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Deoxyribonucleic acid extraction for invertebrate metabarcoding from stream sediment and implication to biotic indices

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ABSTRACT

BACKGROUND AND OBJECTIVES: Environmental deoxyribonucleic acid metabarcoding has become a powerful tool for assessing invertebrate biodiversity in stream sediments, providing a non-invasive approach for ecological monitoring. However, the precision of this method is largely contingent upon the nucleic acid extraction protocol utilized, as it can impact taxonomic recovery and the associated biotic indices. Previous studies have focused on nucleic acid yield and taxonomic composition, but limited research has evaluated the impact of extraction methods on ecological assessments. The objective of this study is to analyze four nucleic acid extraction methods to identify their effectiveness in optimizing metazoan recovery and bolstering the reliability of biotic indices generated from metabarcoding data.

METHODS: Four nucleic acid extraction protocols were tested for invertebrate nucleic acid metabarcoding from stream sediment: a manual extraction and three Qiagen commercial kits (Powersoil, Blood and Tissue, and Plant Mini kit). Amplification of the extracted nucleic acid was performed utilizing cytochrome c oxidase I along with hypervariable markers 4 and 9 from the 18 Svedberg ribosomal ribonucleic acid, and subsequent sequencing was conducted using Illumina sequencing methods. The relative read abundance was analyzed to assess metazoan recovery and biotic index scores were calculated based metazoan community of respective extraction protocol.

FINDINGS: Among the tested methods, PowerSoil kit consistently yielded the lowest metazoan relative read abundance (36.07 to 86.31 percent) from stream sediment and exhibited higher non-target amplification, including bacterial nucleic acid, due to its bead-beating step optimized for prokaryotic nucleic acid extraction. In contrast, different protocols exhibited a greater efficacy in recovering metazoans, achieving percentages between 63.83 and 98.58, and significantly limiting the co-amplification of bacterial DNA. This indicates a pronounced specificity for eukaryotic nucleic acid. Plant Mini kit performed unexpectedly well, aligning closely with the manual extraction in biotic indices, likely due to its effective inhibitor removal of amplification. Conversely, the blood and tissue kit demonstrated the most significant dissimilarity in biotic indices, likely resulting from inhibition that caused a decrease in the detection of sensitive taxa. Biotic indices derived from cytochrome c oxidase I and 18 Svedberg ribosomal ribonucleic acid hypervariable 9 markers were comparable, with cytochrome c oxidase I showing slightly higher scores due to its greater specificity in targeting key taxa used for index calculation. The 18 Svedberg ribosomal ribonucleic acid hypervariable 4 markers, resulted in diminished biotic index scores and was less successful in facilitating the recovery of metazoans, particularly among invertebrates that are vulnerable to ecological stressors.

CONCLUSION: Overall, the choice of extraction method substantially influenced the detection of target metazoan taxa and the reliability of biotic indices, with the manual extraction and Plant Mini kit emerging as the most effective protocols. The findings illustrate the necessity of appropriate protocols selection in the metabarcoding of stream sediments, indicating that refined techniques for the effective recovery of metazoan nucleic acids are imperative for reliable ecological analysis.

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INTRODUCTION

The accelerating loss of global biodiversity, coupled with increasing human pressures on freshwater ecosystems, has intensified the demand for reliable tools to evaluate and monitor ecological communities (Elbrecht *et al.,* 2016; Reid *et al.,* 2019). The group of macroinvertebrates, comprising insects, crustaceans, and mollusks, plays a vital role as bioindicators in the assessment of water quality and the determination of the health status of freshwater ecosystems (Tampo *et al.,* 2021, Hu *et al.,* 2022). Their diversity and distribution offer crucial insights into the ecological status of aquatic habitats, making them an essential component of environmental monitoring programs (Zhao *et al.,* 2019). Established techniques for examining invertebrate biodiversity in environmental samples have proven to be labor-intensive and time-consuming, often yielding data that is incomplete or subject to bias (Gill *et al.,* 2014; Elbrecht and Leese, 2017). However, the emergence of environmental deoxyribonucleic acid (DNA) as environmental DNA (eDNA) metabarcoding has offered a revolutionary approach that can comprehensively assess invertebrate communities by analyzing genetic material shed into the environment (Brantschen *et al.,* 2021; Wang *et al.,* 2021). Metabarcoding utilizes high-throughput sequencing techniques to identify and quantify multiple invertebrate taxa based on their unique DNA barcode markers (Hajibabaei *et al.,* 2019; Macher *et al.,* 2018), allowing researchers to evaluate the diversity and composition of invertebrate communities rapidly, accurately, and cost-effectively (Piper *et al.,* 2019; Wang *et al.,* 2018). This approach has emerged as a prominent strategy in recent years, showcasing its effectiveness in supporting conservation efforts, facilitating biomonitoring, and assessing the environmental consequences within aquatic and terrestrial ecosystems. A pivotal aspect of eDNA metabarcoding is the DNA extraction process, as it constitutes the first and fundamental step in the workflow (Pawlowski *et al.,* 2022). The selection of DNA extraction protocol is critical for effective eDNA recovery from environmental samples, influencing both the yield and purity of genomic DNA as well as shaping the taxonomic profile observed in metabarcoding analyses (Pearman *et al.,* 2020; Lekang *et al.,* 2015). While the importance of DNA extraction in determining the results of eDNA metabarcoding is acknowledged, there exists a limited body of research that investigates how different extraction methodologies influence the biotic indices derived from these datasets. Previous research has primarily evaluated DNA extraction protocols based on yield and diversity metrics (Deiner *et al.,* 2015). However, the implications for biotic index calculations in freshwater ecosystems remain underexplored. Given that biotic indices are integral to ecological assessments, understanding the influence of DNA extraction choices on these indices is essential. The objective of this study was to analyze and contrast the efficiency of multiple DNA extraction techniques, which included manual protocols as well as commercial kits, in the context of metabarcoding invertebrate communities from sediment samples. This study evaluated four protocols: a manual extraction method using guanidine hydrochloride (GuHCl) and three commercial kits, namely DNeasy PowerSoil (DNPS), DNeasy Blood and Tissue (DNBT), and DNeasy Plant Mini (DNP). While DNPS and DNBT are extensively employed for metazoan metabarcoding applications (van der Loos and Nijland, 2021), DNP has demonstrated efficacy in recovering metazoan diversity from bulk samples (Majaneva *et al.,* 2018). Despite being the traditional manual technique, phenol/chloroform extraction leads to considerably diverse community structures (Djurhuus *et al.,* 2017). Consequently, the current research implemented the GuHCl protocol, recognized for its superior taxonomic recovery capabilities in freshwater samples (Amin *et al*., 2024). This study aims to determine which methods yield optimal metazoan diversity profiles by targeting the cytochrome c oxidase I (COI) and 18 Svedberg ribosomal ribonucleic acid (18S rRNA) gene regions. This will support accurate biotic index calculations and enhance the overall evaluation of invertebrate biodiversity derived from environmental samples. The study aims to compare four nucleic acid extraction protocols to determine their effectiveness in maximizing metazoan recovery and improving the reliability of biotic indices derived from metabarcoding data. This study was conducted in Seom Stream, South Korea in 2020.

MATERIALS AND METHODS

Sample area and sediment collection

Sediments were collected in August 2020 from Seom Stream (37.50096111N; 127.9940194 E) using a shovel. The collection site was a run mesohabitat

and had 0.8 meter per second squared (m/s^2) velocity dan 13.5 centimeters (cm) depth, measured by a digital water velocity meter FP111 (Global Water Flow Probe, Texas, United States). The approximately 3 kilograms (kg) of collected sediments were packed in a disposable plastic bag, and stored at −20 degrees Celsius (˚C) until used for further analysis.

DNA Extraction Protocols

Prior to the eDNA extraction process, the sediment was blended with a corresponding volume of deionized water and agitated manually for 3 minutes (min). Larger pebbles were removed by a sieve with a pore size of 2.23 millimeters (mm), and the resulting filtrate was homogenized using a commercial electric hand mixer for an additional 3 min. For DNA extraction, a 2 milliliter (mL) portion of the homogenized mixture was reserved, and the remaining homogenate was aliquoted and kept at −20 ˚C. All equipment was decontaminated with 10 percent (%) commercial bleach solution containing sodium hypochlorite prior to use. Genomic DNA was extracted employing a manual guanidine hydrochloride protocol (MAN) and three distinct commercial kits supplied by Qiagen, including the DNPS, DNBT, and DNP kits. Triplicate sediment subsamples (250 microliters (µL) each) were prepared for each DNA extraction protocol. Commercial kit extractions followed the manufacturer's standard procedures. A modified version of the existing protocol (Amin *et al.*, 2024) was used for manual eDNA extraction, which involved substituting the ceramic sphere homogenization process with a one-minute vortex homogenization step. All extracted DNAs, whether manual or utilizing commercial kits, were eluted in 50 µL of tris-ethylenediaminetetraacetic acid (TE) buffer, assessed using the ND-1000 NanoDrop (Thermo Scientific, United States), and stored at −20°C until library preparation.

Metabarcoding library preparation and high throughput sequencing

Three distinct markers were employed to amplify metazoan eDNA, including mitochondrial COI, nuclear 18 Svedberg ribosomal RNA hypervariable region 4 (18S rRNA-V4), and hypervariable region 9 (18S rRNA-V9) gene (Table 1). The initial round of polymerase chain reaction (PCR) library construction utilized each of the three eDNA subsamples as distinct templates. The first PCR reaction (20 µL total volume) included 2.0 µL of template DNA, 1.0 µL of 10 picomol (pmol) forward and reverse primers (with adapters), 2.0 µL of 2.5 milimolar (mM) deoxynucleotide triphosphates (dNTPs), 2.0 µL of 10X Ex Taq buffer, 0.2 µL of Ex Taq HotStart (HS) DNA Polymerase (Takara Bio. Inc., Shiga, Japan), 0.6 µL of 3% dimethyl sulfoxide (DMSO) to enhance reaction efficiency, and 11.20 µL of deionized water to complete the final volume. Table 1 provides a comprehensive overview of the specific PCR conditions for each marker, and the PCR setup also incorporated negative controls that did not contain template DNA.

Following electrophoresis on a 1.5% agarose gel, the purification of the expected amplicon size was conducted using the AccuPrep® Gel Purification Kit (Bioneer, Daejon, Republic of Korea). Following

Table 1. The primers for metabarcoding employed in this study

Marker	Direction	Primer sequence (5' to 3')	Initial round of PCR setup	Sources
Cytochrome c oxidase I COI	forward reverse	GGDACWGGWTGAACWGTWTAYCCHCC GGDGGRTAWACWGTTCAWCCWGTHCC	94˚C for 3 min.; 6 × (94˚C for 20 seconds (sec.), 60˚C for 15 sec., and 72˚C for 15 sec.); 24 × (94˚C for 20 sec., 48°C for 15 sec., and 72˚C for 15 sec.); 72˚C for 5 min.	Vamos *et al.*, 2017; Leese *et al.*, 2021
18 Svedberg ribosomal RNA hypervariable region 4	forward reverse	GGAATTACCGCRGSTGCTGG ACTTTCGTTCTTGATYRATGA	94˚C for 3 min.; 10 × (94˚C for 20 sec., 44˚C for 15 sec., and 72˚C for 30 sec.); 10 × (94˚C for 20 sec., 62˚C for 30 sec., and 72˚C for 30 sec.); 72˚C for 5 min.	Tragin *et al.*, 2018
18 Svedberg ribosomal RNA hypervariable region 9	forward reverse	TTGTACACACCGCCCGTCGC CCTTCYGCAGGTTCACCTAC	94˚C for 3 min.; 20 × (94˚C for 20 sec., 60˚C for 15 sec., and 72˚C for 15 sec.); 72˚C for 5 min.	Tragin *et al.*, 2018

purification, the first amplicons underwent indexing through a second PCR, in accordance with the previously described method (Amin *et al.,* 2024). Second amplicons were purified, and their quality and quantity were assessed using the Qubit double-strand deoxyribonucleic acid (dsDNA) HS Assay Kit (Invitrogen, California, United States). Sequencing of the amplicons was performed on the Illumina Sequencing platform with a 2 × 300 base-pair (bp) paired-end for high-throughput analysis.

Bioinformatics pipeline and data analysis

Initially, raw reads underwent demultiplexing, after which adapters and indices were trimmed. Sequences with low quality scores (below 20) were subsequently removed (<20) using CLC genomics workbench v8.0 (CLC Bio, Massachusetts, USA). Subsequently, primer sequences were removed by Cutadapt v1.9.1 (Martin, 2011). The trimmed reads were then subjected to the Divisive Amplicon Denoising Algorithm 2 (DADA2) v1.16.1 (Callahan *et al.,* 2016) to produce amplicon sequence variants (ASVs). After ASVs curation using lulu v0.1.0 (Frøslev *et al.,* 2017), curated ASVs were identified through taxonomy assignment using the basic local alignment search tool (BLAST) v2.10.1+ against the National Center for Biotechnology Information (NCBI) nucleotide database (accession date: 04/02/2021), and accession numbers were used for higher taxonomic assignments, ranging from genus to kingdom, using simple exploration of ecological data (SEED) v2.1.1a (Větrovský *et al.,* 2018). An overall community profile related to the specific marker was developed by merging the data collected from all four protocols. The ASV tables were analyzed using phyloseq v1.32 (McMurdie and Holmes, 2013), vegan v2.5.7 (Dixon, 2003), and microeco v 0.12 (Liu *et al.,* 2020a). The calculation of dissimilarity matrices for metazoan communities was performed using relative read abundance (RRA) using Bray-Curtis distance method. Subsequently, eight biotic indices were computed using biotic package v 0.1.2 (Briers, 2016), including Biological Monitoring Working Party, BMWP (Hawkes, 1998) and the Whalley revision (Walley and Hawkes, 1997), Average Score Per Taxon, ASPT (Armitage *et al.,* 1983), Proportion of Sediment-sensitive Invertebrates, PSI (Extence *et al.,* 2013), Lotic-invertebrate Index for Flow Evaluation, LIFE (Extence *et al.,* 1999), and Walley-Hawkes-Paisley-Trigg (WHPT) for presence/absence and abundance data (Paisley *et al.,* 2014The dataset of biotic indices underwent multivariate analysis through Principal Coordinate Analysis (PCoA), utilizing Euclidean distance to effectively visualize the underlying patterns and relationships. Variations in biotic indices across protocols and markers were analyzed using permutational analysis of variance (PERMANOVA) (Anderson, 2014) with the *adonis* function, employing 999 permutations. In instances where PERMANOVA revealed significant differences, a permutational multivariate analysis of dispersion (PERMDISP) was utilized to assess whether these differences were due to variations in group centroids or from variations in sample dispersion in the PCoA analysis. The pheatmap v1.0.12 package (Kolde and Kolde, 2015) was performed to visualize the heatmap of the pairwise distance matrix and its clustering. Data visualizations were produced using the ggplot2 package v3.3.3 (Wickham, 2011) in R v3.6.3.

RESULTS AND DISCUSSION

General community structure and co-amplification of non-target taxa

The raw reads were registered and freely accessed under bioproject PRJNA1126988. After processing raw reads of COI (808,053), 18S rRNA-V4 (566,406), and 18S rRNA-V9 (677,082), the respective marker generated 104, 470, and 472 curated ASVs. Among them, 74 (COI), 123 (18S rRNA-V4), and 89 ASVs (18S rRNA-V9) were classified as metazoan. There were marked variations in taxonomic coverage across the three markers (Fig. 1). Amongst the markers, 18S rRNA-V9 exhibited the most comprehensive taxon coverage, encompassing taxa from six different kingdoms, while 18S rRNA-V4 covered five kingdoms, and COI spanned threeThe analysis comparing various extraction protocols indicated that DNPS consistently resulted in the lowest RRA of metazoan reads for all markers tested. For instance, when considering the COI marker (Fig. 1A), DNPS resulted in a metazoan RRA of 86.31%, in contrast to other methods that attained metazoan RRA percentages between 95.02% and 98.58%. Moreover, the 18S rRNA-V4 marker (Fig. 1B) exhibited a markedly lower metazoan RRA of 19.82% with DNPS, whereas other protocols produced metazoan RRAs ranging from 72.26% to 92.66%. In the 18S rRNA-V9 (Fig. 1C), DNPS again demonstrated a lower metazoan RRA of 36.07% compared to the other protocols, which ranged from

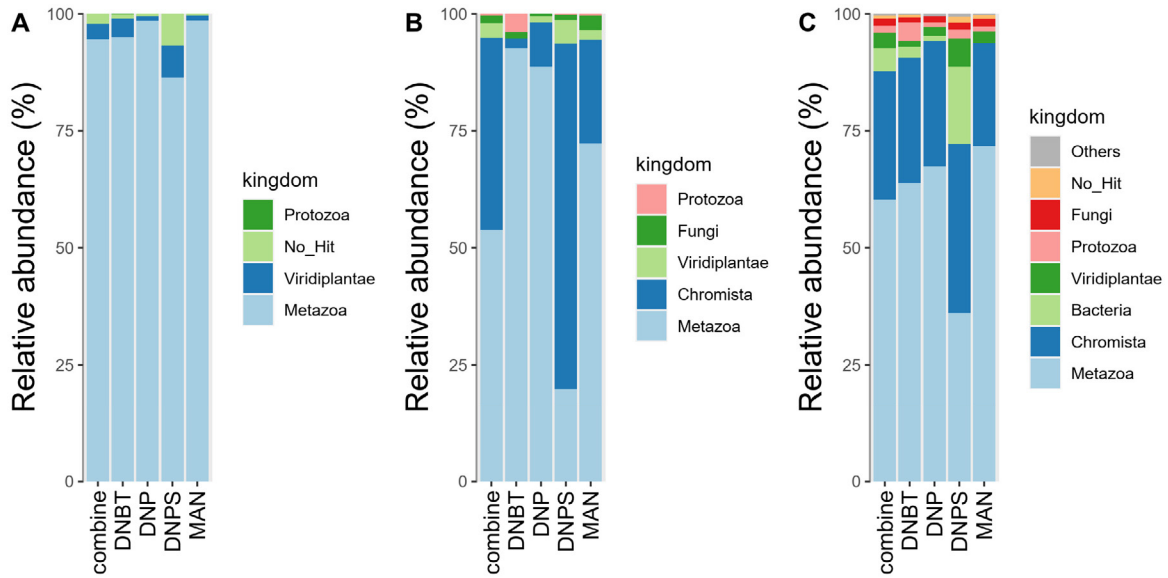

Fig. 1: Relative abundance of (A) COI, (B) eukaryotic 18S rRNA-V4, and (C) eukaryotic 18S rRNA-V9 metabarcoding using four different extraction protocols at the kingdom level

63.83% to 71.66%. All protocols, except for the MAN, demonstrated co-amplification of bacterial sequences when the 18S rRNA-V9 marker was employed. DNPS showed the highest bacterial RRA at 16.58%, whereas DNP and DNBT generated bacterial RRAs of 1.08% and 2.24%, respectively.

The issue of co-amplifying non-target taxa is a persistent challenge in eDNA metabarcoding, potentially leading to a misrepresentation of ecologically vital target taxa. In this study, despite employing a COI primer specifically designed to target freshwater macroinvertebrates from eDNA samples (Leese *et al.,* 2021), the occurrence of non-metazoan taxa was observed across all extraction protocols. Sequences from non-metazoan groups, including Viridiplantae and Protozoa, were observed, with the DNPS protocol resulting in the highest rate of non-target sequences. When examining the taxonomic coverage offered by the 18S rRNA markers, both the V4 and V9 regions amplified a broader diversity of non-metazoan eukaryotic taxa compared to COI. The consistent identification of taxa such as Chromista, Fungi, Protozoa, and Viridiplantae underscores the inherent broader eukaryotic specificity of these markers (Salonen *et al.,* 2018). Nonetheless, both 18S rRNA markers demonstrated a substantial proportion of metazoan reads, at 53.83% for 18S rRNA-V4 and 60.31% for 18S rRNA-V9, indicating the 18S rRNA application in capturing metazoan biodiversity even within mixed-community samples (López-Escardó *et al.,* 2018). This holds significant importance for studies that seek to define complex ecosystems, enabling the simultaneous analysis of metazoan and non-metazoan biodiversity (Guardiola *et al.,* 2016). However, the trade-off was evident, as non-target amplification could decrease the sequencing depth for target taxa, potentially impacting the detection of rare or ecologically significant species. A major limitation associated with the use of broad eukaryotic primers, particularly those that target 18S rRNA, is the likelihood of cross-reactivity with DNA from bacteria. This issue was clearly demonstrated with the 18S-V9 marker, which has a known propensity to co-amplify bacterial sequences in eDNA metabarcoding (Amaral-Zettler *et al.,* 2009; Pawlowski *et al.,* 2011; Maritz *et al.,* 2017), emphasizing the challenges it poses for accurately profiling eukaryotic communities. The findings of this study indicated that bacterial amplification was notably enhanced in the DNPS protocol, designed specifically for optimizing prokaryotic DNA extraction. The DNPS protocol's predisposition to amplify non-target DNA could be

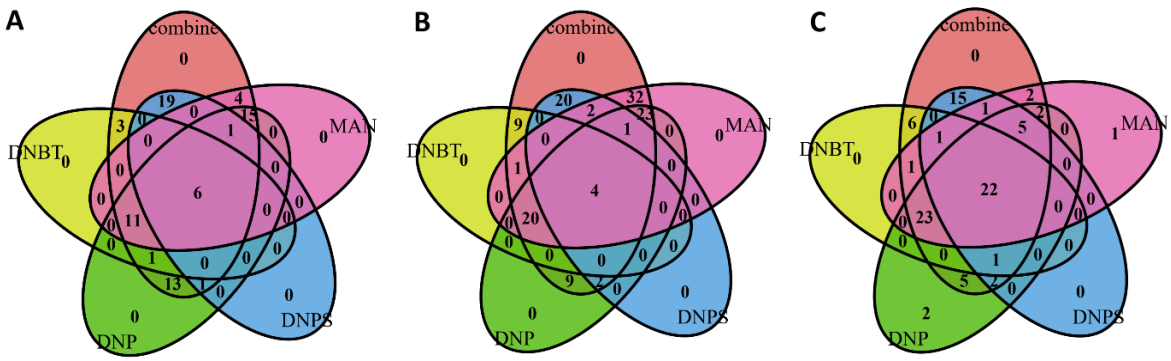

Fig. 2: Shared metazoan amplicon sequence variants (ASVs) amongst eDNA extraction protocols. Number of shared ASVs amongst protocols for the; (A) COI, (B) eukaryotic 18S rRNA-V4, and (C) eukaryotic 18S rRNA-V9 dataset

attributed to its development for prokaryotic DNA extraction, featuring bead-beating steps that are highly effective at lysing bacterial cell walls (Yang *et al.,* 2023). DNP extraction kits yielded the least amount of bacterial sequences compared to other kits, aligning with previous reports (Corcoll *et al.,* 2017). The MAN protocol demonstrated a distinct advantage in this study, as it uniquely avoided the amplification of bacterial sequences, indicating its high efficiency in isolating eukaryotic DNA. This focused recovery highlights MAN's potential as a leading option for eukaryotic metabarcoding, particularly in contexts where bacterial co-amplification might overshadow the amplification of specific metazoan taxa (Collins *et al.,* 2019).

Metazoan community structure

In metazoan datasets, the proportion of the ASV shared amongst all protocols varied considerably, with COI showing a modest proportion of shared ASVs (8.1%) and 18S rRNA-V4 displaying an even lower percentage (3.3%) (Fig. 2). Conversely, the 18S-V9 marker showed a significantly higher rate of shared ASVs, reaching 24.7%. (Fig. 2C). Notably, three ASVs exclusively observed using the 18S rRNA-V9 marker, with no presence using COI or 18S rRNA-V4. Specifically, the MAN protocol revealed a single unique ASV using 18S rRNA-V9, while the DNP protocol identified two distinct ASVs. The combine-DNPS and combine-MAN protocols demonstrated the highest degree of shared ASVs using COI and 18S rRNA-V4 markers, respectively. The findings emphasize the diverse patterns of ASV sharing and uniqueness that exist across the various markers and protocols examined.

Metazoan beta diversity was assesed by analyzing their RRA data, revealing the lowest community dispersal was observed in the 18S rRNA-V9 marker (distance, d = 0.35 ± 0.26), followed by COI (d = 0.56 ± 0.29), whereas 18S rRNA-V4 exhibited the highest community dispersal (d = 0.66 ± 0.21) (Fig. 3A). The analysis reveals that the 18S rRNA-V9 marker leads to a more consistent community structure when compared to other markers, which is supported by the increased number of shared ASVs across different extraction methods (Fig. 2). Cluster analysis indicated that metazoan communities within COI and 18S rRNA-V4 shared a similar cluster topology (Fig. 3B-C), characterized by the following order: [[[[DNP+MAN] combine]DNBT]DNPS]. In contrast, 18S rRNA-V9 displayed a distinct cluster topology , arranged as [[[[DNBT+DNP]combine]MAN]DNPS] (Fig. 3D).

Regarding metazoan community differences concerning the combined dataset, the highest Bray-Curtis dissimilarity values were observed with DNPS (d = 0.71), DNBT (d = 0.84), and DNPS (d = 0.55) when using COI, 18S rRNA-V4, and 18S rRNA-V9, respectively (Fig. 3B-D). Conversely, the highest Bray-Curtis similarity to the combined dataset was observed with MAN (d = 0.41) and DNBT (d = 0.13) in 18S rRNA-V4 and 18S rRNA-V9, respectively. Moreover, the metazoan community present in the combined dataset showed a profile that closely resembles those of DNP and MAN (d = 0.31) regarding COI. Amongst the protocols, DNP exhibited the highest similarity to MAN in COI (d = 0.17) and 18S rRNA-V4 (d = 0.28),

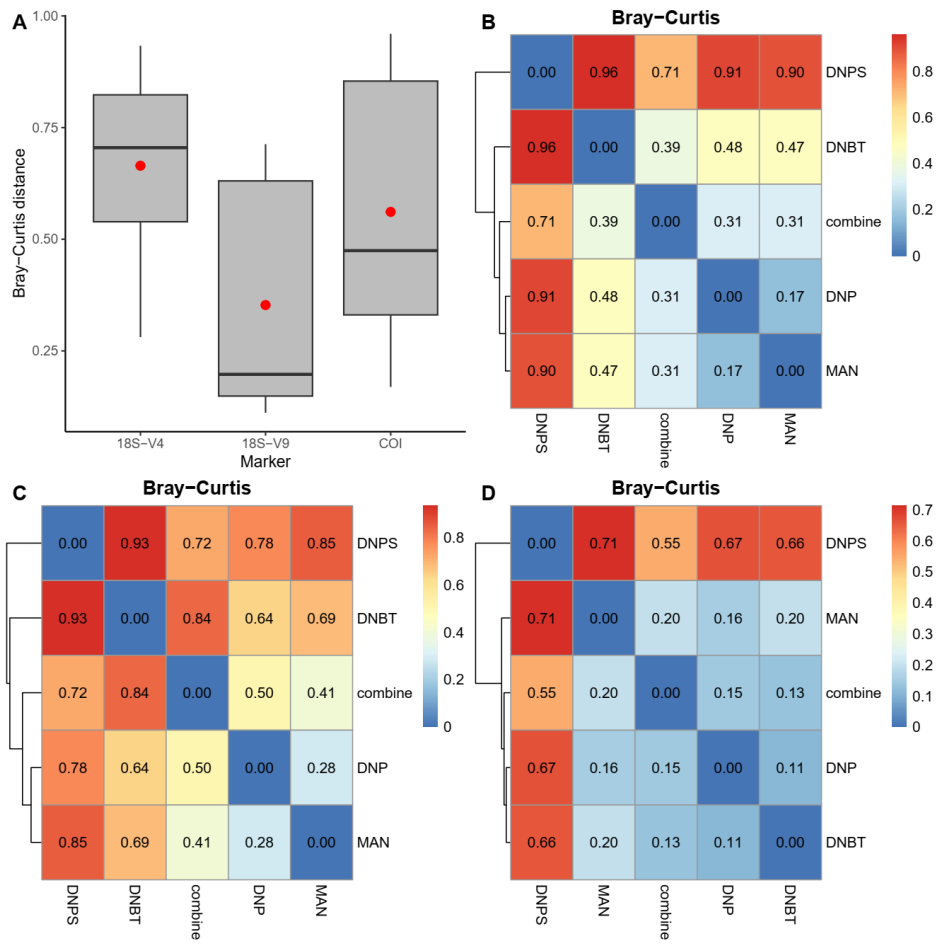

Fig. 3: Metazoan community dispersal was evaluated using COI, 18S-V4, and 18S-V9 markers (A). Pairwise Bray-Curtis dissimilarity of four extraction protocols using COI (B), 18S rRNA-V4 (C), and 18S rRNA-V9 (D) marker. The boxplot displays the means as red circles and the medians as solid lines

while DNP demonstrated the highest similarity to DNBT in 18S rRNA-V9 (d = 0.11). Although DNP was designed primarily for plant DNA extraction and is typically used in non-metazoan metabarcoding (Lear *et al.,* 2018) it proved highly effective for metazoan studies. This observation aligns with prior findings from metabarcoding of seawater samples (Laroche *et al.*, 2020a; Laroche *et al.*, 2020b) and illustrated improved consistency in capturing metazoan diversity (Majaneva *et al.,* 2018). The improved efficacy of DNP can be linked to its capacity to eliminate PCR inhibitors, as noted by Espinosa Prieto *et al*. (2024), which are commonly found in sediment samples (Wei *et al.,* 2018). In contrast, the DNPS and DNBT extraction kits have been widely used for metazoan metabarcoding–(van der Loos and Nijland, 2021). However, this study found that both methods exhibited the lowest similarities to the combined community profile (Fig. 3), indicating suboptimal performance in recovering metazoan sequences (Kang *et al.,* 2022) and capturing the full metazoan diversity in environmental samples (Laroche *et al.,* 2020b). Additionally, the 18S rRNA-V9 marker, with a shorter amplicon length of approximately 129 bp, was more effective for degraded eDNA, as shorter amplicons typically have higher amplification success (Wei *et al.,* 2018), leading to a greater proportion of shared ASVs (Fig. 2) and higher similarity among communities (Fig. 3D). Although mock communities are widely regarded as the gold standard for assessing

the efficiency of DNA extraction and amplification protocols (Hermans *et al.,* 2018) and can offer precise evaluations for bulk sample metabarcoding (Braukmann *et al.,* 2019. However, they inherently lack several vital elements that represent the true state of environmental samples. Specifically, mock communities were artificially constructed (Vamos *et al.,* 2017) and typically underestimated the complex and variable matrix of environmental inhibitors (e.g., humic acids, heavy metals) that can interfere with DNA extraction and amplification. Moreover, mock communities overlooked the natural diversity and abundance of non-target taxa, such as bacteria and protists. These organisms often co-amplify and compete with metazoan DNA during PCR, thereby impacting the representation of target taxa in metabarcoding studies. In contrast, the approach of pooling all datasets such in this study to create a "comprehensive" community representation leveraged the full breadth of the eDNA samples, encompassing the natural variability and competitive interactions present in field conditions. Nevertheless, utilizing mock communities for inferring community composition from bulk samples could be a more appropriate approach. This method yielded a more authentic and ecologically sound evaluation of community composition, effectively illustrating the nuances of the environmental matrix while accounting for possible biases from both biotic elements (non-target taxa) and abiotic conditions (inhibitors).

Biotic indices from metabarcoding data

Utilizing both COI and 18S rRNA markers in metabarcoding presents a balanced and extensive strategy for assessing biodiversity in environmental samples by inferring biotic indices. The COI region used in this study was designed to capture more arthropods taxa as key indicator group from environmental samples (Leese *et al.,* 2021), since the most of previous primer amplified non-arthropods taxa (Hajibabaei *et al.,* 2019). The focus on COI restricted the taxonomic diversity examined in the study, which may have led to the exclusion of non-insect taxa or specific resilient groups critical to biotic index assessments, such as annelids, mollusks, and flatworms (Nahli *et al.,* 2022; Kumari and Maiti, 2020). Furthermore, 18S rRNA markers, particularly the V4 and V9 hypervariable regions, were amplified to overcome the COI limitation due to its effectivity to capturing a wide array of metazoan (Zhao *et al.,* 2021). By integrating COI and 18S rRNA markers, metabarcoding could leverage the high taxonomic resolution of COI while benefiting from the comprehensive taxonomic coverage of 18S rRNA. The synergistic properties of these markers enabled thorough ecological assessments, enhancing both the sensitivity and reliability of biodiversity monitoring and providing deeper insights into ecosystem health from metabarcoding data. This study analyzed biotic indices derived from metazoan communities using COI, 18S rRNA-V4, and 18S rRNA-V9 markers across four different DNA extraction protocols (Table 2), with indices from the combined community profile of all protocols serving as a benchmark. The 18S rRNA-V4 marker yielded significantly lower biotic index scores relative to COI and 18S rRNA-V9 and diverged from the other markers (PERMANOVA, *R*-squared (R^2) = 0.8202, probability value (p) = 0.01), indicating limitations in its effectiveness for accurately assessing ecological status. Analysis of biotic index scores revealed no significant differences across the extraction protocols, regardless of the marker applied (R^2 = 0.09671, p = 0.93). Biotic indices derived from COI were closely aligned with those from 18S rRNA-V9 (Fig. 4A), as

Table 2: Biotic indices generated from COI, 18S rRNA-V4, and 18S rRNA-V9 metabarcoding community using four different protocols and the combination of all protocols

Biotic index	COI	18S rRNA-V4	18S rRNA-V9
BMWP	58/74/48/58/94	1/18/8/18/18	63/58/58/63/68
BMWP-Whalley	64.5/81.2/54.7/61.8/102.1	3.5/22.4/11.8/22.4/22.4	67.6/62.9/62.9/68.4/74.2
ASPT	7.25/6.73/6.86/7.25/7.23	1/4.5/4/4.5/4.5	6.3/6.44/6.44/6.3/6.18
ASPT-Whalley	8.06/7.38/7.81/7.73/7.85	3.5/5.6/5.9/5.6/5.6	6.76/6.99/6.99/6.84/6.75
WHPT (abundance)	7.56/6.56/7.1/6.88/7.1	2.3/4.77/4.3/4.55/4.78	5.9/5.98/5.98/5.92/5.88
WHPT (incidence)	7.59/7.08/7.2/7.27/7.55	2.7/5.15/5.55/5.15/5.15	6.16/6.28/6.28/6.24/6.2
LIFE	8.17/8.33/8.8/8.33/8.18	0/8/10/8/8.33	7.38/7.57/7.57/7.38/7.44
PSI	66.67/77.78/75/66.67/76.19	0/38.46/37.5/40/43.75	53.85/53.85/53.85/57.14/62.5

The numbers within the cells, separated by "/" indicate (i) DNPS/ (ii) DNP/ (iii) DNBT/ (iv) MAN/ (v) combine.

Table 3: Pairwise PERMANOVA analysis comparing biotic indices generated among markers, with R^2 and *p*-values presented in the lower and in upper diagonal, respectively

Biotic index	COI	18S rRNA-V4	18S rRNA-V9
COI		0.01	0.17
18S rRNA-V4	0.8019		0.004
18S rRNA-V9	0.2059	0.8679	

supported by pairwise PERMANOVA analysis, which indicated no significant differences between the two markers (Table 3). This finding suggests comparable performance in assessing ecological status, although 18S rRNA-V9 showed a marginally lower result (Table 2). The study implies that both markers can successfully represent metazoan diversity and offer important information for ecological evaluations. The slight underperformance of 18S rRNA-V9 compared to COI, likely reflected the trade-off between broader taxonomic coverage and finer taxonomic resolution. This emphasizes the value of a dual-marker methodology, which combines the precise specificity of COI with the broad coverage of 18S rRNA, thereby offering a more equitable and robust representation of the community.

Significantly, the 18S rRNA-V4 yielded the poorest biotic indices in DNPS assessments, which can likely be attributed to a lower recovery rate of Metazoan sequences. Longer amplicon of 18S rRNA-V4 might have better resolution for species assignment (Liu *et al.,* 2020b; Hadziavdic *et al.,* 2014), however, it probably generated lower sequence recovery of metazoan from environmental samples (Bylemans *et al.,* 2018; Jo *et al.,* 2017). The detection of trace levels of metazoan DNA within a large background of non-target DNA represents a major challenge, a situation often observed in environmental DNA (eDNA) obtained from environmental samples. These samples are typically rich in DNA from various sources such as bacteria, fungi, phytoplankton, and other single-cell non-target organisms (Zhang *et al.,* 2021; Djurhuus *et al.,* 2017). When the sample contains a predominance of nontarget DNA, including bacterial DNA in 18S rRNA-V9, employing universal primers can result in the amplification of a significant amount of DNA from non-target organisms (Hajibabaei *et al.,* 2019). Consequently, this masking effect demonstrated poor reproducibility because of the non-specific amplification of prokaryotic or non-target eukaryotic DNA (Collins *et al.,* 2019. This limitation affected the ability to detect target invertebrate DNA within the sequenced reads from such samples (Leese *et al.,* 2021), resulting in comparatively lower biotic indices. These limitations suggest that the 18S rRNA-V4 marker may be less suitable for ecological assessments focused on metazoan diversity. To boost its functionality, it might be essential to merge it with more precise markers or to optimize the design of primers to improve specificity for metazoans. Concerning the consistency of biotic indices across different extraction protocols, 18S rRNA-V9 exhibited relatively uniform indices (Fig. 4A and 4D), aligning with the higher proportion of shared ASVs across markers (Fig. 3C), whereas COI showed considerable variability. However, this variability did not influence the overall findings, as indicated by the pairwise PERMANOVA results. Even though short amplicon resulted in weak resolution in lower taxa (Dewi *et al.,* 2024), a higher taxonomic level (family) was sufficient for scoring biotic indices (Chessman *et al.,* 2006; Artemiadou and Lazaridou, 2005) and physicochemical stressors in freshwater ecosystems (Medupin, 2019). In contrast, the COI primer employed in this study was optimized to effectively amplify sensitive arthropods from environmental samples, regardless of its variability (Leese *et al.,* 2021). This is illustrated in Table 4 of the current study, which resulted in elevated scores in biotic indices.

In the context of dissimilarity matrices, a distinct clustering pattern emerged. The biotic indices of the combined dataset exhibited the highest similarity with the DNP in COI dataset (d = 28.99) (Fig. 4B). In contrast, the biotic indices of combine in the 18S rRNA-V4 dataset were most closely clustered with DNP+MAN, revealing the highest degree of similarity to the MAN protocol (d = 3.77), as depicted in Fig. 4C. Remarkably, although the combined dataset was associated with a group encompassing all extraction methods using 18S rRNA-V9, it exhibited a notably higher degree of similarity to the MAN (d = 9.35) when compared to other protocols (Fig. 4D). Additionally, it is remarkable that DNBT (d = 66.07),

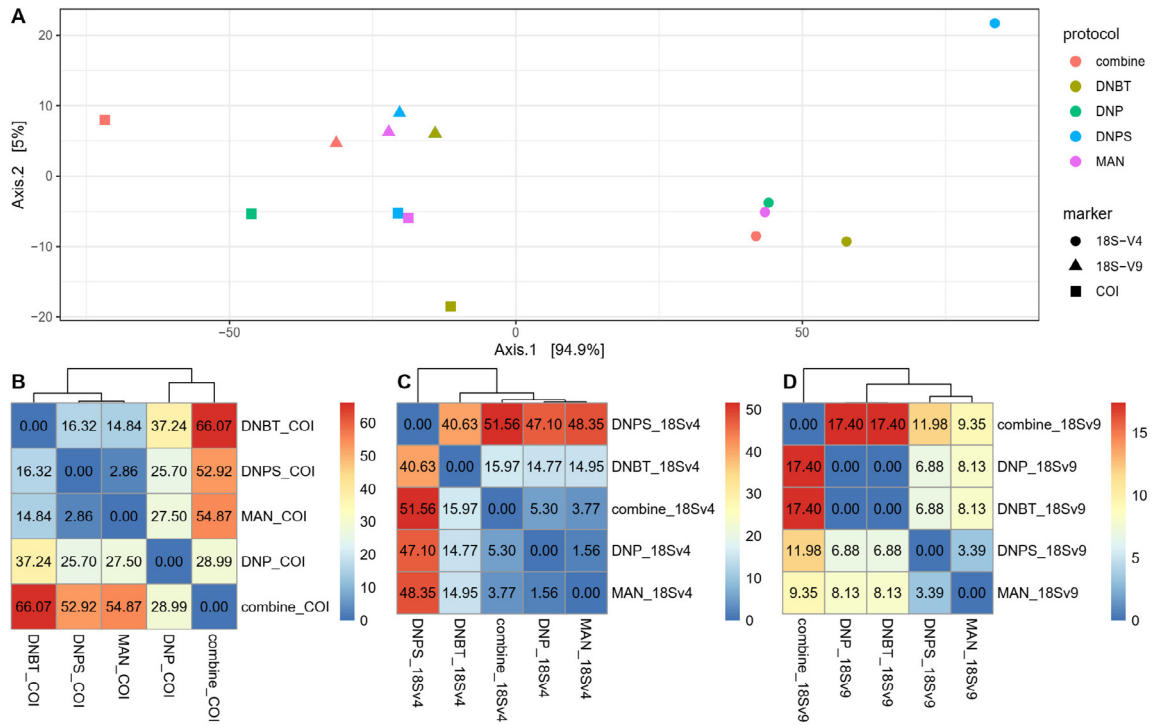

Fig. 4: Principal Coordinate Analysis (PCoA) analysis generated from nine biotic indices comparing four extraction protocols using COI, 18S rRNA-V4, and 18S rRNA-V9 markers (A). Pairwise Euclidean dissimilarity analysis based on the nine biotic indices of four extraction protocols using (B) COI, (C) 18S rRNA-V4, and (D) 18S rRNA-V9 marker.

DNPS (d = 51.56), and DNP+DNBT (d = 17.40) displayed the lowest similarity in terms of biotic indices when compared to the combined protocol within COI, 18S rRNA-V4, and 18S rRNA-V9 dataset, respectively. Metabarcoding studies have generated metacommunities with extensive taxonomic composition and high diversity of benthic invertebrates (Gleason *et al.,* 2023), providing valuable insights for assessing ecosystem health (Wu *et al.,* 2022). Prior studies employing invertebrate COI metabarcoding datasets have successfully reconstructed marine biotic indices, revealing a strong correlation and considerable consistency with traditional biomass-based morphology indices (Aylagas *et al.,* 2018). In contrast, the application of 18S metabarcoding to infer ecological status in invertebrate communities has been limited, as 18S markers are typically employed in diatom-based indices (Pawlowski *et al.,* 2018). Notably, 18S rRNA metabarcoding demonstrated a robust relationship between micro-eukaryotic assemblages in biofilms and water quality (Minerovic *et al.,* 2020). However, deriving biotic indices from metacommunities remains challenging, as methodological factors such as DNA extraction, play a crucial role in accurately capturing taxonomic assemblages from environmental samples (Liu *et al.,* 2020b; Marquina *et al.,* 2022). Beyond the significant costs (Lear *et al.,* 2018), the selected DNA extraction method plays a crucial role in influencing the eDNA results (Hermans *et al.,* 2018). Prior investigations into eDNA extraction methods for invertebrate metabarcoding have largely emphasized the yield of DNA and the composition of taxa (Majaneva *et al.,* 2018). However, there has been a scarcity of studies examining the influence of eDNA extraction methods on the biotic indices that are derived from metabarcoding data. Despite previous efforts to compare eDNA extraction methods for assessing eukaryotic metabarcoding biotic indices in marine aquaculture (Pearman *et al.,* 2020) and offshore benthic sediments (Hestetun *et al.,* 2021), a critical gap remains in understanding how these

Table 4: The number of ASV and unique families in parentheses belong to Ephemeroptera, Plecoptera, Trichoptera, and Diptera (EPTD) taxa from COI, 18S rRNA-V4, and 18S rRNA-V9 metabarcoding communities using four different protocols and the combined of all protocols

Biotic index	DNPS	DNP	DNBT	MAN	combine
COI	17 (8)	27 (11)	13 (6)	22 (7)	40 (13)
18S rRNA-V4	1 (1)	4 (3)	2 (2)	4 (3)	6 (4)
18S rRNA-V9	14 (8)	13 (7)	11 (7)	14 (8)	18 (9)

protocols perform in freshwater ecosystems. This study seeks to bridge the gap by analyzing extraction protocols designed for freshwater samples, delivering critical insights into their effectiveness and the implications for biotic indices and ecological monitoring. The study reveals that the choice of extraction protocol led to variability in the biotic indices. Notably, the MAN and DNP protocols produced biotic indices with greater similarity to the combined dataset. MAN demonstrated a higher species detection rate than commercial kits in freshwater ecosystems (Amin *et al.,* 2024), indicating its high efficiency in generating abundant metazoan reads, which is advantageous for accurate biotic index calculations. The precise elements that lead to the enhanced efficiency of metazoan nucleic acid recovery observed in the manual extraction protocol, in comparison to commercial kits, are still not fully elucidated. However, the relatively simple state of eDNA in environmental samples, unlike the more complex matrix found in tissue samples, suggests that a simple extraction process is sufficient for effective eDNA recovery (Deiner *et al.,* 2015; Renshaw *et al.,* 2015). In other hand, although commercially available column-based extraction kits are effective in isolating contaminant-free DNA from potentially inhibition containing sample, there was still loss of significant amounts of DNA on the column (Abdel-Latif and Osman, 2017; Kemp *et al.,* 2014). Additionally, the application of QIAshredder in DNP effectively mitigated phenolic contaminants during DNA extraction (Forsgren *et al.,* 2017), thereby reducing the potential interference of these compounds, which could otherwise inhibit PCR amplification. Although the DNPS protocol exhibited the most distinct community profiles compared to the combined dataset (Fig. 3), it achieved recovery rates for Ephemeroptera, Plecoptera, Trichoptera, and Diptera (EPTD) as a sensitive group in both COI and 18S rRNA-V9 to that of the MAN protocol (Table 4). This enabled a balanced comparison of biotic indices generated from the COI and 18S rRNA-V9 markers. This finding underscores the potential utility of DNPS for inferring biotic indices from sediment samples (Pearman *et al.,* 2020), provided that appropriate markers are employed. The use of extraction kits with inhibitor-removal capabilities, such as DNP and DNPS, presents notable advantages in tackling sediment heterogeneity and the inconsistencies in water chemistry, particularly within freshwater ecosystems where organic matter levels vary. Sediments high in organic content tend to bind DNA (van Bochove *et al.,* 2020), reducing the yield of recoverable DNA and potentially enriching inhibitors that impede PCR amplification. Additionally, the deployment of these extraction kits contributes to lessening the influence of sample condition variability, which ensures that results remain consistent and reliable, regardless of differing environmental circumstances. On the other hand, DNBT showed the lowest similarity of biotic indices compared to the combined protocol for both COI and 18S rRNA-V9 markers (Fig. 4B and 4D), yielding relatively lower scores, particularly for BMWP and BMWP-Whalley indices (Table 2). These findings may be connected to a lower detection rate of EPTD families (Table 4), which are vital taxa for the purposes of biomonitoring (Laini *et al.,* 2022; Koperski, 2019). Previous studies have reported that DNBT is prone to PCR inhibition in environmental samples (Amin *et al.,* 2021), potentially limiting the amplification of low-abundance sequences. The extraction protocol chosen plays a significant role in determining taxonomic recovery and also influences the biotic indices derived from the data, which are utilized to evaluate the ecological quality of various habitats. Inconsistent recovery of metazoan taxa across different protocols could introduce variability in the indices, complicating the interpretation of ecological data. For instance, the lower biotic index scores observed with DNBT might not accurately reflect poor ecological conditions but rather the protocol's limited efficiency in amplifying

metazoan DNA. Additionally, the choice of inappropriate markers led to an increase in the variability of the index. This methodological bias could lead to false negatives (van der Loos and Nijland, 2021), underestimating the ecological status of a site and obscuring the true biodiversity patterns (Blackman *et al.,* 2024). Conversely, protocols like MAN and DNP, which demonstrated consistent metazoan recovery and produced biotic indices closely aligned with the combined dataset, offer a more reliable basis for ecological interpretation. The reliability of these protocols facilitates their wider use in biomonitoring programs, ensuring trust in the ecological assessments generated from eDNA metabarcoding data. Furthermore, the use of comprehensive protocols can enhance the detection of low-abundance and rare taxa (Stojan *et al.,* 2023), which are often critical for understanding ecosystem complexity and detecting subtle environmental changes in freshwater ecosystems (Shi *et al.,* 2023). The study's reliance on only one mesohabitat type, particularly the run, may constrain the broader relevance of the findings to other ecosystem types. However, the run mesohabitat was selected due to its representation of a balanced invertebrate community which are more characteristic of broader stream ecosystems (Didier and Kestemont, 1996), contrasting with riffles, which are dominated by arthropods, and pools, which are dominated by non-arthropods. Future studies that incorporate a more extensive range of ecosystems, including soil and marine sediments, would yield important comparative insights, facilitating the validation of results in diverse environmental contexts. Due to the unique environmental traits of these ecosystems, especially their varying salinity levels, it is probable that differences in outcomes would be noted. For example, the MAN protocol may not be suitable for high-salinity environments due to potential inhibition of PCR amplification, as previously noted (Amin *et al.,* 2024). Regarding the extraction cost, the MAN protocol offered a significant cost advantage, with extraction costs below 1 United States Dollar (USD) per sample compared to 6.34 USD, 4.48 USD, and 9.80 USD for the DNP, DNBT, and DNPS kits, respectively. Due to its cost-effectiveness, the MAN protocol is exceptionally well-suited for large-scale ecological monitoring and long-term biomonitoring projects, particularly when financial constraints are a consideration. Reduced costs facilitate a wider range of sampling, thereby enhancing the reliability of biodiversity evaluations. However, although the MAN protocol avoids the use of hazardous phenol and chloroform, it employed guanidine hydrochloride (GuHCl) for protein denaturation, necessitating strict safety precautions (Davies *et al.,* 2021). The appropriate management and disposal of GuHCl are vital, requiring compliance with institutional guidelines and environmental safety regulations to minimize any associated risks. Furthermore, the benefit of using the MAN protocol was enhanced when considering its potential to handle larger sample volumes, which has been shown to improve biodiversity estimates for arthropods and other metazoans (Dopheide *et al.,* 2019) and the proportion of metazoan reads (Brannock and Halanych, 2015). Although commercial kits such as the DNeasy Plant Maxi Kit and DNeasy PowerMax Soil Kit enable the handling of larger initial sample volumes, their elevated extraction costs represent a major constraint on scalability for routine or large-scale use.

CONCLUSION

The results of this study underscore the vital role that DNA extraction methods play in the success of eDNA metabarcoding results, demonstrating notable variability in taxonomic recovery, community structure, and biotic index assessments. Across all markers tested (COI, 18S rRNA-V4, and 18S rRNA-V9), the selection of the extraction method played a pivotal role in determining the quality and reproducibility of the datasets, influencing both the relative abundance of metazoan sequences and the identification of ecologically significant taxa. Within the extraction protocols evaluated, DNPS consistently exhibited the lowest metazoan RRA, coupled with an increased proportion of non-target sequences, notably bacterial DNA. This result is probably a consequence of DNPS being optimized for prokaryotic DNA extraction, incorporating a bead-beating step that enhances the bacterial cell lysis and increasing the risk of amplifying non-target DNA. Therefore, DNPS resulted in diminished biotic index scores, notably for metrics like BMWP and ASPT, which are largely contingent upon the accurate detection of sensitive metazoan taxa. This finding underscores the limitations of using protocols designed for broad-spectrum DNA recovery when the primary focus is

on metazoan community profiling. Conversely, the MAN protocol showed improved performance across all indicators, distinguished by increased metazoan RRA and a lower incidence of bacterial sequence co-amplification. The absence of bacterial DNA in MAN-extracted samples suggests greater specificity for eukaryotic DNA, likely benefiting from the protocol's streamlined lysis process and larger sample volume capacity. These characteristics not only facilitated the recognition of target metazoan taxa but also resulted in more uniform biotic index values, which were in close alignment with the aggregated dataset. The efficiency of MAN in generating high-abundance metazoan reads supports its suitability for ecological studies, particularly in freshwater environments where a broad representation of invertebrate taxa is critical. Despite its usual focus on non-metazoan metabarcoding, DNP exhibited impressive results. The protocol's ability to reduce PCR inhibitors likely contributed to its effectiveness in amplifying metazoan sequences, as evidenced by its high similarity in biotic indices to MAN and the combined dataset. Conversely, DNBT showed the greatest dissimilarity in biotic indices, particularly in COI and 18S rRNA-V9 datasets. This inconsistency could be attributed to PCR inhibition, which restricted the amplification of low-abundance sequences and diminished the detection of sensitive taxa essential for biomonitoring. These findings play a crucial role in advancing environmental monitoring efforts for freshwater ecosystems, underscoring the necessity of selecting the right DNA extraction protocol to ensure accurate biotic indices. Our findings highlight that protocols like MAN and DNP offer reliable and consistent recovery of metazoan taxa, which is crucial for assessing ecosystem health. The sensitivity and reliability of biodiversity assessments are enhanced by integrating COI and 18S rRNA markers, enabling more accurate monitoring of freshwater biodiversity. In addition, the economical nature of the MAN protocol renders it a suitable option for large-scale, sustained monitoring efforts, thereby boosting the scalability and operational efficiency of environmental monitoring programs.

AUTHOR CONTRIBUTIONS

M.H.F. Amin, the corresponding author, has contributed to conceptualization, methodology, software, data curation, funding acquisition, writing the original draft, reviewing, and editing. H.W. Kim has contributed to funding acquisition, methodology, resources, and writing the original draft. I.A. Pratiwi has contributed to data curation and funding acquisition. A.R. Kim prepared the figures and interpretation of the results. H.E. Kang prepared the figures and interpretation of the results. N.I. Oktavitri conducted formal analysis and data interpretation. T. Soedarti conducted formal analysis and data interpretation.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript. In addition, the ethical issues, including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy have been completely observed by the authors.

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ABBREVIATIONS

%	Percent
˚C	degrees Celsius
µL	Microliter
18S rRNA	18 Svedberg ribosomal RNA
18S rRNA-V4	18 Svedberg ribosomal RNA hypervariable region 4
18S rRNA-V9	18 Svedberg ribosomal RNA hypervariable region 9
ASPT	Average score per taxon
ASV	Amplicon sequence variant
BLAST	Basic local alignment search tool
BMWP	Biological monitoring working party
bp	Base pair
cm	Centimeter
COI	Cytochrome c oxidase I
d	Distance
DADA2	Divisive amplicon denoising algorithm 2
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNBT	DNeasy blood and tissue
DNP	DNeasy plant
DNPS	DNeasy powersoil
dNTPs	Deoxynucleotide triphosphates
dsDNA	Double-strand deoxyribonucleic acid
eDNA	Environmental DNA
EDTA	Ethylenediaminetetraacetic acid
EPTD	Ephemeroptera, Plecoptera, Trichoptera, and Diptera
kg	Kilogram
LIFE	Lotic-invertebrate index for flow evaluation
m/s^2	Meter per second squared
MAN	Manual guanidine hydrochloride extraction protocol
Min.	Minute(s)
mL	Milliliter
mm	Millimeter
mM	Milimolar
NCBI	National center for biotechnology information
p	Probability value
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
PERMANOVA	Permutational analysis of variance
PERMDISP	Permutational multivariate analysis of dispersion
pmol	Picomol
PSI	Proportion of sediment-sensitive invertebrates
R^2	*R*-squared
RRA	Relative read abundance
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
Sec.	Second(s)
SEED	Simple exploration of ecological data
TE	Tris-ethylenediaminetetraacetic acid
USD	United states dollar
WHPT	Walley-hawkes-paisley-trigg

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