Alpha diversity, as measured by the Shannon index, ranked the stations as follows: estuary > mangrove area > marine sanctuary 1 > marine sanctuary 2. An evident trend from the findings is that some diatom species are associated with salinity levels and can be explained by environmental disturbances, such as water pollution. For instance, *Thalassiosira* and *Chaetoceros* are associated with harmful algal blooms, while *Melosira* has been found to thrive in polluted water and can serve as a water quality indicator. All these observations point to ecological site differences and the need for localized diatom diversity assessments. The research recognizes the potential of diatoms for environmental treatment, disease management, biotechnology, and as nutrient indicators for aquatic life. It provides baseline data on diatom communities and demonstrates the relevance of next-generation sequencing (NGS) and environmental DNA (eDNA) in diatom studies.

Key Words: amplicon sequence variants (ASVs), diatom, eDNA, water monitoring.

Introduction. Diatoms are major contributors to aquatic primary productivity and are the most common phytoplankton. Because they can grow and thrive in diverse environments, they are successful across a wide range of climates and geographies, making them suitable for many applications. Diatoms are of particular interest due to their myriad applications across a broad array of scientific fields, including nanotechnology, biotechnology, environmental studies, biophysics, and biochemistry. Of particular relevance, it emphasizes biosilicification, biomineralization, the application of diatoms in nanomaterials, drug delivery vehicles, optical and immune biosensors, filters, immunodiagnostics, aquaculture feed, lab-on-a-chip technology, metabolites, and biofuels (Mishra et al 2017).

Ecosystem changes are typically tracked by observing physical parameters such as water temperature and pH, which are easily and continuously measurable. Tracing the immense complexity of biodiversity, which includes many uncharacterized organisms, is much more complex than observing physical attributes. The challenge will be on enhanced sensitivity and efficiency of biodiversity measurements especially in water habitats where microscopic life forms like diatoms play critical ecological roles. Diatoms are responsible for providing close to 20% of the oxygen produced worldwide, and they also play crucial roles in carbon fixation, biogeochemical cycling, and nutrient turnover (Falkowski et al

1998; Armbrust 2009). Because they respond quickly to environmental changes, they are good predictors of water quality and ecosystem health. However, identifying and monitoring diatom communities using conventional microscopy-based methods can be time-consuming, require advanced taxonomic skills, and may overlook rare species. It justifies the need for molecular approaches such as environmental DNA (eDNA) metabarcoding, which provides a more comprehensive and non-destructive means of monitoring biodiversity by enabling the identification of species from DNA traces in water or sediment (Thomsen & Willerslev 2015; Pawlowski et al 2018). eDNA refers to genetic material from organisms found in environmental samples such as water and sediment. An organism's eDNA is shed into the environment through passive release from dead cells, shedding cellular components like membranes thereby, providing a rich source for tracking biodiversity (Bohmann et al 2014; Deiner et al 2017). To collect eDNA, a specified volume of water is passed through a membrane, where DNA is trapped, removed, and analyzed using molecular biology techniques for organism detection (Turner et al 2015; Miya et al 2016). Metabarcoding using eDNA enables researchers to identify multiple species simultaneously via high-throughput next-generation sequencing (NGS) (Zimmermann et al 2011; Taberlet et al 2012; Miya et al 2016). It enriches a subset of the target group's eDNA (i.e., diatoms) using universal primers and adds adapters and index sequences to the ends. Using different index sequences, multiple samples could be processed in parallel on the NGS platform (MiSeq system, Illumina Inc., San Diego, CA, USA), producing billions of amplicons from distinct sites. After processing, data are uploaded to assign taxonomy using bioinformatics pipelines (Chen et al 2022).

This study aims to investigate the composition of diatoms in selected research areas within Iligan city and other parts of Lanao del Norte, Philippines and it is among the pioneering works in using NGS and eDNA for diatom analysis. In more detail, the general goals were to identify diatoms using high-throughput eDNA metabarcoding and to establish a pipeline for analyzing amplicon sequencing data. The diatom groups present can indicate environmental disturbances, such as water pollution. In addition, it has potential for use in ecological therapies, disease prevention, biotechnology, and as a nutrient indicator for other aquatic animals.

Material and Method

Study area. This study analyzed diatom composition at four study sites: estuaries (S1, S2) and mangrove areas (S3, S4) in Bayug Island, and marine sanctuaries in Dalipuga, Iligan City (S5), and Kauswagan, Lanao del Norte (S6, S7) (Figure 1). Sampling was conducted nine (9) months from January to September in 2024. Prior informed consent and permits were obtained from local authorities of Bayug Island and Dalipuga, and from the municipal government of Kauswagan.

Diatoms eDNA collection. Samples were processed in triplicate, with 10 L per subsample, for a total of 30 L per sampling site. A combined total of 120 liters of water was collected from all locations, stored in containers, disinfected with bleach, and thoroughly rinsed with distilled water before use. Physico-chemical parameters such as water temperature, pH, salinity, and total dissolved solids (TDS) were measured in situ using a handheld multi-parameter probe. Dissolved oxygen (DO) was measured using a DO meter. All measurements were performed in triplicate at each sampling point. 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 transported to the Molecular Systematics and Conservation Genomics Laboratory at the Center for Biodiversity Studies and Conservation (CBSC), Premier Research Institute of Science and Mathematics (PRISM), MSU-IIT, for eDNA extraction.

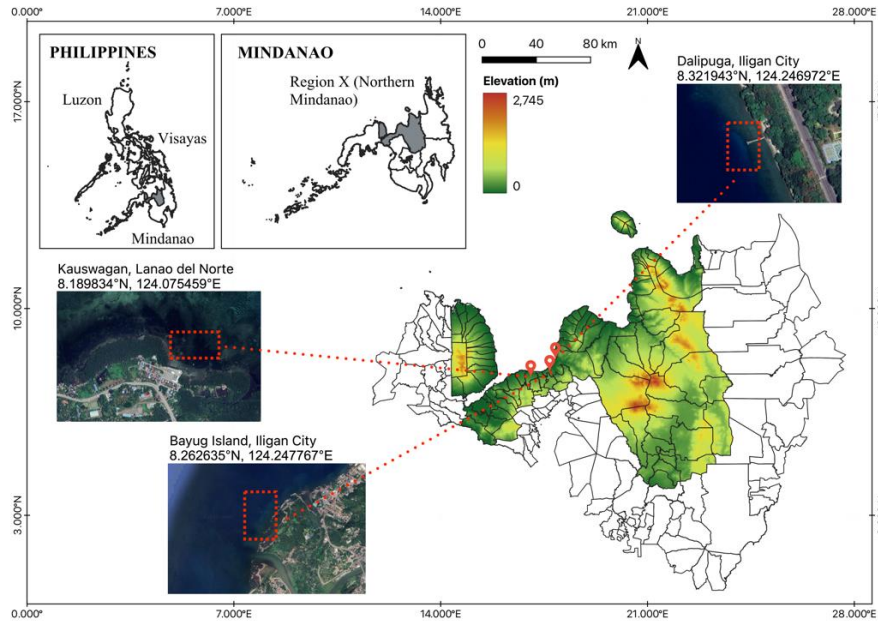


Figure 1. Map of the study area – showing Philippine Archipelago, Mindanao Region, Kauswagan, Lanao del Norte, Bayug Island, Iligan City, and Dalipuga, Iligan City.

DNA extraction, amplification, and MiSeq sequencing. Before eDNA extraction, the collected water samples were centrifuged at 13,000 rpm for 30 min, and the supernatant was removed and used as a starter. eDNA from water samples was 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 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 protocol consisted of an initial denaturation at 94°C for 2 minutes, followed by five cycles of denaturation at 94°C for 45 seconds, annealing at 52°C for 45 seconds, and elongation at 72°C for 1 minute. 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 set between 390 to 410 bp on MiSeq.

Data processing. 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 & Salzberg 2011). Illumina MiSeq paired-end reads were used, and sequences were processed using the Parallel-Meta Suite (PMS) pipeline version 3.7 available from ([https:// github.com/qdu-bioinfo/parallel-meta-suite](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 cutting-edge algorithms that visualize biodiversity indices (Shannon, Simpson, Chao1) providing insights into species relative richness and evenness (Chen et al 2022). Moreover, principal component analysis (PCA) was carried out to visualize beta diversity of amplicon libraries across sampling sites.

Results and Discussion. A total of 36,770 Amplicon Sequence Variants (ASVs) were generated from high-quality metabarcoding reads, representing 80 families and 121 genera. Sequence raw data was deposited online and made publicly available through the following accession numbers: SRR36654427-SRR36654433. These results were based on the V4 region of the 18S rRNA gene across the study areas. ASV richness was used to assess alpha diversity, aligning with species diversity indices (Shannon, Simpson, Chao1) (Figures 2A, 2B, 2C). The findings indicate that Site 1 exhibited the highest species diversity

within the study area, with a value of 2.6. The samples from the estuary areas (S1, S2) show the highest diversity.

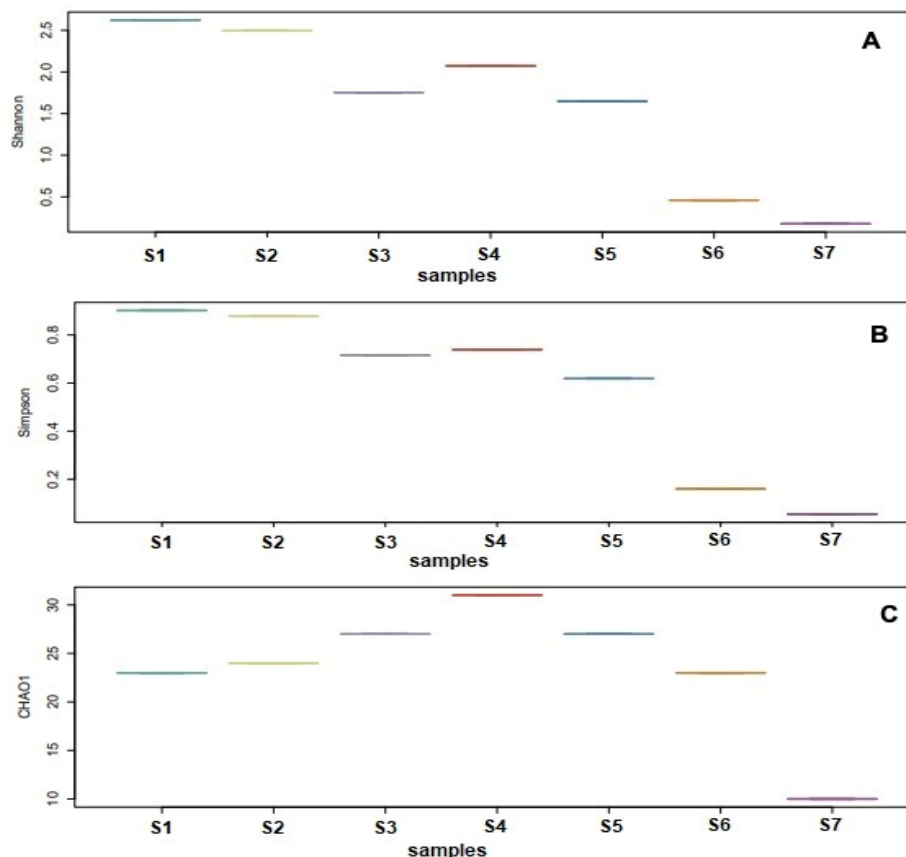


Figure 2. Biodiversity indices of diatoms eDNA from the water samples of four study sites: estuary (S1, S2) and mangrove area (S3, S4) in Bayug Island and marine sanctuaries of Dalipuga, Iligan City (S5) and Kauswagan, Lanao del Norte (S6, S7). A Shannon Index, B. Simpson Index, C. Chao1 index. All indices were analyzed and visually represented from the PMS Pipeline (<https://github.com/qdu-bioinfo/parallel-meta-suite>).

The most abundant genera included *Chaetoceros*, *Melosira*, *Thalassiosira*, and *Navicula* (Figure 3). Alpha diversity, evaluated using the Shannon index, ranked the sites as follows: estuary > mangrove area > marine sanctuary 1 > marine sanctuary 2. Among the genera identified through taxonomic assignment, *Navicula* species were the dominant diatoms found in Bayug Island, Iligan City, Philippines. *Navicula* spp. are halotolerant diatoms known for their sensitivity to chemical pollutants (Rincón & Rivas 2022). Their dominance suggests that the area's water quality is highly polluted, likely due to the untreated discharge of pesticides, industrial waste, and household effluents. It poses a significant threat to the aquatic ecosystem and raises concerns about the region's environmental health.

Genera *Navicula* and *Thalassiosira* have proved to be good examples, demonstrating the complicated interaction between the environmental variables. These genera are capable of flourishing in conditions of high nutrients due to their high correlation with total nitrogen and other organic content (Shen et al 2018). Their ability to adapt enables them to proliferate at a fast rate and take over the colony of diatoms. This competitive advantage can destabilize the balance of species within the ecosystem, possibly impacting the quality of water and causing dramatic shifts in the ecological composition and general health of the region. Other research has indicated that groups of diatoms are excellent water quality indicators. For example, *Navicula* and *Nitzschia* species are commonly recorded to inhabit sites experiencing pollution and enrichment, such as Laguna de Bay, Philippines (Ohtsuka et al 2009). Similarly, metabarcoding surveys in the Rhine River, Germany, reported a well-established link between the density of *Navicula* and anthropogenic stressors such as

industrial effluent and agricultural runoff (Kermarrec et al 2013). These findings support the notion that *Navicula* can serve as a good marker for nutrient loading and chemical stress in aquatic systems.

Melosira dominates in turbid, nutrient-rich water, while *Chaetoceros* and *Thalassiosira* are planktonic centric diatoms with roles in primary productivity and carbon sequestration (Sarhou et al 2005). Their presence in estuarine and mangrove settings is evidence of strong environmental gradients driven by freshwater and marine conditions with active nutrient cycling. *Navicula* domination of Bayug Island may illustrate the effects of human activities on the ecological composition of the area. The transformation may propagate like a ripple effect through the ecosystem, restructuring the population structure and dynamics that use it as habitat and resources (Alvarez et al 2014; Parfait et al 2023). *N. sp. TN-2014* and *N. sp. MAB-2013* lineages are the most abundant *Navicula* species. At the same time, *T. oceanica* and *T. sp. uncultured stramenopile lineage* are the most abundant species of *Thalassiosira*.

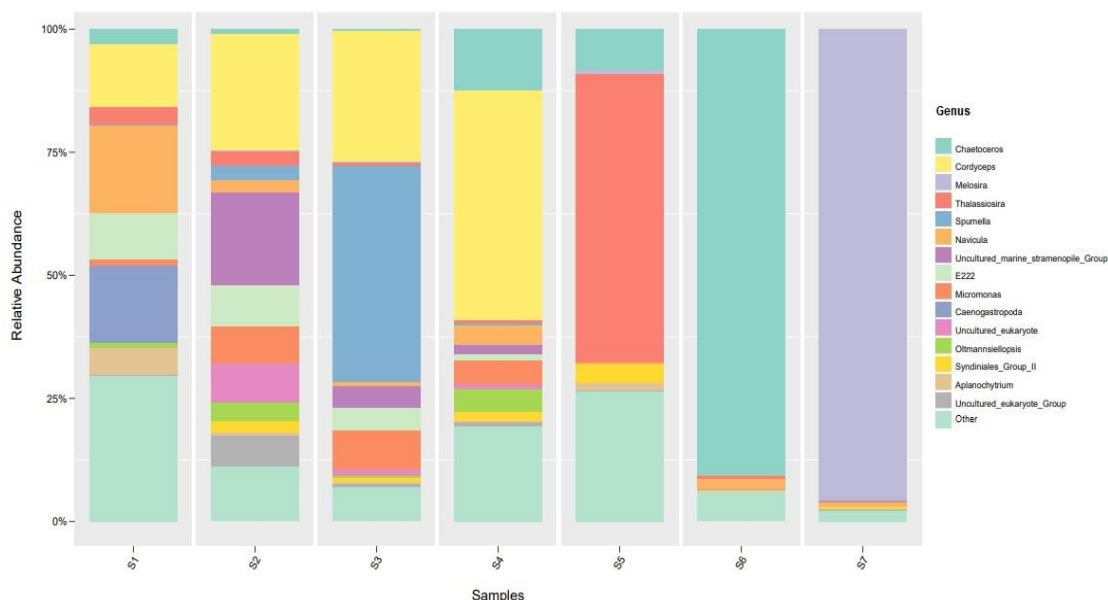


Figure 3. Relative abundance of diatom species from water samples gathered within the four study sites: estuary (S1, S2) and mangrove area (S3, S4) in Bayug Island and marine sanctuaries of Dalipuga, Iligan City (S5) and Kauswagan, Lanao del Norte (S6, S7), Philippines.

The presence of these organic pollution indicators raises serious concerns over declining water quality in certain regions. The excessive nutrient levels can cause eutrophication, a situation where excessive growth of algae results in oxygen depletion and deterioration of water quality, with grave consequences for aquatic life and human activities (Kamboj et al 2022; Rashid & Prakash 2022; Hasan et al 2023). The toxic impacts seen in some *Chaetoceros* species illustrate how susceptible marine ecosystems are, as seen in Cui et al (2023). Such species are linked with toxic algal blooms that can disrupt food webs, lower water quality, and pose a danger to public health, especially through dominant seasonality. For example, *C. indicus*, made up of tiny, hard-to-observe diatoms, plays an important role in food webs. Its role in the export of organic matter and carbon cycling indicates its importance in keeping ecosystems in balance (Daniels et al 2015). The most frequent species of *Chaetoceros*, based on the results, are *C. calcitrans*, *C. sp. YL-2014* and *C. sp. SS628-1* lineage while the rest are referred to as *C. sp. uncultured stramenopiles*.

Despite being a protected location, it was discovered that *Chaetoceros* species predominated at site S6 (marine sanctuary). This location is highly susceptible to human influences due to its proximity to a nearby park and several large factories in the city. High nutrient concentrations, especially those of nitrogen and phosphorus, which are known to promote phytoplankton growth, are probably caused by the ongoing release of household garbage and industrial effluents into the nearby seas. As opportunistic diatoms that react quickly to nutrient enrichment, members of the genus *Chaetoceros* can outcompete other

phytoplankton groups in eutrophic environments. Therefore, their dominance in S6 could be a bioindicator of environmental stress and nutrient loading in the sanctuary. Excessive blooms of this genus can alter food web structures and, in extreme cases, result in harmful algal blooms that affect marine biodiversity and water quality (Daniels et al 2015).

The family Melosiraceae, which includes the diatom *Melosira*, is marked by overall freshwater and marine habitat occurrence. The environmental adaptability, which is the reason why this genus tends to do well under various forms of environmental conditions, makes them well known (An et al 2024). But, as site S7 is near a convention center and a coal-fired power plant, it is rather more subject to anthropogenic contamination from thermal effluents, industrial effluent, and associated nutrient loading. It is noteworthy that *Melosira octogonia* was highly dominant in S7, suggesting that the altered physicochemical parameters of the site may be correlated with its dominance. This species dominance shows that the species is resistant to human-induced disturbance and that it can serve as a bioindicator of aquatic ecosystems which have been disturbed or are nutrient-enriched.

Diatoms are significant in other ways as well. Because of their special qualities, many diatom species have drawn interest in both industrial and medical settings. For example, it has been demonstrated that species of the genus *Thalassiosira* produce substances in their natural environments that inhibit the growth of copepods and other marine animals (Matas et al 2023). Additionally, these species exhibit therapeutic effects with potential healthcare applications (Sabia et al 2018; Sañé et al 2023). Because of their nanoporous and biocompatible architectures, the biosilica frustules of *Navicula* and *Pinnularia* species have also been examined. These frustules can be functionalized for drug delivery, biosensing, and photonic applications (Delalat et al 2015).

In addition, *Chaetoceros* species are being targeted for aquaculture and nutraceutical use in terms of feed, owing to their remarkable long-chain n-3 polyunsaturated fatty acid production, including notably eicosapentaenoic acid, and the presence of antioxidant compounds fucoxanthin and phenolics (Remize et al 2020; Tachihana et al 2020; Shirouchi et al 2023) thus showing how diatom species have an integral importance towards human health, biotechnological, and environmental applications. In the same fashion, *Navicula* genus species have been found to be a source of bioactive compounds (Nieri et al 2023) as well as being antioxidant-rich (Fimbres-Olivarria et al 2018). They are also carotenoid and phenolic producers that are nutritious for food and the food industry, which is also a trait shared by *Chaetoceros* species (Kuppusamy et al 2017; Rattanachitthawat et al 2022). Apart from that, Joseph et al (2016) identified that the *Navicula* species had the most efficient strains for the application of biodiesel production, followed by *Thalassiosira* species. The diatom biomass production has potential uses both in the food and pharmaceutical sectors, making it a promising sustainable resource for a variety of uses.

Principal component analysis (PCA) shows the clustering of amplicon libraries based on beta diversity. The PCA plot separated the amplicon libraries collected from estuary (S1, S2) and mangrove area (S3, S4) in Bayug Island and marine sanctuaries of Dalipuga, Iligan City (S5) and Kauswagan, Lanao del Norte (S6, S7), Philippines, reducing the multidimensional data to distinct clusters. The clusters correspond also to the geographical locations where S6, S7 are expected to be quite distant based on location. To evaluate beta diversity, pairwise distance matrices were generated using Hierarchical Meta-Storms and visualized as a heatmap showing high abundance of species in the estuary (S1, S2), mangroves (S3, S4), marine sanctuary 1 (S5) (Figure 4A-B).

Figure 5 also shows the diatom co-occurrence network. Herewith, the presence of *Chaetoceros* species is highly correlated with *Amphora*, *Nitzschia*, and *Cylindrotheca*, with a Spearman correlation value near 1. Among the species of *Chaetoceros* are: *C. calcitrans*, *C. sp.* YL-2014, *C. sp.* SS628-1 lineage and the rest are identified as uncultured stramenopiles. For *Amphora*, the identified species are *A. coffeaeformis* and *A. sp.* CTM_20023 lineage. Meanwhile, the identified common species of *Nitzschia* were *N. palea*, *N. draveillensis*, *N. microcephala*, *N. sp.* MAB 2013, and *N. sp.* MBIC10099 lineage. Moreover, *Cylindrotheca closterium* is a pennate cosmopolitan diatom. The cells are solitary and motile, with a lanceolate central region and two long, thin, flexible ends. They may exhibit a slightly curved or sigmoid shape. The nucleus and two chloroplasts are

Conclusions. Environmental DNA (eDNA) metabarcoding has proven an effective tool for overcoming the limitations of traditional monitoring techniques, enabling the detection of ecologically and economically significant species. It can serve as a complementary tool to traditional approaches. The approach can make a substantial contribution to biodiversity assessments, species identification, and conservation programs across the estuary, mangrove-reforested areas in Bayug Island, and marine sanctuaries of Dalipuga and Kauswagan in Lanao del Norte. With its potential to revolutionize the field, eDNA metabarcoding is also filling gaps in our understanding of ecological susceptibility and opening new avenues for examining community structure in a much less intrusive manner than traditional sampling techniques. However, it is crucial to acknowledge the limitations of the eDNA approach, including environmental eDNA decay, primer selectivity, and PCR amplification errors. These factors can affect the accuracy and reliability of results and potentially introduce biases or incomplete datasets. As a recommendation for future studies, a combined approach involving multigene eDNA alongside traditional monitoring methods is suggested to achieve improved taxonomic resolution and community analyses. Additionally, it is essential to check the first candidate primer sets before doing any eDNA metabarcoding.

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Conflict of interest. The authors declare that there is no conflict of interest.

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