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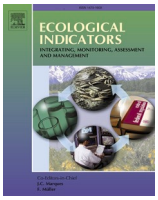

Aquatic invertebrate diversity profiling in heterogeneous wetland habitats by environmental DNA metabarcoding

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ABSTRACT

Invertebrates play vital roles in maintaining biodiversity and food web structure. However, it is difficult to identify invertebrate taxa across complex habitats due to the limitation of traditional morphology. In subtropical wetlands, environmental DNA (eDNA) metabarcoding was used to characterize the composition and diversity of aquatic invertebrates and analyze the environmental impacts on invertebrate community structure. According to the relative abundance (%) of invertebrate OTU richness, 30 sampling sites in wetlands were clustered into six zones, which exhibited significant spatial (i.e., wetland type-specific) differences in taxonomic composition. The relative OTU abundance (%) at the phylum level showed that Cnidaria (48.5%) > Porifera (19.4%) > Rotifera (11.0%) > Mollusca (9.2%) > Annelida (5.3 ± 2.8%) > others (less than 2.2%). Of the five alpha diversity indexes, 'Simpson' was the most effective index to distinguish the spatial differences in invertebrate diversity, and the class-level alpha diversity showed higher recognition than other taxonomic levels. The class-level biomarkers that could indicate the habitat-specific composition of local invertebrates were Insecta, Cnidaria, Hydrozoa, and Gastropoda in the inflow river; Ascidiacea and Demospongiae in the fluvial wetland; Clitellata, Gymnolaemata, and Monogononta in the lacustrine wetland; and Echinoidea in the estuarine wetland. The associations between environmental factors and invertebrate OTU richness based on redundancy analysis showed the taxon-level tendency of phylum (78.5%) > class (73.4%) > order (69.3%) > family (64.9%) > genus (61.2%). Our results demonstrated that aquatic invertebrate diversity profiling by environmental DNA metabarcoding can effectively reflect the composition, diversity and biomarkers of invertebrate communities. In the future, because of its great application potential, the eDNA technique may play an important role in biomonitoring complex water environments.

1. Introduction

Wetlands are one of the world's most important, economically valuable, and diverse ecosystems. A major proportion of wetland biodiversity is composed of aquatic invertebrates, which are an essential group in wetland ecosystems (Havel et al., 2015; Wang et al., 2018). Aquatic invertebrates comprise the main biomass of wetland food webs and are considered a key group of freshwater ecosystems (Vehkaoja et al., 2020). The importance of invertebrates is based on their numbers and diversity (Wang et al., 2020c), in addition to their role in secondary production in both aquatic and terrestrial food webs. Aquatic invertebrates function as both prey and predators, and they take an active part in maintaining community diversity and ecosystem stability (Wang et al., 2019b; Wang et al., 2021a; Wang et al., 2021b). Therefore, the

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distribution and composition of invertebrates, which are affected by environmental changes, may influence the structure of other communities (e.g., fish) as well as the functioning of local food webs (Wang et al., 2020c).

In complex aquatic systems, such as the wetlands connecting lakes, rivers, and estuaries, periodic exchanges of water bodies determine the real-time conditions of water quality (e.g., physicochemical parameters). Therefore, the distribution of aquatic invertebrates in wetlands with fluvial, lacustrine, and estuarine habitats is greatly influenced by the changing water environment (Wang et al., 2021a). For example, during the rising tide, large amounts of brinish pelagic invertebrates drifted into the estuaries or lagoons and changed the community structure of the original freshwater invertebrates (Turschak et al., 2016). In addition, when domestic sewage or industrial wastewater was discharged into the river, invertebrate species that were sensitive to nutrient enrichment and chemical pollutants sharply disappeared, which was reflected by the instant changes in species richness, assemblage biomass, and diversity of invertebrate communities (Lasne et al., 2007). Moreover, given that small pelagic invertebrates (e.g., copepods and cladocerans) are primary food resources for fish larvae and juveniles as well as for filter-feeding consumers (e.g., *Hypophthalmichthys* fish and bivalves), the abundance and biomass of pelagic invertebrates as prey may influence the nursery, breeding, and feeding of fish predators (Mao et al., 2020). Consequently, understanding the taxonomic composition and diversity of pelagic invertebrates is important to gain insight into the trophic networks and energy flows of aquatic food webs.

Traditional methods of identifying species and enumerating individuals based on morphology are costly, time-consuming and require highly trained individuals with expertise (Wang et al., 2020a; Wang et al., 2020b; Wang et al., 2019a). Morphology-based biotic monitoring is only feasible for easily observable species; however, it is difficult to identify assemblages belonging to different taxa (e.g., Arthropoda vs. Cnidaria) in complex habitats (e.g., freshwater river vs. brackish lagoon). DNA-based community analyses have offered some alternatives to traditional methods and have become even more promising with the availability of ultrasequencing platforms now supplanting cloning (Aylagas et al., 2014). Taxon detection from bulk samples can be achieved using PCR amplification followed by deep sequencing of homologous gene regions. Sequences are then compared to libraries of reference barcodes for taxonomic identification. This “metabarcoding” approach has been used as a powerful means to understand the diversity and distribution of meiofauna (Yu et al., 2012).

Metabarcoding technology can be used to characterize the compositions of species by the use of environmental DNA and next generation sequencing (NGS) (Taberlet et al., 2012). Several gene regions have been used in metabarcoding of invertebrate assemblages, such as hypervariable regions of 18S rRNA (Lindeque et al., 2013; Sun et al., 2015), 28S rRNA (Hirai et al., 2013), mitochondrial 16S rRNA (Goetze, 2010) and cytochrome *c* oxidase I (COI) (Zaiko et al., 2015). Mitochondrial COI is one of the most commonly sequenced regions for biodiversity analyses of animals, including invertebrate diversity. However, the standard COI primers target the 658-base pair (bp) barcoding region, whose size is considered too large for high throughput sequencing platforms (e.g., Ion Torrent PGM). Leray et al. (2013) designed a new COI primer set that targets a 313-bp fragment to characterize the gut contents of fish. Given that the target length of this primer is very suitable for NGS and the products have good performance for species identification, it was used to characterize invertebrate diversity from environmental samples.

Metabarcoding has found several applications (Shokralla et al., 2014), such as investigating biological diversity (Hajibabaei et al., 2011), characterizing prey composition in gut contents (Leray et al., 2013), and analyzing food-web dynamics (Leray et al., 2012). Many studies have demonstrated that metabarcoding improves taxonomic resolution and can be useful in assessing the biodiversity of invertebrates. In the present study, metabarcoding of the mitochondrial COI 313-bp region was used to characterize the genetic diversity of invertebrates in the Huayang Lake (HYL) wetland (Dongguan, southern China). As a subtropical littoral ecosystem, the HYL wetland receives inlet water from both freshwater rivers and brackish estuaries. The periodic exchange of water bodies in the HYL wetland determined the complexity of local aquatic communities. The objectives of our study include 1) using a metabarcoding protocol to identify the distribution, diversity, and biomarkers of aquatic invertebrates in the HYL wetland; 2) analyzing the associations between the structure of invertebrate communities and the physicochemical parameters of water quality; and 3) exploring the application of eDNA in the assessment of biodiversity and environmental quality.

2. Materials and methods

2.1. Study area and sampling sites

Huayang Lake (HYL) National Wetland Park is located in northwestern Dongguan city, Guangdong Province, southern China. Dongguan City is located in the south central part of Guangdong Province, the northeast of the Pearl River Delta, with a subtropical maritime climate and an annual average temperature of 23.3 °C. The annual average precipitation is 2042.6 mm, and the precipitation within the jurisdiction is relatively small and well distributed. Except for winter, when precipitation is scarce due to the influence of dry and cold continental air masses, precipitation is abundant most of the time. The topography consists of plains and low hills, mainly flood plains and hilly terraces, and the terrain is high in the southeast and low in the northwest. Overall, the HYL wetland has a mild climate, abundant precipitation, and flat terrain.

The total area of the HYL wetland is approximately 352 hm^2, and the wetland rate reaches 83.91%. The HYL wetland is a delta river network formed by the Pearl River and its tributary, the East River, with HYL as the center and many rivers converging. A number of rivers are intertwined and connected to the HYL, and the main rivers flowing through the area are the northern mainstream of the East River, the Inverted Canal Waterway and the Machong River Waterway. The wetland park has various aquatic plants and trees and shrubs naturally combined with banana forest, which enriches the species diversity and stability. At present, dozens of foliage plants, flowering plants, fruit-bearing plants and aquatic plants, such as white fescue, mullein, plantain and red table lily, are distributed in the scenic area.

Thirty sampling sites, including the inlets and outlets of water exchange, were chosen inside and outside the wetland (Fig. 1). Sites 1–5 were located in the main channel of the Dongjiang River and supplied water to the urban channels, i.e., sites 6–10. Sites 11–15 received water from both outside (e.g., sites 6–10) and inside the wetland (e.g., sites 16–20). Notably, sites 16–20, located near the center of the HYL wetland, formed the main part of the HYL. Site 26 was the opening on the eastern side of the HYL wetland and received water from the Machong Waterway (i.e., sites 22–26). Sites 26–30, located near the south side of the HYL wetland, were the drainage channels of the HYL and released water when the lake level rose high. The physicochemical parameters of water quality (see Table S1 in the Supplementary Material) were sampled in May-June 2021 and determined by a third-party testing agency with China Mandatory Approval qualification.

2.2. Collection of invertebrate eDNA in water

Environmental DNA field methods. — Our field methods for eDNA collection followed those of Leray et al. (2013). Sample bottles were sterilized with a 50–60% bleach solution, rinsed with deionized water, and allowed to dry before being sealed. Sample containers remained closed until field collection took place. Water sampling was conducted in May–June during 2021, and field negatives were not collected. Samples were collected by boat from 5 − 10 cm below the surface by submerging a sterile disposable 3-L plastic bottle (3 replicates). Samples were

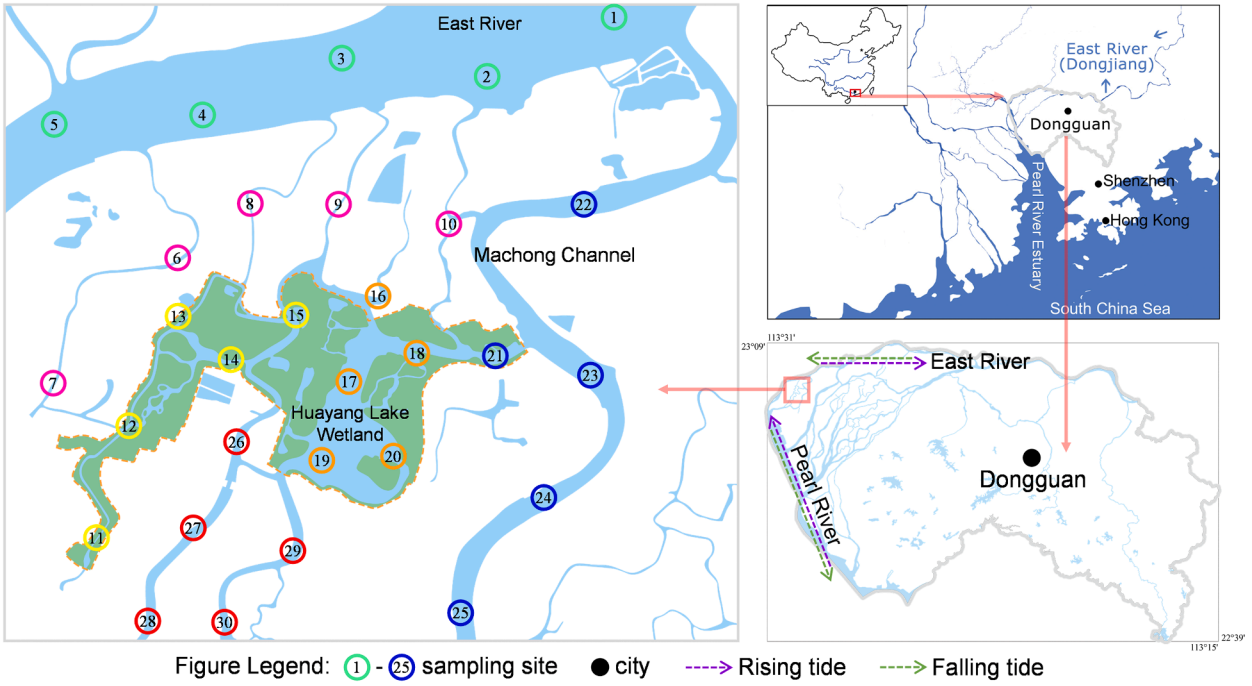

Fig. 1. Location of the sampling sites (1 – 30 in circles) in the Huayang Lake wetland. Sites 1–5, 6–10, 11–15, 16–20, 21–25, and 26–30 were located in the river mainstream, inflow river channel, fluvial wetland, lacustrine wetland, estuarine wetland, and outflow river channel, respectively.

collected by hand, and the researcher wore clean gloves so that all body parts in direct contact with water were covered for each sample. Samples were stored in a cooler with ice during transport (between 1 and 6 h in most cases) and returned to the lab for filtration.

Environmental DNA laboratory methods. — All laboratory equipment was sterilized with a 50% bleach solution, rinsed with deionized water, and allowed to dry between samples to prevent cross-contamination and false positives. A 3-L sample of water was filtered through a 47-mm, 0.45-μm pore size, cellulose, nitrate filter (Whatman International, Little Chalfont, UK). Using sterile forceps, the filters were folded into quarters, rolled, and placed in a vial with ≥ 98% ethanol.

The DNA was extracted from the filters using the phenol–chloroform-isoamyl alcohol (PCI) DNA extraction protocol described in Renshaw et al. (2014). Briefly, the ethanol in which filters were stored was decanted and replaced with 800 μL of lysis buffer (Qiagen GmbH, Hilden, Germany) to increase extraction efficiency. Filters were then incubated at 65 °C for 30 min prior to the addition of 800 μL of PCI (one phase, 25:24:1; Amresco LLC, Cleveland, Ohio). Following a 5-min centrifugation at 15,000 × g, 600 μL of the aqueous phase containing the DNA was transferred to a clean 1.5-mL microcentrifuge tube. An equal volume of chloroform-isoamyl alcohol (24:1; Amresco LLC) was added, and the mixture was again centrifuged at 15,000 × g for 5 min. A 400-μL aliquot of the aqueous layer was transferred to a clean vial and mixed with cold, 100% molecular grade ethanol and 16 μL of 5 M NaCl. The DNA was allowed to precipitate overnight at −20 °C. The resulting precipitate was pelleted by centrifugation for 10 min at 15,000 × g and dried until no visible liquid remained. Pellets were dissolved in 50 μL of low tris-EDTA buffer (10 mM Tris, 0.1 mM EDTA) and frozen until real-time quantitative PCR (qPCR) analysis was performed. We used a previously developed eDNA assay based on qPCR with specific PCR primers and a minor groove-binding (MGB) probe. We used the following conditions: hot start 95 °C for 15 min followed by 55 cycles at 94 °C for 60 s and 60 °C for 60 s (Takahara et al. 2012; Laramie et al. 2015).

2.3. Amplification by PCR and next generation sequencing (NGS)

The eukaryotic mitochondrial COI gene was amplified using degenerate primers (mlCOIintF and gHCO2198) (Leray et al., 2013). Amplification by PCR was performed with a final volume of 50 μL, made up of 1 μL of 10 μM primers, 2 μL of DNA template, 37.8 μL of ultrapure water, 5 μL of 10 × PCR High Fidelity PCR buffer, 2 μL of $MgSO_4$ (50 mM), 1 μL of dNTP mix (10 mM) and 0.2 μL of Platinum Taq DNA polymerase (Invitrogen, USA). To minimize potential bias of the PCR, triplicate PCR was performed for each sample. Amplifications by PCR were performed in 96-well plates using a Sure Cycler 8800 thermal cycler (Agilent Technologies, USA). Although a lower PCR cycle number can help improve the diversity of PCR amplicons from environmental samples, a "touchdown" PCR profile with 41 cycles was used to maximize the products and minimize the probability of nonspecific amplification because of the high level of degeneracy of the primer sequences. PCR was conducted for 16 initial cycles as follows: denaturation for 10 s at 95 °C, annealing for 30 s at 62 °C (1 °C per cycle), and extension for 60 s at 72 °C, followed by 25 cycles at an annealing temperature of 46 °C. The final extension was performed at 72 °C for 10 min. A negative control reaction with no DNA template was included in all experiments. PCR products were detected on a 2% (w/v) agarose gel, and fragments from the gel were purified using the MinElute gel extraction kit (Qiagen, CA, USA). After purification on the gel, PCR products were quantified using Qubit dsDNA HS assay kits (Invitrogen, USA), and the final concentration was adjusted to 10 ng/μL using molecular grade water.

To ensure a homogeneous number of sequencing reads from each sample, amplicons were mixed in equal concentrations (10 ng/μL) in an equimolar pool. One hundred nanograms of the pooled amplicon in a total volume of 79 μL of nuclease-free water was used in end-repair and ligation of adaptors by use of the Ion Plus fragment library kit (Life Technologies, USA) according to the manufacturer's protocols. To eliminate primer dimers and PCR artifacts less than 100 bp, the end-repaired and ligated adaptor DNA was purified with the Agencourt AMPure XP kit (Beckman Coulter, Germany). The purified amplicon library was then transferred to new 1.5-mL Eppendorf LoBind tubes (Eppendorf, Germany) and assessed for region size distribution and DNA concentration using an Agilent 2100 bioanalyzer (Agilent Technologies, USA).

Quantified, size-selected amplicon libraries (467 bp), including

amplification primers, MID tags, and Ion Torrent adaptors, were serially diluted to a final concentration of 100 pM and attached to the surface of Ion Sphere particles (ISPs) using the Ion PGM template OT2 400 kit (Life Technologies, USA). The ISPs were enriched on the Ion OneTouch enrichment system (Life Technologies, USA) and together with the template were sequenced on "318 v2" microchips using the Ion PGM sequencing 400 kit (Life Technologies, USA) with the Ion Torrent PGM (Life Technologies, USA) for 850 flows according to the manufacturer's protocols.

2.4. Statistical analysis

All statistical analyses were conducted in R 4.0.5 (R Core Team, 2021) with the primary packages 'cluster', 'factoextra', 'phyloseq', 'vegan', 'microeco', and 'ggplot2'. Cluster analysis for grouping sampling sites relied on the Bray-Curtis dissimilarities of sequentially aggregated operational taxonomic unit (OTU) abundance of invertebrate assemblages. Statistically significant cluster groupings were identified using a bootstrap randomization technique in which the nonzero values were resampled and used to generate pseudovalues of Bray-Curtis dissimilarities under the null hypothesis.

LEfSe (linear discriminant analysis [LDA] effect size) is a tool for high-dimensional biomarker mining to identify genomic features (e.g., as genes, pathways, and taxonomies) that significantly characterize two or more groups (Liu et al., 2021). LEfSe first provides the list of features that are differential among groups (i.e., wetland zones grouped by cluster analysis in this study) with statistical and biological significance, ranking them according to the effect size, then provides a mapping of the differences to taxonomic trees. Finally, LEfSe produces a histogram visualizing the raw data within the specified problem structure for each relevant feature. The LDA scores of invertebrate communities with significant differences in each group, determined by analyzing gene sequences, were used to find biomarkers. The biomarkers with significant differences in species trees between different classification levels can be observed in the plot.

A frequency distribution of pseudovalues was generated from 1000 randomizations of the data matrix, and the 95th percentile was used as the critical value to determine significance. Environmental factors and invertebrate OTU richness that showed significant variations in their values were used, and stepwise forward selection was performed to linearly reduce the correlated variables along the axes. A permutation limit (with a P value of 0.05) was used to determine which variables to incorporate into the final model. Linear dependencies were explored by computing the variable variance inflation factors to ensure no confounding colinearity. The statistical significance of the axes derived from each analysis was tested with a Monte Carlo test (999 permutations).

Table 1
The dominant assemblages at different taxonomic classifications.

Phylum	Class	Order	Family	Genus	
Cnidaria 48.5 ± 6.80%	Hydrozoa 44.1 ± 6.41%	Anthoathecata 27.7 ± 7.71%	Pandeidae 8.80 ± 5.02%	*Catablema* 8.74 ± 3.25%	
			Hydridae 8.32 ± 5.41%	*Hydra* 8.01 ± 4.71%	
		Leptothecata 7.20 ± 4.61%	Campanulariidae 5.91 ± 3.28%	*Obelia* 4.22 ± 3.31%	
		Siphonophorae 5.02 ± 0.91%	Apolemiidae 4.76 ± 2.33%	*Apolemia* 3.28 ± 1.25%	
	Anthozoa 3.71 ± 1.62%	Scleractinia 3.47 ± 1.33%	Caryophylliidae 2.28 ± 0.95%	*Phyllangia* 2.01 ± 0.45%	
Porifera 19.4 ± 7.87%	Demospongiae 14.6 ± 7.21%	Poecilosclerida 13.4 ± 7.30%	Crellidae 10.9 ± 5.03%	*Crella* 10.1 ± 4.91%	
			Microcionidae 1.88 ± 0.98%	*Clathria* 1.64 ± 0.77%	
	Homoscleromorpha 4.02 ± 1.89%	Homosclerophorida 3.82 ± 1.76%	Plakinidae 3.53 ± 1.65%	*Plakinastrella* 2.94 ± 1.13%	
Rotifera 11.0 ± 5.91%	Monogononta 9.35 ± 5.65%	Ploima 8.97 ± 4.71%	Synchaetidae 3.18 ± 1.45%	*Polyarthra* 2.12 ± 0.53%	
			Brachionidae 3.02 ± 0.94%	*Brachionus* 2.87 ± 0.93%	
	Bdelloidea 1.30 ± 0.89%	Bdelloida 1.28 ± 0.46%	Philodinidae 1.22 ± 0.57%	*Rotaria* 1.19 ± 0.63%	
Mollusca 9.23 ± 2.08%	Gastropoda 9.01 ± 2.12%	Basommatophora 8.75 ± 2.76%	Planorbidae 5.97 ± 1.88%	*Bulinus* 2.87 ± 0.93%	
				Biomphalaria 1.01 ± 0.64%	
	Bivalve 0.19 ± 0.13%	Limoida 0.17 ± 0.10%	Limidae 0.14 ± 0.08%	*Limatula* 0.09 ± 0.03%	
Annelida 5.34 ± 2.79%	Clitellata 4.81 ± 2.19%	Haplotaxida 4.79 ± 2.26%	Naididae 4.71 ± 2.05%	*Limnodrilus* 4.63 ± 1.96%	
Arthropoda 2.21 ± 0.81%	Insecta 1.40 ± 0.65%	Diptera 0.89 ± 0.28%	Culicidae 0.74 ± 0.33%	*Aedes* 0.22 ± 0.20%	
		Ephemeroptera 0.41 ± 0.27%	Baetidae 0.25 ± 0.21%	*Mayobaetis* 0.18 ± 0.14%	
Echinodermata 1.70 ± 0.72%	Crinoidea 1.46 ± 0.81%	Comatulida 1.34 ± 0.56%	Aporometridae 1.21 ± 0.68%	*Aporometra* 1.09 ± 0.69%	

3. Results

3.1. Composition of invertebrate assemblages in the HYL wetland

The composition of pelagic invertebrate assemblages at the 30 sampling sites in the HYL wetland is shown in Fig. S1 of the Supplementary Material. The dominant taxa – the first seven phyla with the highest OTU abundance – are listed in Table 1. The relative OTU abundance (%) at the phylum level showed that Cnidaria (48.5%) > Porifera (19.4%) > Rotifera (11.0%) > Mollusca (9.23%) > Annelida (5.34%) > Arthropoda (2.21%) > Echinodermata (1.70%) > others. The relative OTU abundance at the class level showed that Cnidaria|Hydrozoa (44.1%) > Porifera|Demospongiae (14.6%) > Rotifera|Monogononta (9.35%) > Mollusca|Gastropoda (9.01%) > Annelida|Clitellata (4.81%) > others (less than 4.02%). The relative OTU abundance (%) at the order level showed that Cnidaria|Hydrozoa|Anthoathecata (27.7%) > Porifera|Demospongiae|Poecilosclerida (13.4%) > Rotifera|Monogononta|Ploima (8.97%) > Mollusca|Gastropoda|Basommatophora (8.75%) > Cnidaria|Hydrozoa|Leptothecata (7.20%) > Cnidaria|Hydrozoa|Siphonophorae (5.02%) > others (less than 4.79). These results indicated that the OTU-based invertebrate composition at the phylum and class levels was determined by Hydrozoa

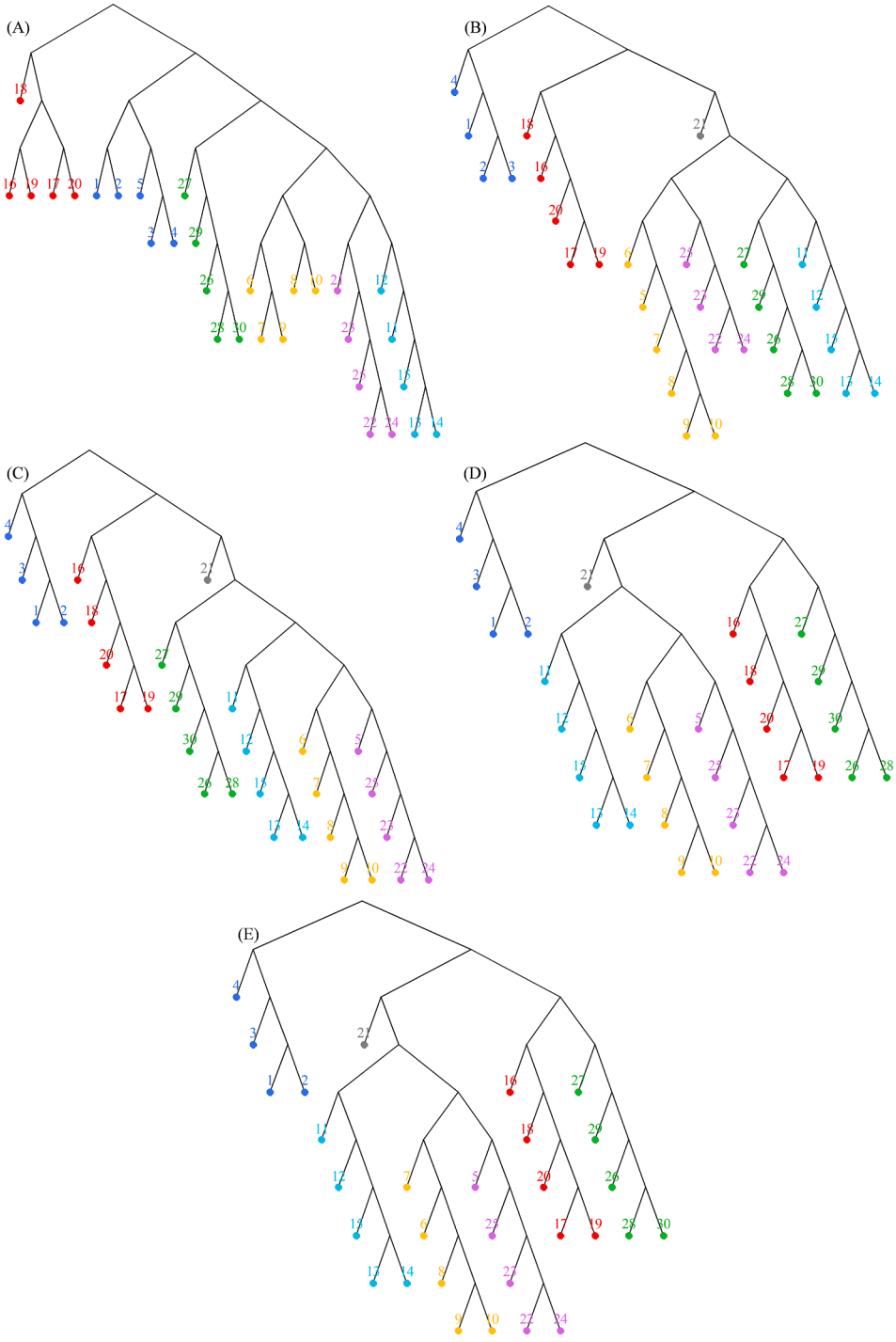

Fig. 2. Cluster analysis based on the OTU abundance of invertebrate assemblages at sites 1–30 in the Huayang Lake wetland. (A), (B), (C), (D), and (E) are cluster analyses at the phylum, class, order, family, and genus levels, respectively.

and Demospongiae, which are commonly found in seawater, followed by Monogononta, Gastropoda, and Clitellata, which live in both seawater and freshwater. Compared with the composition of invertebrate assemblages at the phylum and class levels, those at the order, family, and genus levels were dispersed. Crellidae|*Crella* (10.1%), Pandeidae|*Catablema* (8.74%), and Hydridae|*Hydra* (8.01%) had the highest OTU abundance, followed by Naididae|*Limnodrilus* (4.63%), Campanulariidae|*Obelia* (4.22%), and Apolemiidae|*Apolemia* (3.28%). Generally, the OTU-based invertebrate compositions above the order level (i.e., phylum and class levels) were concentrated on individual taxa, whereas those below the order level (i.e., family and genus levels) belonged to different small taxa. The genus-level OTU abundance was almost less than 10%, indicating that the invertebrate composition at the genus level depended on diverse taxa.

3.2. Spatial variation in the structure of invertebrate assemblages

The clustering analysis based on the relative abundance (%) of invertebrate OTU richness showed that the 30 sampling sites in the HYL wetland could be divided into six zones, which exhibited clear wetland type-specific characteristics (Fig. 2). Regardless of the taxonomic level, sites 1–4, 6–10, 11–15, 16–20, 22–25, and 26–30 were clustered into six zones, with significant ($P < 0.05$) between-group variance observed. The exceptions were sites 5 and 21, which were grouped into different zones when clustered at different taxonomic levels. At the phylum and class levels, the invertebrate compositions in the six zones were all determined by Cnidaria|Hydrozoa, followed by Rotifera|Monogononta in zone IV and by Porifera|Demospongiae in zones I–III and V–VI (Fig. S1 of the Supplementary Material). At the order, family, and genus levels, the composition of invertebrate assemblages in zone I (sites 1–4), zone II (sites 6–10), and zone IV (sites 16–20) was mainly determined by Leptothecata|Campanulariidae|*Obelia* (17.1%–24.8%), Poecilosclerida|Crellidae|*Crella* (24.2%–31.5%) and Anthoathecata|Hydridae|*Hydra* (16.5%–23.8%), and Anthoathecata|Pandeidae|*Catablema* (17.2%–19.3%) and Haplotaxida|Naididae|*Limnodrilus* (13.6%–17.4%), respectively.

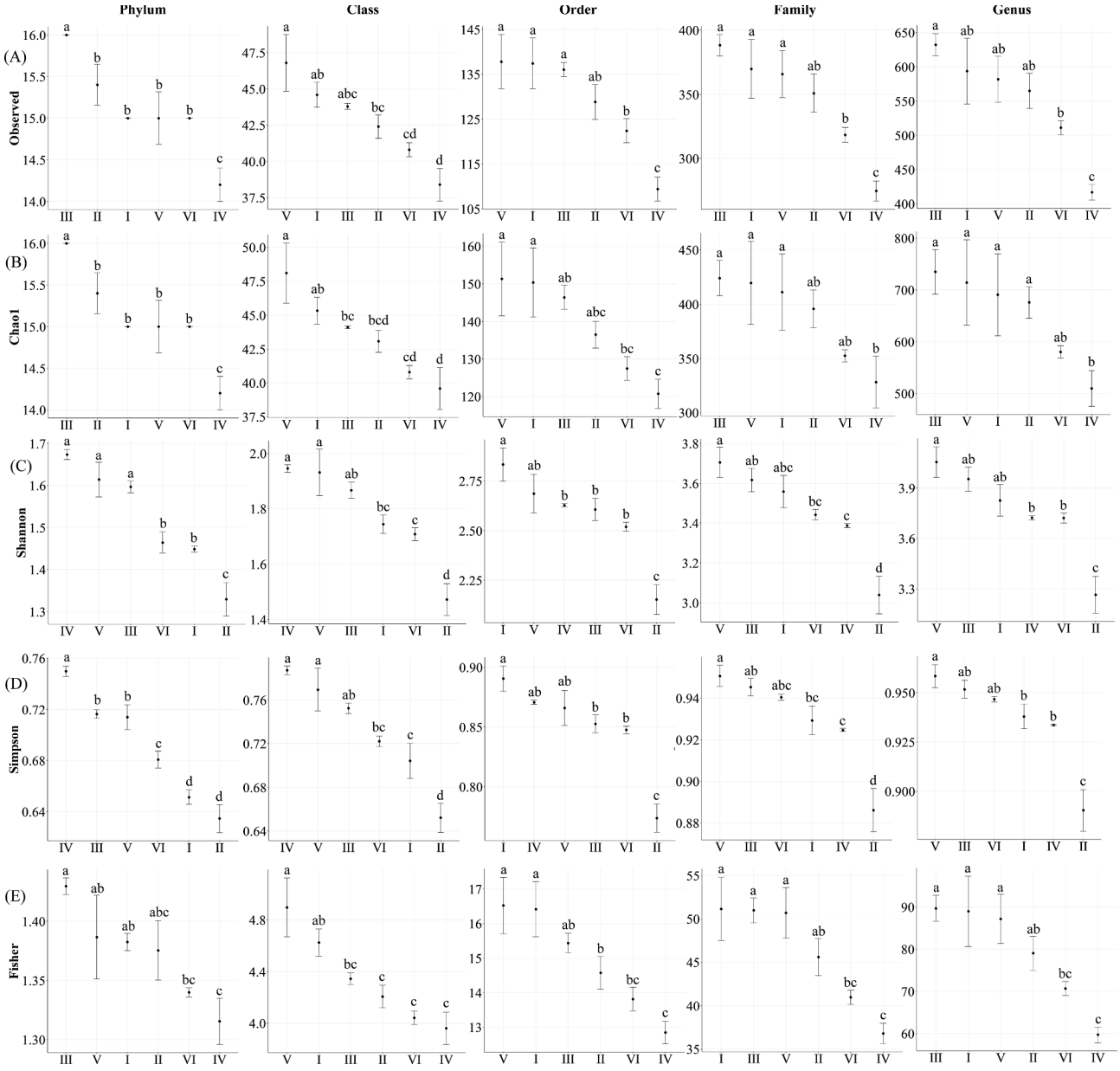

Fig. 3. The variation in alpha diversity of invertebrate assemblages among the six wetland zones. The five diversity indexes include the (A) Observed index, (B) Chao1 index, (C) Shannon index, (D) Simpson index, and (E) Fisher index.

An interesting finding was that in zones I, II, and IV, the dominant invertebrate taxa at the family and genus levels were consistent with those at the phylum and class levels (see details in Fig. S1 of the Supplementary Material). This indicated that the clustering results could be attributed to the key invertebrate taxa at family or genus levels (e.g., Campanulariidae|*Obelia* at sites 1–4 in zone I and Crellidae|*Crella* at sites 6–10 in zone II), which were dominant in determining the invertebrate assemblage composition at the phylum and class levels. In contrast, the family- and genus-level compositions of invertebrate assemblages in zones III, V, and VI were determined by various taxa, which had relatively equal OTU abundances; and thus, there were no dominant taxa that could determine the structure of local invertebrate assemblages.

3.3. Spatial variation in alpha diversity of invertebrate assemblages

Five common indexes, including 'Observed', 'Chao1′, 'Shannon', 'Simpson', and 'Fisher', were used to evaluate the alpha diversity of invertebrate assemblages (Fig. 3). Among the five indexes, 'Simpson' was the most effective index to distinguish the differences in invertebrate diversity among the spatial zones, followed by 'Shannon' and 'Observed'. 'Fisher' and 'Chao1' were less effective than the other three indexes. For example, 'Simpson' and 'Shannon' indexes could distinguish the differences in invertebrate diversity among the zones to at least 3- or 4-level significance (i.e., superscript 'a' to 'c'–'d' of boxplots in Fig. 3), whereas 'Fisher' index could only distinguish the differences to 3-level significance (i.e., superscript 'a' to 'c'). In terms of 'Simpson' and 'Shannon' indexes, the highest invertebrate diversity were observed in zone IV at phylum and class levels, in zone I at order level, and in zone V at family and genus levels. In contrast, in terms of 'Observed' and 'Chao1' indexes, the highest invertebrate diversity were observed in zone III at phylum, family and genus levels but in zone V at class and order levels. An interesting finding was that, regardless of the taxonomic levels, 'Simpson' and 'Shannon' indexes showed similar results in diversity ranking, whereas 'Observed', 'Chao1' and 'Fisher' showed similar results in diversity ranking. Generally, the phylum- and class-level alpha diversity showed higher (at least not lower) recognition than the order-, family- and genus-level alpha diversity, indicating that the eDNA-based taxonomic identification at phylum and class levels was effective in distinguishing the spatial differences in invertebrate diversity. Thus, we suggest that calculating the 'Simpson' and 'Shannon' indexes at the phylum and class levels is effective for evaluating eDNA-based diversity of invertebrate assemblages in field.

3.4. Biomarkers that determined zone-specific invertebrate assemblages

At a spatial scale, the wetland type-specific invertebrate assemblages could be characterized by the biomarkers within different taxa (Fig. 4). At the phylum level, Arthropoda, Cnidaria, and Mollusca were the biomarkers that indicated the invertebrate taxa in zone I. Annelida, Bryozoa, and Rotifer were the biomarkers that indicated the invertebrate composition in freshwater zone IV. Porifera, Chordata, and Echinodermata were the biomarkers that indicated the invertebrate taxa in zones II, V, and VI. At the class level, Insecta, Cnidaria, Hydrozoa, and Gastropoda were the biomarkers that indicated the invertebrate taxa in zone I; Ascidiacea and Demospongiae were the biomarkers that indicated the invertebrate taxa in zone II; Clitellata, Gymnolaemata, and Monogononta were the biomarkers that indicated the invertebrate taxa in zone IV; and Echinoidea was the biomarker that indicated the invertebrate taxa in zone VI. At the order level, Anthomedusae, Leptothecata, and Bdelloidea were the biomarkers that indicated the invertebrate taxa in zone I; Poecilosclerida was the biomarker that indicated the invertebrate taxa in zone II; Haplotaxida, Siphonophorae, and Ploima were the biomarkers that indicated the invertebrate taxa in zone IV; Scleractinia was the biomarker that indicated the invertebrate taxa in zone V; Echinoida, Basommatophora, and Homosclerophorida were the biomarkers that indicated the invertebrate taxa in zone VI. The family- and genus-level biomarkers were similar; Campanulariidae|*Obelia* and Hydractiniidae|*Hydractinia*, Crellidae|*Crella* and Hydridae|*Hydra*, Corynidae|*Sarsia*, Pandeidae|*Catablema* and Naididae|*Limnodrilus*, and Protiaridae|*Halitiara* and Plakinidae|*Plakinastrella* were the biomarkers that indicated the local invertebrate taxa in zones I, II, III, IV, and VI, respectively. Interestingly, there was no biomarker in zone III at phylum, class, and order levels, indicating that the invertebrate taxa above family level in zone III were similar to those in surrounding zones.

3.5. Relationships between environmental factors and the structure of invertebrate assemblages

The environmental factors were first selected to exclude collinearity. Depth (m), flow velocity (m/s), dissolved oxygen (DO, mg/L), salinity (‰), chemical oxygen demand determined by Mn (COD_{Mn}, mg/L), TP (total phosphate, mg/L), TN (total nitrogen, mg/L), NH_3–N (ammonia nitrogen, mg/L) and NO_3–N (nitrate nitrogen, mg/L) of physicochemical water parameters, coliform (most probable number per liter, MPN/L) and fecal coliform (FC, MPN/L) of bacterial community factors, as well as Cu (μg/L) and Hg (μg/L) of heavy metals were selected as the noncollinear environmental factors. Of the selected non-collinear environmental factors, only those had strong explanatory power on invertebrate OTU richness were used in RDA. The RDA results (Fig. 5) showed that the explanatory power of environmental factors on OTU richness exhibited the taxon-level tendency of phylum (78.5%) > class (73.4%) > order (69.3%) > family (64.9%) > genus (61.2%). This indicated that the association between environmental factors and phylum- or class-level OTU richness was stronger than that between environmental factors and other-level OTU richness. Therefore, we suggest that eDNA-based biological monitoring at the phylum or class level is effective in reflecting the conditions of the local environment.

The Pearson correlation analysis showed that DO, flow velocity, FC, and TP were four key environmental factors that had close associations with invertebrate OTU abundance at different taxonomic levels (see Fig. 6 and details in Table S2 of the Supplementary Material). At the phylum level, these factors had significant ($P < 0.05$) positive correlations with Nematoda, Platyhelminthes, and Cnidaria. At the class level, these factors had significant positive correlations with Dorylaimea, Crinoidea, and Bdelloidea. At the order level, these factors had significant positive correlations with Pulmonata, Leptothecata, Rhynchobdellida, and Anthoathecata. At the family level, these factors had significant positive correlations with Campanulariidae, Hydractiniidae, Tetillidae, and Philodinidae. At the genus level, these factors had significant positive correlations with *Lecane*, *Rotaria, Biomphalaria*, *Obelia*, *Adineta*, and *Cinachyrella*. Notably, in addition to the four key environmental factors, salinity, coliform, NO_3–N, TN, and Hg were the primary water quality and heavy metal factors that influenced the invertebrate assemblages regardless of the taxonomic characteristics.

4. Discussion

4.1. The influence of wetland environment on distribution of aquatic invertebrates

Sites 1–4 in zone I were located in the main stream of the East River, and the invertebrate assemblages were indicated by freshwater taxa (e.g., Gastropoda and Bdelloidea). Sites 6–10 in zone II received the estuarial brackish water that was poured back into the urban waterways at high tide, and thus, the invertebrate assemblages remaining in zone II were indicated by marine taxa (e.g., Hydrozoa and Demospongiae). Zone IV was located in the center of the HYL wetland, where a relatively static water body was conducive to freshwater invertebrates (e.g., Monogononta and Clitellata) preferring to live in limnophilic habitats. Sites 26–30 in zone VI were located on the southern side of the HYL wetland and connected directly with the Pearl River Estuary; thus, the OTUs of Echinodermata preferring to live in the sea were detected.

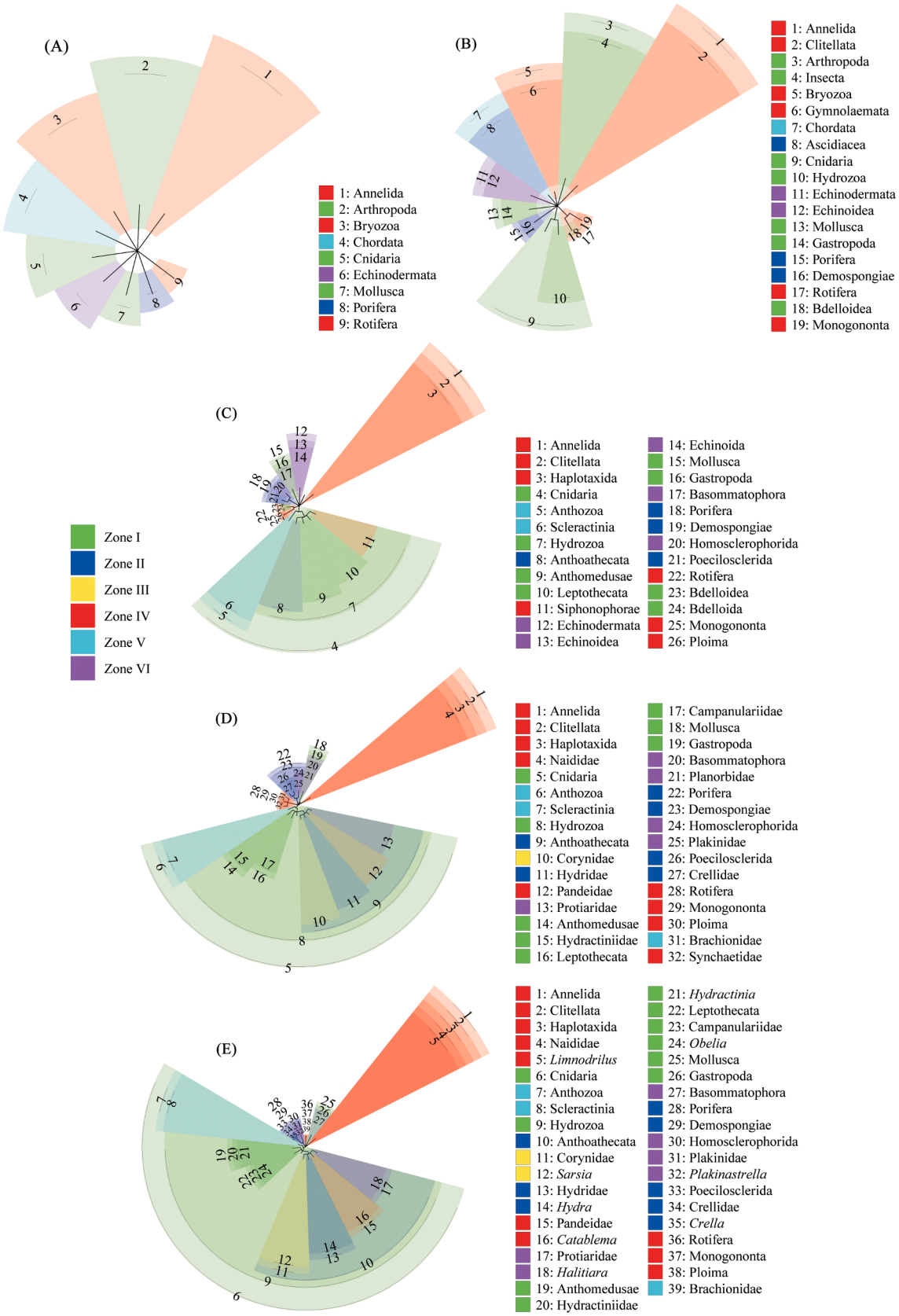

Fig. 4. Invertebrate biomarkers selected by LEfSe from the six biological groups in the Huayang Lake wetland. The circles radiating from inside to outside represent the classification levels from phylum to genus, with the diameter of each species circle representing the level of OTU abundance of each classification. (A) Phylum-level biomarkers, (B) class-level biomarkers, (C) order-level biomarkers, (D) family-level biomarkers, (E) genus-level biomarkers.

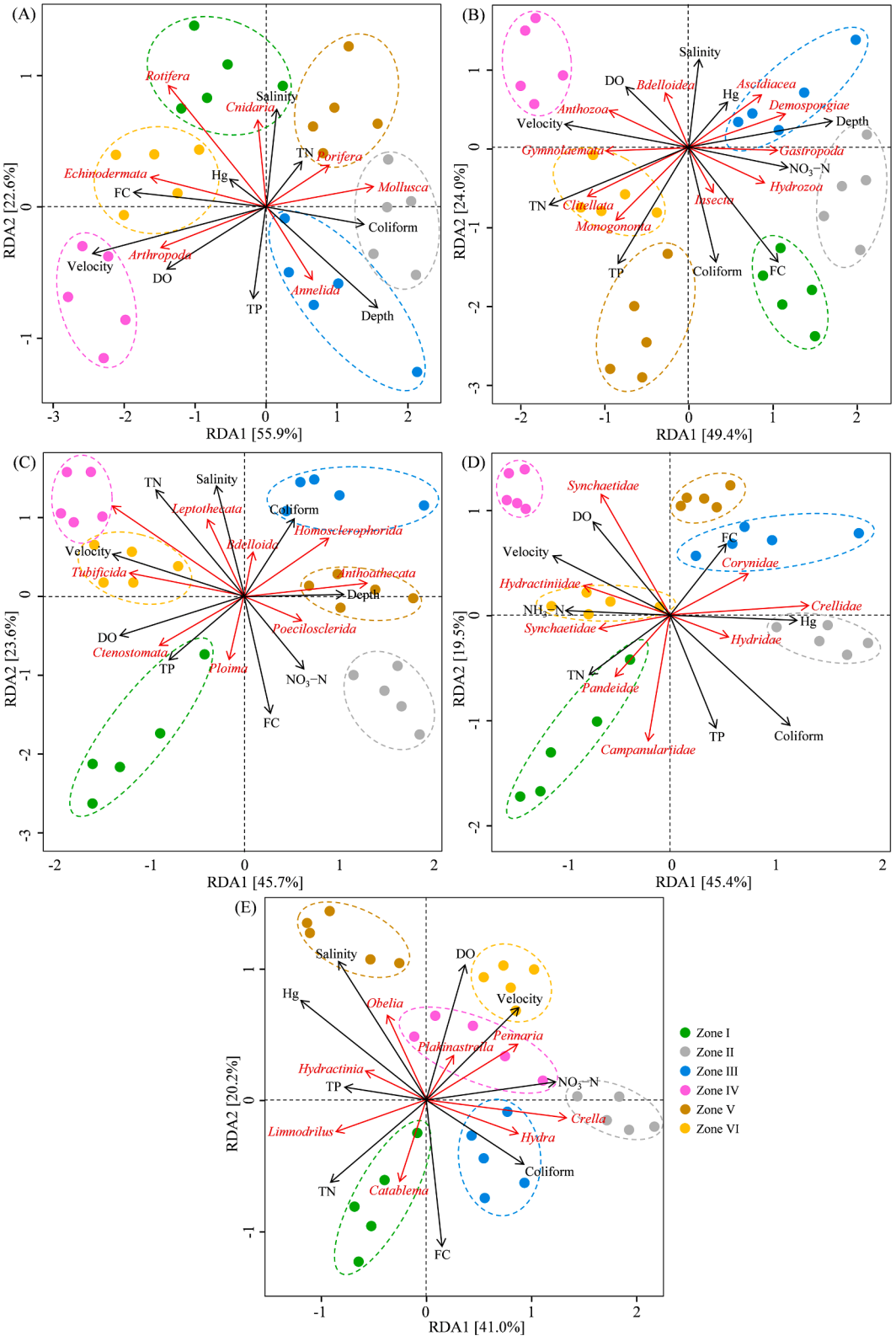

Fig. 5. The redundancy analysis (RDA) between environmental factors and the OTU abundance of invertebrate assemblages. (A) RDA between environmental factors and phylum-level OTU abundance, (B) RDA between environmental factors and class-level OTU abundance, (C) RDA between environmental factors and order-level OTU abundance, (D) RDA between environmental factors and family-level OTU abundance, (E) RDA between environmental factors and genus-level OTU abundance. DO, dissolved oxygen (mg/L); FC, fecal coliform (MPN/L); NH_3–N, ammonia nitrogen (mg/L); NO_3–N, nitrate nitrogen (mg/L); TN, total nitrogen (mg/L); TP, total phosphorus (mg/L).

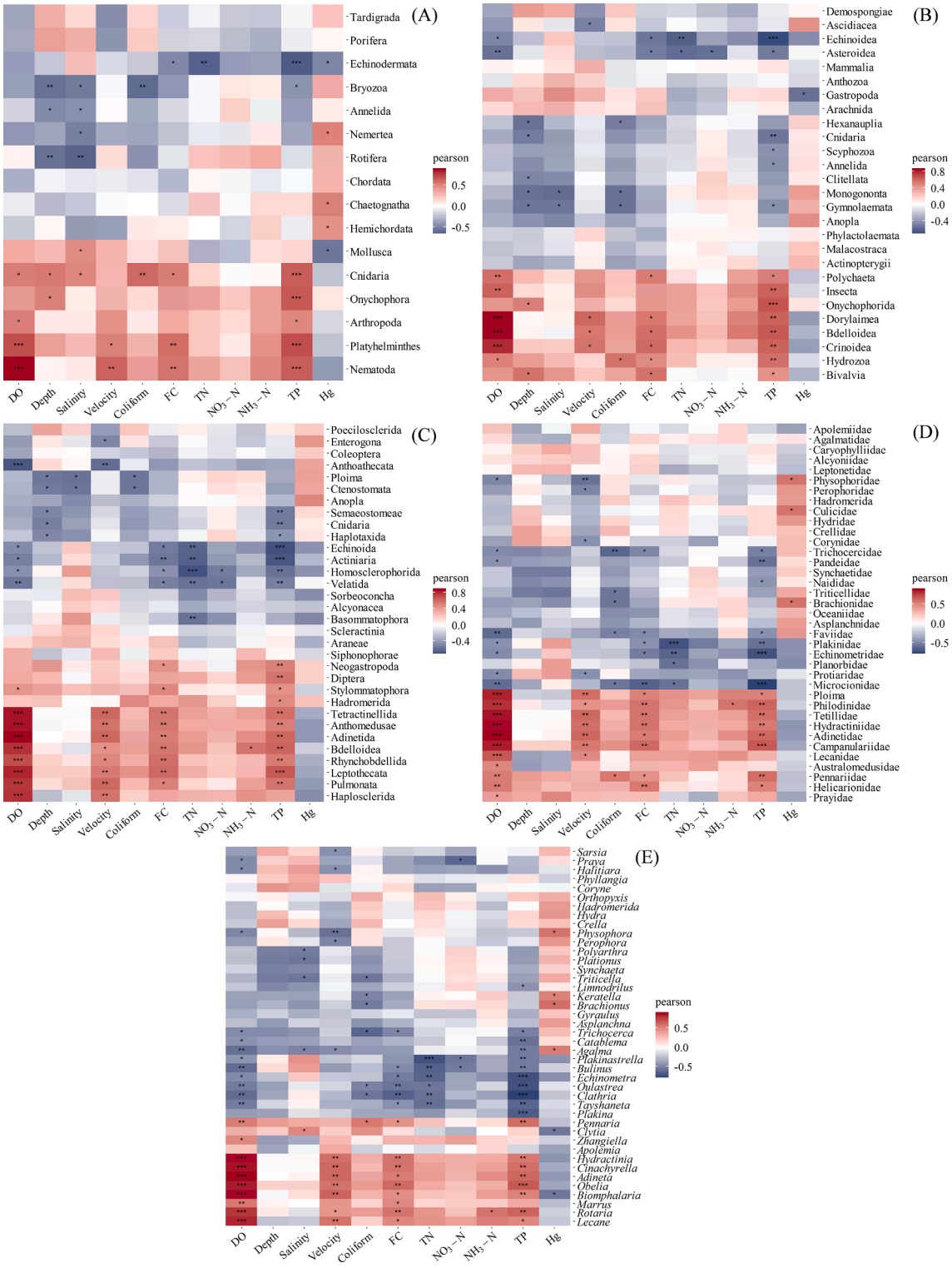

Fig. 6. Pearson correlation between environmental factors and the OTU abundance of invertebrate assemblages in the Huayang Lake wetland. (A) Pearson correlation between environmental factors and phylum-level OTU abundance, (B) Pearson correlation between environmental factors and class-level OTU abundance, (C) Pearson correlation between environmental factors and order-level OTU abundance, (D) Pearson correlation between environmental factors and family-level OTU abundance, (E) Pearson correlation between environmental factors and genus-level OTU abundance. DO, dissolved oxygen (mg/L); FC, fecal coliform (MPN/L); NH_3–N, ammonia nitrogen (mg/L); NO_3–N, nitrate nitrogen (mg/L); TN, total nitrogen (mg/L); TP, total phosphorus (mg/L). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Generally, the habitat pattern of the HYL wetland, such as freshwater vs. brackish and limnetic vs. fluvial, could be reflected by the spatial distribution of invertebrate communities. The high OTU richness of the key taxa with specific environmental adaptation might be used to indicate changes in habitat characteristics or water quality.

4.2. Choosing an appropriate taxonomic level for evaluating biodiversity and the environment

Compared with fish, which had comprehensive DNA information in the gene bank, using eDNA metabarcoding to distinguish invertebrate

diversity faces the problem of lacking information on individual DNA sequences. For example, eDNA metabarcoding was not sensitive enough to identify invertebrates at the species level, which is why we used taxonomic classification from genus to phylum without species. Our results suggested that the capacity of eDNA metabarcoding to identify invertebrate taxa is a key factor influencing monitoring and evaluation effects. For instance, the identification capacity of eDNA for Arthropoda (e.g., Insecta and Arachnida) and Mollusca (e.g., Gastropoda and Bivalvia) was able to precisely distinguish the genus-level differences. In contrast, it is unable to distinguish the order- and family-level differences in Annelida.

In complex conditions, such as the exchange of fresh and brackish waters in the HYL wetland, choosing an appreciate taxonomic level that can be identified by eDNA metabarcoding is important. Using current primers, the identification of dominant invertebrates at the class and phylum levels, such as Cnidaria|Hydrozoa, Porifera|Homoscleromorpha, Rotifera|Monogononta, and Arthropoda|Insecta, is critical for revealing the spatial differences in invertebrate distribution. The phylum- and class-level identification by eDNA could be used to reflect the invertebrate distribution in heterogeneous water bodies, such as running rivers, lentic lakes, and brackish estuaries. Therefore, we recommend that for complex habitats such as the HYL wetland that receives both fresh and brackish waters, analyzing the invertebrate composition or diversity at the phylum or class level (at least to family) is more effective than at other levels.

4.3. Selecting invertebrate biomarkers with biological and environmental indications

Generally, the complex habitats in the HYL wetland can be simulated as a 'river–lake–estuary' continuum, where fresh, brackish, and salty waters keep exchanging. This is a typical multivariate ecosystem that determines multistep changes in biotic communities. As a result, the biomarkers in each zone reflected both the water and habitat characteristics in the local environment. Given that inflow and outflow river channels were influenced by tides, zones I and V retained the DNA information on both freshwater Mollusca and marine Cnidaria and Crella, and the latter became dominant in the upper water layer. Zone II represented the habitats in small channels, where the water exchanges were less than those in zones I and V. During the rising tide, the floodgates of the HYL wetland were open in part of the time, and marine invertebrates were pumped into these channels. When the floodgates were closed, the brackish waters carried by tides remained in the channels, leading to the high richness of Cnidaria. As a result, Cnidaria was the biomarker in zone II. Zone IV is a relatively lentic lake area, where Ploima of Rotifera, which reproduces fast in relatively still waters, is the biomarker in the static water area. Above family level, there were no biomarkers for zone III, indicating that zone III could not be distinguished from surrounding zones II and IV. This is consistent with the local conditions, where the invertebrates in zone III were from zones II and IV. Zone VI was the outlet of the HYL wetland and was connected with the Pearl River Estuary. The high salinity and slow-flowing water in zone VI were suitable for Echinodermata and Cnidaria that preferred to live in marine environment.

4.4. Potential use of eDNA in biodiversity and environment monitoring

We suggest that eDNA-based monitoring of invertebrate communities could be used to 1) analyze the distribution and composition of aquatic invertebrates in different habitats and 2) evaluate the effects of environmental changes (e.g., water pollution and habitat degradation) on invertebrate diversity and integrity. In complex habitats, such as the HYL wetland, it may not be practical to use eDNA to identify invertebrates for detailed taxonomic classification (e.g., at the genus or species level) or to focus on specific functional (e.g., feeding and living) groups. Instead, it is interesting to use eDNA to identify the invertebrate composition at a general taxonomic level and conduct scientific research at a large spatial scale. Our results demonstrated that the phylum- and class-level eDNA analysis was effective enough for comparing alpha diversity, selecting biomarkers, and revealing the relationships between environmental factors and the structure of invertebrate communities.

In this study, a series of environmental factors, such as dissolved oxygen, flow velocity, salinity, fecal coliform, and total phosphorus in the water, had great impacts on the structure of invertebrate communities. However, the environmental impacts on invertebrates at the phylum and class levels are inconsistent with those at the family and genus levels. One reason is that the dominant genus or family based on OTU richness does not represent the overall taxonomic characteristics of the phylum or class. Another reason is that environmental factors directly influence specific genera or families and then indirectly influence classes or phyla. Given that aquatic invertebrates function as both prey and predators, the key environmental factors that influence invertebrate distribution may further influence community diversity and food web stability (Wang et al., 2021a). Our further research will focus on the relationships between the composition and diversity of invertebrate communities and those of fish communities as well as on the structure and functioning of aquatic food webs.

5. Conclusions

Our study used eDNA macrobarcoding technology to investigate the composition and diversity of invertebrate communities in complex wetland habitats with interlaced brackish and fresh waters. The results showed that phylum- or class-level identification was more suitable for the eDNA-based field monitoring of aquatic invertebrates than other-level identification. The relative OTU abundance (%) could effectively distinguish the dominant invertebrate taxa among the spatial zones (i.e., different wetland types). The composition and diversity of invertebrate communities in each spatial zone could be indicated by local biomarkers; furthermore, the living habits of these invertebrate biomarkers could identify the wetland type-specific differences in local water quality and habitat characteristics. The association between environmental factors and phylum- or class-level OTU richness exhibited great strength in indicating the environmental impacts on invertebrate communities. In the future, eDNA macrobarcoding technology has great potential to be used in field monitoring of invertebrates, and even extend to fish and other vertebrates.

CRediT authorship contribution statement

Tuan-Tuan Wang: Conceptualization, Methodology, Investigation, Funding acquisition, Formal analysis, Writing – original draft, Data curation, Resources. **Xiao-Di Wang:** Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft, Data curation. **Ding-Ying Wang:** Methodology, Investigation, Formal analysis, Data curation. **Shi-Di Fan:** Methodology, Investigation, Formal analysis, Resources. **Sai Wang:** Conceptualization, Methodology, Writing – review & editing, Visualization, Supervision, Project administration. **Zhong-Bing Chen:** Methodology, Investigation, Formal analysis, Writing – original draft, Visualization. **En-Ni Wu:** Investigation, Formal analysis, Data curation. **Yang Zhang:** Investigation, Data curation, Resources. **Cong-Cong Jin:** Investigation, Data curation, Resources. **Zhuo-Luo Ma:** Investigation, Data curation, Resources. **Wen-Tong Xia:** Conceptualization, Methodology, Writing – review & editing, Supervision. **Ling Mo:** Visualization, Supervision, Project administration.

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

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecolind.2023.110126.

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