Feeding Selectivity of *Ruditapes philippinarum* on Phytoplankton

Yin Liu [1],[†], Lun Song [2],[*],[†], Guangjun Song [2], Jinhao Wu [2], Kun Wang [2], Zhaohui Wang [2] and Suxuan Liu [3]

[1]   College of Fisheries and Life Science, Dalian Ocean University, Dalian 116023, China
[2]   Key Laboratory of Marine Biological Resources and Ecology, Liaoning Ocean and Fisheries Science Research Institute, Dalian 116023, China
[3]   College of Marine Sciences and Technology and Environment, Dalian Ocean University, Dalian 116023, China
*   Correspondence: lsong2020@yeah.net
†   These authors contributed equally to this work.

Abstract: A monthly survey of the feeding selectivity of *Ruditapes philippinarum* in the Yalu River Estuary in 2020–2021 was conducted using high-throughput sequencing identification and visual grading technology. The results showed that the most-dominant species in the water of the shellfish culture area and in the stomachs of *R. philippinarum* was *Karlodinium veneficum* in those years. The selectivity index (E) indicated that *R. philippinarum* avoided consuming Bacillariophyta, Chrysophyta and Cryptophyta throughout the year and preferentially consumed Dinophyta and Chlorophyta. In 2020, the annual average biomass of Dinophyta, Bacillariophyta, Chlorophyta, Dictyochophyta, Cryptophyta and Chrysophyta in the stomach contents of *R. philippinarum* was 54:14:16:1:10:4; it was 41:12:28:0:1:17 in 2021. The annual average biomass ratio of picophytoplankton, nanophytoplankton and microphytoplankton in the stomachs of *R. philippinarum* was 13:48:39 in 2020; it was 14:66:20 in 2021. *R. philippinarum* actively fed on nanophytoplankton and avoided picophytoplankton. Among the phytoplankton of different sizes and groups that *R. philippinarum* prefer to feed, chemical oxygen demand (COD) and organic phosphorus (DOP) have a significant negative effect on the nanophytoplankton community, pH has a positive effect on the Dictyochophyta community and COD and the inorganic nitrogen to phosphorus ratio (DI-N/P) have a significant positive effect on the Chlorophyta community.

Keywords: eukaryotic phytoplankton; high-throughput sequencing; particle size structure; *Ruditapes philippinarum*; Yalu River Estuary

1. Introduction

Aquaculture, which is one of the fastest-growing food-producing sectors in the world, will play a critical role in narrowing the gap between rising consumer demand for seafood and declines in the supply of wild-caught seafood products. Many countries have thus made the development of aquaculture a national priority. Filter-feeding shellfish have become increasingly common in China's coastal waters because of the continual increase in the scale of filter-feeding shellfish culture. Unlike farmed fish that can be fed diverse feeds, the main food source for filter-feeding shellfish culture is natural phytoplankton [1,2]; however, filter-feeding shellfish are selective feeders [3,4]. Large phytoplankton tend to be selectively ingested via the ctenoid gills of filter-feeding shellfish; after entering the intestinal tract, phytoplankton are assimilated selectively through the cilia. Unassimilated phytoplankton are embedded in the mucus and excreted as feces. Both of these selection mechanisms are affected by various properties of phytoplankton, including their size, shape, mobility, viscosity, toxicity and nutrient composition [4–11]. The selective feeding mechanism of shellfish is complex, and phytoplankton retention rates and preferences vary among regions, seasons and environments [2].

Ruditapes philippinarum, *Mollusca*, *Bivalvia*, *Veneroida*, *Veneridae*, *Ruditapes*, is dioecious. It is one of the four major shellfish species cultured in China, and it is widely distributed

along the coast of China. Its rapid growth and short culture period, coupled with its high adaptability, make it an excellent species for artificial high-density culture. It has great commercial value and has been introduced all over the world. The Yalu River Estuary of Dandong City, Liaoning Province, China, is one of the main regions for *R. philippinarum* culture, and it accounts for 18% of China's total output of *R. philippinarum*. However, *R. philippinarum* yields have decreased steadily in recent years because of slowing growth rates, reductions in meat quality, and increases in mortality, and this poses a major threat to the sustainable and healthy development of the shellfish aquaculture industry. Previous studies have found that the phytoplankton in the Yalu River Estuary has small particle size components and weak nutrient supply capacity, which may affect the nutritional reserve and healthy growth of *R. philippinarum*. The aim of this study was to evaluate the feeding preferences of *R. philippinarum* for phytoplankton of different sizes and from different groups during different periods to evaluate whether nanophytoplankton and Dinophyta have contributed to the decline in *R. philippinarum* yields.

Phytoplankton are typically identified by observing morphological characters with the aid of a microscope. Given the limitations in the resolution of traditional microscopes, many small phytoplankton, especially picophytoplankton, have not been detected in previous studies, and thus are understudied. Here, high-throughput sequencing identification and visual grading technology were used to characterize the monthly feeding preferences of *R. philippinarum* for different types and sizes of phytoplankton in the Yalu River Estuary in 2020 and 2021. Our results provide new insights that could be used to identify possible approaches for ensuring the sustainable development of *R. philippinarum* culture in the Yalu River Estuary.

2. Materials and Methods

2.1. Sample Collection

Samples of *R. philippinarum* and phytoplankton were collected from the fixed stations in March–December 2020 and March–October and December 2021 (in November 2021, due to epidemic, we could not go out to sea for sampling, so there are no data) in the Yalu River Estuary, which is located in the northern part of the Yellow Sea (Figure 1). In 2020, it was only sampled at Station 1, and in 2021 at Station 1 and Station 2. Because the average water depth of our sampling site was approximately 5 m, environmental DNA samples of phytoplankton were only collected from the surface of the seawater at a depth of 0.5 m. Using a 5 L card cover type water collector to collect seawater about 0.5 m below the water surface, at least 1 L of seawater was vacuum-filtered through 0.22-μm cellulose acetate membranes and subsequently folded and flash-frozen at –80 °C until further processing in the laboratory. At the same time, 1 kg of *R. philippinarum* was collected from each station, and 30 stomach contents of *R. philippinarum*, about 0.5 g, were collected from each station every month for high-throughput molecular sequencing.

The pretreatment, preservation, detection and quality control methods for the environmental samples were implemented with reference to "Marine Survey Specifications" (GB/T12763-2007) and "Marine Monitoring Specifications" (GB17378-2007). The measured parameters were seawater depth (Dep), water temperature (T), salinity (SST), pH, dissolved oxygen (DO), chemical oxygen demand (COD), total nitrogen (TN), total phosphorus (TP), silicate (SiO_3), dissolved inorganic nutrients (nitrite (NO_2), nitrate (NO_3), ammonia nitrogen (NH_4), inorganic phosphorus (DIP), and inorganic nitrogen (DIN)), the inorganic nitrogen-to-phosphorus ratio (DI-N/P), organic nitrogen (DON), organic phosphorus (DOP) and the organic nitrogen-to-phosphorus ratio (DO-N/P).

Figure 1. Sampling sites in Yalu River Estuary, China.

2.2. Genomic DNA Extraction

The metagenome of the eukaryotic phytoplankton was extracted using the cetyltrimethylammonium bromide (CTAB) method. The filter membrane and visceral mass of *R. philippinarum* were cut into small pieces and placed in a 1.5-mL centrifuge tube; 500 μL of CTAB lysate (2% CTAB; 100 mmol/L Tris-Cl, pH 8.0; 1.4 mmol/LNaCl; and 10 mmol/L EDTA), 1 μL of β-mercaptoethanol and 5–10 μL of protease were added and left at 55 °C for 1–1.5 h. Following centrifugation, the liquid was removed and placed in a new centrifuge tube; the supernatant was then extracted twice using the phenol-chloroform method. The supernatant was collected, and twice the volume of pre-cooled absolute ethanol was added and left to precipitate for 2–3 h. After collecting the precipitate, it was washed with 75% ethanol to obtain phytoplankton genomic DNA. The concentration and purity of DNA were determined using 1% agarose gel electrophoresis and an ultraviolet spectrophotometer (Unico Instrument Co., Ltd, Shanghai, China); the samples were stored in a refrigerator at –20 °C for subsequent use.

2.3. PCR Amplification of the 18S rDNA V4 Variable Region

Total genomic DNA from the seawater samples was extracted using the CTAB/SDS method. The primers used in this study were V4(F/R), which are self-developed primers for amplification of the 18S rDNA V4 region of eukaryotic phytoplankton. The upstream primer was V4-F (5′-GCGGTAATTCCAGCTCCAATA-3′), and the downstream primer was V4-R (5′-GATCCCHWACTTTCGTTCTTGA-3′) [12]. The primers were designed with different labels and sent to Shanghai Shenggong Biological Company for synthesis. The PCR reaction system was 50 μL, including 5 μL of PCR buffer, 8 μL of dNTP mixture and 2 μL of downstream primers (10 μmol/L) each, 2 μL of template DNA, 2.5 U Taq DNA polymerase and sterile water. The amplification reactions were conducted on a PE 9700 PCR instrument (Perkin Elmer, Waltham, MA, USA). The thermal cycling conditions were as follows: predenaturation at 94 °C for 3 min; 33 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 45 s, elongation at 72 °C for 45 s and a final extension at 72 °C for 5 min. PCR products were detected by 1% agarose gel electrophoresis. Sequencing libraries were generated using the NEB Next® Ultra™ DNA Library Prep Kit for Illumina (NEB, San Diego, CA, USA) per the manufacturer's instructions; index codes were subsequently added. Library quality was assessed on a Qubit@ 2.0 Fluorometer (Thermo Scientific, Waltham, MA, USA) and an Agilent Bioanalyzer 2100 system. The library was sequenced on an Illumina HiSeq 2500 platform, and 250-bp and 300-bp paired-end reads were generated. The experimental reagents were all from Tianjin Kemiou Chemical Reagent Co., Ltd, Tianjin, China.

2.4. Data Analysis

2.4.1. Quality Control and Analysis of Sequencing Data

The raw data obtained were spliced using FLASH software. Quality control of the spliced sequences was carried out in QIIME software to obtain high-quality data. To ensure the accuracy of our sequencing analysis, more than 90% of our dataset comprised high-quality data. Sequence analyses were performed using Uparse software (Uparse v7.0.1001, http://drive5.com/uparse/, accessed on 22 February 2021, Independent Investigator, Edgar, R.C., Tiburon, CA, USA). Sequences with ≥97% similarity with a given operational taxonomic unit (OTU) were assigned to that OTU [13]. The representative sequence for each OTU was screened for further annotation. The Silva Database (http://www.arb-silva.de/, accessed on 2 November 2021) was used to annotate taxonomic information with the RDP classifier algorithm (v2.2, http://sourceforge.net/projects/rdp-classifier/, accessed on 11 April 2022, Microbial Genomics and Bioinformatics Research Group and Ribocon GmbH, Bremen, Germany). Non-algal OTUs were removed given that we were exclusively interested in the abundance and richness of eukaryotic phytoplankton.

2.4.2. Dominance

Previous studies have shown that the relative proportion of eukaryotic phytoplankton sequences is closely related to the biomass ratio of phytoplankton populations [14–17]; thus, the proportion of each eukaryotic phytoplankton sequence can be used as an indicator of the biomass ratio. Dominant species were identified according to the equivalent spherical diameter [14–16,18–21], and species with a phytoplankton dominance over 0.1% in each sample were considered dominant. The sum of the eukaryotic phytoplankton sequences with an equivalent spherical diameter greater than 20 μm was used as the microphytoplankton centralized statistic; the sum of the eukaryotic phytoplankton sequences with an equivalent spherical diameter of 3–20 μm was used as the nanophytoplankton centralized statistics; and the sum of the eukaryotic phytoplankton sequence with an equivalent spherical diameter of less than 3 μm was used as a centralized statistic for picophytoplankton. The proportions of the different size-fractionated phytoplankton were determined according to the numbers of sequences derived from each phytoplankton population.

The dominance index Y represents the distribution of biological individuals within a community and is also used as an indicator of biodiversity (Equation (1)):

$$Y = \frac{nx}{N} f\text{x} \tag{1}$$

where n_x is the sum of the sequences of x algae in all samples; N is the sum of all algae sequences; and f_x is the occurrence frequency of x algae in all samples. Species with a dominance greater than 0.1% at each station were considered overall dominant species according to the cell-size biomass ratio.

Ivlev's selection index E was used to analyze the feeding selectivity of *R. philippinarum* in the Yalu River Estuary.

$$E = \frac{ri - pi}{ri + pi}$$

where *ri* represents the percentage of phytoplankton *i* in the stomachs of *R. philippinarum*, and *pi* represents the biomass percentage of phytoplankton i in the water. The range of E is [−1,1]. When E is −1, *R. philippinarum* avoids consuming phytoplankton *i*; when E is 1, *R. philippinarum* preferentially consumes phytoplankton *i*; and when E is close to 0, *R. philippinarum* neither avoids nor preferentially consumes phytoplankton *i*.

2.5. Statistical Analysis

The sampling method of environmental parameters refers to the Chinese National Standard: Ocean Survey Specification (GB/T 12763.4-2007), and the measurement method refers to the Chinese National Standard: Ocean Monitoring Specification (GB 17378-2007), as shown in Table 1. The dominance of the dominant phytoplankton species was repre-

sented by boxplots; the significant difference between environmental factors and particle size groups was analyzed by the Pearson statistical test; and the statistical results were represented by heatmaps. The formula calculation, data analysis and distribution map were all completed by WPS Office and Origin 2021 software.

Table 1. Sampling methods to measure the environmental parameters.

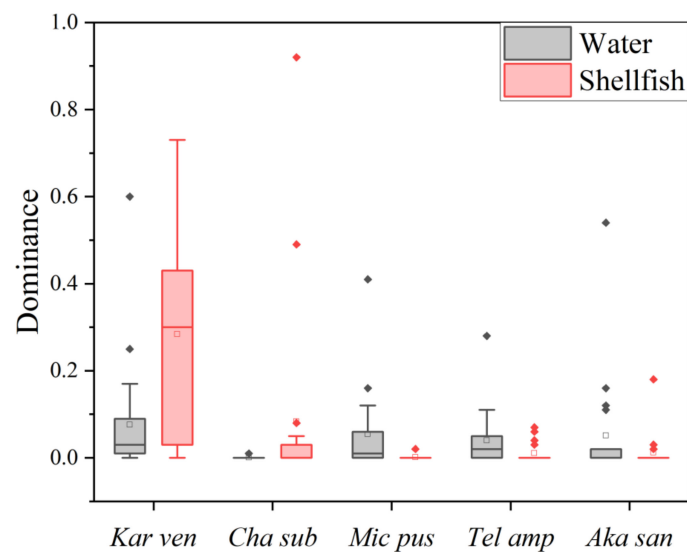| Test items	Detection Method	According to Standards
Silicate (SiO_3)	Silicon content-Molybdenum blue spectrophotometric method	GB17378.4-2007/17.2
Water temperature (T)	Surface water thermometer	GB17378.4-2007/25.1
pH	pH meter method	GB17378.4-2007/26
Salinity (SST)	CTD method	GB17378.4-2007/29.2
Dissolved oxygen (DO)	Iodometry	GB17378.4-2007/31
Ammonia nitrogen (NH_4)	Hypobromite oxidation method	GB17378.4-2007/36.2
Nitrite (NO_2)	Naphthalene ethylenediamine spectrophotometry	GB17378.4-2007/37
Nitrate (NO_3)	Zn-Cd reduction method	GB17378.4-2007/38.2
Inorganic phosphorus (DIP)	Phosphorus molybdenum blue spectrophotometry	GB17378.4-2007/39.1
Total phosphorus (TP)	Potassium persulfate oxidation method	GB17378.4-2007/40
Total nitrogen (TN)	Potassium persulfate oxidation method	GB17378.4-2007/41
Chemical oxygen demand (COD)	Alkaline potassium permanganate method	GB17378.4-2007/32

3. Results

3.1. Phytoplankton Community Structure in the Stomachs of Shellfish and Seawater

3.1.1. Dominant Species

From 2020 to 2021, the top five dominant species and those with dominance in the stomachs of *R. philippinarum* and the water of the Yalu River Estuary are shown in Figure 2. Among them, *Karlodinium veneficum* had the highest average dominance in the two years, and it was the first dominant species in the water and stomachs in 2020 and stomachs in 2021, with an annual dominance of 0.12, 0.21 and 0.36, respectively. In 2021, the first dominant species in the water was *Akashiwo sanguinea*, with an annual dominance of 0.09, of which the highest dominance in autumn was 0.33, and the annual dominance of *K. veneficum* was 0.03. *Chattonella subsalsa* was the second dominant species in the stomachs in 2021, with an annual dominance of 0.18, of which the highest dominance, 0.47, was in summer. *Teleaulax amphioxeia* was the third dominant species in the water in 2021, with the highest dominance in spring at 0.12, followed by *Micromonas pusilla* as the fourth dominant species, with the highest dominance in summer, which was 0.10. According to the two-year investigation results, among the top five dominant species, the average dominance of *K. veneficum* and *C. subsalsa* in the water was lower than that in the stomachs, while the other three dominant species were higher than that in the stomachs.

Figure 2. The top five dominant species and dominance in the stomachs of *R. philippinarum* in the water of the Yalu River Estuary. Vertical bars are the mean ± SE. *Kar van*: *Karlodinium veneficum*; *Cha sub*: *Chattonella subsalsa*; *Mic pus*: *Micromonas pusilla*; *Tel amp*: *Teleaulax amphioxeia*; *Aka san*: *Akashiwo sanguinea*.

3.1.2. Phytoplankton Groups

The dominant species ($Y > 0.02$) of phytoplankton in various groups in the stomachs of *R. philippinarum* and the water in the Yalu River Estuary in 2020 and 2021 are shown in Table 2. In 2020, the most-dominant species of Bacillariophyta in the stomachs of *R. philippinarum* was *Guinardia striata* (its dominance peaked in October at 0.72); the dominance in the water was 0.001, where it had not reached dominance. The most-dominant species of Bacillariophyta in the water was *Thalassiosira nordenskioeldii* (its dominance peaked in May at 0.28); the dominance in the stomachs was 0.002, where it had not reached dominance. In 2021, the most-dominant species of Bacillariophyta in the stomachs of *R. philippinarum* was *Bacillariophyta* sp. GSL077 (its dominance peaked in December at 0.29), and it was not a dominant species in water. The most-dominant species of Bacillariophyta in water was *Chaetoceros* sp. NIES-3971 (its dominance peaked in March at 0.66); the dominance in the stomachs was 0.01.

In 2020, the most-dominant species of Dinophyta in the stomachs of *R. philippinarum* was *K. veneficum* (its dominance peaked in April at 0.73), and the most-dominant species in water was also *K. veneficum* (its dominance peaked in August at 0.60). In 2021, the most-dominant species of Dinophyta in the stomachs of *R. philippinarum* was *K. veneficum* (its dominance peaked in May at 0.69), while the dominance in water was 0.03. The most-dominant species of Dinophyta in water was *A. sanguinea* (its dominance peaked in September at 0.54), and it was not a dominant species in the stomachs.

In 2020, the most-dominant species of Chlorophyta in the stomachs of *R. philippinarum* was *Picochlorum* sp. (its dominance peaked in June at 0.46); the dominance in the water was 0.002, where it had not reached dominance. The most-dominant species of Chlorophyta in water was *Ostreococcus tauri* (its dominance peaked in July at 0.84); the dominance in the stomachs was 0.01, where it had not reached dominance. In 2021, the most-dominant species of Chlorophyta in the stomachs of *R. philippinarum* was *Pterosperma cristatum* (its dominance peaked in March at 0.39); the dominance in the water was 0.003, where it had not reached dominance. The most-dominant species of Chlorophyta in water was *M. pusilla* (its dominance peaked in August at 0.16), and it was not a dominant species in the stomachs.
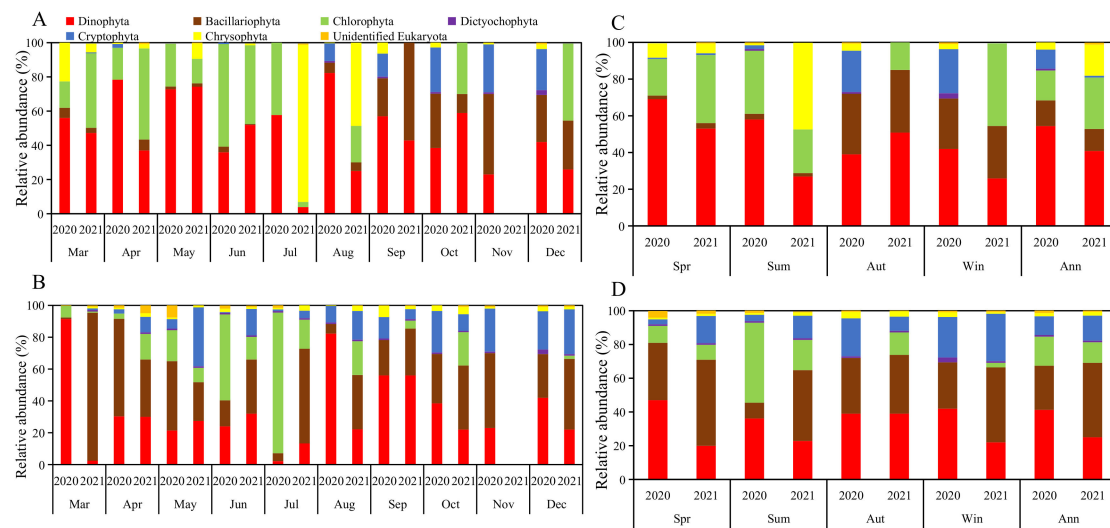
Table 2. Dominant species of various groups of phytoplankton in the stomach contents of *R. philippinarum* and water of the Yalu River Estuary in 2020–2021.

Group	Species Name (Stomach Contents)	Y	Species Name (in Seawater)	Y
Bacillariophyta	*Guinardia striata* *	0.11	*Chaetoceros* sp. NIES-3971 **	0.08
	Rhizosolenia setigera *	0.05	*Thalassiosira nordenskioeldii* *	0.05
	Bacillariophyta sp. GSL077 **	0.04	*Rhizosolenia setigera* **	0.04
			Skeletonema menzellii **	0.04
			Brockmanniella brockmannii *	0.03
			Thalassiosira curviseriata *	0.03
			Chaetoceros calcitrans **	0.03
			Thalassiosira pseudonana **	0.03
			Cyclotella choctawhatcheeana **	0.03
			Thalassiosira concaviuscula *	0.02
			Arcocellulus cornucervis **	0.02
Dinophyta	*Karlodinium veneficum*	0.21 */0.34 **	*Karlodinium veneficum*	0.12 */0.03 **
	Polykrikos kofoidii *	0.07	*Akashiwo sanguinea* **	0.09
	Ansanella granifera *	0.05	*Dissodinium pseudolunula* *	0.06
	Islandinium minutum **	0.03	*Spiniferites belerius* *	0.05
	Akashiwo sanguinea *	0.02	*Ansanella granifera* *	0.04
	Alexandrium affine *	0.02	*Alexandrium hiranoi* **	0.03
	Proterythropsis sp. *	0.02	*Gyrodinium dominans* *	0.02
	Alexandrium leei *	0.02	*Alexandrium affine* *	0.02
Chlorophyta	*Picochlorum* sp.	0.09 */0.04 **	*Ostreococcus tauri* *	0.1
	Pterosperma cristatum **	0.08	*Micromonas pusilla*	0.05 */0.06 **
	Tetradesmus obliquus **	0.03	*Mamiella gilva* **	0.05
	Chlamydomonas raudensis **	0.03		
Cryptophyta	*Cryptomonadales* sp.*	0.02	*Cryptomonadales* sp.*	0.07 */0.03 **
	Teleaulax amphioxeia	0.02	*Teleaulax amphioxeia* **	0.06
			Cryptomonadales environmental sample **	0.03
Chrysophyta	*Chattonella subsalsa* **	0.18	*Fibrocapsa japonica* *	0.01#
	Chrysosphaerella sp.*	0.02		
Dictyochophyta	*Phaeocystis globosa* *	0.02	*Phaeocystis globose* *	0.003 #

* Dominant species of various groups of phytoplankton in the stomachs and water in 2020; ** Dominant species of various groups of phytoplankton in the stomachs and water in 2021; # The dominance of this group of phytoplankton in water did not reach 0.02.

In 2020, the most-dominant species of Cryptophyta in the stomachs of *R. philippinarum* was *T. amphioxeia* (its dominance peaked in September at 0.07), and its dominance in water was 0.02. The most-dominant species of Cryptophyta in water was *Cryptomonadales* sp. (its dominance peaked in November at 0.23), and its dominance in the stomachs was only 0.02. In 2021, the most-dominant species of Cryptophyta in the stomachs was *T. amphioxeia* (its dominance peaked in May at 0.28); however, no Chlorophyta in the stomach contents reached dominance.

Overall, the biomass ratio of Dinophyta, Bacillariophyta, Chlorophyta, Dictyochophyta, Cryptophyta, Chrysophyta and unknown algae in the stomachs was 54:14:16:1:10:4:1 and 41:12:28:0:1:17:1 in 2020 and 2021, respectively. Chlorophyta accounted for the highest proportion in June; Bacillariophyta accounted for the highest proportion in November; and Dinophyta accounted for the highest proportion in other months. In 2021, only March, May, June and October had the highest proportion of Dinophyta. The proportion of biomass in water was 41:26:17:1:11:2:2 and 25:44:12:1:15:2:1, respectively, in 2020 and 2021. In 2020, the proportion of Dinophyta was the highest in March, August, September and December; in 2021, only June and September had the highest proportion of Dinophyta (Figure 3).

Figure 3. The biomass proportion of various groups of phytoplankton in 2020 and 2021: (**A**) monthly in the stomachs; (**B**) monthly in the water; (**C**) seasonal in the stomachs; (**D**) seasonal in the water.

3.1.3. Phytoplankton Sizes

The dominant species ($Y > 0.02$) of phytoplankton of different sizes in the stomachs of *R. philippinarum* and the water in the Yalu River Estuary in 2020 and 2021 are shown in Table 3. In 2020, the most-dominant picophytoplankton species in the stomachs of *R. philippinarum* was *Picochlorum* sp.; the most-dominant picophytoplankton species in water was *O. tauri*. In 2021, the most-dominant picophytoplankton species in the stomachs of *R. philippinarum* was *Bacillariophyta* sp. GSL077; the most-dominant picophytoplankton species in water was *M. pusilla*.

Table 3. Dominant species of phytoplankton of different sizes in the stomach contents of *R. philippinarum* and water of the Yalu River Estuary in 2020–2021.

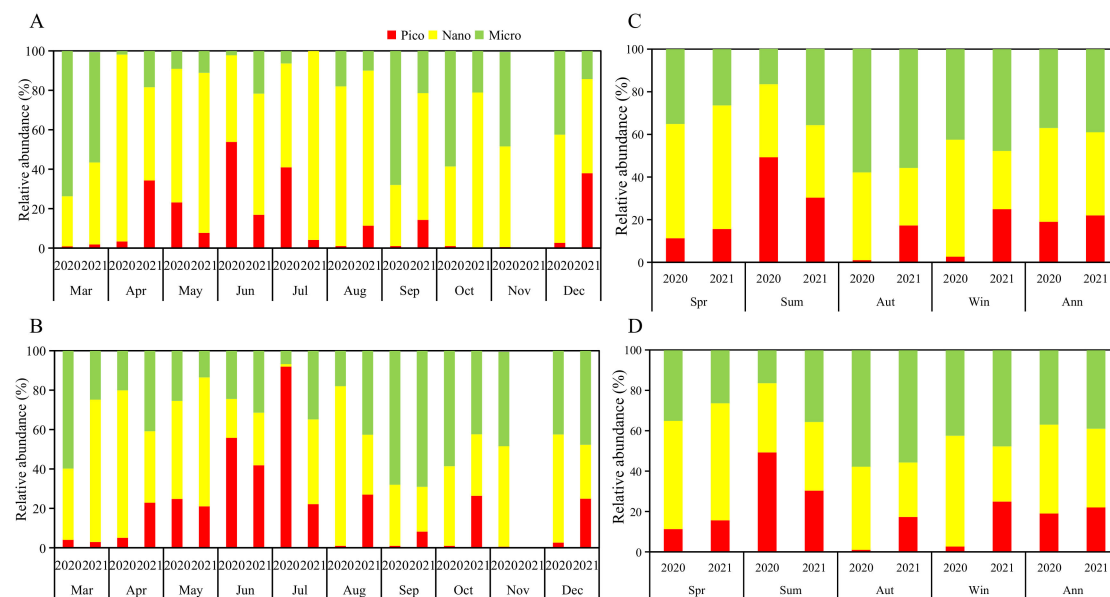| Size	Species Name (Stomach Contents)	Y	Species Name (in Seawater)	Y
picophytoplankton	*Picochlorum* sp.	0.09 */0.04 **	*Ostreococcus tauri* *	0.1
	Bacillariophyta sp. GSL077 **	0.05	*Micromonas pusilla*	0.05 */0.06 **
			Mamiella gilva **	0.05
			Chaetoceros calcitrans **	0.03
			Cryptomonadales environmental sample**	0.03
			Cryptomonadales sp. **	0.03
nanophytoplankton	*Karlodinium veneficum*	0.21 */0.36 **	*Karlodinium veneficum*	0.12 */0.03 **
	Chattonella subsalsa **	0.18	Chaetoceros sp. NIES-3971 **	0.08
	Ansanella granifera *	0.05	*Cryptomonadales* sp. *	0.07
	Chlamydomonas raudensis **	0.03	*Teleaulax amphioxeia*	0.02 */0.06 **
	Proterythropsis sp. *	0.02	*Thalassiosira nordenskioeldii* *	0.05
	Teleaulax amphioxeia *	0.02	*Ansanella granifera* *	0.04
	Phaeocystis globose *	0.02	*Skeletonema menzelii* **	0.04
	Cryptomonadales sp. *	0.02	*Brockmanniella brockmannii* *	0.03
	Chrysosphaerella sp. *	0.02	*Cyclotella choctawhatcheeana* **	0.03
			Telonema subtile *	0.02
			Arcocellulus cornucervis **	0.02
microphytoplankton	*Guinardia striata* *	0.11	*Akashiwo sanguinea*	0.02 */0.09 **
	Pterosperma cristatum **	0.08	*Dissodinium pseudolunula* *	0.06
	Polykrikos kofoidii *	0.07	*Spiniferites belerius* *	0.05
	Rhizosolenia setigera *	0.05	*Rhizosolenia setigera*	0.04
	Tetradesmus obliquus **	0.02 */0.03 **	*Thalassiosira curviseriata* *	0.03
	Islandinium minutum **	0.03	*Thalassiosira pseudonana* **	0.03
	Akashiwo sanguinea *	0.02	*Alexandrium hiranoi* **	0.03
	Alexandrium affine *	0.02	*Gyrodinium dominans* *	0.02
	Alexandrium leei *	0.02	*Thalassiosira concaviuscula* *	0.02
	Navicula sp. 39 **	0.02	*Thalassiosira nodulolineata* *	0.02
			Alexandrium affine *	0.02

* Dominant species of phytoplankton of different sizes in the stomachs and water in 2020; ** Dominant species of phytoplankton of different sizes in the stomachs and water in 2021.

In 2020, the most-dominant nanophytoplankton species in the stomach of *R. philippinarum* and the water was *K. veneficum*. In 2021, the most-dominant nanophytoplankton species in water was also *K. veneficum*, and the most-dominant nanophytoplankton species in water was *Chaetoceros* sp. NIES-3971.

In 2020, the most-dominant microphytoplankton species in the stomach of *R. philippinarum* was *G. striata*; the most-dominant microphytoplankton species in the water was *Dissodinium pseudolunula* (its abundance peaked in March at 0.57), and the dominance in the stomachs was 0.003, where it had not reached dominance. In 2021, the most-dominant microphytoplankton species in the stomachs of *R. philippinarum* was *P. cristatum*; the most-dominant microphytoplankton species in water was *A. sanguinea*.

Overall, the biomass ratio of picophytoplankton, nanophytoplankton and microphytoplankton in the stomachs of *R. philippinarum* was 13:48:39 and 14:66:20 in 2020 and 2021, respectively. In 2020, picophytoplankton accounted for the highest proportion in June, microphytoplankton accounted for the highest proportion in March, September and October, and nanophytoplankton accounted for the highest proportion in other months. In 2021, only March had the highest proportion of microphytoplankton, and nanophytoplankton accounted for the highest proportion in other months. The proportion of biomass in water was 19:44:37 and 22:39:39 in 2020 and 2021, respectively. In 2020, the proportion of nanophytoplankton was the highest in April, May, August, November and December. In 2021, only March, May and July had the highest proportion of nanophytoplankton (Figure 4).
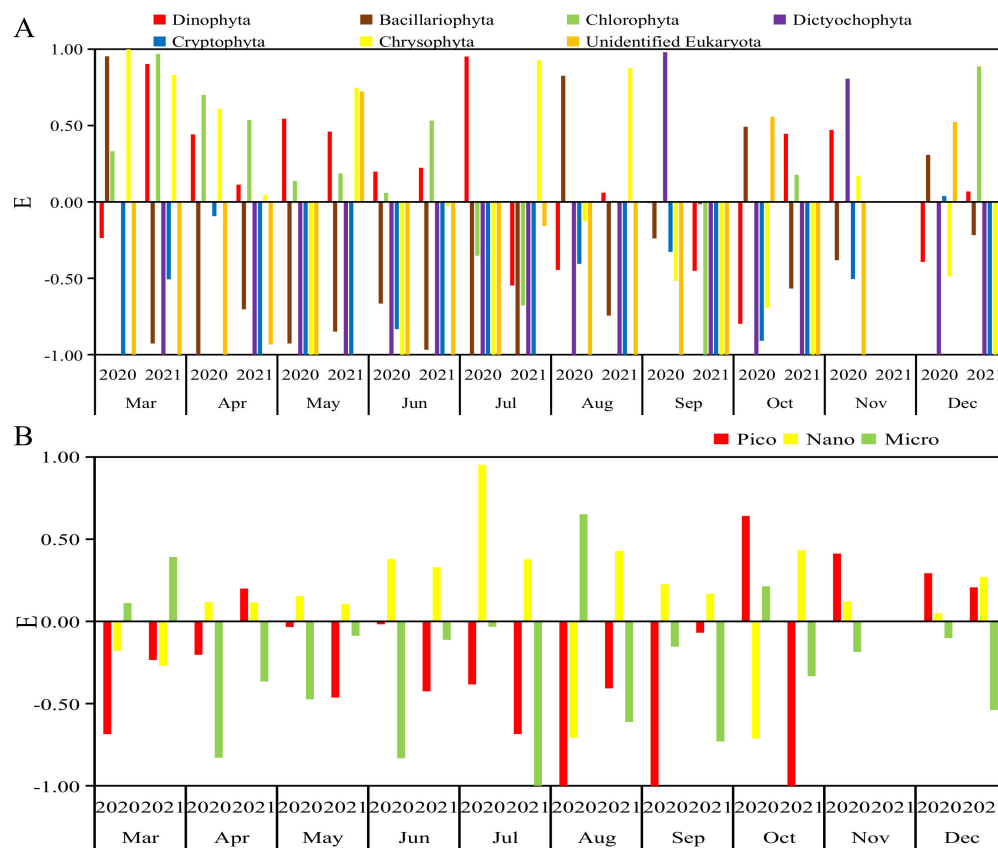

Figure 4. The biomass proportion of phytoplankton of different sizes in 2020 and 2021: (**A**) monthly in the stomachs; (**B**) monthly in the water; (**C**) seasonal in the stomachs; (**D**) seasonal in the water.
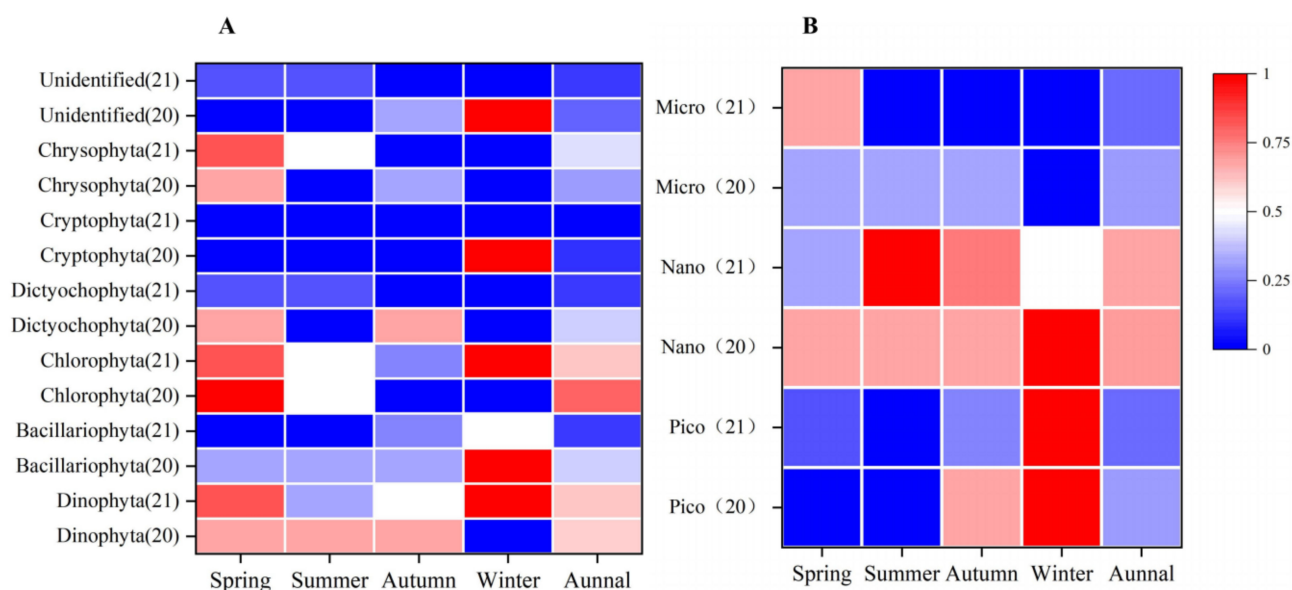
3.2. Selectivity Index (E) Value

3.2.1. Selectivity for Phytoplankton Groups

The selectivity index of *R. philippinarum* to various groups of phytoplankton in the water in the Yalu River Estuary was shown in Figure 5, and the situation of E values greater than 0 is shown in Figure 6. Judging from the proportion of E values greater than 0 in the whole year, *R. philippinarum* preferred to feed on Dinophyta and Chlorophyta; Bacillariophyta, Chrysophyta, Cryptophyta and Dictyochophyta were negative. Among Bacillariophyta, the proportion of E values greater than 0 in winter in 2020 and 2021 was higher than 0.5, so *R. philippinarum* may prefer to eat on Bacillariophyta in winter. Among Dictyochophyta, the proportion of E values greater than 0 in spring and autumn of 2020 was higher than 0.5, so *R. philippinarum* may like eating Dictyochophyta better than others in spring and autumn. Among Cryptophyta, the proportion of E values greater than 0 in

winter in 2020 was higher than 0.5, so *R. philippinarum* may prefer to feed on Cryptophyta in winter. Among Chrysophyta, the proportion of E values greater than 0 in spring of 2020 and spring and summer of 2021 was higher than 0.5, so *R. philippinarum* may prefer to feed on Chrysophyta in spring and summer.

Figure 5. Selectivity index of *R. philippinarum* for phytoplankton in the Yalu River Estuary in 2020–2021: (**A**) various groups; (**B**) various sizes.

Figure 6. Frequency distribution of the situation of E values greater than 0: (**A**) various groups; (**B**) various sizes.

3.2.2. Selectivity for Phytoplankton of Different Sizes

Judging from the proportion of E values greater than 0 in the whole year, *R. philippinarum* preferred to feed on nanophytoplankton; picophytoplankton and microphytoplankton were negative. In the picophytoplankton community, the proportion of E values greater than 0 in autumn and winter of 2020 and winter of 2021 was higher than 0.5, so *R. philippinarum* may like consuming picophytoplankton better than others in autumn and winter. In the spring of 2021, the proportion of E values of microphytoplankton larger than 0 was higher than 0.5, so *R. philippinarum* may prefer to eat on microphytoplankton in spring (Figures 5 and 6).
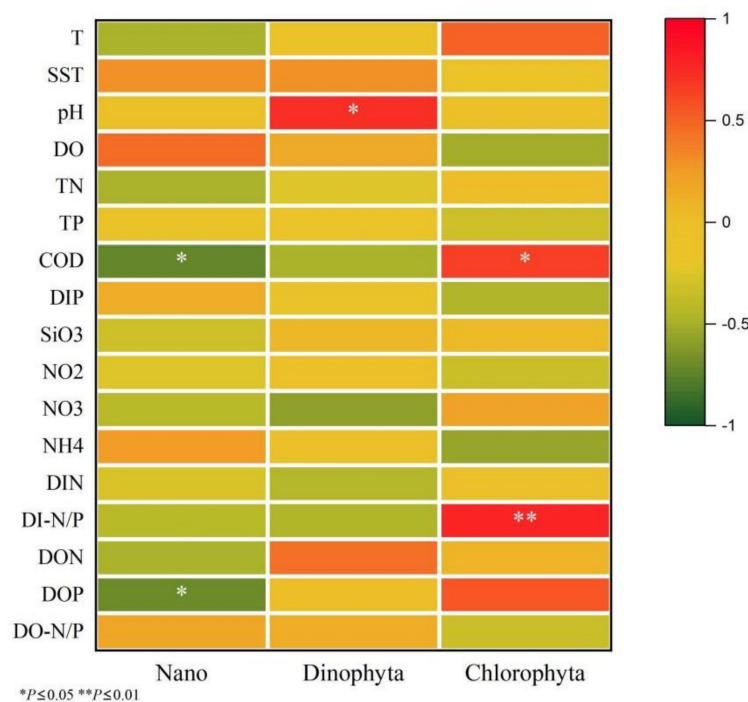
3.3. Relationship between the Environmental Factors and Feeding Selectivity

The average values of environmental parameters for each season in 2020 and 2021 are shown in Table 4. NO_3, NH_4, DIN, TN, and TP gradually increase with the change in seasons, while DON and DOP had little change throughout the year. DI-N/P, COD and T were the highest in summer, followed by spring and autumn, and lowest in winter.

Table 4. The average values of environmental parameters for each season in 2020 and 2021.

Environmental Factors	Spring	Summer	Autumn	Winter
T	8.63	24.27	19.96	4.57
SST	27.56	26.21	24.84	26.56
pH	7.99	8.01	7.93	7.86
DO	10.94	8.01	8.90	10.85
TN	0.300	0.440	0.573	0.570
TP	0.009	0.013	0.027	0.025
COD	1.20	1.70	1.63	1.15
DIP	0.003	0.005	0.020	0.019
SiO_3	0.178	0.930	1.089	0.868
NO_2	0.007	0.009	0.037	0.019
NO_3	0.152	0.240	0.304	0.320
NH_4	0.039	0.036	0.082	0.108
DIN	0.198	0.286	0.422	0.446
DI-N/P	245.49	351.74	76.31	51.08
DON	0.101	0.154	0.151	0.124
DOP	0.006	0.009	0.006	0.006
DO-N/P	38.56	51.78	64.72	48.32

The relationship between the phytoplankton dominance and environmental factors in the water at Station 1 in 2020 and Station 1 and Station 2 in 2021 is shown in Figure 7. Among them, DO, SST and DO-N/P had positive effects on the nanophytoplankton community, but none of them reached significance, while COD and DOP had significant negative effects on the nanophytoplankton community. pH, SST and DON had positive effects on Dinophyta community, and pH had a significant effect on Dinophyta. NO_3, DIN and DI-N/P had negative effects on the Dinophyta community, but none of them reached significance. COD and DI-N/P had significant positive effects on the Chlorophyta community, while NH_4, DO and DIP had negative effects on the Chlorophyta community, but none of them reached significance.

Figure 7. The relationship between the phytoplankton dominance and environmental factors in water.

4. Discussion

The proportion of Dinophyta biomass in the water was higher than that of Bacillariophyta in 2020, and the proportion of Bacillariophyta was higher than that of Dinophyta in 2021, while the proportion of Dinophyta biomass in the stomachs of *R. philippinarum* was far higher than that of Bacillariophyta in 2020 and 2021. At the same time, from the ratio of E values greater than 0, it can be found that the *R. philippinarum* avoided consuming most Bacillariophyta, Chrysophyta, Dictyochophyta and Cryptophyta and preferred consuming Dinophyta, and Chlorophyta throughout the year in the shellfish culture area of the Yalu River Estuary. This is consistent with the feeding habits of wild shellfish in that area as observed by Pan [22], in which feeding selectivity was higher for Dinophyta and Chrysophyta than for Bacillariophyta and Chlorophyta. In this study, *R. philippinarum* avoided feeding on Bacillariophyta in spring. Lavaud et al. stated that *Pecten maximus* cease consuming Bacillariophyta when their density in water is high [23]. Bougrier et al. indicated that *Ostrea chilensis* avoids consuming small Bacillariophyta [24]. The second most-dominant species observed in spring was *T. nordenskioeldii*, and the first dominant species observed was *Chaetoceros* sp. NIES-3971 in the water, which had high cell density in the water and they belong to Bacillariophyta with a relatively small particle size, so *R. philippinarum* chose to avoid eating. In addition, there were great differences between the different Bacillariophyta entering the digestive glands of shellfish [22], which may lead to *R. philippinarum* avoiding Bacillariophyta in Yalu River Estuary.

Langdon and Waldock [25] believed that filter-feeding shellfish ingested less or no toxin-producing phytoplankton, while Jiang et al. stated that shellfish are insensitive to most toxic phytoplankton and can ingest and accumulate toxins without affecting their feeding selectivity [26]. Different species of bivalve mollusks have relatively different feeding choices to toxic phytoplankton. *Mytilus edulis* rarely have negative responses when ingesting paralytic shellfish poisoning (PSP) toxin-producing phytoplankton (*Alexandrium* sp.), but scallops, oysters and clams decrease in different degrees, which may be related to the difference in sensitivity of shellfish neuron cells to PSP [27–29]. In this study, it was found that the first predominant species of *K. veneficum* in the water in the shellfish culture area of the Yalu River Estuary had hemolysin toxin, and was the first dominant species in the stomachs of *R. philippinarum*. *A. sanguinea* with hemolytic toxin was also the dominant

species in the water and stomachs. Therefore, the negative response of *R. philippinarum* to toxic algae may not be great.

This study found that DI-N/P had a positive effect on Chlorophyta, while NH₄ had a negative effect on Chlorophyta. In spring and summer, DI-N/P was significantly higher than that in autumn and winter, and the NH₄ content was significantly lower than that in autumn and winter, so the content of Chlorophyta in water was higher. Langdon and Waldock indicated that filter-feeding juvenile oysters eat phytoplankton containing specific fatty acids [25]. Navarro et al. also found that Dinophyta had a higher fatty acid content than Bacillariophyta, and high fatty acid can promote *Argopecten purpuratus* to feed [30]. Ding et al. also indicated that unsaturated fatty acid was an important factor for the growth of many bivalves [31]. Sun et al. found that *Picochlorum* sp. contains higher unsaturated fatty acids, and this study found that *Picochlorum* sp. was the dominant species in the stomachs of *R. philippinarum* in 2020 and 2021 [32]. All these reasons may make *R. philippinarum* prefer to eat Dinophyta and Chlorophyta.

Molina et al. showed that *Limnoperna fortunei* does not feed on Cryptophyta [33], and Loret et al. found that *Pecten maximus* selectively feeds on Cryptophyta [34]. In our study, *R. philippinarum* avoided consuming Cryptophyta in spring, summer and autumn, but preferentially consumed Cryptophyta in winter. This finding might be explained by differences in the properties of shellfish species. Alternatively, this could be caused by the failure to detect the cells of Cryptophyta in the stomachs of mussels given that their fragile cell walls can be easily broken during digestion [35]. Shumway et al. also indicated that Cryptophyta cells may be completely digested and absorbed by bivalve mollusks during their passage through the intestinal tract [3]. In this study, it was found that the proportion of Cryptophyta in winter was relatively higher compared with other seasons, and the water temperature in winter was low. It is possible that Cryptophyta can stay in the stomach of *R. philippinarum* for a long time, so the detected proportion was higher in winter than other seasons. Therefore, although some fragile Cryptophyta may not be capable of being detected in the stomachs of bivalve mollusks through microscopy, they appear to be an important food source for natural populations of bivalves [3,34–36].

The biomass ratio of picophytoplankton, nanophytoplankton and microphytoplankton in the stomachs of *R. philippinarum* in the shellfish culture area of the Yalu River Estuary was 13:48:39 and 14:66:20 in 2020 and 2021, respectively, which indicated that *R. philippinarum* preferred consuming nanophytoplankton. Cranford et al. found that bivalve mollusks could easily feed on nanophytoplankton and that the retention rate of 2–8 μm phytoplankton was high [1]. Strohmeier et al. found that the retention rate of phytoplankton by *Mytilus edulis* gradually increased with the size of phytoplankton, and the maximum retention rate was observed for 7–35 μm phytoplankton [37]. Rosa et al. found that *M. edulis* preferred algae that were greater than 4 μm in size [38]. Dunphy et al. showed that *O. chilensis* retains 6 μm phytoplankton less efficiently than the 15 μm phytoplankton, which is consistent with the findings of our study [39]. Møhlenberg et al. showed that the retention rate of phytoplankton by *M. edulis* decreased rapidly for phytoplankton less than 4 μm in size [40]. Tammes et al. indicated that *M. edulis* could not effectively retain phytoplankton smaller than 2.5 μm [41]. Zhang measured the rejection rate of 2 μm phytoplankton in *M. edulis*, *Crassostrea gigas* and *Azumapecten farreri* by simulating on-site running water, which was 19%, 17% and 8%, respectively, and the rejection rate of particles below 2 μm was even lower [2]. These findings are consistent with the observation that *R. philippinarum* avoided consuming picophytoplankton in the shellfish culture area of the Yalu River Estuary.

The consumption of bivalve shellfish is determined by the filtration rate and particle-retention efficiency of the gills, which is the main organ for the transportation of seawater and the retention of particles. The feeding of shellfish is closely related to the structure of the gills and the movement of cilia on the gill filaments [42,43]. Jørgensen and Ward et al. found that the retention rate of phytoplankton was severely reduced when the anterior cilia of *M. edulis* were inactivated, which indicates that the anterior cilia are critically important for shellfish feeding [44,45]. Riisgård measured the retention rate of phytoplankton by

six filter-feeding shellfish and found that the retention rate of phytoplankton larger than 4 μm in shellfish with larger anterior cilia, such as *Geukensia demissa*, *Spisula solidissima*, *Brachidontes exustus* and *Mercenaria mercenaria*, was 100%; by contrast, the retention rate of phytoplankton larger than 4 μm in shellfish with small anterior cilia or no anterior cilia, such as *Crassostrea virginica* and *Argopecten irradians*, was 75%–85% [43].

R. philippinarum possesses large anterior cilia; consequently, it preferred consuming large phytoplankton (nanophytoplankton and microphytoplankton). Among the six filter-feeding shellfish species studied by Riisgård [43], only *G. demissa* could significantly retain 0.2–2 μm phytoplankton, which may stem from the greater compactness of the cilia of *G. demissa* compared with the other five species examined. Wang found that the distance between the lateral cilia of *R. philippinarum*, *Argopecten irradians*, *A. farreri* and *C. gigas* was less than 1 μm, which is consistent with the proportion of picophytoplankton detected in the stomachs of *R. philippinarum* in our study (18%) [42]. Some researchers have suggested that filter-feeding shellfish prefer phytoplankton with greater concentrations of organic nutrients, and phytoplankton with low concentrations of organic nutrients are excreted as feces; picophytoplankton are typically not consumed by bivalves because of their small size and low nutritional quality [46,47].

This study found that the retention rate of picophytoplankton by *R. philippinarum* in the Yalu River Estuary was greater in autumn and winter than in summer and *R. philippinarum* preferred feeding on *Chaetoceros calcitrans* and *Syndiniales* sp. among picophytoplankton. Wei argued that *C. calcitrans* was an ideal opening food source for bivalves [48], and Lora-Vilchis also found that *C. calcitrans* was rich in some specific fatty acids; it also has a better effect as an initial feed for the larvae of *Pinna rudis* Linnaeus [49], which indicates that the picophytoplankton *C. calcitrans* can be ingested by juveniles. Ward et al. showed that slender or tri-radial phytoplankton may be more easily trapped by bivalves than spherical phytoplankton of the same volume [4,50]. *C. calcitrans* is mostly chain-like in water and is more likely to be trapped and fed; these considerations might explain why *R. philippinarum* preferentially consumed picophytoplankton in the Yalu River Estuary in autumn and winter [51–55].

The research area in the study was the main breeding area for *R. philippinarum*, in recent years, slow growth, decreased meat quality, and increased mortality have generally occurred, but no diseases, pollution, or red tides have been found in this sea area.The results showed that *R. philippinarum* avoided feeding on picophytoplankton, and the nanophytoplankton that actively fed was *K. veneficum* with low nutrition and toxicity in the study. Due to the quantitative selectivity strategy of *R. philippinarum* on feed microalgae, it filtered and fed on the dominant *K. veneficum* as much as possible in the case of low effective feed concentration. In addition, the increasing density of *R. philippinarum* culture resulted in the slow growth of *R. philippinarum* and increased mortality in the region. Therefore, it is recommended that the cultivation density should be reasonably controlled within the range of cultivation capacity in this region to maintain the healthy and sustainable cultivation of *R. philippinarum*.

5. Conclusions

In the Yalu River Estuary, *R. philippinarum* avoided consuming most Bacillariophyta, Chrysophyta, Dictyochophyta and Cryptophyta and preferentially fed on Dinophyta and Chlorophyta. *R. philippinarum* had a particularly strong preference for consuming *K. veneficum*, which was the most-dominant species in the water.

The annual average biomass ratio of picophytoplankton, nanophytoplankton and microphytoplankton in the stomachs of *R. philippinarum* was 13:48:39 in 2020 and 14:66:20 in 2021. *R. philippinarum* actively fed on nanophytoplankton and avoided picophytoplankton.

Among the phytoplankton of different grain sizes and groups that *R. philippinarum* prefer to feed, COD and DOP have a significant negative effect on the nanophytoplankton community, pH has a positive effect on the Dictyochophyta community, and COD and DI-N/P have a significant positive effect on the Chlorophyta community.

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