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Multiple Effects of Environmental Factors on Algal Growth and Nutrient Thresholds for Harmful Algal Blooms: Application of Response Surface Methodology

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Abstract Systematic understanding of the co-effects of environmental factors on phytoplankton proliferation can enable more effective control of harmful algal blooms in eutrophic lakes and reservoirs. A batch of statistically designed experiments using response surface methodology was recently conducted on mixed algae samples collected from Changtan Reservoir. The central composite designed response surface model was established to evaluate multiple effects of various physical and chemical factors (total nitrogen, total phosphorus, temperature, and light intensity) on algal density and chlorophyll a content. Analysis of variance indicated an excellent correlation between modeling results and experimental responses. Among the selected environmental variables, promotion of the interactive effects of nitrogen and phosphorus together with the optimum total nitrogen/phosphorus mass ratio (between 7.9 and 10.1) was determined to be the most significant stimulating parameter associated with algal blooming development dominated by non-nitrogen-fixing species. The favorable effects of strong illumination were shown to be greater than those of high temperature. The border values of total nitrogen and phosphorus concentrations leading to a critical value of algal density under different water temperatures and light intensities could be predicted as nutrient loading thresholds for harmful algal blooms by our second-order polynomial regression model.

Keywords Harmful algal blooms · Environmental factors · Response surface methodology · Central composite design · Optimum TN/TP ratio · Nutrient thresholds

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1 Introduction

One of the most serious environmental problems impacting inland freshwater bodies is eutrophication, which diminishes water quality by spurring excessive growth of algae and increasing suspended organic materials [1, 2]. At high densities, harmful algal blooms (HABs) produce taste and odor problems in drinking water, release toxins, and kill aquatic biota because of conditions associated with their proliferation and senescence, such as low dissolved oxygen and high ammonia concentrations [3, 4]. Therefore, controlling HABs has become the goal of ecosystem amelioration of lakes and reservoirs.

Algal growth in a water body is a complex activity characterized by a large number of interior physical, chemical, and biological mechanisms and impacted by a series of exterior environmental factors. Total nitrogen (TN), total phosphorus (TP), water temperature, and light intensity have been identified as primary factors influencing algal growth, and their synergic effects contribute to phytoplankton growth [5, 6]. Previous studies have shown that algal growth is strongly influenced by nutrient supply of N and P, which are assumed to be the most important elements restricting the growth of HABs [7, 8]. Most notably, cyanobacterial biomass increases with eutrophication, leading to the HABs in many cyanobacteria-dominant blooming water bodies. Water

temperature and light intensity can also have a direct positive effect on the growth of algae [9, 10].

Previous laboratory studies have only explored the effects of single or coupled environmental influential factors on the abundance or photosynthesis of limited cyanobacterial species, while ignoring the interactive effects of combined environmental factors and the optimum TN/TP ratio for mixed algal growth. For example, Xu et al. [11] studied the phytoplankton growth controlled by nitrogen and phosphorus inputs in Lake Taihu, Liu et al. [12] investigated the effects of temperature and nutrient ratios on *Microcystis* blooms in Lake Taihu, Cao et al. [13] evaluated the effects of light intensity on algal blooms in channel-type reservoirs, and Yang et al. [14] studied the combined effects of nitrogen concentration, temperature, and light intensity on the growth of *Microcystis aeruginosa* in batch culture. However, environmental factors exist individually but occur simultaneously in the nature, and HABs are always accompanied by complicated dynamics of the phytoplankton community in the practical aquatic environment [4]; therefore, investigations of the multiple effects of interactive environmental factors on phytoplankton growth and community structure in mixed culture will provide a better understanding of the formation of HABs in natural eutrophic water bodies. To accomplish this, it is useful to investigate the synergic effects of all influential environmental factors at once using a mathematical modeling method to identify major factors that have strong effects on mixed algal growth and determine the nutrient threshold values of the significant factors influencing HABs.

Apart from some arguments about the uncertainty associated with the proposed fitted multi-response surface model [15], response surface methodology (RSM) as a unique type of statistically based experimental design has been shown to be a more effective approach than investigation of the effects of factors individually. RSM has also frequently been used to screen significant variables from a large number of potential variables with a minimal level of optimized testing [16, 17]. RSM is generally preferred for estimation of the synergic effects among multiple environmental variables and value prediction [18–20]. Jiang et al. [21] used the central composite design to systematically investigate the effects of environmental factors on *Microcystis* growth and predict the optimum values of variables that stimulate rapid *Microcystis* growth. Lintz et al. [22] presented an approach to quantify ecological thresholds from response surface models. Wang and Linker [23] discussed the effects of limited nutrients on algal growth using the RSM. Nevertheless, none of these previous attempts has applied the approach of RSM to identify simultaneous interaction effects of these environmental factors on mixed algal growth and the nutrient thresholds for control of HABs. Accordingly, the present study was conducted to creatively apply a statistically based model designed by RSM to analyze the interaction effects of these significant environmental factors on the growth of mixed algae when they are simultaneously present in water.

Identification of the nutrient threshold values for HABs is useful to nutrient loading control as an effective tool of water quality management [24]. However, prediction of nutrient thresholds based on statistically designed regression models has not been discussed, and the simultaneous effects of various related physicochemical parameters have been ignored in investigations of nutrient thresholds for HABs. Two-dimensional statistical techniques have been employed to investigate effects on relationships between algal-nutrients and chlorophyll-nutrient, and significant breakpoint or threshold models of nutrient input were established to explain the variations in microcystins, algal metrics, or initiation of algal growth [21, 25–27]. The average threshold trophic level index (TLI) has also been discussed as the beginning point of eutrophication [2]. Xu et al. [11] proposed that the limiting levels of nutrient inputs for phytoplankton growth control in Lake Taihu were 0.8 mg/L TN and 0.2 mg/L TP. Most previous empirical models of nutrient thresholds were based on general water quality criteria such as eutrophication or acidification in aquatic ecosystems, rather than algal density or chlorophyll a content [28], while high levels of the latter variable were measured to reflect HABs more directly and adequately [29]. Therefore, the second goal of our research was to extend utilization of the response surface model and determine if targeting a certain value of algal density or chlorophyll a content as the critical border value of HAB could enable trace back to predict the corresponding nutrient-limiting values under various temperature and light intensity levels as alerting thresholds for HABs.

In this research, a batch of central composite designed (CCD) experiments that regulated the levels of four primary selected environmental factors was conducted to establish and analyze a second-order polynomial regression model and corresponding response surface plots for the interactive effects of TN, TP, temperature, and light intensity on algal density and chlorophyll a content. Phytoplankton community compositions with different TN/TP ratios were also identified to explain the correlation between the nutrient effects and the growth of mixed algae. The optimum TN/TP mass ratio and the nutrient loading thresholds of TN and TP for HABs were subsequently assessed by graphical optimization and value prediction of the response surface. Overall, the results presented herein provide valuable science-based theoretical information of HAB formation in eutrophicated freshwater bodies and will facilitate development of strategies for prevention of blooms and water quality monitoring. The optimization

strategy of the mathematical modeling approach established in our study for the evaluation of nutrient thresholds will be useful in similar studies conducted in the future.

2 Materials and Methods

2.1 Study Area

The study and sampling area is the Changtan Reservoir (116° 04′ 13″ E–116° 07′ 56″ E, 24° 42′ 23″ N–24° 50′ 15″ N), a typical subtropical channel-type freshwater reservoir with a total water-collecting area of about 1991 km^2 located in the Shiku River between Jiaoling County in Guangdong Province and Wuping County in Fujian Province (Fig. 1). Poultry wastewater, agricultural fertilizer, and residential sewage are non-point pollution sources with high levels of inorganic nitrogenous and phosphorous complexes that have caused severe eutrophication and frequent HABs in the reservoir. During summer, large-scale HABs are often visible in the downstream area of the reservoir (sampling sites A6–A10), which serves as the major drinking water source for Jiaoling County.

2.2 Sampling and Cultivation

Fresh culture samples of mixed algae and raw surface water samples (top 50 cm) were seasonally collected from 10 sites (A1–A10) in the Changtan Reservoir from October 2011 to December 2012 and analyzed for physicochemical parameters. Samples for quantitative and qualitative analyses were adjusted to a constant volume with Milli-Q distilled water (Millipore™ water purification system, Bedford, MA, USA) and preserved with 1 % acetic Lugol's solution. These cultures were centrifuged at 15,000 rpm for 10 min (Xiangyi™, H2050, Hunan, China), then cultivated in modified BG11 culture medium with an original pH of 7.5.

2.3 Experimental Design

The CCD response surface model was applied to evaluate the interactive effects and optimum values of important operating parameters for algal growth. The interactive effects of four main environmental factors, TN ($NaNO_3$ as the N source), TP (K_2HPO_4 as the P source), T (water surface temperature), and L (light intensity), on algal growth were investigated. The ranges of real values for each factor fixed in the CCD experimental design virtually covered the practical variation of natural environmental conditions observed in the Changtan Reservoir during our sampling period. These values were as follows: (a) nitrogen content 0.1 to 10 mg/L, (b) phosphorus content 0.01 to 1 mg/L, (c) temperature 18 to 34 °C, and (d) light intensity 28 to 142 μE/m^2/s. Although other ambient factors have the potential to influence cyanobacterial blooms, nutrients, temperature, and illumination were found to be the most significant influencing factors in the eutrophicated systems in the Changtan Reservoir.

As shown in Table 1, each independent factor varied over five coded levels ($-\alpha$, −1, 0, +1, $+\alpha$, α=2 for rotatable designs). The actual value of each determined variable i was coded as follows:

$$x_i = \frac{X_i - X_{ci}}{\Delta X_i}, \quad i = 1,\ 2,\ 3,\ \ldots,\ k; \tag{1}$$

where x_i was the coded level, X_i was the real value of an independent variable, X_{ci} was the real value of an independent variable at the central point, and ΔX_i was the step change of variable i.

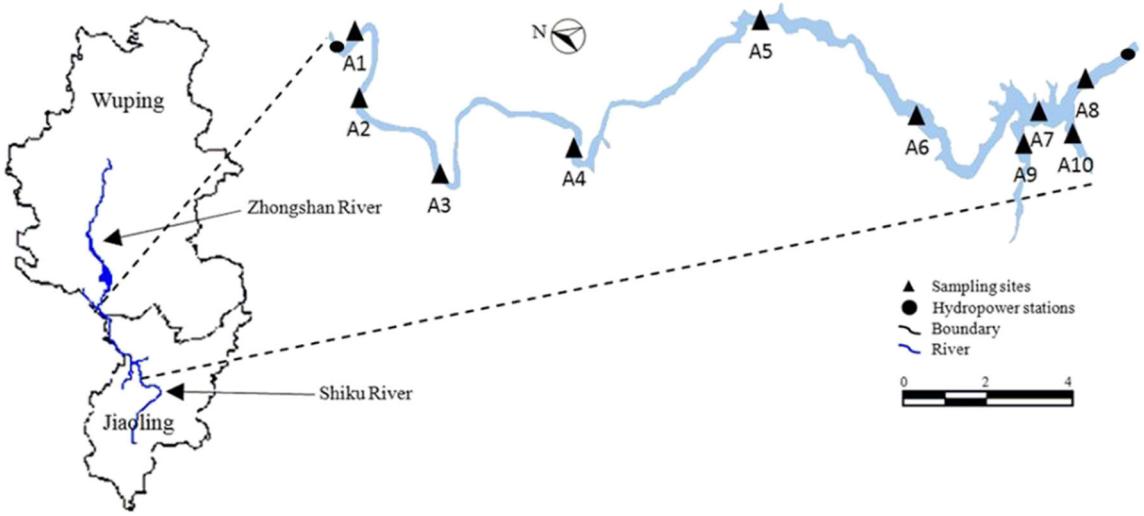

Fig. 1 Map of the study area and sampling sites

Table 1 Variations in real values of selected factors over coded levels for the CCD model

Level	TN (mg/L)	TP (mg/L)	T (°C)	L (μE/m^2/s)
+2	10.00	1.00	18	28
+1	7.53	0.75	22	57
0	5.05	0.51	26	85
−1	2.58	0.26	30	113
−2	0.10	0.01	34	142

The RSM represented these independent factors as process variables in a quantitative form:

$$Y = f(A,\ B,\ C,\ D) \tag{2}$$

where Y is the predicted response (i.e., algal density or chlorophyll a content), and A, B, C, and D are the coded levels of actual variables.

Response values were first approximated by a suitable lower-order polynomial in a linear manner represented by the following equation:

$$Y = k_0 + k_1A + k_2B + k_3C + k_4D \tag{3}$$

where k was the estimated coefficient of the equation.

In our system with curvatures, the following higher second-order polynomial in the form of a quadratic model was employed:

$$\begin{aligned} Y = {} & k_0 + k_1A + k_2B + k_3C + k_4D + k_{12}AB \\ & + k_{13}AC + k_{14}AD + k_{23}BC + k_{24}BD + k_{34}CD \\ & + k_{11}A^2 + k_{22}B^2 + k_{33}C^2 + k_{44}D^2 \end{aligned} \tag{4}$$

The CCD experimental matrix, which was derived from the standard CCD quadratic model using Design Expert (DX) 8.0.0 (Stat-Ease, Inc., Minneapolis, MN, USA), was composed of a central point and different coded levels for each selected factor (Table 2). We executed a total 30 different combinations of runs (including six replicates of the center point for experimental error estimation) to calculate 15 coefficients of the second-order polynomial equation for approximation of the experimental responses. The complex response surface provided by relatively few combinations of variables was obtained by plotting the expected response values.

To confirm the validity of our statistical model, the model prediction values were compared with the real values of algal density and chlorophyll a content reported in previous studies (Paso de las Piedras Reservoir and Lake Taihu) and our previous sampling data in the Changtan Reservoir. The three data sets of environmental factors were reported in the form of TN, TP, T, and L, and the real values of algal abundance were reported along with the standard deviations. The predicted values of algal density and chlorophyll a content were generated by calculation with a quadratic regression model using the average real values of corresponding environmental data reported in the literature. Thus, we can use this comparison to determine whether the obtained response surface model is valid for the practical application of HAB prediction.

Table 2 CCD experimental matrix of four environmental variables in five coded levels

Run	Variables			
	TN	TP	T	L
1	−1	−1	−1	−1
2	+1	−1	−1	−1
3	−1	+1	−1	−1
4	+1	+1	−1	−1
5	−1	−1	+1	−1
6	+1	−1	+1	−1
7	−1	+1	+1	−1
8	+1	+1	+1	−1
9	0	0	0	−2
10	0	0	−2	0
11	0	−2	0	0
12	−2	0	0	0
13	0	0	0	0
14	0	0	0	0
15	0	0	0	0
16	0	0	0	0
17	0	0	0	0
18	0	0	0	0
19	+2	0	0	0
20	0	+2	0	0
21	0	0	+2	0
22	0	0	0	+2
23	−1	−1	−1	+1
24	+1	−1	−1	+1
25	−1	+1	−1	+1
26	+1	+1	−1	+1
27	−1	−1	+1	+1
28	+1	−1	+1	+1
29	−1	+1	+1	+1
30	+1	+1	+1	+1

2.4 Experimental and Analytical Methods

2.4.1 Experimental Methods

Batch experiments were conducted with the previously cultivated mixed algae cultures, which had been diluted with fresh medium to an initial concentration of 750 cells/mL. Algal cultures were all grown at designated temperatures with a light/dark photoperiod of 12 h/12 h in a temperature/illumination-controlled incubator (Xinmiao™, GZX-250BS-II, Shanghai, China). Samples were exposed to photosynthetically active

radiation provided by fluorescent lights at one of five levels during the experiment. Based on the documented [30] and preliminary experimental results, the exponential growth period of the phytoplankton was around 7 to 10 days during incubation, and the specific growth rates were generally positive. Therefore, the experimental period of each run in our study was set to 7 days. Cultures were not absolutely axenic, but samples were autoclaved (Sanyo™, MLS-3750, Japan) at 120 °C (1.2 atm) for 2 h to minimize additional bacterial contamination over the experimental period. Erlenmeyer flasks sealed with cotton and kraft paper were used as experimental containers. All flasks were gently shaken in a thermostatic incubator shaker (Honour™, HNY-111B, Tianjin, China) three times for 30 min each every day to keep the medium well mixed and to disperse densely packed algal colonies.

2.4.2 Determination of Algal Density and Chlorophyll a Content

Algal density and chlorophyll a content were interpreted as responses to the CCD experimental matrix since significant variables for the model of algal density were not completely consistent with those for the model of chlorophyll a content. Phytoplankton samples for enumeration of abundance were collected and observed immediately after each experimental run. Variations in algal cell number and community structure were evaluated to the genus level by observing hemacytometer slides under a standard compound (upright) microscope (Carl Zeiss™ Light Microscopy, Axioskop 40 Pol, Germany) at ×400–×1000 magnification. The cell numbers determined from colonies and single cells were then converted to algal density based on aliquot size. The count precision was ±10 %, assuming that cells were randomly distributed [31]. The phytoplankton species were identified according to Hu's standard protocol [32]. The succession of dominant algal species in different environmental treatments was assessed based on the percentage of each species' contribution to the overall phytoplankton density. Species with a density percentage that was generally higher than 50 % of the average were considered dominant, whereas those with a density percentage higher than 10 % were considered associated species [33].

Statistical results have shown a linear correlation between chlorophyll a content and algal abundance in different freshwater environments during the accumulation of phytoplankton biomass [34]. Water samples for the measurement of chlorophyll a content were collected, after which 50-mL aliquots were filtered through Whatman AH glass fiber filters (0.45-μm pore size). Cell pigments were then extracted in the dark overnight by 90 % acetone solution and centrifuged [35, 36]. Finally, the chlorophyll a content was measured using the double-beam UV spectrophotometric method (Puxi™, TU-1901, Beijing, China).

3 Results and Discussion

3.1 Second-Order Polynomial CCD Model and Statistical Analysis

The estimated coefficients and statistical significance of parameters in second-order polynomial CCD models of algal density and chlorophyll a content are shown in Table 3. The combination of two codes indicates an interaction effect between two parameters. One term was considered significant when its p value was less than 0.05. Certain first-order parameters (A, B, D), second-order parameters (A^2, B^2, D^2), and interactive parameters (AB, AD, BD) showed a relatively significant influence ($p<0.05$) on variation of algal density, while

Table 3 Estimated regression coefficients and their associated p values of coded parameters in CCD models of algal density and chlorophyll a content

Variables	Coefficient estimate	p value
Model of algal density		
Intercept	2.960	<0.0001
A–TN	0.160	0.0243
B–TP	0.460	<0.0001
C–T	0.120	0.0733
D–L	0.350	<0.0001
AB	0.650	<0.0001
AC	−0.040	0.6221
AD	0.350	0.0005
BC	0.059	0.4712
BD	0.250	0.0073
CD	−0.037	0.6439
A^2	−0.320	0.0001
B^2	−0.300	0.0002
C^2	0.037	0.5549
D^2	0.180	0.0087
Model of chlorophyll a content		
Intercept	0.092	<0.0001
A–TN	0.004	0.2186
B–TP	0.012	0.0009
C–T	0.018	0.0530
D–L	0.012	0.0007
AB	0.018	0.0001
AC	0.0003	0.9440
AD	0.013	0.0019
BC	0.003	0.4645
BD	0.009	0.0184
CD	−0.002	0.5526
A^2	−0.010	0.0024
B^2	−0.010	0.0029
C^2	0.0005	0.8540
D^2	0.005	0.0809

others were not significant. Only B, D, A^2, and B^2 and interactive parameters AB, AD, and BD had a significant influence on the model of chlorophyll a content.

The coefficient estimates indicated that the first-order effects of all four environmental parameters were positive, while second-order effects of TN and TP were negative in both models. TN, TP, and light intensity were the most important variables influencing algal density and chlorophyll a content in our study, and these factors showed an interactive effect. The interactive effect between nutrients and light intensity found in this study might have been due to their influences on the photosynthetic processes and nutrient uptake by algae [37].

The parameter coefficients were estimated by RSM based on CCD design. High values of parameter estimates for variables with a high level of significance indicated their importance. These coefficients were fitted in the second-order regression equation in terms of coded factors to describe variations in algal density (Y_a) as follows:

$$Y_a = 2.96 + 0.16A + 0.46B + 0.12C + 0.35D + 0.65AB - 0.04AC + 0.35AD + 0.059BC + 0.25BD - 0.037CD - 0.32A^2 - 0.3B^2 + 0.037C^2 + 0.18D^2 \quad (5)$$

where A=TN, B=TP, C=temperature, and D=light intensity.

The second-order polynomial model of chlorophyll a content (Y_c) in terms of coded factors was also regressed by considering all of the significant terms and was expressed as follows:

$$Y_c = 0.092 + 0.004A + 0.012B + 0.018C + 0.012D + 0.018AB + 0.0003AC + 0.013AD + 0.003BC + 0.009BD - 0.002CD - 0.01A^2 - 0.01B^2 + 0.0005C^2 + 0.005D^2 \quad (6)$$

where A=TN, B=TP, C=temperature, and D=light intensity.

The second-order polynomial equation was applied for response surface optimization to determine the optimum conditions for HABs. The coded response values of the independent variables were entered in the established design to fit the suggested numerical model. Rotatable 3D response surface plots were then visually accessed by plotting the predicted responses according to the final quadratic polynomial equation in terms of coded factors, which explained the regular pattern of experimental data variances.

Multiple regression analysis of variance (ANOVA) was conducted to test the significance of fit of the second-order polynomial equation for our experimental data. As shown in Table 4, the algal density regression model was significant, as indicated by the low probability value of $p<0.0001$ and the Fisher F-test value of 17.98, which meant there was only a 0.01 % chance that a Model F value this large could occur because of noise. The low standard deviation and coefficient of variation (CV) revealed that most variations could be explained by the model. The goodness of regression was also checked by the coefficient of determination R-squared and adjusted R-squared values, which were 0.944 and 0.891, respectively, indicating a highly dependent correlation between the measured and modeled values of response. The adequate precision was a signal to noise ratio that compared the range of predicted values at the design points with the average prediction error. Its value of 17.943 revealed that model discrimination was satisfied and the modeled responses accurately reflected the measured variables.

Similarly, the low probability value of 0.0013 and the Fisher F-test value of 5.39 implied that the model of chlorophyll a content was also significant. The pure error of 0.003 implied that the lack of fit was not significantly relative to the pure error, which indicated good model fitness. The R-squared and adjusted R-squared value of 0.834 and 0.679, respectively, reflected an acceptable fit between the observed and simulated values, but these were not as good as for the model of algal density. The adequate precision value of 9.676 indicated the rationality of the regression model for analysis of the response trends for optimization.

3.2 Phytoplankton Community Composition and Dominant Algal Species

A total of 24 algal species among five phytoplankton phyla with different TN/TP ratios were identified. The observed phytoplankton species included Bacillariophyta (*Melosira*, *Navicula*, *Nitzschia*, and *Surirella*), Chlorophyta (*Chlorella*, *Cladophora*, *Closterium*, *Cosmarium*, *Oocystis*, *Scenedesmus*, *Ulothrix*, and *Volvox*), Chrysophyta (*Ochromonas* and *Synura*), Cyanophyta (*Anabaena*, *Aphanizomenon*, *Chroococcus*, *Coelosphaerium*, *Gomphosphaeria*, *Lyngbya*, *Microcystis*, *Nostoc*, and *Nostocales*), and Euglenophyta (*Euglena*). The phytoplankton community structure at different TN/TP ratios is shown in Fig. 2. The relative abundance of *Microcystis* to the total algae was generally greater than 50 % (except when TN/TP<1) during the bloom period, appearing to be the most dominant cyanobacterial species. Additionally, *Scenedesmus*, *Cladophora*, and *Melosira* were considered to be the three major associated species. The density percentages of the N-fixing species (*Anabaena*, *Aphanizomenon*, and *Nostoc*) at different nutrient ratios were generally less than 9 %. The highest proportion of non-N-fixing species occurred at a TN/TP of 10.

3.3 Effect of Interactive Variables TN-TP

3.3.1 Model of Algal Density and Chlorophyll a Content

Response surface plots were investigated to determine trends in the variation of algal density and chlorophyll a content over

Table 4 Analysis of variance for regression of the CCD response surface model

Sources of variation	Sum of squares	Degree of freedom	*M*-squared	*F* value	*p* value>*F*
Model of algal density					
Model	25.45	14	1.82	17.98	<0.0001
Pure error	0.048	5	0.0096		
Corrected total	26.970	29			
Standard deviation	0.320				
C. V. (%)	12.020				
R-squared	0.944				
Adjusted *R*-squared	0.891				
Adequate precision	17.943				
Model of chlorophyll a content					
Model	0.024	14	0.0017	8.65	<0.0001
Pure error	0.0002	5	0.0005		
Corrected total	0.027	29			
Standard deviation	0.014				
C. V. (%)	17.240				
R-squared	0.890				
Adjusted *R*-squared	0.787				
Adequate precision	12.400				

the interactive variables TN-TP under different temperatures and illumination intensities. As shown in Fig. 3, the 3D saddle-like response surface plots of algal density with varying TN/TP demonstrated the synergic growth-promoting effects of TN and TP on algal growth with a light intensity of 142 μE/m^2/s at 30 °C, which are the average conditions of the Changtan Reservoir during summer. Increasing the TN concentration from 0.5 to 5 mg/L and the TP concentration from 0.05 to 0.5 mg/L facilitated the rise of algal density from 1.23×10^4 to 4.23×10^4 cells/mL. Significant interactive effects of both TN and TP (non-linear positive correlation) on algal density were observed. These findings indicate that the phytoplankton growth was not merely determined by the initial magnitude or the individual variation of N or P. Specifically, at higher P concentration, the increasing growth rate of algal density with increasing TN loading concentration was significant. Similarly, for the response surface plots of chlorophyll a content (Fig. 4), a significant positive interaction effect of both N and P sources was observed. Chlorophyll a content, which reflected total phytoplankton biomass, increased from 0.019 to 0.137 mg/L with TN and TP supplies ranging from 0.5 to 5 mg/L and 0.05 to 0.5 mg/L, respectively.

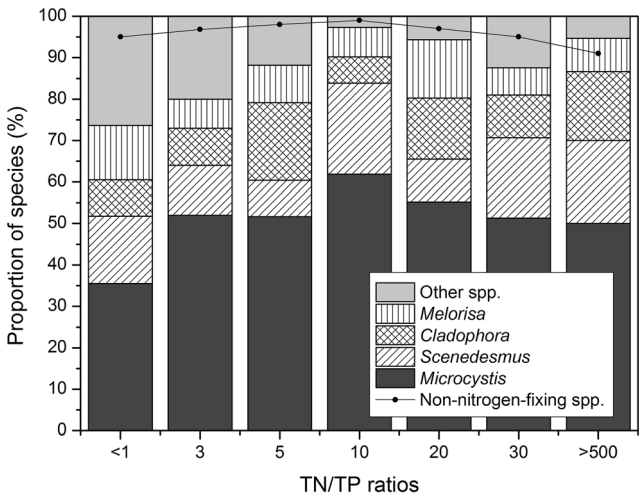

Fig. 2 Phytoplankton community structure at different TN/TP ratios

It is well known that nitrogen and phosphorus are essential nutrients for algal proliferation. The occurrence of HABs is primarily stimulated by excessive increases of TN and TP in aquatic areas. Although a strong positive correlation is assumed to exist between algal blooms and TP concentration in the water, and some arguments have asserted that cyanobacterial blooms are more affected by increases in P than simply by a decrease in the TN/TP ratio [38, 39], recent studies revealed that the effect of P was related to N, and HABs still required N to reach a certain level [3, 11, 40, 41]. Similarly, phytoplankton abundance did not increase significantly in response to nitrogen while the phosphorus level was low. Klausmeier et al. [42] explored whether exponential algal growth favors a greater allocation to P-rich assembly machinery. Although the availability of nitrogen is essential to the proliferation of non-N-fixing species, reductions in heavy P loading are important for controlling the magnitude of HABs [11]. Our modeling results contradicted previous findings that the singular accumulation of nitrogen or phosphorus in water bodies was the key factor stimulating phytoplankton proliferation. High concentrations of both TN and TP were

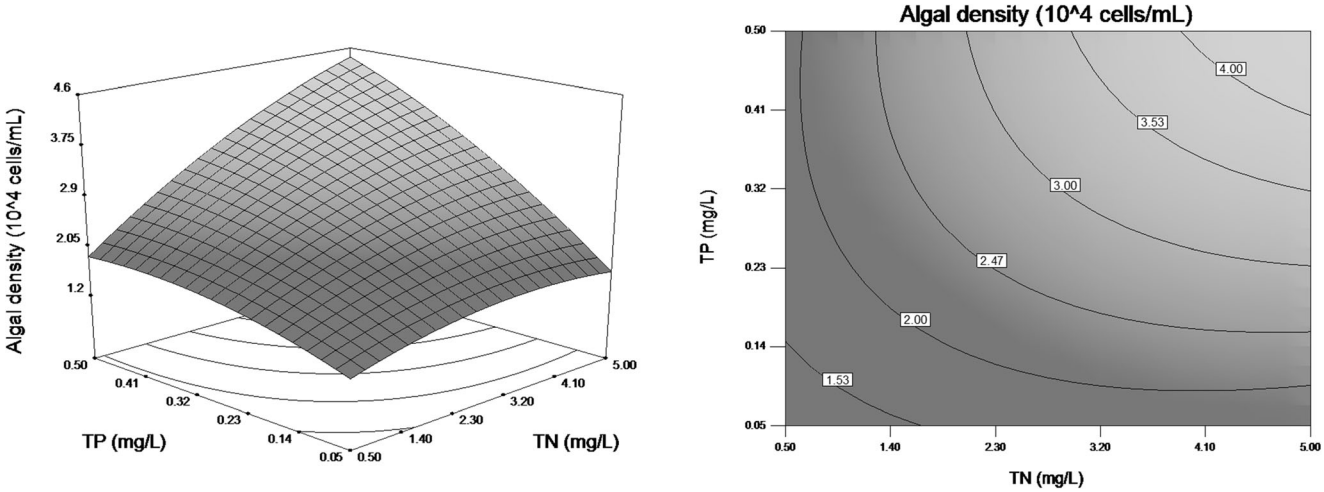

Fig. 3 3D response surface plot of algal density with varying TN/TP and corresponding contour graph at T 30 °C–L 142 μE/m^2/s

significantly correlated for this kind of algal bloom forming dominated by non-N-fixing species such as *Microcystis*.

3.3.2 Optimal TN/TP Loading Ratio for HABs

Graphical optimization of response surface plots by DX analysis enabled determination of the optimal TN/TP loading mass ratio for increases in algal density and chlorophyll a content. The method consisted of setting maximum limits for each response under different temperature and illumination conditions and overlaying the contour curves according to the criteria imposed, followed by calculation of the corresponding TN/TP ratio. The model of algal density indicated that the optimum TN or TP concentration for algal growth might vary under different temperatures or light conditions, and the average optimum TN/TP ratio ranged from 9.5 to 10.1. The optimum TN/TP ratio for algal growth described by the model of chlorophyll a content ranged from 7.9 to 8.6, which was slightly lower than that of the algal density model.

As shown in Fig. 3, algal growth might be restricted upon divergence from the optimum TN/TP ratio when N or P sources are raised or reduced separately, which is similar to the results reported by Chen et al. [3]. The optimum TN/TP ratio was closely related to variations in algal density and chlorophyll a content within certain ranges of nutrient concentrations, but no longer recommended as a decisive predictor for algal bloom control as long as nutrient inputs exceeded threshold values for HABs. Saturation of the algal population by high concentrations of N and P may be complicated by increasing nutrient demand that likely constrains nutrient transport and availability [26]. The optimum TN/TP ratio was considered as a cause rather than a result of large-scale HABs.

Redfield first suggested that the optimum N/P mass ratio for phytoplankton growth was about 16:1, with seasonal variations [43]. Later, experimental whole-lake research [44, 45] established that high concentrations of P and a low N/P supply ratios were favorable for cyanobacterial dominance and that a TN/TP mass ratio of 29:1 differentiates lakes with and without

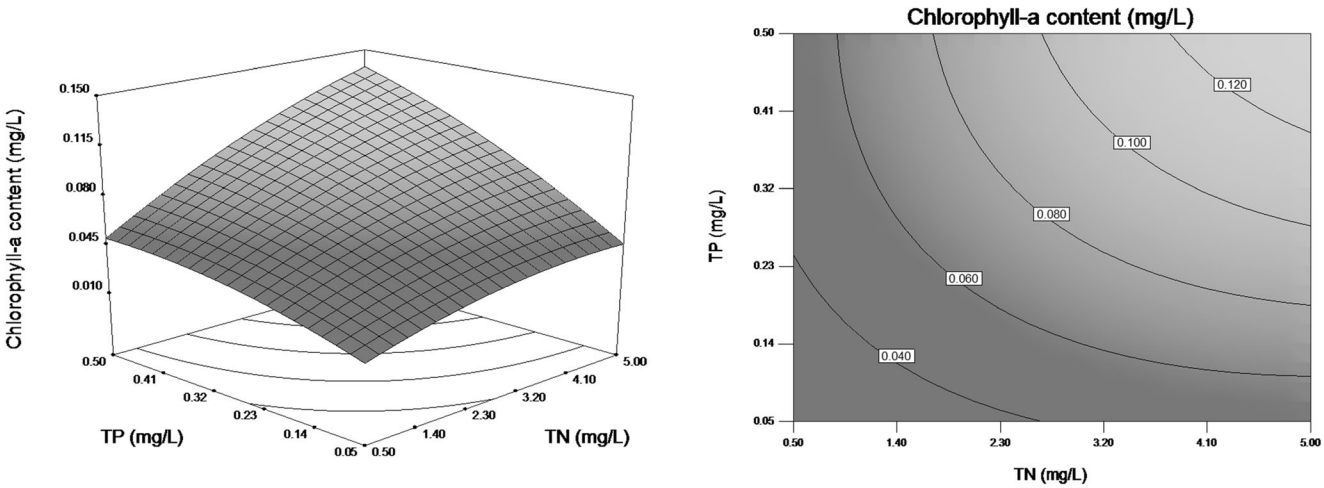

Fig. 4 3D response surface plot of chlorophyll a content in response to TN/TP and corresponding contour graph at T 30 °C–L 142 μE/m^2/s

cyanobacterial dominance. Although Downing et al. [46] suggested that cyanobacterial blooms were more strongly correlated to simple increases in nutrient concentration rather than TN/TP ratio, Klausmeier et al. [42] later used a Droop's model to show that the optimum N/P ratio under exponential growth at saturated nutrient resource levels was 8.2. Further studies predicted that cyanobacterial dominance and its rapid growth were evident at TN/TP ratios of less than 30 [12, 40]. Rather, simple total nutrient ratios (TN/TP) reflected phytoplankton requirement for nutrients [47]. Kim et al. [40] also predicted that algal blooms were favored under relatively low TN/TP ratios in hypertrophic reservoir systems. The range of optimum TN/TP loading ratios under different temperatures or light intensities suggested in the present study (7.9 to 10.1) was generally in accordance with these previous findings.

Cyanobacteria is a diverse group, and only some species (*Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, etc.) are nitrogen fixers. Smith et al. [48] concluded that the best ratio of dissolved N/P ratio for the growth of BGA was 7.2:1 by mass and that a TN/TP ratio of 22:1 provided a distinct boundary for water bodies dominated by N-fixing cyanobacteria and those without such dominance. It has been widely reported that N-fixing species had a higher optimum TN/TP ratio than non-N-fixing species because N fixers need to power N fixation at the expense of P-rich assembly machinery [42]. Typically, the bloom-forming *Microcystis* was a superior competitor with a lower N/P supply ratio (<10) in warm temperatures [12, 40]. These previous reports have been confirmed in the present study. As shown in Fig. 2, the relative abundance of non-N-fixing species to the total algae at different TN/TP ratios was generally greater than 90 % during the entire exponential growth period in the system investigated in the present study. The phytoplankton community was dominated by *Microcystis*, which belongs to a non-N-fixing cyanobacterial genus [4]. Consequently, the average optimum TN/TP ratio proposed by our research does not emerge as a universally applicable value from our theoretical modeling results. Instead, it should be seen as merely a species-specific optimum ratio linked to the prediction of blooming dominated by non-N-fixing cyanobacteria species.

3.4 Effect of Temperature-Illumination Interaction

The response surface plot of the effects of temperature and light intensity is presented in Fig. 5. As shown in this figure, the algal density was interactively affected by temperature and light intensity when TN and TP were set to their optimum levels. Increasing the temperature from 18 to 34 °C facilitated an increase in algal density from 2.76×10^4 to 3.68×10^4 cells/mL. As light intensity decreased, algal density decreased from 3.31×10^4 to 2.28×10^4 cells/mL and then showed a slight rebound at low light.

Many previous experimental studies have shown that high light intensity could accelerate photosynthesis and stimulate the proliferation of phytoplankton, while others have shown that low light levels could suppress dominance of all species of cyanobacteria in favor of other phytoplankton [49, 50]. The range of light intensity in the Changtan Reservoir was slightly higher than the light conditions in the Guadiana estuary (9.3 to 85.6 $\mu E/m^2/s$), where nutrient concentrations were also unlimited and the occurrence of photoinhibition was unlikely due to light-limiting conditions [30]. Positive responses of phytoplankton abundance under high light conditions could therefore be reasonably attributed to light enrichment.

The impact of increasing temperature on algal density appeared to be greater when light intensity was low, but phytoplankton growth was obviously stimulated to a large extent by strong illumination (prior to high temperature) with light at greater than 75 $\mu E/m^2/s$. The different increases shown by the 3D response surface plots of the function of temperature and illumination indicated that light intensity exerted a greater influence on algae growth than temperature in natural environmental systems, which coincided with the results of ANOVA. A high algal density of 3.31×10^4 cells/mL could be reached at 18 °C at a light intensity of 142 $\mu E/m^2/s$, which was not far from the algal density of 3.71×10^4 cells/mL at 34 °C that was observed under the same light intensity. These results indicated that algal blooming might still occur at lower temperatures when there are abundant nutrients in water, and the unfavorable effect of low temperature could be made up for by high luminosity.

As shown in the response surface plots, the biomass peak of algal density occurred when water temperature and light intensity reached their maximum values. These findings agreed with the argument that high light intensity alone was not advantageous to phytoplankton growth, but that a combination of suitable temperature and light intensity resulted in HABs under the same water quality and hydrodynamic conditions [13]. The phytoplankton abundance observed at light intensity lower than 75 $\mu E/m^2/s$ may have been due to the acclimation of several low-energy requiring cyanobacteria (e.g., *Microcystis* or *Lyngbya*) to low-temperature and low-light conditions [21, 30]. These specific cyanobacterial species were assumed to maintain buoyancy and have a competitive advantage over other phytoplankton species by avoiding light limitations [12]. The proliferation of certain photophilic cyanobacterial species and their contribution to the total algal biomass during the entire growth period were relatively limited.

3.5 Threshold Value of Optimized Nutrient Input for HABs

Algae can be considered to be blooming at concentrations of hundreds to thousands of cells per milliliter, depending on the

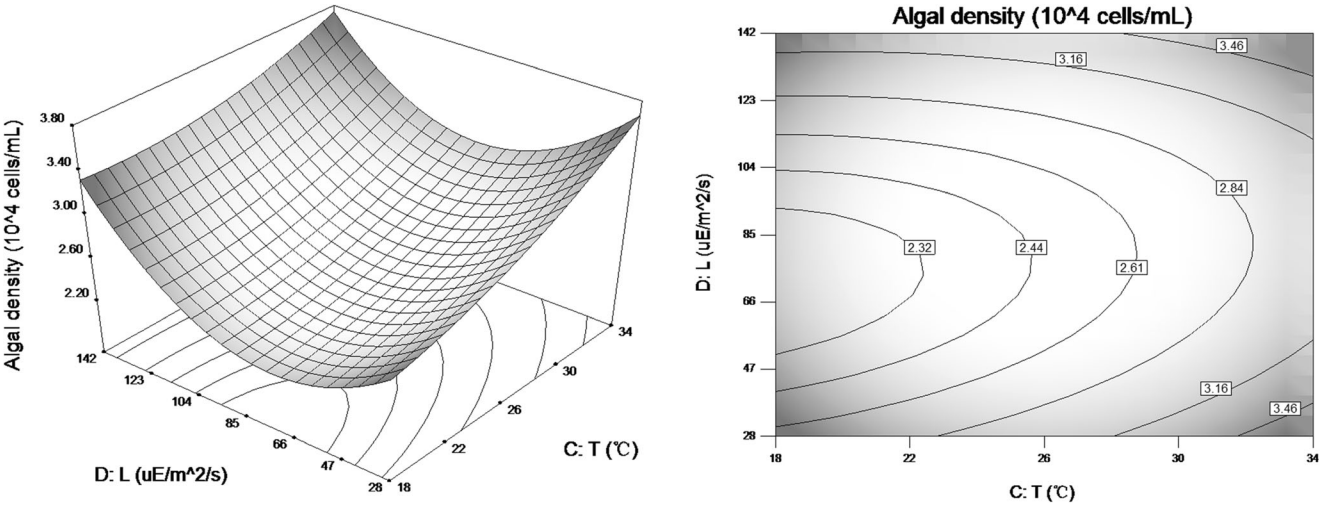

Fig. 5 3D response surface plot of the effects of temperature/light intensity on algal density and corresponding contour graph at TN 2.76 mg/L–TP 0.11 mg/L

severity of the bloom. Since there are no officially recognized criteria, an algal density of 2.00×10^4 cells/mL was taken as the critical sign of large-scale HABs in this study based on our previous sampling experience and discoloration of the water resulting from the assemblage of pigmented cells.

The identification of nutrient threshold values was based on a consistent set of overlaying contour graphs of algal density highlighting an area of operability for HABs in which all environmental criteria were simultaneously satisfied. The consistency of nutrient threshold values measured across various temperature and light intensity levels suggested that the thresholds identified in this study were ecologically relevant. By digitizing the contour curves and coordinating unique curve points with DX analysis, border values of TN and TP for large-scale HABs with different water temperature and light intensities could be identified as nutrient thresholds and described in the 3D regression surface plots shown in Fig. 6 ($p<0.05$). The threshold values for TN ranged from 0.31 to 1.39 mg/L, while those for TP ranged from 0.08 to 0.16 mg/L. The range of nutrient threshold values identified in this study was similar to the thresholds identified by Black et al. [27] using algal metrics within the same environmental impacts (TN 0.59 to 1.79 mg/L, TP 0.03 to 0.28 mg/L), which were based on the criteria of algal community data.

The regression model of nutrient thresholds established in this study used algal density as the alerting criteria rather than chlorophyll a content and explained higher variances of nutrient thresholds under different conditions of temperature and light intensity in a predictable manner. As an example, rapid large-scale algae assemblages were often observed on the

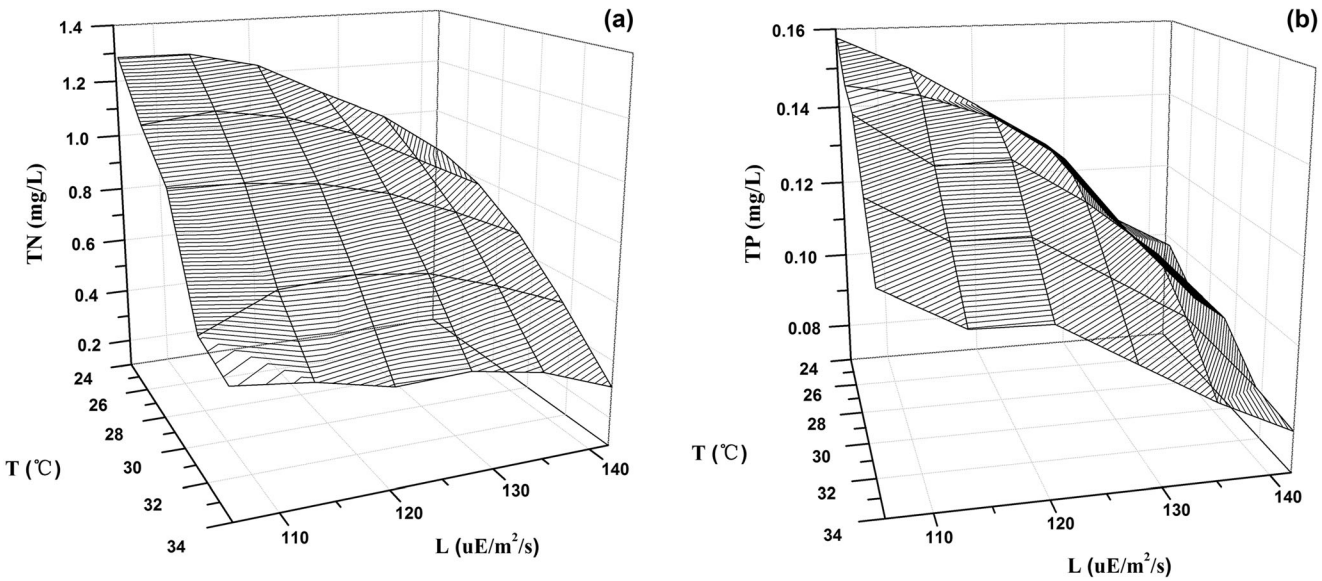

Fig. 6 3D regression surface plots of TN (**a**) and TP (**b**) thresholds for HABs based on the criteria for algal density in response to temperature/light intensity

water surface on hot summer days; therefore, a TN of 0.73 mg/L and TP of 0.07 mg/L should be used as thresholds at 30 °C–142 μE/m^2/s, which are the average conditions in the Changtan Reservoir during summer. Conversely, a TN of 1.19 mg/L and TP of 0.15 mg/L should be used as nutrient threshold values for HABs at 26 °C–113 μE/m^2/s, which are the average conditions in the Changtan Reservoir during spring and autumn. These results agree with the measured algal density during blooming and water quality data (TN> 0.8 mg/L and TP>0.05 mg/L) from downstream sampling sites (A6–A10) in the Changtan Reservoir (Fig. 7a). The nutrient threshold values for HABs under low temperature and poor light availability were generally considered unattainable and are therefore not reflected in Fig. 6a. For example, the nutrient threshold values for winter (TN of 3.33 mg/L and TP of 0.43 mg/L) could not easily be attained at 22 °C with a light intensity of 85 μE/m^2/s. Considering the reported nutrient absorption ability of cyanobacteria [51], we found that the nutrient thresholds obtained by our simulation were feasible.

3.6 Validation of Model Prediction

To confirm the validity of our statistical and experimental strategies, the model was first tested by comparing the results of model prediction and measured values of algal density among all 10 sampling sites of the Changtan Reservoir in July 2012. As shown in Fig. 7a, the modeled values were substantially higher than the actual phytoplankton abundance from sampling sites A1 to A3. The large deviation between the predicted and the real values of sampling sites A1 to A3 could be attributed to the fact that the algal assemblages are significantly dispersed by the high flow rate because of the daily sluice from the upper hydropower station (designated as a "filled circle" in Fig. 1). The predicted values for other sampling sites were slightly underestimated, but fit natural consequences acceptably. The underestimation likely occurred because the flow rate decreased significantly in these downstream sampling sites (A5 to A10) owing to the wider channel, and the algae assemblages were mostly retarded and accumulated on the water surface of these open areas. The increase of algal biomass in these downstream areas was also considered one of the major signs of serious HABs in the Changtan Reservoir. Further investigations are required before evaluating the hydrodynamic effects on the predicted values which can be completed through new modeling approaches.

Model verification was performed using two other cases of algal blooming formation reported in Paso de las Piedras Reservoir of Argentina [33] and Lake Taihu of China [7], which are a typical hypertrophic lake and reservoir in the subtropics, respectively (Fig. 7b, c). The actual values shown in the figure were acquired from the reported results of replicates. The predicted algal density and chlorophyll a content were consistent

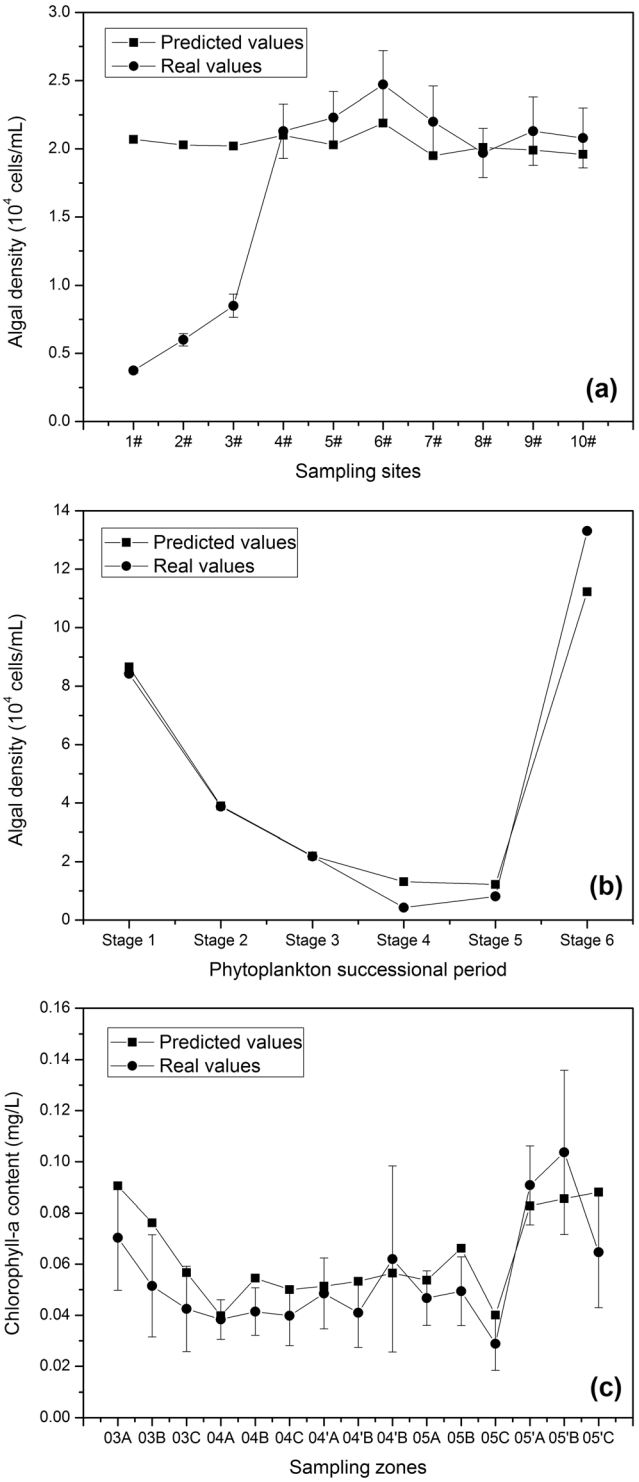

Fig. 7 Comparison of predicted and measured values for the Changtan Reservoir (**a**), Paso de las Piedras Reservoir in Argentina (**b**), and Lake Taihu (**c**)

with the actual values obtained from sampling (standard error <10 %, *R*-squared >90 %). Further testing using optimized medium (TN/TP ratio between 7.9 and 10.1) was conducted under the same cultivation conditions, and the maximum algal

density of 6.2×10^4 cells/mL and chlorophyll a content of 0.138 mg/L were attained. These findings indicate that the predicted values of our second-order polynomial models for algal density and chlorophyll a content agreed well with the experimental results and previously reported practical data, which further confirmed the good fit between our statistical simulation and the actual values.

4 Conclusions

In this study, CCD response surface models were established to investigate the effects of selected significant environmental factors (TN, TP, temperature, light intensity) on algal density and chlorophyll a content. The optimum TN/TP mass ratios and nutrient thresholds under different environmental circumstances for large-scale HABs were also evaluated. The active interaction effect of high TN and TP concentrations together with a TN/TP ratio between 7.9 and 10.1 was determined to be the most significant activating parameters associated with algal bloom development dominated by non-N-fixing species. The positive effects of light intensity on algae growth were greater than those of water temperature.

Establishment of a second polynomial regression model by fitting the experimental data and validation based on comparison to natural values of algal biomass enabled prediction of the nutrient loading thresholds of TN and TP under different water temperatures and light intensities. Accordingly, this model can be used to help alert managers to the eutrophication status of selected water bodies and the critical point of HABs. The results presented here contribute to the theoretical knowledge for the prediction and analysis of algal bloom formation in the Changtan Reservoir and other similarly eutrophic freshwater lakes or reservoirs. However, further research is necessary to clarify the mechanisms by which hydrodynamics and phytoplankton succession affect algal blooms under various physicochemical conditions.

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