

Original Articles

Benefits of combined environmental DNA and microscopy for diversity monitoring in rotifer community: A mesocosm experiment



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ABSTRACT

Rotifers are crucial for the monitoring of aquatic ecosystems because of the sensitive response of rotifer community structure to environmental changes. Comparisons and combinations of *meta*-barcoding (sequence analysis) and traditional morphological identification for rotifer community responses are rare. To better understand the differences between the two approaches, we conducted a study using 48 mesocosms (2500 L) and a combination of warming, nutrient loading, and pesticide to evaluate the applicability of both methods to rotifer communities in order to better understand how rotifer communities respond to multiple environmental stressors. The findings demonstrated that the degree of matching between both techniques was limited, despite the fact that there was a substantial positive association between the morphology abundance determined by morphological identification and the sequence abundances determined by eDNA metabarcoding. Although the two methods had similar responses to various environmental stresses, sequence analysis can identify rotifers that are difficult to identify with traditional methods such as Bdelloidea, while classical morphological methods were more suitable for assessing the effects of various environmental changes on rotifer communities. This study provided valuable implications for monitoring and managing aquatic ecosystems since it enables us to spot changes in the rotifer community structure early on and take the necessary precautions to safeguard these sensitive ecosystems.

1. Introduction

Globally, aquatic ecosystems are at risk from a variety of environmental stressors, including as eutrophication brought on by urban and industrial expansion, changing land use, hazardous chemicals, and climate change (Vörösmarty et al., 2010; Xiong et al., 2019). Studies have shown that eutrophication and toxic pollutants, which are caused by anthropogenic disturbances in water bodies, are the primary stressors responsible for community variation (Desrosiers et al., 2019; Xiong et al., 2019). Climate change, on the other hand, has already altered the phenology and distribution of freshwater species because it can change the physical, chemical, and biological characteristics of water bodies (Scheffers et al., 2016; Cohen et al., 2018). The full scope of these risk factors' effects on aquatic ecosystems is still unknown, despite their growing size.

Aquatic ecosystem biodiversity is crucial for preserving and

sustaining environmental health and ecosystem functions, as well as having great aesthetic and economic value (Comberti et al., 2015). Rotifers in particular are crucial to the structural makeup, material cycling, and energy exchange of aquatic ecosystems. As a group of tiny and slow swimmers, rotifers are perfect open bait for predators for their characteristics of high rate of food resource conversion, quick rate of reproduction (Sun et al., 2019), are slow swimmers, and containing a broad range of amino acids (Thackeray, 2022). Rotifers are the perfect test subjects for studies on water ecotoxicology because of their quick reactions to changes in the water environment (Li et al., 2020; Arreguin-Rebolledo et al., 2023). Rotifers are important biological markers for assessing ecosystem health and play a significant role in ecotoxicology research (Yang & Zhang, 2020).

Traditional taxonomy issues make it difficult to comprehend how environmental change affects rotifer communities (Yang & Zhang, 2020; Qiu et al., 2022). Particularly in large-scale environmental studies and

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monitoring programs, species identification and individual counting based on traditional morphological bases can be time-consuming and expensive, requiring highly trained persons with competence in identifying species (D. W. Yu et al., 2012). The use of metabarcoding technology, which may be utilized to define the taxonomic components that make up environmental DNA (eDNA), presents a chance to get over the restrictions of morphological taxonomy-based biodiversity monitoring (Xie et al., 2017; Kestel et al., 2022). In addition to not requiring extensive taxonomic knowledge, eDNA sequencing methods have a number of advantages for the evaluation of aquatic ecosystems, such as the categorization of a wider biological range, higher detected sensitivity, and rapid efficiency (Kestel et al., 2022; Zhong et al., 2022). However, there are a few significant difficulties with data interpretation (Laakmann et al., 2020). It is still challenging to relate diversity and community organization to molecular estimates of diversity and community organization (Laakmann et al., 2020). Applying eDNA metabarcoding typically involves comparing the quantity and variety of taxa found through morphological classification (Mächler et al., 2019; Heine et al., 2021). Comparing and combining the two approaches is uncommon in control studies, particularly when examining how subjects react to their environment changes.

Through a year-long mesocosmic controlled experiment, we compared the ability of rotifers to respond to environmental pressures using the standard morphological approach and the new generation sequencing method in this study. Based on the species richness, abundance and other characteristics of rotifers acquired by morphological identification and eDNA metabarcoding, objectives of the study were to: (1) clarify the differences between both approaches in the identification of rotifer communities; (2) explore the difference of response of both approaches to the community structure of rotifers under the influence of multiple stressors.

2. Materials and methods

2.1. Method

A total of 48 cylindrical polyethylene mesocosms were utilized to replicate shallow lake ecosystems at Huazhong Agricultural University in Wuhan, China. Each mesocosm had dimensions of 2500 L, 1.5 m in diameter, and 1.4 m in height, and contained four trays (L: 60.5 cm, W: 41.5 cm, and H: 11.5 cm) with 10 cm of sediment from Lake Liangzi (Shi et al., 2022; P. Y. Zhang et al., 2022). Total nitrogen (TN) of 5.5 ± 0.4 mg g⁻¹ and total phosphorus (TP) of 0.42 ± 0.08 mg g⁻¹, dry weight, were the sediment's initial characteristics. Each of the four trays was planted with different amounts of *Vallisneria densiflora* and *Hydrilla verticillata*. Tap water and rainfall progressively enhanced the water line to promote the growth of submerged hydrophytes. In the mesocosms, we included 20 individuals of *Bellamya aeruginosa* and 20 individuals of *Radix swinhonis* as periphyton grazers in an effort to mimic a natural food web. Eight individuals of *Carassius carassius* (approximately 3 cm) and ten individuals of *Macrobrachium nipponense* (3.0 to 7.7 cm) were added as benthic predators that feed on zooplankton, macroinvertebrates, detritus, periphyton and so on. These animals coexist in local water bodies as common species, and their densities and biomasses are within natural ranges (Gong et al., 2009; Zhi et al., 2020; Guo et al., 2021; Mao et al., 2021; J. L. Yu et al., 2021).

We used a mixed-design approach that included a control group (C: ambient), temperature scenarios (W: warming by + 3 °C), and two pollution levels (E: addition of nitrogen and phosphorus; P: addition of pesticides), resulting in a total of 8 treatments (ambient: C; warming: W; nutrient loading: E; pesticide: P; warming and nutrient loading: WE; warming and pesticide: WP; nutrient loading and pesticide: EP; warming, nutrient loading and pesticide: WEP) with repeat six times for each, and the 48 mesocosms received random treatment assignments. Based on the typical temperature in the control, the warmed treatments (W) were administered (T. Wang et al., 2020). Two digital temperature

sensors (DS18B20) and a heating element with a power of 600 W was used in each mesocosm to manage the temperature. To replicate nutrient loading, doses of 0.5 mg/L N (NaNO₃) and 0.05 mg/L P (KH₂PO₄), equivalent to those employed in earlier experiments, were added weekly to the chosen mesocosms (Jeppesen et al., 2007; Coppers et al., 2016). Pesticide treatment was achieved by adding imidacloprid (P) at a dose of 10 µg/L. It was suggested that the dose correspond to a realistic environmental concentration (Hénault-Ethier et al., 2017). Each mesocosm was equipped with a small pump (WP-1880, 1200 L/H) that continuously circulates the water columns of all mesocosms, simulating the natural water cycle and dissolved oxygen in the water column. To account for evaporation, distilled water was introduced to the heated mesocosms. Throughout the trial, the water's average depth in the mesocosms was maintained at about 1.2 m.

2.2. Sample preparation and environmental factors measurement

The experiment ran from April 8, 2021 to November 4, 2021. The samples were collected on November 4, 2021. Depth-integrated homogenized water samples were collected using a transparent plexiglas tube (diameter 70 mm, length 1 m) from every aquatic ecosystem mesocosm. For microscope identification, rotifer abundance was measured by fixing 1000 mL of a depth-integrated water sample with lugol (2% in volume). In the lab, the 1 L of water sample was concentrated to 50 mL for morphological rotifer diagnosis. Immediately after removing a 250 mL depth-integrated water sample, it was pumped back to the lab and put through a 0.22 µm pore size filter membrane. The filter membrane was pumped and filtered before being stored for high-throughput sequencing in an ultra-low temperature refrigerator at -80 °C (Yang, Zhang, Zhang, et al., 2017).

With the use of HACH HQD Portable Metres (HQ60d, HACH, USA), conductivity and pH were measured. A portable WGZ-2B turbidity metre (Xinrui, Shanghai, China) was used to measure turbidity. In order to obtain depth-integrated water samples for the study of TN and TP concentrations as well as phytoplankton biomass (by chlorophyll a (chl a) concentrations), a transparent plexiglas tube with a diameter of 70 mm and a length of 1 m was employed. After being broken down by potassium peroxodisulfate, the concentrations of TN and TP were determined using a spectrophotometric technique. Whatman GF/C filters were used to filter a certain amount of water (between 100 and 500 mL, depending on the amount of solid matter in the water column), which was then extracted with acetone and measured spectrophotometrically to determine the presence of chl a. (Su et al., 2017).

2.3. Morphological analysis

Using Zeiss microscopy (Zeiss Axio Imager 4.2) with 100-1000 magnifications, taxonomic and quantitative determination of rotifers at the lowest feasible categorization level was carried out for the morphological study (J.J. Wang, 1960; Duyen, 2015). To achieve the proper quantity of rotifers, the sample was further concentrated in the lab. The concentrated samples were carefully mixed before identification, and 1 mL of the secondary samples were obtained for individual counting in the Sedgwick Rafter cell counting chamber. To determine its average, each sample is tallied two to three times. Rotifer population density was converted to individuals per volume (ind./L).

2.4. DNA extraction, PCR amplification, and sequencing

Using the E.Z.N.A.® soil DNA Kit (Omega Bio-tek, Norcross, GA, U.S.), total DNA was extracted from a total of samples in accordance with the manufacturer's instructions. All DNA samples underwent quality control, and NanoDrop 2000 spectrophotometers (Thermo Fisher Scientific, Wilmington, DE, USA) were used to measure the concentration. The mitochondrial COI gene was amplified using mlCOIintF (5'-ACTCCTACGGGAGGCAGCAG-3') and gHC02198 (5'-

GGACTTACHVGGGTWTCTAAT-3') primers. The PCR conditions included 30 s at 95 °C, 30 s at 55 °C, and 45 s at 72 °C for 27 cycles. Reactions were carried out with 4 µL TransStart FastPfu buffer, 2 µL 2.5 mM deoxynucleoside triphosphates (dNTPs), 0.8 µL of each primer (5 µM), 0.4 µL TransStart FastPfu DNA Polymerase, 10 ng of extracted DNA, and ultrapure water adding to make up final 20 µL. Agarose gel electrophoresis was used to verify the size of amplicons before paired-end sequencing on the Illumina MiSeq platform using PE300 chemical at Wefind Biotechnology Co., Ltd. (Wuhan, China). Raw reads were deposited into the NCBI Sequence Read Archive (SRA) database (Xie et al., 2017).

Demultiplexing was followed by FLASH (v1.2.11) merging and fastp (Chen et al., 2018) quality filtering of the resultant sequences. Then, the high-quality sequences were de-noised using the Qiime 2 (version 2020.2) (Bolyen et al., 2019) pipeline's recommended parameters and the DADA 2 (Callahan et al., 2016) plugin, which achieves single nucleotide resolution based on error profiles within samples. The number of sequences from each sample was filtered to 4000 in order to reduce the effects of sequencing depth on alpha and beta diversity measures. This nevertheless produced an average good's coverage of 97.90%. The Naive Bayes consensus taxonomy classifier built in Qiime 2 and the NCBI database were used to assign OTUs to their appropriate taxonomic groups.

2.5. Statistical analyses

Diversity analyses were done with R software (version 4.1.0). Unless otherwise noted, all calculations used logarithmically converted data. Community ecology analyses were carried out using the "vegan" package in R software (Oksanen et al., 2015). Using Pearson correlation, the sequence number of DNA metabarcoding data and the species abundance of morphological identification were compared. The Bray-Curtis index (function vegdist) was utilised to create a community dissimilarity data matrix based on relative frequencies of abundances and readings, which was then put to use for non-metric multidimensional scaling (NMDS). Using the function anosim, a similarity analysis (ANOSIM) was carried out to determine the importance of sample grouping. Differences between treatments were assessed using the non-parametric Kruskal-Wallis test, and Pearson correlation was used to examine correlations between the two approaches once again. The relationship between relative abundance and Shannon-Wiener index as well as environmental variables were assessed using the Mantel test (Legendre, 2019).

Chi-squared tests were employed to determine statistical significance, and generalised linear models (glm function in lme4 package) with Gaussian distributions were used to evaluate both the primary and interaction impacts of interventions on response variables (relative abundance of species by morphological identification, sequence number of DNA metabarcoding data, and alpha diversities of two approaches) in the experiment. Taxes play a role in the differences between both approaches. The community data from COI and microscopy was used for these testing.

3. Results

3.1. Assignment of molecular operational taxonomic units (OTUs)

In all samples, 3,250,978 original DNA sequences were generated. 3,244,908 sequences retained after quality control and chimaera eradication, representing 0.19% sequence loss. The sequencing fragment's average read length is 313 bp (Fig. S1). These sequences were grouped into a total of 778 OTUs by setting the pumping depth to 95% of the minimum sample sequence size (Fig. S2). The sequence data was classified and allocated using the NCBI database, and the components from the rotifer phylum were chosen. 63 OTUs data were ultimately obtained.

3.2. Taxonomical composition of molecular and morphological data

By using morphological identification, a total of 40 species of rotifers, belonging to 3 orders, 10 families, and 15 genera, were identified (Fig. 1A). The average sample density was 1503.91 ± 763.97 ind./L (mean \pm S.D, the same below), with a range of 40 to 11,720 ind./L. By using an eDNA metabarcoding technique, 63 species of rotifers, representing 3 orders, 12 families, and 19 genera, were discovered (Fig. 1B). The average read count per sample was $16.78 \times 10^3 \pm 6.25 \times 10^3$ reads and 7.72 ± 1.65 OTUs, ranging from 15 reads with 3 OTUs to 17.9×10^4 reads with 8 OTUs. Although the taxonomic richness with eDNA metabarcoding data was higher than the morphological identification approach, the morphological identification had superior resolution at the species classification level.

Monogononta and Digononta make up the majority of freshwater rotifers. For the Monogononta, the Ploimida make up the majority of the rotifers in both approaches. In the morphological identification approach, there were 35 species (relative abundance accounted for 96.94%), comprising 7 families and 11 genera of Ploimida, of which the Branchionidae accounted for the majority of 13 species. In the eDNA metabarcoding approach, there were 50 species (relative abundance accounted for 98.53%), comprising 9 families and 14 genera of Ploimida of which the Branchionidae accounted for the majority of 25 species. In the Bdelloidea, the outcomes of both methods varied dramatically. Only one Bdelloidea was found with an uncertain order in the morphological identification (relative abundance: 1.87%). The eDNA metabarcoding identified a total of 9 Bdelloidea (relative abundance: 0.64%), including 2 families and 5 genera, and 6 species reported at the species level. The NCBI GenBank provides several COI sequences for each of these 6 species (Fig. S4).

By morphological identification and eDNA metabarcoding, the relative abundance of shared species was lower, while the relative abundance of shared genera was relatively higher. The degrees of matching between both approaches varied depending on the resolution. Only 10 taxa, or 25% of morphological identification species, were shared by both techniques at the species level; however, the ratio rose to 47% at the genus level (7 genera), and to 50% at the family level (5 families) (Fig. 2A and 2B). The same species identified by both approaches was the *Brachionus leydi*, *Brachionus angularis*, *Brachionus quadridentatus*, *Brachionus calyciflorus*, *Keratella cochlearis*, *Platynus patulus*, *Trichotria tetractis*, *Lecane bulla*, *Trichocerca similes*, and *Trichocerca dixon-nuttalli*, with relative abundance of 17.37% for morphological identification and 11.01% for eDNA metabarcoding, respectively. The same genera identified by both approaches were the *Anuraeopsis*, *Brachionus*, *Keratella*, *Platynus*, *Lecane*, *Trichocerca*, and *Trichotria*, with relative abundance of 91.80% for morphological identification and 88.75% for eDNA metabarcoding, respectively (Fig. S3).

Out of the 40 species that were identified using morphological analysis, 32 were allocated at the species level and 7 at the genus level (Table S1). Along with the 10 species shared by both methods, eDNA metabarcoding at the species level missed 22 species identified by morphological identification (Fig. 3). Out of the 22 species, 11 species (relative abundance: 3.85%) had COI sequences in the NCBI GenBank, whereas the 11 species without COI sequences (relative abundance: 19.75%) had only one small subunit ribosomal RNA gene from each of the other 3 species, and the remaining 8 species had no available barcode sequences in the NCBI GenBank. Although *Colurella uncinata* and *Trichocerca pusilla* were frequently present in the samples, eDNA metabarcoding was unable to identify them because they lacked the corresponding COI sequence in the NCBI GenBank.

There were 4 species in the Flosculariaceae, which was solely recognised by morphological identification, representing 10.00% of the total number of species but only 1.19% of the total abundance. These species were divided among 3 families and 4 genera. The NCBI database only had reference sequences for *Testudinella patina*, whereas, the sequencing process missed this. The NCBI GenBank recorded 5 families and 16

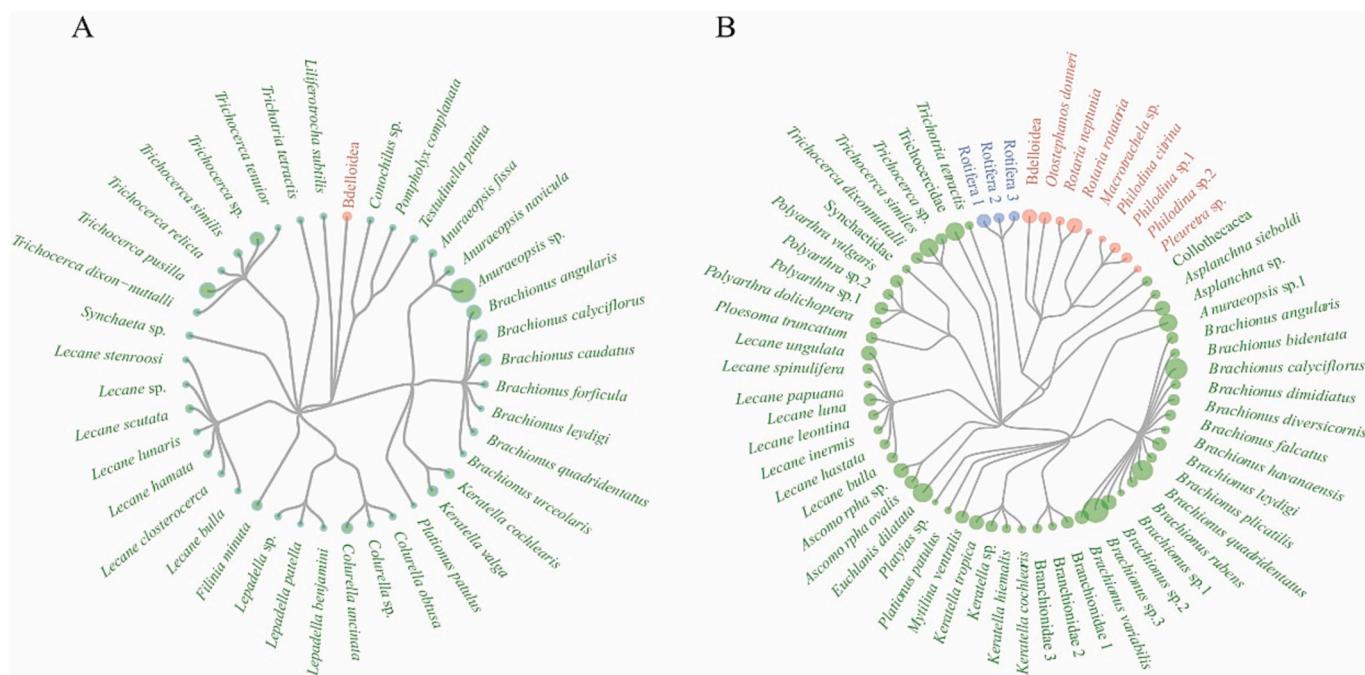


Fig. 1. Rotifer community composition using the morphological identification (A) and eDNA metabarcoding (B) approaches (circles indicate relative species abundance). Green represents Monogononta, red represents Digononta, and blue represents the unclassified rotifers.

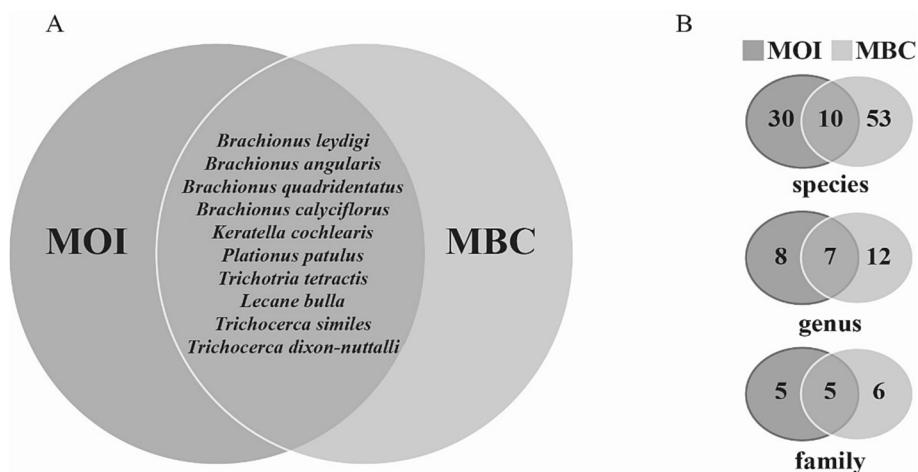


Fig. 2. Venn diagrams showing the list of share species (A) and number of taxa (species, genus, and family) (B) identified by the morphological identification (MOI) and the eDNA metabarcoding (MBC).

genera, while the Flosculariaceae contains 6 families and 14 genera for morphological identification. It's important to note that *Collotheca* was allocated to the family Flosculariidae (Flosculariaceae) in the NCBI GenBank, but in the morphological identification, it was placed in the Collothecidae (Collothecacea) family.

40 OTUs were assigned at the species level (63.5%), 13 at the genus level (20.6%), 5 at the family level (7.9%), 2 at the order level (3.2%), and 3 OTUs were left unclassified out of the 63 allocated OTUs found with eDNA metabarcoding. Metaspecies of family and above taxonomic order have a 4.26% relative abundance (Table S1). Additionally, 30 OTUs (species level) were found by eDNA metabarcoding but not by morphological identification. By eDNA metabarcoding, an unidentified species of *Brachionus* had the highest relative abundance proportion (64.15%), but there were very few sequences of this species recorded in the NCBI GenBank.

There was just one unidentifiable Collothecacea (*Collothecacea*_sp.

_EM-2017, relative abundance accounted for 0.03%) with one COI sequence in NCBI GenBank for the Collothecacea. *Acyclus inquietus*, *Stephanoceros fimbriatus*, and one unidentified order were included in the NCBI GenBank, each with a single barcode sequence. Collothecacea has two families and three genera, according to the morphological identification. The short subunit ribosomal RNA gene sequences of *Acyclus inquietus* and *Stephanoceros fimbriatus* are identical. In both pertinent research and NCBI GenBank, there aren't many interpretations for the function of Collothecacea.

3.3. Response to multiple environmental stresses

The results of morphological identification and eDNA metabarcoding for the responses of rotifer communities to various environmental conditions were different. The non-parametric Kruskal-Wallis test was used to detect the differences between the various treatments results showed

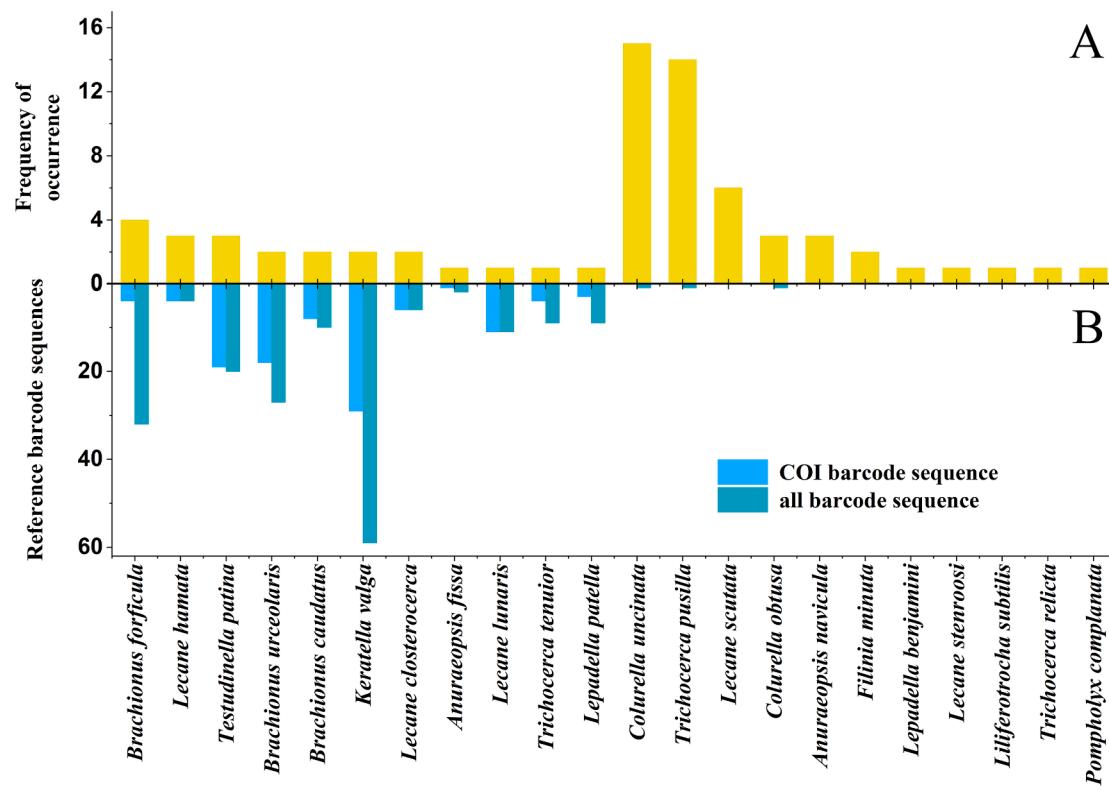


Fig. 3. The frequency of occurrence (A) and the number of barcode sequences (B) (COI and all) of 22 species at the species level that were recognised by the morphological identification and not detected by the eDNA metabarcoding in GenBank.

that there were significant differences between E-P, E-WEP, EP-WEP, and WE-WEP in the number of species determined by morphological identification ($P < 0.05$) (Fig. 4A). The treatment with the highest average species number was WEP group. According to eDNA metabarcoding, there were substantially more species in the EP-P than the P-WEP ($P < 0.05$) (Fig. 4B). The EP group had the highest average number of species. There was no significant divergence in sequence number between the various treatments (Fig. 4D), but E-P, E-WEP, E-WP, and WE-WEP exhibited significant differences for species abundance as determined by morphological identification ($P < 0.05$) (Fig. 4C). Additionally, there was no discernible variation in Shannon-Wiener indices among the various treatments, and the Shannon-Wiener indices acquired by eDNA metabarcoding was comparable to those obtained by morphological identification, with no significant difference (Fig. S5). The species number and Shannon-Wiener indices for both approaches did not exhibit any significant linear correlation (Fig. S6A and S6C), and the correlation between morphological identification abundance and eDNA metabarcoding abundance was $R^2 = 0.08$ ($P = 0.051$) (Fig. S6B).

Although differences across treatments were evident, non-metric multidimensional scaling (NMDS) was unable to clearly separate them (Fig. S7). Under numerous environmental pressures, the makeup of the rotifer communities was different from that under a single pressure. More species could be identified using eDNA metabarcoding when numerous environmental forces are present than when only one environmental factor is present. For morphological identification, this distinction was not discovered (Fig. 5A). For the 10 species shared by both approaches, more species were found under multiple environmental pressures than under a single pressure, and this was also evident in terms of the relative abundance of species. Under a variety of environmental stressors, the relative abundance of common species determined by morphological identification was higher than that determined by eDNA metabarcoding (Fig. 5B). Multiple pressures revealed more species and higher abundances in terms of shared species alone.

Warming has a significant positive effect on the abundance of rotifers (Table S2), and pesticides had a significant positive effect on the abundance and Shannon-Wiener index of rotifers by morphological identification (Table S2). These are the major and interactive effects of the generalized linear models (GLM) on the response variables. However, there was no discernible variation in the Shannon-Wiener index or sequence abundance between the various eDNA metabarcoding treatments.

In addition, the impacts of every individual environmental parameter on the various treatments were taken into account. The comparison between the results from both approaches and the 13 environmental characteristics (Temperature, DO, pH, Conductivity, Secchidepth, Turbidity, TN, NH₄, NO₃, TP, PO₄³⁻, Phytoplankton index and Pesticide) was done Mantel test (Fig. 6). The findings revealed a substantial positive association between morphological identification-based abundance and NO₃ ($P < 0.05$), phytoplankton index and pesticide ($P < 0.01$), and a significant positive correlation between Shannon-Wiener index and Secchidepth ($P < 0.05$) and pesticide ($P < 0.05$). The Shannon-Wiener index and PO₄³⁻ had a substantial positive connection ($P < 0.05$) for eDNA metabarcoding. The analysis results of the phytoplankton index and the outcomes of the two strategies using linear regression models demonstrated that there is good connection between the two ways. (Fig. S9).

Additionally, a correlation study was done between environmental parameters and the genus level (Fig. S8). For the top five genera with morphological identification in terms of relative abundance (Fig. S3), *Anuraeopsis*, *Filinia*, and *Colurella* showed no significant link with the environmental variables. Significant negative correlations between *Trichocerca* and Conductivity ($P < 0.01$) as well as NO₃ ($P < 0.05$) were found. *Brachionus* and Conductivity ($P < 0.05$), TN ($P < 0.001$), Turbidity ($P < 0.01$) as well as TP ($P < 0.001$) were significantly positively correlative, and Secchidepth ($P < 0.01$) showed a significant negative correlation. Additionally, there was a strong positive link between the amounts of phytoplankton and *Brachionus* ($P < 0.001$)

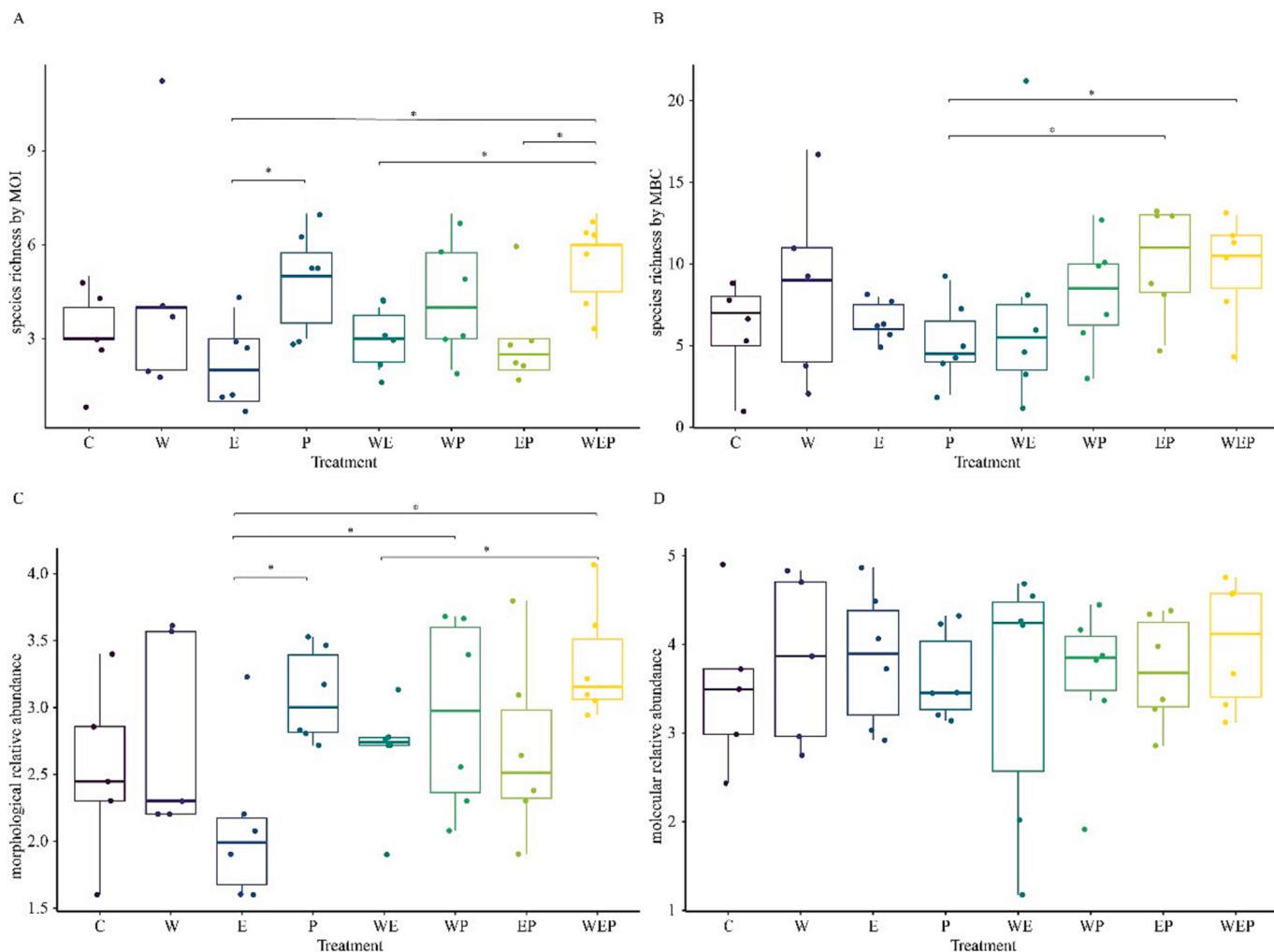


Fig. 4. Boxplots of species numbers (A, B) and relative abundance (C, D) by the morphological identification (MOI) and the eDNA metabarcoding (MBC) compared among treatments (ambient: C; warming: W; nutrient loading: E; pesticide: P; warming and nutrient loading: WE; warming and pesticide: WP; nutrient loading and pesticide: EP; warming, nutrient loading and pesticide: WEP). The asterisk indicates a significant difference between the two treatments (Kruskal-Wallis test, $P < 0.05$).

(Table S3, Table S4). Regarding the top five taxa with the highest relative abundance of eDNA metabarcoding (Fig. S3), *Brachionus* demonstrated outcomes comparable to those attained through morphological identification. *Brachionus* and Conductivity ($P < 0.05$), Turbidity ($P < 0.05$), TP ($P < 0.01$) as well as the abundance of phytoplankton ($P < 0.01$) were significantly positively correlative, except for Secchidepth showing a significant negative correlation ($P < 0.01$). *Trichocerca* was negatively correlative with Secchidepth ($P < 0.05$) and positively correlative with Turbidity ($P < 0.05$). Lecane and pH showed a strong positive correlation ($P < 0.05$) (Table S3, Table S4).

4. Discussion

In order to preserve biodiversity worldwide and supply ecosystem services, aquatic environments are essential (J. J. Wang et al., 2021). The responsiveness of freshwater ecosystems and their predictive significance in management have become a focus of research due to the dual challenges of global climate change and rising eutrophication by anthropogenic activities (Glibert, 2020). Freshwater biodiversity studies and environmental monitoring are based on the identification and classification of freshwater microbiota. Rotifers are an essential part of aquatic ecosystems, and knowledge of these systems' health can be gained from their diversity and abundance. The rotifer communities in the various treatment groups were examined in this work using a classic

morphological technique and a new generation sequencing strategy, and the differences between the two approaches in terms of species identification and environmental response were contrasted. eDNA metabarcoding has identified more species than morphological identification in terms of species identification, and this technology can provide more in-depth ecological information about the rotifer community (Yang, Zhang, Zhang, et al., 2017). The degree of matching between the two techniques is limited, despite the fact that there was a substantial positive association between the morphology abundance determined by morphological identification and the sequence abundances determined by eDNA metabarcoding. The distinct differences in relative abundance between the same species level and the same genus level was due to the low matching degree of species level detected by the two methods, and most genera belong to common genera. For evaluating how rotifers respond to various environmental stressors, the standard morphological identification methods are more appropriate. Compared with other studies, Anna Schroeder's study (Schroeder et al., 2020) in Venice Lagoon showed that both methods revealed a similar spatio-temporal distribution pattern and the sequence abundances and individual counts were significantly correlated for various taxonomic groups. Jianghua Yang's study (Yang, Zhang, Xie, et al., 2017) in Lake Tai Basin showed that the utility of metabarcoding for taxonomic profiling of zooplankton communities was validated by the morphology-based method on a large ecological scale. It is suggested that there are

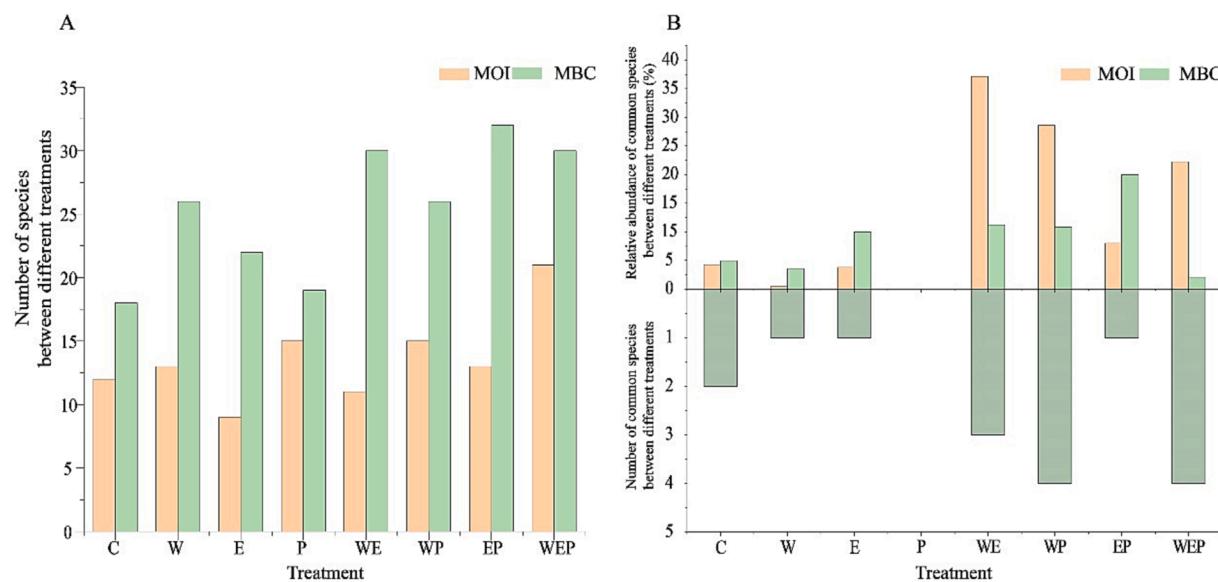


Fig. 5. The number of rotifer species discovered by different treatments in the morphological identification (MOI) and the eDNA metabarcoding (MBC) (A). The variety and comparative abundance of rotifer species in common among different treatments in MOI and MBC (B). Different treatments are respectively ambient: C; warming: W; nutrient loading: E; pesticide: P; warming and nutrient loading: WE; warming and pesticide: WP; nutrient loading and pesticide: EP; and warming, nutrient loading and pesticide: WEP.

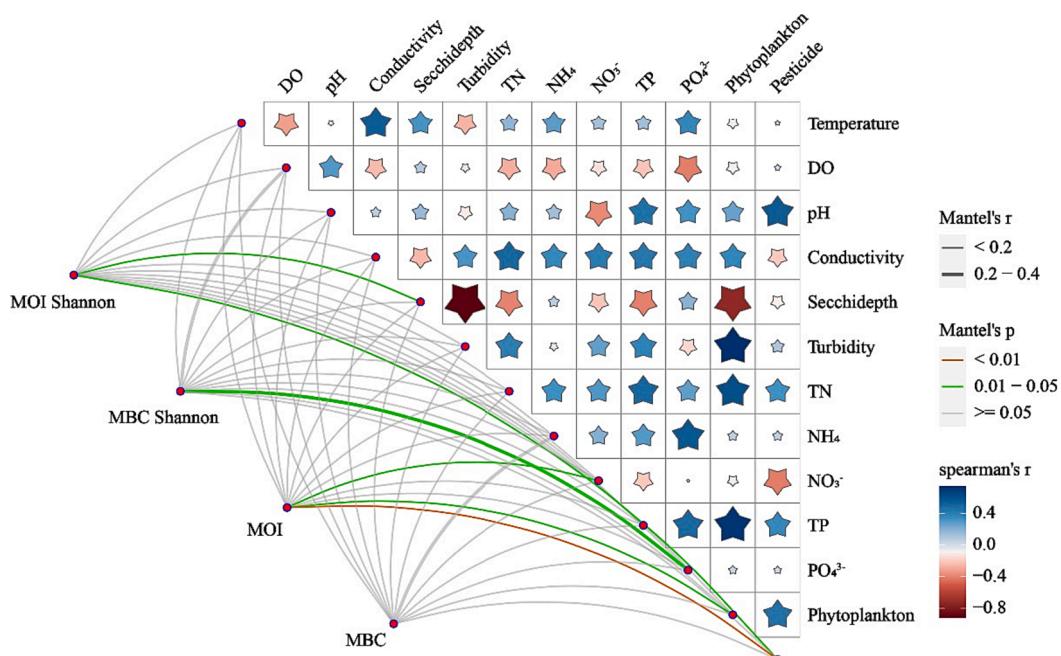


Fig. 6. Water environmental factors in the rotifer ecosystem. Spearman tests were used to compare water environmental parameters on a pairwise basis, and the color gradient shows the Spearman correlation coefficient. Using (geographic distance-corrected) Mantel tests, the relative abundance of species and the Shannon-Wiener index from various datasets (the morphological identification (MOI) and the eDNA metabarcoding (MBC)) were connected to water environmental parameters. Edge width: Mantel's R statistic; edge color: statistical significance.

enormous potential of combining morphological and molecular approaches to comprehend the structuring mechanisms of aquatic ecosystem assemblages.

4.1. Reasons for the differences in results between morphological identification and eDNA metabarcoding

The biggest barrier to the application of metabarcoding is typically thought to be the incompleteness and correctness of databases (Bucklin

et al., 2016). One of the most significant variables influencing the dependability of sequencing technologies is the calibre of the NCBI reference database. Only 10 of the 32 rotifers in this study that have been classified to the species level through morphological identification have been recognised by eDNA metabarcoding, even though 21 of them have COI sequences in GenBank. Additionally, multiple species that were morphologically identified, for instance, were not found using high-throughput sequencing because their species' reference sequences were not present on NCBI. These rotifers mostly came from uncommon

species. There are only reference sequences of other species in the same genus, with the exception of *Liliferotrocha subtilis*, which is not included in the NBCI database.

In addition to the allocation of deletions caused by a lack of reference sequences, substantial intraspecific variability also prevented the detection of reference sequences by sequencing, even though they were present in the NBCI database. Rotifer parthenogenesis shortens generation time and speeds up genetic evolution. It possesses great diffusion ability and good local adaptation, which may promote intraspecific divergence and interspecific gene flow (Gomez et al., 2002; Cristescu et al., 2012). Compared to other mitochondrial and nuclear genes, the COI fragment appears to have a wider range of phylogenetic signal (Hebert et al., 2003). In addition to distinguishing between closely related species, phylogeographic groupings within a single species can also be distinguished because of the rapid evolution of COI (Cox & Hebert, 2001; Wares & Cunningham, 2001). For instance, *Brachionus calyciflorus*, a common Rotifer discovered in 22 lakes in the Netherlands, belongs to up to 15 COI genetic groupings (Papakostas et al., 2016). In China, this species also underwent significant intraspecific divergence (Xiang et al., 2011). 359 COI barcode sequences from Rotifer, *Brachionus plicatilis*, and 924 COI barcode sequences from Rotifer, *Rotaria rotatoria*, were found in this study. These are the perfect species to investigate the genetic makeup and evolutionary history of rotifers. The sequences in the NCBI GenBank were collected from all across the world. The intra-specific differences of the majority of rotifer species were substantial due to the geographically distinct rotifer communities, and the genetic separation between populations grew as geographic distance did (Gómez et al., 2007; Mills et al., 2007). Despite the possibility of references in the database, the sequencing approach failed to detect the genomic structure alterations.

The most important factor for a successful evaluation of DNA metabolism barcode batch samples is a barcoding primer pair amplifying the marker sequence of short length for HTS as much as feasible (Schroeder et al., 2020). The results of biodiversity will vary depending on the barcode used (Clarke et al., 2017; Piñol et al., 2019). According to studies, the 18S V9 primer is more suited for detecting the diversity of protozoa and algae (Amaral-Zettler et al., 2009). OTU can nonetheless accurately reflect the effects of seasons and water types on zooplankton ecosystems even when a significant portion of them cannot be identified (Clarke et al., 2017). Mitochondrial 16S primer is better suited for cladocera and rotifer diversity research because of its higher zooplankton amplification specificity (Zheng et al., 2014; Lindsay et al., 2015). Great taxonomic resolution can be achieved using COI primer amplification products of moderate length, but this comes at the expense of decreased amplification success (Schroeder et al., 2020). Currently, a number of studies have employed a multi-marker approach for precise species identification and discrimination, using group-specific primers to lessen bias resulting from varying amplification success between different taxonomic groups (Bucklin et al., 2010; Bucklin et al., 2016; Clarke et al., 2017). As a result, it is advised to combine multiple primer pairs for the construction of local databases and surveys of variety.

The bioinformatics process also has an impact on the final results of molecular methods. Even between parallel repeats of the same rotifer community, the distribution of sequence readings was statistically different. The main reasons for the deviation were the sampling method, sampling volume, extraction of DNA, selection of similarity threshold, PCR amplification and high-throughput sequencing. Therefore, the accuracy of DNA macrobarcoding can be verified by classical morphological classification. For this study, we adopted the widely used sampling process based on the actual situation of the Middle universe, used commercial DNA extraction kit to extract samples, and selected COI sequences for amplification. the Qiime 2 (version 2020.2) (Bolyen et al., 2019) pipeline's recommended parameters, the DADA 2 (Callahan et al., 2016) plugin for subsequent analysis.

In particular for rotifers whose classification is still ambiguous, the incorrect identification of rotifers may lead to significant discrepancies

in the results of rotifers produced by both approaches (Wallace, 2002). Traditional morphological methods based on morphological feature classification are challenging because of the morphological diversity of species. As with the *Brachionus quadridentatus*, various morphological variations have frequently been classified as distinct species, subspecies, or forms. The majority of morphological identification is performed on fixed samples as well. After the formaldehyde was added, the morphology of Bdelloidea, Notommatidae, and other species without obvious dorsal and ventral envelopes significantly changed. For example, in this work, only one species of Bdelloidea was determined through morphological identification because Bdelloidea contracted after the addition of lugol (2% in volume). While nine species—six of which were at the species level—were determined through eDNA metabarcoding.

4.2. Morphological identification method was more suitable for evaluating environmental stress response.

The findings demonstrate the benefits of conventional morphological techniques are demonstrated by the non-parametric Kruskal-Wallis test and GLM analysis. Despite the fact that eDNA metabarcoding can identify more species, morphological techniques can identify more significant variations across treatments in terms of the number of species and relative abundance. Temperature, nutrition, and pesticide addition could lead to different response mechanisms based on the results of the non-parametric Kruskal-Wallis test ($P < 0.05$), and the results of the GLM demonstrated that the single environmental stress of temperature increases and insecticide addition had a favorable promoting effect on rotifer abundance in comparison to the control group. Rotifers can grow and flourish at the right temperature, and the use of pesticides can indirectly increase their number by affecting Cyclops' predation on them (Rico et al., 2018; Arreguin-Rebolledo et al., 2023). According to Mantel tests, which examine the connections between particular environmental conditions and species abundance as well as the Shannon-Wiener index, morphological methods can demonstrate more substantial connections with environmental variables. In a certain range, an increase in nitrogen, phosphorus, and other nutrient concentrations in water is frequently followed by an increase in phytoplankton abundance, which fosters the growth and development of rotifers that consume algae (Fu et al., 2021). Through their effects on algae, turbidity and transparency have an indirect impact on the abundance of rotifers (Zhao et al., 2020). Using linear regression model, it can be demonstrated that there is good connection between the two ways. By morphological identification, the correlation between phytoplankton index and rotifer abundance is more significant ($P < 0.05$). In the top five genera, both approaches exhibited comparable connections between environmental conditions and the level of rotifers. In addition, the molecular method detects the sum of the biological information in the sample. When intact living organisms die, although these organisms cannot be detected by morphological identification methods, their molecular information is still left in the water column, which may be detected by molecular methods, so the overall change in species number and abundance is not significant. Our study demonstrates that traditional morphological identification techniques are more suited for evaluating how rotifers react to various environmental stressors.

It is not easy to decide between standard morphological identification techniques and metabarcoding when analysing how rotifers respond to environmental stressors because each technique has benefits and drawbacks. In general, the classic morphological identification technique is more suited for evaluating the relative species abundance of rotifers in response to various environmental stresses. However, the diversity of rotifers may be underestimated by conventional morphological identification technique, which may restrict some of the functions of rotifers in adapting to environmental changes (Yang, Zhang, Xie, et al., 2017). Even though metabarcoding is practical and reliable for identifying and tracking rotifers in aquatic ecological environments, it

still has some restrictions and weaknesses, including an incomplete and inaccurate reference database (Visco et al., 2015), technical biases in the PCR reaction, primer specificity, and even sequence analysis (Lee et al., 2012; Taberlet et al., 2012), the bioinformatics processing flow, and the inability to distinguish between different life stages and health conditions. The constraints of conventional morphological identification are its challenging nature and strict professional knowledge requirements. It is advised to create a local species database that combines traditional taxonomies that intuitively reflect morphological functions with metabarcoding techniques to the assessment of rotifers and their response to environmental stressors in order to get around these limitations and provide a more thorough and balanced approach (Bucklin et al., 2016; Harvey et al., 2017; Stefanni et al., 2018).

5. Conclusions

In this study, rotifers communities from various treatment groups were assessed using both traditional morphological techniques and next-generation sequencing techniques. The differences between both approaches were compared in terms of community structure and response to controllable environmental variables. The matching degree between both techniques was limited, despite the fact that there was a substantial positive association between the morphology abundance determined by morphological identification and the sequence abundances determined by eDNA metabarcoding. More species can be identified using eDNA barcoding technology, which can also give detailed ecological data regarding rotifers ecosystems. For assessing how rotifers react to various environmental stressors, traditional morphological identification approach was more appropriate. These findings implied that integrating these two approaches can provide a more thorough evaluation of aquatic ecosystems, enabling us to detect early changes in rotifers community structure and implement the necessary precautions to safeguard vulnerable habitats.

CRediT authorship contribution statement

Yue Chen: Conceptualization, Methodology, Formal analysis, Writing – original draft, Visualization. **Huan Wang:** Conceptualization, Methodology, Formal analysis, Writing – original draft, Supervision, Funding acquisition. **Yingchun Gong:** Conceptualization, Methodology, Writing – review & editing, Supervision, Data curation, Funding acquisition. **Peiyu Zhang:** Methodology, Formal analysis, Writing – review & editing. **Huan Zhang:** Methodology, Formal analysis, Writing – review & editing. **Tao Wang:** Methodology, Formal analysis, Data curation. **Jiayi Xie:** Methodology, Formal analysis, Writing – review & editing. **Jun Xu:** Conceptualization, Methodology, Data curation, Writing – review & editing, Project administration, Supervision, Funding acquisition. **Hongxia Wang:** Methodology, Formal analysis, Writing – review & editing. **Xianghong Kong:** Methodology, Formal analysis, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that has been used is confidential.

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Appendix A. Supplementary data

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References

- Amaral-Zettler, L.A., et al., 2009. A method for studying protistan diversity using massively parallel sequencing of V9 hypervariable regions of small-subunit ribosomal RNA genes. *PLoS One* 4 (7), e6372.
- Arreguin-Rebolledo, U., et al., 2023. Multi-and transgenerational synergistic effects of glyphosate and chlorpyrifos at environmentally relevant concentrations in the estuarine rotifer *Proales similis*. *Environ Pollut* 318, 120708. <https://doi.org/10.1016/j.envpol.2022.120708>.
- Bolyen, E., et al., 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol* 37 (8), 852–857. <https://doi.org/10.1038/s41587-019-0209-9>.
- Bucklin, A., et al., 2010. A “Rosetta Stone” for metazoan zooplankton: DNA barcode analysis of species diversity of the Sargasso Sea (Northwest Atlantic Ocean). *Deep-Sea Research Part II-Topical Studies in Oceanography* 57 (24–26), 2234–2247. <https://doi.org/10.1016/j.dsrr.2010.09.025>.
- Bucklin, A., et al., 2016. Metabarcoding of marine zooplankton: prospects, progress and pitfalls. *Journal of Plankton Research* 38 (3), 393–400. <https://doi.org/10.1093/plankt/fbw023>.
- Callahan, B.J., et al., 2016. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat Methods* 13 (7), 581–583. <https://doi.org/10.1038/nmeth.3869>.
- Chen, S., et al., 2018. fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics* 34 (17), i884–i890. <https://doi.org/10.1093/bioinformatics/bty560>.
- Clarke, L.J., et al., 2017. Effect of marker choice and thermal cycling protocol on zooplankton DNA metabarcoding studies. *Ecol Evol* 7 (3), 873–883. <https://doi.org/10.1002/ee.2667>.
- Cohen, J.M., et al., 2018. A global synthesis of animal phenological responses to climate change. *Nature Climate Change* 8 (3), 224–228. <https://doi.org/10.1038/s41558-018-0067-3>.
- Comberti, C., et al., 2015. Ecosystem services or services to ecosystems? Valuing cultivation and reciprocal relationships between humans and ecosystems. *Global Environmental Change-Human and Policy Dimensions* 34, 247–262. <https://doi.org/10.1016/j.gloenvcha.2015.07.007>.
- Coppens, J., et al., 2016. The influence of nutrient loading, climate and water depth on nitrogen and phosphorus loss in shallow lakes: a pan-European mesocosm experiment. *Hydrobiologia* 778 (1), 13–32. <https://doi.org/10.1007/s10750-015-2505-9>.
- Cox, A.J., Hebert, P.D., 2001. Colonization, extinction, and phylogeographic patterning in a freshwater crustacean. *Mol Ecol* 10 (2), 371–386. <https://doi.org/10.1046/j.1365-294x.2001.01188.x>.
- Cristescu, M.E., et al., 2012. Speciation with gene flow and the genetics of habitat transitions. *Mol Ecol* 21 (6), 1411–1422. <https://doi.org/10.1111/j.1365-294X.2011.05465.x>.
- Desrosiers, M., et al., 2019. Assessing anthropogenic pressure in the St. Lawrence River using traits of benthic macroinvertebrates. *649*, 233–246.
- Duyen, N. V. (2015). *Identification Handbook of Freshwater Zooplankton of the Mekong River and its Tributaries*.
- Fu, H., et al. (2021). Abiotic and biotic drivers of temporal dynamics in the spatial heterogeneity of zooplankton communities across lakes in recovery from eutrophication. *Science of the Total Environment*, 778, 146368. <https://doi.org/ARTN 146368.10.1016/j.scitotenv.2021.146368>.
- Glibert, P.M., 2020. Harmful algae at the complex nexus of eutrophication and climate change. *Harmful Algae* 91, 101583. <https://doi.org/10.1016/j.hal.2019.03.001>.
- Gómez, A., et al., 2007. Persistent genetic signatures of colonization in *Brachionus manjavacas* rotifers in the Iberian Peninsula. *16*.
- A. Gomez et al. The interplay between colonization history and gene flow in passively dispersing zooplankton: microsatellite analysis of rotifer resting egg banks *Journal of Evolutionary Biology* 15 1 2002 158 171 <https://doi.org/DOI 10.1046/j.1420-9101.2002.00368.x>.
- Gong, Z.J., et al., 2009. Population dynamic and production of *Bellamya aeruginosa* (Reeve) (Mollusca: Viviparidae) in Lake Donghu, Wuhan. *Journal of Lake Science* 21 (3), 401–407.
- Guo, Y. M., et al. (2021). Freshwater snail and shrimp differentially affect water turbidity and benthic primary producers.
- Harvey, J.B.J., et al., 2017. Comparison of morphological and next generation DNA sequencing methods for assessing zooplankton assemblages. *Journal of Experimental Marine Biology and Ecology* 487, 113–126. <https://doi.org/10.1016/j.jembe.2016.12.002>.
- Hebert, P.D., et al., 2003. Biological identifications through DNA barcodes. *Proc Biol Sci* 270 (1512), 313–321. <https://doi.org/10.1098/rspb.2002.2218>.

- Heine, P., et al., 2021. Comparing eDNA metabarcoding with morphological analyses: Fungal species richness and community composition of differently managed stages along a forest conversion of Norway spruce towards European beech in Germany. *Agroforestry Systems* 496, 119429.
- Hénault-Ethier, L., et al., 2017. Herbaceous or *Salix miyabeana* 'SX64' narrow buffer strips as a means to minimize glyphosate and aminomethylphosphonic acid leaching from row crop fields. *Agric Ecosyst Environ* 258, 1177–1186.
- Jeppesen, E., et al., 2007. Shallow lake restoration by nutrient loading reduction—some recent findings and challenges ahead. *Limnol Oceanogr* 52, 239–252.
- Kestel, J.H., et al., 2022. Applications of environmental DNA (eDNA) in agricultural systems: Current uses, limitations and future prospects. *Sci Total Environ* 847, 157556. <https://doi.org/10.1016/j.scitotenv.2022.157556>.
- Laakmann, S., et al., 2020. The crossover from microscopy to genes in marine diversity: from species to assemblages in marine pelagic copepods. *Philos Trans R Soc Lond B Biol Sci* 375 (1814), 20190446. <https://doi.org/10.1098/rstb.2019.0446>.
- Lee, C.K., et al., 2012. Groundtruthing Next-Gen Sequencing for Microbial Ecology—Biases and Errors in Community Structure Estimates from PCR Amplicon Pyrosequencing. *Environ Microbiol* 14, 2900–2911.
- Legendre, P., J. E. o. E. (2019). Numerical Ecology.
- Li, X.D., et al., 2020. Progress on the usage of the rotifer *Brachionus plicatilis* in marine ecotoxicology: A review. *Aquat Toxicol* 229, 105678. <https://doi.org/10.1016/j.aquatox.2020.105678>.
- Lindsay, D.J., et al., 2015. DNA barcoding of pelagic cnidarians: current status and future prospects. *Bulletin of Plankton Society of Japan* 62 (1), 39–43.
- Mächler, E., et al., 2019. Assessing different components of diversity across a river network using eDNA.
- Mao, Z., et al., 2021. Pelagic energy flow supports the food web of a shallow lake following a dramatic regime shift driven by water level changes. *Sci Total Environ* 756, 143642. <https://doi.org/10.1016/j.scitotenv.2020.143642>.
- Mills, S., et al., 2007. Global isolation by distance despite strong regional phylogeography in a small metazoan. *BMC Evol Biol* 7, 225. <https://doi.org/10.1186/1471-2148-7-225>.
- Oksanen, J., et al., 2015. Vegan: Community Ecology Package. R Package Version 2.2-1, 2, 1–2.
- Papakostas, S., et al., 2016. Integrative Taxonomy Recognizes Evolutionary Units Despite Widespread Mitonuclear Discordance: Evidence from a Rotifer Cryptic Species Complex. *Syst Biol* 65 (3), 508–524. <https://doi.org/10.1093/sysbio/syw016>.
- Piñol, J., et al., 2019. The choice of universal primers and the characteristics of the species mixture determine when DNA metabarcoding can be quantitative. *Environ Monit Assess* 28, 407–419.
- Qiu, X., et al., 2022. The Effects of Water Level Fluctuation on Zooplankton Communities in Shahu Lake Based on DNA Metabarcoding and Morphological Methods. *Animals (Basel)* 12 (8). <https://doi.org/10.3390/ani12080950>.
- Rico, A., et al., 2018. Effects of imidacloprid and a neonicotinoid mixture on aquatic invertebrate communities under Mediterranean conditions. *Aquat Toxicol* 204, 130–143. <https://doi.org/10.1016/j.aquatox.2018.09.004>.
- Scheffers, B.R., et al., 2016. The broad footprint of climate change from genes to biomes to people. *Science* 354 (6313), aaf7671. <https://doi.org/10.1126/science.aaf7671>.
- Schroeder, A., et al., 2020. DNA metabarcoding and morphological analysis - Assessment of zooplankton biodiversity in transitional waters. *Mar Environ Res* 160, 104946. <https://doi.org/10.1016/j.marenres.2020.104946>.
- Shi, P. L., et al. (2022). The Coupling Response between Different Bacterial Metabolic Functions in Water and Sediment Improve the Ability to Mitigate Climate Change. *Water*, 14(8). <https://doi.org/ARTN 1203. 10.3390/w14081203>.
- Stefanni, S., et al. (2018). Multi-marker metabarcoding approach to study mesozooplankton at basin scale. *Scientific Reports*, 8. <https://doi.org/ARTN 12085. 10.1038/s41598-018-30157-7>.
- Su, J., et al., 2017. Developing surface water quality standards in China. *Resources Conservation and Recycling* 117, 294–303. <https://doi.org/10.1016/j.resconrec.2016.08.003>.
- Sun, Y., et al., 2019. Small-Sized Microplastics Negatively Affect Rotifers: Changes in the Key Life-History Traits and Rotifer-*Phaeocystis* Population Dynamics. *Environ Sci Technol* 53 (15), 9241–9251. <https://doi.org/10.1021/acs.est.9b02893>.
- Taberlet, P., et al., 2012. Towards next-generation biodiversity assessment using DNA metabarcoding. *Molecular Ecology* 21 (8), 2045–2050. <https://doi.org/10.1111/j.1365-294X.2012.05470.x>.
- Thackeray, S.J., 2022. Zooplankton Diversity and Variation Among Lakes. In: Mehner, T., Tockner, K. (Eds.), *Encyclopedia of Inland Waters*. Elsevier, Oxford, pp. 52–66.
- Visco, J.A., et al., 2015. Environmental Monitoring: Inferring the Diatom Index from Next-Generation Sequencing Data. *Environ Sci Technol* 49 (13), 7597–7605. <https://doi.org/10.1021/es506158m>.
- Vörösmarty, C.J., et al., 2010. Global threats to human water security and river biodiversity. *Nature* 467, 555–561.
- Wallace, R. L. (2002). *Rotifers: Exquisite Metazoans* 1. Paper presented at the Integrative and comparative biology.
- Wang, J.J., 1960. *Fauna Sinica: Freshwater Rotifera*. Science Press, Beijing, China.
- Wang, T., et al., 2020. A dynamic temperature difference control recording system in shallow lake mesocosm. *MethodsX* 7, 100930. <https://doi.org/10.1016/j.mex.2020.100930>.
- Wang, J. J., et al. (2021). Ecological indicators for aquatic biodiversity, ecosystem functions, human activities and climate change. *Ecological Indicators*, 132, 108250. <https://doi.org/ARTN 108250. 10.1016/j.ecolind.2021.108250>.
- Wares, J. P., & Cunningham, C. W. (2001). *PHYLOGEOGRAPHY AND HISTORICAL ECOLOGY OF THE NORTH ATLANTIC INTERTIDAL*. Paper presented at the Evolution; international journal of organic evolution.
- Xiang, X.L., et al., 2011. Patterns and processes in the genetic differentiation of the *Brachionus calyciflorus* complex, a passively dispersing freshwater zooplankton. *Mol Phylogenet Evol* 59 (2), 386–398. <https://doi.org/10.1016/j.ympev.2011.02.011>.
- Xie, Y., et al., 2017. Environmental DNA metabarcoding reveals primary chemical contaminants in freshwater sediments from different land-use types. *Chemosphere* 172, 201–209. <https://doi.org/10.1016/j.chemosphere.2016.12.117>.
- Xiong, W., et al., 2019. Biological consequences of environmental pollution in running water ecosystems: A case study in zooplankton. *Pt B* 252, 1483–1490.
- Yang, J., Zhang, X., 2020. eDNA metabarcoding in zooplankton improves the ecological status assessment of aquatic ecosystems. *Environ Int* 134, 105230. <https://doi.org/10.1016/j.envint.2019.105230>.
- Yu, D.W., et al., 2012. Biodiversity soup: metabarcoding of arthropods for rapid biodiversity assessment and biomonitoring. *Methods in Ecology and Evolution* 3 (4), 613–623. <https://doi.org/10.1111/j.2041-210X.2012.00198.x>.
- Yu, J. L., et al. (2021). Changes in Pelagic Fish Community Composition, Abundance, and Biomass along a Productivity Gradient in Subtropical Lakes. *Water*, 13(6). <https://doi.org/ARTN 858. 10.3390/w13060858>.
- Zhang, P. Y., et al. (2022). Heat waves rather than continuous warming exacerbate impacts of nutrient loading and herbicides on aquatic ecosystems. *Environment International*, 168, 107478. <https://doi.org/ARTN 107478. 10.1016/j.envint.2022.107478>.
- Zhao, G., et al., 2020. Phytoplankton in the heavy sediment-laden Weihe River and its tributaries from the northern foot of the Qinling Mountains: community structure and environmental drivers. *Environ Sci Pollut Res Int* 27 (8), 8359–8370. <https://doi.org/10.1007/s11356-019-07346-6>.
- Zheng, L.M., et al., 2014. 16S rRNA is a better choice than COI for DNA barcoding hydrozoans in the coastal waters of China. *Acta Oceanologica Sinica* 33 (4), 55–76. <https://doi.org/10.1007/s13131-014-0415-8>.
- Zhi, Y.W., et al., 2020. Responses of four submerged macrophytes to freshwater snail density (*Radix swinhonis*) under clear-water conditions: A mesocosm study. *Ecology and Evolution* 10 (14), 7644–7653. <https://doi.org/10.1002/ece3.6489>.
- Zhong, W., et al., 2022. Holistic Impact Evaluation of Human Activities on the Coastal Fish Biodiversity in the Chinese Coastal Environment. *Environ Sci Technol* 56 (10), 6574–6583. <https://doi.org/10.1021/acs.est.2c01339>.

Further reading

- Yang, J., et al., 2017a. Zooplankton Community Profiling in a Eutrophic Freshwater Ecosystem-Lake Tai Basin by DNA Metabarcoding. *Sci Rep* 7 (1), 1773. <https://doi.org/10.1038/s41598-017-01808-y>.
- Yang, J., et al., 2017b. Indigenous species barcode database improves the identification of zooplankton. *PLOS One* 12 (10), e0185697.
- Zhang, Z.S.H., X.F., 1995. *Research Methods of Freshwater Plankton*. Science Press, Beijing, China.