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Changes in community structure of active protistan assemblages from the lower Pearl River to coastal Waters of the South China Sea

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Abstract

Protists make up an important component of aquatic ecosystems, playing crucial roles in biogeochemical processes on local and global scales. To reveal the changes of diversity and community structure of protists along the salinity gradients, community compositions of active protistan assemblages were characterized along a transect from the lower Pearl River estuary to the open waters of the South China Sea (SCS), using high-throughput sequencing of the hyper-variable V9 regions of 18S rRNA. This study showed that the alpha diversity of protists, both in the freshwater and in the coastal SCS stations was higher than that in the estuary. The protist community structure also changed along the salinity gradient. The relative sequence abundance of Stramenopiles was highest at stations with lower salinity and decreased with the increasing of salinity. By contrast, the contributions of Alveolata, Hacrobia and Rhizaria to the protistan communities generally increased with the increasing of salinity. The composition of the active protistan community was strongly correlated with salinity, indicating that salinity was the dominant factor among measured environmental parameters affecting protistan community composition and structure.

Keywords: Community structure; Estuary; High-throughput sequencing; Microbial eukaryotes; Salinity gradient; 18S rRNA

Introduction

Protists play important roles in structuring marine ecosystems and global biogeochemical cycles (Azam et al. 1983; de Vargas et al. 2015; Sherr and Sherr 2002; Zubkov and Tarran 2008). Numerous studies have shown that due to various environmental factors, the community structure and diversity of protists varies both spatially and temporally (Caron et al. 2011; Lallias et al. 2015; Lie et al. 2013). For a better understanding of the primary ecological roles played by protists, their biogeographical distribution and response to changing environments represent fundamental questions that need to be addressed (Caron et al. 2016).

The South China Sea is one of the largest semi-enclosed marginal seas (Hu et al. 2000). With large inputs of fresh water and nutrients input from the Pearl River and oceanic water intrusion, the northern South China Sea is characterized by sharp environmental gradients over small spatial scales (Ning et al. 2004). It is thus an ideal environment for examining the spatial variation in microbial community composition and the mechanisms by which this is controlled.

Traditional microscopic methods have significant limitations for the study of protists because: (i) species identification is restricted to those organisms with well-

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documented morphological characteristics; (ii) they require large amounts of resources in terms of time and effort; (iii) species that are rare, cryptic and/or present in low abundance are frequently overlooked; and (iv) a wealth of taxonomic knowledge and expertise is needed (Eland et al. 2012). Given the wide range of protistan groups that exist, these requirements are seldom met. Fortunately, molecular sequencing methodologies have overcome these difficulties resulting in the discovery of previously unexpected patterns of distribution and high diversities of protists (Logares et al. 2014; López-García et al. 2001; Massana et al. 2004). In recent years, the development of 18S rRNA (and its gene)-based high-throughput sequencing technology has enabled us to quickly obtain hundreds of thousands to millions of environmental sequences at low cost, allowing detailed studies of microbial community structure (de Vargas et al. 2015; Hu et al. 2016; Lie et al. 2013), evolutionary relationships (Dunthorn et al. 2014; Sun et al. 2016), and microbiological and ecological relevance (Lallias et al. 2015; Lima-Mendez et al. 2015). In environmental surveys, the 18S rRNA gene has been commonly used as a marker to determine protistan community structure in various aquatic habitats (Guillou et al. 2008; Logares et al. 2014). However, extracellular DNA or DNA from dead/dormant cells can distort the results because this cannot be discriminated from the DNA of living/active cells using DNA-based sequencing alone. Compared with DNA, RNA is much less stable in extracellular conditions. Therefore, most RNA recovered from environmental samples originates from living cells. Estuarine systems are hotspot for a series of biogeochemical processes, harboring a variety of microbial communities adapted to wide ranging of salinity fluctuations (Attrill and Rundle 2002; Bazin et al. 2014a; Vigil et al. 2009). Studies have shown that a large number of dead cells/extracellular DNA exist in estuarine habitats (DeFlaun et al. 1987). Ensuring that nucleic acids originate from living cells is particularly relevant when investigating active protist communities in such environments. Nevertheless, most previous studies of protists were based on DNA sequencing (de Vargas et al. 2015; Decelle et al. 2014; Xu et al. 2017a) and only a few have employed the RNA-based sequencing (Charvet et al. 2014; Hu et al. 2016; Logares et al. 2014; Massana et al. 2015; Xu et al. 2017b). Studies of protist communities in estuarine environments involving RNA sequencing are even rarer (Sun et al. 2017).

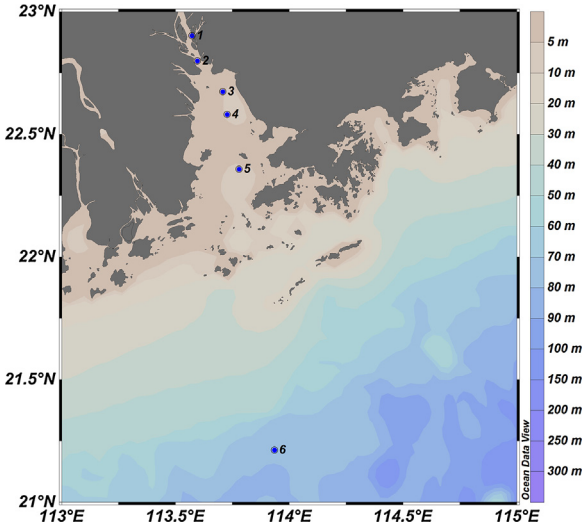

Fig. 1. Geographic locations of the sampling sites along the Pearl River estuary and in the northern South China Sea.

In the present study, we investigate the active protistan communities that occur in the Pearl River estuary, southern China, by high-throughput amplicon sequencing of the V9 region of the 18S rRNA. The main aims are to evaluate the composition of active protistan assemblages along salinity gradients and to increase understanding of the factors that shape their community structure.

Material and Methods

Sample collection and environmental parameters

Fig. 1 shows the locations of the sampling sites in the Pearl River estuary and a near-shore open water site of the SCS. A total of six surface water samples (sites 1-6) and two subsurface samples at sites 1 and 5 were collected from 27 July 2013 to 4 August 2013. Sample IDs corresponding to the sites from where they were collected are shown in Table 1. Samples were collected on Millipore filters (pore size, 0.8 μm) from 1 liter of seawater after passing through 200 μm mesh-size pre-filter in order to reduce the contribution of metazoa to nucleic acids

Table 1. Sampling sites information and physical parameters measured.

Sample ID	Site	Latitude	Longitude	Depth (m)	Salinity (‰)	pH	Temperature (°C)	DO (mg/L)
1S	1	22°54'6.66"	113°34'21.60"	2	0	6.1	29.29	1.2
1D	1	22°54'6.66"	113°34'21.60"	8	0	6.2	29.01	0.8
2S	2	22°47'56.2"	113°35'46.80"	1	1	7.2	30.67	5.6
3S	3	22°40'23.39"	113°42'30.41"	1	1	8.1	30.89	8.1
4S	4	22°34'51.24"	113°43'39.28"	1	4	8.1	30.66	8.2
5S	5	22°21'27.48"	113°46'48.12"	2	18	8	28.00	6.7
5D	5	22°21'27.48"	113°46'48.12"	8	26	8.2	27.63	6.3
6S	6	21°12'42.00"	113°56'09.00"	2	34	8.3	29.09	6.6

extracts. The 0.8 μm filters were covered with RNA stabilization solution (Ambion, USA) and preserved at −20 °C until further processing. Environmental data including salinity (S), pH, water temperature (T) and dissolved oxygen (DO) were obtained using a YSI Professional Plus water quality meter (YSI, USA).

RNA extraction, PCR amplification, and sequencing

AllPrep DNA/RNA Mini Kit (Qiagen, Germany) was used to extract total RNA following Xu et al. (2017b). The RNA concentration and quality were determined using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE) and gel electrophoresis, respectively. RNA was then reverse-transcribed into cDNA using QuantiTect Reverse Transcription Kit and genomic DNA was removed by gDNA Wipeout Buffer supplied within the kit (Qiagen, Shanghai, China). The universal eukaryotic forward (1389F) and reverse (1510R) primers were used to amplify the V9 region of the 18S rRNA according to Amaral-Zettler et al. (2009). PCR was run in six separate reactions for each sample to mitigate reaction-level polymerase chain reaction biases (Bates et al. 2013; Polz and Cavanaugh 1998) and obtain sufficient amplicons for sequencing. The pooled PCR products were purified using Wizard® SV Gel and PCR Clean-Up System (Promega, Beijing, China). All purified hypervariable V9 region amplicons were sent to a commercial company for pair-end sequencing using Illumina MiSeq platform. Sequence data have been submitted to the NCBI Sequence Read Archive and are accessible under the accession number SRP129859.

Sequence and statistical analysis

Quality filtering, demultiplexing and assembly of raw data were done with Trimmomatic (Bolger et al. 2014) and Flash software (Magoč and Salzberg 2011) employing several criteria: (i) reads were truncated at any site that obtained an average quality score of <20 over a 50-bp sliding window, and the truncated reads shorter than 50 bp were discarded; (ii) reads with any mismatch in the barcode, more than two nucleotide mismatches in the primer or containing ambiguous characters were removed; and (iii) overlapping sequences shorter than 10 bp or with a mismatch ratio of more than 0.2, were eliminated. Using the command of identify_chimeric_seqs.py based on *de novo* method in QIIME, potential chimeric sequences were detected and removed (Caporaso et al. 2010). Operational taxonomic units (OTUs) were clustered at a 95% similarity cutoff using Uclust as implemented in QIIME (Caron et al. 2009). Reads present as a single copy (singleton) were removed. Generation of OTU tables and taxonomy assignment of each OTU were done with QIIME using the reference taxonomic database of the Protist Ribosomal Reference database (PR^2) (Guillou et al. 2013). The unwanted OTUs (e.g. Bacteria, Archaea, Metazoa, and plastidial sequences) and OTUs assigned as "Unassigned" were further removed before downstream analysis.

To normalize the sampling effort, all datasets were subsampled at a uniform depth of 25,685 sequences (the lowest number of sequences for all samples). Alpha diversity estimates (Shannon, Chao, ACE, and Simpson) were calculated using Mothur (Schloss et al. 2009), and Phylogenetic Diversity (PD) was generated for each sample using QIIME (Caporaso et al. 2010). SIMPER (similarity percentage) analysis was used to identify taxa primarily responsible for the differences observed among samples using PAST software (Hammer et al. 2001). Non-metric multidimensional scaling (NMDS) was constructed using PRIMER (Clarke 1993). Mantel test was calculated using PASSaGE 2 (Rosenberg and Anderson 2011). The significance of the Mantel statistic was obtained after 1,000 permutations and the results of the statistical tests were considered to be significant at p-values < 0.01.

Results

Physical and chemical properties of the sampling sites

Table 1 summarizes the physical and chemical properties of the eight samples along the Pearl River estuary and the near-shore open water site in the SCS. A salinity gradient existed along the transect, with the lowest (0‰) at the upstream site 1 and the highest (34‰) at the near-shore open water site 6. Salinity zones were named according to the Venice salinity classification system: oligohaline (0.5-5‰), mesohaline (5-18‰), polyhaline (18-30‰), euhaline (30-40‰) and hyperhaline (>40‰). According to this system, site 1 was fresh water, sites 2, 3, and 4 were oligohaline, site 5 was polyhaline, and site 6 was euhaline. The pH ranged from 6.14 to 8.25 and water temperature ranged from 27.6 to 30.9 °C. Dissolved oxygen concentration ranged from 0.8 to 8.2 mg l^{-1}. The geographic distance from site 1 to site 6 was 190.83 km.

Alpha diversity of protists

To evaluate the differences in the protistan community structure in the waters along the Pearl River estuary and in the near-shore open water SCS, the eight water samples (six surface and two subsurface) were assessed by high throughput sequencing of PCR-amplified 18S rRNA. A total of 262,343 quality-screened sequences with an average length of 131 bp were generated. Sequence numbers per sample ranged from 25,685 (sample 5D) to 42,413 (sample 4S) with a mean of 34,049 ± 8,364 (n = 8) (Table 2). Rarefaction curves revealed considerable variation in the OTU diversity of the samples (Fig. S1).

Table 2. Diversity estimates of protistan communities in the sampling sites. Operational taxonomic units (OTUs) were classified at the 95% 18S rRNA V9 gene sequence identity.

Sample ID	Reads	OTU*	ACE*	Chao*	Shannon*	Simpson*	PD*	coverage*
1S	37,707	692	868 (824, 927)	856 (805, 929)	3.76 (3.73, 3.79)	0.909 (0.907, 0.912)	89.3	0.993
1D	30,794	655	796 (758, 848)	778 (737, 837)	3.79 (3.77, 3.82)	0.899 (0.896, 0.902)	79.3	0.994
2S	30,605	497	674 (626, 740)	670 (614, 754)	2.77 (2.74, 2.79)	0.797 (0.793, 0.802)	68.7	0.993
3S	31,997	472	770 (709, 845)	739 (647, 880)	2.87 (2.84, 2.89)	0.815 (0.812, 0.819)	58.6	0.994
4S	42,413	273	421 (382, 474)	348 (317, 402)	2.17 (2.15, 2.19)	0.753 (0.749, 0.767)	37.3	0.997
5S	35,953	905	1118 (1,070, 1,180)	1075 (1,028, 1,141)	4.42 (4.39, 4.44)	0.964 (0.963, 0.964)	123.5	0.991
5D	25,685	775	882 (853, 922)	859 (830, 901)	4.42 (4.40, 4.44)	0.964 (0.963, 0.965)	110.0	0.994
6S	27,189	762	844 (820, 878)	832 (806, 872)	4.97 (4.95, 4.99)	0.982 (0.972, 0.983)	105.5	0.995

*Normalized numbers based on subsampling of 25,685 sequences.

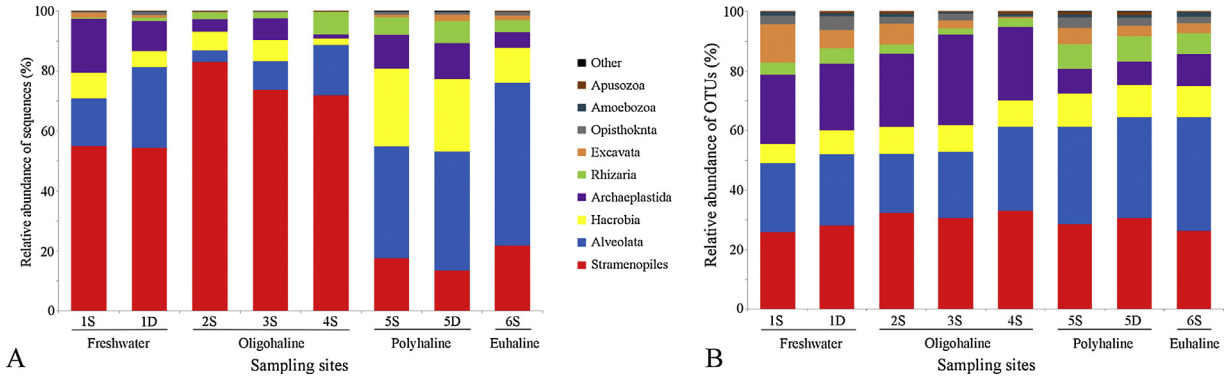

Fig. 2. Relative abundances of sequences (A) and OTUs (B) of protistan assemblages in the Pear River estuary at the supergroup taxonomic level.

Alpha diversity measurements (Ace, Chao, Shannon, Simpson, and OTU richness) are presented in Table 2. Site 5, which belonged to the polyhaline zone, had the highest alpha diversity estimates, followed by site 6 which belonged to euhaline zone and site 1 which belonged to freshwater zone. The oligohaline sites 2, 3, and 4 had the lowest alpha diversity estimates (Table 2). Phylogenetic diversity (PD), which measures the total branch length connecting all OTUs in the SSU rRNA gene phylogeny, showed the same trend (Table 2).

Community composition of protists

The community composition of protistan assemblages changed along the salinity gradients. The contribution of Stramenopiles-affiliated sequences, mostly associated with diatoms, to total protistan sequences reached ca. 72% to 83% in the oligohaline sites followed by the freshwater (ca. 55%), euhaline (ca. 22%) and polyhaline (ca. 16%) sites. Sequences affiliated with Alveolata were most frequently recovered from the euhaline site (ca. 54%) followed by the polyhaline (ca. 38%), freshwater (ca. 21%), and oligohaline (ca. 17%) sites (Fig. 2). The relative abundance of Hacrobia sequences peaked at the polyhaline (ca. 25%) site and their contribution was found to be the lowest at the oligohaline sites (ca. 5%). The contribution of Rhizaria sequences increased from 0% to 8% with increasing salinity. The sequences affiliated with Excavata, Opisthokonta, Amoebozoa and Apusozoa made only minor contributions to the total protistan communities (Fig. 2). OTU richness also showed salinity-specific distribution patterns. The polyhaline and euhaline sites had higher relative OTU richness for Alveolata and Rhizaria but lower relative OTU richness for Archaeplastida (Fig. 2).

The five most dominant OTUs belonged to Bacillariophyta (three OTUs), Ciliophora (one OTU), and Cryptophyta (one OTU), respectively (Table 3). The closest cultured organism of the most dominant OTU, i.e. OTU_1541 was *Cyclotella striata* (99.3% similarity). OTU_1540 and OTU_1542 also belonged to Bacillariophyta and their closest cultured organisms were *Skeletonema costatum* (99.3% similarity) and

Table 3. List of the top five most abundant OTUs with the number of reads (% of total reads), taxonomic identification, and similarity (%-S) with the closest match with a species name in the GenBank database.

OTU ID	Reads	Group	Closest named match	%-S
OTU_1541	19.5%	Bacillariophyta; Stephanodiscaceae	*Cyclotella striata* (JQ217342)	99.3
OTU_1540	11.1%	Bacillariophyta; Skeletonemataceae;	*Skeletonema costatum* (DQ396524)	99.3
OTU_1542	3.7%	Bacillariophyta; Aulacoseiraceae;	*Aulacoseira baicalensis* (AJ535186)	97.3
OTU_1544	3.4%	Ciliophora; Strombidiidae	*Strombidium conicum* (FJ422992)	100
OTU_1546	2.6%	Cryptophyta; Geminigeraceae	*Teleaulax amphioxeia* (AJ007287)	98.6

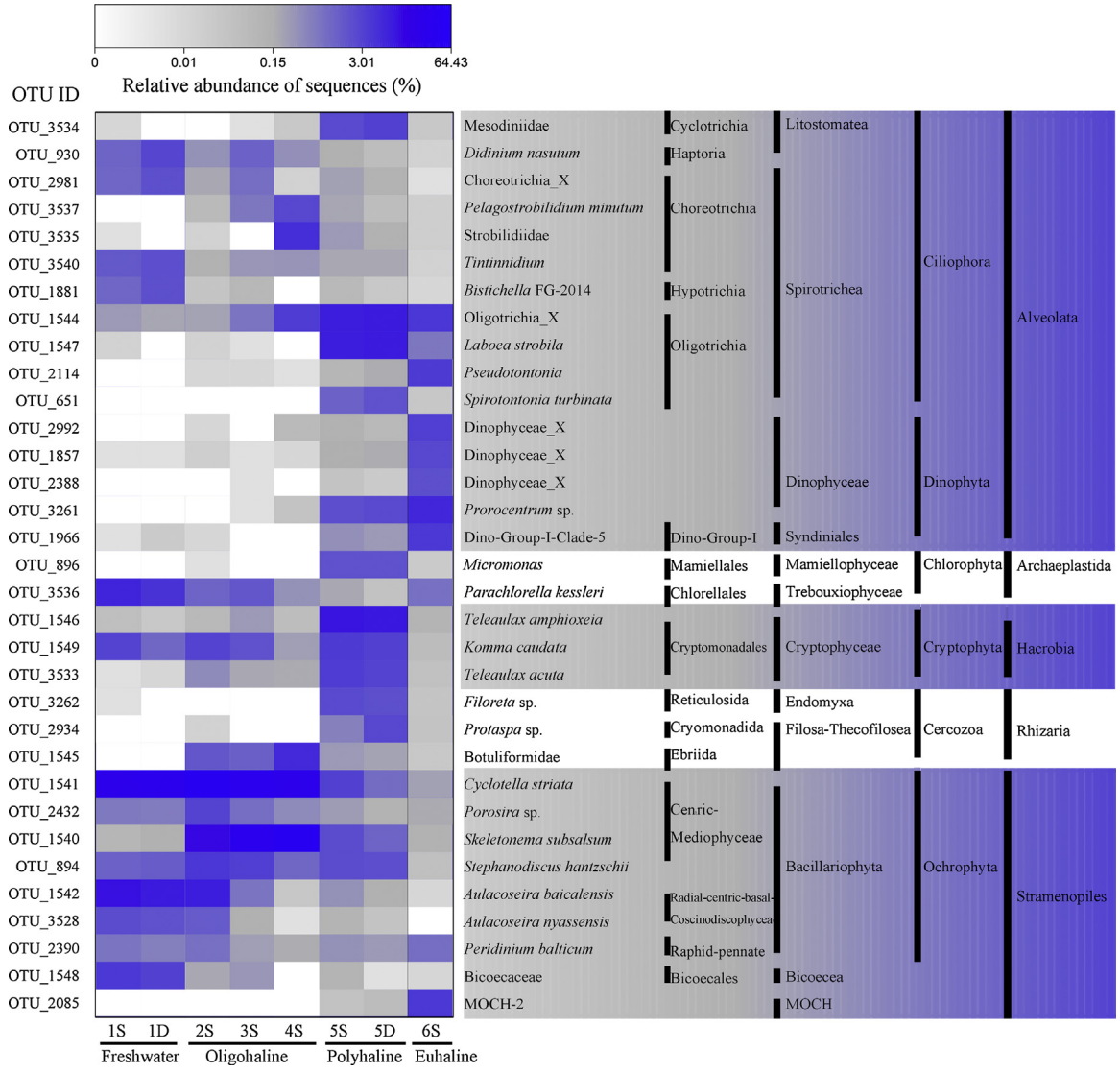

Fig. 3. Heatmap showing the relative sequence abundance of representative OTUs classified at the lowest taxonomic level. Only OTUs occurring in two or more samples with relative sequence abundance > 2% in at least one sample are shown.

Aulacoseira baicalensis (97.3% similarity), respectively. OTU_1544 was a member of Ciliophora and its sequence was identical to *Strombidium conicum*. The closest cultured organism of OTU_1546 was the cryptophyte *Teleaulax amphioxeia* and the similarity was also high (98.6% similarity) (Table 3). To know more about the compositions of dominant taxa, a heatmap showing the relative abundance of representative OTUs identified at the lowest possible taxonomic rank was constructed (Fig. 3). Thirty-three dominant taxa from all OTUs (1,980 in total) that accounted for 80% – 98% of the total sequences in each sample were selected, among which 16 OTUs belonged to Alveolata, nine to Stramenopiles, and the rest to other supergroups, i.e., Rhizaria, Hacrobia, and Archaeplastida. Most OTUs selected

showed salinity-specific distribution patterns. Sixteen dominant OTUs were discovered belonging to Alveolata, primarily Dinophyta and Ciliophora. Among the ciliates, OTUs belonging to the subclass Oligotrichia were found mainly at sites with high salinities. The relative abundance of OTUs identified as *Didinium nasutum*, *Tintinnidium*, and *Bistichella* decreased along the salinity gradient. The Mesodiniidae-affiliated OTU mainly occurred at the polyhaline site. One OTU, *Pelagostrobilidium minutum* was not detected at the freshwater site. The relative contributions of OTUs in Dinophyta, e.g. Dinophyceae, *Prorocentrum* and Dino-Group I, increased from low-salinity to polyhaline and euhaline sites. Within Stramenopiles, OTUs identified as *Skeletonema subsalsum* and *Porosira* sp. were mainly found at the oligohaline sites. The contributions of OTUs identified as *Aulacoseira baicalensis* and *A. nyassensis*, and a member belonging to Bicoecaceae decreased with increasing salinity. An OTU classified as member in MOCH-2 was only found at the polyhaline and euhaline sites. An OTU identified as *Cyclotella striata* was found with high contributions at all sites with its lowest contribution found at the euhaline site. Hacrobia was represented by three OTUs including members of the Cryptophyta, the contributions of which peaked at the polyhaline sites. Rhizaria were also represented by three dominant OTUs. The relative abundance of two OTUs peaked at the polyhaline site while one identified as member of Botuliformidae peaked at the oligohaline sites. Archaeplastida was represented by two OTUs, both of which belonged to Chlorophyta. The relative abundance of *Micromonas* affiliated OTU peaked at the polyhaline site while that of *Parachlorella* affiliated OTU made minimum contributions in the polyhaline site (Fig. 3).

Effects of environmental factors on community structure of protists

Non-metric multidimensional scaling (NMDS) charts were constructed based on Bray-Curtis similarities for the microbial eukaryote communities (Fig. 4). The stress values were <0.05 which gave an excellent representation of the dissimilarities. All the samples were clustered into two groups, one corresponding to high-salinity sites, i.e. sites 5 (polyhaline) and 6 (euhaline) and the other corresponding to low-salinity sites, i.e. sites 1 (freshwater), 2, 3, and 4 (oligohaline) (Fig. 4).

SIMPER analysis selected seventeen OTUs with the largest dissimilarities among the samples, which in total contributed ca. 50.09% of the dissimilarities in protistan communities among freshwater, oligohaline, polyhaline, and euhaline groups (Fig. 5). These OTUs were mainly from Stramenopiles (six OTUs), Alveolata (six OTUs), and Hacrobia (three OTUs), which contributed ca. 30.74%, 10.51% and 5.55% of the community dissimilarities, respectively. Using the Pearson correlation coefficient matrix to analyze the correlation between these 17 OTUs and environmental factors, the abundance of most are significantly correlated with salinity and geographical distance (Table 4).

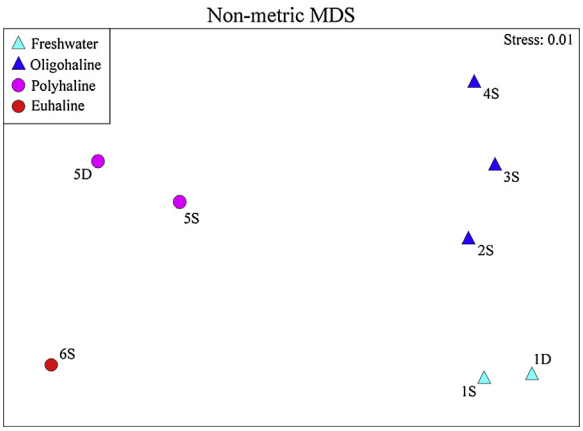

Fig. 4. Plots of nonmetric multidimensional scaling (nMDS) ordination based on community distance matrices.

The influence of environmental factors on the protistan communities was analyzed by the Mantel test. Geographic distance and salinity significantly affected the protistan community structure with salinity being the most dominant ($p = 0.001$, $R^2 = 0.6975$; Table 5).

Discussion

Diversity of protists in the Pearl River estuary

Studies have shown that a large number of dead cells and extracellular DNA exist in estuarine ecosystems (DeFlaun et al. 1987). Compared with DNA, RNA is easily degraded in the extracellular environment. RNA sequencing can ensure that the signals detected are derived from live cells while also reducing the interference of metazoan sequences (Hu et al. 2016; Xu et al. 2017b). Although several studies have addressed the molecular diversity of prokaryotes (Bacteria and Archaea) along the salinity gradients in the Pearl River estuary (Ling et al. 2012; Liu et al. 2014; Xie et al. 2014), the molecular diversity of protists remains largely unknown. The current study used high-throughput sequencing of 18S rRNA to study protists in a complex estuarine ecosystem thereby mitigating the bias introduced by DNA sequencing. Although rarefaction analysis showed that protists were not fully sampled in the present study, the OTU diversity of the samples being unsaturated (Fig. S1), our study nevertheless gives a snapshot of the protistan diversity along the salinity gradient in this subtropical estuary. Polyhaline groups had the highest, and oligohaline groups the lowest, diversity estimates, respectively (Table 2). The diversity distribution pattern of protistan assemblages along the Pearl River estuary salinity gradients revealed in the current study is more consistent with Remane's Artenminimum ('species minimum') model (Remane 1934) than the "protis-

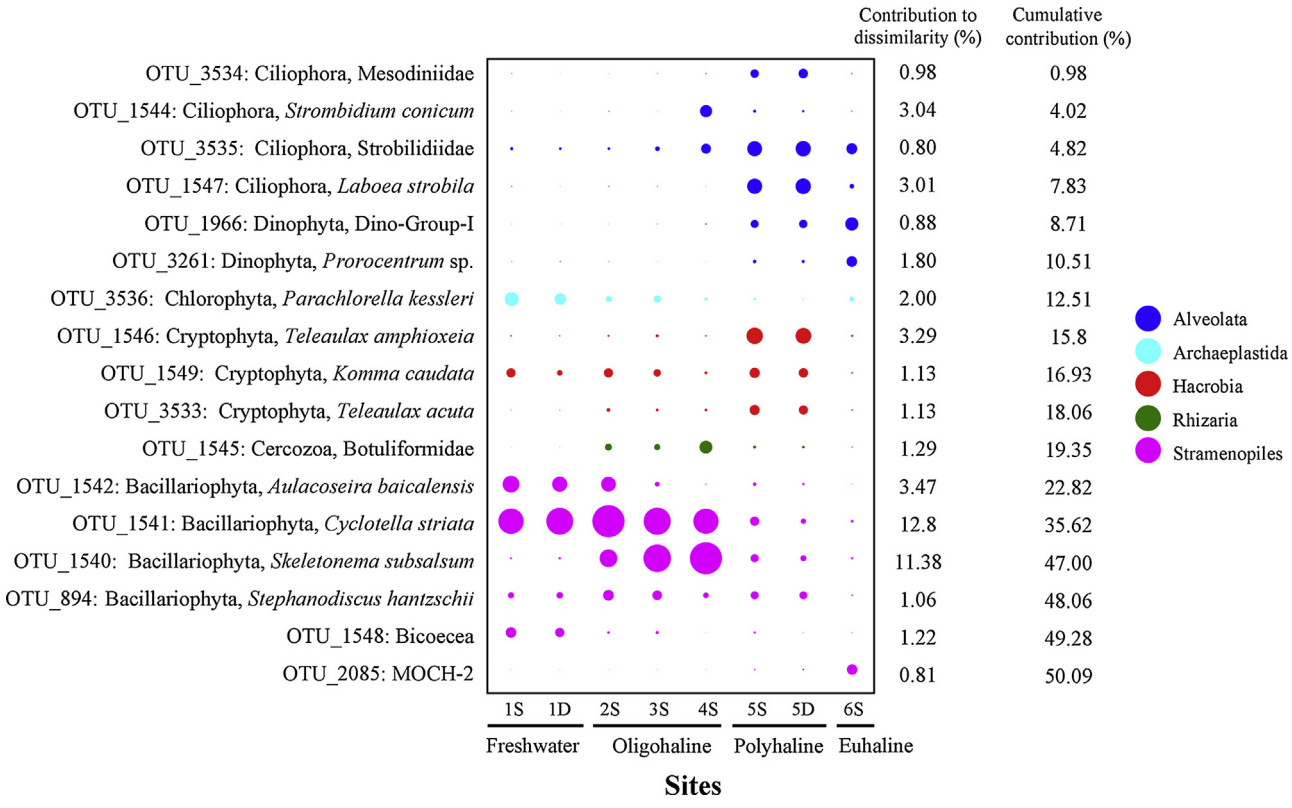

Fig. 5. Taxonomic identities of the top seventeen OTUs that contributed most to community dissimilarities along the salinity gradient with their relative contributions to community dissimilarities. The diameters of the circles are proportional to the abundances of the OTUs, with the size of the circle indicating the average abundance (square root-transformed) of each OTU at a given site.

tan sepecies-maximun" model of Telesh et al. (2011). These two models were, however, based on datasets that differed significantly from that of the present study. Firstly, that data used both by Remane (1934) and Telesh et al (2011) were generated from the Baltic Sea, the world's largest semi-enclosed, brackish water body, with slow water flow and a stable salinity gradient. Secondly, microscopy-based techniques were employed in both cases. Thirdly, samples of mesohaline water were not collected in the present study, so the protist community structure in mesohaline waters of the Pearl River estuary is currently unknown. And fourthly, the dataset used in the current study was generated from one-time sampling in the summer and without replicates. Therefore the temporal diversity distribution pattern of protists along the Pearl River estuary remains unknown so whether it will fit the "Artenminimum" or "protistan species-maximum" models needs to be further tested.

Community composition and relative sequence abundance of protists

In the present study, a significant level of spatial variability of protistan communities among samples was found (Fig. 4). Reads affiliated with Stramenopiles, Alveolata, Hacrobia, and Archaeplastida were most frequently recovered among the samples with different contributions to the total protistan community (Fig. 2). The relative sequence abundance of protists was dominated by Stramenopiles at the freshwater and oligohaline sites, which included the three most dominant OTUs, i.e., OTU_1541, OTU_1540, and OTU_1542 which were identified as *Cyclotella striata*, *Skeletonema subsalsum*, and *Aulacoseira baicalensis*, respectively. OTU_1541 and OTU_1542 were found particularly abundant at the freshwater and oligohaline sites. *Skeletonema subsalsum* (OTU_1540), which is often associated with eutrophic conditions (Balzano et al. 2011; Lee et al. 2016; Sarno et al. 2007) occurred mainly at the oligohaline and polyhaline sites. Although nutrients were not measured in our study, previous studies carried out in the same area in the summer showed that the estuary, especially the low-salinity regions, has high levels of nutrients (Liu et al. 2014; Xie et al. 2014). A previous microscopy-based study has also shown that in the inner part of the estuary where salinity was low, *Skeletonema subsalsum* had high abundance (Qiu et al. 2010). Observations by microscopy have previously confirmed the dominance of diatoms for most of the year in a coastal-estuarine environment (Ansotegui et al. 2003).

As the salinity increased from the upper to the lower parts of the estuary, the contribution of Alveolata-affiliated sequences also increased. Members in Alveolata have evolved a large range of trophic modes enabling them to adapt to many types of habitat. Consequently, they can serve

Table 4. Pearson correlation coefficients between the seventeen dominant OTUs and environmental variables. Only the significant coefficients ($P < 0.05$) are listed.

OTU	Taxa	Salinity		pH		Temperature		DO		Geographic distance	
		ρ	P	ρ	P	ρ	P	ρ	P	ρ	P
OTU3534	Ciliophora, Mesodiniidae	0.768	0.035							0.774	0.034
OTU1544	Ciliophora, *Strombidium conicum*	0.868	0.009							0.88	0.007
OTU3535	Ciliophora, Strobilidiidae										
OTU1547	Ciliophora, *Laboea strobila*										
OTU1966	Dinophyta, Dino-Group-I					-0.743	0.042				
OTU3261	Dinophyta, *Prorocentrum sp.*	0.963	< 0.001	0.878	0.008					0.975	< 0.001
OTU3536	Chlorophyta, *Parachlorella kessleri*	-0.843	0.013							-0.819	0.018
OTU1546	Cryptophyta, *Teleaulax amphioxeia*									0.747	0.04
OTU1549	Cryptophyta, *Komma caudata*										
OTU3533	Cryptophyta, *Teleaulax acuta*										
OTU1545	Cercozoa, Botuliformidae							0.731	0.049		
OTU1542	Bacillariophyta, *Aulacoseira baicalensis*	-0.916	0.003	-0.881	0.007					-0.916	0.004
OTU1541	Bacillariophyta, *Cyclotella striata*	-0.795	0.024							-0.795	0.025
OTU1540	Bacillariophyta, *Skeletonema subsalsum*										
OTU894	Bacillariophyta, *Stephanodiscus hantzschii*										
OTU1548	Bicoecea	-0.819	0.018							-0.783	0.028
OTU2085	MOCH-2	0.883	0.021							0.87	0.024

Table 5. Mantel test comparison between community variability (measured as Bray-Curtis dissimilarity) and DO, pH, depth, temperature, salinity and the geographic distance between sampling sites. When the correlation is significant both ρ-value and R^2 are bold ($p < 0.01$).

Factor	Community distance	
	ρ	R^2
Salinity	**0.001**	**0.6975**
Geographic distance	**0.001**	**0.454**
Temperature	0.026	0.2155
DO	0.289	0.0061
pH	0.152	0.0299
Depth	0.439	0.0038

as important primary producers, consumers, and parasites in marine environments (Guillou et al. 2008). Ciliates of the subclasses Oligotrichia and Choreotrichia (class Spirotrichea) are major components of pelagic marine food webs (Azam et al. 1983; Fenchel 1988). In the Pearl River estuary, contributions of Oligotrichia-affiliated OTUs were generally found to be greater at high- than low-salinity sites, but some members in Choreotrichia had higher relative abundances in low-salinity sites. For example, OTUs identified as *Laboea strobila*, *Pseudotontonia*, and *Spirotontonia turbinata* were found mostly at the polyhaline and euhaline sites, which is consistent with their occurrence in previous studies (Agatha 2011). To date, most tintinnids have been reported from marine waters with only few species reported from freshwaters (Bachy et al. 2012). In the present study, OTUs belonging to tintinnids showed clear freshwater to marine water transition (Fig. S2). For example, the OTUs identified as *Tintinnidium* mainly occurred in freshwater and their contribution to the total community decreased with increasing salinity (Fig. S2). Syndiniales, a group of parasitoids which includes Dino-Group-I to −V, has been mainly reported from marine rather than freshwater environments (Guillou et al. 2008). Parasitism by this group of protists is proposed to be a major force in ocean food webs (Guillou et al. 2008). In the present study, the OTU affiliated with Dino-Group-I was mainly found at sites with high salinity, which is consistent with previous reports (Guillou et al. 2008).

Environmental factors controlling community structure of protists

Assessing how environmental parameters affect the community structure and distribution pattern of organisms underpins our understanding of the relationships between biotic and ecological factors in natural ecosystems. Estuaries are transitional habitats and harbor complex biological communities that are adapted to fluctuations of salinities (Attrill and Rundle 2002). In the present study, using high-throughput amplicon sequencing of the 18S rRNA, we investigated which

local environmental drivers are most strongly associated with protistan diversity across the salinity gradient of the Pearl River estuarine ecosystem. The present study showed that salinity and geographic distance significantly affected protistan community structure (Table 5). The change of community composition of both prokaryotic and eukaryotic communities along salinity gradients has previously been observed (Casamayor et al. 2002). Furthermore, selective factors such as salinity and nutrients are often organized in a gradient in an estuary, resulting in spatial variations in microbial composition (Morris et al. 1978; Zhang et al. 2014). A recent study revealed the spatial and temporal distribution patterns of ciliates, which are important grazers in microbial food webs, in a subtropical estuary and identified salinity as the dominant factor controlling ciliate distribution patterns (Sun et al. 2017). Lallias et al. (2015) compared microbial eukaryote diversity between two rivers and concluded that salinity is one of the strongest factors affecting meiofaunal distribution. Another study examined microbial eukaryotes in several estuarine ecosystems and revealed only a weak degree of relatedness among samples by location and season (Vigil et al. 2009). By contrast, it has been reported that the communities of microbial eukaryotes along a South Australian coastal lagoon were affected more by sampling location than by salinity, with tidal forces being the main factor shaping phytoplankton community structure (Bazin et al. 2014a). Studies of estuarine ciliates have given contrasting results: Bojanić et al. (2012) showed water temperature to be the principle cause of community variability, whereas Doherty et al. (2010) found no obvious relationships between community compositions and measured environmental parameters. Studies on the dynamics of estuarine microbial eukaryotes are usually based either on horizontal surveys across salinity gradients (Balzano et al. 2015; Bazin et al. 2014a,b; Lallias et al. 2015; present work), time series surveys at one to two fixed locations (Vigil et al. 2009), or spatial and temporal surveys of a single assemblage (Dolan and Gallegos 2001; Sun et al. 2017). Variabilities among sampled estuarine ecosystems, differences in sampling strategies and methods used, and limited numbers of environmental parameters measured, may cause the discrepancies summarized above. Comprehensive studies that integrate multi-dimensional approaches, such as temporal and spatial sampling, the application of multiple methods of sample processing (microscopy, high-throughput sequencing, etc), estuarine ecosystems in different geographic locations, and measurements of more environmental parameters, are needed in order to fully characterize microbial eukaryotic communities in estuarine environments and to determine the factors that drive the variability of their structure.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ejop.2018.01.004.

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