



# *In situ* food compositions reveal niche partitioning in small marine cladocerans and copepods in Daya Bay, South China Sea

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**ABSTRACT:** Marine cladocerans and small copepods (<0.7 mm) share similar food resources based on their size and feeding modes but little is known about the mechanisms allowing their coexistence. Using the high-throughput sequencing (HTS) method, the *in situ* eukaryotic diets of the small cladocerans *Penilia avirostris* and *Pseudevadne tergestina* and the calanoid copepod *Parvocalanus crassirostris* during spring and summer in Daya Bay, South China Sea, were analyzed and their dietary preference and potential niche overlap were described and compared. Approximately 195 operational taxonomic units belonging to metazoans, phytoplankton, protists, and fungi were detected in the gut contents of the 3 species. Overall, animal and phytoplankton prey contributed evenly to the sequences of gut contents of *P. avirostris*, with diatoms and cnidarians being the most important prey, whereas arthropods were the major prey resource for *P. tergestina*, with an extremely high percentage (86.2%). *P. crassirostris* contained a high percentage of phytoplankton prey, with dinoflagellates and diatoms as the major prey (24.3 and 12.9%, respectively). Both *P. avirostris* and *P. crassirostris* consumed a higher percentage of phytoplankton with the seasonal transition. Low niche overlap (0.001–0.19) among the 3 small crustaceans highlights their strong dietary niche partitioning and explains their coexistence in Daya Bay.

**KEY WORDS:** Dietary composition · Cladoceran · Copepod · 18S rDNA · High-throughput sequencing · Niche partitioning

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## 1. INTRODUCTION

Niche partitioning among sympatric species associated with habitats and resources facilitates their coexistence and contributes to the diversity of the same guild in various ecosystems. Marine cladocerans are distributed in coastal pelagic systems globally and assert sole dominance or co-dominate with copepod species in mesozooplankton communities in some seasons (Atienza et al. 2006a, 2007). Marine

cladocerans, with body lengths <0.7 mm, are classified as small mesozooplankton. This group and small copepods determine the dynamics of the mesozooplankton community in many coastal and estuary ecosystems (Hopcroft et al. 1998, Turner 2004, Liu et al. 2013, He et al. 2021). Cladocerans are generally less important than copepods in marine ecosystems but may control the trophodynamic pathways of the plankton community during seasons of high abundance (Paffenhöfer & Orcutt 1986, Turner et al. 1988,

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Atienza et al. 2006b). Hence, understanding their dietary composition and food preference bears significance to the function of ecosystems.

A large amount of literature has been published regarding the unsettled feeding pattern of marine cladocerans. Their feeding habits differ among different genera based on the feeding appendages. While *Penilia avirostris* is a typical suspension feeder, podonids perform raptorial feeding (Russell-Hunter 1979, Kim et al. 1989). Early studies using incubation experiments or microscopic examination of gut contents revealed that marine cladocerans, generally regarded as herbivores, may consume diverse prey with sizes ranging from <2 to 170  $\mu\text{m}$ , including diatoms, dinoflagellates, microflagellates, ciliates, and even bacteria (Russell-Hunter 1979, Nival & Ravera 1981, Katechakis & Stibor 2004, Atienza et al. 2006b, 2007, Sánchez et al. 2011) but prefer small and intermediate-sized particles (2–70  $\mu\text{m}$ ; Kim et al. 1989, Katechakis et al. 2004).

The copepod *Parvocalanus crassirostris* is globally ubiquitous and dominant in coastal waters, but only limited studies using traditional methods have addressed its feeding habits, and a consensus has not been reached. Calbet et al. (2000) reported that this species engaged in opportunistic feeding but preferred >5  $\mu\text{m}$  autotrophs over heterotrophs *in situ*. The copresence of remains of protozoa and diatoms in fecal pellets of *P. crassirostris* indicate that this species consumes a considerable proportion of ciliates in addition to algae (Chen et al. 2013).

Marine cladocerans and small copepods appear to have comparable body sizes and similar feeding patterns and may compete with each other for resources, which apparently hampers their cohabitation in coastal waters. Some work has been performed to decode the mechanism allowing their coexistence, but these efforts were confined to laboratory incubations with natural prey assemblages. Katechakis et al. (2004) attributed the coexistence of *P. avirostris* and *Acartia clausi* to their different optimum food particle sizes. However, these 2 species had comparable dietary niche breadth (~0.35), and their food size spectrum overlapped at a level of 62.5%, suggesting weak food separation between them. The feeding tendency toward smaller prey in *P. avirostris* was proposed to lead to niche partitioning from *Oithona nana* (Atienza et al. 2006a), but niche overlap was not quantified clearly. Incubation experiments can simultaneously provide quantitative and qualitative analyses of feeding selection by animals, but the inevitable bottle effect and loss of soft-bodied prey may cause biases in trophic con-

nection construction. Microscopic observation also limits the food spectrum to a narrow range of sizes and species. Therefore, this method most likely cannot provide realistic depictions of the *in situ* diets of animals.

Molecular sequencing has been increasingly used to study biodiversity in various ecosystems. This technique is time-saving, poses relatively minimal professional demands, and, most importantly, offers excellent fine-scale resolution (Cleary et al. 2016). By analysis of gut contents and feces, this technique can identify prey species that cannot easily be identified under microscopy because of their rarity, small size, fragility, or high digestibility (Li 2010). The development of sensitive and high-throughput next-generation sequencing techniques has greatly accelerated the efficiency of molecular sequencing and promoted the coverage of prey communities, and therefore can potentially uncover the complete food spectra of zooplankton (Craig et al. 2014, Hu et al. 2014, Ho et al. 2017).

Daya Bay is one of the coastal ecosystems in China where ecological environments have changed greatly in recent years due to the heavy influence of anthropogenic activities (Qiu et al. 2005, Liu et al. 2012, Wu et al. 2016). According to recent reports, small copepods such as *P. crassirostris* and *Paracalanus parvus* occur perennially in western Daya Bay as the predominant mesozooplankton species (Liu et al. 2013, He et al. 2021). Marine cladocerans, particularly *P. avirostris*, are consistently the seasonally dominant group in this area every year (Fang et al. 2010). Based on the observation of the coexistence of *P. avirostris*, *P. tergestina*, and *P. crassirostris* in Daya Bay with alternating dominance, we hypothesized that dietary niche partitioning exists among these 3 species. In this study, we aimed to characterize and compare the *in situ* eukaryotic diets of these 3 common small crustaceans during their co-occurrence to verify this hypothesis. Their *in situ* dietary compositions were investigated in Daya Bay by means of high-throughput sequencing (HTS) targeting 18S rDNA of gut contents. The 18S rDNA V4 region was chosen because it is one of the most popular markers and shows enough variability to distinguish among taxa (Walters et al. 2019); therefore, it is suitable to study the *in situ* feeding ecology of small marine organisms, the prey of which is usually too small to be observed under a microscope. Furthermore, relative abundance based on sequencing data can provide a semiquantitative estimation of dietary composition and facilitate the evaluation of niche overlap among these crustaceans.

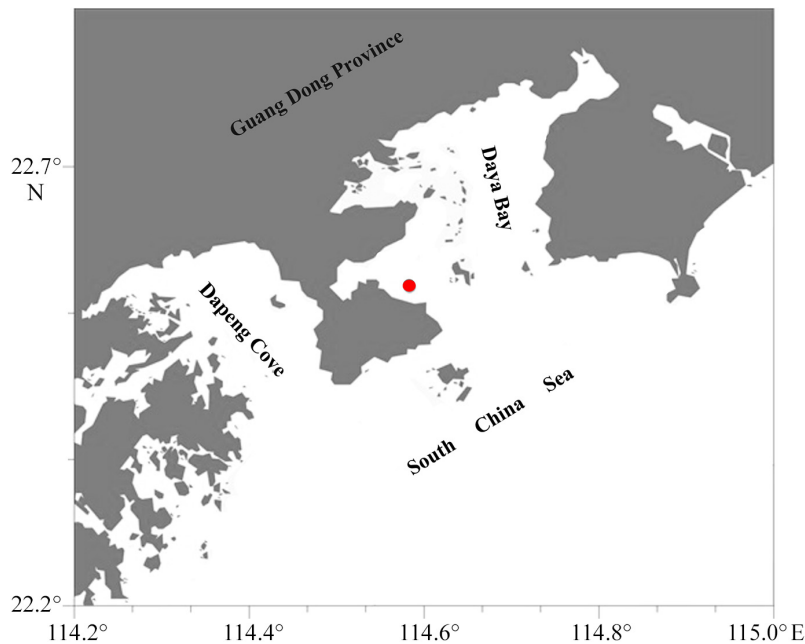


Fig. 1. Sampling site in Daya Bay, South China Sea, during spring and summer 2019

## 2. MATERIALS AND METHODS

### 2.1. Sampling

The sampling site was located nearshore in the western part of Daya Bay at a depth of 10 m (22° 33' 48.6" N, 114° 35' 0.6" E; Fig. 1). Profiles of water temperature, dissolved oxygen, and salinity were measured with an electronic meter (YSI Pro Plus). Mesozooplankton for *in situ* prey spectrum analysis were collected by vertical tows with a conical plankton net (31.6 cm diameter, 169  $\mu$ m mesh size) in April, May, July, and August 2019. Towing was performed slowly and at a constant speed to prevent mesozooplankton from evacuating the area. Animals were gently transferred to sterilized bottles and preserved with neutral Lugol's solution (2% final concentration), which has been shown to be effective in preserving DNA samples of phytoplankton and copepods (Zhang & Lin 2005). These samples were returned to the laboratory within 2 h in darkness at 0°C and were immediately stored at 4°C. A portion of the animals from the tow were transferred to bottles with 0.22  $\mu$ m filtered seawater and brought to the laboratory alive. Under a stereomicroscope, cladocerans, including *Penilia avirostris* and *Pseudevadne tergestina*, and the dominant copepod species *Parvocalanus crassirostris*, were sorted for DNA extraction. The same species were picked out from live subsamples and incubated with

0.22  $\mu$ m filtered seawater for defecation. Then, the same species were picked out for DNA extraction as the controls. Each sample contained 200 individuals. A total of 10 samples were obtained, comprising 4 for *P. avirostris*, 3 for *P. tergestina*, and 3 for *P. crassirostris*. Unfortunately, no samples were successfully prepared for the latter 2 species in April because of their low abundances.

### 2.2. Prey background

For phytoplankton species identification and abundance determination, water samples were collected from the surface and bottom layers using 5 l Niskin bottles, mixed in equal amounts, and preserved with Lugol's solution. The method did not include small-size-fractionization of phytoplankton. Identification was carried out under an inverted microscope (Leica DRB) at magnifications of 400–600 $\times$  according to Yamaji (1966) and Yang & Dong (2006). The total phytoplankton density was calculated as cells  $l^{-1}$ . Mesozooplankton were collected by vertical tows from the bottom to the surface with a conical net (31.6 cm diameter, 169 mm mesh size). Animals were preserved in 5% formaldehyde for later identification and enumeration under a stereomicroscope. Taxa were identified to the species or genus level under a stereomicroscope.

Seawater samples were collected separately in the same way. Water was prefiltered through a sieve with a 100  $\mu$ m mesh and then filtered through 0.7  $\mu$ m GF/F filters. The filters containing particles were decarbonated with 2 N HCl, dried again, and then analyzed using a CNH analyzer (Series II CHNS/O Analyzer 2400; PerkinElmer Instruments) for particulate organic carbon (POC) and nitrogen quantification.

### 2.3. DNA extraction

The sorted cladoceran or copepod samples were rinsed 5 times with sterilized 0.22  $\mu$ m filtered seawater and then twice with Milli-Q water to remove any attachments from the body surface. Cleaned animals were homogenized with classic homogenizers and then incubated in lysis buffer for 2 d at 55°C for thorough cell lysis. DNA was extracted by applying a modified CTAB protocol and finally eluted in 30  $\mu$ l of 10 mM Tris-HCl (pH 8.0) (Zhang et al. 2005).

#### 2.4. PCR amplification and Illumina sequencing

The DNA extracts were amplified using the universal TAREuk454FWD1–TAREukREV3 primer pair (TAREuk454FWD1: 5'-CCA GCA SCY GCG GTA ATT CC-3'; TAREukREV3: 5'-ACT TTC GTT CTT GAT YRA-3'), which targets the V4 region (~380 bp) of eukaryotic 18S rDNA (Stoeck et al. 2010). PCR was carried out in a 20  $\mu$ l reaction volume composed of 4  $\mu$ l 5 $\times$  FastPfu Buffer, 2  $\mu$ l 2.5 mM dNTPs, 0.8  $\mu$ l each of 5  $\mu$ M universal forward and reverse primers, 0.4  $\mu$ l FastPfu Polymerase, and 10 ng genomic DNA. The PCR conditions were as follows: an initial denaturation step at 95°C for 5 min, 27 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s, and final elongation at 72°C for 10 min (Lin et al. 2018). PCR products of each sample were pooled and purified with a Geneaid Gel/PCR fragment Extraction kit. DNA concentration and quality were determined using a NanoDrop 1000 spectrophotometer and Qubit 3 Fluorometer.

Paired-end Illumina sequencing (2  $\times$  300 bp) of all the purified PCR products was performed by GENEWIZ on a MiSeq desktop sequencer system (Illumina) using kits manufactured by GENEWIZ.

#### 2.5. Data analysis

The obtained 18S rRNA reads were processed by the QIIME data analysis package. Quality filtering was performed after the reads were joined, assigned, and truncated, and any sequences failing to meet the following criteria were excluded: sequence length <200 bp, no ambiguous bases, and mean quality score  $\geq$ 20. The remaining sequences were referred to the RDP Gold database using the UCHIME algorithm for the detection and deletion of chimeric sequences. The remaining effective sequences were grouped into operational taxonomic units (OTUs) through clustering against the Silva 138 database at a preclustered 97% sequence identity threshold with the program VSEARCH (v.1.9.6). The taxonomy of all OTUs was assigned by the ribosomal database program (RDP) at a confidence threshold of 0.8. Then, the representative sequences of OTUs were analyzed by the RDP classifier based on the Bayesian algorithm, and the community composition of each sample was calculated under different species classification levels. To decrease the number of false positives, OTUs with a frequency of <10 were removed. Sequences identified as predators (copepods or cladocerans) or parasitic species (ciliates) were also

removed from all data sets (Guo et al. 2012, Hu et al. 2014). The Chao1, Shannon ( $H'$ ), and Simpson indices were calculated using the software Past 3 (Hammer et al. 2001).

Venn diagrams of between-group out were generated using the 'venn.diagram' function from the R package 'VennDiagram'. The frequency distribution of different phyla in gut contents with >1% relative abundance across all samples was compared among samples using the function 'chisq.test' ('vegan' package), as were those in the phytoplankton and mesozooplankton communities. Based on the relative proportion of genus-level reads, niche width and niche overlap indices were calculated using the functions 'niche.width' and 'niche.overlap' from the R package 'spaa' for cladocerans and copepods using the equations by Levins (1968). The niche overlap index ranges between 0 (no overlap) and 1 (complete overlap). Interspecific and intraspecific differences in dietary niche width were analyzed with 1-way ANOVA. Nonmetric multidimensional scaling (NMDS) plots were constructed using the functions 'metaMDS' (package 'vegan') and 'ggplot' (package 'ggplot2') in R based on the Bray-Curtis dissimilarity of species to illustrate patterns of prey use among the 3 species. To test for similarity among species and months, we performed a permutational analysis of variance (PERMANOVA) on the same distance matrix using the function 'adonis' (package 'vegan'), where species and month were the 2 predictor variables with 999 permutations (Brandl et al. 2020). A bipartite network was constructed to visualize interactions between prey taxa and the 3 species using the function 'plotweb' (package 'bipartite') on the relative average abundances of prey OTUs for all seasons (Brandl et al. 2020). This network contains only the OTUs with >0.5% relative abundance across all samples for simplicity.

R v.4.2.1 (R Core Team 2022) was used to perform relevant analyses and visualizations with the 'tidyverse', 'vegan', 'pacman', 'VennDiagram', 'devtools', 'spaa', 'colorRamps', and 'bipartite' packages. The level of significance for all critical ranges was set at  $\alpha = 0.05$ .

### 3. RESULTS

#### 3.1. Hydrology

The sea surface temperature at the sampling site increased from 26.1 to 27.2°C from April to May and remained constant in summer (Fig. 2). Salinity

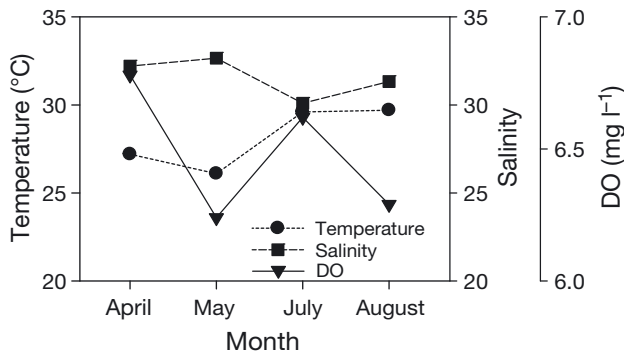


Fig. 2. Water surface temperature, salinity, and dissolved oxygen (DO) concentration at the sampling site in Daya Bay during spring and summer 2019

decreased from 32.2–32.6 to 30.1–31.1 from spring to summer, while dissolved oxygen varied in the range of 6.29–6.78 mg l<sup>-1</sup> regardless of season (Fig. 2).

### 3.2. Prey background

Phytoplankton abundance increased sharply from spring to summer, whereas mesozooplankton abundance experienced a drop in May and then continued to increase through late summer (Fig. 3A). The POC concentration doubled with the seasonal transition, whereas the atomic C:N ratio of particulate organic matter (POM) increased gradually from  $2.82 \pm 0.63$  to  $6.62 \pm 1.37$  (Fig. 3B).

Phytoplankton community structure in terms of phylum composition varied greatly with months (chi-square test,  $p < 0.01$ ) but not between July and August ( $p > 0.05$ ) (Fig. 4A,B). Dinoflagellata and Cryptophyta were the major phytoplankton phyla in April, with contributions of 78.8 and 17.7%, respectively, and the main genera included *Gyrodinium* and *Cryptomonas*. In May, Dinoflagellata and Bacillariophyta were the only 2 phyla identified and accounted for 40.5 and 59.5% of the abundance, respectively, with *Chaetoceros*, *Gyrodinium*, and *Prorocentrum* as the main genera. The advantage of Dinoflagellata was eclipsed and Bacillariophyta became the most advantageous phylum in summer with extremely high percentage of 99 and 98% in July and August, respectively. *Rhizosolenia*, *Pseudonitzschia*, and *Leptocylindrus* were the main diatom genera.

Mesozooplankton community structure changed significantly with month (chi-square test,  $p < 0.05$ ). The components included Arthropoda, Dinoflagellata, Chordata, Annelida, Cnidaria, Ctenophora, or Chaetognatha, and one group of planktonic larvae

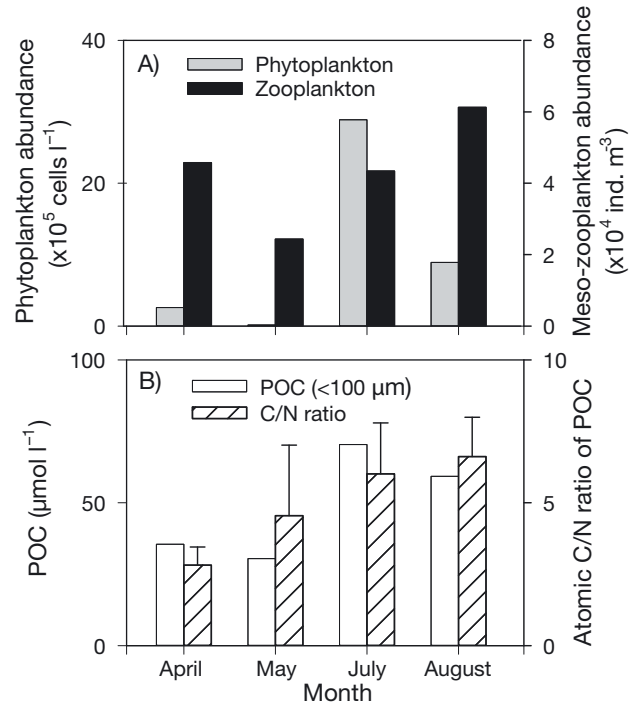


Fig. 3. (A) Phytoplankton and mesozooplankton abundances and (B) concentration and atomic C:N ratio of particulate organic carbon (POC) at the sampling site in Daya Bay during spring and summer 2019

(corresponding to larvae not belonging to the above phyla) (Fig. 5A). On average, Arthropoda was the most important phylum, with percentage ranging from 40.6–94.8% of the total abundance, followed by planktonic larvae and heterotrophic dinoflagellates, with contributions of 1.3–22.9 and 0.1–33.7%, respectively.

A total of 19 genera were identified, including 16 Arthropoda genera and one genus each for Chaetognatha, Chordata, and Dinoflagellata (Fig. 5B). The genera *Parvocalanus* and *Penilia*, to which the 2 predators under study belong, were of the most importance, with extremely high contributions of 23.4 and 16.3%, respectively, followed by *Paracalanus*, *Oithona*, and *Noctiluca*, with average contributions of 10.1–10.9% (Fig. 5B). The genus *Pseudevadne* accounted for 2.3% of the total abundance on average.

### 3.3. Eukaryotic dietary compositions of copepods and cladocerans

A total of 2 127 360 effective reads were obtained from all samples (4 samples of *Penilia avirostris*, 3 samples of *Pseudevadne tergestina*, 3 samples of *Par-*

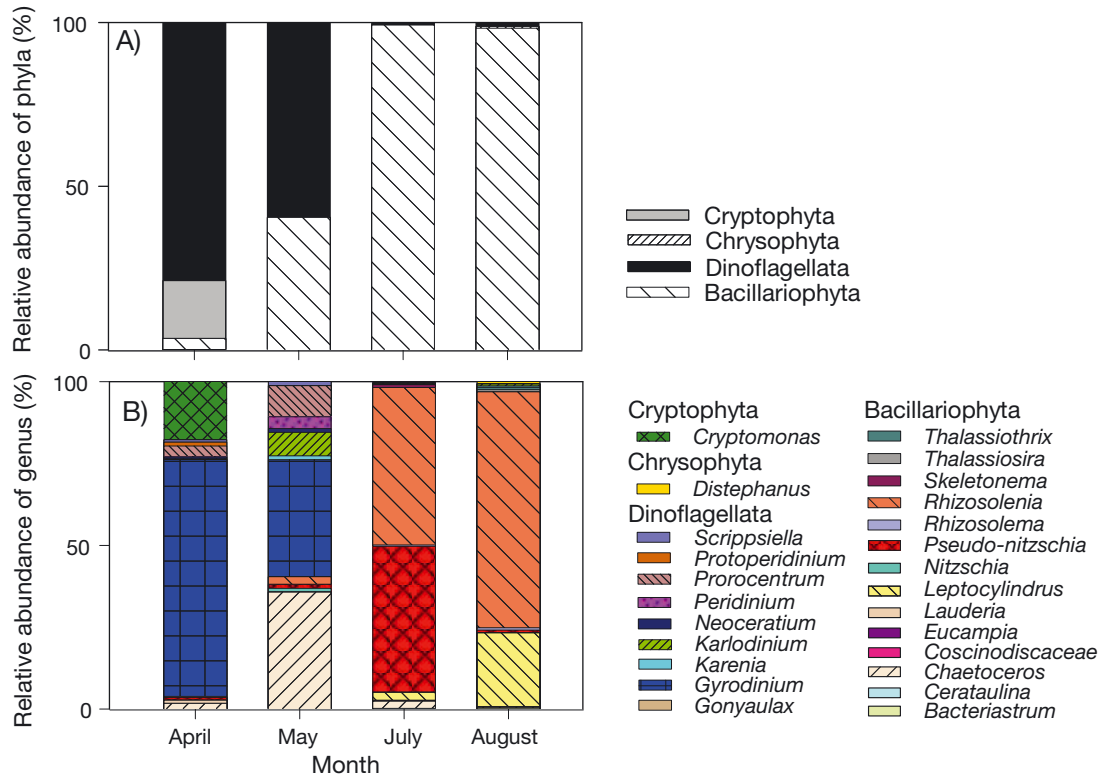


Fig. 4. Relative abundances of eukaryotic phytoplankton (A) phyla and (B) genera in the waters at the sampling site in Daya Bay during spring and summer 2019

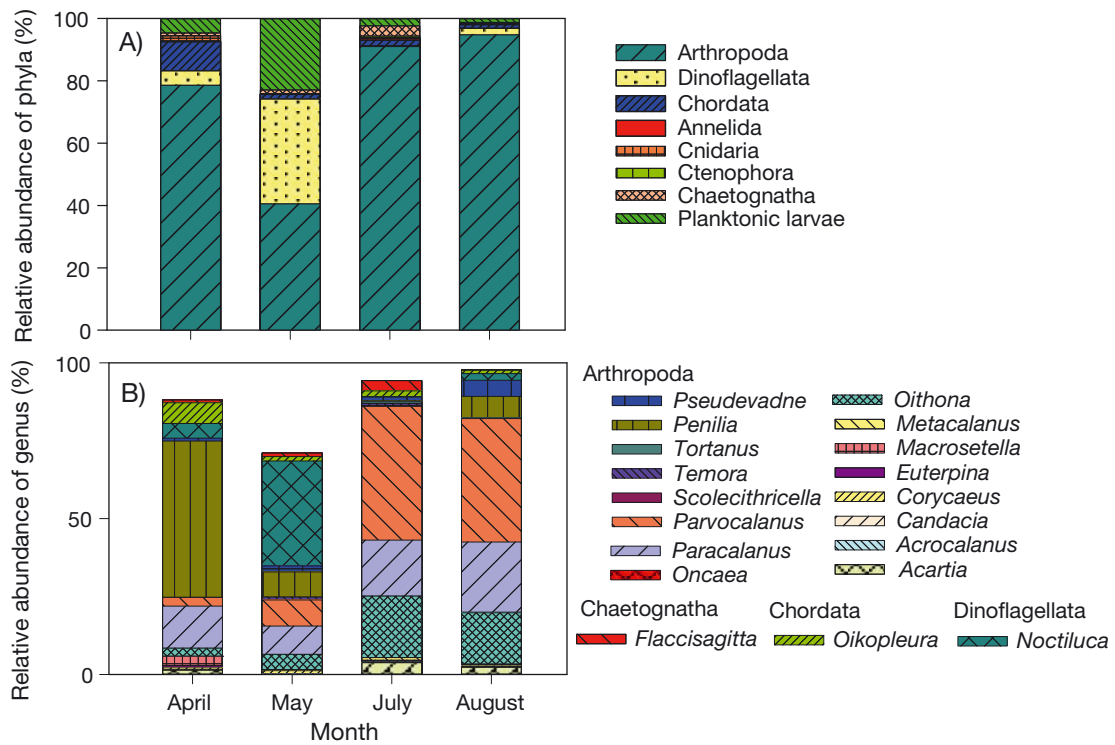


Fig. 5. Relative abundances of mesozooplankton (A) phyla and (B) main genera in the waters at the sampling site in Daya Bay during spring and summer 2019

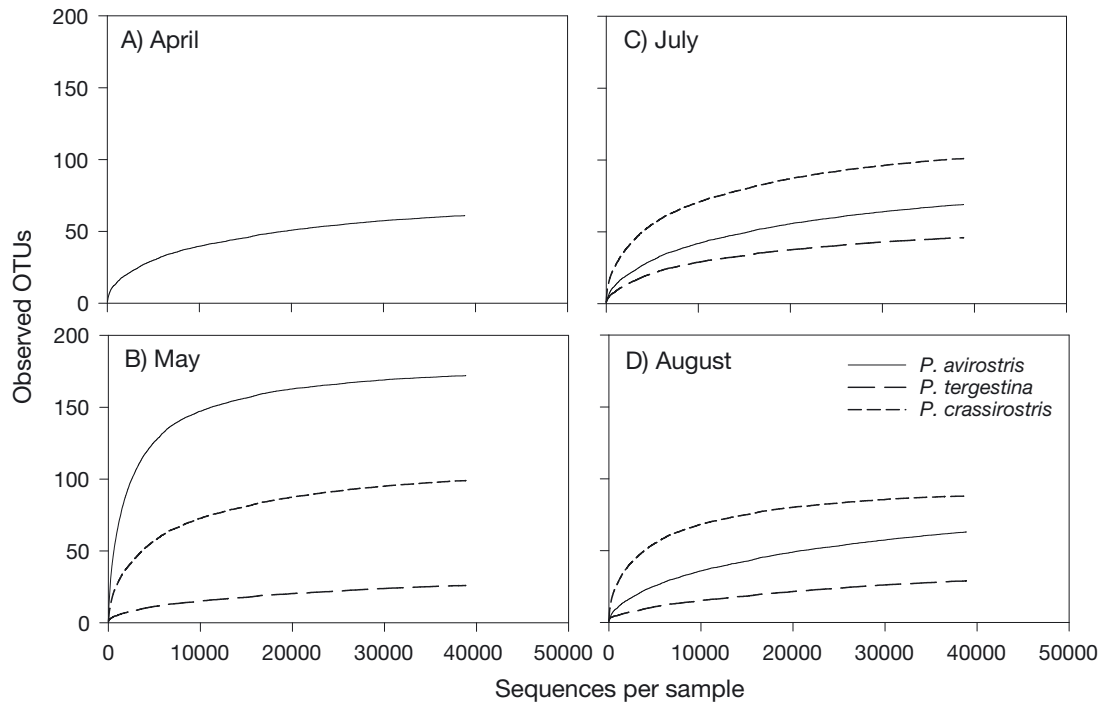


Fig. 6. Rarefaction curves of operational taxonomic unit (OTU) diversity in the gut contents of *P. avirostris*, *P. tergestina*, and *P. crassirostris* in Daya Bay during (A) April, (B) May, (C) July, and (D) August 2019

*vocalanus crassirostris*, and 10 corresponding animal controls), clustering into 195 OTUs. The number of OTUs initially increased with sequencing effort and then reached a plateau, implying that the amount of sequencing data was somewhat reasonable (Fig. 6). Among these OTUs, 89.2, 46.7, and 37.9% can be assigned to phylum, genus, and species, respectively. The Chao1, Shannon, and Simpson indices indicated that the depth of sequencing covered a wide prey species spectrum (Table 1).

The identified OTUs belonged to 24 phyla, and half of the sequences belonged to metazoans (53.2%), such as Arthropoda, Cnidaria, Chaetognatha, and Ctenophora. Cnidaria was the most abundant group, accounting for 21.8% of all sequences, followed by Chaetognatha, with a percentage of 16.4% (Fig. 7). Phytoplankton was also important, accounting for 44.9%, mainly including Bacillariophyta, Dinoflagellata, Chlorophyta, and Chrysophyta. Bacillariophyta and Dinoflagellata were the most abundant microalgae, with average contributions of 29.1 and 13.3%, respectively. There were also a few protozoans and fungi, including Ciliophora, Picozoa, Radiolaria, Ascomycota, and Eumycota, which together accounted for 1.9% of the total sequences of gut contents (Fig. 7). In addition, 16.0% of sequences on average were not identified to phylum across species and months.

Table 1. Dietary diversity indices of *Penilia avirostris*, *Pseudovadne tergestina*, and *Parvocalanus crassirostris* in Daya Bay during spring and summer 2019.  $H'$ : Shannon index; Chao1: estimator of the number of OTUs (operational taxonomic units)

Sample	No. of OTUs	No. of sequences	$H'$	Simpson	Chao1
April					
<i>P. avirostris</i>	51	2109	2.04	0.76	59
May					
<i>P. avirostris</i>	137	20382	1.95	0.64	140
<i>P. tergestina</i>	20	973	0.81	0.31	25
<i>P. crassirostris</i>	62	3639	1.79	0.64	75
July					
<i>P. avirostris</i>	57	690	2.93	0.91	78
<i>P. tergestina</i>	39	1180	1.47	0.52	46
<i>P. crassirostris</i>	67	5534	2.01	0.74	85
August					
<i>P. avirostris</i>	55	1290	1.86	0.66	91
<i>P. tergestina</i>	27	1266	1.06	0.54	43
<i>P. crassirostris</i>	56	5557	1.87	0.70	64

#### 3.4. Interspecific differences in dietary compositions

The 3 small crustaceans exhibited different eukaryotic dietary diversities in gut contents. *P. avirostris* had the highest diversity index values, followed

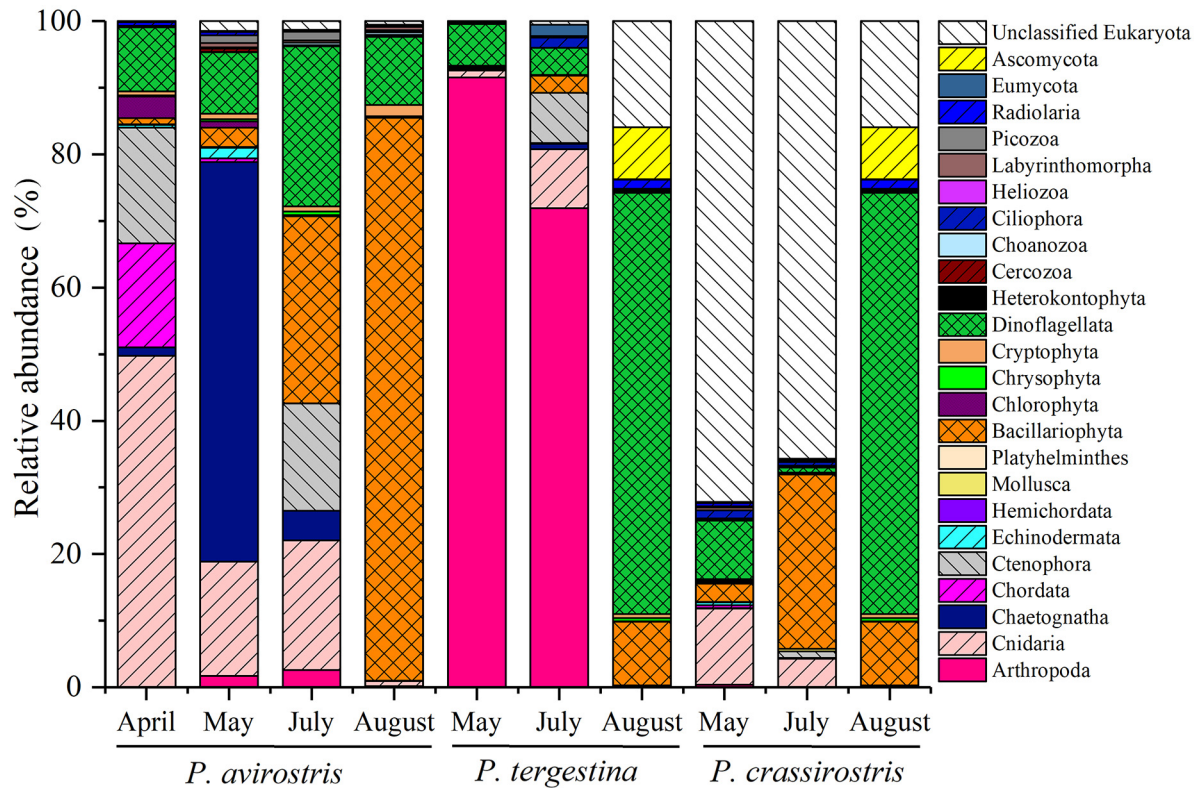


Fig. 7. Relative read abundances of eukaryotic phyla in gut contents of *P. avirostris*, *P. tergestina*, and *P. crassirostris* in Daya Bay in spring and summer 2019

by *P. crassirostris*, and *P. tergestina* had the lowest OTU values (Table 1, Fig. 7). Gut contents had a distinct frequency distribution of phyla across predators and months (chi-square test,  $p < 0.001$ ). The bipartite network of all prey items further indicates distinct relative prey contributions across the 3 species (Fig. 8). *P. avirostris* contained nearly half of the sequences originating from animals, and the other half belonged to phytoplankton (53.2 and 44.9%). On average, phytoplankton and gelatinous animals were important prey for *P. avirostris*. Diatoms alone accounted for 29.1% of prey sequences, followed by cnidarians and arrow worms, with average percentage of 21.8 and 16.4%, respectively. Dinoflagellates and ctenophores contributed 13.3 and 8.4% to prey sequences, respectively. In contrast, arthropods were the most important prey for *P. tergestina*, with an extremely high contribution of 86.2%, whereas other phyla, including dinoflagellates, cnidarians, and ctenophores, together constituted 10% of the total prey sequences. *P. crassirostris* contained a high proportional contribution of unidentified eukaryotic sequences (51.3%), and phytoplanktonic dinoflagellates and diatoms accounted for 24.3 and 12.9% of the total prey sequences, respectively. Cnidarians made up the remaining 5.3% of sequences.

### 3.5. Temporal dietary variation

The dietary compositions of the 3 species also showed seasonal variations. Gelatinous animals were the major resources for *P. avirostris* in spring, in that cnidarians and arrow worms made up 49.7 and 60.0% of the total prey sequences, respectively. At the genus level, the cnidarians *Muggiaea*, ctenophores *Bolinopsis*, and arrow worms *Flaccisagitta* were the main genera. In July, diatoms, dinoflagellates, cnidarians, and ctenophores accounted for 16.1–28.1% of the total prey sequences, whereas phytoplankton represented the overwhelmingly dominant prey, with an extremely high percentage of prey sequences (94.7%) in August, mainly composed of the genera *Rhizosolenia* and *Leptocylindrus*.

Arthropods, making up 71.9–91.6% of prey sequences, were consistently the main dietary constituents of *P. tergestina* in spring and summer, but most OTUs were unidentified with respect to class. The copepods *Temora* and *Paracalanus* were the main genera in May and August, respectively. Dinoflagellates, cnidarians, and ctenophores were minor food resources.



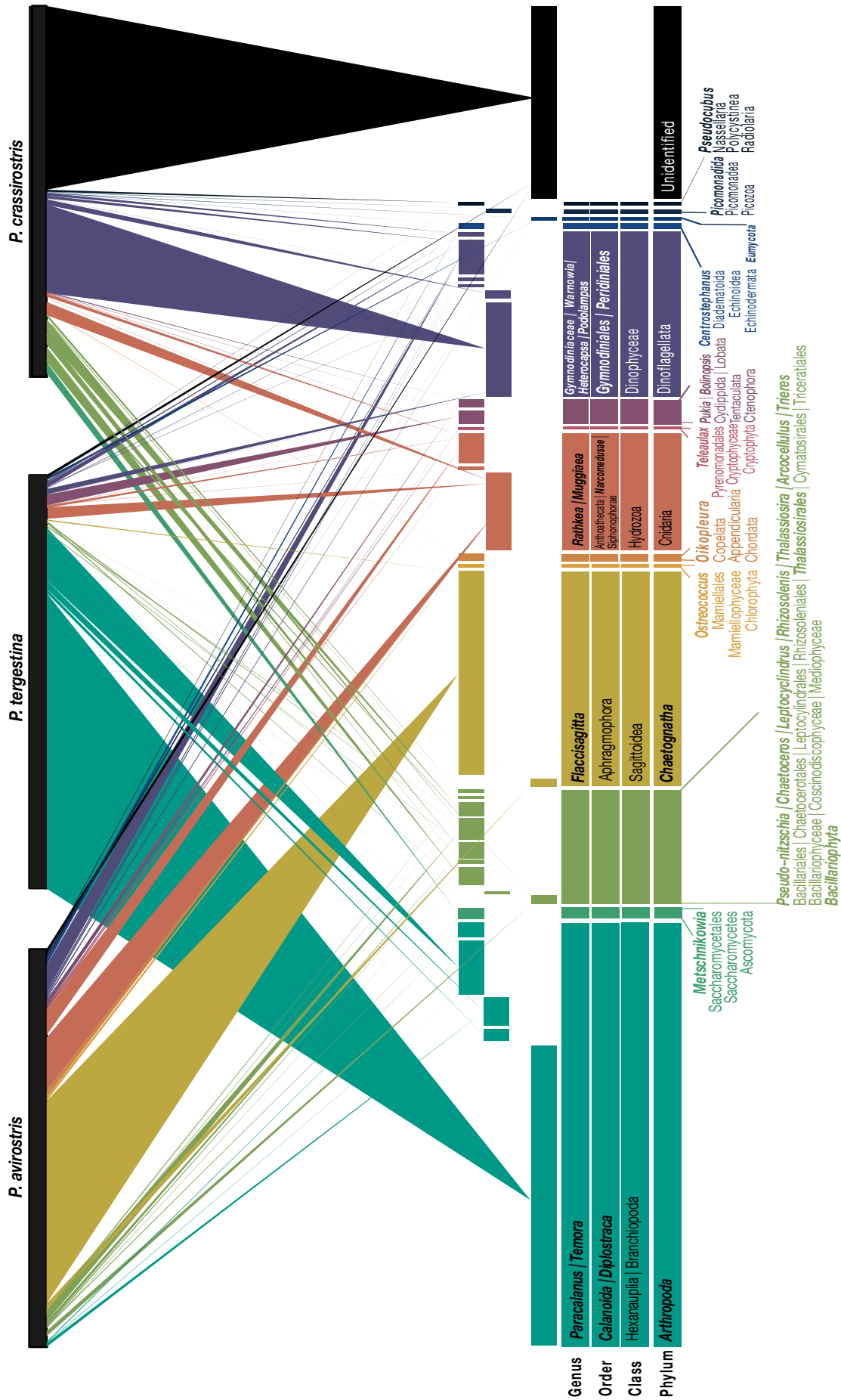


Fig. 8. A bipartite graphic of trophic interactions among *P. avirostris*, *P. tergestina*, *P. crassirostris*, and prey items in Daya Bay during spring and summer 2019. Colors reflect prey phyla. Connecting arrow width is proportional to the relative abundance of each prey taxon in the species' gut contents. The vertical position of the prey rectangles and labels reflects the level of taxonomic resolution, from phylum (lowest) to class, order, and genus (highest). Prey rectangles correspondingly represent the taxa labeled in **bold italic** letters from left to right in the network

High percentage (65.7 and 72.2%) of prey sequences of *P. crassirostris* belonged to a few OTUs unidentified to phylum in May and July, respectively. Cnidarians were the most abundant identified food resource in May, followed by dinoflagellates and diatoms. In summer, diatoms and dinoflagellates alternatively represented the predominant identified food resources with high percentage of 26.2 and 63.3%, respectively. The main diatom genera included *Pseudo-nitzschia*, *Thalassiosira*, *Leptocylin-drus*, and *Rhizosolenia*, whereas the main dinoflagellate genus was *Heterocapsa*.

### 3.6. Niche partitioning

Dietary niche width was greatest in *P. avirostris* (9.08), followed by *P. crassirostris* (6.61) and *P. tergestina* (1.97), as determined by pooling all OTUs over the 2 seasons. Specifically, the greatest niche width occurred in *P. avirostris* in July (11.5), and the lowest occurred in *P. tergestina* in May (1.45) (Table 2). The dietary niche overlapped negligibly among these 3 species (Table 3). The highest niche overlap occurred between *P. avirostris* and *P. tergestina* in July (0.19), whereas the lowest overlap occurred between *P. tergestina* and *P. crassirostris* in August (0.001) (Table 3, Fig. 9).

NMDS plots showed separations among groups and seasons (stress = 0.110; Fig. 10). *P. tergestina* was clearly separated from the other 2 species based on the locations of the top 30 dietary OTUs in the plot. For *P. avirostris* and *P. crassirostris*, dietary composition was clearly differentiated between spring (May) and summer (July and August). The distinctness of species groupings was reinforced by the PERM-

Table 2. Dietary niche breadth of *P. avirostris*, *P. tergestina*, and *P. crassirostris* in Daya Bay during spring and summer 2019

Sample	Niche breadth
April	
<i>P. avirostris</i>	4.11
May	
<i>P. avirostris</i>	2.8
<i>P. tergestina</i>	1.45
<i>P. crassirostris</i>	2.79
July	
<i>P. avirostris</i>	11.5
<i>P. tergestina</i>	2.07
<i>P. crassirostris</i>	3.85
August	
<i>P. avirostris</i>	2.91
<i>P. tergestina</i>	2.16
<i>P. crassirostris</i>	3.3

Table 3. Dietary niche overlap indices of *P. avirostris* (PA), *P. tergestina* (PT), and *P. crassirostris* (PC) in Daya Bay during spring and summer 2019

	May		July		August	
	PA	PT	PA	PT	PA	PT
May						
PA	-	-	-	-	-	-
PT	0.01	-	-	-	-	-
PC	0.02	0.01	-	-	-	-
July						
PA	-	-	-	-	-	-
PT	-	-	0.19	-	-	-
PC	-	-	0.08	0.01	-	-
August						
PA	-	-	-	-	-	-
PT	-	-	-	-	0.01	-
PC	-	-	-	-	0.05	0.001

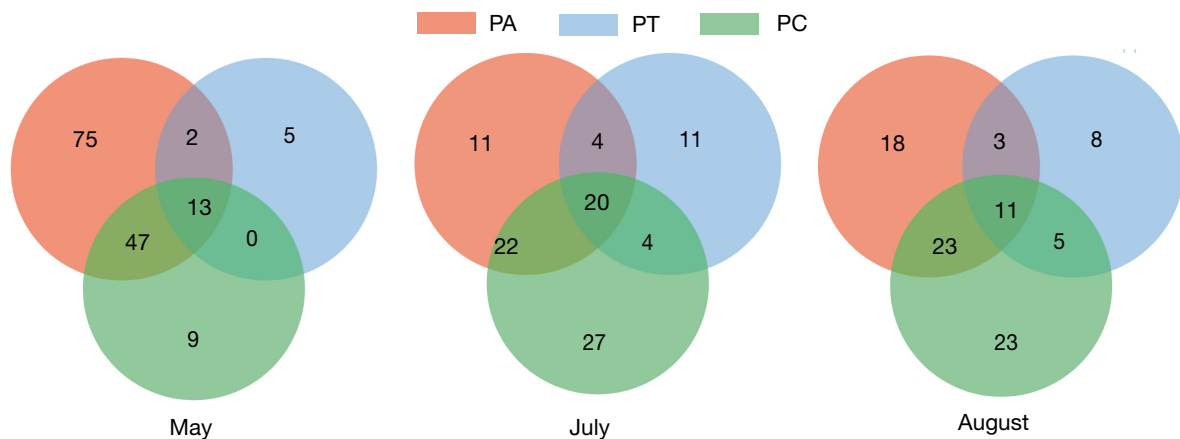


Fig. 9. Venn diagram of overlap in the operational taxonomic units of gut contents of *P. avirostris* (PA), *P. tergestina* (PT), and *P. crassirostris* (PC) in Daya Bay in spring and summer 2019

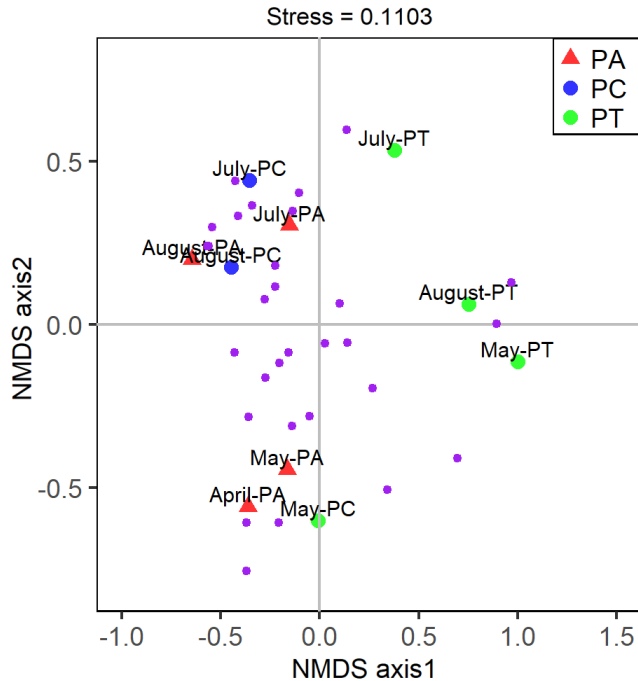


Fig. 10. Nonmetric multidimensional scaling (NMDS) plot based on the prey found in the gut contents of *P. avirostris* (PA), *P. tergestina* (PT), and *P. crassirostris* (PC) in Daya Bay during spring and summer 2019. Purple dots: top 30 operational taxonomic units

ANOVA, which showed high explanatory power for species as a grouping variable ( $F = 3.20$ ,  $df = 2$ ,  $p = 0.001$ ,  $R^2 = 0.478$ ), but not for season ( $F = 0.8917$ ,  $df = 3$ ,  $p = 0.626$ ,  $R^2 = 0.308$ ).

#### 4. DISCUSSION

In this study, HTS technology greatly improved the analysis of the food spectra of 3 common small marine crustaceans: *Penilia avirostris*, *Pseudevadne tergestina*, and *Parvocalanus crassirostris*. These 3 species are all omnivorous generalists but exhibit distinct dietary compositions and diversities based on the relative abundance of detected food items. Strong dietary niche partitioning among these species is suggested by low dietary niche overlap, which allows for their coexistence in Daya Bay.

Compared with the traditional microscopic method, a large amount of sequence data obtained by HTS could provide comprehensive information on the diet of marine organisms, especially for small individuals, for which stomach dissection and food identification are difficult. Moreover, the relative abundance of sequences can reflect the relative percentage of each food item ingested. It is undeniable that the contribu-

tion of metazoans to the gut contents was overrepresented to some extent in this study, but the fluctuation in the major prey groups in terms of the phylum and genus levels nearly matched the seasonal change in the prey community in the ambient water, indicating that the relative abundance of sequences can provide semiquantitative information for diet analysis (Lin et al. 2018), such as the niche partitioning of 3 small crustaceans. In this study, only the universal primer set (18S rDNA) was used, and some rare prey sequences may not have been detected due to the large amount of DNA fragments from the predator itself (reviewed by Santoferrara 2019). However, the semiquantification of relative abundance among samples was sufficient for the analysis of dietary differences (e.g. Deagle et al. 2009), as was also indicated in the present study.

#### 4.1. Dietary diversity

The *in situ* food spectrum of the ubiquitous marine cladoceran *P. avirostris* was first studied using the HTS technique and showed the highest diversity in May and greatest niche width in July compared with the co-occurring species *P. tergestina* and *P. crassirostris*. Overall, gelatinous zooplankton, including cnidarians, ctenophores, and chaetognaths, were important to the total prey sequences, comparable to phytoplankton. Early studies combining microscopic observation and incubation methods indicated that *P. avirostris* can graze on phytoplankton or ciliates with cell sizes in a wide range of  $<2$  to  $70 \mu\text{m}$  (Katechakis et al. 2004). The present study widens the food spectrum of this species. The importance of gelatinous zooplankton in prey composition was highlighted in spring, when cnidarians or arrow worms alone accounted for nearly half of the prey sequences. Two important genera were arrow worm *Flaccisagitta* (e.g. *Flaccisagitta enflata*) and cnidarian *Muggiaea* (e.g. *Muggiaea atlantica*), which are commonly dominant zooplankton in Daya Bay (Lian et al. 2011, Du et al. 2013). Our observations showed that the former genus accounted for 1.39% of the total mesozooplankton abundance during the study period. However, *Muggiaea* and *Bolinopsis* were not observed in the samples for microscopic identification, possibly because they were damaged or destroyed during towing in plankton nets or during preservation. Two possible underlying mechanisms may explain gelatinous zooplankton consumption by *P. avirostris*, which are traditionally thought to be predators. Larvae or eggs of these groups fall within the size range

accessible to mesozooplankton and may be collected by feeding appendages of *P. avirostris*. In Daya Bay, the chlorophyll *a* concentration is usually low in spring (Wu et al. 2017, Song et al. 2004), and phytoplankton accounted for a small proportion of potential food particles, as the C/N ratio of POM was significantly lower during the spring sampling (~3.7, Fig. 1B; Ke et al. 2017). Detritus and eggs originating from gelatinous zooplankton may provide surplus nutrition for *P. avirostris* and support its population recruitment in spring. Reproduction of prolific cnidarians and arrow worms usually occurs in spring and summer, which have appropriate temperatures and abundant prey (Alvariño 1992, Lo et al. 2014). In addition, arrow worms usually die after egg laying, and consequently, many carcasses will be generated during reproductive seasons (Alvariño 1992), which may greatly promote the detrital food chain in water columns. Nevertheless, *P. avirostris* fed on diatoms and dinoflagellates in response to the proliferation of phytoplankton in summer. The genera *Rhizosolenia* and *Leptocylindrus*, the main contributors to the ambient phytoplankton assemblage, also showed high proportional percentages in the gut contents of *P. avirostris*. Therefore, this species could be defined as an opportunistic omnivorous generalist, and feeding flexibility facilitates its seasonal dominance in Daya Bay and other coastal waters.

*P. tergestina* had the lowest dietary diversity and narrowest niche breadth of all 3 species but is also an omnivorous generalist. It showed strong specialization for animal resources during spring through summer, with arthropods as the most important prey. Microscopic observation revealed that *P. tergestina* is a raptorial herbivore with a preference for diatoms (Jagger et al. 1988). In contrast, Li (2010) found that ciliates accounted for ~60% of prey sequences in the gut contents in addition to phytoplankton by using a DNA sequencing approach. In this study, the detected dietary diversity of *P. tergestina* was further largely broadened based on the HTS technique, and arthropods constituted major food sources for this species. At the genus level, the calanoid copepods *Temora* and *Paracalanus* contributed considerably to the diet of *P. tergestina*. In the sampling area, the genus *Temora* co-occurred with *P. tergestina* in May, whereas *Paracalanus* co-occurred in May and August with percentages of 9.1 and 22.5%, respectively. Cnidaria and Ctenophora were also present in the gut contents of *P. tergestina*. It is speculated that the trophic connection between this species and other zooplankton occurs through egg- or larval-feeding or detritus food chains involving fragments

of carcasses, exuviae, or fecal pellets of the sequenced organisms. The extension of food spectrum species to diverse zooplankton in both cladocerans suggests that marine cladocerans may contribute to the functioning of marine pelagic ecosystems by participating in detritus food chains apart from microbial loops and planktonic food chains (Turner et al. 1988, Sánchez et al. 2011).

*P. crassirostris* exhibited high dietary diversity with a medium niche breadth compared with the other 2 species. Phytoplankton, including dinoflagellates and diatoms, were the main prey, whereas cnidarians were also important. The proportion of phytoplankton as food resources increased during the seasonal transition from spring to summer, paralleling the seasonal change in phytoplankton abundance in Daya Bay, which indicates an opportunistic feeding mode in this species (Eskinazi-Sant'Anna 2013, Calbet et al. 2000). The strong trophic interaction between *P. crassirostris* and cnidarians in spring in this study is consistent with early reports for *Calanus sinicus* in the Bohai Sea and the Taiwan Strait (Ho et al. 2017, Yi et al. 2017). *P. crassirostris* consumed detritus or eggs originating from cnidarians, and this intraguild carnivorous feeding may facilitate animals to achieve nutrition identical to their bodily needs. Small copepods exerted great grazing pressure on dinoflagellates in Daya Bay based on gut pigment analysis (He et al. 2021). In the study by Eskinazi-Sant'Anna (2013), dinoflagellates were not observed in fecal pellets of *P. crassirostris* beyond diatoms and protozoa under microscopy, probably because dinoflagellate species lack digestion-resistant cellulosic cell walls. However, the full food spectrum of *P. crassirostris* remains undetermined because a few abundant prey could not be identified in May and August. A more comprehensive gene database in typical coastal waters is needed to decode the food spectrum of this important ubiquitous marine copepod in the future. In addition, cannibalism cannot be manifested using the sequencing technique because self-sequences are usually excluded in data processing.

#### 4.2. Niche partitioning

These 3 small planktonic crustaceans are all omnivorous generalists but showed contrasting feeding tendencies and flexibilities. Seasonal transitions in dietary composition in *P. avirostris* and *P. crassirostris* suggest their opportunistic feeding modes and feeding flexibilities. In contrast, *P. tergestina* exhibited a static feeding tendency toward arthro-

Pods. Overall, there was low niche overlap among these 3 species (0.001–0.19), indicating clear niche partitioning between them. In the only previous study on diet overlap between copepods and cladocerans, the overlap index for *Acartia clausi* and *P. avirostris* was determined based on their selectivity-size profiles, and the observed value was as high as 0.63 (Katechakis et al. 2004). Diet overlap decreased with the resolution of the method, and herein, the HTS technique with high resolution for identifying prey taxa reveals the niche separations across these mesozooplankton species, which are usually undetected with visual methods due to their small sizes.

The extent of niche partitioning depends on differences in the inherent feeding mode and food environment. *P. avirostris* and *P. crassirostris* both perform filter feeding, whereas *P. tergestina* performs raptorial catching, partially explaining their niche overlap situations. The size of the respective appendages and the discarding behavior after food collection may determine the prey composition of filter feeders. Alongside gelatinous animal resources and unidentified OTUs, specific trophic linkages between the main phytoplankton groups (diatoms and dinoflagellates) and consumers contributed to niche partitioning between *P. avirostris* and *P. crassirostris*. The dinoflagellate *Heterocapsa* and diatom *Leptocylindrus* occurred in the food spectrum of *P. avirostris* at high frequencies and percentage, whereas chain-forming diatoms such as *Pseudonitzschia* and *Thalassiosira* were present in the gut of *P. crassirostris*.

Dietary niche partitioning among consumers at the same trophic level promotes their coexistence and endows an ecosystem with high taxonomic diversity and system stability. *P. avirostris* and *P. crassirostris* usually coexist in Daya Bay during spring through summer every year. During our sampling events, these 2 species were consistently the dominant mesozooplankton species, with a greater advantage for the latter except in April, although *P. avirostris* had comparable or higher niche breadth compared to *P. crassirostris*. In particular, the extremely wide dietary niche breadth in July did not endow *P. avirostris* with an abundance advantage. This finding contradicts the niche breadth hypothesis, which proposes that the local abundance of a species in sites of occurrence is higher for species with large niche breadths (Brown 1984). In contrast, *P. crassirostris* appeared to be more successful in Daya Bay and other coastal waters. According to Sheth et al. (2014), a species that is a resource generalist and specializes in environmental conditions that are common in a

region may have a high local abundance. Both *P. avirostris* and *P. crassirostris* are omnivorous generalists that are able to adapt their feeding modes well in response to variable food conditions and can exploit non-phytoplankton prey in situations of phytoplankton rarity. Nevertheless, *P. crassirostris* can thrive on diatoms and dinoflagellates when phytoplankton abundance was high in July and August better than *P. avirostris*. Apart from this, other factors, such as the life history traits of cladocerans and environmental biotic (predation) and abiotic physicochemical (temperature, salinity, and so on) factors also contribute to the variation in the population dynamics of marine cladocerans and copepods. As reported by Almeida et al. (2012), *P. crassirostris* can maintain high reproduction over a wide range of water temperatures and salinities.

### 4.3. Implications

The 3 small crustacean species *P. avirostris*, *P. tergestina*, and *P. crassirostris* usually play linking roles between the microbial loop and classic planktonic food chain, as their prey spectra can range from picosized particles to micro-sized protists based on incubation experiments (Calbet et al. 2000, Atienza et al. 2007, Sánchez et al. 2011, Chen et al. 2013). In this study, their *in situ* food spectrum was found to be broader, ranging from phytoplankton and microzooplankton to other groups, including arthropods and gelatinous zooplankton. Trophic interactions among these 3 species and other zooplankton may occur through egg consumption, common detritus feeding, or scavenging. It seems that detritus derived from different sources is a supplementary input to the pelagic food web in coastal and estuarine waters; therefore, the detritus food chain should be considered in the energy flows of these ecosystems (Thresher et al. 1992, Harfmann et al. 2019).

## 5. CONCLUSIONS

The *in situ* eukaryotic food spectra of the common and coexistent marine cladocerans *P. avirostris* and *P. tergestina* and the copepod *P. crassirostris* in Daya Bay were elucidated by means of HTS of 18S RNA in this study. They are all categorized as omnivorous generalists but with different diet diversities and feeding preferences. Animal and phytoplankton prey contributed evenly to the sequences of gut contents of *P. avirostris*, whereas Arthropoda were the

major prey resource of *P. tergestina*. Phytoplankton, including dinoflagellates and diatoms, were the major prey (24.3 and 12.9%, respectively) of *P. crassirostris*. Both *P. avirostris* and *P. crassirostris* consumed a higher proportion of phytoplankton with the seasonal transition from spring to summer. Trophic interactions between these crustaceans and arthropods and gelatinous zooplankton indicated detritus consumption and intraguild predation or scavenging. *P. avirostris* had the broadest niche breadth, followed by *P. crassirostris*, and *P. tergestina* had the narrowest niche breadth. The marginal dietary niche overlap among these 3 species facilitated their coexistence in this ecosystem. The HTS technique, presenting a wider range of trophic connections, greatly improved our understanding of the *in situ* feeding modes of these small important mesozooplankton in Daya Bay. However, a high proportion of OTUs in gut contents were not identified (e.g. *P. crassirostris*), which constrained the application of this technique. Therefore, more informative gene databases are needed in the future to resolve the full-scale food spectra of additional species.

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