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Assessing Fish Diversity in the Chishui River Using Environmental DNA (eDNA) Metabarcoding

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Abstract: Since 2017, a fishing ban in the Chishui River (China) has reduced human disturbances, yet the early-stage recovery of fish resources remains unquantified. Here, we applied environmental DNA (eDNA) metabarcoding to assess fish diversity and restoration status across its upper, middle, and lower reaches. An analysis of operational taxonomic units (OTUs) revealed higher unique than shared OTUs among reaches, indicating significant spatial partitioning of fish communities. The upper reaches exhibited the highest diversity due to reduced human activity, attributed to greater species richness, while the middle and lower reaches showed similar community structures. Key findings include the following: (1) the detection of rare endemic species (*Schizothorax*) and non-native *Oreochromis* DNA, suggesting invasion risks; (2) the investigation revealed a predominance of small-bodied fish species, indicating that large-bodied fish populations in the Chishui River (China) remained relatively scarce; (3) the recovery of demersal fish DNA from surface waters, confirming eDNA's broad detection capacity. The results indicate that the fishing ban has contributed to the partial recovery of the fish community in the Chishui River (China). However, risks of biological invasion (e.g., *Oreochromis* species) remain, and large-bodied fish are still relatively scarce. To ensure effective conservation, it is critical to strengthen the monitoring and evaluation of the fishing ban's effectiveness and implement timely measures to prevent invasive species proliferation.

Keywords: Chishui River; fish diversity; environmental DNA; metabarcoding; invasive species; fish size reduction

Key Contribution: This study demonstrates the efficacy of environmental DNA metabarcoding in non-invasively assessing post-ban fish diversity, and identifies critical ecological risks such as Nile tilapia invasion and the limited recovery of large fish. These findings provide a scientific basis for evaluating the fishing ban's effectiveness and guiding targeted conservation strategies in the Yangtze River Basin.

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1. Introduction

The Chishui River, a major first-class tributary on the right bank of the upper Yangtze River, persists as the last undammed watercourse in this region, maintaining its natural hydrological regime. Originating from Chishuiyuan Town in Zhenxiong County, Yunnan Province, this river system traverses 13 counties across Yunnan, Guizhou, and Sichuan provinces, extending 444.5 km with a drainage basin of 20,440 km^2 [1]. In April 2005, the General Office of the State Council officially designated the Chishui River's main channel and key tributaries as a part of the Upper Yangtze River Rare and Endemic Fish Species National Nature Reserve. This conservation framework specifically targets the protection of critical habitats and population sustainability for endangered endemic species, including *Psephurus gladius*, *Acipenser dabryanus*, and *Myxocyprinus asiaticus*. Since then, the primary function of the Chishui River has been to preserve these unique fish species and their environments.

Recent ichthyological inventories document over 160 fish species within the Chishui River system [2]. Notably, studies indicate significant anthropogenic pressures—including overfishing, pollution, and shipping activities—have led to a marked reduction in fish sizes and overall fishery resources, with consequential impacts on the Yangtze River's biodiversity and ecological security [3]. Further research by Liu Fei et al. [4] has demonstrated significant interannual variability in the fish community structure of the Chishui River over the past decade, marked by a pronounced decline in both the size and number of endemic and larger fish species. The biodiversity index of the river basin has continued to decrease, severely affecting the fish resources. The intrusion of non-native species, particularly the fishes *Rhynchocypris oxycephalus* and *Barbatula toni* as documented by Tang Rui et al. [2], has further aggravated biodiversity decline in the Chishui River. These invasive species, originating from northeastern China, exhibit strong ecological adaptability.

In 2017, the Ministry of Agriculture instituted comprehensive fishing restrictions in the Chishui River Basin to facilitate ecological restoration and aquatic biodiversity conservation. This regulatory framework was strengthened through a complete four-year fishing moratorium implemented in January 2021, which has effectively mitigated immediate anthropogenic impacts on fish populations. However, there have been no published studies on the fish community in the early stages of the fishing ban, and the extent of recovery of natural fish resources in the Chishui River remains unknown. Conventional fish monitoring approaches (e.g., electrofishing and gillnetting) may inadvertently undermine the ban's outcomes: their invasive nature (e.g., physical harm to fish and habitat disruption during sampling) can alter community structures, thereby biasing assessments of restoration progress. Environmental DNA (eDNA) metabarcoding has emerged as a non-invasive alternative, enabling biodiversity detection through water sampling with minimal ecosystem impact [5]. This method has proven effective in assessing post-disturbance recovery in sensitive habitats, detecting cryptic species, and mapping spatial community dynamics [6–8]. For example, Sales et al. [9] applied environmental DNA (eDNA) metabarcoding to analyze water and sediment samples from the Jequitinhonha River Basin (Brazil) during pre- and post-rainfall seasons, demonstrating significant spatiotemporal dynamics in fish communities and the pronounced influence of rainfall events on species richness. Addressing these challenges, this study aims to accomplish the following: (1) quantify post-ban fish diversity patterns across the upper, middle, and lower reaches of the Chishui River using eDNA metabarcoding; (2) identify persistent threats to ecological recovery, including invasive species proliferation and prevalence of small-bodied fish taxa in community composition.

2. Materials and Methods

2.1. Study Area and Sample Collection

The Chishui River, extending 444.5 km through Yunnan, Guizhou, and Sichuan provinces, displays pronounced anthropogenic activity gradients along its longitudinal axis [1]. The upper reaches are characterized by a concentration of coal and sulfurous iron ore resources; the middle reaches are dominated by liquor production as a key industry; and the lower reaches are densely populated and navigable year-round [10–12].

Sampling was conducted along the Chishui River over a four-day period from 28 to 31 January 2021. Ten sampling stations (CS1–CS10) were strategically positioned along the upper, middle, and lower river sections (Figure 1). At each station, we collected 1.5 L of surface water using pre-sterilized sampling equipment, followed by vacuum filtration through 0.2 µm polycarbonate membranes (Whatman, Maidstone, UK). The filtration assembly underwent sodium hypochlorite sterilization and thorough rinsing between each use. Filter membranes were immediately cryopreserved in liquid nitrogen for subsequent laboratory analysis.

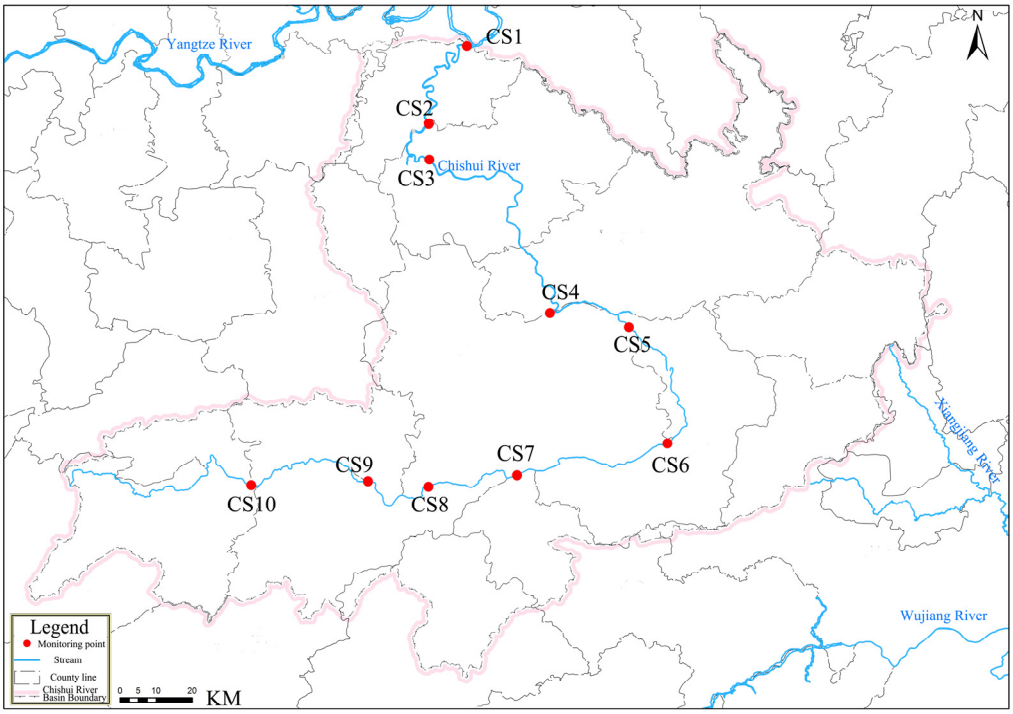


Figure 1. The sampling sites in the Chishui River classified by reaches: lower (CS1–CS4), middle (CS5–CS7), and upper (CS8–CS10).

2.2. DNA Extraction, PCR Amplification, and High-Throughput Sequencing

DNA was extracted from filters using a Mobio DNA extraction kit and stored in liquid nitrogen. The targeted amplification of the mitochondrial cytochrome b gene (Cytb) was performed to investigate fish diversity in the Chishui River. The Cytb gene was amplified using primers L14912-CYB (5′-TTCCTAGCCATACAYTAYAC-3′; Y=C or T) and H15149-CYB (5′-GGTGGCKCCTCAGAAGGACATTTGKCCYCA-3′; K=G or T, Y=C or T) [13]. The PCR reaction mixture (total volume 50 µL) included 4 µL of 10× PCR Buffer, 1 µL of dNTPs, 1 µL of each forward and reverse primer, 5 µL of DNA template, and ddH_2O to adjust the volume. The PCR conditions were as follows: initial denaturation at 94 °C for 5 min; followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 1.5 min; with a final extension at 72 °C for 7 min. Each sample was subjected to three PCR replicates, with sterile water used as a negative

control showing no visible amplification. PCR products from triplicate reactions were pooled, purified using the AxyPrep DNA Gel Extraction Kit (Axygen, Corning, NY, USA), and subsequently sequenced using the TruSeq Nano DNA LT Library Prep Kit (Illumina, San Diego, CA, USA). Sequencing was performed on the Illumina MiSeq platform using paired-end sequencing after passing quality checks.

2.3. Data Analysis

Primer sequences were removed using cutadapt v2.3 [14], and the sequences with non-matching primers were discarded. The sequences were merged using the fastq_mergepairs module of Vsearch v2.13.4 and quality-controlled using the fastq_filter module [15]. Duplicate sequences were eliminated with the derep_fulllength module. Clustering of the unique sequences at a 97% similarity threshold was performed using the cluster_size module, and chimeras were removed using the uchime_denovo module to yield high-quality sequences, which were outputted as representative sequences and operational taxonomic unit (OTU) abundance matrices. The OTU sequences were annotated using the BROCC algorithm [16] based on blastn comparisons to NCBI's NT database [17], with the annotations subsequently manually verified. Sequencing depth was analyzed through dilution curves in QIIME2 [18]. Diversity analyses were conducted on leveled OTU tables using the vegan package in R 4.3.1 [19], with the minimum number of sequences per sample. Fish species occurrence frequency maps were generated using Krona Tools 2.7 [20]. A Non-metric Multidimensional Scaling (NMDS) analysis was performed using R 4.3.1.

3. Results

3.1. Sequencing Statistics

The initial sequencing yielded data volumes ranging from 87,762 to 150,601 reads. Following bioinformatic processing including primer trimming, sequence assembly, quality filtering, and redundancy removal, refined datasets containing 57,780–117,785 high-quality reads were obtained. Subsequent clustering based on 97% sequence similarity, along with the removal of chimeras and singleton sequences, resulted in the identification of 821 representative OTUs. The rarefaction curve based on OTU accumulation (Figure 2) revealed asymptotic stabilization of the observed OTU numbers with increasing sequencing depth, confirming adequate sampling completeness for robust diversity characterization and the subsequent analytical procedures.

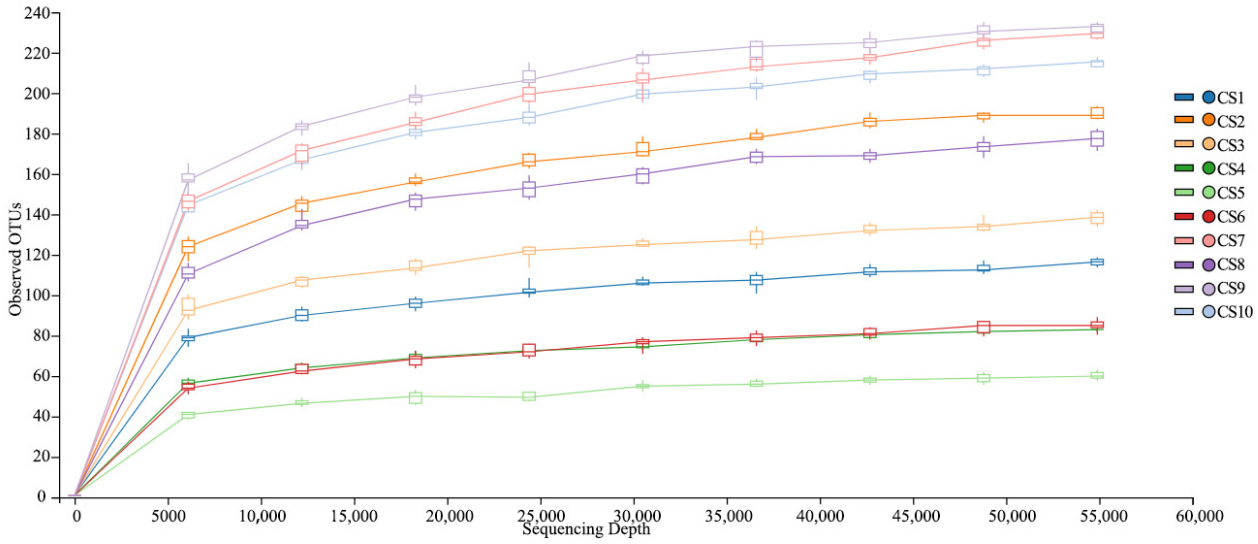


Figure 2. Rarefaction curve of the observed species in Chishui River.

The OTUs were taxonomically annotated, and their distribution across the upper, middle, and lower reaches of the Chishui River was analyzed using the OTU abundance matrix (Figure 3). Notably, only 9.74% of the OTUs were common across all three regions. In contrast, region-specific OTUs were much more prevalent, comprising 30.21%, 16.08%, and 28.26% of the OTUs in the upper, middle, and lower reaches, respectively. These differing human activities in each region influence the aquatic ecology [21], resulting in high proportions of endemic species and low proportions of shared species across the regions.

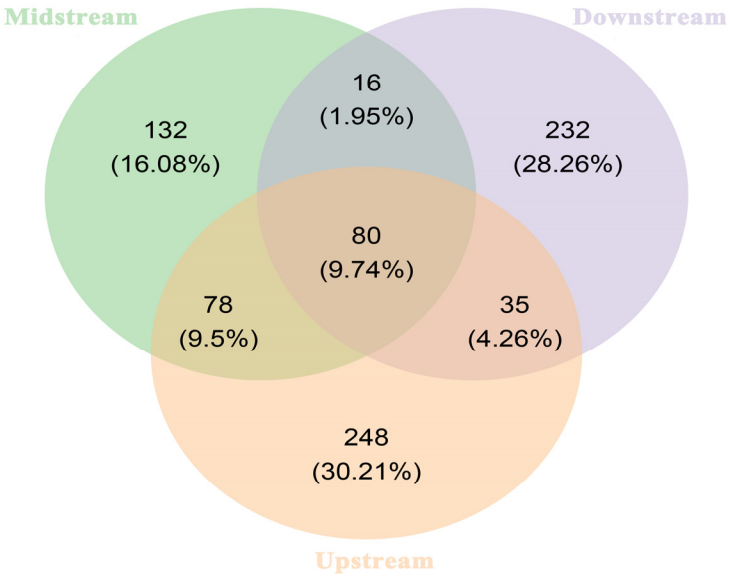


Figure 3. Venn diagram for shared and unique OTU of upper, middle, and lower areas in Chishui River.

3.2. Alpha Diversity in the Chishui River Fish Community

The alpha diversity of the fish community in the Chishui River is illustrated in Figure 4. The Shannon–Wiener index represents the overall diversity of the fish community, the Pielou evenness index measures the numerical proximity of different fish species, and species richness denotes the number of species at each sampling site.

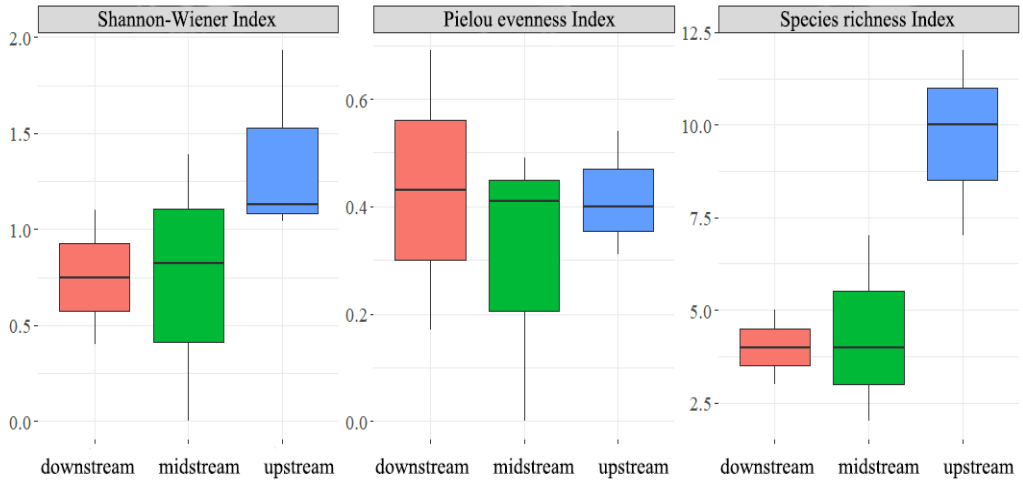


Figure 4. The α diversity of each sampling area in Chishui River.

The Shannon–Wiener index exhibited distinct spatial patterns, with the values ranging 1.04–1.93 in the upper reach, 0.01–1.39 in the middle reach, and 0.40–1.10 in the lower reach.

This gradient demonstrates superior fish community diversity in the upper river sections compared to the downstream regions.

The Pielou evenness index varied from 0.17 to 0.69 downstream, 0.01 to 0.49 in the middle reaches, and 0.31 to 0.54 in the upper reaches, indicating a relatively even distribution of fish communities across the regions.

Species richness also showed a notable gradient, with the number of fish species increasing from downstream to upstream—from 3 to 5 species in the lower reaches, 2 to 7 in the middle, and 7 to 12 in the upper reaches. This gradient underscores the enhanced species richness and consequent higher community diversity observed in the upper reaches compared to the middle and lower sections of the river.

In conclusion, while fish communities in the middle and lower reaches exhibited similar compositions, the upper reaches harbored a richer and more diverse fish community, highlighting the significant ecological gradient along the Chishui River.

3.3. Fish Community Composition

We analyzed fish community composition across ten sampling sites, excluding CS1 and CS4 where the fish OTU abundances fell below the 0.005% detection thresholds (Figure 5). The survey identified a diverse range of species including tilapia (*Oreochromis* sp.), rainbow trout (*Oncorhynchus mykiss*), cut-tailed anemonefish (*Pseudobagrus truncatus*), plateau loach (*Triplophysa* sp.), Xichang huashui loach (*Sinogastromyzon sichangensis*), *Sinibotia superciliaris*, *Acrossocheilus* sp., grass carp (*Ctenopharyngodon idella*), carp (*Cyprinus* sp.), *Garra imberba*, and lip (*Hemibarbus labeo*), bighead carp (*Aristichthys nobilis*), horse mackerel (*Opsariichthys* sp.), naked-bellied sheet-lip trevally (*Platysmacheilus nudiventris*), springbok (*Pseudogyrinocheilus prochilus*), kissing trevally (*Rhinogobio typus*), snake trevally (*Saurogobio dabryi*), and schizothorax (*Schizothorax* sp.). Fish community compositions varied significantly among sites within the same reach (Figure 5).

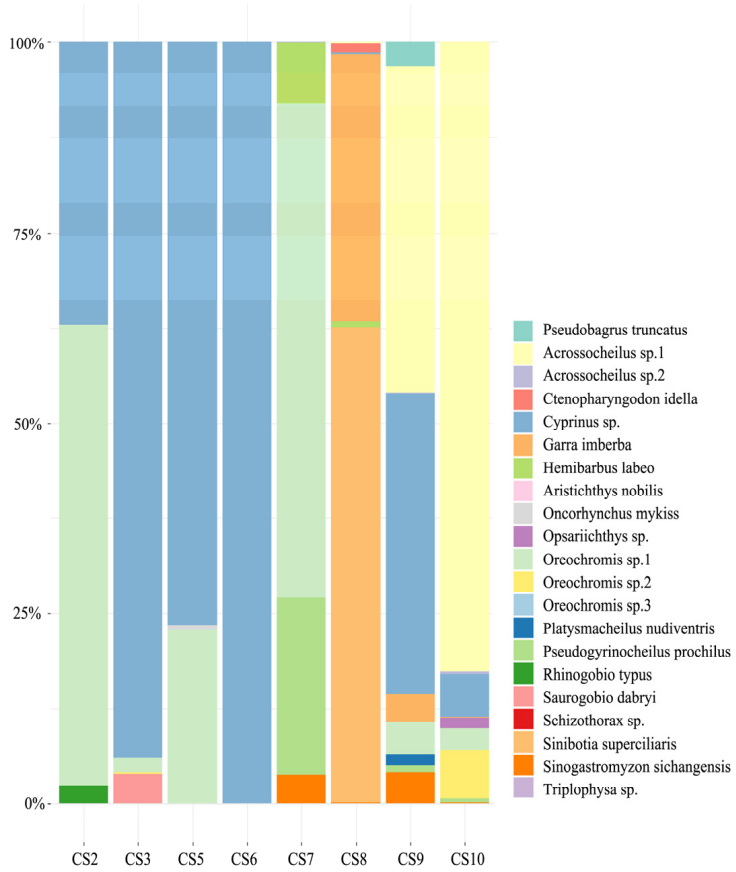


Figure 5. The fish community composition of each sampling site in Chishui River.

The results showed that the downstream section of Lianyu Creek (CS2) was dominated by tilapia and carp species, while the Fuxing section (CS3), the midstream Tongzi River inlet (CS5), and Moutai Township (CS6) were primarily populated with carp. Midstream Qingchi (CS7) hosted a mix of tilapia and springbok, while upstream Datun Township (CS8) was noted for its significant populations of cuttlefish and Zhonghua sand loach. The upstream Qingshuipu (CS9) was rich in fish species diversity, predominantly glossy-lipped fish and carp. Similarly, the main species at the upstream Chahe Ferry (CS10) were glossy-lipped fish. Based on the above results, tilapia, carp, springbok, cuttlefish, Zhonghua sand loach, and glossy-lipped fish were identified as the most abundant species throughout the Chishui River.

3.4. Fish Species Composition

We analyzed fish distribution patterns in the Chishui River using Krona diagrams (Figure 6). Our findings show that tilapia (*Oreochromis* sp.) was the most widespread fish species throughout the river. The survey also identified several specialized species, including *Pseudobagrus truncatus*, *Triplophysa* sp., and *Schizothorax* (Table 1).

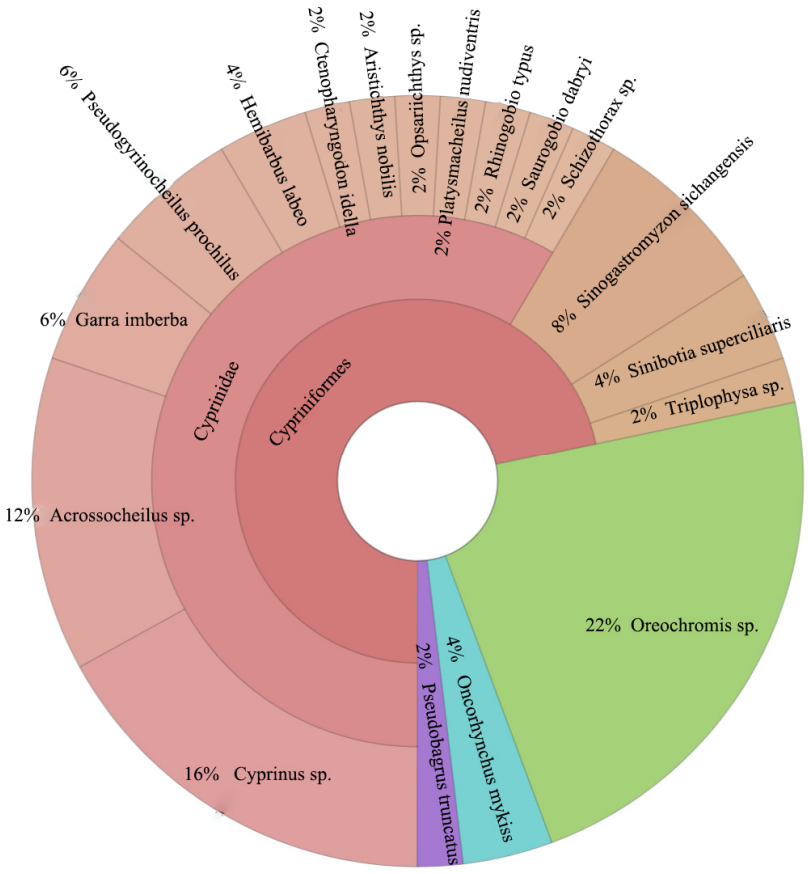


Figure 6. The observed frequency of each fish species in the Chishui River.

The river supports various common fish species: *Cyprinus* sp., *Acrossocheilus* sp., *Sinogastromyzon sichangensis*, *Sinibotia superciliaris*, *Rhinogobio typus*, *Opsariichthys* sp. and *Saurogobio dabryi* [4]. Notably, the survey predominantly found smaller fish species, such as kissing trevally, snake trevally, horse-mouthed fish, lip trevally, Xichanghua sucker loach, Zhonghuasha loach, and glossy-lipped fish, with fewer large fish species being recorded.

Our survey found mostly small-sized species like the *Rhinogobio typus*, *Saurogobio dabryi*, *Opsariichthys* sp., *Sinogastromyzon sichangensis*, *Sinibotia superciliaris*, and *Acrossocheilus* sp., with larger fish species being notably rare.

Table 1. Summary table of fish species.

Species	Native/Invasive	Characteristic
Oreochromis sp.	Invasive	Tropical species
Oncorhynchus mykiss	Invasive	Cold-water species, rarely captured by manual fishing
Pseudobagrus truncatus	Native	Uncommon; captured annually in low numbers
Triplophysa sp.	Native	Rare
Sinogastromyzon sichangensis	Native	Seasonally abundant; often used as duck feed
Sinibotia superciliaris	Native	Moderately abundant
Acrossocheilus sp.	Native	Common species
Ctenopharyngodon idella	Native	Rare in natural rivers; likely escapees from aquaculture; scarce in the Chishui River
Cyprinus sp.	Native	Rare in natural rivers; likely escapees from aquaculture; scarce in the Chishui River
Garra imberba	Native	Rare
Hemibarbus	Native	Common species
Hypophthalmichthys nobilis	Native	Rare in natural rivers; likely escapees from aquaculture; scarce in the Chishui River
Opsariichthys sp.	Native	Uncommon
Platysmacheilus nudiventris	Native	Rare
Pseudogyrinocheilus prochilus	Native	Uncommon; widely distributed in upper tributaries of the Pearl River Basin, China
Rhinogobio typus	Native	Highly abundant
Saurogobio dabryi	Native	Highly abundant
Schizothorax	Native	Uncommon

The proportion of *Oreochromis* sp. exhibited significant spatial heterogeneity among the sampling sites (Figure 7, Figure A1). Higher proportions were observed at CS2 and CS7, accounting for 60.43% and 65.03% of the total species, respectively, whereas the lowest proportions occurred at CS6 and CS8. Overall, *Oreochromis* sp. accounted for 9.68% of all the fish species. The invasion intensity of Nile tilapia varied significantly across habitats, and its distribution may be closely associated with local environmental stressors or anthropogenic disturbances.

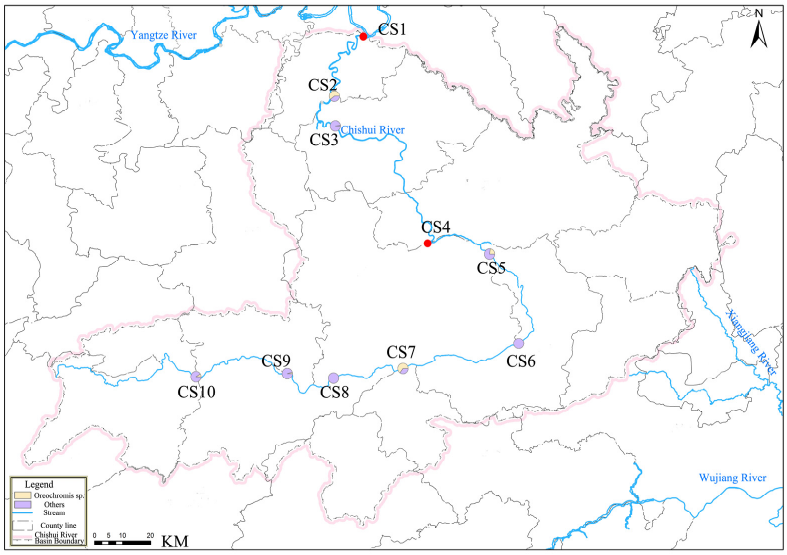


Figure 7. Percentage of tilapia and other fish at different points (red dots as before for sampling points).

4. Discussion

4.1. Prospects of Investigating Fish Communities Using Environmental DNA Technology

Traditional surveys of fishery resources primarily rely on conventional methods such as seine netting, trawling, fixed gear, and diving assessments [22]. While these techniques are established, they often fail to provide a comprehensive and accurate depiction of fish community diversity. Furthermore, they are time-consuming, labor-intensive [23], and can adversely affect the fish populations, potentially undermining fishery closure policies. In contrast, we investigated the diversity of fish communities in the Chishui River by using environmental DNA technology, which not only does not damage fish communities in the water body, but also has high sensitivity and is able to investigate more fish species in a more detailed way [23]. For instance, the rare and endemic *Schizothorax*, native to the upper Yangtze River, was successfully detected in this study without any impact on its survival [24].

In this study, we conducted an investigation using eDNA filtered from surface water samples. The results revealed the presence of fish species such as *Pseudobagrus truncatus* and *Triplophysa*, which typically inhabit the bottom layers of rivers [25]. Given that our samples were collected from surface waters, this indicates that eDNA from surface waters can partially uncover the biodiversity of benthic species. Previous studies have suggested that eDNA from surface waters can reflect the community diversity of bottom waters [26]. Our study further confirms this conclusion, demonstrating the significant potential of eDNA technology in assessing species diversity.

4.2. Diversity and Spatial Structure of Fish Communities in the Chishui River Based on Environmental DNA

Using a combination of alpha diversity metrics (Shannon–Wiener index, Pielou evenness index, and species richness index) and β-diversity analysis (Bray–Curtis dissimilarity with NMDS ordination) (Figure A2), this study provides a comparative analysis of the fish community diversity along the river, highlighting significant ecological variations. The spatial heterogeneity of fish diversity observed in the Chishui River reflects a complex interplay between natural habitat gradients and region-specific anthropogenic pressures. Contrary to traditional downstream-increasing diversity patterns, our eDNA results revealed the highest species richness in the upper reaches, followed by the middle and lower reaches. This inversion aligns with the intensity and type of human activities across the basin. In the upper reaches (CS8–CS10), localized coal and sulfur mining activities, while present, appear spatially confined to specific valleys, leaving the surrounding headwater habitats relatively undisturbed. The midstream area (CS5–CS7) is dominated by the Baijiu production industry, and more adaptable species (such as *Cyprinus* sp.) have been found [27], which indicates that organic sewage discharge may homogenize the community and create a living environment for invasive species. Downstream areas (CS1–CS4), subjected to dense urbanization and year-round navigation, exhibited the lowest diversity. Previous studies have shown that human activities can affect fish diversity [28]. Therefore, it is inferred that human interference led to lower fish diversity in the lower reaches of the Chishui River compared to the middle and upper reaches in this survey. While all the reaches face anthropogenic pressures, the upper river's retained habitat complexity appears to buffer biodiversity loss.

4.3. Potential Risks to the Chishui River Fish Community

This study, employing environmental DNA analysis for species composition, has identified two primary concerns regarding the fish community of the Chishui River: (1) the threat of biological invasion and (2) a predominance of small fish species in the findings.

Biological Invasion Risks: The most significant invasive risk identified is the tilapia, previously recorded in Guizhou Province [29]. Tilapia DNA was detected at the majority of the sample sites surveyed, and tilapia accounted for 9.68% of all fish species, known for its adaptable feeding habits, rapid growth, and high reproductive capacity [30]. Wan et al. documented the presence of 38 non-native fish species in the natural waters of the Yangtze River basin, with Nile tilapia (*Oreochromis niloticus*) and Mozambique tilapia (*Oreochromis mossambicus*) classified as high-risk invasive species. These tilapias exhibit broad distributions across critical hydrological zones, including the mainstream of the Three Gorges Reservoir, Chishui River, Han River, and interconnected lacustrine ecosystems [31]. Tilapia can expand through escape from aquaculture and subsequent natural reproduction [32]. Should tilapia successfully establish itself, it could lead to the extinction of native fish species, a loss of biodiversity, and irreversible damage to the ecosystem structure and function [33]. Therefore, the ongoing monitoring of fish diversity in the Chishui River is imperative to mitigate the risks of tilapia bioinvasion.

Prevalence of Small Fish Species: The survey revealed that the fish community in the Chishui River basin primarily consists of smaller species. Liu Fei et al. have attributed this trend to overfishing based on a decade-long survey from 2007 to 2016 [4], which has resulted in a directional selection that favors genes for slower growth rates. This genetic shift may have long-term, profound impacts on the structure of the fish community [34]. Although a comprehensive fishing ban has been in effect for four years, the dominance of smaller fish suggests that its efficacy needs further evaluation. It is crucial to continue in-depth surveys and monitor the succession of fish communities in the Chishui River basin to provide insights into the impacts of these conservation measures.

5. Conclusions

This study systematically investigated the fish community diversity in the Chishui River during the early stages of the fishing ban using eDNA metabarcoding technology, revealing the current status of fish resource recovery and potential ecological risks following policy implementation. The results indicate that human activities exert significantly divergent impacts across river sections. In the upstream region, where human disturbance is minimal, species richness and community diversity are significantly higher than in the middle and lower reaches, underscoring the critical role of natural habitats in fish resource recovery. In contrast, the middle and lower reaches, subject to intensive human activities (e.g., liquor production and shipping), exhibit a simplified fish community structure, markedly differing from the upstream region. Notably, the widespread detection of *Oreochromis* sp. across multiple areas highlights an ongoing threat posed by invasive species to native fish community stability. Additionally, the surveyed species composition exhibited a persistent predominance of small-bodied fish taxa, with no notable recovery observed for large fish populations.

Author Contributions: Conceptualization, J.Z.; methodology, J.G.; software, C.P. and Y.H.; validation, Y.W. (Yajie Wu); formal analysis, S.X.; data curation, M.H. and Y.H.; writing—original draft, J.G.; writing—review and editing, J.G. and Y.W. (Yingru Wang); supervision, W.L. and Y.W. (Yingru Wang). All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: This study employed environmental DNA (eDNA) methods for ecological analysis. All the samples were collected non-invasively without direct animal interaction, tissue extraction, or ecosystem disturbance. As eDNA sampling falls outside the scope of ethical review requirements for animal research, no institutional approval was necessary. Fieldwork complied with local environmental regulations to minimize ecological impact.

Data Availability Statement: Data will be made available upon request.

Conflicts of Interest: Author Yajie Wu was employed by the company Huaneng Lancang River Hydropower Co., Ltd. The remaining authors declare that this research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflicts of interest.

Appendix A

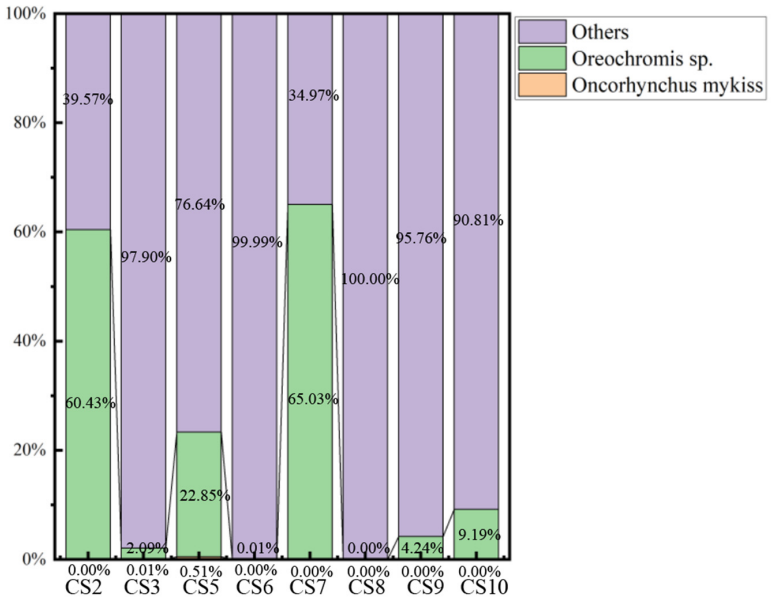


Figure A1. Proportion of invasive species and other species in the Chishui River.

The presence of the invasive species *Oncorhynchus mykiss* was found only at point CS3 and CS5 sites, and the relative abundance was very small.

Appendix B

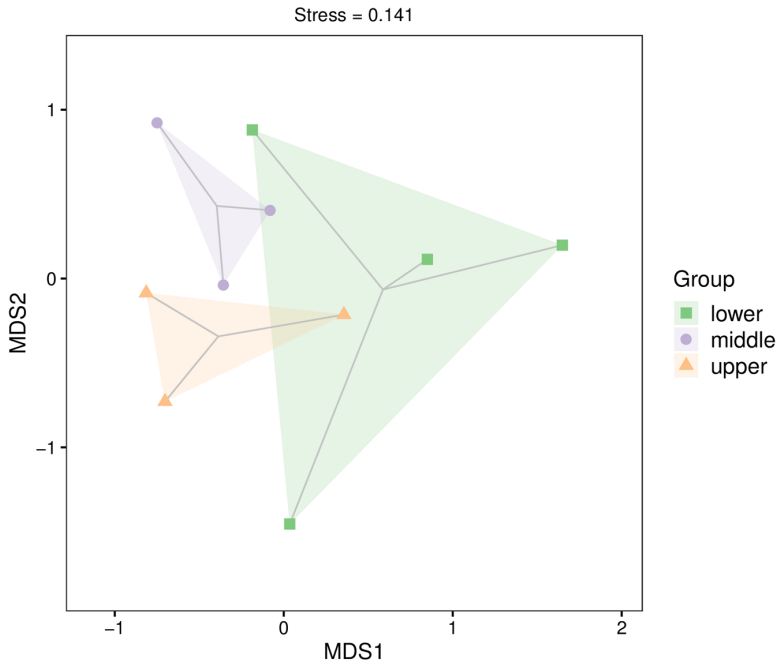


Figure A2. Non-metric Multidimensional Scaling (NMDS) analysis of fish communities in the upper, middle, and lower reaches of the Chishui River (Bray–Curtis distance).

The NMDS analysis based on Bray–Curtis distance revealed significant spatial divergence in fish community structures among the upper, middle, and lower reaches of the Chishui River. Samples from each reach exhibited clear segregation in the two-dimensional ordination space, indicating distinct differences in fish composition across the river sections. Notably, communities in the lower reaches diverged markedly from those in the middle and upper reaches, likely due to anthropogenic disturbances.

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