FISHERIES ECOLOGICAL ENVIRONMENT IN SOUTH CHINA SEA

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Zhenhua Ma and Chao Song

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frontiers Research Topics

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FISHERIES ECOLOGICAL ENVIRONMENT IN SOUTH CHINA SEA

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Editorial: Fisheries ecological environment in South China Sea

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Editorial on the Research Topic
Fisheries ecological environment in South China Sea

The South China Sea is a marginal sea of the Western Pacific Ocean, encompassing around 3.5 million km^2. The sea is of abundant fishery resources and provides many important sites for the coastal aquaculture industry. Most of the countries in the coastal fringes of the South China Sea are developing or underdeveloped economies that are home to about 270 million people. Therefore, the fisheries and aquaculture industry is a crucial sector for the development of coastal countries. Millions of people derive their main income directly or indirectly from fisheries. The development of fisheries and aquaculture in the South China Sea has driven significant increases in food and nutrition and has improved livelihoods, employment, and local economic development among the coastal countries.

However, the intensive anthropogenic activities have posed dramatically negative impacts on the fisheries and environment in the South China Sea and weakened the development potential of fisheries and aquaculture around the coastal countries and the people's livelihoods in the future. To make fisheries, ecosystems, and resident livelihoods sustainable, there is a need for new ideas to face the challenges of fisheries and the South China Sea environment. This particular Research Topic of Frontiers in Environmental Science Section Fisheries Ecological Environment in the South China Sea includes thirteen original research articles, two methods articles, and two reviews.

Pressures from human activities not only decrease the diversity of community structure and environmental pollution to make the ecosystem worsen but also introduce hazards into the environment. With rapid industrial and economic development, the coastal bays around the South China Sea are also subjected to the potential risk of heavy metal pollution arising from anthropogenic activities. Wang et al. found that hazardous elements such as Cd, Cr, Hg, As, Cu had been all detected in Daya Bay. Their concentrations are affected by human activities such as sewage outlets, water channel dredging, and shellfish farming, which has made Daya bay at moderate ecological risk or a considerable ecological risk. Special concerns should be paid to the rare Earth elements. Elements like La, Nd, Sm, Ho, Er, and Lu has been detected in the seawater environment of the South China Sea (Li et al.). These rare Earth elements have been extensively used in several industries and high-tech devices, such as petrochemical, ceramic, metallurgy, laser, and fiber optic industries. However, little is known about their function in the ecosystem.

Antibiotics are an effective approach for the prevention and treatment of various bacterial diseases in aquaculture. However, the overuse of antibiotics leads to increased antibiotic residues in the environment. Teicoplanin, a glycopeptide antibiotic, has been detected in environmental water (Jin et al.). The introduction of antibiotics into the water environment would not only increase drug resistance of microorganisms but also affect the growth of miniature aquatic organisms, such as zooplankton and phytoplankton. Shan et al. found that the number of phytoplankton increased but their biodiversity reduced when sulfamethoxazole and enrofloxacin were fed into the culture environment. These two antibiotics cause water pollution by affecting the species and quantity of phytoplankton and changing the dominant species and community structure, which would endanger the living environments of aquatic organisms and increases the dietary risk from fishery products. Therefore, to protect the fishery ecological environment should be paid more attention to antibiotics residue.

Xu et al. analyzed the data collected by four trawl surveys during 2016–2017 in Daya Bay, a typical bay on the coast of the South China Sea. The fish structure in the bay presented miniaturization and a low-weight trend, and the fish community structure was dominatingly composed of smaller size fishes. Rao et al. also found that small body sizes, short longevity, and high tolerance animals were more abundant in macrobenthic communities in the disturbed area of Daya Bay. Similar results have been found in the Rongjiang River estuary reaching the South China Sea. Li. et al. found that nearly 90% of animals in benthic communities were Mollusca and Arthropoda species in the sediment of the Rongjiang estuary. And in eutrophic regions, the survival of sensitive benthic species is restricted, and they are gradually replaced by tolerant species.

As shown above, anthropogenic activities have imposed great pressure on the ecological environment of the fisheries of the South China Sea. The degradation of fishery resources and environment may result in a wide range of social and economic consequences in the countries around the South China Sea. Sustainable fisheries contribute to the marine ecosystem functioning and the livelihoods of the millions of fishermen. Therefore, much innovative thinking has been proposed to solve the challenges of the ecological environment of the fisheries of the South China Sea.

He and Zhang proposed to revamp the fisheries law of China to combat illegal, unreported, and unregulated (IUU) fishing. They gave three main design and operation features. Firstly, an unbroken and immutable electronic information capture, transmission, and verification system linked to the nationwide fisheries legality label and its local subsidiaries, to ensure traceable and trackable seafood movement inside and outside China. Secondly, a precautionary and market-responsive approach to engaging public, private, and societal partnerships in adopting sustainable seafood production and consumption practices, and incentivizing nationwide awareness and whistleblowing of IUU catches, especially from imported sources. Finally, a collaborative inter-agency model of information sharing, regulatory coordination, and legal enforcement to embed seafood traceability in multiple relevant legal regimes and across all responsible governmental agencies in China. Through the technology-enabled traceability and market-responsive solutions, it may help to mitigate illicit capturing and transaction events and decrease the potential of overfishing. I think it is suitable for all countries around the South China Sea.

Solutions to fishery resource proliferation and environment restoration were also proposed. In order to fishery resource proliferation, the summer fishing moratorium system began in the South China Sea in 1999. Now the system has become the most important and influential fishery resource conservation system in China (Zhu, 2009), and made a great contribution to alleviating the enormous pressure on marine fishery resources caused by the excessive fishing intensity and protecting China's offshore fishery resources, especially the spawning fish and juvenile fish. However, the system still has much more space to be improved. Wang et al. investigated the life history of the estuarine fish from the western Pearl River estuary. They found that the spawning period of the fish is from March to September, which was longer than the summer fishing moratorium of Pearl River. Therefore, if this species is to be better protected and managed, the moratorium period needs to be extended. Zhang et al. found that the three-and-a-half-month fishing moratorium has led to an income reduction for fishermen and illegal fishing boat poaching increase. Therefore, the summer fishing moratorium system should be improved by adjusting the subsidies for fishermen and moratorium duration to find a balanced system between fishermen, people, and the system so that it can be sustainable.

Fish stock enhancement is another effective tool in fishery resource proliferation, which could enhance the wild population, increase food fish supplies, and improve the fishermen's livelihood (Booth and Cox, 2003; Leber, 2011; Kitada et al., 2019). Marine fish release is one important method of fish stock enhancement. However, the success of fish release is affected by many factors such as release timing, habitat environment, fish species, and fish health condition (Camp et al., 2013). Zhang et al. proposed an optimizing release strategy of black sea bream in the northern South China Sea. Firstly, the period from June to October was recommended for black sea bream releasing. Secondly, multiple release sites and release batches could reduce the drastic increase in mortality rate due to over-high stocking density. This improvement in fish release would increase the survival rate of fish as well as the fish stock enhancement.

Another important measure for fish stock enhancement is protecting the fish population. The continental shelf of the northern South China Sea is a key habitat for nearly 1,500 fish species (Sun and Chen, 2013). This region is an important spawning and nursing ground for numerous fishes (Zhang et al., 1985). Therefore, the protection of spawning zones is also very important for fisheries ecology and fishery resource proliferation. Xu et al. found the population of *E. cardinalis* concentrated in the northern Beibu Gulf to spawn from November to February. In early spring, parent fish mainly occur in the northeast of the gulf after spawning and juveniles concentrate in the shallow nearshore of this area in late spring. Hou et al. identified the eggs and spawning zones of Hairtail fish in the Northern South China Sea. They found that the spawning grounds of the fish shifted with hydrological conditions. In spring, eggs of *T. japonicus* and *T. nanhaiensis* occurred at central and southern Beibu Gulf water mass and South China Sea surface water mass, while in late summer-autumn, their eggs mainly occurred in the waters of South China Sea surface water mass. The results could help us to adjust the timing and spawning zone of the fish and enhance the protection.

The restoration of the environment could have a great positive effect on the benefit to fishery resource proliferation and the ecology system of the South China Sea. Ren et al. investigated the fish assemblages in subtidal seagrass meadows surrounding the West Sand, South China Sea. A total of 843 individuals from 25 fish species belonging to 22 genera, 11 families, and ten orders were recorded in seagrass meadows. And the fish abundance was highly correlated with the seagrass coverage. It indicated that seagrass meadows could greatly enhance the biological diversity and biomass in the sea environment. Moreover, 80.36% and 46.13% of total individuals collected by beach seine and underwater visual census methods were earlier-stage juveniles, less than half the sizes of their total length at maturity, respectively. It indicated that the seagrass meadows at West Sand were very important fish nursery grounds and have played a very important ecological service.

Wu et al. investigated the composition of fish intestinal microbiota from waters along the Pearl River Estuary and tried to reveal water microbiota influences fish intestinal microbiota in different estuary habitats. The microbial exchange showed an increasing tendency from the upstream to downstream points. It indicated the potential of fish adaption to the environment increased. It provides a new view to restore the fish population in the estuary zone.

Meng et al. studied the interaction effects of environmental factors on beneficial algae and harmful algae. It was found that temperature, light, nutrients, and pH significantly influenced the competition inhibition parameters of Chlorella vulgaris and Anabaena sp. strain PCC 1042. Therefore, the growth of Chlorella vulgaris and Anabaena sp. strain PCC can be controlled by changing the environmental factors so as to mediate the water quality.

Sun et al. have studied the changes in microbial ecology driven by the culture environment. The study demonstrated that aquaculture considerably altered physicochemical factors, induced changes in bacterial community composition, and increase the metabolic capabilities of the bacterial Communities in pond water. It provides a scientific basis for the management of aquaculture environments and could be helpful to the water quality management of aquaculture areas.

As mentioned above, the scientists in the c have dedicated to fishery resource proliferation and ecological environment protection. However, these are far from enough, because it is a very complicated project to restore the ecological environment of the fisheries of the South China Sea. It needs long-term endeavors from many scientists from different research fields. Therefore, the aim we proposed such a Research Topic is to raise concerns about the ecological environment of the South China Sea and work together to protect the sea we live on.

Author contributions

CK wrote the manuscript of the Editorial, the other guest editors of the Research Topic reviewed and revised the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The Composition of Intestinal Microbiota From *Collichthys lucidus* and Its Interaction With Microbiota From Waters Along the Pearl River Estuary in China

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By their nature and geographical location, estuaries shape different marine habitats via freshwater and seawater interactions. Thus, fish intestinal microbiota, as mediated by estuary habitat fluctuations, are fundamentally important but rarely studied. Similarly, it is unclear how, and to what extent, water microbiota influences fish intestinal microbiota in different estuary habitats. In this study, the euryhaline fish species, *Collichthys lucidus* from three different habitats in the Pearl River estuary (PRE) was investigated to determine the influence of habitat fluctuation on intestinal microbiota. The three water environments selected for sample collection were very different, particularly for chlorophyll-a, suspended solid, and nutrient constituents. Using high-throughput sequencing of 16S rRNA gene amplicons, we observed that dominant microbial genera in surrounding estuary waters or fish intestines were seldom shared. The most dominant genera in water samples were Candidatus *Actinomarina* and HIMB11, while *Bifidobacterium*, *Stenotrophomonas*, *Escherichia-Shigella* and *Rhodopseudomonas* were more abundant in fish intestines. Fish hosts can shape fish intestinal microbiota. However, microbial exchange was also found between fish intestines and water samples. The frequency of microbial exchange between fish intestines and water samples was increased from upstream to downstream estuary points, and was influenced by changes in seawater salinity in the estuary. Finally, core intestinal microbiota from *C. lucidus* was analyzed, and showed that *Bifidobacterium*, *Rhodopseudomonas*, *Escherichia-Shigella*, *Acinetobacter,* and *Stenotrophomonas* were highly abundant. These microbiota were theoretically implicated in immune responses, nutrient metabolism, probiotics, and potential pathogen behaviors. Overall, these data highlighted the composition of *C. lucidus* intestinal microbiota in different habitats across the PRE.

Keywords: microbial exchange, water environment, host effects, water microbiota, habitat transition

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INTRODUCTION

Estuaries are transition zones between the land and the sea, and represent a dynamic system where freshwater meets seawater. These interactions make estuaries unique habitats in terms of habitat diversity and species productivity (Mitra, 2015; Kamrani et al., 2016; Zhou et al., 2019b). It was previously shown that protective estuary environments may facilitate the generation of over half of all marine fish (Mitra, 2015). Moreover, spatial differences in fish assemblages in estuaries were primarily attributed to unique salinity, water temperature, primary productivity, turbidity, and water nutrient conditions (Eick and Thiel, 2014; Zhou et al., 2019b). However, insights on how fish adapt to variable physico-chemical features in these environments requires further investigation (Molina et al., 2020).

The intestinal microbiota is considered an "extra organ", and plays a key role in fish adaption to the environment, mediating nutrient metabolism, immune responses, and gut homeostasis (Li et al., 2015; Egerton et al., 2018; Butt and Volkoff, 2019). Fish intestinal microbiota originate from the eggs, the first feed, and the surrounding waters, and develop a complex, habitat-driven composition (Dehler et al., 2017a; Egerton et al., 2018). Generally, factors affecting intestinal microbiota are categorized as: 1) environmental factors, 2) diet, and 3) host-associated factors (Talwar et al., 2018). Previous studies have focused on fish intestinal microbiota in aquaculture, its relationship with the environment, and demonstrated intestinal microbiota roles for host health and welfare (Dehler et al., 2017a; Egerton et al., 2018; Sun et al., 2019). However, the intestinal microbiota from wild fish requires more investigation (Egerton et al., 2018).

In estuaries, tidal action mixes inland freshwater with seawater, generating spatial variations in salinity, nutrients, oxygen, turbidity, and organic pollutants (Fu et al., 2003; Mitra, 2015; Wu et al., 2017). However, little is known how fish intestinal microbiota respond to these physico-chemical variations. Also, spatial distributions of water microbial communities in estuaries are different, e.g., Proteobacteria classes vary significantly between freshwater and saltwater environments, whereas Actinobacteria are more abundant in freshwater areas (Kirchman et al., 2005; Zhang et al., 2006; Feng et al., 2009). Furthermore, the main sources of microbes in fish come from surrounding waters and their diet (Wu et al., 2012; Dehler et al., 2017a; Egerton et al., 2018). However, limited attention has been given to the exchange of microbial communities between fish intestines and estuary waters. Previous studies have suggested that microbial taxa/operational taxonomic units (OTUs), recognized as core microbiota, are constant in fish, regardless of fish populations or geographical locations (Ghanbari et al., 2015; Givens et al., 2015; Kokou et al., 2019).

The Pearl River estuary (PRE) is a subtropical estuary, located on the south coast of China, with an annual rainfall of 1600–2300 mm (Huang et al., 2003). The PRE is divided into different regions, comprising the Shiziyang Channel, Lingding Bay, and the northern South China Sea (Wu et al., 2014). The Pearl River is the second largest river in China, with an annual average river discharge of 10,524 $m^3 s^{-1}$ (Zhao, 1990; Huang et al., 2003). Approximately 20% of the total flow appears in the dry season from October to March, with 80% in the wet season, from April to September (Zhao, 1990; Huang et al., 2003). In the dry season, seawater covers most of the estuary (Ying, 1994).

Collichthys lucidus is an economically important fish species, widely distributed across the PRE. *C. lucidus* is a small-sized species and often lives in the benthic zones of coastal waters (Liu et al., 2015). This fish is short-lived with a life span of about 3 years and becomes first sexually mature when it is about 80 cm (Zhuang, 2018). *C. lucidus* usually feeds on zoobenthos, small fishes and mysidacea (Zhuang, 2018). In 1986, catch levels in the PRE were reported at 4,000 tons/year, accounting for 26.0% of the total fish biomass by bottom trawling (He and Li, 1988). However, *C. lucidus* populations and biomass have decreased rapidly in recent years (Huang et al., 2018). In this study, we collected *C. lucidus* samples from three different habitats along the PRE, to investigate intestinal microbiota composition, following variations in the water environment. Furthermore, we also investigated relationships between fish intestinal microbiota and water microbiota from individual PRE sites. These data provided key insights on *C. lucidus* intestinal microbiota interactions with estuary water.

MATERIALS AND METHODS

Sample Collection and Measurements

Fish (intestines) and water samples were collected in triplicate at three sampling sites along the PRE, in the dry season (December 2019) (**Supplementary Figure S1**). Site A1 was situated near the Shiziyang Channel, while sites A2 and A3 were located in Lingding Bay and the northern South China Sea, respectively, (**Supplementary Figure S1**). *C. lucidus* was collected from each site using bottom trawling. Three adult fish (average body length; 9.7–10.3 cm) from each site were chosen for intestinal sampling. The whole intestine was aseptically dissected *in situ* using sterile scissors. Then, the contents were squeezed out, collected into sterile plastic cryotubes, and stored in liquid nitrogen for DNA extraction. Water samples were taken from the surface and bottom layers of each individual site, and mixed. Mixed water from each site (1 L) was filtered *in situ* through a 0.2 μm pore-size membrane (Millipore, United States), and the membrane immediately stored in liquid nitrogen for DNA extraction. The remaining water was used for physicochemical analysis following previously published protocols ["The specification for marine monitoring" GB 17378.4 (2007), China]; and Wu et al. (2017). Salinity, temperature, pH and dissolved oxygen (DO) were determined *in situ* using the YSI Pro Plus meter (YSI Inc. Yellow Springs, United States). Chlorophyll a (Chl a) was extracted in 10 ml 90% acetone in the dark for 24 h in a refrigerator and measured using spectrophotometer (Shimadzu, Japan). Oil from the seawater was extracted with n-hexane and measured using UV-spectrophotometer according to the standard curve. The suspended solids from 0.5 L seawater were measured gravimetrically on a pre-weighted Whatman GF/C filter. NO_2-N, NO_3-N, NH_4-N, and PO_4-P were analyzed using a continuous flow injection analyzer (AA3, Seal Analytical, UK).

Water environmental parameters are shown (**Supplementary Table S1**), and indicated differences in chlorophyll a (Chl a) concentrations, suspended solids (SS), and nutrients (NO_2-N, NO_3-N, NH_4-N, and PO_4-P) along the PRE.

DNA Extraction and High-Throughput Sequencing

Total genomic DNA from filter membranes and intestinal contents (0.2 g) were extracted using the E. Z.N.A® Water DNA Kit (Omega, United States) and QIAamp® Fast DNA Stool Mini Kit (Qiagen, United States), respectively, according to manufacturer's instructions. PCR was performed on DNA by targeting the V3–V4 region of the microbial 16S rRNA gene. The following primers were used; 341F (5′-CCTACGGGNGGCWGCAG-3′) and 806R (5′-GGACTACHVGGGTATCTAAT-3′) which were synthesized by Sangon Biotech (Shanghai, China). A final 50 μL PCR reaction volume consisted of; 5 μL 10 × KOD buffer, 1.5 μL each primer (5 μM), 5 μL 2.5 mM dNTPs, 1 μL KOD polymerase, and 20 ng DNA template. PCR amplifications (95°C for 2 min, followed by 27 cycles at 98°C for 10 s, 62°C for 30 s, and 68°C for 30 s and a final extension at 68°C for 10 min) were conducted in triplicate. Amplified PCR products were then extracted and purified with the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, United States). These purified amplicons were pooled in equimolar amounts and sequenced on an Illumina HiSeq 2500 platform, using a 2 × 250 base pair (bp) paired-end strategy (Gene Denovo Co., Guangzhou, China). Raw reads from intestine and water samples were deposited into the Sequence Read Archive (SRA) database of the NCBI, under accession numbers; PRJNA647310 and PRJNA647485, respectively.

Sequence Analysis

After sequencing, reads containing >10% unknown nucleotides and <50% bases with quality Q-values > 20, were removed to generate clean reads. Paired-end clean reads were then merged as raw tags using FLASH (V1.2.11), with a minimum overlap of 10 bp, and mismatch error rates of 2% (Chen et al., 2018). Raw tags were then received after merging paired-end clean reads using FLASH (V1.2.11) (Magoc and Salzberg, 2011). Raw tags were processed with QIIME (V1.9.1) software to generate clean tags under specific filtering conditions (Caporaso et al., 2010). Subsequently, UCHIME algorithms were used to filter clean tags, remove chimeric tags, and derive effective reads (Edgar et al., 2011). Finally, these effective reads were clustered into operational taxonomic units (OTUs) using UPARSE (V9.2.64), with 97% sequence similarity (Edgar, 2013). A dominant sequence was chosen to represent each OTU, and taxonomic assignments analyzed using the RDP classifier (V2.2) Wang et al. (2007) in the SILVA database (V128) (Pruesse et al., 2007). QIIME (V1.9.1) was used to calculate α-diversity indices, including Shannon, Simpson, and Chao1 indices (Caporaso et al., 2010).

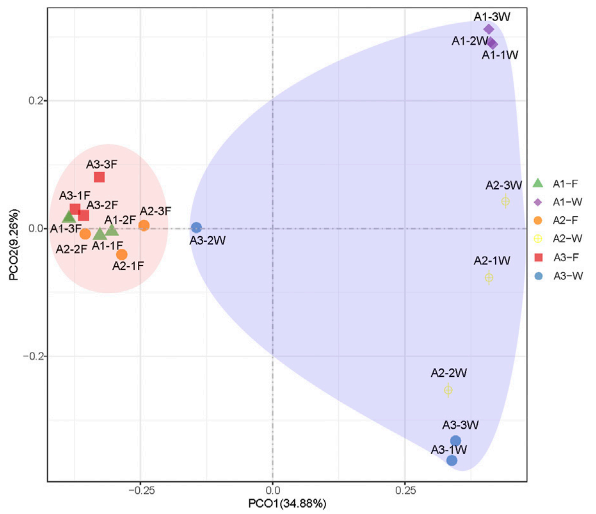

FIGURE 1 | PCoA of microbial communities in intestine (F) and water (W) samples from the Pearl River estuary, based on Bray-Curtis distance.

Statistical Analysis

Principal coordinates analysis (PCoA) was conducted based on the Bray-Curtis distance of microbial phylogeny, using R (V4.0.0) software. The relative abundance of dominant bacterial phyla/classes (top 20) was performed using Microsoft Excel. A heat map was constructed using STAMP (V2.1.3) software Parks et al. (2014), where samples were analyzed using the relative abundance of dominant bacterial communities at genus levels (top 40). Significant dominant microbial community differences between fish intestines and water samples at the phylum/class (top 20) and genus level (top 40) were compared and analyzed using a two-sided Welch's *t*-test ($p < 0.05$) by STAMP (V2.1.3) software (Parks et al., 2014). Analysis of similarities (ANOSIM) was used to test for differences in microbial communities between intestine and water samples, based on the Bray-Curtis distance matrix ($p < 0.05$). The R value from ANOSIM was used to determine any overlaps in microbial communities (Buttigieg and Ramette, 2014). Mantel tests and Pearson's correlation analyses were used to examine correlations between microbial communities and environmental factors, using the ggcor R package (V3.6.1).

RESULTS

High-Throughput Sequencing Data and Operational Taxonomic Units

The Illumina HiSeq sequencing platform produced a total of 1,698,194 raw tags from intestine and water samples. In total, 3,321 OTUs (intestine: 1,217, water: 2,349) were identified at 97% sequence similarity. OTUs in water samples were much higher than intestine samples at the same site. Specifically, average OTU numbers in water samples ranged between 1,822 and 2,797 across the three sites, while this range was 841 and 1,047 for intestine samples. The α-diversity (Shannon and Chao1) index of

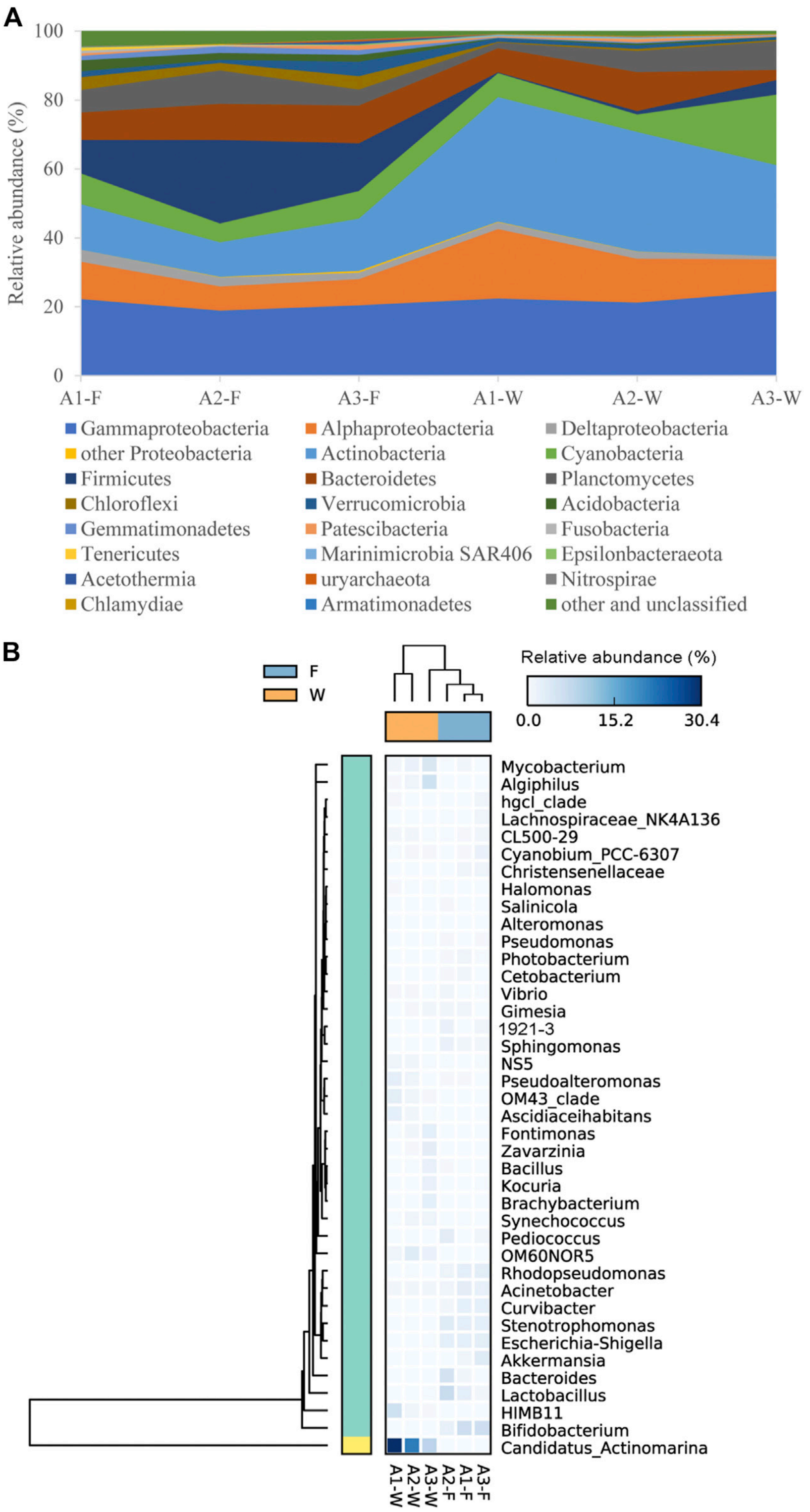

FIGURE 2 | Relative abundance of dominant microbial communities in intestine (F) and water (W) samples from different sites along the Pearl River estuary, at phylum/class **(A)** and genus **(B)** levels.

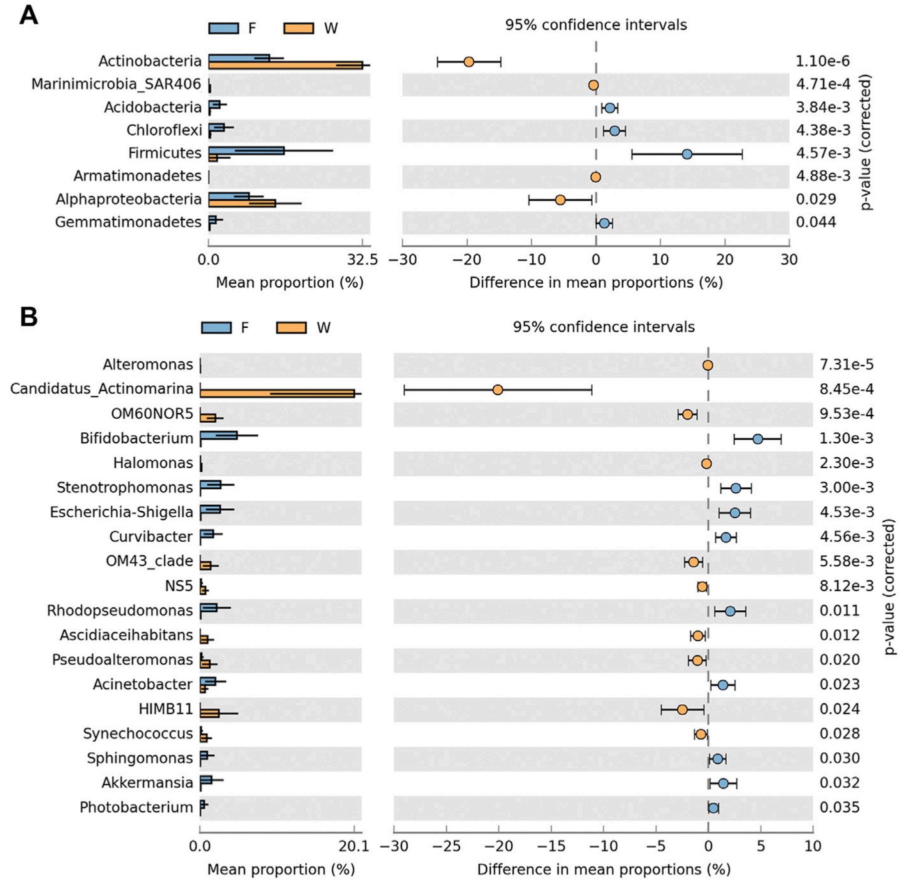

FIGURE 3 | Dominant microbial community significant differences between intestine (F) and water (W) samples from the Pearl River estuary at phylum/class **(A)** and genus **(B)** levels (Welch's *t*-test, $p < 0.05$).

microbial communities in water samples was also higher. In addition, for individual intestine or water samples, OTU numbers and the Shannon index from site A2 were the highest, but no significant differences were observed between sites ($p > 0.05$). The bacterial coverage of each sample was >98.0%, indicating most bacteria in samples were represented and identified (**Supplementary Table S2**). PCoA based on Bray-Curtis distances of OTU abundance of each sample, indicated that intestine and water samples were separated from each other (**Figure 1**). However, intestine samples from different sites were closely clustered together, with a high similarity. Water samples from the three sites were clustered into another group, but exhibited low similarity between sites. Our ANOSIM data further confirmed that microbial communities between intestine and water samples from the same site were well separated (R > 0.9). However, microbial community distribution between intestine samples were barely separated (**Supplementary Table S3**).

Microbial Composition Between Intestine and Water Samples

The microbial composition of the top 20 phyla in each sample is shown (**Figure 2A**). The relative abundance of these phyla from individual samples accounted for >95% of all sequences. Proteobacteria was the most abundant taxa in all samples. In addition, Actinobacteria, Cyanobacteria, Firmicutes, Bacteroidetes, and Planctomycetes were also dominant in intestine and water samples. Within Proteobacteria, Gammaproteobacteria was the most dominant class, followed by Alphaproteobacteria and Deltaproteobacteria in all samples. However, the dominant phyla and proteobacterial classes between fish intestine and water samples (**Figure 3A**) were analyzed to test whether there were significant differences ($p < 0.05$) in the two groups. Actinobacteria in water samples (mean; 32.5%) were more abundant than intestine samples (mean; 12.8%), while, Alphaproteobacteria, Marinimicrobia SAR406, and Armatimonadetes were more abundant in water samples, with means of 14.0, 0.4, and 0.02%, respectively. When compared with water samples, Firmicutes, Acidobacteria, Gemmatimonadetes, and Chloroflexi displayed a significantly higher abundance in intestine samples ($p < 0.05$).

Using a heat map, the top 40 genera were selected to show the microbial composition of intestine and water samples (**Figure 2B**). Candidatus *Actinomarina*, HIMB11, *Algiphilus*, and *Mycobacterium* were the dominant genera in water samples (>2%), especially Candidatus *Actinomarina* which

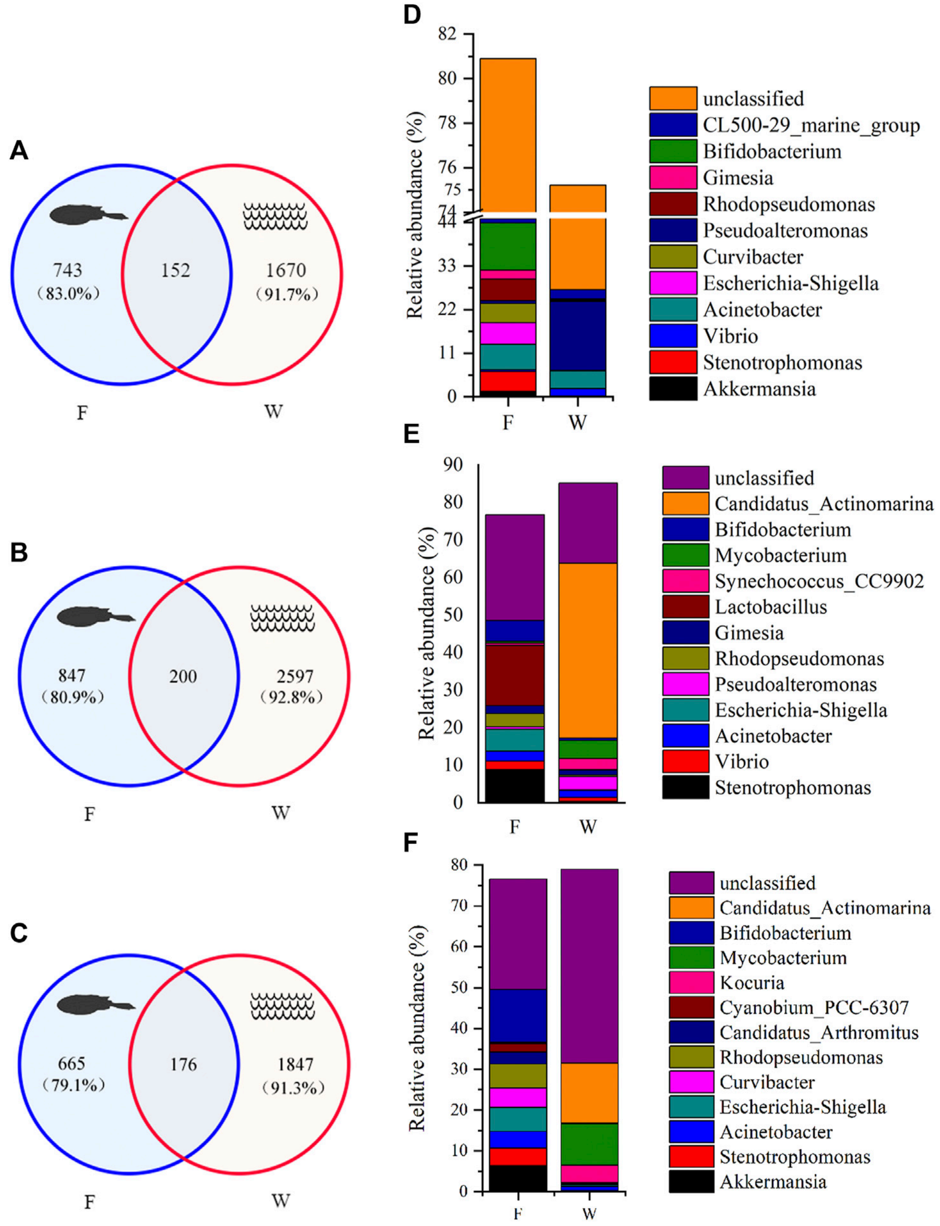

FIGURE 4 | Microbial composition between fish intestine (F) and water (W) samples from the Pearl River estuary. **(A–C)** Venn diagrams of OTU composition in intestine and water samples from individual sites, A1, A2, and A3 (correspond to A–C). The percentage in parentheses indicates the contribution of unique OTUs to total OTUs in each sample; **(D–F)** The relative abundance of dominant microbial genera based on shared OTU analysis for the three sites.

belonged to the Actinobacteria phylum, and showed the highest abundance (20.1%). However, the most dominant genera in intestine samples (>2%) included *Bifidobacterium*, *Lactobacillus*, *Stenotrophomonas*, *Escherichia-Shigella*, *Rhodopseudomonas,* and *Acinetobacter*. Moreover, 19 of the top 40 genera exhibited significant differences between intestine and water samples ($p < 0.05$) (**Figure 3B**). The nine genera; *Bifidobacterium*, *Stenotrophomonas*, *Escherichia-Shigella*, *Curvibacter*, *Rhodopseudomonas*, *Acinetobacter*, *Sphingomonas*, *Akkermansia,* and *Photobacterium* were significantly higher in intestine samples ($p < 0.05$), while the remaining 10, including Candidatus *Actinomarina*, HIMB11, OM60NOR5, OM43 clade, *Pseudoalteromonas*, etc., were much higher in water samples ($p < 0.05$).

Microbial Community Relationships Between Intestine and Water Samples

Microbial exchange between intestine and water samples from the same site was analyzed (**Figure 4**). The number of shared OTUs at sites A1, A2, and A3 were 152, 200, and 176, respectively. These OTUs accounted for 17.0–20.9% of total OTUs in intestinal

TABLE 1 | Composition of shared OTUs in intestine and water samples from different Pearl River estuary sites (A1, A2, and A3).

Sampling sites	Sample	Number of shared OTUs	% Of total OTUs numbers	% Of total sequences
A1	intestine	152	17.0	46.0
	water	152	8.3	13.8
A2	intestine	200	19.1	39.4
	water	200	7.2	33.4
A3	intestine	176	20.9	47.0
	water	176	8.7	44.9

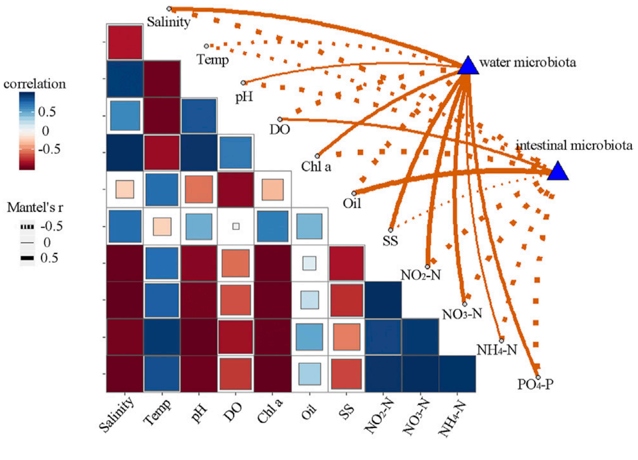

FIGURE 5 | Relationship between microbial communities (Bray-Curtis distance) and environmental factors, using Mantel tests. Temp, temperature; DO, dissolved oxygen; Chl a, chlorophyll a; SS, suspended solids.

samples from the three sites, while this was 7.2–8.7% in water samples (**Figures 4 A–C**). However, shared OTUs represented a higher proportion of total sequences in intestinal samples (39.4–47.0%) (**Table 1**). In water samples, shared OTUs at sites A1, A2, and A3 corresponded to 13.8, 33.4, and 44.9% of total sequences (**Table 1**). Therefore, the exchange of microbial communities between intestine and water samples was more pronounced at downstream PRE sampling sites.

At each site, the taxonomic assignment of shared OTUs was also analyzed, and dominant genera taxa identified (**Figures 4 D–F**). We observed differences in the relative abundance of dominant genera between intestine and water samples at the same site, due to the high abundance of special genus (*Pseudoalteromonas*, Candidatus *Actinomarina*, *Mycobacterium*) in water samples, but not in intestine samples. For example, *Pseudoalteromonas* had a very high abundance in water samples at site A1, accounting for 17.3% of total shared sequences. Candidatus *Actinomarina* (46.6%) was the most dominant genus in water samples at site A2. Similarly, Candidatus *Actinomarina* (14.8%) and *Mycobacterium* (10.1%) were more abundant at site A3. Nevertheless, microbial exchange between intestine and water samples was also observed. *Acinetobacter* and CL500–29 marine group were the dominant groups in both intestine and water samples at site A1, while *Vibrio*, *Acinetobacter* and *Gimesia* were exchanged frequently between samples at site A2. Intestine and water samples at site A3 shared the dominant genus, *Acinetobacter*. In general, this genus was shared by both intestine and water samples, and showed a constant dominance at all sites. Moreover, the relationship between microbial communities and environmental factors, using Mantel tests (**Figure 5**), revealed that intestinal microbiota exhibited a good correlation with oil, whereas water microbiota were correlated with suspended solids (SS), salinity, NO_2-N, and NO_3-N.

The Intestinal Microbiota of *C. lucidus*

The microbial composition of *C. lucidus* intestine samples from all sites was also analyzed (**Figure 6**). The shared OTUs of intestine samples from all sites were 412, and were identified as core intestinal microbiota. These OTUs at sites A1, A2, and A3 corresponded to 46.0, 39.4, and 49.0% of the total OTUs in each sample. However, shared OTUs accounted for 76.7, 72.6, and 80.6% of total sequences in intestine samples from sites A1, A2, and, A3, respectively, (**Figure 6A**). Furthermore, microbial composition was demonstrated based on the analysis of shared OTUs (**Figure 6B**). These were mainly assigned to 14 dominant genera, which represented 30.2–32.9% of total shared sequences from the three individual intestine samples. Also, 5 of the 14 genera showed low variability levels between each sample, including *Bifidobacterium*, *Rhodopseudomonas*, *Escherichia-Shigella*, *Acinetobacter,* and *Stenotrophomonas*. In addition, a high proportion of sequences belonging to unclassified taxa were found in shared sequences (30.1–43.9%).

DISCUSSION

By investigating fish intestinal microbiota and its association with estuary waters, we can improve our understanding of how fish adapt to dynamic estuary environments. In this study, *C. lucidus,* which was widely distributed in the PRE, was selected to investigate changes in intestinal microbiota across estuary transitions, using Illumina high-throughput sequencing. Furthermore, microbial exchange between intestine and water samples along the PRE was also analyzed.

We observed that Proteobacteria, Actinobacteria, Cyanobacteria, Firmicutes, Bacteroidetes, and Planctomycetes were highly abundant in *C. lucidus* intestines. These phyla also dominate other fish species (Givens et al., 2015; Gao et al., 2020). We also observed differences between water microbiota and *C. lucidus* intestinal microbiota from the PRE, while no significant

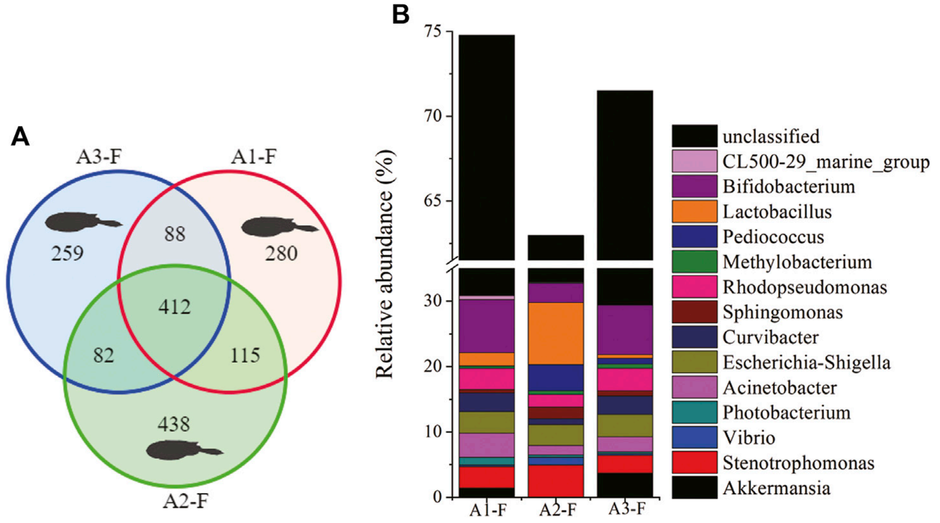

FIGURE 6 | Composition of microbial communities in fish intestine samples from different Pearl River estuary sites (A1, A2, and A3). **(A)** Venn diagrams show OTU composition (fish: F); **(B)** The relative abundance of dominant microbial genera based on shared OTU analysis.

differences were observed in microbial composition in intestines at different habitats in the PRE. Candidatus *Actinomarina* and HIMB11 genera were most dominant in water samples, but seldom discovered in intestines. In contrast, *Bifidobacterium*, *Stenotrophomonas*, *Escherichia-Shigella*, and *Rhodopseudomonas* genera were abundant in intestine samples, but rare in water samples. Previous studies similarly reported that dominant microbial groups in surrounding waters were not observed in the intestines of habitant fish (Schmidt et al., 2015; Yan et al., 2016). A possible reason could be that fish intestinal microbiota are substantially shaped by the host, and that host effects decrease microbial interactions with surrounding environments (Schmidt et al., 2015; Yan et al., 2016). Importantly, this is the first report characterizing such host effects in *C. lucidus* in the PRE. In addition, host effect was also identified during the migration of the *Salmo salar* (Schmidt et al., 2015; Llewellyn et al., 2016; Dehler et al., 2017b; Rudi et al., 2018). However, host effects may be weakened by selective variations, such as host immunity, physiology, and development (Bolnick et al., 2014; Schmidt et al., 2015; Yan et al., 2016).

Surrounding waters are important sources of intestinal microbiota for fish (Wu et al., 2012; Dehler et al., 2017a; Egerton et al., 2018). However, it is unclear how, and to what extent, water microbiota influences fish intestinal microbiota in different estuary habitats. The OTUs of water samples shared with intestinal samples at sites A1, A2, and A3, accounted for 13.8, 33.4, and 44.9% of total sequences, respectively, showing an increasing microbial exchange tendency from PRE upstream to downstream points. This observation may be related to changes in salinity. The aforementioned *S. salar* study indicated that fish drink continuously to compensate for water loss during freshwater to seawater transitions, thus microbial exchange between intestinal and water samples were increased when *S. salar* was transferred to a seawater environment (Dehler et al., 2017b). Meanwhile, the dominant taxa no matter in fish intestinal samples or in water samples showed a low frequency of exchange from each site of the whole estuary (**Figure 4**). Similar results for dominant taxa in hosts did not share preferences with these in rearing environment was observed in aquaculture environment (Sun et al., 2019; Xiong et al., 2019). However, *Acinetobacter* constantly appeared in both intestine and water samples along the estuary. *Acinetobacter* is a dominant taxa in marine fish intestines (Wang et al., 2018; Givens et al., 2015; Gomez and Balcazar, 2008). Some *Acinetobacter* species play important roles in fish digestive processes Ray et al. (2012), Ringo et al. (2016), while other species are opportunistic pathogens and induce fish intestinal inflammation (Zhou et al., 2019a). In addition, we observed a large proportion of unclassified taxa which had colonized fish intestines from the surrounding water, but the function and relevance of this group requires further investigation. Moreover, the relationship between microbial communities and environmental variables indicated that oil pollutants affected fish intestinal microbiota (**Figure 5**). It was suggested that pollutants may act as environmental stressors to weaken host immune systems, thereby influencing intestinal microbiota (Hansen and Olafsen, 1999; Zeglin, 2015). However, water microbiota was associated with suspended solids (SS), salinity, NO$_2$-N, and NO$_3$-N levels (**Figure 5**). These common constituents influence the water microbiota in aquaculture (Vasemagi et al., 2017; Sun et al., 2019).

When compared with water samples, shared OTUs accounted more for proportion of either the number of total OTUs or the total sequences in the intestinal samples (**Table 1**), indicating the importance of core intestinal microbiota *for C. lucidus*. Several highly abundant genera were consistently observed in the core intestinal microbiota, including *Bifidobacterium*, *Rhodopseudomonas*, *Escherichia-Shigella*, *Acinetobacter,* and *Stenotrophomonas* (**Figure 6**). *Bifidobacterium* is a Gram-positive obligate anaerobe which

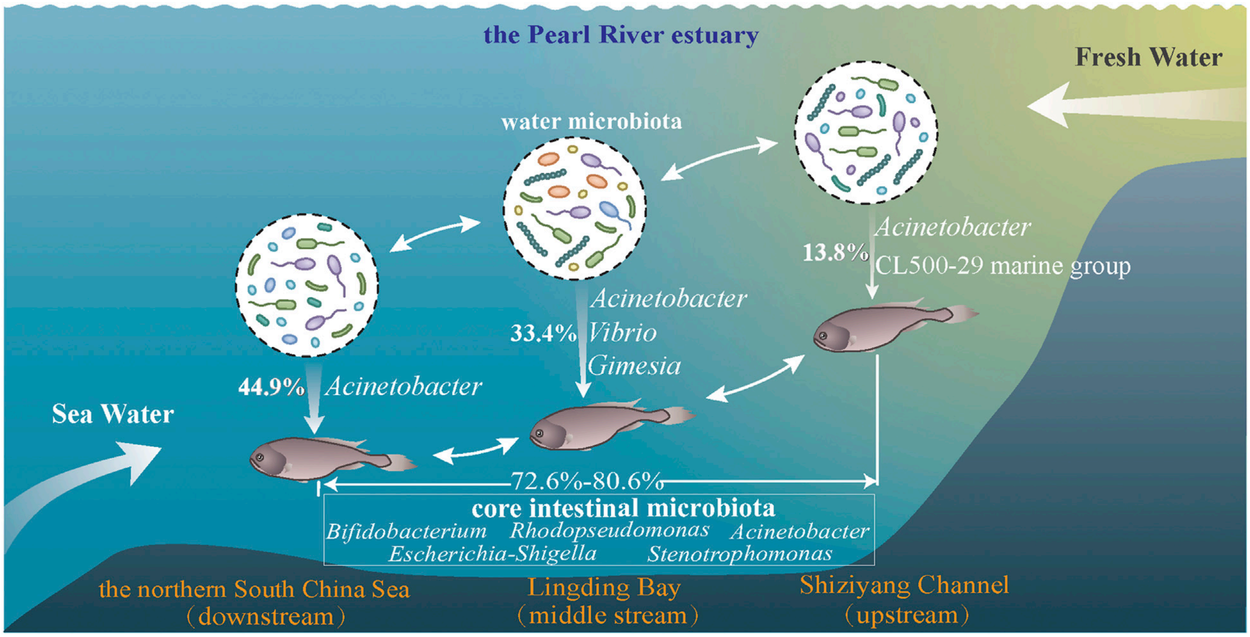

FIGURE 7 | Schematic diagram showing the composition and interactions of intestinal microbiota from *C. lucidus* with surrounding study sites (A1, A2, and A3) along the Pearl River estuary. The fresh water movement is represented from right to left.

prevents pathogenic bacterial invasion into the intestinal environment of humans and animals (Sekirov et al., 2010; Fukuda et al., 2011). However, it is not common in marine fish, whereas *Bifidobacterium* is more common in some freshwater fish (Kopecny et al., 2010; Vlkova et al., 2012). *Rhodopseudomonas* is a photosynthetic bacteria, often recognized as a probiotic species, and associated with growth promoters or immune responses in fish, e.g., *R. palustris* (Zhou et al., 2010; Wang, 2011; Feckaninova et al., 2017). *Escherichia-Shigella* is frequently identified in fish intestinal samples, and is known as a potential pathogen (Sun et al., 2019; Zheng et al., 2019; Gao et al., 2020). *Acinetobacter* is common in fish intestines, the functions of which have been outlined above. Most of the *Stenotrophomonas* species have key roles in nitrogen and sulfur cycles Ryan et al. (2009), but *S. maltophilia* is often identified as an opportunistic pathogen in aquaculture (Geng et al., 2010; Abraham et al., 2016). The presence of such opportunistic pathogens in fish intestines suggests the intestinal tract is a potential pathogen access route (Roeselers et al., 2011; Li et al., 2015). Core intestinal microbiota components were also observed in *Salmo salar* Dehler et al. (2017a), Rudi et al. (2018), zebra fish Roeselers et al. (2011), European sea-bass (Kokou et al., 2019), carps (grass carp, crucian carp and bighead carp) Wu et al. (2012), Li et al. (2015), rainbow trout Wong et al. (2013), and several other marine fish (Givens et al., 2015; Gao et al., 2020). Based on our data, the core intestinal microbiota of *C. lucidus* harbored bacteria associated with immune response, nutrient metabolism, probiotic actions, and potential pathogen behaviors.

CONCLUSION

Interactions between the intestinal microbiota of an euryhaline fish species and water environments along an estuary were investigated in this study. Dominant genera in intestine samples or water samples were seldom exchanged. While differences existed between the water microbiota and intestinal microbiota, some microbial taxa, e.g., *Acinetobacter* were constantly exchanged. Furthermore, these microbial exchanges were increased from upstream to downstream estuary points. Salinity changes may have influenced these microbial exchanges between samples (**Figure 7**). In conclusion, we analyzed the core intestinal microbiota of *C. lucidus* across different estuary points, and identified potential microbiota functions, incorporating immune responses, nutrient metabolism, probiotic actions, and potential pathogen behaviors.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by Committee on Laboratory Animal Welfare and Ethics of South China Sea

Fisheries Research Institute, Chinese Academy of Fishery Sciences (nhdf2020-03).

AUTHOR CONTRIBUTIONS

Conceptualization, PW, YL, and CL; investigation, PW, YX, LL, and YX; methodology, PW, YX, and YX; formal analysis, PW; writing original draft, PW and YX; writing-review and editing, PW, YL, CL, and TW; data curation, YLand LL; funding acquisition, CL and PW.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2021.675856/full#supplementary-material.

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Evaluation of Fish Communities in Daya Bay Using Biomass Size Spectrum and ABC Curve

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Based on the data collected by four trawl surveys during 2016–2017, we applied biomass size spectrum (BSS) and abundance–biomass comparison (ABC) curve to assess the status of fish communities' status in Daya Bay, China. Our findings indicated a unimodal pattern and biomass size ranged from −2 to 10 grain levels and the pattern of the Sheldon-type BSS of fish in Daya Bay. Moreover, fishes in the range of four to eight size class were relatively abundant. The highest peak belonged to the two to four grain level ($\log_2$ size bins), mainly consisting of *Leiognathus brevirostris*, *Callionymus meridionalis*, *Callionymus koreanus*, *Evynnis cardinalis*, *Trachurus japonicus*, and other small fishes. The curves of the BSS in spring and winter were relatively flat and comprised a large curvature. The summer and autumn curves were comparatively steep, and the seasonal curvature was small. The curvatures of the curve were mainly related to a large number of small *Evynnis cardinalis* and a small number of large-sized *Harpadon nehereus* and *Leiognathus ruconius*. In our study, it was observed that the number and the size of the breeding population, trophic levels, migration habits, and other life history characteristics, as well as anthropogenic disturbances (especially overfishing), significantly affected the peak shape, slope, or curvature of the fish BSS, with overfishing being the main factor. The ABC curve exhibited that Daya Bay was in a critical state of disturbance throughout the year. The spring, summer, and autumn were in severe disturbance, while the winter was in moderate disturbance.

Keywords: fish communities, biomass size spectrum, ABC curve, Daya Bay, trawl surveys

INTRODUCTION

Understanding how fish communities respond to natural disturbances is fundamental to assess ecosystem resistance and resilience mechanisms (Romero et al., 2020). The body size compositions of communities can be modeled using a size spectrum framework (Kerr and Dickie 2001). Changes in the fish size influence the ecosystem (Peterson and Wroblewski 1984; Scharf et al., 2000; Woodward et al., 2005). Therefore, the ecological and biological characteristics of fishes can be determined according to their size. Fishes throughout their life cycle traverse a few trophic levels that are positively associated with their size (Jennings et al., 2001). The size spectrum is extensively applied to study the characteristics of the fish community, particularly in the aspects of production and abundance (Sheldon et al., 1977; Boudreau and Dickie 1992; Cyr and Peters 1996).

The size spectrum expresses the relation between organism size and biomass or abundance and is used as an indicator of organisms' mass or abundance, which is described within the logarithmic body size interval (Trebilco et al., 2013). It allows for understanding the distribution of biomass or abundance in the various size intervals within a biological community and also assists in realizing the impact of ecosystem productivity and the energy on that relation (Macpherson et al., 2002). According to the general rule, the spectrum line of the size spectrum is straight with a slope of −1 in a relatively stable ecosystem state (Jung and Houde 2005); however, when the community is disturbed by any outside factors, the spectrum line displays a "dome" parabolic shape (Kerr and Dickie 2001). Overfishing is one of the main factors that influence the slope or curvature. Therefore, the size spectrum can be used as an indicator to evaluate the impacts of human activities and environmental changes on ecological systems (Guiet et al., 2016). The biomass size spectrum (BSS) has been widely used to estimate mortality in aquatic ecosystems, particularly in fish species. This predicts the effects of various human disturbances on aquatic ecosystems and analyzes the population structure and dynamics of aquatic ecosystems (Sheldon et al., 1972; Peterson and Wroblewski 1984; Sprules et al., 1991). At present, there are many studies on the fish BSS overseas that mainly focused on the fish community structure, fishery biology, nutrition level, fishing intensity, mortality and so on (Jennings et al., 2002; Graham et al., 2005; Jung and Houde 2005; Emmrich et al., 2011). However, only few studies have applied fish BSS in China.

The abundance–biomass comparison (ABC) curve method was proposed by Warwick in 1986 to monitor the impact of disturbances (mainly pollution impact) on benthic invertebrate communities (Warwick 1986). This method is then used for internal comparisons of species abundance and biomass distribution in various samples to predict the level of environmental stress based on the responses of species distribution (Warwick et al., 1987; Warwick and Clarke 1994; Pagola-Carte 2004; Wijeyaratne and Bellanthudawa 2018). When the biomass dominance curve is above the abundance dominance curve, it indicates that the community is in a stable state, and species composition is dominated by large-size species with slow growth and late sexual maturation. When the curves intersect, the community is in a moderately disturbed state. When the biomass dominance curve is below the abundance dominance curve, it indicates that the community is in a severely disturbed state, and species composition is dominated by small-size species with fast growth and early sexual maturity (Sprules and Munawar 1986). In recent years, the ABC curve method has been widely used in fishery research (Blanchard et al., 2004; Yemane et al., 2005; Li 2008; Wijeyaratne and Bellanthudawa 2018). Also, the trends of biomass dominance curves and abundance dominance curves were compared to analyze the characteristics of fish communities under different disturbance statuses (Warwick 1986). The ABC method can be applied to analyze the impact of human disturbances such as fishing on fish communities and determine the expected response of biological communities to disturbances, which is of great significance to the research and development of fishery resources (Li 2008).

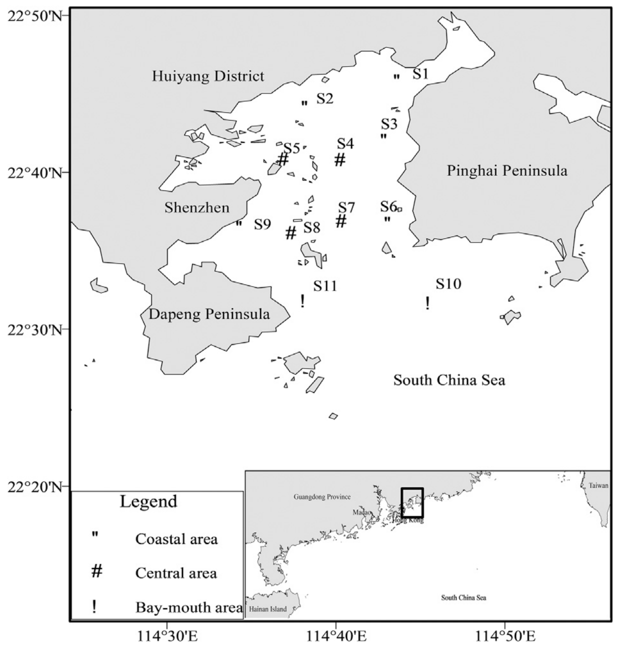

FIGURE 1 | Map of sampling stations in Daya Bay.

Daya Bay is a subtropical semi-closed bay, in Guangdong Province, China, in the northern part of the South China Sea, surrounded by mountains from three sides (Xu 1989). It is a suitable habitat for spawning, feeding, and growing fish and rich in commercial fishes with vast biodiversity (Wang et al., 2005). However, the rapid socioeconomic development, human activities, and natural disturbances have caused damage to Daya Bay's ecosystem, which decreased the biodiversity level and resources significantly (Wang et al., 2010; Li et al., 2015). Since the 1980s, there have been numerous reports on the study of fish communities in Daya Bay; however, all of them were conventional research methods based on taxonomical studies. The use of the size spectrum to study the characteristics of fish communities has not been reported yet. Therefore, in this study, based on the data of the four submarine trawl surveys from 2016 to 2017, we constructed the size spectrum of fishes in Daya Bay in order to provide a scientific basis for ecological protection, sustainable development, and utilization of fish resources.

MATERIALS AND METHODS

Study Area and Sampling

Daya Bay is located at 113°29′42″–114°49′42″E and 23°31′12′–24°50′00″N in Guangdong Province, China, with an area of 600 km^2 (**Figure 1**). It is one of the largest semi-closed bays in China and the greatest entrance in Guangdong Province (Wang et al., 2006). It is not linked to any main rivers, and a majority of its water comes from the South China Sea. It is poured mainly by tides as its water depth ranges from 6 to 21 m, with an average of 11 m (Xu 1989), and about 60% of water in Daya Bay has a depth lower than 10 m (Wang et al., 2006).

Eleven stations were set in Daya Bay, among which the coast consists of five stations, namely, S1, S2, S3, S6, and S9; the middle of the bay consists of four stations, including S4, S5, S7, and S8; and the mouth of the bay comprises two stations, namely, S10 and S11 (**Figure 1**). Four voyage bottom trawl surveys were carried out in August 2016 (summer), December 2016 (winter), March 2017 (spring), and October 2017 (autumn). Along with fish collection, data on four environmental variables were obtained simultaneously from the stations. The water depth (m) of each station was obtained by a ship-borne sounder. The physical environment data such as sea surface salinity, sea surface temperature (°C), dissolved oxygen (DO, mg/L), and pH were measured by a YSI Pro Series multifunctional water quality meter. The environmental parameters and fishery resources investigations were based on "the specifications for the oceanographic survey" (GB/T12763-2007, China), "the specifications for marine monitoring" (GB17378-2007, China), "the specifications for coastal waters environmental monitoring norms" (HJ442-2008, China), and "the specifications for marine fishery resources survey" (SC/T9403-2012, China) and standardized the data. The survey vessel was a steel fishing boat with a power of 135 kW that was operated during the day. The sampling net was a single-vessel winged single-bag bottom trawl with a net mouth circumference of 102 m, net length of 50 m, on the framework of 51 m, under the outline of 51 m, and net mesh size of 2 cm. Each station was trawled one time and dragged 1 h, with an average hauling speed of 3.4 knots for all surveys. The catches were classified and identified on site, and the biological parameters such as the body length (BL, mm) and body weight (BW, 0.1 g) of each individual were immediately measured, and then the samples were frozen, stored on site, and transferred to the laboratory. Sampling and analysis were carried out according to the "Marine Biological Survey Code" (National Standardization Administration of the People's Republic of China General Administration of Quality Supervision 2007).

Analytical Methods

Abundance–Biomass Comparison Curve

The ABC curve method analyzes the characteristics of the community under different disturbance conditions by calculating the distribution of species abundance and biomass using the W-statistic as a statistic of the ABC curve method (Yemane et al., 2005):

$$W = \sum_{i=1}^{s} \frac{(B_i - A_i)}{50\,(S-1)},$$

where A_i and B_i represent the cumulative numbers of the species number i and the cumulative percentage of biomass in the ABC curve, respectively, and S is the total number of species.

Sheldon-type BSS

In Sheldon-type BSS analysis, if the size of the smallest fish species is V, then the first particle size of fish ranges from V to 2V, the second size ranges from 2 to 4V, and so on. The fish size interval follows the base 2 logarithm and divides the fish size into different size levels (Sheldon et al., 1972). The Sheldon-type BSS is divided by the $\log_2$ conversion size interval as the abscissa, and the total biomass per unit area (m^2) corresponding to each size group is converted by $\log_2$ as the ordinate (Jung and Houde 2005). This allows the biomass distribution to not rely on the fish size intervals, allowing comparisons of different fish communities, regardless of fish size (Macpherson et al., 2002). The fish community's structural characteristics are reflected by the "peak shape" formed by the crests and troughs on the curve (Kerr and Dickie 2001).

Normalized Biomass Size Spectrum

In the NBSS, the horizontal axis is consistent with the Sheldon-type model, but the vertical axis is different. The vertical axis is the ratio of the total biomass (wet weight) of organisms in a specific weight category to the interval of weight changes in the category, all expressed on a logarithmic scale (Sprules and Munawar 1986). This logarithmic scale describes the characteristics of fish community structures mainly using the linear regression equation or parabolic equation parameters, which compare the differences among various fish communities and predict the future development of fish community conditions (Sprules and Munawar 1986; Kerr and Dickie 2001). When the community is in a stable state, the NBSS is a straight line ($y = ax + b$), where a represents the slope, indicating the relationship between biomass and the particle size of fish. Once a equals −1, it demonstrates that the fish biomass is consistent with the increase in the particle size. When a is less than −1, it indicates that the fish biomass decreases as the size increases. On the other hand, a greater than −1 reveals that the fish biomass increases with the increase in the grain size, while b represents the intercept, expressing the abundance and productivity of the fish community. The difference in b value represents the variance in the productivity level of different fish communities, for which the magnitude of the productivity level is positively correlated with the b value (Platt and Denman 1977; Guo et al., 2017). On the other hand, when the community is in an unstable state, the NBSS is a parabolic equation ($y = ax^2+bx+c$), and a represents curvature which is affected by various factors such as the nutritional level, fishing, and breeding population (Macpherson and Gordoa 1996; Trebilco et al., 2013). R^2 represents the determination coefficient of the fitting equation obtained by the linear or nonlinear model and describes the extent to which fish communities deviate from their steady state.

Data Analysis

The data of the survey were calibrated and analyzed by ArcGis10.3 software and SPSS19.0 statistical analysis software. According to the survey results of each station, the average body weight of fish of more than 9 g and less than 9 g at each station was calculated. Also, to map the fish body size distribution, an ArcGIS10.3 raster interpolation was used, which helps

TABLE 1 | Seasonal changes of the fish community structure in Daya Bay.

Season	Catch (kg/h)	Body weight ranges (g)	Average body weight (g)	Dominant species composition
Spring	15.2	2–1,200	9	*Evynnis cardinalis*
—	—	—	—	*Callionymus richardsoni*
—	—	—	—	*Clupanodon punctatus*
—	—	—	—	*Trachurus japonicus*
Summer	67.8	1.5–2,012	6	*Thamnaconus hypargyreus*
—	—	—	—	*Leiognathus brevirostris*
—	—	—	—	*Apogon lineatus*
Autumn	37.3	0.4–238.8	27	*Leiognathus brevirostris*
Winter	12.4	0.5–693	24	*Leiognathus brevirostris*

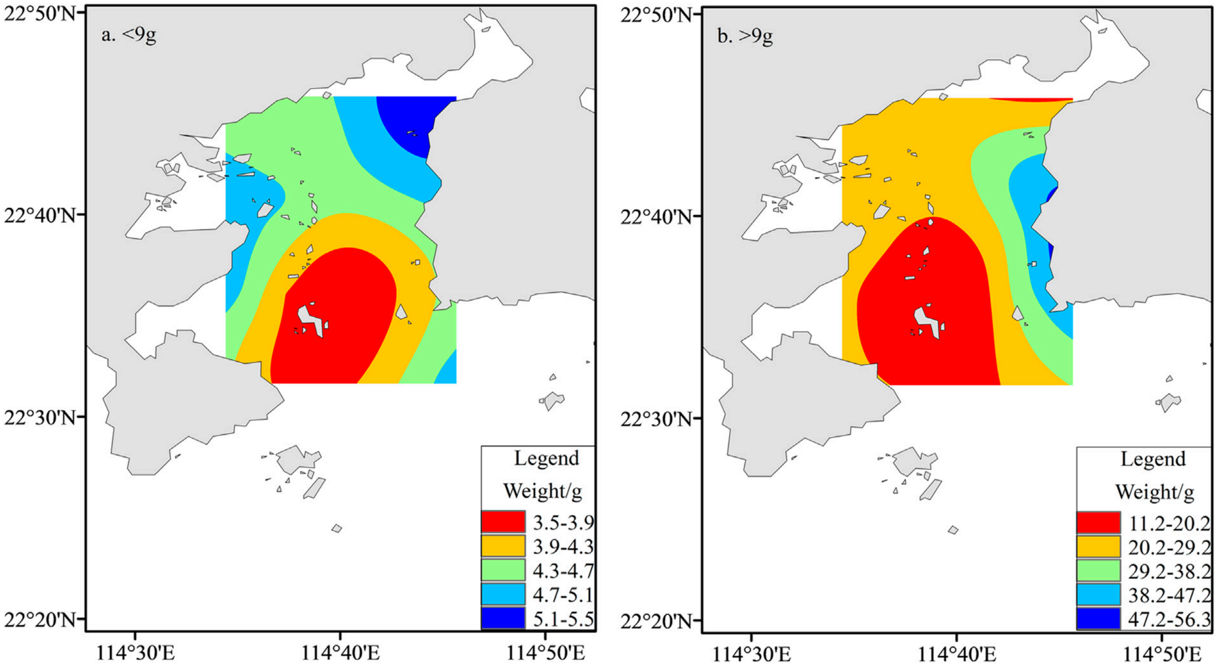

FIGURE 2 | Spatial distribution of fish physically in two designated size classes in Daya Bay; **(A)** small fish <9 g wet weight; **(B)** large fish >9 g wet weight.

understand the characteristics of the distribution within the seascape.

RESULTS

Fish Community Structure

A total of 131 fish species were collected, belonging to 13 orders, 53 families, and 84 genera, all of which belonged to the Osteichthyes class. Perciformes contained the largest number of species, with 71 species, accounting for 54.20% of the total species. The fish community of Daya Bay demonstrated significant seasonal changes, which are summarized in **Table 1**. The fish community mainly composed of *Evynnis cardinalis* and *Callionymus richardsoni*, which feed on benthic organisms; *Trachurus japonicus* and *Apogon lineatus*, which feed on plankton organisms; and *Leiognathus brevirostris* and *Clupanodon punctatus* larvae, which feed on plankton and benthic organisms. Overall, the fish structure presented miniaturization and a low-weight trend. The average individual mass of fish in the whole bay was 9 g. The distribution of the body size of small fish (<9 g) and large fish (>9 g) after grid interpolation showed that the abundance of large-sized fish reduced progressively from the east to the west coast of the gulf. On the other hand, the small-sized fish overall showed an increasing trend from the bay mouth area to the northern coastal waters. Moreover, the fish in the coral reef area and the bay mouth were the smallest in size, while the high-value area was in the eastern coastal waters. Overall, the fish in the littoral waters were comparatively large, while the fish in the middle and the mouth of the bay were relatively small (**Figure 2**).

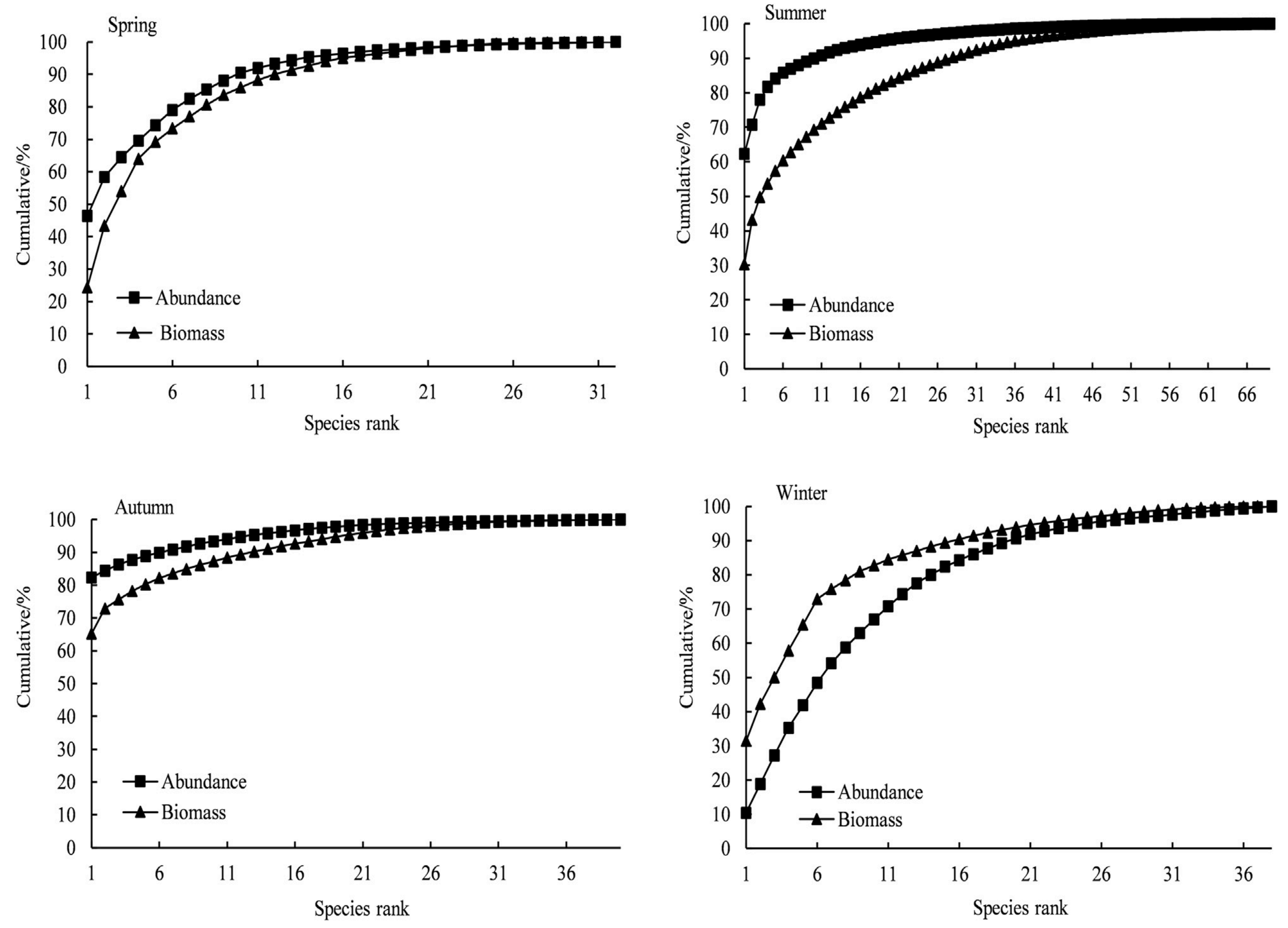

FIGURE 3 | ABC curves of fish community in every season in Daya Bay.

Abundance–Biomass Comparison Curve

There were significant seasonal variations and spatial differences in fish communities' ABC curves of Daya Bay (**Figure 3** and **Figure 4**). In the seasonal changes, the fish communities in spring, summer, and autumn were all in a critical disturbance state. The fish community consisted mainly of small-sized fish, while the winter fish community was in a moderate disturbance state. The fish community structure was better in winter than the other three seasons, and the *W* values of the four seasons were −0.07, −0.17, −0.08, and 0.17, respectively. In the spatial distribution, the coastal waters and the central waters were in a critical disturbance state, while the mouth of the bay was moderately disturbed. The *W* values of the coastal waters, central waters, and mouth waters were −0.10, −0.15, and −0.09, respectively. The coastal area was the most disturbed, followed by the central waters. Furthermore, the entire sea area was in a critical disturbance state throughout the year.

Fish BSS

Fish BSS in Daya Bay

According to the survey results of four voyages, the BSS of fish in Daya Bay was constructed (**Figure 5**). The Sheldon-type BSS of fish was a single-peaked pattern with the grain size ($\log_2$ size bins) ranging between −2 and 10, where the fish were predominantly in the range of four to eight grain level. The size spectrum dome was located on the two to four grain level. Its corresponding body mass of fish ranged from 8 to 16 g, mainly composed of *Leiognathus brevirostris*, *Callionymus meridionalis*, *Callionymus koreanus*, *Evynnis cardinalis*, *Trachurus japonicus,* and other small fishes. Also, the curvature of the NBSS was −0.23 in Daya Bay.

Seasonal Changes of Fish BSS

The Sheldon-type BSS of the four seasons in Daya Bay showed an irregular sawtooth shape with evident seasonal variations (**Figure 6**, $p < 0.05$). In general, all four seasonal curves showed a unimodal pattern. Commonly in spring and winter, the peak and trough were interdependent, and the curve of BSS slightly fluctuated. Only one peak appeared in summer and autumn, without trough occurrence, and the curve fluctuated marginally. The fish size ranged from −2 to 10 in four seasons, ranging from −2 to 10 in spring, summer, and winter, while from −2 to 8 grain level in autumn.

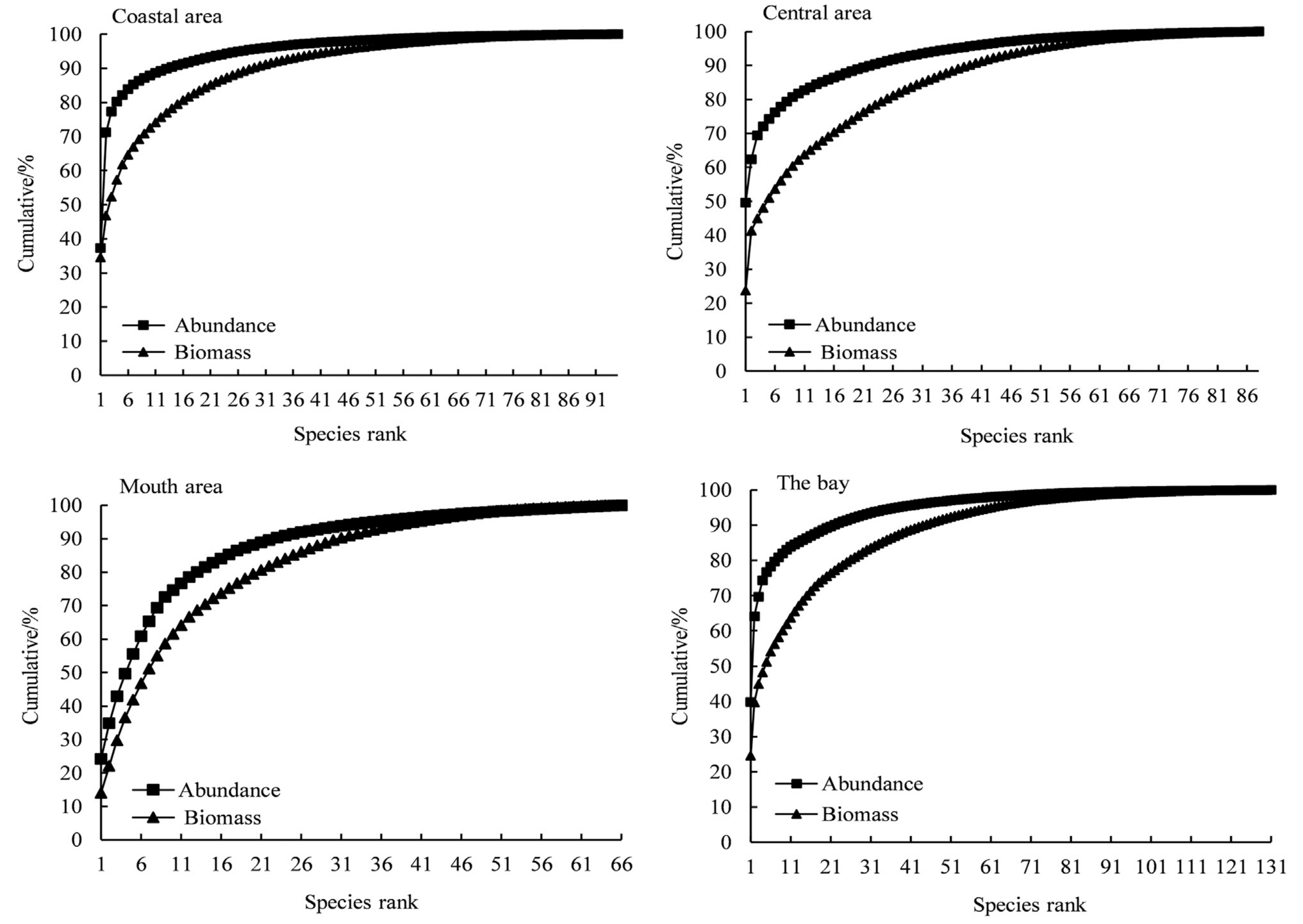

FIGURE 4 | ABC curves of fish community in the region in Daya Bay.

The NBSS of fish for all seasons was designed based on the analysis of fish biology data. All the regression results were significant ($p < 0.05$), and there were seasonal differences (**Figure 7**; **Table 2**). The curvature ranges of the NBSS of fish for the four seasons ranged from −0.33 to −0.19, of which spring curvature was the largest, followed by winter and autumn, and summer curvature was the smallest. The R^2 ranged from 0.67 to 0.95, exhibiting the largest value in summer and the smallest in winter.

Spatial Changes of Fish BSS

The Sheldon-type BSS and NBSS of fish were constructed based on the survey data, the coastal area, the central region, and the bay mouth in Daya Bay with evident spatial changes (**Figure 8** and **Figure 9**, $p < 0.05$). The Sheldon-type BSS in the three regions was a single peak, while the highest peaks in the coastal and central sea areas were in the two to four size range, and the highest peak in the bay waters was in the four to six size range. The fish size ranged from 2 to 10 grains, of which the coastal and bay mouth waters ranged from −2 to 10 grains, while the central waters ranged from 2 to 8 grains. The curvature of fish NBSS was in the range from −0.39 to −0.19, of which the coastal area was the largest, followed by the bay mouth (−0.27). The central area was the smallest; R^2 ranged from 0.81 to 0.98, with the highest in the central region and the lowest in the coastal zone (**Table 3**).

DISCUSSION

Ecological Indication of ABC Curve

The fish biomass dominance curve in Daya Bay was lower than the abundance dominance curve, indicating that the fish community structure was severely disturbed, and the fish community composed of small, fast-growing species that mature early. Throughout the year, spring, summer, and autumn seasons were in a severe disturbance state; however, winter was in a moderate state (Yemane et al., 2005). This was consistent with the findings of a previous study that the fish composition was mainly dominated by small-size fish such as *Evynnis cardinalis*, *Callionymus koreanus*, *Clupanodon punctatus*, *Trachurus japonicus*, *Thamnaconus hypargyreus*, and *Leiognathus brevirostris*, where the fish size structure showed a trend of miniaturization and low weight (Guo et al., 2018). Studies have also shown that the status of fish abundance is

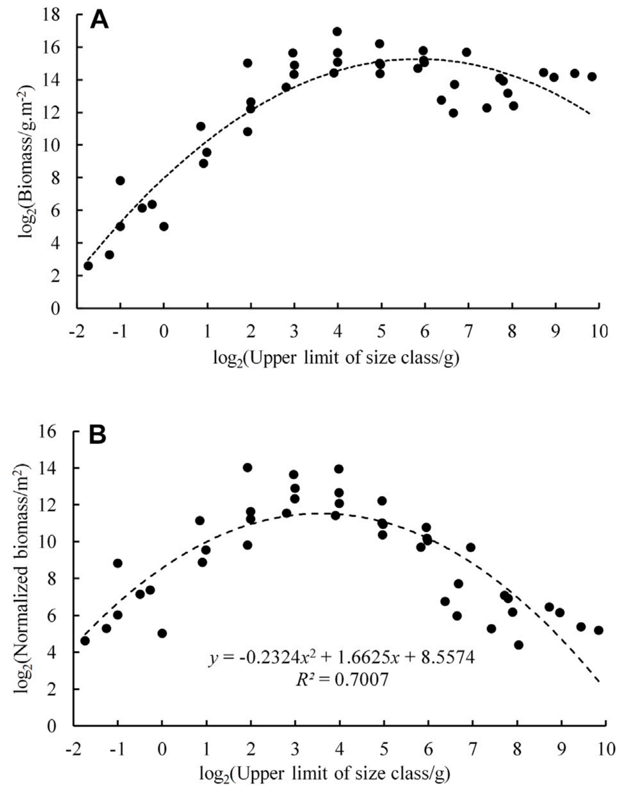

FIGURE 5 | Biomass size spectrum of fish in Daya Bay; a. Sheldon; b. normalized.

related to its reproductive rate, growth habits, seasonal differences in the living environment, and human activities (especially fishing) (Fenberg and Roy 2008; Li 2008; Rochet and Benoit 2012; Guo et al., 2018; Xu et al., 2020; Marrakchi et al., 2021). Considering the time of the investigation, the spring and summer seasons were before and after the South China Sea fishing moratorium, but summer was the most disturbed, while the fish community structure in spring was less disturbed than summer. The average fish weight (9 g) in spring was higher than that in summer (6 g). Besides, the spring and summer seasons were the main spawning periods for most fish in Daya Bay. Some fishes such as *Clupanodon punctatus*, *Trachurus japonicus*, *Trichiurus lepturus*, and *Thryssa dussumieri* began reproduction considerably, which resulted in both fecundity and juveniles to reach the maximum during summer (Xu 1989; Lin et al., 2010; Cao et al., 2021; Lakshmikandan et al., 2021). In addition, the summer survey was conducted in the second week after the end of the fishing season. Overfishing by coastal fishers resulted in the caught being dominated by the high abundance of small-size fish in summer, in which their weight and quantity accounted for 47.5 and 72.8% of the total catches, respectively. Besides, the biomass dominance curve was lower than the abundance dominance curve, and the fish community was in a severe disturbance state. In winter, a small quantity of *Acanthopagrus schlegelii*, *Gymnothorax hepaticus*, *Johnius distinctus*, *Pampus chinensis*, *Johnius belangerii*, and other large species were captured, with the average individual weighed more than 50 g, which was significantly higher than that in the other three seasons. Also, the community structure showed lower disturbances in winter than in the other three seasons, and the fish community was in a moderate state of disturbance due to the impact of low temperature, fish migration, and low fishing pressure. Remarkably, the coastal waters were affected by overfishing, resulting in a severe disturbance. In the middle of the bay, several island reefs provided a natural sanctuary for reef fish breeding (Wang and Zhao 2001), which was not conducive to bottom trawl fishing. Therefore, the degree of disturbance was relatively low, and moderate disturbance was observed.

Characteristics of Fish BSS in Daya Bay

Composition of Fish

The particle size of organisms is highly associated with the material and energy flow in marine ecosystems, the structure and function of biological communities, and physical and chemical processes (Belgrano and Brown 2002; Li 2002). Therefore, biologic particle size spectroscopy can be employed to reflect the structural composition of biologic communities (Marquet et al., 2005). The results of fish compositions of this study are shown in **Table 4**.

Ecological Characteristics of Fish Community Structure

The curve was larger in spring and winter and smaller in summer and autumn. The regression coefficient, R^2, was small in spring and winter and was large in summer and autumn. The size ranges were wide in spring and winter and slightly smaller in summer and autumn, indicating that the fish community structure in spring and winter was composed of smaller size fishes than summer and autumn. In winter, the fish community structure was the least disturbed by human activities, while summer showed the highest human disturbances. Particularly, the coastal areas were the most affected areas by human activities, followed by the central bay area. The lowest degree of human disturbance was observed in the mouth of the bay, indicating that the fish community structure was unstable in the coastal zones, and the fish community structure was relatively stable in the central region and mouth of the bay.

The particle size is a prominent structural feature of fish communities and significantly reflects fish movement (Bainbridge 1958), predation (Lundvall et al., 1999; Scharf et al., 2000), reproduction (Woodward et al., 2005), the mortality rate (Peterson and Wroblewski 1984), and fishing (Fenberg and Roy 2008). The trophic level of organisms is closely related to the fish size (Jennings et al., 2001; Trebilco et al., 2013). Mainly, the fish grain size structure is influenced by the productivity level of the area, fishing intensity (especially overfishing), species compensation ratio (number of breeding population), and environment, all of which influence the BSS of fish (Jennings and Reynolds 2007; Fenberg and Roy 2008; Wang et al., 2013). In this study, the main fish species in the smallest grain size (2–0 grain size) during spring, summer, autumn, and winter seasons were *Glossogobius olivaceus*, *Apogon lineatus*,

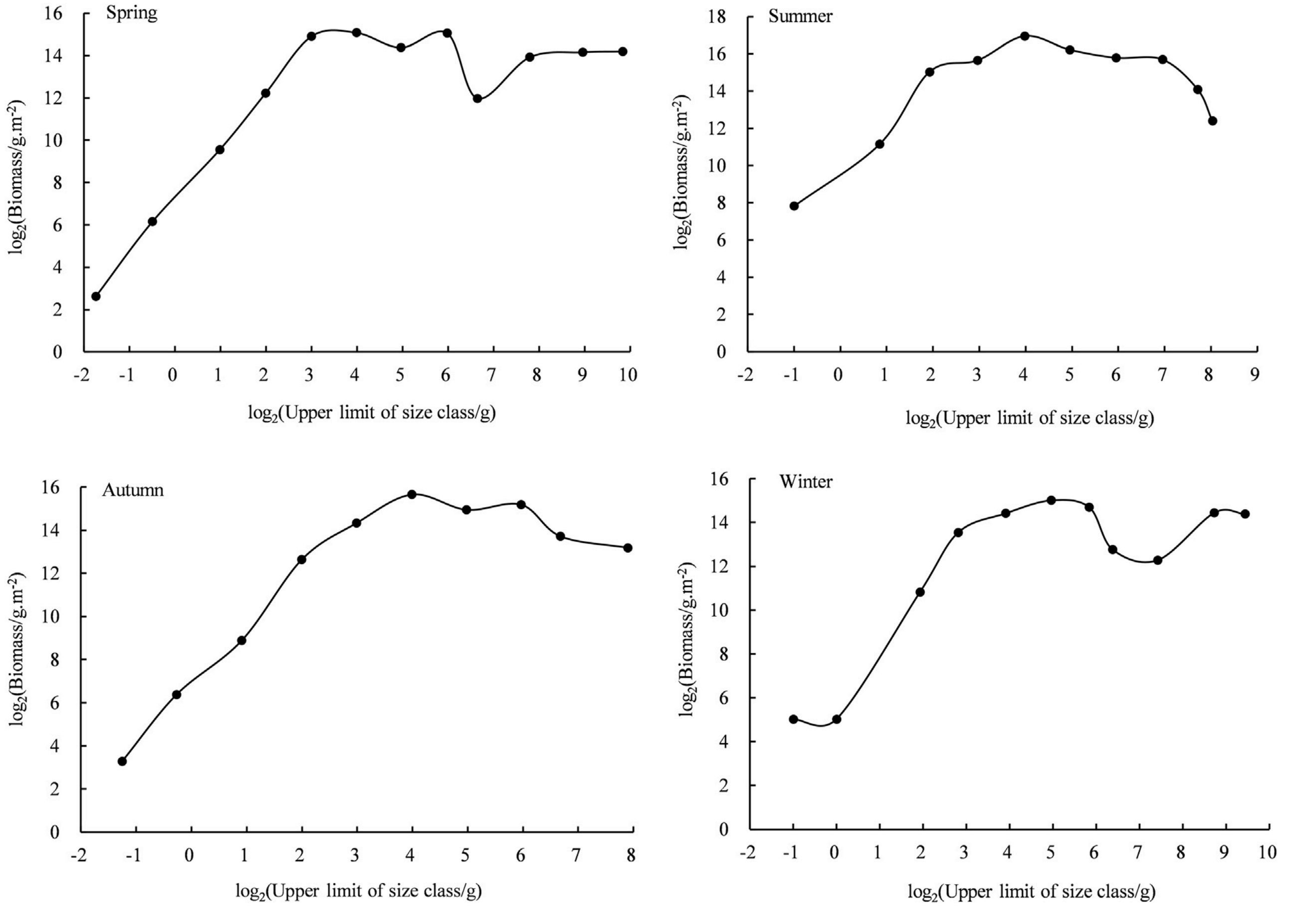

FIGURE 6 | Biomass size spectra of fishes in every season in Daya Bay.

Argyrosomus pawak, and *Parachaeturichthys polynema*, respectively. Since the biomass size was small at this grain level, it significantly influenced the curvature of its NBSS. Predominantly, the biomass grain level in the coastal waters, the middle bay area, and the mouth area was the smallest, and the fish species with the highest biomass contribution rates were *Apogon lineatus*, *Argyrosomus pawak,* and *Pterois volitans*. Consequently, this affected the curvature of the NBSS. In summer and autumn, Daya Bay shows high productivity, and spawning fishes begin to spawn abundantly in summer, resulting in both fecundity and juveniles to reach their peaks (Xu 1989; Lin et al., 2010). Besides, from June to August, due to the fishing moratorium period in the South China Sea, the protection of fish resources resulted in highest fish abundance, and biomass size and upwelling were observed on the east coast of Guangdong (Xu 1989). Also, the South China Sea invasion trend showed increased nutrition supplementation in the bay, which provides a well-nourished environment for fish and plankton growth (Wu et al., 2007; Lakshmikandan et al., 2020; Barati et al., 2021; Lakshmikandan et al., 2021), resulting in the larger mean biomass size of fish in summer and autumn than in spring and winter. However, after summer fishing moratorium, the increase in fishing intensity reduced the amount of large-size fishes (Guo et al., 2018), coupled with the replenishment of spawning fishes (Xu 1989), and resulted in a fish community comprising small-sized fishes. And overfishing was the main reason for the relatively steep curvature of the NBSS. In spring and winter, the productivity of Daya Bay was comparatively low, and fishing was moderately less, wherein fishes such as *Clupanodon punctatus* began spawning predominantly. In winter, most commercial fishes such as *Trachurus japonicus*, *Pagrosomus major*, *Formio niger*, and *Decapterus maruadsi* migrated from the bay to the deep waters outside the bay for overwintering (Xu 1989), while more large fishes such as *Sparus macrocephalus* were captured, which resulted in a comparatively uniform distribution of fish size and a relatively flat NBSS curvature. Also, the particle size structure was superior to other seasons as the grain size range was larger. Coastal waters were disturbed by fishing, and the coastline and fish habitats were damaged (Wang et al., 2010). The degree of disturbance was highest, and there was a lack of small-size species ranging from −2 to −1, which led to a steeper curve of the BSS. In the middle of the bay, several island reefs were not easy to catch on the bottom trawling. The degree of disturbance was rather small, and the community was reasonably stable, resulting in relatively flat curvature. The water depth was higher in bay mouth than the

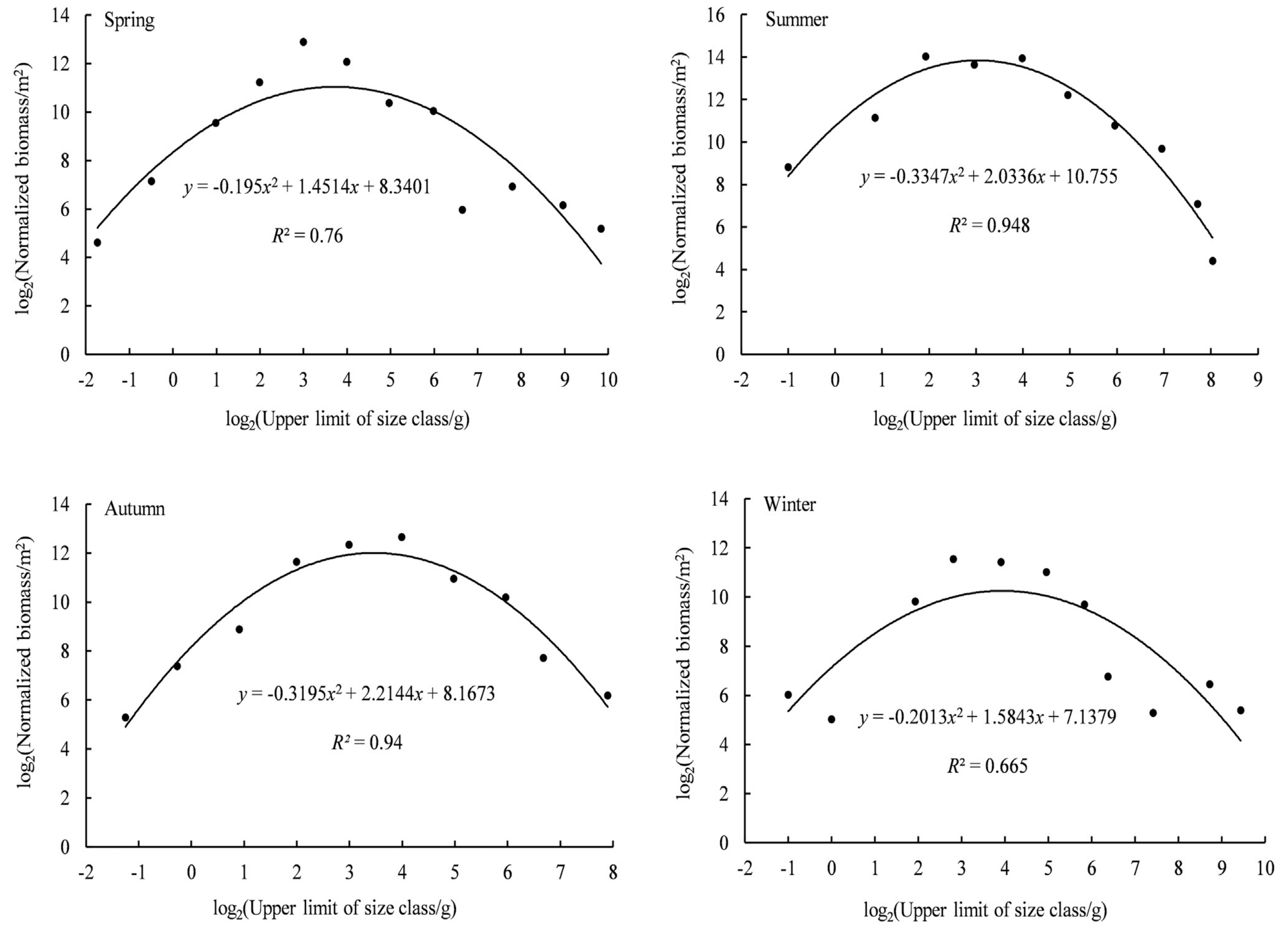

FIGURE 7 | Normalized biomass spectra of fishes in every season in Daya Bay.

TABLE 2 | Seasonal comparison of the normalized biomass particle size spectrum of fish in Daya Bay.

Season	Size range	Biomass (g/m^2)	Curvature	R^2
Spring	−2 to 10	182670.21	−0.20	0.76
Summer	−2 to 9	421909.57	−0.33	0.95
Autumn	−2 to 8	170995.99	−0.32	0.94
Winter	−2 to 10	151221.58	−0.20	0.67

nearshore and the middle, and human activities were relatively low, resulting in a more stable fish community structure. Ma et al. (2014) studied the number size spectra (NSS) of planktons in Daya Bay during summer and winter. The result indicated that the hot water discharges from nuclear power plants and agricultural wastewater releases significantly affected the plankton biomass size distribution and NSS patterns. The study demonstrated the application of BSS in studying the impacts of human disturbances on the marine ecosystem and also indicated that the particle size can be used as an ecological indicator to reflect the stability of the community structure.

Peaks are usually formed in small- and large-size species, and troughs are formed at their junctions (Schwinghamer 1981). It was suggested that the distribution of peaks and troughs is not a fixed pattern (Dolbeth et al., 2014). Their formation mainly depends on the appearance of key species in different seasons, rather than the living environments of the communities. In this study, the wave peak corresponds to a large number of small species or a low number of large species, and the troughs were at the intersection of small and large species. Also the capture of specific species in the community affected the peak shape of the NBSS, which was consistent with a previous study (Shen 2010). The corresponding fish species in the two to four size class exhibited the highest biomass abundance, which accounted for 24.76% of the total biomass, mainly consisting of highly abundant small fishes. For instance, the catch quantity and weight of *Leiognathus brevirostris* were the highest, accounting for 37.96 and 37.85% of the total catch, respectively. Also, the fish biomass on the two to four grain level exhibited an increasing trend, while the fish biomass on the 4–10 scales showed a decreasing trend, resulting in a single-peak pattern of the Sheldon-type BSS in Daya Bay. In summer, largely small-sized fishes, such as *Evynnis cardinalis* and *Leiognathus brevirostris*,

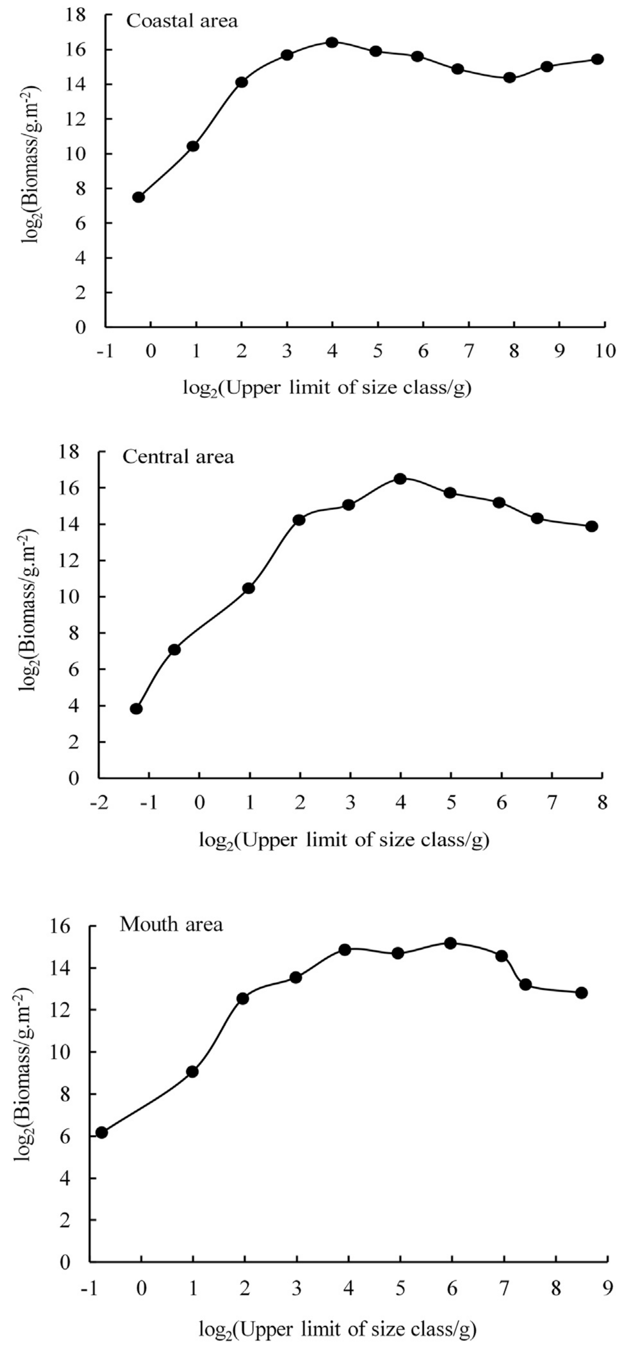

FIGURE 8 | Sheldon type biomass size spectrum of fish in different areas of Daya Bay.

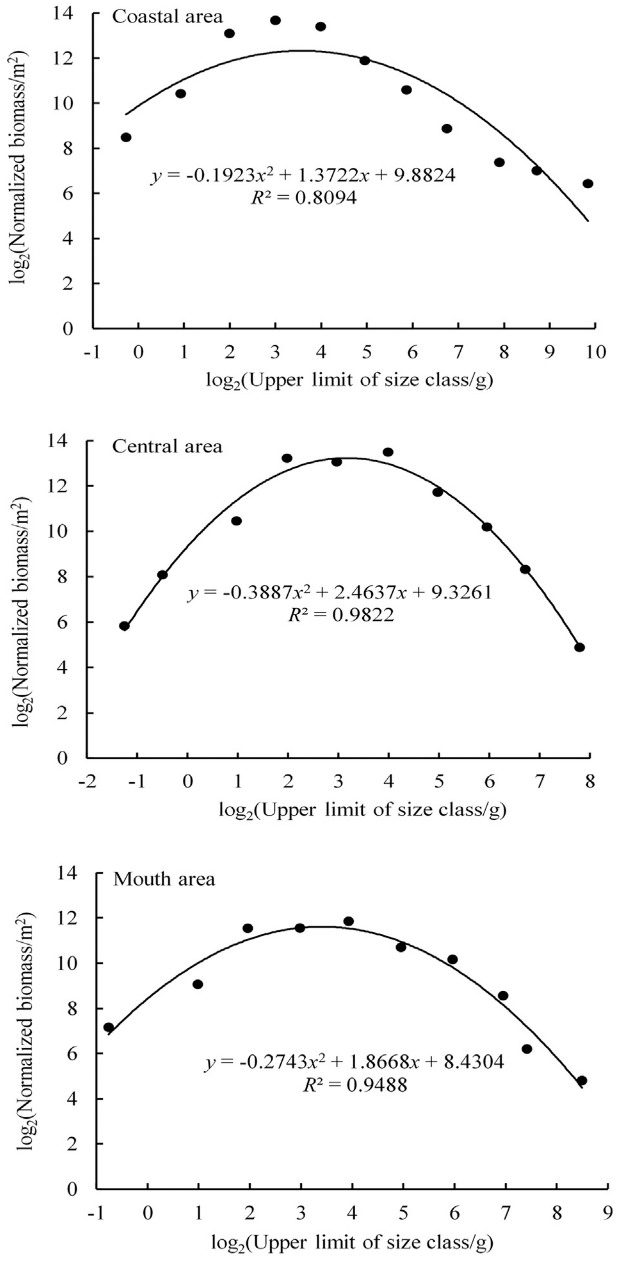

FIGURE 9 | Normalized biomass size spectra of fish in different regions of Daya Bay.

TABLE 3 | Spatial changes of the normalized biomass particle size spectrum of fish in Daya Bay.

Region	Size ranges	Biomass (g/m^2)	Curvature	R^2
Coastal area	−1 to 10	397123.97	−0.19	0.81
Central area	−2 to 8	274829.11	−0.39	0.98
Bay mouth	−1 to 9	151712.77	−0.27	0.95

were captured, and the catch weight reached the maximum, making it the highest peak in the two to four grains. In winter, one *Acanthopagrus schlegelii* and one *Pampus chinensis* were captured on the six to eight scale, and the biomass size was the smallest, resulting in a trough occurrence.

The continuous fish size spectrum analysis provides a straightforward mechanism to understand the condition of the aquatic ecosystem (Benoît and Rochet 2004; Maury et al., 2007). It lowers the intricacy of food webs, indicates the slope of NBSS, and demonstrates that the intercepts are useful to describe the characteristics of ecosystem status (Guiet et al., 2016). It is

TABLE 4 | Fish composition according to the particle size.

Particle size	Biomass (g)	Composition of fish	Trophic structure
−2 to −1	0.25–0.5	One *Glossogobius olivaceus* and one *Argyrosomus pawak* larvae	Planktivorous
−1 to 0	0.5–1	More small *Apogon lineatus*	Planktivorous
0–1	1–2	A large number of *Thamnaconus hypargyreus*, *Apogon lineatus*, *Glossogobius olivaceus*, and small fishes	Planktivorous
1–2	2–4	A large number of *Thamnaconus hypargyreus*, *Leiognathus brevirostris*, *Apogon lineatus*, *Oxyurichthys papuensis*, *Parachaeturichthys polynema*, and other small fishes	Planktivorous and benthic
2–3	4–8	Smaller individuals such as *Thamnaconus hypargyreus*, *Leiognathus brevirostris*, *Apogon lineatus*, *Oxyurichthys papuensis*, *Parachaeturichthys polynema*, *Evynnis cardinalis*, and *Trachurus japonicus*	Planktivorous and benthic
3–4	8–16	A large number of small fishes such as *Evynnis cardinalis*, *Trachurus japonicus*, *Leiognathus brevirostris*, *Callionymus meridionalis*, and *Acentrogobius caninus*	Planktivorous and benthic
4–5	16–32	A large number of small fishes such as *Umbrina russelli*, *Evynnis cardinalis*, *Sillago japonica*, and *Odontamblyopus rubicundus*	Benthic and detrital
5–6	32–64	Low count of *Clupanodon punctatus*, *Harpadon nehereus*, *Muraenesox cinereus*, *Saurida tumbil*, *Gerres filamentosus*, *Rhabdosargus sarba*, and *Nemipterus virgatus*	Benthic and nekton
6–7	64–128	A small number of larger *Sparus berda*, *Pisodonophis boro*, *Fugu niphobles*, and *Scatophagus argus*	Benthic and nekton
7–8	128–256	Four tails *Scatophagus argus*, one-tail *Plotosus lineatus*, *Acanthopagrus schlegelii*, *Takifugu poecilonotus*, *Dysomma anguillaris*, *Pampus chinensis*, and other individual large fish composition	Benthic and nekton
8–9	256–512	One *Evynnis cardinalis*, *Scatophagus argus*, *Muraenesox cinereus*, four *Sparus macrocephalus*, and other individual large fish	Benthic and nekton
9–10	512–1,024	One large-tailed *Parachaeturichthys polynema*, *Acanthopagrus schlegelii*, and *Gymnothorax hepaticus*	Benthic, detrital, and nekton

different from traditional research methods and does not rely on taxonomy knowledge. Moreover, it minimizes the calculation errors caused by the uncertainty of classification and identification, while enhances our understanding of marine ecosystem characteristics and fish species in ecological and physiological processes. Additionally, it forecasts and evaluates productivity, production, and distribution of fish community and assists in monitoring environmental changes (Borgmann 1982; Boudreau and Dickie 1992; Cyr and Peters 1996; Trebilco et al., 2013; Guo et al., 2017). It is an innovative method to study fish communities. It can be applied to study the influences of fishing, reproduction tactics, or environmental alternation at various times on the fish community. Also, evaluating the influences of climatic variations on the marine ecosystem can be considered another application (Woodworthjefcoats et al., 2013; Guénette et al., 2014). On the other hand, the structure of species diversity in fish communities considerably influences the marine ecosystem's performance, sensitivity to disturbances, and resilience (Blanchard et al., 2011), which urges the development of size-based uniqueness of the fish size spectrum (Guiet et al., 2016). In future research, it is essential to improve and advance the fish size spectrum method for further development and protect marine fishery resources.

CONCLUSION

The Sheldon-type BSS of fish in Daya Bay was a unimodal pattern, consisting of the fish size ranging from −2 to 10 grain level. Fishes in the range of four to eight size class were relatively abundant. The highest peak belonged to the two to four grain level and predominantly composed of *Leiognathus brevirostris*, *Callionymus meridionalis*, *Callionymus koreanus*, *Evynnis cardinalis*, *Trachurus japonicus*, and other small fishes. The curve of the BSS was relatively flat with large curvature in spring and winter. The curves in summer and autumn were comparatively steep, and the curvature was small. The characteristics of fish BSS were significantly affected by the number and size of the breeding population, the trophic level and migratory habits of fish, and other life history characteristics as well as human disturbance (fishing), among which overfishing was the dominant factor. The ABC curve showed that the fish community in Daya Bay was in a state of serious disturbance throughout the year.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because the study was conducted by only measuring the size and identifying fish type by their appearance in the ecosystem. No experiment was conducted on the studied fishes.

AUTHOR CONTRIBUTIONS

SX, JF, TX, and CL contributed to the conception and design of this study. JG, KW, TX, BB, and YL developed the method, acquired the data, and wrote the first draft of the manuscript. JF and YXu ran the analyses. SX and BB provided feedback on analysis. SX, BB, and CL provided revisions and editing. All authors read and approved the submitted version.

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Interaction Effects of Temperature, Light, Nutrients, and pH on Growth and Competition of *Chlorella vulgaris* and *Anabaena* sp. Strain PCC

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Interaction effects of temperature, light, nutrients, and pH on growth and competition of *Chlorella vulgaris* and *Anabaena* sp. strain PCC were evaluated using an orthogonal design method to elucidate how these environment factors promote the growth of beneficial algae and limit the growth of harmful algae. The optimal conditions for the growth of *C. vulgaris* in the mono-culture system were as follows: temperature, 35°C; light, 660 lx; N concentration, 0.36 mg L^{-1}; P concentration, 0.1 mg L^{-1}; and pH, 9.0; and those for *Anabaena* were as follows: temperature, 30°C; light, 6,600 lx; N concentration, 0.18 mg L^{-1}; P concentration, 0.1 mg L^{-1}; and pH, 7.0. The optimal conditions for the growth of *C. vulgaris* in the co-culture system were as follows: temperature, 25°C; light, 4,400 lx; N concentration, 0.18 mg L^{-1}; P concentration, 0.5 mg L^{-1}; and pH, 6.0; and those for *Anabaena* were as follows: temperature, 35°C; light, 4,400 lx; N concentration, 0.36 mg L^{-1}; P concentration, 0.5 mg L^{-1}; and pH, 6.0. Both competition-inhibition parameters of *Anabaena* against *C. vulgaris* and those of *C. vulgaris* against *Anabaena* were the largest under the following conditions: temperature, 30°C; light intensity, 6,600 lx; N concentration, 0.36 mg L^{-1}; P concentration, 0.025 mg L^{-1}; and pH, 8.0. According to the Lotka–Volterra competition model, *Anabaena* won in the competition in the co-culture system with the following conditions: 1) temperature, 15°C; light, 660 lx; total N (TN), 0.18 mg L^{-1}; total P (TP), 0.025 mg L^{-1}; pH, 6; 2) temperature, 15°C; light, 2,200 lx; TN, 0.36 mg L^{-1}; TP, 0.025 mg L^{-1}; pH, 7; 3) temperature, 15°C; light, 6,600 lx; TN, 3.6 mg L^{-1}; TP, 0.5 mg L^{-1}; pH, 9; 4) temperature, 30°C; light, 4,400 lx; TN, 0.18 mg L^{-1}; TP, 0.05 mg L^{-1}; pH, 9; 5) temperature, 35°C; light, 660 lx; TN, 3.6 mg L^{-1}; TP, 0.05 mg L^{-1}; pH, 8; and 6) temperature, 35°C; light, 2,200 lx; TN, 0.72 mg L^{-1}; TP, 0.025 mg L^{-1}; pH, 9. However, *C. vulgaris* could not win in the competition in the co-culture system under all conditions tested.

Keywords: *Chlorella vulgaris*, *Anabaena* sp. strain PCC, environment factors, interspecies competition, temperature, light, nutrients, pH

INTRODUCTION

Harmful cyanobacterial blooms are becoming increasingly common in eutrophic water bodies, and they may result in a wide range of environmental, social, and economic consequences. For example, cyanobacterial blooms can increase oxygen demand, which may lead to localized incidents of hypoxic or anoxic conditions causing fish deaths (Anderson et al., 2002; Paerl, 2008). Surface blooms can also block light from reaching benthic primary producers, which may adversely affect food web dynamics depending on lake-bottom habitats (Bricelj and Lonsdale, 1997; Gallegos and Bergstrom, 2005). The social and economic effects of cyanobacterial blooms include negative effects on recreational opportunities due to the closure of affected areas, declining local fisheries, and increased water treatment costs (Hoagland et al., 2002; Paerl, 2008). In addition to their wider ecological effects, cyanobacteria are known to produce a suite of secondary metabolites that include hepatotoxins, neurotoxins, and dermatotoxic compounds. These toxins have been linked to reduced water quality and detrimental effects at higher trophic levels (Leonard and Paerl, 2005; Ferrão-Filho et al., 2009), small-animal illness and even mortality (Boyer, 2007; Jacoby and Kann, 2007), and adverse health risks in humans (Paerl, 2008).

Anabaena is a microscopic, bloom-forming, harmful cyanobacterium that has been reported to cause harmful algal blooms (Bouma-Gregson et al., 2017) and produce algal toxins in waters worldwide (Cai et al., 2006; Lawton and codd, 2010). It also causes ecological damage through high biomass and other related effects (Al-Mamoori et al., 2020). To improve water quality, protect aquatic animals, and ensure the safety and quality of aquatic products, scientists have carried out extensive research on how to control the growth of *Anabaena* in water bodies, especially in aquaculture ponds (Yue et al., 2006; Yu et al., 2016; Peng X. et al., 2020). *Chlorella vulgaris* is a typical microalga in water bodies. It can be easily digested by fish and other aquatic animals and is considered to be useful in water bodies (Meng et al., 2017). Therefore, domination by *C. vulgaris* in a water body is considered an indicator of good water quality (Yang and Lu, 2014; He et al., 2019).

Several environmental factors can influence the growth of algae (Zhao et al., 2014), including abiotic factors such as hydrodynamics (i.e., turbulence, wind-driven currents, and stratification) (Hotto et al., 2007; Fortin et al., 2010), nutrients (Downing et al., 2001; Heisler et al., 2008; Elliott, 2012), temperature (Cires et al., 2011), light availability (Cires et al., 2011; Renaud et al., 2011), and biotic interactions such as competition and grazing (Gobler et al., 2007; Ger et al., 2010). Temperature, light, nutrients, and pH are important ecological factors influencing algal growth (Mao et al., 2007), but there are only a few reports on their interactive effects on *C. vulgaris* and *Anabaena*. Changes in any of the factors may alter the phytoplankton community structure (Mei et al., 2003; Meng et al., 2013). It is important to understand how environmental factors can be manipulated to promote the growth of beneficial algae and limit the growth of harmful algae. Therefore, using an orthogonal design, in this study, we aimed to evaluate whether the interactions between temperature, light, nutrients, and pH influence the growth and competition of *C. vulgaris* and *Anabaena*, and to determine the optimal conditions for the dominance of *C. vulgaris* over *Anabaena* in water. The results might be valuable in guiding the control of environmental factors in pond and tank aquaculture.

TABLE 1 | Levels of each factor.

Level	Factor				
	T (°C)	L (lx)	TN ($mg·L^{-1}$)	TP ($mg·L^{-1}$)	pH
1	15	660	0.18	0.025	6
2	25	2,200	0.36	0.05	7
3	30	4,400	0.72	0.1	8
4	35	6,600	3.6	0.5	9

MATERIALS AND METHODS

Algae and Growth Conditions

Chlorella vulgaris and *Anabaena* sp. strain PCC 1042 were obtained from the Institute of Hydrobiology, Chinese Academy of Sciences. All algae were cultured in BG11 medium (Meng et al., 2015) in a plant growth chamber (PGX-150B; Wuxi Woxin Apparatus Co., Ltd., China). The culture conditions were as follows: temperature, 25°C; light intensity, 2.2×10^3 lx (using a fluorescent lamp as the light source); and photoperiod, 12-: 12- h light/dark.

The experiments were conducted in 250 ml Erlenmeyer flasks with 200 ml of BG11 medium (Meng et al., 2015). The cultures were shaken gently once every 2 h during the light period and allowed to stay still during the dark period. Before sampling, they were shaken again to ensure homogeneous cell distribution.

Experimental Design

An orthogonal array L_{16} 5^4) with five environmental factors (temperature (T), light (L), N concentration (TN), P concentration (TP), and pH) and four levels (**Table 1**) were set using software Minitab 15 (The water temperature that good for the growth of most fish species is higher than 15°C, and the highest water temperature is about 35°C in summer, so the four temperature of 15°C, 25°C, 30°C, 35°C were chosen. The phosphorus concentration of 0.02 mg L^{-1} is critical point for eutrophication happening (Shi et al., 2004), and that of 0.5 mg L^{-1} is the first level standard value for the waste water discharged from aquaculture pond (Ministry of Agriculture and Rural Affairs of the PRC, 2007), so the four phosphorus concentrations of 0.025 mg L^{-1}, 0.05 mg L^{-1}, 0.1 mg L^{-1}, 0.5 mg L^{-1} were chosen. Our other test showed that the nitrogen and phosphorus ratio for the best growth of *Chlorella vulgaris* is 7.2:1 (Wang et al., 2015), so the four nitrogen concentrations of 0.18 mg L^{-1}, 0.36 mg L^{-1}, 0.72 mg L^{-1}, 3.6 mg L^{-1} were chosen based on the four phosphorus concentrations. According to the "Environmental quality standards for surface water" (Ministry of Ecology and Environment of the PRC, 2002), the pH should be between 6 and 9, so the four pH levels of 6, 7, 8, 9 were chosen. Although the natural light intensity in summer is as high as 100,000 lx in summer, the best light intensity for the growth of most fish species is lower than 7,000 lx (Xu et al., 2014), so the four light

TABLE 2 | The orthogonal test program.

Treatment	T (˚C)	L (lx)	TN ($mg{\cdot}L^{-1}$)	TP ($mg{\cdot}L^{-1}$)	pH
1	15	660	0.18	0.025	6
2	15	2,200	0.36	0.05	7
3	15	4,400	0.72	0.1	8
4	15	6,600	3.6	0.5	9
5	25	660	0.36	0.1	9
6	25	2,200	0.18	0.5	8
7	25	4,400	3.6	0.025	7
8	25	6,600	0.72	0.05	6
9	30	660	0.72	0.5	7
10	30	2,200	3.6	0.1	6
11	30	4,400	0.18	0.05	9
12	30	6,600	0.36	0.025	8
13	35	660	3.6	0.05	8
14	35	2,200	0.72	0.025	9
15	35	4,400	0.36	0.5	6
16	35	6,600	0.18	0.1	7

intensity levels of 660, 2,200, 4,400, 6,600 were chosen.); there were 16 groups in total, as shown in **Table 2**. There were three types of algal culture in every group, namely, mono-culture of *C. vulgaris* (treatment C), mono-culture of *Anabaena* (treatment A), co-culture of *C. vulgaris* and *Anabaena* (treatment CA). Three replicates for each group were prepared.

The initial density of *C. vulgaris* and *Anabaena* in each group was 5×10^5 cell ml^{-1}, and the inoculations were carried out according to the method of Meng et al. (2015).

General Analyses

During the algal culture period, the liquid medium in each group was sampled with a 0.45-μm filter needle to determine total N and total P concentrations. Subsequently, $NaNO_3$, KH_2PO_4, and ammonia-free water were added to increase the N and P concentrations and liquid medium volume to the initial level according to the determination results.

The cells were counted every 24 h after the beginning of the test. The test was terminated when a negative growth of algae was found, and the density 1 day before the negative growth was used as the maximum density of each algal species. The algal density was determined according to the methods described in the *Standard Methods for the Examination of Water and Wastewater* (Chinese National Environmental Protection Agency, 2002).

Data Analysis Methods

Specific Growth Rate

Specific growth rate was calculated using the following formula (Meng et al., 2012):

$$\ln\frac{Nn}{\mathrm{N}n-1} = \mu_n \cdot (tn - tn-1)\ (n = 1, 2, 3, 4, \ldots) \tag{1}$$

where, μ_n is the growth rate on day n, N is cell density (cells ml^{-1}), and t is the culture time (d). The average specific growth rate (μ) is the algal growth rates from day 1 of the test to the day when the maximum cell density appeared, and μ was used to compare the growth rate of algal cells in different treatments.

Algae Growth Curve Fitting

The growth of algae was fitted by the logarithmic form of a logistic equation, and the least squares method was used for regression analysis to obtain the intercept and slope rate of the equation as estimated values for a and r respectively:

$$\ln\frac{K-N}{N} = \text{a-rt} \tag{2}$$

where, N is the biomass of the algae (count the biomass of algae in each treatment every day, and this biomasses are N values), K is the maximum biomass of the algae (using the maximum biomass of the algae in each treatment as its K value), r is the intrinsic growth rate, t is culture time.

Calculation of Competition–Inhibition Parameter

The Lotka–Volterra competition model was adopted to calculate the competition–inhibition parameters of *C. vulgaris* and *Anabaena* (Meng et al., 2012):

$$\frac{Nc, n - Nc, n-1}{tn - tn-1} = \frac{rcNc, n-1\,(Kc - Nc, n-1 - \alpha Na, n-1)}{Kc}\ (n = 1, 2, 3, 4, \ldots) \tag{3}$$

$$\frac{Na, n - Na, n-1}{tn - tn-1} = \frac{raNa, n-1\,(Ka - Na, n-1 - \beta Nc, n-1)}{Ka}\ (n = 1, 2, 3, 4, \ldots) \tag{4}$$

where, N_c and N_a are the biomass ($\times 10^4$ cells ml^{-1}) of *C. vulgaris* and *Anabaena* in the co-culture system at time t, respectively; r_c *and* r_a are the intrinsic growth rate of *C. vulgaris* and *Anabaena* in the mono-culture system, respectively; K_c and K_a are the maximum biomass of *C. vulgaris* and *Anabaena* in the mono-culture system, respectively; α is the competition–inhibition parameter of *Anabaena* against *C. vulgaris* in the co-culture system, and β is the competition–inhibition parameter of *C. vulgaris* against *Anabaena* in the co-culture system.

The restraint beginning point and inhibition parameter were calculated according to Chen et al. (1999).

Statistical Analysis

Statistical analyses were performed using SPSS 15.0. Significant differences in algae biomass caused by the environment factors combination of T, L, TN, TP and pH, that is to say between the different treatments, were analyzed using the one-way analysis of variance (ANOVA), and Tukey's multiple comparisons were used for statistical comparisons. Data were tested for normality of distribution (Shapiroe–Wilk test) and homogeneity of variance (Levene's test) before analysis. Data that did not meet the assumptions of normality and homoscedasticity were transformed (lg), and then analyzed using the one-way ANOVA. Student's t-test was used for testing the significance of the coefficients in the regression equations. Results with $p < 0.05$ were considered significant.

RESULTS

Growth of *C. vulgaris* and *Anabaena*

The growth curves of *C. vulgaris* and *Anabaena* are shown in **Figure 1** and their growth rates in **Table 3**. The main effect

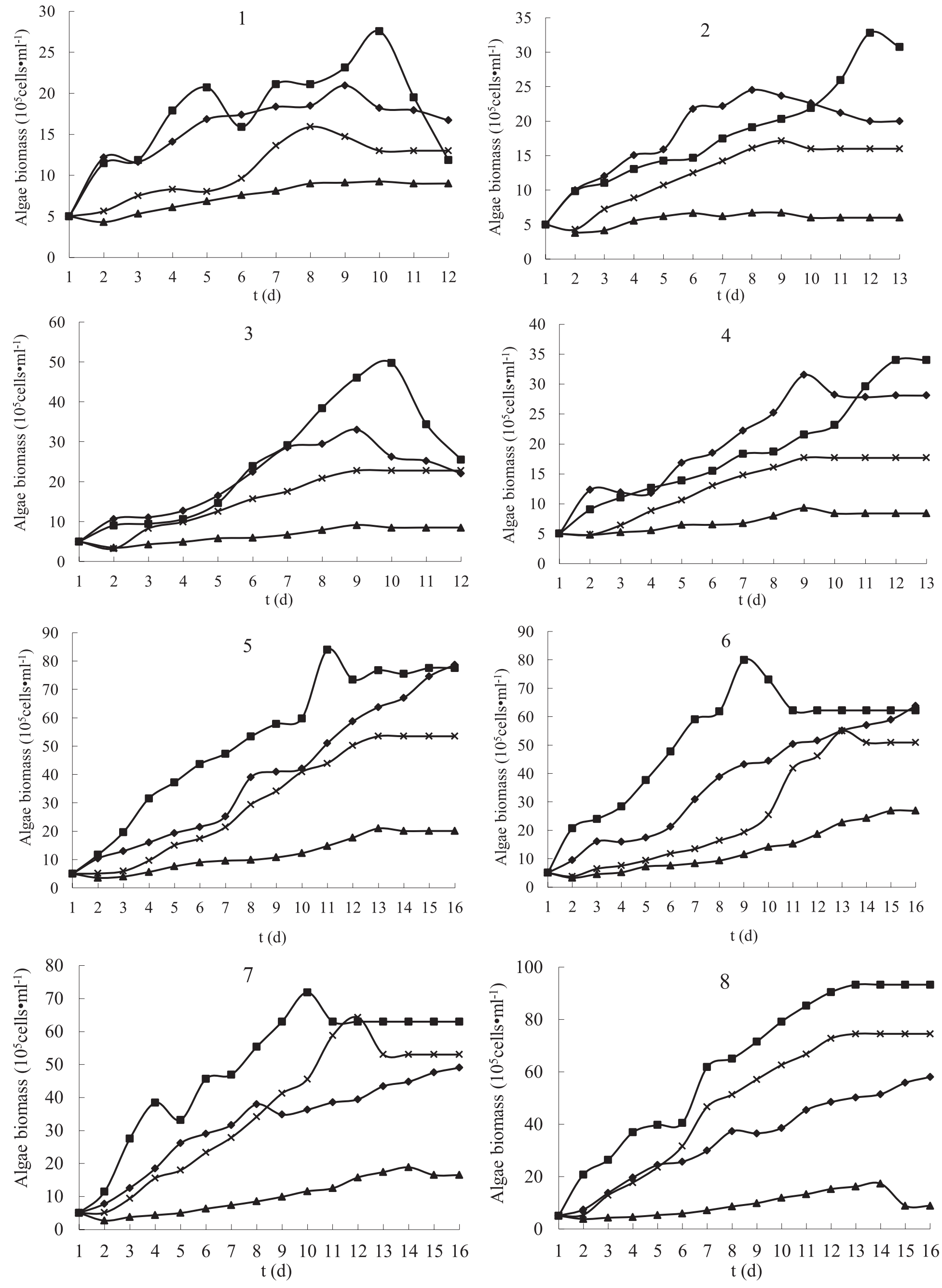

FIGURE 1 | Growth curves of Chlorella vulgaris and Anabaena sp. strain PCC in mono-culture and co-culture systems at different conditions (from Treatment 1 to Treatment 16).

analysis plots (**Supplementary Figures S1, S2**) showed that the optimal growth conditions for *C. vulgaris* in the mono-culture system were as follows: temperature, 35°C; light intensity, 660 lx; N concentration, 0.36 mg L^{-1}; P concentration, 0.1 mg L^{-1}; and pH, 9.0; and those for *Anabaena* were as follows: temperature, 30°C; light intensity, 6,600 lx; N concentration, 0.18 mg L^{-1}; P concentration, 0.1 mg L^{-1}; and pH, 7.0. As shown in **Supplementary Figures S3, S4**, the optimal growth condition for *C. vulgaris* in the co-culture system were as follows: temperature, 25°C; light intensity, 4,400 lx; N concentration, 0.18 mg L^{-1}; P concentration, 0.5 mg L^{-1}; and pH, 6.0; and those for *Anabaena* were as follows: temperature, 35°C; light intensity, 4,400 lx; N concentration, 0.36 mg L^{-1}; P concentration, 0.5 mg L^{-1}; and pH, 6.0.

The maximum cell density of *C. vulgaris* in the mono-culture system was observed in treatment 5 (temperature, 25°C; light

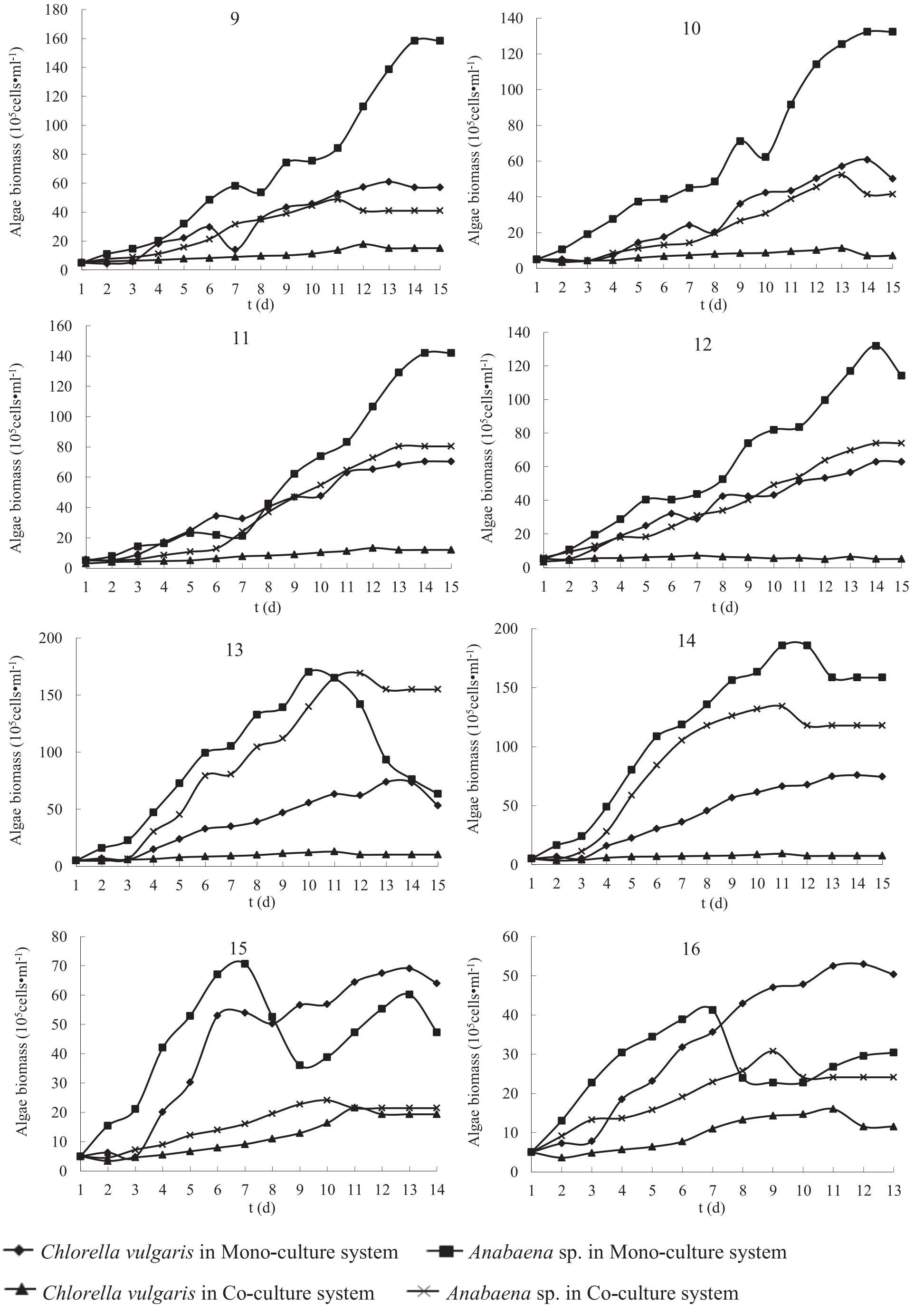

FIGURE 1 | (Continued).

intensity, 660 lx; N, 0.36 mg L^{-1}; P, 0.1 mg L^{-1}; pH, 9.0); under these conditions, the maximum cell density was 787.0×10^4 cells L^{-1}. The maximum cell density of *Anabaena* in the mono-culture system was found in treatment 14 (temperature, 35°C; light intensity, 2,200 lx; N, 0.72 mg L^{-1}; P, 0.025 mg L^{-1}; pH, 9.0); under these conditions, the maximum cell density was $1{,}854.9 \times 10^4$ cells L^{-1}. The maximum cell density of *C. vulgaris* in the co-culture system was in treatment 6 (temperature, 25°C; light intensity, 2,200 lx; N, 0.18 mg L^{-1}; P, 0.5 mg L^{-1}; pH, 8.0); under these conditions, the maximum cell density was 268.5×10^4 cells·L^{-1}. The maximum cell density of *Anabaena* in the co-culture system was in treatment 13 (temperature, 35°C; light intensity, 660 lx; N, 3.6 mg L^{-1}; P, 0.05 mg L^{-1}; pH, 8.0); under these conditions, the maximum cell density was $1\ 690.2 \times 10^4$ cells·L^{-1}.

TABLE 3 | Mean specific growth rate (μ) of Chlorella vulgaris and Anabaena sp. strain PCC at different conditions.

Algae	Treatment	Mean specific growth rate	
		Mono-culture	Co-culture
Chlorella vulgaris	1	0.179	0.096
	2	0.227	0.112
	3	0.236	0.076
	4	0.157	0.094
	5	0.184	0.119
	6	0.200	0.126
	7	0.143	0.138
	8	0.163	0.127
	9	0.208	0.108
	10	0.207	0.106
	11	0.193	0.135
	12	0.192	0.116
	13	0.215	0.106
	14	0.204	0.108
	15	0.218	0.205
	16	0.199	0.166
Anabaena sp.	1	0.213	0.173
	2	0.191	0.167
	3	0.278	0.189
	4	0.174	0.158
	5	0.276	0.198
	6	0.359	0.200
	7	0.247	0.242
	8	0.244	0.243
	9	0.266	0.226
	10	0.241	0.233
	11	0.257	0.231
	12	0.252	0.195
	13	0.392	0.336
	14	0.418	0.369
	15	0.441	0.175
	16	0.352	0.174

The growth curves of both *C. vulgaris* and *Anabaena* in the mono-culture system fitted an "S" shape (**Figure 1**), indicating that the growth curve of the two algae in the mono-culture system could be fitted with a logistic model, and the inflection point could be calculated using a logistic equation (**Supplementary Materials**). To calculate the inflection point time, the growth curves of *C. vulgaris* and *Anabaena* in the co-culture system were also fitted by a logistic model (**Supplementary Materials**).

Competition–Inhibition Parameters Between *C. vulgaris* and Anabaena

The competition–inhibition parameters of *C. vulgaris* against *Anabaena* and *Anabaena* against *C. vulgaris* in the co-culture system are shown in **Table 4**. The results revealed that the competition–inhibition parameters of *Anabaena* against *C. vulgaris* (α) were higher than those of *C. vulgaris* against Anabaena (β) in treatments 1, 2, 4, and 11. On the contrary, the competition–inhibition parameters of C. vulgaris against Anabaena (β) were greater than that of Anabaena against C. vulgaris (α) in other treatments. Both α and β reached the highest value in treatment 12. As shown in **Table 4**, the inflection point time of *C. vulgaris* was later than that of *Anabaena* in the mono-culture system in most treatments, but it was complicated, and there were no change trends for the inflection point time of *C. vulgaris* and *Anabaena* in the co-culture system.

DISCUSSION

Interaction Effects of Environmental Factors on the Growth of *C. vulgaris* and Anabaena in Mono-Culture System

The optimal growth conditions are different for different species of algae (Nakajima et al., 2020; Toth et al., 2020; Li et al., 2021). Long et al. (2011) reported that the optimal growth conditions for spring algal species are as follows: temperature, 20°C; light intensity, 4,700 lx; N concentration, 3.5 mg L^{-1}; P concentration, 0.3 mg L^{-1}; and water flow rate, 0.1 m s^{-1}. Our analyses showed that the maximum cell density of *C. vulgaris* increased with temperature (**Supplementary Figure S1**) and reached the peak point at 35°C. The optimal growth conditions for *C. vulgaris* were as follows: temperature, 35°C; light intensity, 660 lx; N concentration, 0.36 mg L^{-1}; P concentration, 0.1 mg L^{-1}; and pH, 9.0; and those for *Anabaena* were as follows: temperature, 30°C; light intensity, 6,600 lx; N concentration, 0.18 mg L^{-1}; P concentration, 0.1 mg L^{-1}; and pH, 7.0. Song et al. (2013) observed an apparent exponential growth phase of *Chlorella* when the temperature increased to 20°C or 25°C, and its specific growth rate and maximum cell density also increased with the increase in temperature. In the present study, we found a similar relationship between maximum cell density and temperature.

As shown in **Supplementary Figure S2**, the maximum cell density of *Anabaena* at a light intensity of less than 2,200 lx was below the mean level, indicating that its growth was inhibited under such light conditions. A long period under light-limited conditions could increase the precipitation of *Anabaena* cells and result in cell loss (Wu et al., 2012), which might explain the phenomenon observed in our experiment.

Through regression analysis, Liang et al. (2010) found that water temperature and total P concentration are two key factors affecting the growth of *Anabaena*. Wang et al. (2005) reported that the effect of water temperature on algal biomass is closely related to the nutrient concentration in the water. They found that the algal biomass at a low temperature was greater than that at a high temperature in the water with low nutrient concentrations (inorganic N, 0.2–0.4 mg L^{-1}; organic N, 0.2–0.4 mg L^{-1}; and total P, 0.005–0.01 mg L^{-1}), and the algal biomass at a low temperature was less than that at a high temperature in water with high nutrient concentrations (inorganic N, 0.3–0.65 mg L^{-1}; organic N, 0.4–0.7 mg L^{-1}; and total P 0.01–0.03 mg L^{-1}). We obtained a similar result in our experiment, that is, the maximum cell density of *C. vulgaris* in treatments with a high temperature and low nutrient concentrations was lower than that in treatments with a low temperature and high nutrient concentrations. The activities of the algae increased with the increase in temperature, leading to a higher metabolic rate and greater nutrient consumption; thus, the

TABLE 4 | Inhibition parameters of *Chlorella vulgaris* and *Anabaena* sp. strain PCC after inflection point at different conditions.

Treatment	Inhibition parameter	Time (d)													
		2	3	4	5	6	7	8	9	10	11	12	13	14	Average
1	α	0.89	1.08	1.05	1.09	1.03	0.54	0.72	0.75						0.89
	β		2.10	3.60	0.42	–-2.15	−0.02	1.98							0.79
2	α		3.93	0.86	1.59	1.43	1.66	1.05	1.11						1.95
	β			−1.05	−0.83	−0.02	0.28	0.13	1.16						0.14
3	α			2.42	1.69	2.04	1.23	0.87	0.73						1.49
	β			5.43	2.98	2.43	3.84	2.21	2.56						3.24
4	α	3.89	3.33	1.38	2.30	1.67	0.65	0.57							1.97
	β			−0.70	−1.49	0.19	0.98	0.43							−0.12
5	α			2.72	1.89	1.31	1.33	0.90	0.48	0.15					1.25
	β			−2.13	−9.04	8.70	7.36	5.60	6.25						2.79
6	α						0.64	0.43	1.34	0.15	0.07	0.51	0.38		0.50
	β						3.75	0.85	3.39	1.43	0.18				0.57
7	α				2.17	1.85	1.02	0.54							1.40
	β	6.84	7.25	5.85	4.37	3.64	3.40								5.23
8	α				0.32	0.47	0.23	0.41	0.28	0.44	0.40				0.36
	β			−5.70	3.78	2.27	1.72	1.47	0.66	1.03					0.75
9	α					2.72	1.89	1.31	1.33	0.90	0.48	0.15			1.25
	β					−2.13	−9.04	8.70	7.36	5.60	6.25				2.79
10	α			2.11	2.89	3.32	2.91	2.36	1.85	1.23	1.08	0.77			2.06
	β							−1.96	5.62	0.85	3.27	3.30			2.22
11	α					2.50	2.41	2.20	1.30	0.86	0.84	0.51			1.52
	β							−8.62	1.48	3.89	2.39	3.00	2.50		0.77
12	α	3.24	1.76	4.11	2.36	2.59	1.70								2.63
	β							9.30	5.05	1.82	8.55	2.40	6.93	6.26	5.76
13	α		7.74	0.93	1.15	0.70	0.62	0.38	0.43	0.38					1.54
	β					9.95	−0.01	4.45	−1.55	−2.04	−0.15				1.77
14	α	−1.63	1.04	1.94	1.54	1.73	2.03								1.11
	β	5.69	1.67	2.20	1.60										2.79
15	α						0.96	1.34	0.42	0.67	0.04	0.56			0.66
	β						4.81	2.91	2.66	1.93	1.36				2.73
16	α			1.95	1.77	1.66	1.03	0.70	0.54	0.82					1.21
	β				3.59	3.59	3.38	2.54	1.85	1.58					2.76

available nutrients in the water depleted and finally limited the algal growth.

Interaction Effects of Environmental Factors on the Competitive Growth of *C. vulgaris* and Anabaena in the Co-culture System

Different microalgal species compete with one another for light, nutrients, and other resources (Cao et al., 2012); therefore, competition is a crucial factor promoting changes in the predominant algae in water bodies (Piazzi and Ceccherelli, 2002; Litchman, 2003; Burrows et al., 2021). Our experiment revealed that competition–inhibition parameters of *C. vulgaris* against *Anabaena* and those of *Anabaena* against *C. vulgaris* in the co-culture system differed under different conditions, indicating that the predominant positions of the two algae alternated with the environment. Chen et al. (2010) reported that competition–inhibition parameters between *Oscillatoria* and *Microcystis* changed at 20, 25, and 30°C. Moreover, Chen et al. (2009) showed that *Microcystis* is more competitive than *Scenedesmus* at light intensity of below 6,600 lx, and the vice versa at higher light intensity. In our analyses, the maximum cell densities of both *C. vulgaris* and *Anabaena* in the co-culture system were lower than in the mono-culture system; this indicated that the growth of both algae was inhibited in the co-culture system.

The algae growing together compete for light, nutrients, and other resources and even secrete growth-inhibition substances to inhibit each other (Xu et al., 2004; Peng Y. et al., 2020; Stn and Bykiik, 2020). When the growth of one algal species is inhibited due to the deficiency of some nutrient elements, another species less dependent on these nutrient elements might grow quickly and become the dominant species (Wang et al., 2010; Xu et al., 2011). Hu et al. (2006) showed that *Chlorella* reduced the N and P concentration in the environment by absorbing them. Therefore, it could be inferred that the growth of *C. vulgaris* changes the N-to-P ratio in a co-culture system to a certain extent. This effect might explain why the competition–inhibition parameters of *C. vulgaris* against *Anabaena* were higher than those of *Anabaena* against *C. vulgaris* in the present study, even when the growth of *C. vulgaris* had been inhibited in treatments 1, 2, 4, 10, 11, 12, 13, and 16. Zhao H. T. et al. (2011) showed that, under non-steady-state conditions, *Microcystis aeruginosa* quickly adopted the dominant position when nutrients were added continuously, but *Scenedesmus obliquus* occupied the dominant position when the nutrient level was low. In our experiment, the nutrients in the medium were measured and restored to the initial level every 24 h. Under such conditions, the cell density of *Anabaena* was always higher than that of *C. vulgaris*, indicating that *Anabaena* had a greater affinity for nutrients. Zhao X. D. et al. (2011) revealed that under conditions of low N and P concentrations, *M. aeruginosa* became the dominant species. Zhao H. T. et al. (2011) found that *Microcystis* sp. could adapt to low-nutrient conditions by changing cell size. Therefore, it could be inferred that another species of cyanobacteria, namely *Anabaena*, might be able to absorb nutrients and inhibit the growth of *C. vulgaris* by changing cell size according to its cell density and the nutrient concentration in the environment. This would also explain why the competition–inhibition parameters of *Anabaena* against *C. vulgaris* were greater in treatments with lower N and P concentrations.

Competitive Growth of *C. vulgaris* and Anabaena in the Co-culture System

According to the algal competition ending described in the Lotka–Volterra competition model (Meng et al., 2012), the relationships between competition–inhibition parameters α, β, and environmental capacities K_c and K_a are shown in **Table 5**. As shown in **Table 5**, in treatments 1, 2, 4, 11, 13, and 14, $1/K_a < \alpha/K_c$ and $1/K_c > \beta/K_a$, indicating that *Anabaena* was dominant in the competitions; in treatments 3, 5, 9, 10, 12, 15, and 16, $1/K_c < \beta/K_a$ and $1/K_a < \alpha/K_c$, indicating that *C. vulgaris* and *Anabaena* could coexist, but not stably; in treatments 6, 7, and 8, $1/K_c > \beta/K_a$ and $1/K_a > \alpha/K_c$, indicating that *C. vulgaris* and *Anabaena* could coexist stably (**Supplementary Figure S5**). The phenomenon in our experiment showed that low phosphorus conditions are good for the victory of *Anabaena* in the competitions, and high phosphorus conditions are beneficial for *C. vulgaris*. Just as *M. aeruginosa*, the other harmful blue-green algae, could become the dominant species under conditions of low phosphorus concentration by the way of changing cell size to adapt to low-nutrient conditions. So maybe maintain a suitable condition with high phosphorus concentration (no less than 0.1 mg L^{-1}) is useful to promote growth of beneficial algae such as *C. vulgaris* and inhibit growth of harmful algae such as *Anabaena*.

TABLE 5 | Parameters of Chlorella vulgaris and Anabaena sp. strain PCC in Lotka-Volterra Model.

Treatment	$1/K_c$	$1/K_a$	α/K_c	β/K_a
1	0.048	0.036	0.043	0.029
2	0.041	0.030	0.080	0.004
3	0.030	0.020	0.045	0.065
4	0.032	0.029	0.062	-0.004
5	0.013	0.012	0.014	0.017
6	0.016	0.013	0.008	0.007
7	0.020	0.014	0.007	0.003
8	0.017	0.011	0.006	0.008
9	0.016	0.006	0.021	0.018
10	0.016	0.008	0.034	0.017
11	0.014	0.007	0.022	0.005
12	0.016	0.008	0.042	0.044
13	0.014	0.006	0.021	0.010
14	0.013	0.005	0.025	0.009
15	0.014	0.014	0.020	0.074
16	0.019	0.024	0.027	0.059

CONCLUSION

The optimal growth conditions for *C. vulgaris* in the mono-culture system were as follows: temperature, 35°C; light intensity, 660 lx; N concentration, 0.36 mg L^{-1}; P concentration, 0.1 mg L^{-1}; and pH, 9.0. The optimal growth conditions for *Anabaena* in the mono-culture system were as follows: temperature, 30°C; light intensity, 6,600 lx; N concentration, 0.18 mg L^{-1}; P concentration, 0.1 mg L^{-1}; and pH, 7.0. The optimal growth conditions for *C. vulgaris* in the co-culture system were as follows: temperature, 25°C; light intensity, 4,400 lx; N concentration, 0.18 mg L^{-1}; P concentration, 0.5 mg L^{-1}; and pH, 6.0. For *Anabaena* in the co-culture system, the optimal growth conditions were as follows: temperature, 35°C; light intensity, 4,400 lx; N concentration, 0.36 mg L^{-1}; P concentration, 0.5 mg L^{-1}; and pH, 6.0. Light was not the most critical factor affecting the growth of *C. vulgaris*, and pH was not the most critical factor affecting the growth of *Anabaena*.

Temperature, light, nutrients, and pH significantly influenced the competition–inhibition parameters of the two algal species. Both competition–inhibition parameters of *Anabaena* against *C. vulgaris* and *C. vulgaris* against *Anabaena* were the highest under the following conditions: temperature, 30°C; light intensity, 6,600 lx; N concentration, 0.36 mg L^{-1}; P concentration, 0.025 mg L^{-1}; and pH, 8.0. According to the Lotka–Volterra competition model, *Anabaena* won in the competition in co-culture system under the following conditions: 1) temperature, 15°C; light, 660 lx; TN, 0.18 mg L^{-1}; TP, 0.025 mg L^{-1}; pH, 6; 2) temperature, 15°C; light, 2,200 lx; TN, 0.36 mg L^{-1}; TP, 0.025 mg L^{-1}; pH, 7; 3) temperature, 15°C; light, 6,600 lx; TN, 3.6 mg L^{-1}; TP, 0.5 mg L^{-1}; pH, 9; 4) temperature, 30°C; light, 4,400 lx; TN, 0.18 mg L^{-1}; TP, 0.05 mg L^{-1}; pH, 9; 5) temperature, 35°C; light, 660 lx; TN, 3.6 mg L^{-1}; TP, 0.05 mg L^{-1}; pH, 8; and 6) temperature, 35°C; light, 2,200 lx; TN, 0.72 mg L^{-1}; TP, 0.025 mg L^{-1}; pH, 9.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

JC, PX, and SM designed the study. SM and XC planned the research activities. SM, XC, and JW performed the study, and collected and analyzed the data. SM wrote the manuscript. LF, LQ, and YZ revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2021.690191/full#supplementary-material

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Identification of Eggs and Spawning Zones of Hairtail Fishes *Trichiurus* (Pisces: *Trichiuridae*) in Northern South China Sea, Using DNA Barcoding

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Commercially important hairtails, *Trichiurus* spp., are widely distributed throughout China's marine waters. Because eggs of these species are difficult to identify and the taxonomy of the group has recently been resolved, their spawning grounds in the northern South China Sea (SCS) are not well known. We identified three *Trichiurus* species (*T. japonicus*, *T. nanhaiensis*, and *T. brevis*) using DNA barcodes in spring and late summer–autumn ichthyoplankton surveys of 2019 in the northern SCS. Egg distributions reveal that the spawning grounds of *T. japonicus* and *T. nanhaiensis* occur mainly from the central and southern Beibu Gulf, along Hainan Island, to the waters off the Pearl River Estuary, and that egg densities are higher in spring than in late summer-autumn. Spawning of *T. japonicus* commonly occurs along the continental shelf, *T. nanhaiensis* along the continental shelf to slope (over seabed depths of 42–380 m), and *T. brevis* mainly in shallow water. Considering the salinity and temperature data, we found that the eggs of *Trichiurus* were associated with specific water masses. In spring, eggs of *T. japonicus* and *T. nanhaiensis* occurred at central and southern Beibu Gulf water mass (CSBGWM) and South China Sea surface water mass (SCSWM). While in late summer-autumn, eggs of *T. japonicus* and *T. nanhaiensis* mainly occurred in the waters of SCSWM. Our findings reveal that the occurrence of *Trichiurus* eggs and their spatial and temporal distribution are determined by hydrological conditions.

Keywords: *Trichiurus* spp., fish eggs, DNA barcode, spawning ground, hydrological characteristic, northern South China Sea

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INTRODUCTION

Hairtails, *Trichiurus* spp., also known as Cutlassfishes, are caught throughout China's waters. These species are among the most important commercial fishes in China, the number one fishery in terms of catch, and the third most marine catches in the South China Sea (SCS) (China Agriculture Press, 1997-2019; Lu et al., 2008). The taxonomy of *Trichiurus* sp in the SCS has been long confused. Prior to the 1990s only one species [*T. lepturus* (synonym *T. haumela*)] was reported from this region. In the 1990s two additional species were recognised (*T. nanhaiensis* and *T. brevis*) (Wang, 1992; Wang, 1992). However, it is now widely accepted that two species that were once considered conspecific, *T. japonicus* and *T. lepturus*, are distinct (Wang, 1992; Wang, 1993; Wang et al., 1993; Wang et al., 1994; Chakraborty et al., 2006; Tzeng et al., 2007; Hsu et al., 2009; Tzeng and Chiu, 2012), brings to four the number of species now recognised from this region, in which *T. lepturus* is supposedly rare.

The importance of the hairtail fishery has resulted in considerable fisheries research effort being directed towards it (Ye and Rosenberg, 1991; Kwok and Ni, 1999; He et al., 2014; Wang et al., 2017). These fishes in the Bohai, Yellow Sea, East China Sea and South China Sea have all been overfished. To protect fish spawning stocks and juveniles, SCS fisheries were closed for 2 months in June and July from 1999 to 2008, and prolonged to 3.5 months in May and August from 2017 to now. Despite these efforts, there's been little research on the early life stages and spawning of hairtail species (*T. japonicus, T. nanhaiensis* and *T. brevis*) in the SCS. Understanding early life stages of species is vital if recruitment is to be understood, and fisheries are to be managed (Chambers and Trippel, 1997; Fuiman, 2002; Checkley et al., 2009). More than 3,400 fish species—over 10.4% of the world fish species—occur in the SCS, which lies in the centre of 25 world biodiversity hotspots, surrounded by the Philippines, Wallacea, Sundaland, and Indo-Burma (Myers et al., 2000). The high diversity and low density of fishes in this region renders identification of adults and early life stages challenging.

The continental shelf of the northern SCS is a key habitat for nearly 1,500 fish species (Sun and Chen, 2013). This region is an important spawning and nursing ground for numerous fishes, such as hairtails, Golden threadfin bream, and Japanese scad (Zhang et al., 1985). Recent progress in understanding the reproductive biology and fishery ecology of these economic fishes has been made (Zhang et al., 1985; Chen et al., 2003; Yan et al., 2011; Shi et al., 2020). However, due to the difficulties identifying early life stages of fish, information of their spawning grounds based on ichthyoplankton surveys is limited in the SCS (Zhang et al., 1985; Zhou et al., 2011; Li et al., 2014; Chen et al., 2018).

DNA barcoding, an identification method using molecular maker (e.g., cytochrome c oxidase subunit I, COI), enables identification of fish eggs and larvae, regardless of their developmental stage or morphological plasticity (Valdez-Moreno et al., 2010; Frantine-Silva et al., 2015; Hubert et al., 2015). Many fish sequences (to June 2020, 21,800 + fish species, 342,000 + specimens) have been deposited in the BOLD (Barcode of Life Data System, http://www.barcodinglife.org, http://www.boldsystems.org) database (Ratnasingham and Hebert, 2007), which can be used to facilitate identification of both adult and larval fishes.

To identify spawning grounds of fishes in the northern SCS, we undertook ichthyoplankton surveys in the spring and late summer–autumn of 2019. Our aims were to: 1) differentiate eggs of *Trichiurus* species collected in these surveys using DNA barcodes, and to describe their morphology, 2) determine the spatial and temporal distribution patterns of eggs of *Trichiurus* species in the study period, and 3) analyse the hydrological conditions of spawning grounds of *Trichiurus* species. Our findings should contribute to improved management and conservation of these species and their spawning grounds in the northern SCS.

MATERIALS AND METHODS

Sampling

Fish eggs were widely collected in the northern SCS (16.75–23.15°N, 107.25–117.21°E, **Figure 1**) during the spring (March–April) and late summer–autumn (August–October 2019). Eggs were collected with a 2.7 m long, 80 cm diameter zooplankton net, with a 0.505 mm mesh, and cod-end container of 400 μm. Nets were fitted with a General Oceanics flowmeter to estimate filtered water volume. In the represent study, two zooplankton nets were dragged simultaneously in horizontal trawls, and two vertical trawls were also conducted. For horizontal trawls, nets were dragged 10–15 min at 1.5–2.2 knots; for vertical trawls, nets were hauled from the bottom (if less than 200 m seabed depth) or from 200 m (if greater than 200 m seabed depth) to the surface at 1–1.5 m/s. The samples were preserved with one sample fixed in ~75% ethanol/seawater, and the other sample in 5% formaldehyde solution/seawater. Temperature and salinity at different depths were recorded with a conductivity, temperature and depth (CTD) (SSTAML Plus X) from the surface to the near the bottom.

Egg Morphology

All eggs from each station were sorted under a stereomicroscope in a laboratory. If available, up to 15 eggs of different morphologies were randomly selected (when there were more than 15 eggs at a station, otherwise all eggs were selected), then photographed. Selected eggs were individually numbered, immersed in hydrogen peroxide for ~8 min for cleaning and to rehydrate them so that their diameter was comparable to fresh eggs, and measured to 0.001 mm after being photographed using Zeiss microscope (Axioplan 2 imaging E). Their DNA was then extracted. Egg morphology descriptions follow Shao et al. (2002) and Ikeda et al. (2015). In the present study, pairwise *t*-test was used to analysis the difference among the egg diameters of genus Trichiurus species.

DNA Extraction and Polymerase Chain Reaction Amplification

Total genomic DNA was extracted from each numbered egg using an Axygen Genomic DNA Miniprep Kit (Axygen, Shanghai, China). COI sequences (~648 p) were amplified and sequenced using universal primers FishF1 and FishR1 (Ward et al., 2005). The polymerase chain reaction (PCR) contained 20 μl Tsingke TM Master Mix, 1 μl of each primer (10 pmol), 1–10 μl template DNA, and 8–17 μl ddH_2O (total template DNA + ddH_2O = 18 μl) to make a total volume of 40 μl. PCR conditions were 94°C for 3 min, 35 cycles at 94°C for 30 s, 51°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 10 min. Amplification products of PCR reactions were purified using 1% low-melting agarose electrophoresis and sequenced bidirectionally on an ABI 3730 XL DNA system following manufacturer protocols.

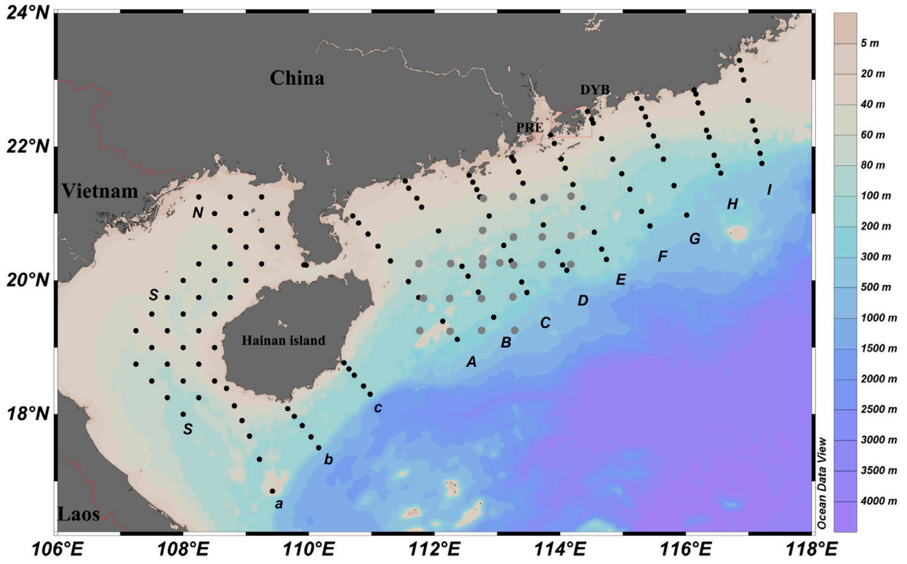

FIGURE 1 | The sampling stations in northern South China Sea. The black dots indicated the horizontal stations; the gray dots indicated the horizontal and vertical stations. PRE, Pearl River Estuary; DYB, Daya Bay.

High-quality DNA sequences were edited with MEGA v 6.0 (Tamura et al., 2013).

Sequence Analysis

Egg sequences were initially checked the tracer files and assembled by SEQMAN in Lasergene version 7.0 (DNASTAR Inc., Madison, WI, United States), and then delimited by Blast searches in BOLD (Barcode of Life Data system, http://www.boldsystems.org/). Sequences exceeding a 98% similarity and 2% genetic distance threshold with the nearest neighbour species were tagged with the taxon name using criteria of Hubert et al. (2015). Local and adjacent oceanic fish COI sequences of Trichiurus spp. were cited from reliable literatures for secondary analysis should the aforementioned criteria not be met (Hsu et al., 2009; Tzeng and Chiu, 2012; Chang et al., 2017; Hou et al., 2018). A neighbor-joining (NJ) tree was reconstructed to illustrate lineage diversity via phylogenetic topology, based on the Kimura 2-parameter model (K2P, Kimura, 1980) with 1,000 bootstrap replicates by MEGA v6.0 (Tamura et al., 2013). Genetic distance was also calculated in MEGA v6.0 based on K2P model.

Hydrological Characteristic and Egg Distribution Analysis

The temperature-salinity diagrams were conducted to analyse the water mass type in the study area (Liu et al., 2001; Lei et al., 2002; Zhu et al., 2019). Data View 4.5 (Schlitzer, R., http://odv.awi.de, 2015) was used to draw the contour maps of SST and SSS of CTD data in spring and later-summer-autumn surveys. In the present study, k-means clustering method was applied to classify the surface sea temperature (SST) and surface sea salinity (SSS), and analysis the relationship with the hydrological requirments and occurrence of *Trichiurus* eggs.

Trichiurus spp. egg catch rates per station are presented as numbers of eggs per 100 m^3 of filtered water (flowmeter data). Catch rates at stations with *Trichiurus* eggs were plotted using Surfer (version 15.0, Golden Software Inc., Golden, CO, United States). As the incubation period of *Trichiurus* spp. egg is 74–80 h at 21–24°C, and higher water temperature can reduce the time of duration (Zhang et al., 1985). Thus, we supposed the occurrence locations of eggs can represent the spawning ground. The extent of the spawning ground of *Trichiurus* spp. was indicated by the egg distribution pattern, by using a confocal ellipse of the distribution area and the density at each station, and calculated as follows:

$$(\overline{X}, \overline{Y}) = \left(\frac{\sum_i D_i X_i}{\sum_i D_i}, \frac{\sum_i D_i Y_i}{\sum_i D_i}\right), (\overline{X}, \overline{Y}) = \left(\frac{\sum_i D_i X_i}{\sum_i D_i}, \frac{\sum_i D_i Y_i}{\sum_i D_i}\right).$$

Where X and Y are the longitude and latitude of the station i, and the X and Y bars are the confocal of the ellipses. The estimation followed the description of Sokal and Rohlf (1995) and Kim et al. (2005).

RESULTS

Hydrological Characteristics

The distinct seasonal variation of SST and SSS in spring and late-summer-autumn of 2019 is shown in **Figure 2**. In summary, the distributions of SST and SSS in spring were different from those in late-summer-autumn. In spring, the SST showed regular trends of northeastward reducing from the sea areas of mouth of Beibu Gulf to the coast of main land, and the lowest SST was occurred in the coastal area from Pearl River Estuary (PRE) eastward to Shantou sea areas (**Figure 2**). The SSS showed four different distributional regions, and the lowest SSS was detected in the

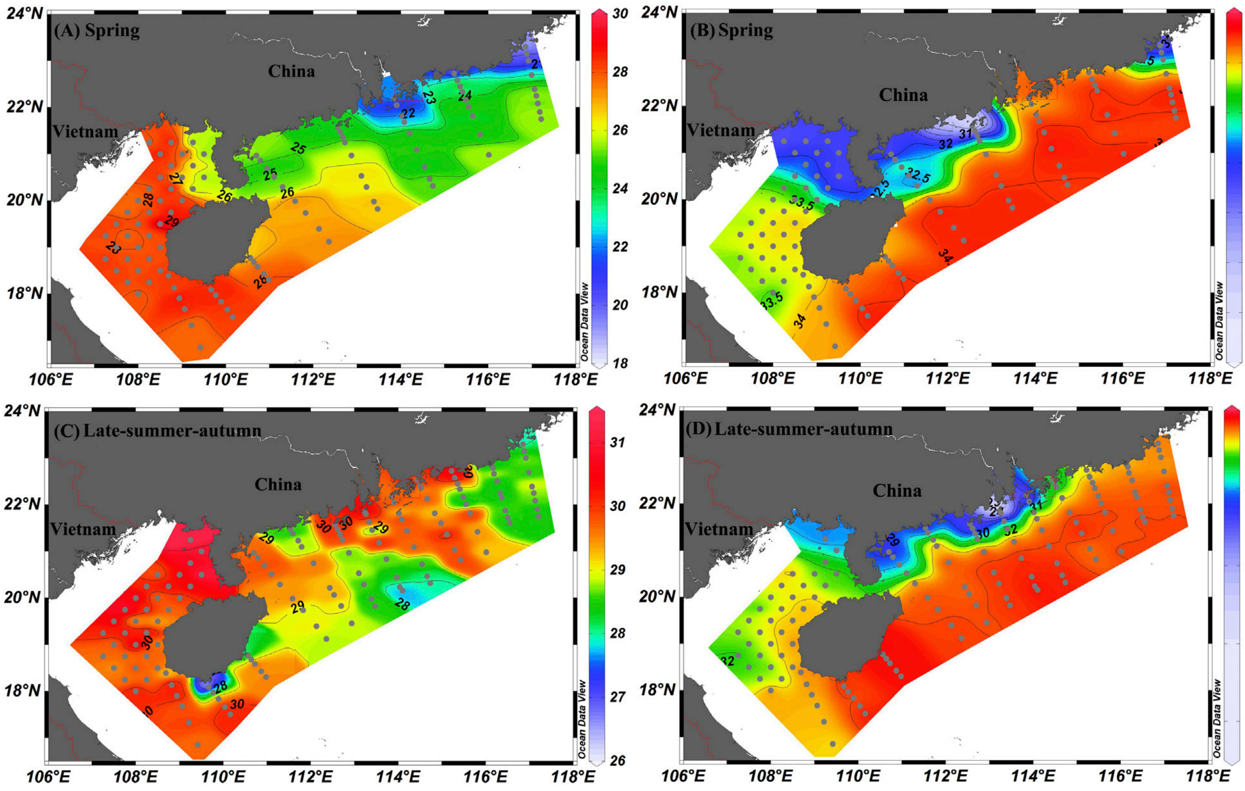

FIGURE 2 | Spatial distribution of surface temperature [shading in ˚C, **(A,C)**], and salinity [shading in PSU, **(B,D)**] during spring and late-summer- autumn in 2019.

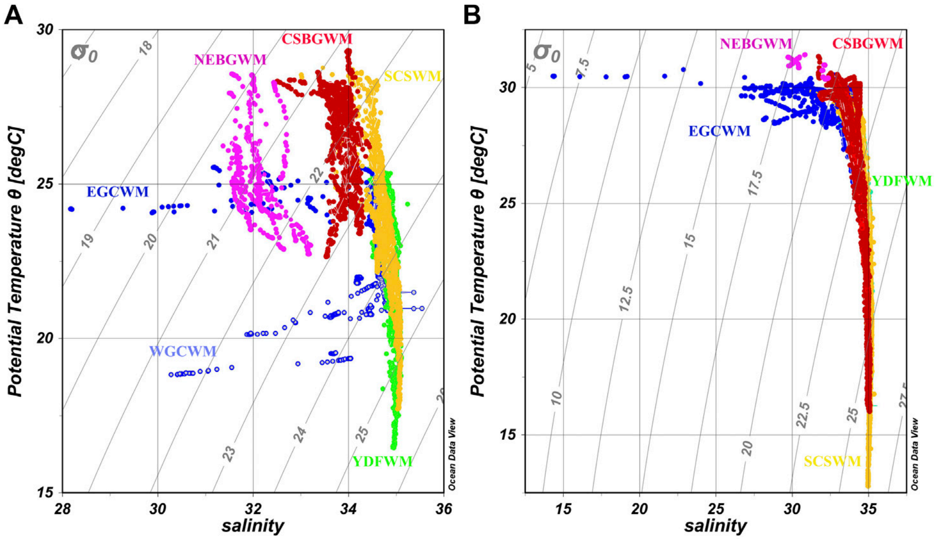

FIGURE 3 | Potential temperature-salinity (T-S) diagrams in spring **(A)** and late-summer-autumn **(B).** The water masses are as follows: northeastern Beibu Gulf water mass (NEBGWM), central and southern Beibu Gulf water mass (CSBGWM), South China Sea surface water mass (SCSWM), western Guangdong coastal water mass (WGCWM), eastern Guangdong coastal water mass (EGCWM), and Yuedong offshore water mass (YDFWM).

southwest coast of the PRE. In addition, the salinity front was clearly detected from the PRE southwestward to the central waters of Beibu Gulf, indicating the river plume from PR spread over westward over the coast and continental shelf area (**Figure 2**). In late-summer-autumn, the SST in the study area increased significantly than that in spring, especially the coastal

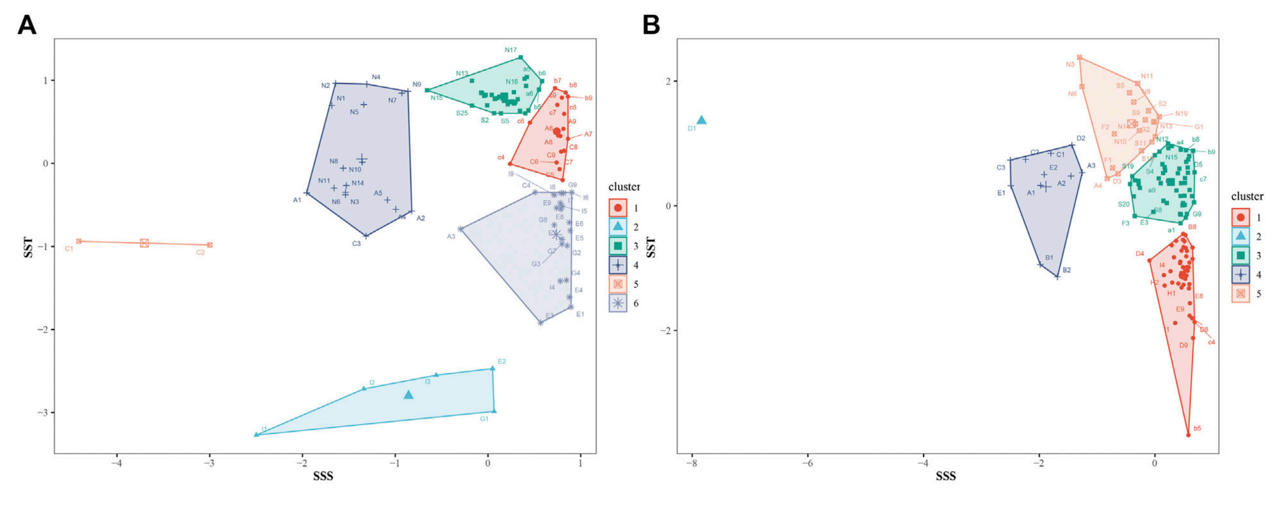

FIGURE 4 | K-mean cluster analysis of SSS and SST in spring **(A)** and late-summer-autumn **(B)**.

and offshore area of PRE. The lowest SST and high SSS was occurred in southeastern nearshore sea areas of Hainan island, indicating a coastal upwelling probably occurred in this sea area (**Figure 2**). The low SSS sea area was observed to form as an elongated river plume over the northern SCS shelf, and the salinity front was extend to the nearshore of Daya Bay and shifted northwardly to coastal water, which was affected by diluted water from PR (**Figure 2**).

T-S diagrams were conducted to determine the water mass types. In summary, six categories of water masses were mainly detected in the study area in spring, i.e., two water masses in Beibu Gulf and four water masses in the east of Hainan island of northern SCS (**Figure 3**). The water masses in the Beibu Gulf is obviously different from those in east of Hainan island of northern SCS. In Late-summer-autumn, five categories of water masses were mainly detected in the study area (**Figure 3**). Northeastern Beibu Gulf water mass (NEBGWM) and eastern Guangdong coastal water mass (EGCWM) can be differentiated from other water masses. However, central and southern Beibu Gulf water mass (CSBGWM), South China Sea surface water mass (SCSWM) and Yuedong offshore water mass (YDFWM) mixed, indicating that the southwest monsoon in the SCS induced the high temperature of sea water in the region. In order to link the distributional pattern of fish eggs in horizontal trawls with water mass, k-means clustering analysis was used to group SSS and SST of CTD in each station. It indicated that six categories of potential water masses could be distinguished in spring (**Figure 4A**), and five categories of water masses in late summer-autumn (**Figure 4B**).

Fish Eggs Delimitation by DNA Barcode

Of the successfully amplified and obtained good quality sequences, 76 sequences of 615 bp partial COI gene fragment (following alignment and trimming of noisy sites lacking apparent insertions/deletions and stop codons) were referred to *Trichiurus* spp. or Trichiuridae with 98% similarity and 2% genetic divergence among species after a blast search in BOLD; 75 sequences were 98–100% similar, with most nearest neighbours being 98.42–100% similar (one sequence was 96.5% similar). Both best match and nearest neighbour best match fell below

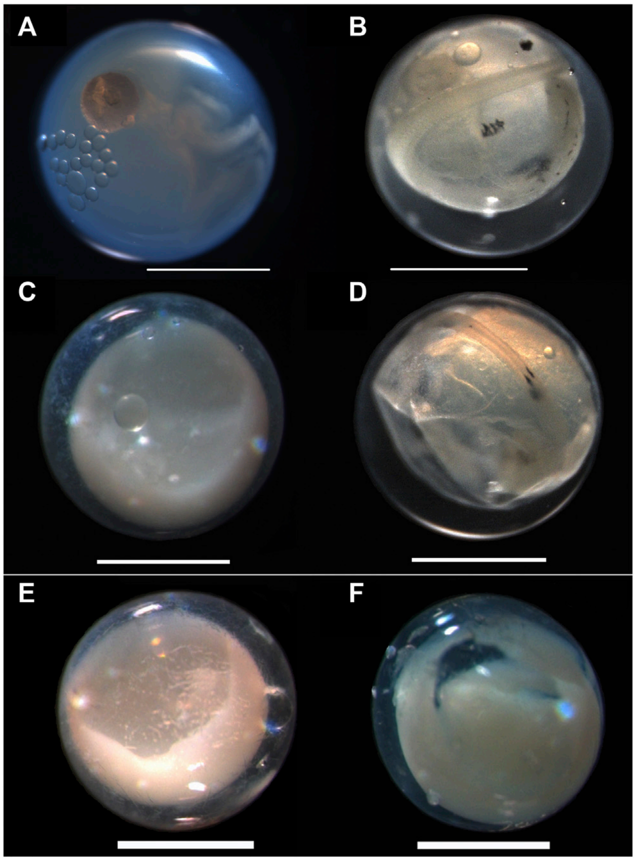

FIGURE 5 | Photographs of three *Trichiurus* species eggs identified by COI: **(A,B)** *T. japonicus*; **(C,D)** *T. nanhaiensis*; **(E,F)** *T. brevis*.

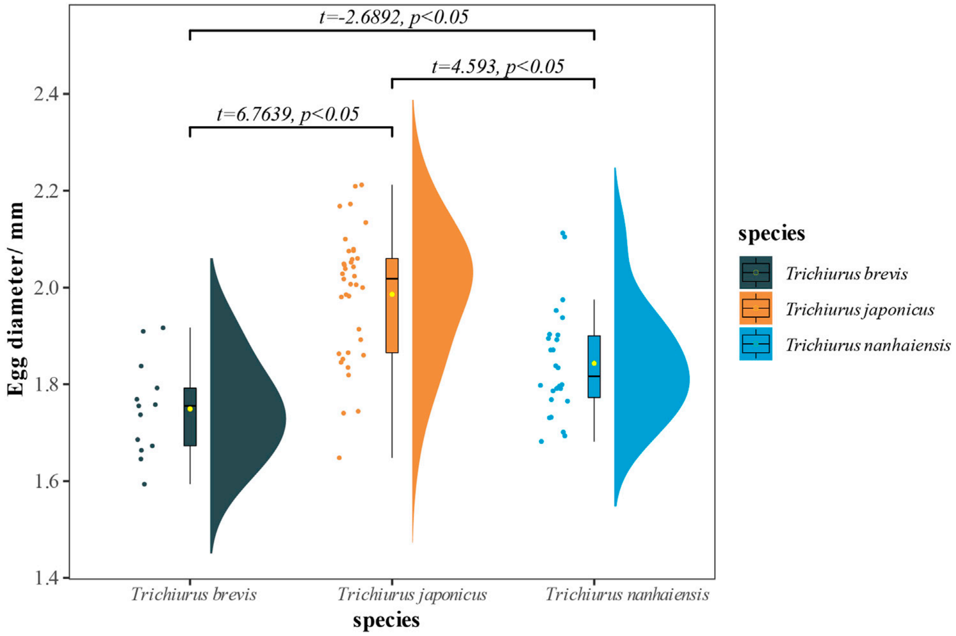

FIGURE 6 | Diameters (mm) of eggs of three *Trichiurus* species from the northern South China Sea.

thresholds, indicating samples were identifiable only to genus *Trichiurus* or family Trichiuridae (**Supplementary Table S1**). Secondary analysis combined our own and downloaded sequence data (**Supplementary Table S2**). A neighbour-joining tree of combined data produced three clusters supported by bootstrap values ≥99%, indicating individual clusters were single species (**Supplementary Figure S1**). In addition, average genetic distances within the three lineages were 0.007, 0.003 and 0.003, the eggs can be referred to *T. japonicus*, *T. nanhaiensis* and *T. brevis*, respectively.

Egg Morphology

Eggs of each species are pelagic, solitary, spherical, and have a smooth chorion and narrow perivitelline space (**Figure 5**, **Supplementary Table S3**). Their diameters ranged 1.65–2.21 mm, with the smallest (*T. brevis*) averaging 1.65–1.92 mm (n = 8; mean ± SE = 1.75 ± 0.09), and the largest (*T. japonicus*) averaging 1.65–2.21 mm (n = 37; mean ± SE = 1.986 ± 0.13). Egg diameters of *T. nanhaiensis* ranged 1.68–2.11 mm (n = 26; mean ± SE = 1.84 ± 0.11, **Figure 6**). The t-tests indicate that, among the egg diameters of three genus *Trichiurus* species, *T. japonicus* and *T. nanhaiensis* have a significant difference ($t = 4.593, p < 0.05$), similarly, *T. nanhaiensis* and *T. brevis* ($t = -2.6892, p < 0.05$), and *T. japonicus* and *T. brevis* ($t = 6.7639, p < 0.05$). Oil globule shape was somewhat distorted, rendering diameter measurement difficult in developmental stages. Only diameters of regular oil globules were measured (0.29–0.57 mm), with the smallest (*T. brevis*) averaging 0.29–0.30 mm, distinct from those of *T. japonicus* (0.34–0.57 mm) and *T. nanhaiensis* (0.31–0.42 mm) (**Supplementary Table S3**). The diameter of the smallest oil globule and that of the egg differentiates of *T. brevis* from those of *T. japonicus* and *T. nanhaiensis*, but not the latter two from each other. Multiple oil globules in early stage eggs of *T. japonicus* began to merge into a single globule after fertilisation (**Figure 5A**); after blastopore closure, two large melanophores occurred on the otic capsule, and sporadic melanophores occurred from the trailing of otic capsule to ventral side of midpiece of embryo (**Figure 5B**); when the embryo encircled 5/6 of yolk-sac it thickened and the perivitelline space significantly increased, indicating increased density, which ultimately will lead to the egg sinking from surface layers deeper into the water column. Two melanophores in the tail region were found to shift to fin folds. We observed no black melanophores on the single oil globule in alcohol-preserved specimens, which we regard as an artefact of preservation. Developmental stages for *T. nanhaiensis* and *T. japonicus* were similar, with two large melanophores occurring on the otic capsule after blastopore closure (**Figures 5C,D**). No melanophores occurred on the otic capsule of *T. brevis* following closure of the blastopore (**Figure 5F**), differentiating eggs of this species from those of others.

Spawning Grounds and Hydrological Conditions

Eggs of *Trichiurus* in spring occurred mainly in the southern Beibu Gulf, along Hainan Island, and off the Pearl River Estuary; their densities in spring were higher than in late summer–autumn (**Figure 7**). In spring, eggs of *T. japonicus* occurred mainly in the middle-to-mouth of Beibu Gulf, and off the PRE, from 38.1 to 88.3 m, 25.4–28.5°C SST, and 32.2–34.6 salinity (**Table 1**; **Figure 7**). In late summer–autumn, eggs of *T. japonicus* were widely dispersed east of Hainan Island, but occurred nowhere in

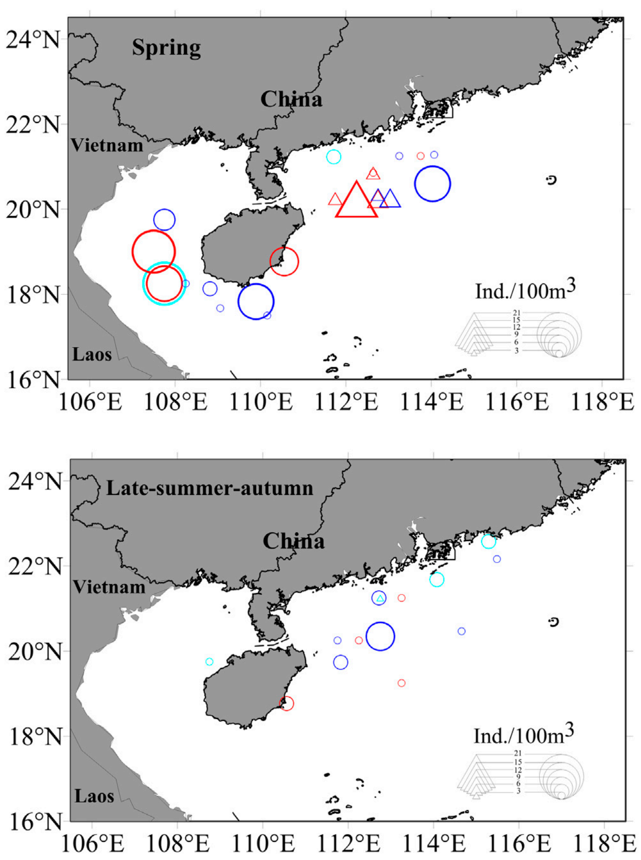

FIGURE 7 | Distributions of eggs of three *Trichiurus* species in the northern South China Sea in spring and late-summer–autumn of 2019. Egg abundances: circles, horizontal; triangles, vertical. Red, *T. nanhaiensis*; blue *T. japonicus*; cyan *T. brevis*.

Beibu Gulf. In spring, eggs of *T. nanhaiensis* occurred in the same general areas as those of *T. japonicus*, but were found together at only one station. In late summer–autumn, eggs of *T. nanhaiensis* occurred at four stations at low density, over a wide depth range (55–380 m). In spring, eggs of *T. brevis* occurred at two stations, outside Beibu Gulf, and an inshore station in western coastal sea area of Guangdong, while during late summer–autumn, they occurred at four inshore stations (**Figure 7**). The horizontal distribution patterns of eggs of *T. japonicus* and *T. nanhaiensis* indicated by confocal ellipses with axes and slope are also shown in **Figure 8**. It is obvious that the spawning ground changed northeastward in late-summer-autumn in comparison to that in spring for *T. nanhaiensis*. However, for *T. japonicus*, the spawning ground off the PRE in spring changed southwestward and obviously enlarged in late-summer-autumn.

The occurrence sites of *Trichiurus* eggs during spring and late summer–autumn of 2019 ranged 25.4–29.2°C SST and 32.4–34.6 salinity (**Figures 2**, **7**), although in spring it was mainly 25.4–28.5°C SST and 33.5–34.6 salinity. Eggs of *T. japonicus* occurred over a wider salinity range than those of *T. nanhaiensis* or *T. brevis*. Hydrological requirments in which *Trichiurus* eggs occurred were observed to correlate with water mass conditions. Eggs of *T. japonicus* and *T. nanhaiensis* mainly occurred in the water masses of CSBGWM and SCSWM in spring, and occurred in SCSWM in late summer-autumn (**Figures 3**, **7**). The salinity front restricted the spatial distribution along the coastal sea area of northern continental shelf of SCS (**Figures 2B,D**, **7**). For *T. brevis*, eggs occurred one station in CSBGWM and the other station in EGCWM in spring, and occurred one coastal station in CSBGWM and three coastal stations in EGCWM along the salinity front, indicating different hydrological conditions for the spawning event.

DISCUSSION

Molecular Identification and Egg Morphology

In the present study, we combined DNA barcoding (partial COI sequences) and morphology to unequivocally identify eggs of co-occurring *Trichiurus* species and their occurrence sites. Our eggs originated from samples taken during spawning-ground surveys conducted in spring and late summer–autumn of 2019 in the northern SCS. Most samples contained eggs of other species, which is not surprising given the high fish diversity in this area. A lack of detailed descriptions of fish eggs makes it difficult to identify them. Eggs of *Trichiurus* can be easily identified to family (Trichiuridae) in that they are spherical, similarly sized, have a smooth chorion, and a narrow perivitelline space.

Four species of *Trichiurus* (*T. japonicus*, *T. lepturus*, *T. nanhaiensis*, and *T. brevis*) occur in the SCS, but the early life stages are known for only *T. haumela* (as *T. lepturus*). While we found no eggs of *T. lepturus* in 2019, this species is supposedly rare in the northern SCS. We did, however, identify eggs of the three other species, which not only demonstrates the value and importance of DNA barcoding in fish egg identification, but for identifying spawning grounds of commercially important fish species also.

TABLE 1 | Egg distributional information of three *Trichiurus* spp. species in the northern SCS.

Species	Season	Depth of waters (m)	SST (°C)	salinity	Latitude	Longitude
T. japonicus	Spring	38.1–88.3	25.4–28.5	32.2–34.6	17.50–21.27°N	107.75–114.07°E;
	Late summer-Autumn	44.4–118.0	27.0–29.8	32.7–34.6	20.25–22.16°N	111.75–115.48°E
T. nanhaiensis	Spring	42–55.8	25.4–28.1	32.4–34.5	18.25–21.25°N	117.50–113.75°E
	Late summer-Autumn	55–380	27.2–29.2	32.6–34.4	18.68–21.25°N	110.64–113.25°E
T. brevis	Spring	30.5–45.2	27.9–27.9	32.8–34.2	18.25–21.23°N	107.75–111.72°E
	Late summer-Autumn	10.3–51.0	28.5–30.5	33.7–33.9	19.75–22.57°N	108.75–115.48°E

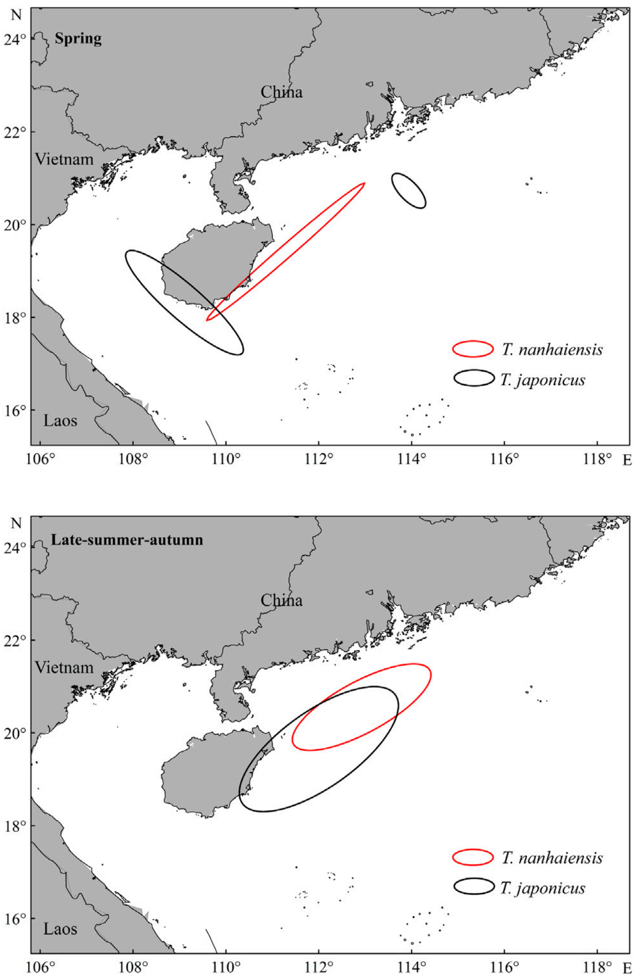

FIGURE 8 | Horizontal distribution patterns of eggs of *T. nanhaiensis* and *T. japonicus* indicated by confocal ellipses of distribution areas, weighted by the density at each station, during spring and late-summer autumn in 2019.

Egg Size Variation and Reproductive Strategies

The size of *T.* japonicus eggs in the SCS (1.65–2.21 mm) is greater than those from Japan (1.59–1.88 mm; Ikeda et al., 2015) and Korea (1.66–1.84 mm; Lee and Kim, 2014), suggesting regional variation. Because only T. hamela (as T. lepturus) was recognised from the SCS prior to the 1990s, we combine egg sizes of the three Trichiurus species we report from this region (1.59–2.21 mm) and compare them to T. hamela data from the SCS reported by SCSFRI (Institute, 1966) (1.61–1.83 mm) and Zhang et al. (1985) (1.50–1.90 mm). Eggs of Trichiurus in the SCS today appear to be larger than they were from the 1960s to 1980s.

We speculate that reproductive strategies of Trichiurus species have shifted in this region in response to changed oceanographic conditions or increased fishing pressure. The egg size-fecundity trade-off, and hypothesis that "bigger is better" for offspring fitness, regards larger maternal size to be preferable to produce larger offspring (Steiger, 2013). Had mature female fish size decreased in size because of fishing pressure, this combined with other environmental changes might result in production of smaller offspring, thereby reducing population biomass (Conover and Munch, 2002). However, Régnier et al. (2013) argued that bigger was not always better, because small brown trout (Salmo trutta) eggs can survive at higher rates than larger eggs throughout incubation (Chambers and Leggett, 1996; Régnier et al., 2013; Farmer et al., 2015; Neuheimer et al., 2015). Although size at hatching is directly correlated with egg size, and larger fish may produce larger eggs, the relationship between adult size and egg size among 309 North Atlantic fishes was weak (Chambers and Leggett, 1996). In subtropical to tropical waters of the northern SCS, *T. japonicus* and *T. nanhaiensis* might change reproductive strategy to produce larger eggs in response to fishery pressure and ocean warming, especially given both short and warm winter events in recent years. This may provide some insight into sharp declines in northern SCS stocks.

Spawning Ground Distributions and Hydrological Conditions

We describe the spatial and temporal distribution of *T. japonicus*, *T. nanhaiensis* and *T. brevis* eggs in the northern SCS in spring and late summer–autumn. DNA provides unequivocal evidence for *T. brevis* spawning in shallow waters, over seabed depths of 10.3–51.0 m, *T. japonicus* to spawn along the continental shelf over seabed depths of 38.1–118.0 m, and *T. nanhaiensis* to spawn along the continental shelf and slope over seabed depths of 42–380 m. Stations at which these three species co-occurred were rare (two stations in spring and one in late summer–autumn (**Figure 7**). Meanwhile, we found that the occurrence sites of *T. japonicus* eggs well matched the spatial and temporal distribution of the fishery resource in the surveyed seasons (Shi et al., 2020). It indicated that the egg distribution pattern can reflect the adult fish distribution and reproductive traits. In addition, the central spawning sea area of *T. japonicus* and *T. nanhaiensis* was east of Hainan Island, where a high catch rate of adult Japanese scad *Decapterus maruadsi* occurred (also the main spawning ground of this species (Institute, 1966). As *D. maruadsi* was the dominant prey (34% by weight) in stomachs of *T. nanhaiensis*, and second-most dominant prey in stomachs of *T. lepturus* (as *T. japonicus* now), both species likely fed during spawning, with prey availability inducing formation of spawning grounds (Yan et al., 2011; Yan et al., 2012).

Several studies have suggested that hydrological conditions, i.e., temperature, salinity and primary production were considered to significantly affect the occurrence and distribution patterns of ithchyoplankton (Hillgruber and Kloppmann, 1999; Franco-Gordo et al., 2002; Aceves-Medina et al., 2004; Lee and Go, 2005; de Macedo-Soares et al., 2014; Huang et al., 2017). In the present study, the eggs of *Trichiurus* were associated with specific water masses. In spring, eggs of *T. japonicus* and *T. nanhaiensis* occurred at CSBGWM and SCSWM. The SST in these two water masses increased earlier than other regions in the monsoon transition period, which were firstly affected by the southwest monsoon in the region

(**Figures 2A,C**). In late summer-autumn, eggs of *T. japonicus* and *T. nanhaiensis* mainly occurred in the waters of SCSWM, south the salinity front (**Figures 2C,D**). The lower temperature sea and high salinity brought by Qiongdong upwelling have obviously benefited the spawning activities of the two speicies in the region. Meanwhile, the high temperature induced by strong southwest monsoon limit the spawning activity in the Beibu Gulf. Thus, we propose that the southwest monsoon promote the reproductive activities of *T. japonicus* and *T. nanhaiensis* in spring and restrict them in late summer-autumn.

Compared with a survey east of Hainan Island in 1964 in the same survey months (Institute, 1966), the spawning ground appears to have migrated west towards Qiongzhou Strait (19.8–21.0°N, 110.7–113°E), and south, away from the PRE. The coastal spawning habitat (<30 m) suitable for Trichiurus has degraded, corresponding with SST and salinity thresholds in spring (**Table 1**, **Supplementary Figures S2, S3**). Spawning ground shifts have been reported for other fish species also (e.g., Japanese sardine, Arctic and Atlantic cods, chub mackerel) as being induced by fisheries and climate change (Kikuchi et al., 1992; Van Der Meeren and Ivannikov, 2006; Opdal, 2010; Kanamori et al., 2019). Throughout the SCS, average SST has increased by about 0.348°C from 1982 to 2010, and has been projected to increase by about 0.36°C over 9 decades from 2010 to 2,100 (Atkinson et al., 2016). The annual mean SST in the central SCS has increased by 0.92°C from 1950 to 2006 (Cai et al., 2009). In continental shelf shallow waters (0–200 m) in the northern SCS that we surveyed, average SST increases may be greater.

Ocean warming can result in fishes shifting their distributions to higher latitudes, and local biodiversity loss. This can change the composition of fisheries, shift spawning grounds, and even increase the possibility of natural hybridisation (Perry et al., 2005; Peter et al., 2011; Cheunge et al., 2013; Neira et al., 2015; Verges et al., 2016; Kanamori et al., 2019; Takahashi et al., 2020). High temperatures can also lead to physiological changes in reproduction, with male fishes raised at stressful temperatures having shorter, slower sperm than fishes raised at more benign temperatures, decreasing fertilisation rates and affecting recruitment (Breckels and Neff, 2013). The tendency for *Trichiurus* eggs to concentrate in eastern areas of Qiongzhou Strait (110.7–113°E) may be an adaption to oceanic environmental change. Changes in distributions of eggs shall cause the attention in the fish protection and fishery management.

Fishery Conservation and Management

Because of coastal habitat degradation and/or loss, coupled with over-fishing, fishery resources have declined. A pressing need exists to protect these fish resources and improve fishery management in the northern SCS (Qiu, 2002). The timing of spawning seasons and locations of spawning grounds are key parameters in fishery management strategies. Using DNA barcodes, we identify eggs of specific fish species and demonstrate how this technology can assist with morphological identifications of eggs in spawning ground surveys. Meanwhile, under the background of ocean warming, potential shift spawning grounds may occur under the change of hydrological conditions. Our study provides important information on spawning ground areas that can be used to advance their protection, and how adjusting the timing of fishing moratoria in the SCS can facilitate sustainable fisheries management.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI [accession: MT762604- MT762679]

ETHICS STATEMENT

The animal study was reviewed and approved by the South China Sea Fisheries Research Institute, Chinese Academy of Fishery Science.

AUTHOR CONTRIBUTIONS

GH and ZC analyzed the data and completed the first draft. GH, YX, and JW performed the field survey. GH, WH, JW and JZ conducted the experiments. KZ and ZC provided the guidance on data analysis and structure. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2021.703029/full#supplementary-material

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Threadfin Porgy (*Evynnis Cardinalis*) Haplotype Pattern and Genetic Structure in Beibu Gulf, South China Sea

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Threadfin porgy (*Evynnis cardinalis*) is one of the important commercial fishing targets of bottom trawl fishery in the northern South China Sea. It is mainly threatened by overexploitation and listed as endangered species in the IUCN Red List. To investigate the demographic history and genetic structure of *E. cardinalis* population, partial sequences of the mitochondrial cytochrome c oxidase subunit I (COI) gene were obtained from 162 individuals collected from Beibu Gulf, South China Sea. In total, 44 different haplotypes were identified, and the dominant haplotype was found in all sampling sites. Across the dataset, nucleotide diversity was low, whereas haplotype diversity was high. Low pairwise comparisons of Φ_{ST} and high gene flow among sampling sites revealed a genetically homogeneous population structure in Beibu Gulf, indicating a single panmictic stock of *E. cardinalis* in this area. The star-like haplotype network, unimodal mismatch distribution, and significantly negative Tajima's D and Fu's Fs values indicated recent population demographic expansion of *E. cardinalis*. The mismatch distribution and Bayesian skyline plot results indicated that *E. cardinalis* from Beibu Gulf might have experienced colonization and demographic expansion due to sea level fluctuations during the late Pleistocene.

Keywords: haplotype pattern, genetic differentiation, demographic expansion, COI, South China Sea

INTRODUCTION

Marine fish are generally deemed to have high dispersal potential because of their high moving capability at both larval and adult stages, and the absence of obvious physical barriers to dispersal (Caley et al., 1996; Hellberg, 2009). Theoretically, a species with the higher dispersal capability, the lower the genetic structure between populations. Therefore, in marine fish, especially migratory fish, the signal of population differentiation is weak and difficult to observe because of high levels of gene flow (Gandra et al., 2020). Understanding the genetic diversity of commercially important species is critical to implement protection policies and management regulations (Araki and Schmid, 2010). Genetic diversity (both within and between populations) greatly influences the adaptive potential of species to environmental changes, and ultimately determines their long-term resilience to ecological disturbances (Pauls et al., 2013). Additionally, knowledge of genetic structure is useful for understanding the migration routes, areas, and seasons of fish spawning. Such information can help fisheries managers define the spatiotemporal scales over which they can implement effective stock management and conservation plans (Bradbury et al., 2008). Therefore, from the resource management perspective, understanding the levels of gene flow among populations and patterns of genetic diversity is a fundamental issue. The effectiveness of marine protected areas depends on both their ability to self-recruit (reproductive potential) and the spillover of adults and export of larvae to nearby fished areas (Harrison et al., 2012; Le Port et al., 2017). Over the last 30 years, population genetic studies have become an essential tool for stock management and conservation of coral reef, estuarine, and coastal populations because population genetic studies are useful for estimating genetic diversity and the ability to survive anthropogenic activities such as overfishing, habitat degradation, eutrophication, invasive species, and pollution (Ryman et al., 1995; Ruzzante et al., 1998; Gill and Kemp, 2002; Gaither et al., 2010; Machado et al., 2020). Mitochondrial DNA markers (mtDNA) are widely employed to detect population structure in marine species because they have large number of copies, high mutation rates, generally maternal inheritance, and almost nonexistent recombination. The primary advantages of mtDNA are the inheritance pattern and nonexistent recombination: speciation events over multiple generations can be traced through maternal cloning, and male dispersal does not homogenize the population (Prugnolle and de Meeus, 2002). These factors make mtDNA markers particularly suitable indicators of the genetic structure of marine populations with high gene flows, such as zooplankton and migratory fishes (Wang et al., 2013; Xu et al., 2019; Machado et al., 2020).

Threadfin porgy, *Evynnis cardinalis* (Lacepède 1802), occurs in the Indo-West Pacific from Japan, Korea, and China to Vietnam and Indonesia (Chen and Qiu, 2005). The species is mainly distributed at depths of 30–60 m, but also can occur to 100 m depth (Iwatsuki and Carpenter, 2014). *E. cardinalis* can be found in various types of bottoms, but is more common close to coral reefs or rough bottoms. Small individuals are very common in shallow, such as sheltered bays, while larger fish often live in deeper water (Eggleston, 1974). *E. cardinalis* is one of the main commercial fishing targets of bottom trawl fishery in the northern South China Sea and is thought to have three geographical stocks in the northern South China Sea: the Taiwan Strait, South China Sea, and Beibu Gulf stocks (Zhang et al., 2020). According to the surveys data, more than 90% catches of the total of sparids is *E. cardinalis* in Beibu Gulf. *E. cardinalis* is a migratory fish that is captured all year round with significant seasonal differences among different fishing areas. In Beibu Gulf, *E. cardinalis* undergoes seasonal migration and is found toward the northeastern shallow area of the gulf in late autumn and early winter. Then, spawning takes place in northwest Weizhou Island in early spring, and the recruits disperse widely in the northern area of the gulf and begin to disperse to the south in late spring (Iwatsuki and Carpenter, 2014). However, this species exhibits late maturity and longevity, which predispose it to impacts from heavy exploitation. In the northern South China Sea, the Beibu Gulf stock of *E. cardinalis* declined by 58% from 2001 to 2005, whereas the Taiwan Strait stock declined by 62% from 1993 to 2008. Therefore, a recent IUCN Red List assessment reported *E. cardinalis* as endangered, and this species is mainly threatened by overexploitation (Iwatsuki and Carpenter, 2014). Previous studies of *E. cardinalis* in Beibu Gulf have focused on feeding habits, growth and mortality, ecological distribution, phylogeny, and stock density (Chen and Qiu, 2003; Chen and Qiu, 2005; Zhang et al., 2007; Zhang et al., 2014; Cai et al., 2017; Zhang et al., 2020). However, no information is available to date on the population genetic structure of *E. cardinalis* in the northern South China Sea, and this prevents reliable stock assessments and protection policy formulation. The objective of this study was to provide a population genetic analysis using a portion of the mitochondrial cytochrome c oxidase subunit I (COI) gene to assess the population genetic diversity pattern and historical demography, and estimate *E. cardinalis* expansion time in Beibu Gulf.

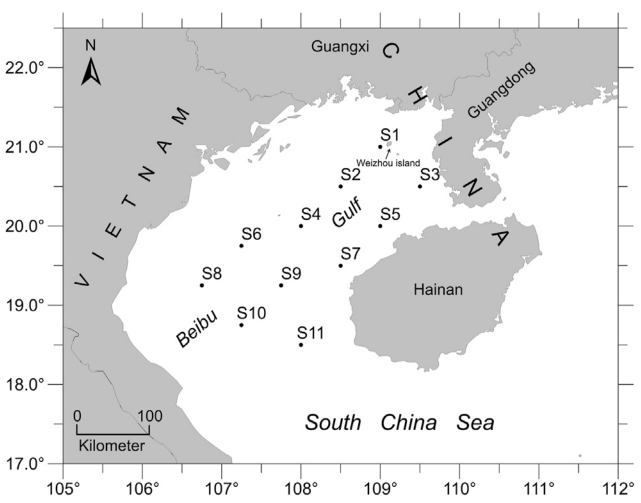

FIGURE 1 | Map showing study area and 11 sampling locations in Beibu Gulf, South China Sea.

MATERIALS AND METHODS

Study Area

Beibu Gulf (17°–22°N, 105°–110°E) is located in the northwestern part of the South China Sea and has a long coastline that belongs to China and Vietnam. It is an approximately 128,000 km^2 of semi-closed gulf that ranges from Leizhou Peninsula, Qiongzhou Strait, and Hainan Island to east Vietnam coast, and extends to the Guangxi coast in the north (Ma et al., 2010). The bottom of Beibu Gulf is flat and deepens from the northwest to the southeast, with a depth of typically less than 100 m (average depth, 42 m). The surrounding climate of this gulf is subtropical and monsoonal. Moreover, this gulf contains numerous estuaries from which rivers discharge nutrients. Beibu Gulf is a traditional fishing ground and important source of fishery products for coastal areas because of high productivity and rich biodiversity, which benefit from its unique geographic location and climatic conditions (Chen et al., 2009).

Sample Collection

All *E. cardinalis* specimens were collected from fishery surveys carried out by the South China Sea Fisheries Research Institute; these surveys were conducted by the commercial fishing vessel "Beiyu60011" in the northern South China Sea using bottom trawler nets in September 2018. We set eleven sampling sites in the study area of Beibu Gulf, which covered over 45,000 km^2 (**Figure 1**). In total, 11–16 specimens of each sampling locations were used for the DNA analyses after morphological identification. The dorsal fin or muscle were removed from each specimen and preserved in absolute ethanol at −20°C.

DNA Extraction, Amplification, and Sequencing

Genomic DNA were extracted from the dorsal fin or muscle tissue using the TIANamp Marine Animals DNA Kit (TIANGEN, China). The concentration used as PCR template was adjusted to an A260 of approximately 0.05–0.2. Fragments of the mtDNA cytochrome c oxidase subunit I gene were amplified from total genomic DNA by the polymerase chain reaction. The primer sets FishF1 and FishR1 (Ward et al., 2005) were used for PCR amplification. Each 50 µl PCR tube contained of 5 µl PCR buffer, 4 µl of 25 µM $MgCl_2$, 2.5 µl DNA template, 5 µl CoralLoad concentrate, 0.5 µl of 25 µM solution of each primer, 1 µl of 10 µM dNTPs, 0.25 µl TopTaq DNA polymerase, and 31.25 µl ddH_2O (QIAGEN, Germany). The procedure for PCR amplification were in turn as follows: an initial step of 95°C for 3 min; 35 cycles with 95°C for 30 s (denaturation), 54°C for 30 s (annealing), and 72°C for 1 min (extension); then followed by 5 min at 72°C (final extension) on a 2,720 Thermal Cycler (Applied Biosystems, United States). PCR products were visualized on 1.2% agarose gels and the most intense bands were selected for sequencing. All PCR products were bidirectionally sequenced on an ABI 3730XL automated sequencer with both forward and reverse primers.

Genetic Analysis

The authenticity of all COI sequences was first verified by BLAST search in GenBank (BLASTn, megablast algorithm) and compared with the highest match (99–100%).

Then all sequences were assembled in Bioedit (Hall, 1999) and aligned using the CLUSTALW multiple algorithm under default options. Ambiguous sequences were trimmed after alignment. Molecular diversity from COI sequences was measured using DnaSP 5.0 (Librado and Rozas, 2009) with the following variables: number of haplotypes (H), polymorphic sites (S), haplotype diversity (h), and nucleotide diversity (π).

In order to visually represent the relationships between the mtDNA haplotypes of the sampled *E. cardinalis* individuals, we performed a haplotype network analysis using HAPLOVIEWER, which turns traditional phylogenetic tree into haplotype genealogies network (Salzburger et al., 2011). The phylogenetic tree used for this visual representation was obtained by employing a maximum likelihood approach in PhyML 3.0 (Guindon et al., 2010) using a GTR model with four gamma-distributed rate categories as the substitution model.

Population genetic differentiation was estimated by pairwise Φ_{ST} values among 11 sampling sites using the Tamura–Nei model of nucleotide substitution in Arlequin 3.5 (Excoffier and Lischer, 2010). The model was assessed as most suitable for our data using jModeltest 2.1 (Darriba et al., 2012). The significance of the pairwise Φ_{ST} values was tested by 10,000 permutations. To estimate migration rates between sampling sites, we performed maximum likelihood analysis using the coalescence method in Migrate 3.2.1 (Beerli and Felsenstein, 1999). The estimated parameters were $P_{ij} = \theta_i M_{ij}$, where P_{ij} is the number of effective migrants from i to j, θ is mutation-scaled population size, and M_{ij} is m_{ij}/μ (where m_{ij} is the immigration rate from population i into j, μ is the mutation rate per generation).

Inferring the Historical Demography

Signatures of population demographic patterns (bottlenecks or expansions) in *E. cardinalis* were first examined by Tajima (1989) and Fu (1997) statistics with 10,000 permutations in Arlequin 3.5 to determine whether *E. cardinalis* in the Beibu Gulf data conformed to or departed from neutral theory model expectations because of factors such as a population bottleneck or expansion. For neutral markers, significant negative Fu's Fs and Tajima's D values can be expected under population expansion.

Then, we calculated the mismatch distribution among sampling sites under the sudden expansion model. This measure quantified the smoothness of the observed mismatch distribution. We used parametric bootstrapping (1,000 replicates) as implemented in Arlequin 3.5 to test the goodness of fit of the observed mismatch distribution to the expected under the sudden expansion model using raggedness index (R index) and the sum of squared deviations (Schneider and Excoffier, 1999; Ray et al., 2003; Excoffier, 2004). Populations that underwent substantial expansion are expected to display unimodal mismatch distributions with a low R index.

Furthermore, we used two methods to estimate the population expansion time of *E. cardinalis* in Beibu Gulf. First, the population expansion time was directly calculated from the mismatch distribution of τ (tau) statistic and converted to the absolute time in years (t) using the equation $\tau = 2\,ut$, where u is the mutation rate of the sequence and is calculated as $u = 2\,\mu k$; k is the nucleotides number of the sequence and μ is the mutation rate of the mtDNA gene per generation (Rogers and Harpending, 1992). Following Cantatore et al. (1994), mutation rates between 1 and 3% per million years were selected for our mitochondrial analysis.

Subsequently, we inferred historical demography from effective population size estimates over time using the Bayesian skyline plot (BSP) method in BEAST 1.8.0 (Drummond et al., 2005; Drummond and Rambaut, 2007). This calculation used a strict molecular clock with an HKY model assumed with among-site rate heterogeneity across all branches. Markov chains were run for 2.5×10^7 generations and sampled every 1,000 generations, with the first 2,500 samples discarded as burn-in. Three replicates were performed and combined to analyze COI datasets, respectively. The rest of parameters were set as default values. TRACER 1.5 was

TABLE 1 | Descriptive statistics of genetic diversity of *Evynnis cardinalis* sampled from Beibu Gulf based on COI sequence data.

Sampling sites	*N*	*H*	*S*	*h*	*π*
S1	15	8	9	0.79 ± 0.105	0.00231 ± 0.0006
S2	15	9	9	0.886 ± 0.069	0.0025 ± 0.0005
S3	15	9	10	0.886 ± 0.069	0.00253 ± 0.0005
S4	15	6	7	0.705 ± 0.114	0.00185 ± 0.0005
S5	15	8	7	0.79 ± 0.105	0.00203 ± 0.0004
S6	15	7	7	0.657 ± 0.138	0.00188 ± 0.0005
S7	15	8	9	0.79 ± 0.105	0.0025 ± 0.0006
S8	15	10	12	0.857 ± 0.09	0.00277 ± 0.0006
S9	15	7	9	0.724 ± 0.121	0.00231 ± 0.0007
S10	16	10	11	0.825 ± 0.098	0.00222 ± 0.0005
S11	11	7	7	0.873 ± 0.089	0.00277 ± 0.0006
Overall	162	44	44	0.788 ± 0.033	0.00232 ± 0.0002

N, *sample size;* H, *number of haplotypes;* S, *number of segregating sites;* h, *haplotype diversity (±S.D.);* π, *nucleotide diversity (±S.D.).*

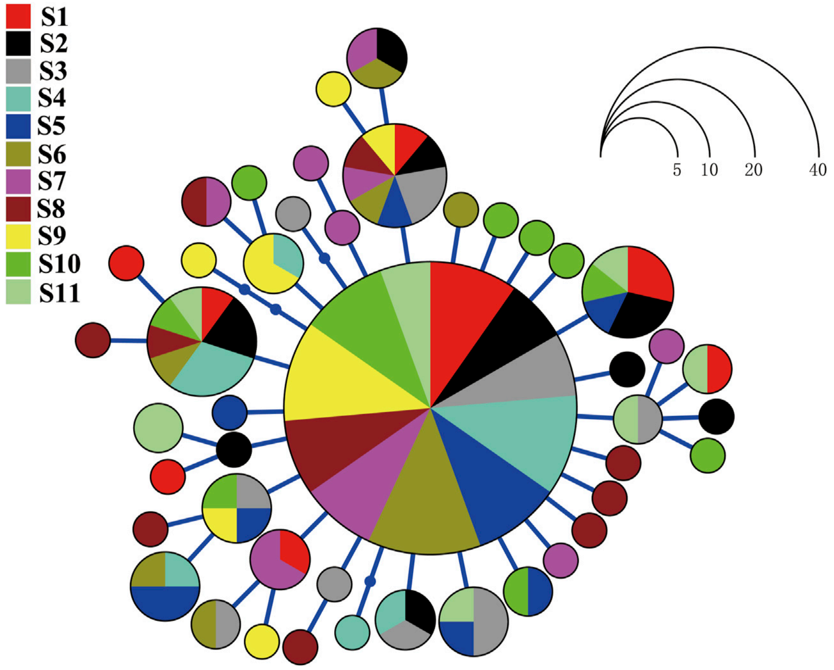

FIGURE 2 | Haplotype network for *Evynnis cardinalis* on the basis of nucleotide variation at the mitochondrial cytochrome c oxidase subunit I gene. Each circle represents a unique haplotype whose size is proportional to the number of individuals sharing that specific haplotype.

employed to visualize the posterior probabilities of the Markov chain statistics and to estimate a statistical summary of the genetic parameters.

RESULTS

Genetic Diversity

In total, 162 high-quality sequences were obtained after alignment and trimmed to 618 bp. The average base composition were C = 28.8%, A = 22.3%, T = 30.1%, G = 18.8%. In total, we found 44 different haplotypes in our sampling area (**Table 1**; **Figure 2**). All 44 haplotype sequences were deposited in GenBank (accession numbers MW881382–MW881425). Overall, the nucleotide diversity was low (average, 0.00232; range, 0.00185–0.00277), while the haplotype diversity was relatively high and heterogeneous (average, 0.788; range, 0.657–0.886) (**Table 1**).

The haplotype network analyses of the mtDNA from *E. cardinalis* in Beibu Gulf showed a typical star-like pattern, with the most common haplotype, H1, located at the center of the haplotype network and surrounded by many low-frequency haplotypes that were divergent from H1 by only one or two mutations (**Figure 2**). Most of the haplotypes had low-frequency, and 27 haplotypes were uniquely present at only one sampling site. Only 11 out of the 44 haplotypes were observed at more than two sampling sites. However, several of the abundant haplotypes were present at multiple sampling sites. For example, the dominant widespread haplotype H1 distributed in all 11 sampling sites (**Figure 3**); the largest distance between two sampling sites harboring this haplotype was more than 300 km. H6 and H8 were distributed at eight and seven sampling sites, respectively. Moreover, between six and ten haplotypes were found per sampling site, but there was no clear pattern of geographical variation (**Figure 3**). The highest number of haplotypes (H = 10) was observed at sites 8 and 10 (**Table 1**).

The average genetic differentiation, Φ_{ST}, of *E. cardinalis* within Beibu Gulf was 0.031 (range, −0.0657–0.0671). All pairwise Φ_{ST} values among the sampling sites were small and not significant

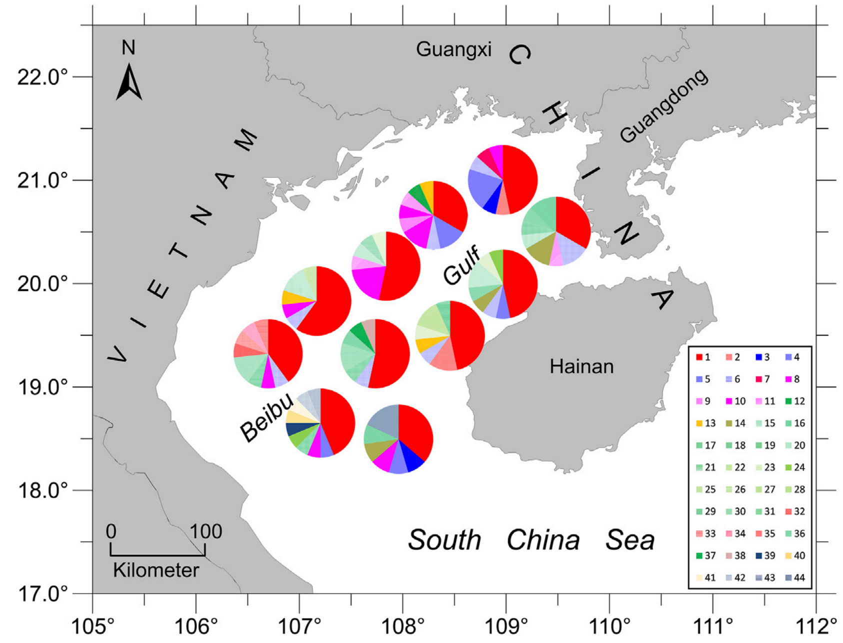

FIGURE 3 | Pie diagrams showing the relative abundance of different haplotypes at the eleven sampling sites (total number of different haplotypes = 44). The size of each pie fraction is proportional to the proportion of individuals with a given haplotype.

TABLE 2 | Pairwise differentiation Φ_{ST} of *Evynnis cardinalis* sampled from Beibu Gulf based on COI sequence data.

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
S1	0										
S2	−0.0597	0									
S3	−0.0203	−0.0456	0								
S4	−0.0213	−0.0364	−0.0322	0							
S5	0.0041	−0.039	−0.0386	−0.0397	0						
S6	−0.0204	−0.0442	−0.0421	−0.0424	−0.0567	0					
S7	−0.033	−0.0403	−0.0276	−0.0246	−0.0079	−0.0509	0				
S8	−0.0283	−0.0275	−0.0197	−0.0505	−0.0113	−0.0239	−0.0323	0			
S9	−0.008	−0.0312	−0.0295	−0.0212	−0.0228	−0.0588	−0.0657	−0.0266	0		
S10	−0.0274	−0.0289	−0.0205	−0.0443	−0.0337	0.006	−0.0098	−0.0249	0.0014	0	
S11	−0.0476	−0.0141	0.0107	0.0281	0.0367	0.0671	0.0335	0.0302	0.0598	0.0037	0

TABLE 3 | Migration rates (number of migrants per generation) among *Evynnis cardinalis* sampling sites from Beibu Gulf (receives/exports).

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
S1	0										
S2	19.34/12.78	0									
S3	17.37/45.76	13.73/43.34	0								
S4	20.67/8.33	25.16/16.59	25.61/10.87	0							
S5	17.26/12.19	11.62/10.72	38.16/7.41	9.08/14.31	0						
S6	15.36/18.03	23.55/19.19	42.58/18.06	17.02/22.18	6.01/16.28	0					
S7	5.98/30.87	17.41/45.36	21.88/30.68	12.38/22.22	12.13/37.6	18.83/30.66	0				
S8	15.67/39.67	20.4/36.59	14.36/36.54	7.69/35.23	11.57/18.65	22.88/33.07	24.08/9.91	0			
S9	20.77/22.94	25.72/9.85	31.27/19.78	20.88/15.8	14.99/33.7	34.08/14.1	41.58/36.78	37.02/29.12	0		
S10	23.62/40.04	7.89/30.56	41.27/28.81	8.65/38.6	6.98/36.74	20.98/26.3	22.18/28.17	31.06/24.21	22.95/30.58	0	
S11	9.6/45.44	23.9/32.68	37.6/38.86	11.78/30.71	10.46/36.42	13.41/18.61	28.33/33.01	16.41/40.41	15.93/27.67	34.03/31.68	0

TABLE 4 | Mismatch distribution, Tajima's D and Fu's Fs statistics for *Evynnis cardinalis*.

sites	Tajima's D		Fu's Fs		Mismatch distribution				
	D	***p***	**Fs**	***p***	**τ (95% CI)**	**R index**	**R index *p***	**SSD**	**SSD *p***
S1	−1.471	0.05	−6.441	0	2.719 (0.758, 3.865)	0.151	0.27	0.042	0.2
S2	−1.591	0.04	−8.46	0	2.379 (0.84, 3.629)	0.221	0.08	0.072	0.04
S3	−1.844	0.015	−8.333	0	2.455 (0.955, 3.832)	0.235	0.05	0.08	0.04
S4	−1.39	0.044	−3.927	0.001	2.607 (0.852, 4.15)	0.178	0.4	0.051	0.34
S5	−1.359	0.102	−7.415	0	2.234 (0.932, 3.813)	0.352	0.07	0.105	0.04
S6	−0.863	0.248	−5.146	0	2.658 (1.123, 4.184)	0.15	0.33	0.041	0.25
S7	−1.219	0.132	−6.157	0.001	3.006 (1.061, 4.217)	0.115	0.41	0.024	0.36
S8	−1.796	0.014	−9.165	0	2.893 (0.963, 4.479)	0.163	0.23	0.05	0.13
S9	−1.319	0.12	−4.663	0	2.945 (0.459, 4.707)	0.068	0.74	0.009	0.78
S10	−1.944	0.003	−10.011	0	2.428 (0.781, 3.863)	0.275	0.05	0.096	0.06
S11	−0.73	0.26	−4.09	0.004	2.617 (1.654, 4.047)	0.154	0.37	0.042	0.25
total	−2.446	0	−28.378	0	1.531 (1.281, 1.941)	0.055	0.26	0.002	0.24

SSD = sum of squared deviation; R index = Raggedness index.

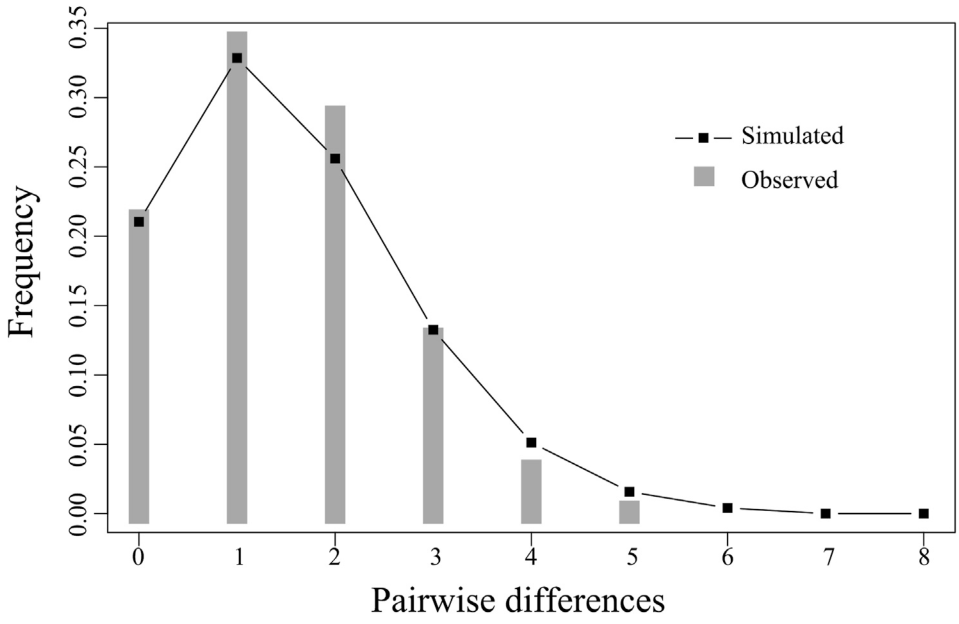

FIGURE 4 | Observed mismatch distributions (bars) and expected mismatch distributions under sudden expansion model (line) of COI for *Evynnis cardinalis* from Beibu Gulf.

(**Table 2**). The effective number of migrants per generation among sampling sites ranged from 5.98 to 45.76. The rates of migration from S7 to S1 were the lowest, whereas those from S1 to S3 were the highest (**Table 3**).

Demographic History

The Fu's Fs and Tajima' D tests of *E. cardinalis* in Beibu Gulf were significantly negative (Fu's Fs = −28.378, $p < 0.01$; Tajima's D = −2.446, $p < 0.01$); this indicated that *E. cardinalis* may have experienced population expansion (**Table 4**). The mismatch distribution appeared to be unimodal (**Figure 4**), which was consistent with the expected distribution under a sudden expansion model (R index = 0.055, $p = 0.26$).

On the basis of the mutation rate of 1–3% of mitochondrial genes per million years (Cantatore et al., 1994) and τ value of all data (1.531, 95% confidence interval: 1.281–1.941), the expansion time for *E. cardinalis* in Beibu Gulf was estimated to have occurred from approximately 62–21 ka.

The BSP analysis indicated that the *E. cardinalis* haplotypes in Beibu Gulf coalesced approximately 40 ka when a mutation rate of 2% of mitochondrial genes per million years was used for analysis. However, the BSP pattern revealed a coalescence time of 30–70 ka when using a mutation rate of 1–3% per million years (**Figure 5**). Despite these different coalescence times, the BSP patterns showed similar tendencies and indicated that a steady population expansion took place between 5 and 30 ka.

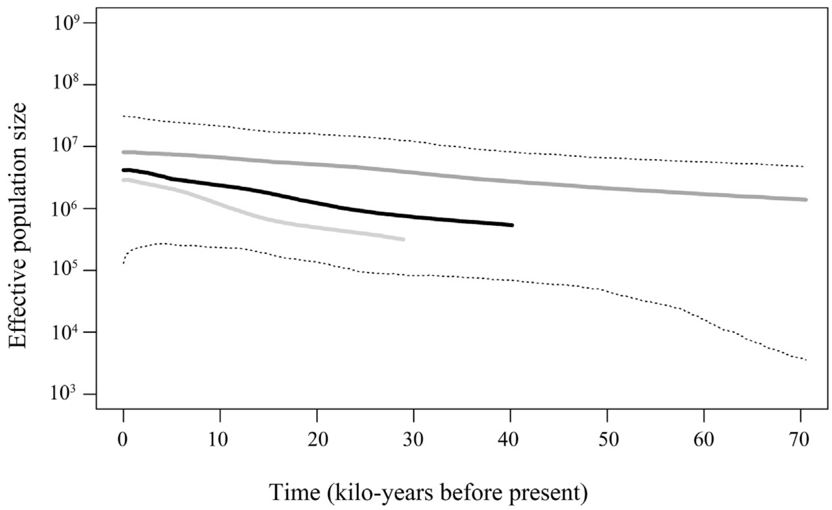

FIGURE 5 | Bayesian skyline plot revealing the demographic trends of *Evynnis cardinalis* in Beibu Gulf. The dark solid line is median estimate under the assumption of a 2% mutation rate per million years. The dark gray solid line shows the median given the assumption of a 1% mutation rate per million, while the light gray bottom line shows the median obtained under the assumption of a 3% mutation rate per million years, and the dashed lines show the 95% highest posterior density (HPD) limits.

DISCUSSION

Studies of population connectivity with mitochondrial markers provide critical information on gene flow and genetic relationships between neighboring populations (Turner et al., 2004; García et al., 2008). Many studies showed that mitochondrial markers are highly effective for revealing marine fish genetic diversity and population connectivity (Lavergne et al., 2014; Gao et al., 2019; Machado et al., 2020). In this study on *E. cardinalis*, mtDNA sequence analysis of specimens from Beibu Gulf revealed no significant genetic differentiation among sampling sites, with low Φ_{ST} values indicating genetic homogeneity.

In contrast to freshwater fish, marine fish are generally expected to show relatively low genetic differentiation across their distribution. It is mainly attributed to genetic exchange being maintained by adult mobility throughout Beibu Gulf during reproduction, and through the passive dispersal of eggs and larvae due to the lack of noticeable physical barriers in pelagic oceans (Grant and Bowen, 1998; Hellberg, 2009; Machado et al., 2020). The dominant widespread haplotype H1 was found in all 11 sampling sites, which also indicated high dispersal potential of *E. cardinalis* at planktonic egg, larval, or adult stages in Beibu Gulf.

Previous studies suggested that *E. cardinalis* breed once a year in Beibu Gulf (Chen and Qiu, 2003; Hou et al., 2008). *E. cardinalis* gonads begin to develop in November and begin to spawn from December to February. The population concentrated in the northern Beibu Gulf during spawning. In early spring, parent fish mainly occur in the northeast of the gulf after spawning, and juveniles concentrate in shallow nearshore of this area in late spring. Thereafter, juveniles gradually migrate southwest and widely disperse in deep waters of Beibu Gulf in summer or early autumn (Zhang et al., 2020).

In addition, the dispersal pattern of *E. cardinalis* was also impacted by circulation in Beibu Gulf. In spring, the density gradient and monsoon wind drive the ocean current from northeast to southwest in the gulf. The surface current velocity reaches 30 cm/s, and the current in the middle layer is approximately 5–10 cm/s (Gao et al., 2017). The direction of the spring currents roughly coincides with the migration route of *E. cardinalis*. Therefore, the seasonal migration and ocean current may be responsible for gene exchange between different locations, and *E. cardinalis* shows low levels of genetic differentiation in Beibu Gulf. If we refer to the biological description of a single stock as given by Ihssen et al. (1981), "a stock is an intraspecific group of randomly mating individuals with temporal and spatial integrity," then the lack of distinct spatial boundaries and genetic substructure (low Φ_{ST} values) revealed by genetic analyses indicated that *E. cardinalis* in Beibu Gulf belong to a single stock.

The presence of a single stock in Beibu Gulf indicates that geographical isolation might block gene exchange between the Beibu Gulf stock and the other 2 *E. cardinalis* stocks, the Taiwan Strait and South China Sea stocks. In Beibu Gulf, the circulation, Hainan Island, and Leizhou Peninsula could act as barriers that impede free dispersal of *E. cardinalis* into this gulf from other areas of the South China Sea (Gao et al., 2017). Our findings and similar investigations conducted elsewhere demonstrated that marine fish that inhabit coastal waters usually constitute a single panmictic stock. For example, Rodrigues et al. (2008) revealed that *Cynoscion acoupa* from northern Brazil belongs to a single stock, even though it covers at least 1,260 km of coastline. Gao et al. (2019) reported a high level of genetic homogeneity in the *Pholis fangi* population around Yellow Sea and Bohai Sea, and suggested it should be considered as a single panmictic stock. Hoolihan et al. (2006) also reported a homogeneous distribution of Spanish mackerel (*Scomberomorus* *commerson*) throughout the Gulf of Oman, Arabian Gulf, and Arabian Sea on the basis of mtDNA analyses.

In addition, mtDNA sequence regions are particularly suitable for inferring historical processes that might be responsible for the contemporary geographical distribution of marine species because they are more prone to genetic drift than nuclear genes and have a relatively small effective population size (Slatkin and Hudson, 1991; Avise, 1994). In our study, the haplotype network of *E. cardinalis* from Beibu Gulf exhibited a star-like and unstructured pattern with a predominance of scattered. The dominant haplotype (carried by 45% of the specimens) was at the center of the haplotype network and surrounded by many haplotypes that diverged from the dominant haplotype by only few mutations. Most surrounding haplotypes were unique to each sampling site and showed few differences among them (**Figure 2**). Similar star-like haplotype networks have been observed for other species in different coastal areas: *Terapon jarbua*, which consists of a panmictic stock from the Socotra Archipelago to the Hadhramout Coast along the wider Gulf of Aden (Lavergne et al., 2014); and *Pogonias courbina*, which did not display distinct structure along the coast of the southwestern Atlantic Ocean (Machado et al., 2020).

A star-like haplotype network pattern, low nucleotide diversity, and high haplotype diversity are often considered consequences of recent population expansion linked to the Pleistocene environmental changes (Craig et al., 2007; Pereira et al., 2009; Liu et al., 2011). The recent demographic expansion of *E. cardinalis* from Beibu Gulf is also supported by the unimodal mismatch distribution and significantly negative Fu's Fs and Tajima's D values. The population expansion of *E. cardinalis* in Beibu Gulf, which was directly estimated from the mismatch distribution, started 62–21 ka before present, corresponding to the late Pleistocene. BSP analysis indicated steady population expansion that started around 30 ka. Both two methods of estimated period of population expansion are consistent with the environment changes of the northern South China Sea in the Pleistocene.

E. cardinalis is mainly distributed from 30 to 60 m depth, and spawns in coastal habitats and shallow shorelines. Therefore, the *E. cardinalis* distribution is closely related to historical sea level fluctuations. When sea level was 120 m lower than the present level during the last glacial maximum of the Pleistocene, the northern South China Sea included Beibu Gulf, which was part of the South China continent, Hainan Island, and Taiwan Island were connected to mainland China, and the entire South China Sea was separated from the Indian Ocean to form a semi-closed basin (Wang and Sun, 1994; Voris, 2000). Similar to other terrestrial species, *E. cardinalis* may have moved and survived in a potential glacial refuge during this period, such as the semi-closed South China Sea (Hewitt, 1999). In the late Pleistocene, the sea level was still 30 m lower than the present level, but the glaciation began to disappear and the sea water gradually poured into Beibu Gulf through the Qiongzhou Strait (Lu et al., 2003). An initial population of *E. cardinalis* may have immigrated to Beibu Gulf from neighboring areas after it was filled with sea water and sufficiently deep. This initial panmictic stock quickly colonized the empty novel environment under the founder and priority effects, and might have experienced rapid population expansion when favorable conditions occurred (Shulman et al., 1983; Boileau et al., 1992).

CONCLUSION

A homogeneous population structure with low genetic diversity and star-like haplotype pattern was revealed for *E. cardinalis* in Beibu Gulf of the northern South China Sea. Pairwise comparisons of Φ_{ST} and the effective number of migrants between all sampling sites in Beibu Gulf indicated a single panmictic stock of *E. cardinalis*. This is mainly attributed to the free genetic exchange of *E. cardinalis* during reproduction. However, *E. cardinalis* from the eastern South China Sea cannot disperse into Beibu Gulf because of the circulation patterns and ocean geographical features such as the semi-closed sea of Beibu Gulf. This molecular evidence revealed that *E. cardinalis* from Beibu Gulf might have experienced colonization and population expansion during the late Pleistocene due to sea level fluctuations.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: National Center for Biotechnology Information (NCBI) BioProject database under accession numbers: MW881382-MW881425.

ETHICS STATEMENT

The animal study was reviewed and approved by South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences.

AUTHOR CONTRIBUTIONS

LX: Conceptualization, Methodology, Formal analysis, Data curation, Writing—original draft, Writing—review and editing. LW, JN, DH, YL, and SL: Methodology, Formal analysis. FD: Funding acquisition, Project administration, Resources.

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Sulfamethoxazole and Enrofloxacin Antibiotics Affect Primary Productivity of Phytoplankton in Fishery Environment

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The antibiotics sulfamethoxazole (SMZ) and enrofloxacin (ENF) are commonly used in aquaculture in China, but their effects on the primary productivity of phytoplankton are unclear. On the basis of the fishery drug use standards (SMZ, 100 mg kg^{-1} d^{-1}; ENF, 15 mg kg^{-1} d^{-1}), these antibiotics were put into culture ponds to observe their effects on the phytoplankton community and primary productivity. The results showed that the changes in phytoplankton species at SMZ and ENF sites were different. At the ENF sites, Bacillariophyta species increased from 7 to 9, euglenophyta from 8 to 12 and cyanophyta from 11 to 9. Chlorophyta species at the SMZ sites increased from 10 to 15 and euglenophyta from 7 to 4. SMZ and ENF significantly promoted the number of cyanophyta ($p < 0.05$). The promoting effect of SMZ was more obvious than that of ENF. SMZ was more effective than ENF in promoting cyanophyta. The maximum number of cyanophyta at SMZ sites was 52.39 million L^{-1}, and the concentration of SMZ was positively correlated with the number of cyanophyta. The maximum number of cyanophyta at ENF sites was 33.13 million L^{-1}, and the promoting effect of low concentrations was more significant than that of high concentrations. Both SMZ and ENF promoted increased phytoplankton biomass, consistent with the residual time of the antibiotics in the aquaculture environment. The greatest biomass at the SMZ sites was 129.31 g m^{-3}, and that at the ENF sites was 117.85 g m^{-3}. The changes in the α diversity index showed that both SMZ and ENF led to a decrease in phytoplankton biodiversity, and that SMZ was more harmful to it. There were significant differences in the Shannon–Wiener, Pielou and Simpson indexes of the SMZ sites within groups ($p < 0.05$). The β diversity index showed that both antibiotics could change the phytoplankton habitat, but the effect of ENF on the habitat was recoverable, while that of SMZ prevented its restoration. These data will be valuable in protecting the ecological environment of fisheries and ensuring the safety and stability of fishery aquatic ecosystems.

Keywords: sulfamethoxazole, enrofloxacin, phytoplankton, primary productivity, biodiversity

INTRODUCTION

In recent years, antibiotics have been widely used in the prevention and treatment of a variety of bacterial diseases (Bialk-Bielinska et al., 2011; Johnson et al., 2015). However, antibiotics are usually not completely metabolized (Zheng et al., 2017). Some of them are excreted into the culture environment through the urine and feces of aquatic animals in the form of crude drugs or metabolites (Aks et al., 2006; Gao et al., 2018). The antibiotics sulfamethoxazole (SMZ) and enrofloxacin (ENF) have been found most often (Xu et al., 2006; Yan et al., 2020), and their residues in the greatest amounts in water (Zhang et al., 2019). The release of residual antibiotics into the environment may affect the phytoplankton community and endanger the safety and stability of the ecosystem.

Algae are an important part of aquatic ecosystems as primary producers, providing oxygen and organic matter to other aquatic organisms through photosynthesis (Laurens et al., 2017; Farooqui et al., 2021). Changes in the number of algae may cause changes in the dominant species and biomass of the community, affect the stability and complexity of the community structure, lead to habitat changes and even harm the structure and function of the whole ecosystem (Rakowski and Cardinale, 2016). Algae are also an important indicator in an environmental quality assessment of an aquatic ecosystem, and so helpful in a comprehensive and effective evaluation of the comprehensive effects of pollutants on phytoplankton and the whole aquatic ecosystem (Li et al., 2008; Khalil et al., 2021). The diversity and complexity of aquatic ecosystems can be effectively understood by observing the species composition and quantitative changes in algae.

α and β diversity indexes are commonly used to describe the diversity of a community. The α diversity index is mainly concerned with the number of species in local and uniform habitats, so describes within-habitat diversity. The β diversity index refers to the differences in species composition or the replacement rate of species along the environmental gradient between different habitat communities, and so describes between-habitat diversity, and emphasizes the change in species diversity within the community (Baczkowski et al., 1998). The diversity index is positively correlated with the number of species. If a region is rich in species, the value of the diversity index is high. If the region is disturbed by external forces, resulting in a reduction in the number of organisms and in the ecological positions of the species remaining, it is easier for other species to replace them and so to maintain the stability and balance of the ecosystem (Peng et al., 2020).

The algae growth inhibition test is one of the most common aquatic ecological toxicity tests. Significant differences in the effects of different antibiotics have been found in the physiological and biochemical indexes of different algae (Aderemi et al., 2018; Fu et al., 2017). Barsha Roy (Br et al., 2020) reported that different concentrations of tetracycline inhibited the growth of Scenedesmus obliquus, and (González-Pleiter et al., 2019a) found that Microcystis was highly sensitive to quinolones (ciprofloxacin). The characteristics of the algae community in the culture environment of the shallow lakes (Peng et al., 2021), the marine (Chong et al., 2020) and the river (Tian et al., 2021) have been reported in the literature, but its structure and the potential risk of SMZ and ENF in the culture environment are not clear. There is an urgent need for research into the risk these antibiotics pose to product and environmental quality. We studied the effects of SMZ and ENF on primary productivity of phytoplankton in the culture environment, to obtain basic experimental data and a theoretical basis to promote the sustainable development of fisheries and to protect the ecological environment.

MATERIALS AND METHODS

Experiment Strategy

Seven tilapia cultivation ponds with the same conditions were selected (**Table 1**). One blank control site (A), three ENF sites (B, C, D) and three SMZ sites (E, F, G) were set up. According to the "Guidelines for the use of fishery drugs" (Ministry of Agriculture of the People's Republic of China, NY5071-2002) and the "Code for the use of Enrofloxacin in aquaculture" (Ministry of Agriculture of the People's Republic of China, SC/T1083-2007), SMZ (100 mg kg^{-1} d^{-1}) and ENF (15 mg kg^{-1} d^{-1}) were fed for 5 consecutive days, once a day for SMZ and twice a day for ENF (Mix the material feeding). According to the national drug use standard, the SMZ withdrawal period was 30 days and the ENF withdrawal period was 10 days. Tests at the SMZ sites lasted for 40 days and at the ENF sites they lasted for 20 days. Eight samples of aquaculture water, sediment, tilapia muscle, liver and intestinal contents were collected from each pond at 1, 6, 10, 15, 20, 25, 32 and 40 days. The first sample was the blank, and the second sampling time was the second day after continuous feeding for 5 days. At the same time, to ensure the

TABLE 1 | Basic information of *Tilapia* pond.

	Experimental cycle (days)	Geographical location	Average temperature (°C)	Pond area (m^2)	Water depth (m)	*Tilapia* population	Initial fish weight (g)
A	40	119.87443 E 31.44171 N	33.1	1667	1.8	3600–4000	350 ± 17
B	20				1.8		360 ± 19
C	20				1.7		350 ± 14
D	20				1.8		350 ± 23
E	40				1.7		340 ± 15
F	40				1.6		340 ± 20
G	40				1.6		350 ± 17

samples representative, samples of culture water, sediment and tilapia in each pond were collected by the five-point method. The muscle, liver and intestinal contents were separated and packed into plastic food bags and transported to the laboratory where they were frozen at −20°C.

Sample Collection, Counting and Determination of Plankton Biomass

The qualitative samples were collected with a No. 25 plankton net and were taken back to the laboratory for observation and classification under a 10 × 40 optical microscope (BM2000, Jiangnan, China). A quantitative sample was collected in a 1000 ml plexiglass water collector; After standing for 24 h, 30 ml was taken for microscopic examination. The taxonomy of the phytoplankton was recorded according to the Freshwater biota of China (ISBN:7-5027-3729-4).

The phytoplankton counting method was obtained according to the formula as follows:

$$M = \frac{C}{F_S \times F_N} \times \frac{30}{0.1} \times P_N$$

In the formula: M is the number of phytoplankton in 1 L of water; C is the area of the counter box, the unit is mm^2; F_S is the area per field of view, the unit is mm^2; F_N is the number of fields passed per slice count; P_N is the number of phytoplankton actually counted by counting each sheet.

The content of Chlorophyll a was determined by the 90% acetone method to calculate the cumulative biomass of phytoplankton. 200 ml of the water sample was filtered and were refrigerated (–4°C) for more than 12 h. We added 3–4 ml 90% acetone solution, used a homogenate machinea (Pro200, United States) to break the filter paper, then centrifuged (2–16P, Sartorius, Germany) the tube for 10 min at 2460×g, Continued to volume with 90% acetone to 10 ml. The absorbance values were measured at 630, 645, 663 and 750 wavelengths by spectrophotometer (MCL8, Shangai).

The biomass was recorded and calculated, the formula as follows:

$$B = \frac{[11.64\,(D_{663} - D_{750}) - 2.16\,(D_{645} - D_{750}) + 0.10\,(D_{630} - D_{750})] \times V_1}{V \times \delta}$$

In the formula: B is the amount of chlorophyll A (mg/m3); V is water volume (ml); D is the absorbance value at different wavelengths; V_1 is constant volume (ml); δ is the cuvette thickness.

The Detection of Sulfamethoxazole and Enrofloxacin Antibiotics

Aquaculture water sample: 200 ml samples were taken for extraction and concentration. The HLB solid phase extraction column (ENF:60 mg/3 ml, Anpel, Shanghai; SMZ:500 mg/6ml, Anpel, Shanghai) was selected and activated with 5 ml methanol and 5 ml ultra pure water successively. Then the water sample was added. After the water sample was drained, 5 ml methanol was used for two times (3 ml + 2 ml) for elution, and 10 ml centrifuge tube was used to collect the elution. The eluent was diluted with methanol to a certain scale. After shaking well, part of the eluent was taken through a 0.22 μm aperture filter membrane and transferred to the sample bottle for testing.

TABLE 2 | Formula principle and evaluation criteria of α diversity index and β diversity index.

Diversity index	Formula	Evaluation criteria
H	$H = -\sum_{i=1}^{s} p_i \ln p_i$	<1.00 Heavy pollution 1.00–1.99 Serious pollution 2.00–2.49 Moderate pollution 2.50–3.50 Mild pollution >3.50 No pollution
D	$D = \frac{S-1}{\ln N}$	<1.00 Heavy pollution 1.00–1.99 Serious pollution 2.00–3.99 Moderate pollution 4.00–6.00 Mild pollution >6.00 No pollution
E	$E = \frac{H}{\ln S}$	0.00–0.30 Heavy pollution 0.31–0.50 Moderate pollution 0.51–0.80 Mild pollution 0.81–1.00 No pollution
C	$C = \sum_{i=1}^{s} \frac{N_i\,(N_i-1)}{N(N-1)}$	0.01–0.10 Main dominant species >0.1 Absolutely dominant species
C_J	$C_J = \frac{c}{a+b-c}$	0 Completely dissimilar 0.01–0.25 Extremely dissimilar 0.26–0.50 Mild similarity 0.51–0.75 Moderate similarity 0.76–0.99 Very similar 1 Complete similarity

In the formula: P_i is the frequency of group I, $P_i = \frac{N_i}{N}$; S is the number of phytoplankton, N_i is the number of individuals of group I, N is the total number of individuals of all groups, an is the number of phytoplankton groups in sample a, b is the number of phytoplankton groups in sample b, c is the number of public phytoplankton groups in sample an and sample b.

Sediment samples: 2 ± 0.01 g sample was weighed in a 50 ml centrifuge tube, 10 ml 0.1% formic acid acetonitrile was added and mixed by shaking. The sample was rotated at 2000 r min^{-1} for 10 min (Henry Troemner, United States), and centrifuged at 10680×g for 5 min (GL-22MS, Bioridge, Shanghai). The supernatant of 5 ml after centrifugation was added to the enhanced lipid removal purification tube activated by 5 ml ultra pure water, and then was rotated at 2000 r min^{-1} for 5 min and centrifuged at 2460×g for 5 min. After centrifugation, the supernatant was transferred to the Bond Elu EMR-Lipid tube (Agilent) containing 1.7 g MgSO4, which was vibrated at 2000 r min^{-1} for 5 min, and centrifuged at 2460×g for 5 min. The upper organic phase was passed through a 0.22 μm aperture filter membrane and transferred to a sample bottle for testing.

An ultra performance liquid chromatography tandem mass spectrometry (LC-MS/MS, Agilent 6420, United States) was used to analyze the two selected antibiotics. Standard curves were plotted with the gradient concentrations of 1, 5, 10, 20 and 50 μg L^{-1}. The regression coefficients of SMZ and ENF were all greater than 0.998 and 0.9996, respectively. The recovery rates of SMZ and ENF were 78.3–98.4% and 81–112% respectively. The limits of detection and quantification were less than 0.01 μg L^{-1} for sulfamethoxazole and 0.2 μg L^{-1} for enrofloxacin.

TABLE 3 | Phytoplankton species composition in a typical tilapia pond.

Species name
Cyanophyta
Microcystis incerta
Microcystis marginata
Microcystis pseudofilamentosa
Microcystis flos-aquae
Chroococcus minutus
Chroococcus turgidus
Chroococcus limneticus
Anabaena circinalis
Anabaena osicellariordes
Anabaenavi gueri
Parasitophyta microphylla
Merismopedia punctata
Merismopedia tenuissima
Merismopedia convolute
Phormidium tenue
Oscillatoria princeps
Anguina oscillatoria
Anabaenopsis.sp
Chrysophyta
Synuraceae urelin
Xanthophyta
Tribonema.sp
Bacillariophyta
Cyclotella catenata
Cyclotella asterocostata
Cyclotella meneghiniana
Synedra ulna
Synedra pulchella
Synedra acusvar
Navicula avenacen
Navicula exigua
Melosira varians
Melosira granulata
Melosira granulata var. spiralis
Nitzschia longissima
Nitzschia lorenziana
Nitzschia acicularis
Cymbella cymbiformis
Stephanodiscus medius
Surirella ovalis
Pyrrophyta
Gymnodinium aerucyinosum
Peridinium umbonatum
Mitratum cyaneum
Gymnodinium cyaneum
Euglenophyta
Trachelomonas felix
Trachelomonas pulcherrima
Trachelomonas abrupta
Trachelomonas curta
Euglena geniculata
Euglena virids
Euglena deses
Euglena thinophila
Euglena acus
Euglena spirogyra
Phacus pleuronectes
Phacus tortifolius
Phacus tortus
Phacus longicauda
Phacus helicoides
Phacus anomalus
Phacus ovalis
Phacus triqueter
Khawkinea acutecaudata
Khawkinea variabilis
Lepocinclis ovum
Lepocinclis marssonii
Strombomonas rotunda
Strombomonas acuminata
Strombomonas fusiformis
Peranema.sp
Cryptophyta
Chroomonas caudata
Chroomonas acuta
Cryptomons erosa
Cryptomons ovata
Chlorophyta
Chlorella vulgaris
Scenedesmus armatus
Scenedesmus quadricauda
Scenedesmus bicaudatus
Scenedesmus bijuba
Scenedesmus dimorphus
Scenedesmus javaensis
Pediastrum simplex
Pediastrum duplex
Pediastrum tetras
Pediastrum boryanum
Pediastrum biradiatum
Crucigenia tetrapedia
Crucigenia quadrata
Crucigenia rectangularis
Chodatella wratislaviensis
Coelastrum reticulatum
Coelastrum microporum
Cosmarium obsoletum
Cosmarium laeve
Arthrodesmas convergens
Staurodesmus cuspidatus
Staurastrum manfeldtii
Tetraedron trilobulatum
Tetraedron hastatum
Closterium parvulum var. angustum
Closterium gracile
Closterium gracile var. elongatum
Westella botryoides
Selenastrum bibraianum
Chlamydomonas.sp
Oocystis.sp
Micractinium pusillum
Actinastrum hantzohii
Schroederia spiralis
Dictyosphaerium ehrenbergianum
Gonium.sp
Kirchneriella lunaris

Analysis of Ecological Diversity Index

The biodiversity index is used to express the relationship between the number and species of mixed biological communities composed of many kinds of organisms, and reflects the complexity of biological communities or habitats (Diaz et al., 2020). The effects of SMZ and ENF on the species and quantity of phytoplankton were analyzed by α and β diversity indexes, and the ecological characteristics of phytoplankton in the culture environment were evaluated comprehensively (Ers et al., 2020).

The α diversity index, including Shannon–Wiener index (H), Pielou index (E), Simpson's diversity index (C) and Margalef richness index (D). The β diversity index included the Jaccard index (C_J). The calculation formulas and evaluation criteria of the above indexes are shown in **Table 2**.

Data Processing and Statistical Analysis

The data were analyzed by Microsoft Excel 2013 (United States) and SPSS 25 software (Chicago, United States), and plotted by Origin 2021 (Northampton, United States) function and Heml Heatmap drawing software (United States).

RESULTS

Effects of Sulfamethoxazole and Enrofloxacin on Phytoplankton Species in an Aquaculture Environment

We found 109 species in the eight phyla Chlorophyta, Cyanophyta, Bacillariophyta, Euglenophyta, Cryptophyta, Pyrrophyta, Xanthophyta and Chrysophyta. The species composition of the phytoplankton is shown in **Table 3**. Among them were 38 Chlorophyta species, accounting for 34.9% of the total phytoplankton species; Euglenophyta were second with 26 species, accounting for 23.9% of the total phytoplankton species. The numbers of species from other phyla were 18, 17, 4, 4, 1 and 1 in Cyanophyta, Bacillariophyta, Cryptophyta, Dinoflagellata, Chrysophyta and Xanthophyta, respectively. The number of algae species at the SMZ sites was similar to that at the ENF sites. We found 101 species of algae at the SMZ sites, including 36 species of Chlorophyta, 23 species of Euglenophyta, 17 species of Bacillariophyta and 15 species of Cyanophyta. The ENF sites held 98 species of algae, including 34 species of Chlorophyta, 24 species of Euglenophyta, 15 species of Bacillariophyta and 15 species of Cyanophyta, and the species number of cryptomophyta, dinoflagellata, chrysophyta and xanthophyta remained the same at both sites. Four phytoplankton species related to the two antibiotic sites were 4, 4, 1 and 1, respectively.

After SMZ and ENF entered the aquaculture environment, there were significant differences in the number of phytoplankton species of Cyanophyta, Chlorophyta, Bacillariophyta and Euglenophyta (**Figure 1**). After adding ENF for 5 days, the Bacillariophyta and Euglenophyta species increased on the 6th day, the number of Bacillariophyta species increased from 7 to 9 and returned to their initial levels on the 10th day, while the number of Euglenophyta species increased from 8 to 12 on the 15th day. This indicated that ENF promoted the growth of Bacillariophyta and Euglenophyta and was positively correlated with them. In the early stage of ENF dosage there was no significant effect on Cyanophyta species, but the number

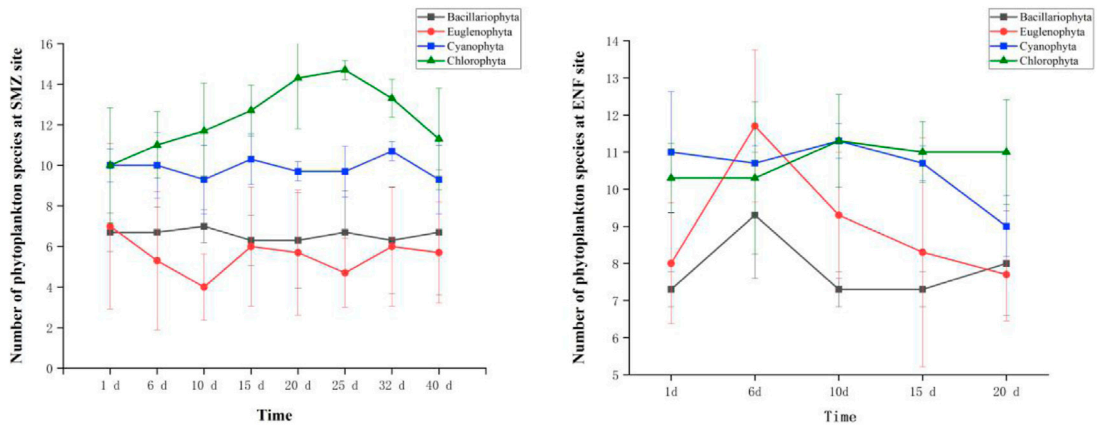

FIGURE 1 | Effects of sulfamethoxazole and enrofloxacin on phytoplankton species.

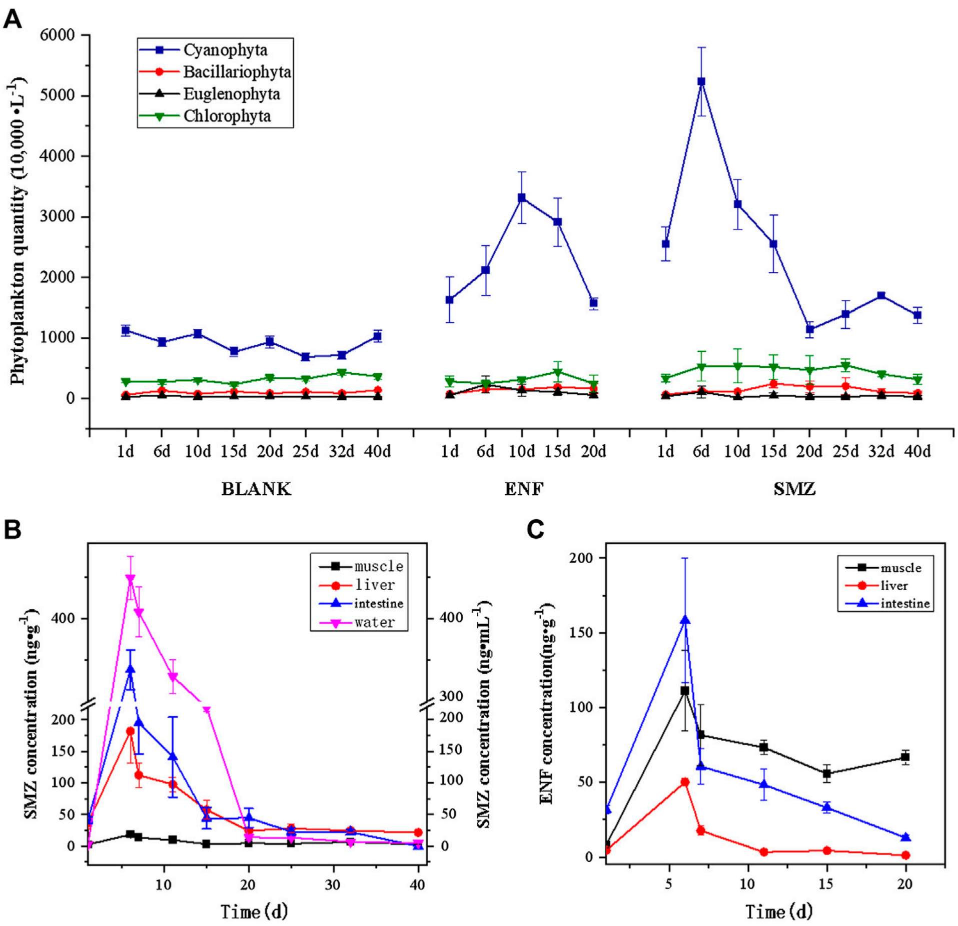

FIGURE 2 | Effects of sulfamethoxazole and enrofloxacinon on phytoplankton population changes.

of Cyanophyta species decreased from 11 to 9 on the 10th day as antibiotic concentrations fell. ENF had no significant effect on Chlorophyta species. The effect of SMZ was opposite to that of ENF. We found that Cyanophyta and Bacillariophyta species remained at 10 and 7, respectively, with no significant change. The number of Chlorophyta species increased from 10 species on day 1–15 species on day 25, then declined. SMZ inhibited the growth of Euglenophyta, and the number of Euglenophyta decreased from seven species on day 1–4 species on day 10.

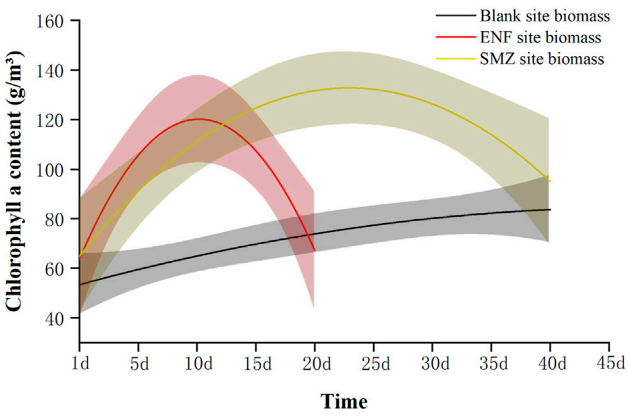

FIGURE 3 | Effects of sulfamethoxazole and enrofloxacinon on phytoplankton biomass.

Effects of Sulfamethoxazole and Enrofloxacin on the Number of Phytoplankton in an Aquaculture Environment

There were significant differences between the two antibiotics on the number of Cyanophyta compared with Bacillariophyta, Chlorophyta and Euglenophyta. The main results were: both SMZ and ENF greatly increased the number of Cyanophyta. The promoting effect of SMZ was more obvious than that of ENF, and the promoting effect of antibiotics on Cyanophyta was related to its concentration in the culture environment. Taking into account the difference in the initial habitat of each pond, pond A was selected as a blank group without any treatment. The results showed that the numbers of four species of algae in the blank site remained stable throughout the experimental period, and the number of Cyanophyta was stable at 7–11 million L^{-1} (**Figure 2A**). The number of Cyanophyta at the SMZ sites almost doubled and was 25.54 million L^{-1} on the first day. The number began to decrease after increasing to 52.39 million L^{-1} on the sixth day, and fell to its lowest value on the 20th day. At this time, the number of Cyanophyta stabilized at about 12 million L^{-1}, which was half of the initial level. The number of Cyanophyta at the ENF sites almost doubled, from 16.31 million L^{-1} on the first day to a peak of 33.13 million L^{-1} on the tenth day, then began to decrease slowly and returned to the initial level on the 20th day.

The concentrations of the two antibiotics in different media in the aquaculture environment varied with time (**Figures 2B,C**). The concentration of antibiotics was its highest on the sixth day after feeding. SMZ was completely degraded in tilapia after about 20 days, and ENF was completely degraded in tilapia after about 10–12 days. Analysis of specific algae species revealed that the sites tested with the two antibiotics were mainly characterized by the outbreak and growth of *Anabaena circinalis*; *Chroococcus turgidus*; *Chroococcus limneticus*; *Merismopedia punctata*; *Merismopedia tenuissima and Merismopedia convolute*, but there were differences. The number of Cyanophyta at the SMZ sites was at its highest on the sixth day, and the concentration of SMZ was the highest at this time; while the number of Cyanophyta at the ENF sites was highest on the tenth day, and the withdrawal period of ENF was 10 days. The results showed that there was a positive correlation between the concentration of SMZ and the number of Cyanophyta; that is, the higher the concentration of SMZ, the better its promoting effect on Cyanophyta. Although ENF was also able to increase the number of Cyanophyta, the effect of low concentrations was more significant than that of high concentrations.

The biomass of phytoplankton changed with the alterations in species and quantity. Compared with the blank site, the phytoplankton biomass at the SMZ and ENF sites increased significantly, and the cumulative biomass at the SMZ sites was its highest on the 20th day, with the highest value of 129.31 g/m³ (**Figure 3**). The cumulative biomass at the ENF sites reached its highest value of 117.85 g/m³ on the tenth day, and then began to decrease. The phytoplankton biomass on the 20th day at the ENF sites was 63.77 g/m³, which was close to the initial level (62.49 g/m³), but the phytoplankton biomass at the 40th day at the SMZ sites was 91.91 g/m³, which was different from the initial level (63.28 g/m³). The change in phytoplankton biomass was consistent with the residual time of antibiotics in the culture environment, and the cumulative biomass increased because antibiotics greatly promoted the growth of Cyanophyta in the culture environment. When the promoting effect of antibiotics on Cyanophyta disappeared after their complete degradation, the cumulative biomass of phytoplankton decreased.

Effects of Sulfamethoxazole and Enrofloxacin on α Diversity of Phytoplankton in an Aquaculture Environment

After SMZ and ENF were fed into the culture environment, the α diversity index of the phytoplankton decreased (**Table 4**). The higher the diversity index, the richer the biodiversity of the species, and vice versa. The results showed that both SMZ and ENF were able to reduce the biodiversity of phytoplankton. At the same time, we found that the values of the Shannon–Wiener, Margalef richness and Pielou indexes at the SMZ sites were lower than those at the ENF sites, which indicated that SMZ was more harmful to phytoplankton biodiversity. Moreover, the values of the three indexes at the SMZ sites were their lowest on the sixth day; the lowest values were 2.32 ± 0.43, 3.93 ± 0.92 and 0.65 ± 0.09, respectively. The lowest values at the ENF sites, however, were 2.67 ± 0.14, 5.03 ± 0.98 and 0.71 ± 0.04, respectively, on the 10th and 15th days, which was consistent with the maximum number of Cyanophyta. Analysis of α diversity index showed that the Shannon–Wiener, Pielou and Simpson diversity indexes at the SMZ sites were significant different within groups ($p < 0.05$), but there was no significant difference in each index at the ENF sites. The difference between SMZ groups verified the conclusion that the antibiotics aggravated the degree of water pollution by changing the structure of the algae community. According to the evaluation criteria of the α diversity index, SMZ will exacerbate the degree of water pollution of culture ponds, and many indexes

TABLE 4 | α diversity index of culture water at SMZ site and ENF site.

	ENF				SMZ			
	H	**D**	**E**	**C**	**H**	**D**	**E**	**C**
1 d	🟨 2.87±0.12	🟨 5.91±0.55	🟨 0.79±0.04	0.09±0.02	🟨 2.94±0.13	🟨 5.34±0.27	🟦 0.80±0.04	0.08±0.02
6 d	🟨 2.80±0.11	🟨 5.50±0.30	🟨 0.75±0.05	0.10±0.02	🟥 2.32±0.43	🟥 3.93±0.92	🟨 0.65±0.09	🟩 0.16±0.06
10 d	🟨 2.67±0.14	🟨 5.32±0.12	🟨 0.71±0.04	🟩 0.11±0.01	🟨 2.59±0.21	🟨 4.27±0.33	🟨 0.73±0.06	🟩 0.11±0.02
15 d	🟨 2.73±0.22	🟨 5.03±0.98	🟨 0.74±0.04	🟩 0.11±0.02	🟨 2.78±0.36	🟨 4.74±0.50	🟨 0.76±0.09	0.10±0.04
20 d	🟨 2.65±0.40	🟨 5.06±0.74	🟨 0.72±0.08	🟩 0.12±0.05	🟨 2.98±0.20	🟨 4.84±0.45	🟦 0.82±0.04	0.07±0.02
25 d	The drug withdrawal period is over				🟨 3.01±0.22	🟨 5.12±0.38	🟦 0.82±0.05	0.07±0.03
32 d					🟨 2.90±0.06	🟨 5.34±0.50	🟨 0.78±0.04	0.08±0.01
40 d					🟨 2.74±0.22	🟨 4.83±0.82	🟨 0.77±0.05	0.10±0.02

Note: red indicates moderate pollution; yellow indicates mild pollution; blue indicates no pollution; green indicates the existence of absolutely dominant species.

show that the water quality of culture ponds changes from mild pollution to moderate pollution after SMZ enters the culture environment. ENF does not intensify the degree of water pollution of culture ponds, and ENF sites always maintain mild levels of pollution. When the Simpson diversity index of the SMZ sites exceeded 0.1 on the 6th and 11th days, the Simpson diversity index of the ENF sites exceeded 0.1 on the 10th day, which was consistent with the time when the number of Cyanophyta reached their peak at both the SMZ and the ENF sites. At this time, Cyanophyta was absolutely dominant in phytoplankton, other species with narrow nutritional niches had died out, the community structure tended to be simple, and both stability and biodiversity decreased.

Effects of Sulfamethoxazole and Enrofloxacin on β Diversity of Phytoplankton in a Culture Environment

The habitats of phytoplankton communities at different times at the sites tested with the two antibiotics are shown in **Figure 4**. Compared with the blank site, the comprehensive Jaccard index of the SMZ and ENF sites on the sixth day was 0.30–0.46 (mild similarity); that of the ENF sites increased to 0.60–0.75 with the passage of time (moderate similarity), while that of the SMZ sites was still 0.30–0.50. The results showed that both antibiotics could affect the community structure of phytoplankton and change the habitat, but that the community could recover from the effects of ENF. The Jaccard index value increased gradually with the passage of time, remaining unchanged until the end of the recommended withdrawal period. The changes in habitat and biomass of phytoplankton at the SMZ and ENF sites were consistent. At the ENF sites they returned to their initial levels. Those at the SMZ sites, however, could not be completely restored and the phytoplankton community structure changed, so the cumulative biomass was different from the initial level.

DISCUSSION

The aquaculture industry has long been plagued by a variety of bacterial diseases. The emergence of antibiotics to avoid the impact of such diseases on aquaculture species can effectively improve the economies of fisheries (Ming et al., 2020). Their widespread use, however, has also caused many environmental problems. These include the phenomenon of antibiotic residues in the culture environment, which is becoming increasingly serious (Zhao et al., 2019). Attention is often only paid to the food health and safety problems caused by the residues of antibiotics in the cultured species, and their harmful effects on the culture environment is ignored (Ana et al.,1987). Phytoplankton are the cornerstones of aquatic ecosystems but, at present, research into the effects of antibiotics on phytoplankton primary productivity in culture environments has not yielded clear results.

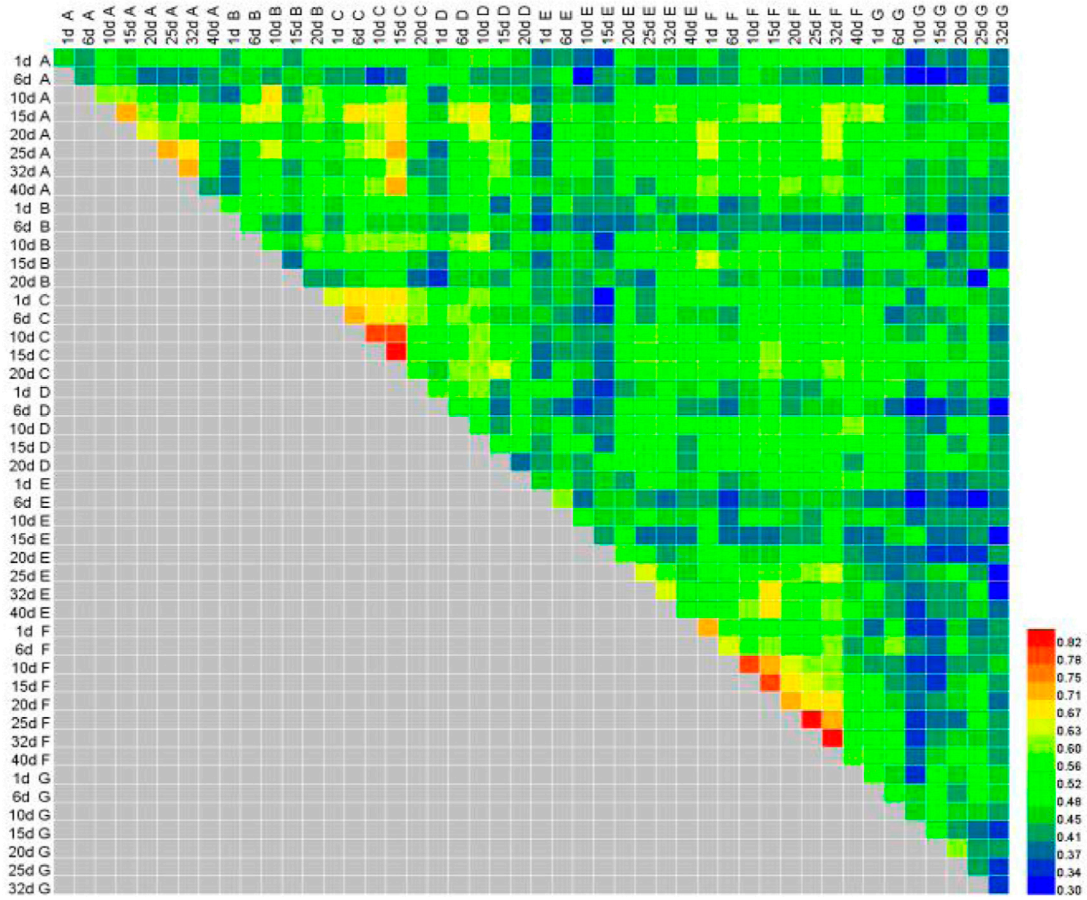

FIGURE 4 | Effects of sulfamethoxazole and enrofloxacinon on habitat of culture ponds.

Sulfamethoxazole and Enrofloxacin Cause the Outbreak Mechanism of Cyanobacteria Population

We found that both SMZ and ENF can affect the primary productivity of phytoplankton to different degrees. Change in primary productivity was mainly due to the more obvious increase in the number of Cyanophyta than of other phytoplankton. The reason for this phenomenon may be that neither SMZ nor ENF can induce the formation of reactive oxygen species (ROS) (Ling et al., 2015). Chenshan (Shan et al.) found that ROS is an intracellular by-product of aerobic metabolism, and that the toxicity of antibiotics is related to the increase in ROS formation at an early stage. The formation of ROS and the imbalance in cellular antioxidant defense mechanisms will lead to oxidative stress and to protein, lipid and DNA damage, so SMZ and ENF have no inhibitory effect on cyanobacterial cells (Gomes et al., 2016; Mullineaux et al., 2018). EC50 (the median effective toxicity concentration with a 50% effect in the blank group) is a standardized parameter for evaluating different compounds to clarify their toxicity (González-Pleiter et al., 2019b) found that the EC50 value of ENF in *Microcystis aeruginosa* cells was lower than 1 mg L^{-1}, indicating that, at the current environmental level, the antibiotic may lead to its ecological risk being classified as "toxic to aquatic organisms". The EC50 values of SMZ and other antibiotics are 0.985–630 mg L^{-1}, which are higher than those of quinolones, macrolides and tetracyclines (Rajaniemi et al., 2005). Therefore, it is an antibiotic with less cytotoxicity to cyanobacteria. Our results showed that the number of cyanobacteria at the SMZ sites was higher than at ENF sites, which was consistent with the experimental results.

Sulfamethoxazole and Enrofloxacin Aggravate the Eutrophication of Aquaculture Water Environment

After SMZ and ENF entered the aquaculture water body, the dominant species of the algae community changed. This affected the community structure, caused the habitat to change and finally led to a change in the nutritional status of the water body. There is a certain correlation between the dominant population and the nutritional status of the water body (Wyta et al.): for example, the appearance of a large number of Chrysophyta often reflects that the water body is Oligotrophic; and Cyanophyta, Chlorophyta and Chlorophyta indicate a eutrophic water body (Dokulil et al., 2003; Parus and Karbowska, 2020). The results showed that cyanobacteria comprised the dominant community at SMZ and ENF sites, and we inferred that aquaculture water using SMZ and ENF was strongly eutrophic. At the same time, and by evaluating the water bodies by indicator species, the emergence of a large number of Anabaena (Rajaniemi et al., 2005) species at the initial stage of SMZ and ENF entry led to a deterioration in water quality. Water quality improved with the natural degradation of the antibiotics and the self-purification capacity of indicator species such as *Scenedesmus* (Rosas et al., 1993) and *Spirulina* (Liu et al., 2018). The α and β diversity indexes showed that the biodiversity of the phytoplankton community decreased and the water quality deteriorated at the sites tested with the two antibiotics, which was consistent with the evaluation results of indicator species.

CONCLUSION

SMZ and ENF have significant–and different–effects on the primary productivity of phytoplankton in the culture environment. These two antibiotics cause water pollution by affecting the species and quantity of phytoplankton and changing the dominant species and community structure. The degree of water pollution caused by SMZ was higher than that of ENF, and the habitat change caused by ENF was not restored during the drug withdrawal period. This endangers the living environments of aquatic organisms and increases the dietary risk from fishery products. Our test results and actual production management needs indicate that we should pay more attention to, and better manage, these two antibiotics in the fishery culture environment. This will help in reducing the environmental risk caused by drug residues, and protect the fishery ecological environment, as well as ensuring the quality and safety of aquatic products.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because *Tilapia* is a cultured species.

AUTHOR CONTRIBUTIONS

XS designed and completed experiments, wrote articles, etc. Others offered help and participated in the experiments.

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Life History Traits, Elasticity Analyses, and Phenotypic Plasticity of *Squaliobarbus curriculus* in the Pearl River Estuary, China

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Squaliobarbus curriculus, commonly known as red-eye fish, is widely distributed in East Asia. It is one of the important aquatic germplasm resource and economic species in the Pearl River. To give suggestions for better protection and management, we investigated its life history and conducted elasticity analyses. Samples (n = 451) were collected between 2016 and 2017 from the western Pearl River estuary. There were no significant differences between the length-weight relationships of females and males ($W = 0.00001\mathrm{SL}^{3.121}$). The von Bertalanffy growth function was $L_t = 553.2\,[1 - e^{-0.111(t+1.009)}]$. The estimated length at 50% sexual maturity for females was 209.6 and 200.0 mm for males, both at 3 years of age. Oocyte size-frequency distribution suggested batch spawning. Fecundity ranged between 9,407 and 175,086 eggs per fish (mean = 51,040, or 143.9 eggs/g of fish weight). To better understand the ecological phenotypic plasticity of *S. curriculus* we conducted meta-analyses on all available life history data for this species. Our results showed that the standard lengths at ages 2 and 3 in the estuary were significantly smaller than in the upper reaches of the Pearl River basin, and there were also obvious differences in fecundity and oocyte size. For more, the standard lengths at ages 2 and 3 were correlated negatively with latitude. Elasticity analysis showed that juveniles' (aged 1–3) survival had the largest contribution to the population growth rate, which suggests that management efforts should focus on the early life stages.

Keywords: growth, reproductive biology, salinity, ectotherms, latitude

INTRODUCTION

Due to its popularity with consumers and resilience to unfavorable environmental conditions, the importance of barbel chub *Squaliobarbus curriculus* (Richardson, 1846) for freshwater aquaculture and capture fisheries in southern China has been steadily growing during the last few decades (Lei et al., 2012). Belonging to the family Cyprinidae (Leuciscinae subfamily), and commonly known as red-eye fish for its red spots on the superior border of eyes, *S. curriculus* is mainly distributed in Asia, including China, western Korea, Vietnam, and Amur River drainage in Russia. As it is one of the dominant species in the western Pearl River Estuary (according to our unpublished 2016–2017 survey), it is also an important commercial fishing species in this area. Importantly, the National Aquatic germplasm resources protection area for *S. curriculus* (and *Erythroculter pseudobrevicauda*) is also located in the Xijiang River, only 130 km upstream from our studied area.

Life history characteristics such as age, growth, and reproduction of fish populations are crucial for determining the fish recruitment and understanding the fish population dynamics (Quinn and Deriso, 1999; Lowerre-Barbieri et al., 2017). Therefore, life history studies are the most important prerequisite required for the assessment of exploited fish populations (Campana, 2001), and essential for fish population management and conservation (Copeland et al., 2017). By analyzing the life history of *S. curriculus*, we aim to improve our understanding of its population dynamics and produce data necessary for designing conservation measures to prevent the decline of its aquatic germplasm resources.

Elasticity analysis quantifies the relative importance of vital life history traits (fecundity, growth, and survival) for the overall population growth rate. This, in turn, provides guidance for management efforts, which should generally focus on demographic parameters with the highest elasticity (Caswell, 2000). Elasticity analysis is one of the most widely used tools in demographic studies, evolutionary and population ecology studies, and particularly in studies aimed at the conservation of exploited and endangered species (Heppell et al., 2000; Manlik et al., 2018). It has been used to guide wildlife management (de Kroon et al., 2000; Manlik et al., 2018), not only in vertebrates (Sæther and Bakke, 2000; Heppell et al., 2000, Gerber and Heppell, 2004, Wang et al., 2017), but also in invertebrates (Sommerville et al., 2014) and plants (Crone, 2016).

In order to better protect and manage *S. curriculus* populations, it is also important to understand their phenotypic plasticity in different environments (Noss 2001). Salinity and temperature (latitude) are two important environmental factors that will affect the variation of the life history characteristics of *S. curriculus*. In estuaries, freshwater meets seawater, so the salinity in estuary habitats is affected by a number of factors, most notably sea tides and freshwater influx, and therefore it can vary notably over relatively short time periods (Bricheno et al., 2021). Due to the demands of maintaining osmotic balance, this variation causes metabolic stress in fish (Nordlie, 2006). Therefore, these environmental factors (salinity fluctuation) might be producing observable impacts on the life history traits of wild *S. curriculus* populations inhabiting the estuary of the Pearl River. The body sizes of organisms tend to be inversely correlated with latitude and temperature (Meiri, 2011; Rypel, 2014), but studies in ectotherms (including fish) often produce conflicting results (Bauer, 1992; Mousseau, 1997, Belk and Houston, 2002, Angilletta and Dunham, 2003, Ashton and Feldman, 2003, Heibo et al., 2005, Pincheira-Donoso et al., 2008, Chucholl, 2011, Rypel, 2014). Thus a general explanation for the variation in size-latitude relationships of ectotherms has remained elusive (Angilletta and Dunham, 2003; Chucholl, 2011; Rubalcaba et al., 2019).

To propose appropriate fisheries management measures, we sampled the *S. curriculus* populations inhabiting the western part of the Pearl River estuary over a 1-year period, inferred their life history traits (age, growth, sex ratio, size at maturity, and fecundity), and then conducted elasticity analyses. Following this, the ecological phenotypic plasticity was studied to allow us to propose better protection and management measures for *S. curriculus*.

MATERIALS AND METHODS

Study Site

The Pearl River (length = 2,214 km, drainage basin = 452,000 km^2) is the second largest river in China in terms of annual water discharge, with $3.26 \times 1011\ m^3\ yr^{-1}$. Although they merely share a common delta, Xijiang, Beijiang, and Dongjiang Rivers are considered tributaries of the Pearl River. The largest, Xijiang, accounts for ~70% of the total Pearl River freshwater discharge (China Bureau of Hydrology, Ministry of Water Resources, http://sqqx.hydroinfo.gov.cn/websq/). All samples for this study were collected in the Xijiang part (or the western part) of the Pearl River estuary, located in the vicinity of Jiangmen City, Guangdong Province, China (**Figure 1**).

Sample Collection and Processing

Sampling was conducted on 10 sites between December 2016 and September 2017. Each site was sampled thrice in each season: 22–24th December 2016, 24–26th March, 12–14th July, and 19–21st September 2017 (**Figure 1**). The sampling was conducted using 5 drift gillnets (100 m long × 1.5–2.3 m high with mesh sizes ranging from 4 to 7 cm) and 2 trap nets (20 m long × 0.6 m high, with mesh size 0.2 cm) set in the evening for 5 h. In total, 451 specimens were collected. The standard length (SL 1 mm) and weight (W 0.01 g) were recorded. The sexual characteristics were macroscopically analysed on 358 specimens (after discarding too small and damaged samples). Gonads were surgically removed, weighed (precision = 0.01 g), and preserved in 10% formalin for subsequent determination of the fecundity and oocyte size-frequency distributions. Gonad maturity stages, determined using a dissection microscope, were identified as stage I - inactive stage, stage II - recovering stage, stage III - early maturing stage, stage IV - late maturing stage, stage V - mature stage, and stage VI - spent stage (Bancroft and Stevens, 1996). Oocyte diameter was calculated as the average of the major and minor axes. Oocytes that did not contain yolk were not measured.

Length-Weight Relationships

We examined the difference in SL distributions between sexes using the Kolmogorov–Smirnov test (K–S test). The standard length and weight relationship ($W = aSL^b$) was converted into the logarithmic form $\ln W = \ln a + b \ln L$ (Ricker, 1975), where a and b parameters were calculated using least-squares regression. SL–W relationships between sexes were compared using the analysis of covariance (ANCOVA). To test whether the growth of fish was isometric, the student's T-test was applied to assess whether the b parameter significantly differed from the expected value of 3 (Pauly, 1984).

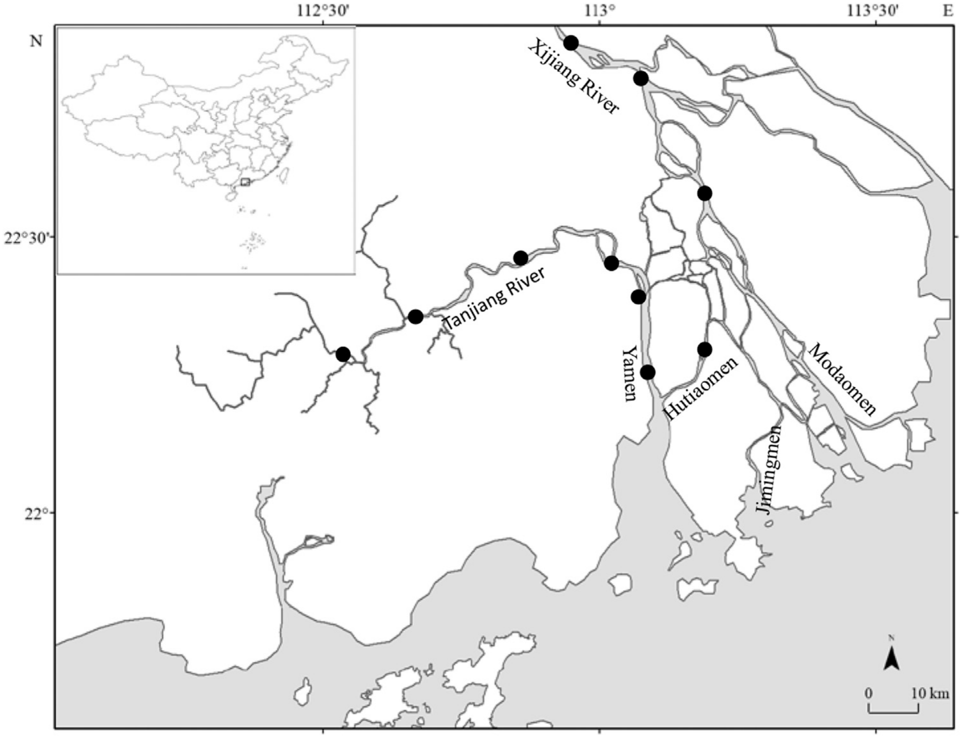

FIGURE 1 | Sampling locations of *S. curriculus* in the western Pearl River estuary. Black dots represent sampled sites.

Age Estimates and Growth

The annual growth of scales was used to estimate the age of fish, needed for subsequent growth estimates. Scales were taken from the end of the pectoral fin and the beginning of the dorsal fin of 437 specimens and analysed under a dissecting microscope as described before (Wang et al., 2015). Each scale was interpreted twice by one reader, with an interval of at least 2 weeks between the two counts. The reader did not have any prior information on the sex, length, or capture time of specimens. Counts were accepted as correct if they were in agreement. If the two counts differed, a recount was conducted and accepted as correct only if it matched any of the previous two counts. If the count did not match any of the previous two, the specimen was not used for downstream analyses. Specimens in different age groups were all assigned a birth date of the 1st of May. The von Bertalanffy growth function (VBGF) based on length-at-age from all age readings were fitted by non-linear regression: $L_t = L_\infty [1 - e^{-k(t-t_0)}]$. Lt is the length at age t, L_∞ is the asymptotic length, k is the growth coefficient, and t_0 is the age at length 0.

Reproduction

Chi-squared (χ^2) test was used to determine whether the sex ratio deviated from 1:1 (Zar, 1999). Sexually mature specimens for both sexes were defined as possessing stage III to stage VI gonads during the spawning season. The SL at 50% maturity ($L50$) was calculated by fitting the logistic curves to the proportion of fish designated as mature in each 10 mm length class using a non-linear least-squares procedure (the Marquardt method). The logistic equation used is $P = 100/\{1 + \exp[-a \times (\text{SL}-L_{50})]\}$, where P is the percentage of mature specimens at SL, SL is the standard length (mm), and a is the slope of the curve.

Phenotypic Plasticity

To better understand the ecological phenotypic plasticity of *S. curriculus*, we tested the impacts of varying salinity on life history traits and correlation between size and latitude by conducting a meta-analysis using all available life history data for this species, across a broad range of latitudes and habitat types (**Table 1**). We obtained all published literature on the life history of *S. curriculus*, and calculated the length at the ages of 2 and 3 according to VBGF. If there was data available for the length at the ages of 2 and 3 in the literature, we directly used the data from the literature. We obtained latitude data from Google Maps. In the analysis of length and latitude, we excluded the relevant data for the estuary.

Survivorship, Mortality Rates, and Elasticity Analysis

The population catch curve was established from the age-frequency distribution to estimate the total mortality rate (Z), corresponding to the sum of natural mortality (M) and fishing mortality (F). The natural log of the number of specimens (ln N) was plotted against the age. The slope (b) of the descending right limb of the age-frequency distribution curve was found through

TABLE 1 | Length at age and sampling site for *S. curriculus* populations from China analysed in this study. L2 and L3 indicate length at the ages of 2 and 3 respectively.

Site	L2 (mm)	L3 (mm)	Latitude (N)	Sample size	Sampling year	References
Jialing river, Chongqing	238.43	297.50	31.16	122	1976–1977	He and Yang (1997)
Fuhe river, Hubei	186.00	236.12	30.86	267	2003–2005	Yang et al. (2006)
River in Guangxi	258.29	311.37	23.53	892	1981–1984	GXFRI[a] 1984
Hujiang river, Guangxi	259.00	308.00	22.77		1981–1984	GXFRI 1984
Xijiang river, Guangxi	254.00	294.00	23.44		1981–1984	GXFRI 1984
Liujiang river, Guangxi	258.00	309.00	24.26		1981–1984	GXFRI 1984
Youjiang river, Guangxi	231.00	281.00	23.75		1981–1984	GXFRI 1984
Lake Baiyangding, Hebei	165.12	217.95	38.87	30	??	Li and Yang (1992)
Lake Suyahu, Henan	229.00	270.00	33.04		1978	XDF[b] 1984
Songhua river, Heilongjiang	118.00	154.00	46.18		1901	Nicolsky (1960)
Lake Dongting, Hunan	208.00	271.00	28.89	179		HFRI[c] 1977
Lake Nandagang, Hebei	147.50	250.60	38.50		1958–1959	Li (2009)
Haihe river, Tianjin	167.80	220.76	39.20			Li (2011)
Hanjing river, Hubei	188.00	253.00	32.05	166	1977	Deng et al. (1981)
Changjing river, Anhui	191.54	218.31	31.29	338	2006–2007	Guo et al. (2009)
Beijing river, Guangdong	218.00	279.00	24.40	70	1981–1983	Pan (1989)
Dongjiang river, Guangdong	185.00	245.20	24.25	304	1981–1983	Lu (1990)
Xijiang river, Guangxi	197.00	258.00	23.42	892	1981–1983	Lu (1990)
[d]Xijiang river/estuary, Guangdong	172.00	225.00	23.05	307	1981–1983	Lu (1990)
[d]Xijiang river, Guangdong	171.30	227.85	23.04	296	2006–2008	Zhu et al. (2013)

[a]GXFRI: Guangxi Zhuang Autonomous Region Fisheries Research Institute.
[b]XDF: Xinxiang Normal University, Department of Biology, Fish Writing Team.
[c]HFRI: Hunan Fisheries Research Institute.
[d]Estuary area.

least-squares linear regression, with b estimating the Z (Ricker, 1975).

The methodology described by Chen and Watanabe (1989) was used to estimate the M (natural mortality) for each age-class t:

$$M_{(t)} = \frac{k}{1 - e^{-k(t-t_0)}} \text{for } t \le t_{mat};$$

and

$$M_{(t,\ t_{max})} = \left[\frac{1}{t_{max} - t}\right] \times \ln\left[\frac{e^k\, t_{max} - e^k t_0}{e^k\, t - e^k\, t_0}\right] \text{for } t > t_{mat}$$

in which t_{mat} is the age at 50% sexual maturity, t_{max} is the maximum age in a population (life span), k and t_0 are the von Bertalanffy growth curve parameters.

Survival rates of the age zero (S_0) can be calculated according to the population growth rate (λ).

Survival values (S) for each mortality rate were estimated using the formula described by Ricker (1975): $S = e^{-z}$. For the cases when $t \le t_{mat}$, age-specific survivorship and mortality rates were estimated using the methodology described by Chen and Watanabe (1989); for $t_{mat} < t$, age-specific survivorship and mortality rates were estimated using the population catch curve.

The mean number of female offspring per female was multiplied by 1/2 of the fecundity. Fecundity was then converted to age-specific fecundity, according to the relationship between fecundity and the age of the fish species.

According to the above data, elasticity analyses were calculated using the projection matrix A, or Leslie matrix (Caswell 1989):

$$A = \begin{vmatrix} F_1 & F_2 & \dots & F_i \\ P_1 & 0 & 0 & 0 \\ 0 & P_2 & 0 & \vdots \\ 0 & 0 & \ddots & 0 \\ 0 & 0 & \cdots & P_i \end{vmatrix}$$

F_i is age-specific fecundity multiply by S_0, Pi is age-specific survivorship, and λ is the dominant eigenvalue of A.

We calculated the elasticity matrices E from the eigenvectors (λ) of each projection matrix A (de Kroon et al., 1986):

$$e_{ij} = \frac{a_{ij}}{\lambda} \frac{v_i w_i}{<w, v>} = \frac{a_{ij}}{\lambda} \frac{\partial \lambda}{\partial a_{ij}} = \frac{\partial log \lambda}{\partial log a_{ij}}$$

where a_{ij} is the (i, j) element of the matrix A, v and w are the left and right eigenvectors of the projection matrix A, and $<w, v>$ is the scalar product of the two vectors $\{v_1 \times w_1 + v_2 \times w_2 \ldots\}$. From there, fertility elasticity, juvenile survival elasticity and adult survival elasticity were calculated according to Heppell et al. (2000) and Wang et al. (2017). Elasticity analyses were performed using "Popbio" R package (http://cran.r-project.org/web/packages/popbio/).

In the present paper, we defined juvenile as age-1 to age at maturity, and adult as age after maturation.

RESULTS

Length-Weight Relationships

The standard length ranged from 40 to 350 mm (mean ± SD = 185.1 ± 53.4 mm), and weight from 1.00 to 986.21 g (mean ± SD = 147.3 ± 127.0 g). The K–S test found a significant difference between the SL distributions of females and males (H = 1.448,

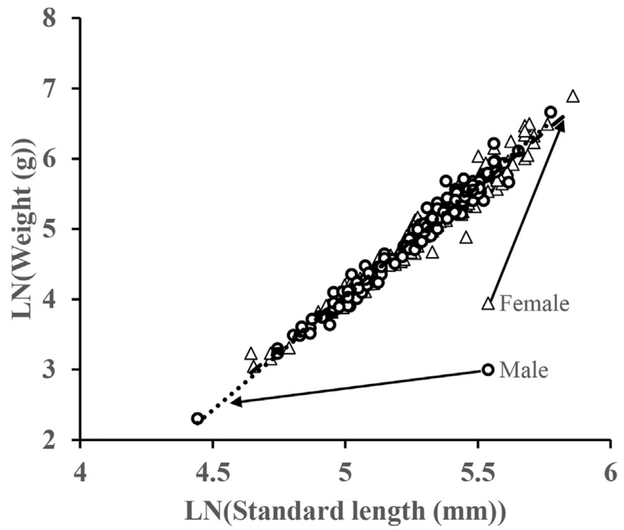

FIGURE 2 | Log-scale visual representation of the length-weight relationship of *S. curriculus* in the western Pearl River estuary, China.

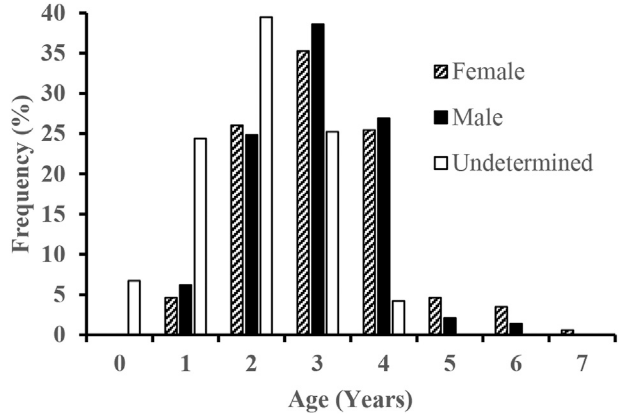

FIGURE 3 | Age-frequency composition of the *S. curriculus* in the western Pearl River estuary, China.

p = 0.030), but the difference was not significant after removing 11 individuals larger than 290 mm (H = 1.121, p = 0.162; among these 11 individuals, there was only one male specimen, which skewed the data distribution).

Length–weight relationships were calculated separately for females and males. The regression equations were $W = 0.00001SL^{3.095}$ ($r^2 = 0.966$, $n = 174$) for females and $W = 0.00001SL^{3.215}$ ($r^2 = 0.974$, $n = 147$) for males. No statistically significant differences were detected for SL–W relationships between sexes (ANCOVA after log-transformation, $n = 321$, $F = 3.641$, $p = 0.057$). The regression equation derived from pooled data was $W = 0.00001SL^{3.121}$ ($r^2 = 0.986$, $n = 451$). The allometric index value (b = 3.121) obtained from the function was significantly larger than 3 (t-test,

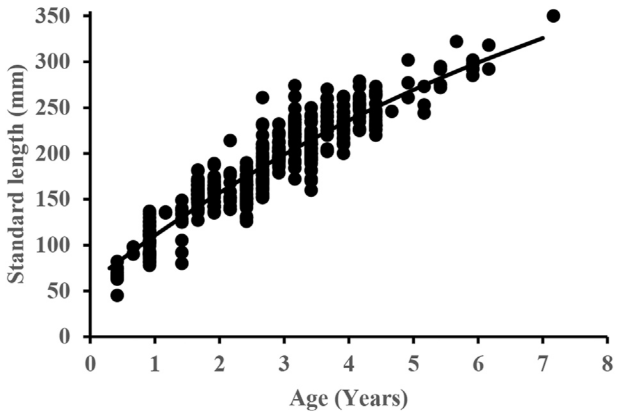

FIGURE 4 | The von Bertalanffy growth curve of *S. curriculus* based on the observed standard length at age in the western Pearl River estuary, China.

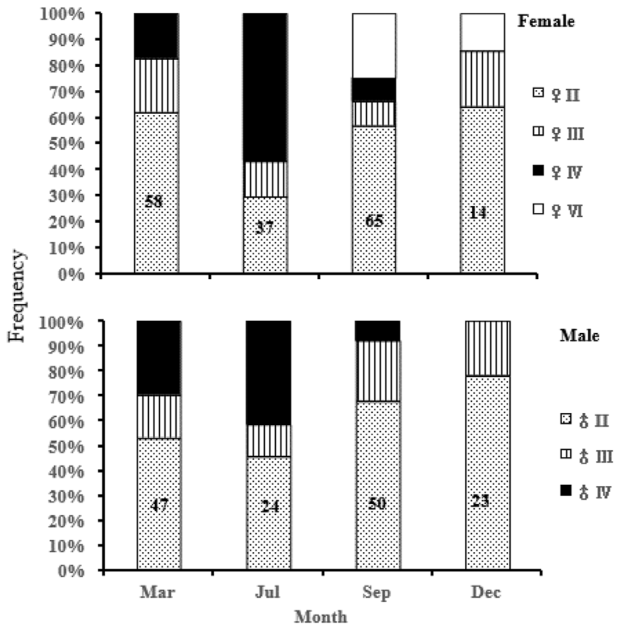

FIGURE 5 | Seasonal gonad stages for female and male *S. curriculus* in the western Pearl River estuary, China.

df = 450, t = 6.94, $p < 0.01$), which indicates a positive allometric growth (**Figure 2**).

Age Structure and Growth

The age varied from 1 to 7 years for females and 1–6 years for males (**Figure 3**). The VBGFs fitted to length-at-age data were described as $L_t = 552.2\ [1 - e^{-0.111(t+1.123)}]$ for females (n = 174) and $L_t = 616.5\ [1 - e^{-0.091(t+1.21)}]$ for males (n = 147). As there were no significant differences in SL–W relationships between sexes, the VBGFs fitted to all of the length-at-age data together was: $L_t = 553.2\ [1 - e^{-0.111(t+1.009)}]$ (**Figure 4**).

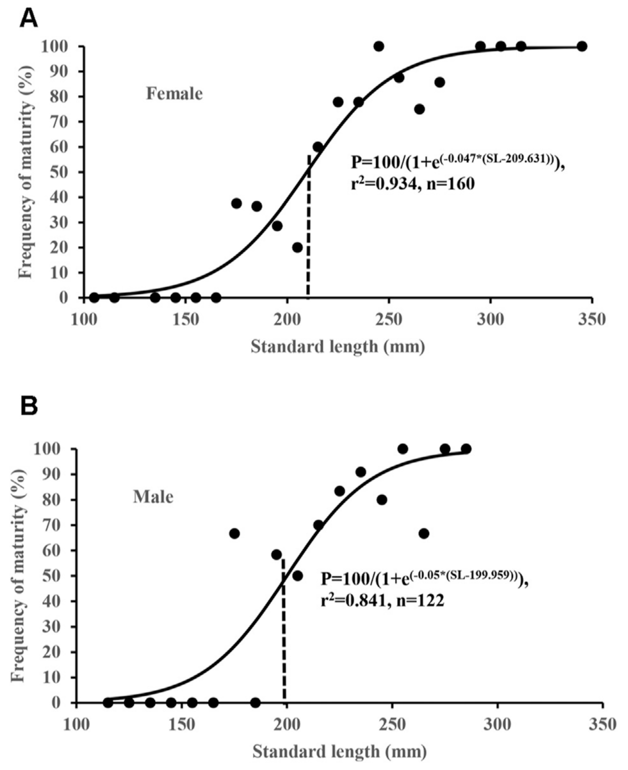

FIGURE 6 | Logistic curve fitted to the percentage contributions of *S. curriculus* to determine the L_{50} for both sexes in the western Pearl River estuary, China.

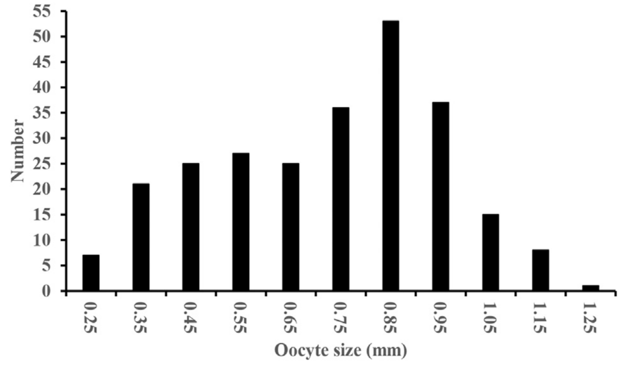

FIGURE 7 | Oocyte size-frequency distribution in a randomly chosen *S. curriculus* specimen with stage IV gonads (period: July, SL = 252 mm, weight = 379.03 g, No. of eggs = 255).

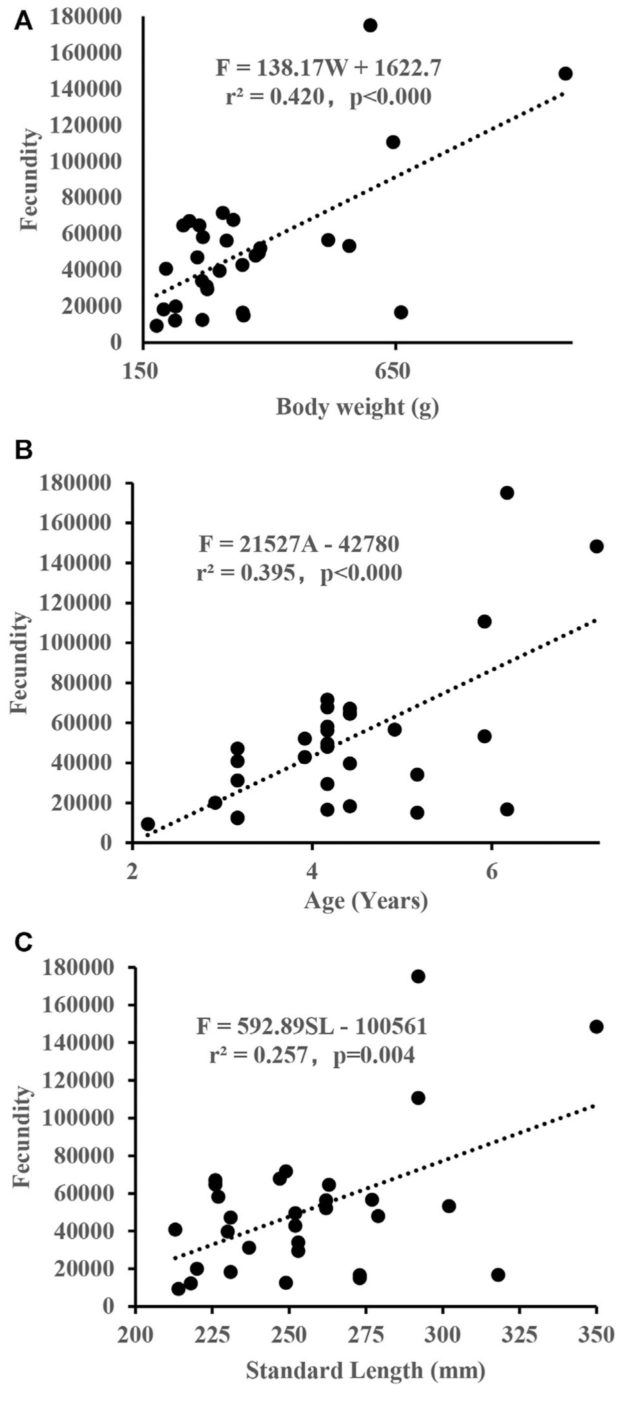

FIGURE 8 | The relationships between body weight **(A)**, age **(B)**, standard length **(C)**, and fecundity of *S. curriculus* in the western Pearl River estuary, China.

Reproduction

Among the 321 successfully sexed specimens, 174 were females and 147 were males. This puts the overall sex ratio (F/M) at 1.18, not significantly different from the expected 1:1 ratio ($\chi^2 = 2.271$, df = 1, $p = 0.132$). Analysis of seasonal gonadal maturation stages variation showed that maturity stage IV of females and males was found between March and September, with the highest frequency in July (summer), whereas stage III was found throughout the year (**Figure 5**).

Size-at-maturity estimates were based on the examination of the 282 specimens collected during the spawning period (March to September). Logistic curves describing the proportion of mature specimens in each 10 mm interval of SL were expressed as $P = 100/[1 + e^{(-0.047(SL-209.6))}]$ ($r^2 = 0.934$, $n = 160$) for females and $P = 100/[1 + e^{(-0.050(SL-200.0))}]$ ($r^2 = 0.841$, $n = 122$) for males (**Figure 6**). The estimated length at 50% sexual maturity (SL_{50}) was 209.6 mm for females and 200.0 mm for males, both at 3 years of age according to the VBGFs. The SLs of the smallest sexually mature female and male were 175 mm (aged 2) and 172 mm (aged 3), respectively.

Oocyte size-frequency distribution in stage IV consisted of up to two cohorts of oocytes, which indicates two batches in the spawning period (**Figure 7**). Fecundity was estimated from 30 mature females that ranged from 213 to 350 mm SL. The estimated values of fecundity ranged between 9,407 and 175,086 eggs per fish, and the mean was 51,040 eggs (SD 37,945). The relative fecundity was between 25.4 and 291.8 eggs/g of fish body weight, with the mean of 143.9 eggs/g (SD 75.4). The fecundity of *S. curriculus* increased linearly with increasing standard length and total weight (**Figure 8**), and the fitted regression equation was $F = 592.89SL–100561$, $r^2 = 0.2573$ and $F = 138.17W + 1622.7$, $r^2 = 0.4198$ respectively. The fecundity also increased linearly with increasing age (**Figure 8**), and the best function was described as $F = 21527A - 42780$, $r^2 = 0.3949$.

Phenotypic Plasticity

The standard lengths at ages 2 (157.00 mm) and 3 (199.00 mm) determined in the present study are significantly smaller than in the upper reaches of the Pearl River basin (**Table 1**): 254.00 and 294.00 mm in the Xijiang River, 258.00 and 309.00 mm in the Liujiang River, 231.00 and 281.00 mm in the Youjiang River (all three in the Guangxi province, China, and all are tributaries of the Pearl River). The corresponding two values were also somewhat larger in a study of this species conducted in the years 1981–1983 in the Xijiang River/estuary (Guangdong province): 172.00 and 225.00 mm. The fecundity of 3- (21,801 eggs) and 4-year-olds (43,328 eggs) is also significantly lower than in the upper reaches of the river (54,238 and 126,639 eggs, respectively) (Pearl River Fishery Resources Survey Editorial Committee, 1985). As opposed to this, the oocyte size in stage IV (0.70–1.20 mm **Figure 7**) is larger than in the upper reaches of the river (0.37–1.09 mm) (Pearl River Fishery Resources Survey Editorial Committee, 1985).

Our meta-analysis of *S. curriculus* populations in China indicates that standard lengths at ages 2 ($L_2 = -4.7809N + 348.93$, $r^2 = 0.6643$, $p < 0.01$) and 3 ($L_3 = -4.6586N + 399.43$, $r^2 = 0.6422$, $p < 0.01$) were correlated negatively with latitude (the latitude ranged from 23.04 to 46.18; **Table 1**).

Elasticity Analyses

According to the relationship between fecundity and age of *S. curriculus* in this study, and the fact that it breeds twice a year, the age-specific fecundities of 3–7 year-old females were 21,801, 43,328, 64,855, 86,382, and 107,909 eggs per fish, respectively. We divided the number of specimens aged 2 and 3 in September 2017 by the number of specimens aged 2 and 3 in December 2016, thereby calculating the finite rate of population increase: $\lambda = 1.069$. Using this number, the survival rate of the age zero (S_0) was 0.000035 $year^{-1}$. Thus, F_i values of 3–7 year-old females were 0.766, 1.521, 2.277, 3.033, 3.789 offsprings per fish, respectively. Age-specific survival rates of 1–6 year-olds were 0.499, 0.838, 0.944, 0.290, 0.290, 0.290 $year^{-1}$, respectively. Based on the above results, the inferred Leslie matrix (age-based projection matrix model) is:

$$\begin{vmatrix}
0 & 0 & 0.766 & 1.521 & 2.277 & 3.033 & 3.789 \\
0.499 & 0 & 0 & 0 & 0 & 0 & 0 \\
0 & 0.838 & 0 & 0 & 0 & 0 & 0 \\
0 & 0 & 0.944 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 0.290 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 0.290 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 & 0.290 & 0
\end{vmatrix}$$

Elasticity analyses based on the age-based projection matrix model showed that: fertility elasticity (age 0–1 elasticity) of *S. curriculus* was 0.242, juvenile (specimens aged 1–3) survival elasticity was 0.485, and adult (specimens aged > 3) survival elasticity was 0.273.

DISCUSSION

S. curriculus is a medium-sized fish (its maximum total length is 48.8cm according to the FishBase) with a short lifespan (the maximum age in this study was 7 years old). It has early sexual maturity (the earliest sexual maturity was 2 years old), high fecundity, long breeding period, and it can reproduce twice a year. This life history characteristics underlie its strong adaptability to environmental and human disturbances. This study showed through meta-analysis that the life history characteristics of *S. curriculus* were affected by fluctuations in environmental factors (such as salinity and temperature), and elasticity analysis proved that the early life history stage was the most critical life history stage to maintain its population growth.

Estuarine Fish Life History Characteristics

The estuary is a region that is affected both by the sea tides and widely varying freshwater influx, which can result in a strong variation in salinity (Bricheno et al., 2021). In such an environment, maintaining homeostasis can be energetically very costly, and affect the amount of energy available for growth and reproduction (Schreck, 2010). Therefore, fluctuating salinity often has highly significant effects on life history traits, such as growth and reproduction (Stearns, 1992; Martin et al., 2009). The *S. curriculus* populations studied in this research also demonstrate this effect, for the individuals of the same age in the estuary were significantly smaller in size and fecundity than individuals in the upper reaches, but the oocyte size in stage IV was larger. Although there could be a large number of variables synergistically producing these observed phenomena, we hypothesise that they may be at least partially attributed to increased metabolic costs of osmoregulation in a fluctuating-salinity environment (Gan et al., 2016). For example, a similar decrease in growth and reproduction parameters with increasing salinity has been reported in *Oreochromis niloticus* and *Colossoma macropomum* (Lowe et al., 2012; Fiúza et al., 2015).

Intriguingly, the "environmental stability hypothesis" predicts that more environmentally variable habitats select for organisms that have higher reproductive allotment (per unit body mass), larger brood sizes, and smaller offspring size relative to organisms from more stable habitats (Stearns, 1983, 1992). Examples of species fitting this pattern are the sailfin molly *Poecilia latipinna*, the western mosquitofish *Gambusia affinis*, and the least killifish *Heterandria formosa* (Martin et al., 2009). The discrepancy between our results and this hypothesis may be caused by different adaptation characteristics of the fish to the environment. We should also mention that some of the differences in fecundity and oocyte size may be partially caused by the comparison of studies spanning several decades, so changes in climate and water environment may also have an impact.

Size-Latitude Relationships

Variation in life-history traits with latitude (temperature) can be explained by phenotypic plasticity (Roff, 2002; Ren et al., 2020), and a ubiquitous rule is that body sizes of organisms tend to be smaller at high temperatures and low latitudes, and larger at low temperatures and high latitudes (Meiri, 2011; Rypel, 2014). Our meta-analysis of *S. curriculus* populations in China indicates exactly the opposite. This rule was also disproven in many other freshwater fishes (Belk and Houston, 2002; Heibo et al., 2005, Rypel, 2014), as well as other ectotherm animals, such as insects (Mousseau, 1997), snakes (Ashton and Feldman, 2003), and lizards (Pincheira-Donoso et al., 2008). However, many ectotherms are known to exhibit a larger size in colder environments (Angilletta and Dunham, 2003), such as the freshwater pearl mussel *Margaritifera* (Bauer, 1992), chelonian turtles (Ashton and Feldman, 2003), some crayfish (*Procambarus clarkii*) (Chucholl, 2011). A general explanation for the variation in size-latitude relationships of ectotherms remains elusive (Angilletta and Dunham, 2003; Chucholl, 2011; Rubalcaba et al., 2019).

Intriguingly, among the freshwater fishes on the North American continent, cool- or cold-water species were in agreement with the rule, whereas the opposite was found for warm-water species (Rypel, 2014). Temperature is perhaps the most important environmental factor for fish growth, as fish have a thermal optimum (T_{opt}, Zarco-Perello et al., 2012): at lower temperatures, metabolic rate declines and growth rate decreases (Reynolds, 2002; Chung et al., 2021), but temperatures higher than T_{opt} are likely to lead to declines in growth (Zarco-Perello et al., 2012). Since the environmental temperature decreases with latitude, our meta-analysis of *S. curriculus* populations in China shows that size is positively correlated with the average temperature. As our data comprised only localities north of the 23 degrees north latitude, they don't comprise the T_{opt} of *S. curriculus*; this explains the positive association between growth and temperature found in this study.

Elasticity Analysis

Elasticity analysis quantifies the contributions of different variables to the population growth rate (Heppell et al., 2000), so it can help us predict the response of populations to disturbance and decide which life stages should be protected (Heppell et al., 2000; Manlik et al., 2018). Our results indicate that management efforts should focus on protecting the juvenile *S. curriculus,* as this life stage had the highest contribution to the population growth rate (0.485). In the context of mostly overfished fishery resources in China (Chen et al., 2009), these life history characteristics of *S. curriculus* might be advantageous. This may be the explanation for the increase in the relative abundance of this species in recent decades (Wang et al., 2017), with the finite rate of population increase of 1.069 inferred in our study. Although this indicates that *S. curriculus* should be able to withstand the current fishing pressure, excessive overfishing would still be likely to produce negative impacts on this population. At present, a yearly fishing ban from March to June is the main protection and management measure employed in the Pearl River Basin. This measure is primarily aimed at protecting the fish during the reproductive season and early life history stages. According to our results, the contribution of the 0–1-year-old life stage of *S. curriculus* to population growth is merely 0.242, whereas the contribution of the 1–3-year-old stage is much higher (0.485). This indicates that protection (or fishing intensity regulation) measures aimed at juveniles are more likely to produce positive outcomes on population growth and stability than the measures currently employed. Thus, implementing a minimum catch size and/or minimum mesh size is particularly important. In the study area, the minimum catch size should not be less than 200 mm (3 years old), and the minimum mesh size of the fishing net should also be adjusted accordingly.

According to our research, the spawning period of *S. curriculus* is from March to September, which may actually be longer than the fishing ban period mentioned above. Therefore, if this species is to be better protected and managed, the fishing ban period needs to be extended. The unique environment of the estuary has a significant impact on the growth of this species, which directly shows that changes in the aquatic habitat, such as environmental pollution, are likely to affect its growth. In summary, only by formulating reasonable management methods based on the life history characteristics of *S. curriculus* can we ensure the permanent sustainability of this resource.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Key Laboratory of South China Sea Fishery Resources Exploitation & Utilization, Ministry of Agriculture and Rural Affairs, South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Guangzhou, China.

AUTHOR CONTRIBUTIONS

Conceptualization, TW, YL, and C-HL; investigation, TW, Y-YX, LL; methodology, TW, Y-YX; formal analysis, TW; writing original draft, TW, IJ, and LL; writing-review & editing, TW, YL, IJ, C-HL, and PW; data curation, YL, IJ, and LL; funding acquisition, C-HL and PW.

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Physicochemical Factors Drive Bacterial Communities in an Aquaculture Environment

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The role of microbial ecology in aquaculture is becoming increasingly significant; however, studies on the changes in microbial ecology driven by the culture environment are limited. In the present study, high-throughput sequencing and chemical analysis was used to explore changes in physicochemical factors, bacterial communities, and their relationships between a water source (Bay) and an aquaculture area located in a reclaimed area. Statistical analysis results revealed that operational taxonomic units levels in inlet water and pond water varied significantly ($p < 0.05$). Non-metric multidimensional scaling (NMDS) analysis revealed the distribution characteristics of bacterial communities with water properties. The abundance of Alphaproteobacteria, Actinobacteria, and Flavobacteria in pond water increased significantly when compared to inlet water. The abundance of heterotrophic bacteria, such as *Candidatus Actinomarina*, *Candidatus Aquiluna*, *Marivita*, and *Vibrio* genera in pond water was significantly higher (Welch's t-tests, $p < 0.05$) than inlet water. Functional prediction analysis primarily revealed an increase in the function that was associated with carbon and nitrogen metabolism in the pond environment. Canonical correlation analysis revealed that the bacterial communities was predominantly influenced by inorganic nutrients. Nitrate-nitrogen (N), nitrite-N, ammonium-N, and phosphate-phosphorous (P) were the key factors influencing bacterial communities in pond environment. A significant correlation was observed between inorganic N and phosphorus (P), and dominant bacterial genera ($p < 0.05$), demonstrating the potential mechanism of regulation of nutrients in bacterial communities. The present study described the microbial ecology of aquaculture ponds in detail and provides a scientific basis for the management of aquacultural environments.

Keywords: aquaculture, bacterial community, physicochemical factors, correlation, high-throughput sequencing

INTRODUCTION

Over the last few decades, offshore aquaculture and ocean fishing have not satisfied the dietary requirements of populations due to an increase in demand for seafood globally. In China, offshore aquaculture has developed rapidly, and large-scale aquaculture has become the most widespread offshore fish production system. The number of aquaculture species and levels of production have increased significantly. Aquaculture systems are relatively complex ecosystems with highly diverse bacterial communities, which influence nutrient cycling and energy flow in aquaculture ecosystems (Moriarty, 1997; Austin, 2006).

With the rapid development of coastal aquaculture, water quality is increasingly attracting the attention of stakeholders. During aquaculture activities, only a portion of the nutrients in the feed is consumed and assimilated by the culture animals, and most of the nutrients are retained in the aquaculture water environment (Thakur and Lin, 2003; Sahu et al., 2013). The residual nutrients could induce changes in the aquatic environment, for example, in the form of shifts in pH, dissolved oxygen (DO), and particulate organic matter, in addition to eutrophication (Martin et al., 1998), which could facilitate bacterial settlement and proliferation. Previous studies have revealed that the physical and chemical properties of water [pH, chemical oxygen demand (COD), temperature, salinity, phosphorus (P), and nitrogen (N)] influence bacterial communities in aquaculture environments (Zhang et al., 2016; Li et al., 2017; Alfiansah et al., 2018; Yang et al., 2018). The culture environment has been reported to exert considerable influence on the composition of intestinal bacteria and the occurrence of disease in aquatic species (Wang et al., 2014; Li et al., 2017; Yukgehnaish et al., 2020). Therefore, studying microbial ecology in aquaculture ecosystems is an effective approach to monitor the functioning of aquaculture systems and it provides valuable information that could facilitate the sustainable management of aquaculture systems (Sun et al., 2020b).

Although studies have examined the relationship between the aquaculture environment and bacterial communities (Zhang et al., 2016; Alfiansah et al., 2018; Sun et al., 2019; Sun et al., 2020b), the difference of bacterial communities between water source and aquaculture systems, and the factors driving this difference remain unclear. For aquaculture process, influent water quality and microbe have an important impact on the aquaculture area. Therefore, studying the changes and driving factors of microbial community structure is of great significance for disease prevention and water quality management. In the present study, combination of analytical chemistry and high-throughput sequencing to compare variations in physicochemical parameters and bacterial communities between the bay water and the aquaculture area. Furthermore, this study analyzed the correlation between physicochemical factors and bacterial communities, and finally determined the factors driving bacterial communities in the aquaculture systems.

MATERIALS AND METHODS

Study Area and Sample Collection

Samples were collected from the Dengfeng mariculture in Fujian, China in December 2018. All ponds had the same water inlet from an adjacent Bay through which water entered the aquaculture area (**Supplementary Figure S1**). The area of each pond (shrimp-crab polyculture) is 33,300 m^2-66,600 m^2. Pond water in the reclaimed area was changed every 2 days, and subsequently comes out through the water outlet. The water inlet and outlet were far from each other to prevent water pollution. Six samples were collected near inlet area in the bay and pond water samples (n = 26) were collected from different ponds using a glass water hydrophore at 0.5 m depth. A total of 500 ml of water was filtered through 0.22-μm polycarbonate membranes (EMD Millipore, Billerica, MA, United States). The membranes were placed in 1.5 ml sterile microcentrifuge tubes. Filter membranes were stored in liquid nitrogen for subsequent DNA extraction.

Another 500 ml of each of the remaining water samples was filtered through a 0.45-μm filter and used to analyze inorganic nutrients. Nitrite-nitrogen (N), nitrate-N, ammonium-N, phosphate-phosphorus (P), and DO concentrations were determined according to the procedure described in "The specialties for marine monitoring" (GB17378.4–1998, China). Water salinity and pH were measured in the field using a YSI 6600 V2 Sonde water quality monitoring system (YSI Incorporated, Yellow Springs, OH, United States).

Illumina MiSeq Sequencing of Bacterial Communities

Total DNA was extracted from the filter membranes using a DNA Extraction Kit (Omega Bio-Tek, Norcross, GA, United States) according to the manufacturer's instructions. The V3 and V4 regions of the 16S rRNA gene were amplified with the 319F (5′- ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVG GGTWTCTAAT-3′) primers (Mateos-Rivera et al., 2016). The total volume of the PCR reaction system was 25 μL: 5×*FastPfu* buffer 4 μL, 2.5 mmol/L dNTPs 2 μL, 5 U/μL *FastPfu* polymerase 0.5 μL, primer (5.0 μmol/L) 1.0 μL and 10 ng template DNA. PCR reaction conditions were 95°C for 3 min, 30 cycles at 95°C for 30 s, 55°C for 30 s, 72°C for 45 s, and 72°C for 10 min. Different barcodes at both ends of the forward and reverse primers were used to distinguish samples. After amplification, the PCR products were purified and paired-end sequenced (2 × 250) on an Illumina MiSeq platform (Illumina, San Diego, CA, United States) according to standard protocols.

Data Analysis

For pair-ended reads obtained by Illumina MiSeq sequencing, the sequences were first classified according to barcode information and then spliced according to the overlap relationship. Barcodes and primers were trimmed from paired-end sequences and then assembled using FLASH (Magoč and Salzberg, 2011). Low quality sequences with ambiguous bases and average quality scores <30 was removed. All chimeric tags were removed using UCHIME algorithm (Edgar et al., 2011). Tags with sequence similarity greater than 97% were defined as OTUs clusters using the CD-HIT tool (Huang et al., 2010). The RDP classifier used the SILVA database (version 132), which had taxonomic categories predicted at the species level. Alpha diversity indices were calculated in QIIME 1.9.1 (Caporaso et al., 2010) from rarefied samples, and assessed using Shannon's index and Chao1 index.

T-tests were used to assess the significant differences ($p < 0.05$) in bacterial OTUs between samples. NMDS (non-metric multi-

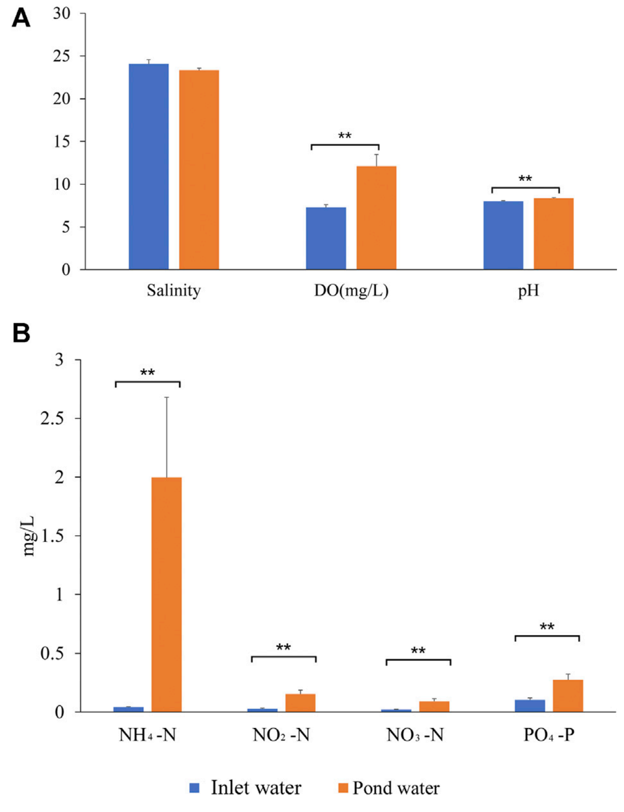

FIGURE 1 | Physico-chemical parameters of pond water and inlet water. **(A)** comparison of salinity DO, and pH between inlet water and pond water; **(B)** comparison of nitrite-N, nitrate-N, ammonium-N and phosphate-P concentrations between inlet water and pond water; Asterisks represent significance analysis (p < 0.01).

dimensional scaling) of unweighted unifrac distances was generated in R Vegan package (version 2.5.3) (Oksanen et al., 2015) and plotted in R project ggplot2 (version 2.2.1) package (Kahle and Wickham, 2013). Canonical correspondence analysis (CCA) was executed in R project Vegan package (version 2.5.3) to clarify the influence of environmental factors on bacterial community composition. Pearson correlation analysis between genera abundance and environmental factor was calculated in R project psych package (version 1.8.4). PICRUSt2 (Douglas et al., 2020) was used to predict the functional profile of the bacterial communities in inlet water and pond water based on 16S rRNA sequences. Welch's t-test ($p < 0.05$) was calculated in R project Vegan package (version 2.5.3) to determine if there were statistically significant differences in the prediction function of bacterial communities between inlet water and pond water.

RESULTS

Variations in Key Physicochemical Factors Between Inlet Water and Pond Water

Water quality parameters are summarized in **Figure 1** and **Supplementary Table S1**. Overall, t-test showed that pond water quality parameters exhibited distinct variations when compared to inlet water. Notably, nitrite-N (p = 0.001), nitrate-N (p = 0.008), ammonium-N (p = 0.008), phosphate-P (p = 0.003) and DO (p = 0.002) concentrations were significantly higher in pond water than in inlet water, especially ammonium-N concentrations. pH was significantly higher in pond water than in inlet water. However, salinity had no significant difference between pond water and water inlet.

High-Throughput Sequencing Data and OTU Analysis

Based on Illumina sequencing analysis, a total of 2,994 OTUs (n = 32) were observed at a 97% similarity level. t-test analysis revealed that there were statistically significant differences ($p < 0.05$) in OTU types and abundance between inlet water and pond water (**Figure 2A**). The relative abundance of 169 OTUs increased, while that of 211 OTUs decreased in pond water when compared to inlet water. The NMDS results revealed similarity in bacterial communities between inlet water and pond water (**Figure 2B**). Inlet water and pond water samples clustered separately with large distances between each cluster. However, the distances within pond water samples had higher variability compared to inlet water. The Shannon's diversity and Chao1 index value of bacterial communities in pond water were lower than in inlet water (**Figures 2C,D**). A Venn diagram revealed that the number of bacterial-specific OTUs was higher in inlet water than in pond water. The total number of common OTUs in inlet water and pond water was 771 (**Figure 2E**).

Bacterial Communities in the Aquaculture Environment

The abundance of bacterial taxa corresponding to different samples was compared by annotating sequences information. The dominant taxa in each group at the class and genus levels are presented in **Figure 3**, **Supplementary Table S2** and **Supplementary Table S3**. The dominant bacterial classes in inlet water and pond water (**Figure 3A**) were Alphaproteobacteria (20.63 ± 1.74% and 24.00 ± 2.26%), Actinobacteria (3.65 ± 0.58% and 17.57 ± 2.30%), Gammaproteobacteria (20.98 ± 4.18% and 13.05 ± 1.05%), Acidimicrobia (16.63 ± 2.89% and 12.52 ± 2.18%) and Flavobacteriia (4.87 ± 0.62% and 8.77 ± 0.79%). The results of the Welch's t-tests ($p < 0.01$, **Figure 3B**) revealed that the abundances of Actinobacteria and Flavobacteriia were significantly higher in pond water than in inlet water. In contrast, the abundance of Gammaproteobacteria, Betaproteobacteria, and Deltaproteobacteria was significantly lower in pond water than in inlet water.

A significant difference ($p < 0.01$) was also observed in bacterial genera among the samples, with specific genera observed in inlet water, and pond water (**Figures 3C,D**). The dominant genera (average abundance >1%) in inlet water were *Candidatus Actinomarina* (12.41 ± 2.88%), NS5 marine group (1.32 ± 0.20%), *Candidatus Aquiluna* (1.09 ± 0.12%), and *Salinihabitans* (0.91 ± 0.13%). The dominant genera (relative abundance >1%) in pond water were *Candidatus Actinomarina* (8.29 ± 1.80%), *Candidatus Aquiluna* (7.99 ± 1.19%), *Marivita* (2.25 ± 0.65%), *Salinihabitans* (2.21 ± 0.41%), NS5 marine group

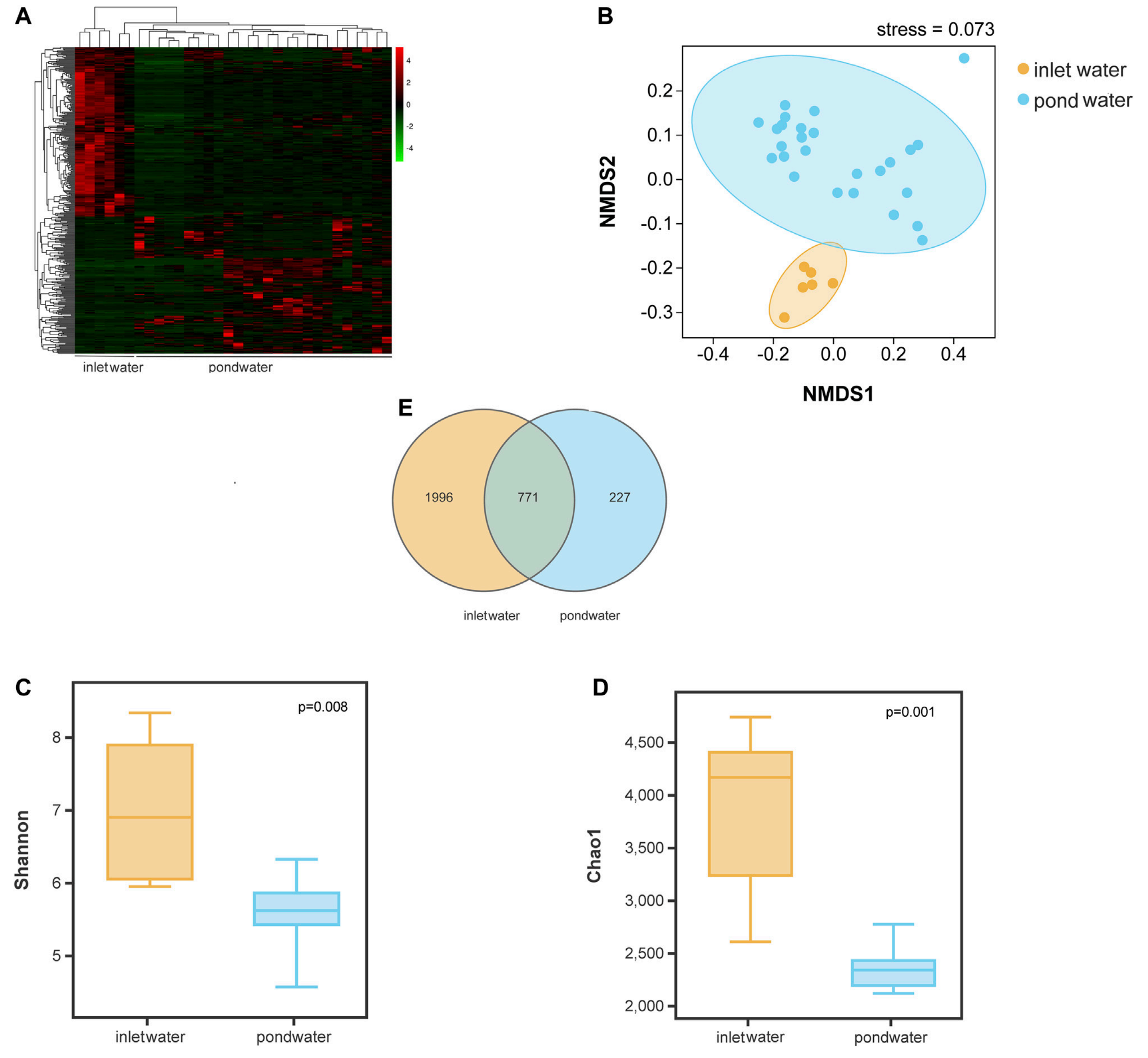

FIGURE 2 | Analysis of significant differences in OTUs between inlet water and pond water **(A)**; NMDS analysis **(B)** of bacterial communities between inlet water and pond water samples; Welch's *t*-test analysis of Shannon's diversity index **(C)** and Chao1 index **(D)** of bacterial communities; Venn diagram analysis of OTU numbers in inlet water and pond water **(E)**.

(2.14 ± 0.41%), and *Planctomyces* (1.98 ± 0.85%). Furthermore, the results of the Welch's t-tests revealed that the abundance of *Candidatus Aquiluna*, *Salinihabitans*, and *Roseibacillus* was enriched in pond water than inlet water (**Figure 3D**).

Potential Functions Prediction of Bacterial Communities in Aquaculture Area

PICRUSt2 analysis revealed variations of prediction functions among samples, which were primarily associated with carbon and nitrogen metabolism (**Figure 4**). Of all 34 functional families, the majority of them belonged to Amino acid metabolism (13.91 ± 0.06%), Carbohydrate metabolism (13.12 ± 0.07%), Metabolism of cofactors and vitamins (12.30 ± 0.09%), Metabolism of terpenoids and polyketides (9.04 ± 0.07%), Metabolism of other amino acids (8.37 ± 0.04%), Xenobiotics biodegradation and metabolism (6.83 ± 0.11%), Lipid metabolism (6.78 ± 0.08%), and Energy metabolism (5.43 ± 0.07%). Welch's *t*-test showed that almost all of these functions of bacterial communities were significantly higher ($p < 0.05$) in pond water than in inlet water.

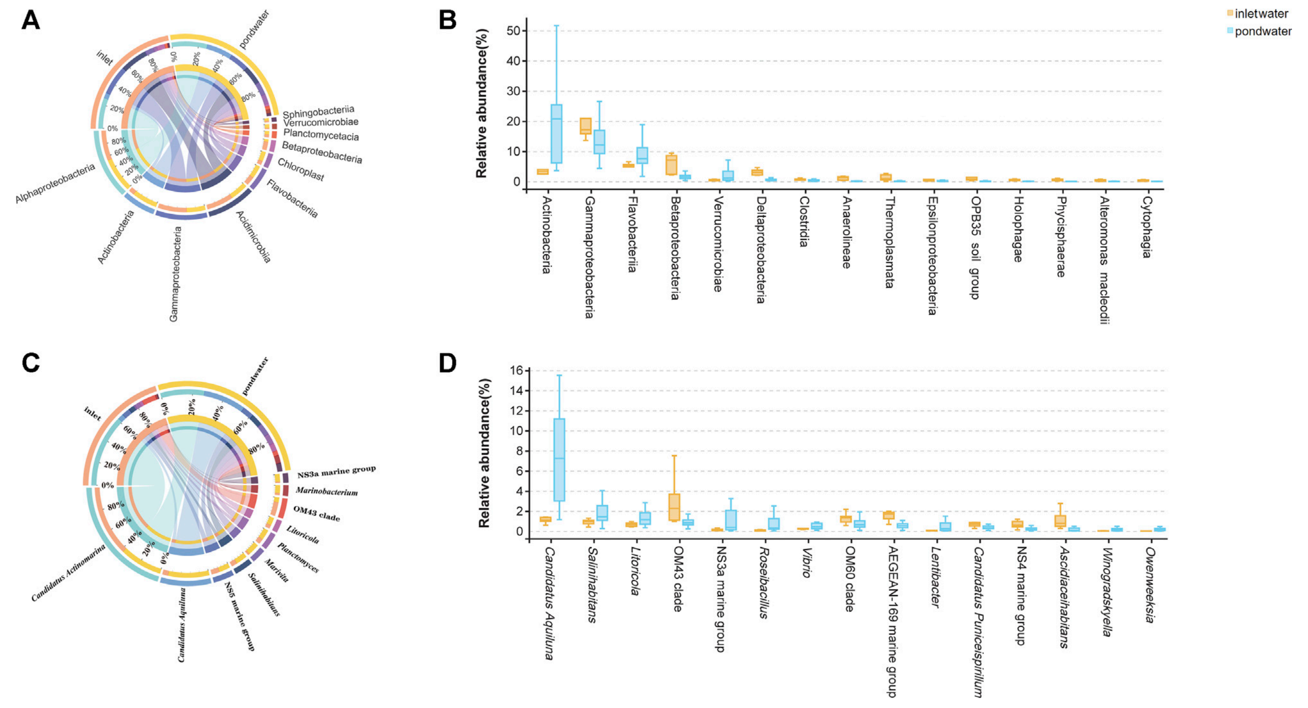

FIGURE 3 | Analysis of bacterial communities in inlet water and pond water; dominant bacterial taxa in the samples at the class **(A)** and genus **(C)** levels; statistical analysis of significant differences in bacterial class **(B)** and genera **(D)** (Wilcoxon rank sum test, $p < 0.05$).

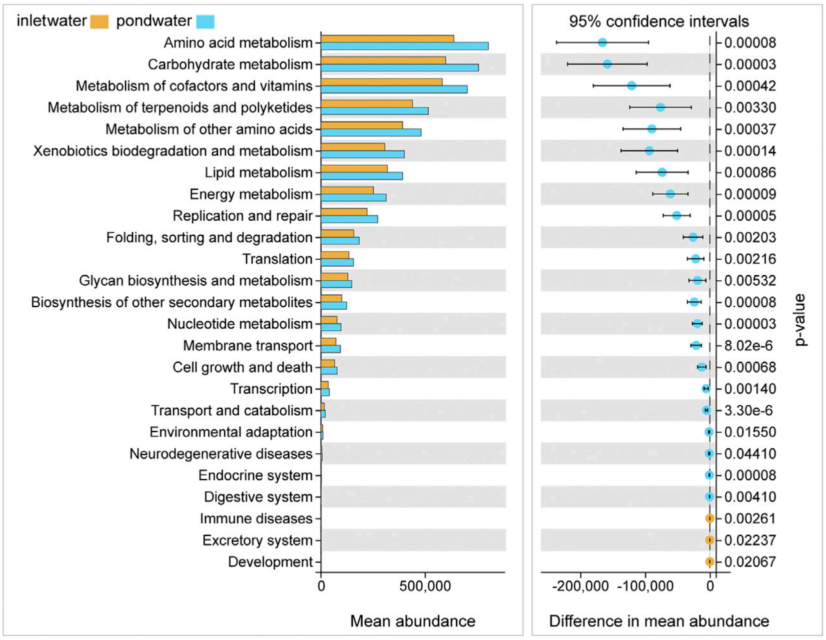

FIGURE 4 | Predicted potential functions of bacterial communities and significant difference ($p < 0.05$) analysis of functions in inlet water and pond water.

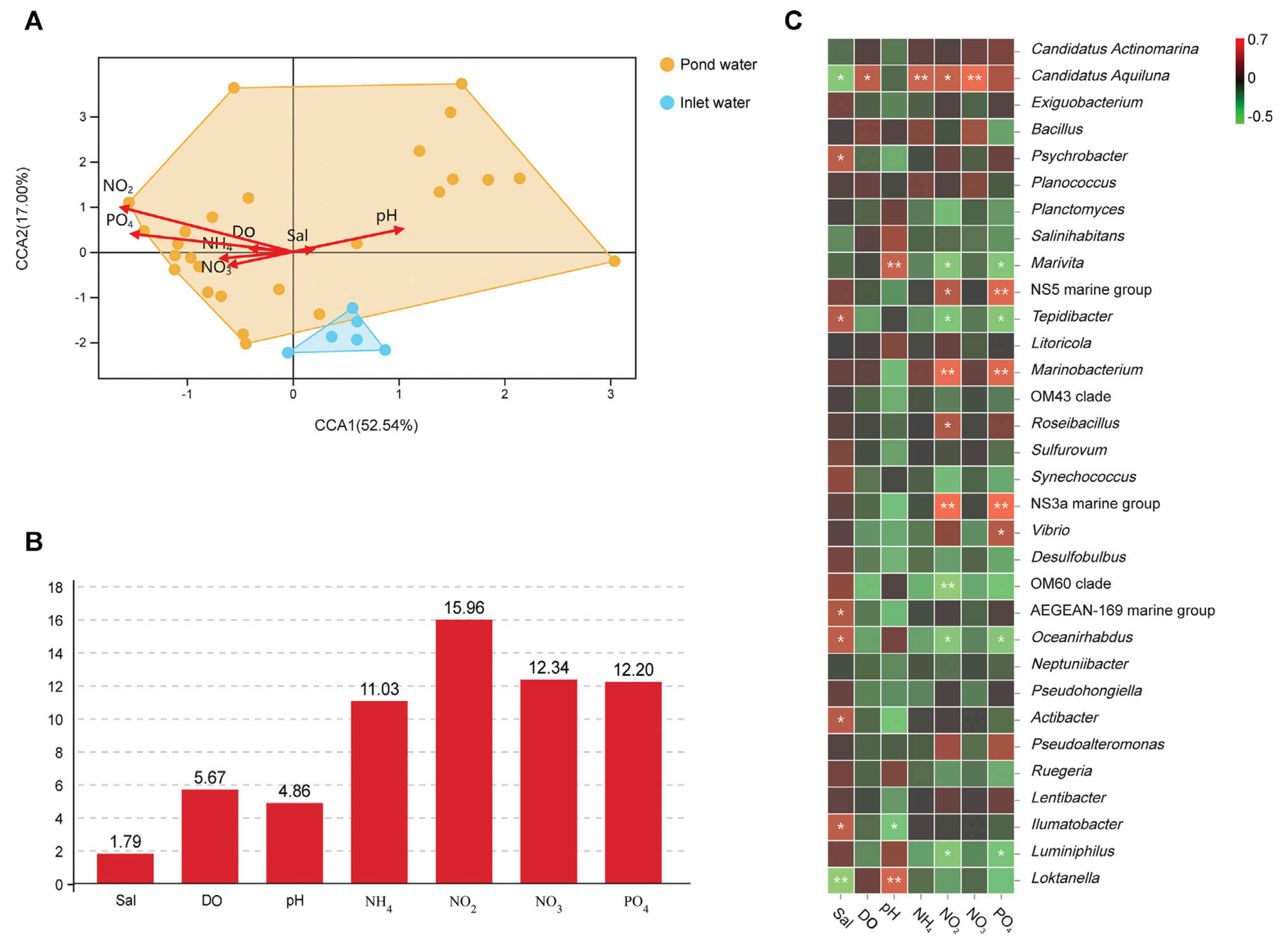

FIGURE 5 | CCA analysis of physicochemical factors and bacterial communities **(A)**; environmental contribution analysis **(B)**; correlation analysis between dominant bacterial taxa and physicochemical factors **(C)**.

Canonical Correlation Analysis Between Physicochemical Factors and Bacterial Communities

Canonical Correlation Analysis (CCA) was conducted to investigate the relationships between physicochemical factors (salinity, pH, DO, nitrate-N, ammonium-N, nitrite-N, and phosphate-P) and bacterial communities in inlet water and pond water (**Figure 5A**). The results revealed that nitrate-N, nitrite-N, phosphate-P, and ammonium-N were the key factors influencing bacterial communities. The bacterial communities in pond water was largely influenced by nitrate-N, nitrite-N, phosphate-P, and ammonium-N. Analysis of physicochemical factors contribution on bacterial communities exhibited that Nitrite-N (15.60%), nitrate-N (12.34%), phosphate-P (12.20%), and ammonium-N (11.03%) influenced bacterial communities the most in pond water (**Figure 5B**).

Figure 5C illustrated the correlation between dominant bacteria genera and physicochemical factors. A significant correlation was observed between inorganic N and P nutrients, and dominant bacterial genera within the aquacultural environment. Nitrate-N, nitrite-N, phosphate-P, and ammonium-N exhibited significant positive correlations with dominant genera, such as *Candidatus Aquiluna*, NS5 marine group, *Marinobacterium*, NS3a marine group, and *Vibrio* ($p < 0.05$ or $p < 0.01$); however, they were negatively correlated with *Marivita*, *Tepidibacter*, *Oceanirhabdus*, and *Luminiphilus* ($p < 0.05$ or $p < 0.01$). The types of bacterial genera that were significantly correlated with salinity were quite distinct from bacterial genera correlated with nutrients.

DISCUSSION

The present study investigated variations in physicochemical factors and bacterial communities between inlet water and pond water, and analyzed the relationships between bacterial communities and physicochemical factors. There were distinct bacterial communities and functions between inlet water and pond water, as well as in the physicochemical factors driving the difference in the aquacultural environments.

Aquaculture Decreases the Diversity and Richness of Bacterial Communities in Pond Water

Bacterial diversity and richness significantly decreased in pond water than in inlet water. Previous studies have revealed that bacterial communities in different environmental media (aquaculture water, sediment, and animal intestines) comprise specific bacterial taxa, which is attributed to environmental factors (Sun et al., 2019; Sun et al., 2020b), and suggests that the bacterial communities in such ecosystems is influenced by the environment. Eutrophication in aquaculture ponds water is attributed to animal feeding activities (Thakur and Lin, 2003; Sahu et al., 2013), and environmental factors such as nutrients and organic matter induce significant changes in bacterial communities, with more notable impacts in aquaculture pond environments than in water inlet environments. The relatively rich nutrients in pond water induce rapid propagation of microbe that are susceptible to such environmental changes, which, in turn, decreases diversity and richness of microbial community, as observed in a previous study (Wang et al., 2014). Bacterial diversity increases under relatively low nutrient conditions, while high nutrient levels promote eutrophication, which inhibits the growth of environmentally sensitive bacteria (Chrzanowski et al., 1995), and decreases the diversity of bacterial communities in water. Environmental variation influences the changes of bacterial communities in aquaculture systems, which suggests that microorganisms exhibit sensitive responses to shifts in external environments.

Aquacultural Environment Significantly Alter the Bacterial Communities

Mariculture has a great impact on nutrient concentrations due to the addition of feed, thus shaping the bacterial communities of the pond. A previous study reported that only 36% of N and 33% of P were assimilated and absorbed by cultured animals on average, and the remaining nutrient proportions were lost in various forms in the culture environment (Bouwman et al., 2013). The residual nutrients could induce changes in the aquatic environment, for example, in the form of shifts in pH, dissolved oxygen (DO), high N and Ps concentrations and organic matter. Bacterial community diversity in the eutrophic environment changed significantly compared with those before nitrogen and phosphorus nutrient addition (Haukka et al., 2006). In the present study, the regulation of inorganic nutrients by bacteria in aquaculture ponds was significantly enhanced. Inorganic N and P (ammonium-N, nitrate-N, nitrite-N, and phosphate-P) substantially promoted changes in bacterial communities.

Previous studies have revealed that bacterial communities are influenced by temperature, salinity, chlorophyll a, total N, inorganic N and P, C/N ratio, total P, and feed sources (Xiong et al., 2014; Zhang et al., 2014; Lin et al., 2017; Sun et al., 2019; Sun et al., 2020c). Among the parameters evaluated in the present study, inorganic nutrients, such as N and P, were the primary factors influencing bacterial communities in aquaculture environments, which is consistent with the findings of previous studies (Sun et al., 2019; Sun et al., 2020c). The physicochemical factors could directly alter bacterial communities by impairing microbial physiological activities, or indirectly, by establishing conditions that affect microorganisms adversely.

In addition, there were great variability in physicochemical parameters and bacterial community structure among pond water samples. This is due to differences in farming processes between ponds, such as feed application and feeding amount, which lead to different pond water parameters and nutrient structure. Differences in these physicochemical factors lead to changes in bacterial community structure and diversity. These results further demonstrated the driving effect of environmental parameters on bacterial community in aquaculture.

Correlation analysis of physicochemical factors and bacterial communities suggested that inorganic nitrogen and phosphate exhibited significant positive correlations with Candidatus *Aquiluna*, NS5 marine group, *Marinobacterium*, NS3a marine group, and *Vibrio*. These microorganisms adapt to the eutrophication of aquaculture ponds and their abundance is increased. In the present study, the dominance of heterotrophic bacteria could result in high uptake of inorganic nutrients such as nitrate-N, ammonium-N, and nitrite-N. *Candidatus Aquiluna* has been previously reported to be the predominant bacterial species in the study area (Lin et al., 2017; Sun F. et al., 2019), and it has the capacity to fix carbon and conduct rhodopsin-based phototrophy (Kang et al., 2012). *Marivita* abundance is often highest near phytoplankton blooms or in numerous marine and saline environments associated with organic particles (Slightom and Buchan, 2009), and could be a key factor influencing the transformation and removal of N in aquaculture water (Lin et al., 2017). Furthermore, the abundance of *Vibrio* significantly increased in culture environments in the present study. *Vibrio* is common genera of marine bacteria, and play important roles in utilizing many carbon, nitrogen, and phosphorus substrates (Roux et al., 2009). *Marinobacter* contains nitrous oxide reductase (*nosZ*) genes and has the capacity to reduce nitrite-N and nitrate-N to produce N_2 via aerobic denitrification (Liu et al., 2016). These bacteria have the ability to transform inorganic nitrogen and phosphorus, and have the potential to enhance environmental quality, which is crucial in aquaculture.

Aquaculture Increase the Metabolic Capabilities of the Bacterial Communities in Pond Water

Metabolic capabilities of the bacterial communities in pond water increased significantly than in inlet water based on prediction functions analysis. Functional prediction demonstrated that carbon and nitrogen metabolism were the dominant processes in the aquaculture area. Overall, aquacultural environments increase C and N metabolic capacities of bacteria, particularly Amino acid metabolism and Carbohydrate metabolism. Enrichment function involved in carbohydrate and amino acid metabolism indicated that microbes prefer the pond environments with rich in carbohydrates and proteins. Previous studies have revealed that Proteobacteria, Bacteroidetes, and Actinobacteria are the dominant functional phyla associated with N and P removal, and COD reduction in water (Sun et al., 2019; Sun et al., 2020a). Aquacultural environments could increase phytoplankton diversity and abundance and generate substantial amounts of carbohydrates, amino acids, and other substances. Such substances would promote the growth of bacterial groups such as Rhodobacterales and Flavobacteriales (Osterholz et al., 2016), which increases bacterial metabolic capacity. In addition to nutrient metabolism, bacteria in the ponds also increased their capacity to degrade some harmful substances in the water, such as xenobiotics, terpenoids and polyketides. Actinobacteria could secrete a variety of enzymes and obtain nutrients from a wide variety of nutrient sources, including various complex polysaccharides and refractory biomaterials (Barka et al., 2016).

In conclusion, the present study has demonstrated that aquaculture considerably altered physicochemical factors and induced changes in bacterial community composition and function. Furthermore, this study identified dominant bacterial taxa and potential factors influencing bacterial communities in pond water and established that bacterial communities characteristics were correlated with environmental status. This study was helpful to the water quality management of aquaculture area.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: NCBI Sequence Read Archive Database with project number PRJNA758780.

AUTHOR CONTRIBUTIONS

Conceptualization, FS, CW and HY; investigation, CW and FS; methodology, FS; formal analysis, FS; writing original draft, FS; writing-review and editing, FS; data curation, CW and HY; funding acquisition, FS and HY.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2021.709541/full#supplementary-material

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Assessment of the Ecological Status of Rongjiang Estuary (China) Under Human Pressure, Using Biotic Indices Based on Benthic Macroinvertebrates

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Rongjiang River, the second largest river system in Guangdong Province, flows through the main urban areas of Jieyang and Shantou cities before reaching the South China Sea. Human activities in the surrounding area pose significant threats to this aquatic ecosystem. The ecological status (ES) of the benthic ecosystem of the Rongjiang River estuary has not yet been conducted using indices based on the macrobenthic fauna, which is important for evaluating environmental health. Here, we used four biotic indices (the AZTI's Marine Biotic Index (AMBI) and Multivariate AZTI's Marine Biotic Index (M-AMBI), and taxonomic distinctness indices (average taxonomic distinctness Δ^+ and variations in taxonomic distinctness Λ^+) to appraise the current ES of benthic communities in Rongjiang estuary. Samples were taken from 11 sampling sites located in six general regions: western aquaculture zones, aquaculture zones, Hanjiang River water channel, Shantou City, Shantou Port, and near the ocean. The benthic ecosystem of this estuary is greatly disturbed: the ES of the aquaculture zones and the sites near the Hanjiang River water channel, Shantou City, and close to the ocean was poorer compared with that of other areas; ES was also poorer in winter than in summer. Generalized linear models revealed that Shannon-Wiener index was negatively correlated with dissolved inorganic nitrogen ($p < 0.01$), M-AMBI was negatively correlated with temperature and dissolved inorganic nitrogen ($p < 0.05$ and $p < 0.01$, respectively), and Λ^+ was negatively correlated with pH ($p < 0.05$). The AMBI, M-AMBI, Δ^+, and Λ^+ indices were suitable for assessing the ES of the benthic ecosystem in an anthropogenically disturbed estuary.

Keywords: biotic indices, ecological status, Rongjiang estuary, human disturbance, benthic macroinvertebrates

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INTRODUCTION

With their complex hydrodynamics, estuaries have been important areas for human settlement, and they also represent some of the most productive natural ecosystems on Earth (Costanza et al., 1997; Lotze et al., 2006; van der Linden et al., 2012). Their health is key to the development of human society, but anthropogenic and natural stressors have increased estuarine ecosystem deterioration and biodiversity loss (Barbier et al., 2011; Gao et al., 2019; Hillman et al., 2020; Spreitzenbarth and Jeffs, 2021). For example, many anthropogenic pollutants such as dissolved inorganic nitrogen (DIN) can lead to eutrophication, which decrease the survival of sensitive species, reduces species diversity, and simplifies food webs (Alexander et al., 2017; Gao et al., 2019; Ji et al., 2020). In addition, high variability in the natural environment (e.g. hydrodynamics) can also affect the structure of biological communities because only a few highly dominant species are capable of thriving in such environments (Elliott and Quintino 2007; Haase et al., 2012; Van der Linden et al., 2017).

Macrobenthic invertebrates are an important component of aquatic ecosystems, and they have been commonly used to assess the ecological status (ES) of the benthos (Díaz et al., 2004; Pinto et al., 2009). The population dynamics of benthic taxa are sensitive to stresses such as human disturbance because benthic taxa show high site fidelity, have long life spans, and often reside at the sediment-water interface where many pollutants concentrate (Dauer et al., 1993; Dauvin et al., 2007; Dauvin et al., 2012). Selection of the most suitable macrobenthos indices for use in marine ecological assessments remains a major challenge given the high diversity of biotic indices that have been developed. The AZTI's Marine Biotic Index (AMBI) (Borja et al., 2000) and Multivariate AZTI's Marine Biotic Index (M-AMBI) (Muxika et al., 2007) are determined by classifying species into one of five ecological groups (EGs), and these indices were shown to be the most effective for evaluating marine ecological quality among 35 benthic indices examined (Patrícioet al., 2009; Borja et al., 2015). The other two indices of taxonomic distinctness, average taxonomic distinctness Δ^+, and variations in taxonomic distinctness Λ^+ (Clarke and Warwick 1998; 1999; Warwick and Clarke 1995) are based on diversity and related attributes and have also been shown to be useful for terrestrial and marine ecological assessments (Hu and Zhang 2016; Arbi et al., 2017). All of the aforementioned indices have been used in the seas around China (Hu and Zhang 2016; Arbi et al., 2017; Qiu et al., 2018; Li et al., 2021). However, these indices might not be effective in some circumstances (Borja et al., 2000; Salas et al., 2006; Muxika et al., 2007; Patrícioet al., 2009; Li et al., 2021). For example, the robustness of AMBI and M-AMBI is reduced if only 1–3 taxa or individuals can be identified in a sample, or more than 20% of taxa in a sample are not assigned to an EG (Borja and Muxika, 2005; Muxika et al., 2007). In addition, too much interference information for the taxonomic diversity (TD) indices–Δ^+ and Λ^+ is added by tolerant taxa, such as some tolerant Oligochaeta and Mollusca, because these species contribute the greatest path lengths, which lead to equal or even higher scores in disturbed sites than in undisturbed sites (Hu and Zhang 2016). Given the complex nature of biological systems, robust ES evaluations require the use of several biotic indices (Salas et al., 2006; Wetzel et al., 2012; Martinez-Haro et al., 2015).

Rongjiang River, the second largest river system in Guangdong Province, flows through the urban environment between Jieyang and Shantou cities before entering the South China Sea (Wang et al., 2016). Aquaculture ponds on this river between these two cities cover approximately 21,297 ha (Gu et al., 2018). The aquaculture, industrial pollution, agriculture, domestic sewage, and other human activities surrounding this river and estuary all pose significant threats to the aquatic ecosystem (Gu et al., 2018; JYEPB Jieyang Environmental Protection Bureau 2018; STEPB 2018). Furthermore, the estuary ecosystem of this river is affected by other areas, through the water channel between this river and the Lianjiang River and Hanjiang River. The ES of the benthic ecosystem of the Rongjiang River estuary has not yet been fully evaluated because no ES assessment of the Rongjiang River estuary has been conducted using indices based on the macrobenthic invertebrate fauna, which is important for evaluating environmental health.

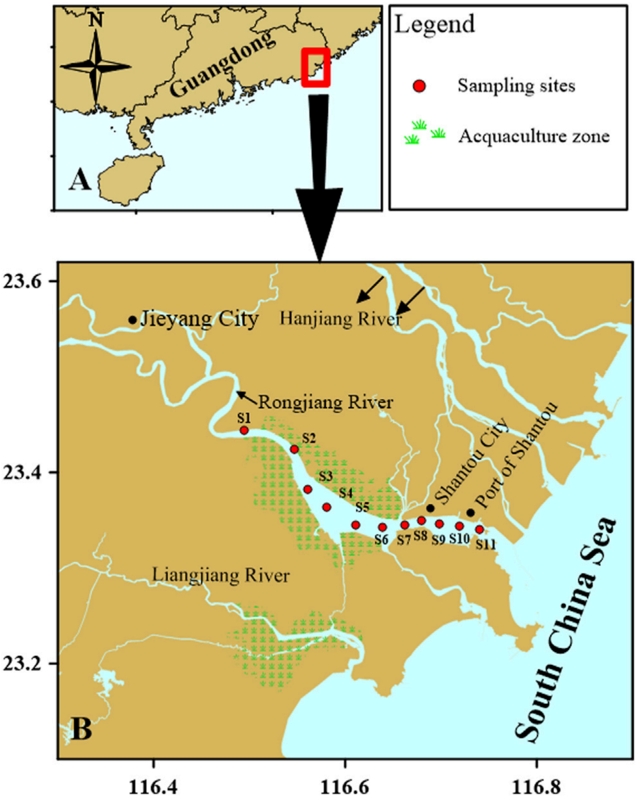

FIGURE 1 | Local geography, sampling stations (red spots), and aquaculture areas (green symbols), Rongjiang River, South China Sea.

The aim of this study was to 1) evaluate the ES of the benthic ecosystem of Rongjiang estuary using the AMBI, M-AMBI, Δ^+, and Λ^+ indices; 2) assess the suitability of the AMBI, M-AMBI, Δ^+, and Λ^+ indices for evaluating the ES of an anthropogenically disturbed estuary system; and 3) explore the environmental factors affecting the ES of the benthic ecosystem.

MATERIALS AND METHODS

Study Area

Rongjiang estuary includes Niu Tian Yang (116°32′–116°39.6′E, 23°18′–23°24′ N) and Shantou Port (116°39.6′–116°44.8′E, 23°19.4′–23°21′N). Niu Tian Yang is surrounded by the alluvial belt of the estuary. It is a reclamation area and one of the most important aquaculture areas in Shantou City; the pond aquaculture area is more than 2000 ha. Shantou Port is one of China's key ports in the Belt and Road Initiative, which exchanges cargo with 268 other ports in 57 countries (Li, 2013). Rongjiang estuary has a mild, wet subtropical climate with prevailing northeasterly and southwesterly monsoons from November to May and April to September, respectively. Thus, the area experiences wet and dry (70 and 30% freshwater discharge, respectively) seasons (Chen, 2010).

Sampling sites were located within six general regions: western aquaculture zones (S1 and S2), aquaculture zones (S3–S6), Hanjiang River water channel (S7), Shantou City (S8 and S9), Shantou Port (S10), and near the ocean (S11).

Sampling and Measurements

Samples were taken from 11 sampling sites (S1-S11) from the upper to lower reaches of Rongjiang River (**Figure 1**). The macrobenthos was sampled in June 2017 (wet season) and January 2018 (dry season). Benthic invertebrates were sampled using a grab sampler in an area of 0.1 m^2. Three replicates were taken at each site to ensure the reliability of the data, and the grab sample was retained if it was more than 75% full. Samples were gently washed and sieved through 0.5 mm mesh; they were then preserved in 4% borax-buffered formalin before identification and counts.

We measured 18 environmental factors associated with various ecological factors. These environmental factors were grouped into two classes–natural variables and anthropogenic pollutants–each with similar attributes. The natural variables were the temperature, pH, salinity, and dissolved oxygen (DO) of the bottom water and sediment grain size. The anthropogenic pollutants were chemical oxygen demand (COD_{Mn}), suspended matter concentration (SS), chlorophyll a (Chla), dissolved inorganic phosphorus (DIP), dissolved inorganic nitrogen (DIN) of the bottom water, metal concentrations (As, Pb, Zn, Cd, Cr, Cu and Mn) and the soil organic matter (SOM) content of the sediment.

The temperature, pH, salinity, and DO of the bottom water were measured in the field using a multiparameter water quality monitor (YSI 6600, Brannum Lane Yellow Springs, United States). COD_{Mn} was measured by the permanganate method, Briefly, NaOH was added to water samples, followed by $KMnO_4$ digestion solution. The sample were heated for 10 min in a water bath at 96–98°C. After digestion, the sample was cooled immediately in an icebox to quench the reaction. The remaining permanganate in the sample solution was determined by titration. SS samples were filtered through pre-weighed Whatman GF/F fiber filters (25 mm). Chla was determined by filtering 100–200 ml of seawater onto GF/F fiber filter by a cascading filtering device under low vacuum pressure. After extraction with 90% acetone, Chla was determined by a Turner Design fluorometer (TD Trilogy) (GB/T17378.4-2007; GB/T12763.6-2007) (General Administration of Quality Supervision, 2008a; 2008b). Water column nutrient variables were analyzed following the methods of Kirkwood et al. (1996). Measurement of the SOM content, metal concentrations and grain size was based on the methods of Gu et al. (2014, 2018).

TABLE 1 | Threshold levels and ecological status (ES) for the AZTI's Marine Biotic Index (AMBI) and Multivariate AZTI's Marine Biotic Index (M-AMBI).

Site pollution classification	Ecological status	AMBI	M-AMBI
Normal	High	0–1.2	>0.77
slightly polluted	Good	1.2–3.3	0.53–0.77
moderately polluted	Moderate	3.3–5.0	0.38–0.53
highly polluted	Poor	5.0–6.0	0.20–0.38
Azoic	Bad	6.0–7.0	<0.20

Requested Potential Ecological Risk Index and Mean Effects Range-Median Quotient Based on the Metal Concentrations in Sediments

Heavy metals always occur in sediments as complex mixtures; therefore, the potential ecological RI and mean ERM quotient (MERMQ) method were used to determine the comprehensive biological effect of combined toxicant groups (Hakanson, 1980; Long, 2006). The equation (**Eq. 1**) is as follows:

$$C_f^i = C_{surface}^i / C_n^i \;\; \mathrm{RI} = \sum_{i=1}^{n} Er^i = \sum_{i=1}^{n} Tr^i \times C_f^i ;\ \text{mean ERM quotient} = \sum \left(\mathrm{C}_i / ERM_i / \mathrm{n}\right), \tag{1}$$

where C_f^i is the contamination factor, $C_{surface}^i$ is the measured concentration of the examined component (i) in surface sediment, C_n^i is the standard preindustrial reference level, RI is the requested potential ecological risk index, Er^i is the potential ecological risk factor for the metal i, Tr^i is the "toxic-response" factor for the given metal i, C_i is the measured concentration of the examined component (i) in sediment, ERM_i is the ERM for metal i, and n is the number of metals.

Benthic Indicators and Their Relationships to Environmental Factors

Species richness (S), Shannon-Wiener index (H'), AMBI, and M-AMBI were calculated using AMBI index software (version 5.0, http://ambi.azti.es), and the assignment of the identified species to the five EGs was based on the species list (2017 June) in the AMBI index software (Borja and Muxika, 2005). Reference conditions play a crucial role in calculations of M-AMBI, and these vary naturally with location, water type, and habitat. Following Borja et al. (2008), the highest H' and S values increased by 15% used as the reference conditions in our study. Threshold levels and ES for AMBI and M-AMBI are shown in **Table 1** (Borja et al., 2000; Borja and Tunberg, 2011).

Δ^+ and Λ^+ indices were calculated by PRIMER 6 (v. 6.1.12) software using six taxonomic levels (species, genus, family, order, class, and phylum). Pollution status can determined by the location of Δ^+ in a funnel plot. Disturbed sites are usually located far from the 95% confidence interval, whereas undisturbed sites are the 95% confidence interval. In some situations, Δ^+ results were complemented by those of Λ^+ reflecting taxonomic unevenness.

Statistical Analysis

A bivariate correlation analysis based on Spearman's correlation coefficient of all environmental variables was conducted to filter the variables; when two variables were highly correlated within the same variable class ($r > 0.75$, $p < 0.05$), one was discarded. A heatmap was made to visualize the spatial–temporal distribution patterns of standardized environmental factor data. Generalized linear models (GLMs) were used to select the predictor variables that could significantly explain the ES of the benthic ecosystem. A step-wise multiple regression was then used to identify the best-fit models (variable selection and model evaluation were both based on Akaike information criterion (AIC) values).

The bivariate correlation analysis was performed by PRIMER 6 with PERMANOVA + add-on (v. 6.1.12), and the heatmap of environmental factors and GLMs were conducted in R 3.6.2. Sampling maps were plotted using Surfer 14.0 software.

RESULTS

Environmental Conditions

DO was significantly correlated with temperature and salinity ($r = -0.84$, $p < 0.01$; $r = 0.80$, $p < 0.01$, respectively), and SOM was significantly correlated with grain size and MERMQ ($r = 0.79$, $p < 0.05$; $r = 0.87$, $p < 0.05$, respectively). DO and SOM were thus omitted from subsequent analyses.

The temperature, Chla, RI, and SS in the bottom water were higher in summer than in winter; pH, salinity, DIN, DIP, and MERMQ showed the opposite pattern. COD_{Mn} in the aquaculture zones was lower in summer than in winter; in all other areas, the opposite pattern was observed. Temperature decreased from the upper river reaches to a site near the Hanjiang River water channel and then increased; pH showed a similar pattern to temperature in summer but the opposite pattern in winter. Salinity increased from the upper to the lower river reaches; the site near the Hanjiang River water channel was the only exception to this pattern. Chla and RI were higher in the aquaculture zones and areas near Shantou City. The patterns of DIN and MERMQ varied in different periods. In summer, DIN and MERMQ were lower in the aquaculture zones; in winter, DIN and MERMQ were higher in the aquaculture zones and areas near Shantou City. COD_{Mn} was higher in the aquaculture zones than in the other areas. SS was higher in the western aquaculture zones and lower reaches; DIP was lower in the western aquaculture zones. Surface sediments were generally dominated by silts, and the silt, sand and clay contents being 88.76%–99.13%, 0.84%–14.24% and 0–0.04%, respectively (**Supplementary Appendix SA**).

Macrobenthic Faunal Composition

A total of 22 species from 6 phyla, 9 classes, 16 orders, 19 families, and 21 genera were identified. In both summer and winter, the greatest number of species detected belonged to the phylum Annelida (8 species), followed by Mollusca and Arthropoda (5 species each). However, Annelida species only comprised 9.4% of all specimens sampled; Mollusca and Arthropoda species comprised 57.0 and 32.3% of all specimens sampled, respectively. We identified 13 species from these phyla in both summer and winter. Most species belonged to Annelida in winter and Arthropoda and Mollusca in summer (**Supplementary Appendix SB**).

AMBI and M-AMBI

One (7.7%) species was not initially assigned to an EG in either summer or winter, and this species remained unassigned in both seasons after assignment (**Supplementary Appendix SB**).

Four sites (S1, S3, S10 and S11) could not be sampled in summer because of inclement weather. The mean AMBI for summer ranged from 0.75 to 5.99: the western aquaculture zones (S2) were slightly disturbed (good ES). The aquaculture zones (S4–S6) with extensive pond aquaculture and multiple water channels from the Rongjiang and Lianjiang Rivers were slightly to heavily disturbed (poor ES). The site near the Hanjiang River water channel (S7) was slightly disturbed, and areas near Shantou City (S8 and S9) were moderately disturbed and undisturbed, respectively. In winter, the mean AMBI ranged from1.50 to 5.25, and the ES of each region was as follows: the western aquaculture zones (S1 and S2) were moderately disturbed (moderate ES); the aquaculture zones (S3–S6) were slightly disturbed (good ES); S8 (near Shantou City) and S10 (near Shantou Port) were slightly disturbed (good ES); S7 (near the Hanjiang River water channel) was heavily disturbed (poor ES); and both S9 and S11 were moderately disturbed (moderate ES) (**Figures 2A,B**).

According to the M-AMBI, in summer, the ES of the western aquaculture zones (S2) was high, and that of the aquaculture zones S4, S5, and S6 was good, high, and bad, respectively. The ES of the site near the Hanjiang River water channel (S7) and areas near Shantou City (S8) was poor, and the ES of S9 was good. The ES was poorer in winter than in summer. The ES of S1 and S2 in the western aquaculture zones was good and moderate, respectively; the ES of aquaculture zones (S3, S5, and S6) was poor; the ES of (S4 and S7) was poor; the ES of S10 was high; and the ES of S8, S9, and S11 was poor, moderate, and bad, respectively (**Figures 2C,D**).

Taxonomic Distinctness Indices: Δ^+ and Λ^+

Confidence plots for summer revealed that most Δ^+ values were within the 95% confidence interval, and S4 and S8 were close to the upper and lower limit of the 95% confidence intervals, respectively; S7 fell outside the 95% confidence limits. In winter, more sites (S2, S3, S5, S6, S8, and S11) fell outside the lower 95% confidence limit; S9 was close to the lower limit; and S1 and S4 were close to the upper 95% confidence limit. In some cases, the Δ^+ results were complemented by those of Λ^+, reflecting taxonomic unevenness. The Λ^+ plot differed from the Δ^+ plot because as S4, S6, S7, and S8 in summer were near the lower 95% confidence limit (**Figure 3**).

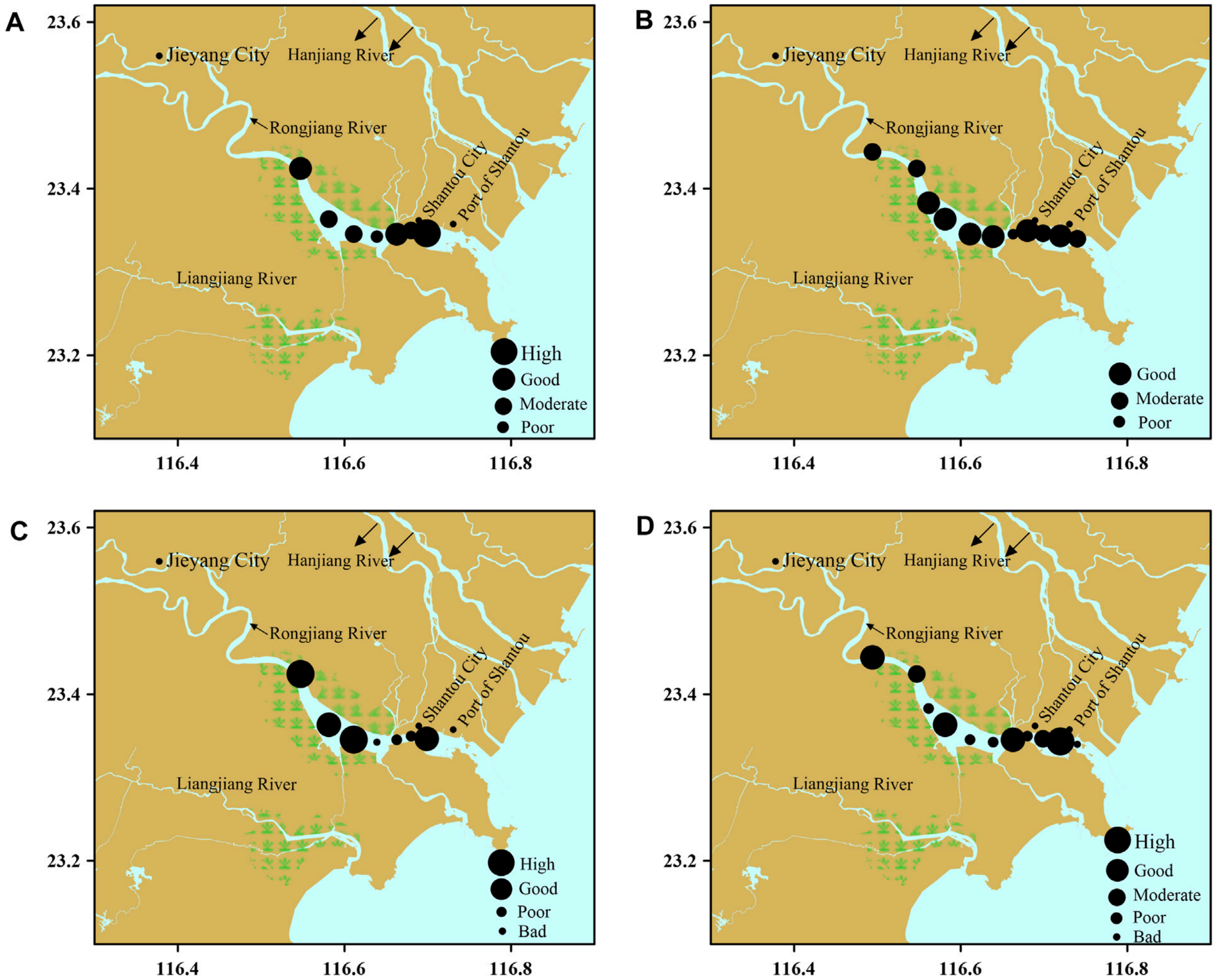

FIGURE 2 | Temporal and spatial variation in AMBI and M-AMBI indices, Rongjiang River. AMBI: **(A)** summer, **(B)** winter; M-AMBI: **(C)** summer, **(D)** winter.

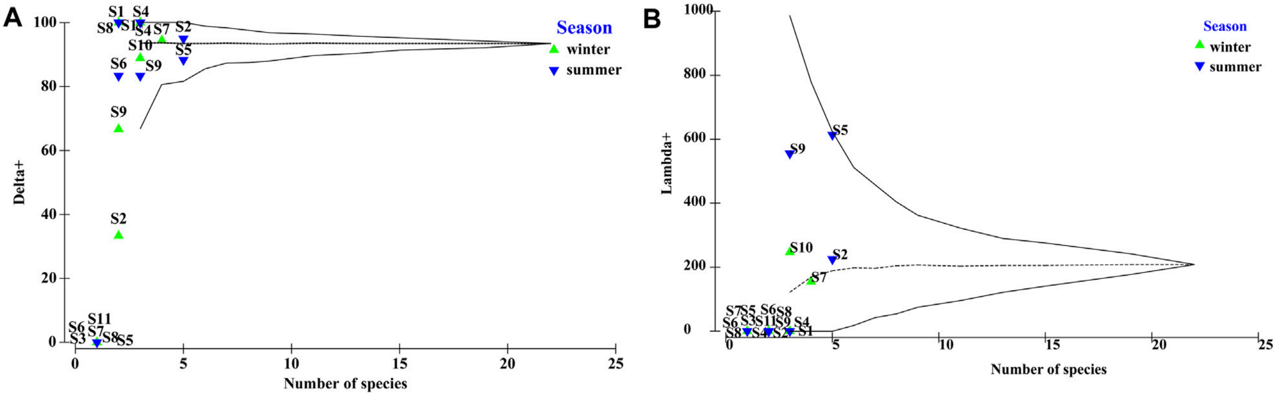

FIGURE 3 | Funnel plots of **(A)** averages (Δ^+ [alpha]), and **(B)** variations (Λ^+ [lambda]) in taxonomic distinctness indices for different sites and seasons.

TABLE 2 | Results of regression analysis based on the generalized linear models (GLMs) between H', AMBI, M-AMBI, Δ^+, Λ^+ and environmental factors.

Environmental factors	AMBI	M-AMBI	Δ^+	Λ^+	H'
Temperature	1.463	–2.194*	0.786	–1.433	–1.015
Salinity	–0.145	0.094	0.554	1.807	0.590
pH	1.792	–1.294	0.120	–2.663*	–0.522
COD_{Mn}	–1.713	2.056	0.571	2.401	2.140
SS	0.127	–0.735	–0.749	–1.963	–0.440
Chla	1.277	–0.118	0.521	–1.380	0.139
DIP	–1.044	–0.967	–1.577	0.100	–1.418
DIN	1.102	–3.610*	–0.675	–2.571	–3.006*
MERMQ	–1.261	1.921	0.004	2.266	1.417
RI	–0.402	–1.309	–1.868	0.207	–1.574
Sand	0.705	–1.945	0.362	–1.132	–1.293
Silt	0.219	–0.849	–0.451	–2.104	–0.790
Clay	0.912	–0.085	0.347	–0.260	–0.870

*$p < 0.05$.

Note: environmental factors including temperature, pH, salinity, chemical oxygen demand (COD_{Mn}), suspended matter concentration (SS), dissolved inorganic phosphorus (DIP), dissolved inorganic nitrogen (DIN) of the bottom water, sediment grain size, the potential ecological risk index (RI) and mean effects range-median (ERM) quotient (MERMQ) based on the metal concentrations in sediments.

Relationships Between Benthic Indicators and Environmental Factors

Relationships between H', AMBI, M-AMBI, Δ^+, Λ^+ and environmental factors were analyzed using GLMs (**Table 2**). H' was negatively correlated with DIN ($p < 0.01$), M-AMBI was negatively correlated with temperature and DIN ($p < 0.05$ and $p < 0.01$, respectively) and Λ^+ was negatively correlated with pH ($p < 0.05$).

DISCUSSION

ES of the Benthic Ecosystem and its Relationship With Environmental Factors

Estuaries are transitional zones between rivers and oceans and are characterized by complex hydrodynamics and chemical conditions (Van der Linden et al., 2017); they are also some of the most anthropogenically disturbed aquatic systems and are sensitive to environmental changes (Wetzel et al., 2012; Gao et al., 2019; Hillman et al., 2020). High variability in this physicochemical has a substantial effect on biological communities, and only a few highly dominant species are capable of thriving in such environments (Elliott and Quintino 2007). Hydrodynamics imposes strong selective forces on the macrobenthos through direct physical stress (Warwick and Uncles 1980); top–down control (Leonard et al., 1998), regulation of larval or juvenile transport (Haase et al., 2012); and control of marine waters from entering the estuary, creating salinity gradients (Wetz et al., 2013). Human activities also affect estuarine environments and estuarine dynamics (Dutertre et al., 2013; Wetz et al., 2013; Gao et al., 2019; Hillman et al., 2020).

Our results showed that the ES of the Rongjiang estuary was higher in summer than in winter, and both anthropogenic pollutants (DIN) and natural variables (temperature of bottom water) play a crucial role in determining the ES of this area. The growth and development of benthic communities are known to be significantly affected by water temperature (Day et al., 1989; Vaquer-Sunyer and Duarte 2011; Spreitzenbarth and Jeffs, 2021). The reproductive activity of species (e.g., *Limnodrilus hoffmeisteri* and *Branchiura sowerbyi*) were high, and the proportion peaks during the winter (Li et al., 2012). Additionally, the study area has a mild, wet subtropical climate with northeasterly (winter) and south-westerly (summer) monsoons. Given that winter is characterized by low minimum rainfall and river flow (Chen 2010), the capacity of the Rongjiang River to dilute incoming substances is almost six times lower in winter than in summer, and this facilitates the accumulation of pollutants; this also explains why the concentrations of DIN were three times higher in winter than in summer (Huang et al., 2015). Consequently, the ES was poorer in winter. In winter, the dominance of larger and longer-lived species such as the polychaete *Micronephthys oligobranchia* decreased, and small and pollution-resistant species with short life spans (e.g., the polychaete *Heteromastus filiformis* and the oligochaetes *L. hoffmeisteri* and *B. sowerbyi*) which were assigned to EG IV and EG V, became dominant (Zhen et al., 1985; Li et al., 2012; Lin et al., 2013). This is a common feature of disturbance in estuaries that have experienced the loss of large macrofaunal animals (Pearson and Rosenberg 1978; Hillman et al., 2020).

Overall, the ES of the aquaculture zones, the site near the Hanjiang river water channel, areas near Shantou City on the Rongjiang River, and the site closest to the ocean was poor or bad. The pond aquaculture surrounding the Rongjiang River results in the rapid deterioration of the aquatic ecosystems (Gu et al., 2018) because the discharge of aquaculture wastewater contributes to nutrient enrichment in this area (He et al., 2005). The low ES of the site near the Hanjiang River water channel and in areas near Shantou City on the Rongjiang River were also due to man-made wastewater discharge (DIN concentration were still high although slightly lower than the aquaculture zones) and all samples met the class III criteria based on the Chinese Sea Water Quality Standard (0.4 mg/L), which is used for harbors (SEPA 1997). High concentrations of nutrients result in eutrophication; in eutrophic regions, the survival of sensitive benthic species is restricted, and they are gradually replaced by tolerant species. The accumulation of nutrients can provide abundant food sources for tolerant species, which allows them to reach high densities, and this increase the unevenness of benthic assemblages, decrease species diversity, and simplifying food webs (Alexander et al., 2017; Ji et al., 2020). Our results were consistent with this observation: species diversity was lower in areas with higher DIN concentration, where species (*P. laevis*) tolerant of excess organic matter enrichment and second-order opportunistic species (Borja 2000) represented more than 50% of taxa. In addition to eutrophication, the poor ES of the lower Rongjiang estuary was driven by intense hydrodynamic disturbance, which can limit the initial colonization of species (Haase et al., 2012; Van der Linden et al., 2017). This is consistent with the hypothesis proposed by previous studies, that hydrodynamics is a stress to most macrofaunal species in estuaries (Van der Linden et al., 2012; Van der Linden et al., 2017).

Value of Using Multiple Indices to Assess the ES of Benthic Ecosystems and Recommendations for Environmental Protection

The composition and distribution of macrobenthic communities in estuaries are shaped by high variability in the physicochemical environment (Day et al., 1989; Leonard et al., 1998; Vaquer-Sunyer and Duarte 2011; Haase et al., 2012; Dutertre et al., 2013). These communities are characterized by the high dominance of a few species (Elliott and Quintino 2007). A single ecological indicator is thus often of limited use in ES assessments. For instance, the robustness of results based on AMBI and M-AMBI is reduced if only 1–3 taxa or individuals can be identified in a sample, or more than 20% of taxa in a sample are not assigned to an EG (Borja and Muxika 2005; Muxika et al., 2007). In addition, in case of the taxonomic diversity indices (TD) indices–average taxonomic distinctness Δ^+ and variations in taxonomic distinctness Λ^+, too much interference information is added by tolerant taxa, such as some tolerant Oligochaeta and Mollusca, because these species contribute the greatest path lengths, which lead to equal or even higher scores in disturbed sites than in undisturbed sites (Hu and Zhang 2016). In this study, 66.7% of taxa were assigned to EG II at S4 in winter, and the ES of S4 was classified by AMBI and M-AMBI as good; however, only two taxa were identified. Healthy ecosystems are not dominated by a single functional group or by a few species (Peng, 2013). Classification of the ES by AMBI and M-AMBI indices was relatively accurate. At S7 in winter, more than 90% of taxa were assigned to EG III, EG IV, and EG V, and the ES of S7 was classified as good by the TD index. Thus, the use of multiple biotic indices can provide a more effective evaluation of the ES of certain areas, and especially in estuaries, where physicochemical environments are highly variable. We found that the AMBI, M-AMBI, Δ^+, and Λ^+ indices were suitable for assessing the ES of benthic ecosystems in aquatic areas experiencing anthropogenic disturbance, and that the use of these indices may enhance our understanding of the effects of environmental variables on the distribution of species.

These results indicate that some measures need to be implemented, such as strict national and local standards to control the discharge of terrestrial pollutants and sewage into Rongjiang, Lianjiang, and Hanjiang Rivers. There is also a need to improve the arrangement of cages, implement controls on the scale of breeding, and improve the breeding structure in multi-species operating models to promote the healthy, stable, and sustainable development of aquaculture.

CONCLUSION

The main conclusions of this study are outlined below:

1) The ES of the benthic ecosystem in Rongjiang estuary has been seriously affected by anthropogenic activities. Several measures need to be implemented to prevent the loss of benthic habitat.
2) The ES of Rongjiang estuary differed in summer and winter.
3) The use of multiple ecological indices permits more robust assessments of the ES of benthic ecosystems in anthropogenically disturbed estuary systems.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

YL: Methodology, Data curation, Writing Original draft preparation; LW: Resources, Software, Visualization; JN, LX, DH, and SL: Reviewing and Editing; FD: Supervision, Conceptualization, Software, Funding acquisition.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2021.728196/full#supplementary-material

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Responses of Functional Traits of Macrobenthic Communities to Human Activities in Daya Bay (A Subtropical Semi-Enclosed Bay), China

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The biological trait analysis (BTA) is regarded as a promising approach to unravel how ecosystem functions respond to human-induced disturbances. This study considered the four sampling locations associated with different human activities in Daya Bay, that is, the domestic and industrial sewage discharge area (SED), mariculture area (MRC), nuclear power plants thermal discharge area (NTD), and an area with relatively low human disturbance as a reference (REF). Thirty modalities of nine traits were selected in BTA. Our results showed a clear shift in the functional structure of macrobenthic communities between the sampling locations, except for the case between NTD and REF. The trait composition in the communities did not highlight any seasonal patterns. Bioturbation, longevity, tolerance, body size, feeding habit, and environmental position were the key traits to characterize the functional structure of macrobenthic communities and demonstrated predictable responses along the environmental gradients. Water depth, DO, Chl-*a*, NH_4^+, and petroleum contaminants in sediments were the main variables influencing the trait composition. In addition, the taxonomic index (H') and functional diversity index (Rao's Q) showed clear differences among the sampling locations. Although there were no significant differences between NTD and REF in terms of the trait composition and functional diversity, a potential function loss in NTD still can be detected through the integrated analysis with taxonomic diversity. We suggest that the traits (except for fragility, larval development, and living habits) selected and the diversity indices (H' and Rao's Q) could serve as promising indicators of ecological conditions in Daya Bay.

Keywords: biological trait analysis, macrobenthic community, functional diversity, human activity, Daya Bay

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INTRODUCTION

Macrobenthic fauna are key components in marine ecosystems and drive important processes such as sediment reworking, bio-irrigation, nutrient cycling, and organic matter decomposition (Widdicombe et al., 2004; Lindqvist 2014; Bonaglia et al., 2017). As their limited migration ability, relatively long life span, and sensitivity to environmental changes, benthic macrofauna are usually regarded as effective indicators for ecosystem health assessment (Snelgrove 1998; Patricio et al., 2009; Borja et al., 2010). Traditionally, biodiversity and how it is related with environmental change and ecosystem health have often been measured and quantified through taxonomy-based approaches, which include taxonomic diversity indices and multivariate methods, based on species richness, abundance, and biomass (Vandewalle et al., 2010; Strong et al., 2015). But these approaches do not take ecosystem functions into account and ignore that different species may perform similar functional roles in communities and ecosystems (Frid et al., 2000; Dolédec and Bonada 2013). Furthermore, ecosystem processes and services are primarily determined by the functional identity, rather than by taxonomic identity (Bellwood et al., 2012; Naeem et al., 2012). Since the beginning of this century, the trait-based approach, characterizing organisms by a suite of functional identities (functional traits), has received an increasing recognition in the marine community ecology (Bremner et al., 2003; Gagic et al., 2015; Barton et al., 2016; Beauchard et al., 2017; Degen et al., 2018).

A representative trait-based approach is biological trait analysis (BTA) which uses a set of biological traits of organisms to depict the variation patterns of the functional structure of communities along environmental gradients (Bremner et al., 2006). Generally, the functional structure of a community consists of two components, which can be quantified by different indices (Dias et al., 2013). The first component, the "community-weighted mean trait value" (CWM), can be calculated for each trait as the average trait value in a community weighted by the relative abundance/biomass of the species carrying each value (Garnier et al., 2004). This index is usually used to identify the dominant traits in a community and can be particularly useful to understand the response of the biotic communities to the changes in disturbances and environmental gradients due to the environmental selection for certain traits (Vandewalle et al., 2010; Ricotta and Moretti 2011). The other component, the functional diversity (FD), describes the extent of trait differences among coexisting species within a community (Petchey and Gaston 2006). A general assumption is that the stressed communities would lead to a decrease in FD (Mason et al., 2013), thereby decreasing the diversity in resource use strategies (Strong et al., 2015). FD can be further decomposed into three complementary subcomponents, that is, functional richness, functional evenness, and functional divergence (Mason et al., 2005). An increasing number of indices have been proposed or are being developed for the quantification of one or more of these subcomponents, for example, FRic, FEve, FDiv, FDis, FR, and Rao's Q. FRic (functional richness index), FEve (functional evenness index), and FDiv (functional divergence index), respectively, measure the three subcomponents of functional diversity (Mason et al., 2005; Villeger et al., 2008). Functional dispersion index (FDis) measures the mean distance in the multidimensional trait space of individual species to the centroid of all species (Laliberte and Legendre 2010). FR (functional redundancy index) corresponds to the average number of species sharing similar sets of traits (Mouillot et al., 2014). Rao's Q (Rao's quadratic entropy) incorporates the pairwise functional differences of species weight by their relative abundance (Schmera et al., 2017). Among these indices, CWM and Rao's Q (Rao's quadratic entropy) are most frequently used in previous studies (Ricotta and Moretti, 2011).

Coastal areas host more than half of the planet's human population, depending on the ecosystems and the services they provide (Barbier et al., 2008; Halpern et al., 2008). Most coastal ecosystems have been somewhat altered by human activities at all spatial scales (Lotze et al., 2006; Halpern et al., 2008; Halpern et al., 2015; Cloern et al., 2016). Benthic communities are extremely susceptible to human disturbances, which include domestic and industrial sewage and extensive mariculture, resulting in eutrophication, oxygen deficiency, and contaminant enrichment in sediments (Villnas et al., 2011; Franzo et al., 2016). This situation could become worse, given the weak hydrodynamic force in enclosed and semi-enclosed bays; therefore, the concentration of pollutants in sediments often reaches up to the orders of magnitude higher than in the overlying water (Trannum et al., 2004; Cardellicchio et al., 2007). Recently, more and more nuclear power plants (NPPs) have been or are being constructed for the surge in energy demand in China (Ye et al., 2017). Thermal discharge and the use of radionuclides pose substantial risks to aquatic organisms living nearby after the operation of NPP (Shiah et al., 2006; Teixeira et al., 2009; Thinova and Trojek 2009). BTA has been applied in the marine macrobenthic community to assess human impacts such as bottom trawling (Tillin et al., 2006; Bolam et al., 2014), pollution (Oug et al., 2012; Gusmao et al., 2016; van der Linden et al., 2016; Nasi et al., 2018; Egres et al., 2019; Nunes de Souza et al., 2021), dredging, and dredged material disposal (Cooper et al., 2008; Bolam et al., 2016). Most of the research studies focus on Europe, South America, and Arctic region; the application of BTA on the macrobenthic community is relatively scarce in Asia. Above all, previous studies only consider the impact of singular human activities, while the comparisons on the effects between different human activities are rarely involved.

Daya Bay is embedded in the key economic development zone in south China. It is surrounded by petrochemical industries, mariculture, NPP, and urban development (Wu et al., 2009; Liu et al., 2018), which makes it an ideal paradigm for studying the response of macrobenthic communities to multiple human activities. Numerous previous studies demonstrated that human activities had severely changed the biotic and abiotic environment in Daya Bay (Gao and Chen 2008; Wu et al., 2009; Wang et al., 2011; Ma et al., 2014; Arbi et al., 2017). The mariculture area in Daya Bay has expanded from 440 ha in 1988 to 14,000 ha in 2005, a nearly 600-fold growth over the past decades (Wu et al., 2009). The annual average Chl-*a* concentration increased by 1.9 mg/m^3, and harmful algal blooms increased significantly (Song et al., 2009). The cooling sea water from NPP has been discharged at a rate of 95 m^3/s and with a temperature of 65°C since 2002, and sea water +1°C area can cover approximately 56 km^2 (Yu et al., 2010). The thermal discharges decreased the abundance and diversity of phytoplankton and zooplankton and consequently declined the fishery production (Hao et al., 2016). With the rapid development

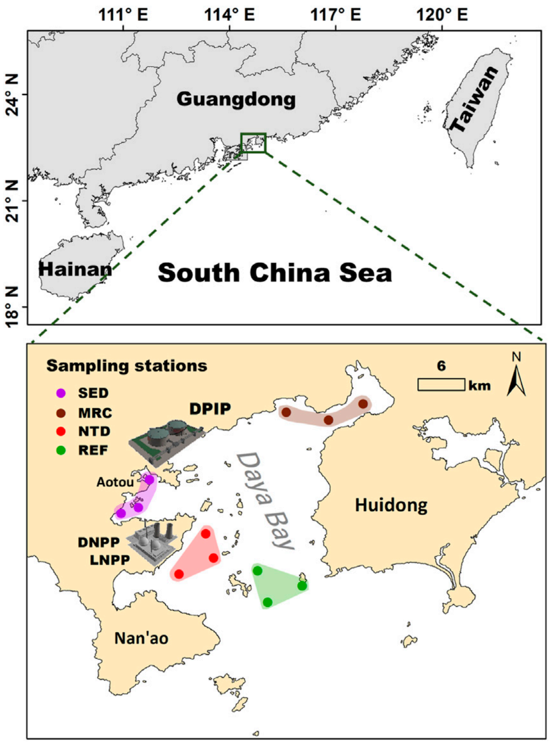

FIGURE 1 | Location of sampling stations in Daya Bay. Stations are classified as sewage discharge (SED, dots in the purple shadow), mariculture (MRC, dots in the brown shadow), NPP thermal discharge (NTD, dots in the red shadow), and reference (REF, dots in the green shadow). DPIP, Daya Bay Petrochemical Industry Park; DNPP, Daya Bay Nuclear Power Plant; LNPP, Ling'ao Nuclear Power Plant.

of the petrochemical industries in Daya Bay, around 1,150 m^3/h sewage, containing COD, petroleum hydrocarbons, heavy metals, sulfide, ammonia, and so on, is directly discharged into the bay (Xu et al., 2014).

In this study, BTA was used to illustrate the functional responses of macrobenthic communities to human activities. We targeted three major human activities (domestic and industrial sewage discharge, mariculture, and NPP thermal discharge) in Daya Bay and aimed to 1) evaluate potential differences in the functional traits of macrobenthic communities influenced by different types of human activities; 2) identify the most influential environmental variables impacting the functional trait patterns; and 3) discern the most responsive traits to environment stress.

MATERIALS AND METHODS

Study Area

Daya Bay is a semi-enclosed bay located in the northern edge of the South China Sea, with a subtropical marine climate. It covers an area of 600 km^2 and has an average water depth of 10 m. The minimal sea surface temperature occurs in winter (average 17.3°C) and the maximum in summer (average 29.3°C). It is mainly influenced by the East Asian Monsoon system, whereby southwest winds prevail in summer and northeast winds in winter. The average annual rainfall is 1984 mm, 80% of which happens in spring and summer (wet season) and 20% in autumn and winter (dry season) (Wang et al., 2008). Tidal current is the dominant current component in Daya Bay which is of the irregular semi-diurnal type. Despite the low velocity (2–6 cm/s), there also exist two types of seasonal circulation: cyclonic circulation in summer and anticyclonic circulation in winter (Wu et al., 2007). There are mariculture areas (shellfish farming that has lasted over the past decades) in the northeast coast, petrochemical industries (operated since 2001) in the northwest coast, and two nuclear power plants (operated since 1994 and 2002, respectively) in the western coast. Aotou, located in the northwest coast, is the most densely populated town in Daya Bay, with more than 60,000 residents. Over the past decades, the ecological environment of Daya Bay has been severely influenced by the aforementioned types of human activities (Wang et al., 2008; Chen et al., 2010; Qu et al., 2018; Ke et al., 2019).

Field Sampling and Sample Processing

Three stations were set in the four sampling locations associated with different human activities, respectively, resulting in a total of 12 stations (**Figure 1**). These locations are domestic and industrial sewage discharge area (SED), mariculture area (MRC), sea water +1°C area induced by NPP thermal discharge (NTD), and an area in the mouth of the bay with relatively low human disturbance as a reference (REF). Sampling surveys were conducted seasonally (November in 2017, January, April, and July in 2018, representing autumn, winter, spring, and summer, respectively). At each station, four replicate sediment samples were collected using a 0.05-m^2 Van Veen grab and washed through a 0.5-mm mesh sieve, and the residues were transferred to the sample containers with 5% formalin buffer *in situ* for faunal analysis. In the laboratory, animals were identified to the lowest possible taxon and enumerated under a dissecting microscope. Taxonomic data from the four replicates were pooled to obtain faunal composition at each sampling station.

The biological and physicochemical samplings were carried out simultaneously. A set of human activity–related environmental variables were selected. One extra sediment sample from the surface up to 10 cm depth was obtained at each station for the analyses of grain size, total organic carbon (TOC), petroleum contaminants (PCs), and heavy metals including As and Hg, which were mainly influenced by the anthropogenic input (Zhao et al., 2016). A total of 1,000 ml water sample was collected from the bottom layer per station for measurements of phosphate (PO_4^{3-}), ammonia (NH_4^+), and chlorophyll a (Chl-*a*). The water samples were stored in PE bottles with special fixatives. All samples were preserved under 4°C prior to analyses. Water depth (Dep) was measured by a handheld depth sounder (Hondex PS-7). Temperature (T), salinity (Sal), and dissolved oxygen (DO) were measured *in situ* using a handheld multiparameter meter (YSI Pro Plus). In the laboratory, the sediment grain size was determined using a granulometer (Mastersizer 2000) capable of measuring particle

TABLE 1 | Biological traits and modalities used in the macrobenthic community of Daya bay.

Trait	Modality	Trait code	Ecosystem functioning
Morphology trait			
Body size	Small (<10 mm)	S1	Body size is important for the amount of oxygen and nutrient flux across the sediment–water interface
	Small to medium (10–50 mm)	S2	
	Medium to large (50–100 mm)	S3	
	Large (>100 mm)	S4	
Fragility	Fragile	F1	Indicative of sensitivity to physical disturbance and prey accessibility and ease of ingestion
	Intermediate	F2	
	Robust	F3	
Life history traits			
Larval development	Pelagic	LD1	Ability of a species to disperse and recover from a population decline
	Benthic	LD2	
Longevity	Short (<2 years)	A1	An indicator for the stability of population over time
	Medium (2–5 years)	A2	
	Long (>5 years)	A3	
Behavioral traits			
Living habit	Attached	LH1	Foraging mode, ability to escape from predation, migratory requirements, dispersal, and the way in biogeochemical process
	Tube-dweller	LH2	
	Burrower	LH3	
	Crawler/swimmer	LH4	
Feeding habit	Deposit feeder	FH1	Indicates hydrodynamic conditions, food acquisition, and nutrient cycling
	Filter/suspension feeder	FH2	
	Opportunist/scavenger	FH3	
	Predator	FH4	
Bioturbation	Surficial modifier	B1	Impact on biogeochemistry, organic matter re-distribution, and habitat provision
	Biodiffusor	B2	
	Upward conveyor	B3	
	Downward conveyor	B4	
	None	B5	
Tolerance	Low	T1	Indicates vulnerability or resistance/resilience of a species towards pollution
	Intermediate	T2	
	High	T3	
Environmental position	Infauna	EP1	Affects carbon fixation and subsurface oxygenation
	Epifauna	EP2	

sizes from 0.02 to 2,000 μm. Shepard's sediment classification method was used to describe the types of sediment (Shepard, 1954). The analytical methods of remaining variables refer to the Marine Monitoring Specification (GB 17378.4-2007; GB 17378.5-2007).

Biological Trait Analysis

Nine biological traits subdivided into 30 modalities were used to describe the functional properties of macrobenthic communities in Daya Bay (**Table 1**). The traits included morphological (body size and fragility), life history (larval development and longevity), and behavioral (living habit, feeding habit, bioturbation, tolerance, and environmental position) characteristics. The selection of these traits and their implications of ecosystem functions were based on the recent reviews (Beauchard et al., 2017; Degen et al., 2018; Degen and Faulwetter 2019; Lam–Gordillo et al., 2020). Information regarding the biological traits were gathered from literature (Oug et al., 2012; Queirós et al., 2013; Jumars et al., 2015), online databases (http://www.marlin.ac.uk/biotic/; http://www.marinespecies.org/; https://www.univie.ac.at/arctictraits/; http://www.marinespecies.org/traits/index.php; http://polytraits.lifewatchgreece.eu/polytraits), and local specialists. When little or no information is available for a taxon, we referred to the information from the same genus or family. A "fuzzy coding" procedure was used to code for each taxon according to their affinity for the modalities of each trait (Chevenet et al., 1994; Bremner et al., 2003). Fuzzy-coding procedure was used to account for multiple trait modalities of an individual taxon, that is, a scoring range of 0–3 was adopted, where 0 expresses no affinity for the given trait category, 1 or 2 express partial affinity, and 3 expresses exclusive affinity. In present study, to avoid bias among different traits, the affinity scores for each trait were standardized so that the sum was equal to 1 (Darr et al., 2014).

Data Analysis

The principal component analysis (PCA) was used to investigate the variation in environmental variables (Z-score normalized) among the sampling locations. The quantification of the functional structure of communities was proceeded after obtaining the taxa matrix (table L, taxa by stations) and the fuzzy coded trait matrix (table Q, traits by taxa). CWM was calculated for each of the 30 trait modalities. The trait modality standardized scores of each taxon were multiplied by its abundance for every station and then summed over all taxa present at each station to generate a CWM table (traits by stations) with the overall frequency of each trait modality per station. The trait composition among the different sampling locations was explored with the fuzzy correspondence analysis (FCA) based on the CWM table. FCA is an ordination method appropriate for fuzzy coded data (Bremner et al., 2006). In FCA, the correlation ratio (cr) indicates the contribution of each trait to the overall variance, whereas traits with a CR value greater than 0.1 can be considered as the traits with the greatest influence on variance between stations (Liu et al., 2019). Then the CWM table was calculated by Bray–Curtis measure to construct the resemblance matrix. Based on this resemblance matrix, the permutational multivariate analysis of variance (PERMANOVA main test, with 9,999 permutations) was performed to test whether the trait composition of macrobenthic communities showed significant differences among the sampling locations/seasons. The similarity percentage (SIMPER) analysis based on the CWM table and taxa matrix with Bray–Curtis measure was performed to determine the dominant traits and species/taxonomic unit characterizing the dissimilarity among the communities.

FD was estimated by Rao's quadratic entropy (Rao's Q) because it incorporates functional richness, functional evenness, and functional divergence (Mouillot et al., 2013). To compare the relationship between functional diversity and taxonomic diversity, the classical taxonomic diversity index, that is, the Shannon–Wiener diversity index (H', based on log2), was also calculated.

In order to unravel the trait-environment relationships, the BIOENV analysis was used to assess which environmental variables correlate best with the CWM values. The RLQ and fourth-corner methods were further applied to assess which traits response to environmental gradients, based on the following three tables, that is, R table (environmental variables by stations), L table, and Q table (Dray et al., 2014).

Non-parametric Kruskal–Wallis tests and Wilcoxon tests were performed to test whether the median values of environmental variables and indices (functional and taxonomic) showed significant differences between the sampling locations and between seasons. Kruskal–Wallis tests, Wilcoxon tests, PCA, FCA, and the calculation of functional and taxonomic diversity indices were run in R-3.5.3 (R Core Team, 2019) using the packages "stats," "factoextra," "vegan" (Oksanen et al., 2019), "ade4" (Dray and Dufour, 2007), and "FD" (Laliberté et al., 2014). PERMANOVA main test, SIMPER, and BIOENV were performed in PRIMER 7 (with PERMANOVA+).

RESULTS

Environmental Variables

For environmental variables, notable differences among the sampling locations and among seasons were observed (**Supplementary Table S1**). Compared with the stations in SED and MRC, the stations in NTD and REF had higher values of water depth and salinity, whereas they showed a lower content of PCs and Hg in sediments ($p < 0.05$). SED showed a higher concentration of Chl-*a* than the other sampling locations ($p < 0.05$) and a higher content of TOC in sediments than MRC. Salinity, temperature, DO, Chl-*a*, PO_4^{3-}, and NH_4^+ differed significantly between seasons ($p < 0.05$). The highest value of salinity occurred in autumn. The highest content of PO_4^{3-} and NH_4^+ occurred in spring, while the highest concentration of Chl-*a* occurred in summer. The sediment types in the four sampling locations were all silty clay.

The first two principal components of the PCA accounted for 51.0–56.0% of the total variation in environmental conditions over the studied stations (**Figure 2**). The seasonal variation of the environmental conditions of the sampling locations was clear. But, in general, SEDs were associated with higher concentration of Chl-*a* and higher contents of petroleum PCs and Hg in sediments, NTD had greater percentages of silt and clay, REF was characterized by greater water depth. MRC had relatively large intra-group variations and did not show concordant characteristics among seasons.

Functional Structure of Macrobenthic Communities

A total of 163 taxa, including 80 polychaetes, 50 molluscs, 25 crustaceans, 3 echinoderms, 2 sipunculid, 1 platyhelminth, 1 nemertean and 1 chordate, were identified during the four sampling surveys in Daya Bay. Taxa matrix and fuzzy coded trait matrix are shown in **Supplementary Tables S2, S3**. FCA depicted the patterns in the trait composition for the stations grouped by the sampling locations and seasons (**Figures 3A,B**). The first two FCA axes accounted for 56.4% of the variance in the trait composition between the stations, 38.1% along axis 1, and 18.3% along axis 2. The traits, that is, bioturbation, longevity, tolerance, body size, and feeding habit were significantly related to axis 1, and environmental position was strongly correlated with both axes (**Table 2**).

For the trait composition of macrobenthic communities, a significant difference among the sampling locations was confirmed by the PERMANOVA main test (Pseudo-F = 9.587, $p < 0.001$). However, no significant difference among seasons was detected (Pseudo-F = 1.329, $p = 0.215$). Therefore, the comparisons of the trait composition between seasons will not be carried out in the following analyses. Pair-wise tests showed that there were significant differences between the sampling locations ($t = 2.031–4.455$, $p < 0.01$), excluding the case between NTD and REF ($t = 1.268$, $p = 0.152$).

The average values of CWM for each sampling period were plotted to visualize the functional composition of macrobenthic communities in the sampling locations (**Figure 4**). The SIMPER test performed on CWM values showed that the macrobenthic communities of the four sampling locations were all characterized by the following trait modalities (**Table 3**), that is, fragile (F1), pelagic larval development (LD1), short longevity (A1), borrower (LH3), deposit feeder (FH1), and high tolerance (T3). Nevertheless, SED and MRC exhibited a higher percentage of

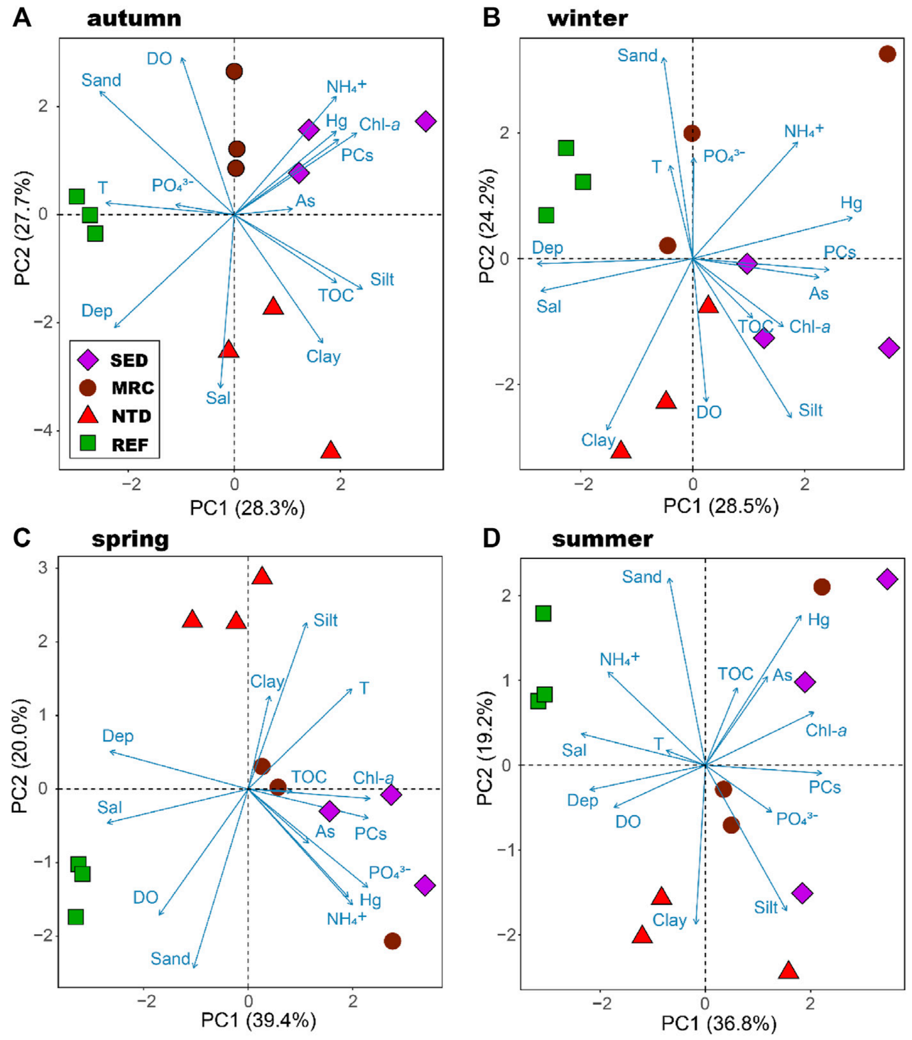

FIGURE 2 | Principal component analysis of the relevant environmental variables in Daya Bay. SED, sewage discharge area; MRC, mariculture area; NTD, NPP thermal discharge area; REF, reference area. The Bering Sea.

small body size (S1) than NTD and REF, and MRC, NTD, and REF had higher percentages of small-medium body size (S2) than SED; NTD, and REF had higher percentages of medium longevity (A2) and biodiffusor (B2) than SED and MRC, while SED was dominated by upward conveyor (B3) and MRC was dominated by surficial modifier (B1); REF had a higher percentage of intermediate tolerance (T2) than the other sampling locations; SED had a higher percentage of epifauna (EP2) than the other sampling locations. The SIMPER test performed on taxa matrix showed that (**Table 4**) the macrobenthic communities of SED were represented by two Polychaeta species (*Polydora* sp. and *Prionospio polybranchiata*); MRC were represented by one Nemertea species and six Polychaeta species (Cirratulidae, *Goniada maculata*, *Mediomastus chinensis*, *Notomastus latericeus*, *Paraprionospio cristata*, and *Sigambra hanaokai*); NTDs were represented by one Nemertea species and five Polychaeta species (*Aglaophamus lyrochaeta*, Cirratulidae, *Cossura dimorpha*, *M. chinensis*, and *S. hanaokai*); REFs were represented by one Nemertea species, nine Polychaeta species (*A. dibranchis*, *A. lyrochaeta*, Cirratulidae, *Glycera* sp., *Laonice cirrata*, *Listriolobus brevirostris*, *Lumbrineris* sp., *M. chinensis*, and *S. hanaokai*), one Crustacea species (*Photis hawaiensis*), and two Echinodermata species (*Amphiodia* (*Amphispina*) *obtecta* and *Protankyra bidentata*).

There were significant differences in Shannon–Wiener diversity (H') and functional diversity (Rao's Q) among the sampling locations ($p < 0.01$). The highest H' and Rao's Q were both observed in REF, and the lowest values both occurred in SED (**Figures 5A,B**). The H' was significantly higher in REF than that in NTD ($p = 0.013$), but there was no significant difference in Rao's Q between them (**Figures 5A,B**). The H' was slightly higher in MRC than that in NTD, and Rao's Q was slightly higher in NTD than that in MRC (**Figures 5A,B**). However, there were no significant differences between MRC and NTD for both indices. Additionally, Rao's Q responded rapidly as H' increased in SED, MRC, and NTD, but for REF with relatively

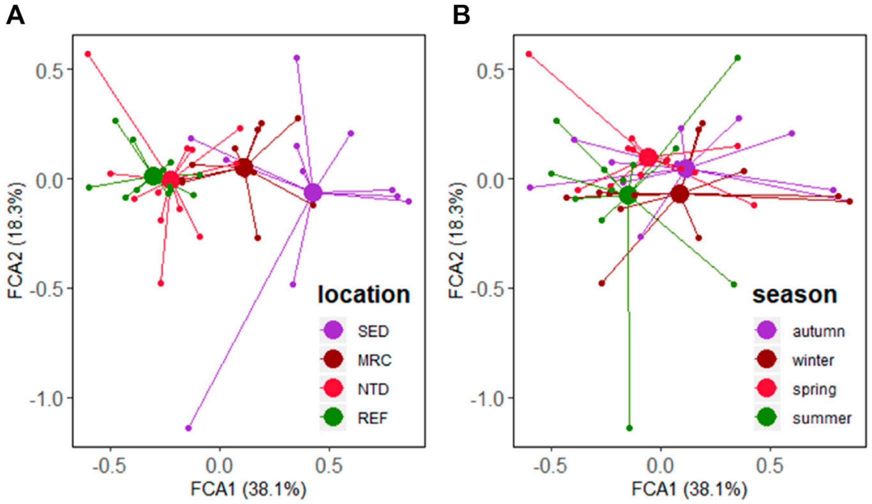

FIGURE 3 | FCA ordinations of the trait composition of macrobenthic communities group by **(A)** location and **(B)** season in Daya Bay. SED, sewage discharge area; MRC, mariculture area; NTD, NPP thermal discharge area; REF, reference area.

TABLE 2 | Correlation ratios (cr) between the first two axes of the FCA and the biological traits.

Trait	Axis 1	Axis 2
Body size	**0.186**	0.091
Fragility	0.086	0.018
Larval development	0.035	0.001
Longevity	**0.225**	0.037
Living habit	0.058	0.089
Feeding habit	**0.110**	0.082
Bioturbation	**0.247**	0.099
Tolerance	**0.191**	0.059
Environmental position	**0.113**	**0.124**

Bold figures indicate cr >0.1.

higher species diversity, the variation of Rao's Q was small (**Figure 5C**).

Relationships Between Traits and Environmental Variables

According to the BIOENV analysis, functional trait pattern was best correlated with the combination of Dep, DO, Chl-*a*, NH_4^+, and PCs ($\rho = 0.470$). The combination of RLQ and fourth-corner analysis revealed 14 out of the 30 trait modalities were significantly correlated ($p < 0.05$) with the environmental variables (**Figure 6**). Specifically, the frequency of small body size (S1) was positively associated with the contents of NH_4^+, As, Hg, and PCs, and negatively associated with water depth, salinity, and the percentage of clay. Small-medium (S2) was negatively associated with the contents of NH_4^+ and Hg. Pelagic larval development (LD1) was more abundant in higher percentage of clay sediments and benthic larval development (LD2) had the opposite trend. Animals with short longevity (A1) were more abundant in PCs/Hg enriched sediments but less abundant in greater water depth, salinity, and clay percentage environments. The frequency of medium longevity (A2) was positively influenced by water salinity, and negatively influenced by PCs, silt, Hg, and NH_4^+. Filter/suspension feeder (FH2) was positively correlated with NH_4^+, and predator (FH4) was positively correlated with water salinity. Upward conveyor (B3) and downward conveyor (B4) were more abundant in DO, Chl-*a*, and PCs enriched environments, while the surficial modifier (B1) was less abundant in DO enriched environments, and downward conveyor (B4) was less abundant in PO_4^{3-}-enriched environments. Animals with high tolerance (T3) were scarce in deeper waters. Epifauna (EP2) was more abundant in NH_4^+-, As-, and Hg-enriched environments and in fauna (EP1) had the opposite trend.

DISCUSSION

Effect of Anthropogenic and Natural Drivers on Environmental Conditions in Daya Bay

Previous studies have confirmed the significant impacts of human activities on Daya Bay environments, for example, petrochemical sewage discharge (Xu et al., 2014; Liu et al., 2018), mariculture (Qi et al., 2019), and thermal discharge (Yu et al., 2010; Hao et al., 2016). In the present study, water depth was associated with environment stress. When water depth increased, the content of nutrients (PO_4^{3-}, NH_4^+) in water columns and contaminants (PCs, Hg) in sediments decreased, and vice versa. It can be partly attributed to the human activities such as sewage discharge and mariculture that increase the chemical pollution load located in shallow waters. However, the relatively low Chl-*a* and TOC contents in MRC may be due to its higher sea water renewal and the dilution and dispersion rates of contaminants. MRC is located in the eastern part of Daya Bay, which is the main channel for water exchange with the outer sea (Wu et al., 2007). Due to the

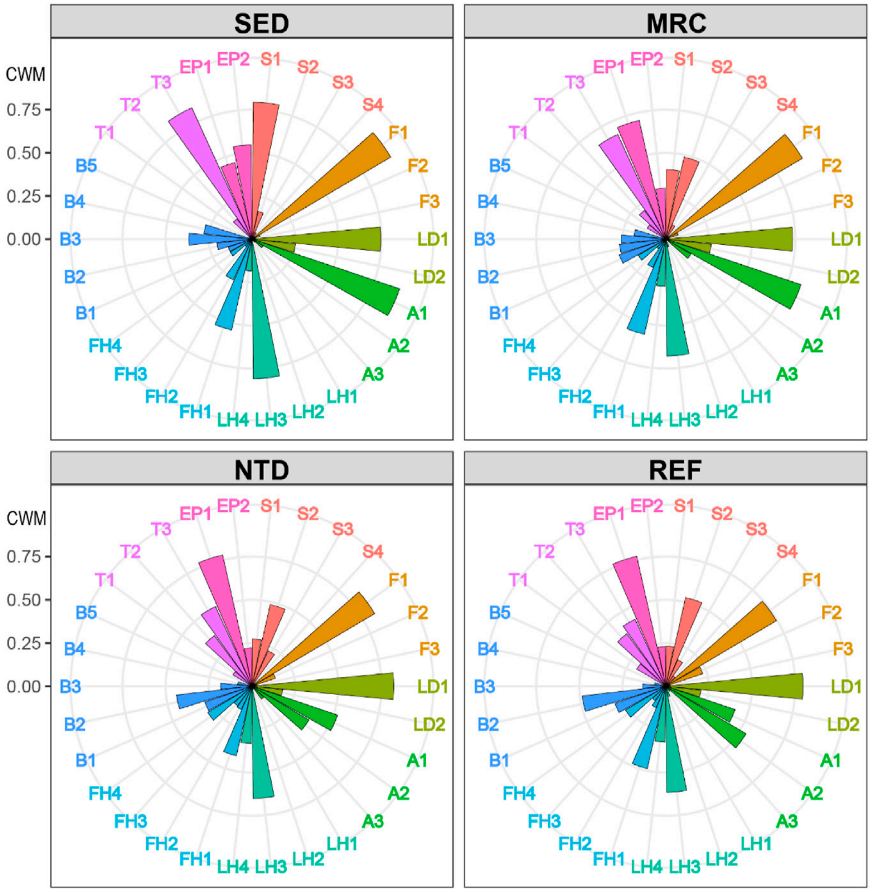

FIGURE 4 | Community-weighted means of trait modalities (CWMs), average values for sampling locations. SED, sewage discharge area; MRC, mariculture area; NTD, NPP thermal discharge area; REF, reference area. See **Table 1** for the meaning of the trait codes.

diurnal fluctuations in water temperature, the heating effect of thermal discharge was not reflected from our *in situ* surveys. Furthermore, the precipitation and the East Asian Monsoon also have an important influence on water quality in Daya Bay. We observed that the higher content of PO_4^{3-}, NH_4^+, and Chl-*a* occurred in wet season (spring and summer). When the East Asian Monsoon prevails, the nutrients are washed out from the land into the bay, causing differences in the water quality between the wet season and dry season (Wu et al., 2009). Additionally, the eastern Guangdong coastal upwelling promotes primary productivity explaining the high Chl-*a* concentration in summer (Gu et al., 2012). As a result, the environmental conditions of Daya Bay were determined by both anthropogenic and natural factors.

Functional Response of Macrobenthic Communities to Human Activities

Assessments of benthic community responses to human activities are an important task when considering the significance of the impacts on the sustainability of marine ecosystem functions. Trait distribution emerges through evolution and natural selection and is mediated by the environment, biological interactions, and anthropogenic drivers (Barton et al., 2016). Given that the sensitivity of traits to changes in habitat disturbance, the order of extinction of traits matters for assessing the decline in ecosystem functioning (Boersma et al., 2016; Kenny et al., 2018). For instance, organic pollution affects mainly organisms sensitive to oxygen depletion, whereas acidification affects those sensitive calcium depletion (Bolam et al., 2017).

We explored the functional structure of macrobenthic communities in Daya Bay subject to the potential impacts from different human activities, in particular, assessing the potential effects of contaminants and other environmental variables on macrofaunal functional identity and diversity. The FCA carried out on trait composition highlighted the differences in the sampling locations associated with different human activities (see **Figure 3A**), a result corroborated also by the PERMANOVA main test (Pseudo-F = 9.587, $p < 0.001$). However, no significant differences were observed between NTD and REF ($t = 1.268$, $p = 0.152$). We found that bioturbation, longevity, tolerance, body size, feeding habit, and environmental position, were key traits in characterizing the functional structure of macrobenthic communities (See

TABLE 3 | Average values and contributions of community-weighted means of trait modalities (CWMs) to the similarity of macrobenthic communities in sewage discharge area (SED), mariculture area (MRC), NPP thermal discharge area (NTD), and reference area (REF), based on SIMPER analysis (cutoff applied at 70%).

Modality	CWM					Contribution (%)		
	SED	MRC	NTD	REF	SED	MRC	NTD	REF
Body size								
Small (S1)	0.79	0.40	0.27	0.23	10.6	4.2	—	—
Small to medium (S2)	0.16	0.49	0.48	0.53	—	5.4	5.4	6.3
Fragility								
Fragile (F1)	0.93	0.92	0.83	0.74	14.4	12.3	10.7	8.9
Larval development								
Pelagic (LD1)	0.75	0.73	0.82	0.79	10.0	9.0	11.1	9.8
Longevity								
Short (A1)	0.90	0.83	0.52	0.42	13.5	10.6	5.9	4.4
Medium (A2)	0.08	0.17	0.38	0.54	—	—	4.4	5.9
Living habit								
Burrower (LH3)	0.81	0.68	0.65	0.61	10.8	8.4	8.1	7.6
Feeding habit								
Deposit feeder (FH1)	0.54	0.56	0.41	0.49	—	6.5	4.4	5.7
Bioturbation								
Biodiffusor (B2)	0.21	0.27	0.44	0.49	—	—	4.9	5.4
Tolerance								
Intermediate (T2)	0.15	0.21	0.36	0.38	—	—	—	4.5
High (T3)	0.84	0.67	0.51	0.43	11.5	8.1	5.8	4.7
Environmental position								
Infauna (EP1)	0.45	0.70	0.78	0.77	—	8.3	10.0	9.5

TABLE 4 | Average abundance and contributions of taxonomic units to the similarity of macrobenthic communities in sewage discharge area (SED), mariculture area (MRC), NPP thermal discharge area (NTD), and reference area (REF), based on SIMPER analysis (cutoff applied at 70%).

Taxonomic unit	Abundance (ind./m^2)				Contribution (%)			
	SED	MRC	NTD	REF	SED	MRC	NTD	REF
Nemertea	0.8	5.8	4.6	12.8	—	6.5	7.4	4.5
Polycheata								
Aglaophamus dibranchis	0.0	1.7	11.7	21.9	—	—	—	6.22
Aglaophamus lyrochaeta	4.6	6.7	27.9	31.0	—	—	34.8	11.2
Cirratulidae	0.4	23.8	4.6	21.2	—	13.4	8.1	6.1
Cossura dimorpha	1.3	3.8	3.3	6.8	—	—	4.1	—
Glycera sp.	0.8	1.7	2.1	10.1	—	—	—	8.0
Goniada maculata	1.7	13.3	3.3	3.6	—	7.96	—	—
Laonice cirrata	0.0	0.8	3.3	8.2	—	—	—	2.1
Listriolobus brevirostris	0.0	0.0	2.5	17.2	—	—	—	4.9
Lumbrineris sp.	0.0	0.8	0.4	18.8	—	—	—	7.5
Mediomastus chinensis	0.8	12.9	12.9	11.8	—	5.3	4.2	3.9
Notomastus latericeus	0.0	18.8	6.3	18.3	—	5.2	—	—
Paraprionospio cristata	0.4	63.8	21.7	10.9	—	20.2	—	—
Polydora sp.	37.1	5.8	2.1	0.8	52.0	—	—	—
Prionospio polybranchiata	31.3	16.7	4.2	0.0	26.8	—	—	—
Sigambra hanaokai	4.6	12.1	10.8	12.9	—	11.8	14.5	3.6
Crustacea								
Photis hawaiensis	0.4	20.0	1.3	11.3	—	—	—	2.5
Echinodermata								
Amphiodia (*Amphispina*) *obtecta*	0.0	0.4	3.8	19.3	—	—	—	5.1
Protankyra_bidentata	0.0	0.4	1.3	9.1	—	—	—	4.7

Table 2). Small body size (<10 mm), short longevity (<2 years), and high tolerance animals were more abundant in SED and MRC (higher proportions in SED), which were represented by the species of Spionidae, that is, *Polydora* sp., *P. polybranchiata*, and *P. cristata*. These are typical features of opportunistic species within nutrient-enriched or stressed habitats (Oug et al., 2012; Nasi et al., 2018). Although fragile animals were dominant in Daya Bay, this was more evident in SED and MRC, indicating they were not suitable for animals with shells, for example, mollusks and crustaceans. The dominant bioturbation mode in SED was upward conveyor (B2). Upward conveyors are efficient at moving deep horizon particles to the sediment surface

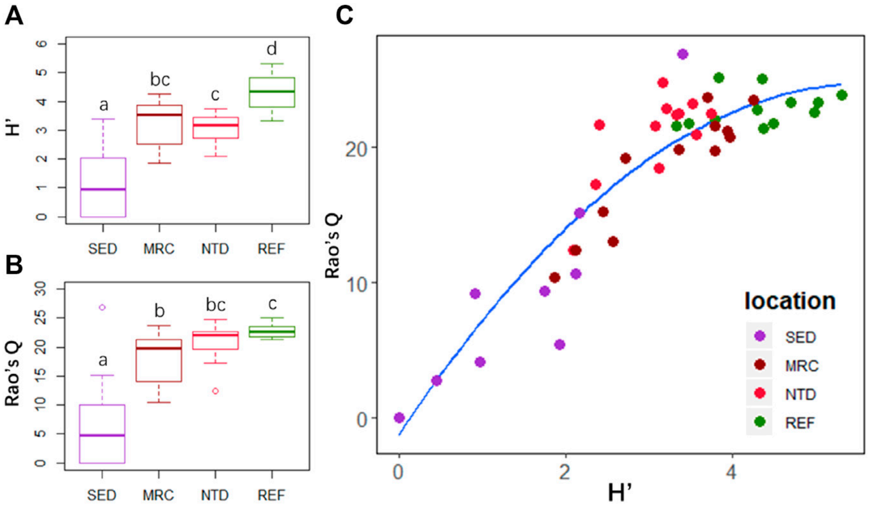

FIGURE 5 | Taxonomic and functional diversity indices of macrobenthic communities in the four sampling locations: **(A)** boxplot of Shannon–Wiener diversity index (*H′*) and **(B)** Rao's quadratic entropy index (Rao's Q); **(C)** relationship between *H′* and Rao's Q. SED, sewage discharge area; MRC, mariculture area; NTD, NPP thermal discharge area; REF, reference area. Different lowercase letters indicate significant differences (Wilcoxon test after Kruskal–Wallis test: $p < 0.05$).

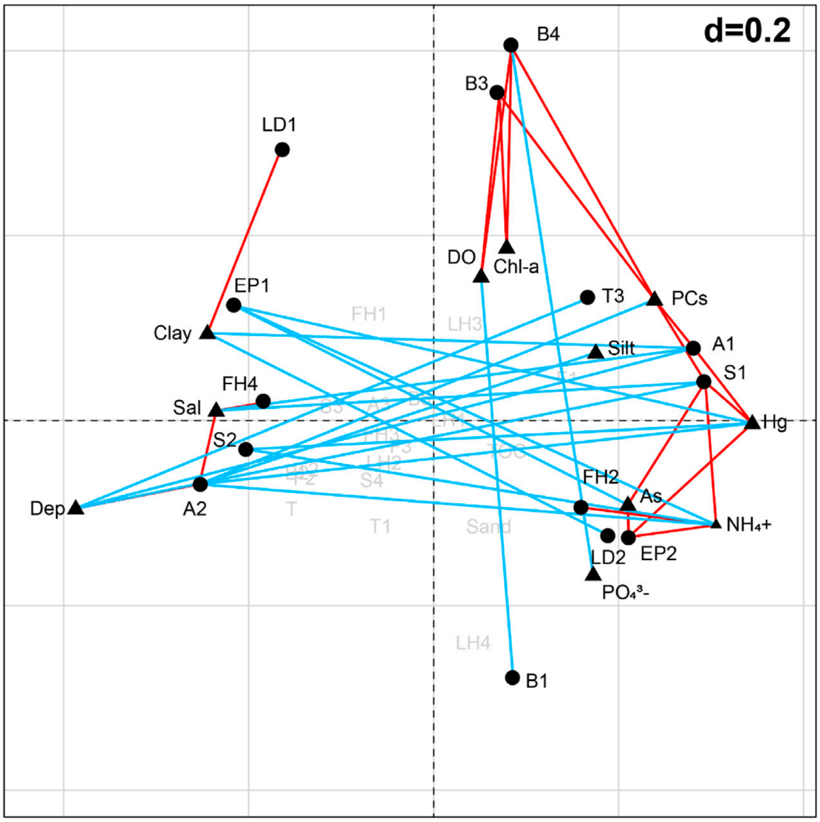

FIGURE 6 | Correlations between the environmental variables and trait modalities carried out by fourth-corner on the factorial map of RLQ analysis. Red/blue lines represent positive/negative correlations. Triangles represent the environmental variables; circles represent the trait modalities. See **Table 1** for the meaning of the trait codes.

(Kristensen et al., 2012). Surficial modifiers (B1) in general with filter/suspension feeding habit, a greater amount of food provided by mussel biodeposition can explain why they were more abundant in MRC (Lacoste et al., 2019). The activities of surficial modifiers are restricted to the surficial layer (0–2 cm) of the sedimentary profile (Queirós et al., 2013); therefore, the limited bioturbation potential could lead to the deterioration of sediment quality in MRC. Less infauna (EP1) found in SED was supposedly related to the anoxic sediments there. Comparatively, small-medium body size (S2), medium longevity (A2), and intermediate tolerant (T2) species were more abundant in NTD and REF (higher proportions in REF), indicating their relatively good environmental status. A two-decade study demonstrated that the body size of the macrobenthic fauna became smaller and biodiversity was increasingly simplified in Daya Bay (Wang et al., 2008). It is true, from our findings, that a small Polychaeta species *Sigambra hanaokai* was formerly rare in Daya Bay and has recently become ubiquitous.

On the other hand, we found a similar functional structure between seasons (Pseudo-F = 1.329, p = 0.215), indicating low temporal variability for the trait composition in macrobenthic communities. While the taxonomic composition was quite different between seasons (Pseudo-F = 1.682, p = 0.006). Most benthic organisms reproduce and settle at specific times of the year (Reiss and Kroncke, 2005), which makes it difficult to compare works conducted in different seasons. Yet the seasonal stability of functional structure seems to make up for this deficiency.

The taxonomic and functional diversity indices showed clear differences between different human activities (**Figures 5A,B**). The relatively lower taxonomic and functional diversity were found in the disturbed areas (i.e., SED, MRC, and NTD). It is consistent with previous studies which noted that the inner bay of the Daya Bay disturbed by mariculture and sewage pollutions had lower taxonomic richness and diversity (Arbi et al., 2017), and the biomass and species richness near nuclear power plants had dropped dramatically relative to the results of pre-operational surveys (Wang et al., 2008). Compared with MRC, NTD had relatively lower taxonomic diversity but higher functional diversity. This result suggested that thermal discharge might cause a decline in taxonomic diversity but not affect the functional diversity, if the reduced species contribute little to function in the community. Thermal discharge have been shown to affect the abundance, richness, and feeding habit of freshwater macroinvertebrate (Worthington et al., 2015; Han et al., 2017). However, most previous studies have demonstrated that the influence of thermal discharge on marine macrobenthic community was insignificant (Lardicci et al., 1999; Kwon et al., 2017; Jung et al., 2018; Lin et al., 2018), possibly benefitting the huge bulk of seawater. Kwon et al. (2017) assumed that if thermal discharge could exert an influence on the macrobenthic community depending on the season, summer may be a bad one. Lin et al. (2018) admitted that the elevated seawater temperature at the bottom might not be high enough to significantly affect the macrobenthos, but the stratification and elevated temperature could affect the phytoplankton at the bottom. In fact, the shift of pelagic food web in thermal discharge areas has been identified (Chen et al., 2015; Hao et al., 2016). The relatively low proportion of deposit feeder (FH1) in NTD might be a response to this shift. Deposit feeders ingest sediments and use organic matter and microbial organisms in sediments as food, such as benthic diatoms. However, the effects of thermal discharge on benthic diatoms have been demonstrated (Ingleton and McMinn, 2012). Despite the higher level of taxonomic diversity, REF showed little increase in functional diversity over NTD. In other words, there were a larger number of functionally similar species in REF. This indicates that this community potentially has a higher functional redundancy than the other communities. Functional redundancy is a policy that insures against changes in the functioning of the ecosystem due to the loss of species (Dolédec and Bonada, 2013). Although NTP and REF are similar in functional structure, a potential decrease in functional diversity in NTP can be expected when environmental degradation continues. Therefore, the combination of taxonomic and functional diversity can be a reliable method for ecological risk assessment.

In the present study, the trait composition was primarily influenced by water depth, DO, Chl-*a*, NH_4^+, and petroleum contaminants (PCs) in sediments. Generally, water depth and sediment type are considered as the main natural factors that obviously influence the distribution of macrobenthic fauna (Bolam et al., 2017; Kenny et al., 2018; Liu et al., 2019). Although natural factors showed influence on the contaminants, given the consistency of the sediment type (silty clay) and the small water depth range (4–16 m) in Daya Bay, we assumed that the human-induced environmental changes were the main factors affecting macrobenthic communities. Combined with the correlations between the environmental variables and trait modalities (**Figure 6**), we suggested that the traits "body size," "longevity," "feeding habit," "bioturbation," "tolerance," "environmental position," and the diversity indices H' and Rao's Q could be served as the promising indicators of how macrobenthic fauna react to the stress caused by human activities in Daya Bay.

Ecosystem Functioning Effects

The present study on macrobenthic communities in Daya Bay showed that the BTA method detected specific functional features correlated with human disturbances, for instance, the change from small body size organisms to larger body size organisms along pollution gradients. Of particular interest is the demonstration of the impact of human activities on body size, feeding habit, and bioturbation. All these traits express the aspects of biological activities that are important for sediment reworking, and nutrient uptake and turnover. It is a first step toward understanding how human disturbances can affect ecosystem functioning. However, the accurate assessment of the magnitude of biogeochemical processes loss in response to functional structure shifts still requires further studies (Gusmao et al., 2016).

Cautions and Limitations

The performance of the BTA relies on the selected traits and metrics used during the analytical process (Bremner, 2008). In the present study, a priori selection of traits was based on a trade-off between their efficacy for describing ecological functioning and the effort required to gather information on the selected traits. Some traits, such as reproductive type and mobility, are especially important to analyze recolonization when disturbances are absent. At present, it seems unrealistic to incorporate all possible functional features that could be of importance. Darr et al. (2014) suggested that biomass-based BTA represent an informative tool to describe the functional structure of macrobenthic community. In contrast, Gusmao et al. (2016) considered that abundance is a more suitable metric than biomass. In fact, the discrepancy may depend on the selected traits. Because some traits (e.g., larval development) are linked to abundance and others (e.g., bioturbation) are directly related to biomass. However, how to effectively integrate abundance and biomass into the corresponding traits is still a challenging question.

CONCLUSION

In the present study, BTA was used to discern shifts in the functional structure of macrobenthic communities associated with different human activities. The effects of contaminants and other environmental variables on macrofaunal functional identity and diversity were observed. However, the seasonality of trait expressions in communities was not significant. Bioturbation, longevity, tolerance, body size, feeding habit, and environmental position were the traits most affected by the human disturbances. And water depth, DO, Chl-*a*, NH_4^+, and petroleum contaminants (PCs) in sediments were the main factors related to the trait composition. Small body size, short longevity, and high tolerance species were more abundant in the contaminated areas (SED and MRC). The taxonomic and functional diversity indices revealed obvious differences among the sampling locations. Although NTD and REF were similar in trait composition, a potential function loss in NTD can be detected through the integrated analysis with taxonomic diversity.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**; further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

LC conceived of the study and obtained funding. LC, YR, XC, XZ, SF, and HH were responsible for field and laboratory work. LC conducted the identification of benthic macrofauna and created many of the manuscript elements. YR led the data analysis and writing effort, with significant contributions to these by all authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2021.766580/full#supplementary-material

Supplementary Table S1 | Environmental variables (mean ± SD) for four seasons at the four sampling locations. Dep, depth; Sal, salinity; T, temperature; PCs, petroleum contaminants in sediments; TOC, total organic carbon in sediments; As, arsenium in sediments; Hg, mercury in sediments.

Supplementary Table S2 | List of abundance of benthic macrofauna recorded at each station of the four sampling locations of Daya Bay in four seasons from 2017 to 2018 (ind./m^2).

Supplementary Table S3 | Fuzzy coded trait data of each taxa of benthic macrofauna recorded at the four sampling locations of Daya Bay in four seasons from 2017 to 2018.

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METHODS
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Multielemental Determination of Rare Earth Elements in Seawater by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) After Matrix Separation and Pre-concentration With Crab Shell Particles

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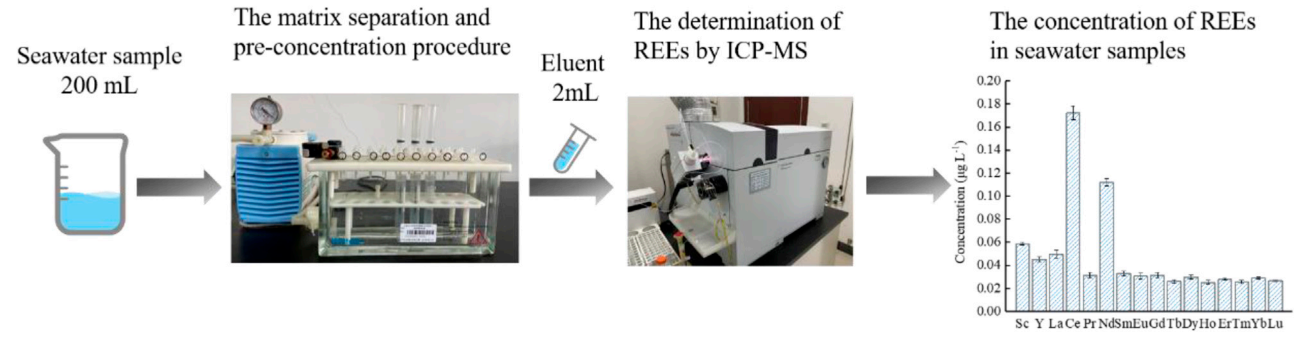
Considering the unique characteristics of rare earth elements (REEs), the presence of REEs beyond specific limits will adversely affect the environment and it can be employed as a powerful probe for investigating hydrogeochemical processes. This requires sensitive determination of REEs in natural seawater. A matrix separation and pre-concentration technique using the mini-column packed with crab shell particles (CSPs) by inductively coupled plasma mass spectrometry (ICP-MS) as a means of determination has been developed. The aim of the proposed method was to simultaneously determine 16 REEs (Sc, Y, La, Ce, Pr, Nd, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb, and Lu) at trace or ultra-trace concentrations in seawater. The biosorption capacity of CSPs was found to achieve 1.246–1.250 mg g^{-1} for all elements. In order to optimize performance of the method, the effects of analytical parameters concerning oscillation time, solution pH, salt concentration and eluent concentration were explored. Under the optimal conditions, the detection limits of REEs ranged 0.0006–0.0088 μg L^{-1}, and relative standard deviations (n = 7) varied between 0.55 and 1.39%. The accuracy of developed method was evidenced by applying it to the analysis of REEs in seawater samples, with the overall recoveries at a level of 95.3 and 104.4%. Together, this work provides a promising and cost-effective CSPs-based pretreatment approach for REEs detection in sea environment.

Keywords: rare earth elements, seawater, inductively coupled plasma mass spectrometry, separation, preconcentration, crab shell particles

INTRODUCTION

In recent years, rare earth elements (REEs: consisting of La, Ce, Pr, Nd, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb, Lu, Y, Sc, and Pm) are extensively used in several industries and high-tech devices, such as petrochemical, ceramic, metallurgy, laser and fiber optic industries (Balaram, 2019). With the ever-increasing industrialization, concerns on the accumulation and release of REEs, and their adverse effects on ecological environment have already been observed worldwide. On one hand, REEs in seawater originate from various anthropogenic and natural processes including river runoff, eolian



GRAPHICAL ABSTRACT | xxx

dust, hydrothermal activity along with glacier discharge, which occur as a valuable tracer for investigating hydrological and geological acivities (Dubinin, 2004; Crocket et al., 2018). Furthermore, the data of REEs provide significant information about hydrogeochemical processes, covering ocean circulation, water physical mixing, external water transport and water-rock interface interactions (Hathorne et al., 2020). As a consequence, it is essential to propose an accurate, rapid and selective method for the sensitive measurement of REEs at trace or ultra-trace concentrations in natural environment with complicated matrix.

Among several analytical techniques employed for measuring multi-elements in various environmental samples, neutron activation analysis (NAA), inductively coupled plasma-optical emission spectrometry (ICP-OES) and inductively coupled plasma mass spectrometry (ICP-MS) have gained increasing attentions (Li et al., 2017; Zhu and Zheng, 2018; Montalvan-Olivares et al., 2021). It is worth noting that ICP-MS is a most powerful and sensitive technique with high precision, wide linear dynamic range and low detection limits for REEs determination, which only requires a small amount of samples (Zhu et al., 2009; Wysocka and Vassileva, 2017; Wysocka, 2020). Nevertheless, the direct determination of REEs by this technique suffers from the interferences of complicated matrix and the ultra-low concentration (e.g., ng L^{-1} or pg L^{-1}) of target elements. Albeit the significant progress in the development of analytical technologies, direct determination of REEs in a highly saline water still remains a challenge. To circumvent these difficulties, a variety of approaches including co-precipitation (Zhu, 2020), ion-exchange (Barrat et al., 2020), liquid-liquid extraction (Guo et al., 2014) and solid phase extraction (SPE) (Pyrzynska et al., 2016; Chen et al., 2019) have been used for matrix separation and analyte pre-concentration, aiming to improve detection capability of REEs. In terms of these techniques, SPE has been preferred as a sample preparation approach owing to its flexibility of adsorbent, simple operation, low risk of contamination and high recovery (Tazoe et al., 2021). However, the certain limitation of SPE technique is attributed to the high cost of commercially available adsorbent. Therefore, the development of cost-effective SPE adsorbents has drawn special attention in the filed of analyte pretreatment.

Crab shell, a by-product from seafood processing, shows superior binding capacity for various heavy metal ions such as lead, cadmium, chromium, and cobalt, which has been identified as an effective SPE adsorbent (Boulaiche et al., 2019; Jeon, 2019; Richards et al., 2019). Attributed to its abundant contents of calcium carbonate, chitin along with proteins, crab shell was successfully applied to adsorb REEs in aqueous solution (Vijayaraghavan and Balasubramanian, 2010; Cadogan et al., 2014). Micro-precipitation of REE carbonate followed by adsorption to the chitin on the surface of crab shell was found to be the major mechanism responsible for REEs extraction by crab shell (Vijayaraghavan et al., 2009). Being a straight-chain polymer comprised of β-1,4-N-acetylglucosamin, chitin has available acetamido groups (Zhou et al., 2021). In particular, acetamido groups of chitin exhibit a high potential for non-specific metal chelation. Due to its good biosorption capacity, excellent mechanical stability, ease in desorption and ability of withstanding extreme conditions, crab shell is suited for column applications involving REEs extraction and pre-concentration prior to their detection (Su et al., 2019). Recycling crab shell is capable to potentially eliminate the disposal problem and convert useless waste into high value-added products. Furthermore, in the case of high biocompatibility, cost-effectiveness and great availability, crab shell can serve as a powerful REEs adsorbent. However, studies related to the adsorption and extraction of crab shell for rare earth elements are still scanty.

Accordingly, the objective of the present work is to develop a simple and cost-effective method for the extraction and pre-concentration of 16 REEs in seawater samples using a mini-

TABLE 1 | Instrumental and operating conditions for ICP-MS measurement.

Parameter	Value
RF power (W)	1,550
Deflect voltage (V)	19
Analog voltage (V)	1703
Pulse voltage (V)	1,115
Plasma Ar gas flow rate (L min^{-1})	15
Nebulizer gas flow rate (L min^{-1})	0.8
Auxiliary Ar gas flow rate (L min^{-1})	1.0
Collision cell gas (ml min^{-1})	7% H_2 in He at 4.3
Sample uptake rate (L min^{-1})	1
Sampling depth (mm)	7.8
Mass resolution (at 10%)	0.65–0.85
Data acquisition mode	Time resolved mode

column packed with crab shell particles (CSPs) prior to their measurement with ICP-MS. To explore the applicability of this approach, the effects of oscillation time, pH, salt concentration and eluent concentration were investigated by batch experiments. The analytical performances including limits of detection (LOD) and relative standard deviations (RSD) were also studied. Additionally, validation of the analytical procedure has been carried out, which was applied to the determination of REEs in spiked and unspiked natural seawater samples. To the best of our knowledge, crap shell particles have rarely been used as an adsorbent for the separation and pre-concentration of REEs at ultra-low level in seawater samples.

EXPERIMENTAL

Instruments

Mutielemental determination of REEs was carried out utilizing the quadrupole ICP-MS (Agilent 7,700, Agilent, America) equipped with a concentric nebulizer, a quartz injector (a sample tubing of 0.3 mm i.d. along with 1.6 mm o.d.), and a quartz cyclonic spray chamber including a third-generation octopole reaction system (ORS3) using helium gas. The resolution adjustement and mass calibration were weekly performed with the AutoTune function to stabilize the sensitivity of ICP-MS instrument. To correct instrument drift and signal fluctuation, the internal standard solution including indium (In) and rhodium (Rh) was mixed on-line with the samples. The optimized instrumental parameters of ICP-MS measurements are provided in **Table 1**.

Reagents and Solutions

All chemicals used in the experiment were of ultrapure grade. Ultra-pure water supplied by Guangzhou Chemical Reagent Factor was used throughout the present work. HNO_3 and NaOH solutions were used for pH adjustment. China Series Standard Seawater D191 (R_{15} = 0.9995) was employed to evaluate the effect of salt concentration on this method. The working standard solutions of REEs were prepared by gradual dilution of multi-element calibration standard (10 mg L^{-1}, Agilent Technologies, United States) with the matrix of 5% HNO_3. The glassware employed in this work was immersed in 30% (v/v) HNO_3 overnight and rinsed with ultra-pure water before use.

Synthesis of the Column Packed With Crab Shell Particles

Waste shells of *Portunus sanguinolentus* were obtained from the seafood market located in Zhoushan, Zhejiang. The crab shells were firstly rinsed adequately with distilled water to remove flesh and other impurities on the surface, then sun-dried for several days, crushed to particles, and sieved with 100 mesh. The crab shell particles were placed in a tube furnace for activation by calcining at 700°C for 2 h under nitrogen. The other chemical pretreament was not performed and CSPs were kept in the desiccators. A polyethylene mini-column (10 × 0.46 cm) was packed with CSPs, which was used in an off-line mode. A suitable glass wool was placed onto the bottom of column, and then 0.30 g of CSPs were placed upper part with the bed length of 5 mm. Another glass wool was fitted closely above the layer of CSPs.

TABLE 2 | Effect of oscillation time on the recoveries of REEs.

Analyte	Recoveries					
	0	2 min	4 min	6 min	8 min	10 min
Sc	95.9	91.2	93.6	93.3	94.7	99.3
Y	95.8	94.1	98.9	96.2	96.0	97.1
La	96.5	97.3	91.5	93.5	98.4	93.4
Ce	97.1	97.2	91.5	95.6	98.5	94.9
Pr	98.4	96.1	99.8	93.7	96.3	92.9
Nd	96.3	94.3	95.8	100.8	95.8	92.5
Sm	96.6	93.6	95.2	100.2	96.6	95.6
Eu	95.4	98.3	94.4	94.6	99.0	96.7
Gd	95.8	95.6	97.2	95.6	96.6	92.8
Tb	95.6	99.4	95.9	93.2	100.1	93.8
Dy	97.6	95.1	95.7	93.4	95.5	93.9
Ho	97.6	97.5	94.7	94.5	100.0	93.2
Er	95.9	97.7	96.5	94.0	94.3	92.7
Tm	96.4	97.7	97.4	94.3	97.4	95.6
Yb	96.2	94.7	97.4	95.4	95.5	96.0
Lu	95.6	98.2	97.0	94.4	99.4	95.0

Note: pH: 6.0; CSPs dosage:0.4 g L^{-1}; initial concentration of REEs: 20 µg L^{-1}; sample volume: 20 ml; sample flow rate: 1.0 ml min^{-1}; eluent: 4 ml of 0.1 M HNO_3; eluent flow rate: 1.0 ml min^{-1}; n = 3.

Procedure

Biosorption Studies

The biosorption capacity of the CSPs was conducted by means of a batch procedure. The CSPs with a dosage of 0.4 g L^{-1} were contacted with 20 ml of 500 µg L^{-1} REEs standard solution at pH 6.0 in a 50 ml centrifuge tube, which was kept on a rotary shaker at 300 rpm and at room temperature (24 ± 2 °C) for a period of 2 h, and then CSPs were separated from aqueous solution by filtration. The experiment was performed in triplicate.

The amount of REEs sorbed by CSPs was calculated using the following equation:

$$Q = \frac{V\left(C_o - C_f\right)}{M} \tag{1}$$

Where Q is the REEs uptake (mg g^{-1}); C_o and C_f are the initial and equilibrium concentrations of REEs in solution (mg L^{-1}), respectively; V is the volume of solution (L) and M is the mass of CSPs (g).

Off-Line Preconcentration and Elution Procedure

The sample solution was passed through the CSPs mini-column at a flow rate of 1.0 ml min^{-1} with a peristaltic pump. After the loading period, ultra-pure water was pumped through the column at a flow rate of 1.5 ml min^{-1} for 20 s to remove any un-retained matrix ions. The REEs retained on the sorbent were eluted utilizing HNO_3 at a flow rate of 1.0 ml min^{-1}. The eluent solution was collected for analyte concentrations measurement by ICP-MS.

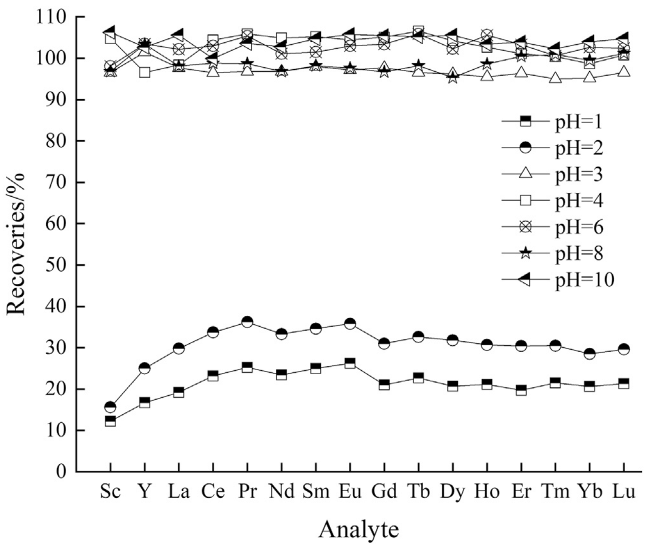


FIGURE 1 | Effect of solution pH on the recoveries of REEs.

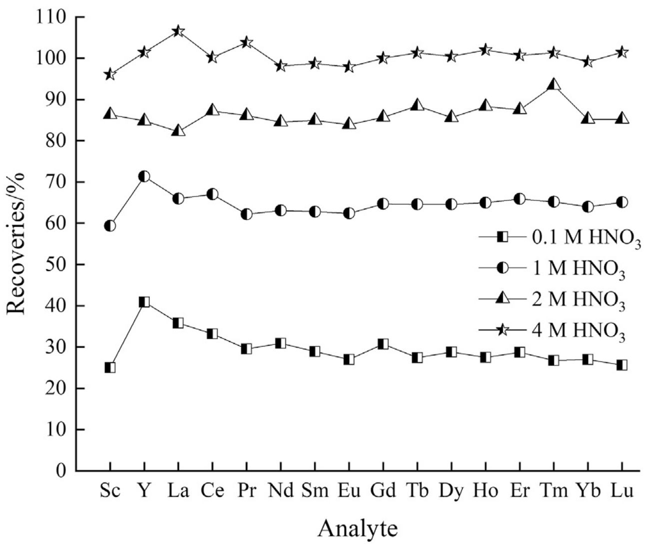


FIGURE 2 | Effect of eluent concentrations on the recoveries of REEs.

Applications to Natural Seawater Samples

In order to assess accuracy of the proposed method, it was applied to the analysis of natural seawater samples spiked with standard reference REE solutions. The natural seawater samples were collected from Xisha sea area (113°E, 13°N). Before performing the extraction steps, 200 ml of the seawater sample was firstly filtered through a cellulose membrane with a pore size of 0.45 μm and then adjusted the pH to the optimum value if necessary. The recovery tests were carried out using 2 ml 4 M HNO_3 for eluting analyte, and then pre-concentration by a factor of 100 was therefore acheieved. To obtain reliable high-quality data, the eluent solution was diluted (corresponding to a dilution factor of 20) before the analysis. Basically, due to its non-existence in natural environment except uranium minerals (Wysocka, 2020), radioactive promethium (Pm) is not determined in natural waters.

RESULTS AND DISCUSSION

Biosorption Capacity of Crab Shell Particles

To estimate the biosorption capacity of CSPs towards REEs, the proposed method was conducted under the condition as described in *Biosorption studies*. According to **Equation 1**, it can be calculated that the sorption capacity of CSPs for all elements were in the range 1.246 mg g^{-1}–1.250 mg g^{-1} (**Supplementary Table S1**), denoting that CSPs possessed high affinity towards each REE. It should be mentioned that the present approach could extract 1.246–1.250 μg REE in 1 ml aqueous solution when the SPE column was packed with 1 g CSPs. Undoubtedly, the biosorption capacity of CSPs was sufficient to extract REEs from aqueous solution in following experiments.

Optimization of Analytical Parameters

Effect of Oscillation Time

Biosorption of REEs onto CSPs was possibly connected with oscillation time. The effect of oscillation time on the recoveries of REEs was investigated by performing equilibrium sorption experiments at varying time intervals (0, 2, 4, 6, 8, and 10 min). As shown in **Table 2**, it should be noted that direct passing through the column packed with CSPs possessed high recoveries (95.6–98.4%) of REEs, which almost unchanged with oscillation time. Obviously, biosorption of REEs from aqueous solutions onto CSPs was observed to be a rapid process. Therefore, sample solutions could be directly pumped through the column packed with CSPs without the oscillation procedure, improving the simplicity and efficiency of the present work.

Effect of Solution pH

As the main constituent of crab shells, calcium carbonate favors micro-precipitation of REEs by dissociating to Ca^{2+} and CO_3^{2-}, which is related to the pH of solution. In this respect, one of the most significant variables on preconcentration procedure is the pH level of samples. The effect of pH on the extraction of REEs with CSPs was evaluated within the range from 1.0 to 10.0. Beyond pH 10, noticeable precipitation of REEs was found, thus the range of pH was not explored further. Unfortunately, the results (**Figure 1**) indicated that the overall recoveries of REEs were in the range 12.2–26.2% and 15.6–36.2% at the pH of 1 and 2 respecetively, which could be attributed to the low level uptake of REE ions by CSPs. On one hand, the lowering of solution pH can cause the surface charge on CSPs to become positive, which will inhibit the attraction of positively charged REE ions by means of electrostatic repulsion (Cadogan et al., 2014). On the other hand, it is worth noting that the competition among the REE ions enhances due to the limited quantity of available adsorption sites when the pH decreases (Ramasamy et al., 2017). As solution pH increased, it was clearly observed that the quantitative recoveries of REEs were close to 95.0–106.3% at the pH ranges of 3.0–10.0.

TABLE 3 | Effect of salt concentrations on the recoveries of REEs.

Analyte	Recoveries			
	5‰	10‰	20‰	35‰
Sc	95.8	96.7	96.2	96.9
Y	100.3	102.8	100.6	101.9
La	98.5	100.1	97.5	99.5
Ce	103.7	105.2	104.2	103.5
Pr	95.5	98.9	103.8	99.1
Nd	97.5	99.5	99.5	100.9
Sm	95.1	99.2	99.9	99.8
Eu	95.3	99.6	98.9	100.3
Gd	96.1	101.3	100.0	99.9
Tb	97.6	100.8	96.1	99.9
Dy	96.4	100.3	100.4	99.8
Ho	97.3	100.3	101.4	99.0
Er	96.6	99.5	100.2	99.1
Tm	96.1	99.0	99.0	101.9
Yb	96.5	97.4	100.2	99.8
Lu	97.4	98.5	96.1	100.3

Note: pH: 6.0; initial concentration of REEs: 20 μg L^{-1}*; sample volume: 20 ml; sample flow rate: 1.0 ml* min^{-1}*; eluent: 2 ml of 4 M* HNO_3*; eluent flow rate: 1.0 ml* min^{-1}*;* n = 3.

Namely, the proposed method had a high applicability over a wide pH range. All further experiments were carried out at pH 6.

Effect of Eluent Concentration

Desorption procedure is of utmost importance to recover the retained REEs in concentrated form and recycle the biomass. Generally, appropriate selection of eluent solutions is required for a successful desorption process. In order to enhance sequential stripping of REEs retained by CSPs, the effect of HNO_3 solution at different strengths (0.1, 1, 2 and 4 M) was explored using series standard seawater containing $20\ \mu g\ L^{-1}$ REEs ions with the salt concentration of 5‰. Results concerning the influence of salt concentration are depicted in **Figure 2**. The obtained results revealed that 4 M HNO_3 exhibited effective in desorption with the elution efficiencies of 96.1–106.5%. By contrast, the overall recoveries of REEs were in the range 25–40.9%, 59.4–71.3% and 82.2–93.4%, while uziling 0.1, 1 and 2 M HNO_3 solution as eluent, respectively. It could be speculated that REEs in seawater were intensely bound onto the surface of sorbent and thus resistant to eluting with mild acid solution. Hence, 4 M HNO_3 solution was selected as the optimum eluent for further experiments.

Effect of Salt Concentration

The salt content in seawater can pose the problems of clogging the inlet of cones to ICP-MS instrument, resulting in signal drifting and memory effect. The effect of salt concentrations on biosorption and elution procedure of REEs was studied using series standard seawater containing $20\ \mu g\ L^{-1}$ REEs ions with the salt concentration in the range of 5–35‰. Results concerning the influence of salt concentration are provided in **Table 3**. The obtained results indicated that the overall recoveries were between 95.1 and 105.2% (95.1–103.7%, 96.7–105.2%, 96.1–104.2% and 96.9–103.5% at salt concentration of 5, 10, 20 and 35‰, respectively). From the high recovery of all REEs in varying salt concentrations, it could be concluded that the separation process effectively eliminated severe matrix interferences from seawater and the proposed method could successfully extract REEs from highly saline seawater samples.

Analytical Performance of the Proposed Method

The detection limit of individual REE was affected by a variety of factors including atomic mass, natural abundance of isotopes, sample preparation steps, potential contamination and performances of ICP-MS system. The blank test was prepared using 20 ml ultra-pure water, and water was

TABLE 4 | Analytical parameters of the proposed method.

Element	Regression equation[a]	Correlation coefficient (R)	LOD (ng L^{-1})	RSD (%)
Sc	$y = 0.0352x+0.0022$	0.9998	8.81	1.37
Y	$y = 0.0603x+0.0023$	0.9997	6.95	0.90
La	$y = 0.0688x+0.0003$	0.9995	7.55	0.63
Ce	$y = 0.0715x+0.0029$	0.9993	8.44	0.55
Pr	$y = 0.0809x+0.0002$	0.9994	1.62	1.20
Nd	$y = 0.0140x+0.00004$	0.9994	8.60	1.35
Sm	$y = 0.0118x+0.00003$	0.9996	4.26	0.85
Eu	$y = 0.0441x+0.00006$	0.9996	1.74	1.26
Gd	$y = 0.0138x+0.00004$	0.9994	4.48	1.18
Tb	$y = 0.0867x+0.0002$	0.9994	1.48	0.88
Dy	$y = 0.0212x+0.00003$	0.9997	3.28	0.85
Ho	$y = 0.0840x+0.00007$	0.9995	0.89	1.19
Er	$y = 0.0279x+0.00003$	0.9996	1.35	1.25
Tm	$y = 0.0853x+0.00006$	0.9996	0.71	0.84
Yb	$y = 0.0190x+0.00002$	0.9997	2.21	1.39
Lu	$y = 0.0856x+0.0001$	0.9997	0.59	1.18

[a] *y, ratio; x, concentration (μg* L^{-1}*).*

Note: pH: 6.0; sample volume: 20 ml; sample flow rate: 1.0 ml min^{-1}*; eluent: 2 ml of 4 M* HNO_3*; eluent flow rate: 1.0 ml* min^{-1}*;* n = 11.

TABLE 5 | Applications of the proposed method for determination of REEs in seawater.

Analyte	Added (μg L^{-1})	Found (mean ± SD, μg L^{-1})	Recovery (%)
Sc	0.00	0.059 ± 0.001	—
	1.00	1.078 ± 0.002	101.9
	2.50	2.636 ± 0.019	103.1
Y	0.00	0.045 ± 0.002	—
	1.00	1.007 ± 0.006	96.2
	2.50	2.630 ± 0.022	103.4
La	0.00	0.050 ± 0.003	—
	1.00	1.012 ± 0.003	96.3
	2.50	2.594 ± 0.006	101.8
Ce	0.00	0.172 ± 0.006	—
	1.00	1.125 ± 0.007	95.3
	2.50	2.661 ± 0.038	99.5
Pr	0.00	0.031 ± 0.002	—
	1.00	1.025 ± 0.003	99.4
	2.50	2.580 ± 0.031	102.0
Nd	0.00	0.112 ± 0.003	—
	1.00	1.074 ± 0.016	96.2
	2.50	2.633 ± 0.022	100.8
Sm	0.00	0.033 ± 0.002	—
	1.00	1.064 ± 0.009	103.2
	2.50	2.642 ± 0.037	104.4
Eu	0.00	0.031 ± 0.002	—
	1.00	0.993 ± 0.002	96.3
	2.50	2.545 ± 0.038	100.6
Gd	0.00	0.032 ± 0.002	—
	1.00	1.047 ± 0.010	101.5
	2.50	2.590 ± 0.008	102.3
Tb	0.00	0.026 ± 0.002	—
	1.00	1.012 ± 0.008	98.6
	2.50	2.625 ± 0.024	104.0
Dy	0.00	0.030 ± 0.002	—
	1.00	1.052 ± 0.004	102.2
	2.50	2.563 ± 0.023	101.3
Ho	0.00	0.025 ± 0.002	—
	1.00	0.100 ± 0.003	97.2
	2.50	2.642 ± 0.017	104.7
Er	0.00	0.028 ± 0.001	—
	1.00	0.996 ± 0.004	96.8
	2.50	2.546 ± 0.015	100.7
Tm	0.00	0.026 ± 0.001	—
	1.00	1.041 ± 0.002	101.5
	2.50	2.593 ± 0.025	102.7
Yb	0.00	0.029 ± 0.001	—
	1.00	1.027 ± 0.002	99.8
	2.50	2.592 ± 0.034	102.5
Lu	0.00	0.026 ± 0.001	—
	1.00	1.030 ± 0.016	100.3
	2.50	2.563 ± 0.028	101.4

Note: pH: 6.0; sample volume: 200 ml; sample flow rate: 1.0 ml min^{-1}*; eluent: 2 ml of 4 M HNO*$_3$*; eluent flow rate: 1.0 ml min*$^{-1}$*;* n = 3.

passed through the CSP column followed by elution with 2 ml 4 M HNO_3. Based on the results of 11-set of replicated blank test, the detection limits of REEs were calculated as the concentration corresponding to three-fold standard deviation of the blank signal. The standard curves of 16 REEs are shown in **Supplementary Figure S1**, and the results of regression equation, correlation coefficient and LOD are given in **Table 4**. Under the optimum conditions, the detection limits of the method ranged from 0.59 ng L^{-1} (Lu) to 8.81 ng L^{-1} (Sc), which were low enough for the measurement of REEs in seawater samples. It should be noted that monoisotopic elements such as Pr, Tb, Ho, and Tm possessed lower detection limits than most polyisotopic ones (La, Ce, Nd, Sm, Eu, Gd, Dy, and Yb), which was in accordance with the results of previous studies (Hathorne et al., 2013; Rousseau et al., 2013; Zheng et al., 2014; Wysocka and Vassileva, 2017). The detection limits of REEs achieved in this work lowered than those reported values for REEs (e.g., 2.0–10.3 ng L^{-1}; 2.0–60.0 ng L^{-1}) in seawater determined by ICP-MS (Karada et al., 2011; Zhu et al., 2021). A relatively lower value for LOD might be attributed to lower background signal intensity, higher sensitivity, accurate blank correction, optimization of pretreating steps or a combination of some of these parameters.

The relative standard deviation tests were carried out by seven preconcentration and determination cycles using the solutions with the concentration of 1 μg L^{-1} for REEs. The analytical reproducibility was among 0.55–1.39% (RSD). The precisions were higher than most of reported values in the literatures (e.g., 2.8–11%; 1.2–18%) (Freslon et al., 2011; Arslan et al., 2018). These results demonstrated that the low detection limits and high precision of the developed method were sufficient to quantify ultra-trace REEs in seawater.

Overall, in terms of the detection limits and precision, the proposed approach provided data that were comparable with those of other methods. Compared with other techniques of SPE in the reported literatures (e.g., optimum pH range of 5–8) (Karada et al., 2011; Kumar et al., 2011), the process in this work was no need for careful pH adjustment (optimum pH values ranging 3–10). Indeed, the proposed method did not require any hazardous or harmful organic solvents which were indispensable in solvent extraction. Further, in comparison with the method of co-precipitation (Hsieh et al., 2011; Arslan et al., 2018), one of its demerits was that the relatively high concentration of co-precipitate carrier might interfer with the determination of REEs, while the elemental impurities in CSPs were negligible for REEs measurement. The present method apparently improved the capability for the determination of REEs in sea environment, and exhibited the advantages of relatively simple pretreatment procedure, high sensitivity, cost-effectiveness along with eco-friendliness.

Application of the Proposed Method to Natural Seawater Samples

In order to verify the applicability and accuracy of the developed method, this method was applied to the measurement of REEs in spiked and unspiked seawater samples under the optimum condition described above. The analytical results of REEs concentrations in seawater samples collected from Xisha sea area are presented in **Table 5**. The total REEs concentrations in seawater samples ranged from 0.025 μg L^{-1} to 0.172 μg L^{-1}. It could be noted that the mean concentrations of light REEs (ranging from La to Nd) were between 0.031 μg L^{-1} and 0.172 μg L^{-1}, while the medium REEs (ranging from Sm to Ho) concentrations were between

$0.025\ \mu g\ L^{-1}$ and $0.033\ \mu g\ L^{-1}$, and the level of heavy REEs (ranging from Er to Lu) were among $0.026–0.029\ \mu g\ L^{-1}$. Generally, cerium ($0.172\ \mu g\ L^{-1}$) was the most abundant element, followed by neodymium ($0.112\ \mu g\ L^{-1}$). The obtained results were compared with those obtained using other SPE method reported previously (e.g., $0.007–0.138\ \mu g\ L^{-1}$) (Karada et al., 2011), which were well within one order of magnitude. The recoveries of the varying spiked samples were between 95.3 and 104.4%, indicating that there was no significant loss of REEs during the extraction and elution process with the present method. The results clearly demonstrated the successful application of proposed method in the determination of ultra-low REEs in natural seawater samples.

CONCLUSION

After optimization of the separation and pre-concentration of REEs with crap shell particles as a unique sorbent, the results of accuracy and sensitivity for 16 REEs were sufficient for their precise determination in seawater samples. Based on the obtained results, it can be summarized that: 1) Biosorption experiments indicated that CSPs exhibited a high biosorption capacity towards REEs ($1.246–1.250\ mg\ g^{-1}$), which was capable to meet the demand of subsequent experiments. 2) The preconcentration of REEs by CSPs was found to be pH dependent, with optimal recoveries occurring at pH 3.0–10.0. Furthermore, desorption process revealed that REEs retained on CSPs could be eluted with 2 ml 4 M HNO_3 solution. The developed method was successfully applied to the analysis of high salinity samples (35‰), since severe matrix interference could be effectively eliminated. 3) The detection limits of REEs achieved $0.0006–0.0088\ \mu g\ L^{-1}$ and the precision of REEs values was in the range of 0.55–1.39% (RSD). 4) The proposed method has been validated by analysis of natural seawater samples to confirm its sensitivity and reliability, with the recoveries ranging from 95.3 to 104.4%. Overall, rapid sorption process, high biosorption capacity, and performance of simplicity, accuracy coupled with cost-effectiveness make crab shell particles-based pretreatment procedure a powerful and attractive approach for the determination of ultra-low REEs in seawater.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

DL designed and performed the experiments, analyzed the data and completed the articles. XW and KH provided the guidance on the conception and design of this study. ZW provided the guidance on data analysis and structure. XW, KH, and ZW contributed to the revision and editing of manuscript. All authors conducted the experiments and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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METHODS
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Simultaneous Determination of Active Clinical Components of Teicoplanin and Ramoplanin in Environmental Water by LC-MS/MS Coupled With Cascade Elution

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A simple, sensitive, and simultaneous method was established and validated for the active clinical components of teicoplanin and ramoplanin in environmental water by LC-MS/MS coupled with cascade elution. Moreover, a cascade elution method, which was rapid, solvent-less, and high-extraction efficient was successfully proposed to realize the extraction and purification of seven targets in one step. Under optimized conditions, the method showed excellent linearity with the correlation coefficient (R^2) ≥0.998 in the range of 1.0–100.0 ng L^{-1}. Low matrix effects and good recoveries which ranged from 86 to 114% were reached with RSDs lower than 3.0% for most targets. The limits of detection and limit of quantification were 0.1–1.3 and 0.3–4.0 ng L^{-1}, respectively. This method was successfully applied for the determination of teicoplanin and ramoplanin in water samples from the Pearl River and the South China Sea. TA2-2,3 was quantified in only one sample with the concentration of 8.0 ng L^{-1}.

Keywords: teicoplanin, LC-MS/MS, cascade elution, environmental water, ramoplanin

INTRODUCTION

Antibiotics are an effective approach for the treatment of various bacterial infections and animal growth promotion. However, the overuse of antibiotics leads to increased antibiotics residues in the environment, thereby increasing drug resistance, which has become a global issue (Carvalho and Santos, 2016; United Nations (UN), 2016). In the past 10 years, glycopeptide antibiotics have been incrementally used as the last resort for the clinical treatment of serious Gram-positive bacterial infections (Wilson, 2000; Binda et al., 2014). Among glycopeptide antibiotics, teicoplanin (TEC) and ramoplanin (RAM) are commonly used and studied antibiotics in clinical settings (Farver et al., 2005; Tanwar et al., 2014). TEC, extracted from *Actinoplanes teichomyceticus,* is used to treat various serious Gram-positive bacterial infections, especially methicillin-resistant *Staphylococcus aureus* (MRSA) infections (Cavalcanti et al., 2010). Similarly, RAM is a novel antibiotic with unique antibacterial mechanisms and significant activities against MRSA and vancomycin-resistant *Clostridium difficile* (Farver et al., 2005). However, its widespread clinical applications pose a potential threat to environmental water. The glycopeptide was present in environmental water from 12.68 to 24.25 μg L^{-1} (Soran et al., 2017). TEC and RAM are transferred to the environmental water through various transfer pathways, including the pharmaceutical factory wastewater, laboratory waste, hospital wastewater, and domestic sewage (Feng et al., 2020). The TEC contents in patient feces (de Lalla et al., 1992) and urine (Riva et al., 1987) were 118–2413 and 0.1–10 mg L^{-1}, respectively. The residues of TEC and RAM could induce antibacterial resistance through agricultural irrigation, aquaculture, and direct drinking (Sartelli, 2010; WHO, 2014). These residues, even at a lower concentration, are a threat to the microbial flora and aquatic animals, increasing the risk of aquatic ecological imbalance (Roose-Amsaleg and Laverman, 2016; Grenni et al., 2018). To the best of our knowledge, very few methods have been reported for the analysis of TEC and RAM in environmental water. Therefore, it is of great significance to establish a reliable detection method for TEC and RAM in environmental water.

TEC and RAM have high molecular weight and multicomponent substances, which primarily contain cyclic polypeptides (connected by some amino acids), glycosyl groups, and lipophilic side chains. TEC, mainly composed of TA2-1, TA2-2, TA2-3, TA2-4, TA2-5, and TA3-1, has similar structures but diverse antibacterial activities (Parenti et al., 1978). Ramoplanin is a mixture of three components, namely, ramoplanin A1–A3 (RA1, RA2, and RA3). RA2 is used individually in the clinical application due to its unique antibacterial activities (Cavalleri, 1984). Thus, TA2-1, TA2-2, TA2-3, TA2-4, TA2-5, and TA3-1 in TEC and RA2 in RAM were selected as the analytical objects to improve the practicality and efficiency of the analysis.

So far, many analytical methods have been developed for the determination of TEC and RAM, including amicrobiological assay (Awni et al., 1991), fluorescence polarization immunoassay (Xu and Käll, 2002; Ali et al., 2020), nano-gold fluorescence assay (Teepoo et al., 2013), micellar electrokinetic chromatography (Tsai et al., 2009), HPLC-UV (Riva et al., 1987), HPLC-ELSD (Song et al., 2018), and LC-MS/MS (Ewles et al., 2011; Begou et al., 2017). Among these methods, LC-MS/MS has the unique advantages of high sensitivity, high selectivity, and stability for multicomponent glycopeptide antibiotics. Hence, it is regarded as the gold standard for traceability and quality (Castro-Puyana et al., 2017). Begou et al. (2017) introduced the LC-MS/MS method for determining teicoplanin (TA2-2). Ewles et al. (2011) validated a bioanalytical method for the quantification of RAM using LC-MS/MS. Thus, LC-MS/MS was selected in this study.

The solid phase extraction method is a simple, efficient, and mature pretreatment technology for trace-level analysis of antibiotics in environmental water, enriching the analytes and removing impurity interference (Ongay et al., 2012; Sadutto and Picó, 2020). However, a simple elution usually cannot obtain a good elution rate for all targets simultaneously in terms of different multicomponent or multi-type antibiotics (Kang et al., 2010; Wei et al., 2014; Botero-Coy et al., 2018). The cascade elution is based on a profound understanding of all targets. First, all targets are classified and ranked in advance according to some attributes, such as polarity and pKa, and then one or a series of solvent systems is designed for accurate extraction of targets. Secil Yilmaz Turan classified the ingredients in wheat bran, ensured the extraction sequence of these compounds, and used a cascade method to obtain good extraction of proteins and feruloylated arabinoxylans (Yilmaz-Turan et al., 2020). Paola Imbimbo also separated active phycocyanin and fatty acids from *Galdieria phlegrea* through the cascade method (Imbimbo et al., 2019). The cascade method is rapid, solvent-less, and highly efficient, which was successfully proposed to realize the extraction and purification in one step. In this study, the conditions of instrument and pretreatment were optimized. The cascade elution method was developed for detecting the active clinical components of teicoplanin and ramoplanin in environmental water by LC-MS/MS. This method was applied to real environmental samples, including river water, lake water, aquaculture water, and sea water.

MATERIALS AND METHODS

Reagents and Materials

Acetonitrile (ACN), methanol (MeOH), ethyl acetate (EAC), and cyclohexane (CYH) of LC-MS grade were obtained from Merck (Darmstadt, Germany). Other solvents, including formic acid (FA), ammonium acetate, and ammonium hydroxide solution (25–28%), were obtained from Tokyo Chemical Industry (Tokyo, Japan), Aladdin (Shanghai, China), and Macklin (Shanghai, China), respectively. Glass microfiber filters (GF/F grade) were purchased from Whatman (Buckinghamshire, United Kingdom). Ultrapure water (18.2 μs cm^{-1} at 25°C) was prepared by the Genie 15 system of RephiLe (Shanghai, China). Sep-pak@vac C_{18} SPE cartridge (200 mg, 3 ml), Oasis HLB SPE cartridge (200 mg, 6 ml), and Oasis WCX SPE cartridge (200 mg, 6 ml) were supplied by Waters (Milford, MA, United States). Superlclean SCX SPE cartridge (500 mg, 3 ml) was supplied by Superlco Corporation (Beffefonte, United States). BE Carbon-300NH_2 SPE cartridge (500 mg, 6 ml) and Bond Elut-SAX (500 mg, 6 ml) were purchased from Agilent technologies (CA, United States). The solid-phase extraction procedure was performed using 24-port Visiprep SPE vacuum manifold with a minipump from Agela (Tianjin, China).

Standards and Stock Solution

Teicoplanin (TEC, purity 98%, containing TA3-1, TA2-1, TA2-2, TA2-3, TA2-4, and TA2-5) was purchased from Standards (Shanghai, China) and ramoplanin (RA2, purity 99%) was purchased from TRC (Toronto, Canada). The internal standard polymyxin B sulfate (PMB, purity 91%) was obtained from Dr. Ehrenstrofer GmbH (Augsburg, Germany). The stock solutions (1.0 mg ml^{-1}) for RA2, TEC, and PMB were prepared by 0.1% FA aqueous solution and stored in the dark for 3 months at −20°C. The mixed standard working solution (10.0 μg ml^{-1} for TEC and RA2) and the internal standard (1.0 μg ml^{-1}) were prepared by diluting each stock solution with MeOH-0.1% FA aqueous solution (50:50, v/v) in a brown glass bottle and stored at 4°C for a month.

Mass Spectrometry

The Agilent 6470B triple quadrupole mass spectrometric system was employed for mass spectrometry condition analysis. The

TABLE 1 | MRM parameters of all target peaks and the internal standard.

Compound name	Formula	RT	Precursor ion (m/z)	Product ion(m/z)	FV (V)	CE (V)	CAV(V)
Teicoplanin A3-1	$C_{72}H_{68}Cl_2N_8O_{28}$	1.446	782.4	204.1[a] 137.8	135	11 48	5
Teicoplanin A2-1	$C_{88}H_{95}Cl_2N_9O_{33}$	1.815	939.9	316.2[a] 298.1	150	10 26	5
Teicoplanin A2-2 and A2-3	$C_{88}H_{97}Cl_2N_9O_{33}$	1.813	940.3	316.3[a] 204.1	150	10 17	5
Teicoplanin A2-4 and A2-5	$C_{89}H_{99}Cl_2N_9O_{33}$	1.991	947.7	330.1[a] 203.9	145	10 20	5
Ramoplanin A2	$C_{106}H_{170}ClN_{21}O_{30}$	2.222	1277.7	1196.7[a] 1115.6	170	45 44	5
Polymyxin B (IS)	$C_{55}H_{96}N_{16}O_{13}$	1.448	402.1	101.1[a] 123.1	115	29 37	5

[a] *means quantitative ion.*

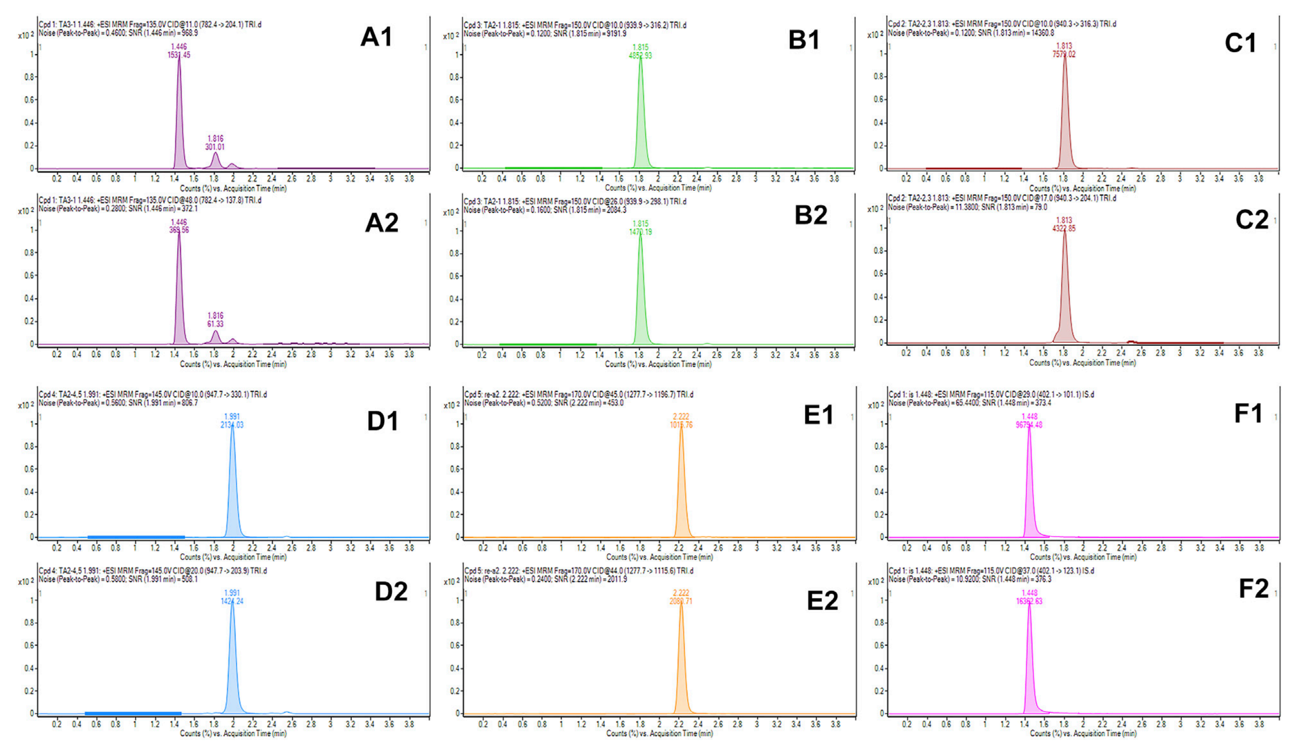

FIGURE 1 | Representative chromatograms obtained from the calibrator standard at 100 ng ml^{-1} for teicoplanin, ramoplanin, and the internal standard. **A1**, **A2**, **B1**, **B2**, **C1**, **C2**, **D1**, **D2**, **E1**, **E2**, **F1**, and **F2** refer to TA3-1 (782.4/204), TA3-1 (782.4/137.8), TA2-1 (939.9/316.2), TA2-1 (939.9/298.1), TA2-2,3 (940.3/316.3), TA2-2,3 (940.3/204.1), TA2-4,5 (947.7/330.1), TA2-4,5 (947.7/203.9), RA2(1277.7/1196.7), RA2 (1277.7/1115.6), IS (402.1/101.1), and IS (402.1/123.1), respectively.

electrospray ionization (ESI) source in the positive ion mode was selected for the analytes. The following mass spectrometer parameters were adopted: drying gas temperature, 300°C; the flow rate of drying gas, 5 L min^{-1}; nebulizer gas pressure, 35 psi; sheath gas temperature, 325°C; the flow rate of sheath gas, 9 L min^{-1}; capillary positive voltage, 3500 v; and nozzle-positive voltage, 500 v. The multiple reaction monitoring (MRM) mode was used to analyze each component. The detailed optimal parameters of the mass spectrum, including fragmentor, collision energy, cell accelerator voltage, and mass transitions, are listed in **Table 1**. The data were handled by Agilent MassHunter qualitative analysis 10.0 and Agilent MassHunter quantitative analysis for QQQ 10.1 equipped with the system.

Liquid Chromatography

The Agilent 1290 UHPLC system was employed to optimize the liquid chromatography conditions. The SB C_{18} REHD 2.1 × 100 mm (1.8 μm) column (Agilent Technologies, United States) was applied for chromatographic separation. The injection volume was 10 µL, and the autosampler tray temperature was stabilized at 20°C, whereas the column oven was maintained at 35°C. The mobile phase consisted of water (A) and acetonitrile (B), containing 0.1% (v/v) formic acid (FA). The total analysis time was 4 min at a flow rate of 0.35 ml min^{-1}. All the analytes were well-separated sequentially under the following linear gradient: 0 ~ 0.5 min, 5% B; 0.5 ~ 1.5 min, 5–30% B; 1.5 ~ 2.4 min, 30–40% B; 2.4 ~ 3 min, 95% B; and 3 ~ 4 min, 5% B. The chromatogram of each component is shown in **Figure 1**. (The chromatograms of real water matrix at the spiked concentration of LOQs for each target are shown in **Supplementary Figure S1**).

Sample Preparation

The HLB cartridge was selected to enrich and purify the sample after filtering by 0.7-µm glass microfiber filters. First, the HLB cartridge was activated with 1 ml methanol and equilibrated with 1 ml water. Then, the 1-L sample with 20 ng L^{-1} internal standard was automatically loaded onto the SPE device through a vacuum pump at a flow rate of 2 ~ 3 ml min^{-1}. After sample loading, it was washed with 1 ml of water and eluted with 1 ml of methanol and 1 ml of 20% (v/v) methanol water (containing 0.5% formic acid) sequentially at a flow rate of 0.5 ml min^{-1}. Finally, the eluent was determined by the LC-MS/MS method after being vortexed.

Matrix Effect

The matrix effect (ME) is usually caused by the matrix components extracted with the analyte, which could inhibit or enhance the ionization response of the analyte under ESI conditions. It is calculated by comparing the slope of the standard curve ($R_{standard}$) with the slope of the matrix standard curve (R_{matrix}), as per the formula $[(R_{matrix}-R_{standard})/R_{standard}] \times 100\%$. A positive value of the ME refers to the signal enhancement of targets, while a negative value indicates signal suppression. Overall, the ME around −20~20, −20 ~ −50, or 20–50% and > ±50% shows weak, moderate, and strong matrix effects, respectively (Economou et al., 2009).

RESULTS AND DISCUSSION

Mass Spectrometry Optimization

Jaewan Jung (Jung et al., 2019) used PMB as the internal standard of TEC due to lack of hydrogen isotope internal standard of TEC and RAM. Based on the similar structures of TEC and RAM, PMB was selected as the internal standard for this study. The electrospray ionization (ESI) source operating conditions were optimized by injecting 1.0 µg ml^{-1} of the analyte solution. First, the most abundant m/z value was selected as the precursor ion through the full-scan mass spectrum. The positive ESI mode was selected due to weak signal or no signal of targets in the negative ESI mode (Peteghem et al., 2003). Unlike some single small molecules, TEC and RAM produced abundant interfering fragments, interference between components, and relatively low molecular ions with different charges. It was inferred that they do not exist in the standard substance or do not completely dissolve in the solvent. Furthermore, the 0.1% formic acid aqueous solution, water, methanol, and acetonitrile were compared to obtain a fine solvent of targets. The results showed that the 0.1% formic acid aqueous solution was the best. Then, the $[M + H]^+$ ion, [M + 2H]2+ ion, and [M + 3H] 3+ ion of each target were compared to obtain the most abundant ion. The $[M + 2H]^{2+}$ ion was found with a best response toward TEC and RAM, and the $[M + 3H]^{3+}$ ion was most suitable for PMB among these three ions due to its strong response intensity. Since each mass spectrum has different resolutions, the ions with the closest mass-charge ratio to the theoretical precursor ions and the highest response value were selected as the actual precursor ions. At the same time, the full scanning spectrum generated by multiple injections was confirmed. Later, these precursor ions were used to produce the daughter ions, and each analyte was monitored by one precursor ion and two daughter ions. The fragmentor, collision energy, cell accelerator voltage, and other parameters were optimized. Also, the best ESI conditions, such as sheath gas temperature and the flow rate of sheath gas, were acquired. It was worth noting that TA2-2 and TA2-3 were a pair of isomers with the same mass spectrum performance and TA2-4 and TA2-5 were also the same.

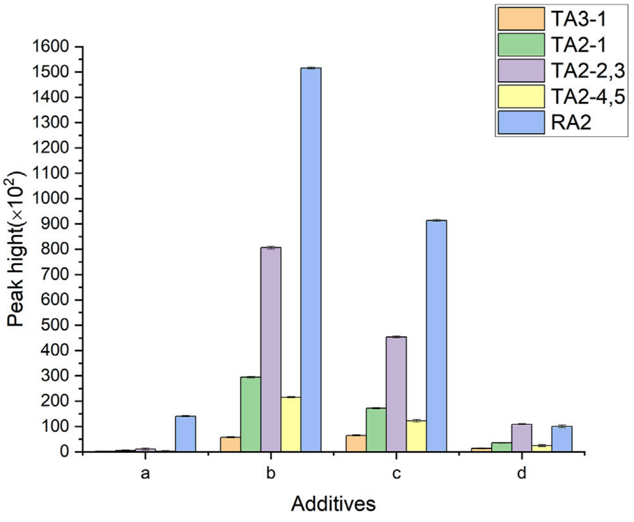

FIGURE 2 | Effect of different additives on the sensitivity of the analytes. **(A–D)** refer to the mobile phases without formic acid, the mobile phases with 0.1% formic acid, the mobile phases with 0.2% formic acid, and the mobile phases with 10 mM ammonium acetate.

Chromatographic Optimization

SB-C_{18} 1.8 μ m 2.1 × 100 mm was selected to obtain good sensitivity and peak shape. Then, methanol and acetonitrile were contrasted for the elution experiment. Acetonitrile exerted a better separation effect for each component. It was showed that 0.1% formic acid improved the peak shape in **Figure 2**, by comparing with 0.2% formic acid and 10 mM ammonium acetate. The best elution procedures were obtained by adjusting the elution ratio and gradient, testing the column equilibrium time, washing time, and the stability of the column separation. The flow rate of the mobile phase and column temperature was

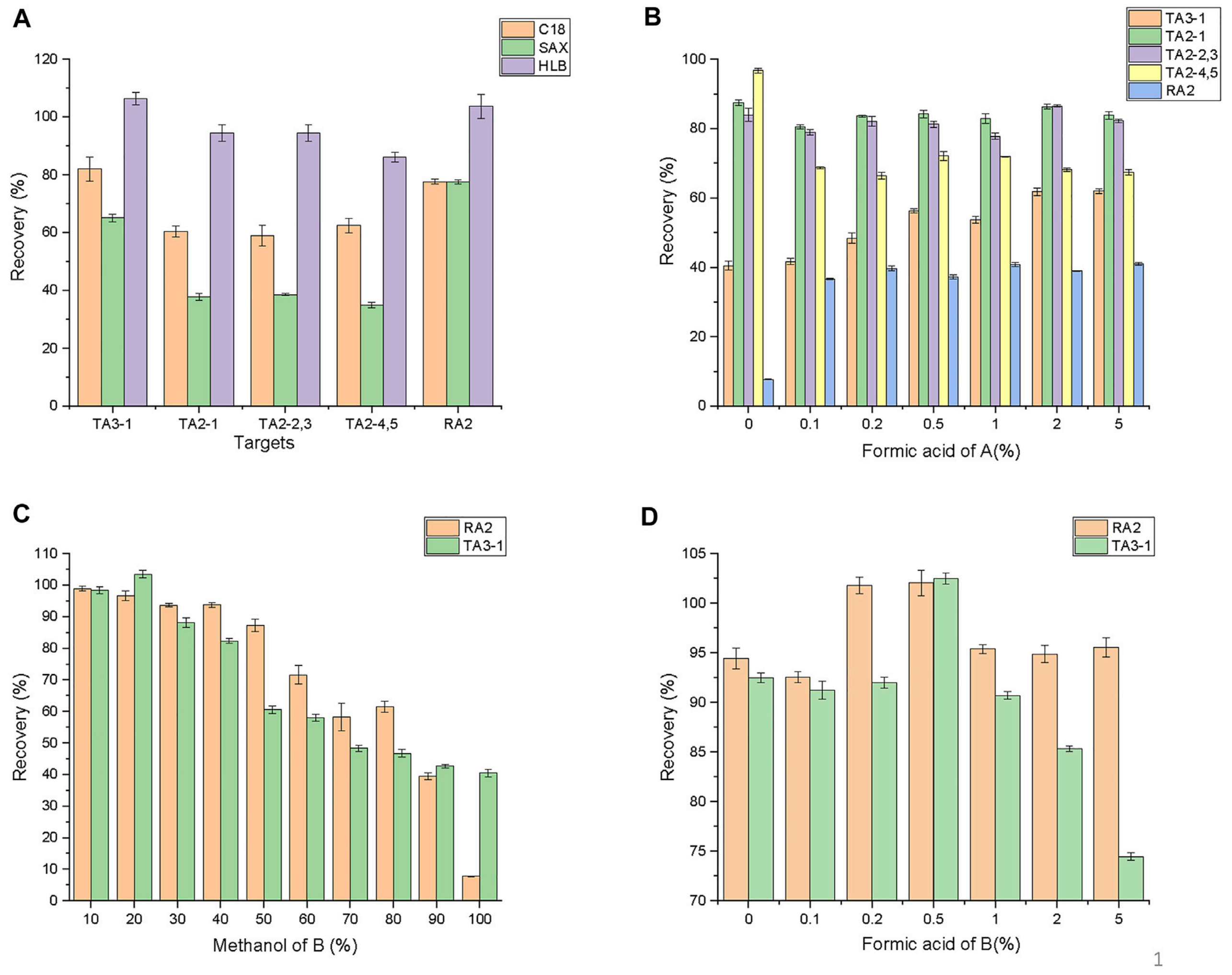

FIGURE 3 | Optimization of SPE procedures. **(A)** for SPE columns; **(B)** for concentrations of formic acid in eluent A, formic acid of A means the concentrations of formic acid in methanol; **(C)** for contents of methanol in eluent B, methanol of B mean the concentrations of methanol in water; **(D)** for contents of formic acid in eluent B, formic acid of B means the concentrations of formic acid in 20% methanol water.

also obtained to ensure the efficiency of the analysis and the separation ability.

Optimization of Sample Pretreatment

Selection of the SPE Column

TEC and RAM are amphoteric compounds with strong polarity, which are easily soluble in water and hardly dissolve in nonpolar solvents. The pK_a of teicoplanin is 5.66, but of RAM is 8.0 (Bardone et al., 1978; Cavalleri et al., 1984). Six kinds of SPE columns, including C_{18} SPE cartridge, HLB SPE cartridge, SCX strong cation exchange cartridge, WCX weak cation exchange cartridge, carbon-300NH_2 forward amino extraction cartridge, and SAX weak anion exchange cartridge, were selected to extract 5 ml of the 10-μg ml^{-1} mixed standard solution. SCX, WCX, and carbon-300NH_2 had low recovery (less than 30%) as teicoplanin was slightly acidic and lost loading. As shown in **Figure 3A**, the adsorption and elution ability of the HLB SPE cartridge (86.09–106.4%) was better than that of the C_{18} SPE (58.91–81.91%) and SAX SPE cartridges (34.89–65.01%) for all targets. Thus, the HLB SPE cartridge was selected for the SPE column. The optimal activation (in **Supplementary Figure S2**) and washing (in **Supplementary Figure S3**) procedures of SPE are summarized in the supplementary information.

Optimization of Cascade Elution

The cascade elution was designed carefully according to the TEC and RAM characteristics. TEC and RAM showed good water solubility and strong polarity. TA3-1 has strong polarity due to the lack of long lipophilic side chains in the six main components of TEC. Compared with TEC, RAM possesses good hydrophilic property on account of dispersed benzene rings and more hydrophilic groups, including amino, imino, carbonyl, and phenolic hydroxyl. Thus, TA2-1, TA2-2, TA2-3, TA2-4, and TA2-5 with low polarity were eluted as the first type of the targets (A). Then, RA2 and TA3-1 were eluted as the second type of the targets (B). Methanol possesses a strong elution ability

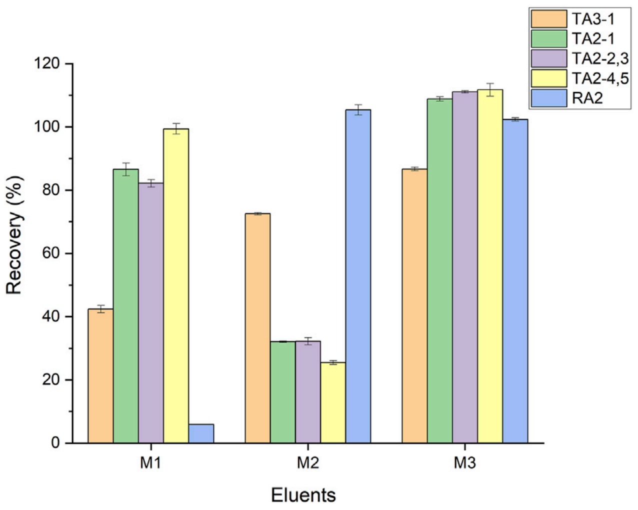

FIGURE 4 | Comparison of the cascade elution and other simple modes of SPE procedures. M1, M2, and M3 means methanol, 0.5% FA in 20% methanol, water, and cascade elution (methanol and 0.5% FA in 20% methanol water), respectively.

compared with that of other pure solvents in SPE. Formic acid and water have good polarity and could produce competitive hydrogen bonding with the targets. Hence, one or a series of solvent systems similar to the polarity of the target substance was prepared by adjusting the proportions of methanol, formic acid, and water to ensure good purification.

First, methanol and different acidic concentrations of methanol were compared to achieve good elution efficiency of TA2-1, TA2-2, TA2-3, TA2-4, and TA2-5. As shown in **Figure 3B**, methanol was the best (83.9–96.74%). Additionally, with the increase in acidity, the elution efficiency of TA2-4 and TA2-5 dropped from 96.7 to 67.4%, whereas the elution efficiency of TA2-1, TA2-2, and TA2-3 was stable at around 82%. Notably, the elution efficiency of TA3-1 increased from 41.7 to 61.9%, and the elution efficiency of RAM increased from 7.7 to 41.0% under this condition, indicating that formic acid improved the elution efficiency of TA3-1 and RAM. The reason could be the acidity of the solvent close to pK_a of phenolic hydroxyl or carboxyl in the molecule. It is noteworthy that the excessive use of the extractant could be helpful to the elution efficiency of the targets and might extract more impurities. With the increase in methanol, the elution efficiency of all the components increased slowly with less than 2% growth, as shown in **Supplementary Figure S4**. Therefore, 1 ml was considered as the most suitable elution volume.

Then, RA2 and TA3-1 were isolated from the HLB SPE cartridge. Remarkably, the increase in formic acid might lead the elution efficiency of RAM and TA3-1 to 80%. Meanwhile, it might cause their degradation and not reach satisfactory recovery (Wang et al., 2020). Thus, a solvent with suitable polarity was explored by adjusting the proportion of methanol, water, and formic acid. As shown in **Figure 3C**, with the increase in methanol proportion, the elution efficiency of TA3-1 increased and then declined from 103.4 to 40.4%, while the elution efficiency of RA2 dropped after the methanol proportion was increased by 50%. Moreover, RA2 and TA3-1 acquired the best elution efficiency by 20% methanol aqueous solutions. Afterward, different concentrations of formic acid were added with 20% methanol aqueous solutions. As shown in **Figure 3D**, the elution efficiency of RA2 and TA3-1 increased by 0–0.5% formic acid and decreased by 0.5–5% formic acid, especially TA3-1. Furthermore, the 20% methanol aqueous solution with 0.5% formic acid had the optimal recoveries (102.0–102.5%). Hence, it was confirmed as the second eluent of the cascade elution (optimization of the elution volume is shown in **Supplementary Figure S5**). Finally, the cascade elution was validated and it achieved the best elution, compared with 20% methanol (containing 0.5% formic acid) and methanol (**Figure 4**). The whole elution process takes 4 min, and only 1.2 ml of methanol is used.

Method Validation

The method was evaluated by the linearity, sensitivity, accuracy, and precision in real samples under the best conditions. Ultimately, the effectiveness and applicability of the method were ensured.

Linearity and Sensitivity

The linearity of this method was assessed by the correlation coefficient obtained from the calibration equation. As summarized in **Table 2**, the correlation coefficient (R^2) of all components was greater than 0.998, with the linear range of 1–100 ng ml^{-1}. The sensitivity was evaluated by the limit of detection (LOD) and limit of quantification (LOQ). Specifically, the LOD is defined by the signal-to-noise ratio (SNR) of 3 and the LOQ is calculated by the SNR of 10. The results suggested that the LODs and LOQs of all analytes were in the ranges of 0.1–1.3 and 0.3–4.0 ng L^{-1}, respectively. The detailed procedures are demonstrated in **Table 2**, which indicated that this method had good selectivity, satisfactory linearity, and significant sensitivity.

TABLE 2 | Linearity, sensitivity, and matrix effects of the developed method for analytes.

Analytes	R2	Linear range (ng ml-1)	The real sample (ng L^{-1})		ME%	
			LOD	LOQ	Sea water	River water
TA3-1	0.999	1–100	0.1	0.3	−20	−23
TA2-1	0.998	1–100	0.6	1.8	−23	−31
TA2-2,3	0.999	1–100	1.0	2.9	−23	−33
TA2-4,5	0.998	1–100	0.3	1.1	−17	−34
RA2	0.999	1–100	1.3	4.0	−10	−13

TABLE 3 | Precision and recovery of analytes in two samples (n = 9).

Sample type	Target	Spiked levels (ng L^{-1})			Intra-day						Inter-day					
		Low	Middle	High	Low		Middle		High		Low		Middle		High	
					Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Sea water	TA3-1	6	12	30	102.7	1.5	105.9	3.0	102.6	1.4	102.7	1.5	106.9	2.6	102.6	1.4
	TA2-1	6	12	30	93.7	2.2	97.8	2.6	102.4	1.5	92.8	1.5	98.6	1.8	102.3	1.4
	TA2-2,3	6	12	30	90.4	2.7	95.6	2.5	101.9	0.7	90.9	1.5	96.7	2.3	101.8	0.8
	TA2-4,5	6	12	30	90.7	2.4	93.5	2.4	95.5	1.1	90.6	1.9	93.6	2.3	95.3	1.2
	RA2	4	8	40	106.8	2.7	107.4	1.8	110.9	0.4	108.7	1.5	107.7	1.9	111.4	0.7
River water	TA3-1	6	12	30	100.7	1.8	111.0	2.0	108.1	0.6	101.1	2.0	110.9	1.9	108.1	0.6
	TA2-1	6	12	30	89.1	2.6	100.5	1.6	102.3	0.9	88.7	2.8	99.9	2.3	102.2	1.0
	TA2-2,3	6	12	30	86.0	1.1	98.0	1.5	101.9	0.5	86.1	1.3	98.2	1.6	101.9	0.4
	TA2-4,5	6	12	30	93.9	1.6	86.2	1.6	95.0	1.1	94.2	1.8	86.5	2.4	95.0	1.1
	RA2	4	8	40	114.5	1.7	105.0	1.0	101.8	1.2	113.9	2.3	104.7	1.3	101.5	1.3

Precision and Accuracy

The precision and accuracy of this method were expressed as the relative standard deviations (RSDs) and recoveries, respectively. The spiked samples with three different concentrations were prepared using two different spiked samples, namely, sea water and river water. They were measured repeatedly after sample pretreatment. In **Table 3**, the intra-day and inter-day precision of all components in different samples were 0.5–3.0% and 0.4–2.8%, respectively. Furthermore, the recoveries of all targets in the intra-day and inter-day were 86.0–114.5% and 86.1–113.9%, respectively. Thus, this method had good precision and high accuracy.

Matrix Effect

In trace analysis, the influence of the matrix effect is not negligible. In the environmental water, water usually dissolves many organic and inorganic substances, including human medicines, nursing products, veterinary medicines, and industrial products. Therefore, two kinds of matrix samples were used to prepare a calibration working curve to determine, and the results are shown in **Table 2**. The matrix effect range of all components of TEC in sea water and river water was −17 ~ −34%, indicating the presence of medium ion inhibition in the enriched samples. The matrix effect range of RAM in sea water and river water was −10 ~ −13%, illustrating the presence of weak ion inhibition. The cascade elution method used a small volume of the solvent to efficiently elute the targets, leaving most of the impurities in the HLB SPE cartridge. Thus, a low matrix effect was obtained in this method.

Applications to the Real Sample

Since the coastal cities have developed industries, large urban populations and high consumption of antibiotics and their water resources are seriously threatened, affecting the entire water ecological environment once they flow into the sea. Thus, the samples were randomly collected from water resources, such as river water, lake water, aquaculture water, and sea water. The aquaculture water was collected from the Pearl River Basin (including Pearl River inlet, middle Pearl River, and Pearl River outlet). These water samples were collected in a 1-L brown glass bottle and stored at 4°C in the laboratory. Every sample was acquired by a 2-L professional sampler in accordance with the principles of random sampling. TA2-2,3 was found, and the concentration was 8 ng L^{-1} in one of these samples. The results showed that the method could be used for determining real complex samples.

CONCLUSION

In this study, a new method, based on the cascade elution procedure, has been developed for the simultaneous determination of seven active clinical components of TEC and RAM in environmental water. Additionally, a cascade method was successfully applied for elution and purification in only 4 min, showing selectivity and effectiveness. Moreover, the combination of the cascade elution with LC-MS/MS is fast and accurate for environmental water, as the consumption of the organic solvent is reduced in one step of the method. The proposed method was applied for the quantitative analysis of multiple environmental water samples. To the best of our knowledge, this is the first time that TA2-2,3 was detected in the lake of China with the concentration of 8 ng L^{-1}.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: Figshare [DOI: 10.6084/m9.figshare.16704544, 10.6084/m9.figshare.16704643, 10.6084/m9.figshare.16704664, and 10.6084/m9.figshare.16704670].

AUTHOR CONTRIBUTIONS

YY contributed to the conception and design of the study. HJ and CZ organized the database. HJ and CZ performed the statistical analysis. HJ wrote the first draft of the manuscript. CZ wrote sections of the manuscript. HJ and CZ contributed equally to this study. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2021.785408/full#supplementary-material

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Optimization of a Marine Fish Release Strategy: A Case Study of Black Sea Bream *Acanthopagrus schlegelii* in the Zhanjiang Estuary, Northern South China Sea

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The release strategy (choice of the species and locations stocked, releasing mode, and stocking size and times) is an important part of quantitative evaluations of marine fish stock enhancement, while optimization of the release strategy can contribute to assess the stocking success intended to alleviate declining fishery resources and to increase the income of fishers. In this study, a typical fish species released in the northern South China Sea, black sea bream *Acanthopagrus schlegelii*, was taken as the research object. The biological characteristics of this sparid were determined from samples collected from waters in the Zhanjiang estuary during June, July, and September 2020 to April 2021, and the data were applied to estimate its length frequency and its growth parameters using the ELEFAN I run in FiSAT II. We then simulated and evaluated the stocking effects of five scenarios under different release strategies, while assuming a fixed total quantity of released fish and a constant of mortality rate at different life stages. The results showed that (1) the breeding season of black sea bream in this region is mainly from December to March of the next year, which is also the period when most significant sexual reversal in this species occurs, and (2) the relationship between standard length and weight in black sea bream is $W = 5.092 \times 10^{-5} L^{2.906}$, $L_\infty = 54.39$ cm, $K = 0.15$, and $t_0 = -0.967$. (3) The recommended period to release black sea bream in Zhanjiang waters is from June to October. It appears more productive if the total quantity of fish released can be divided into two batches. The growth potential of released juvenile fish in this study was evaluated based on a density-dependent theory, and the stocking effect of released stocks was simulated with the consideration for biological parameters and field sampling technique. This study provides a reference for the optimization of fish release strategies in coastal waters.

Keywords: stock enhancement, simulation model, optimization of releasing strategy, *Acanthopagrus schlegelii*, northern South China Sea

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INTRODUCTION

Overfishing, habitat degradation, and climate change exert continuous pressure on the global fishery (Jackson, 2001), with 34.2% of fisheries worldwide estimated to be overexploited in 2017 (FAO, 2020) and the proportion of fisheries deemed sustainable having decreased to 65.8% from 90% in 1974 (FAO, 2020). Sustainable fisheries contribute to the marine ecosystem functioning and to the livelihoods of the millions of fishermen, and provide a vital source of high quality of animal protein for human consumption. Ecosystem-based fishery management and the framework of responsible approach to stocking (Blankenship and Leber, 1995) hold tremendous potentials for increasing production for some capture fisheries, and many active steps such as the regulation of fishing efforts (Hammer and Truitt, 1942; Crutchfield, 1979), habitat restoration (Zaharia et al., 2014; Adams et al., 2019), and stock enhancement (Lorenzen et al., 2010; Johnston et al., 2018) have been put into practice.

Fish stock enhancement, which has been conducted worldwide since the 1970s, has been one of the most popular tools in fisheries management, but most studies have been in experimental stages (Kitada, 2018) and few reach their set goals (Li and Huang, 2011). The desired goals for stock enhancement include the enhancement of wild population, increase in food fish supplies, and improvement of fishermen's livelihood (Ingram and De Silva, 2015). The outcomes of stock enhancement will in turn affect the efforts of ranching activities and financial supports to the artificial propagation (Kellison and Eggleston, 2004; Garlock et al., 2017).

It is impossible to realize the desired stocking goals if simply releasing large quantities of larvae/juvenile fish, as the success of stock enhancement is affected by factors such as stocking density, age and size of fish released, condition and health of fish, the timing of stocking, and the habitat suitability in an active adaptive management framework (Camp et al., 2013). Since the evaluation of stocking success (including ecological and economic aspects) (Bell et al., 2006; Kitada, 2018) is time-consuming and expensive, modeling the stocking programs with simulation can help us assess and improve the stocking programs (Ingram, and De Silva, 2015). The evaluation of stocking effects is also a part of fish stock assessment; most of the stock assessment techniques can be used to estimate the stocks and biological parameters of released populations, such as natural mortality, release mortality (Benaka et al., 2014), post-release survival, and recapture rate (Kitada et al., 1992; Støttrup et al., 2002; Lyu et al., 2021). The optimization of the release strategy must consider the technique for breeding the stocked species and fishing, and also the trade-offs between the costs of breeding and the benefits of stocking (Kellison and Eggleston, 2004). Research has dealt with the release strategies such as white sturgeon *Acipenser transmontanus* (Botsford and Hobbs, 1984), optimal release scenarios for summer flounder *Paralichthys dentatus* (Kellison and Eggleston, 2004), choosing the candidate stocking species (Garlock et al., 2017), and optimal stocking quantities and management actions to be taken after the release (Liu et al., 1994). Using the example of black seabream *Acanthopagrus schlegelii*, this study aims to contribute more effective stock enhancement using fish growth parameters. Most stocking programs are biomass oriented, whereby increases in catch and revenue are considered the most important expectations among fisheries and fishers, especially in developing countries/regions.

The black seabream is a warm-water demersal sparid found mainly in sand/silt-bottom or rocky-reef habitats of shallow coastal waters (Wu et al., 2011). It is a prominent aquaculture species owing to its rapid growth rate, high adaptability in coastal waters, and high-quality protein, which commands a higher market price (Wu et al., 2011). A sharp decrease in sparid catches has prompted the stocking of black seabream in Chinese waters (Guo et al., 2021), with the quantity reaching 70.15 million individuals in 2017 (Ministry of Agriculture and Rural Affairs of the People's Republic of China et al., 2018), accounting for 0.27% of the annual total quantities of China (Tang, 2019). Previous studies have reported on the capture–recapture analyses of black seabream in Daya bay, northern South China Sea (SCS) (Lin et al., 2001; Liu et al., 2019). However, the growth potential of released juvenile fish and their contributions to population recruitment are largely unknown. Using examples of the wild stocks of black seabream in the coastal waters around the Zhanjiang estuary, northern SCS, we applied a fish growth model and the density-dependent theory to simulate release scenarios and the recruitment/stocking effects of the released stocks, with an aim to describe an optimal release scenario. This study offers an improved framework for quantitative analysis of marine fish stock enhancement.

MATERIALS AND METHODS

Sample Collection and Processing

A total of 273 wild black seabream *A. schlegelii* were collected from the wharf landings of fishermen who used gillnets, fishing tackle, or trawls in the sea near the Zhanjiang estuary, in June, July, and September of 2020 to April 2021. The fish samples were taken back to the laboratory for biological analysis, the standard length (L) and body mass (W) of the black sea bream were measured, and the stages of gonadal maturity were determined (Holden and Raitt, 1974). The length was accurate to 1 mm, and the body mass was accurate to 0.1 g.

Simulation Model

Estimation of the increase in body mass of released fish was based on biological data from black sea bream collected in the field. Simulation of the number and biomass of released stocks were based on the relationship between stocking density and mortality rates. To optimize the stocking effects of constant total quantity of released fish, we simulated five stocking scenarios with the months as the interval unit (**Table 1**). For simplicity of calculation, we assumed that the total number of fish released is 50,400, and the stocking times is 1, 2, 3, 4, and 7, respectively, in scenarios I, II, III, IV, and V, and then simulated the stocking effect in a maximum 6-month period and six more months after

TABLE 1 | Simulated stocking scenarios under different intervals of release with the constant of total quantity of fish released.

Scenario	Intervals of release/month	Events of stocking	Number of fish per release
I	0	1	50,400
II	6	2	25,200
III	3	3	12,600
IV	2	4	6,300
V	1	7	3,150

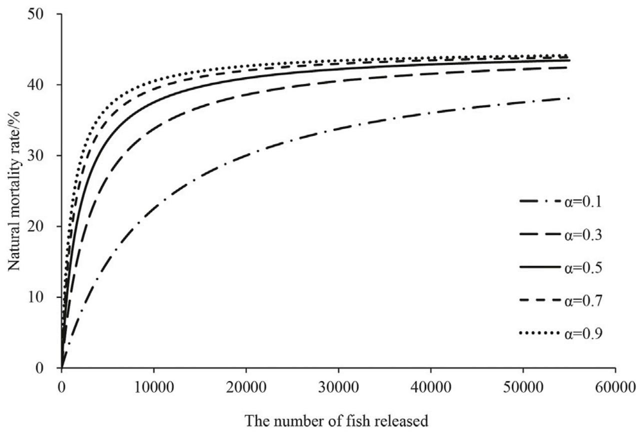

FIGURE 1 | The relationship between the natural mortality rate and the number of fish released based on a density-dependent theory. *α* is the constant of the specific habitat.

release. Density-dependent theory in ecology usually maintains a linear, inverse relationship between the population mortality and population density (Roughgarden, 1971; Herrando-Pérez et al., 2012; Lee and Strauss, 2016). Therefore, differences in stock density during a single release may lead to varied mortality rates. Combined with the total number of fish released and their estimated natural mortality rates, the changes of the number of released fish stocks and their corresponding weight in each period could be simulated. Refer to the Ricker model to predict exponential yield (Ricker, 1975). Finally, the total biomass (weight/quantity) of the released fish was accumulated, and the effects of the stocking scenarios were evaluated.

Hypothesis of the Simulation Model

The model assumes the following:

1) The mortality rate of released fish is restricted only by density. Although studies have shown that the mortality rate of released fish in wild conditions can vary with age and size (Lorenzen, 2000), in this study, the release interval of different batches was relatively short (months), and therefore, the effect of the size difference between the batches of released fish on mortality was ignored.
2) There is no significant interaction between wild and released fish stocks in stocking waters during this study.

Data Analysis

Estimation of Biological Parameters

The power function was used to fit the body length–weight relationship of the black sea bream stock (Ricker, 1973) as follows:

$$W = aL^b \tag{1}$$

where W is body mass (g), L is the length (mm), a is the condition factor, and b is the growth index. The larger value of a indicates the good growth conditions of fish and the value of b can test if the fish grow uniformly, and changes in the value may be related to the nutritional conditions and life stages of the fish.

Following the Snedecor statistical method of grouping by body length (Snedecor, 1980; Chen et al., 2008):

$$CI = \frac{R}{\left(\frac{R}{SD}*4\right)} \tag{2}$$

where CI is class interval, R is the range between the maximum and the minimum of body length, and SD is the standard deviation of body length. The body length data were grouped in increments of 18.5 cm, and the dominant group accounted for more than 10% of the total sample.

The ELEFAN I method of FiSAT II was used to analyze the body length frequency, estimate the growth parameters such as asymptotic body length (L_∞) and growth rate (K), and fit the von Bertalanffy growth equation of black sea bream (Munro and Pauly, 1983) (**Eqs 3**, **4**) is obtained by combining **Eqs 1–3**.

$$L_t = L_\infty \left(1 - e^{-K(t-t_0)}\right) \tag{3}$$

$$W_t = W_\infty \left(1 - e^{-K(t-t_0)}\right)^b \tag{4}$$

The t_0 value was estimated using Pauly's (Pauly, 1979) empirical equation:

$$\log_{10}(t_0) = -0.3922 - 0.2752\log_{10}L_\infty - 1.038\log_{10}K \tag{5}$$

Natural Mortality Rate of Released Fish Stocks

In certain areas, the natural mortality rate increased with an increase in juvenile fish density (Minto et al., 2008). The functional response curve (Lindholm et al., 2001) reflects the density–mortality relationship (**Figure 1**) and the number of juvenile fish released, then:

$$m = \frac{\alpha x}{1 + \alpha x} \mathrm{z} \tag{6}$$

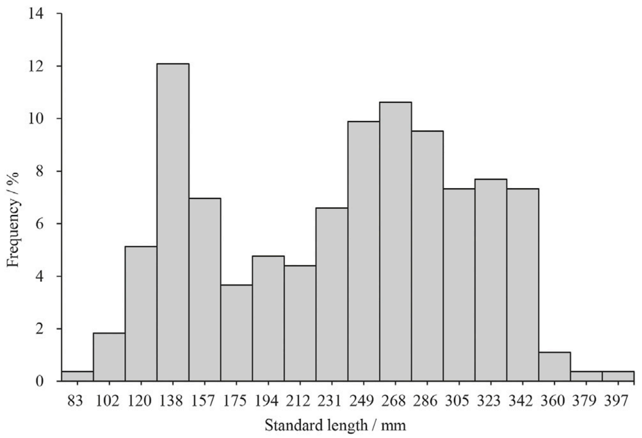

FIGURE 2 | Frequency distribution of *Acanthopagrus schlegelii* body length from Zhanjiang estuary waters, northern South China Sea.

where m is the monthly natural mortality rate. α is the constant of the specific habitat (which has little effect on the research results and thus 0.5 is taken for simplicity), x is the number of fish released/1,000, and z is the theoretical maximum mortality that can be achieved under the curve. The variable x is the density of released fish, but it is difficult to quantify due to the random and rapid dispersal of released fish in stocking waters. Therefore, the number of fish released is used to replace x, and the formula is modified accordingly. According to a simulated 30-day survival rate of black sea bream experiment (Zhou et al., 2020), the initial monthly mortality rate of 2,400 black sea breams was 24.5%, and thus z is approximately 0.45 in **Eq. 6**.

The number of released fish was calculated as:

$$N_t = (1 - m)N_{t-1} \tag{7}$$

where N_t is the survived number of fish at month (t), and $N_{t\text{-}1}$is the number of fish released in previous month (t–1).

Calculation of Resource Quantity

Biomass (B) is related to individual body mass and population at a given time (Zhan, 1995) (**Eq. 8**):

$$B_t = W_t * N_t \tag{8}$$

From **Eqs 2–6**, the biomass of black seabream released was calculated as:

$$B_t = W_\infty \left(1 - e^{-K(t-t_0)}\right)^b * \left(1 - \frac{\alpha x}{1 + \alpha x} z\right) * N_{t-1} \tag{9}$$

For scenarios with multiple releases, the previously released population will impact the calculation of mortality for the post-release population. When calculating the resource change under a multi-stage release strategy, the number of fish released should be added to the number of surviving fish in the previously released groups:

$$B_t = W_\infty \left(1 - e^{-K(t-t_0)}\right)^b * \left(1 - \frac{\alpha (x_i)}{1 + \alpha (x_i)} z\right) * N_{t-1} \tag{10}$$

where B_t is biomass at time t, N_t is the survived number of fish at month (t), $N_{t\text{-}1}$ is the number of released fish that survived in the previous month (t–1), and x_i is the total number of live fish in multiple batches in the last month/1,000.

RESULTS

Standard Length and Body Mass

A total of 273 black sea bream samples were collected in this study. The standard length (SL) range was 74–406 mm, and the average was 235.65 SL. The dominant body-length groups were 129–148 mm (12.09%) and 259–277 mm (10.62%) (**Figure 2**).

Gonadal Development and Sex Determination

The gonadal stages of the black seabream were evaluated and a coefficient of sexual maturity was calculated for fish in the sea area near the Zhanjiang estuary. The breeding period of the fish was from December to March of the next year. Among the 273 fish sampled, 131 females, 88 males, and six hermaphrodites were visually identifiable as the gonad differentiation was obvious. Monthly variation in the stages of gonadal development denoting males and females is shown in **Figure 3**. Gonads in stages IV and V appeared from December to March.

Length–Weight Relationship

The relationship between the body length and body mass showed a more-or-less equivalent increase in black sea bream in this sea area. The annual body mass relationship of black sea bream was $W = 5.092 \times 10^{-5} L^{2.906}$ ($R^2 = 0.971$) (**Table 2**). Comparing the body length and weight parameters in different seasons, the condition factor a was winter > spring > autumn > summer. The power index b is summer > autumn > winter > spring (**Table 2**). The b value in summer is greater than 3, reflecting positive allometric growth; the b value in autumn and winter is less than 3, indicating negative allometric growth.

Biological Parameter

The von Bertalanffy growth parameters were obtained by the ELEFAN I method using the time-series and body length frequency data of black sea bream (**Figure 4**) as follows: asymptotic body length $L_\infty = 54.39$ cm, growth rate $K = 0.15$, and $t_0 = -0.967$.

Variation of the Biomass Under Release Scenarios

Changes in the number and biomass of black sea bream (**Table 3**; **Figures 5**, **6**) indicated that dividing the release of black seabream into two batches was best. Five kinds of release scenarios were simulated (**Figure 7**), with each releasing a total of 50,400 juvenile black sea bream but at different intervals. The survival and biomass of the twice release strategies (dividing the total quantity of released fish into two

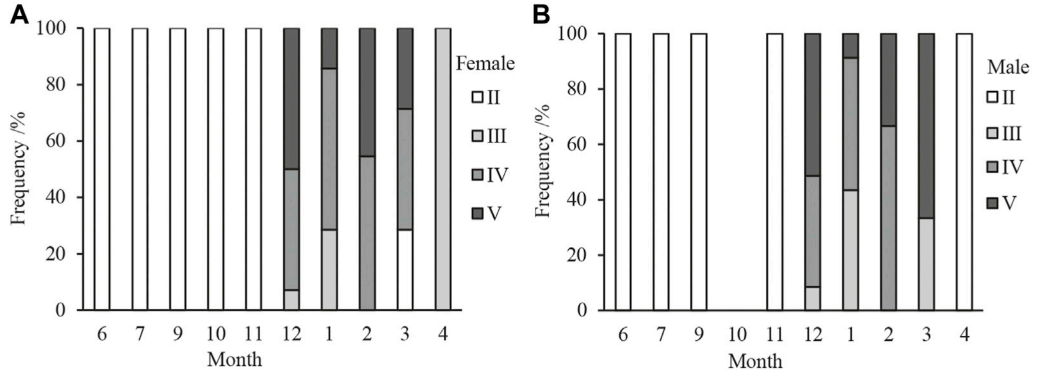

FIGURE 3 | Monthly variations in gonad stages denoting males and females of the *Acanthopagrus schlegelii* sampled from Zhanjiang estuary waters. **(A,B)** represented the males and females respectively.

TABLE 2 | Parameters of the length–weight relationship for *Acanthopagrus schlegelii*.

Season	*a*	*b*	R^2
Annual	5.092×10^{-5}	2.906	0.971
Spring	3.151×10^{-4}	2.578	0.853
Summer	1.390×10^{-5}	3.167	0.981
Autumn	6.602×10^{-5}	2.866	0.977
Winter	1.154×10^{-4}	2.758	0.918

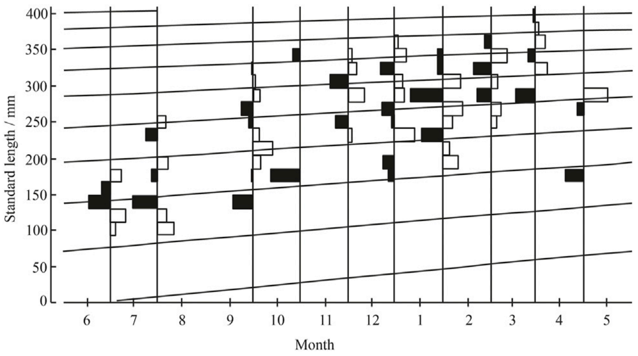

FIGURE 4 | Length frequency distribution data and growth curves estimated using ELEFAN method for *Acanthopagrus schlegelii*.

TABLE 3 | Simulation of percentages of the survived fish and stock size to the total amount of fish, respectively, 6 months after release finished under scenarios I–V.

Scenario	Number of survived fish/%	Stock size/%
I	1.78	10.57
II	5.50	15.81
III	4.82	14.70
IV	4.56	14.36
V	4.29	14.05

batches) in December accounted for 15.81% of the total release, and the remaining strategies decreased with more release events. A one-time release strategy had the worst effect because of the high mortality rate of released stocks, and the remaining amount is 1.78 and 10.57%. Trends in the biomass and the quantity of released juvenile fish were similar. Because the individual's natural growth rate is lower than the rate of natural mortality, all release curves showed a downward trend. One release–effect curve decreased monthly after a maximum in the first month, and the other release–effect curves exhibited peaks because of multiple release events. After the last releasing (in July), the stocks and biomass of the juvenile fish under the different release scenarios decreased at different rates because of no further supplement to the existing stocks.

DISCUSSION

Optimization of Stocking Strategies

In this study, the effects of different scenarios were compared based on growth parameters and the estimated mortality rate. The stocking effect of twice release strategy is the best among the five releasing strategies (**Figure 7**). In a practical fish stocking program, in order to achieve the effect of population replenishment, the number of released fish is usually very large (Tang, 2019), and the preferable released strategy needs to be optimized on species accordingly. The simulation model of Yamashita et al. (2017) of an optimum stocking density of juvenile flounder *Paralichthys olivaceus* showed that the most important factors were prey density and the number of wild juvenile flounder. If the population size is relatively high in a certain sea area, the natural regulation within the population would lead to a slower growth rate of the fish and an increase in mortality (Liu et al., 1994). In the stocking practice, multiple release sites can be selected in the target stocking sea area to reduce the drastic increase of mortality rate due to overhigh stocking density.

Studies have shown that the survival rate of released marine fish varied with seasons (Mckinnell and Lundqvist, 2000). Most of the release work of black seabream in the northern

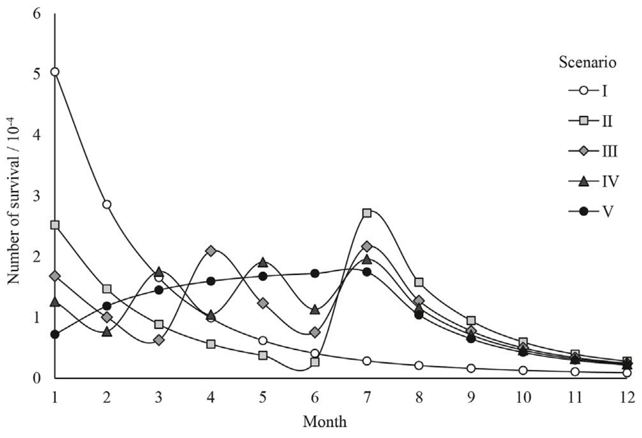

FIGURE 5 | Changes in the number survival of released fish under scenarios. Scenario I to V indicated that the total number of fish were released in one, two, three, four, and seven events, respectively, with different intervals during 6 months.

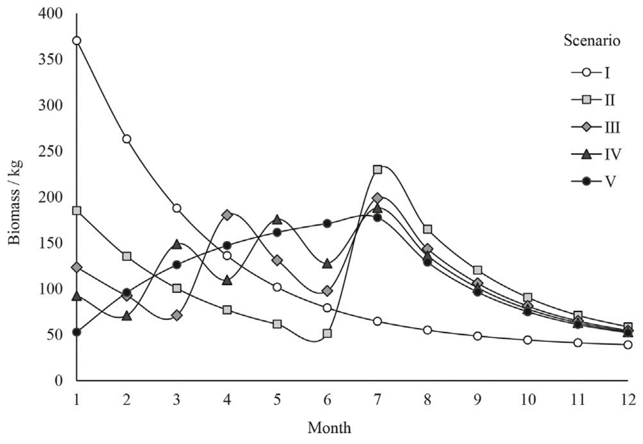

FIGURE 6 | Changes in the biomass of survived fish under scenarios. Scenario I to V indicated that the total number of fish were released in one, two, three, four, and seven events, respectively, with different intervals during 6 months.

SCS has begun in summer, and is expected to be finished in autumn every year. However, insufficient attention is paid to the actual effects of both the stocking interval and the quantity of fish released in a single event, with fish being released once to many times every year (Liang et al., 2010; Liu et al., 2019). Zhou (2004) summarized the releases of black seabream in the Xiangshan estuary from 1992 to 1994, which were mainly released from June to October, and the recapture rate was about 4.4%–6.05%. In all, the total released quantity of black sea bream subsequently increased year by year, with good economic benefits. However, few quantitative studies on costs and benefits of stock enhancement have been reported, with some qualitative reports. The recapture rate of black seabream in Daya bay in November 2014 was 7.76%, exceeding that record in June 2015 (3.66%) and July 2016 (2.49%), but that the better recapture effect in November was mainly due to the larger size of the released stocks (Liu et al., 2019). Xu et al. (2008) suggested that to avoid the phenomenon of black sea bream being caught immediately after stocking, releases are more appropriate in the closed fishing season, generally in summer and autumn—a time when the development of the released fish corresponds with the natural population. Based on the body length–weight relationship in this study, the *b* value was higher in summer, following the closed season for fishing in the SCS, which begins in May (Lin and Cheng, 2009). Therefore, June to October would be more appropriate for the release of black sea bream in Zhanjiang estuary waters. The swimming and disperse ability of black sea bream after release is strong, and the number of recaptures appears to be highest at 3–5 months after release, and decrease sharply thereafter (Liu et al., 2019). In combination with the optimal release season (June–October), the optimum release interval of black sea bream in the Zhanjiang estuary waters would be 5 months, with the fish first released in June, and again in October.

Factors Affecting the Stocking Effects

In this study, the release interval and natural mortality were considered to be the two most important factors on stocking effects. As for the release interval, a longer release cycle was expected to be better stocking results. The release interval constituted the biggest difference between the release strategies. Short intervals would amount to a more continuous release of the juvenile fish into the natural water, with each release event involving a relatively small number. However, shortened time intervals and insufficient dispersal of the previous batch of released fish allows for greater intraspecific competition among successive batches, with implications for survival. According to the density-dependent theory (Lorenzen and Enberg, 2002), increases in population density of the released fish lead to higher mortality in certain waters (Liu et al., 1994). With repeated releases, the increase in population density of the released fish leads to an overall high mortality rate, and thus the natural mortality for early released fish is high. In contrast, although secondary releases may involve a large number of fish, there will be less interaction owing to the longer interval, because half of them are released later, with a short period of natural morality, resulting in a larger number of final survivors. Using a yield-per-recruit model, Xu (2015) simulated a release and management strategy of Chinese white prawn *Penaeus chinensis*, and proposed that a postponed release time somewhat could achieve greater fishing yields. Therefore, appropriately extending the time interval between two releases but within the appropriate release period can reduce the impact of natural mortality on the released fish biomass.

Density-dependent mortality is a key consideration in the optimization of a release strategy, as the natural mortality importantly acts on the released fish. The natural mortality rates of fish populations vary greatly at different life stages

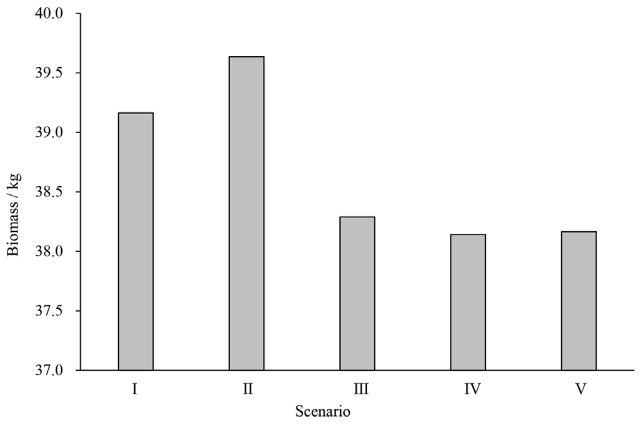

FIGURE 7 | Stocking effects of the released fish on biomass replenishing under scenarios. Scenarios I to V indicated that the total number of fish were released in one, two, three, four, and seven events, respectively, with different intervals during 6 months.

and are difficult to estimate accurately (Zhan, 1995). Some research believed that the rates of natural mortality were underestimated for released fish. The release strategy simulation by Xu (2015) deployed a constant mortality rate and assumed that the natural mortality coefficient did not conform to actual changes in the parameter, and this could affect the strategy optimization results. Lindholm (2001) studied the response of cod *Gadus morhua* larvae to changes in sea-bottom habitat based on three types of response curves depicting mortality and population density, and found that the relationship between survival rate and density of the cod was similar regardless of the type of curve. The purpose of the present study was to optimize the releasing strategy for stocking black seabream, and thus only change in one type of mortality rate was selected. There is an obvious upper limit in a mortality curve. In the case of a large quantity of released fish, the mortality rate increased, but this change did not affect the increasing trend of the amount after releasing. Therefore, although the quantity of released fish stocked in two events (scenario II in **Table 1**) is still high, the mortality rate will not be significantly higher than that with the other release strategies. In addition, factors affect the mortality rate of released fish, and will exert stocking effects even in circumstances of unknown mortality of released fish, but it will affect the actual stocking effects. Thus, simulation results can still provide a reference for the optimization of the release strategies.

Implications of Condition Factors for Fish Stocking

The results indicate that the growth of black seabream is better in waters of the Zhanjiang estuary area than in Daya bay because of the value of parameter *a* (**Eq. 1**) was higher for fish in the Zhanjiang estuary (4.37×10^{-5}), compared with that for those in Daya bay (1.55×10^{-5}) (Chen et al., 2019). Seasonal variation in *a* revealed the trend winter > spring > fall > summer. In general, the highest value of *a* reflects the stage of gonad maturity (Zhan, 1995). The gonad with maturity stage IV or V in the black seabream was observed mainly from December to March of the next year, when the inflated gonad occupied three quarters or more of the body cavity.

The values of the growth parameters reflect the impacts of environmental changes on life conditions of fish (Froese and Pauly, 2021). The *a* value of the stocks correspondingly varied with the different life stages of the black seabream. The growth index *b* (2.906) indicates the growth of fish expressed as isauxesis for length and body mass. The value of *b* was close to that for the black seabream in Daya bay (Chen et al., 2019) and in Taiwanese waters (Chu et al., 2011). Seasonal variations in *b* revealed the descending trend from summer, fall, winter, to spring in order. The higher *b* value in summer was possibly attributable to the rapid growth in body mass and faster development of gonads (Zhan, 1995; Chen et al., 2019). This research found a lower *b* value in winter compared with that in summer and fall, which was possibly caused by a shortage of food and the high energy costs in the winter. Furthermore, higher seasonal fishing pressure may lead to allometric growth as well as a decrease in the quantity of bait fish (Stergiou and Moutopoulos, 2001).

Gonad Development and Sex Change

Male-to-female sex change in black seabream is possibly related to the body shape (Todd et al., 2016). The black seabream is male in the first 2 years (Lee et al., 2001). The popular theory for explaining the gynandromorphy is the size-advantage hypothesis (Munday et al., 2006; Kazancıoğlu and Alonzo, 2010), which hypothesizes that the sex change of fish stocks is adaptive with the total reproductivity of the whole population, and that the body shape determines the direction of the sex change. Under this assumption, males that change to females gain more individual advantages, such as a bigger body shape and higher fecundity, which are beneficial for survival of the whole population (Gemmell et al., 2019). It is imperative to take a systematic consideration, incorporating the reproductive characteristics into the optimization of stocking practice, to attain the desired stocking goals.

CONCLUSION

We put forward a method for optimizing the fish release strategies based on the biological characteristics, growth parameters, and mortality rates of the stocking population, which is constructive to the quantifying of fish stocking effects. Using the example of the black seabream released into the Zhanjiang estuary and adjacent waters, we established a framework to improve the stock enhancement practice. Further research is needed on the contribution of the released stocks to the replenishment of fish population, as well as their interactions with the wild stocks.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Experimental Ethics Committee of Guangdong Ocean University, China.

AUTHOR CONTRIBUTIONS

SZ: Methodology and writing original draft preparation; LL: Fieldwork and experiments, data curation, and resources; XW: Supervision, conceptualization, reviewing and editing, and funding acquisition.

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Heavy Metals in Marine Surface Sediments of Daya Bay, Southern China: Spatial Distribution, Sources Apportionment, and Ecological Risk Assessment

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Daya Bay, especially in the northwestern region, which is a nature reserve with larval economic fish and shrimp populations, is no longer an unpolluted marine environment due to the recent increases in anthropogenic activities. This study collected seasonal surface sediment samples from 20 sites in northwestern Daya Bay to evaluate pollution and ecological risks and to identify possible sources and transport pathways of heavy metals (Cd, Pb, Cr, Cu, Zn, Hg, As). The results showed that all the heavy metal concentrations were below the established primary standard criteria, except for concentrations of Cr in spring, as well as Cu and Zn in autumn at several sampling stations, which had excess rates of 35, 4.76, and 4.76%, respectively. The geoaccumulation index (Igeo) values of heavy metals indicated that all sites had unpolluted to moderate pollution levels. In comparison to the samples collected in autumn, those in spring experienced a higher degree of pollution, particularly Cr and As. The ecological risk indices of heavy metals in sediments ranged from 225.86 to 734.20 in spring and from 196.69 to 567.52 in autumn, suggesting that most sites had a moderate ecological risk or a considerable ecological risk, and very few a had high ecological risk. Moreover, ArcGIS10.2 software was used to visualize their spatial distribution, and the results were similar in both spring and autumn. The results of the Pearson correlation analysis and principal component analysis showed that Cu, Hg, and Pb might be affected by anthropogenic activities, and As might be derived from natural sources such as atmospheric inputs. A cluster analysis showed that heavy metals were mainly affected by the negative impacts of human beings on the environment.

Keywords: heavy metals, marine surface sediments, sources apportionment, ecological risk assessment, daya bay

INTRODUCTION

Surface sediments are a significant component of aquatic environments. In many areas, the heavy metals in the surface sediment are gradually increasing and have become a growing topic of interest worldwide (Ranjbar Jafarabadi et al., 2017; Nel et al., 2020). This is because of their enrichment, nonbiodegradability, or long biological half-life, as well as their inherent toxicity, extensive sources, biogeochemical recycling, and ecological risks (Ranjbar Jafarabadi et al., 2017; Shakouri and Gheytasi, 2018; Xu et al., 2018). Most heavy metals are incorporated into the sediment once they enter the marine environment. Therefore, sediments are considered a source of metals, and they also record anthropogenic impacts (Kang et al., 2017). With rapid industrial and economic development, many coastal bays are subjected to severe heavy metal pollution arising from anthropogenic activities (Qian et al., 2014; Lu et al., 2017; Zhang P. et al., 2017; Liang et al., 2018; Pan et al., 2020).

Marine conservation areas are intertidal or subtidal environments and encompass the overlying water, other geographical features (such as the coast, estuary, wetlands, and islands), and associated flora and fauna (Ervin et al., 2010; Gaines et al., 2010; Zhang Y. et al., 2017). Marine protected areas are of great significance in the protection of biodiversity and can enhance ecological function and reduce the decline of marine biomass (Ervin et al., 2010). The “Baguang-Aotou-Xiachong” area of Daya Bay, which acts as a buffer zones between the land and the sea, is vulnerable to natural processes and anthropogenic activities. Since the 1980s, there have been large-scale anthropogenic activities along the coastline, such as mariculture, ports, nuclear power stations, and petrochemical industries (Yu et al., 2010; Gu et al., 2016). Various kinds of pollution enter Daya Bay, causing serious heavy metal contamination.

The source, migration, transformation, and destination of heavy metals are hot topics in the study of sedimentary environments. Rivers are an important nonpoint source of pollution and have attracted much research attention. There are five main rivers in northwestern Daya Bay, namely, the Danao River, the Zao River in the south, the Yanqian River, the Baigang River, and the Xiayong River. The upstream industries on these rivers are intensive and include processing and metal industries. Although there have been several previous studies on heavy metal contamination in Daya Bay in recent years, these studies mainly investigated the present situation of heavy metal pollution in the bay as a whole (Yu et al., 2010; Zhao et al., 2016), and there was no specific research on the effects of terrestrial pollutants on heavy metals in marine surface sediments. Most previous researchers divided heavy metal pollution sources into either natural or anthropogenic (Lu et al., 2017; Lü et al., 2018) and lacked a specific relationship between the heavy metals and the sampling stations. Moreover, the effect of sewage outfall on marine protected areas was ignored when identifying heavy metal sources. Therefore, the objectives of this study were as follows: 1) to study the spatial distribution of heavy metals in the Daya Bay area based on the GIS interpolation method; 2) to estimate the degree of heavy metal contamination using the geoaccumulation index (I_{geo}) and potential ecological risk index (RI); and 3) to identify the sources and transport pathways of heavy metals.

MATERIALS AND METHODS

Study Area and Sample Collection

Daya Bay (latitude 22°25’~22°50’N, longitude 114°30’~114°55’E) has a subtropical climate, high temperatures, rainy conditions, and the prevailing monsoon dominates. It is a submerged valley in a mountainous area, which is more than 30 km from south to north. The gulf of Daya Bay has the characteristics of a subsidence mountain coast. The seabed topography of Daya Bay gradually deepens from the top of the bay, with an average depth of 11 m. The study area includes areas of petrochemical industrial, ports, seawater aquaculture, and densely populated towns. It can be used as a typical study area for the effects of human activities on heavy metal pollution in Daya Bay.

The research area, also known as the Baguang-Aotou-Xiayong area, is located on the northwestern side of Daya Bay. Twenty surface sediment samples were collected in the bay in late spring (April) and early autumn (September) of 2016 (**Figure 1**).

Analytical Methods

Sediment samples were collected using an oceanic 50 mud collector. For each sample, the top 5 cm of sediment was sampled. The samples were placed in precleaned polyethylene bags and stored at 4°C in the laboratory until analysis.

The sediment samples were freeze-dried. Decomposition of the sample was performed through ultrasonic oscillation before instrumental measurement. Grain size samples were analyzed using the laser diffraction method (Mastersizer2000 Malvern, United Kingdom). The measurement scope of the appliance was 0.02–2000 μm, and the relative error of repeated measurements was less than 3. A sieve analysis method was used for particle sizes larger than 2000 μm. Dried and homogenized samples were analyzed using microwave digestion. After weighing 0.3 g of each sample in a high-pressure Teflon bomb, 8 ml concentrated HNO_3 (65%) and 2 ml H_2O_2 (30%) were added. The sample was covered with a watch glass for 24 h to prevent interference. Samples were then digested using the Ethos Plus microwave laboratory station (Milestone, Sorisole, Italy) with the following temperature stages, all at 1000 W: 30–200°C for 15 min, 200°C for 40 min, followed immediately by ventilation at room temperature for 18 min. Extracts were then transferred into 50 ml flasks and heated until 1 ml remained. After cooling, the concentrated extract was diluted to 5 ml with ultrapure water and mixed well.

The concentrations of mercury (Hg) and arsenic (As) were measured with an atomic fluorescence (AFS, 830, Hitachi, Japan). Copper (Cu), lead (Pb), cadmium (Cd), and chromium (Cr) in the sediments were determined with flame free atomic absorption spectrometry (AAS, Z2000, Hitachi, Japan), and zinc (Zn) by flame atomic absorption spectrometry (AAS, Z2000, Hitachi, Japan).

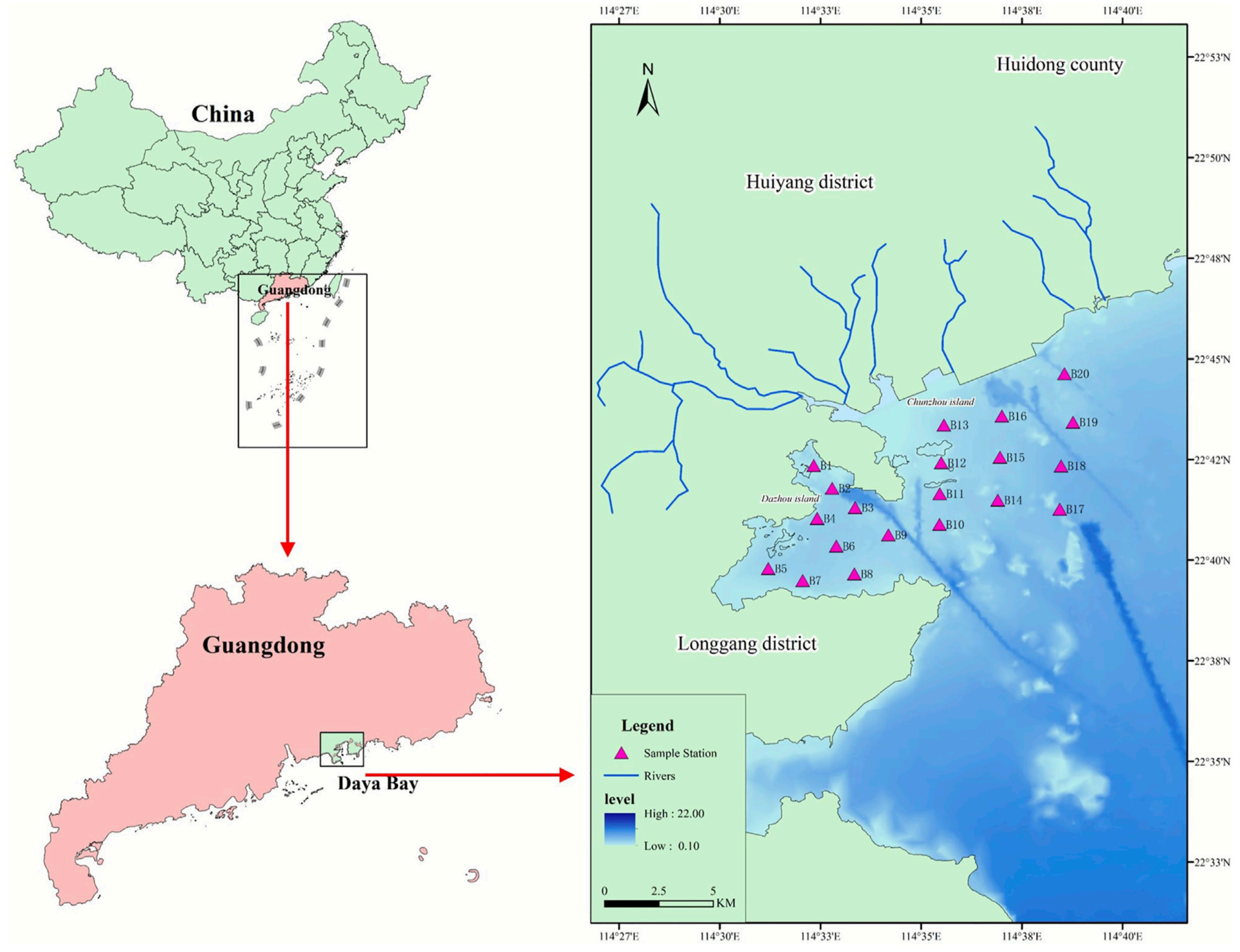

FIGURE 1 | Sampling sites of surface sediment in Daya Bay.

Quality Assurance and Quality Control

The quality assurance of the analytical process for the dissolving heavy metals in the sediments was implemented using the methods released by the National Research Center for standard GB17378-2007. Each sample was measured three times or more to determine the precision and accuracy. The measured blank values were not greater than the corresponding method detection limit. In addition, the deviation of these values was less than 6% in all analyses. Before operation, all applied glassware (bottles, tubes, etc.) in the lab were soaked with 10% HNO_3 (w/w) for more than 2 days and rinsed in deionized water.

Evaluation of Heavy Metals Contamination

Geoaccumulation Index

The useful and common index to define and estimate metal contamination in sediment (Müller, 1979) is the geoaccumulation index (*Igeo*). It is defined as:

$$I_{geo} = log_2\left(\frac{C_i}{1.5B_i}\right)$$

where C_i stands for the concentration of the examined metal i and B_i stands for the geochemical background concentration of the metal i (Zhang, 1991). The selection of background value is an important aspect in the assessment of pollution status (Naifar et al., 2018). Factor 1.5 stands for the background matrix correction factor because of lithogenic effects (Müller, 1979). The I_{geo} values are divided into the following 7 categories: unpolluted level (<0); unpolluted to moderate level (0–1); moderate level (1–2); moderate to strong level (2–3); strong level (3–4); strong to extremely strong level (4–5); extreme level (>5).

Single Pollution Indices (C_f)

The C_f is a commonly used index to evaluate the degree of contamination for sediments in aquatic ecosystems and to reveal the contamination level for an individual metal (Loska et al., 1997). Its calculation is shown with equation below:

$$C_f^i = \frac{C_i}{B_i}$$

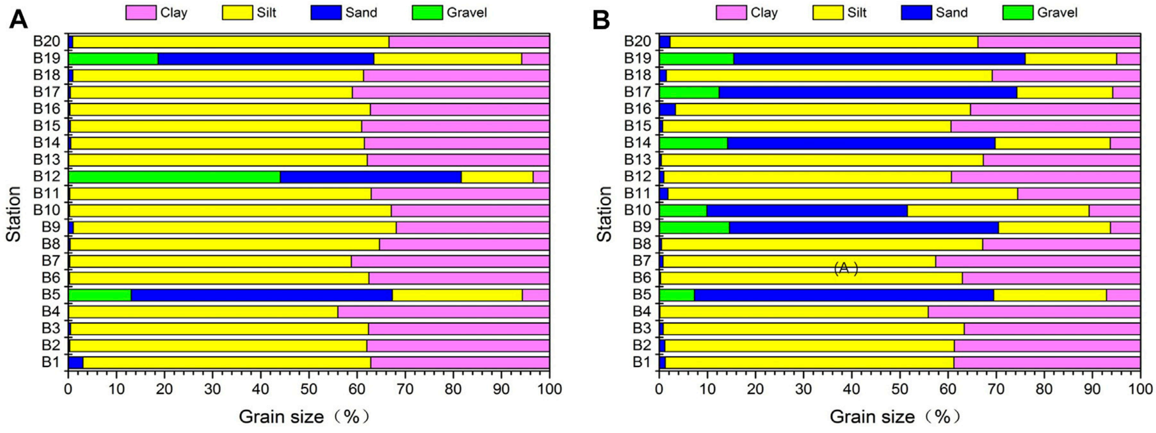

FIGURE 2 | The grain size of surface sediment in Daya Bay in spring **(A)** and autumn **(B)**.

where C_i is the concentration of the examined metal i and B_i is the geochemical background concentration of the metal i.

Potential Ecological Risk Index (*RI*)

To explore ecological risk in the heavy metals, the *RI* index is introduced (Hakanson, 1980), and its calculation formula is as follows:

$$RI = \sum_{i=1}^{n} E_r^i = \sum_{i=1}^{n} T_r^i \times C_f^i$$

$$C_f^i = \frac{C_s^i}{C_n^i}$$

where E_r^i stands for the potential ecological risk factor for each heavy metal, andT_r^istands for the toxic-response factor for individual heavy metal i. The toxic response factors for Cu, Pb, Zn, Cd, Cr, Hg, and As are 5, 5, 1, 30, 2, 40, and 10, respectively (Hakanson, 1980). The *RI* is divided into 4 categories: (Hakanson, 1980): <150 (low ecological risk), $150 \leq RI < 300$ (moderate ecological risk), $300 \leq RI < 600$ (considerable ecological risk), and >600 (very high ecological risk). C_f^i is the contamination factor for individual examined metal i, C_s^i stands for measured actual concentration for metal i in the surface sediment, and C_n^i is the background value for metal i.

Statistical Analysis and Geochemical Mapping

The data normality was assessed by the Kolmogorov–Smirnov and Shapiro–Wilk normality tests. Additionally, Pearson correlation analysis, principal component analysis (PCA), and cluster analysis (CA) were conducted in SPSS 22.0 and R3.2.2 to explore the possible sources of the heavy metal elements and their relevance to other metals, respectively. Ultimately, the Kriging interpolation method was introduced to interpolate the values of the calculated indices and heavy metals concentration (Karim and Williams, 2015; Tian et al., 2017), which revealed the visualization of the heavy metal index and their spatial distribution by ArcGIS10.2 software.

RESULTS AND DISCUSSION

Concentration of the Heavy Metals in Marine Surface Sediments of Daya Bay

As shown in **Figure 2**, the grain size of the surface sediment at each station was quite different. Generally, the surface sediments were primarily composed of silt and clay in spring and autumn with mean sizes of 6.78φ and 6.18φ, respectively. The percentage of silt varied between 14.94 and 67.10%, and the percentage of clay ranged from 3.42 to 44.02% in spring. Similarly, the percentage of silt varied between 18.98 and 78.66%, and the percentage of clay ranged from 5 to 44.12% in autumn. The results showed that the average grain size in spring was smaller than that in autumn.

The average amount of organic carbon in autumn was lower than that in spring (**Table 1**), and the decomposition of total organic carbon was closely related to the migration and transformation of heavy metals in sediments.

As shown in **Table 1**, the concentrations of Cd, Pb, Cr, Cu, Zn, Hg, and As were 0.07–0.42, 28–55, 62–101, 13–82, 79–141, 0.01–0.07, and 6.7–11 mg/kg in spring, respectively. Moreover, in autumn, the respective concentration ranges for individual heavy metals were as follows: Cd, 0.05–0.27 mg/kg; Pb, 24–49 mg/kg; Cr, 24–62 mg/kg; Cu, 12–35 mg/kg; Zn, 90–182 mg/kg; Hg, 0.02–0.06 mg/kg; As, 6.9–8.6 mg/kg. According to their average concentrations, the examined metals in the sampled sediment can be arranged in the following order for both spring and autumn: Zn > Cr > Pb > Cu > As > Cd > Hg. Except for Hg and Zn being equal, the mean concentrations were higher in spring than in autumn for individual heavy metals. Additionally, the coefficients of variation (CVs) of the heavy metals in spring were 58.08, 17.59, 11.93, 53.19, 13.76, 39.68, and 12.10%, respectively. Correspondingly, the heavy metal CVs in autumn were

TABLE 1 | Descriptive statistics of heavy metal concentrations in surface sediments (mg/kg) in Daya Bay.

Element	Spring					Autumn				
	Min	Max	Mean	SD	CV (%)	Min	Max	Mean	SD	CV (%)
Cd	0.07	0.42	0.13	0.08	58.08	0.05	0.27	0.10	0.06	57.55
Pb	28.00	55.00	41.95	7.38	17.59	24.00	49.00	38.75	6.01	15.50
Cr	62.00	101.00	79.55	9.49	11.93	24.00	62.00	52.90	8.25	15.60
Cu	13.00	82.00	27.35	14.55	53.19	12.00	35.00	23.25	6.82	29.33
Zn	79.00	141.00	116.25	16.00	13.76	90.00	182.00	116.25	21.83	18.78
Hg	0.01	0.07	0.04	0.02	39.68	0.02	0.06	0.04	0.01	38.20
As	6.70	11.00	8.59	1.04	12.10	6.90	8.60	7.69	0.60	7.79
TOC	1.23	3.15	1.88	0.37	19.68	1.50	2.50	1.80	0.28	15.56
TS	21.80	388.00	98.37	92.46	93.99	10.10	420.00	156.76	127.52	81.35

TABLE 2 | Concentration (mg/kg) comparative summary for heavy metals in surface sediment of the research region and other relevant areas.

Location	Season	Cd	Pb	Cr	Cu	Zn	Hg	As	References
Study area	Spring	0.13 ± 0.08	41.95 ± 7.38	79.55 ± 9.47	27.35 ± 14.55	116.25 ± 16.00	0.04 ± 0.02	8.59 ± 1.04	—
—	Autumn	0.10 ± 0.06	38.75 ± 6.01	52.90 ± 8.25	23.25 ± 6.82	116.25 ± 21.83	0.04 ± 0.01	7.69 ± 0.60	—
Daya Bay, China	—	0.07 ± 0.02	37.01 ± 8.47	59.03 ± 16.62	16.46 ± 6.34	87.81 ± 26.43	0.04 ± 0.01	8.16 ± 1.99	Zhao et al. (2016)
South China Sea	—	0.40 ± 0.40	23.6 ± 8.9	105 ± 86	38.1 ± 24.6	87.4 ± 47.7	ND	ND	Zhu et al. (2011)
Zhelin Bay, South China	—	0.063 ± 0.30	35.69 ± 11.96	23.07 ± 9.27	7.95 ± 4.11	74.95 ± 9.79	ND	ND	Gu, (2017)
Mai Po Marshes Nature Reserve, Hong Kong	—	1.05 ± 0.823	52.6 ± 12.2	22.4 ± 7.86	42.8 ± 16.7	149 ± 53.6	ND	ND	Liang and Wong, (2003)
Beibu Bay, South China Sea	—	0.16 ± 0.05	27.99 ± 8.69	53.65 ± 19.73	58.26 ± 20.17	67.28 ± 26.56	0.06 ± 0.03	9.53 ± 3.99	Dou et al. (2013)
Mangrove-aquaculture wetland in Shenzhen	—	0.187 ± 0.053	46.6 ± 9.88	50.8 ± 13.3	34.54 ± 16.4	137 ± 51.6	ND	16.43 ± 3.33	Feng et al. (2017)
Background value	—	0.027	21.97	22.35	6.44	21.67	0.011	2.61	Zhang, (1991)
MSQ-1	—	0.5	60	80	35	150	0.2	20	Marine sediment Quality standards (GB18668-2002)
MSQ-2	—	1.5	130	150	100	350	0.5	65	
MSQ-3	—	5.0	250	270	200	600	1.0	93	

57.55%, 15.50%, 15.60, 29.33, 18.78, 38.20, and 7.79%, respectively. The results demonstrated that the concentrations of heavy metals (Cd, Pb, Cu, Hg, As) were relatively discrete in spring.

Compared with the marine sediment quality standards (GB18668-2002) (China), the concentration of Cr (excess rate: 35%) in spring and copper (excess rate: 4.76%) and zinc (excess rate: 4.76%) in autumn exceeded the primary standard criteria (MSQ-1), and all the others were below the primary standard criteria. The mean and standard deviations of all the heavy metals in the research area and other relevant regions were calculated and are displayed in **Table 2**. The average concentration of these examined metals in the research area was higher than that in other related regions in spring, with the exception of mangrove areas and Beibu Bay. However, in autumn, all examined heavy metals were in the mean interval of other relevant areas except Zn, Pb, and Cu.

The Spatial Distribution of Heavy Metals

The spatial distributions of heavy metal concentrations in the research region were displayed in **Figures 3** and **4**, and they showed similar overall patterns. The highest concentrations occurred near Chunzhou and Dazhou Island in spring and autumn, decreasing to the periphery (except for As in spring). Cd, Zn, and Hg showed the same spatial distribution in both spring and autumn, with their highest values occurring near Dazhou Island, especially east of Yalin Bay. The lowest concentrations occurred in the northeastern part of the research area. The maximum values of As occurred in the northeastern part of the study area in spring, and in the central regions in autumn, which was different than observed in a previous study (Dou et al., 2013). Since there is no major rivers discharge into Daya Bay and human activities are concentrated in the vicinity of the continental shelf, higher concentrations of these heavy metals are expected to be inside

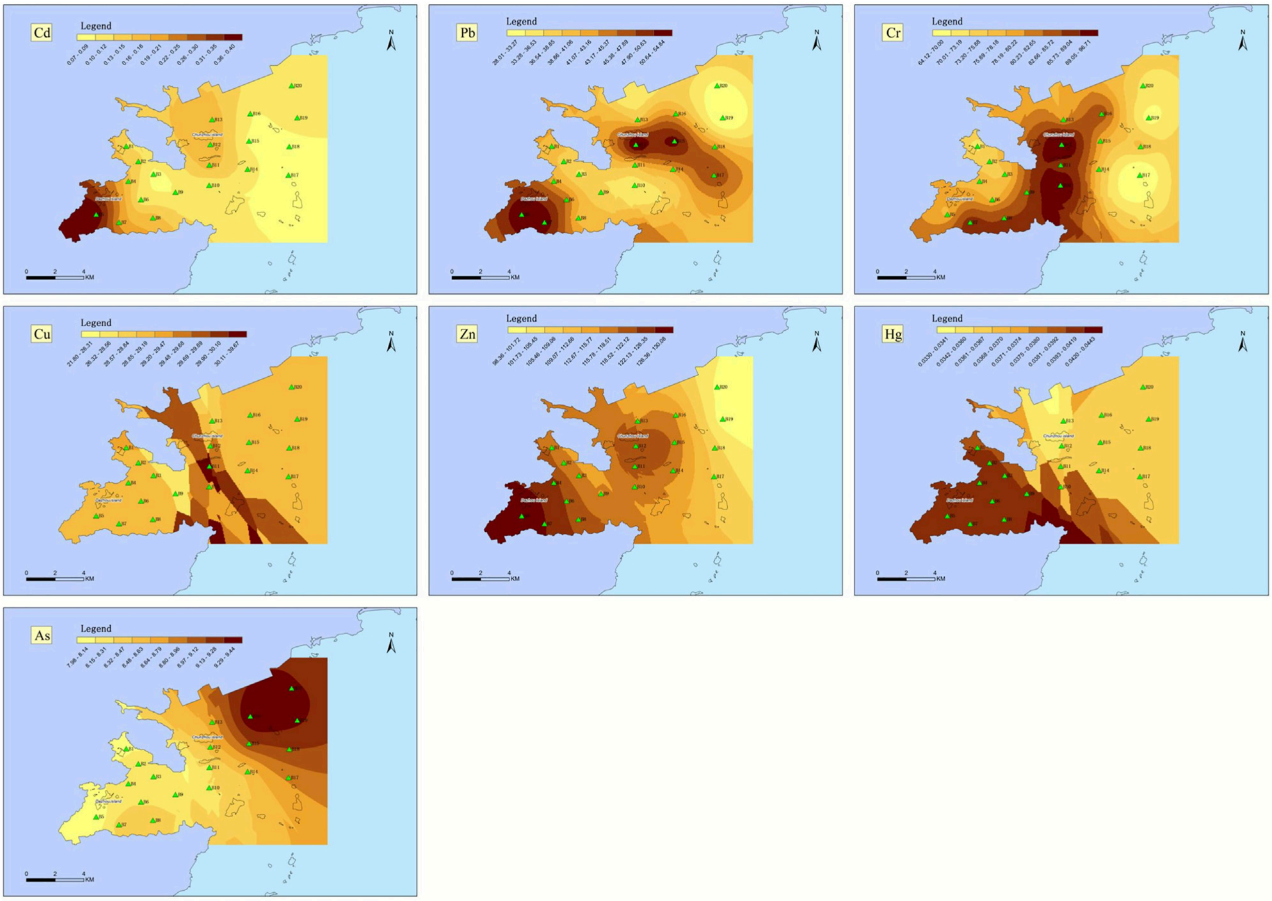

FIGURE 3 | Spatial distribution of heavy metals in surface sediments of the study area in spring.

of the bay (Yu et al., 2010). In general, all heavy metals changed unobviously in spring and autumn except As, which might be due to the small seasonal variation of outlet input volume, and the significant changes of As in spring and autumn might be related to the influence of atmospheric deposition (Lü et al., 2018). Due to the southerly and easterly wind blowing in spring and the northerly wind in autumn in Daya Bay, precipitation in the bay is greater in summer and autumn, explaining the greater seasonal variation in As.

Heavy Metals Contamination and Their Ecological Risk

As illustrated in **Figure 5**, the I_{geo} values of the measured heavy metals were: 0.79 to 3.37 (average 1.57) for Cd, -0.24 to 0.74 (average 0.33) for Pb, 0.89 to 1.59 (average 1.24) for Cr, 0.43 to 3.09 (average 1.37) for Cu, 1.28 to 2.12 (average 1.82) for Zn, -0.24 to 1.98 (average 1.12) for Hg, 0.78 to 1.49 (average 1.12) for As in spring, and 0.30 to 2.74 (average 1.10) for Cd, -0.46 to 0.57 (average 0.22) for Pb, -0.48 to 0.89 (average 0.63) for Cr, 0.31 to 1.86 (average 1.21) for Cu, 1.47 to 2.49 (average 1.82) for Zn, 0.28 to 1.93 (average 1.03) for Hg, 0.82 to 1.14 (average 0.97) for As in autumn. These values show that there was a significant difference between spring and autumn. For these metals, their average pollution level can be ranked in descending order in spring: Zn > Cd > Cu > Cr > As = Hg > Pb, while in autumn the order is Zn > Cu > Cd > Hg > As > Cr > Pb. According to Müller's (Müller, 1981) criteria, the I_{geo} values of heavy metals indicated unpolluted to moderate pollution in the study area. However, the I_{geo} values during spring indicated a higher level of pollution than those in autumn, especially for Cr and As. Previous studies (Zhao et al., 2016) have demonstrated that the mean I_{geo} values for Cd, Cr, Cu, Hg, and Zn were less than zero ($I_{geo} < 0$), indicating limited pollution in Daya Bay and the adjacent shelf. In contrast, the mean I_{geo} values for As and Pb reached 0.07 and 0.49, respectively, suggesting moderate pollution levels by these metals in the study area in 2008. The results showed that the environmental quality of sediments in Daya Bay deteriorated. In general, the distribution trends in spring and autumn were very similar, which might be due to the effect of sewage on heavy metals being greater than that of river-derived pollutants.

Risk assessment of heavy metals in sediments is particularly important for environmental management (Qu et al., 2016). The *RI* values fluctuated greatly, and ranged from 225.86 to 734.20 in spring, and from 196.69 to 567.52 in autumn, respectively. As shown in **Figure 6**, the highest ecological risk of all heavy metals

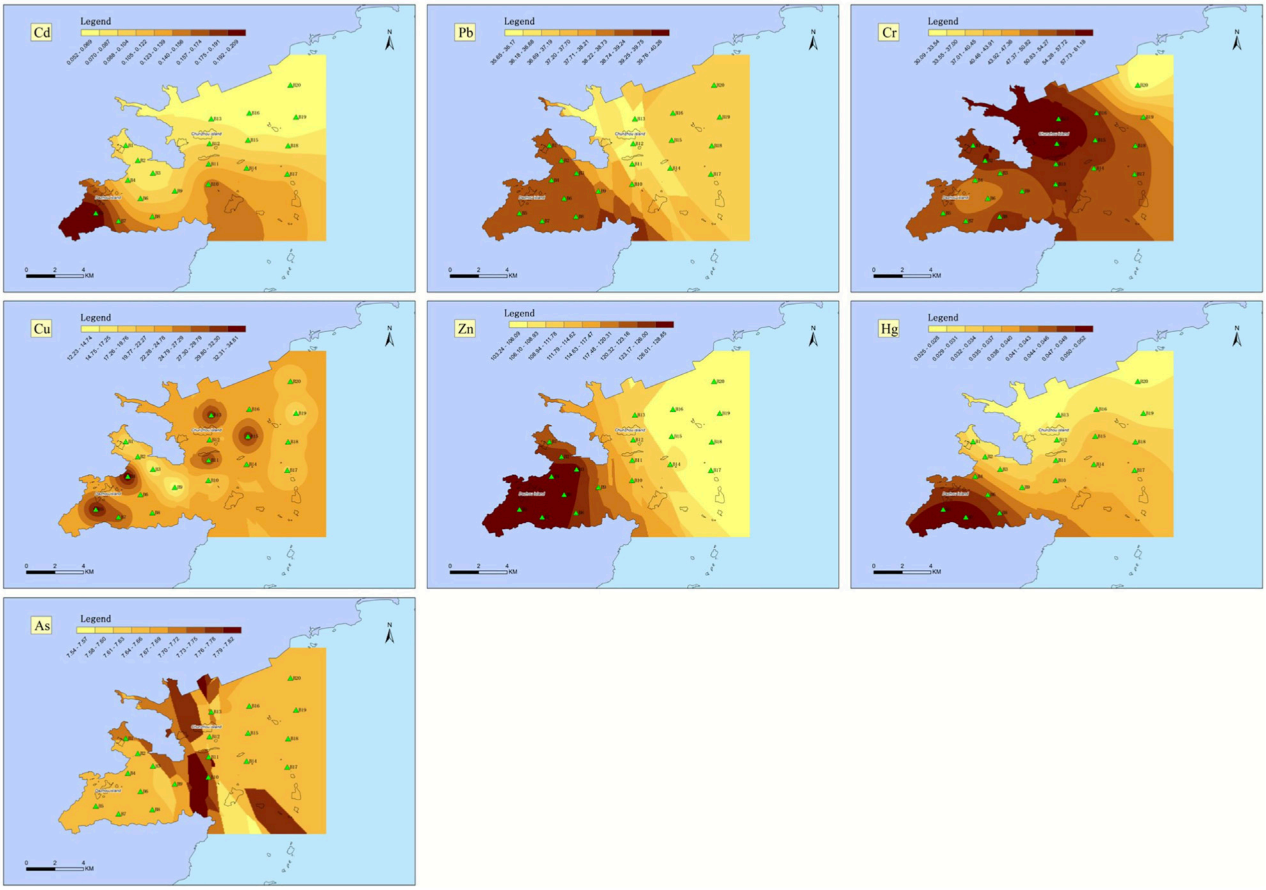

FIGURE 4 | Spatial distribution of heavy metals in surface sediments of the study area in autumn.

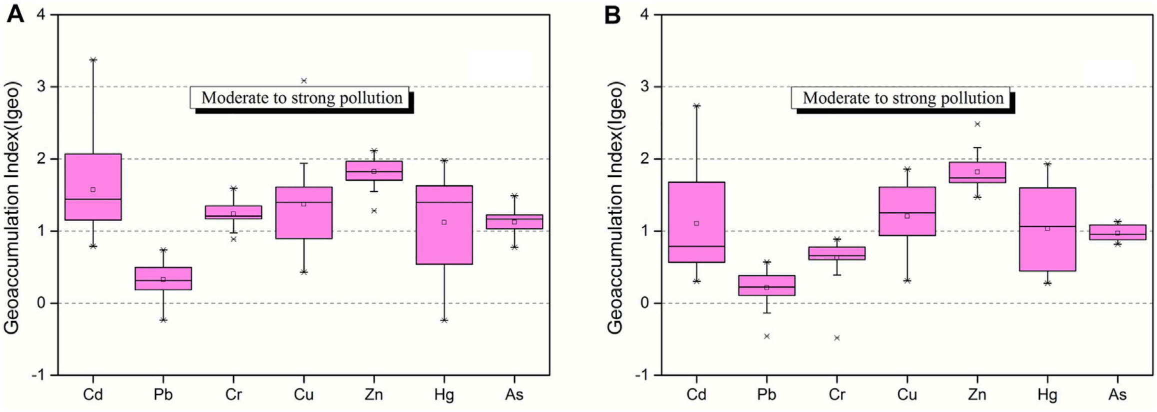

FIGURE 5 | Geoaccumulation index (I_{geo}) of heavy metals in surface sediments during spring **(A)** and autumn **(B)**.

occurred in the area near Dazhou Island in both spring and autumn, decreasing to the periphery. However, the *RI* values in spring were obviously higher than those in autumn. On the basis of Hakanson's classification (Hakanson, 1980), the *RI* values of the heavy metals in our study suggest that most sites have a moderate ecological risk or a considerable ecological risk, and very few had a high ecological risk. Overall, the distribution trend of *RI* in spring and autumn was very similar to the I_{geo} evaluation results, and the impact of outlets on heavy metals is greater than that of terrestrial pollutants, i.e., rivers. However, the *RI* values in

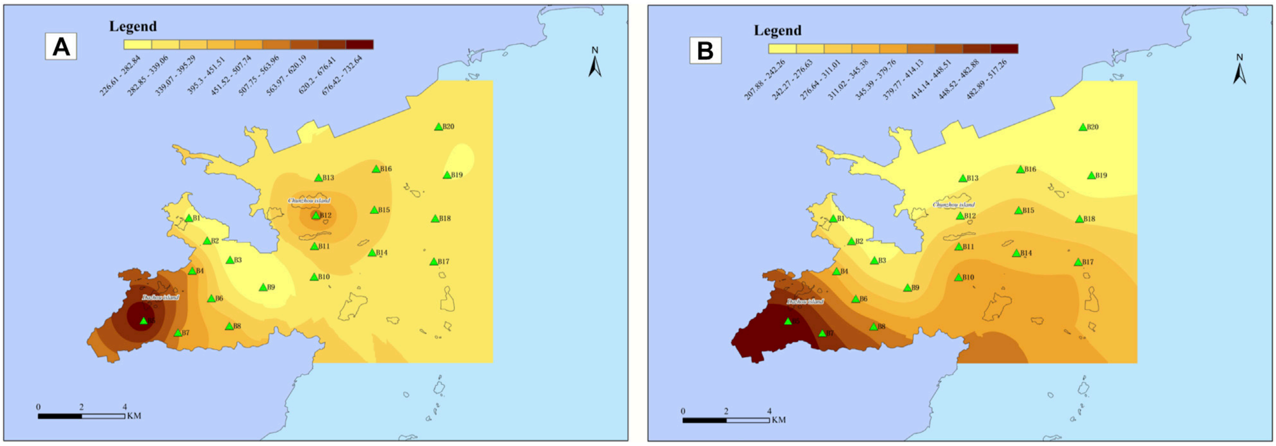

FIGURE 6 | The potential ecological risk index (*RI*) of heavy metals in surface sediments during spring **(A)** and autumn **(B)**.

TABLE 3 | Heavy metal communality of rotated component matrix for Cd, Pb, Cr, Cu, Zn, Hg, and As of surface sediments in Daya Bay.

	Component	
	1	2
Cd	0.640	0.797
Pb	0.860	0.240
Cr	0.206	−0.156
Cu	0.717	0.046
Zn	0.754	0.292
As	0.810	−0.878
Hg	−0.071	−0.057
% of variance	45.45	27.22
% of cumulative	45.45	68.22

Notes: 1) Extraction method: principal component analysis; 2) Rotation method: varimax with Kaiser normalization; 3) Rotation converged in 3 iterations.

spring were significantly higher than those in autumn. This may be due to the smaller sediment size in spring and more heavy metals being adsorbed.

Identification of Heavy Metals Sources

Pearson correlation analysis (**Figure 7**) indicated that there were no relatively significant correlations between heavy metals (Cd, Pb, Cr, Cu, Zn, Hg, As) and the gravel, sand, silt, and clay measurements. A large number of studies (Swarnalatha et al., 2014; Han et al., 2017; Xu et al., 2018) have shown that changes in heavy metal contents are closely related to changes in sediment particle size. An increase in clay minerals, and their specific surface area, results in increased surface free energy and therefore greater heavy metal adsorption (Lu et al., 2017; Naifar et al., 2018). Moreover, organic matter is highly related to heavy metals in the aquatic environment through adsorption and complexion (Dou et al., 2013; Liu et al., 2017). The results of the Pearson correlation matrix show that there is a significant correlation between total organic carbon (TOC) and Cd, Pb, Hg, and Zn. Moreover, As and Cr were also strongly correlated, suggesting that they might have a common source.

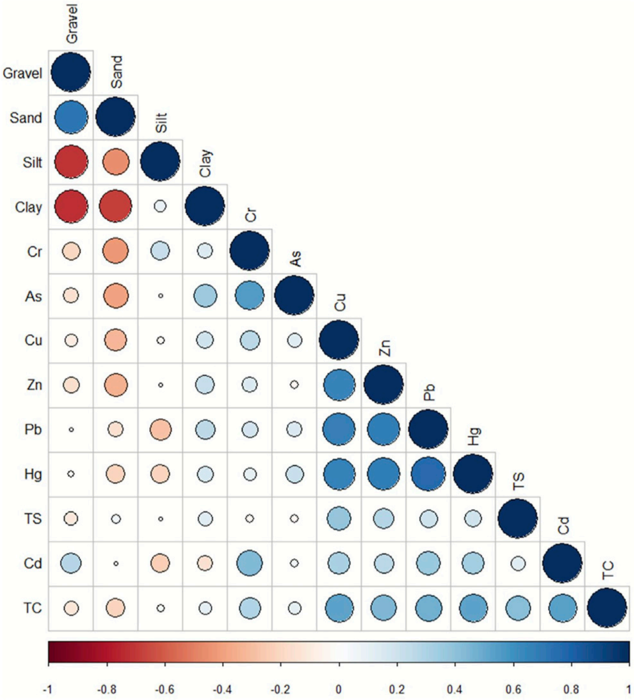

FIGURE 7 | Matrices of Pearson correlation among heavy metals (Cd, Pb, Cr, Cu, Zn, Hg, and As), gravel, sand, silt, and clay contents. Red and blue dots correspond to negative and positive correlations, respectively. Small dots with light color intensity represent low correlations, while large dots with darker colors correspond to higher correlations. The rows in the correlation matrices refer to parameters in water, and bivalves are included in a rectangular box.

Principal component analysis (PCA) processed by SPSS 22.0 was used in this research. The rotated component matrices of

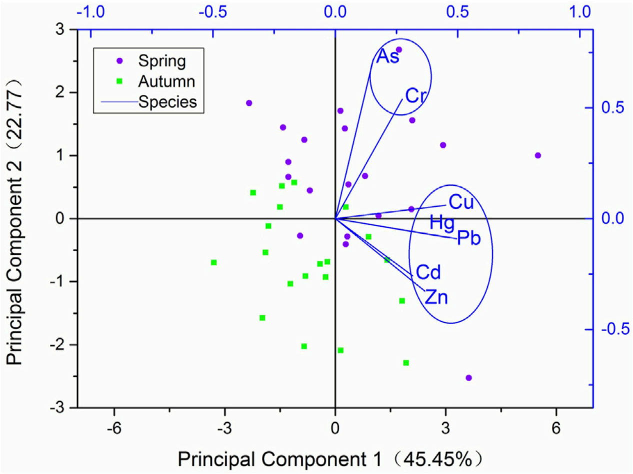

FIGURE 8 | Component scores for the first two principal components (PC1 vs. PC2).

the PCA are shown in **Table 3**. The Kaiser-Meyer-Olkin (KMO) and Bartlett's results were 0.650 and 105.62 (df = 21, Sig b < 0.001), respectively, suggesting that PCA might be useful in reducing the dimensionality. The PCA indicated that the proportion of the first two principal components among the total variants was 68.22 (**Figure 8**). The first principal component (PC1) with high loadings of Cu, Cr, Zn, Hg, and Pb accounted for 45.45% of the total variance, suggesting that they might have similar sources. Concentrations of Cu, Cr, Zn, Hg, and Pb were higher than their corresponding background values, and they were also present at moderate pollution levels based on their I_{geo} values, demonstrating that these five heavy metals were moderately affected by anthropogenic activities. The second principal component (PC2) accounted for 22.77% of the total variance, with the strongest loadings from As. According to spatial interpolation analysis, As is mainly affected by atmospheric deposition, and PC2 may represent natural sources.

Before clustering, the data were processed with a single factor pollution index to reduce the error, so that the clustering result was closer to reality. Cluster analysis was conducted to examine the relationships among variables (heavy metals in the surface sediment) and sample sites (**Figure 1**). As shown in **Figure 9A**, in spring, the left vertical dendrogram (left side of the box) exhibited clustering of the sampling sites in line with heavy metal concentrations in the surface sediment. The horizontal dendrogram indicated clustering of heavy metals in line with their likenesses. In the vertical dendrogram, all the sampling sites in Daya Bay were divided into four clusters. Cluster I was composed of 8 sampling sites (B11, B13, B19, B20, B3, B9, B1, B2), cluster II contained 10 sample stations (B8, B4, B7, B15, B16, B17, B18, B6, B10, B14), cluster III contained 1 sampling site (B5), and the last site (B12) near Chunzhou Island was located in cluster IV. The results indicated that the distribution of the heavy metals was somewhat different in the 20 sampling sites. Obviously, there were distinct common characteristics in each cluster. In cluster I, Pb, Cu, and Hg in sites B19, B20, B3, B9, B1, and B2 had relatively low concentrations. These stations should have high concentrations because they are near waterways or sewage outfalls. This phenomenon might be due to the negative impact that of humans have on the environment, such as the regular cleaning of waterways (Jiang et al., 2007). In clusters II and III, all heavy metal concentrations were high, especially the Cd, Cu, and Hg concentrations. According to the spatial distribution diagram, these sites are mainly concentrated in the vicinity of Yalin Bay, suggesting that the high concentrations of Cd, Cu, and Hg were related to shellfish farming. Similar to cluster III and cluster IV, all their concentrations were also relatively higher, but Cu was notably higher. This might be due to the inflow of rivers which have plenty of industrial activities upstream, such as the Danao River and Xiachong River. Industrial activities, including the chemical production industry, cotton industry, paper industry, dyeing

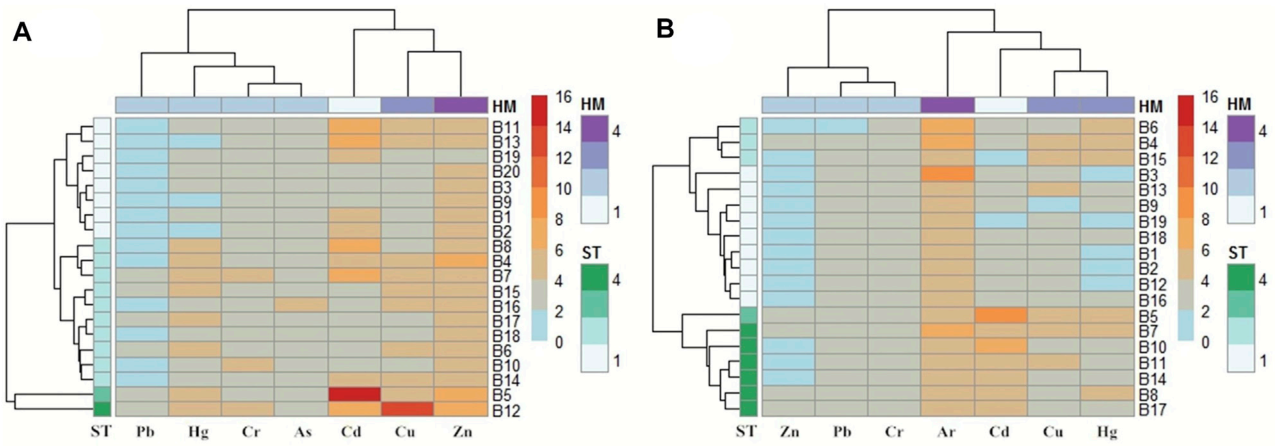

FIGURE 9 | Dendrogram obtained based on hierarchical agglomerative cluster analysis (HACA) for **(A)** the relationship between heavy metals in surface sediments and contributing sites in spring and **(B)** the relationship between heavy metals in surface sediments and contributing sites in autumn, in Daya Bay in 2016.

industry, and metalworking industry, may augment the concentrations of Cu, Pb, and Zn (Liang et al., 2018). There were 4 different clusters for the heavy metals in the horizontal dendrogram. Cluster I incorporated Cd, cluster II included Pb, Cr, As, and Hg, cluster III was made up of Cu, and the only member of cluster IV was Zn.

As shown in **Figure 9B**, in the vertical dendrogram, the Daya Bay sampling sites can be divided into four clusters in autumn, which were the same as those in spring. Cluster I was composed of nine sampling sites (B3, B13, B9, B19, B18, B20, B1, B2, B12, B16), cluster II contained three sample sites (B6, B4, B15), cluster III contained one sampling site (B5), and finally, the remaining sites (B10, B11, B14, B8, B17) were included in cluster IV. For cluster I and cluster 3, the above sampling site clustering analysis in spring can also be applied in autumn. There were 4 different clusters for the heavy metals in the horizontal dendrogram. Cluster I consisted of Cd, cluster II included Pb, Cr, and Zn, cluster III was made up of Hg and Cu, and only member of cluster IV was As. This result is different from the results in spring, which also proves that As is affected by atmospheric deposition.

CONCLUSION

In spring and autumn of 2016, grain size and heavy metal (Cd, Pb, Cr, Cu, Zn, Hg, and As) concentrations were analyzed in surface sediment samples collected from a marine protected area with larval economic fish and shrimp populations in Daya Bay. For the physicochemical properties of the sediments, generally, the surface sediments primarily consisted of silt and clay in spring and autumn, and the average particle size in spring was smaller than that in autumn. For the heavy metal analysis, all the heavy metal concentrations were below the primary standard criteria, except for the concentrations of Cr in spring and Cu and Zn at autumn in several sampling stations. The spatial distributions of heavy metal concentrations in the study area were the same in spring and autumn, which might be due to the small seasonal variation in sewage discharge. The highest heavy metal values were found near Dazhou Island, especially east of Yalin Bay, and the lowest concentrations occurred in the northeastern part of the research area. Most of sites had a moderate ecological risk or a considerable ecological risk level, and very few had high ecological risk levels. Heavy metals are mainly derived from natural sources such as human activities and atmospheric input, and man-made sources are mainly affected negatively by human activities, such as sewage outlets, channel dredging, and shellfish farming.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

HW wrote the article and did the laboratory analysis. HW and HH contributed to the field analysis, while the latter also conceptualized the research. ZF, ZK, YY, and HL provided conceptual and editorial inputs on the manuscript and discussed field methodology. All authors contributed to the article and approved the submitted version.

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The Satisfaction of the Fishermen in the South China Sea With the Summer Fishing Moratorium System and Its Influencing Factors

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This study investigated the satisfaction of fishermen with the summer fishing moratorium system in the three provinces of the South China Sea through a questionnaire survey and explored the degree of its influencing factors. A multistage random sampling method was used to issue questionnaires to fishermen involved in the summer fishing moratorium. A total of 258 valid questionnaires were collected, and the factors were analyzed them using the factor analysis method by SPSS 23.0 software. The results showed that the variance contribution rate of the first five factors reached 61.859%, and the 11 factors that have potential commonality in influencing fishermen's satisfaction with the fishing moratorium can be summarized and classified into five more significant factors, such as "economic income during fishing moratorium (16.396)," "illegal fishing boat (12.095)," "fishing moratorium time (12.012)," "law enforcement credibility (11.853)," and "summer fishing moratorium effect (9.503)." According to the factor score calculation, the fishermen's satisfaction with the fishing moratorium was F = 3.53, and the overall satisfaction was relatively high. This study reveals the satisfaction degree of the fishermen in the South China Sea with the summer fishing moratorium system and its influencing factors, which can provide a basis for improving the summer fishing moratorium system and ensuring the lives of fishermen.

Keywords: fisherman, summer fishing moratorium system, satisfaction, factor analysis, South China Sea

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INTRODUCTION

The fishing moratorium system of China started in 1995. The implementation area is the sea area of the East, Yellow, and Bohai seas from 35°N south to 27°N north. In 1999, the summer fishing moratorium system also began in the South China Sea. During the period, both the time of fishing closure and the type of operation were adjusted several times (Wang, 2008). Now the system has become the most important and influential fishery resource conservation system in China and is also the most suitable fishery resource conservation system for China's national conditions (Zhu, 2009). In 2017, the summer fishing moratorium was adjusted from May 1 to August 16 in the South China Sea. In addition to fishing gear, all types of operations, including fishing vessels and fishing support services for fishing vessels, are listed as closed to fishing, which is the biggest adjustment since the implementation of the system in the South China Sea. At present, the marine summer fishing moratorium system is still under continuous adjustment. In the current national policy of China's marine summer fishing moratorium, local fishery management agencies are allowed to implement a special fishing license system for special economic fish stocks after being approved by the Ministry of Agriculture and Rural Affairs. The summer fishing moratorium system of China has made a great contribution to alleviating the enormous pressure on marine fishery resources caused by excessive fishing intensity and protecting China's offshore fishery resources, especially the spawning fish and juvenile fish. Fishermen are the main body of the implementation of the summer fishing moratorium, and they have made important contributions to the protection of China's maritime rights and interests and fishery resources (Qian, 2018). The guarantee of fishermen's own rights and interests and their satisfaction with the summer fishing moratorium will affect the implementation of the system, which is also the premise to ensure the smooth implementation of the system. The quantitative and intuitive analysis of fishermen's satisfaction and existing opinions on the summer fishing moratorium system are of great significance to improve the system and fishermen's satisfaction.

At present, the studies on the relationship between fishermen and the fishing moratorium system mainly focus on basic descriptive statistical analysis or fishermen-particular factor analysis (Tyler and Degoey, 1995; Hamm et al., 2013; Liu et al., 2019). For example, the field survey in the fishing ports of Dongguan Xinwan and Shantou Nan'ao in Guangdong Province showed that fishermen, as a special group, are at a natural disadvantage in terms of land management rights and social and economic status (Fwg. 2018). The survey based on the perspective of fishermen in Hainan Province suggested that fishermen need to be constantly optimized and improved in terms of institutional settings, resource allocation, information communication, policy support, and social participation (Zhou and Wan, 2017; Yue et al., 2018). Based on the statistical description method, an empirical analysis of fishermen explored the impact of the fishing moratorium system and oil price subsidy policy adjustment on the family income of marine fishermen (Song and Huang, 2015). But the research on fishermen's satisfaction with the fishing moratorium system, the degree of problems encountered and the impact of various factors influencing satisfaction with the fishing moratorium are still lacking. Therefore, it is necessary to conduct a comprehensive analysis and evaluation from various subjective and objective aspects, such as the needs of the fishermen and the problems of the management department and the system itself.

Factor analysis, as a multivariate statistical analysis method (Gorsuch, 1983), is widely used in studies of decision-influencing factors (Dangi and Kohli, 2018), or support and satisfaction, etc. (Xu and Xu, 2010). For example, investment institutions classify and analyze investors so that they can accurately allocate assets for their investments (Wei and Chongchong, 2018). The factors influencing the purchase decision of new energy vehicles were analyzed (Brunetti et al., 2020). Jia and Chen (2015) analyzed the influencing factors of the electronic safety system operation to obtain the potential factors affecting the load, which is of great significance to the operation of the electronic safety system (Jia and Chen, 2015). Jie and Shi-Long (2016) obtained the factors that affect fishermen's fishing insurance satisfaction and their evaluation using factor analysis (Jie and Shi-Long, 2016). Wang et al. (2013)) used questionnaires to conduct principal component analysis on factors affecting residents' satisfaction with hypermarkets (Wang et al., 2013). Galeoto et al. (2018) used the factor analysis method to analyze the customer service satisfaction of second-hand bookstores and determine which factors will affect customers' repurchase intentions (Galeoto et al., 2018).

Therefore, in this study, we aimed to investigate fishermen's satisfaction with the fishing moratorium in the South China Sea and its influencing factors. To accomplish this, we used a multistage random sampling survey to distribute questionnaires to fishermen in the three provinces of the South China Sea during the fishing moratorium. A total of 258 questionnaires were collected, and the principal component analysis was conducted based on factor analysis. The result of this study can provide a theoretical basis for improving the fishing moratorium system and enhancing the livelihood security of fishermen.

MATERIALS AND METHODS

Obtaining Factors Influencing Fishermen's Satisfaction With the Fishing Moratorium System

In order to truly and effectively obtain the factors that affect fishermen's satisfaction with the fishing moratorium system, we conducted a preliminary survey of experts, fishery law enforcement officers, and a small number of fishermen involved in the fishing moratoriums. Everyone was asked to complete an open-ended questionnaire with only one question: What factors do you think affect fishermen's satisfaction with the fishing moratorium system? These items (including duplicate items) were compiled and scored on a 5-point Likert scale, including 5 points for "strongly agree," 4 points for "agree," 3 points for "not necessarily," 2 points for "disagree," and 1 point for "strongly disagree" (Matell and Jacoby, 1971; Laerhoven et al., 2004).

Survey Method of Satisfaction of Fishing Moratorium System

A questionnaire survey is a quantitative research method based on positivist methodology. Standardized questionnaires are distributed or mailed to relevant personnel, collected, and sorted out, and the research results are obtained through statistical analysis (Wu et al., 2003; Zheng, 2014). Therefore, this study used a questionnaire survey to collect the influencing factors of fishermen's satisfaction with the voluntary closed season fishing system. The questionnaire included 1) basic information about fishermen, 2) the collection of factors affecting fishermen's satisfaction with the fishing moratorium, and 3) fishermen's awareness of the fishing moratorium system.

TABLE 1 | Impact factors of fishermen's satisfaction with the summer fishing moratorium system.

Impact factor	Variable
Fishing moratorium subsidies	X1
Fishing moratorium time	X2
Impact on full-year revenue	X3
Effect of fishery resource restoration	X4
Management efforts of fishery law enforcement officers	X5
Degree of fairness of law enforcement officers	X6
Effectiveness of fishery law enforcement management	X7
Three no fishing boats—illegal fishing	X8
Fishing encroachment by fishing vessels of other countries	X9
Rationality of the voluntarily resting system	X10
Publicity training for the fishing moratorium	X11

The questionnaire survey was conducted from May 1 to August 16, 2020. Three hundred questionnaires were distributed to fishermen in Guangdong, Guangxi, and Hainan provinces in China, and 258 valid samples were obtained.

Analysis Method of Satisfaction of Fishing Moratorium System

Factor analysis is a multivariate statistical analysis method that reduces some complicated variables into a few new and unrelated compound factors. The basic idea of factor analysis is to group variables according to the magnitude of correlation, so that the correlation between variables in the same group is stronger, while the correlation between variables in different groups is smaller (You, 2003).

The specific steps of factor analysis generally include the following three aspects: 1) estimate the factor loading matrix based on the original data and determine the common factors; 2) classify and name each common factors according to the total variance contribution of each original factors; and 3) establish the factor score function and calculate the score of each common factor according to the factor score coefficient matrix.

RESULTS

Factor Screening

The Delphi method and the core group discussion method were adopted, and the factors were screened by referring to the actual scores of experts. The mean value and coefficient of variation were set, and the factors with a mean value no less than 4.0 and a coefficient of variation no greater than 0.15 were determined to meet the requirements. Finally, a total of 11 question items were determined (**Table 1**).

Reliability Test of the Questionnaire

A total of 300 questionnaires were distributed, and 281 were actually collected. After eliminating invalid samples, 258 valid samples were obtained, and the effective recovery rate was 86%. The survey results were in line with the actual situation. The overall reliability of the obtained 258 questionnaires was tested using SPSS 23.0, with an overall Cronbach's reliability coefficient of 0.644. The analysis showed that the questionnaire had high overall reliability, and the results obtained through the analysis of the questionnaire were reliable. The overall validity of the questionnaire was analyzed, and the sample Kai–Meyer–Olkin (KMO) index was 0.652, which was greater than the empirical value of 0.500. Bartlett's spherical approximate chi-square value was 314.216, with 55 degrees of freedom and 0.000 significance (**Table 2**). The results showed that this study met the conditions for factor analysis, and there were common factors, so it was suitable for factor analysis.

TABLE 2 | KMO and Bartlett's test.

KMO (Kai–Meyer–Olkin measure of sampling adequacy)		0.652
Bartlett's test of sphericity	Approx. chi-square	314.216
	df	55
	Sig	0.000

The value of KMO is usually between 0 and 1. The closer to 1, the stronger is the correlation between variables. If it is greater than 0.6, it is suitable for factor analysis.

Descriptive Analysis of Individual Characteristics of the Samples

Through a general descriptive analysis of the respondents' gender, age, education level, and average monthly income, it was possible to understand the composition of the fishermen participating in the fishing moratorium in the South China Sea. These analyses could prepare for the subsequent question of whether the demographic characteristics of fishermen and fishermen's satisfaction with the measured indicators had a significant impact. Among the 258 valid samples obtained in this study, 50.5% of them were 50 years or older, and the overall population was aging. The education level was generally junior high school and primary school, and only 3.5% of the respondents

TABLE 3 | Basic characteristics of the sample

Sample characteristic	Topic options	Number of people	Percentage (%)
Age (years)	Over 60	34	13.3
	50–59	96	37.2
	40–49	75	29.2
	30–39	46	17.6
	16–29	7	2.7
Educational background	College and above	9	3.5
	High school	30	11.5
	Junior high school	121	46.9
	Elementary school and below	98	38.1
Average monthly income (yuan)	Over 5,000	25	9.8
	3,000–4,999	100	38.9
	1,000–2,999	110	42.5
	Less than 1,000	23	8.8
Working time (years)	Over 40	55	21.2
	30–39	89	34.6
	20–29	68	26.5
	10–19	41	15.9

had a college degree or above; 82.3% of the respondents had been engaged in fishery production for 20 years or more. Some families earn a living from fishing, with an average monthly income of 1,000–5000 yuan. The survey showed that 63.2% of the respondents fully understood the fishing moratorium system, while only 5% of the respondents were unclear about the system, and 84% of the respondents agreed with the effectiveness of the system (**Table 3**).

Basic Characteristics of Influencing Factors

Fishermen scored each influencing factor according to the required degree of importance, variables, and statistical characteristics of the investigated influencing factors (**Table 4**). The average value of 11 factors was greater than 3, and the minimum value of 3.388 was "publicity and training during the fishing moratorium," indicating that the factors listed in the questionnaire were indeed the factors considered by fishermen in their satisfaction with the fishing moratorium. Among them, fishing moratorium subsidies ranked first, with an average of 4.806; second to fifth places were the length of the fishing moratorium, the illegal fishing by three non-fishing vessels, the impartiality of law enforcement personnel, and the impact on the restoration of fishery resources, with an average of greater than 4.000. These results showed that fishermen considered these five factors to be more important, while the fishing moratorium subsidies and the impact on annual income were the most important factors for fishermen.

Influencing Factors Analysis of Fishermen's Satisfaction With Seasonal Fishing Moratorium System

The 11 influencing factors were subjected to factor analysis using SPSS 23.0 (**Table 5**). The results showed that the variance contribution rate of the first five factors reached 61.859%, and the effect was more obvious, indicating that these five common factors could reflect most of the information of the original variable. So the 11 influential factors could be divided into five factors for further analysis.

Component Naming

According to the results of rotation factor loading, the expressions of each factor were obtained (**Table 6**). In the first factor load, the fishing moratorium subsidy had a large influence coefficient on annual income, with an average score of 4.806, ranking first, indicating that it had a decisive influence on the satisfaction of the fishing moratorium system. According to the survey results, Guangxi and Hainan had not received fishery

TABLE 4 | Analysis of variable characteristic.

Impact factor	Variable	Mean	Maximum	Minimum	S.E.
Fishing moratorium subsidies	X1	4.806	5	4	0.3960
Fishing moratorium time	X3	4.721	5	2	0.5782
Impact on full-year revenue	X8	4.628	5	2	0.6841
Effect of fishery resource restoration	X9	4.391	5	2	0.8263
Management efforts of fishery law enforcement officers	X6	4.112	5	3	0.5357
Degree of fairness of law enforcement officers	X4	3.919	5	1	0.6025
Effectiveness of fishery law enforcement management	X2	3.678	5	2	0.8092
Three no fishing boats—illegal fishing	X5	3.574	5	1	0.6210
Fishing encroachment by fishing vessels of other countries	X7	3.473	5	1	0.6785
Rationality of the voluntarily resting system	X10	3.411	5	2	0.6436
Publicity training for the fishing moratorium	X11	3.388	5	1	0.7822

TABLE 5 | Total variance explained of impact factors on fishermen's satisfaction of the summer fishing moratorium.

Component	Initial eigenvalues			Rotating load sum of squares		
	Total	Variance contribution ratio (%)	Cumulative contribution ratio (%)	Total	Variance contribution ratio (%)	Cumulative contribution ratio (%)
1	1.965	17.860	17.860	1.804	16.396	16.396
2	1.362	12.384	30.244	1.330	12.095	28.491
3	1.301	11.832	42.075	1.321	12.012	40.503
4	1.150	10.450	52.526	1.304	11.853	52.356
5	1.027	9.333	61.859	1.045	9.503	61.859
6	0.970	8.817	70.676	—	—	—
7	0.841	7.648	78.324	—	—	—
8	0.818	7.439	85.763	—	—	—
9	0.703	6.390	92.153	—	—	—
10	0.624	5.670	97.823	—	—	—
11	0.239	2.177	100.000	—	—	—

Extraction method: principal component analysis.

TABLE 6 | Rotated component matrix (a) of factors affecting the satisfaction of the summer fishing moratorium.

Index	Principal component				
	1	2	3	4	5
Fishing moratorium subsidies	0.754	0.228	0.089	−0.051	0.050
Fishing moratorium time	−0.030	−0.022	0.782	0.061	−0.151
Impact on full-year revenue	0.832	−0.064	−0.045	−0.006	−0.022
Effect of fishery resource restoration	−0.104	0.132	0.066	-0.099	0.855
Management efforts of fishery law enforcement officers	−0.027	0.102	0.173	0.653	0.063
Degree of fairness of law enforcement officers	−0.002	−0.064	−0.316	0.689	0.120
Effectiveness of fishery law enforcement management	−0.029	0.198	0.238	0.560	−0.172
Three no fishing boats—illegal fishing	0.071	0.897	0.030	0.133	−0.050
Fishing encroachment by fishing vessels of other countries	0.076	0.910	−0.071	0.073	0.047
Rationality of the voluntarily resting system	0.067	−0.021	0.709	0.056	0.217
Publicity training for the fishing moratorium	−0.202	0.207	0.033	−0.218	−0.435

Extraction method: principal component analysis. Rotation method: Varimax with Kaiser normalization. a, rotation converged in 6 iterations.

subsidies during the fishing moratorium, leaving fishermen with almost no source of income during the fishing moratorium. The average impact score of the annual income was not high indicating that annual income fluctuations were not high, due to various fish species' ability to grow during the fishing moratorium and the increased production and income of fishermen following the fishing moratorium. So the first factor could be named "the economic income factor during the fishing moratorium."

In the second factor load, the coefficient between illegal fishing of three vessels and fishing invasions by other countries was larger. Among them, the average score of illegal fishing by three vessels was 4.628, ranking third. This is an important factor affecting the satisfaction of fishermen who normally participate in the fishing moratorium system. The average score of fishing encroachment by other countries was 3.473, with a relatively low ranking. In the course of investigation, it was found that Vietnamese fishing boats were the main culprits in some areas of Hainan and Guangxi provinces in China, so the second factor could be named the "illegal fishing boat poaching factor."

In the third factor load, the coefficients of the length and the rationality of the fishing moratorium system were larger. In the survey of fishermen, 43% of fishermen thought that the fishing moratorium was too long, 21.6% of fishermen thought that the moratorium should be advanced and the corresponding end time should be advanced, but there were not many opinions on the fishing moratorium system itself. So the third factor could be named "the length of the fishing moratorium factor."

In the fourth factor load, the intensity of management and law enforcement, the fairness of law enforcement personnel, and the coefficient of management effect of law enforcement agencies were relatively large. Among them, law enforcement officers ranked fourth, with an average score of 4.391 in fairness, which is a more important factor for fishermen's satisfaction with the fishing moratorium. So the fourth factor could be named the "law enforcement credibility factor."

In the fifth factor, the influence coefficient of fishery resources restoration was larger, with an average score of 4.112. This showed that fishermen were more concerned about the catches after the fishing moratorium ends, which was also an important factor affecting fishermen's satisfaction with the fishing moratorium system. So the fifth factor could be named the "effect factor of the fishing moratorium."

Comprehensive Score of Fishermen's Satisfaction With the Fishing Moratorium

In order to more intuitively reflect the fishermen's satisfaction with the fishing moratorium, five main factors were set as F1, F2, F3, F4, and F5 when calculating factor scores. According to the factor score coefficient matrix (**Table 7**), the factor score expression was obtained.

Illegal fishing boats poaching factor is given as $F_1 = 0.075X_1 - 0.029X_2 - 0.104X_3 + 0.102X_4 - 0.102X_5 - 0.107X_6 + 0.051X_7 + 0.497X_8 + 0.514X_9 - 0.032X_{10} + 0.156X_{11}$ Similarly, the expressions of F2, F3, F4, and F5 could be obtained. Then, the variance contribution of each common factor was divided by the total variance contribution, and the expressions of fishermen's satisfaction with the fishing moratorium and the combined factor were obtained by weighted average given as follows:

$$F = \frac{F_1W_1 + F_2W_2 + F_3W_3 + F_4W_4 + F_5W_5}{W_1 + W_2 + W_3 + W_4 + W_5}.$$

In the aforementioned equation, W1, W2, W3, W4, and W5 are the variance contribution of each common factor.

The original variable data of the 11 influencing factors were substituted into the aforementioned 5 expressions. The fishermen's satisfaction with the fishing moratorium was calculated as F = 3.53, and the scores of the 5 main factors were F1 = 2.07, F2 = 3.27, F3 = 3.66, F4 = 4.11, and F5 = 3.52.

DISCUSSION

The influencing factors of fishermen's satisfaction with the summer fishing moratorium system include fishery management (Chen, 2007), deficiencies in the system itself (Yang, 2018), fishermen's income (Liu and Chen, 2001), and many other aspects. These major aspects also include various

TABLE 7 | Factors score coefficient matrix.

Index	Composition				
	1	2	3	4	5
Fishing moratorium subsidies	0.554	0.075	0.057	−0.055	0.028
Fishing moratorium time	−0.025	−0.029	0.594	0.012	−0.150
Impact on full-year revenue	0.642	−0.104	-0.044	0.023	−0.046
Effect of fishery resource restoration	−0.120	0.102	0.050	−0.121	0.826
Management efforts of fishery law enforcement officers	−0.019	−0.102	0.095	0.494	0.043
Degree of fairness of law enforcement officers	0.019	−0.107	−0.277	0.566	0.098
Effectiveness of fishery law enforcement management	−0.024	0.051	0.150	0.414	−0.178
Three no fishing boats—illegal fishing	−0.015	0.497	0.002	0.009	−0.047
Fishing encroachment by fishing vessels of other countries	−0.015	0.514	−0.072	−0.037	0.049
Rationality of the voluntarily resting system	0.038	−0.032	0.535	0.004	0.200
Publicity training for the fishing moratorium	−0.164	0.156	0.039	−0.190	−0.403

Extraction method: principal component analysis. Rotation method: Varimax with Kaiser normalization.

factors. These factors have different degrees of influence on different investigators in different regions and in the same region. These effects cannot be measured directly with one measure (one question). A set of measurements must be used and then combined with the results to be more accurate. Therefore, by issuing questionnaires to quantify the influence degree of the influencing factors and scoring them, more influencing factors can be grouped into several new comprehensive factors by using the factor analysis method. These comprehensive factors can well cover each item of the original data and, at the same time, simplify the analysis process into the analysis of factor items, making the research process more concise and clear and reducing the intensity of research. However, factor analysis can only be used for comprehensive evaluation, and it also needs the quantity and composition of the data. When the respondents are fishermen, their age, education, and the dialects of each region are very challenging for the collection of respondents. When designing the questionnaires, the questions should be designed specifically and filled in and understood as simply as possible to ensure that the data can be used for molecular analysis.

From the perspective of the contribution degree of each common factor, the five common factors analyzed by SPSS 23.0 software had the highest contribution to the satisfaction of the summer fishing moratorium closure period (16.396), and the overall satisfaction score was the lowest (F1 = 2.07). All these indicated that this factor was the main factor influencing fishermen's satisfaction during the fishing moratorium, and it was also the worst factor. For fishermen, gaining economic resources is a profession, and the three-and-a-half month fishing moratorium means that it is impossible to obtain benefits from fishing. Previous studies have shown that the implementation of the summer fishing moratorium ensures the reproductive and growth of most fish (Musiello-Fernandes et al., 2017), and that fishing efficiency is improved and the corresponding cost is reduced after the fishing moratorium. Thus, the annual income fluctuation of fishermen is not significant (Chen et al., 2008). However, others believe that the increased cost of fishing at sea, the increase in production without an increase in income. In addition to the implementation of the summer fishing moratorium system, the subsidies are insufficient, and the heavy economic burden makes it difficult for fishermen to solve the production and living problems during the summer fishing moratorium (Hou et al., 2009). According to our survey, the subsidies for the summer fishing moratorium are only issued in some areas of Guangdong Province, and there is almost no fishing moratorium subsidy in Guangxi and Hainan provinces in China. This led to the fact that these fishermen, who were already living on the sea, had no source of income during the three and a half months of the moratorium, and some families rely on the income from fishing to pay school fees for their children, while some fishermen's bank loans for fishing vessels were not repaid, making life very difficult for these fishermen during the moratorium. Therefore, fishermen have a higher demand for appropriate subsidies during the summer fishing moratorium, and the government should consider increasing fishery subsidies to meet the basic needs of fishermen during this period.

The factor with the second highest contribution rate is the illegal fishing boat poaching factor (12.095) and the overall satisfaction score (F2 = 3.27), and this factor has a greater impact on fishermen's satisfaction and a higher degree of satisfaction with this factor. In this study, based on the research project "Assessment of the order of marine fishing moratorium in the South China Sea" of the South China Sea Fisheries Research Institute, several batches of fixed-point cruises were conducted in the South China Sea. The survey found that 73% of the fishing boats operating illegally during the moratorium period were small "three-no" fishing boats, and the main type of illegal operation was gillnetting. The existence of "three-no" fishing boats not only violates Chinese laws and regulations and hinders the implementation of the reduction policy but also destroys the fishery ecological environment, disrupts the fishing moratorium system and the normal fishery production order, and infringes upon the legitimate rights and interests of the majority of fishermen (Xu, 2007). Some studies have shown that in the face of the current scarcity of fishery resources in China, cleaning up and banning fishing-related "three-no" boats are crucial to restoring the original order and relieving the pressure of marine fisheries, restoring fishing grounds, and eliminating the “tragedy of the commons” (Li, 2015). In some fishing areas, some seasonal aquatic economic species will disappear if not fished for a certain period of time. For example, in the Duntou fishing port of Dongfang city in Hainan Province, most of the local fishermen especially use small gillnet boats to catch a kind of seasonal crab. However, they are not allowed to operate during the seasonal fishing moratorium, resulting in the waste of these crab resources. A similar situation exists in the Yunao fishing port of Nan’ao county of Guangdong Province. A species of small squid is present in this sea during the fishing moratorium and if not harvested, will disappear after the moratorium. All of these problems in the system will affect fishermen’s satisfaction with the fishing moratorium. Therefore, local government fishery agencies should issue special fishing permits for special economic fish stocks. In addition, it is necessary to strengthen the joint coordination with the fishery administration and the marine police and other aspects to implement the crackdown on Vietnamese fishing vessels infringing on fishing and to defend the rights and interests of our fishermen.

The length of the fishing moratorium factor (12.012) also has a great influence on the satisfaction of the fishing moratorium. The survey found that about 56% of the fishermen hope to appropriately shorten the duration of the fishing moratorium and 72.3% of the fishermen hoped that the moratorium would be brought forward due to the frequent typhoons in the South China Sea every August, which will affect the fishermen’s fishing at the end of the moratorium. Some studies have shown that most of the typhoons in the South China Sea are concentrated from July to September (He and Ding, 2007), which marks the end of the fishing moratorium; fishermen cannot catch fish due to frequent typhoon weather. The current time of the three major sea areas in China is unified for the beginning of May. The development of the fishing moratorium time is measured according to the breeding period of the main economic fish in China’s major sea areas, the growth period (Ling et al., 2004; Fu, 2008) and China’s regions can be unified management and other comprehensive situations (Lu, 2005). It reduces the pressure of fishery law enforcement, increases the operability of law enforcement, and facilitates unified supervision. Some studies have concluded that the current fishing moratorium in the South China Sea is still unable to cover the growth period of juvenile fish, suggesting that the current fishing moratorium should be advanced by half a month or from April 1 every year, or even try to close fishing in spring in the future (Feng et al., 2019), so as to protect the growing juveniles and spawning brood stock. However, whether to adjust the time of the fishing moratorium in China’s sea season still needs further study.

CONCLUSION

The satisfaction score of the summer fishing moratorium system was F = 3.53, which was still an overall system that made fishermen more satisfied. However, there are still some regulations that do not match the reality and overestimate the responsibility of fishermen to the fishing moratorium system. This system is too idealistic and neglects operability to accommodate different contradictions. In addition to various subjective factors and macro-conditions, the actual implementation has encountered many new situations and problems. The improvement process should be biased toward the actual needs to find a balanced system between fishermen, people, and the system so that it can be sustainable.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material; further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

LZ and QW designed the study. LZ, YZ, and SM collected the fishery data. LZ analyzed the data. SM and YZ helped with data collection and analysis. LZ wrote the article. All authors have read and agreed to the published version of the manuscript.

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Fish Assemblages in Subtidal Seagrass Meadows Surrounding the West Sand, South China Sea

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Both beach seine (BS) and line transect-based underwater visual census (UVC) methods were applied to investigate fish assemblages of the West Sand (Xisha Islands, South China Sea) seagrass meadows and their relationship with seagrass coverage. The West Sand seagrass meadows were divided into four survey areas, two (areas A and B) to the north and two (areas C and D) to the south. The average seagrass coverages (%) in the north were significantly higher than those in the south ($p < 0.05$). The dominant seagrass species is *Thalassia hemprichii*, contributing to over 70% substrate coverage in all four areas. A total of 843 individuals from 25 fish species belonging to 22 genera, 11 families, and ten orders were recorded, with five species obtained by both BS and UVC methods. The labrids *Halichoeres trimaculatus* and *Stethojulis strigiventer* were the only two species that dominated (index of relative importance $IRI > 500$) in both BS and UVC methods. Fish assemblages among the four areas were significantly different (BS, $p = 0.002$; UVC, $p = 0.002$); area A was significantly different from areas B–D ($p < 0.05$), which was mainly driven by the variation in *H. trimaculatus* density. Furthermore, fish assemblages of the four areas (abundance data) revealed by both BS ($r^2 = 0.8455$) and UVC ($r^2 = 0.9354$) methods showed high correlation with the seagrass coverage. The majority of the individuals (>93%) recorded by BS and UVC methods were shorter than the total length at maturity (*TL*m), with 80.36 and 46.13% of individuals less than half the sizes of their *TL*m, respectively. Our results, for the first time, proved the importance of seagrass meadows at the West Sand as fish nursery grounds. The ecological roles of seagrass habitats in South China Sea merit further investigation.

Keywords: beach seine, underwater visual census, juvenile fish, nursery ground, species composition, vegetative habitat, Xisha islands

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INTRODUCTION

Seagrass meadows, as an important part of coastal and estuarine ecosystems, mainly form in tropical and subtropical coastal and shallow reef regions (Short et al., 2011; Syukur et al., 2017; Carmen et al., 2019). Seagrass habitats support over 20% of the global largest industrial and small-scale fisheries as nursery grounds (Unsworth et al., 2018). The colonization of seagrass meadows has been proved to promote the increase in sediment mud and organic matter contents, as well as the abundance and richness of macrofaunal communities (Lundquist et al., 2018). Seagrass meadows are beneficial to the retention of eggs and spawning by mitigating the turbulent hydrodynamics (Short et al., 2011; Costa et al., 2020).

Fishes, especially coral reef fishes, usually utilize different habitats at different life stages due to the requirement of food and shelter (Galaiduk et al., 2018; Sambrook et al., 2019; Berkström et al., 2020). The high overlap (40–43%) in fish richness between coral reefs and vegetative habitats (seagrass and macroalgal habitats) indicates their important roles in homing fishes (Sambrook et al., 2019). Many coral reef fishes spend part of their lifetime in seagrass meadows as nursery, refuge, and foraging grounds (Honda et al., 2013; Whitfield, 2016; Parsons et al., 2018). Compared with coral reefs, juvenile fishes have higher densities and survival and growth rates in seagrass meadows, indicating the important nursery function of this habitat (Nagelkerken et al., 2002; Nakamura et al., 2012; Moussa et al., 2020). Seagrass meadows provide structural complexity as shelter, rich small food, and protection for juvenile fishes (Cullen-Unsworth and Unsworth, 2013).

Different seagrass meadow structures harbored distinct fish assemblages. For example, larger fishes could benefit from the open space below an *Amphibolis griffithii* leaf canopy, while small fishes could conceal in the foliage of *Posidonia sinuosa* (Hyndes et al., 2003; Sambrook et al., 2019). Meanwhile, fishes are also beneficial to the seagrass habitats through their ecological niche, especially in the transportation of nutrients and energy between different habitats and ecosystems (Burkepile et al., 2013). However, seagrass meadows have suffered great degradation from anthropogenic influences and natural disasters due to their vulnerability (Halpern et al., 2008), and attempts and measures for their conservation and restoration are essential (Carmen et al., 2019; Orth et al., 2020).

In Chinese waters, 22 seagrass species (four families and 10 genera) have been recorded among the 72 species being recorded globally (Short et al., 2011; Zheng et al., 2013; Huang et al., 2016). Seagrass meadows in China were divided into two geographic bioregions, i.e., the South China Sea Bioregion in southern China and the Bohai Sea and Yellow Sea Bioregion in northern China. The former was greater in terms of the number of species and cover area (Shi et al., 2010; Zheng et al., 2013). In the South China Sea Bioregion, 15 seagrass species were recorded, with *Thalassia hemprichii* dominant in Hainan and Taiwan, and *Halophila ovalis* was the most widely distributed in Hainan, Guangdong, Guangxi, Taiwan, and Hong Kong (Shi et al., 2010; Zheng et al., 2013). Seagrass meadows have been threatened by anthropogenic activities in southern China, including Hainan, Guangdong, and Guangxi (Huang et al., 2006). At Xisha Islands (15°47′–17°08′ N, 110°10′–112°55′ E), South China Sea, five seagrass species were recorded, namely, *T. hemprichii*, *H. ovalis*, *H. minor*, *H. beccarii*, and *Thalassodendron ciliatum*, and there was no information on Nansha Islands (Fan et al., 2009; Zheng et al., 2013).

Despite the important ecological services of seagrass meadows, studies regarding the fish nursery function of this important habitat in the South China Sea are few. To our best understanding, there was only one study that presented a photo evidence to document the importance of seagrass habitats as a juvenile fish refuge at Xisha Islands (Du et al., 2020). Clearly, further studies such as detailed and statistical data on fish assemblage are essential to provide robust evidence to support the importance of seagrass meadows for juvenile fishes.

The Qilianyu cluster of Xisha Islands consists of eight islands, with the West Sand being the only one significantly surrounded by a large area of seagrass meadows. The aims of this study were to examine the current status of the seagrass meadows at the West Sand in terms of both seagrass species diversity and coverage, to evaluate the fish assemblages in seagrass meadows using beach seine (BS) and line transect-based underwater visual census (UVC) methods, and to analyze the correlation between seagrass coverage and fish assemblage. The fish nursery function of the seagrass meadows at the West Sand is also discussed.

MATERIALS AND METHODS

Study area background

The Qilianyu cluster (Xisha Islands, South China Sea) consists of eight islands, namely, the West Sand, Zhaoshu Island, North Island, Middle Island, South Island, North Sand, Middle Sand, and South Sand (**Figure 1A**). The Qilianyu cluster is mainly influenced by a tropical oceanic monsoon climate with an average annual temperature of approximately 26.5°C (24–29°C) and characterized by irregular semidiurnal tides (Huang et al., 2008; Wu et al., 2018).

The West Sand and Zhaoshu Island are grouped into the same reef (Wu et al., 2018; Sun et al., 2021). The West Sand is an uninhabited island that has an estimated land area of 0.26 km^2 and 2.1-km coastline (Jia et al., 2019) and is significantly surrounded by dense seagrass meadows. The seagrass meadows extend at least 100 m from the shore.

Survey method and design

Around the West Sand, four survey areas (A–D) were set with each approximately 27,500 m^2, and the separation between each survey area was at least 200 m (**Figure 1B**). The fish assemblages in the seagrass meadows were investigated using BS and UVC methods in each survey area in December (winter) 2019. Samplings were conducted during daytime when the tide started to rise and the water depth was less than 1.5 m.

A trapezoid-shaped BS was used, with 6 m in upper side width, 4.5 m in lower side width, 0.9 m in height, and 4 mm in mesh size. A total of twelve 20-m line transects were randomly set in each survey area, and the distance between two transect lines was about 20 m. The BS was manually hauled by two people along each transect line against the coastline (**Figure 1C**). The sampling area of each haul was about 90 m^2 (4.5 m × 20 m). Before every haul, there was a waiting time of at least 5 min to minimize any disturbance to fishes. All collected fishes were put in labeled plastic hermetic bags and immediately stored in cooler boxes with ice. In the laboratory, each individual was measured for the total length (TL, in mm), standard length (SL, in mm), and body weight (BW, in g). Each individual was identified to the species or genus level if possible according to the external morphological features (Fu, 2014; Allen et al., 2015; Fang and Lv, 2019) and the classification system followed Nelson et al. (2016).

UVC surveys were conducted using a GoPro Action video recorder. A total of fourteen 10-m line transects were randomly deployed in each survey area. The diver swam above the 10-m line transect and recorded the types of substrates at every 10-cm interval, i.e., a total of 100 recording points on each 10-m line

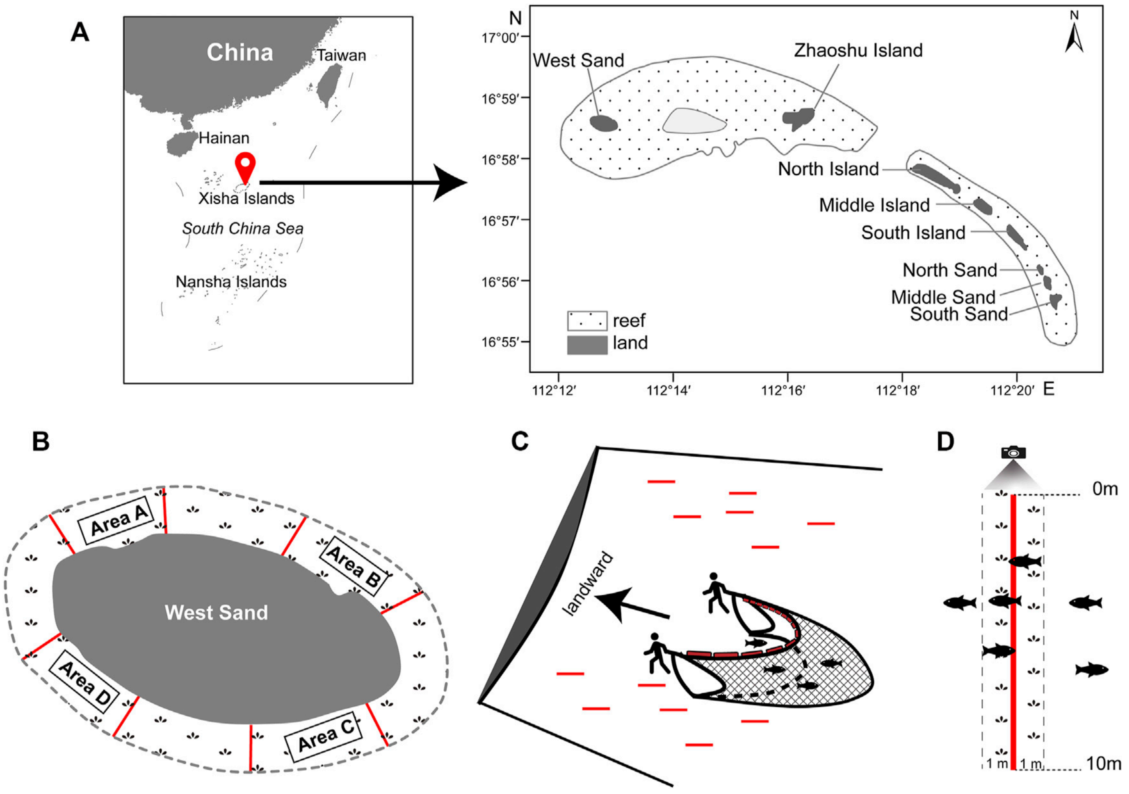

FIGURE 1 | (A) Location of the Qilianyu cluster (red balloon), Xisha Islands, South China Sea. **(B)** The four survey areas A–D at West Sand. **(C)** Beach seine method (BS). Red lines indicate the 12 random 20-m line transects in a survey area. **(D)** Line transect-based underwater visual census method (UVC). The red line indicates one of the fourteen 10-m line transects in a survey area.

transect. The seagrass coverage (%) was then calculated as the number of points with seagrass/100 points × 100. Following the substrate line transect, there was at least 5 min of waiting time to minimize the disturbance to fishes, and then the fish recording was performed. The diver swam above the 10-m line transect slowly with a speed controlled at 3–5 m per minute and recorded all fishes with a recording area of about 20 m^2 (i.e., 1-m width on both sides of the 10-m line transect) (**Figure 1D**). The fish density (ind./m^2) per 10 m line transect was calculated as the number of individual fishes/20 m^2. Fish identification was conducted to the lowest taxonomic level as possible (see above), and the body sizes of recorded fishes were estimated with TL and categorized into size at every 5-cm TL interval (e.g., 0–5.0 cm, 5.1–10.0 cm, 10.1–15.0 cm).

For each species obtained by BS and UVC methods, the body size (TL) at sexual maturity (*TL*m) was collected from FishBase (www.fishbase.org). For the species only available with standard length (SL) at sexual maturity (*SL*m), the transformation to *TL*m was based on the relationship of TL and SL (www.fishbase.org). For the species only identified at the genus level, *TL*m was not applicable.

DNA Barcoding to Identify Juvenile Fish Species

DNA barcoding was also applied to ascertain the species identification of the early the juvenile fish samples. At least one specimen of each of the morphologically identified species that were collected from beach seine was randomly selected for DNA barcoding using partial sequences of cytochrome *c* oxidase subunit I (COI) gene. A piece of skeletal muscle was isolated from each specimen for genomic DNA extraction using Chelex$^{®}$ 100 resin following the instruction manual (Bio-Rad Laboratories, Inc., United States). Polymerase chain reaction (PCR) was conducted in a total volume of 25 µl, including 20 ng DNA template, 1 unit of Ex Taq DNA polymerase (Takara), 0.5 µM reverse and forward primers, 0.2 mM mixed dNTPs, 1.5 mM MgCl$_2$, and 1 × PCR buffer (pH 8.4, 200 mM Tris-HCl and 500 mM KCl). Partial sequences of cytochrome c oxidase subunit I (COI) gene were amplified using three forward primers, VF1 (5′-TCTCAACCAACCACAAAGACATTGG-3′, Ivanova et al., 2006), Fish F1 (5′-TCAACCAACCACAAAGACATTGGCAC-3′, Ward et al., 2005), and Fish F2 (5′-TCGACTAATCATAAAGATATCGGCAC-3′), in combination with two reverse primers, Fish R1 (5′-TAGACTTCTGGGTGGCCAAAGAATCA-3′) and Fish R2 (5′- ACTTCAGGGTGACCGAAGAATCAGAA-3′) from Ward et al. (2005). PCR cycling conditions are as follows: 95°C for 2 min; 39 cycles of 95°C for 30 s, 51°C for 30 s, and 72°C for 45 s; and 72°C for 2 min. PCR products were purified and sequenced by BGI Ltd. (Hong Kong) with the Sanger sequencing method. Raw sequences were assembled using Geneious Prime v11.0.911 (Biomatters Ltd.). Identification

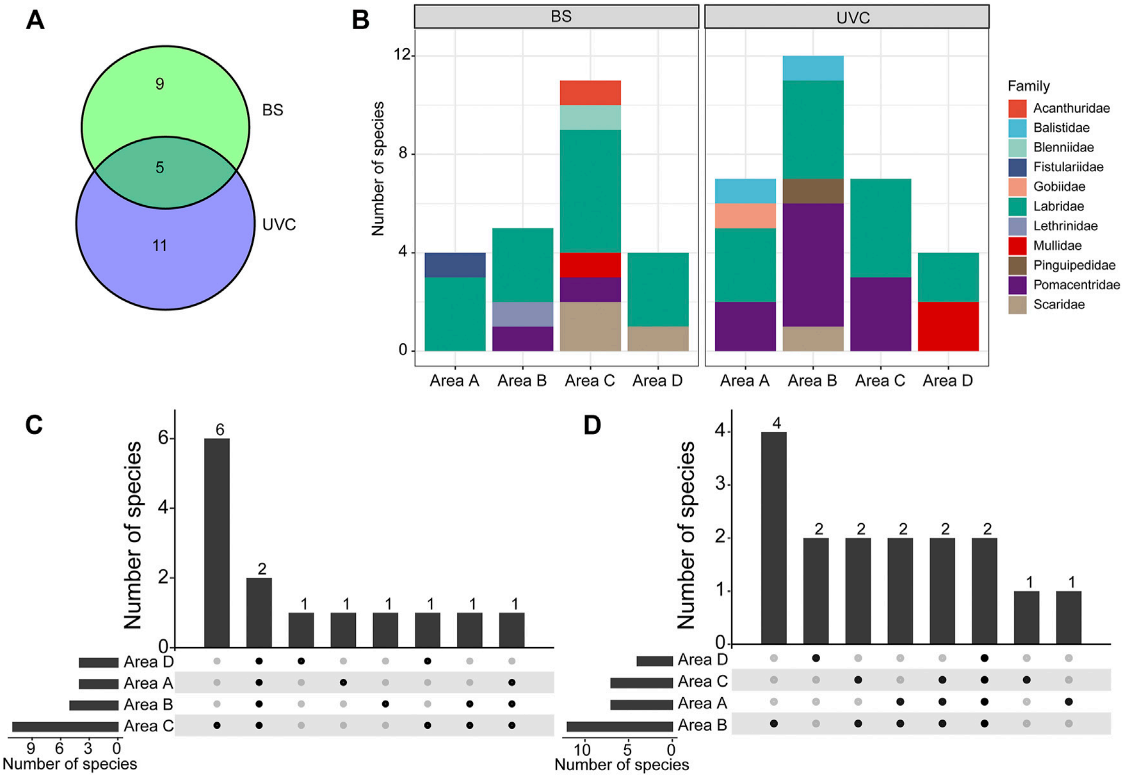

FIGURE 2 | Fish species compositions in the four survey areas A–D using BS and UVC methods at West Sand. **(A)** Species composition. **(B)** The number of species at the family level. **(C)** Species specificity and similarity among the four areas using the BS method. **(D)** Species specificity and similarity among the four areas using the UVC method. BS, beach seine; UVC, underwater visual census. The numbers above bars and the solid black spots in **(C)** and **(D)** indicate the specificity and similarity in the number of species that occurred.

TABLE 1 | Dominant species determined by the index of relative importance (*IRI*) ≥ 500.

Survey area	Survey method	Dominant species (*IRI* ≥ 500)		
		Halichoeres trimaculatus	***Stethojulis bandanensis***	***Stethojulis strigiventer***
Area A	Beach seine	–	–	7,700
	Underwater visual census	1,635	–	2,356
Area B	Beach seine	13,995	518	979
	Underwater visual census	6,126	–	–
Area C	Beach seine	12,816	1,391	–
	Underwater visual census	4,800	–	–
Area D	Beach seine	12,717	–	–
	Underwater visual census	3,462	–	–

–, IRI < *500 or not recorded.*

of the assembled COI partial sequences was performed using BLAST to search against the GenBank nucleotide database of the National Center for Biotechnology Information (NCBI) and Barcode of Life Data System (BOLD).

Data analysis

The number of individual fishes per haul (x) collected using the BS method was log(x + 1) transformed to meet the assumptions of homogeneity of variance and normality prior to univariate analysis. Then, one-way ANOVA was applied to detect the spatial difference among survey areas. A non-parametric test (Kruskal–Wallis ANOVA) was used for comparisons of other variables/datasets, i.e., the number of individual fishes per 10-m line transect recorded using the UVC method, density (ind./m^2) by BS and UVC, biomass (g/m^2) by BS, and seagrass coverage (%), because these datasets were not normally distributed even after numerous transformations (Kolmogorov–Smirnov normality test, $p < 0.05$).

The index of relative importance (*IRI*) was applied to determine the dominant species (Pinkas et al., 1971). A species is considered dominant when *IRI* ≥ 500 (Wei and Huang, 2020).

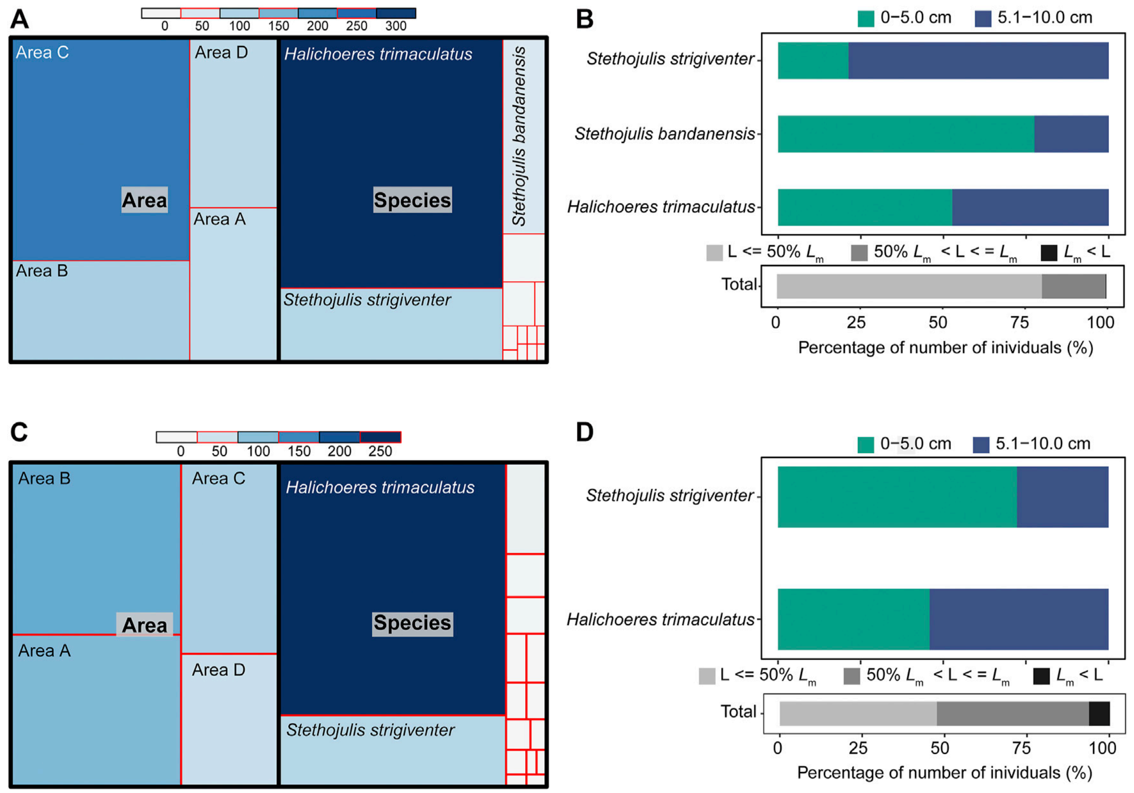

FIGURE 3 | (A) The number of individuals by area and by species and **(B)** the proportion of individuals with sizes (total length TL, cm) compared to the size at 50% of sexual maturity (*TL*m) using the BS method. **(C)** The number of individuals by area and by species **(D)** and the proportion of individuals with sizes (TL, cm) compared to the size at 50% of sexual maturity (*TL*m) using the UVC method.

For the BS method, *IRI* was calculated as (*N*% + *W*%) × *F*%. For the UVC method, *IRI* was calculated as *N*% × *F*%. *N*%, *W*%, and *F* % are the relative individual, weight, and frequency of occurrence, respectively.

A Bray–Curtis similarity matrix was constructed based on the log(x + 1)-transformed fish density to reduce the influence of rare and dominant species (Clarke and Green, 1988). Subsequently, a permutational multivariate analysis of variance (PERMANOVA) was performed to test the difference in fish communities between survey areas and methods, which was visualized using non-metric multidimensional scaling (nMDS) ordination plots (Clarke, 1993; Anderson, 2005). Similarity percentage (SIMPER) analysis was used to compare the species and their contributions to the variations in fish communities. The relationship between seagrass coverage and fish abundance was detected using canonical analysis of principal coordinates (CAP).

RESULTS

Fish species

A total of 843 individuals from 25 species belonging to 22 genera, 11 families, and ten orders were recorded (**Supplementary Table S1**). Fourteen species (belonging to 13 genera and eight families) were captured using the BS method, and 16 species (belonging to 14 genera and seven families) were observed using the UVC method; five species were recorded in both methods, namely, *Chrysiptera biocellata*, *Halichoeres trimaculatus*, *Parupeneus barberinoides*, *Stethojulis bandanensis*, and *S. strigiventer* (**Figure 2A**; **Supplementary Table S1**). Labridae was the most dominant family with eight species recorded.

All specimens collected by the BS method in the four survey areas A–D were identified to species level via morphological and molecular approaches, except for a single specimen of *Petroscirtes* which could only be identified to the genus level. The partial COI sequences obtained were deposited in NCBI GenBank with accession numbers MZ539887–MZ539934 (**Supplementary Table S1**); they were 98–100% identical to the corresponding reference sequences on NCBI GenBank and BOLD. *H. trimaculatus* and *S. bandanensis* were captured in all four survey areas (**Supplementary Table S1**). Labridae consisted of 42.86% of the total number of species collected (**Figure 2B**). The highest species number (11 species) was recorded in area C; six species were collected only in area C, and one species was collected only in areas A, B, and D, respectively (**Figure 2C**).

By the UVC method, *H. trimaculatus* and *S. strigiventer* were observed in all four survey areas (**Supplementary Table S1**). Labridae and Pomacentridae accounted for the highest

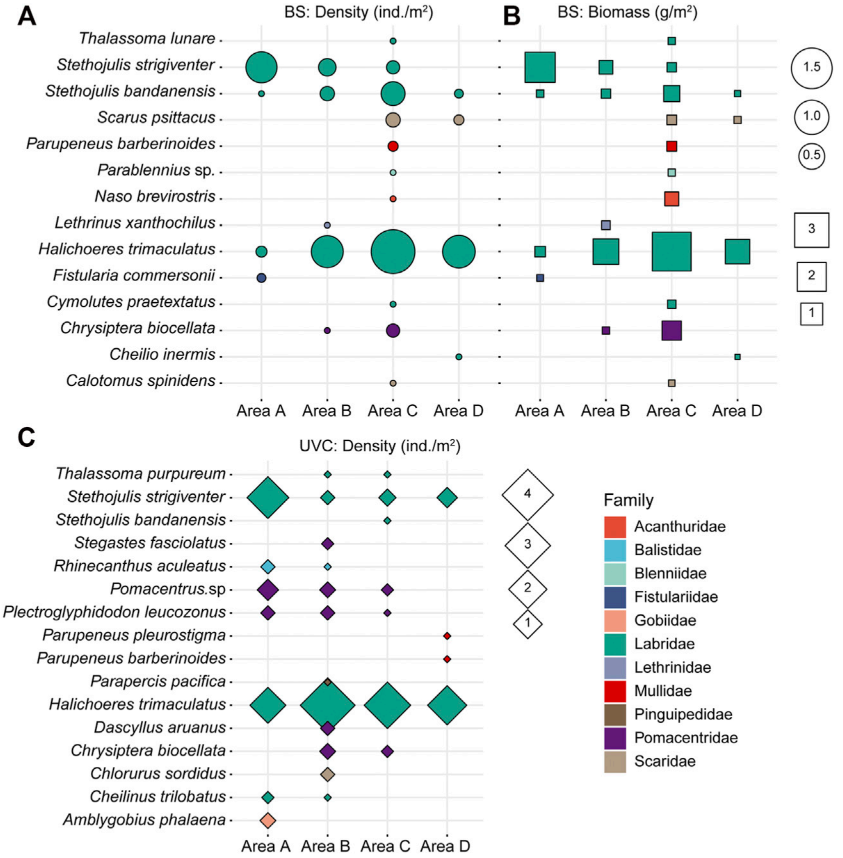

FIGURE 4 | (A) Density (ind./m^2) and **(B)** biomass (g/m^2) of fish species collected using the BS method. **(C)** Density (ind./m^2) of fish species using the UVC method.

proportion of the total number of species, together up to 100% in area C (**Figure 2B**). Area B was the highest in species number (12 species); four species were recorded only in area B, two species were found only in area D, and one species was found only in area A and area C (**Figure 2D**).

Three dominant species, *H. trimaculatus*, *S. bandanensis*, and *S. strigiventer*, were determined with *IRI* > 500 (**Table 1**). In particular, *H. trimaculatus* was the primary dominant species in three of the four survey areas of the BS method and in all four survey areas of the UVC method.

Abundance and body size

A total of 494 individuals were collected from the four survey areas A–D using the BS method (**Figure 3A**; **Supplementary Table S1**). *H. trimaculatus* (319 individuals), *S. bandanensis* (49 individuals), and *S. strigiventer* (94 individuals) together contributed 93.52% of the total number of individuals. Area C had the highest number of individuals (18.92 ± 24.78 ind./haul), which was significantly higher than that in area A (6.50 ± 11.66 ind./haul) (one-way ANOVA, $p < 0.05$). No significant difference was detected among other areas ($p > 0.05$ or $p > 0.15$). Nearly all the individuals (99.80%) were shorter than their *TL*m, and 80.36% of the total individuals were less than half the sizes of their *TL*m (**Supplementary Table S2**). For the three dominant species, many individuals were smaller juveniles (≤5.0 cm TL), i.e., 52.66% for *H. trimaculatus*, 77.55% for *S. bandanensis*, and 21.28% for *S. strigiventer* (**Figure 3B**).

A total of 349 individuals were recorded using the UVC method (**Figure 3C**; **Supplementary Table S1**). Two species, *H. trimaculatus* (231 individuals) and *S. strigiventer* (65 individuals), contributed 84.81% of the total number of individuals. The mean number of individuals per 10-m line transect was 7.43 ± 8.08 in area A, 8.43 ± 8.63 in area B, 5.36 ± 5.97 in area C, and 3.71 ± 6.71 in area D. No significant difference in the number of individuals per transect was found among the four survey areas A–D (Kruskal–Wallis ANOVA, $p > 0.15$). Most individuals (93.70%) were shorter than their *TL*m, and 46.13% of the total individuals were less than half the sizes of their *TL*m (**Figure 3D**; **Supplementary Table S3**).

Density and biomass

The mean fish densities using the BS method were 0.07 ± 0.13 ind./m^2 in area A, 0.09 ± 0.09 ind./m^2 in area B, 0.21 ± 0.28 ind./m^2 in area C, and 0.08 ± 0.12 ind./m^2 in area D. Fish density was not significantly different among the four survey areas A–D (Kruskal–Wallis ANOVA, $p > 0.05$, **Figure 4A**). The mean biomasses were 0.20 ± 0.38 g/m^2 in area A, 0.16 ± 0.16 g/m^2

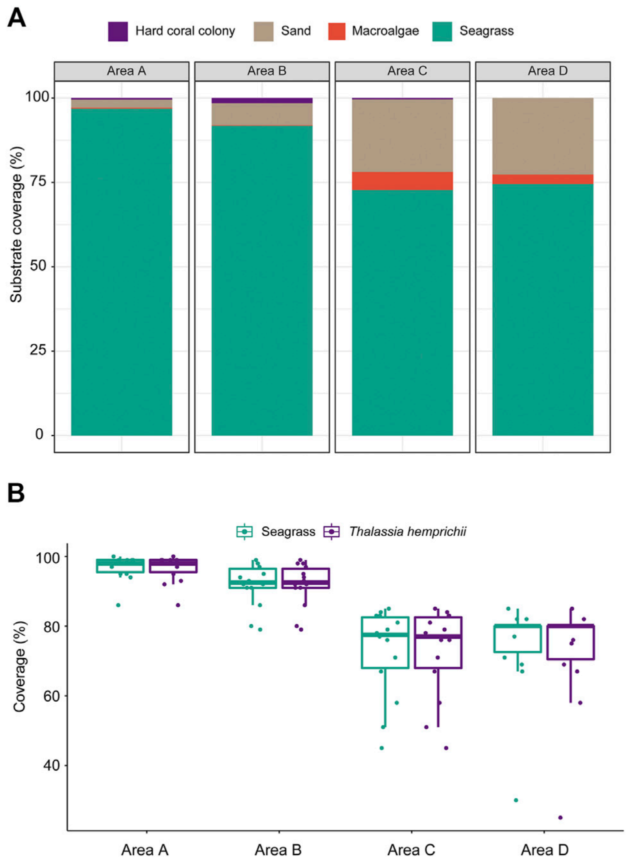

FIGURE 5 | (A) The average substrate coverages (%) and **(B)** variations in seagrass coverage and *T. hemprichii* coverage of 10-m line transects (N = 14) in the four survey areas A–D at West Sand.

in area B, 0.48 ± 0.66 g/m^2 in area C, and 0.11 ± 0.17 g/m^2 in area D. Spatial variations in biomass were also not significantly different among the four areas A-D (Kruskal–Wallis ANOVA, $p > 0.05$, **Figure 4B**).

The mean densities using the UVC method were 0.37 ± 0.40 ind./m^2, 0.42 ± 0.43 ind./m^2, 0.27 ± 0.30 ind./m^2, and 0.19 ± 0.34 ind./m^2, in areas A, B, C, and D, respectively. No significant differences in fish density were found among the four areas A-D (Kruskal–Wallis ANOVA, $p > 0.15$, **Figure 4C**).

Overall, Labridae had the highest density and biomass, contributing over 90% in density and 79% in biomass using the BS method and over 80% in density using the UVC method. For both BS and UVC methods, *S. strigiventer* had the highest density and biomass in area A, while *H. trimaculatus* had the highest density and biomass in areas B–D.

Seagrass coverage and spatial structure of fish assemblages

There are two seagrass species at the West Sand, namely, *H. ovalis* and *T. hemprichii*. The mean seagrass coverages (%) in area A (96.86 ± 3.61%) and area B (91.79 ± 6.27%) were significantly higher than those in area C (72.71 ± 12.85%) and area D (74.50 ± 13.84%) (Kruskal–Wallis ANOVA, $p < 0.05$, **Figure 5A**). The absolute dominant species was *T. hemprichii*, contributing to >99% seagrass coverage in areas A and B and >97% in areas C and D (**Figure 5B**). Other substrates included sandy bottom, macroalgae, and coral colonies (**Figure 5A**).

Fish assemblages recorded using BS and UVC methods had significant difference (PERMANOVA pairwise test, $p = 0.001$). Fish assemblages had significant differences among the four areas (PERMANOVA; BS, $p = 0.002$; UVC, $p = 0.002$), with area A being significantly different from areas B–D in both BS and UVC methods (PERMANOVA pairwise tests: $p < 0.05$) (**Figures 6A,B**). Furthermore, the fish assemblages of the four areas from both BS ($r^2 = 0.8455$) and UVC ($r^2 = 0.9354$) showed high correlation with the seagrass coverage (%) using CAP analysis (**Figures 6C,D**).

SIMPER analysis revealed the fish species contributing the difference between areas. High dissimilarity of fish assemblages between areas was detected in BS (>61%) and UVC (>58%) methods (**Table 2**). More than 90% of dissimilarity across areas was contributed by five and 12 species from BS and UVC, respectively. Variations in fish assemblages between areas were mainly driven by the changes in the density of *H. trimaculatus* (BS: > 43%; UVC: > 40%).

DISCUSSION

Survey methods

Multiple stereo video-based and net-based methods have been commonly applied to quantify fish assemblages in nearshore waters and to further understand the ecological characteristics of fishes (Bortone et al., 1989; Saleh et al., 2020). However, fish assemblages collected by different survey methods, e.g., baited and unbaited remote underwater video (BRUV; URUV), diver-operated video (DOV), UVC, BS and beam trawling, have marked differences due to their intrinsic selection (Guest et al., 2003; Horinouchi et al., 2005; French et al., 2021). Thus, application of multiple methods has been suggested to complement each other to better assess fish assemblages, e.g., in seagrass habitats (Harmelin-Vivien and Francour, 1992; Baker et al., 2016; French et al., 2021).

This study showed that BS and UVC methods were able to sample different fish assemblages within the same area of seagrass meadow, supporting the necessity of using combined sampling methods. Our samplings took place over a relatively large surveyed area (approximately 27,500 m^2 per survey area) and separated by a sufficient distance apart from each haul and each line transect; therefore, the sampling made the replicates to be not likely performed repeatedly over the same point and thus minimized the operating impact on seagrass habitats. UVC combined with line transect and video application is believed to pose less disturbance to the ecosystem; however, it has constraints in size estimation (Edgar et al., 2004), which is crucial in evaluating the nursery services of seagrass meadow. The relatively clear water (visibility about 15 m) and short length of seagrass leaves (*H. ovalis* and *T. hemprichii*) at the West Sand could reduce the potential of missing secretive species.

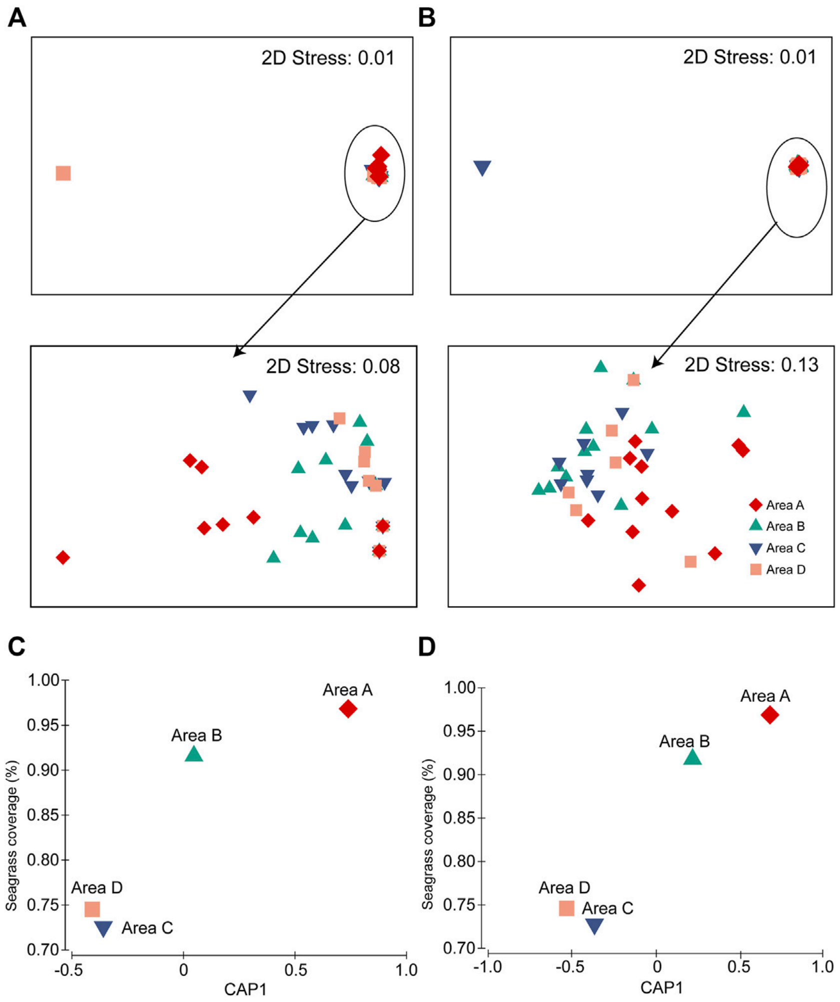

FIGURE 6 | Differences in fish assemblages among the four areas A–D: **(A)** BS method and **(B)** UVC method. CAP plot showing the correlation between seagrass coverage (%) and fish abundance: **(C)** BS method and **(D)** UVC method.

Seagrass meadows and fish assemblages

Two seagrass species were recorded at the West Sand, with *T. hemprichii* identified as the dominant species. *T. hemprichii* was also reported as a dominant seagrass species in the southern Philippines (Espadero et al., 2021). Interestingly, both studies reported that the family Labridae was the dominant fish taxon in these "*T. hemprichii*-dominating" seagrass meadows. In another study, the canopy height of seagrass meadows was proved as the paramount indictor for the density and species richness of juvenile fishes in the east coast of Zanzibar Island, Tanzania (Gullström et al., 2008). The seagrass diversity and 3-dimensional seagrass structure have great influence on fish assemblages (Hori et al., 2009; Park and Kwak, 2018). The species-specific association between the certain fish species and seagrass species would deserve further investigations.

Fish abundance can be associated with seagrass coverage. For example, in the Venice Lagoon (Italy), the abundance of *Nerophis ophidion* and *Syngnathus typhle* (Syngnathidae) was strongly correlated with seagrass coverage as revealed by canonical correspondence analysis (CCA) (Franco et al., 2006). Our results also proved the high correlation between seagrass coverage and fish abundance using both BS and UVC methods. Compared with unvegetated habitats, seagrass meadows showed not only higher fish abundance and species richness but also higher growth and survival rates (Heck et al., 2003). The morphological characteristics and the structural complexity of seagrass meadows had great influence on regulating fish assemblages (Gullström et al., 2008). Fish density and species diversity are much higher in seagrass meadows than those in unvegetated sandy bottoms (Larkum et al., 1989). According to an experiment conducted on Barker Inlet/Port River Estuary (Australia), the fish abundance in seagrass meadows even after the removal of seagrass canopy was higher than that in unvegetated areas (Connolly 1994). Hori et al. (2009) proved that the seagrass species diversity, vegetation, and canopy height of seagrass meadows affected greatly the abundance, biomass, and richness of associated fish assemblages using canonical correlation analysis (CCoA).

TABLE 2 | Contributory influence of fish species on the dissimilarity between fish assemblages using BS and UVC methods (cumulated contribution >90%).

Survey method	Species	Survey area					
		A vs B	A vs C	A vs D	B vs C	B vs D	C vs D
Beach seine	Average dissimilarity	82.12	86.55	88.02	64.86	63.83	61.89
	Halichoeres trimaculatus	43.69	47.79	55.37	62.00	71.89	70.65
	Stethojulis bandanensis	9.74	13.49	–	20.61	10.82	17.39
	Stethojulis strigiventer	40.47	30.38	34.14	9.78	10.45	–
	Cheilio inermis	–	–	–	–	–	3.14
	Fistularia commersonii	–	–	4.10	–	–	–
Underwater visual census	Average dissimilarity	78.09	76.23	74.28	59.41	64.13	58.70
	Halichoeres trimaculatus	41.25	44.94	40.36	61.41	60.27	69.08
	Stethojulis strigiventer	26.96	28.13	32.99	7.55	13.71	16.35
	Pomacentrus sp.	8.50	8.88	10.82	5.91	5.32	2.43
	Plectroglyphidodon leucozonus	4.44	2.75	–	4.87	4.51	–
	Chrysiptera biocellata	–	–	–	4.22	2.52	–
	Stethojulis bandanensis	–	–	–	3.30	–	4.48
	Stegastes fasciolatus	–	–	–	2.42	2.56	–
	Thalassoma umbrostigma	–	–	–	2.19	–	–
	Parapercis pacifica	–	–	–	–	2.35	–
	Amblygobius phalaena	4.21	4.46	5.40	–	–	–
	Rhinecanthus aculeatus	3.54	3.00	3.51	–	–	–
	Cheilinus trilobatus	2.61	–	–	–	–	–

–, not applicable.

Furthermore, fishes inhabiting the surface, inside, and bottom of seagrass meadows greatly correlated with seagrass meadow characteristics (e.g., biomass, richness, and canopy height of seagrass species) (Hori et al., 2009). This interesting topic merits further investigation.

The variations in fish assemblages between daytime and nighttime were reported. For example, higher species richness, abundance, and biomass of piscivorous fishes were found in nighttime in a seagrass bed of northern Japan (Tanaka et al., 2018). Some piscivorous fishes, *Eleginus gracilis* (Gadidae), *Sebastes schlegelii* (Sebastidae), and *Myoxocephalus brandtii* (Cottidae), swam to seagrass habitats from adjacent ecosystems for feeding in the night and consequently increased the predation risk for small fishes in seagrass beds (Tanaka et al., 2018). A higher proportion of large individuals were collected using seine nets in nighttime compared to that in the daytime in *Zostera capricorni* seagrass habitats in Moreton Bay, Australia (Guest et al., 2003). In the seagrass habitats dominated by *T. testudinum* in the coast of Quintana Roo, Mexico, higher fish abundance and species richness were collected in daytime using visual surveys (Yeager and Arias-Gonzalez, 2008). In this study, dominant species, *H. trimaculatus*, *S. bandanensis*, and *S. strigiventer*, all from Labridae, contributed most to the variations of fish assemblages in the daytime at the West Sand, all feeding on benthic invertebrates (e.g., crustaceans, mollusks, polychaetes). The comparison of fish assemblages between daytime and nighttime needs to be taken into account in future studies.

Ecological services of seagrass meadows

This study documented the importance of seagrass meadows at the West Sand as fish nursery grounds. A high number of juvenile fishes were detected in seagrass meadows of the West Sand; i.e., approximately 99.80% and 93.70% individuals were juveniles using BS and UVC methods, respectively. Moreover, 80.36% and 46.13% of total individuals collected by BS and UVC methods were earlier-stage juveniles, less than half the sizes of their *TL*m, respectively. Three dominant fish species, *H. trimaculatus*, *S. bandanensis*, and *S. strigiventer*, were realized to be primarily dominant with the major proportion of juveniles <5 cm TL. In a previous study using the UVC method in the lagoon, the coral reef habitat, between the West Sand and Zhaoshu Island with a water depth of 2–5 m, *H. trimaculatus* was also one of the dominant species, with some individuals beyond the size of sexual maturity based on the external morphology (Yang et al., 2018, data not shown). *H. trimaculatus* was, however, not a dominant species outside the lagoon, the same coral reef habitat, with 9–12 m in depth (Yang et al., 2018).

The fish nursery function of seagrass meadows has raised attention (Nordlund et al., 2018; Fulton et al., 2020). For example, 83.23% juveniles of *H. trimaculatus* collected from the seagrass and seaweed beds in New Caledonia were of small sizes, i.e., 1/3 to 2/3 of their *TL*m (Mellin et al., 2007). In the northern Sulawesi, Indonesia, over 93% individuals sampled from the seagrass meadows using the BS method were juveniles (Du et al., 2018). Using the UVC method, Moussa et al. (2020) reported that 73% of the observed individual fishes were juveniles in the seagrass beds at the coast of Mayotte Island, Indian Ocean. Gerard et al. (2015) showed that about 98% of *Lutjanus griseus* juveniles inhabited seagrass habitats based on carbon- and nitrogen-stable isotope and otolith analyses.

Other ecological services of seagrass meadow, such as breeding grounds and foraging grounds, were also reported. In Liusha Bay, southern China, *Stethojulis trilineata* laid their eggs on the entire leaf of *H. ovalis* and primarily fed on seagrass in winter, indicating the important spawning and early life stage feeding roles of seagrass for fishes (Jiang et al., 2020). In Plaridel and Laguindingan, the Philippines, at least 17 species foraged in seagrass beds with rising tides (Espadero et al., 2020). Fishes are also beneficial to seagrass habitats through their ecological niche, especially in the transportation of nutrients and energy between different habitats and ecosystems (Burkepile et al., 2013). These ecological services mentioned above may be also provided by seagrass meadows around the West Sand and could be analyzed in subsequent research studies.

CONCLUSIONS

The status of seagrass meadows and its association with fish assemblages in the South China Sea have been largely unknown. In this study, we applied for the first time two complementary methods (non-destructive method: UVC; destructive method: BS) to analyze fish assemblages in seagrass meadows at the West Sand. Our results revealed that more than 90% of the fish samples were undersized at sexual maturation, and most of them (about 80% by the BS method and 46% by the UVC method) were smaller juveniles (i.e., less than half the sizes of their *TL*m). Fish assemblage data collected from BS and UVC methods were significantly different. However, both methods have detected Labridae as the dominant group; the three dominant species, *H. trimaculatus*, *S. bandanensis*, and *S. strigiventer*, were nearly all juveniles. High correlations were detected between fish assemblage and seagrass coverages from both BS and UVC methods. The significant spatial difference in fish assemblage among areas A–D was mainly driven by the spatial variations in the density of dominant species. Our results support the ecological importance of seagrass meadows as nursery grounds for coral reef fishes. The roles of seagrass meadows as fish spawning grounds and the habitat shifts from seagrass meadows to nearby coral reefs with the developmental life stages merit further evaluation.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI [accession: MZ539887–MZ539934].

ETHICS STATEMENT

The animal study was reviewed and approved by Sansha Civil Government of Hainan Province, China.

AUTHOR CONTRIBUTIONS

Q-qR wrote the first draft. Q-qR, PL, and ML revised the manuscript. Q-qR, QX, J-hW, and ML conducted fieldwork. Q-qR, PL, G-hY, QX, and ML did the data analysis. PL, MY, and J-hW performed molecular analysis. All authors contributed to the article preparation and approved the submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2021.765702/full#supplementary-material

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POLICY BRIEF
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China Revamping Decades-Old Fisheries Law to Combat Illegal, Unreported, and Unregulated Fishing: Stimulating the Intersection of Law, Technology, and Markets

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As the world's leading fish producer, exporter and consumer State, China must act decisively to eradicate national and international commerce in wild-capture species derived from illegal, unreported, and unregulated (IUU) fishing. In 2019 China launched an overhaul of its 1986 Fisheries Law with a draft statute subject to the deliberation of the National People's Congress. In this policy brief, we argue that China should not miss the opportunity but proactively align with the global paradigm shift toward responsible and sustainable fisheries management. To amend the patchy legal framework and implement the rule of law ahead, we suggest technology-enabled traceability and market-responsive solutions to help the country mitigate illicit capturing, processing, and transaction events infiltrating from bait to plate.

Keywords: IUU fishing, China, Fisheries Law, supply chain management, digital traceability, seafood market

INTRODUCTION

Illegal, unreported, and unregulated (IUU) fishing is defined by the Food and Agricultural Organization (FAO) of the United Nations (UN) to encompass fishing activities: (1) in violation of applicable national, regional, and international laws, regulations or measures on the conservation and management of fisheries; (2) not reported or misreported to relevant national authorities or regional fisheries management organizations (RFMOs); and (3) conducted by stateless vessels, vessels (re)flagged to non-parties to RFMOs, for stocks or in areas where no competent RFMOs function, except for general international law contravened (FAO, 2001). Despite the broad consensus, IUU fishing continues to proliferate in pattern and magnitude worldwide, posing a daunting challenge for qualitative assessment on a global scale. One of the most cited estimates is Agnew et al. (2009) that IUU-caught fish was 11–19% of reported catches, translating to 11–26 million tons valued at US$10-23.5 billion in 2003. Packard Foundation (2020) provides the latest account, adapted from Watson and Tidd (2018), that IUU catches accounted for approximately 25% of global landings or 32 million tons in 2015, which had stabilized in more recent years.

In need of an updated analysis, the FAO calls for more robust and transparent estimates, with two critical suggestions provided to member States: (1) Efforts are more likely to be usefully focused on generating estimates at sub-national, national or regional levels; and (2) Indicators to monitor progress could be reflected by the numbers of vessels on RFMOs' IUU fishing vessel lists and included in the European Union (EU) "yellow" and "red" card lists, respectively (Macfadyen et al., 2016). Furthermore, a requisite toolbox of guiding principles and technical methodologies are developed to help monitor IUU fishing down to the state level (FAO, 2018).

Orientating toward a locally nuanced understanding of IUU fishing is opportune for China as the world's largest fisheries economy in terms of capture, production and export (FAO, 2020). Since it is the most important fishing nation on the planet, China must evolve from a significant cause to a potential solution to illegal and unsustainably sourced seafood on the global arena. Existing studies are not lacking in stressing China's fisheries management improvement on the supply side (Xue, 2006; Mallory, 2013; Pauly et al., 2014; Gutiérrez et al., 2020; Shen and Huang, 2020). This policy paper extends to a full chain lens, drawing upon the rapid development of information and communication technologies as well as seafood market dynamics. China's top fisheries statute and supportive sub-laws are being carefully refined, incorporating new mechanisms to ensure seafood's origin and transactions are increasingly transparent. We see this as a promising start to advance to government-led and even digital supply chain management to integrate seafood safety, security, and sustainability concerns into the fabric of traceability. Despite the lack of a universally agreed notion, we define seafood traceability governance for this study as the system or tool that makes the flow of seafood information possible through specified stages of production, processing, distribution, and sale (i.e., trackable), and allows for such records of production and product movement to be accessible at a future date and distant places (i.e., traceable).

A WIDESPREAD AWAKENING TO ILLEGAL, UNREPORTED, AND UNREGULATED FISHING INSIDE AND OUTSIDE CHINA

China's involvement in and level of due diligence to IUU supplies were not well documented until the last 10 years, building upon a growing mixture of publicly available (e.g., governmental, institutional websites, and publications), independently collected (e.g., expert surveys and interview-based fieldwork) and technically generated (e.g., Vessel Monitoring System/VMS, Automatic Identification System/AIS, and related satellite inputs) sources of materials. Although IUU fishing has troubled China's marine fisheries management as early as the 1950s, it is only from 2006 that China's law enforcement authorities began to seriously combat domestic IUU fishing through the "Special Law-Enforcement Campaigns for Marine Fisheries during the Summer Moratorium" (Zhang and Vincent, 2020). Such effort was further supported by China's Supreme Court which started launching criminal suits specifically against IUU fishing in 2016. In 2020, about 280 criminal cases of domestic IUU fishing were heard, leading to approximately 1,700 fishing boats confiscated or punished (China Coast Guard, 2021). However, such legal responses merely covered the summer moratorium for a long time. Without a central state repository, it remains dubious if China adequately comprehends the incidence and scale of IUU fishing that falls under its jurisdiction, let alone properly discharges international publicity obligations. Distinct terminologies (e.g., species names), data collection methodologies (e.g., bottom-up or top-down) and geographical demarcation applied in fisheries management (e.g., China's claimed share of exclusive economic zones/EEZs in disputed seas) have further complicated knowledge sharing. With few officially verified profiles, most existing studies remain fragmented and speculative. Bearing in mind those restraints, they can still be regarded as valuable and complementary estimates to complete the puzzle altogether. Provided below is a straightforward synthesis of pre-existing international scorings and surveys pertinent to China's performance in combatting IUU fishing:

- China received the worst IUU score out of 152 coastal states based on 40 indicators and assessed by fisheries experts for the IUU Fishing Index to benchmark countries' vulnerability, prevalence and response to IUU fishing. Its performance was considered particularly poor in discharging flag, port and overall State responsibilities (Macfadyen et al., 2019).
- China received an overall score of 0.37 in 2018, placing it at the 35th place among 40 countries assessed under the Fisheries Management Index which relied on expert surveys to rate fisheries management effectiveness on a stock-by-stock basis (Packard Foundation, 2020).
- China received a failing score among over half of 33 countries representing the top 90% of the world fish catch and evaluated for ecosystem-based fisheries management across the three parameters of principles, criteria and implementation steps (Pitcher et al., 2009).
- With data extracted from the FishSpektrum Krakken database and AIS for 2017 and 2018, at least 183 vessels in China's distant-water fishing (DWF) fleet were suspected or confirmed to be involved with IUU fishing. Numbers included state-owned DWF vessels and 104 vessels flagged outside China (Gutiérrez et al., 2020).
- Five Chinese-flagged or -owned vessels remain, as of the time of this writing, under the Combined IUU Fishing Vessel List which aggregates IUU vessel listings from twelve RFMOs and the International Criminal Police Organization (Trygg Mat Tracking, 2022).
- Drawing data from global institutions and existing reviews, China was considered to have provided the largest sum of harmful capacity-enhancing subsidies (e.g., fuel subsidies, tax exemptions, and vessel construction) valued at $16,637.7 million, contributing to 47.5% of global subsidy value in 2016 (Arthur et al., 2019).
- The United States identified China as a third country with flagged vessels engaged in IUU fishing in 2009, issuing a yellow card that stayed for 22 months (Hosch, 2016). The 2019 report of the United States reserved concerns about China's fishing practices though it was no longer listed (National Oceanic and Atmospheric Administration, 2019).

Here we present a brief bibliometric survey to facilitate the understanding of how IUU fishing has been studied and addressed by China, while a more in-depth and systematic review is warranted but beyond the scope of this brief. Because of non-transparency and data discrepancies, most scholarly studies have focused on specific sectors or species where China leads the world in production, processing or consumption (e.g., Hanson et al., 2000; Sadovy et al., 2017). The FAO recommends that more diverse and credible indicators must be explored to benchmark a state's relative status within a broad spectrum of IUU-related activities. In China's case, first, we conducted an online search to identify how its regulatory responsibilities to curb IUU fishing have been recognized in official documents. We used relevant keywords in Chinese ("'海洋非法捕捞', '海洋违 规渔船', '禁止捕捞', '海洋三无渔船'" and "IUU") to search national political and legal documents from the website of China' Ministry of Agriculture and Rural Affairs,[1] and the online law archives hosted by Peking University.[2] The search resulted in a total of 193 documents from which we identified 19 IUU-relevant policies ad laws. Second, we searched in Chinese and international peer-reviewed literature to examine the extent of issue awareness by the number of studies and the interest scope by keyword. To this end, we used "IUU" and "China's policy/policies" as the topic keywords to search peer-reviewed English literature from the Web of Science and the same keywords in title from Google Scholar. The search ended up with a total of three papers we found that directly addressed China's policy on IUU fishing. Additionally, we used similar topic keywords in Chinese to search peer-reviewed Chinese literature (Full text = "三无渔船" or "非法捕捞" + Full text = "海洋" and "我国" + Full text = "政策" or "管理" + Full text = "IUU" and "我国") from China National Knowledge Infrastructure. The search reverted with 129 articles, where we found 27 articles that addressed the same topic of interest.

Apart from a numeric illustration in **Figure 1**, the keywords applied by the identified literature sources are summarized by frequency in **Figure 2**. We also categorize the identified Chinese official documents into 12 keyword clusters with frequency shown in **Figure 3**. Viewed together, the surveys present three main findings. Firstly, both Chinese governmental and scholarly awakening to IUU fishing is growing fast, outstripping international studies focused on China's role in IUU fishing (*N* = 19, 27, and 3 in China's policy documents, Chinese and international literature, respectively in **Figure 1**). Although this is not surprising, it highlights the importance to comprehensively review Chinese policy documents and literature to truly understand how China has been viewing and addressing IUU fishing. Secondly, despite a growing interest in various management tools, China's academic studies and national policies have paid limited attention to international regulatory alignment to date (e.g., traceability, port state measures, and international agreement ratification in **Figure 2**). Thirdly, it follows that China has been keener on developing fishery management tools to address illegal fishing, as a subset of IUU fishing, in violation of its own national rules in domestic waters (e.g., fishing gear regulation, vessel management, and summer moratorium in **Figure 3**) than on regulating its distant-water fishing (DWF).

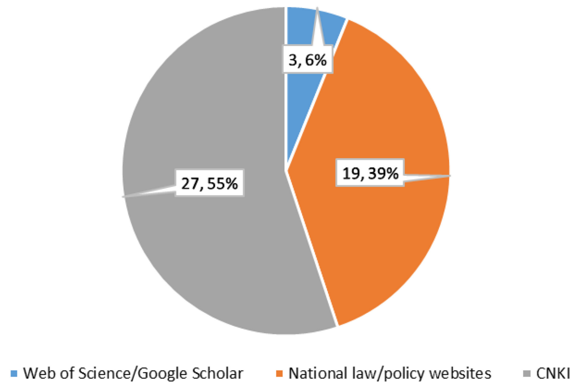

FIGURE 1 | Illustration of the number of articles, laws, and policies related to Chinese policy on IUU fishing.

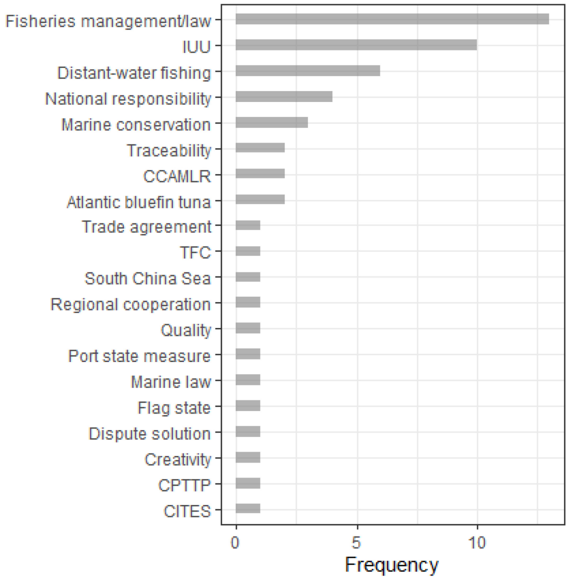

FIGURE 2 | Illustration of keyword frequency in surveyed Chinese and English scholarly literature. CCAMLR, Commission for the Conservation of Antarctic Marine Living Resources; TFC, Transferable Fishing Concessions; CPTTP, Comprehensive and Progressive Agreement for Trans-Pacific Partnership; CITES, Convention on International Trade in Endangered Species of Wild Fauna and Flora.

A CRITICAL ASSESSMENT OF AMENDED LEGAL PROVISIONS TO COMBAT ILLEGAL, UNREPORTED, AND UNREGULATED FISHING

For the passing decade, international legal liabilities, political tensions and ecological degradation realities have pressed hard

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[1] www.moa.gov.cn

[2] www.pkulaw.com

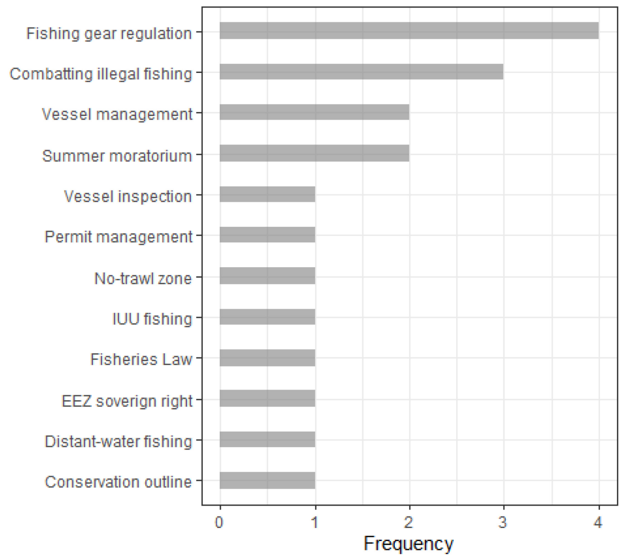

FIGURE 3 | Illustration of keyword frequency in surveyed Chinese laws and policies.

for China to consider rebalancing its agricultural modernization goals ("three rural" policy: agriculture, rural areas, and farmers) with ecological civilization imperatives. The latter were formally endorsed in the 13th National Five-Year Plan (2016–2020), echoed by a specific Marine Ecological Civilization Plan and the pledge for a Maritime Community with a Shared Future under the Belt and Road Initiative (BRI) (Packard Foundation, 2019). The 2015 UN Sustainable Development Goals underpinned by economic, environmental and social evaluation pillars are set to influence the modernization of China's sectoral laws. The top Fisheries Law, administered by the Fisheries Bureau under the Ministry of Agriculture and Rural Affairs (MARA), came to life in 1986. It has been revised four times between 2000 and 2013. Having undergone reassessment and revision since mid-2019, the new statute was later submitted for the deliberation of the National People's Congress (MARA, 2019b). Pending its final adoption, it is noteworthy that sustainability and conservation are explicitly enshrined as the new compass for direction to transform Chinese fisheries (MARA, 2019b, Arts 1 and 4). The old principle of rational exploitation exists to unleash the potential of aquaculture, mariculture, and recreational fisheries to satisfy a growing consumer demand (MARA, 2019b, Art 3). Conversely, freshwater and marine capturing, especially DWF, are brought under more stringent scrutiny and capacity controls than before. A shifted strategy aims to charter a new course of legal action to identify and punish IUU fishing involving Chinese actors (MARA, 2019b, Arts 65 and 74; MARA, 2020c, Art 33).

In all, the proposed amendment will convert the statute into eight chapters that span a total of 83 provisions. Among the others, Chapter 4 provides for targeted measures at IUU fishing, and Chapters 6 and 7 clarify supervision and management bodies and legal liabilities for IUU violators, respectively. Despite a missing national anti-IUU plan of action to echo the FAO's call (He, 2016), the article investigates the new normative space for China to actively respond to IUU fishing ahead. In line with the FAO's integrity approach (FAO, 2001), the normative survey is embedded in China's coastal, flag, port and market State responsibilities to prevent, deter, and eliminate IUU fishing. The legal provisions most relevant to China's state responsibilities to curb IUU fishing are classified in **Table 1** below.

The illustration below reveals that, to some extent, China has begun to re-think its attitude and approach to the widespread problem of IUU fishing. Yet, limitations are still conspicuous in light of a requisite fisheries management toolbox recommended as best policy practice (Costello et al., 2019). The amendment verifies that, in comparison, China aims to regulate territorial and EEZ fisheries more rigorously than its DWF expanding and operating overseas. Firstly, despite a level of regulatory consistency, China is operating in several sub-law carve-outs to continue promoting DWF suited to its economic-technological but not ecological modernization priorities (Fabinyi and Liu, 2014). It explains why TAC sees no near promise of implementation into the DWF sector, except for prolonged piloting programs in designated local fisheries since 2017 (MOA, 2017a). The statute continues to excuse DWF from any output limit, implying that Chinese vessels would make the most of their quotas granted by RFMOs and coastal countries. Secondly, DWF vessels are subject to bespoken landing, reporting, input restrictions, and at-sea transshipment regulations. They were initially exempted from the "two zeros" (vessel number and aggregate horsepower) growth policy (MOA, 2017b) until the 13th National Five-Year DWF Development Plan targeted a cap of 3,000 DWF vessels by 2020 and an amount of DWF enterprises fixed at the year 2016 level (MOA, 2017c). Still, existing DWF vessels are not required to abstain from destructive fishing practices, e.g., pair bottom trawls, stow nets, and Danish seine nets that have started phasing out of domestic waters (MOA, 2017b). Instead, a recent MARA amendment recognizes DWF's eligibility to continue existing trawling practices even though new trawlers are not allowed to be constructed (MARA, 2020b). And thirdly, China as a flag State does not hold legal entitlement to intervene in foreign coastal jurisdiction on operative matters, such as TAC, fishing gear, and onboard inspection at sea. Even if it intends to discharge certain "moral" accountability to the harvested area, nowadays 70% of the Chinese DWF fleet is composed of privately own vessels (Gutiérrez et al., 2020). Unlike state-owned enterprises' diligent execution of government policy, resource conservation is not an accepted priority of most profit-driven firms. About half of China's DWF vessels were found to concentrate in West and East Africa, overlapping with its BRI investment plan in some key fishing grounds and landing ports of the world (Pauly et al., 2014). However, like the EU, China was considered to have significantly underreported local catches to the FAO, including from both legal and illegal sources (Belhabib et al., 2015). A deficit in accountability to partner African countries triggered pervasive conflicts, legal violations, and even violence in distant waters where Chinese vessels operated from time to time (Li and Amer, 2015; Gutiérrez et al., 2020). The current input-based regulatory framework thus

TABLE 1 | Amended Fisheries Law and sub-law provisions to address IUU fishing.

Management tools in amended Fisheries Law (by provision)	Coastal State responsibilities		Flag State responsibilities	Port and market State responsibilities
	IUU fishing in territorial sea, enclosed national EEZs	IUU fishing in co-managed bordering EEZs	IUU fishing in distant foreign EEZs and the high seas	Offloading, import and export of IUU-caught products
Input (e.g., vessels, engines and net devices) restrictions (Art 9)	√		√ (Separately in Art 30)	N.A.
Total allowable catch (TAC) (Art 28)	√ (Local pilot programs)	√ (Bilateral fishery agreements)	N.A.	N.A.
Enterprise approval and annual audit (Art 30)	√ (By municipal and provincial fisheries bureaus)		√ (By national fisheries bureau)	N.A.
Fishing permit (Art 31)	√ (Paper or electronic)			N.A.
Fishing logbook (Art 33)	√ (MLVs only in MARA, 2018, Art 8)		√ (Electronic logbooks encouraged in MARA, 2018, Art 51)	N.A.
At-sea transshipment (Art 33)	√ (Prohibited in MARA, 2018, Art 52)		√ (MLVs post-transshipment logbook reporting in MARA, 2018, Art 50)	N.A.
Vessel safety (Art 34)	√		√ (Mandatory VMS in MARA, 2020c, Art 35)	N.A.
Designated port entry, exit and reporting (Arts 35 and 36)	√ (MLVs only in MARA, 2019a)		√ (Separately in Art 30)	√ (RFMOs catch certifications verified by customs in MOA, 2010; entry denied for IUU vessels listed by RFMOs in MARA, 2019c)
Product legality label (Arts 35 and 52)	√ (MLVs only; local pilot programs)		×	√ (purchase, processing and sale of illegally caught fish prohibited)
Onboard observer (Art 37)	√ (MLVs only, national observers)		√ (National and RFMOs observers in MARA, 2020c, Art 35)	N.A.
Prohibited fishing zones, seasons (Art 41)	√		N.A.	N.A.
Prohibited fishing methods, gear, species (Arts 42 and 48)	√		√ (Coastal State and RFMOs rules in MARA, 2020c, Art 39)	N.A.
Personal blacklist and business embargos (Art 74)	×		√	N.A.

√, existent; ×, not-existent; N.A., not applicable.
Medium and large fishing vessels (MLVs) are between 12–24 m and 24 m and above in length, respectively. Small fishing vessels are 12 m and below in length.

prompts us to explore innovative and effective countermeasures to assist in empowering China's fisheries management in the implementation phase afterward.

ACTIONABLE RECOMMENDATIONS: COMBINING TRACEABILITY TECHNOLOGY AND MARKET LEVERAGES

As identified in **Figures 1–3** above, the universally reproached phenomenon of IUU fishing is no longer a strict taboo in China's political, legal, and academic circles. Since 2006, combating illegal capture fisheries has become more pronounced as a policy goal of the Ministry of Agriculture (MOA, renamed as MARA after March 2018) (MOA, 2006). Various promising indicators have emerged, such as ministerial circulars to target punishment (e.g., fines, blacklisting, and revocation of licenses) at DWF vessels and enterprises notified as lawbreakers from overseas (Shen and Huang, 2020). Proportional to its fisheries and economic size, China has gradually taken a critical move to respond to international norms and guidelines that call for collaborative efforts to eradicate IUU fishing. There is no lack of diversity in the regulatory tools the MARA has deliberated in the new statute. Implementation and enforcement coordination, nevertheless, remain the hardest crux in a renovated legal web to be. To complement and support traditional monitoring, surveillance, and control measures at sea, the transformative role of technological and marketplace-based measures warrants further exploring under the amended statute. There is potential for China to transition to government-led and even digital seafood traceability governance, so that stronger synergies are generated among different fisheries management tools acting on seafood legality and sustainability.

When canvassing the legal provisions above, we see the seeds of digital traceability governance already planted within. China's restrictive DWF policy has taken roots in the overarching textual design, where the most noteworthy include the mandated installment of VMS, a more extensive use of electronic fishing logs (to enable at least hourly reporting to the central Fisheries Bureau), and a blacklisting system to drive large fleets to perform higher transparency obligations (MARA, 2020a). Enhanced technological intelligence stands a chance to capture and transmit almost real-time complex fisheries profiles back to national authorities based faraway. Digital evidence, including VMS data and audio-visual images, once collected by government officials, carries the same legal weight as physical proof in prosecution and administrative sanctions (Art 54). Such modernization of DWF fleets means the industry can be expected to set the motion for strengthening industrial fishing operators' reporting and legal accountability to national and international jurisdictions. Furthermore, the envisioned legality labeling scheme operated at the point of market entry provides the safety valve for a point-to-point government-administered traceability system with regular inputs contributed by fishers and importers. In practice, two critical developments have unfolded to experiment the idea of government-led and digital seafood traceability within China.

For one thing, with its incomparable scale and pace in market digitalization, in 2018 China started a local government-pioneered and electronic legality labeling scheme in Taizhou, Zhejiang Province. An initial set of 110 local fishing vessels tested the use of a mobile application ("渔港通") to report all vessel movement and landing information automatically to nearshore governmental monitoring stations upon entry and exit (ZJOL, 2019). As a time-efficient and government-funded traceability program, it shows the promise to lower reporting costs and drive greater compliance motivation among local capture fishers. At the next stage, as the initiative does not explicitly exclude otherwise, Chinese DWF vessels and incoming foreign vessels may also be engaged in the information system, along with China's ratification of the Agreement on Port State Measures (PSMA) (FAO, 2009). In the long run, transnational inter-state cooperation in digital traceability governance can be explored in neighboring and distant foreign EEZs. Incidents of domestic fisheries law and international legal violations by Chinese DWF vessels necessitate increased data availability, access, and sharing to enable transparent fisheries monitoring across international borders (Park et al., 2020). Presumably, international supply chain cooperation toward common traceability goals can function as a stabilizer to political and economic relations in disputed fishing areas.

For another, in response to the food contamination crisis amid the ongoing COVID-19 pandemic, Beijing was the first to launch in October 2020, followed by more than ten provinces and cities in China, a cold chain and fully digital traceability platform for imported food products. Foreign refrigerated seafood cannot be sold to local consumers if product traceability information is not uploaded, verified, and accessible to both businesses and consumers through the unified online platform (Global Times, 2020). The implementation of traceability fundamentally hinges on the coordination mechanism established between local fisheries, Customs, and market surveillance agencies. This movement thus echoes precisely the new statute's call for inter-departmental monitoring and enforcement of the proposed legality labeling scheme (Article 52). Moreover, such policy innovation functions as vivid laboratory testing of how to strengthen institutional cooperation and integration to better inform China's future implementation of PSMA commitments (Wang and Tang, 2017).

Globally, seafood traceability governance is being enthusiastically embraced as an intelligent and cost-sharing management model by corporate and regulatory bodies. In several RFMOs, Europe, North America and more recently Japan (Japan National Diet, 2020), the transition from a paper-based to centralized digital traceability system is set in motion. Predominant exemplars of traceability governance include: (1) the EU's and several RFMOs' government-to-government (G2G) catch documentation schemes (CDS) to trace catch movement from unloading through to the first point of sale; (2) the United States' government-to-business (G2B) import permit administered under the 2016 Seafood Import Monitoring Program to collect fisheries data through reporting and recordkeeping obligations (He, 2018); and 3) various B2C chain-of-custody certifications and ecolabels bestowed by third-party organizations such as the Marine Stewardship Council (Hosch, 2016). In March 2020, the Global Dialogue on Seafood Traceability (to which China's Aquatic Products Processing and Marketing Alliance is a member) publicized the first industrial standards to lead seafood companies into developing business-to-business (B2B) digital and interoperable traceability chain relations (GDST, 2020). A common backbone to those diverse traceability frameworks is a requisite and comparable group of Key Data Elements (i.e., who, where, what, and how fish were caught, processed, and distributed) that must be documented and transmitted throughout a chain (The Environmental Justice Foundation et al., 2020). Significant strides taken by communication and information technologies, such as smart contracts, the Internet of Things, Blockchain and Virtual Reality, have lent the key driving force (Fung Business Intelligence, 2017). The EU's government-led traceability paradigm has been found most instrumental in raising global alarms on IUU fishing vessels and their supportive states (Rafols, 2020), while contributing domestically to a "rather low" rate of mislabeling across "a considerable portion of the European seafood market" (Mariani et al., 2015).

The potential of seafood traceability governance can be further tapped by major consumer states like China to drive behavioral changes society-wide. Exporting to the United States and EU markets implies that Chinese companies must have already fulfilled advanced traceability requirements to secure market access. Currently, for the export-oriented sector, the MARA implements various CDS templates in conformity with the EU's, RFMOs' and Chile's traceability regulations at the border. For fish imports originating from Russia, a bilaterally agreed CDS exists to identify and eliminate any gray area of opportunities for foreign captured fish to be laundered through China's processing plants (MARA Government Service Platform). Moreover, the central Fisheries Bureau is steadily advancing China's ratification of the PSMA sooner rather than later, so that import controls will be considerably strengthened across all major points of entry along the national coastline (Woody, 2019). When traceability is combined with the use of the legality labeling scheme stipulated in Article 35, the government should not rest on simple label affixation to check the legal provenance of numerous local and foreign landings on Chinese ports. The recent COVID-19 pandemic permeating into the seafood market (e.g., salmon and shrimp packaging contamination scandals in northern China) demonstrates that the government's reactive ruling philosophy and paper-based working style have gone outmoded and resource-constrained. Making seafood supplies digitally traceable and trackable from any single link back to the origin will offer a more dynamic, efficient, and targeted solution to identify loopholes and legal liabilities. Hence, in designating ports for capture landings, the goal of traceability is expressly recognized in the draft revision (Art 36).

Given China's increasing seafood import dependency, governmental enforcement cooperation and information sharing should be seen as the next must-do. An undeniable challenge for combating IUU fishing is that millions of fishers and fish-processing workers rely on marine capture fisheries for their livelihoods in China. Nevertheless, law enforcement has been systematically compromised by the fragmented authority in fisheries management and local protectionism in coastal provinces (Su et al., 2020). To address such challenge head-on, China has employed short-term economic incentives, such as vessel buyback, fishing license retirement, and other capacity-reduction subsidies (He, 2015). However, traceability implementation requires more long-term investment in education, technical upskilling, and infrastructure building in coastal communities to enable local fishers to mitigate the opportunity cost before they can reap real gains. Thus, the understaffed Fisheries Bureau warrants institutional capacity building to renovate human resources and enforcement equipment before it can take a central coordinator role in executing the traceability agenda.

Apart from technological and institutional breakthroughs, there is also the need to mainstream sustainability literacy in one of the world's biggest seafood markets. China's DWF expansion has generated worldwide concerns and alerts (Pauly et al., 2014; Gutiérrez et al., 2020). It comes with the imperative to ensure the transparency and legal credibility of the supply chain reaching numerous Chinese retailers and consumers. Currently, no specific international norm applies to processing and consumer State responsibilities against IUU fishing. Since port entry is considered the readiest point to cast IUU catches out of commerce, most existing traceability chains stop at the border and rarely engage downstream economic actors (e.g., processors, distributors, retailers, and final consumers) (He, 2018). In the way forward, a whole-of-government and society approach is highly warranted. The potential of China to develop a traceability system accountable to the public cannot be underestimated. While food safety remains a perennial domestic concern, accumulated consumer surveys highlight a growing preference among middle-class and younger generations for correctly labeled seafood by origin, species name, and even catching method (Marine Stewardship Council, 2018; GoalBlue, 2019). Hence, China can be well advised to leverage its massive online and offline trading outlets, highly efficient distributional, and Internet networks to engage all stakeholders in verifying seafood's legal and traceable qualities. Distant communication and feedback technologies, such as Quick Response codes, radio-frequency identification, and interactive mobile applications, have seen mounting popularity among technology-savvy chain stores, supermarkets, and younger consumer groups in contemporary China (Fung Business Intelligence, 2017). When legality and sustainability information is integrated into the reporting cycles of a plethora of e-commerce platforms such as Alibaba and JD.com, local consumers will be rewarded with the ease of making the most conscious and sensible purchasing decisions (Gutierrez and Khizhniak, 2019).

In the future, the seafood traceability system may even proceed to allow public or at least mixed portfolios in user access, for example, through permissioned consortium Blockchains (Blaha and Katafono, 2020), to avoid frauds and encourage a broader group of information contributors. The Beijing-championed cold chain traceability platform discussed above is already open to the public, where consumers can proactively inquire and check product traceability information through WeChat and Alipay scan codes (China News, 2020). A full-chain traceability framework will only be as strong as its weakest link. Collaborative monitoring of information streams will be critical for ensuring data authenticity, integrity, and equal knowledge sharing. China's mission to manage the massive inflow and outflow of seafood through traceability mechanism is becoming essential to safeguard the sustainability of global fisheries, which has a long-lasting impact on people's food security, environmental, and social wellbeing.

CONCLUSION

The literature, policy, and legal surveys carried out above reinforce the emerging perception of China's rapidly transforming fisheries economy and its regulating environment. The proposed amendments to the Fisheries Law are a formal oath taken by the legislator to commit to a multitude of international and regional legal pacts against IUU fishing. While it may be regarded as an effort of internal adaption, we consider it the first important step for China to contribute globally to a sustainable seafood future for humanity. To empower the rule of law ahead, we recommend seafood traceability governance to help bring China's proposed anti-IUU policies into reality. Traceability governance should be operated as a transparent, interactive, and preventive strategy to consolidate all business and State parties' responsibilities for fisheries sustainability and the health of marine ecosystems. A steady progression from piloting projects to a truly nationwide framework will hinge upon the optimization of three main design and operation features below:

- An unbroken and immutable electronic information capture, transmission, and verification system linked to the nationwide fisheries legality label managed by the MARA and its local subsidiaries, to ensure traceable and trackable seafood movement inside and outside China, including under-regulated DWF and fishery processing entities.
- A precautionary and market-responsive approach to engaging public, private, and societal partnerships in adopting sustainable seafood production and consumption practices, incentivizing nationwide awareness and whistleblowing of IUU catches, especially from DWF and imported sources.
- A collaborative inter-agency model of information sharing, regulatory coordination and legal enforcement to embed seafood traceability in multiple relevant legal regimes and across all responsible governmental agencies in China.

AUTHOR CONTRIBUTIONS

JH designed the study and drafted the abstract, main text, **Table 1**, and list of references. XZ conducted the literature, policy, and law surveys in both Chinese and English, drafted the **Figures 1–3**, and provided comments and suggestions on initial drafts. Both authors contributed to the article and approved the submitted version.

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