



## Original Research

## A bacteria-based index of biotic integrity indicates aquatic ecosystem restoration

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## ABSTRACT

Intensive ecological interventions have been carried out in highly polluted shallow lakes to improve their environments and restore their ecosystems. However, certain treatments, such as dredging polluted sediment and stocking fish, can impact the aquatic communities, including benthos and fishes. These impacts can alter the composition and characteristics of aquatic communities, which makes community-based ecological assessments challenging. Here we develop a bacteria-based index of biotic integrity (IBI) that can clearly indicate the restoration of aquatic ecosystems with minimal artificial interventions. We applied this method to a restored shallow lake during 3-year intensive ecological interventions. The interventions reduced nutrients and heavy metals by 27.1% and 16.7% in the sediment, while the total organic carbon (TOC) increased by 8.0% due to the proliferation of macrophytes. Additionally, the abundance of sulfur-related metabolic pathways decreased by 10.5% as the responses to improved ecosystem. The score of bacteria-based IBI, which is calculated based on the diversity, composition, and function of benthic bacterial communities, increased from 0.62 in 2018 to 0.81 in 2021. Our study not only provides an applicable method for aquatic ecological assessment under intensive artificial interventions but also extends the application of IBI to complex application scenarios, such as ecosystems with significantly different aquatic communities and comparisons between different basins.

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## 1. Introduction

Shallow lakes are irreplaceable in maintaining water supply, flood control, drought resistance, pollution alleviation, and biodiversity preservation. Shallow lake ecosystems, composed of various communities, such as plankton, macrophytes, fish, and microorganisms, play an important role in improving water quality, providing habitats, and offering human food and economic benefits. However, the impacts of climate change and increasing human activities, including water scarcity, area loss, overexploitation, pollution, and ecological degradation, have significantly threatened lakes [1,2]. For decades, various measures, such as increasing irrigation efficiency to reduce lake water utilization [3] and planting macrophytes [4], have been implemented worldwide to restore lake ecosystems. Recently, in response to the Chinese government's

emphasis on environmental protection and ecological restoration, intensive ecological interventions have been implemented in many heavily polluted aquatic ecosystems in China. Interventions such as water replenishment, stringent regulation of industrial, agricultural, and domestic waste disposal, restriction of aquaculture activity, polluted sediment dredging, macrophyte planting, reed harvesting, fishing prohibition, and fish stocking have been used to effectively maintain water levels and areas, reduce internal and external pollution, and facilitate the restoration of aquatic ecology. These interventions have successfully improved water quality and aquatic ecosystems. For example, the proportion of lake and reservoir drinking water sources has increased from 33% to 40% since 2018 because of improved water quality [5]. However, in addition to environmental variations, some ecological interventions, such as polluted sediment dredging, large-scale macrophyte planting, and fish stocking, also directly change the diversity and biomass of corresponding communities [6,7]. These effects lead to fluctuations in associated communities, which may inadequately reflect variations in an aquatic environment and its ecological restoration. However, microorganisms, which are widely

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present in many environments, are generally not selected as target organisms for ecological restoration. Microorganisms, such as bacteria, are characterized by their adaptability and sensitivity to environmental variations. These characteristics are based on their rapid reproduction and transportation by water flow [8]. Therefore, bacterial communities are considered potential precise indicators of ecological improvement in lakes.

Sediment is an essential component of shallow lake ecosystems. The sediment–water interface not only physically affects material transport, adsorption, and desorption [9,10] but also influences the migration and transformation of materials such as carbon and nitrogen, which play important roles in the cycling of material between water and sediment [11,12]. Sediment provides habitats as well as organic and inorganic nutrients for benthic bacterial communities. Therefore, the nutrients and pollutants in sediment shape benthic bacterial communities [13]. The activities of benthic bacterial communities, such as nitrification and denitrification, sulfur oxidation and reduction, and the degradation of organic phosphorus pesticides, are important for aquatic ecosystems because they participate in processes such as nutrient cycling, organic matter transformation, pollutant degradation, and self-purification. Moreover, microorganisms in lake sediment are sensitive to environmental variations [14].

Spatial and temporal variations in benthic microorganisms have been extensively investigated in aquatic ecosystems [15–19]. Benthic microbial communities are strongly influenced by variations in pH [20], ambient temperature [21], nutrients [22], and heavy metals [23]. Over the past two centuries, anthropogenic disturbance has significantly altered the biodiversity and biogeochemical circulation of lake ecosystems. The abundance, diversity, and predominance of bacterial groups, as well as functional groups of benthic microbial communities, have been severely impacted by anthropogenic disturbances, such as external nutrients and pollutants [16,24]. Compared with other components of aquatic ecosystems, benthic bacterial communities are directly linked to biogeochemical processes and are characterized by high diversity and high sensitivity to environmental variations [25]. Therefore, benthic bacterial communities are considered potential indicators of ecological health in freshwater systems.

Although obtaining accurate information from bacterial communities is still challenging, it has been facilitated by the advent of modern sequencing technology, which has reduced the price of sequencing and improved the detection and classification of microbial taxa. In addition, many errors in morphological identification due to a lack of experience can be avoided. High-throughput sequencing has been applied in numerous studies of bacterial communities in shallow lakes in China. For example, Zhang et al. [26] reported a correlation between microbial communities and 2-methylisoborneol occurrence in lake landscapes in Beijing. Wang et al. [27] found differences in bacterial community composition and functional gene structure between macrophyte- and phytoplankton-dominated regimes in Taihu Lake. Data obtained through sequencing can be further mined using various methods. In practical applications of ecological assessment, the index of biological integrity (IBI) is an intuitive method that screens various indices in a particular community and constructs an evaluation system based on the collected data. Because the IBI provides a direct, comprehensive view of ecological restoration, it is a useful tool for assessing aquatic ecological health. This assessment is directly based on the variations in a particular population in aquatic ecosystems rather than on the impacts of measured and unmeasured environmental factors. Fish [28], macroinvertebrates [29], and plankton [30] are general indicators of IBI in the assessment of aquatic ecological health. However, artificial interventions, such as

the definition of reference areas and the selection of candidate matrices, are often included in traditional biological integrity assessments. The definition of reference areas is usually based on the comparison of environmental parameters to those of impaired areas, which is feasible in both theory and practice [31–33]. Because of the spatiotemporal specificity of ecosystems, each assessment must define its specific reference area. A reference area is usually defined by local water quality or anthropogenic intensity. However, the measurement of limited indicators may not adequately represent comprehensive environmental conditions. For example, heavy metal content has rarely been included in the definition of a reference area [34]. Furthermore, in ecological assessments based on different communities, candidate matrices used in the IBI have been artificially selected, weighted, and scored [29,30]. Thus, the lack of unified standards has limited the accuracy and universal application of the IBI across different regions. A biological integrity index developed based on the intrinsic properties (i.e., composition, diversity, and function) of benthic microbial communities have the potential to reduce the need for artificial interventions and be extended to other freshwater environments for the aquatic ecosystem assessment due to the strong adaptability and high sensitivity to environmental variations.

Our study aimed to determine variations in benthic bacterial communities as a potential indicator of lake ecological improvement under intensive ecological intervention. Baiyangdian Lake is a shallow macrophyte-dominated lake in northern China that has been heavily polluted by wastewater discharge from catchments and surrounding residential areas [35]. Cadmium accumulates in sediment, which causes moderate to high ecological risks [36,37]. Accumulated nitrogen and phosphorus are released at the water–sediment interface [38,39], and excessive nitrite and phosphorus concentrations limit the biomass of submerged macrophytes [40]. The ammonium nitrogen and organic matter in sediment significantly influence benthic macroinvertebrate communities [35]. Since 1997, environmental flows have been created to maintain the aquatic ecological health of Baiyangdian Lake by releasing water from upstream reservoirs [41]. Constant water supplies have maintained water levels, improved water quality [41], and increased vegetation coverage [42].

In April 2017, the construction of the Xiong'an New Area (including Baiyangdian Lake) was announced. In 2018, strict regulations and treatments were implemented. The waste discharge in the entire catchment was regulated, especially from the Fu River, which flows through Baoding City and carries agricultural, industrial, and residential waste into Baiyangdian Lake. According to the ecological water replenishment plan, clean water from the Baigouyin and Xiaoyi Rivers was released into the lake. Domestic aquacultural farms were also strictly limited, including the shutdown of fish farms, duck farms, and lotus farms to reduce pollution from fertilizers, feed, and feces. After the amount of nutrient and pollution input was limited, the heavily polluted sediment in the lake was dredged to reduce nutrient release and pollution from the sediment. Furthermore, large-scale macrophyte planting and fish stocking were conducted to restore the damaged ecosystem. These measures were expected to control and improve the aquatic ecosystems of Baiyangdian Lake, but their restorative effects have not been reported. Therefore, Baiyangdian Lake was considered a promising area for this study.

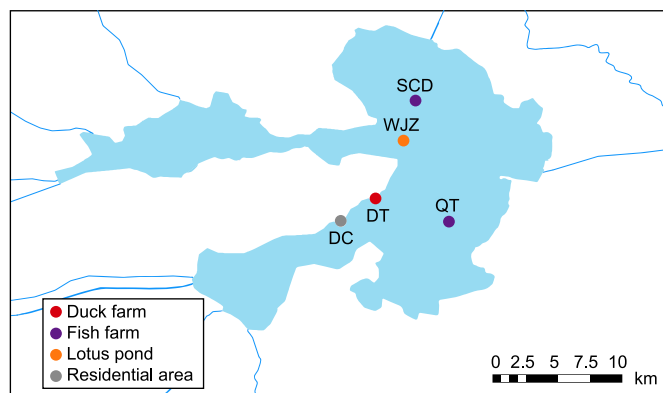
The objectives of this study are as follows: (1) to detect variations in aquatic environmental factors (e.g., heavy metals, total nitrogen, total phosphorus, and total organic carbon); (2) to characterize the composition, distribution, variation, and function of benthic bacterial communities; (3) to develop a novel index of biological integrity (nBa-IBI) based on the intrinsic properties of

benthic bacterial communities that minimize artificial intervention; and (4) to evaluate its effectiveness in ecological restoration assessment by comparing it with the traditional bacteria-based index of biotic integrity (tBa-IBI). This study provides new measures for lake ecosystem assessment that extend universality and improve the understanding of the bacterial response to intensive ecological interventions in a typical shallow lake ecosystem. Furthermore, this study provides a method for developing an ecosystem monitoring network that contributes to water governance in the Beijing–Tianjin–Hebei urban agglomeration in northern China [43].

## 2. Materials and methods

### 2.1. Study area and sampling

Baiyangdian Lake is located in the lower reaches of the Daqing River system of the Hai River Basin (38°43′–39°02′ N, 115°45′–116°07′ E, Fig. 1). It is the largest shallow lake in the North China Plain, with an approximate area of 366 km<sup>2</sup> at its average water level [44]. Baiyangdian Lake has a typical temperate monsoon climate with an annual mean temperature of 12.2 °C and an annual mean precipitation of 442.5 mm, 70–80% of which occurs between June and August. In this study, sampling was designed and conducted in July 2018 (nine sampling sites) and August 2020 (11 sampling sites). Additional sampling was conducted in August 2021 (ten sampling sites) to provide sufficient data. Sediment samples were collected from four distinct habitat types characterized by different degrees of anthropogenic disturbance (i.e., a lotus pond, fish farm, duck farm, and residential area). However, because of local governmental constraints, only five comparable sampling sites were permissible after 2018, which are DC (Duancun), DT (Diantou), QT (Quantou), SCD (Shaochedian), and WJZ (Wangjiazhai). The sediment temperature was measured directly using an EXO2 Multiparameter Sonde (YSI, Inc., USA). A grab sampler was used to collect approximately 500 g of sediment from the top layer, which was stored in sterilized plastic bags before being promptly transported to the laboratory in an ice box with ice. The collected sediment samples were divided into two parts for different purposes. The sediment samples for high-throughput sequencing were stored at –20 °C. The sediment samples for the analysis of environmental parameters were air-dried and sieved to remove stones and root fragments.



**Fig. 1.** Sampling sites and main water flow direction of the Baiyangdian Lake. Sampling sites were named according to their local names (DC: Duancun; DT: Diantou; QT: Quantou; SCD: Shaochedian; WJZ: Wangjiazhai).

### 2.2. Determination of environmental parameters

The sediment samples for the analysis of environmental parameters were divided into three parts: (1) 10 g of sediment was mixed with ultrapure water (1:2.5) to make a slurry for pH measurement using a pH meter (ST3100/F, USA); (2) 5 mm of HCl (1 mol L<sup>-1</sup>) was added to 2 g of sediment and heat-dried to remove inorganic carbon for the measurement of total organic carbon (TOC) and total nitrogen (TN) by an elemental analyzer (VARIO EL, Germany); (3) 1 g of sediment was digested with nitric acid–hydrofluoric acid–hydrochloric acid (HNO<sub>3</sub>–HF–HCl) for the measurement of total phosphorus (TP) and heavy metals (As, Cd, Cr, Cu, Ni, Pb, and Zn). The samples collected in 2018 were measured using inductively coupled plasma atomic emission spectrometry (Spectro Arcos EOP, USA). The samples collected in 2020 and 2021 were measured using an inductively coupled plasma mass spectrometer (Thermo Fisher Scientific, USA). An ANOVA (Analysis of Variance) was performed to determine the statistically significant differences between the study years and sites.

### 2.3. DNA extraction and Illumina MiSeq amplicon sequencing

Total genomic DNA was isolated from 0.5 g sediment samples stored at –20 °C for high-throughput sequencing using an E.Z.N.A.® Soil DNA Kit (Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer's instructions. The extracted genomic DNA was assessed on 1% agarose gel, and the DNA concentration and purity were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA).

The V3–V4 hypervariable region of the bacterial 16 S rRNA gene was amplified using the primer pairs 338 F (5'-ACTCCTACGG-GAGGCAGCAG-3') and 806 R (5'-GGACTACHVGGGTWCTAAT-3') from the extracted total genomic DNA. The PCR mixture consisted of 4 µL of 5 × TransStart FastPfu buffer, 2 µL of 2.5 mM dNTPs, 0.8 µL of forward primer (5 µM), 0.8 µL of reverse primer (5 µM), 0.4 µL of TransStart FastPfu DNA Polymerase, and 10 ng of template DNA. PCR (polymerase chain reaction) was performed in triplicate on an ABI GeneAmp® 9700 PCR thermocycler (ABI, CA, USA) according to the following protocol: 3 min at 95 °C; 27 cycles of 30 s at 95 °C; 30 s at 55 °C; and 45 s at 72 °C; and a final extension step of 10 min at 72 °C. The PCR products were separated on 2% agarose gel, purified using an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA), and quantified using a Quantus™ Fluorometer (Promega, USA). The purified PCR products were pooled in equimolar amounts and sent for sequencing.

### 2.4. Bioinformatics and statistical analysis

The frozen sediment samples were subsequently sent to Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China) for sequencing. According to standard protocols, sequencing was performed on an Illumina MiSeq PE300 platform (Illumina, San Diego, CA, USA). The raw 16 S rRNA gene sequencing reads were demultiplexed, quality-filtered using FastP (version 0.20.0) [45], and merged using FLASH (version 1.2.7) [46]. Operational taxonomic units (OTUs) with 97% similarity were clustered using UPARSE (version 7.1) [47] with the identification and removal of chimeric sequences. The taxonomy of each OTU representative sequence was analyzed using the RDP Classifier (version 2.2) [48] and identified in the Silva database (version 138) at a confidence threshold of 0.7. Singletons were removed at the end of the analysis pipeline.

The data obtained from the triplicates were merged for downstream analysis. The alpha diversity and evenness of each sample were assessed by calculating the Chao1 index, Shannon–Wiener index, and Shannon's evenness index, which were calculated at

the OTU level using the *phyloseq* package in R [49]. An ANOVA was performed to determine statistically significant differences in environmentally sensitive bacterial indicators between the study periods and the study sites. A PERMANOVA (permutational multivariate analysis of variance) was performed with the Bray–Curtis Similarity Index to determine statistically significant differences in the bacterial communities among the study periods and sites. Beta diversity was determined by principal coordinate analysis (PCoA) based on the Bray–Curtis Similarity Index calculation. Because the accuracy of the 95% confidence ellipses in the PCoA results increased as the data sets were added, unmerged triplicates were used in the PCoA. An ANOVA, PERMANOVA, and PCoA were performed using PAST3 [50]. The functions of the bacterial communities were predicted using PICRUSt2 in R against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database [51]. A redundancy analysis (RDA) rather than a canonical correspondence analysis (CCA) was conducted to investigate the relationships between the benthic bacterial communities and the measured environmental factors because the gradient length was 2.1 according to the detrended correspondence analysis. The RDA was calculated using CANOCO 5 [52]. The sequencing data were submitted to NCBI under BioProject ID: PRJNA850031.

### 2.5. Development of *nBa-IBI* and *tBa-IBI*

The experimental results described in sections 2.1 to 2.4 indicated that after ecological restoration, the variations in intrinsic properties of benthic bacterial communities had (1) increased diversity, (2) decreased relative abundance of bacterial taxa related to nutrients (especially nitrogen and sulfur), and (3) decreased predicted functions associated with nutrients. Therefore, in this study, the *Ba-IBI* was developed based on candidate metrics that consisted of three categories: diversity metrics, composition metrics, and function metrics that included the variations in intrinsic attributes of microbial communities (Table S1). The diversity metrics included alpha diversity indices (i.e., the Chao1 index, the Shannon–Wiener index, and the Shannon's evenness index). The indices used in the composition metrics were the relative abundances of bacteria involved in sulfur and nitrogen cycle-related metabolism. The functional metrics included indices of the predicted metabolic pathways related to sulfur and nitrogen cycles.

The calculation of *tBa-IBI* followed the general protocols established by Tan et al. [53] with modifications in selecting candidate metrics. According to the ecological restoration progress in Baiyangdian Lake and the measured environmental data, the samples taken in 2021 had lower nutrients and lower heavy metal contents than those taken in 2018 and 2020. Therefore, the samples taken in 2021 were considered less disturbed and were defined as a reference. In addition to the three categories described above (Table S1), the dominance metrics included indices of the relative abundance of each dominant phyla, classes, orders, families, and genera within the top 10 phyla and the top 12 genera. The representative indices with significant discrimination were screened using a Mann–Whitney *U* test ( $p < 0.05$ ) and box-plot tests. The discrimination power of the indices was examined by their degrees of interquartile (IQ) overlap in the box-plot tests. The box plots were based on the values between the reference sites and impaired sites for each index, and the 25% quartile (Q1), median values (Q2), and 75% quartile (Q3) of each “box” were compared. The indices were considered to have significant discrimination if the Q2 of each “box” was not between Q1 and Q3 of the other “box”. Spearman's correlation analysis was conducted to identify redundant indices. Indices with a significant correlation ( $r > 0.75$ ,  $p < 0.01$ ) were considered redundant. One of the redundant indices was selected as a representative based on applicability. Mann–Whitney *U* tests

were calculated using PAST3.

The 5th and 95th percentile scaling system was applied to calculate the score of each metric [54]. In metrics that decreased with restoration, the lower threshold was the 5th percentile of metric values and the score = (Maximum – Site value)/(Maximum – Lower threshold). For metrics that increased with restoration, the upper threshold was the 95th percentile of metric values, and the score = Site value/Upper threshold. The *nBa-IBI* and *tBa-IBI* were indicated by the median scores of each index.

## 3. Results

### 3.1. Characterization of environmental parameters

The environmental parameters of the lake sediment are shown in Fig. 2. In general, compared with those taken in 2018 and 2020, the samples taken in 2021 had lower nutrient and heavy metal contents, indicating an improvement in the environment. The TN and TP concentrations ranged between 1.57 and 0.18 g kg<sup>-1</sup> and between 6.15 and 0.58 g kg<sup>-1</sup>, respectively. TN and TP decreased from 2018 to 2021, except for TN at site SCD, which increased from 2.86 to 5.97 g kg<sup>-1</sup> and had the highest TN values in 2021. The highest TN value was 6.15 g kg<sup>-1</sup> in 2018 at site WJZ, which decreased to 1.65 g kg<sup>-1</sup> in 2021. The highest TP value was 1.57 g kg<sup>-1</sup> in 2018 at site DT, which decreased to 0.70 g kg<sup>-1</sup> in 2021. The TOC content ranged between 17.35 and 76.57 g kg<sup>-1</sup>. The TOC values at sites DC, QT, and SCD increased from 2018 to 2021, while the TOC values at sites DT and WJZ decreased from 2018 to 2021. The TN and TOC values exhibited similar trends at sites SCD and WJZ, which indicated that the TOC value at site SCD increased to the highest value (76.57 g kg<sup>-1</sup>) in 2021 and that the TOC value at site WJZ decreased from the highest value (60.31 g kg<sup>-1</sup>) in 2018.

The concentrations of heavy metals decreased from 2018 to 2021 (0.18–0.50 mg kg<sup>-1</sup> for Cd, 28.77–62.43 mg kg<sup>-1</sup> for Cr, 12.96–67.00 mg kg<sup>-1</sup> for Cu, 14.46–70.75 mg kg<sup>-1</sup> for Ni, 9.65–26.63 mg kg<sup>-1</sup> for Pb, and 58.18–390.07 mg kg<sup>-1</sup> for Zn), except for As. The concentration of As in the sediment ranged between 6.04 and 14.98 mg kg<sup>-1</sup> and increased from 2018 to 2021 at all sampling sites. There were several exceptions: Cr at site QT increased to 43.75 mg kg<sup>-1</sup> in 2021, and Pb at sites DC and DT increased to 29.28 and 18.24 mg kg<sup>-1</sup>, respectively, in 2021. The ANOVA results indicated that pH, TP, Cd, Cu, and Zn differed significantly among the study periods ( $p < 0.05$ ), and Pb differed significantly among the study sites ( $p < 0.05$ ; Table S2). Zn had significantly high concentrations in 2020, but no natural or artificial pollution events that could have increased the Zn concentration by two to four times in the sediment of Baiyangdian Lake have been reported. Furthermore, the results for the samples taken in 2018 and 2021 were comparable to those of other studies [36,55,56]. Therefore, the high Zn content in 2020 was considered an instrumental error.

### 3.2. Characterization of benthic bacterial communities

In total, 1,634,443 sequences were obtained after demultiplexing, merging, and excluding chimeric sequences. The read numbers ranged from 25,045 to 42,008, with a mean value of 34,128. A filtering process was conducted to merge the 16 S rRNA gene sequencing libraries from 2018, 2020, and 2021 into one gene library for downstream analysis and 18,647 reads were randomly filtered from each sample.

Fig. 3 shows the alpha diversity measured by the Chao1 index, the Shannon–Wiener index, and the Shannon's evenness index. In general, alpha diversity increased from 2018 to 2021: Chao1 index from 4365.72 to 5743.09 to 5181.22–6204.27; Shannon–Wiener



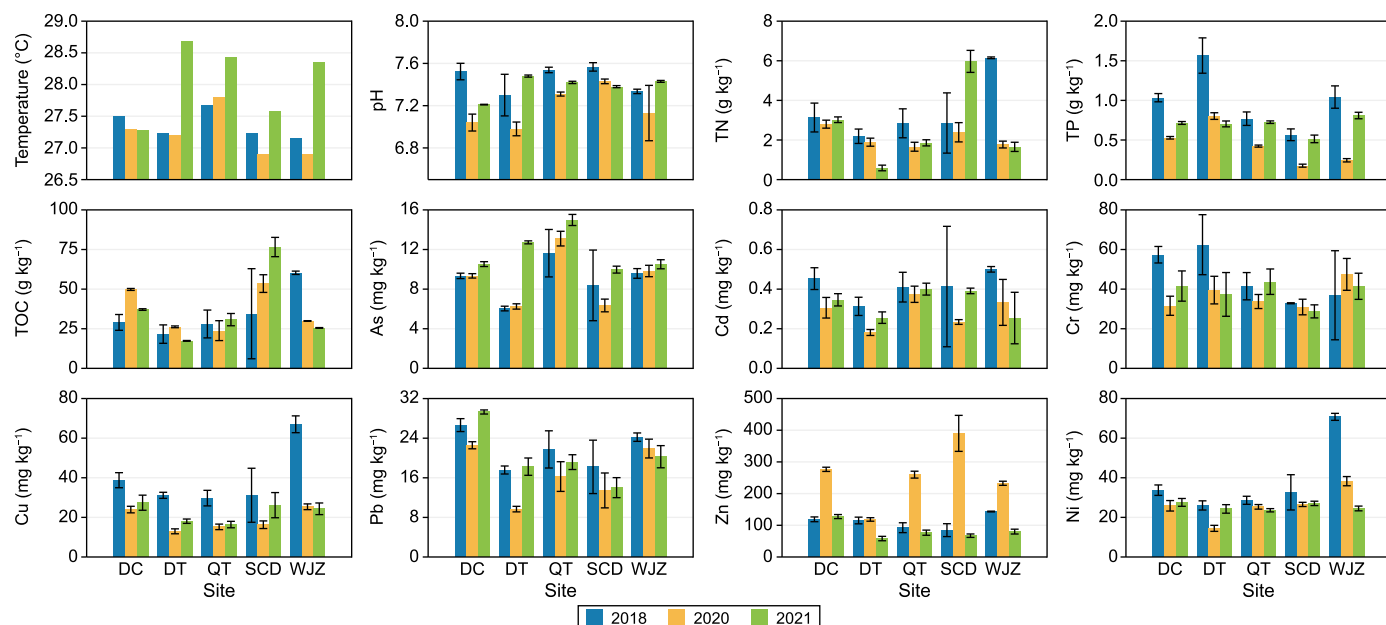


Fig. 2. Water-sediment interface temperature, pH, total nitrogen (TN), total phosphorus (TP), total organic carbon (TOC), and heavy metal contents (As, Cd, Cr, Cu, Pb, Zn, and Ni) at all the sampling sites. Parameters measured from samples collected in 2018, 2020, and 2021 are colored blue, yellow, and green, respectively.

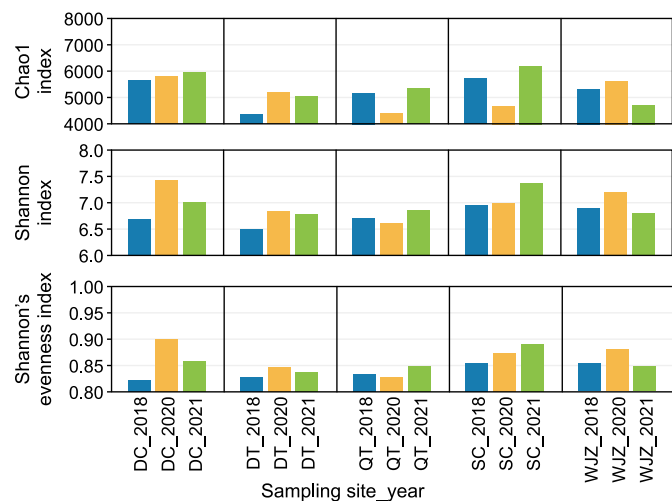


Fig. 3. Alpha diversity represented by the Chao1 index, Shannon–Wiener index, and Shannon’s evenness index at all sampling sites in 2018, 2020, and 2021.

index from 6.57 to 6.96 to 6.79–7.37; and Shannon’s evenness index from 0.82 to 0.85 to 0.84–0.89, except at site WJZ, where the Chao1 index, Shannon–Wiener index, and Shannon’s evenness index measurements decreased from 5338.99 to 4738.85, from 6.90 to 6.80, and from 0.853 to 0.847, respectively. The SCD site had the highest alpha diversity in 2018 (Chao1 index: 5743.09; Shannon–Wiener index: 6.96; Shannon’s evenness index: 0.85) and 2021 (Chao1 index: 6204.27; Shannon–Wiener index: 7.37; Shannon’s evenness index: 0.89). The DT site had the lowest alpha diversity (Chao1 index: 4365.72; Shannon–Wiener index: 6.57; Shannon’s evenness index: 0.83) in 2018. The alpha diversity at sites DC, DT, and WJZ increased from 2018 to 2020 and decreased until 2021. The highest alpha diversity in 2020 was observed at site DC (Chao1 index: 5805.61; Shannon–Wiener index: 7.44; Shannon’s evenness index: 0.90).

Fig. 4 shows the relative abundance of the dominant phyla

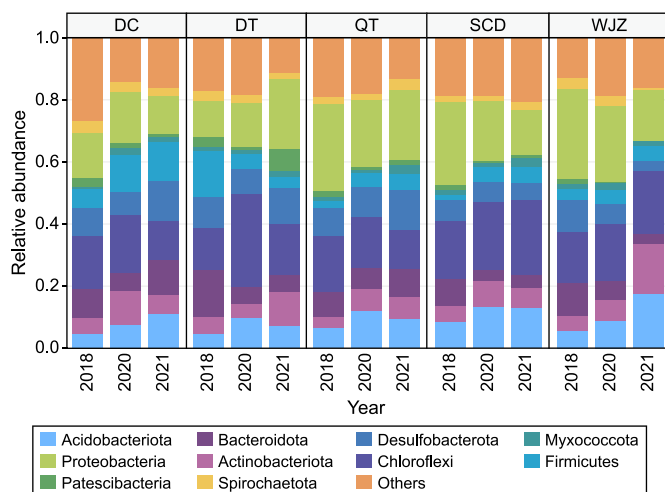
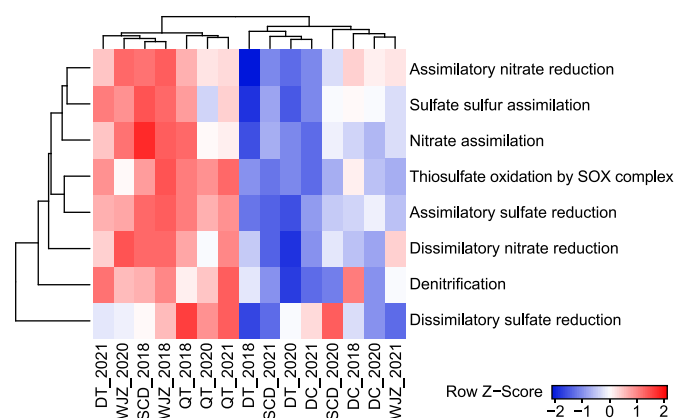


Fig. 4. Bacterial community structures at the phylum level (most abundant ten phyla) at all sampling sites (DC, DT, QT, SCD, and WJZ) from 2018 to 2021.

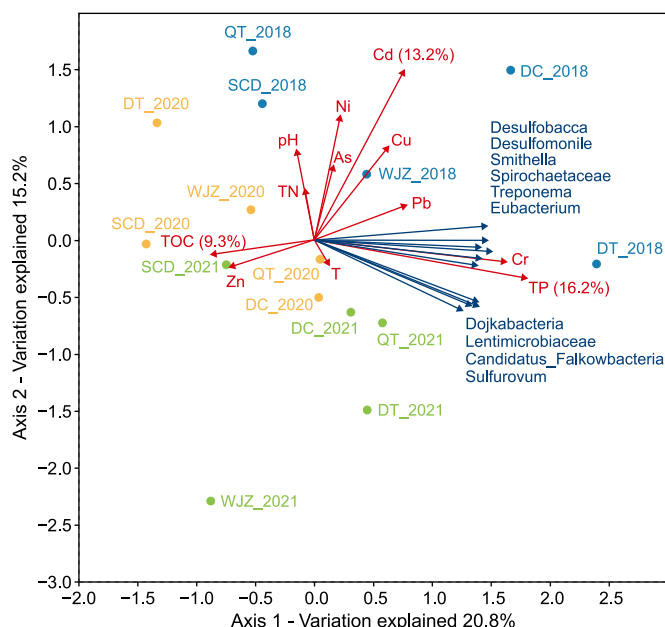
according to the percentage of total sequence reads. Proteobacteria (19.62%), Chloroflexi (18.29%), Acidobacteriota (9.33%), Desulfobacterota (8.63%), and Bacteroidota (7.49%) were the five most abundant phyla. A total of 63.4% of the total relative abundance consisted of these five phyla. The relative abundance of Proteobacteria decreased from 2018 to 2021 (DC: 14.50–12.02%; QT: 28.11–22.42%; SCD: 26.94–14.58%; WJZ: 29.01–16.42%), except at site DT (11.58–22.51%). Chloroflexi decreased at sites DC (17.17–12.46%) and QT (17.96–12.48%) and increased at sites DT (13.81–16.53%), SCD (18.68–24.11%), and WJZ (16.21–20.00%) from 2018 to 2021.

Fig. 5 shows the relative abundance of the dominant genera, with relative abundances higher than 1.5%. Unknown taxa in the family Anaerolineaceae (3.08%), unknown taxa in the order Aminicenantes (2.50%), unknown taxa in the order SBR1031 (2.44%), unknown taxa in the family Bacteroidetes vadinHA17 (2.12%), and





**Fig. 8.** Heatmap of sulfur-related metabolism and nitrogen-related metabolism predicted by PICRUSt2. The relatively high and low values are indicated by red and blue, respectively. The samples, and sulfur- and nitrogen-related metabolism data were organized by the hierarchical clustering method.



**Fig. 9.** RDA plots of the measured environmental factors. Samples from 2018, 2020, and 2021 are indicated by blue, yellow, and green dots, respectively. The measured environmental parameters are indicated by red arrows, and the parameters that had a significant influence ( $p < 0.05$ ) exhibit the percentages of explained variation. The top ten fittest bacterial genera are indicated by dark blue arrows.

2018 to 2021 (nBa-IBI: 0.62–0.81; tBa-IBI: 0.31–0.90), indicating the successful ecological restoration of Baiyangdian Lake. The differences between nBa-IBIs and tBa-IBIs were the indices applied in the calculation and the significant discrimination of the final scores. Regarding the calculation of tBa-IBI, the results of the Mann–Whitney  $U$  test and the box-plot test indicated that *Desulfatiglans*, *SEEP-SRB1*, *Sulfurimonas*, *Thioalkalispira-Sulfurivermis*, and *Sulfur\_oxidation\_groups* had significant discrimination (Table S1; Fig. S1). The Spearman's correlation test results indicated that *Sulfurimonas* and *SEEP-SRB1* were redundant indices (Table S4). Therefore, the *Desulfatiglans*, *Thioalkalispira-Sulfurivermis*, and sulfur oxidation groups were considered indices for calculating the Ba-IBI. The Mann–Whitney  $U$  test indicated that the tBa-IBI scores in 2018, 2020, and 2021 had significant discrimination ( $p < 0.05$ ). However, most indices in the calculation of the nBa-IBI and the nBa-IBI scores

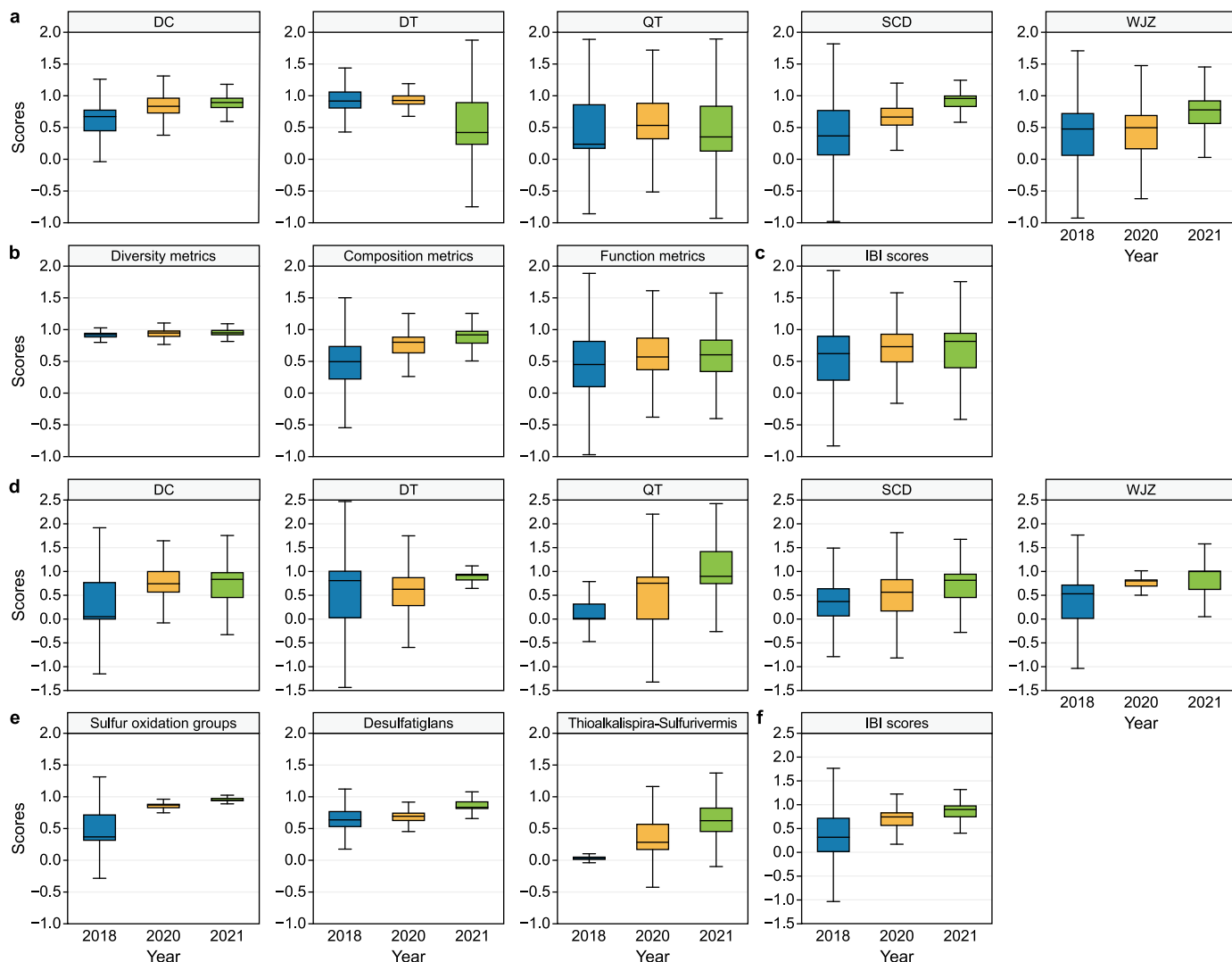
in 2018, 2020, and 2021 had no significant discrimination ( $p > 0.05$ ; Table S1). The nBa-IBI scores of sites DT and QT also showed different trends compared to the tBa-IBI scores. The median value of the nBa-IBI scores at site DT decreased from 0.92 in 2018 to 0.42 in 2021, but the median value of the tBa-IBI scores at site DT decreased from 0.81 in 2018 to 0.63 in 2020 and increased to 0.92 in 2021 afterwards. The median value of the nBa-IBI scores at site QT increased from 0.24 in 2018 to 0.53 in 2020 and decreased to 0.35 in 2021, but the median value of the tBa-IBI scores at site QT increased from 0.01 in 2018 to 0.90 in 2021.

## 4. Discussion

Intensive ecological interventions have significantly improved the environment of Baiyangdian Lake. However, the temporary influence of some treatments, such as fish stocking, may have disrupted corresponding communities and negatively affected ecological assessments. The results of this study indicate that using benthic bacterial communities as ecological indicators is feasible and practical. Furthermore, based on the intrinsic properties of benthic bacterial communities, a novel index of biological integrity (nBa-IBI) was developed. This index can also be extended to other freshwater environments for the aquatic ecosystem assessment.

### 4.1. Improvement of the aquatic environment

General decreases in nutrients and pollution were observed, except for As, pH, TP, Cd, Cu, and Zn, which exhibited significant differences during the study period, as indicated by the ANOVA results ( $p < 0.05$ ; Table S2). The environmental parameters measured in 2018 were similar to those reported in previous studies [57,58]. The contents of TOC, TN, TP, As, Cd, Cu, Zn, and Mn were reported to be derived from industrial and agricultural activities in the catchment of Baiyangdian Lake [58]. There were fewer TN, TP, and heavy metals during the sedimentation process because the lake water contained fewer nutrients and less pollution after implementing environmental interventions in 2018. Additionally, the sediment released accumulated nutrients and pollutants into the lake water due to reduced external discharge [59]. In addition to the environmental treatments that directly influenced the decrease in the total amount of phosphorus in the sediment, the natural growth and artificial planting of submerged macrophytes could also absorb phosphorus and nitrogen from the sediment and above-sediment in lake water [42]. Furthermore, the heavily polluted sediment in Baiyangdian Lake had been dredged, which removed sediment containing high amounts of nutrients and pollutants. In contrast, As concentration increased from 2018 to 2021 at all sampling sites. The sources of As were probably villages along and inside Baiyangdian Lake [60], which might indicate that local sewage treatment facilities are defective. Interestingly, the TOC values increased from 2018 to 2021 except at sites DT and WJZ. The main sources of TOC accumulation in sediment are macrophytes and algal residue. However, aquacultural farms can have substantial numbers of animals. Their feed and stool could also influence the TOC content of the sediment. Anthropogenic disturbances at different sites influenced variations in the TOC values. Since 2018, a TOC/TN ratio  $>9$  indicated that vascular plants were the dominant primary producers in the lake. The increases in the TOC/TN ratio from 2018 to 2021 may also indicate an increasing proportion of macrophytes. Therefore, the increase in TOC was attributed to the natural growth or artificial planting of macrophytes after the shutdown of aquacultural farms at sites DC, QT, and SCD. However, replacing highly regulated anthropogenic environments with natural ecosystems caused a decrease in TOC values at site DT and site WJZ, a former duck farm and a lotus farm,



**Fig. 10.** Box plots of nBa-IBI scores at different sampling sites (DC, DT, QT, SCD, and WJZ) (a), nBa-IBI scores from different metrics (b), nBa-IBI scores among different years (c); tBa-IBI scores at different sampling sites (d), tBa-IBI scores of screened indices (e), and tBa-IBI scores among different years (f).

respectively. The decreases in organic carbon imports, such as feed, stool, and plant debris, accounted for the decreases in TOC values. In 2021, the growth of macrophytes became the main source of organic matter in lake water [61], rather than livestock discharge and municipal domestic sewage [62].

#### 4.2. Influence on benthic bacterial communities

Sediment properties and pollutants strongly influence microbial communities. Additionally, trophic status can majorly shape benthic bacterial communities in freshwater lakes [63]. Zn, TP, Cu, and Pb were key environmental factors influencing bacterial community variation in Baiyangdian Lake in November 2017 [56]. According to the RDA results (Fig. 9), TP, Cd, and TOC were significant influences ( $p < 0.05$ ) that explained 16.2%, 13.2%, and 9.3%, respectively, of the variations among all the measured environmental parameters. Alpha diversity was positively correlated with TOC (Fig. 6), indicating that organic matter may be an essential nutrient for heterotrophic microbial communities in Baiyangdian Lake. Phosphorus is an essential nutrient for organisms because it participates in numerous crucial cellular processes, such as

photosynthesis, aerobic and anaerobic respiration, energy storage and transfer, and cell division [64]. Variations in phosphorus caused obvious variations in the communities, which was indicated by the positive correlation with the phosphate-accumulating organism *Dechloromonas* [65], indicating that phosphorus has been a limiting factor for benthic bacterial communities in Baiyangdian Lake since 2018. Because the lake is a macrophyte type, the growth of macrophytes cannot be ignored. Macrophytes may play a vital role in determining sediment microorganisms [66]. Restoring macrophytes enhances the heterogeneity of sediments and increases microbial diversity by increasing the number of potential niches [67]. Furthermore, a higher macrophyte biomass increases the availability of substrates for microorganisms [68]. In addition to the influence of trophic status on microbial communities, long-term exposure to high heavy metal pollution can cause a significant decline in microbial diversity [23,69]. Cd was the most concerning pollutant and the most common heavy metal in Baiyangdian Lake [55]. Thus, the ecological risk associated with Cd is considerable. Cd influences the bacterial community in rhizosphere soil by changing the soil pH, total nitrogen content, and ammonium content [70]. A previous study indicated that the sediment in Baiyangdian Lake



was heavily polluted by Cd at approximately  $0.5 \text{ mg kg}^{-1}$  but had little effect on the habitat and seasonal variations in bacterial communities [71]. Our study revealed the interannual influence of Cd concentration on benthic bacterial communities in Baiyangdian Lake (Fig. 9).

#### 4.3. Variations in benthic bacterial communities

Because benthic bacterial communities have greater temporal stability than aquatic bacterial communities [72,73], water flow has little direct influence on sediment bacterial communities. Influenced by environmental properties, the bacterial communities in this study exhibited distribution patterns that represented variations among years (Fig. 7). Specifically, Proteobacteria (19.62%) and Chloroflexi (18.29%) dominated the bacterial communities at the phylum level (Fig. 4). They are widespread in lake sediment worldwide, and their abundance has been reported under a variety of environmental conditions [74]. Proteobacteria and Chloroflexi contain essential bacteria for degradation and metabolism in lake sediment [17]. Proteobacteria play a key role in the degradation and metabolism of sediments [75], and Chloroflexi is considered an important participant in the degradation of pollutants in Baiyangdian Lake [27]. Gammaproteobacteria was the dominant class within the phylum Proteobacteria, accounting for 79.67% of Proteobacteria; its relative abundance decreased from 2018 to 2021. Gammaproteobacteria are abundant in organic-rich sediments, such as shrimp aquaculture systems and fish farm sediments [76,77]. Furthermore, Gammaproteobacteria is considered the most representative class of Proteobacteria in eutrophic lakes [78]. Baiyangdian Lake is in a state of mild to moderate eutrophication [79], and a decrease in Gammaproteobacteria is a potential indicator of ecological restoration. Highly abundant unclassified taxa in Baiyangdian Lake sediment, such as unknown taxa in the family Anaerolineaceae and unknown taxa in the family Bacteroidetes vadinHA17, have also been reported in similar lakes worldwide [17,19,80]. The Anaerolineae class, especially the family Anaerolineaceae, was abundant in the bacterial communities. The Anaerolineaceae family includes strict anaerobes and chemoheterotrophs [81] involved in organic matter degradation and influenced by the TOC content in sediment from Baiyangdian Lake.

In this study, the benthic bacterial communities also had high relative abundances of *Thiobacillus* and *Sulfuricurvum*, which are chemolithoautotrophic sulfur oxidizers that obtain energy by oxidizing hydrogen sulfide, pyrite, and elemental sulfur to sulfuric acid [82,83]. The relative abundance of *Thiobacillus* increases with sufficient sulfur compounds [84]. Thus, because of the decrease in sulfur imports since 2018, such as residential sewage discharge, feed, stool, and fertilizer, after anthropogenic environmental treatments, the abundance of the genera *Thiobacillus* and *Sulfuricurvum* decreased from 2018 to 2021 (Fig. 5), especially at site DC, where the sampling site was close to residential sewage and had the highest relative abundance of *Sulfuricurvum* (8.94%) in 2018. Compared to sulfur oxidizers, sulfur-disproportionating and sulfate-reducing bacterial groups (e.g., *Desulfatiglans*) had low abundance but played important roles in the sulfur cycle [85,86]. Organic matter is often a limiting factor in sulfate-reducing bacteria, and higher contents of both sulfur compounds and organic matter are required for sulfate-reducing bacteria to increase in abundance [87]. In our study, the increase in TOC did not cause an increase in *Desulfatiglans*, which indicated the decrease in sulfur compounds in the sediment of Baiyangdian Lake. The prediction of bacterial function with PICRUST2 also indicated a decline in functional bacterial groups related to sulfur metabolism and nitrogen metabolism (Fig. 8). Sulfur metabolism and nitrogen metabolism exhibited similar fluctuating patterns because the genus

*Thiobacillus* also has a strong denitrification capacity [82]. Other taxa related to nitrogen metabolism, such as *Nitrospira*, had low abundance compared to *Thiobacillus*. However, the relative abundance of *Thiobacillus*, *Desulfatiglans*, and functional bacterial groups related to sulfur and nitrogen metabolism increased at site DT, a former duck farm (Figs. 6 and 8). A possible reason is that the extensive use of antibiotics in former duck farms might have disrupted the local bacterial community in the sediment [42]. Previous studies examined seasonal variations, sediment–water exchange of antibiotics [88], and distribution of quinolone antibiotics [26] in Baiyangdian Lake. Zhang et al. [24] indicated that the sediment at site DT contained a greater concentration of quinolone antibiotics than at the other sampling sites in our study. After the duck farm was shut down, bacterial communities varied in decreasing antibiotic pressure. These variations can be observed in Fig. 5, which shows that the bacterial community at site DT in 2021 was much closer to that at the other sampling sites than in 2018. The variance explained (total: 36.0%) by the RDA axes in Fig. 9 was low, indicating the low distinctness of the bacterial communities in the RDA. The explanation may be that some unmeasured factors, such as sulfur content or antibiotics, had a greater influence on the bacterial communities than did the measured environmental factors. Because information on antibiotics after ecological interventions in Baiyangdian Lake is lacking, determining how the distribution of and variation in antibiotics influence the microbial community is a promising avenue for future research.

#### 4.4. Application of bacterial indicators

Ecological treatments include harvesting emergent plants, planting submerged plants (i.e., *Hydrilla verticillata*, *Potamogeton wrightii*, and *Myriophyllum spicatum*), and releasing fish and benthic macroinvertebrates such as *Bellamyia aeruginosa*. These treatments reduce the total nutrient content, restore damaged benthic ecosystems after large-scale dredging, and revitalize aquatic ecosystems [4,89–93]. However, these treatments, which are applied annually, directly affect corresponding communities and reduce their ability to reflect environmental variations [6,7]. Compared with the disrupted routine indicators, the variations in benthic bacterial communities were sensitive indicators of environmental improvement. Furthermore, whether the variations in benthic bacterial communities could be applied to environmental assessments was clarified by calculating the Ba-IBI (Fig. 10), which is directly based on changes in a certain population in aquatic ecosystems rather than on the impacts of measured and unmeasured environmental factors [94]. In this study, both the nBa-IBI scores and tBa-IBI scores increased from 2018 to 2021, indicating that intensive ecological interventions effectively improved the aquatic ecosystem in Baiyangdian Lake. All three core indices (relative abundance of *Desulfatiglans*, *Thioalkalispira-Sulfurivermis*, and sulfur oxidation groups) in the tBa-IBI were related to the sulfur cycle and can be interpreted, indicating that the sulfur-related groups were sensitive to environmental variations in Baiyangdian Lake. These core indices of the tBa-IBI, as well as the high relative abundances of *Thiobacillus* and *Sulfuricurvum*, are sulfur-related genera, which indicates that the potentially most influential environmental factor might be sulfur-related, such as sulfur, sulfate, or sulfide contents. This finding also indicated that the sulfur content in highly disturbed aquatic ecosystems may explain the low explanation of other measured environmental factors and axes in the RDA results (Fig. 9).

Traditional ecological assessments by the IBI are regionally specific because of scientific interventions, such as the definition of reference areas and the selection of candidate matrices [31,32]. The lack of unified standards limits the accuracy and universality of the

IBI, which makes it difficult to extend applications, especially comparisons among different aquatic ecosystems [95]. Ecological assessment using microbial community-based IBI is promising because of its strong adaptability and high sensitivity to environmental variations. Microbial community-based IBIs were established and applied in Taihu Lake and the Three Gorges Reservoir in response to the local extinction of traditional indicator macroorganisms [96] and special environmental conditions caused by reservoir operation [97]. However, the indices used differed among previous studies. Five core indices related to nitrogen-cycling antibiotic resistance genes were identified in Taihu Lake. Still, the selected core indices in the Three Gorges Reservoir were random and distributed across multiple bacterial communities, such as Acidobacteria, Gemmatimonadetes, Geobacter, Methanotrophs, and Phototrophs. The differing core indices applied in previous studies indicate that the traditional development of microbial community-based IBIs with artificial interventions are regionally specific because of differences in environmental influences. In this study, two statistical tests were applied in the calculation of tBa-IBIs to remove a large number of low discrimination indices. These tests increased the discrimination of the tBa-IBIs but reduced its universality because it was impossible to filter out the same indicators across different studies. Considering that the intrinsic properties of the bacterial communities, such as diversity, composition, and function, fluctuate regularly with environmental variations, we established a novel IBI based on the intrinsic properties of benthic bacterial communities to reduce artificial interventions. The nBa-IBI exhibited a trend similar to that of the tBa-IBI, indicating the effectiveness of the ecological assessment (Fig. 10). However, the nBa-IBIs at site DT showed a decreasing trend, which differed from the tBa-IBI. Unlike the other indicators of IBI (i.e., fish and macroinvertebrates), bacteria can be significantly impacted by antibiotics. The release of antibiotic pressure at site DT may explain the overall decline in composition metrics and function metrics, resulting in abnormally low scores in 2021 (Fig. 10). The vital impact of antibiotics considerably limits the application of Ba-IBI in highly anthropogenically influenced areas, such as aquacultural farms and residential areas.

Unlike bacteria, microeukaryotes are less affected by antibiotics, but they have high ecological plasticity and are extremely environmentally adaptable [98]. Because of these characteristics, microeukaryotes such as fungi are promising indicators for ecological assessment. However, the relationships between eukaryotic communities and environmental factors require further study. Although the nBa-IBI indicates the recovery of aquatic ecosystems and proves the effectiveness of this method for lake health assessment, the spatial and temporal variations in benthic microbial communities resulting from human activities [36], different habitat types, and seasonal variations [71] also need further clarification. Another limitation of this study is the lack of quantified results. Because the quantification of 16 S rRNA and functional genes, such as the *dsrB* gene, *nirS* gene, and *nirK* gene, is directly linked to nutrient variations [99], it could be a potential metric for calculating nBa-IBI.

## 5. Conclusion

Our investigation of Baiyangdian Lake demonstrated that the variations in benthic bacterial communities indicated the remarkable restoration of the aquatic ecosystem under intensive ecological treatment from 2018 to 2021.

- (1) The environment of Baiyangdian Lake was successfully restored. The content of nutrients and heavy metals in the sediment decreased, while the TOC content increased with

increasing environmental improvements due to the growth of macrophytes.

- (2) Temporal variations in benthic bacterial communities were strongly influenced by environmental factors, especially total amounts of phosphorus, cadmium, and total organic carbon. The abundance of sulfur-related taxa and phosphate-accumulating bacteria, such as *Thiobacillus*, *Sulfuricurvum*, and *Dechloromonas*, was decreased by environmental improvements, as was the abundance of sulfur- and nitrogen-related metabolic pathways.
- (3) A novel bacteria-based index of biotic integrity with low artificial interventions was developed for practical applications involving ecological assessments. The results indicated a clear recovery of the aquatic ecosystem with accessibility, interpretability, and the potential for universal application.

Therefore, the variations in benthic bacterial communities were determined to be potential indicators of lake ecological improvement under intensive ecological interventions. However, antibiotic pollution needs to be investigated before bacterial indicators are applied. Furthermore, eukaryotes, such as fungi, could represent a promising research direction, but the interpretation of fungal indicators remains a challenge because the fungal genome database is incomplete.

## CRediT authorship contribution statement

**Qi Liu:** Data curation, Investigation, Methodology, Visualization, Writing - Original Draft, Writing - Review & Editing. **Senlu Yin:** Investigation, Visualization. **Yujun Yi:** Funding acquisition, Methodology, Project Administration, Resources, Supervision, Data Curation, Investigation.

## 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.

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## Appendix A. Supplementary data

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