

## Article

# eDNA Biomonitoring of Macroinvertebrate Communities for the Bioassessment of a River's Ecological Status

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**Abstract:** Environmental DNA (eDNA) becomes a promising technology for macroinvertebrate monitoring worldwide. In recent decades, with increasing humanization processes, such as water pollution and habitat fragmentation, the richness and abundance of macroinvertebrates show a dramatic decline, which is particularly evident in tropical or subtropical rivers. The high-throughput and rapid monitoring of species' survival and the ecological status of their habitats are relevant to river management. Here, we used the eDNA technology to detect macroinvertebrates in the Dongjiang River—a typical subtropical river in Southern China, to assess the ecological status, based on eDNA datasets. Our data showed a total of 640 OTUs detected by eDNA technology, belonging to three phyla, five classes, 13 orders, 33 families and 71 genera of macroinvertebrates, and these taxa had a 36.6% coverage rate with historical data at the genus level. The traditional water quality index (WQI) showed that the upstream of Dongjiang River were mainly levels I–II, the middle stream were levels II–III, and the downstream were levels IV–V. The eDNA-based biotic indices showed almost the same findings, that is, the overall ecological status of Dongjiang River was: upstream > middle reaches > downstream. Overall, this study provides important datasets and technical support for eDNA technology in macroinvertebrate monitoring and ecosystem management in the subtropical rivers.

**Keywords:** eDNA; bioassessment; water quality; river health; Dongjiang River



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## 1. Introduction

Macroinvertebrates are an important part of the structure and function of the river ecosystem, which is sensitive to environmental changes, due to their relatively long life cycles and weak migration abilities [1–3]. In recent decades, the richness and abundance of macroinvertebrates have been sharply reduced, driven by human activities, such as water pollution and climate change [4–6]. As we know, macroinvertebrates can accelerate the decomposition of benthic debris, promote the material exchange at the terrestrial-aquatic interface and the self-purification of water, and maintaining the biodiversity of macroinvertebrates is directly related to the key ecosystem processes, such as nutrient circulation, energy flow, material decomposition and migration in the river biological system [7–9]. Currently, macroinvertebrates have been widely adopted by countries around the world to indicate the river ecological status [10–13]. However, most current projects for macroinvertebrate monitoring, especially in developing countries, are still based on traditional morphological methods. Many deficiencies, such as the long monitoring cycle, that is time-consuming and laborious, makes it difficult to carry out large-scale macroinvertebrate monitoring projects at the watershed scale.

Recent advances in the eDNA technology provide new opportunities for improving the throughput, accuracy and standardization of macroinvertebrate monitoring [14–16]. Compared with the traditional methods, eDNA technology does not need a lot of manpower and material resources, it is low cost and easy to operate [17–19]. More importantly, this

technology is highly sensitive and can accurately detect the presence of the target species, even at very low species densities [17,20,21]. Recently, eDNA technology is considered as one of the most promising methods to achieve the rapid, large-scale and large-sample species monitoring of aquatic ecology [22–24]. For example, Blackman et al. [25] used eDNA to characterize the spatio-temporal patterns of the multi-trophic biodiversity and food-web characteristics uncovered across a river catchment; Liu et al. [26] compared the structure and diversity of macroinvertebrates in the Okinawa Trough and Mariana Trench, by eDNA metabarcoding; Serrana et al. [27] studied the ecological consequences of sediment bypass tunnels on macroinvertebrates in dam-fragmented rivers, by DNA metabarcoding. Although the methodology, based on eDNA, is becoming more and more mature, there is still a lack of sufficient evidence to directly apply the eDNA datasets to assess the river's ecological status, especially in tropical or subtropical river systems, compared with temperate regions [28].

Here, we used the eDNA technology to monitor macroinvertebrates in the Dongjiang River. Dongjiang River is a typical subtropical waterway, which is one of the four important water systems in Southern China. Dongjiang River supports an important drinking water source for the Guangdong-Hong Kong-Macao Greater Bay Area (the Greater Bay Area), which is also a natural habitat for macroinvertebrates and other aquatic species [29]. In recent decades, with the rapid development of the social economy in the Greater Bay Area, some important tributaries of Dongjiang River have undergone great changes, such as water attenuation and water pollution, resulting in a significant reduction of the habitat diversity and biodiversity, and the habitat of many important biological groups has been destroyed and degraded [30–32]. Therefore, the accurate and comprehensive monitoring of the dynamic changes of the aquatic species resources in Dongjiang River is the basis for biodiversity protection. Our objectives include: (1) to compare and analyze the consistency between the eDNA datasets and the historical records of the Pearl River basin; (2) to evaluate the ecological status of the Dongjiang River, based on the eDNA dataset, and to compare the consistency with the traditional water quality index.

## 2. Material and Methods

### 2.1. Study Area and eDNA Sampling

Dongjiang River is one of the three major tributaries in the Pearl River basin, located between 113°30'–115°45' E and 22°45'–25°30' N, which supplies a major source of drinking water for nearly 40 million people living in six cities, including Hong Kong, Shenzhen, Guangzhou, Dongguan, Huizhou and Heyuan, in the Greater Bay Area [25,30]. In recent decades, with the rapid economic development of the Dongjiang River, human-driven land use changes led to a serious deterioration of the eco-environmental quality and water security [33]. As one of the indicators widely adopted in the river ecological status assessment [34–36], the rapid and complete monitoring of macroinvertebrates and their living spaces was relevant to the ecological protection and management in the Dongjiang River. To identify and verify the reliability of the eDNA technology in distinguishing habitats with different human impacts, this study selected three different river systems in the Dongjiang River, according to the different densities of human land use, such as cropland and impervious cover [33], six sites were set up for each river system, namely the upstream group (DJ 01-06), midstream group (LJ 01-06) and the downstream group (SM 01-06).

Field eDNA samples were collected in October 2021, referring to the published protocols [33,37]. Briefly, three 1-liter samples were sampled at each site using the Thermo Fisher sterile bottles, then the samples were transferred and stored in a dark closed incubator (0~4 °C) and filtered within 6h. In the pretreatment chamber, 300–500 mL volumes of water were filtered through 0.45 µm hydrophilic nylon membranes (Merck, Rahway, NJ, USA), until 3L of surface water was filtered, and finally, six replicates (or subsamples) were obtained at each site. To monitor the possible contaminants between the different sites, blank controls were prepared at each site. All duplicate eDNA samples and blank

controls were individually placed in 5.0 mL centrifuge tubes and stored at  $-20\text{ }^{\circ}\text{C}$  before the eDNA extraction.

## 2.2. Determination of the Water Quality Parameters

Eight water quality parameters were measured at each site, including total nitrogen (TN, mg/L), total phosphorus (TP, mg/L), ammonia nitrogen ( $\text{NH}_3\text{-N}$ , mg/L), total dissolved particles (TDS, mg/L), water temperature (WT,  $^{\circ}\text{C}$ ), pH, dissolved oxygen (DO, mg/L) and the potassium permanganate index ( $\text{COD}_{\text{Mn}}$ , mg/L). Specifically, TDS, WT, pH, and DO were recorded by AP-2000 multiparameter water quality instruments (Aquaread, Broadstairs, UK) on-site. One liter of surface water was collected at each site, and five or six drops of concentrated sulfuric acid were added into the bottles, then they were brought back to the laboratory in dark and sealed conditions, to measure the TN, TP and  $\text{NH}_3\text{-N}$ , following standard methods (NEPB, 2002). The other one liter of surface water was stored in low temperatures and in dark conditions ( $0\text{ }^{\circ}\text{C}$  to  $4\text{ }^{\circ}\text{C}$ ), then sent to the laboratory to measure the  $\text{COD}_{\text{Mn}}$  within 24 h.

## 2.3. eDNA Extraction and the High-Throughput Sequencing

All eDNA replicates and blank controls were extracted using a DNeasy PowerWater Kit (QIAGEN, Hilden, Germany). The polymerase chain reaction (PCR) assays were performed using a specific COI primer for detecting macroinvertebrates [38]. The primer sequences are as followed: forward:  $5'\text{-AACGACGCTAGCAAACAAATARDGGTATTCGDTY-3}'$ ; reverse:  $5'\text{-GGDACWGGWTGAACWGTWTACCHCC-3}'$ . The reverse primer was carried with a 12-nt unique short nucleotide sequence to label the different replicates. Each reaction included  $10\text{ }\mu\text{L}$  of 2x Taq Plus Master MixII,  $2\text{ }\mu\text{L}$  of the template DNA,  $1\text{ }\mu\text{L}$  of upstream and downstream primers each, and  $6\text{ }\mu\text{L}$  of enzyme-free water in a total reaction volume of  $20\text{ }\mu\text{L}$ . The PCR amplification was performed under the following conditions:  $95\text{ }^{\circ}\text{C}$  pre-denaturation for 30 s;  $95\text{ }^{\circ}\text{C}$  denaturation for 5 s,  $54\text{ }^{\circ}\text{C}$  renaturations for 30 s,  $72\text{ }^{\circ}\text{C}$  extensions for 10 s, 40 amplification cycles;  $72\text{ }^{\circ}\text{C}$  extensions for 5 min;  $4\text{ }^{\circ}\text{C}$  preservation. The PCR products were further purified after detection by 2% agarose gel electrophoresis. The equipment and reagents used in the DNA extraction and the PCR amplification were all sterile in the state or sterilized to avoid cross-contamination during the operation.

## 2.4. Bioinformatics Analysis and Biodiversity

The PCR products obtained from the DNA amplification were pooled into a single tube with equimolar quantities for NGS sequencing on the Illumina MiSeq PE150 platform (Illumina, San Diego, CA, USA). First, the forward and reverse sequences were merged using the “-fastq\_mergepairs” script in the VSEARCH pipeline [39]. Then, FASTQ files were converted to FASTA format using SeqIO in Python version 2.7. The merged sequences were filtered for the low-quality reads using the “split\_libraries.py” script with “-w 50 -s 25 -l 100” parameters in the QIIME toolkit v1.91 [40]. All cleaned sequences were clustered into operational taxonomic units (OTUs), using the USEARCH 11 pipeline with a similarity threshold of 97% [41]. The representative OTUs were aligned to a custom reference database (NCBI NT Genbank database downloaded in March 2022, and an indigenous database), and the similarity threshold was set at 85%. Three taxonomic  $\alpha$  diversity indices (i.e., Shannon–Weiner Index, Simpson Index and Margalef Index) were calculated using the “alpha\_diversity.py” script in the QIIME toolkit.

## 2.5. Bioassessment of the River's Ecological Status

### 1. Calculation of the comprehensive water quality index (WQI)

The single factor water quality index  $P_i$  of each main pollution index was calculated according to the monitoring water quality parameters, which was composed of whole digits and one decimal number, represented as:

$$P'_i = X_1.X_2 \quad (1)$$

Among them,  $X_1$  and  $X_2$  were calculated,  $X_1$  is the overall water quality category of the river;  $X_2$  is the position of the monitoring data in the water quality variation range of class  $X_1$ , and the value was determined according to the principle of rounding. Then we calculated the comprehensive water quality identification index WQI. The calculation formula was given in Equation (2).

$$WQI = \frac{1}{m} \sum (P'_1 + P'_2 + \dots + P'_m) \quad (2)$$

where,  $m$  is the number of the single water quality index for the comprehensive water quality evaluation;  $P'_1, P'_2, P'_m$  is the single factor water quality index for the 1st and 2nd...  $m^{\text{th}}$  water quality index, respectively. The water quality evaluation grade, based on the comprehensive water quality identification index method was shown in Supplementary Materials Table S1.

## 2. Biotic Pollution Index

The biotic pollution index (BPI) was used to evaluate the water quality and characterize the proportion of the pollution-resistant and sensitive species. The BPI index values increased as the water quality conditions deteriorated, the calculation formula was shown in Equation (3).

$$BPI = \frac{\log(N_1 + 2)}{[\log(N_2 + 2) + \log(N_3 + 2)]} \quad (3)$$

where  $N_1$  is the number of individuals of Oligochaeta, Hirudinea and Chironomidae larvae,  $N_2$  is the number of Polychaeta, Malacostraca and other aquatic insects, except Chironomidae larvae, and  $N_3$  is the number of individuals of mollusks. In this study, the sequence number of OTUs corresponding to each taxon was used to represent the number of individuals to calculate the biological pollution index of each site, and the criteria for evaluating the water quality were shown in Supplementary Materials Table S2.

## 3. Biotic Indices

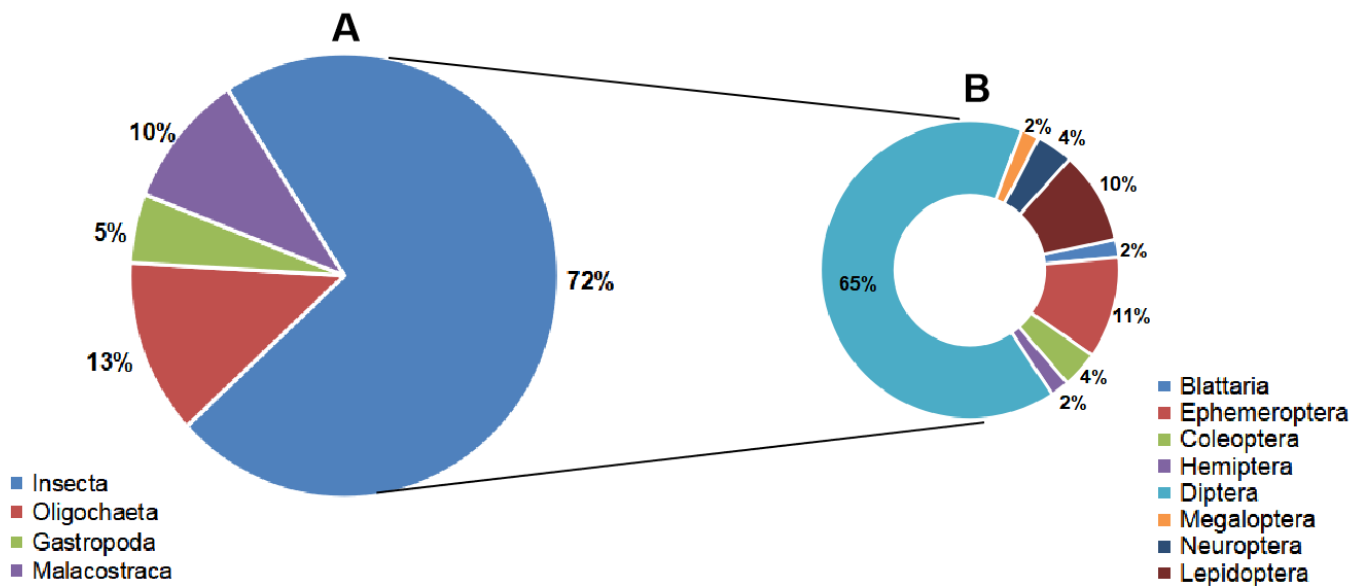
The biotic indices (BIs) were originally proposed by Chutter [42] in 1972 and were mainly used to evaluate the water quality after organic pollution. Currently, it has no longer been limited to a single organic pollution factor, but could be used to evaluate the water quality degradation caused by various pollution factors (physical and chemical). The smaller BI values indicated a better ecological status, the calculation formula was shown in Equation (4).

$$BI = \sum_{i=1}^n n_i t_i / N \quad (4)$$

Among them,  $n_i$  is the number of individuals (genus or species level) of the  $i^{\text{th}}$  taxon, and  $N$  is the total number of individuals in the sample,  $t_i$  is the tolerance value. The tolerance value refers to the tolerance of living organisms to the environmental stress in time and space, reflecting the relative ability for live organisms to survive and reproduce under the interference of environmental stress [43]. The criteria for evaluating the water quality were shown in Supplementary Materials Table S3.

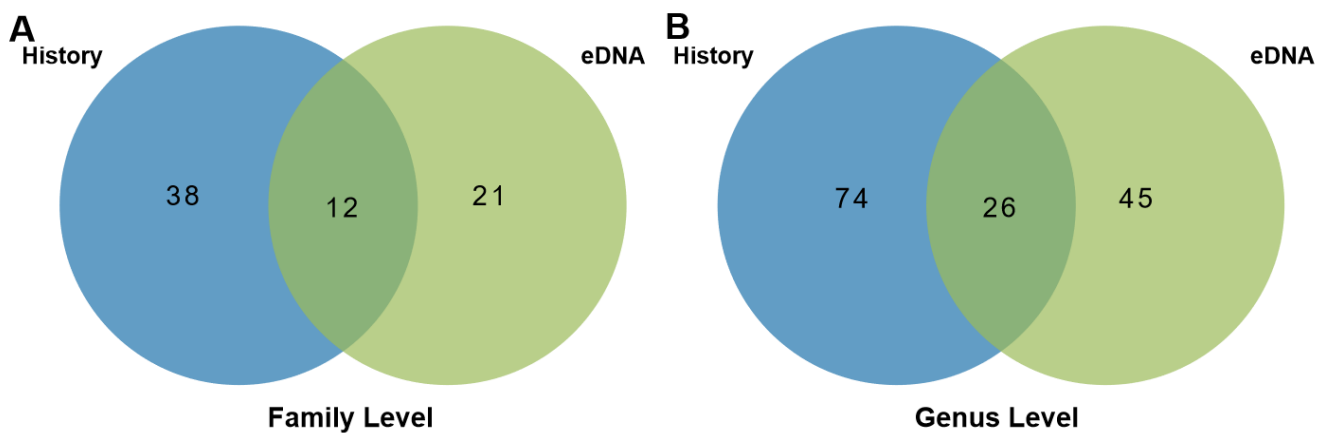
## 3. Results and Discussion

A total of 640 OTUs were detected by the eDNA technology in the Dongjiang River, which was annotated to three phyla, five classes, 13 orders, 33 families and 71 genera (Table S4). Specifically, there were 57 genera of Arthropoda (accounted for 81.2% of the total species), nine genera of Annelida and five genera of Mollusca. Arthropoda, Insecta and Diptera accounted for the largest proportion of macroinvertebrate species at the class and order level, respectively (Figure 1).



**Figure 1.** Community composition of macroinvertebrates at the class (A) and order (B) levels detected by the eDNA technology in the Dongjiang River.

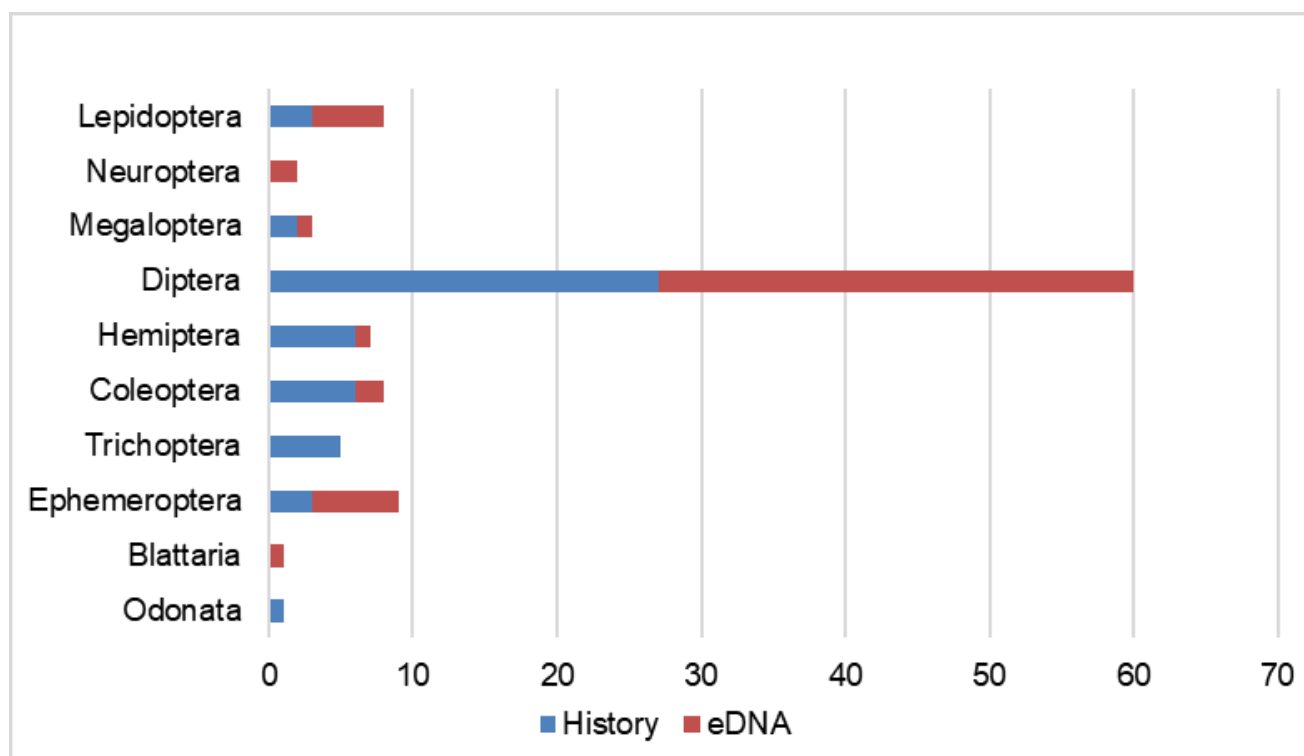
We found 12 coverages at the family level between the eDNA datasets and historical records, and 21 families did not coincide with the historical records. At the genus level, there were 26 coverages at the genera level, and there were also 45 genera that were not covered in the historical records (Figure 2). Although the number of taxa detected by the eDNA technology was less than the historical records, this may be because the historical morphology-based investigation was conducted in four quarters. In this study, we only had one time or a snapshot of eDNA monitoring datasets, but it still showed the advantages of the new technology in biomonitoring, over traditional methods [44,45]. In general, habitat and hydrological changes in different seasons had a huge impact on the structure of aquatic communities. For example, Cai et al. [46] found that the community structure and spatial pattern of macroinvertebrates were strongly correlated with the nutrient status, wind-induced disturbances and habitat complexities. Weerakoon et al. [47] showed that seasonal water-level fluctuations and changes in tropical reservoirs had significant effects on the structure of macroinvertebrates’ communities.



**Figure 2.** Paired comparison of the eDNA monitoring datasets and the historical records at the family (A) and genus (B) levels in the Dongjiang River.

We found that among the taxa detected by the eDNA technology across different orders, there were obvious differences from the historical records (Figure 3). Specifically,

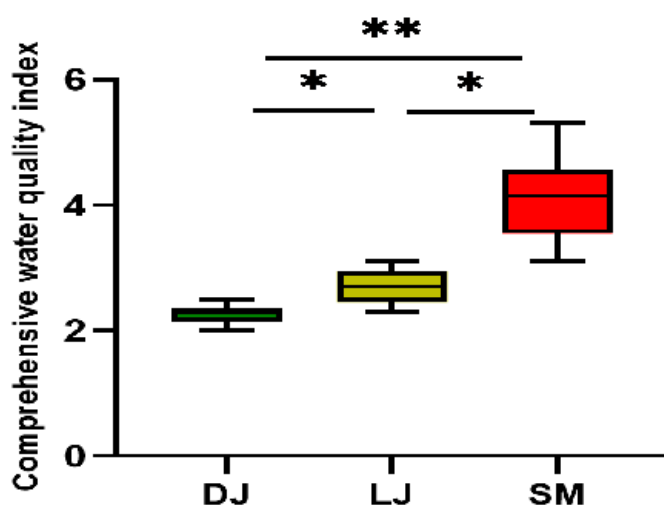
the number of Diptera, Neuroptera and Lepidoptera detected by the eDNA technology was higher than in the historical records, which may be because the eDNA technology performs a species annotation at the molecular level and could monitor some species that were not easily detected using conventional techniques [48]. However, it was also slightly insufficient in the monitoring of other macroinvertebrates, such as Megaloptera, Hemiptera and Trichoptera. This may be due to a primer bias [49], for example, to amplify enough species, the universal primer was required to have a high degeneracy [50], it may lead to great differences in the amplification ability for different species, and the effects were obvious to amplify the mixed DNA of multiple species. The DNA of some species could not or was difficult to be amplified, which led to the subsequent high-throughput sequencing and bioinformatics analysis failure to annotate some species. Therefore, some studies suggested that different primers should be used for different species monitoring [51]. Apart from that, despite the scientific maturity of the eDNA techniques, several factors affect the eDNA extraction and results, such as the source of eDNA, the shedding rate, the degradation rate, the translocation, and sedimentation [52].



**Figure 3.** Differences between the historical records and the eDNA approach of different orders.

Based on the comprehensive water quality index, the upstream water quality of Dongjiang River was better than that in the mid- and downstream, and mainly belonged to Levels I~II, among which, site DJ05 was Level I, basically meeting the requirements of the source water and surface water source first-class protection zone; the water quality of the midstream was mostly Levels II~III, among which, site LJ02 was Level III; there were three Level IV sites and two Level III sites in the downstream, among which SM01 had the highest comprehensive water quality index (i.e., 5.3), belonging to Level V (Figure 4).



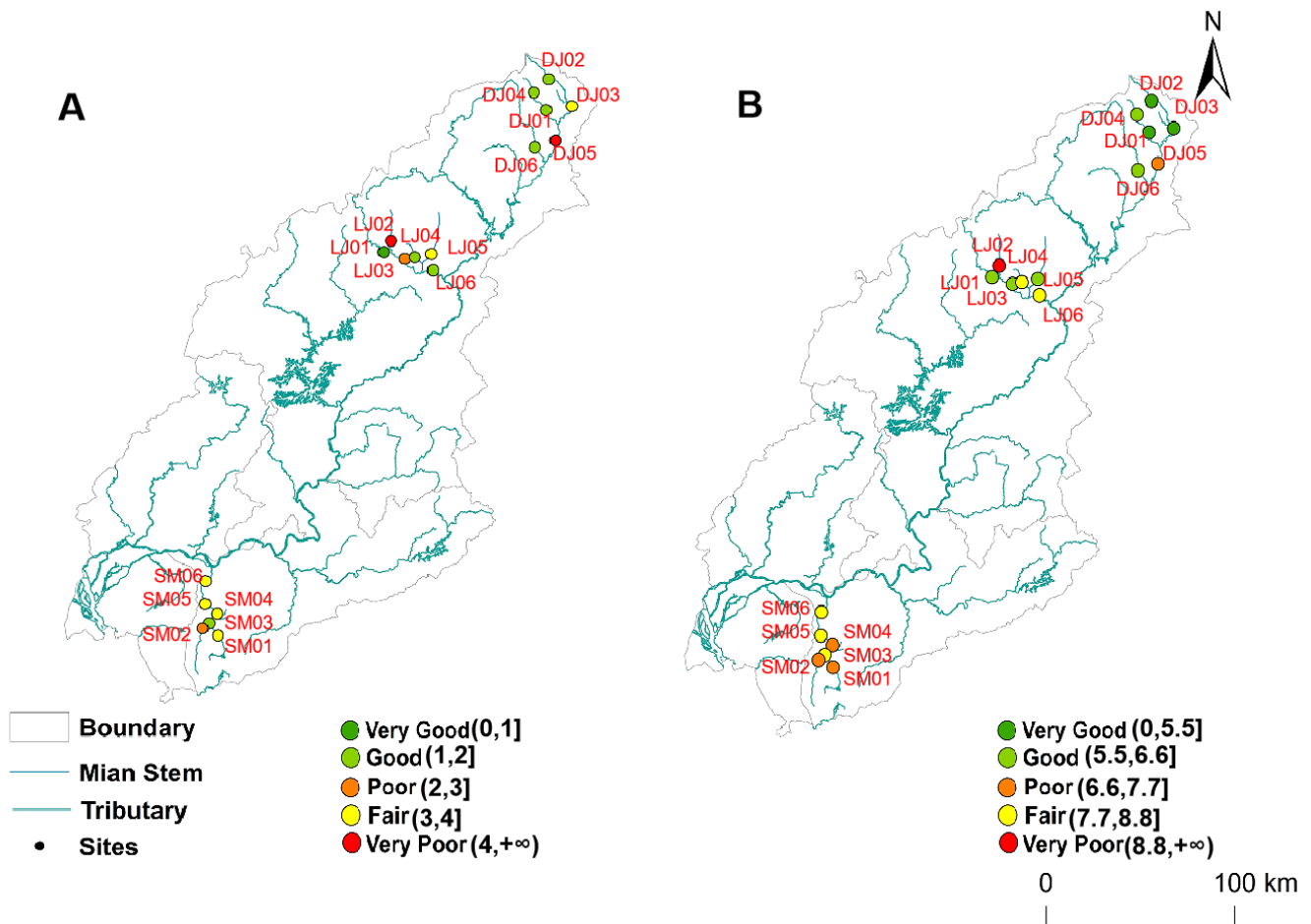


Site	Comprehensive water quality index	Water quality assessment grade	Site	Comprehensive water quality index	Water quality assessment grade
DJ01	2.3	II	LJ04	2.5	II
DJ02	2.3	II	LJ05	2.3	II
DJ03	2.2	II	LJ06	2.7	II
DJ04	2.3	II	SM01	5.3	V
DJ05	2	I	SM02	3.7	III
DJ06	2.5	II	SM03	4.3	IV
LJ01	2.9	II	SM04	3.1	III
LJ02	3.1	III	SM05	4.2	IV
LJ03	2.7	II	SM06	4.1	IV

**Figure 4.** Results of the water quality evaluation, based on the comprehensive water quality index in Dongjiang River. “\*” indicates significant at the 0.05 level “\*\*\*” indicates significant at the 0.01 level.

The eDNA-based biotic indices showed almost the same findings as the comprehensive water quality index (Figure 5). For example, the eDNA-based Shannon–Wiener index showed that the ecological status of Dongjiang River was between clean and slightly polluted, as a whole. The ecological status of the upstream area was better than that of the midstream and downstream. The clean sites were concentrated in the upstream reaches, while there was only one clean site in the downstream, and other sites in the downstream were lightly or moderately polluted. The results of BI showed the three cleanest sites, namely DJ01, DJ02 and DJ03, and the five clean sites were mainly concentrated in the mid and upper reaches. Site LJ02 had the highest BI value, which was consistent with the Shannon–Wiener index, both of which were heavily polluted. The ecological status in the downstream was mainly light pollution and medium pollution, with three light pollution sites and three medium pollution sites. The average BPI was the highest in the downstream (i.e., 0.72), followed by the upstream zone (i.e., 0.65) and the midstream (i.e., 0.61). Based on the results of the BPI, the ecological status of Dongjiang River was  $\beta$ -medium pollution.

This was mainly due to our data showing that the monitored sites were dominated by Oligochaeta, Chironomidae and other pollution-resistant species.



**Figure 5.** Classification of the ecological status, assessed by the eDNA-based Shannon–Weiner index (A) and the biotic indices (B) in Dongjiang River.

Although the eDNA-based biological index (e.g., the Shannon–Wiener index and BI) was consistent with the traditional WQI in the overall results, there were also slight differences in a few sites. For example, site DJ05 was moderately or heavily polluted, based on the Shannon–Wiener index or BI, while the evaluation results of the comprehensive WQI were Level I. These results would be attributed to the fact that Dongjiang River was an important rare earth mining area [53], the water upstream of site DJ05 may be polluted by heavy metals and other pollutants, the species and diversity of macroinvertebrates could be reduced, due to the heavy metals or salinity loads [53–56]. In addition, the Shannon–Wiener index of site SM01 showed that it was lightly polluted, but its comprehensive WQI was Level V, and the concentrations of ammonia nitrogen, total nitrogen and total phosphorus all reached above the fifth grade. These results may be because the Shannon–Wiener index was only focused on the level of the species diversity [57], the diversity and uniform distribution of the tolerant species may also lead to the discrepancy between the Shannon–Wiener index and the actual water quality [58]. The total nitrogen and total phosphorus of site SM01 were selected as candidate parameters to calculate the comprehensive trophic state index, and the result was 91.4, which was in a severe eutrophication state. The result of the BI showed that it was moderately polluted and that it was closer to the actual water quality. Currently, the biological index has been adopted by countries around the world for the ecological status assessment, such as In North America, where multiple biological indices were used to comprehensively assess the ecological status, as early as the early



1990s [59]. Although the physical and chemical parameters of the water quality could reflect the concentration of pollutants, the traditional water quality only paid attention to a few common variables. As we know, the river flow velocity, climate, bed sediment composition and other physicochemical variables beyond the monitoring range should also affect the community structure of macroinvertebrates, which lead to the difference between the biological index and the traditional physicochemical evaluation [60].

In general, eDNA technology is revolutionizing the era of biomonitoring, especially for developing countries with a backward monitoring technology. New technologies provide high-throughput datasets, which are crucial for the protection and management of subtropical rivers with fewer study efforts. It is believed that this study was only the first attempt at a new technology in a developing country's subtropical river, and some surprising findings were uncovered, such as the only one-time eDNA datasets that could provide the same scale as the historical records, and the results the eDNA -based biological index, were generally consistent with the traditional WQI. However, many efforts still need to be made before new technologies can be popularized, such as the ecological process of eDNA in tropical rivers and the primer bias. It is mainly because of the differences in a natural context, such as the water temperature and sunlight. Biodiversity and eDNA itself are obviously different in tropical or subtropical rivers and in cold or temperate rivers.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/w15020308/s1>, Table S1. Classification of water quality based on the comprehensive water quality index (WQI), Table S2. Classification of ecological status based on the Biotic Pollution Index (BPI), Table S3. Classification of ecological status based on the Biotic Index (BI), Table S4. Comparison of species composition of macroinvertebrates between historical records and the eDNA approach in the Dongjiang river.

**Author Contributions:** F.W. and F.L. designed the study and wrote the first draft of the manuscript, F.L. and Y.Z. (Yuan Zhang) provided the funding, Y.Z. (Yanting Zou), F.W. and S.Q. collected the data and conducted the statistical analyses, and Y.Z. (Yuan Zhang) contributed to the discussion and revisions. All authors have read and agreed to the published version of the manuscript.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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