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# Characterization of ultraphytoplankton pigments and functional community structure in Xiangxi Bay, China, using HPLC-CHEMTAX

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## ABSTRACT

To characterize the ultraphytoplankton community structure and understand its succession, we investigated photosynthetic pigment concentration and composition; algae phyla composition and abundance; and environmental factors in Xiangxi Bay (XXB) from February to December 2013. High-performance liquid chromatography (HPLC) analyses revealed eight diagnostic pigments: Fucoxanthin, Neoxanthin, Violaxanthin, Alloxanthin, Lutein, Zeaxanthin, Chlorophyll b and Chlorophyll a. The temporal differences between seven of the pigments (all excepting Neoxanthin) were significant ( $P < 0.05$ ), and there were no significant spatial differences between the eight pigments ( $P > 0.05$ ). A chemical taxonomy (CHEMTAX) calculation identified seven phyla of ultraphytoplankton: cryptophytes, chlorophytes, cyanobacteria, diatoms, euglenophytes, chrysophytes, and dinoflagellates. The temporal differences between five of the phyla (except chlorophytes and euglenophytes) were significant ( $P < 0.05$ ), and there was no significant spatial difference between the seven phyla ( $P > 0.05$ ). The redundancy analyses demonstrated that the key environmental factors for the succession were  $\text{N-NH}_4^+$  and  $\text{P-PO}_4^{3-}$ . Application of the HPLC-CHEMTAX method has provided the first analysis of ultraphytoplankton community structure in XXB, and our findings also show that environmental factors influence the succession of ultraphytoplankton over time.

## ARTICLE HISTORY

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## KEYWORDS

Xiangxi Bay; photosynthetic pigments; ultraphytoplankton; HPLC-CHEMTAX; environmental factors

## Introduction

Xiangxi Bay (XXB) is the largest tributary of the Three Gorges Reservoir (TGR) in Hubei Province, with a total area of 3183 km<sup>2</sup>. Impoundment of the TGR has had a great impact on the ecosystem, biodiversity, and water pollution of XXB. In particular, algal blooms have been frequent since June 2003 (Lian et al. 2014; Mao et al. 2015), and the bay has been the subject of much research (Wang et al. 2014; Yang et al. 2015). The current research focuses attention on outbreaks of single-species algal blooms, and on monitoring the related environmental factors. Little research has been carried out into community structure characteristics and phyla composition, or into investigation of temporal and spatial changes.

Phytoplankton participate in the material cycle and energy flow as the main contributors of primary productivity, and they play an important role in the freshwater ecological system. Phytoplankton

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consist of microphytoplankton (20–200  $\mu\text{m}$ ), nanophytoplankton (2–20  $\mu\text{m}$ ), picophytoplankton (0.2–2  $\mu\text{m}$ ), and ultraphytoplankton (<5  $\mu\text{m}$ ) (Robineau et al. 1999), and each of them makes different contributions to primary productivity and biomass. In XXB, the current research only focuses attention on algal blooms and community structure of microphytoplankton (about 20  $\mu\text{m}$ ), Zhou et al. (2012) explored the effects of rainfall on spring phytoplankton community structure in the XXB, and the results indicated that the phytoplankton structure and abundance in the XXB were significantly affected by the physicochemical factors changed by heavy rainfalls. Liu (2012) conducted a one-year water quality study (November 2009 to October 2010) in XXB, and proposed a conceptual model for a management strategy to control phytoplankton blooms in tributary bays via controlled releases from TGR. In order to fill the void for research of the algae with different sizes except for the aforementioned phytoplankton (about 20  $\mu\text{m}$ ) in XXB, we first selected the ultraphytoplankton (defined as phytoplankton passing through a 5  $\mu\text{m}$  filter) as the study subject.

Ultraphytoplankton are autotrophic organisms and include all the picophytoplankton (0.2–2  $\mu\text{m}$ ) and some of the nanophytoplankton (2–20  $\mu\text{m}$ ). They consist of both prokaryotic and eukaryotic phytoplankton (Sieburth et al. 1978). The global distribution of ultraphytoplankton is extremely wide, and they are found in polar, temperate, and tropical waters (Dishon et al. 2012; Girault et al. 2013). Their quantity, biomass, and productivity can be used as a quality indicator of water ecosystem (Silva et al. 2009). Therefore, it was seen as ‘invisible main character’, and there were several reasons for this. First, a lot of studies (Li et al. 1983; Ines et al. 2012) shown that ultraphytoplankton were the main primary producers, and their distribution density in the water was large, which constitutes most of the phytoplankton quantity. Second, their rapid growth, large number, and high turnover rate can overcome the disadvantage of small individual biomass, and they could remain quite high in productivity. Third, ultraphytoplankton were eaten by heterotrophic flagellates and ciliates, and so indirectly becoming a food source for zooplankton. As part of the classical food web they ultimately affect fishery production. However, previous studies have mainly focused on the oceans (Denis et al. 2010), and the characteristics and succession of the ultraphytoplankton community structure in freshwater ecosystems have been overlooked, mainly because of difficulties in their taxonomic identification.

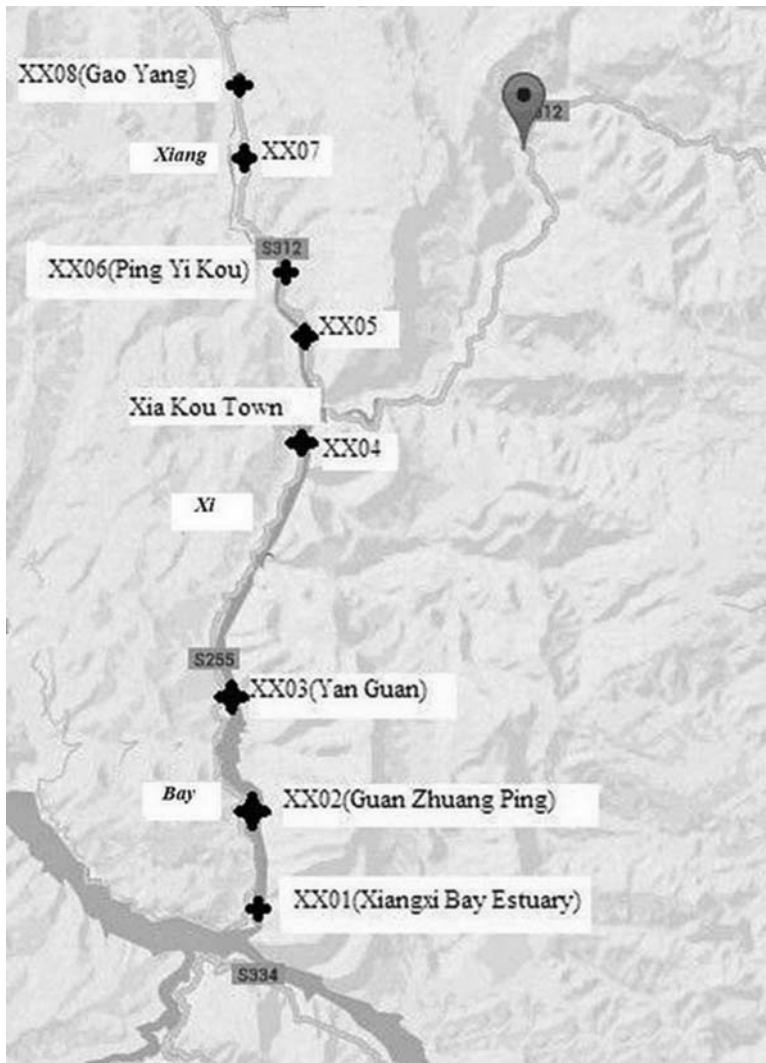
High-performance liquid chromatography (HPLC) is a faster, reproducible method of analyzing a large set of samples (Jeffrey et al. 1997a), compared with classical microscopy. It also has the advantage of detecting ultraphytoplanktonic organisms, which are normally difficult to identify by light microscopy (Bautista & JimenezGomez 1996). Several algal phyla may contain the same kind of pigments; for example Fucoxanthin (Fuco), which is a major pigment in diatoms, is also found in chrysophytes and prymnesiophytes. The development and use of statistical tools such as CHEM-TAX (Mackey et al., 1996) can prevent confusion between phyla by applying matrix factorization to pigment data to evaluate the contribution from phytoplankton groups to total Chl a. HPLC-CHEM-TAX is considered to be a suitable method for determining the abundance of phytoplankton in the ocean (Madhu et al. 2014; Agirbas et al. 2015), but there have been no reports on characterization and succession of the ultraphytoplankton community structure through the use of HPLC-CHEM-TAX analysis in XXB.

The present study was developed to understand ultraphytoplankton succession and its ecological significance in the freshwater XXB during 2013. Specifically, our objectives were: (i) to discover the contribution of ultraphytoplankton to total phytoplankton biomass; (ii) to illustrate the ultraphytoplankton community structure by using HPLC-CHEMTAX; and (iii) to understand the temporal and spatial variation of dominant ultraphytoplankton taxonomic phyla in relation to environmental factors.

## Methods

### *Study area and sampling strategy*

The XXB is the largest tributary of the TGR, with a main course of 94 km and a watershed of 3099 km<sup>2</sup>. Samples were taken bimonthly from our eight sampling sites with depth less than 50 m



**Figure 1.** Location of the sampling sites in the study area.

(from Gaoyang to XXB estuary; see [Figure 1](#)) from February to December 2013, and there were three replicates per site. The volume of 750–1000 mL water samples (the volume was different according to the initial ultraphytoplankton concentrations at different sampling sites, and the samples volume data were entered in the formula to calculate pigments concentrations) were brought into our laboratory for the analysis of environmental factors within 24 h. The samples used for the photosynthetic pigment measurement were collected and filtered through a 10- $\mu\text{m}$  sieve to remove sediment and larger-sized phytoplankton, and were then filtered through a 5- $\mu\text{m}$  membrane. Finally, the samples were filtered through 0.7  $\mu\text{m}$  Whatman GF/F glass fiber filters. In general, the volume of initial samples was 750–1000 mL, and it would lose 1–2 mL in every filtration step. The final samples were stored in liquid nitrogen for HPLC analysis.

### **HPLC photosynthetic pigment analysis**

The HPLC method was established as follows: nine standard pigments were prepared in the laboratory of Jianjun Hou, and a patent was applied to cover the related technique (Chinese number

201410022083.4). These standard pigments were Peridinin (Peri), Fucoxanthin (Fuco), Neoxanthin (Neox), Violaxanthin (Viol), Alloxanthin (Allo), Lutein (Lute), Zeaxanthin (Zeax), Chlorophyll b (Chl b) and Chlorophyll a (Chl a), which were analyzed using Infinity 1260 Series HPLC (Agilent, USA) comprising a solvent delivery module, a temperature control system, and a diode array detector (DAD). Chromatographic separation was carried out using a C<sub>8</sub> column for reverse phase chromatography (ZORBAX Eclipse XDB-C<sub>8</sub> 4.6 × 150 mm, 3.5 μm, Agilent, USA) and a 36-min elution program. The solvent system was composed of phase A (methanol:1 M ammonium acetate = 4:1, v/v) and phase B (methanol). Gradient conditions: 0–2 min, 100% A; 2–16 min, 100%–55% A; 16–27 min, 55%–0% A; 27–32 min, 0% A; 32–36 min, 0%–100% A and equilibration of 30 min was made afterward, and the flow rate was 1.0 mL/min. A series of concentration of pigments was injected for HPLC analysis, and the corresponding peak areas were calculated from the signals in the DAD (λ: 440 nm). The peak area appeared on the horizontal axis and the amount of pigment on the vertical axis, linear equations were obtained through linear regression, and the slope of the straight line fitting represented the response factor ( $f_p$ ).

The filter containing ultraphytoplankton was then cut into pieces and stored in 2-mL Eppendorf tubes. The photosynthetic pigments were extracted with 1 mL *N,N*-dimethylformamide (DMF) for 30 min at –20 °C, in the dark. The samples were then centrifuged at 6000 r/min for 15 min at 4 °C, and the supernatant was mixed with an equal volume of ammonium acetate (1 M). The mixtures were filtered through 0.22 μm Nylon 66 filter membranes (JinTeng, China), immediately injected into the HPLC, and pigment composition analyzed as above. Pigment peak areas were calculated from the signals in the DAD (λ: 440 nm) and the concentration of pigments was calculated according to the  $R_f$  and the following formula (Hu et al. 2011). The spatial and temporal differences were analyzed with ANOVA statistical analysis.

$$C_s = \frac{A_p f_p v_{\text{ext}} 10^3}{v_{\text{inf}} v_{\text{fit}} B}$$

Where  $C_s$  is the photosynthetic pigment concentration, ng/L;  $A_p$  the photosynthetic pigment elution peak area, mAU\*s;  $f_p$  the slope of the linear regression equation between the photosynthetic pigment concentration and peak area, mg/mAU;  $V_{\text{ext}}$  the volume of pigment extraction liquid, mL;  $V_{\text{fit}}$  the total volume of the water filtered through the filter, mL;  $V_{\text{inj}}$  the sample volume for HPLC analysis, mL;  $B$  the dilution factor.

### CHEMTAX analysis of pigment data

The initial pigment ratio is key to CHEMTAX processing as it can directly affect the calculation (Mackey et al. 1996). In studies of different habitats, including an estuary (Lionard et al. 2008), a bay (Madhu et al. 2014), lagoons (Sarmiento & Descy 2008), and freshwater (Guisande et al. 2008), many adjustments were made to the choice of pigment matrix and the initial ratio. The intent was to isolate and incubate the phytoplankton in the laboratory to discover the pigment/Chl a ratio, but finding the ratio for every phylum was complex. In this research, the optimal initial pigment ratios (Table 1) were obtained from the literature (Furuya et al. 2003; Schluter et al. 2006) and HPLC analysis. The pigment concentration data and initial pigment ratio data were inputted using CHEMTAX software version 1.95, and the new pigment ratio data were obtained from the first running. The second CHEMTAX running used this output pigment ratio as an input pigment ratio. After five to seven repetitions, the output results of algae phyla composition showed no change, indicating that the final results had been obtained. According to a previous report (Latasa 2007), multiple operations of the CHEMTAX analysis reduced the dependence on the initial pigment ratio, and the results converged to the ‘true value,’ thus improving the reliability and accuracy of the results. The relative abundance of ultraphytoplankton contributing to the total ultraphytoplankton Chl a (UPChl a)

**Table 1.** CHEMTAX pigment initial ratio table, derived from the analysis of all samples, and combined with references.

	Peri	Fuco	Neox	Viol	Allo	Lute	Zeax	Chl b	UPChl a
Euglenophytes	0	0	0.035	0	0	0	0	0.0195	1
Dinoflagellates	0.4920	0	0	0	0	0	0	0	1
Cryptophytes	0	0	0	0	0.3840	0	0	0	1
Chrysophytes	0	0.2880	0	0.0713	0	0	0.0490	0	1
Chlorophytes	0	0	0.0410	0.0280	0	0.1500	0.0303	0.3700	1
Cyanobacteria	0	0	0	0	0	0	0.2836	0	1
Diatoms	0	0.5210	0	0.0090	0	0	0	0	1

biomass was calculated. The spatial and temporal differences were analyzed by using ANOVA statistical analysis.

### Analysis of environmental factors

The factors detected comprised on-site environmental factors: water temperature (WT), above and below water irradiance, dissolved oxygen (DO), transparency, pH, conductivity, air temperature, turbidity, and oxidation–reduction potential (ORP). Chemical factors that affect water samples include total nitrogen (TN), total phosphorus (TP),  $P-PO_4^{3-}$ ,  $N-NO_3^-$ ,  $N-NH_4^+$ , Chl a, and chemical oxygen demand (COD). The WT, pH, DO, ORP, and conductivity were measured using YSI (Professional Plus); transparency was measured with a Secchi disk (SD); and water irradiance was measured with LightScout quantum meters in situ. Chemical factors such as TP, TN,  $P-PO_4^{3-}$ ,  $N-NO_3^-$ ,  $N-NH_4^+$ , and COD were analyzed according to the standard methods (SEPB 2002). Total Chl a was measured by filtering water (0.2–2.5 L), until the filter (Whatman GF/F) showed color. This filter was cut and dissolved in acetone (5 mL, 90%) for 24 h in dark and cold conditions. Then, the mixture was centrifuged at 6000 r/min for 15 min at 4 °C, and the supernatant was used for total Chl a detection with UV spectrophotometry.

### Redundancy analysis of the relationship between environmental factors and ultraphytoplankton community structure

First, the variables of species were analyzed by detrending correspondence analysis (DCA). If the longest length of gradient is less than or equal to 3.0, it is appropriate to use the linear model (principal component analysis or redundancy analysis [RDA]). RDA was then used to analyze the relationship between environmental factors and ultraphytoplankton community structure. By using previous selection and Monte Carlo tests, a minimal subset of the environmental factors were used for RDA analysis, which explained a significant ( $P < 0.05$ ) variation within species data, and only these environmental factors are shown on the biplots. Ordination plots were made by Canoco for Windows 4.5 software.

## Results

### $f_p$ of the nine photosynthetic pigments

The standard curves of the photosynthetic pigments (Peri, Fuco, Neox, Viol, Allo, Lute, Zeax, Chl b, and UPChl a) were obtained by using HPLC. The  $f_p$  of the nine different photosynthetic pigments were 0.2481, 0.1132, 0.0864, 0.0709, 0.0897, 0.0784, 0.0811, 0.2806, and 0.2843, respectively, and the linear correlation coefficient of the standard curve was between 0.9954 and 1. The relationship between the  $f_p$  values of photosynthetic pigments and literature-reported values (Jeffrey et al. 1997b; Hu et al. 2011) was significantly correlated except for UPChl a and Chl b. Then the photosynthesis pigments concentrations were calculated by inputting the data and using the above formula at different sampling sites.

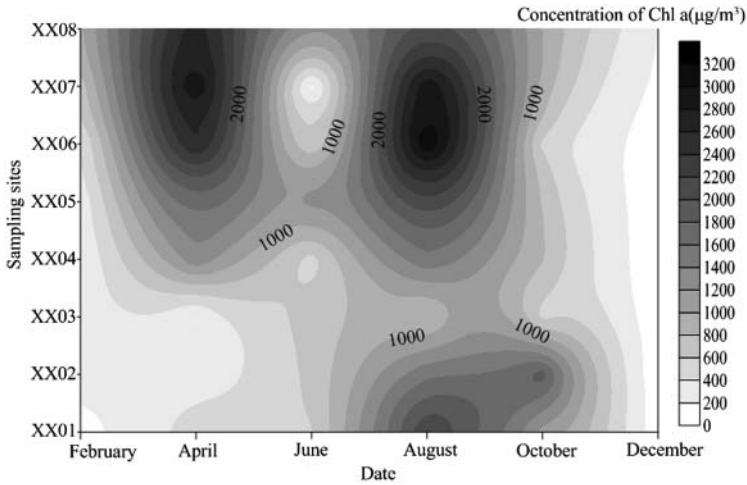


Figure 2. Biomass of ultraphytoplankton.

### Ultraphytoplankton biomass

The UPChl a concentration was analyzed to evaluate the ultraphytoplankton biomass at eight sampling sites in XXB in 2013, and the spatial and temporal distribution of biomass was clarified. Results are shown in Figure 2. In August (summer) the UPChl a concentration was the highest ( $3171.42 \mu\text{g}/\text{m}^3$ ), and the corresponding sampling site was XX06. Meanwhile, in December (winter) the UPChl a concentration was the lowest ( $53.47 \mu\text{g}/\text{m}^3$ ), and the corresponding sampling site was XX02. The spatial and temporal differences were analyzed by using ANOVA statistical analysis. The results showed that the temporal difference in biomass was significant ( $P < 0.05$ ), and the spatial difference of biomass was not significant ( $P > 0.05$ ). Overall, the spatial and temporal distributions show that upstream biomass is greater than downstream biomass, and it is higher in August and April than in the other four months.

### Concentration and composition of photosynthetic pigments

The ultraphytoplankton photosynthetic pigments were analyzed by using HPLC at eight sampling sites in XXB. There were no Neox at the sampling site XX06 in February or at XX02 in April. In all the other sampling sites eight photosynthetic pigments (Fuco, Neox, Viol, Allo, Lute, Zeax, UPChl a, and Chl b) were detected on all dates. The two main photosynthetic pigments were Fuco and Allo; these are the diagnostic pigments of diatoms and cryptophytes, respectively, which may be the dominant ultraphytoplankton in XXB. The spatial and temporal distribution for seven pigments (except UPChl a) is shown in Figure 3. The concentrations of Fuco (Figure 3(a)) were higher during April and August (summer), with values rising as distance between sites and estuary increased. The highest concentration of Fuco was  $1018.49 \mu\text{g}/\text{m}^3$  at XX07 in April. The concentrations of Neox (Figure 3(b)) were very low, and the highest concentration of this pigment was  $40.82 \mu\text{g}/\text{m}^3$ , which was observed at XX02 in August. The concentrations of this pigment were mostly greater upstream and downstream in August, and upstream in April. The concentrations of Viol (Figure 3(c)) were also low, the highest being  $55.35 \mu\text{g}/\text{m}^3$  at XX06 in April. In general, concentrations of this pigment were higher upstream than downstream, and were higher in April than in other months. The concentrations of Allo (Figure 3(d)) were high; the highest concentration was  $647.69 \mu\text{g}/\text{m}^3$  at XX02 in October. Concentrations of this pigment were higher in April, August, and October than in other months, and higher upstream than downstream. The highest concentration of Lute (Figure 3(e))

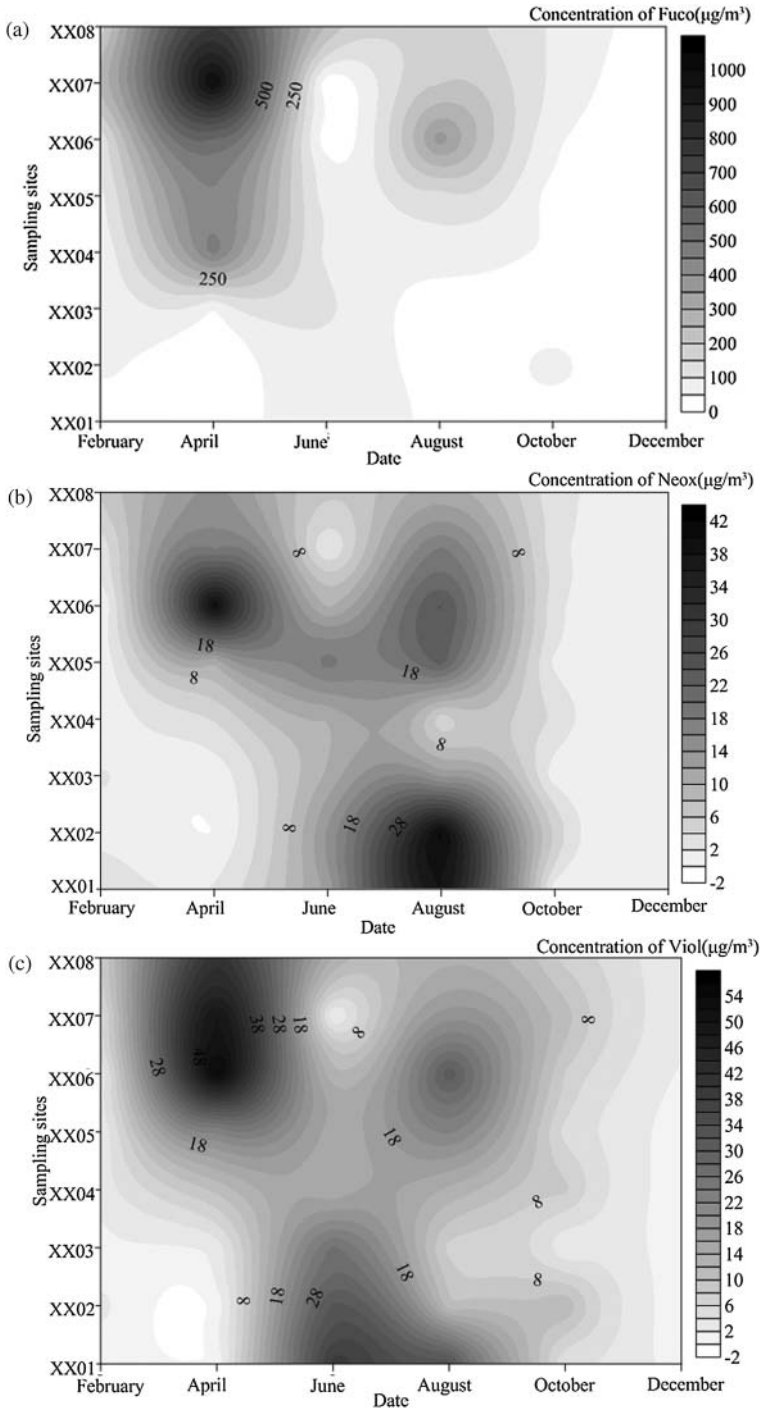


Figure 3. Temporal and spatial distribution of photosynthetic pigments: (a) Fuco, (b) Neox, (c) Viol, (d) Allo, (e) Lute, (f) Zeax, (g) Chl b.



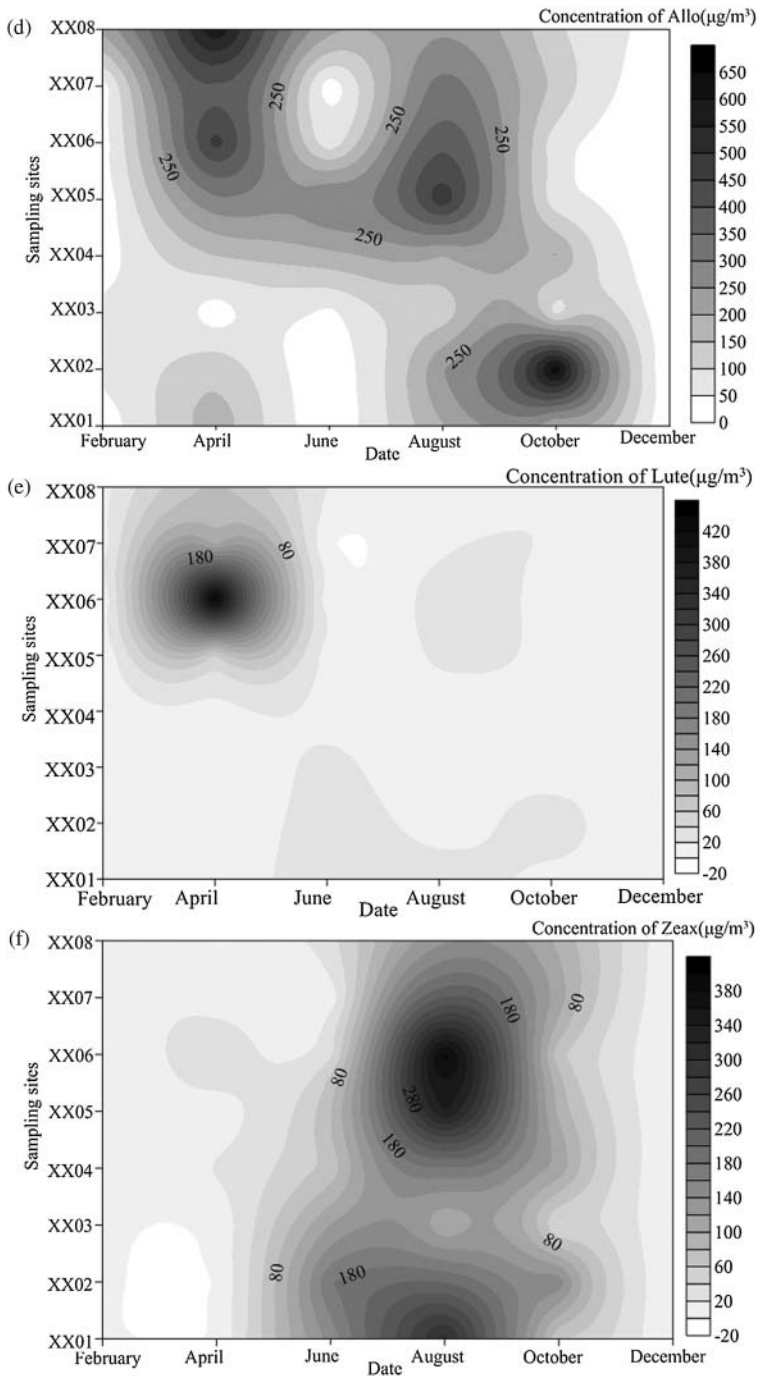


Figure 3. (Continued).

was  $443.10 \mu\text{g}/\text{m}^3$  at XX06 in April. The concentrations of this pigment were higher at XX05, XX06, and XX07 in April than at other sampling sites in any other months. The highest concentration of Zeax (Figure 3(f)) was  $390.38 \mu\text{g}/\text{m}^3$  at XX06 in August. Concentrations of this pigment were higher at XX05, XX06, and XX07 in April than at other sampling sites in any other months; and they were higher upstream than downstream in August than at other sampling sites in any other months.

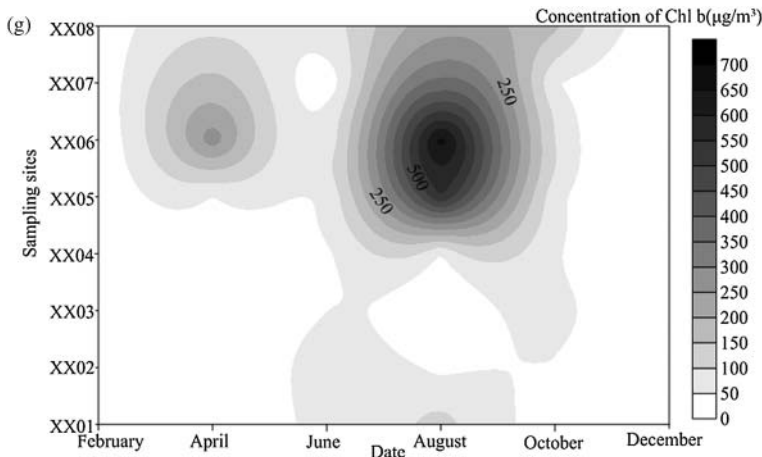


Figure 3. (Continued).

The highest concentration of Chl b (Figure 3(g)) was  $671.69 \mu\text{g}/\text{m}^3$  at XX06 in August. Concentrations of this pigment were higher at XX05, XX06, and XX07 in August than at other sampling sites in any other months. The spatial and temporal differences between the seven photosynthetic pigments (except UPChl a) were analyzed using ANOVA statistical analysis. Results showed that six photosynthetic pigments (apart from Lute) had significant differences ( $P < 0.05$ ) at different months. Then, a multiple comparison test following up on the ANOVA indicated the dates were significantly different from other dates, and the details were as follows: For Fuco, April was significantly different ( $P < 0.05$ ) from the other five months. For Neox, August was significantly different ( $P < 0.05$ ) from February, October, and December. For Viol, April was significantly different ( $P < 0.05$ ) from February and December, and June was significantly different ( $P < 0.05$ ) from December. For Allo, February and December were significantly different ( $P < 0.05$ ) from April and August. For Zeax, February, April, and December were significantly different ( $P < 0.05$ ) from June, August, and October, and August was significantly different ( $P < 0.05$ ) from June and October. For Chl b, August was significantly different ( $P < 0.05$ ) from the other four months except April. The photosynthetic pigments (Fuco, Neox, Viol, Allo, Lute, Zeax, Chl b) showed no significant differences ( $P > 0.05$ ) at different sampling sites.

### Composition and succession of ultraphytoplankton community structure

The ultraphytoplankton community structure was identified with CHEMTAX based on pigment concentration data and initial pigment ratio. The composition of the ultraphytoplankton community structure is shown in Figure 4. Seven ultraphytoplankton phyla were found in the research area: cryptophytes, diatoms, chlorophytes, cyanobacteria, chrysophytes, euglenophytes, and dinoflagellates. Succession in the ultraphytoplankton community structure appears in Figure 5. This shows that succession occurred in cryptophytes, diatoms, chlorophytes, and cyanobacteria, in the following sequence.

In February, cryptophytes dominated at all sampling sites except at XX01 and XX03, where diatoms and cyanobacteria dominated, respectively; In April, chlorophytes dominated at sampling sites XX01 and XX06. Diatoms dominated at sampling sites XX04 and XX05. The cryptophytes dominated at the other four sampling sites. However, compared with February, the percentage of cryptophytes was lower at XX02 and XX07, and higher at XX08. In June, cyanobacteria dominated at sampling sites XX03 and XX05, and diatoms dominated at sampling site XX07. The cryptophytes dominated at the other four sampling sites. In August, cryptophytes dominated at all sites except

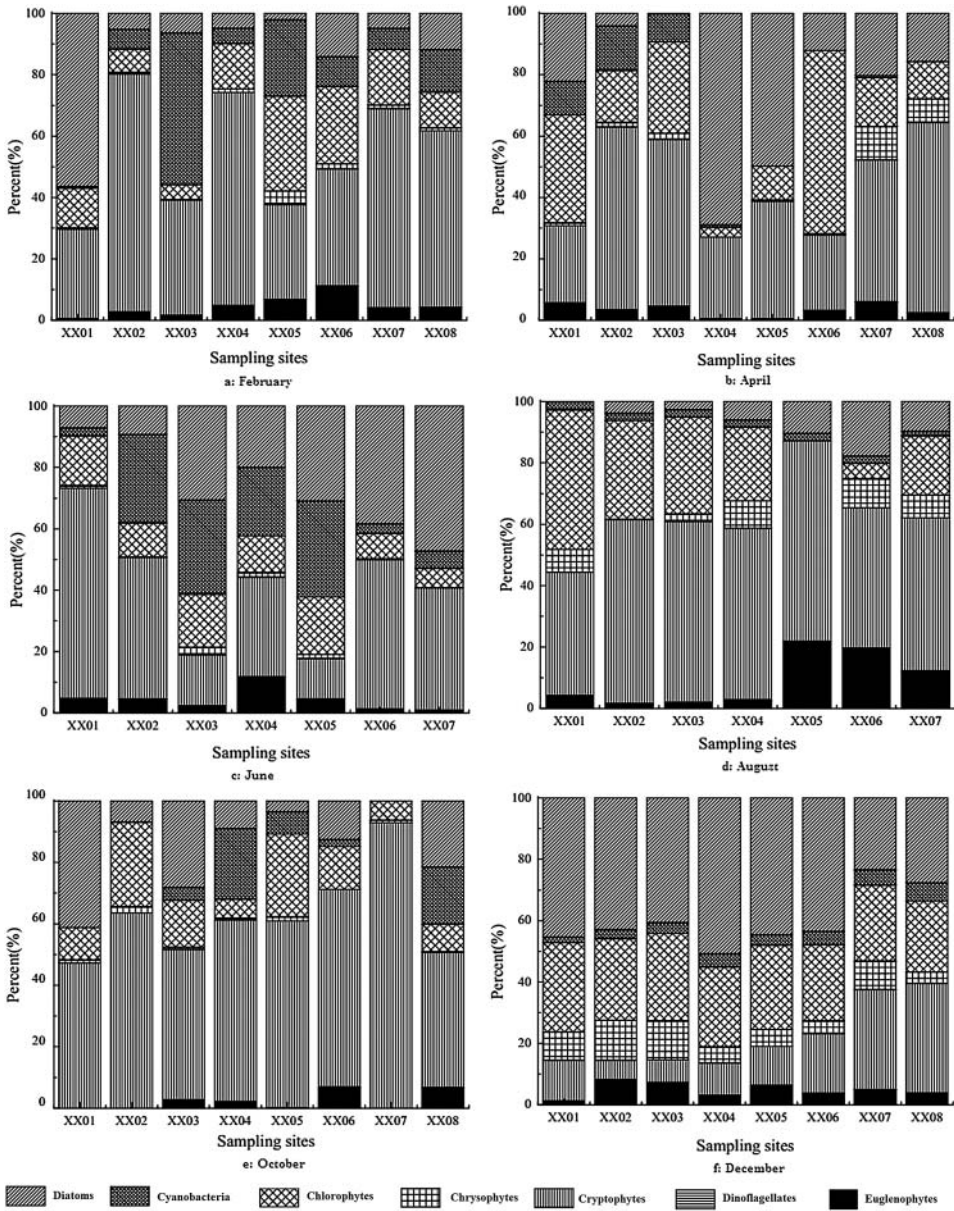


Figure 4. Ultraphytoplankton community structure at different months.

XX01 where chlorophytes dominated. However, compared with June, the percentage of cryptophytes was higher at XX02, and it was the same at XX06. In October, cryptophytes dominated at all sampling sites. In December, diatoms dominated at all sampling sites except XX07 and XX08 where cryptophytes dominated, but compared with October, the percentage of cryptophytes was lower, which were 32.52% and 35.72%, respectively. The spatial and temporal differences were analyzed by using ANOVA statistical analysis. The results indicated that cryptophytes, cyanobacteria, diatoms, chrysophytes, and dinoflagellates had significant differences at different months ( $P < 0.05$ ), and chlorophytes and euglenophytes had no obvious significance ( $P > 0.05$ ) at different months. Then, a multiple comparison test following up on the ANOVA indicated the dates were significantly

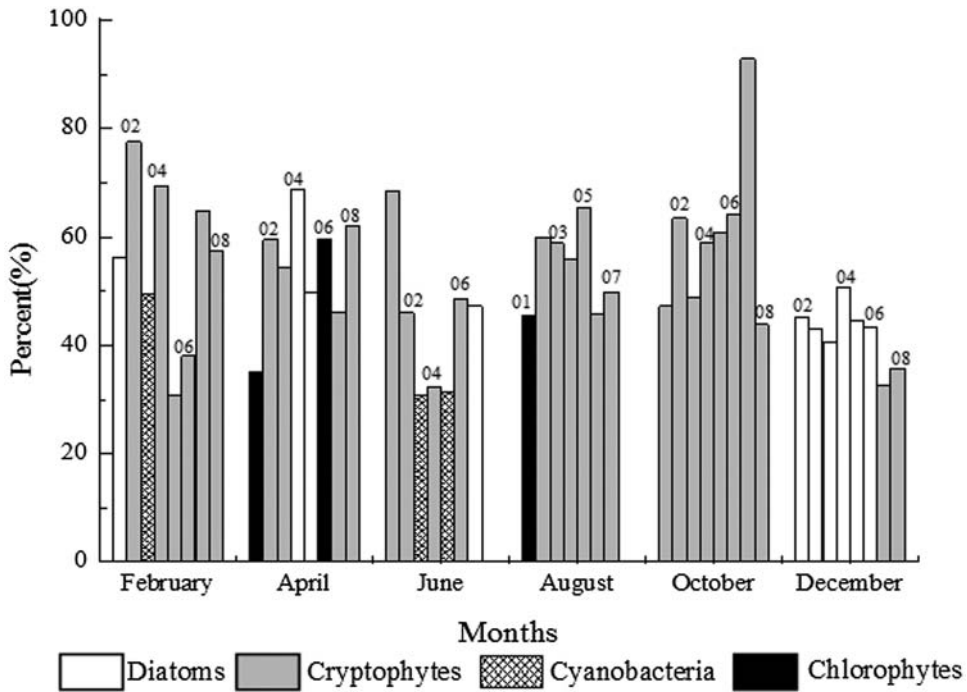
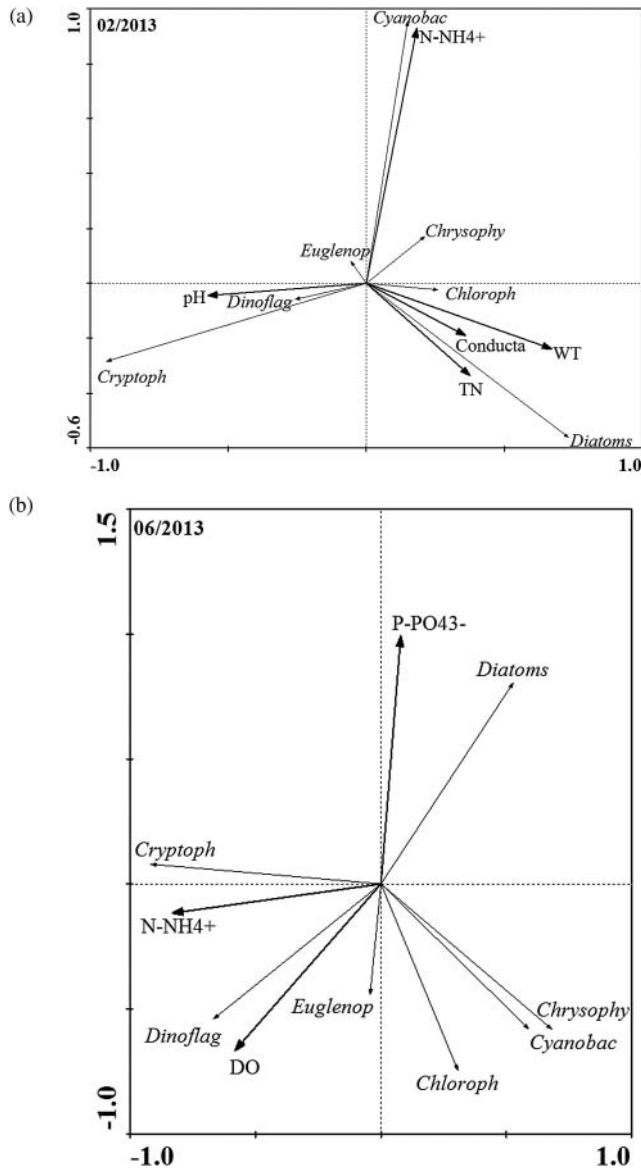


Figure 5. Succession of dominant ultraphytoplankton community.

different from other dates, and the details were as follows: For cryptophytes, December was significantly different ( $P < 0.05$ ) from other four months except June. For diatoms, December was significantly different ( $P < 0.05$ ) from February, August, and October. For chrysophytes, December was significantly different ( $P < 0.05$ ) from other four months except August. For dinoflagellates, August was significantly different ( $P < 0.05$ ) from April and December. The seven kinds of ultraphytoplankton (cryptophytes, diatoms, chlorophytes, cyanobacteria, chrysophytes, euglenophytes, and dinoflagellates) community structure showed no significant difference ( $P > 0.05$ ) at different sampling sites.

### Relationship between ultraphytoplankton community structure and environmental factors

The environmental variables were first analyzed using DCA. It suggested that the greatest length of gradient was less than 3, which is suitable for RDA analysis (Figure 6). By using previous selection and Monte Carlo tests, the environmental factors explained no significant ( $P > 0.05$ ) variation within species data at all months except February and June. Therefore, the relationship between ultraphytoplankton community structure and environmental factors was clarified by RDA on February and June, and the environmental factors did not significantly affect the ultraphytoplankton community structure at the other four months. The RDA results for February and June were as follows: in February (Figure 6(a)), the eigenvalues ( $\lambda$ ) for RDA axis 1 and RDA axis 2 were 0.492 and 0.352, respectively; 89.1% of variance of the phyla-environment relationship was explained by RDA axis 2. WT ( $R = 0.669$ ) and pH ( $R = -0.571$ ) were strongly connected to axis 1, and  $\text{N-NH}_4^+$  ( $R = 0.912$ ) was significantly associated with axis 2. The dominant phylum was cryptophyta, which was negatively correlated with  $\text{N-NH}_4^+$  and WT. The ordination biplot indicated that  $\text{N-NH}_4^+$  was the most significant environmental factor in February. In June (Figure 6(b)) the eigenvalues ( $\lambda$ ) for RDA axis 1 and RDA axis 2 were 0.542 and 0.275, respectively; 98.6% of variance of the phyla-environment relationship was explained by RDA axis 2. DO ( $R = -0.537$ ) was strongly connected to



**Figure 6.** Ordination biplot of environmental variables and ultraphytoplankton species assemblages obtained by RDA for different months: (a) February and (b) June.

axis 1, and  $P-PO_4^{3-}$  ( $R = 0.914$ ) and DO ( $R = -0.613$ ) were significantly associated with axis 2. The dominant phylum was cryptophyta, which was positively correlated with DO and negatively correlated with  $P-PO_4^{3-}$ . The ordination biplot indicated that  $P-PO_4^{3-}$  was the most significant environmental factor.

## Discussion

### *Ultraphytoplankton contribution to the total biomass*

Various observations have been made on correlations between algal biomass and Chl a concentration (Alvarez-Fernandez & Riegman 2014). So, in this study, we found that the Chl a concentration

of ultraphytoplankton ( $<5 \mu\text{m}$ ) ranged from 53.47 to 2951.65  $\mu\text{g}/\text{m}^3$ , and its contribution to total phytoplankton biomass was up to 0.73%–19.43% (expressed as Chl a), which was lower than that previously reported (0.2%–99.7%; Silva et al. 2009) at the EPEA coastal station. The result suggested that the ultraphytoplankton may have different contribution at marine environment and freshwater. As no information is available for comparison in the study sites, this result provides a reference for ultraphytoplankton contribution to the total biomass in XXB. According to the UPChl a distribution in XXB, the ultraphytoplankton upstream is higher than downstream, and it is higher in August and April than in the other four months. These results indicate that eutrophication can easily occur upstream in the region between Xia Kou and Gao Yang, and may bring the threat of algal blooms. According to a previous report (Yao et al. 2012), a serious algal bloom occurred near Gao Yang, which is located between our sampling sites XX05 and XX07. We were therefore able to monitor the contribution of ultraphytoplankton to total phytoplankton biomass to evaluate environmental conditions.

### **Ultraphytoplankton community structure**

The HPLC-CHEMTAX method was used successfully for the first time to analyze the ultraphytoplankton community structure in XXB. The predominant biomarker pigments were Fuco, Allo, Zeax, and Chl b, which signified abundances of diatoms, cryptophytes, cyanobacteria, and chlorophytes, respectively. Earlier studies have shown that diatoms dominate under conditions of high nutrients (Mikaelyan et al. 2015). In the present in situ investigation, a similar ultraphytoplankton succession was observed in XXB throughout the study period. In general, the dominating diatoms were present in February, April, June, and December (Figure 5). The water may be rich in  $\text{N-NO}_3^-$  and  $\text{P-PO}_4^{3-}$ , which would have enhanced diatom growth. According to a previous report (Supraha et al. 2014), as an important primary producer, cryptophytes are widely distributed in freshwater and oceans, and even formed bad-tasting blooms. We also found that the dominant cryptophytes were present every month in the in situ samples, which may have two reasons. First, this may be because they are better adapted to air temperature and illumination; second, there may have been a succession of different species which adapted to the changes in environmental parameters. Chlorophytes play an important role in freshwater ecosystems, and their optimum growth temperature is 20–30 °C (Evans 1997). In this study, the water was rich in dominant chlorophytes because of high conductivity, high nutrient concentration, and low pH. Furthermore,  $\text{N-NO}_3^-$  was present at moderate levels, and this promoted the growth of chlorophytes in XXB. The predominance of Zeax in XXB signifies the abundance of cyanobacteria, which proliferated under conditions of low nutrient and high temperature (Wang et al. 2015). In this research,  $\text{N-NH}_4^+$  was present at low levels, and this promoted the growth of cyanobacteria in XXB.

### **The relationship between environmental factors and ultraphytoplankton**

Phytoplankton succession was affected by environmental factors and nutrients. Based on previous reports on lakes and reservoirs, oligotrophic systems have  $<0.2 \text{ mg}/\text{L}$  TN and  $<0.01 \text{ mg}/\text{L}$  TP, while eutrophic systems have  $\text{TN} > 0.5 \text{ mg}/\text{L}$  and  $\text{TP} > 0.02 \text{ mg}/\text{L}$  (Peng et al. 2013). During 2013, the lowest values of TN and TP were 0.71 and 0.05  $\text{mg}/\text{L}$ , respectively, which indicated that the XXB falls into the category of eutrophicated water bodies. It was therefore necessary for us to clarify the relationship between environmental factors and ultraphytoplankton. In this research, the relationship between environmental factors and ultraphytoplankton had no obvious significant ( $P > 0.05$ ) during April, August, October, and December, which indicated environmental factors did not affect the ultraphytoplankton community structure at these months. On the other hand, the relationship between environmental factors and ultraphytoplankton had no obvious significant ( $P < 0.05$ ) during February and June, and the most significant environmental factors were  $\text{N-NH}_4^+$  and  $\text{P-PO}_4^{3-}$ , which were in negative correlation with cryptophytes. Generally the most limiting nutrient

for algal growth in the freshwater ecosystem is phosphorus; Harder (1968) found that phosphorus is the most significant factor influencing phytoplankton in freshwater. In our study, nitrogen also played an important role in ultraphytoplankton temporal dynamics. This result was consistent with the previous report by Koike et al. (1986), and it found that Antarctic nanophytoplankton (<10  $\mu\text{m}$ ) take up  $\text{NH}_4^+$  as a function of irradiance. There were another two important environmental factors including WT and DO, which were in negative and positive correlations with cryptophytes, respectively. As always, WT was considered to be the most important environmental variable affecting the succession of phytoplankton (Farinas et al. 2015). Oxygen is an important environmental factor of the reservoir which is essential to the metabolism of all aquatic organisms that possess aerobic respiration. Concentration of DO indicated water quality and its relation to the distribution and abundance of various algal species (Verma et al. 2012). In the present study, the DO of XXB water samples ranged from 6.73 to 18.00 mg/L. The minimum DO value was observed in October (6.73 mg/L) and the highest peak was observed in April (18.0 mg/L).

## Conclusions

We were able to apply the HPLC-CHEMTAX method successfully, identifying eight biomarker pigments and seven ultraphytoplankton (groups or classes) in XXB for the first time. The dominant ultraphytoplankton were cryptophytes, diatoms, cyanobacteria, and chlorophytes.

During the study period, XXB was found to belong to the category of eutrophicated water bodies. Upstream biomass was higher than downstream biomass, and was higher in August and April than the other four months. Action should be taken to improve the water quality in TGR to reduce the risk of harmful algal blooms.

In XXB, ultraphytoplankton succession showed obvious temporal characteristics. Different dominant phyla were driven by corresponding key factors.  $\text{N-NH}_4^+$  and  $\text{P-PO}_4^{3-}$  were the key factors affecting ultraphytoplankton succession in February and June, respectively.

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