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Risk Screening of Invasive Aquatic Species and a Survey of Fish Diversity Using Environmental DNA Metabarcoding Analysis in Shanghai

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Abstract: As the largest coastal city in China, Shanghai's rapid development in transportation, tourism, trade, and commerce has facilitated the spread and invasion of non-native aquatic organisms. Aquatic organisms are highly elusive, and once established, eradicating them becomes a challenging task. Currently, our understanding of the invasion risk posed by non-native aquatic species in Shanghai is limited. Therefore, it is imperative to investigate the pathways of introduction, distribution, and dispersion and the invasion risk and impacts of non-native aquatic organisms in Shanghai. This study investigated aquatic organisms in Shanghai's primary water bodies, including Huangpu River, Suzhou River, and Dianshan Lake. The risk assessment was conducted using the Aquatic Species Invasiveness Screening Kit (AS-ISK), and field monitoring was performed with environmental DNA (eDNA) technology. Results of the risk assessment indicate that among the 21 evaluated species, 9 fall into the medium-to-high-risk category with scores ≥26, while 12 are classified as low-risk with scores <26. The top four species with the highest invasion risk are *Gambusia affinis*, *Pomacea canaliculata*, *Lepomis macrochirus*, and *Coptodon zillii*. This study identified 54 fish species belonging to seven orders, 16 families, and 42 genera at 16 sampling sites in Shanghai, among which *Channa maculata*, *Micropterus salmoides*, and *Misgurnus bipartitus* are non-native. The results suggest that Shanghai faces a high invasion risk of aquatic species, necessitating enhanced scientific prevention and control measures. Early monitoring is essential for species with medium-to-high invasion risk, and a further evaluation and analysis of the risks associated with introduced fish species already present in Shanghai are recommended for aquaculture practices.

Keywords: freshwater fish; AS-ISK; field monitoring; aquaculture; non-native species

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1. Introduction

Biological invasions have led to a series of negative impacts on ecological security, native species, the social economy, and more [1–6]. Currently, it is listed as one of the five major global environmental issues of the 21st century [7]. The Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services (IPBES), in its 2019 report, highlighted that with the increase in global trade volume, population, and climate change, the number of non-native species has increased by approximately 40% since 1980, with about one-fifth of the world facing the risk of biological invasion. The proportion of newly introduced invasive species has reached historic highs and shows no signs of slowing down [8].

Over 5000 species are non-native in China, among which more than 660 have successfully invaded, representing a 35% increase compared with a decade ago [9–11]. In the list of 100 of the World's Worst Invasive Alien Species announced by the International Union for Conservation of Nature (IUCN), over 50 species have already invaded China, with approximately 20% of them being aquatic organisms. Most non-native aquatic species are introduced into China by human activities, and they establish natural populations due to China's unique climate, geography, and complex habitat types, subsequently impacting local ecosystems and native species [12,13]. For example, the introduction of *Gambusia affinis* led to a significant decline in the population of native *Oryzias sinensis* [14]. The introduction of *Trachemys scripta elegans* in Henan province resulted in the disruption of aquatic ecosystems and potentially the local extinction of native turtles [15]. Clearing the introduced *Atractosteus spatula* in Ruzhou City, Henan Province, took a month and caused substantial economic losses. In light of this, an urgent need exists for the risk assessment and field monitoring of non-native aquatic species.

As the largest coastal city in China, Shanghai's rapid development in transportation, tourism, trade, and commerce has provided convenient conditions for the spread and invasion of non-native aquatic species, accelerating the ecological process of their invasion [16,17]. Zhang et al. [17] compiled a list of non-native invasive species in Shanghai in 2012, indicating a total of 212 non-native species, of which nine (4.25%) are aquatic animals. These include *Procambarus clarkii*, *Pomacea canaliculata*, *Rana catesbeiana*, *G. affinis*, *Micropterus salmoides*, *Colossoma brachypomus*, *Oreochromis aureus*, *Ictalurus punctatus*, and *Oncorhynchus mykiss*. Among these, *P. canaliculata* can produce up to 300,000 eggs in a year and has a strong reproductive capacity, with wide distribution across ten districts in Shanghai [18,19]. Due to its preference for rice crops and aquatic vegetation, it poses varying degrees of harm to invaded waterways and threatens fish biodiversity [20,21]. Aquatic organisms have strong concealment and once successfully invaded, they are challenging to eradicate. Therefore, understanding the pathways, distribution status, dispersion routes, invasion risk, and impact of non-native aquatic species in Shanghai is of utmost importance.

With the acceleration of the invasion of non-native species and the progress of industrialization and urbanization, the native fish diversity in Shanghai faces numerous threats and challenges. For example, a survey in 2019 by Yu et al. [22] found that the number of fish species in the Suzhou River has decreased by 17 species compared with the 1960s, with a total of only 45 species [23]. To maintain the health of aquatic ecosystems and protect fish diversity, the Shanghai Municipal Agricultural and Rural Affairs Commission issued a notice on implementing a five-year fishing ban in the Yangtze River Estuary and other inland waters, which came into effect on 10 February 2021. Additionally, Shanghai has allocated significant funds for continuous stocking activities to protect fish diversity. However, the current state of fish diversity in Shanghai and the impact of related policies on its diversity are not yet fully understood. Therefore, it is necessary to conduct a survey of fish diversity in the three main rivers of Shanghai and study the current situation and trends.

This study utilizes the Aquatic Species Invasiveness Screening Kit (AS-ISK), a widely applied pre-assessment tool for invasive aquatic species [24]. It is suitable for almost any climate/marine ecological zone and for any aquatic species (excluding parasites and pathogens) [25] and has been used in more than 26 risk assessment (RA) regions, including China [26–28], Turkey [29–31], the United Kingdom [32], India [33], Russia [34], and Italy [35–37], to effectively assess the invasion risk of non-native aquatic species.

The emergence and application of environmental DNA (eDNA) metabarcoding analysis have brought unprecedented innovation to the monitoring of aquatic organisms. Compared with traditional survey methods (such as direct observation, baited traps, and trawl fishing [38]), this technology offers higher detection capability, cost-effectiveness, environmental friendliness, and non-invasiveness. It overcomes many limitations of traditional survey methods [39,40] and has been widely used in species monitoring. For example, Liu et al. [41] used eDNA metabarcoding analysis to investigate fish diversity in Beijing and detected a total of 52 freshwater fish species, including 13 non-native species.

Jeunen et al. [42] monitored biodiversity in Belarusian waters using eDNA metabarcoding analysis and detected nine non-native fish species. In the early stages of invasive aquatic species, when species density and abundance are low, monitoring these low-density target organisms in complex communities is critical for building an early detection and warning system and preventing the further spread of invasive species [41]. Due to its high sensitivity, eDNA metabarcoding analysis can detect the presence of a species in water bodies at low densities [43], making it highly advantageous in monitoring non-native aquatic species.

This study encompasses both modeling assessment and field surveys. It utilizes AS-ISK for risk assessment of non-native aquatic species in Shanghai and eDNA metabarcoding analysis for field monitoring to gain a comprehensive understanding of fish diversity, the composition of non-native species, and their invasion risk. This study provides recommendations for the management of non-native aquatic species in Shanghai and serves as a reference for control measures and biodiversity protection in nearby river basins.

2. Materials and Methods

2.1. Risk Assessment Modeling of Non-Native Aquatic Species

In this study, 21 non-native aquatic species were selected for risk assessment. The selection criteria were based on four categories: (1) non-native aquatic species that have already been confirmed to have invaded Shanghai (three species); (2) non-native aquatic species found in Shanghai but have not yet established (three species); (3) non-native aquatic species that have invaded surrounding water bodies in Shanghai (four species); and (4) non-native aquatic species introduced to Shanghai due to trade activities, aquaculture, and the aquarium industry (eleven species). The non-native aquatic species that were assessed included intentionally or unintentionally introduced species. Once the selection was completed, the Aquatic Species Invasiveness Screening Kit (AS-ISK, available at www.cefas.co.uk/nns/Tools/, accessed on 21 September 2021.) was used to assess the invasion risk of potentially invasive species in the RA region.

AS-ISK consists of 49 factors about life history characteristics, biology, ecology, biogeography, and introduction history, along with an additional 6 factors regarding climate change, to predict the likelihood of species invasion under current and future climate conditions [26,29,30,44]. To effectively screen species in the RA region, assessors must provide responses to each question in the toolkit, as well as the confidence level and reasoning for each response based on the relevant literature or field survey results. Based on the assessor's responses, each species receives both a Basic Risk Assessment (BRA) score (score range: −20 to 68) and an Integrated Assessment under Climate Change (BRA + CCA) score (score range: −32 to 80).

The accuracy of the assessment results was evaluated using the area under curve (AUC) of the receiver operating characteristic (ROC) curve analysis. The threshold score for the RA region was determined using Youden's index. When a species' invasion score is greater than or equal to the threshold, it is classified as a species with medium to high invasion risk; otherwise, it is classified as a species with low invasion risk. Confidence factors were calculated based on the confidence level for each response, with the formula as follows:

$$\mathrm{CF} = \sum (CQ_i)/(4 \times 55)(i = 1, 2 \ldots 55)$$

where CF represents the confidence factor and CQ_i is the confidence level for each assessment question (1 = low, 2 = moderate, 3 = high, 4 = very high), with values ranging from 0.25 (low confidence for all questions) to 1 (high confidence for all questions).

2.2. Field Monitoring Using eDNA Metabarcoding Analysis

2.2.1. Water Sample Collection

This survey was conducted from 16 September to 20 September 2022, based on the geographical features of the main river basins in Shanghai and the composition of non-native aquatic species. The survey area was located between 120°55′12″ E–121°7′48″ E and 30°56′24″ N–31°8′24″ N, with a total of 16 sampling points. Among them, seven sites were set along the Huangpu River, six sites in Dianshan Lake, and three sites along the Suzhou River (Figure 1). At each sampling point, we collected one liter of surface water. To prevent DNA degradation, 1 mL of benzalkonium chloride (BAC) solution (concentration 10%) [45] was added to the water samples at each point. The samples were then stored at low temperatures until filtration.

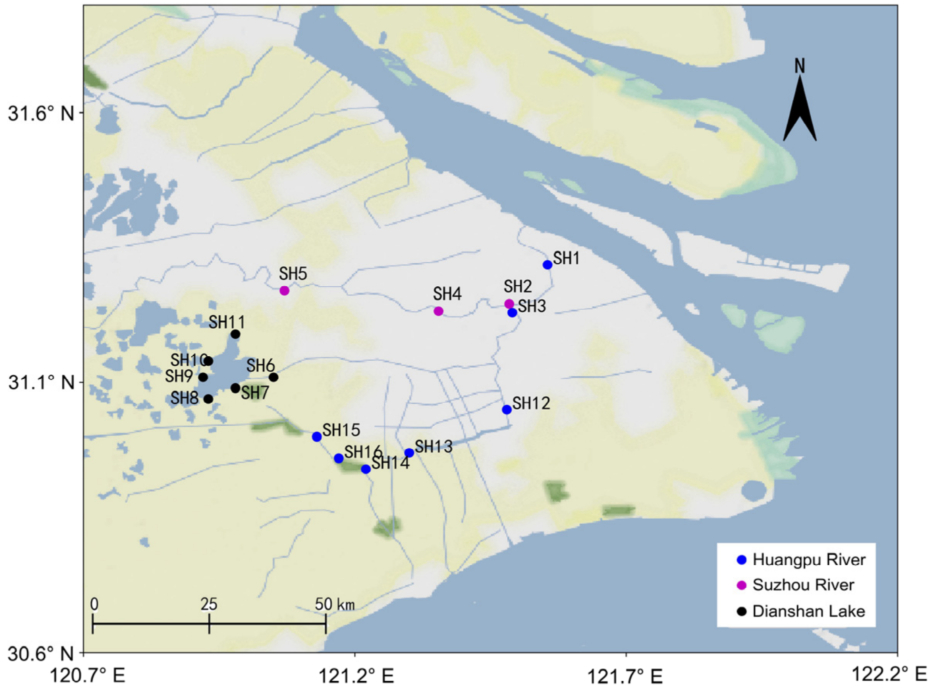


Figure 1. Schematic map of the sampling points in the major rivers of Shanghai.

2.2.2. Water Sample Processing and eDNA Extraction

Glass fiber filters with a pore size of 0.7 μm (Shanghai Xingya, China) were used for vacuum filtration of the collected samples. Filters were stored in 2 mL cryotubes (Sangon Biotech, Shanghai, China) and kept in a laboratory at −20 °C until eDNA extraction. All filtration equipment was soaked in a 10% sodium hypochlorite solution for five minutes before and after use to remove residual DNA, followed by thorough rinsing with tap water and a final rinse with commercially available distilled water (Hangzhou wahaha group co., LTD, Hangzhou, China). Disposable gloves were used for each sample filtration. Pre-processing of eDNA was carried out according to Xia et al. [46]. After pre-processing, total eDNA was extracted from the filters using the DNeasy Blood and Tissue kit (Qiagen, Dusseldorf, Germany). After extraction, the total eDNA was eluted in 50 μL of double-distilled water (ddH_2O) and stored at −20 °C.

2.2.3. Amplicon Library Preparation and Sequencing Run

The amplicon libraries for fish eDNA metabarcoding were prepared using MiFish-U primers [47]. First-round PCR reaction, purification products, and sample dilutions were completed according to Wu et al. [48]. The second-round PCR was performed to add the adapter and 8 bp indices to both ends of the amplicons using the diluted samples [49]. The products of the 2nd PCR were pooled into one tube to include all samples. Using the E-Gel Precast Agarose Electrophoresis System (Thermo Fisher Scientific, Waltham, MA, UAS) and E-Gel SizeSelect 2% (Thermo Fisher Scientific, Waltham, MA, USA), the target size amplicons were extracted. An Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) was subsequently used to verify whether only DNA of the target length (approximately 370 bp) was obtained. The DNA library concentration was measured with a Qubit fluorometer 3.0 (Thermo Fisher Scientific, Waltham, MA, USA) using Qubit dsDNA HS assay kit. The concentration of the DNA library was adjusted to 4 nM. Finally, the library was sequenced using an Illumina HiSeq 4500 with 150 bp paired-end (Illumina, San Diego, CA, USA).

2.2.4. Data Analysis

The raw reads obtained from HiSeq sequencing were pre-processed and analyzed using USEARCH v10.0.240 [50], according to the method of Wu et al. [48]. Based on the distribution information of aquatic organisms in Shanghai, the sequences of fish and other species not distributed in Shanghai were manually calibrated or deleted. All analyses were performed using R ver. 4.1.2, and the results were visualized. In all analyses, the reads were converted to either presence or absence [51].

3. Results

3.1. Risk Assessment of Non-Native Fish Species

The assessment results based on AS-ISK are presented in Table 1. The AUC values of the BRA and BRA + CCA curves obtained from ROC curve analysis were both greater than 0.5 (Figure 2), indicating that AS-ISK can reasonably distinguish between invasive and non-invasive species. The maximum Youden's index values for the RA region corresponded to threshold scores of 26 (BRA) and 28.75 (BRA + CCA). This means that species with BRA scores below 26 were classified as low-invasion risk, while those with scores equal to or greater than 26 were classified as medium-to-high-invasion risk. Similarly, species with BRA + CCA scores below 28.75 were classified as low-invasion risk, while those with scores equal to or greater than 28.75 were classified as medium-to-high-invasion risk.

Table 1. The invasiveness results of the 21 exotic aquatic organisms in Shanghai assessed with the Aquatic Species Invasiveness Screening Kit.

Species			Outcome				Confidence Factor		
Criteria	Common Name	Scientific Name	BRA Score	Invasion Risk	BRA + CCA Score	Invasion Risk	BRA	CCA	BRA + CCA
1	Mosquito fish	*Gambusia affinis*	32	high	32	high	0.55	0.25	0.52
1	Channeled applesnail	*Pomacea canaliculata*	32	high	40	high	0.53	0.25	0.50
3	Bluegill	*Lepomis macrochirus*	31	high	33	high	0.56	0.25	0.53
3	Redbelly tilapia	*Coptodon zillii*	31	high	41	high	0.59	0.33	0.56
3	Green sunfish	*Lepomis cyanellus*	29.5	high	35.5	high	0.56	0.25	0.53
1	Red swamp crayfish	*Procambarus clarkii*	29.5	high	29.5	high	0.55	0.25	0.51
2	Bullfrog	*Rana catesbeiana*	28.5	high	34.5	high	0.54	0.25	0.50
4	Suckermouth catfish	*Hypostomus plecostomus*	28	high	36	high	0.47	0.38	0.46
4	Largemouth black bass	*Micropterus salmoides*	27	high	39	high	0.60	0.25	0.56
4	Red-eared slider	*Trachemys scripta elegans*	25	low	25	low	0.47	0.25	0.45
2	North African catfish	*Clarias gariepinus*	24	low	32	high	0.55	0.25	0.51
4	American shad	*Alosa sapidissima*	23.5	low	23.5	low	0.54	0.25	0.50
4	Channel catfish	*Ictalurus punctatus*	23.5	low	31.5	high	0.49	0.33	0.48
4	Pirapitinga	*Piaractus brachypomus*	23.5	low	33.5	high	0.58	0.25	0.55
4	Amur sturgeon	*Acipenser schrenckii*	21	low	17	low	0.51	0.29	0.49
4	Blue tilapia	*Oreochromis aureus*	20.5	low	22.5	low	0.58	0.25	0.55
3	Red drum	*Sciaenops ocellatus*	20	low	28	low	0.49	0.25	0.46
4	European eel	*Anguilla anguilla*	20	low	24	low	0.51	0.25	0.48
2	Rainbow trout	*Oncorhynchus mykiss*	18.5	low	10.5	low	0.56	0.38	0.54
4	Tench	*Tinca tinca*	15.5	low	21.5	low	0.53	0.33	0.50
4	Roho labeo	*Labeo rohita*	15	low	13	low	0.53	0.25	0.50

Under current climate conditions, nine species (score range [26, 32]) were classified as medium-to-high-invasion risk, while twelve species (score range [15, 26)) were classified as low-invasion risk. The top four species with the highest scores were *G. affinis*, *P. canaliculata*, *Lepomis macrochirus*, and *Coptodon zillii*. The other five species with medium-to-high-invasion risk included *L. cyanellus*, *Procambarus clarkii*, *R. catesbeiana*, *Hypostomus plecostomus*, and *M. salmoides*. The three species with the lowest scores were *O. mykiss*, *Tinca tinca*, and *Labeo rohita*.

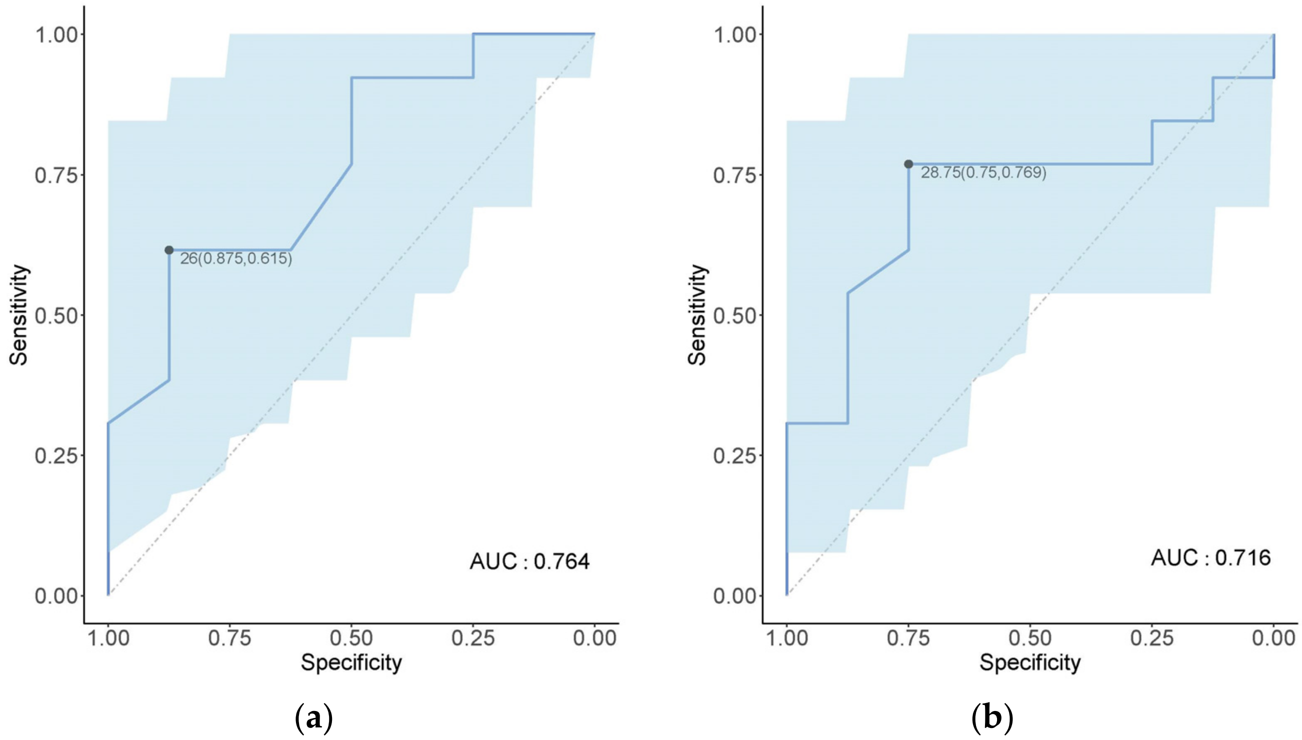


Figure 2. Receiver operating characteristic curve of Aquatic Species Invasiveness Screening Kit scores for 21 exotic aquatic organisms in Shanghai: (**a**) ROC curve for Basic Risk Assessment (BRA) and (**b**) ROC curves for Integrated Assessment under Climate Change conditions (BRA + CCA).

Under climate change conditions, twelve species (range [28.75, 41]) were classified as medium-to-high-invasion risk, while nine species (range [10.5, 28.75)) were classified as low-invasion risk. The top three species with the highest invasion risk were *Pomacea canaliculata*, *C. zillii*, and *M. salmoides*. Furthermore, the invasion risk increased for fourteen species, decreased for three species, and remained unchanged for four species, with *M. salmoides*, *C. zillii*, and *Piaractus brachypomus* exhibiting the most significant score increase from BRA to BRA + CCA.

3.2. eDNA Sampling Results

3.2.1. Fish Species Diversity in Shanghai

In this survey, a total of 1,210,756 different Amplicon Sequence Variants (ASVs) were obtained from 16 sequencing samples. These ASVs were subjected to database matching and annotation and then merged for analysis. The survey revealed a total of 54 fish species in Shanghai, belonging to seven orders, 16 families, and 42 genera (Appendix A). The Cypriniformes order had the highest number of species at 33, accounting for 61.11% of the total fish species in Shanghai. Perciformes was the second most abundant order with 14 species. Siluriformes had three species, while Clupeiformes, Anguilliformes, Cyprinodontiformes, and Sciaenidae each had one species. In terms of families, Cyprinidae had the most species, with 29 in total, followed by Gobiidae and Cyprinodontidae, each having 4 species (Figure 3).

With regard to species distribution, the survey detected 51 fish species in the Huangpu River, 36 in the Suzhou River, and 43 in Dianshan Lake (Figure 4). Fish composition in the Huangpu River, the Suzhou River, and Dianshan Lake was very similar, and 33 fish species were detected in all three rivers, while 2 species were only detected in Dianshan Lake, which were *Sarcocheilichthys sinensis* and *Siniperca chuatsi*. One species, *Macropodus ocellatus*, was exclusively found in the Suzhou River, and eight species, including *Megalobrama terminalis*, *Mugilogobius_myxodermus*, *Channa maculata*, *Lateolabrax maculatus*, *Eleotris* *oxycephala*, *Anguilla japonica*, *Tachysurus nitidus*, and *Mugil cephalus*, were only detected in the Huangpu River (Figure 4).

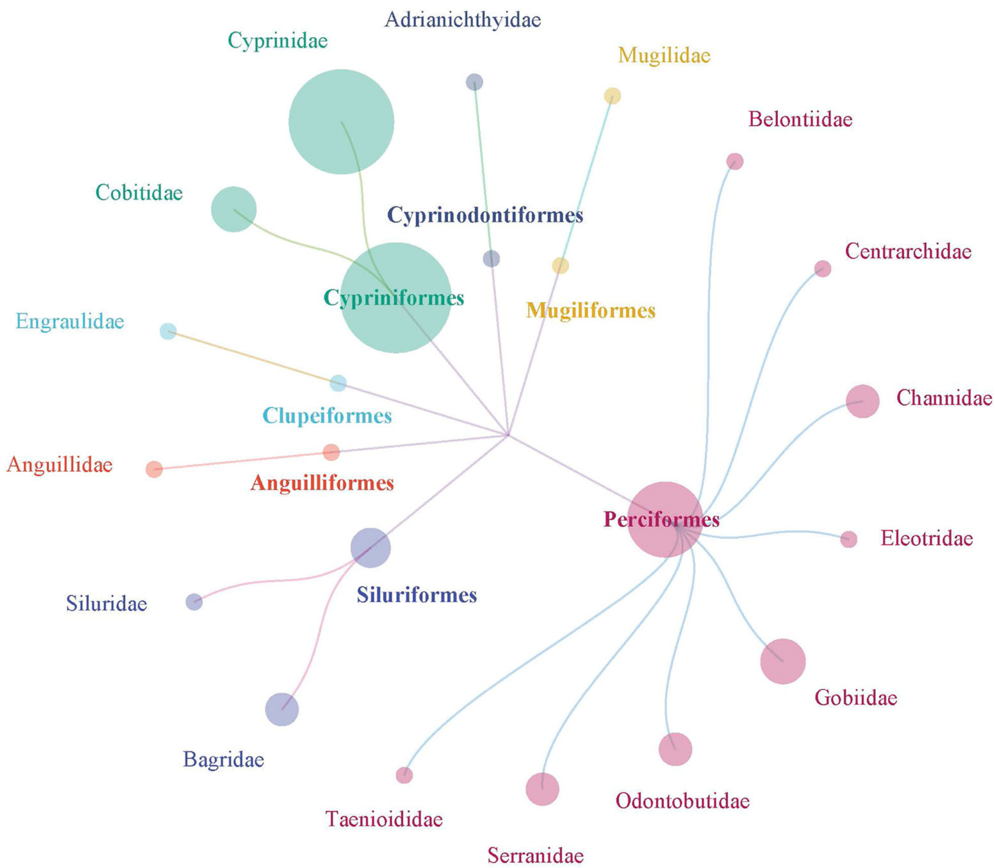


Figure 3. Fish species composition in Shanghai based on eDNA metabarcoding analysis. In this picture, different colors represent different orders, and the size of the circle reflects the number of fish species in that order or family.

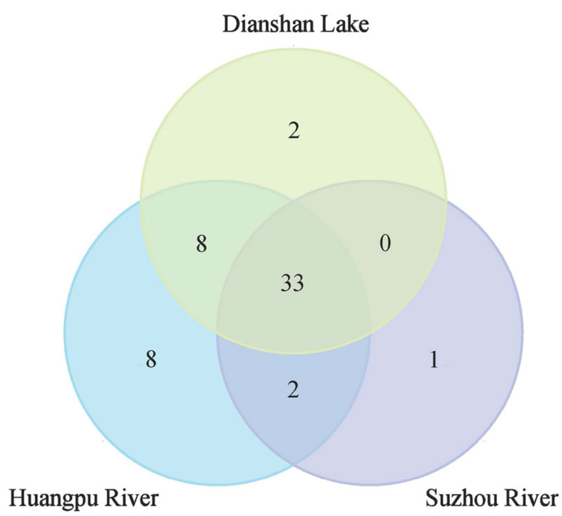


Figure 4. Number of fish species in the three major rivers of Shanghai.

3.2.2. Native and Non-Native Fish Species

The eDNA survey revealed that there was a total of 54 fish species in Shanghai. Among these, 51 species (94.44%) were native to the region, while 3 species (5.56%) were non-native. The presence of non-native fish species at different survey sites is depicted in Table 2. The dominant species in Shanghai, including *Abbottina rivularis*, *Acheilognathus chankaensis*, *A. barbatulus*, *C. argus*, *Odontobutis potamophila*, *Pseudorasbora parva*, *Rhodeus ocellatus*, *Zacco acanthogenys*, and *Misgurnus anguillicaudatus*, were detected in all water samples collected from the survey sites. The non-native fish species included *Micropterus salmoides*, *C. maculata*, and *Misgurnus bipartitus*, with *C. maculata* being restricted to the Huangpu River, while *M. bipartitus* and *Micropterus salmoides* were found in the Huangpu River, the Suzhou River, and Dianshan Lake, which are the three major river systems in the region.

Table 2. List of alien fish species at each site monitored using eDNA metabarcoding analysis.

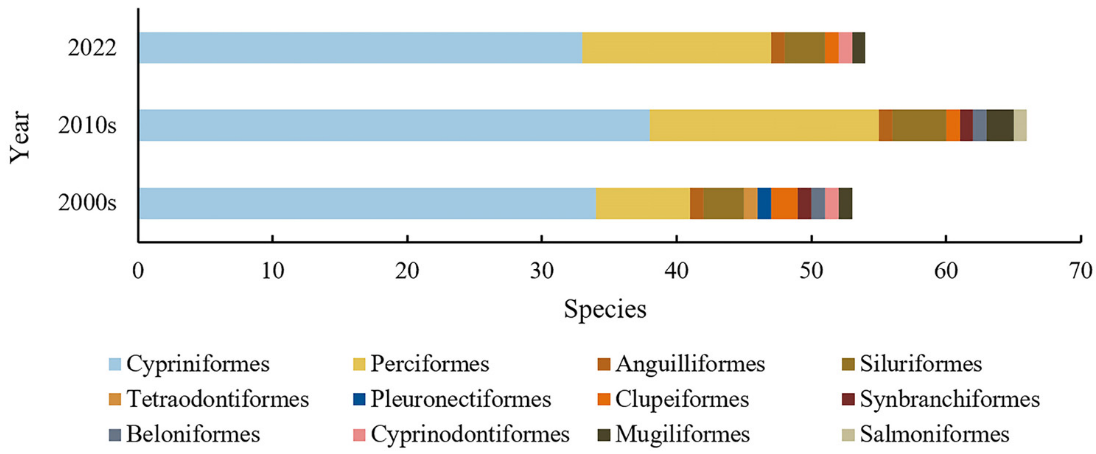
Drainage Basin	Site	Species		
		Channa maculata	*Misgurnus bipartitus*	*Micropterus salmoides*
Huangpu River	SH1	√	√	√
	SH3		√	√
	SH12		√	√
	SH13		√	√
	SH14		√	√
	SH15		√	
	SH16		√	√
Suzhou River	SH2		√	
	SH4			
	SH5		√	√
Dianshan Lake	SH6		√	
	SH7		√	√
	SH8		√	√
	SH9		√	
	SH10		√	√
	SH11		√	√

3.2.3. Comparison with Historical Data

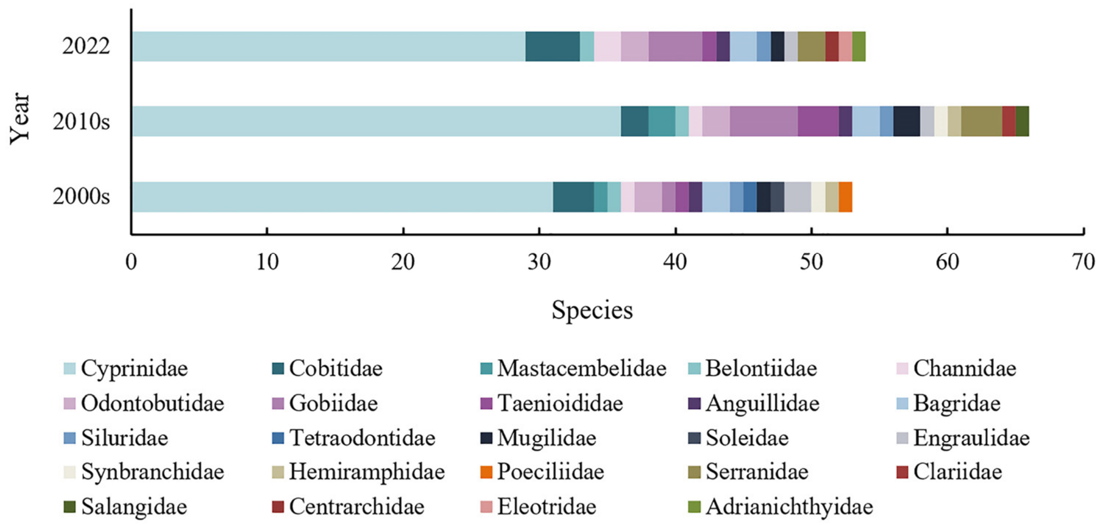
To compare the eDNA survey results with historical data obtained using traditional sampling methods [22,23,52–55], the number of fish species in Shanghai at different time periods was compiled. The survey results from the early 21st century, the 2010s, and the present study revealed 56, 68, and 54 fish species, respectively. However, it is important to note that there were cases of taxonomic changes and synonymous names for certain fish species. For example, *Culter dabryi*, *Chanodichthys dabryi*, and *Erythroculter dabryi* were different names for the same species. Similar cases of synonymous names were observed for *C. mongolicus* and *Culter mongolicus*, *C. alburnus* and *Cultrichthys erythropterus*, *Micropercops swinhonis* and *H. swinhonis*, and *T. nitidus* and *Pelteobagrus nitidus*. Furthermore, the classification of *Chanodichthys erythropterus* and *Culter alburnus* has changed in recent years, with *C. alburnus* being reclassified as *Chanodichthys erythropterus* and *C. erythropterus* as *Culter alburnus*. As a result, the data were recompiled for a comprehensive comparison, as shown in Appendix B.

From Appendix B, it can be observed that the early 21st century, the 2010s, and the present study reported 53, 66, and 54 fish species in Shanghai, respectively. The overall composition of fish species in these different time periods is depicted in Figure 5.

In comparison with the historical data, the present study detected 38 fish species that were previously recorded and discovered 16 fish species that were not identified using traditional methods. Additionally, 42 fish species had historical records but were not identified with the eDNA analysis used in this study (Figure 6).



(**a**)



(**b**)

Figure 5. Composition of fish species in different time periods. (**a**) Composition at the order level and (**b**) composition at the family level. The results for 2022 are from this study.

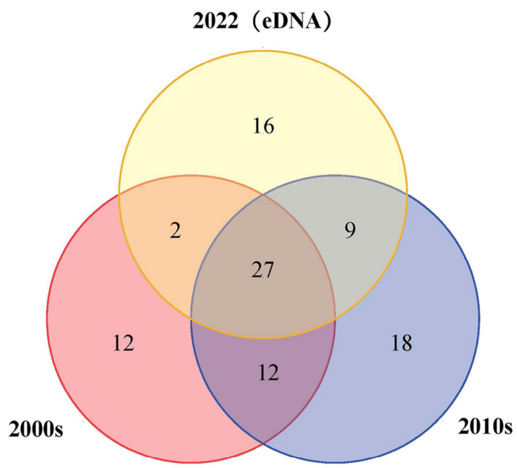


Figure 6. The number of fish species detected in Shanghai in each year. The results for 2022 are from the eDNA analysis conducted in this study.

4. Discussion

4.1. Invasion Risk Factors of Non-Native Fish Species in Shanghai

4.1.1. Introduction and Geographic Factors

The rapid development of the economy, trade, and shipping has led to an increasing frequency of transnational or trans-basin introductions of organisms [17]. For instance, Shanghai's annual consumption of aquatic products is approximately 850,000 tons, with 80% of the products consumed coming from outside Shanghai. The introduction of fish species across regions could contribute to the invasion risk of non-native fish species in Shanghai [3]. The largemouth black bass, due to its rapid growth, disease resistance, short breeding cycle, high yield, and delicious taste, has become one of the most extensively introduced non-native freshwater fish in China over the past five years [56,57]. As of 2021, largemouth black bass had been introduced for cultivation in 29 provinces and cities, including Guangdong, Jiangsu, Zhejiang, Shanghai, and Shandong. The continuous expansion of aquaculture areas and operational scales increases the likelihood of largemouth black bass entering risk assessment areas [58–60]. The results of this eDNA survey indicate that largemouth black bass are found at 11 sites (Table 2). In addition, in the fall of 2023, we observed the presence of live largemouth black bass at SH4, leading to speculation that the species may have undergone dispersal in Shanghai.

Furthermore, Shanghai shares a similar climate type with the native habitat of largemouth black bass, which is a crucial factor of high invasion risk. The native habitat of largemouth black bass is located in the eastern United States, where the climate is mostly subtropical and humid (Cfa), similar to the climate type in Shanghai [61]. Therefore, after being introduced to Shanghai, largemouth black bass can adapt to local climatic conditions and maintain populations in the region. Similarly, the high risk of invasion of the mosquitofish is partly attributed to its introduction to a region with a climate type similar to its native habitat.

4.1.2. Life History Traits

The invasion process of non-native fish species consists of five stages: introduction, establishment, latency, spread, and outbreak [62]. Successfully invading freshwater fish often possess life history traits favorable for invasion, such as high reproductive capacity, broad diet, rapid growth, and physiological tolerance [63–66]. Of the evaluated fish species, the mosquitofish demonstrate strong ecological adaptability and reproductive capacity [67]. It outcompetes other fish species for living space, preys on their food, and directly suppresses these fish species. The Shanghai native *Oryzias sinensis* has been particularly affected by the mosquitofish. Their ecological niches are nearly identical, but the mosquitofish exhibits higher reproductive capacity and aggression; therefore, it may crowd out *O. sinensis*. In addition to the mosquitofish, green sunfish have been found to prey on the eggs and fry of other fish species, posing a significant threat to the survival and reproduction of other fish species [68]. The suckermouth catfish (*H. plecostomus*) exhibits high environmental tolerance and can even survive temporarily without water [69]. The assessment results indicate that these species are categorized as having a medium-to-high invasion risk. Therefore, high reproductive capacity and environmental tolerance are considered to be contributing factors to the accelerated invasion of fish species.

4.1.3. Climate Change

Climate change may interact with other environmental factors, thereby influencing the distribution and reproduction of non-native fish species [60,70,71]. It is anticipated that by the mid-21st century, the national average temperature will exhibit a consistent warming trend, with a temperature increase ranging from 1.2 to 2.8 °C. The annual average precipitation is also expected to increase, with a variation range of −0.1 to 1.1 mm/d in the summer and −0.2 to 0.2 mm/d in the winter. In the Yangtze River's middle and lower reaches, there will be a consistent increase in annual average precipitation between the winter and summer [72]. The temperature rise is likely to enhance the invasion risk of warm-water species such as green sunfish (*Lepomis cyanellus*), North African catfish (*Clarias gariepinus*), redbelly tilapia (*Coptodon zillii*), and largemouth black bass (*Micropterus salmoides*). On the other hand, the invasion risk of cold-water species like rainbow trout (*Oncorhynchus mykiss*) is expected to decrease. Increased precipitation may trigger flood disasters, providing conditions for the spread of some introduced fish species. For instance, during flood events, *I. punctatus* and *H. plecostomus* are known to escape [69,72,73]. Therefore, under future climate change conditions, the invasion risk of these two fish species in Shanghai may increase.

4.2. Discrepancies and Factors in Fish Diversity Survey Results between Traditional Sampling Methods and eDNA Metabarcoding Analysis

From Appendix B, it can be seen that this eDNA survey found some fish species that cannot be identified as known species. This could be one of the reasons for the deviation between the eDNA survey results and historical data. These fish species belong to the genera *Carassius*, *Hemibarbus*, *Cobitis*, *Micropercops*, and *Coilia*. After analysis and investigation by fishery experts, it is inferred that these fish species are likely to be *Carassius auratus*, *H. maculatus*, *Cobitis taenia*, *M.* sp., and *Coilia nasus*. This is because each genus mentioned above is likely to correspond to only one species in the water bodies in and around Shanghai. For these species, we can redesign primers specifically for these groups in the future, increasing the amplicon length to enhance primer specificity and identify specific species. Regarding the other fish species with differences between this study and historical traditional survey results, we will discuss them in two parts.

4.2.1. Fish Species Detected with eDNA Metabarcoding Analysis but Not with Traditional Methods

There are three main reasons why some fish species can be detected with eDNA metabarcoding analysis but not with traditional methods:

(1) Environmental impact

In terms of the environment, we found that changes in water quality may be a key factor affecting fish diversity. According to the "Statistical Data on the Protection of Water Environment in Shanghai" released by the Shanghai Ecological and Environmental Bureau, around 2008, the discharge of industrial wastewater in Shanghai reached 419 million tons. This pollution could lead to the continuous depletion of dissolved oxygen in the water, further harming the ecosystem and causing a reduction in fish diversity. For example, some perciforms have a high requirement for dissolved oxygen [74,75]. In the early 21st century, the absence of perciform fish species may have been attributed to low dissolved oxygen conditions in the water. To address this issue, Shanghai has adjusted its industrial structure and layout, closing a large number of enterprises that discharged pollutants along the river. Substantial funds have been continuously invested in sewage treatment projects, steadily improving the water quality in Shanghai. By 2021, the discharge of industrial wastewater in Shanghai had decreased to 321 million tons. Therefore, in this survey, we observed the presence of *Lateolabrax maculatus* and *S. chuatsi*, species belonging to Perciformes. This may be a result of improved water quality.

(2) Policy impact

To protect fish diversity in water bodies, Shanghai has periodically conducted artificial breeding and releases [76]. According to the "2020–2022 Catalog of Aquatic Resources Artificial Breeding and Release in Natural Water Bodies in Shanghai" released by the Shanghai Agriculture and Rural Affairs Committee in 2019, species such as *S. chuatsi*, *Sarcocheilichthys sinensis*, and *L. maculatus* were listed as target species for artificial breeding and release. Looking at the comparative results in Appendix B, it can be seen that these three fish species were not found in the early 21st century but were discovered in this study. These data indirectly demonstrate the positive effect of Shanghai's measures for artificial breeding and release on fish diversity.

(3) Methodology impact

Historical surveys often utilized traditional sampling methods, using tools such as gill nets, trawl nets, and traps. While these methods directly observe the targeted species, their sensitivity is relatively low [77]. In contrast, the eDNA metabarcoding analysis used in this survey has a relatively higher sensitivity [78,79] and is capable of detecting target organisms at low densities within complex biological communities [43]. However, the high sensitivity of eDNA metabarcoding analysis also makes it susceptible to the influence of the surrounding environment. For example, fish species such as *Cobitis sinensis*, *Rhynchocypris oxycephalus*, *Opsariichthys uncirostris*, and *Microphysogobio microstomus*, which were detected in this survey, are only distributed upstream of the Huangpu River in Huzhou, making it unlikely for them to exist in the wild waters of Shanghai. Their DNA might have been introduced into the monitoring area with the discharge of wastewater from aquaculture facilities or carried by water flow. Additionally, artificial hybridized individuals of the introduced species *Channa maculata* and the local Shanghai species *C. argus* are common in aquaculture [80]. Therefore, the DNA detected in this study is highly likely to be from hybridized *C.* and not from pure *C. argus*. The differences in sensitivity and accuracy between the two methods contribute to discrepancies in the monitoring results.

4.2.2. Fish Species Detected with Traditional Methods but Not with eDNA Metabarcoding Analysis

There are two main reasons why some fish species can be detected with traditional methods but not with eDNA metabarcoding analysis:

(1) Manual identification in traditional sampling methods

One limitation of traditional sampling methods is the potential for subjective biases during the classification process by monitoring personnel, which may affect the accuracy of the monitoring results. In the historical survey results, *E. ilishaeformis*, *Odontobutis obscura*, *Odontamblyopus rubicundus*, *Coilia brachygnathus*, *M. fukiensis*, and *Tridentiger trigonocephalus* were all recorded. However, after identification by fishery experts, it was judged that these species are unlikely to be found within Shanghai. The historical results might be attributed to past identification errors. For example, based on FishBase records, *O. rubicundus* is widely distributed in the Indo-West Pacific region, with records of its distribution found only along the coastal areas of Liaoning in China [81], and there is no record of its distribution in Shanghai or its surrounding areas. Many scholars in the past have misidentified *O. lacepedii* as *O. rubicundus*. Therefore, the records of *O. rubicundus* from the early 21st century and the 2010s may also be the result of identification errors.

(2) Sampling intensity and location

Statistically, among the 41 fish species not detected in this study, 32 (78.05%) are demersal fish species, including *Culter oxycephalus*, *Taenioides cirratus*, *Synechogobius hasta*, and *Rhodeus lighti*. The phenomenon of a large number of demersal fish species not being detected may be due to the lack of stratified sampling in this study. In addition, our sampling sites were mainly concentrated in the main river channel, where riverbanks were cement-filled; however, *G. affinis* were mainly found in swamps with natural banks and aquatic plants, which may have resulted in the non-detection of *G. affinis* in this study. In terms of sampling intensity, this study only conducted surveys for a single season and had a limited number of sampling points. In contrast, the historical data integrated year-round data, and previous studies did not simultaneously monitor fish in the three main rivers of Shanghai. Instead, they focused mainly on specific watersheds and set more sampling points. For instance, in studies on the fish diversity of the Suzhou River, this survey only set 3 sampling points, while previous studies set 10 and 13 sampling points, respectively. This may be the reason for the lower number of fish species detected in the Suzhou River in this study (Figure 7).

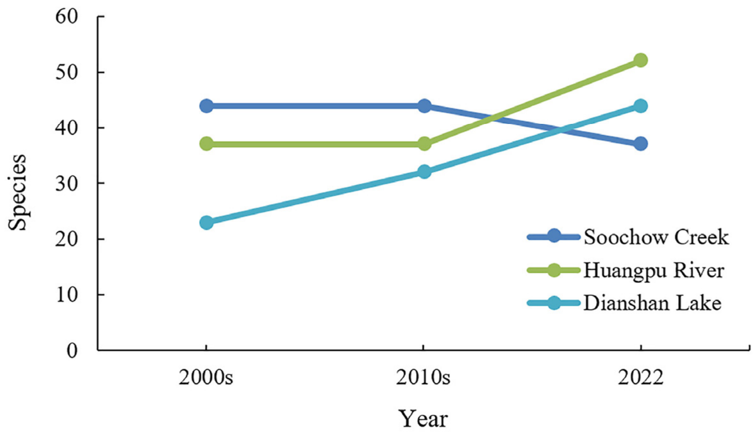


Figure 7. Interannual variation in the number of fish species in the three main rivers of Shanghai.

4.3. Strategies for Prevention and Control of Invasive Fish Species

4.3.1. Enhancement of Regulatory Framework

In recent years, China has recognized the hazards of biological invasions, including those by fish species. Various departments have jointly formulated laws and regulations related to alien species to strengthen the prevention and control management and hazard governance of invasive species. For instance, the "Biosafety Law of the People's Republic of China" issued in 2020 provides comprehensive arrangements for biosafety risk prevention and control [82]. The "Management Measures for Alien Invasive Species" released in 2022 establishes and improves the management system for alien invasive species, strengthening joint prevention and control, and upgrading the national management level for invasive species. Although China has achieved some positive results in the prevention and control of invasive species, legislation and research in this regard are still in their early stages, and policies and regulations for preventing and controlling invasive species are still inadequate and preliminary [83]. In the prevention and control management of invasive fish species, legislative authorities should subsequently formulate targeted quarantine standards and norms based on the invasion process of alien fish species (such as introduction pathways, possible sources, and hazard levels) and establish a systematic prevention and control management system [84]. Additionally, in the compilation of the list of invasive fish species, potentially harmful alien fish species should be considered to reduce blind spots and gaps in subsequent quarantine processes.

4.3.2. Strengthening Responsibilities of All Parties

China still faces challenges regarding the inadequate management intensity in the prevention and control of invasive fish species. To address this issue, efforts should be made to enhance the training of personnel in industries such as quarantine, international trade, and transportation, increasing their vigilance against early fish invasions [12]. On the other hand, specialized institutions should be established to supervise the work of various management departments, strengthen communication between departments, and ensure the coordination and consistency of management processes [84].

4.3.3. Improvement of Key Technologies

Currently, China lacks a unified standard for the risk assessment of invasive fish species [85]. There is an urgent need to establish an effective and operational risk assessment protocol for the invasion of alien fish species in the Chinese context. Additionally, the establishment of an expert committee to provide technical support for the risk assessment of invasive fish species is necessary. In the field of monitoring invasive fish species, technologies such as eDNA should be improved to establish a mature monitoring system to understand the invasion process and dispersal pathways of alien fish species. Moreover, for key processes such as port quarantine, biological control, and ecological restoration, practical technologies, products, and equipment for rapid identification, efficient capture, and biological control of invasive fish species should be developed to swiftly identify and eliminate invasive species [86].

4.3.4. Raising Awareness and Education Quality

The principle of public participation forms the basis of the early warning mechanism for invasive species and is a crucial driver of the early warning mechanism [12]. On the one hand, using media such as television broadcasts and the internet, online popular science campaigns on the prevention and control of invasive fish species should be conducted. On the other hand, institutions such as museums and schools can organize related science activities to explain to the public the biological characteristics, transmission pathways, hazard levels, and elimination methods of invasive fish species. This can enhance public awareness of ecological security and ignite societal interest in the protection of our natural waterways from the invasion of alien species.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d16010029/s1, Table S1. Species composition of fishes at various sites in Shanghai based on the eDNA metabarcoding analysis.

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Appendix A

Table A1. Composition of fish species in Shanghai.

Order	Family	Genus	Species	Dianshan Lake	Huangpu River	Suzhou River
Cypriniformes	Cyprinidae	*Parabramis*	*Parabramis pekinensis*	√	√	√
		Chanodichthys	*Chanodichthys dabryi*	√	√	
			Chanodichthys erythropterus	√	√	√
			Chanodichthys mongolicus	√	√	
		Culter	*Culter alburnus*	√	√	
		Hemiculter	*Hemiculter leucisculus*	√	√	√
		Ctenopharyngodon	*Ctenopharyngodon idella*	√	√	√
		Megalobrama	*Megalobrama terminalis*		√	
		Elopichthys	*Elopichthys bambusa*		√	√
		Rhynchocypris	*Rhynchocypris oxycephalus*	√	√	√
		Hemibarbus	*Hemibarbus* sp.	√	√	√
		Carassius	*Carassius* sp.	√	√	√
		Cyprinus	*Cyprinus carpio*	√	√	√

Table A1. *Cont.*

Order	Family	Genus	Species	Dianshan Lake	Huangpu River	Suzhou River
		Hypophthalmichthys	*Hypophthalmichthys molitrix*	√	√	
			Hypophthalmichthys nobilis	√	√	
		Opsariichthys	*Opsariichthys bidens*	√	√	√
		Pseudorasbora	*Pseudorasbora parva*	√	√	√
		Rhodeus	*Rhodeus ocellatus*	√	√	√
		Mylopharyngodon	*Mylopharyngodon piceus*		√	√
		Sarcocheilichthys	*Sarcocheilichthys nigripinnis*	√	√	√
			Sarcocheilichthys sinensis	√		
		Pseudobrama	*Pseudobrama simoni*	√	√	√
		Microphysogobio	*Microphysogobio* sp.	√	√	√
		Acheilognathus	*Acheilognathus imberbis*	√	√	√
			Acheilognathus macropterus	√	√	√
			Acheilognathus barbatulus	√	√	√
			Acheilognathus chankaensis	√	√	√
		Abbottina	*Abbottina rivularis*	√	√	√
		Zacco	*Zacco acanthogenys*	√	√	√
	Cobitidae	*Paramisgurnus*	*Paramisgurnus dabryanus*	√	√	√
		Cobitis	*Cobitis* sp.	√	√	√
		Misgurnus	*Misgurnus bipartitus* *	√	√	√
			Misgurnus anguillicaudatus	√	√	√
Perciformes	Belontiidae	*Macropodus*	*Macropodus ocellatus*			√
	Centrarchidae	*Micropterus*	*Micropterus salmoides* *	√	√	√
	Serranidae	*Lateolabrax*	*Lateolabrax maculatus*		√	
		Siniperca	*Siniperca chuatsi*	√		
	Channidae	*Channa*	*Channa maculata* *		√	
			Channa argus	√	√	√
	Eleotridae	*Eleotris*	*Eleotris oxycephala*		√	
	Odontobutidae	*Odontobutis*	*Odontobutis potamophila*	√	√	√
		Micropercops	*Micropercops swinhonis*	√	√	√
	Gobiidae	*Rhinogobius*	*Rhinogobius giurinus*	√	√	√
			Rhinogobius cliffordpopei	√	√	√
		Mugilogobius	*Mugilogobius myxodermus*		√	
		Tridentiger	*Tridentiger bifasciatus*	√	√	
	Taenioididae	*Odontamblyopus*	*Odontamblyopus lacepedii*	√	√	
Anguilliformes	Anguillidae	*Anguilla*	*Anguilla japonica*		√	
Siluriformes	Bagridae	*Tachysurus*	*Tachysurus fulvidraco*	√	√	
			Tachysurus nitidus		√	
	Siluridae	*Silurus*	*Silurus asotus*	√	√	√
Mugiliformes	Mugilidae	*Mugil*	*Mugil cephalus*		√	
Clupeiformes	Engraulidae	*Coilia*	*Coilia* sp.	√	√	√
Cyprinodontiformes	Adrianichthyidae	*Oryzias*	*Oryzias sinensis*	√	√	√

"*" indicates that the fish is exotic.

Appendix B

Table A2. Number of fish species detected in Shanghai each year.

Order	Family	Genus	Species	2000s	2010s	2022
Cypriniformes	Cyprinidae	*Parabramis*	*Parabramis pekinensis*	√	√	√
		Chanodichthys	*Chanodichthys dabryi*	√	√	√
			Chanodichthys mongolicus		√	√
			Chanodichthys erythropterus	√	√	√
		Culter	*Culter oxycephalus*		√	
			Culter alburnus		√	√
		Erythroculter	*Erythroculter ilishaeformis*	√		

Table A2. *Cont.*

Order	Family	Genus	Species	2000s	2010s	2022
		Hemiculter	*Hemiculter bleekeri*	√	√	
			Hemiculter leucisculus	√	√	√
		Ctenopharyngodon	*Ctenopharyngodon idella*	√	√	√
		Squaliobarbus	*Squaliobarbus curriculus*	√		
		Megalobrama	*Megalobrama mantschuricus*	√	√	√
			Megalobrama amblycephala	√	√	
		Elopichthys	*Elopichthys bambusa*			√
		Xenocypris	*Xenocypris microlepis*		√	
			Xenocypris davidi		√	
			Xenocypris argentea		√	
		Rhynchocypris	*Rhynchocypris oxycephalus*			√
		Hemibarbus	*Hemibarbus maculatus*	√	√	√
		Carassius	*Carassius auratus*	√	√	√
		Cyprinus	*Cyprinus carpio*	√	√	√
			Cyprinus carpio var. mirror *	√		
		Hypophthalmichthys	*Hypophthalmichthys molitrix*	√	√	√
			Hypophthalmichthys nobilis	√	√	√
		Opsariichthys	*Opsariichthys bidens*			√
		Pseudorasbora	*Pseudorasbora parva*	√	√	√
		Rhodeus	*Rhodeus lighti*	√		
			Rhodeus ocellatus	√	√	√
		Pseudolaubuca	*Pseudolaubuca engraulis*	√	√	
			Pseudolaubuca sinensis	√	√	
		Mylopharyngodon	*Mylopharyngodon piceus*	√		√
		Sarcocheilichthys	*Sarcocheilichthys nigripinnis*	√	√	√
			Sarcocheilichthys sinensis		√	√
		Pseudobrama	*Pseudobrama simoni*	√	√	√
		Paracanthobrama	*Paracanthobrama guichenoti*		√	
		Toxabramis	*Toxabramis swinhonis*	√	√	
		Microphysogobio	*Microphysogobio fukiensis*		√	
			Microphysogobio microstomus			√
		Acheilognathus	*Acheilognathus taenianalis*	√		
			Acheilognathus imberbis			√
			Acheilognathus macropterus	√	√	√
			Acheilognathus barbatulus	√		√
			Acheilognathus chankaensis	√	√	√
			Acheilognathus tonkinensis	√		
		Distoechodon	*Distoechodon hupeinensis*		√	
			Distoechodon tumirostris		√	
		Squalidus	*Squalidus argentatus*		√	
		Abbottina	*Abbottina rivularis*	√	√	√
		Zacco	*Zacco acanthogenys*			√
	Cobitidae	*Paramisgurnus*	*Paramisgurnus dabryanus*	√	√	√
		Cobitis	*Cobitis taenia*	√		√
		Misgurnus	*Misgurnus bipartitus* *			√
			Misgurnus anguillicaudatus	√	√	√
Perciformes	Mastacembelidae	*Mastacembelus*	*Mastacembelus aculeatus*	√	√	
		Sinobdella	*Sinobdella sinensis*		√	
	Belontiidae	*Macropodus*	*Macropodus ocellatus*	√	√	√
	Centrarchidae	*Micropterus*	*Micropterus salmoides* *			√
	Serranidae	*Lateolabrax*	*Lateolabrax maculatus*		√	√
		Siniperca	*Siniperca knerii*		√	
			Siniperca chuatsi		√	√
	Channidae	*Channa*	*Channa maculata* *			√
			Channa argus	√	√	√
	Eleotridae	*Eleotris*	*Eleotris oxycephala*			√

Table A2. *Cont.*

Order	Family	Genus	Species	2000s	2010s	2022
	Odontobutidae	*Odontobutis*	*Odontobutis potamophila*		√	√
			Odontobutis obscura	√		
		Micropercops	*Micropercops swinhonis*	√	√	√
	Gobiidae	*Rhinogobius*	*Rhinogobius giurinus*	√	√	√
			Rhinogobius cliffordpopei		√	√
		Synechogobius	*Synechogobius hasta*		√	
		Mugilogobius	*Mugilogobius myxodermus*			√
		Tridentiger	*Tridentiger trigonocephalus*		√	
			Tridentiger bifasciatus		√	√
	Taenioididae	*Odontamblyopus*	*Odontamblyopus lacepedii*		√	√
			Odontamblyopus rubicundus	√	√	
		Taenioides	*Taenioides cirratus*		√	
Anguilliformes	Anguillidae	*Anguilla*	*Anguilla japonica*	√	√	√
Siluriformes	Bagridae	*Tachysurus*	*Tachysurus fulvidraco*	√	√	√
			Tachysurus nitidus	√	√	√
	Siluridae	*Silurus*	*Silurus asotus*	√	√	√
	Clariidae	*Clarias*	*Clarias gariepinus* *		√	
Tetraodontiformes	Tetraodontidae	*Takifugu*	*Takifugu obscurus*	√		
Mugiliformes	Mugilidae	*Liza*	*Liza haematocheila*		√	
		Mugil	*Mugil cephalus*	√	√	√
Pleuronectiformes	Soleidae	*Cynoglossus*	*Cynoglossus gracilis*	√		
Clupeiformes	Engraulidae	*Coilia*	*Coilia nasus*	√	√	√
			Coilia brachygnathus	√		
Synbranchiformes	Synbranchidae	*Monopterus*	*Monopterus albus*	√	√	
Beloniformes	Hemiramphidae	*Hyporhamphus*	*Hyporhamphus intermedius*	√	√	
Salmoniformes	Salangidae	*Salangichthys*	*Salangichthys tangkahkeii*		√	
Cyprinodontiformes	Adrianichthyidae	*Oryzias*	*Oryzias sinensis*			√
	Poeciliidae	*Gambusia*	*Gambusia affinis* *	√		

"*" indicates that the fish is exotic.

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