Eukaryotic diversity and ecosystem health assessment of the Man-an River, Sinacaban, Misamis Occidental, Philippines using 18S rRNA eDNA metabarcoding

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Abstract. Freshwater ecosystems are increasingly threatened by anthropogenic activities that alter biodiversity and compromise ecological integrity. Environmental DNA (eDNA) metabarcoding has emerged as a powerful non-invasive approach for high-resolution biodiversity assessment in aquatic ecosystems. This study characterized freshwater eukaryotic diversity and evaluated ecological condition along the Man-an River, Sinacaban, Misamis Occidental, Philippines using 18S rRNA eDNA metabarcoding. The V4 region of the 18S rRNA gene was amplified and sequenced using the Illumina MiSeqi100 Plus platform and analyzed through the Parallel-Meta Suite (PMS) pipeline. Sequencing generated 786,884 raw reads and 97,281 high-quality amplicon sequence variants (ASVs), representing 767 species, 537 genera, and 291 families across multiple eukaryotic groups. Dominant taxa included *Aegagropila linnaei*, sp. 1 TK-2012, *Tetrahymena* sp. Dorphan, uncultured eukaryote, and uncultured marine ascomycete, highlighting both ecologically important freshwater organisms and substantial undescribed biodiversity. Alpha diversity analyses revealed that the midstream station consistently exhibited the lowest Shannon and Simpson diversity values, whereas upstream and downstream stations supported relatively higher diversity. Beta diversity analyses further demonstrated clear compositional separation of the midstream community from upstream and downstream stations. Physico-chemical parameters including temperature (27.5°C), pH (7.92), and dissolved oxygen (6.7 mg L^{-1}) met DENR DAO 34 Class C standards across all sampling sites. However, elevated temperature and evidence of agricultural runoff, indicated by the detection of *Colletotrichum musae*, were associated with reduced diversity and altered community composition at the midstream station. This study establishes the first molecular baseline in Man-an River, demonstrating the utility of eDNA metabarcoding as a high-resolution tool for evidence-based conservation and sustainable management of tropical freshwater resources.
Keywords: molecular baseline, river system, sequencing, sustainable management.

Introduction. Freshwater rivers and streams are among the most ecologically important ecosystems on Earth, serving as critical sources of water, biodiversity, and ecosystem services for both human and natural communities (Ward 1998). These systems support diverse aquatic organisms, regulate nutrient cycling, and sustain fisheries, agriculture, and recreational activities. However, increasing anthropogenic pressures such as agricultural runoff, habitat alteration, deforestation, and pollution have contributed to widespread declines in freshwater biodiversity and ecological integrity worldwide (McKinney 2002; Shrestha et al 2018). In tropical river systems, land-use changes associated with agriculture and expanding human settlements can significantly alter water quality and disrupt aquatic community structure.

Monitoring freshwater ecosystem health is therefore essential for effective conservation and sustainable resource management. Traditionally, aquatic biodiversity assessments have relied on capture-based approaches such as netting, trapping, and morphological identification of organisms. Although widely used, these methods are often labor-intensive, invasive, taxonomically challenging, and may fail to detect cryptic, microscopic, or low-abundance taxa (Taberlet et al 2012). In addition, conventional physicochemical monitoring provides only a snapshot of environmental conditions and may not fully capture the biological responses of aquatic communities to environmental stressors (Pawlowski et al 2018).

Environmental DNA (eDNA) metabarcoding has emerged as a powerful non-invasive tool for biodiversity assessment and biomonitoring in aquatic ecosystems. This approach enables simultaneous detection of multiple organisms from DNA fragments suspended in environmental samples such as water, sediment, or soil using high-throughput next-generation sequencing technologies (Zimmermann et al 2011; Taberlet et al 2012; Miya et al 2015). Among commonly used molecular markers, the 18S rRNA gene is a universal eukaryotic marker (Hadziavdic et al 2014) capable of detecting a broad range of freshwater organisms, including algae, fungi, protists, meiofauna, and macroinvertebrate-associated taxa. Consequently, the 18S rRNA gene contains conserved regions interspersed with hypervariable regions (V1–V9), which allow both broad eukaryotic coverage and taxonomic discrimination across multiple phyla simultaneously (Fonseca et al 2010; Hadziavdic et al 2014).

Despite the growing application of eDNA metabarcoding globally, molecular biodiversity assessments of Philippine freshwater ecosystems remain limited, particularly in smaller river systems outside major watersheds. One such understudied system is the Man-an River, a freshwater river embedded within an agroforestry landscape in Misamis Occidental. Although the river supports local communities and surrounding agricultural activities, no existing molecular baseline data are available regarding its freshwater biodiversity and ecological condition. Current environmental monitoring in the area relies primarily on physicochemical measurements, which may not adequately reflect the true biological integrity of the river ecosystem.

Therefore, this study aimed to characterize the spatial distribution of freshwater eukaryotic diversity in the Man-an River using high-throughput 18S rRNA eDNA metabarcoding. Specifically, the study sought to (1) characterize eukaryotic community composition, including macroinvertebrate-associated taxa, across upstream, midstream, and downstream stations; (2) compare alpha diversity indices among sampling stations; and (3) examine relationships between physicochemical parameters and spatial patterns of eukaryotic diversity. By establishing the first molecular biodiversity baseline for the Man-an River, this study highlights the potential of eDNA metabarcoding as a high-resolution biomonitoring tool for freshwater ecosystem assessment, conservation planning, and sustainable river management in the Philippines.

Material and Method

Study area. The study sampling was done within the one-month (December 2025) period, along Man-an River, located in Barangay Señor, Sinacaban, Misamis Occidental, Philippines (Figure 1). Prior informed consent and the necessary research permits were obtained before sampling. Sinacaban is a municipality situated in the northeastern portion of Misamis Occidental, bounded by mountainous terrain that drains into river systems. Man-an River is a freshwater system that traverses agricultural and secondary forest landscapes. This study looked into diversity along three sampling sites established along the river: upstream (A1), which is characterized by a moderately strong current and a stony riverbed, with a nearby spring contributing to the local hydrology. A carabao bathing area is situated slightly below this site, representing a source of periodic organic input and physical disturbance. The midstream (A2) reach is notably wide and shallow, flowing through a landscape influenced by agricultural activities in the higher elevations. This area is used by local communities who install traditional branch traps, locally known as 'bisig', to capture aquatic fauna during periods of high flow. The downstream (A3) section exhibits the strongest current velocity and is dominated by large boulders as substrate. A natural spring (tubod) located beneath a large tree serves as a vital water source for the local residents. Human activity at this site is primarily domestic, including water fetching and occasional clothes washing (Table 1).

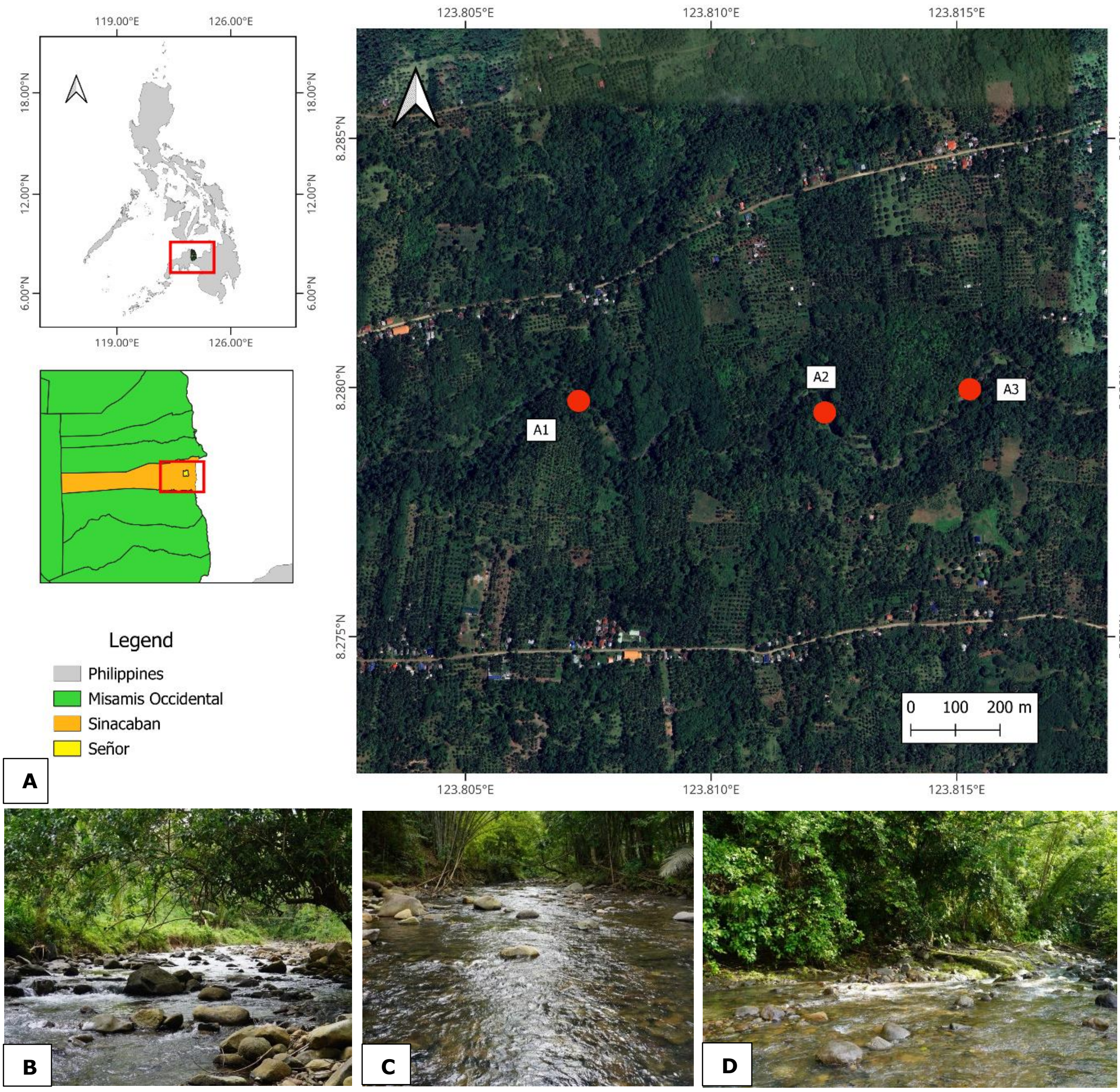

Figure 1. Map of the study area showing the location of a) sampling sites; b) photo of upstream portion (A1); c) photo of midstream portion (A2); and d) photo of downstream portion (A3) along the Man-an River in Barangay Senor, Sinacaban, Misamis Occidental.

Table 1

GPS location of sampling sites with a description of each site

Sites	*Coordinates*	*Descriptions*	*Human influence*
Area 1: Upstream	8°16'47.5"N, 123°48'26.4"E	Moderately strong water current, substrate dominated by small stones and cobbles, presence of a natural spring (tubod) along the riverbank	Localized physical disturbance from a carabao bathing area located in the slightly lower portion of the site
Area 2: Midstream	8°16'46.3"N, 123°48'44.4"E	Wide and shallow river channel, reduced water depth compared to other reaches	Agricultural practices in the surrounding mountainous terrain, use of traditional branch traps (bisig) for subsistence fishing
Area 3: Downstream	8°16'48.8"N, 123°48'54.7"E	Strong current velocity, substrate characterized by large boulders, presence of a large riparian tree and a natural spring (tubod)	Domestic use of the spring by locals for fetching water and occasional laundry activities

Water sampling collection. A total of six sampling stations were established across three sites along Man-an River, with two replicates assigned to each site: upstream (MUR1 and MUR2), midstream (MMR1 and MMR2), and downstream (MDR1 and MDR2). At every station, water samples were collected in triplicate (3L per subsample), for a total of 9L per replicate and 54L from all sites. Prior to use, all collection containers were disinfected with bleach and rinsed thoroughly with distilled water to prevent contamination. Water samples were filtered on-site using a 0.22 μm pore-size filter membrane in a vacuum filtration apparatus. Filter membranes were stored in capped, sterile containers, placed in a portable cooler at approximately 4°C, and immediately transported 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. Water quality parameters, including temperature, pH, and dissolved oxygen (DO), were recorded *in situ* at each sampling station.

DNA extraction, amplification, and MiSeq sequencing. Total genomic DNA was extracted from each filter membrane following the manufacturer's protocol using the HiPurA™ Water DNA Extraction Kit (HiMedia Laboratories, Wagle Industrial Estate, Maharashtra, India). Prior to extraction, filter membranes were centrifuged to recover any precipitated DNA as a pellet. The supernatant was carefully discarded and the remaining pellet was air-dried before proceeding with extraction. All extraction steps were performed using filter tips to minimize the risk of cross-contamination between samples and to prevent contamination of extraction reagents. The quality and quantity of extracted eDNA were evaluated before being sent to Macrogen, South Korea, for Metagenome Custom Amplicon high-throughput sequencing on the Illumina MiSeqi100 Plus platform, using two methods. First, extracted DNA was visualized by gel electrophoresis on a Certified Molecular Biology Agarose gel (BIO-RAD) in 1× TBE buffer using a Cleaver Scientific electrophoresis system (MSMINIONE) to confirm the presence of high-molecular-weight DNA bands. Second, DNA concentration and purity were assessed using a NanoDrop spectrophotometer to verify that the extracted solution contained sufficient DNA of acceptable quality before proceeding to sequencing. Six amplicon libraries were prepared corresponding to the six sampling units: downstream (MDR1, MDR2), midstream (MMR1, MMR2), and upstream (MUR1, MUR2). The V4 region of the nuclear small subunit ribosomal DNA (18S rRNA) gene was amplified using the universal primer set Uni18S-F (forward: 5'-AGGGCAAKYCTGGTGCCAGC-3') and Uni18S-R (reverse: 5'-GRCGGTATCTRATCGYCTT-3') (Zhan et al 2013). PCR amplification was performed under the following thermal cycling conditions: an initial denaturation step at 95°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 52°C for 30 seconds, and extension at 72°C for 90 seconds, with a final extension step at 72°C for 8 minutes. The expected amplicon size ranged from 400 to 600 base pairs. Amplification success was confirmed by gel electrophoresis prior to library submission.

Data processing. To check the quality of sequences Fast QC was used. The resulting sequences were deposited to NCBI GenBank and made publicly available under the accession nos. SRR38838700-SRR38838705. Raw paired-end reads were merged using the FLASH (Fast Length Adjustment of Short Reads) tool, which facilitates the accurate adjustment of short read lengths to produce high-quality merged sequences (Magoč & Salzberg 2011). Reads that were incorrect, unreliable, or failed quality thresholds were discarded during this step. The merged high-quality reads were subsequently analyzed using the Parallel-Meta Suite (PMS) pipeline, version 3.7, accessible at https://github.com/qdu-bioinfo/parallel-meta-suite (Estor & Tabugo 2023). This pipeline offers a wide array of functionalities, including data processing, statistical analysis, and visualization, through a user-friendly graphic user interface (GUI), thereby significantly enhancing usability. Furthermore, the pipeline enables the visualization of several biodiversity indices, such as Shannon, Simpson, and Chao1, that provides a valuable insight into the evenness and richness of species. While the Simpson index emphasizes the relative abundance of species, the Shannon index focuses on quantifying the species richness within a given community. In parallel, Chao1 provides a non-parametric approach to estimate the number of species present in the community (Chen et al 2022). Moreover, beta diversity was assessed using hierarchical clustering with heatmap visualization and principal component analysis (PCA) to examine compositional differences in eukaryotic community structure among sampling stations.

Results and Discussion. Freshwater rivers are more than a water source. They are living ecosystems that support biodiversity, sustain communities, and reflect the ecological health of the surrounding landscape. Their water carries not only sediment and nutrients but also the DNA of every organism living within and around them. In the Man-an River, this molecular archive had never been explored before. This study opens it for the first time, revealing a rich and ecologically informative eukaryotic community across the downstream, midstream, and upstream reaches of the river. From the entire set of high-quality reads obtained through metabarcoding, 97,281 amplicon sequence variants (ASVs) (Figure 2) were produced, encompassing 767 species, 537 genera, and 291 families. These results were obtained from the V4 region of the 18S rRNA gene across the three sampling stations of the Man-an River.

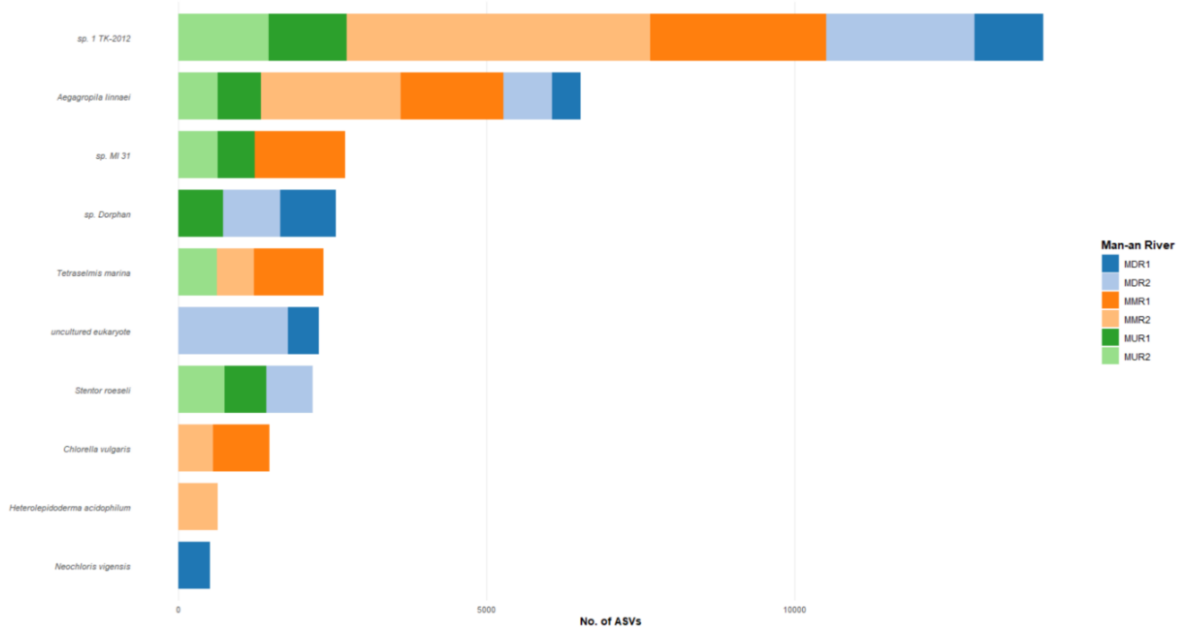

Figure 2. Amplicon sequence variant (ASV) count comparison between sites (downstream-MDR1, MDR2; midstream-MMR1, MMR2; upstream-MUR1, MUR2) of the Man-an River in Sinacaban, Misamis Occidental.

The ten most dominant species detected across all sampling area were sp. 1 TK-2012, *Aegagropila linnaei*, uncultured eukaryote, *Tetrahymena* sp. Dorphan, uncultured marine ascomycete, *Neochloris vigensis*, *Heterolepidoderma acidophilum*, *Colletotrichum musae*, *Poikilospermum lanceolatum*, *Cladochytrium replicatum*. The relative abundance of each eukaryotic group across sampling stations is shown in Figure 3.

Among the species obtained after taxonomic assignment, sp. 1 TK-2012 was the most dominant taxon across Man-an River recording over 12,000 reads across all stations. This designation is likely temporarily code assigned to an uncharacterized eukaryote first detected in a 2012 metagenomic survey, and it currently lacks a formal taxonomic description in the scientific literature. Its consistent dominance across all three stations suggests ecological significance, though its biological identity and functional role in the river ecosystem remain unknown and warrant further taxonomic investigation.

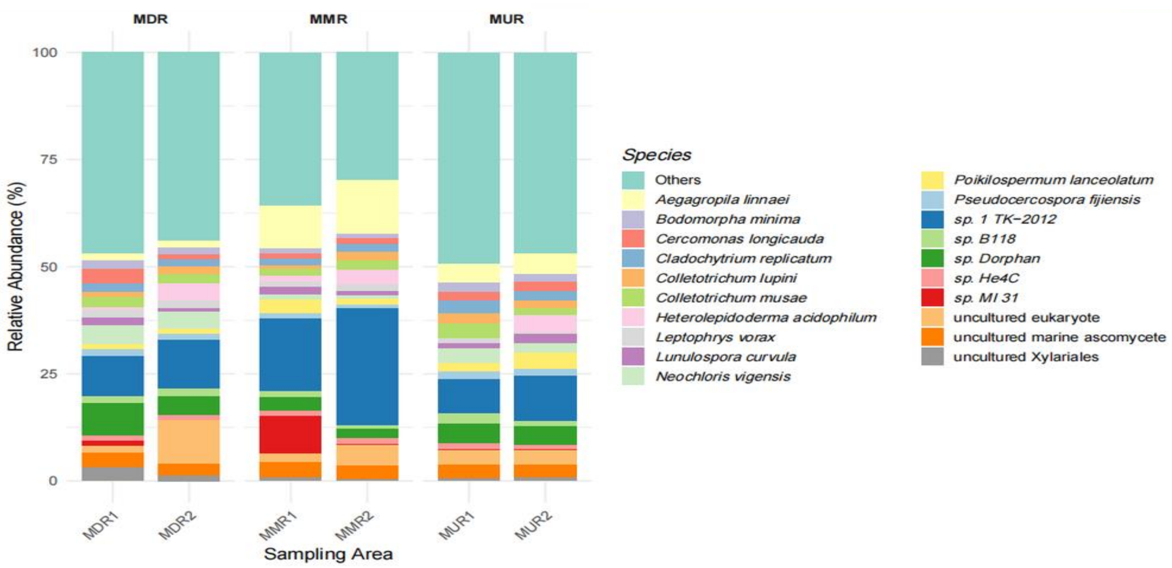

Figure 3. Relative abundance of macroinvertebrate-associated eukaryotic species from water samples gathered within three sampling stations of the Man-an River in Sinacaban, Misamis Occidental.

A. linnaei, commonly known as "Marimo" or "lake balls" (Boedeker et al 2010), was consistently detected across all three sampling stations and ranked as the second most identified species. This filamentous green alga is widely recognized for its distinctive unattached ball-form often called "*Cladophora* balls" or "marimo" in Japan, which develops under specific hydrographic and topographic conditions (Acton 1916; Niiyama 1989). *A. linnaei* is particularly sensitive to eutrophication, increased turbidity, and acidification, conditions typically associated with anthropogenic degradation of freshwater bodies, and declining or locally extinct populations have been documented in connection with these stressors in multiple studies (Pankow 1985; Boedeker et al 2010). Its growth and survival require clear, well-oxygenated water with alkaline pH (generally 7-9), low to moderate nutrient concentrations, and stable hydrological conditions (Boedeker et al 2010). Consequently, the persistent detection of *A. linnaei* across all three stations of the Man-an River serves as a positive ecological signal, indicating that the ambient water quality conditions are sufficient to support pollution-sensitive aquatic organisms.

Another taxon detected is the uncultured eukaryotes. The term "uncultured eukaryote" refers to organisms whose genetic sequences have been recovered from environmental samples but have never been successfully grown in laboratory culture and therefore lack formal taxonomic description (Pawlowski et al 2012). Their consistent detection across downstream, midstream, and upstream stations suggests that these uncharacterized organisms are integral and persistent members of the Man-an River eukaryotic community. The prevalence of uncultured eukaryotes in eDNA datasets is a well-documented phenomenon in metabarcoding studies of freshwater ecosystems, and is widely regarded as evidence of the vast undescribed microbial eukaryotic diversity that remains beyond the reach of culture-dependent methodologies (Berney et al 2004; Massana & Pedrós-Alió 2008). Their detection in the Man-an River underscores the unique advantage of eDNA metabarcoding in capturing biodiversity that would be entirely invisible to traditional morphological surveys. Taxonomic profiling through the PM Suite bioinformatics pipeline revealed that the sequences classified under the "uncultured eukaryote" label predominantly aligned with the phylum Gastrotricha (order Chaetonotida). These findings indicate that the "uncultured eukaryote" designation in the Man-an River encompasses a mixed assemblage of sequences, the dominant component of which belongs to the phylum Gastrotricha, a group of free-living, meiobenthic microinvertebrates that play important roles in benthic nutrient cycling and microbial food-web dynamics (Balsamo et al 2015). The gastrotrich affiliation of these sequences suggests that this phylum constitutes a functionally significant and previously overlooked component of the Man-an River eukaryotic community, potentially representing a vast reservoir of undescribed taxonomic diversity within the local riverine benthos.

Tetrahymena sp. Dorphan was detected as the fourth most abundant taxon across the Man-an River sampling stations. Because "dorphan" typically refers to "developmental orphan" or refers to an informal species isolate, it exists in genetic repositories like the NCBI Taxonomy Browser rather than as a formally named species similar to sp. 1 TK-2012. This designation represents an environmental sequence that has been provisionally coded but never formally described as a species in peer-reviewed literature. The sequence is also catalogued in reference databases such as SILVA under a temporary identifier, suggesting it was previously recovered from an environmental survey but remains taxonomically unresolved (Quast et al 2013). The ecological role and biological identity of *Tetrahymena* sp. Dorphan are currently unknown. However, its consistent detection across all three sampling stations alongside the similarly uncharacterized sp. 1 TK-2012 indicates that unnamed eukaryotic taxa constitute a significant proportion of the living community in the Man-an River. Taxonomic profiling through the PM Suite pipeline provided further resolution, demonstrating that *Tetrahymena* sp. Dorphan belongs to the phylum Ciliophora, class Oligohymenophorea, order Hymenostomatida, within the supergroup Alveolata/SAR. Ciliates of the class Oligohymenophorea are predominantly bacterivorous or microphagous consumers known to play critical roles in microbial food webs, regulating bacterial and picoplankton populations and mediating energy transfer to higher trophic levels in freshwater ecosystems (Zhao & Langlois 2022). Their prevalence in the Man-an River therefore suggests active microbial-loop dynamics and indicates that the river supports a functionally diverse protistan community engaged in nutrient regeneration and energy flow. The detection of this ciliate lineage further highlights that the eukaryotic diversity of the Man-an River extends well beyond the macroorganisms conventionally targeted in morphological bioassessment surveys.

Moreover, the detection of uncultured marine ascomycete sequences in the Man-an River, a freshwater system, is a noteworthy and ecologically intriguing finding. Marine ascomycetes are typically associated with saline and estuarine environments, where they play important roles as saprotrophic decomposers of organic matter, particularly woody and plant debris (Cunliffe 2022; Peng et al 2024). Their presence in the Man-an River, which is a non-tidal, inland freshwater system, may be attributed to several mechanisms. First, airborne dispersal of fungal spores and eDNA from coastal environments is a well-documented phenomenon, and the geographic proximity of Misamis Occidental to coastal zones may facilitate atmospheric deposition of marine fungal propagules into inland waterways (Klepke et al 2022). Second, the detection may reflect limitations in current reference databases, where some freshwater ascomycete sequences are incorrectly annotated as "marine" due to insufficient freshwater fungal representation in taxonomic databases such as SILVA (Quast et al 2013). Third, some ascomycete lineages previously classified as marine have been increasingly reported in freshwater and terrestrial environments, suggesting broader ecological flexibility than previously recognized (Richards et al 2015). Regardless of the origin of these sequences, the detection of uncultured marine ascomycetes in the Man-an River contributes to a growing body of evidence that fungal diversity in freshwater systems is far more complex and taxonomically diverse than current classification systems reflect.

N. vigensis was also detected across all three sampling stations of the Man-an River. *Neochloris* is a genus of unicellular green algae, characterized by its spherical to ovoid cell morphology and chlorophyll-based photosynthetic metabolism (Safi et al 2021). As a primary producer, *N. vigensis* contributes to the autotrophic base of the river food web through oxygenic photosynthesis, carbon fixation, and oxygen production (Reynolds 2006). Members of the genus *Neochloris* are known to thrive in a broad range of environmental conditions, from clean oligotrophic waters to moderately nutrient-enriched systems, and some strains exhibit considerable tolerance to fluctuating light intensities and temperatures (Safi et al 2021). The consistent detection of *N. vigensis* across all stations suggests that primary production via microalgal communities is active throughout the length of the studied river reach, and that light and nutrient conditions remain sufficient to support photosynthetic microalgae even at the ecologically stressed midstream station. The presence of *N. vigensis* is generally associated with moderate to good water quality conditions, as excessive organic pollution typically suppresses microalgal growth through light attenuation and oxygen depletion (Reynolds 2006).

Another species found in the Man-an River was the free-living gastrotrich *Holopedium acidophilum*, which inhabits the interstitial spaces of aquatic sediments, biofilms, and submerged plant surfaces in freshwater environments (Balsamo et al 2015). *H. acidophilum* occupies a critical ecological position at the interface between the microbial and macrofaunal communities of the river benthos. As a microphagous consumer, it feeds on bacteria, microalgae, diatoms, fungal spores, and fine organic detritus, making it an important link in the transfer of energy from the microbial loop to higher trophic levels (Nascimento et al 2012). Gastrotrichs contribute to the breakdown and mineralization of fine particulate organic matter in sediments, accelerating nutrient regeneration and supporting benthic productivity (Bonaglia et al 2014). Their high surface-area-to-volume ratio and rapid reproduction rates make them particularly responsive to changes in microbial food availability, and their population dynamics often reflect shifts in benthic organic matter quality and quantity (Schratzberger & Ingels 2018). The detection of *H. acidophilum* in the Man-an River is a significant finding from a water quality perspective. The species epithet *acidophilum*, indicating an affinity for neutral to slightly acidic conditions, is consistent with the near-neutral pH values recorded across all sampling stations (7.82-7.99) (Table 2). Gastrotrichs as a group are generally sensitive to severe organic enrichment, anoxic conditions, and heavy metal contamination that reduce both their abundance and species richness (Schratzberger & Ingels 2018). Their detection in the Man-an River therefore provides a positive indicator of benthic ecological integrity and acceptable DO conditions, consistent with the DO values of 6.4-6.9 mg L^{-1} recorded across stations (Table 2).

Concurrently, another interesting species encountered was *Colletotrichum musae*, a filamentous ascomycete fungus and a primary causative agent of anthracnose and crown rot disease in banana - one of the most economically devastating postharvest diseases of banana production globally (Kuyu & Tola 2018; Mangoba & de Guzman Alvindia 2023). Its detection in the eDNA water samples of the Man-an River constitutes an ecologically and agriculturally significant finding. As a terrestrial plant pathogen of agricultural origin, *C. musae* does not occupy a native functional role in the aquatic ecosystem of the Man-an River. Rather, its detection represents the passive introduction of terrestrial fungal genetic material into the river system through agricultural runoff, surface water flow, rainfall-driven soil erosion, and overland transport from adjacent banana plantations and agroforestry lands (Deiner et al 2016). The detection of *C. musae* in river eDNA serves as a powerful molecular tracer of agricultural runoff and catchment-scale land-use impact on the Man-an River. The presence of this banana pathogen provides direct biological evidence that surface runoff from banana cultivation areas is entering the river system. This finding has dual implications. First, it signals potential deterioration of water quality through inputs of agricultural chemicals, fertilizers, and organic matter associated with banana farming, which can contribute to nutrient enrichment and biological oxygen demand in the receiving water body (Allan et al 1997). Second, the transport of viable *C. musae* propagules through river water raises potential concerns regarding the downstream dispersal of banana disease inoculum - a biosecurity consideration relevant to local agricultural management, particularly in regions where river water is used for crop irrigation. The detection of *C. musae* therefore constitutes an actionable water quality and land management signal that should be brought to the attention of local government units and agricultural extension services in Sinacaban, Misamis Occidental.

Polystichum lanceolatum, is a woody flowering plant belonging to the family Urticaceae, native to Southeast Asian tropical forests and commonly found in humid lowland and montane riparian environments, including monsoon forests and wet places near streams at elevations of 700-1800 m (Chew 1963). As a terrestrial vascular plant, *P. lanceolatum* is not an aquatic organism; its detection in river eDNA most likely reflects the passive introduction of plant-derived genetic material into the water column from surrounding riparian and forest vegetation through pollen deposition, leaf litter inputs, root exudates, or the decomposition of plant tissue in or adjacent to the river channel (Taberlet et al 2012; Thomsen & Willerslev 2015). The detection of *P. lanceolatum* in the Man-an

River eDNA dataset is ecologically informative in two respects. First, it provides indirect evidence of intact riparian vegetation along the river corridor, as terrestrial plant eDNA detected in river water is typically sourced from adjacent bank vegetation rather than distant upland sources (Bohmann et al 2014). The persistence of riparian plant cover is critical for river health, as it moderates water temperature through shading, contributes allochthonous organic matter as an energy source for aquatic communities, stabilizes riverbanks against erosion, and filters agricultural runoff before it reaches the water channel (Naiman & Decamps 1997). Second, *P. lanceolatum* and related Urticaceae species are known to provide important habitat and food resources for a variety of forest fauna, and their presence in the riparian zone of the Man-an River suggests that the surrounding terrestrial ecosystem retains ecological integrity consistent with supporting diverse plant and animal communities.

Another species found was *Cladochytrium replicatum*, which functions primarily as a saprotrophic decomposer of plant-derived organic matter in freshwater environments, secreting extracellular cellulases and other hydrolytic enzymes to break down cellulose, hemicellulose, and other structural carbohydrates from submerged leaves, woody debris, and algal cell walls (Sime-Ngando 2012; Wurzbacher et al 2010). Through this decomposition activity, *C. replicatum* mineralizes complex organic substrates and releases dissolved inorganic nutrients (nitrogen and phosphorus) back into the water column, driving nutrient regeneration and supporting downstream microbial and algal productivity. Additionally, chytrid zoospores constitute a high-quality dietary resource for zooplankton and small invertebrates, and their production forms a critical energetic pathway known as the "mycoloop", through which fungal biomass channels energy from recalcitrant terrestrial organic matter to higher trophic levels in the aquatic food web (Kagami et al 2014). The detection of *C. replicatum* across the three sampling stations therefore indicates that active fungal decomposition and energy transfer processes are functioning within the river ecosystem. From a water quality perspective, the presence of *C. replicatum* in the Man-an River is consistent with the agroforestry land use of the surrounding catchment, which contributes substantial quantities of leaf litter, woody debris, and plant residues as allochthonous organic inputs to the river channel. The activity of saprotrophic chytrid fungi in processing these organic inputs is ecologically beneficial, as it prevents the excessive accumulation of organic matter that could drive hypoxic conditions through elevated biological oxygen demand. However, under conditions of excessive organic loading, such as may arise from intensive agricultural activity, chytrid populations can proliferate disproportionately, potentially indicating organic enrichment stress (Wurzbacher et al 2010). In the Man-an River, the DO values recorded across all stations (6.4-6.9 mg L^{-1}) (Table 2) remain above the DAO 34 Class C threshold of 5.0 mg L^{-1}, suggesting that organic decomposition processes are currently balanced and not driving hypoxic stress. The detection of this aquatic chytrid therefore serves as an indicator of active but ecologically balanced organic matter processing.

Alpha diversity analyses revealed consistent spatial patterns in eukaryotic community diversity across the three sampling stations of the Man-an River. The upstream station (A3) recorded the highest Shannon diversity index (H' approximately 3.85-3.90) and Simpson index (1-D approximately 0.965-0.970), reflecting both the greatest species richness and the highest community evenness among the three stations. The downstream station (A1) showed slightly lower but comparable values (Shannon H' approximately 3.80; Simpson 1-D approximately 0.960), while the midstream station (A2) consistently recorded the lowest median values for both indices (Shannon H' approximately 3.35; Simpson 1-D approximately 0.910), with the widest interquartile range observed among all three stations (Figure 4).

The Shannon index weighs both species richness and evenness within a community, while the Simpson index emphasizes the relative abundance of species, with values approaching 1.0 indicating very high diversity and low dominance by any single taxon (Shannon & Weaver 1949; Simpson 1949). The higher Shannon and Simpson values at the upstream station suggest that the upstream reach of the Man-an River supports a more diverse and evenly distributed eukaryotic community, consistent with the generally cleaner, less disturbed conditions expected at headwater reaches (Allan & Castillo 2007). The wider interquartile range at the midstream station for both Shannon and Simpson indices indicate greater variability in community diversity between the two midstream replicates (MMR1 and MMR2), suggesting that ecological conditions at the midstream reach are more spatially heterogeneous or temporally unstable compared to the upstream and downstream stations.

Meanwhile, Chao1, a non-parametric estimator used to assess the total number of species present in a community (Chao 1984), showed a similar spatial trend, with the downstream station recording the highest median estimate (approximately 104-105), followed by the upstream station (approximately 102-105), and the midstream station recording the lowest estimate (approximately 101) with the narrowest interquartile range (Figure 4). However, it is important to note that the absolute differences in Chao1 estimates across stations were very small, spanning only approximately four species units between the highest (downstream, approximately 105) and the lowest (midstream, approximately 101), indicating that estimated total species richness was broadly similar across the three stations at the species taxonomic level. This narrow Chao1 range suggests that while community evenness and relative abundance differed markedly between stations - as reflected by the Shannon and Simpson indices - the estimated total number of species present was relatively uniform throughout the river reach at the time of sampling. Kruskal-Wallis tests revealed no statistically significant differences in Shannon ($p = 0.1561$), Simpson ($p = 0.1561$), or Chao1 ($p = 0.2765$) indices across stations, most likely attributable to the limited number of sampling stations ($n = 3$) and replicates ($n = 2$ per station) rather than the absence of true ecological differences (Gotelli & Colwell 2001).

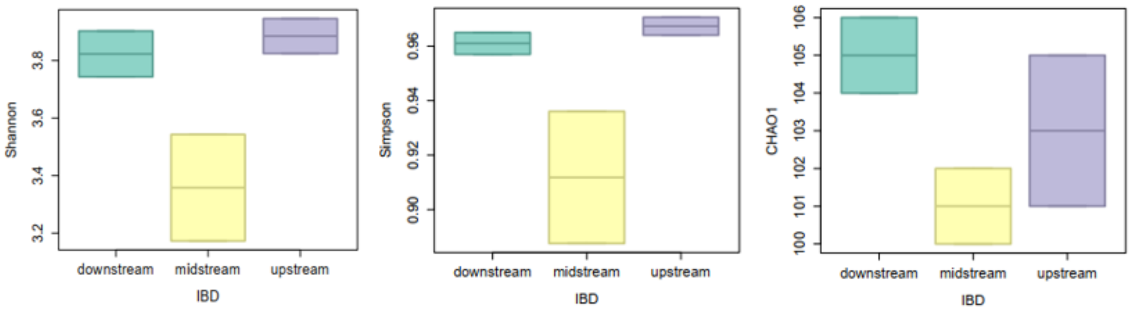

Figure 4. Biodiversity indices of macroinvertebrate-associated eukaryotic species eDNA from the water samples of three study sites: downstream, midstream, and upstream of Man-an River, Sinacaban, Misamis Occidental. A Shannon Index, B. Simpson Indes, C. Chao1 index. All indices were analyzed and visually represented from the PMS Pipeline (https://github.com/qdu-bioinfo/parallel-meta-suite).

Patterns in beta diversity, as represented by the principal component analysis (PCA) (Figure 5A) and hierarchically clustered heatmap (Figure 5B), revealed strong compositional differentiation among the sampling stations of the Man-an River. The heatmap showed clear clustering by river zone. Midstream replicates (MMR1 and MMR2) formed a distinctly separate cluster from the downstream (MDR1, MDR2) and upstream (MUR1, MUR2) replicates, with the highest dissimilarity values (0.3 to 0.4) observed between the midstream and all other samples. In contrast, the downstream and upstream clusters grouped together on the same branch of the dendrogram, indicating greater compositional similarity between these two stations than either share with the midstream station. The PCA ordination corroborated these findings. PC1 and PC2 collectively explained 86.6% of the total community variance (PC1 = 63.0%; PC2 = 23.6%), confirming that the two-dimensional ordination effectively captured the major axes of community differentiation in the dataset. Sampling units were widely distributed across ordination space, with MMR2 positioned at the extreme end of PC1, the most divergent position, consistent with the heatmap finding of high midstream dissimilarity. These spatial trends indicate that the midstream station harbors the most compositionally distinct eukaryotic community along the Man-an River, possibly driven by the combined influence of elevated temperature, agricultural runoff inputs, and localized hydrodynamic conditions at that reach.

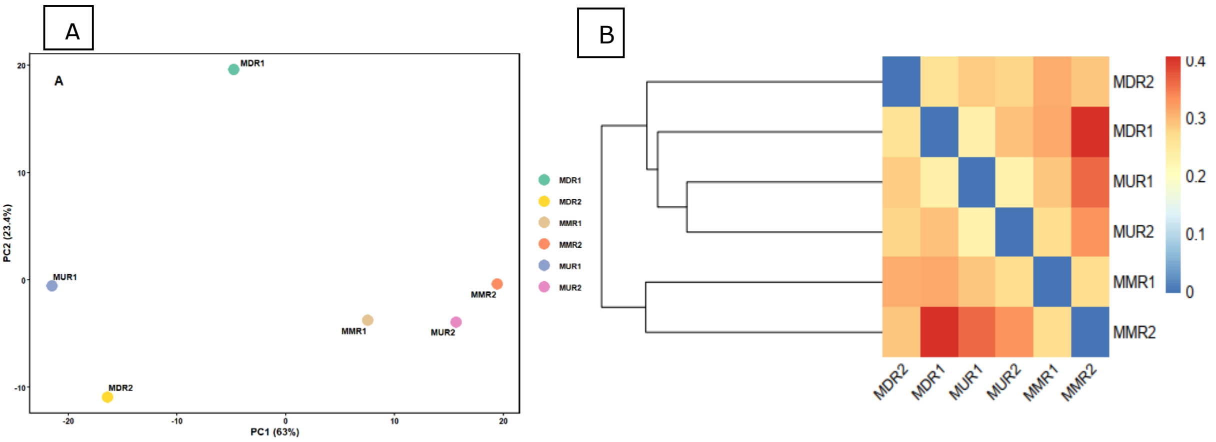

Figure 5. A) Principal component analysis show amplicon library clustering in reduced dimensions based on beta diversity analysis; B) Heatmap showing the beta diversity pairwise distance matrices between sites downstream, midstream, and downstream of Man-an River in Sinacaban, Misamis Occidental, Philippines.

Relationship between physicochemical conditions and eukaryotic community structure. The physicochemical parameters measured at the three sampling stations of the Man-an River are summarized in Table 2. Water temperature ranged from 26.8°C at the downstream station (A1) to 28.0°C at the midstream station (A2) and 27.7°C at the upstream station (A3). pH values ranged from 7.82 (downstream) to 7.94 (midstream) and 7.99 (upstream), all within the neutral to slightly alkaline range. DO concentrations were 6.4 mg L^{-1} at the downstream station and 6.9 mg L^{-1} at both the midstream and upstream stations. All three measured parameters fell within the water quality standards prescribed by the Department of Environment and Natural Resources (DENR) Administrative Order No. 34 (DAO 34), Series of 1990 specifically the Class C criteria for Fishery Water, which stipulate acceptable temperature ranges of 25-31°C, pH of 6.5-9.0, and minimum DO of 5.0 mg L^{-1} (DENR 1990).

Average water temperature ranged from 26.8°C at the downstream station (A1) to 27.7°C at the upstream station (A3) and 28°C at the midstream station (A2) - all within the DAO 34 Class C acceptable range of 25-31°C. The midstream station recorded the highest temperature among the three stations, which spatially coincided with the lowest alpha diversity values and the most compositionally distinct eukaryotic community observed at that reach. Water temperature is a fundamental driver of aquatic community composition, regulating the metabolic rates, physiological tolerances, and reproductive cycles of aquatic organisms (Allan & Castillo 2007). Even modest temperature increases within the physiologically sensitive range of aquatic eukaryotes can alter community structure through thermal filtering, selectively excluding sensitive species while favoring thermotolerant generalists, thereby reducing overall species richness and community evenness (Dossena et al 2012). In the Man-an River, the elevated temperature at the midstream station (28.0°C) is consistent with the reduced relative abundance of temperature-sensitive taxa at that reach including *A. linnaei*. Additionally, the reduced abundance of *H. acidophilum* which is sensitive to thermal and organic stress at the midstream station further supports the interpretation that elevated temperature contributes to the lower diversity and altered community composition observed there (Zeppilli et al 2018). In contrast, the downstream station recording the lowest temperature (26.8°C) showed higher relative abundances of thermally sensitive primary producers including *N. vigensis* and *A. linnaei*, consistent with the more thermally stable conditions at that reach.

Moreover, average pH values ranged from 7.82 at the downstream station to 7.94 at midstream and 7.99 at the upstream station, all within the neutral to slightly alkaline range and well within the DAO 34 Class C standard of 6.5-9.0. The upstream station recorded the highest pH value (7.99), which coincided with the highest Shannon and Simpson diversity values among the three stations. pH is a critical water quality variable that influences nutrient availability, metal solubility, and the physiological functioning of aquatic organisms across all trophic levels (Wetzel 2001). The neutral to slightly alkaline pH conditions recorded throughout the Man-an River are reflected in the community composition detected at each station. The persistent detection of *A. linnaei* across all three stations is consistent with its known optimal pH range of 6.5-8.5 (Boedeker et al 2010), confirming that pH conditions throughout the Man-an River remain within the acceptable range for this pollution-sensitive alga.

The average DO concentrations were 6.4 mg L^{-1} at the downstream station and 6.9 mg L^{-1} at both the midstream and upstream stations, all exceeding the DAO 34 Class C minimum threshold of 5.0 mg L^{-1}. DO is essential for the aerobic respiration of virtually all multicellular eukaryotic organisms, and concentrations above 5.0 mg L^{-1} are generally considered adequate for supporting diverse aquatic communities (Wetzel 2001). The DO values recorded across the Man-an River are reflected in the types of organisms detected at each station. The presence of *A. linnaei* which requires clear, well-oxygenated water for growth and survival (Boedeker et al 2010), across all three stations is consistent with the acceptable DO conditions recorded throughout the river. Similarly, the detection of *H. acidophilum*, a gastrotrich sensitive to anoxic and low-oxygen conditions (Schratzberger & Ingels 2018) further confirms that DO levels in the Man-an River are sufficient to support aerobically dependent meiofaunal organisms. Similarly, the consistent presence of *N. vigensis*, a green microalga that thrives under neutral to alkaline conditions across all stations reflects the broadly favorable pH environment of the river (Safi et al 2021). The slightly higher pH at the upstream station (7.99) may contribute to the greater relative abundance of pH-sensitive clean-water indicator organisms at that reach including *A. linnaei* and *H. acidophilum*, as these organisms exhibit optimal physiological performance under neutral to slightly alkaline conditions (Wetzel 2001). Conversely, the detection of *C. replicatum*, an aquatic chytrid fungus involved in cellulose decomposition, across all stations is consistent with the broadly neutral pH range of the Man-an River, as chytrid enzymatic activity is generally optimal within the pH 6.5-8.0 range (Wurzbacher et al 2014). The slightly lower DO at the downstream station (6.4 mg L^{-1}) may partly reflect this increased organic decomposition activity, as the downstream reach receives allochthonous organic inputs from upstream agricultural runoff, evidenced by the detection of *C. musae* and *P. lanceolatum* eDNA throughout the dataset (Carraro et al 2018). Additionally, the detection of *C. musae*, a banana plant pathogen of agricultural origin across all stations suggests that agricultural runoff entering the river may introduce additional organic loading that contributes to localized oxygen consumption, particularly at the downstream reach (Civade et al 2016).

Table 2

Recorded physico-chemical parameters from three sampling sites in the Man-an River

Parameters	*DAO 34 Class C Standard*	*Sampling stations*		
		Downstream (A1)	*Midstream (A2)*	*Upstream (A3)*
Temperature (°C)	25-31°C	26.8	28	27.7
pH	6.5-9.0 pH	7.82	7.94	7.99
Dissolved oxygen (mg L^{-1})	> 5 mg L^{-1}	6.4	6.9	6.9

Note: Class A-Public Water Supply, Class B-Recreational Water, Class C-Fishery Water; DAO-34 Department of Environment and Natural Resources (DENR) Administrative Order No. 34, Series of 1990, Philippines; Recorded number are average values.

Taken together, the physicochemical data and community composition results suggest that while the Man-an River meets all DENR DAO 34 Class C water quality standards, the measured parameters alone do not fully explain the spatial pattern of eukaryotic diversity observed across the three stations. The midstream station, despite recording water quality values within acceptable limits, consistently showed the lowest alpha diversity and the most compositionally distinct eukaryotic community, highlighting the ecological principle that standard physicochemical monitoring may be insufficient to detect the full extent of biological stress in aquatic ecosystems (Pawlowski et al 2018). Temperature emerged as the most ecologically meaningful physicochemical correlate of diversity in the Man-an River, with the highest temperature at the midstream station (28.0°C) (Table 2) spatially coinciding with the lowest community diversity and the reduced relative abundance of temperature-sensitive organisms including *A. linnaei* and *H. acidophilum*. Meanwhile, the detection of *C. musae* across sampling stations provides biological evidence of land-use impacts on the river that are not captured by physicochemical measurements alone. These findings reinforce the value of complementing physicochemical water quality assessment with biological monitoring using 18S rRNA eDNA metabarcoding, which provides a more sensitive and ecologically informative picture of freshwater river health (Pawlowski et al 2018).

Conclusions. This study provides the first molecular characterization of freshwater eukaryotic diversity in the Man-an River using 18S rRNA eDNA metabarcoding. A total of 767 species across 537 genera, and 291 families were detected from 97,281 high-quality ASVs, revealing a diverse freshwater eukaryotic community composed of algae, fungi, protists, plants, and macroinvertebrate-associated taxa. The consistent detection of ecologically informative taxa such as *Aegagropila linnaei* and *Heterolepidoderma acidophilum* suggests generally favorable ecological conditions within the river system, while the detection of the agriculturally associated fungal pathogen *Colletotrichum musae* indicates potential agricultural runoff influence on the watershed. The midstream station consistently exhibited the lowest alpha diversity and the most compositionally distinct eukaryotic community despite all measured physicochemical parameters remaining within DENR DAO 34 Class C standards. These findings demonstrate that eDNA-derived biological communities may reveal ecological stress patterns not readily detected through physicochemical monitoring alone. Elevated temperature at the midstream station appeared to be associated with reduced diversity, while agricultural land-use inputs likely contributed to shifts in community composition. The abundance of taxonomically unresolved organisms, particularly sp. 1 TK-2012, highlights substantial gaps in current knowledge of tropical freshwater eukaryotic biodiversity and underscores the need for continued taxonomic and ecological investigation of Philippine freshwater systems. Future studies should incorporate seasonal sampling, expanded environmental measurements, hydrological assessments, and multi-gene metabarcoding approaches combining 18S rRNA and COI markers alongside traditional morphological surveys to improve taxonomic resolution and strengthen ecological interpretation. Overall, this study demonstrates the value of eDNA metabarcoding as a sensitive and high-resolution biomonitoring tool for freshwater biodiversity assessment, conservation planning, and sustainable river management in the Philippines. Integrating eDNA metabarcoding into local water governance frameworks and community-based monitoring programs could further enhance conservation efforts, support evidence-based policy development for river preservation, and ultimately help safeguard both the biodiversity and the communities that depend on the Man-an River.

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

Data Availability. The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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