



Environmental status assessment using biological traits analyses and functional diversity indices of benthic ciliate communities



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ABSTRACT

In this study, we tested the hypothesis that the functional diversity of benthic ciliates has high potential to monitor marine ecological status. Therefore, we investigated the spatial and temporal variation of functional diversity of benthic ciliates in the Yangtze Estuary during one year using biological traits analyses and functional diversity indices. Traits and community compositions showed clear spatial and temporal variations. Among a variety of biological traits, feeding type and body size emerged as strongest predictable variables. Functional divergence (FDiv) had an advantage over two other functional diversity indices, as well as over classical diversity measures (i.e. richness, evenness, Shannon-Wiener) to infer environmental status. Significant correlations between biological traits, FDiv and environmental variables (i.e. nutrients, temperature, salinity) suggested that functional diversity of benthic ciliates might be used as a bio-indicator in environmental status assessments. Further mandatory researches need to implement functional diversity of ciliates in routine monitoring programs were discussed.

1. Introduction

As a component of biodiversity, functional diversity generally considers the range of biological traits (“things that organisms do”) in communities and ecosystems (Petchey and Gaston, 2006). Therefore, functional diversity is an important aspect of biodiversity to study and understand ecosystem processes (Díaz and Cabido, 2001). Several definitions exist for functional diversity, but there is a general agreement that functional diversity studies always consider organisms as “dynamic entities that interact with their environment” (Calow, 1987; Laureto et al., 2015). A wealth of trait information is available for different groups of organisms (Petchey and Gaston, 2006). The choice of traits to be used for the measurement of functional diversity depends on the specific aims of a particular study.

Traditional methods to measure functional diversity rely on functional group analysis such as feeding types and body size. These individual measures, however, ignore the functional difference among species within certain groups (Petchey and Gaston, 2002). Therefore, Bremner et al. (2003) introduced an alternative approach, first applied to marine benthic communities. This technique of biological traits analysis considered the variation patterns of a wide range of characteristics shared by different taxa based on multivariate ordinations.

Taking this strategy one step further, different authors introduced various functional diversity indices that estimate the extent, dispersion and relative abundance of species biological traits (Mouchet et al., 2010; Petchey and Gaston, 2006). Among these measures, FRic, FEve, and FDiv measure the three primary aspects of functional diversity (Mason et al., 2005): functional richness, functional evenness and functional divergence, respectively. These measures are complementary and independent to all the other indices (Mason et al., 2005; Mouchet et al., 2010; Villéger et al., 2008). Because species are not equal in their responses to environmental changes these measures have the potential to explore relationships between biodiversity and environmental conditions (Mouchet et al., 2010). Indeed, there is good experimental and analytical evidence that functional diversity can provide a link between organisms and ecosystem properties. For example, biological traits analyses and functional diversity indices were successfully applied to assess environmental changes across habitat gradients or anthropogenic impacts on macrobenthic organisms (Bady et al., 2005; Bremner et al., 2003; Dolbeth et al., 2013; Gusmao et al., 2016; Hussin et al., 2012; Tillin et al., 2006; Veríssimo et al., 2012; Wong and Dowd, 2015).

The vast majority of such studies in benthic ecosystems focused on macrofauna, even though microbial organisms such as unicellular eukaryotes (protists) may have better qualities as environmental

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indicators (Stoeck et al., 2018). This is because protists are highly sensitive to environmental changes, including for example organic enrichment (Stoeck et al., 2018), heavy metal contaminations (Madoni, 2000), acidification (Davidson et al., 2016; Meakin and Wyman, 2011), oxygen depletion (Orsi et al., 2012), hydrocarbon pollution (Echeveste et al., 2010), and UV-radiation (Sonntag et al., 2017). Also, they have very short generation times, resulting in much higher species turnover rates after environmental changes compared to macrofauna with longer generation times. Thus, protists can indicate environmental changes much faster than macrofauna. Furthermore, they have high population densities, which are characterized by an extremely high biodiversity, even under typically azoic environmental conditions (Stoeck et al., 2018). This provides a large choice of potential environmental indicators even in situations when most or all larger organisms have disappeared (Stoeck et al., 2018). Among protists, especially ciliates are used as environmental indicators (Berger and Foissner, 2003; Bick, 1972; Chen et al., 2008; Feng et al., 2015; Foissner, 1999, 2016; Foissner and Berger, 1996; Jiang et al., 2013; Xu et al., 2016). They play key roles in microbial food webs where they predate bacteria, algae, other protists and even some metazoans, while being prey by larger zooplankton (Hamels et al., 2005; Porter et al., 1979; Sherr and Sherr, 1988; Weisse, 2003, 2006; Weisse and Sonntag, 2016; Weisse et al., 1990). Because they are relatively large compared to other protists ciliates are well-studied for more than two decades and have a good taxonomic framework documented in many identification keys (Berger and Foissner, 2003; Bick, 1972; Carey, 1992; Foissner and Berger, 1996). In addition, a wealth of literature is available describing their biological traits (Lynn, 2008; Lynn and Small, 2002; Pratt and Cairns, 1985; Weisse, 2017), many of which are related to their roles in aquatic food webs (Caron and Goldman, 1988; Lischke et al., 2016; Sanders and Wickham, 1993). Accordingly, first studies showed the potential of functional groups of planktonic ciliates to assess water quality (Jiang et al., 2013; Xu et al., 2016, 2017).

Here, we investigate functional diversity of benthic ciliates in the Yangtze Estuary in relation to environmental conditions. We chose this proof-of-concept habitat because coastal and estuarine ecosystems belong to the most productive but also most exploited natural habitats on Earth. Increasing pressure on coastal environments come for example from land use, accelerated urbanization, eutrophication, global warming, aquaculture and tourism (Lotze et al., 2006). Our study was conducted at three contrasting sampling regions over a time course of 12 months to test the following hypothesis: The functional diversity of benthic ciliates has high potential to monitor marine ecological status. To test our hypothesis, we addressed the following specific questions: (1) how do the benthic ciliate community structure and its biological traits composition vary across environmental gradients?; and (2) how well do three standard functional diversity indices resolve the relationship between environmental status and ciliate communities?

2. Materials and methods

2.1. Study sites and sampling

Three typical stations with environmental gradients (e.g. salinity and sediment grain size) were selected in Dongtan wetland in Yangtze Estuary (Fig. 1): Station 1 (31°27'1.88"N, 121°53'40.03"E), which is close to the South Branch of the Yangtze Estuary and mostly influenced by the fresh water of Yangtze River, has sandy soil. Station 2 (31°30'35.00"N, 121°57'33.00"E) is about 10 km northwest of station 1 and facing the East China Sea. This station is characterized by mixed water masses from the Yangtze River and the China Sea. Station 3 (31°34'46.00"N, 121°54'28.16"E) is located ca. 15 km north of station 1, close to the North Branch and mainly influenced by marine waters from the China Sea (Dai et al., 2011). Station 2 and 3 have saline-alkaline clay soil.

Sampling of each station took place once during ebb tide in March

(spring), June (summer), September (autumn), and December (winter) of 2015. At each sampling, ten replicates were collected at each station along a short transect parallel to the water line in the mid-intertidal zone at low tide (120 samples in total). The upper 2 cm of the sediment (ca. 4 ml) were collected using a syringe and then mixed with glutaraldehyde (2% final concentration). For granulometric analyses, total organic carbon (TOC) and total nitrogen (TN) measurements, three additional samples were collected from the top 2 cm of the sediment surface. For analyses of nutrients, three sediment pore water samples were taken using Millipore syringe filters with a pore size of 0.45 μ m. After addition of 1.5% v/v saturated HgCl₂ to the filtered pore water, samples were stored at 4 °C until further processing.

2.2. Ciliate identification

Ciliates were extracted and stained from all 120 samples according to Xu et al. (2010). Enumeration and identification were conducted using microscopy (Olympus BX53) at 200 \times to 1000 \times magnifications. Identifications followed mainly Carey (1992), Corliss (1979), and Lynn and Small (2002). For each taxon, cell lengths of at least five individuals (or all individuals encountered if fewer than five were found) were measured with an ocular micrometer. The species abundance dataset was reduced to retain only those taxa found in at least three samples and contributing at least 10% of total ciliate abundance in any sample, thus selecting taxa that were dominant in each sample and widely distributed over the regions.

2.3. Measurement of environmental parameters

Salinity and temperature of sediment pore water were measured in situ three times at each station using a multi-parameter kit (WTW Multi 3430). Sediment grain size was determined with a laser size analyzer (BECKMAN COULTER LS13 320). TOC and TN contents were determined using a Vario EL CHNOS Elemental Analyzer (as % dry weight of the sediment) according to Ryba and Burgess (2002). Nutrients (ammonia, nitrate, nitrite, phosphate and silicate) were determined using a segmented flow analyzer (Model: SKALAR San^{plus} System, Netherlands) according to Gao et al. (2009).

2.4. Biological trait analysis

Six biological traits sub-divided into 15 categories were chosen according to Xu et al. (2018) for our analysis (Suppl. Table 1). Information on biological traits were gathered both from our own observations and from the literature (Lynn, 2008; Lynn and Small, 2002; Pratt and Cairns, 1985). Fuzzy-coding procedure was used to account for multiple trait categories of an individual taxon, i.e. a scoring range of 0 to 3 was adopted, where 0 expresses no affinity for the given trait category, 1 or 2 express partial affinity and 3 expresses exclusive affinity (Bremner et al., 2003). For example, *Frontonia microstoma* feeds both on microalgae and bacteria but has a preference for the former, so it was coded 2 (algivores), 1 (bacterivores), and 0 (predators) for the “feeding type” trait.

After obtaining “taxa abundance by samples” and “taxa by traits” data matrices, we then calculated the “traits by samples” matrix using the *dbFD* function in the “*FD*” R package (Laliberté et al., 2014).

2.5. Diversity indices

Three classical diversity indices, i.e. Margalef's richness (*d*), Pielou's evenness (*J'*) and Shannon-Wiener (*H'*) indices, were calculated using the submodule DIVERSE in the PRIMER v7.0.11 package (Clarke et al., 2014). Three indices of functional diversity, i.e. FRic, FEve, and FDiv, were computed using the *dbFD* function in the “*FD*” R package (Laliberté et al., 2014). The Bray-Curtis (BC) index was used as a measure of similarity between the samples (beta-diversity).

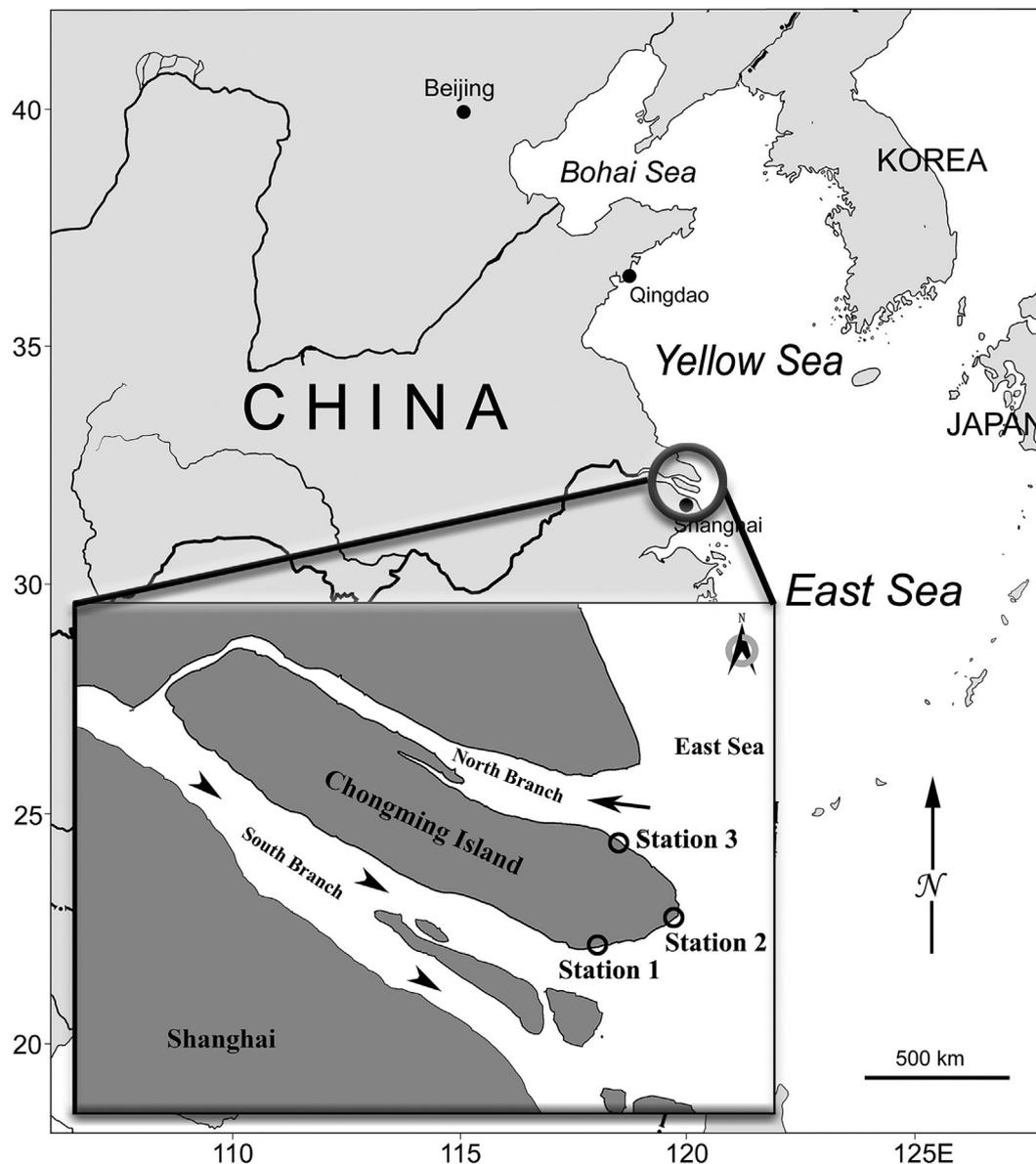


Fig. 1. Map of the three sampling stations in Dongtan wetland, Yangtze Estuary, China. Station 1 ($31^{\circ}27'1.88''\text{N}$, $121^{\circ}53'40.03''\text{E}$), station 2 ($31^{\circ}30'35.00''\text{N}$, $121^{\circ}57'33.00''\text{E}$), and station 3 ($31^{\circ}34'46.00''\text{N}$, $121^{\circ}54'28.16''\text{E}$) are marked in circle. Arrowheads show the direction of freshwater flow of Yangtze River, and arrow indicates the direction of salt water coming from East Sea.

2.6. Statistical analysis

BC similarity values were transformed to distance matrices for non-metric multidimensional scaling (NMDS) analyses. Vectors were fitted to the ordination using the *envfit* function of the “*vegan*” package in R v. 3.3.3 (R Development Core Team, 2008). The fit (R^2) of each variable to the ordination using the *envfit* function was assessed with a Monte Carlo analysis of 10,000 permutations. One-way analyses of similarities (ANOSIM) were used to signify the differences in taxa composition, biological traits composition as well as diversity between samples. RELATE was used to test the significance of agreement between diversity indices and environmental conditions. BIO-ENV was used to identify the multivariate combination of environmental variables best matched to functional diversity. Second-stage matrix-based NMDS ordination and clustering analysis were used to summarize the relationship between biotic dataset (taxon composition, biological traits composition and diversity) and environmental data. These multivariate analyses were conducted using PRIMER v7.0.13 (Clarke et al., 2014).

Data were tested for normality using the Shapiro-Wilks test. Spearman correlations between each environmental variable and biological trait category were calculated using the function *rcorr* in the R package “*Hmisc*” (Harrell Jr and Dupont, 2017).

3. Results

3.1. Environmental variables

Environmental variables were notably different between seasons and sampling sites (Suppl. Table 2). In particular, station 1 usually had lower salinity, nitrate concentrations, TN and TOC contents, but larger grain size values compared to the other two sites. Furthermore, summer and autumn seasons were in general characterized by higher temperatures, nitrite and silicate concentrations, but lower salinities, compared to spring and winter.

3.2. Ciliate community structures and biological trait analyses

A total of 93 ciliate species representing 57 genera, 44 families, 20 orders, and 9 classes were recorded. A complete species list is provided as supplemental material (Suppl. Table 3). Generally, in summer and autumn, higher species number and abundances were recorded compared to spring and winter (Suppl. Fig. 1). Lowest species numbers and abundances were recorded in spring (Suppl. Fig. 1). Among the three sampling stations, station 3 had the lowest numbers of species and abundances compared to the other two stations except in winter (Suppl. Fig. 1). ANOSIM revealed significant differences among the three stations ($R = 0.778$, $p < 0.01$) and four seasons ($R = 0.803$, $p < 0.01$).

Ciliate community structures clustered according to stations and sampled seasons (stress of NMDS analysis: 0.2662598; Fig. 2). For example, all ciliate communities of the spring sampling are located in the negative range of NMDS axis 1, while all but three ciliate communities of the winter sampling are in the positive range of NMDS axis 1. Ciliate communities of station 1 clustered predominantly in the negative range of NMDS axis 2, and ciliate communities of station 3 predominantly in the positive range of this axis. Several biological traits correlated significantly with the NMDS axes (correlation values are shown in Suppl. Table 4). NMDS axis 1 correlated significantly with crawlers and swimmers ($p < 0.01$ in both cases). NMDS axis 2 correlated significantly with feeding type (bacterivores vs. algivores), cell flexibility, size spectrum (small vs. medium) and oxygen preferences.

This shows strong biological trait preferences for community assembly dependent on stations and seasons, which is confirmed by 33 significant Spearman correlations between biological traits and environmental variables (Table 1). Notably feeding type and body size appeared as strong predictable variables.

3.3. Seasonal patterns of ciliate communities and biological traits

In spring, ciliate community structures are clearly separated from each other in NMDS analysis with station 1 communities being located in the negative range of axis 1 and communities of stations 2 and 3 in the positive range (stress: 0.1873894; Fig. 3A). Communities of stations 2 and 3 are separated by NMDS axis 2. Several significant vector correlations with NMDS axes characterize the individual communities and explain cluster patterns. Communities of station 2 in spring are characterized by mostly flattened, flexible and crawling cells in the intermediate size range (50–150 μm). With one exception, communities from stations 3 include a significantly higher proportion of large algivores. Most communities of station 1 included a significantly higher proportion of smaller and attached ciliates, as well as organisms with a cylindrical body shape. In summer, stations can still be separated from each other based on ciliate community structures (stress: 0.1760957; Fig. 3B). However, the trait properties have changed from spring to summer. Station 1 communities include higher proportions of flattened crawlers and predators. Oxygen preferences separate station 1 communities from station 2, while station 3 communities are equally distributed along the oxygen preference vector. As in spring, station 3 still stands out with a higher proportion of large algivores, whereas the size spectrum of the station 2 community shifted towards the smaller organisms. NMDS analysis of autumn samples only distinguishes station 1 communities from station 2 communities (stress: 0.2138701; Fig. 3C), with the main explaining trait variables being feeding type and cell size. In winter (stress: 0.2105011; Fig. 3D), ciliate communities of the three study stations are again better separated from each other. Communities of stations 1 and 3 are predominantly located in the negative and positive range of axis 2, respectively. However, no trait shows a strong

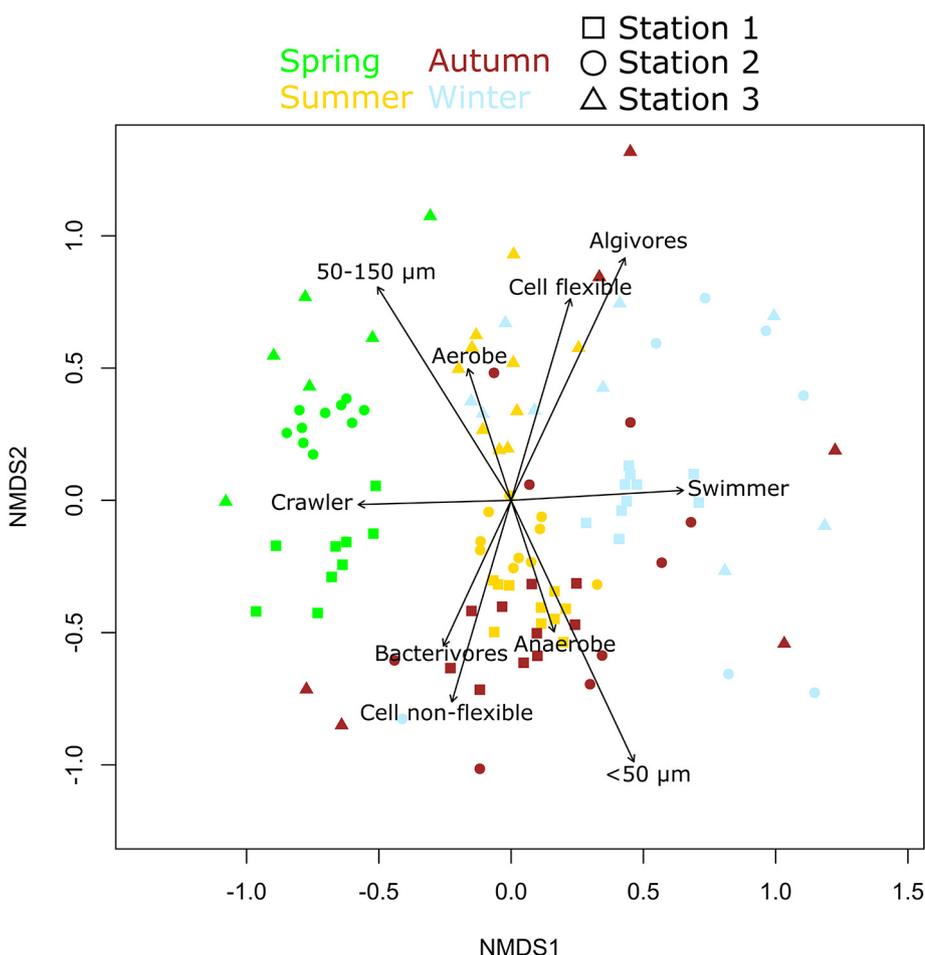


Fig. 2. NMDS ordination plot of benthic ciliates communities from the three sampling stations over four seasons based on species abundance dataset. The length and direction of each vector indicate the strength and significance of the relationship between biological trait categories and axes based on environmental fitting (only vectors with significant correlation were represented, see Suppl. Table 4 for more information).

Table 1

Spearman Rank correlations between environmental variables and biological trait categories. Number = Spearman R; SED, Sediment grain size; SAL, salinity; T, temperature; TN, total nitrogen; TOC, total organic carbon. Significance levels of the correlations are shown at $p < 0.1$ (*), $p < 0.05$ (**) and $p < 0.01$ (***).

Biological traits	Environmental variables									
	NO ₂ ⁻	NO ₃ ⁻	NH ₄ ⁺	TN	PO ₄ ³⁻	SiO ₃ ²⁻	TOC	SED	T	SAL
Bacterivores	0.00	-0.32*	-0.02	0.09	-0.07	-0.26	-0.06	-0.30**	0.15	0.17
Algivores	-0.33*	-0.24	-0.01	0.09	-0.18	-0.24	0.19	-0.26	-0.10	0.45***
Predators	0.26	0.28*	0.17	-0.04	0.29*	0.29*	-0.02	0.33**	0.09	-0.47***
< 50 μm	-0.33*	0.13	-0.34**	-0.27	-0.12	0.08	-0.33*	0.44***	-0.45***	-0.11
50–150 μm	0.10	-0.11	0.15	-0.02	0.09	-0.09	0.06	-0.18	0.22	0.08
> 150 μm	0.25	-0.10	0.48***	0.48***	0.33*	0.08	0.41**	-0.53***	0.52***	0.03
Attached to substrate	0.08	-0.09	-0.22	-0.16	-0.21	-0.29*	-0.28	0.11	-0.14	-0.23
Swimmer	0.01	0.09	-0.25	-0.09	-0.33*	0.11	-0.10	-0.06	-0.20	0.04
Crawler	-0.02	-0.10	0.31*	0.17	0.39**	-0.07	0.21	0.00	0.26	0.00
Anaerobe	0.40**	0.15	0.02	0.22	0.15	0.28*	0.26	0.05	0.42***	-0.30*
Aerobe	-0.40**	-0.15	-0.02	-0.22	-0.15	-0.28*	-0.26	-0.05	-0.42***	0.30*
Flattened	0.15	0.10	0.17	0.14	0.25	0.12	0.18	0.17	0.14	-0.19
Cylindrical	-0.15	-0.10	-0.17	-0.14	-0.25	-0.12	-0.18	-0.17	-0.14	0.19
Cell non-flexible	0.12	-0.22	0.02	0.01	0.01	-0.11	-0.16	-0.01	0.21	0.00
Cell flexible	-0.12	0.22	-0.02	-0.01	-0.01	0.11	0.16	0.01	-0.21	0.00

($R^2 > 0.7$) correlation with this axis. Ciliate communities of station 2 are spread along the positive and negative range of axis 2, but are restricted to the positive range of axis 1, which correlates significantly positive with swimming behavior and significantly negative with crawling lifestyle, large size fraction (> 150 μm) and predatory lifestyle. It is noteworthy, that some traits are not uniform among communities of station 2, which include for example feeding type (bacterivores) and cell shape (cylindrical).

3.4. Relating diversity indices and environmental conditions

Spatial and temporal difference of all diversity indices were significant based on ANOSIM ($p < 0.01$). The NMDS ordination and clustering analyses based on second-stage matrices showed that community similarity matrices based on FDiv and biological traits composition clustered with those based on environmental conditions, while matrices based on the three classical diversity indices, FRic, FEve and taxa composition clustered together (Fig. 4). RELATE analyses confirmed that FDiv was the exclusive index among all indices tested, that significantly correlated with environmental conditions ($p = 0.393$, $p < 0.01$). The top 10 matches between FDiv and the multivariate combination of environmental variables determined by BIO-ENV analysis are presented in Table 2, with the best correlation being a combination of ammonia, TN, temperature and salinity ($p = 0.563$, $p < 0.05$).

4. Discussion

4.1. Biological traits of benthic ciliates

Numerous previous studies demonstrated the power of ciliated protists as bioindicators in aquatic ecosystem using either morphospecies analyses (Berger and Foissner, 2003; Bick, 1972; Chen et al., 2008; Feng et al., 2015; Foissner, 1999, 2016; Foissner and Berger, 1996; Jiang et al., 2013; Xu et al., 2016) or eDNA metabarcoding (Stoeck et al., 2018). The organismic properties, which make ciliates such good indicators, are, however, largely unknown. The biological traits of ciliates are at least one factor, which notably contributes to the qualities of ciliates as environmental indicators.

Feeding type has been the most widely used biological trait to allocate species into trophic groups when describing community functions and ecosystem processes since this trait is closely linked to food resources, energy flow, nutrient cycling and food web interactions (Coûteaux and Darbyshire, 1998; Paganelli et al., 2012; Pratt and Cairns, 1985). Likewise, body size is considered a key trait of ciliates

functional diversity (Weisse, 2017). In his review, Weisse (2017) noted that major morphological and physiological features are correlated with cell size including moving speed, reproduction rate, and vulnerability to starvation and other unfavorable environmental conditions. Therefore, it is reasonable to assume that changes in these two traits composition may have implications for ecosystem processes. These could, for example, include variations in the ratio of bacterivores and algivores indicating changes in the main carbon supply sources (Fenchel, 1968; Wetzel, 1983), and a shift towards smaller-sized species is indicative of organic enrichment (Connell, 1978; Huston, 1979). In our study, these two traits emerged as the strongest predictable parameters among all the traits analyzed, which could explain why functional approach considering single trait (i.e. feeding type or body size) has been approved to be effective in detecting environmental status (Burkovsky and Mazei, 2010; Fernandez-Leborans and Fernandez-Fernandez, 2002; Xu et al., 2016; Zhang et al., 2012; Zhao et al., 2016).

Besides feeding type and body size, we identified oxygen preference as another trait with strong predictive powers. The pattern of this biological trait was mainly affected by the appearance of metopid species in summer and autumn, which are free-living anaerobes. Previous studies suggested that oxygen is a dispersal barrier for strict anaerobes (Forster et al., 2012; Pacheco et al., 2011). Therefore, the distribution of anaerobic ciliates was strongly correlated with those environmental factors affecting oxygen concentrations, such as temperature. Higher air and water temperatures in summer contribute to an accelerated decomposition of organic material under consumption of oxygen, while the solubility of oxygen decreases in warmer waters (Patterson et al., 1989). This may create anaerobic niches in the sediment, selecting for a higher proportion of anaerobic ciliates.

We found the trait mobility, which is assumed to reflect the transfer of energy and matter between the benthic and pelagic realm (Verissimo et al., 2012), associated exclusively with some nutrients (i.e. ammonia, phosphate and silicate). Hamels et al. (2004) reported that sessile ciliates were dominant in freshwater tidal reaches due to the intense hydrodynamic conditions of that benthic environment. However, in our study, no significant correlation between mobility and sediment grain size as a measure of hydrodynamic activity was detected. Thus, this relationship suggested by Hamels et al. (2004) warrants further investigations.

Body form and cell flexibility affect the vulnerability of ciliates from predation and constrain the mobility of ciliates in benthic and interstitial habitats (Weisse, 2017). Although Hamels et al. (2005) as well as Carey (1992) noted that the cell shape of interstitial ciliates is an adaptation to life in the interstitial space among the sand particles, we, however, could not relate these biological traits to corresponding

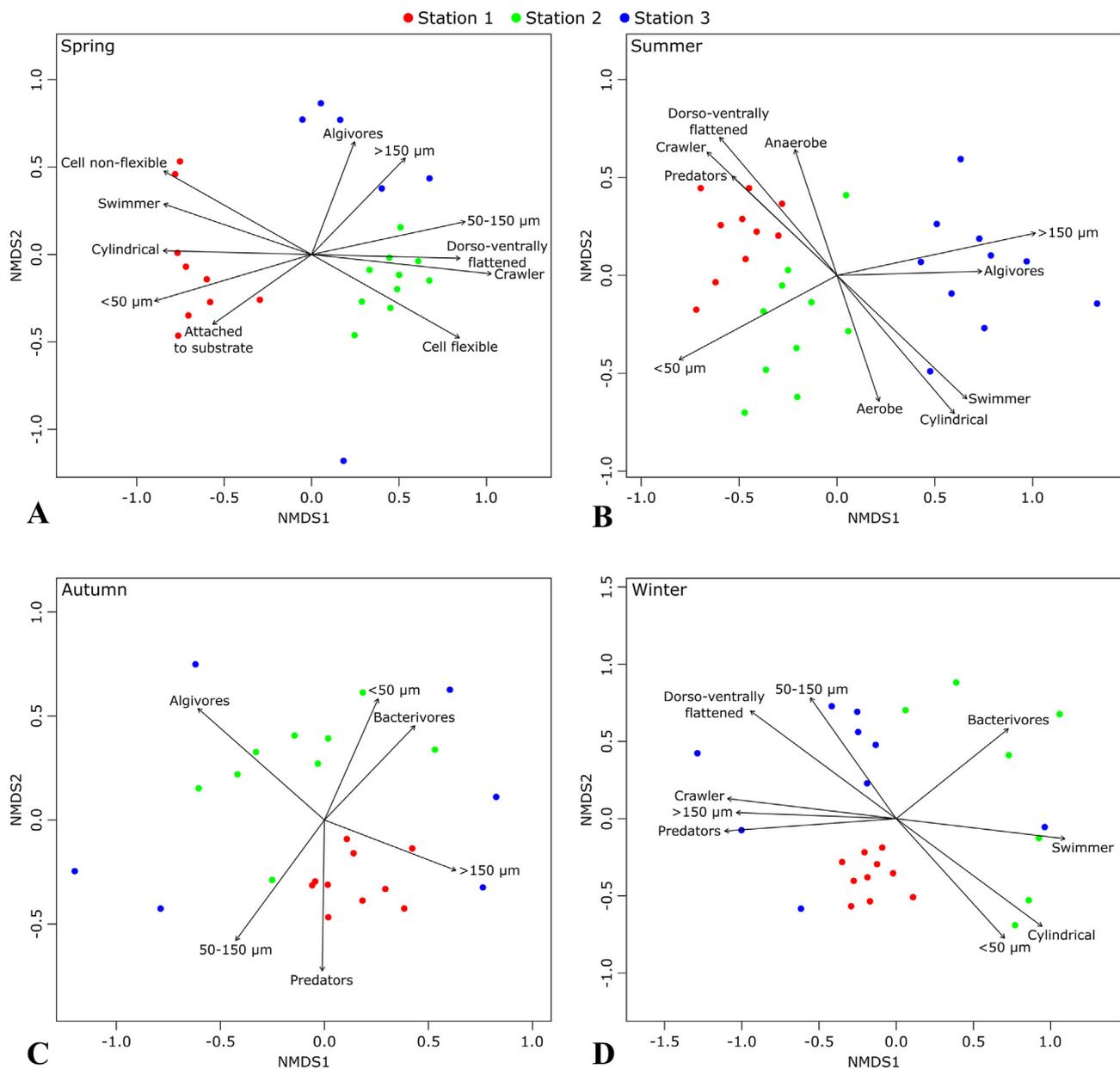


Fig. 3. NMDS ordination plots coded for three sampling stations based on ciliate abundance dataset for each season (A, spring; B, summer; C, autumn; D, winter). The length and direction of each vector indicate the strength and significance of the relationship between biological trait categories and axes based on environmental fitting (only vectors with significant correlation were represented, see Suppl. Table 4 for more information).

environmental variables. No typical interstitial ciliates, i.e. long and worm-shaped trachelocercids were found in current study. This could be due to the habitats we studied were from freshwater to brackish water with the salinity too low for these marine ciliates.

4.2. Functional diversity indices comparison

The three functional diversity indices considered in our study were incongruent in detecting relationships between functional structure and environmental conditions. Although these measures are indispensable to provide a complete quantification of functional diversity (Mouchet et al., 2010), FDiv has an advantage over FRic and FEve, as well as classical diversity indices such as d , J , and H' for environmental status assessments based on our study. Also, Gusmao et al. (2016) failed to reveal differences in community functional structure across pollution gradients using FRic and FEve of macrobenthic communities, while FDiv performed well to characterize the pollution impact. According to

former studies (Mason et al., 2005; Mouchet et al., 2010), FRic is mostly affected through the addition or removal of species with unique biological trait categories, and tends to monotonically change with species richness (see also Weisse, 2017). However, under certain circumstances, species abundance is more likely to be affected than the presence or absence of a species, e.g. at moderate level of disturbance, limiting the power of FRic (Gusmao et al., 2016). FEve, which measures the regularity of the distribution of abundance in the functional space (Villéger et al., 2008; Weisse, 2017), tends to have similar variation trend as species evenness index (Xu et al., 2018). FDiv is highly influenced by the functional dissimilarities among numerically dominant species and has important corollary for ecosystem dynamics (Mouchet et al., 2010; Weisse, 2017). Therefore, it could be more sensitive to detect environmental changes.

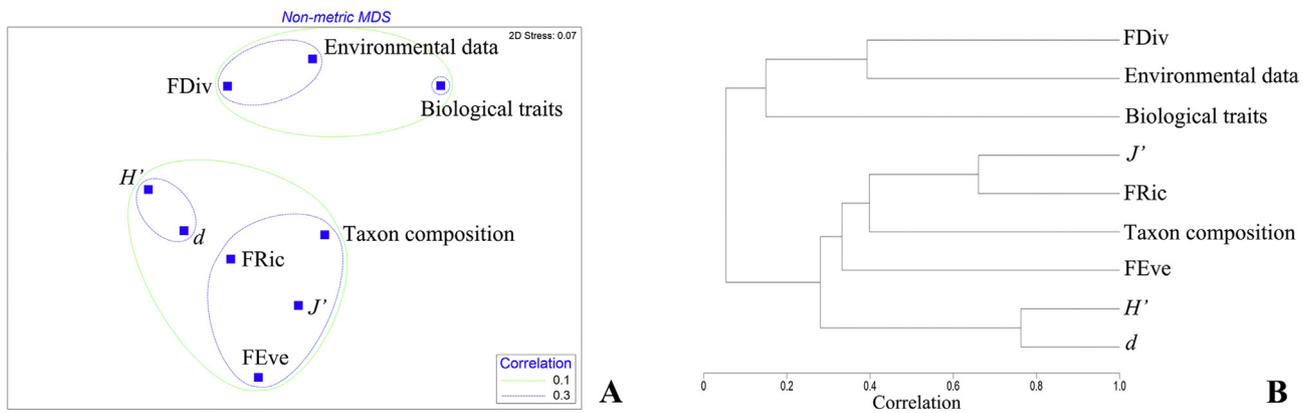


Fig. 4. Second-stage NMDS ordination (A) and clustering analyses (B) of Spearman matrix correlations between every pair of nine resemblance matrices, calculated from environmental data, species abundance, biological traits, three classical diversity indices, and three functional diversity measures. *d*, species richness; FDiv, functional divergence FEve, functional evenness FRic, functional richness; *H'*, Shannon-Wiener index; *J'*, species evenness.

Table 2

Ten best results of BIO-ENV analysis between environmental factors and FDiv of benthic ciliate communities obtained from the three sites of the Yangtze Estuary over four seasons. p value, Spearman correlation coefficient; SED, Sediment grain size; SAL, salinity; T, temperature; TN, total nitrogen; TOC, total organic carbon. $p < 0.05$ (global BEST test).

p Value	Environmental factors
0.563	NH ₄ ⁺ , TN, T, SAL
0.552	TN, SED, T, SAL
0.549	TN, T, SAL
0.548	SiO ₃ ²⁻ , TN, T, SAL
0.548	NO ₃ ²⁻ , NH ₄ ⁺ , TN, SED, T, SAL
0.544	NH ₄ ⁺ , TN, SED, T, SAL
0.534	NH ₄ ⁺ , SiO ₃ ²⁻ , TN, SED, T, SAL
0.529	NH ₄ ⁺ , SiO ₃ ²⁻ , TN, T, SAL
0.529	NH ₄ ⁺ , TN, TOC, SED, T, SAL
0.526	NO ₃ ²⁻ , NH ₄ ⁺ , SiO ₃ ²⁻ , TN, T, SAL

4.3. Response of functional diversity to environmental factors

In current study, the distinct patterns of traits and community compositions in different seasons is indicative of an environmental selection, and both biological traits analysis and FDiv indicated that functional diversity of benthic ciliate communities were strongly correlated to environmental variables, i.e. salinity, temperature, and nutrients (especially ammonia and TN).

Salinity is a key factor in regulating fishes, macrobenthos and protozoa community structure, species composition and distribution patterns, especially in estuarine environment (Darr et al., 2014; Forster et al., 2012; Hamels et al., 2004; Kchaou et al., 2009; Mazei and Burkovsky, 2006; Mouillot et al., 2007). Our research revealed that the relationship between salinity and functional diversity of ciliates were mainly affected by the feeding type structure. This gives reason to assume that the regulation of the functional structure of ciliate communities can be ascribed to the available food sources changing along salinity gradients in estuarine environments.

Temperature has also been documented to be strongly related to protists species distribution (Seeleuthner et al., 2018; Zhao et al., 2016) and functional diversity of fish communities (Mason et al., 2008) due to tightly link to primary productivity and metabolic activity (Patterson et al., 1989). Our findings suggested that temperature/functional diversity relationships of ciliates were mainly affected by the composition of oxygen preference (see the discussion above) and body size. In experimental studies, considerable changes in cell size of ciliates with temperature have been noticed for some time (Atkinson, 1994; Lee and Fenchel, 1972; Weisse, 2017), and increasing in temperature changed

ciliates cell size by a factor of 2–4 (Weisse and Montagnes, 1998) which may explain the positive correlation noticed in our study.

Based on our study, the relationship between nutrients, such as ammonia and TN, and functional diversity of benthic ciliates was affected by traits including feeding type, body size, movement type, and oxygen preference. During studying on functional diversity of testate amoebae community, Arrieira et al. (2015) found that nutrients, e.g. ammonia and TN, were the main predictors of FDiv and supposed this can be attributed to the association of those nutrients with the environment's productivity. Another potential connection could be the function of protozoa in benthic metabolism. Several laboratory experiments in microcosms suggested that protozoa, like ciliates, could have an important effect on the nitrogen cycle through mineralizing nitrogen and excreting ammonia (Clarholm, 1985; Coûteaux and Darbyshire, 1998; Palka, 1988; Stout, 1980; Woods et al., 1982).

In general, it is a major challenge to identify the link between environmental variables and biological traits even if strong correlations between tested parameters are detected (Bremner et al., 2006). Therefore, further experimental investigations are needed to provide solid causal conclusions (Weisse, 2017).

4.4. Implementation of functional diversity of ciliates in routine monitoring programs

In current study, both second-stage NMDS ordination and RELATE analyses revealed the close relationship between functional diversity measured by FDiv and environmental conditions. Former studies also pointed out that functional diversity of ciliates could be higher in a turbid lake compared with clear water lake and high FDiv may be indicative of disruptive selection (Coutinho et al., 2016; Weisse, 2017). Although FDiv index has the potential to be used as environmental indicator, researches on functional diversity of ciliates are still scarce and available cases and data are inadequate to develop a mature monitoring framework. To implement functional diversity of benthic ciliates in routine environmental assessment, we suggest that future studies on using ciliate communities as environmental bio-indicators to include functional diversity, especially FDiv as a parameter and compare it to benchmark indices obtained from macrofauna of the same samples, such as AMBI (AZTI Marine Biotic index, Borja et al., 2000) and taxonomic diversity indices (Warwick and Clarke, 1995). Gusmao et al. (2016) compared the functional diversity indices obtained from macrobenthic communities in polluted sites to the AMBI index. In their study, a significant relationship between functional diversity and AMBI was revealed, which indicates functional diversity of macrobenthos is closely related to benthic environmental health. While, Stoeck et al. (2018) found that benthic ciliates could serve better in detecting the

degree of impact at fine-scale than AMBI index through metabarcoding method. Therefore, further research on comparing functional diversity of benthic ciliates to AMBI index would be necessary to fully exploit its indicator potential.

In future, we suggest to use multivariate techniques to develop the monitoring system instead of univariate index. The advantages of multivariate approaches have been discussed thoroughly by Clarke et al. (2014) including genuine reflecting of changing in biodiversity and compatibility across studies from widely separated locations and with different sampling effort. One example is the taxonomic diversity indices based on macrofauna developed by Warwick and Clarke (1995). Xu et al. (2016) has shown that taxonomic diversity of planktonic ciliates can be used in discriminating water quality status. Although there is a close relationship between functional diversity and taxonomic diversity since wider taxonomic trees should support a wider range of species functions (Dimitriadis and Koutsoubas, 2011), compared with taxonomic diversity based on Linnean classification tree, functional diversity measuring biological traits directly should be more closely related to ecosystem functions and environmental status. Referring to the monitoring framework using taxonomic diversity indices, a master inventory employed from global or local geographic species list will be used to set the background context (Clarke et al., 2014). Then, compare the true functional diversity against the expect range constructed from master species list and falling outside the central 95% of the simulated values indicates departing significantly from expectation (Clarke et al., 2014). In general, these further researches will have to be done before the functional diversity of benthic ciliates can be applied for environmental status assessments in routine monitoring programs.

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