Depth shapes $\alpha$- and $\beta$-diversities of microbial eukaryotes in surficial sediments of coastal ecosystems

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Summary
Little is known about the relative influence of historic processes and environmental gradients on shaping the diversity of single-celled eukaryotes in marine benthos. By combining pyrosequencing of 18S ribosomal RNA genes with data on multiple environmental factors, we investigated the diversity of microeukaryotes in surficial sediments of three basins of the Yellow Sea Large Marine Ecosystem. A considerable proportion (about 20%) of reads was affiliated with known parasitoid protists. Dinophyta and Ciliophora appeared dominant in terms of relative proportion of reads and operational taxonomic unit (OTU) richness. Overall, OTU richness of benthic microeukaryotes decreased with increasing water depth and decreasing pH. While community composition was significantly different among basins, partial Mantel tests indicated a depth–decay pattern of community similarity, whereby water depth, rather than geographic distance or environment, shaped $\beta$-diversity of benthic microeukaryotes (including both the abundant and the rare biosphere) on a regional scale. Similar hydrographic and mineralogical factors contributed to the biogeography of both the abundant and the rare OTUs. The trace metal vanadium had a significant effect on the composition, diversity patterns and underlying mechanisms of single-celled eukaryote distribution in surficial sediments of coastal oceans.

Introduction
Microbial eukaryotes (protists and single-celled fungi) are major components in microbial food webs, playing important roles in primary production and biogeochemical cycles in marine ecosystems (Azam and Malfatti, 2007; Falkowski et al., 2008; Anderson et al., 2013). In the past decade, with the application of culture-independent (especially rRNA gene-based) approaches, much effort has been made to study marine microeukaryote diversity and to unveil novel lineages in deep ocean environments (e.g. Dawson and Pace, 2002; Moreira and López-Garcia, 2002; López-García et al., 2003; Edgcomb et al., 2011a; Pawlowski et al., 2011). The biogeography of microorganisms can be shaped both by the environment and by the history (geographical separation) (Martiny et al., 2006). Relative to water column microbiota, less is known about large-scale diversity patterns of benthic microbial eukaryotes as a whole (Scheckenbach et al., 2010), and about the relative importance of seasonal, chemical and physical factors for determining $\alpha$-diversity (the richness and/or evenness of taxa contained within an individual community) and $\beta$-diversity (community composition or structure changes across space and/or time) in coastal sedimentary environments.

The geographical distribution of rare taxa (the so-called ‘rare biosphere’, Sogin et al., 2006; Pedrós-Alió, 2006) is important for understanding overall protist diversity (Foissner, 1999; Telford et al., 2006). Recent high-throughput sequencing projects have examined the biogeography and temporal dynamics of the rare biosphere of marine bacteria and archaea (e.g. Galand et al., 2009; Gobet et al., 2012; Hugoni et al., 2013), revealing that the distribution patterns of rare and abundant (as defined by relative proportion of sequences $>1\%$ in a sample) taxa have seldom been similar. Some studies of pelagic microbial eukaryote diversity have provided insight into the rare biosphere, e.g. for lake protists (Nolte et al., 2010; Lepère et al., 2013), and marine planktonic microeukaryotes (Dawson and Hagen, 2009; Pawlowski et al., 2011; Bachy...
and Worden, 2014; Edgcomb and Pachiadaki, 2014; Logares et al., 2014). However, it remains to be tested whether the composition of the rare biosphere is influenced more by spatial factors (e.g. geographic distance) than is true of the most abundant microeukaryotes. Furthermore, little is known about the relative importance of geographic distance and water depth on the distributions of benthic marine microeukaryotes (Gooday and Jorissen, 2012).

In this study, we investigated α- and β-diversity of benthic microeukaryotes in the Yellow Sea Large Marine Ecosystem, a semi-enclosed subsection of the northwest Pacific Ocean. We set our cut-offs at > 1% here for ‘abundant’ and < 1% for ‘rare’ operational taxonomic units (OTUs), so as to more likely correspond to those frequently detected and to those missed and less abundant groups in previous molecular surveys utilizing fingerprinting or Sanger clone libraries (Pedrós-Alió, 2006). While also providing a baseline for monitoring and conservation purposes for this degraded ecosystem, this study primarily aimed to examine: (i) seasonal and spatial patterns and underlying mechanisms of the diversity of benthic microbial eukaryotes; and (ii) the relative importance of geographical distance, depth and selected environmental factors on the β-diversity of rare and abundant microeukaryotic communities.

**Results**

**Spatial and seasonal variations of environmental factors**

The depth of sampling sites ranged from 14 to 75 m, increasing from Bohai Sea (BHS, minimum 14 m, median 22.8 m, maximum 30 m, \( n = 14 \)) to North Yellow Sea (NYS, minimum 20 m, median 54 m, maximum 75 m, \( n = 15 \)) and South Yellow Sea (SYS, minimum 50 m, median 72 m, maximum 75 m, \( n = 10 \)) (Fig. 1, Table S1). Physicochemical factors of bottom waters generally showed clear spatial patterns and correlated with water depth. From BHS to SYS, water temperature (3.8–20.4°C) and pH (7.57–7.94) gradually decreased with depth, whereas salinity increased from about 29.2 to 33.2 practical salinity units. The concentration of dissolved oxygen (DO) showed a seasonal pattern, with much higher values during summer (216.5 ± 5.38 \( \mu \)g l\(^{-1} \)) than in the winter (112.1 ± 4.28 \( \mu \)g l\(^{-1} \)). Chlorophyll a (Chl-a) concentrations differed both regionally and seasonally, with the highest values in the NYS in summer (10.49 \( \mu \)g l\(^{-1} \)) and the lowest in the SYS in winter (0.21 \( \mu \)g l\(^{-1} \)). Regional, but no seasonal, differences in sediment characteristics [grain size (GS), C:N ratio, and all measured metals] were recorded. For example, SYS samples were richer in organic carbon (C:N ratio 5.7 ± 0.18), had the finest GS (8.0 ± 0.25 \( \mu \)m) and had the highest concentrations of all...
measured metals except for arsenic (As) and cadmium (Cd), which, instead, both peaked in BHS.

α-Diversity and community composition of benthic microbial eukaryotes

After quality filtering, a total of 279 179 reads were obtained for the 39 sediment samples. Removal of metazoan reads resulted in 171 324 (34.0%) sequences retained for protists and fungi, with 1581–8804 (median 4209) reads per sample (Table S2). At 97% sequence identity, a total of 6220 OTUs were detected, with 964 OTUs (15.5%) shared among all three basins (Fig. S1). Higher values for community richness were observed in the winter in the BHS, and in the summer in the SYS; however, no seasonal difference was observed in NYS samples (Fig. S2).

Sequences were normalized by randomly resampling 1500 reads per sample, which minimized bias associated with sequencing coverage and allowed for comparison of results for all samples. Overall, the pyrotags derived from benthic microeukaryotic communities in the Yellow Sea Large Marine Ecosystem were dominated by the superphyla Alveolata (relative abundance: 67.1%) and Stramenopiles (15.4%), followed by Fungi (3.5%), Chlorophyta (3.0%), Rhizaria (2.9%) and Apusozoa (1.0%), whereas the relative abundances of other taxa (e.g. Amebozoa, Mesomycetozoa, Haptophyta, Choanoflagellida, Telonemia, Streptophyta, Centroheliozoa and Cryptophyta) were each on average less than 1%. Alveolata (principally Ciliophora and Dinophyta in these samples) and Stramenopiles accounted for the majority of OTU numbers observed in all samples, and 50.2% of Alveolata sequences were unclassified Dinophyta (Figs 2 and S1).

Based on the classification of pyrotags and existing knowledge of lifestyles of microeukaryotic taxa (Hausmann et al., 2003), at least seven parasitic groups can be recognized from our data. Sequences affiliated with the dinoflagellate group Syndiniales (also referred to as novel MALV lineages) were the most abundant parasitic group (ranging from 0.5% to 29.9% in individual samples, and representing 4.7% of the overall data set), mostly represented by sequences associated with Dino-Group-I. Sequences of other parasites infecting animals, diatoms and other phytoplankton, including Oomyceta (3.6%), Labyrinthula (3.4%), Pirsonia (2.8%), Apicomplexa (2.5%) and Chytridiomycota (2.1%), appeared in lesser proportions in our data sets (Figs 2 and S1).

Correlations between α-diversities of the total community and environmental factors

α-Diversities of benthic microeukaryotes were high and varied greatly among samples, with OTU richness ranging from 230 to 504, Simpson indices of 0.823–0.988, Shannon indices of 4.49–7.70 and Chao1 indices of 421–1051 (Table S2). Correlations between α-diversities and selected environmental factors exhibited similar patterns (data not shown). OTU richness had the strongest correlation with depth ($r = -0.702$, $P < 0.001$),
followed by longitude \( r = -0.623, P < 0.001 \), pH \( r = 0.603, P < 0.001 \), salinity \( r = -0.595, P < 0.001 \) and temperature \( r = 0.497, P < 0.001 \) (Fig. 3). Among the sedimentary properties measured in this study, several trace metals had significant correlations with richness, including manganese (Mn, \( r = 0.522, P < 0.001 \)), As \( r = 0.484, P = 0.002 \) and zinc (Zn, \( r = -0.411, P < 0.001 \)). OTU richness increased with latitude \( r = 0.379, P = 0.017 \) and decreased with distance from land \( r = -0.488, P = 0.002 \), although correlation coefficients were lower for these two variables in comparison to other factors.

OTU numbers within the 16 major taxonomic groups were significantly correlated with at least one parameter determined in this study (Table S3). With increased water depth, the OTU richness of Dinophyta increased \( r = 0.436, P < 0.01 \), whereas OTU richness of Ciliophora \( r = -0.436, P < 0.01 \) and Choanoflagellida \( r = -0.438, P < 0.01 \) decreased. The correlation between richness of Katablepharidophyta and water depth was weakly supported \( r = -0.336, P < 0.05 \). Notably, the most highly significant correlations \( P < 0.001 \) between OTU richness for particular groups and environmental factors were detected for ciliates and pH \( r = 0.516 \); Choanoflagellida and temperature \( r = 0.614 \), As \( r = 0.530 \) and nitrite \( r = 0.600 \); Chlorophyta and DO \( r = -0.492 \); Apicomplexa and Chl-a \( r = 0.498 \); and Bacillariophyta and nitrite \( r = 0.641 \).

Rare and abundant OTUs

Of the 6220 OTUs observed for benthic microbial eukaryotes in our samples, 151 OTUs appeared to be abundant (as defined by relative abundance of sequences > 1%) in at least one sample and these OTUs represented 50.8% of the total number of sequences retrieved. There were 6218 rare OTUs (defined as OTUs comprised of < 1% of sequences in a sample) comprising 49.2% of the sequence abundance. Two of the 151 abundant OTUs, one related to the parasitic dinoflagellate Duboscquodinium collinii and one related to the bloom-forming Heterocapsa rotundata, were always abundant in samples \( n = 39 \), all three basins) and never rare. Conversely, 149 of the OTUs that were found to be abundant in some samples were rare in others. These included 11 OTUs that were found in all the three basins, and 45, 34 and 19 OTUs that were only detected in BHS, NYS and SYS, respectively.

Dinophyta, Ciliophora and Stramenopiles were highly represented in both the abundant and rare assemblages (Fig. 4). Among the rare OTUs, sequences affiliating with Dinophyta consistently dominated. The proportions of
sequences affiliating with *Ciliophora* and *Stramenopiles* in the rare OTUs were similar to their proportions within the abundant OTUs. Based on relative abundance of sequences, *Fungi*, *Chlorophyta*, *Rhizaria*, *Mesomycetozoa*, *Perkinseas*, *Haptophyta*, *Apusozoa*, *Amoebozoa* and *Choanoflagellida* were a larger proportion of the rare biosphere within individual basins and across all three basins (Fig. 4).

α-Diversity and β-diversity patterns of the total, the abundant and the rare assemblages

Two-way analysis of variance (ANOVA) analyses indicated that α-diversity estimators (OTU richness, Simpson, Shannon and Chao1 indices) of total and rare OTUs followed a similar pattern: α-diversities were significantly different among basins (*P < 0.01*) but not between summer and winter (*P > 0.07; Table 1). In contrast, Simpson and Shannon indices of the abundant OTUs showed both regional (*P < 0.01*) and some seasonal differences (*P < 0.04*), albeit no significant difference was found for richness or Chao1 (*P > 0.9*). For the total and the abundant OTUs, the same pattern was observed for the combined effects of region and season (Table 1).

In the principal coordinate analysis (PCoA) plots, both the total community and the rare OTUs of microeukaryotes exhibited distinct geographic patterns of community composition, with samples collected in two seasons from a given basin clustering together. However, basin-to-basin separation was not as distinguishable for

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*Fig. 4.* Comparisons of relative abundance of abundant and rare OTUs of major groups of benthic microbial eukaryotes in two-season samples in each basin and across all the three basins. Asterisks indicate significant differences (t-tests, *P < 0.05*).
the abundant OTUs, with SYS and NYS overlapping in the sample space (Fig. 5). Hypothesis testing showed that there were significant basin-to-basin changes, but only minor seasonal variations in community composition of either abundant, rare or total OTUs across basins (analysis of similarity [ANOSIM], \( P < 0.001 \)). The rare OTUs showed the greatest spatial variation \( (R = 0.584) \), whereas the spatial variations of the more abundant OTUs were relatively weaker \( (R = 0.340) \) (Table 2).

Geographic distance, depth and environmental controls on \( \beta \)-diversity of the total community

We distinguished the effects of horizontal (geographic distance) and vertical (depth) spatial factors and environmental variables on community composition using Mantel tests (Table 3). The \( \beta \)-diversity of the total community based on Sørensen distance was significantly correlated with depth, geographic distance and environment distance (simple Mantel tests, \( r > 0.54 \), \( P < 0.001 \)). In partial Mantel tests, however, the correlation between geographic distance and \( \beta \)-diversity appeared insignificant when depth was controlled \( (r = 0.139, P = 0.013, \text{ but lower than the confidence level of } 0.05/3 \text{ after Bonferroni correction;} \) Table 3). The effect of depth remained the highest \( (r = 0.588 \text{ and } 0.493, P < 0.001) \) when geographic distance or environment distance was controlled. This indicates that depth, rather than geographic distance or environmental heterogeneity, was the predominant factor shaping the \( \beta \)-diversity of benthic microbial eukaryotes (Table 3).

Simple Mantel tests of individual environmental variables showed that the hydrography of overlying waters (salinity, pH and temperature), trace metal (As, Zn, Cr and V) concentrations in sediments and distance from land were important factors influencing the total community of benthic microeukaryotes (Table 3). We examined how these individual environmental variables might affect local communities by controlling for water depth in partial Mantel tests. The analyses yielded insignificant \( P \)-values (Table 3), indicating the effects of these individual environmental variables on the total community are depth dependent. The use of Bray–Curtis distances for \( \beta \)-diversity characterizations produced similar results (data not shown).

To explore the possible effects of environmental factors on major taxonomic groups, Spearman’s correlation analyses were performed (Fig. 6). The most abundant taxa Dinophyta and Ciliophora responded to environmental factors in opposite ways, with the former being more abundant in deeper (and more distant from the coastline) sites where DO, pH and Chl-a were lower, sediments were finer and higher trace metal concentrations were observed. Most other groups were less abundant
in deeper sites. Haptophyta, Radiolaria, Oomyceta, Labyrinthula and Raphidophyceae were more abundant in coarser sediments with lower concentrations of some trace metals. The relative abundance of Fungi was only positively correlated with the concentration of As in sediments.

Factors driving \( \beta \)-diversity: a comparison of the total, the abundant and the rare biosphere

Separate Mantel tests for the total, the abundant and the rare biosphere OTUs revealed similar patterns (Table 3), that is, community similarity decreases with depth difference, which we here designate as the ‘depth–decay relationship’. In simple Mantel tests, most individual parameters that were found to be significant determinants of community composition for the whole community (total OTUs) were also found to be significant for the abundant OTUs and for the rare OTUs. The only differences were observed in results for two parameters: the concentration of vanadium (V) in sediments was weakly correlated with \( \beta \)-diversity of the rare OTUs \((r = 0.218, P = 0.003)\) and total OTUs \((r = 0.218, P = 0.003)\); the concentration of nitrite was correlated with only the abundant OTUs \((r = 0.246, P = 0.003)\).

When the ‘depth effect’ was controlled in partial Mantel tests, only two environmental parameters (arsenic and nitrite) were found to be significantly correlated with \( \beta \)-diversity (Table 3). At similar depths, arsenic concentration was weakly correlated with community dissimilarity for the abundant \((r = 0.200, P = 0.003)\) and the total OTUs \((r = 0.225, P = 0.003)\). The concentration of nitrite in sediments affected only the abundant OTUs \((r = 0.244, P = 0.003)\). No parameters were found to be significantly correlated with \( \beta \)-diversity of the rare OTUs in these surficial sediments (partial Mantel tests, Bonferroni corrected \(P > 0.05/14\)).

Discussion

Dominance of Alveolata and Stramenopiles sequences in the 18S rDNA libraries

Our results showed that the microeukaryote communities in these coastal sediments were mostly represented by Alveolata and Stramenopiles in terms of either relative abundance of rDNA sequences or OTU (97% similarity)

<table>
<thead>
<tr>
<th>Grouping by</th>
<th>Total</th>
<th>Abundant</th>
<th>Rare</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( R )</td>
<td>( P )</td>
<td>( R )</td>
</tr>
<tr>
<td>Basin (global test)</td>
<td>0.469</td>
<td>(&lt; 0.001)</td>
<td>0.340</td>
</tr>
<tr>
<td>BHS versus NYS</td>
<td>0.469</td>
<td>(&lt; 0.001)</td>
<td>0.352</td>
</tr>
<tr>
<td>NYS versus SYS</td>
<td>0.166</td>
<td>(&lt; 0.022)</td>
<td>0.099</td>
</tr>
<tr>
<td>BHS versus SYS</td>
<td>0.824</td>
<td>(&lt; 0.001)</td>
<td>0.588</td>
</tr>
<tr>
<td>Season (global test)</td>
<td>0.103</td>
<td>0.019</td>
<td>0.130</td>
</tr>
</tbody>
</table>

Community turnover was based on the Bray–Curtis distance.

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numbers (Figs 2 and 6). The high proportion of *Alveolata* sequences in our surveys is comparable with clone library analyses of microeukaryotes in other marine habitats, such as, the water column (e.g., Countway *et al.*, 2007; Schnetzer *et al.*, 2011; Pernice *et al.*, 2013), anoxic deep waters (Stoeck *et al.*, 2009; Edgcomb *et al.*, 2011a) and abyssal sea floor sediments (Scheckenbach *et al.*, 2010). These results are also largely in line with previous morphology-based ecological studies, which have shown that many dinoflagellates form cysts as a part of their life cycle which could remain viable for several years (Lewis *et al.*, 1999). Abundant benthic ciliate morphospecies were also previously observed in Yellow Sea sediments (Meng *et al.*, 2012).

Although there were high proportions of sequences affiliated with alveolates and stramenopiles in our rDNA libraries, we argue that this should be interpreted cautiously, as the relative abundance of rDNA in libraries may not reflect abundance in nature. Many dinoflagellates and ciliates have much higher copy numbers of rDNA genes (up to tens or hundreds of thousands of copies per cell) than many other eukaryotic taxa (e.g., fungi with only 60–220 copies per cell; reviewed in Gong *et al.*, 2013). This can lead to their overrepresentation (not reflective of *in situ* cell abundance) (Prescott, 1994; Galluzzi *et al.*, 2004; Godhe *et al.*, 2008; Gong *et al.*, 2013), and conversely the underrepresentation of fungi in sequence libraries.

Low proportions of fungal sequences have been described for surface marine waters (0.8%, Massana and Pedrós-Alió, 2008), deep anoxic waters (1–4%, Edgcomb *et al.*, 2011a) and coastal surface sediments (3.5%, this study), which contrasts with deep subsurface sediments, where fungi appear to dominate the microbial eukaryotic community (Edgcomb *et al.*, 2011b). Protists may generally be more ecologically important than fungi across most marine habitats aside from deep subsurface sediments; however, this hypothesis requires further testing using consistent methodologies for comparisons. Additionally, the percentages of fungal rDNA sequences can be high in some locations or at certain sampling times. For example, up to 10% of total sequences were affiliated with *Fungi* in samples collected from an estuarine site (BH65) in the BHS during winter. Eleven per cent of sequences were also previously observed in Yellow Sea sediments (Stoeck *et al.*, 2009; Edgcomb *et al.*, 2011a). Protists may gener-

Considerable proportions of eukaryotic parasitoid sequences present in coastal benthos

We recovered a high proportion (overall 20%) of sequences of parasitic microeukaryotes from these sediment samples (Figs 6 and S1). The dominance in this study of Dino-Group-I sequences affiliating to the parasitic Syndiniales is consistent with a meta-analysis of this parasitic group (Guillou et al., 2008). Similarly, a variety of sequences from parasitic protists was discovered in deep-sea vent sediments, and are considered to be important for understanding deep-sea vent ecology and population dynamics (Moreira and López-García, 2003). It is interesting that dinoflagellate phylotypes related to *D. collinii* were always abundant in sediment samples across two seasons. Members of *Duboscquodinium* are known parasites of planktonic tintinnid ciliates; however, the ecology of these parasites remains largely unknown (Coats et al., 2010). Our study illustrates that, even in the best-studied coastal ecosystems, there is a large diversity, and potentially a large population of parasitoid protists and fungi. Some of these taxa are ubiquitously distributed, serving as important players in trophic interactions and ecological processes in coastal marine ecosystems. Taken together,

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previous data and the results of this study suggest that parasitism of microeukaryotes in marine plankton and surface sediments warrants further investigation.

**Depth diversity gradient and pH effect**

Our results demonstrate a distinct depth gradient of α-diversity for benthic microbial eukaryotes as a whole (Fig. 3), which is consistent with recent observations for microeukaryotes and/or meiofauna in the deep sea (Bik et al., 2012; Sevastou et al., 2013). In our study, since most of the sediment samples were collected within the euphotic zone, sunlight that reaches the sediments at shallower sites will undoubtedly support higher productivity of benthic autotrophs, which in turn could sustain more species of heterotrophic eukaryotes within those sediments. Furthermore, among the environmental factors, our analysis shows that pH produced the strongest correlation with OTU richness (Fig. 3). The pH of the bottom water is therefore a major physiochemical factor affecting the α-diversity of benthic microeukaryotes. Given that acidification of coastal bottom water could be enhanced by eutrophication (Cai et al., 2011), our observation has ecological implications. Eukaryotic community diversity declined in our data sets in response to decreasing water pH from about 7.9 to 7.6 (Fig. 3).

**Depth–decay relationship of community composition of benthic microeukaryotes**

Both spatial and environmental effects were significant in shaping the community of benthic microeukaryotes on a regional scale (Table 3). This suggests limited distributions of some benthic microeukaryotes and a ubiquitous distribution for others given the sequencing depth used in this study. Thus, our results favour the moderate endemcity model for protists (Foissner, 1999). This is consistent with previous morphology-based studies showing geographic distributions and limited dispersal of benthic protists, e.g. foraminifera in coastal sediments (Gooday and Jorissen, 2012) and benthic diatoms in lakes (Telford et al., 2006; Vyverman et al., 2007). In contrast, marine planktonic microalgae and some uncultured heterotrophic picoeukaryotes, such as MAST-4 detected at depths within the photic zone (upper < 250 m of the water column), do not appear to demonstrate a distance–decay pattern whereby community similarity decreases with geographic distance (Cermeño et al., 2010; Rodríguez-Martínez et al., 2013). This suggests that benthic and pelagic eukaryotic microbes might have fundamentally different biogeographic patterns. According to a review of processes shaping microbial biogeographic patterns by Hanson and colleagues (2012), we speculate that at least two processes, dispersal limitation and selection, could create and maintain the biogeographic differences observed in these studies. Both benthic and pelagic protists have specialized body plans and structures for their lifestyles. Movements of microeukaryotes are obviously more restricted in sediments than in water columns, where microbes can disperse passively with ocean currents to distant locations. However, the diversity and community composition of planktonic protists along a greater depth gradient may reveal different distributional patterns (Countway et al., 2007; Schnetzer et al., 2011).

Our study advances the current understanding of benthic protist communities by providing evidence that in coastal shallow-water (15–75 m water depth) sediments, depth overrides both geographic distance and environmental heterogeneity in determining community composition. A similar depth–decay relationship was found for deep-sea sediment meiofauna in the Mediterranean Sea (Sevastou et al., 2013) and bacterial communities (Jacob et al., 2013) on a regional scale. Little variation was observed in microeukaryote communities from abyssal plains located thousands of kilometres apart, but at similar depth ranges (5033–5655 m) (Scheckenbach et al., 2010). This supports our observations, and suggests that the depth–decay effect for benthic microbes is likely valid for a wide range of ocean depths, from coastal down to deep ocean sediments.

The apparent importance of water depth for determining diversity of benthic microeukaryotes could be explained by the fact that water depth is a good proxy for many physical and chemical variables in the ocean. With increased water depth, bottom waters become generally colder, more saline and acidic and sediments tend to have higher ratios of organic carbon to nitrogen and higher concentrations of many trace metals (Table 3; Table S1). Light and oxygen, which decrease with water depth, also impact trophic functions and the structure of protist communities. In fact, partitioning of diversity and community composition with depth has also been observed for planktonic protists, which could be related to the delivery of sinking particulate organic matter from productive surface waters (e.g., Countway et al., 2007; Schnetzer et al., 2011). Since sediments represent a sink for falling particulate organic matter, it is understandable that depth also shapes the diversity of microbenthos, as confirmed in this study.

Transportation and sedimentation might have also contributed to the depth–decay pattern of benthic microeukaryotes in this study, as regional sedimentation in coastal oceans is usually depth dependent (Cisne, 1985). The Yellow River and other small rivers input large amounts of sandy and muddy materials to the Yellow Sea Large Marine Ecosystem, delivering terrestrial matter,
pollutants and debris-associated microbes. It cannot be ruled out that some of these microbes may persist in marine benthos. According to estimated sedimentation rates (Liu et al., 2003; Sun et al., 2012), surface sediments of the same thickness (5 cm) should have taken about 12–33 years to accumulate in the shallower BS, and 50 years to accumulate in the deeper NYS. This putatively leads to a differential colonization history of microbial communities in the surface sediments of these ocean systems.

**The rare biosphere of microbial eukaryotes**

We showed that the rare biosphere (rare OTUs) of benthic microeukaryotes follows the same general biogeographic patterns seen for abundant OTUs. This is largely in accordance with recent studies of bacteria or planktonic protists (Galand et al., 2009; Lepère et al., 2013; Logares et al., 2013, 2014). We also observed that the rare OTUs were more strongly affected by depth–decay than were the abundant OTUs (Table 3), suggesting that the rare taxa are more diverse than the abundant taxa in sediment samples collected from different depths.

The hydrographic factors and trace metals influencing β-diversity of the surficial sediment rare OTUs detected in our samples are similar to those affecting total and abundant OTUs. Some trace metals are well known as micronutrients that regulate primary productivity and species composition of marine phytoplankton and microbial communities in surface waters (Morel and Price, 2003; Sunda, 2012). Trace metals may also have toxic effects on benthic protists in the sediments (e.g., Fernandez-Leborans et al., 2007). Coastal sediment particles can adsorb large amounts of trace metals, which means these are less likely to be limiting the growth of microphytobenthos. Hence, it is more likely that toxic effects of trace metals contribute to the depth–decay of community similarity of benthic microeukaryotes, including the rare OTUs.

It is interesting that the effect of vanadium seemed to be stronger on the rare OTUs than on the abundant OTUs (Table 3). There are two types of V-dependent enzymes, V-dependent haloperoxidases known from fungi, lichens, marine macroalgae and *Streptomyces* bacteria, and V nitrogenases in *Proteobacteria* and *Cyanobacteria*. However, little is known about the relationship between vanadium and microbial communities in the environment (reviewed in Rehder, 2013). Vanadium may be utilized by some eukaryotic groups (e.g. haloperoxidase-producing fungi) within the rare biosphere. Vanadium may also have indirect effects on microbial eukaryotes through its effect on bacteria with V-dependent biochemical functions. Further studies are needed to explore these ecological hypotheses.

**Technical remarks**

Due to the complex nature of benthic biota, it was difficult to morphologically identify many eukaryotic microbes in our sediment samples. Next Generation Sequencing of a hypervariable region of the eukaryotic SSU rDNA provided a broad picture of benthic microbial eukaryote diversity in different seasons and across a broad geographic area. However, we recognize that there are advantages and potential caveats of this approach for characterizing α- and β-diversities of microbial eukaryotes. It has been demonstrated that reads of a single hypervariable region might not be appropriate for identifying OTUs at the species level (Lie et al., 2014). Our study targeted a single fragment containing two hypervariable regions (V2 and V3), which may have enhanced the robustness of our classification resolution for our OTUs. The ‘denoising’ step may eliminate morphospecies of ciliates from the sequence data known to contain homopolymers. This may underestimate OTU richness in individual samples (Santoferrara et al., 2014). However, processing our data using a range of denoising parameters did not change the anti-correlation between richness and water depth (data not shown). Furthermore, our study comprised many samples (*n* = 39), which improves reproducibility during quantification of β-diversity (Zhou et al., 2011), and hence the reliability of observed relationships between OTUs and geographic and environmental factors. Nonetheless, sequencing rRNA genes cannot indicate activity (Pawlowski et al., 2011). Further studies will be needed to confirm *in situ* activities of selected taxa. Finally, rarefaction of our data sets to 1500 sequences removed information for many samples. Future deeper sequencing targeting rRNA will be very useful to verify active community members and to unveil a more detailed picture of benthic microeukaryotic diversity over space and time.

**Conclusions**

The results of this study demonstrate that, like soil protists (Bates et al., 2013), microeukaryotes in coastal sediments are not ubiquitously distributed. We found a large diversity of microeukaryotes and we identified a distinct depth gradient of α-diversity for these benthic microbes in the neritic coastal ecosystems. Sediment microeukaryote community similarity in the northern China Seas changes with water depth, a pattern we designate as the ‘depth–decay relationship’, which contrasts with the well-known distance–decay of similarity in biogeography and ecology (Nekola and White, 1999). Similarly there is also evidence for the influence of depth on distributions of microbial eukaryotes in the water column. This begs the question whether biogeographical patterns of pelagic and benthic
microbial eukaryotes in the ocean can be unified by the depth–decay relationship. Our exploration of abundant and the rare OTUs in northern China Seas demonstrates similar patterns of biodiversity and impacts of hydrographic and mineralogical factors on biogeography for both groups, with a slightly higher effect of the trace metal vanadium on the rare OTUs. The depth–decay pattern of community similarity should be further tested for microbial eukaryotes in both planktonic and benthic habitats, using data sets from other geographic regions, on larger or even global scales. Future work investigating temporal patterns of both the whole community and the rare biosphere (Caron and Countway, 2009) may be important for understanding the composition and functional changes of microbenthos facing increasing environmental changes in coastal zones. Such changes include bottom water acidification, hypoxia, temperature changes and pollution events. Furthermore, the prevalence of parasite sequences within benthic communities points to the need to examine the ecology and associations of parasites and hosts (e.g. interactions within the microeukaryotic community, and with macroorganisms in benthic food webs), and the impact of environmental changes on their functioning.

Experimental procedures

Study area, sampling and environmental variables

The Yellow Sea includes the BHS, the NYS and SYS basins, and represents one of the largest shallow continental shelves in the world. The depth gradually increases from north to south. The northern portion of the Yellow Sea Large Marine Ecosystem, the semi-enclosed BHS, is shallow (depth 15–30 m), receiving discharges carrying mineral-rich soil and pollutants from the Yellow River and from many other small rivers of the Bohai Economic Rim. The seafloor is dominated by sand and silt. The depths of the SYS vary from 40 to 150 m. Summers are wet and warm, with a cold and saline water mass in the deepest waters of the SYS and NYS. Winters are cold and dry, with strong northerly monsoons. The seafloor is dominated by sand and silt. The depths of the SYS vary from 40 to 150 m. Summers are wet and warm, with a cold and saline water mass in the deepest waters of the SYS and NYS. Winters are cold and dry, with strong northerly monsoons blowing from late November to March (Tang, 2003).

Sampling was conducted during two seasonal cruises at BHS, NYS and SYS with the R/V Dong Fang Hong 2 in the summer (14–26 June) and winter (21 November–4 December) of 2011. Forty sediment samples were collected from 20 stations across the three seas (Fig. 1). Sediments were obtained using a box-corer and the surface-layer subsamples (top 0–5 cm) were subcored with a custom-made corer (inner diameter 1.5 cm) and well mixed within a plastic bag by hand; core material was put into cryovials, and stored immediately in liquid nitrogen for DNA extraction. Sediment subsamples were archived at 4°C for physicochemical analyses. A total of 25 variables were recorded (Table S1). Temperature and salinity measurements for the water overlying each sediment core were obtained using a Seabird 911 Plus CTD rosette (USA) fitted with 10 l Niskin bottles. The concentrations of Chl-a and DO of bottom waters were measured on site with a probe (Hydrolab MS5, HACH, USA). Water pH was measured according to Zhai et al. (2014). Nitrate, nitrite and ammonium in sediment samples were extracted with 2 M KCl using a 1:10 sediment: extractant ratio from lyophilized sediments, and then measured with a nutrient AutoAnalyser (Seal, Germany). Total organic carbon and nitrogen of sediments were determined with a Vario Micro Cube Elemental Analyser (Elementar, Germany). Sediment GS was analysed with a Marivem Mastersizer 2000F granulometer (Malvern, England). Surface sediments were treated with 1 M HCl and then trace metals were determined with an ELAN DRC II plasma mass spectrometer (ICP-MS) (PerkinElmer, Hong Kong). Pairwise geographic distances of sampling sites were calculated according to the National Oceanic and Atmospheric Administration website (http://www.nhc.noaa.gov/gccalc.shtml). The geographic distance from the sampling site to coast was calculated with Spatial Analyst tools of ArcGIS v.10.0.

DNA extraction, polymerase chain reaction (PCR) amplification and pyrosequencing

Extraction and purification of DNA from sediment samples were carried out using the FastDNA SPIN Kit for Soil (Q-BioGene) according to the manufacturer's instructions. The integrity of extracted DNA was assessed by gel electrophoresis, and the DNA concentrations were measured using a Nanodrop 2000c spectrophotometer (Thermo-Fisher, USA).

The universal eukaryotic primers 82F (5′-GGAATTGTCCTC-3′, López-García et al., 2003) and 516R (5′-ACCAGACTTGCCCTCC-3′, Casamayor et al., 2002) were used to amplify 480 bp fragments covering the complete V2 and V3 regions of the eukaryotic small subunit ribosomal RNA (18S rRNA) gene. A 26 bp adapter, a 4 bp key (TCAG) and a 10 bp barcode specific to each sample were added to the sequences of the forward primers while no barcode was added for the reverse primers (for a list of barcodes see Table S4). Reaction solutions for PCR were made according to standard conditions for Platinum Pfx DNA polymerase (Invitrogen) with 20 ng of environmental DNA as a template. PCR reactions included: 95°C for 2 min, followed by 20 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 1 min and finally, an extension at 72°C for 7 min. Pyrosequencing was performed with GS FLX Titanium LV emPCR Kit (Lib-L) on a Roche 454 GS FLX Titanium sequencer by the BGI Company (Shenzhen, China). Half a sequencing plate was used for this study. One wintertime sample from NYS (NY01W) failed to be amplified and was not included for subsequent analysis.

Processing and analyses of raw pyrosequencing data

Raw data (503 987 reads) were processed and analysed using QIME v.1.7.0 (Caporaso et al., 2010b) and programs (e.g. Mothur v.1.30.0, Schloss et al., 2009) integrated within this platform. Quality filtering retained sequences that satisfied the following criteria: (i) no N's; (ii) quality score > 25 when averaged across the read after trimming adapters and primers; (iii) no sequencing mismatches within the PCR primer regions; (iv) minimum sequence length of 200 bp
maximum length of 500 bp (including PCR primers); and (v) homopolymers \(< 6\). The remaining sequences were denoised using the denoiser\_wrapper.py script with the default settings in QIME. Sequences were clustered into OTUs using UCLUST v.1.2.2 (Edgar, 2010) with defined pairwise sequence identity cut-off of 97% (command: pick\_otus.py -i split\_library\_output/seqs.fna -o uclust\_picked\_otus/ -s 0.97 -m uclust). Representative sequences in each OTU were aligned against the core Silva aligned ribosomal RNA sequence database of the Silva 104 release using PYNAST v.1.2.2 (Caporaso et al., 2010a). Chimera detection was performed with the UCHIME module of USEARCH v.6.0.203 (Edgar et al., 2011) without a reference database and then additional chimera checking was performed with Chimera Slayer (Haas et al., 2011) based on the Silva 104 release database. Putative chimeric sequences and singletons (OTUs containing a single read across all samples) were discarded prior to further analysis. Taxonomy was assigned against the Protist Ribosomal Reference Database (PR2 database, a version based on GenBank v.191) (Guillo et al., 2013) using the BLASTn with default settings. Each cluster was assigned to the lineage with the most taxonomic detail and highest BLAST score meeting the criterion of \( \geq 90\% \) threshold match to sequences in the database. Since Metazoa might account for a large proportion of biomass in some samples, this would obviously distort the relative abundance of eukaryotic sequences, and hence, the community structure of microbial eukaryotes. Reads assigned to Metazoa were therefore excluded from analyses in this study.

The relative abundance of an individual taxon (e.g. phylum) within each sample was estimated by comparing the number of reads assigned to each taxon for individual samples. To normalize the sampling effort, we rarefied all data sets to 1500 sequences (the lowest number of sequences recovered for our 39 samples) for community diversity analysis. We calculated \( \alpha \)-diversity using estimators that included the number of OTUs (richness), and Simpson and Shannon indices. \( \beta \)-Diversity was calculated on Hellinger-transformed Bray-Curtis distances and Sørensen distance matrices. Visualization of community dissimilarities (\( \beta \)-diversity) was made using PCoA in the VEGAN v.2.0–10 package in R v.2.8.1 (R Development Core Team, 2006).

Rare and abundant OTUs

In addition to analysis of the whole communities in each sample, we also performed separate analyses of the rare and the abundant assemblages, and calculated their richness and composition, patterns of community structure and the relative contributions of biogeography and environmental factors to those variations. It is generally recognized that protophytes present at a relative abundances \( > 1\% \) can be detected using traditional community profiling and Sanger sequencing methods, but that these methods are less efficient at or even unable to detect low-abundance protophytes (Pedró-Alío, 2006). Pyrosequencing allowed us to explore the rare biosphere that might not have been detected in previous studies of the northern China Sea sediments. Here, we considered OTUs to be abundant when they comprised more than 1% of the pyrotags in a sample, and rare OTUs were those comprising \( < 1\% \) of the reads in a sample. Separate tables of abundant and rare OTUs were constructed. To normalize these data sets for this comparison, random subsets of 600 and 900 sequences per sample were selected from abundant and rare communities respectively. Analyses of \( \alpha \)-diversity, PCoA and ANOSIM of \( \beta \)-diversity of rare and abundant protophytic communities were performed as described above for the whole communities.

Statistical analyses

Two-way ANOVA was performed to examine the effect of seasonality and region on the OTU richness of microbial communities. Spearman’s rank correlation coefficients were calculated to explore the associations between these \( \alpha \)-diversities or relative abundance of an individual taxon and environmental factors using the package SPSS v.11.5 (SPSS, Chicago, IL, USA). Global and pairwise differences among groupings of samples were tested by ANOSIM (Clarke, 1993) within the PRIMER v.6.0 package (PRIMER-E, Plymouth, UK). Mantel tests were used to explore correlations between spatial factors, environmental parameters and \( \beta \)-diversity with 1000 permutations (Legendre and Legendre, 1998). As geographic distance, depth and environment can be intercorrelated, partial Mantel tests were used to assess their individual effects on \( \beta \)-diversity after controlling for geographic distance, depth or environment (Martiny et al., 2006). Standardization of environmental data, and Mantel tests were conducted in R with Vegan and Picante packages (v.0.6).

Accession number and data availability

SFF files containing total raw sequences have been submitted to the NCBI Sequence Read Archive under the accession number SRA157723.

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References


**Supporting information**

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Fig. S1.** An overview of the numbers of OTU and reads of benthic microbial eukaryotes detected from the BHS, NYS and SYS basins. A Venn diagram showing the total numbers of OTUs detected from the three basins (A), and summary of the relative abundances of major taxa (at superphylum or phylum levels) in all samples (B), of classes within *Alveolata* (C), and *Stramenopiles* (D).

**Fig. S2.** Seasonal variations in OTU richness in each basin. Asterisks indicate significant differences (*t*-tests, *P* < 0.05).

**Table S1.** Summary of sampling and environmental variables of the sediment and overlying water samples collected from the Bohai Sea, South and North Yellow Sea basins.

**Table S2.** Summary of the pyrotags and γ-diversity of microbial eukaryotes in sediment samples.

**Table S3.** Correlations between the OTU number of major taxonomic groups of benthic microbial eukaryotes and environmental variables.

**Table S4.** A list of barcodes for samples.