

Contrasting responses of intertidal microphytobenthos and phytoplankton biomass and taxonomic composition to the nutrient loads in the Jiulong River Estuary

Yuyuan Xie , Lei Wang,[†] Xin Liu, Xin Li,[‡] Yuanyong Wang and Bangqin Huang*

Key Laboratory of the Coastal and Wetland Ecosystems, Ministry of Education/The Fujian Provincial Key Laboratory for Coastal Ecology and Environmental Studies, Xiamen University, Xiamen, China

SUMMARY

Microphytobenthos (MPB) and phytoplankton are important primary producers in the estuarial ecosystem, and their functions are critical to the ecosystem's biodiversity and environmental safety. The aim of this study was to compare the response of MPB and phytoplankton to the nutrient loads in a eutrophic estuary, which has seldom been studied. We used high-performance liquid chromatography (HPLC) and CHEMTAX software to examine the biomass and taxonomic composition of both MPB and phytoplankton at Da-yu Island (DYI) and Ji-yu Island (JYI) in the Jiulong River Estuary from July 2010 to March 2012. The results showed that MPB chlorophyll *a* was low in the summer and high in the winter at both DYI and JYI, indicating a unimodal pattern. However, the phytoplankton chlorophyll *a* showed a mirrored pattern. Diatoms were the dominant class in both benthic and pelagic environments. Although redundancy analysis indicated that the effects of different environmental factors could not be easily separated, it is likely that phosphate and temperature were the most important factors regulating the seasonal patterns of MPB and phytoplankton diatoms, respectively. MPB and phytoplankton cyanobacteria was co-limited by salinity and temperature. The high N/P ratio and low phosphate favored chlorophytes and cyanobacteria. Our study demonstrates the use of HPLC and CHEMTAX in an integrated survey of the spatial and temporal distribution patterns of MPB and phytoplankton in an estuarial ecosystem. The contrasting responses of MPB and phytoplankton to nutrient loads indicate the critical role of MPB in subtropical estuarial ecosystem function. The relationship between nutrients and MPB may indicate a significant contribution to carbon and nutrient cycling.

Key words: CHEMTAX, estuary, HPLC pigments, intertidal zone, Jiulong River, Microphytobenthos, nutrients, phytoplankton.

INTRODUCTION

The estuarial ecosystem plays an important role in regulating biogeochemical carbon cycling. Most estuaries are heterotrophic because external supplies of organic matter from the land are respired there (Cai 2011). However, the magnitude of CO₂ degassing is determined by both respiration and primary production (Cai *et al.* 2006). Phytoplankton in the ocean contributes nearly half of annual primary production in the world, with only 0.2% of the autotrophic biomass (Field *et al.* 1998). However, in estuaries, phytoplankton primary

production is often light-limited when the water column turbidity is high (Gameiro *et al.* 2011; Cloern *et al.* 2014). Microphytobenthos (MPB) living in the sedimentary environment is the major primary producer in the intertidal zones (MacIntyre *et al.* 1996). It has been shown that MPB can develop a biomass exceeding that of phytoplankton by up to a factor of 10 and contribute more than half of the primary production throughout the water column in many estuaries (MacIntyre *et al.* 1996; Underwood & Kromkamp 1999; Perissinotto *et al.* 2002). A data compilation has been published of phytoplankton primary production in 131 estuaries all over the world, showing very high variability across the sites, within an ecosystem or from year to year (Cloern *et al.* 2014). However, there are fewer data on MPB, and thus it is difficult to assess the distribution pattern of MPB primary production and its contribution to estuarial ecosystem services. River estuaries tend to be zones of high productivity for a large number of organisms (Underwood & Kromkamp 1999). It has been suggested that MPB other than detrital organic matter is a major food source in the benthic food web, and benthic primary production is important for sustaining biodiversity and ecosystem health (Yokoyama *et al.* 2005). Within estuaries, both phytoplankton and MPB take up nutrients through photosynthesis, and a previous study estimated that benthic primary production removed about 4% of the nitrogen load in the Severn estuary in the United Kingdom (Underwood 2010). This can be a significant amount of nutrient reduction when the eutrophication level is very high. Phytoplankton and MPB can also act as filters of toxicants (Veltman *et al.* 2005). Thus, it is necessary to conduct integrated research on phytoplankton and MPB in estuaries, and primary production can then be estimated at the system level, which is crucial to a good understanding of estuarial ecosystem diversity and functioning.

High-performance liquid chromatography (HPLC) has been used as an efficient tool for measuring the taxonomic composition of phytoplankton and MPB (Barranguet *et al.* 1997;

*To whom correspondence should be addressed.

Email: bqhuang@xmu.edu.cn

Communicating Editor: Nishihara Gregory

Received 24 July 2017; accepted 25 October 2018.

[†]Present address: Third Institute of Oceanography SOA, Xiamen, China

[‡]Present address: Patent Examination Cooperation Center of the Patent Office, SIPO, Guangdong, China

Huang *et al.* 2010). With this method, the concentrations of tens of pigments including chlorophylls, carotenes, and carotenoids can be determined. Some of these pigments are indicative of particular phytoplankton or MPB classes. The MPB community usually consists of species of diatoms, dinoflagellates, Cyanophyta, Euglenophyta, Chlorophyta, and Cryptophyta (Barranguet *et al.* 1997; Brotas & Plante-Cuny 1998). For example, fucoxanthin is the diagnostic pigment of diatoms, alloxanthin of Cryptophyta, and zeaxanthin of Cyanophyta. By assuming the diagnostic pigment to chlorophyll *a* ratios are constant, the chlorophyll *a* concentrations of all phytoplankton or MPB classes can be calculated using CHEMTAX software (Mackey *et al.* 1996). Another study showed that chlorophyll *a* concentrations calculated with CHEMTAX have good agreement with microscopy count data, and this method is applicable to research on the change in MPB taxonomic composition with environmental factors (Mendes *et al.* 2017). Cartaxana *et al.* (2006) found that the MPB chlorophyll *a* concentration was higher in muddy sediment than in sandy sediment, but the results from pigment analysis did not show significant differences in taxonomic composition, and diatoms dominated in both sites. Nutrients are a major limiting factor that controls MPB biomass and primary production. Treatment experiments showed that enrichment with nitrogen and phosphorus increased the MPB standing crop and altered taxonomic composition across sites with different nitrogen loading rates (Hillebrand & Sommer 1997; Lever & Valiela 2005).

Increased inputs of nitrogen and phosphorus to the sea have resulted in eutrophication becoming a serious risk along the China coast (Stokal *et al.* 2014). In the Jiulong River Estuary located on the southeast China coast, macronutrients had reached eutrophication levels owing to significant human-induced nutrient enrichment of the Jiulong River over the past 30 years (Yan *et al.* 2012; Chen *et al.* 2013). The increasing frequency of harmful algal blooms has been correlated with the elevated riverine nutrient input (Chen *et al.* 2013). However, the impacts of eutrophication on the benthic ecosystem have seldom been studied. Some previous studies were conducted in the Jiulong River estuary and adjacent Xiamen Bay using HPLC and CHEMTAX to examine the phytoplankton composition (Xu *et al.* 2001; Chen *et al.* 2003; He & Peng 2012), but there has been no similar study conducted on the MPB. In this study, we aimed to compare the different responses of intertidal MPB and phytoplankton biomass and taxonomic composition with the nutrient loads in the Jiulong River Estuary. This improved knowledge will be crucial for understanding the processes of carbon and nutrient cycling within this subtropical estuary, which is a key question relating to the ocean carbon budget in marginal seas.

MATERIALS AND METHODS

Study sites and sampling

Xiamen Bay is a semi-enclosed gulf that is adjacent to the Taiwan Strait. More than 2 million people live on the central island (Xiamen Island) and in the northern coastal area. The Jiulong River flows into the bay from the southwest with an

average discharge of about $469 \text{ m}^3 \text{ s}^{-1}$. A high nutrient load is carried by the river water: $120\text{--}230 \mu\text{mol L}^{-1}$ of nitrate, $1.2\text{--}3.5 \mu\text{mol L}^{-1}$ of phosphate, and $200\text{--}340 \mu\text{mol L}^{-1}$ of silicate (Yan *et al.* 2012). Ji-yu Island (JYI) and Da-yu Island (DYI) are located near the river mouth (Fig. 1); the straight-line distance between the two islands is 4.4 km, and the areas of JYI and DYI are 0.36 and 0.18 km^2 , respectively. Both islands are uninhabited and serve as a protected egret natural reserve according to legal regulations (The Standing Committee of Xiamen City People's Congress, 1995). The tidal cycle is fortnightly in this area. The tidal velocity is higher around JYI than DYI with the ebb and flow of the spring tide (Ji 2006). The wet seasons in the Jiulong River were from May to September, and the dry seasons were between October and April of the following year (Chen & Hong 2010; Yan *et al.* 2012).

To assess phytoplankton, 100 mL of river water samples were collected at both JYI and DYI once a month during the spring tide. These were filtered through 25-mm-diameter glass fiber filters, and the filters were stored at $-80 \text{ }^\circ\text{C}$ before pigment analysis. The auxiliary parameters were measured as follows. Water temperature and salinity were determined with a multi-parameter water quality monitoring instrument (YSI 6600-D, Xylem Analytics, USA). One hundred milliliters of water samples were also collected and filtered through $0.45 \mu\text{m}$ polycarbonate filters (Millipore, USA). Then the concentrations of nitrate and phosphate were measured using a nutrient auto-analyzer (Bran Luebbe AA3, GmbH, Germany) with standard colorimetric methods. We did not measure silicate. Based on the results of Yan *et al.* (2012), the concentrations of silicate were higher than nitrate and silicate is not supposed to be a limiting factor in the Jiulong River Estuary.

For MPB, sediment samples were collected in the mudflats on the ebb. The top 1 cm surface samples were collected for pigment analysis, using a PVC collector (7 cm in diameter), and preserved in an ice box then a $-20 \text{ }^\circ\text{C}$ freezer in the lab. Previous studies showed that the distribution of MPB is vertically heterogeneous and that MPB is able to migrate vertically in relation to light and tidal cycles (Mitbavkar & Anil 2004). However, the photic zone is very thin in the sedimentary environment (MacIntyre *et al.* 1996), and thus a 1 cm layer should be sufficient to collect most of the photosynthetically

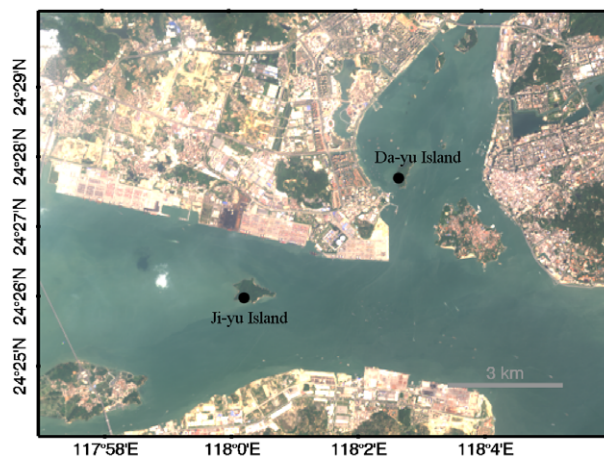


Fig. 1. The locations of sampling sites. [Color figure can be viewed at wileyonlinelibrary.com]

active MPB. In addition, replicate sediment samples were collected every 6 months for grain size analysis conducted with a laser granulometer (Malvern Masterizer 2000, Malvern Instruments, UK).

Pigment analysis

First, a slice of sediment (about 3–5 g) was taken from the preserved sample and freeze-dried at $-70\text{ }^{\circ}\text{C}$ for 15–20 h. Afterwards, the sample slice was immersed in 5 mL *N,N*-dimethylformamide (DMF), stirred and sonicated before 2 h of extraction. Meanwhile, the filters (phytoplankton samples) were also immersed in 5 mL DMF for 2 h of extraction. The extraction was then centrifuged at 1000 g for 3 min, and the supernatant was carefully transferred and filtered through a glass-fiber filter (Whatman, USA) and then mixed 1:1 (v:v) with 1 mol L^{-1} ammonium acetate. Then the mixture was injected into an Agilent 1100 Series system with a $3.5\text{ }\mu\text{m}$ Eclipse XDB C8 column (Agilent Technology, USA) for pigment analysis. The ingredients of solvents and gradient elution procedure were determined following the method of Huang *et al.* (2010). Briefly, solvent A was 80:20 (v/v) of methanol and 1 mol L^{-1} ammonium acetate, and solvent B was methanol. The gradient elution procedure was set to reach 45% solvent B at 16 min, and 100% solvent B at 100 min. Both the retention time and absorption spectra shape of each peak were recorded to determine the type of pigment. The peak areas were corrected to the pigment concentrations based on the standard curves of the purchased standards (DHI Water and Environment, Denmark) that were determined before the sample analysis. In this study, the concentrations of chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), fucoxanthin (Fuco), alloxanthin (Allo), zeaxanthin (Zeax), and peridinin (Peri) were above detection limits, and their concentrations were determined for the following analysis.

Compared with a previous similar study conducted in Xiamen Bay in 2001 (He & Peng 2012), we detected fewer types of pigment, while they also detected 19'-hexanoyloxyfucoxanthin and 19'-butanoyloxyfucoxanthin. But these two pigments were not found in another similar study conducted in 1995 (Xu *et al.* 2001), or at least not all the stations in Xiamen Bay (Chen *et al.* 2003). This difference may imply great spatial and temporal variation among phytoplankton classes in the coastal area. We also used a relatively large volume (5 mL) of DMF for the pigment extraction, because of the high load capacity for the sediment sample. This could be detrimental to the phytoplankton sample, as it lowers the final amount of pigments examined by HPLC and thus lowers the sensitivity to some less abundant pigments.

MPB and phytoplankton species composition calculated by CHEMTAX

CHEMTAX software was used to calculate the relative contribution of phytoplankton and MPB classes to total Chl *a* (Mackey *et al.* 1996). Based on the six pigments identified above, five phytoplankton or MPB classes were loaded on CHEMTAX: diatoms, cryptophytes, chlorophytes, cyanobacteria, and dinoflagellates. The seed ratios between diagnostic pigment and Chl *a* used as input for the CHEMTAX calculation were obtained from the literature. Latasa (2007) found that

successive runs of CHEMTAX using the output ratios as the input ratios could achieve convergent output ratios approaching true values, and therefore he suggested this procedure to achieve adjusted seed ratios before calculating the classes. We followed the procedure of 10 successive runs using different initial seed ratio matrices for the adjusted seed ratios (data not shown). However, some of the convergent ratios largely deviated from the adequate ranges that were summarized by Higgins *et al.* (2011). Moreover, for phytoplankton, two ratios among others could not reach convergence and became unreasonably large or small. Mackey *et al.* (1996) has pointed out that CHEMTAX calculation optimizes the major classes at the expense of the minor classes. The successive runs even magnify the errors in the minor classes that is reflected on the strange seed ratios. Alternatively, Wright *et al.* (2009) proposed another approach to obtain a reliable CHEMTAX result, and in the present study, we followed a modified procedure (Xie *et al.* 2018) as follows. With the average (**Avg**), maximum (**Max**), and minimum (**Min**) seed ratios in Table 1, we randomly generated 60 seed ratio matrices with the equation $\text{SR} = \text{Avg} + \text{D} \times \text{R}$, where **R** is the random number between -1 and 1 , and **D** is $\text{Max} - \text{Avg}$ or $\text{Avg} - \text{Min}$ depending on whether **R** is positive or negative. Then we obtained 60 results using the 60 seed ratio matrices as input for MPB and phytoplankton separately. The six best results were averaged to obtain final class concentrations, and averaged output ratios were given for quality assessment of CHEMTAX results.

Statistical analysis

Paired *t*-test or one-way ANOVA was used to compare the data means between two groups. The correlation between the environmental factors and pigment concentrations was measured by Pearson's correlation coefficient at the significance level of 0.05. The effects of environmental gradients on the community structure of MPB and phytoplankton was examined by redundancy analysis (RDA). All statistical analyses were conducted with R software (version 3.3.1).

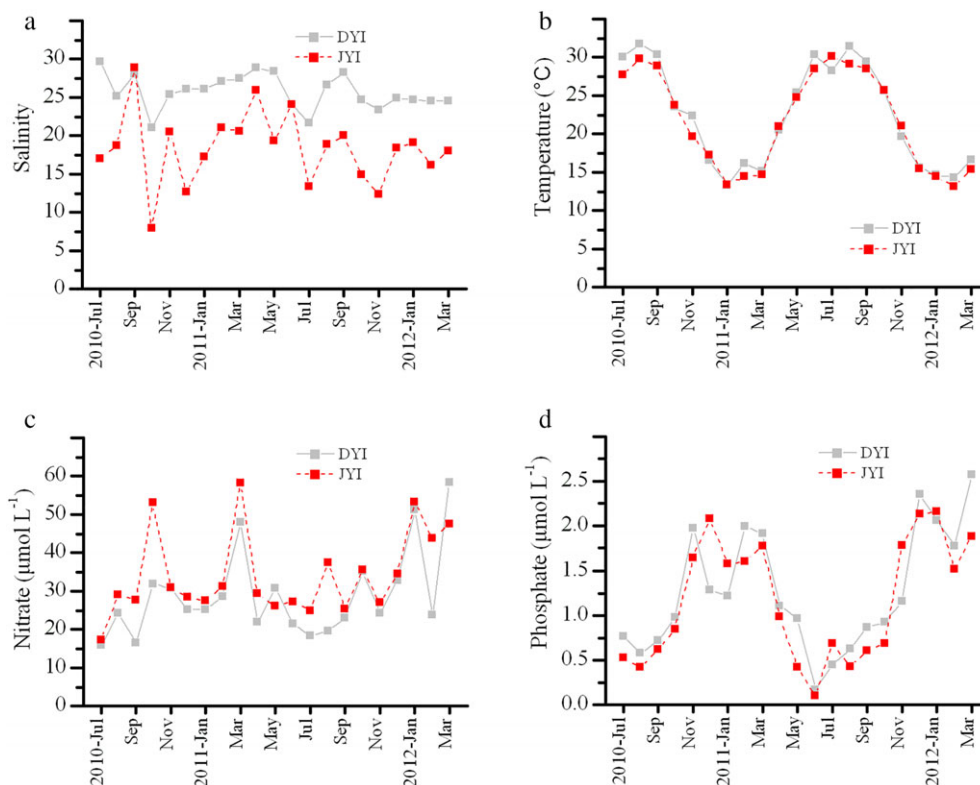
RESULTS

Environmental settings

Grain size analysis showed that there was little difference in the composition of surface sediments between the two sampling sites. They both had a high content (more than two thirds) of silty sand and very little sand (Table 2). Water salinity varied at around 25.7 (between 21.1 and 29.7) at DYI and around 18.3 (between 7.9 and 28.9) at JYI (Fig. 2a), which reflects the upstream location of JYI in the Jiulong River Estuary (Fig. 1). However, the water temperature was similar between DYI and JYI, and showed clear seasonal cycles with the lowest (around $15\text{ }^{\circ}\text{C}$) in the winter and highest (around $30\text{ }^{\circ}\text{C}$) in the summer (Fig. 2b). The nitrate concentrations were lower at JYI than at DYI in some months (Fig. 2c), while the phosphate concentrations were quite similar between DYI and JYI (Fig. 2d). Considering the data together, nitrate varied between 15.7 and $59.3\text{ }\mu\text{mol L}^{-1}$ (Fig. 2c), and phosphate

Table 1. The seed ratios used for CHEMTAX calculation

	Fuco	Allo	Zeax	Chl <i>b</i>	Peri	Chl <i>a</i>
a. Average (Higgins <i>et al.</i> 2011)						
Diatoms	0.620	0	0	0	0	1
Cryptophytes	0	0.380	0	0	0	1
Chlorophytes	0	0	0.032	0.320	0	1
Cyanobacteria	0	0	0.640	0	0	1
Dinoflagellates	0	0	0	0	0.560	1
b. Maximum (Higgins <i>et al.</i> 2011)						
Diatoms	1.710	0	0	0	0	1
Cryptophytes	0	0.791	0	0	0	1
Chlorophytes	0	0	0.138	0.546	0	1
Cyanobacteria	0	0	1.721	0	0	1
Dinoflagellates	0	0	0	0	1.028	1
c. Minimum (Higgins <i>et al.</i> 2011)						
Diatoms	0.191	0	0	0	0	1
Cryptophytes	0	0.163	0	0	0	1
Chlorophytes	0	0	0.001	0.178	0	1
Cyanobacteria	0	0	0.076	0	0	1
Dinoflagellates	0	0	0	0	0.285	1

**Fig. 2.** The changes of (a) salinity, (b) temperature, (c) nitrate, and (d) phosphate during the study period. [Color figure can be viewed at wileyonlinelibrary.com]

varied between 0.08 and 2.58 $\mu\text{mol L}^{-1}$ (Fig. 2d). Generally, nutrients—especially phosphate—were higher from October to April of the following year. The N/P (nitrate/phosphate) ratios varied between 13.5 and 86.4 at JYI, and between 13.4 and 42.2 at DYI, both excluding the point in June 2011 when the N/P ratio was 276.0 and 126.7, respectively, owing to very low phosphate concentrations. The N/P ratios at JYI were lower from October to April of the following year than in the period between May and September.

Variation of MPB pigments

The Chl *a* concentrations of MPB varied between 12.30 and 108.20 mg m^{-2} at DYI and between 4.19 and 48.97 mg m^{-2} at JYI. The former ($41.88 \pm 29.77 \text{ mg m}^{-2}$) was significantly higher than the latter ($22.16 \pm 14.59 \text{ mg m}^{-2}$) over the sampling period ($P < 0.001$, paired *t*-test; Fig. 3). Both showed higher Chl *a* from December to April of the following year than in the other months ($F = 29.49$, $P < 0.001$ and

Table 2. Compositions of surface sediment (top 1 cm) in the two sampling sites

Site	Date	Sand (>63 μm)%	Silty sand (4–63 μm)%	Clay (<4 μm)%
DYI	July 2010	0.51	69.5	22.99
	January 2011	8.72	73.82	17.47
	July 2011	1.68	71.74	26.57
	January 2012	8.1	69.29	22.61
JYI	July 2010	7.46	74.04	17.49
	January 2011	8.43	73.72	17.85
	July 2011	15.79	68.11	16.1
	January 2012	5.71	76.96	17.32

$F = 65.75$, $P < 0.001$ at DYI and JYI, respectively; one-way ANOVA). The Fuco concentrations shared a similar seasonal pattern with Chl *a* (Figs 3 and 4a), and Fuco was also significantly higher at DYI than at JYI ($P < 0.001$, paired *t*-test). The Allo concentrations were mostly lower than 1.0 mg m^{-2} , an order of magnitude lower than those of Fuco, and occasionally absent (Fig. 4b). Although Allo was also relatively high during the period between February 2011 and April 2011, this pattern did not repeat in the same period of 2012. The Zeax concentrations were also mostly lower than 1.0 mg m^{-2} , but peaked in the months between May and October in the case of JYI (Fig. 4c). Chl *b* was only detected in May 2011 and June 2011 at both DYI and JYI; however, the concentrations were all $>2.0 \text{ mg m}^{-2}$ (Fig. 4d).

Comparison between MPB and phytoplankton pigments

Phytoplankton Chl *a* concentrations were high in the summer and low in the winter at both DYI and JYI (Fig. 3). The highest

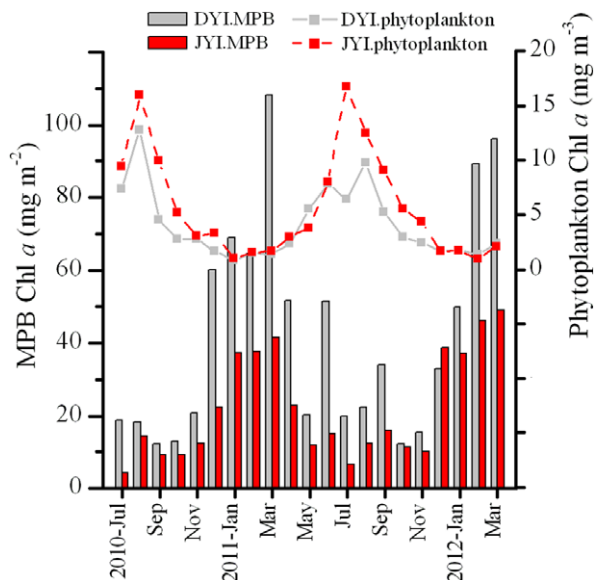


Fig. 3. The changes of chlorophyll *a* concentrations of microphytobenthos and phytoplankton during the study period. [Color figure can be viewed at wileyonlinelibrary.com]

concentrations were 12.77 and 16.73 mg m^{-3} at DYI and JYI, respectively. The lowest concentrations were 0.84 and 0.94 mg m^{-3} , respectively. Fuco showed a similar seasonal pattern to Chl *a* (Fig. 4a). The Fuco concentrations at DYI were from 0.24 to 1.56 mg m^{-3} , while at JYI they were from 0.19 to 1.93 mg m^{-3} . Allo did not show a clear seasonal pattern, with the highest concentrations at $<0.4 \text{ mg m}^{-3}$, and it was absent in some months especially at JYI (Fig. 4b). Zeax was absent in more than half of the months at both DYI and JYI, and it showed high concentrations in July and August only at JYI (Fig. 4c). In contrast to MPB, phytoplankton Chl *b* was detected more frequently, but the concentrations were low (Fig. 4d). Peri was detected in phytoplankton, but not in MPB, and only between March and May of 2011 (Fig. 4e).

MPB Chl *a* was negatively correlated with phytoplankton Chl *a* at DYI ($P < 0.05$, Pearson's correlation), while there was no significant correlation for the other four pigments. At JYI, Chl *a* and Fuco showed significant negative correlations between MPB and phytoplankton while Zeax and Chl *b* showed significant positive correlations ($P < 0.05$, Pearson's correlation).

CHEMTAX output ratios

Compared with the average ratios (Table 1), MPB diatoms in this study showed the lower Fuco/Chl *a* ratio, while cryptophytes, chlorophytes, and cyanobacteria all had higher than average diagnostic pigment to Chl *a* ratios (Table 3). In contrast, for phytoplankton, the Allo/Chl *a* ratio of cryptophytes and the Zeax/Chl *a* ratio of chlorophytes were lower than average. The Chl *b*/Chl *a* ratio of chlorophytes and the Zeax/Chl *a* ratio of cyanobacteria were close to the averages, but the Fuco/Chl *a* ratio of diatoms was still lower. Most ratios were within the ranges shown in Table 1, except the Chl *b*/Chl *a* ratio of MPB chlorophytes and the Peri/Chl *a* ratio of phytoplankton dinoflagellates were dramatically high.

MPB and phytoplankton class composition and relationships with environmental factors

The CHEMTAX calculation showed that the MPB at both DYI and JYI consist mostly of diatoms, while the other three classes were higher than 1 mg m^{-2} Chl *a* in some months (Fig. 5a,b). Diatoms was the most abundant phytoplankton class at both DYI and JYI; however, cryptophytes and chlorophytes also contributed to a significant portion of total phytoplankton Chl *a* throughout the year (Fig. 5c,d). Cyanobacteria appeared only in some months but contributed up to 20% of total phytoplankton Chl *a* in July or August at JYI (Fig. 5d). Dinoflagellates only appeared between March and May in 2011 (Fig. 5c,d).

Table 4 shows the correlation coefficients between environmental factors and the concentrations of MPB and phytoplankton classes. Temperature, phosphate, and N/P ratio are among the most influential factors. RDA analysis showed that the first environmental axis (ENV1) was primarily correlated with temperature, phosphate, and N/P ratio, while the second axis was related to the N/P ratio (Fig. 6). The ENV1 can explain 31.4% of the total variance. For MPB, diatoms showed positive correlations with the ENV1 at both DYI and

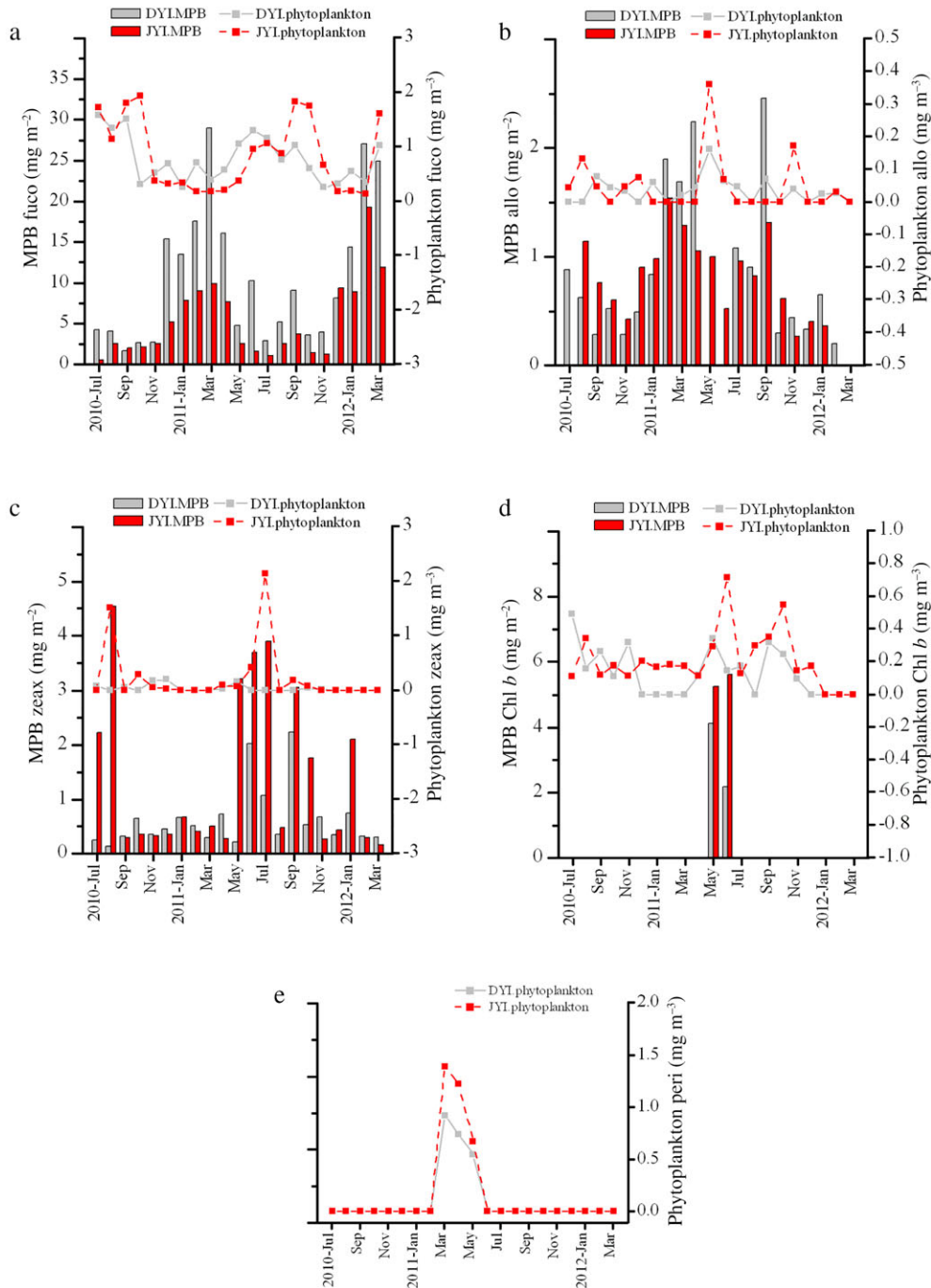


Fig. 4. The changes of (a) fucoxanthin, (b) alloxanthin, (c) zeaxanthin, (d) chlorophyll *b*, and (e) peridinin of microphytobenthos and phytoplankton during the study period. [Color figure can be viewed at wileyonlinelibrary.com]

JYL ($P < 0.05$, Pearson's correlation), while cyanobacteria negatively correlated with the ENV1 at JYL ($P < 0.05$, Pearson's correlation) (Fig. 7). For phytoplankton, diatoms, cryptophytes, and chlorophytes were negatively correlated with the ENV1 ($P < 0.05$, Pearson's correlation), and the slopes were similar between DYL and JYL; cyanobacteria changed with the ENV1 only at JYL (Fig. 8). The correlation between dinoflagellates and the ENV1 was insignificant.

DISCUSSION

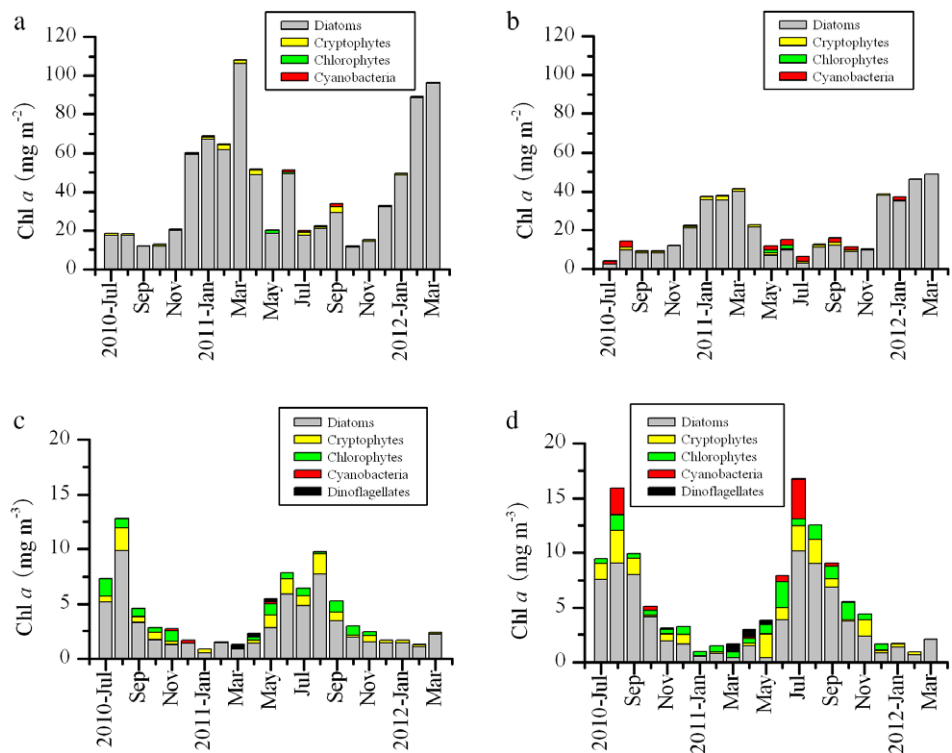
Diagnostic pigment to Chl *a* ratios for MPB and phytoplankton

Compared with the optical microscopy method, pigment chemotaxonomy does not require additional examination for small-sized MPB or phytoplankton (Mendes *et al.* 2017).

Table 3. CHEMTAX output ratios. The number inside the bracket is the standard deviation

	Fuco	Allo	Zeax	Chl <i>b</i>	Peri	Chl <i>a</i>
a. MPB						
Diatoms	0.249 (0.001)	0	0	0	0	1
Cryptophytes	0	0.832 (0.059)	0	0	0	1
Chlorophytes	0	0	0.085 (0.057)	3.000 (0.220)	0	1
Cyanobacteria	0	0	1.349 (0.031)	0	0	1
Dinoflagellates	—	—	—	—	—	—
b. Phytoplankton						
Diatoms	0.327 (0.153)	0	0	0	0	1
Cryptophytes	0	0.140 (0.068)	0	0	0	1
Chlorophytes	0	0	0.008 (0.006)	0.318 (0.061)	0	1
Cyanobacteria	0	0	0.858 (0.462)	0	0	1
Dinoflagellates	0	0	0	0	2.059 (0.438)	1

Fig. 5. The changes of taxonomic composition during the study period, (a) microphytobenthos in DYI, (b) microphytobenthos in JYI, (c) phytoplankton in DYI, and (d) phytoplankton in JYI. [Color figure can be viewed at wileyonlinelibrary.com]



By using HPLC and CHEMTAX, less abundant MPB classes like cyanobacteria, chlorophytes, and cryptophytes have been easily discovered (Barranguet *et al.* 1997; Brotas & Plante-Cuny 1998). There may be a trade-off between the examination of bulk composition and the reliable identification of large-cell species. However, previous studies have shown significant correlations between optical microscopy counts and CHEMTAX calculated concentrations of MPB (Mendes *et al.* 2017) and between flow cytometry counts and CHEMTAX calculated concentrations of phytoplankton (Liu *et al.* 2012). For CHEMTAX, the optimization of diagnostic pigment to Chl *a* ratios is a key issue for obtaining quality output data. The ratios of accessory pigments to Chl *a* are associated with the photoacclimation process and the photoprotection function of MPB and phytoplankton (MacIntyre *et al.* 2002). Comparison

of diagnostic pigment to Chl *a* ratios was made between MPB and phytoplankton in this study (Table 3).

Allo and Zeax play important roles in photoprotection (MacIntyre *et al.* 2002), which can explain their higher ratios to Chl *a* in the intertidal environment than in the water. When the sediment surface is exposed to a high dose of sunlight, the synthesis of photoprotective pigments will protect MPB in maintaining photosynthesis at a relatively steady rate. In contrast, the lower than average Allo/Chl *a* and Zeax/Chl *a* ratios of phytoplankton may imply a state of photoacclimation to the low light availability in the turbid estuarine water. The dramatically high Chl *b* to Chl *a* ratio of MPB chlorophytes was suspicious. It may not reflect the actual ratio in living cells because the excess Chl *b* could come from the terrestrial debris. Similarly, the dramatic high Peri/Chl *a* ratio of phytoplankton

Table 4. The Pearson's correlation coefficients between the environmental factors and the concentrations of MPB and phytoplankton classes

	Temperature	Salinity	Nitrate	Phosphate	N/P
DYI-MPB					
Diatoms	-0.697*	0.094	0.522*	0.582*	-0.097
Cryptophytes	0	0.408	-0.163	-0.058	-0.241
Chlorophytes	0.226	0.160	-0.042	-0.273	0.480*
Cyanobacteria	0.287	-0.162	-0.227	-0.398	0.594*
DYI-Phytoplankton					
Diatoms	0.809*	0.020	-0.403	-0.640*	0.474*
Cryptophytes	0.745*	-0.077	-0.419	-0.688*	0.502*
Chlorophytes	0.741*	0.315	-0.429	-0.539*	0.165
Cyanobacteria	0.110	0.273	-0.138	-0.049	-0.127
Dinoflagellates	-0.167	0.442*	0.211	0.078	-0.079
JYI-MPB					
Diatoms	-0.885*	0.079	0.570*	0.738*	-0.325
Cryptophytes	0.132	0.236	-0.121	-0.195	0.003
Chlorophytes	0.264	0.249	-0.223	-0.450*	0.772*
Cyanobacteria	0.623*	0.031	-0.364	-0.615*	0.489*
JYI-Phytoplankton					
Diatoms	0.858*	0.012	-0.363	-0.678*	0.226
Cryptophytes	0.740*	0.024	-0.504*	-0.603*	0.237
Chlorophytes	0.622*	0.147	-0.338	-0.659*	0.792*
Cyanobacteria	0.491*	-0.182	-0.226	-0.369	0.148
Dinoflagellates	-0.160	0.348	0.238	0.018	-0.076

The bold and asterisk indicate a significant correlation at the level $p < 0.05$. The number of sample (n) is 21

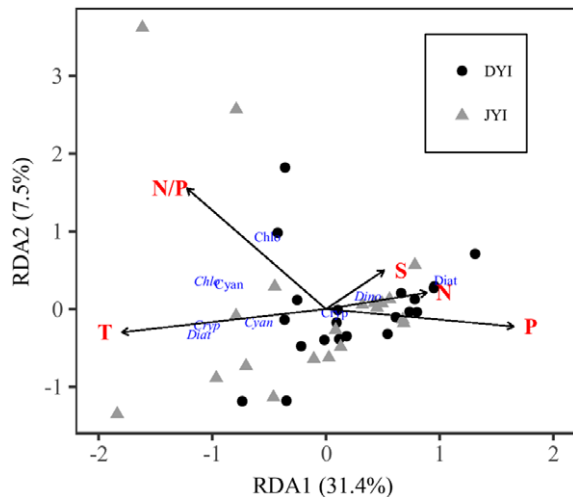


Fig. 6. The redundancy analysis (RDA) for microphytobenthos and phytoplankton (italic). The groups variables are: diatoms (Diat), cryptophytes (Cryp), chlorophytes (Chlo), cyanobacteria (Cyan), and dinoflagellates (Dino). The environmental variables are: temperature (T), salinity (S), nitrate (N), phosphate (P), and N/P ratio (N/P). [Color figure can be viewed at wileyonlinelibrary.com]

dinoflagellates may not be a true value, as CHEMTAX may output an unreliable result for minor classes (Mackey *et al.* 1996). The Fuco to Chl *a* ratio of diatoms is quite similar between MPB and phytoplankton, so Fuco can be used as a feasible indicator of diatoms. The lower than average Fuco to

Chl *a* ratio of diatoms is close to a previously reported ratio in the South China Sea (Wang *et al.* 2015).

The appropriate division of data is strongly recommended in previous studies (Mackey *et al.* 1996; Wang *et al.* 2015). The criterion to separate data can be sampling depth, season or nutrient regime, and this work can also be done with the assistance of principal component analysis. In this study, our results confirmed that it is necessary to separate MPB and phytoplankton pigment data before the CHEMTAX calculation, as the differences in diagnostic pigment to Chl *a* ratio between MPB and phytoplankton were much larger than differences between sites (data not shown). Further division based on seasonal variations in pigment composition could be made depending on the research purposes.

Seasonal variation of MPB and phytoplankton classes

Nitrate and phosphate concentrations measured in this study indicated a eutrophic state at both DYI and JYI. Data from a previous study also revealed heavy eutrophication in the Jiu-long River estuary (Yan *et al.* 2012). Both studies showed that nutrient concentrations are higher in the dry seasons than in the wet seasons. Nutrient regulation of MPB Chl *a* has been previously demonstrated (Hillebrand & Sommer 1997; Hillebrand *et al.* 2000; Hillebrand & Kahlert 2001; Stelzer & Lambert 2001). Those studies showed that nutrient enrichment increases MPB Chl *a* and supports some diatom species. In this study, MPB diatoms showed a significant positive correlation with phosphate. On the contrary, phytoplankton diatoms were negatively correlated with phosphate (Table 4). However, RDA analysis indicated a high degree of co-variance among environmental factors (Fig. 6). The mechanism that regulates the seasonal patterns of MPB and phytoplankton diatoms may be sophisticated. MPB and phytoplankton diatoms distributed oppositely along the environmental gradient (ENV1) (Figs 7a and 8a). A previous study showed that phytoplankton diatoms were mainly regulated by the nutrient concentrations although change in temperature had a significant effect on photosynthesis (Xie *et al.* 2015). Neither of the effects of nutrient and temperature could simply explain why phytoplankton diatoms peaked during the summer in this study. We proposed two possible mechanisms: first, the phytoplankton diatoms are limited by the retention time of water in the estuary (Odebrecht *et al.* 2015), the increased photosynthesis by temperature resulted in faster accumulation of biomass during the summer; second, the seasonal factor with stronger wind and less river discharge during the dry seasons enhances water convection (Van De Kreeke & Robaczewska 1989) that limited phytoplankton diatoms by reduced light (Domingues *et al.* 2011). MPB photosynthesis is also limited by light in the sedimentary environment as light only penetrates the top several centimeters (MacIntyre *et al.* 1996). However, many MPB species have the ability of vertical mitigation, so they can optimize their photosynthesis. Additionally, the increase in temperature promotes the growth of the grazers during the summer, and thus the decreased MPB diatoms might be the result of a balance between growth and loss (Hillebrand *et al.* 2000, 2002; Hillebrand & Kahlert 2001).

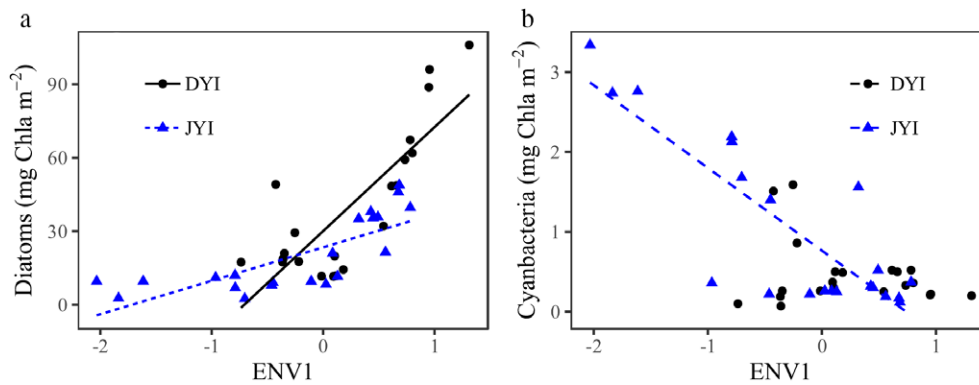


Fig. 7. The responses of microphytobenthos (a) diatoms and (b) cyanobacteria to the first environmental axis of the RDA. [Color figure can be viewed at wileyonlinelibrary.com]

It seems that the temperature range during the winter did not suppress MPB diatoms, because diatoms can adapt to a wide temperature range and usually dominate in a cold and nutrient-replete environment (Irwin *et al.* 2012). The minimum temperature required by most cyanobacteria limits its growth during the cold seasons (Partensky *et al.* 1999), which can explain the absence of cyanobacteria in the dry seasons in this study (Table 4). In the summer, the apparent increase in MPB and phytoplankton cyanobacteria only occurred at JYI, where the salinity is lower than at DYI (Figs 5, 7b and 8d). These results indicate that salinity and temperature co-limit the growth of cyanobacteria in the Jiulong River estuary (Huang *et al.* 2000). The cyanobacteria at JYI could be mostly freshwater species, the spread

of which is constrained by their tolerance range to salinity (Moisander *et al.* 2002).

Phytoplankton chlorophytes and cryptophytes were also negatively correlated with the ENV1 (Fig. 8b,c). The RDA analysis also showed the higher range of N/P ratio favored chlorophytes and cyanobacteria (Fig. 6). The greatly increased alkaline phosphatase activity may be a competitive advantage of chlorophytes in the phosphate-depleted environment (Rengefors *et al.* 2003). Phytoplankton dinoflagellates correlated only with salinity in DYI (Table 4), but the correlation does not imply causation because the variability of salinity was very small (Fig. 2a). The bloom of dinoflagellates is harmful to the environments, its development is regulated not only by temperature but also by water stability (Xie *et al.* 2015).

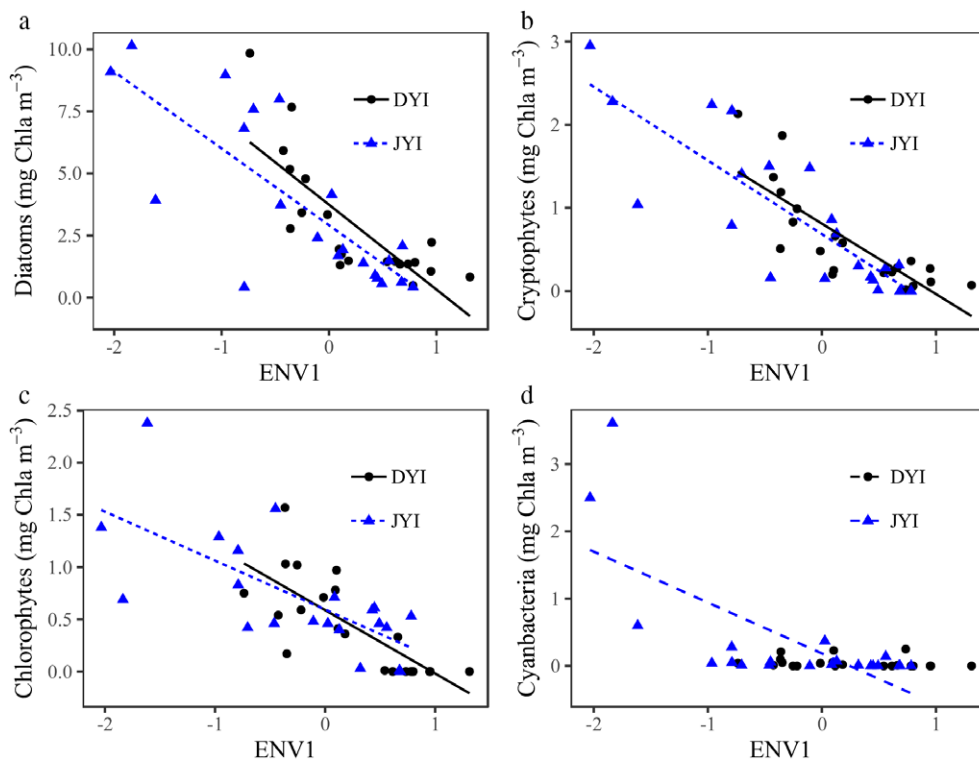


Fig. 8. The responses of phytoplankton (a) diatoms, (b) cryptophytes, (c) chlorophytes, and (d) cyanobacteria to the first environmental axis of the RDA. [Color figure can be viewed at wileyonlinelibrary.com]

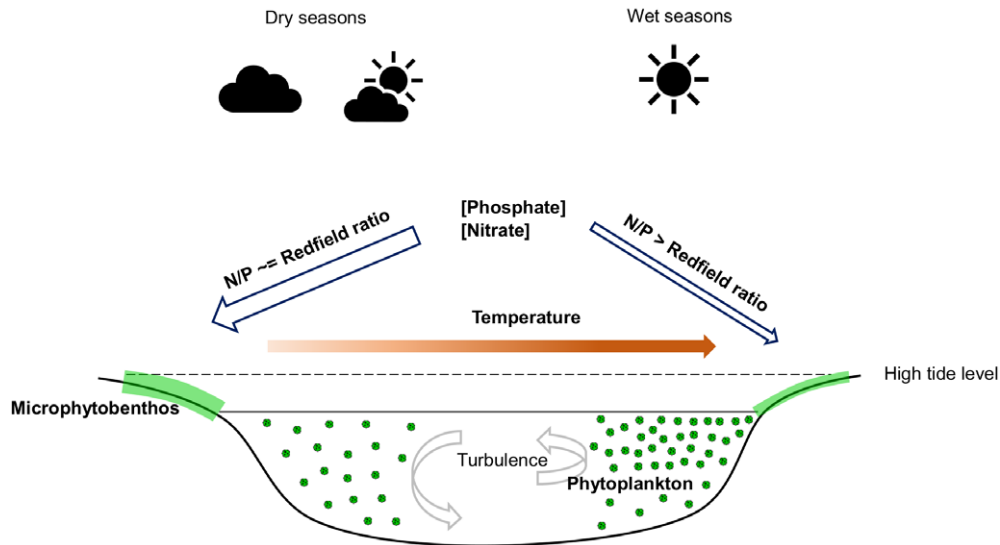


Fig. 9. Conceptual diagrams of contrasting responses of MPB and phytoplankton in Jiulong River Estuary. [Color figure can be viewed at wileyonlinelibrary.com]

DYI is closer to the sea than is JYI, but the phosphate concentrations were quite similar between the two sites (Fig. 2d). The source of additional phosphate may be desorption from particles (Yan *et al.* 2012). However, MPB Chl *a* levels were higher at DYI than at JYI while phytoplankton Chl *a* was quite similar between the two sites. Suspension is not a negligible physical process in controlling MPB biomass (MacIntyre *et al.* 1996), and the loss of MPB Chl *a* may be much higher at JYI than at DYI owing to the turbulent environment from Jiulong River runoff and the tidal current (Sawai *et al.* 2017). Thus, the balance between growth and loss of MPB resulted in a lower MPB Chl *a* at JYI, and the analysis showed differential responses of MPB diatoms to the ENV1 between the two sites (Fig. 7a). Suspended organic matter have been suggested to be a very important food source for zooplankton in pelagic environments (MacIntyre *et al.* 1996).

Chen *et al.* (2013) reported on the overall trend of increasing total nutrient flux from the Jiulong River along with decreasing N/P ratio starting in the 1990s. The expansion of pig farming and the use of phosphate fertilizers were blamed. Phosphate is usually a limiting nutrient along the Chinese coasts (Harrison *et al.* 1990); however, in this study, the N/P ratio was closer to the Redfield ratio of 16:1 in the dry seasons. The Redfield ratio is optimal for MPB growth (Hillebrand & Sommer 1999). Based on our results, the increased phosphate would result in increased MPB Chl *a*, especially in diatoms. But the decreased N/P ratio would also increase the risk of harmful algal blooms along the coasts, because the absolute nutrient concentrations were high (Chen *et al.* 2013). The comprehensive impact of an increasing phosphate influx in the Jiulong estuary zone should be further investigated.

Implication: The contributions of MPB and phytoplankton to subtropical estuarial ecosystem function

In the estuarial ecosystem, MPB and phytoplankton play important roles through their functions in primary

production, food web structure, and removal of nutrients and toxicants, among others. In temperate areas, studies have shown that MPB Chl *a* in intertidal zones is highest during the summer and lowest in the winter (Hubas *et al.* 2006; De Jonge *et al.* 2012) while the peaks of phytoplankton Chl *a* are in the spring and the early autumn (Barnes *et al.* 2015). But in subtropical areas, diatoms were the dominant class in the Jiulong River estuary in this study, and our results showed that MPB diatoms were sensitive to riverine nutrients, especially phosphate, but phytoplankton diatoms were likely regulated by temperature and other mechanisms. Their opposing relationships with environmental factors result in the different contributions of MPB and phytoplankton over spatial and temporal scales (Fig. 9). The relationship of nutrients and primary producers in the intertidal zone suggests that the function of MPB is critical in the subtropical estuarial ecosystem.

Our study also demonstrates the use of HPLC and CHEMTAX in an integrated study of the taxonomic composition of primary producers in both pelagic and benthic environments. Compared with optical microscopy, the advantage of this method is not only the reduction of time-consuming work but also the unified unit of biomass that can be easily interpreted and connected to other research on estuarial carbon and nutrient cycling.

ACKNOWLEDGMENTS

We thank Lin Qi who produced the map for us. This work was supported by the Key Laboratory of the Coastal and Wetland Ecosystems of Xiamen University (WELRI201601), the State Key Laboratory of Marine Environmental Science of Xiamen University (MELRI1603), and the National Natural Science Foundation of China (No. 41466001, 41406158, 41776146, and 41706160). We also thank the Outstanding Postdoctoral Scholarship, State Key Laboratory of Marine Environmental Science at Xiamen University (to Y. X.).

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