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Eutrophication drives regime shift via changes in stoichiometric homeostasis-based submerged macrophyte assemblages

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Homeostasis for phosphorus (HP) in submerged macrophytes may influence the susceptibility of lakes to regime shifts; however, the mechanisms linking submerged macrophyte HP to regime shifts remain unclear. We conducted an in situ mesocosm experiment to compare the dynamic responses of a high-HP species, *Potamogeton maackianus*, and a low-HP species, *Hydrilla verticillata*, to different phosphorus (P) level gradients, as well as their effects on phytoplankton inhibition. The biomass of *P. maackianus* under mesotrophic P (MP; P concentration 0.05 mg L⁻¹) and eutrophic P (EP; P concentration 0.10 mg L⁻¹) conditions was either non-significantly different from, or lower than that under oligotrophic P conditions (OP; P concentration under detection limit of 0.01 mg L⁻¹). Conversely, *H. verticillata* biomass under EP was significantly higher than that under MP on day 90, whereas it died under OP. This variable response of submerged macrophyte species to P level gradients increased the relative growth advantage of *H. verticillata* compared to *P. maackianus* during eutrophication. The inhibition ratio of phytoplankton (IRP) for *P. maackianus* was ~15 times higher than that for *H. verticillata* under EP. Our study demonstrated a trend that submerged macrophyte assemblage IRP increased along with its assemblage HP. Thus, the changes in submerged macrophyte assemblages from high-HP species-dominance to low-HP species-dominance would erode its phytoplankton inhibition capacity, and further promote the regime shift from a clear-water state to a turbid state. Our results advance the regime shift theory from an ecological stoichiometry perspective and highlight the importance of high-HP submerged macrophyte species in the restoration of eutrophic lakes.

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INTRODUCTION

Eutrophication, caused by excessive loading of phosphorus (P) or nitrogen (N), can drive a regime shift in aquatic ecosystems from a clear-water state dominated by submerged macrophytes to a turbid state dominated by phytoplankton. High nutrient levels not only cause physiological damage to the submerged macrophytes (i.e., by generating oxidative stress and disturbing metabolism of carbon [C] and [N]), but also induce excessive proliferation of phytoplankton and periphyton, which can shade the submerged macrophytes^{1–3}. Since the anthropogenic eutrophication of lakes was recognized as a severe ecological problem in mid-twentieth century⁴, an increasing number of shallow lakes have experienced regime shift, thus threatening the ecological health of shallow lakes and human drinking water sources worldwide^{5–7}. The susceptibility of lakes to regime shifts is largely determined by the aquatic assemblages within the lake, particularly submerged macrophyte assemblages⁸. In addition, ecological stoichiometry, the balance between multiple chemical elements in ecological processes, is related to multiple ecological mechanisms (e.g., eutrophication and trophic interactions) that are critical for submerged macrophyte assemblages^{9–11}, thus emphasizing its potential for increasing our understanding of the submerged macrophyte assemblage and regime shifts in shallow lakes.

Stoichiometric homeostasis, which is a basic theory of ecological stoichiometry, refers to the degree to which organisms maintain a stable element content in their tissues under various

ambient nutritional conditions¹². Thus, it reflects the physiological responses of organisms to external environments^{13–15} and is a key mechanism linking abiotic factors and ecological processes within ecosystems¹⁰. Varied stoichiometric homeostasis, especially stoichiometric homeostasis for phosphorus (HP), which is the key macroelement triggering aquatic eutrophication¹⁶, is related to different growth strategies of plant species. Previous studies have demonstrated that high-HP species, which can maintain a relatively constant P level in tissues regardless of fluctuations in the ambient P level, generally display a slow-growth strategy and high resistance to altered external environments (e.g., light availability)¹⁷. Conversely, low-HP species, in which the tissue P content increases with an increase in the ambient P level, generally display fast-growth strategy and weak resistance to altered external environments^{18,19}.

Species-specific growth strategies lead to niche differentiation, which, together with interspecies relationships, further induce changes in submerged macrophyte assemblages along environmental gradients²⁰. Previous studies have demonstrated variations in the relative importance of plant growth strategies (as indicated by species-specific responses to environmental gradients) and interspecies relationships, for the changes in plant assemblages^{21,22}. Previous field studies have demonstrated that high-HP species generally dominate oligotrophic ecosystems with low productivity but high biodiversity²³, whereas low-HP species are common in eutrophic ecosystems²⁴. However, the key role of different growth strategies in the changes in submerged

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Table 1. Results of the two-way analysis of variance.

	d.f.	PM						d.f.	HV					
		Day 30		Day 60		Day 90			Day 30		Day 60		Day 90	
		F	p	F	p	F	p		F	p	F	p	F	p
P	2	4.53	0.03	5.99	0.02	1.39	0.29	2	2.85	0.10	7.59	0.01	57.88	0.00
S	1	0.66	0.43	0.73	0.41	1.16	0.31	1	0.95	0.35	4.55	0.05	2.16	0.19
P*S	2	1.17	0.34	1.97	0.18	0.49	0.63	2	0.35	0.71	2.88	0.10	1.06	0.34

	d.f.	Chl a						d.f.	IRP					
		Day 30		Day 60		Day 90			Day 30		Day 60		Day 90	
		F	p	F	p	F	p		F	p	F	p	F	p
P	2	97.72	0.00	29.90	0.00	17.50	0.00	2	15.09	0.00	38.33	0.00	6.728	0.01
S	3	2.57	0.08	10.46	0.00	10.57	0.00	2	4.81	0.02	14.48	0.00	16.08	0.00
P*S	6	1.63	0.18	1.90	0.12	1.09	0.40	3	2.29	0.10	3.67	0.02	4.08	0.03

The effects of phosphorus level (P), submerged macrophyte species assemblage (S), and their interaction (P*S) on the *Potamogeton maackianus* biomass per plant (PM), *Hydrilla verticillata* biomass per plant (HV), chlorophyll a level (Chl a), and inhibition ratio of phytoplankton (IRP) at each day of measurement. Significant effects ($p < 0.05$) are indicated in bold text.

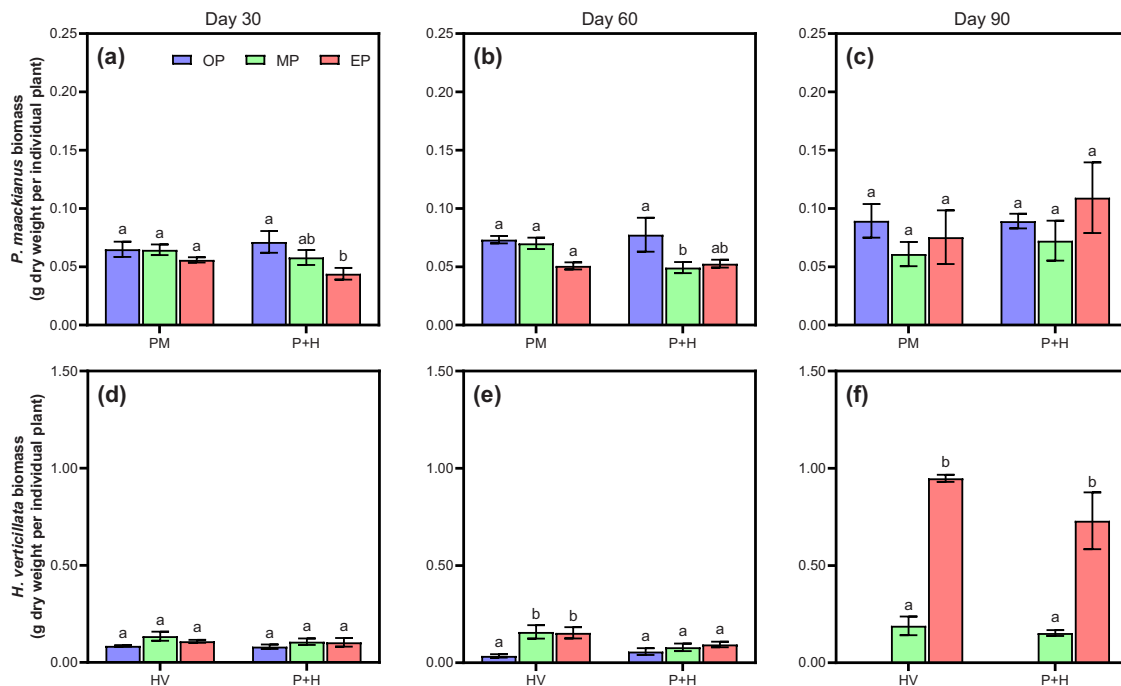


Fig. 1 Biomass of submerged macrophytes. **a–c** Biomass of *Potamogeton maackianus*, **d–f** biomass of *Hydrilla verticillata*. Error bars indicate standard errors ($n = 3$). Values with different lowercase letters are significantly different among phosphorus (P) levels in the same submerged macrophyte assemblages at $p < 0.05$. Note that *H. verticillata* died under OP treatment on day 90. OP, MP, EP indicated three P levels of oligotrophic P (P concentration under detection limit and no P addition), mesotrophic P (0.05 mg L^{-1}), eutrophic P (0.10 mg L^{-1}), respectively. PM, HV, P + H indicated four levels of treatment for submerged macrophyte species assemblage of monoculture *P. maackianus*, monoculture *H. verticillata*, a mixture of the two plants (1:1 plant individuals), respectively.

macrophyte assemblages along nutrient levels could not be confirmed because interspecific relationships were seldom quantified in these studies. Regarding studies on interspecific relationships, a stress gradient hypothesis has been proposed, whereby increasing environmental stress can shift interspecific relationships from competition to facilitation; however, this hypothesis remains controversial^{25,26}.

Field studies have demonstrated that submerged macrophyte assemblages dominated by high-HP species are more capable of maintaining a clear-water state than those dominated by low-HP species^{12,18}. Therefore, the changes in submerged macrophyte assemblages from high-HP species-dominance to low-HP species-dominance can be hypothesized as an important mechanism of regime shift in eutrophic lakes. However, the theoretical basis for

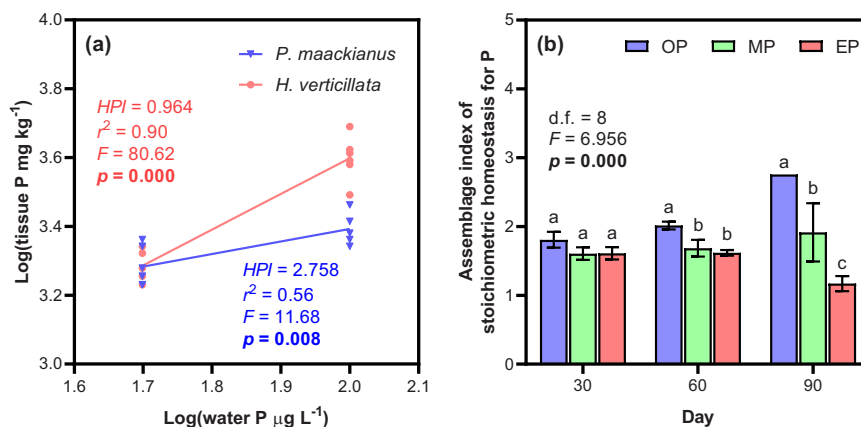


Fig. 2 Stoichiometric homeostasis for phosphorus (P) of the submerged macrophytes. **a** Relationship between the water total P and plant tissue P contents in submerged macrophytes, **b** assemblage stoichiometric homeostasis for P (HPI) under P + H. HPI was obtained using the following equation: $\log(y) = \log(c) + (1/\text{HPI}) \times \log(x)$; where y is the P content in plant tissues, x is the P level in the water column, and c is a constant. Error bars demonstrate standard errors ($n = 3$). Different lowercase letters before the slash are significantly different among P levels on the same day ($p < 0.05$). OP, MP, EP indicated three P levels of oligotrophic P (P concentration under detection limit and no P addition), mesotrophic P (0.05 mg L^{-1}), eutrophic P (0.10 mg L^{-1}), respectively.

this hypothesis has not been fully established. Although several mechanisms (e.g., nutrient competition, allelopathy) have been confirmed to be critical for the clear-water state maintenance of submerged macrophytes^{27,28}, their relative importance under various conditions has not yet been determined, as experimental evidence to compare the effect of submerged macrophytes with varied assemblages on clear-water state maintenance is largely lacking²⁹. To the best of our knowledge, systematic experimental studies have not been undertaken on the changes in submerged macrophyte assemblages and clear-water state maintenance in response to eutrophication based on HP.

To obtain direct evidence of the mechanism by which eutrophication drives regime shift via changes in stoichiometric homeostasis-based-submerged macrophyte assemblages, we conducted an in situ mesocosm experiment. We used different submerged macrophyte assemblages and nutrient levels to compare the dynamic responses of a high-HP species, *Potamogeton maackianus*, and a low-HP species, *Hydrilla verticillata*, as well as phytoplankton, to eutrophication. The relative growth advantage and inhibition ratio of phytoplankton (IRP) were used to assess changes in submerged macrophyte assemblages and clear-water state maintenance, respectively. We hypothesized that (1) low-HP-submerged macrophytes have a growth advantage with eutrophication due to their fast-growth strategy, and that (2) high-HP-submerged macrophytes more effectively inhibit phytoplankton and maintain a clear-water state. The findings of this study can improve our understanding of regime shift theory and contribute to the effective restoration of submerged macrophytes for the control of excessive phytoplankton proliferation in shallow eutrophic lakes.

RESULTS

Dynamic growth of submerged macrophytes

The biomass of *P. maackianus* was significantly affected by the P level on days 30 and 60, whereas the *H. verticillata* biomass was significantly affected by the P level on days 60 and 90 (Table 1). There were no significant differences in *P. maackianus* biomass among the three P levels for PM treatments throughout the experiment (Fig. 1a–c). However, in treatment P + H, the *P. maackianus* biomass under OP was significantly higher than that under EP on day 30 (Fig. 1a), and significantly higher than that under MP on day 60 (Fig. 1b). There were no significant differences in *H. verticillata* biomass among the three P levels on

day 30 (Fig. 1d), nor under the P + H treatment on day 60 (Fig. 1e). Under the HV treatment, the *H. verticillata* biomass under MP and EP was 4.49 and 4.35 times higher than that under OP on day 60, respectively (Fig. 1e). Furthermore, *H. verticillata* plants died under OP on day 90 but grew well under MP and EP (Fig. 1f). The mortality of *H. verticillata* under OP demonstrated its low tolerance to continuous phosphate starvation.

The P content in the tissues of both submerged macrophytes gradually increased with increasing TP levels in the water column. According to Eq. (4), the HPI values of *P. maackianus* and *H. verticillata* were 2.758 and 0.964, respectively (Fig. 2a), further confirming that *P. maackianus* is a high-HP species and *H. verticillata* is a low-HP species. The submerged macrophyte assemblage HPI in the P + H treatment was significantly lower under MP and EP than that under OP on days 60 and 90, especially on day 90 when *H. verticillata* plants died under OP (Fig. 2b). There were significant differences in submerged macrophyte assemblage HPIs at all P levels on day 90 (Fig. 2b).

Interspecific interactions of submerged macrophytes

The P level had significant effects on RYT and RY_{HV} , but not on RY_{PM} (Fig. 3a–c). On days 30 and 90, the RYT values in all treatments were close to 1, indicating a neutral interaction between species. However, on day 60, the RYT under OP and MP was significantly greater than 1 and significantly less than 1, respectively (Fig. 3a), indicating that the interaction between the two species changed from negative (competition) and neutral to positive with P starvation. For individual species, as the RY_{PM} values in all treatments were close to 1 during the whole experiment (Fig. 3b), the RY_{HV} showed the same trends as the RYT (Fig. 3c), especially on day 60 when the RY_{HV} under both MP and EP was significantly less than 1 (Fig. 3c). These results suggest the importance of *H. verticillata* in determining the submerged macrophyte assemblage composition and show that its growth was impaired by the presence of *P. maackianus* under MP and EP.

The RGA of *H. verticillata* over *P. maackianus* was significantly affected by the P level (Fig. 3d); it was significantly greater than 0 under all P levels in the early stages (0–30 days) but was reduced to slightly less than 0 under all P levels by days 30–60. In the late growth stage (days 60–90), the RGA of *H. verticillata* over *P. maackianus* remained slightly below 0 under MP but was significantly greater than 0 under EP. Further, *H. verticillata* died under OP.

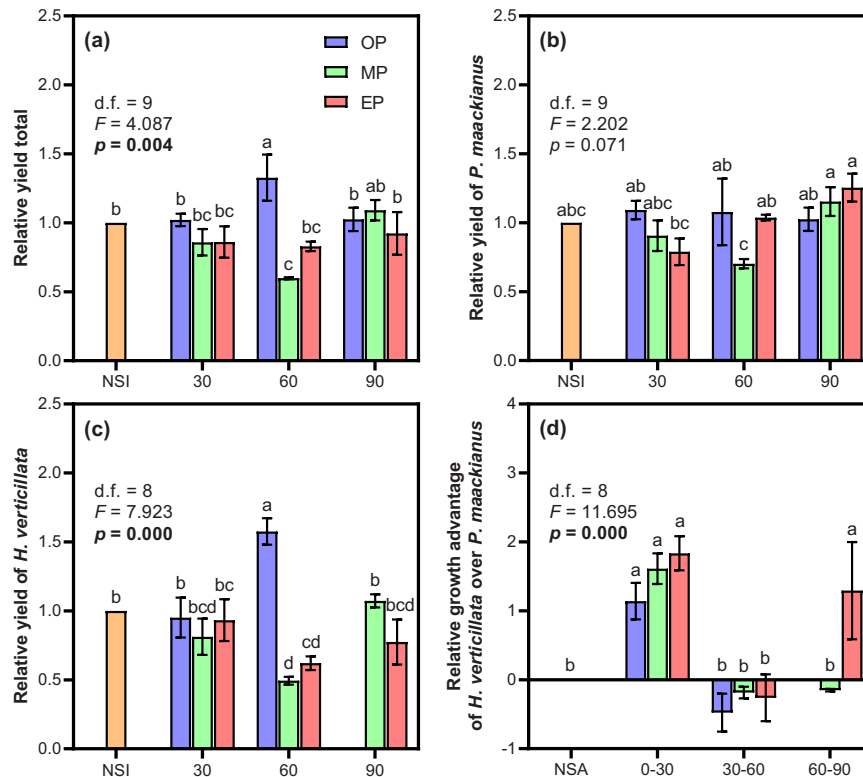


Fig. 3 Relative yield and relative growth advantage of the submerged macrophytes. **a** Relative yield total, **b** relative yield of *Potamogeton maackianus*, **c** relative yield of *Hydrilla verticillata*, **d** relative growth advantage of *H. verticillata* over *P. maackianus*. Error bars indicate standard errors ($n = 3$). Data were not obtained for *H. verticillata* under OP on day 90, as the plants died. Values with different lowercase letters are significantly different among P levels on the same day ($p < 0.05$). OP, MP, EP indicated three P levels of oligotrophic P (P concentration under detection limit and no P addition), mesotrophic P (0.05 mg L^{-1}), eutrophic P (0.10 mg L^{-1}), respectively. NSI indicates neutral species interaction (RY or RYT = 1); NSA indicates that there was no significant advantage (RGA = 0).

Phytoplankton in the water column

The Chl a level was significantly affected by the P level throughout the experiment and was significantly affected by the submerged macrophyte species assemblage on days 60 and 90 (Table 1 and Fig. 4). On day 30, there were no significant differences in Chl a level among submerged macrophyte species assemblages under the same P level, except for Chl a levels in PM and P + H, which were significantly lower than those in NSP. On days 60 and 90, there were no significant differences in Chl a levels among the submerged macrophyte species assemblages under OP; however, Chl a levels in PM and HV were significantly lower than those in NSP under MP and EP.

The two-way repeated-measures ANOVA showed that the P level, submerged macrophyte species assemblage, and their interaction accounted for 89.87%, 89.63%, and 83.60% of the variations in Chl a level on days 30, 60, and 90, respectively (Table 1 and Fig. 5). The percentage of Chl a level variation explained by the P level decreased from 82.49% on day 30 to 39.95% on day 90. In contrast, the percentage of Chl a level variation explained by the submerged macrophyte species assemblage increased from 3.25% on day 30 to 36.20% on day 90.

Inhibition ratio of phytoplankton

The IRP was significantly affected by all P levels, the submerged macrophyte species assemblage, and their interactions, except on day 30 (Table 1). The IRP was generally above 0, except under OP on day 30 (Fig. 6a–c). Non-significant differences among the submerged macrophyte assemblages were found under OP. The IRP of PM was significantly higher than that of HV under MP and EP throughout the experiment, whereas non-significant

differences were observed between PM and P + H under MP on day 30 and day 60. Along with an increase in the submerged macrophyte assemblage HPI, the IRP exhibited a trend of linear increase on day 60 ($r^2 = 0.161$, $p = 0.038$; Fig. 6e) and day 90 ($r^2 = 0.259$, $p = 0.016$; Fig. 6f); however, the linear increase trend on day 30 was non-significant ($r^2 = 0.123$, $p = 0.073$; Fig. 6d). As P level interference on submerged macrophytes and Chl a, the r^2 of linear regression was too low to draw a definite conclusion; however, submerged macrophyte assemblage IRP tended to increase along with HPI. This suggests that a decrease in the submerged macrophyte assemblage HPI induced by a change in the submerged macrophyte assemblage from *P. maackianus*-dominant to *H. verticillata*-dominant would reduce the overall IRP.

Figure 7 illustrates the dynamics of the interspecific relationships of primary producers in mesocosms with oligotrophic and eutrophic P levels. *Hydrilla verticillata* exhibited a growth advantage over *P. maackianus* in the early stages (0–30 days) (Fig. 7a); however, this growth advantage was non-significant by days 30–60 (Fig. 7b). In the late growth stage (days 60–90), *P. maackianus* had a growth advantage under OP as *H. verticillata* died, whereas *H. verticillata* had a growth advantage under EP (Fig. 7c). The IRP for *P. maackianus* was significantly higher than that for *H. verticillata* under EP throughout the experiment (Figs. 6 and 7), particularly on day 90 when the IRP for *P. maackianus* was nearly 15 times higher than that for *H. verticillata* (Fig. 7c). The IRP for *P. maackianus* under EP was significantly higher than that under OP; however, there were non-significant differences between the IRP for *H. verticillata* under OP and that under EP on day 30.

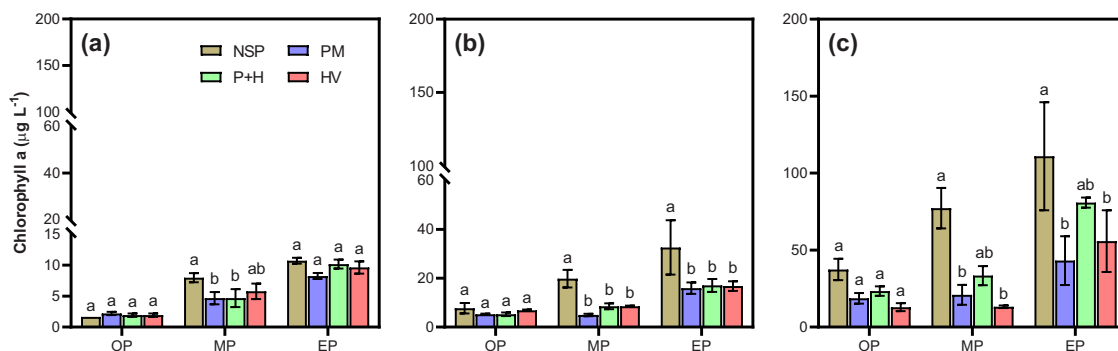


Fig. 4 Chlorophyll a levels of treatments. **a** Chlorophyll a levels on day 30, **b** chlorophyll a levels on day 60, **c** chlorophyll a levels on day 90. Error bars demonstrate standard errors ($n = 3$). Different lowercase letters are significantly different among submerged macrophyte species assemblages in the same P level ($p < 0.05$). OP, MP, EP indicated three P levels of oligotrophic P (P concentration under detection limit and no P addition), mesotrophic P (0.05 mg L^{-1}), eutrophic P (0.10 mg L^{-1}), respectively. NSP, PM, HV, P + H indicated four levels of treatment for submerged macrophyte species assemblage of no submerged plant, monoculture *Potamogeton maackianus*, monoculture *Hydrilla verticillata*, a mixture of the two plants (1:1 plant individuals), respectively.

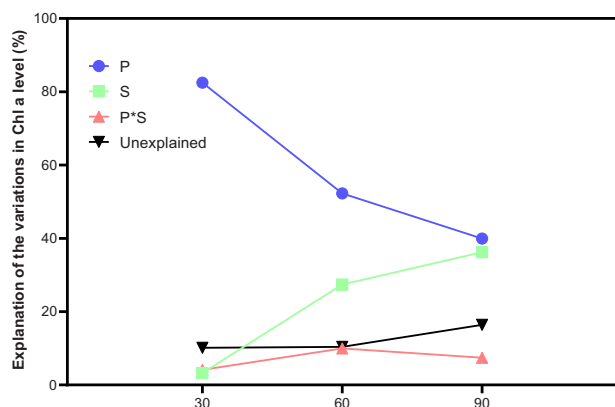


Fig. 5 Explanation of the variations in Chl a level. Phosphorus levels (P), submerged macrophyte species assemblage (S), and their interaction (P*S) affect the Chl a levels. Statistical results of the two-way analysis of variance are detailed in Table 1.

DISCUSSION

Our results confirmed that *P. maackianus* is a high-HP species and *H. verticillata* is a low-HP species; the biomass of *P. maackianus* and *H. verticillata* was significantly affected by P levels on days 30 and 60, and on days 60 and 90, respectively. Biomass of *P. maackianus* under MP and EP was either non-significantly different from or lower than that under OP, suggesting that poor nutrients in OP, in addition to a few dry deposited nutrients, satisfied its metabolism and growth requirements. For *H. verticillata*, the biomass under MP and EP was higher than that under OP in the HV treatment on day 60, whereas on day 90 it died under OP and the biomass under EP was higher than that under MP. These results are consistent with previous findings on the species-specific responses of common submerged macrophytes to eutrophication^{26,30}, indicating varied growth strategies between high- and low-HP-submerged macrophytes. Fast-growing and low-HP species (e.g., *H. verticillata*) can adapt to different nutrient levels by altering their nutrient utilization efficiency, i.e., higher nutrient utilization efficiency occurs during oligotrophication, whereas higher uptake of nutrients occurs during eutrophication¹³. This flexible nutrition utilization strategy affords low-HP species tolerance to temporary oligotrophication during early growth. Over the long term, the fast-growing strategy can result in high plant biomass under EP, which requires active photosynthesis and abundant light availability. Thus, low-HP

species lengthen their stems to reach the water surface to obtain more light, while ensuring efficient light utilization through an adequate nutrient supply³¹. These two mechanisms result in long canopy-forming or erect shoots and nutrient dependence, respectively. Further, low-HP species showed low tolerance to continuous phosphate starvation. Conversely, slow-growing and high-HP species (e.g., *P. maackianus*) adapt to different nutritional levels by maintaining relatively constant element levels through multiple mechanisms (e.g., the developed root system and selective uptake), thus reducing their metabolic activity, as well as their nutrient availability demands, further enhancing their tolerance to P levels after early adaptation¹⁵.

The interaction between *H. verticillata* and *P. maackianus* was neutral on day 30, as germination and the early-stage growth of the two submerged macrophytes involved relatively small resource requirements; thus, an adequate niche space can support their independent growth³². In contrast, on days 60 and 90, the interaction was negative (competition) or neutral under MP and EP, whereas it was positive (facilitation) under OP. This result agrees with the stress gradient hypothesis; phosphate starvation transformed the interaction from negative and neutral to positive as the slow-growing *P. maackianus* alleviated the nutrient limitations of *H. verticillata* under OP. Although the shading inhibition of excessive phytoplankton proliferation on submerged macrophytes, as well as that of low-HP species on high-HP species, is important for primary producers in natural shallow lakes^{15,17}, this was not the case in our study. The non-significant inhibition of shading was likely due to the relatively shallow water, as well as the low initial phytoplankton biomass. In contrast, RY_{HV} under both MP and EP were significantly less than 1, indicating that *P. maackianus* inhibited the growth of *H. verticillata* on day 60, whereas fast-growing *H. verticillata* offset this inhibition on day 90^{33,34}.

Different growth strategies of plants, as well as their inter-specific interactions, lead to RGAs between plants, and further changes in submerged macrophyte assemblages. Our results revealed that *H. verticillata* had a RGA compared to *P. maackianus* under EP, thus confirming our hypothesis (1). However, the relative growth advantage was reversed under OP, as *H. verticillata* died when it was affected by continuous phosphate starvation. This indicates that the mechanism of different growth strategies, rather than interspecies relationships, was the primary mechanism of changes in submerged macrophyte assemblages. Continuous eutrophication can alter a submerged macrophyte assemblage composition from *P. maackianus*-dominance to *H. verticillata*-dominance, indicated by the reduced submerged macrophyte

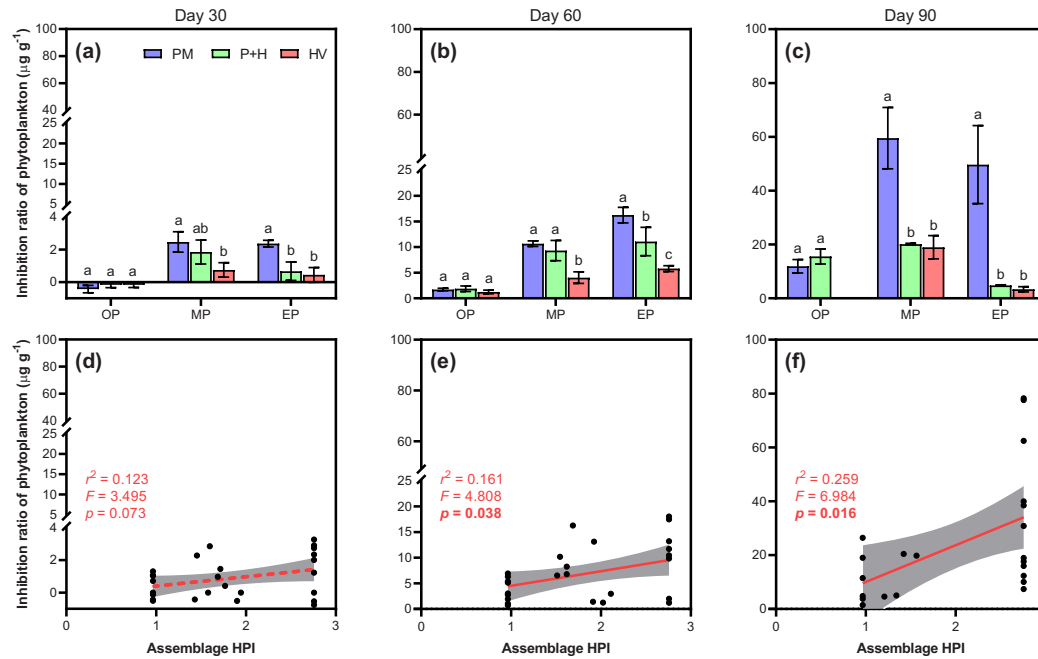


Fig. 6 Inhibition ratio of phytoplankton (IRP) by submerged macrophytes. **a** IRP by submerged macrophytes on day 30, **b** IRP by submerged macrophytes on day 60, **c** IRP by submerged macrophytes on day 90, **d** the relationship between submerged macrophyte assemblage stoichiometric homeostasis for P (HPI) and its IRP on day 30, **e** the relationship between submerged macrophyte assemblage HPI and its IRP on day 60, **f** the relationship between submerged macrophyte assemblage HPI and its IRP on day 90. Error bars demonstrate standard errors ($n = 3$). Values with different lowercase letters are significantly different among submerged macrophyte assemblages in the same P level ($p < 0.05$). Scatter points represent the submerged macrophyte assemblage HPI and IRP ($\mu\text{g g}^{-1}$) value of each mesocosm. Red solid lines indicate significant fitting ($p < 0.05$), whereas red dotted lines indicate non-significant fitting ($p > 0.05$). Shaded areas show a 95% confidence interval of the fit. OP, MP, EP indicated three P levels of oligotrophic P (P concentration under detection limit and no P addition), mesotrophic P (0.05 mg L^{-1}), eutrophic P (0.10 mg L^{-1}), respectively. PM, HV, P + H indicated four levels of treatment for submerged macrophyte species assemblage of monoculture *Potamogeton maackianus*, monoculture *Hydrilla verticillata*, a mixture of the two plants (1:1 plant individuals), respectively.

assemblage HPI, consistent with decades of field observations in Yangtze River Plain lakes³⁵. Changes in submerged macrophyte assemblages from high-HP species-dominance to low-HP species-dominance form a negative feedback loop with eutrophication in the water²⁸; considering the fast-growing strategy and massive P absorption of low-HP species.

Although the difference in Chl a levels were not significant among the three treatment groups with submerged macrophytes, the IRP in PM was higher than that in HV due to the lower biomass of *P. maackianus* than *H. verticillata*, thus confirming our hypothesis (2). This result suggests that nutrient competition was not the key mechanism by which submerged macrophytes inhibit phytoplankton under eutrophication, as *P. maackianus* is less nutrient competitive than *H. verticillata*. In addition to nutrient competition, two other mechanisms, including interference competition (e.g., allelopathy) and enhanced grazing pressure from herbivores (e.g., crustaceans, zooplankton)^{7,27}, possibly explain the higher IRP of *P. maackianus*. As herbivores were excluded at the beginning of our experiment, grazing pressure had a negligible effect, suggesting strong allelopathic inhibition of *P. maackianus* on phytoplankton and *H. verticillata*. This result was consistent with those from previous studies on the species-specific allelopathic effects of common plants^{33,36,37}, as some secondary metabolites (e.g., polyphenols) released to maintain plant stoichiometric homeostasis may have allelopathic effects^{18,34}. Field studies have shown that the biomass of high-HP-submerged macrophytes can be equal to or even greater than that of low-HP-submerged macrophytes in lakes^{8,38}. Thus, the former can better stabilize a clear-water state.

After compiling the above findings, we developed a conceptual model describing the stoichiometric homeostasis mechanism by

which eutrophication drives regime shift via changes in submerged macrophyte assemblages (Fig. 8). Under oligotrophication, exploitation competition involves nutrition, and the competitive edge decreases in the following order: high-HP-submerged macrophyte species > low-HP-submerged macrophytes species > phytoplankton, as submerged macrophytes, especially well-rooted high-HP macrophytes, can take up nutrients from sediment. Under eutrophication, exploitation competition involves underwater light, and the competitive edge is reversed due to the buoyant characteristics of phytoplankton, as well as the tall low-HP macrophytes⁷. However, high-HP-submerged macrophyte species can effectively inhibit phytoplankton, helping to maintain a clear-water state until the proportion of these species in the submerged macrophyte assemblage biomass drops below a threshold. Although the inhibition of *P. maackianus* by the shading of *H. verticillata* was not demonstrated in this study due to the relatively short experimental period and shallow water conditions, a reduction of high-HP-submerged macrophytes that was caused by the shading of low-HP-submerged macrophytes should not be neglected under natural conditions. This conceptual model provides insights into regime shifts in shallow lakes, i.e., elevated nutrient levels in shallow lakes may induce the change in submerged macrophyte assemblages from high-HP species-dominance to low-HP species-dominance. This would erode the phytoplankton inhibition effect with no decline in submerged macrophytes, further increasing the risk of excessive phytoplankton proliferation and regime shift from a clear-water state to a turbid state³⁹. This conceptual model could also be useful for explaining the different regime shift directions in shallow lakes with similar nutrition levels. Lakes dominated by high-HP-submerged macrophyte communities tend to shift to a clear-

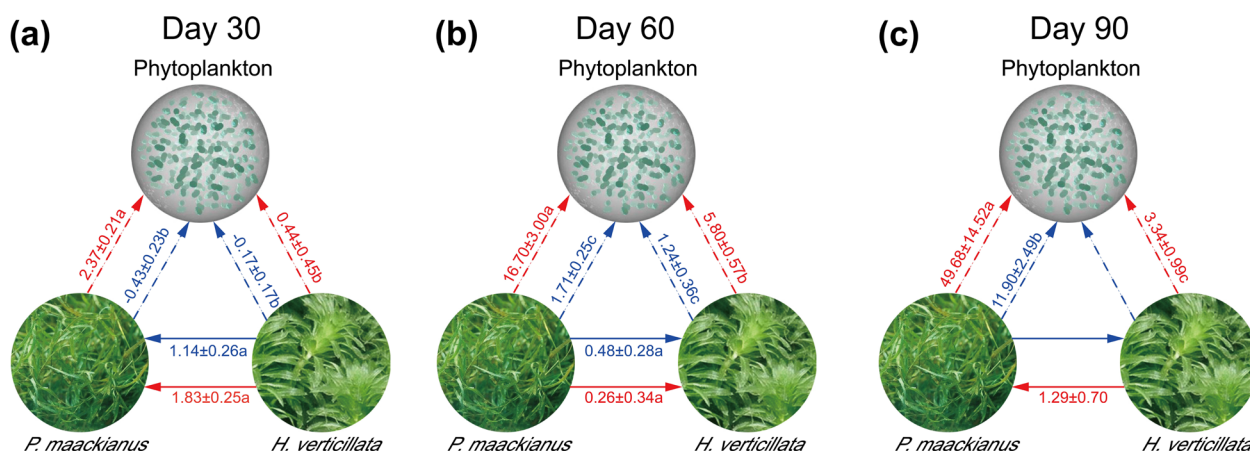


Fig. 7 Interspecific relationships between primary producers. **a** Interspecific relationships between primary producers on day 30, **b** interspecific relationships between primary producers on day 60, **c** interspecific relationships between primary producers on day 90. The solid arrows indicate the relative growth advantage between *Potamogeton maackianus* and *Hydrilla verticillata* in treatment P + H, and the arrows point from advantaged to disadvantaged species; values indicate the specific relative growth advantage (mean ± standard errors). The dotted arrows indicate the inhibition ratio of phytoplankton (IRP) by submerged macrophytes in PM and HV, the values indicate the IRP ($\mu\text{g g}^{-1}$) or the specific RGA. Red indicates eutrophic conditions and blue indicates oligotrophic conditions. Different lowercase letters represent significantly different RGAs or the IRP between oligotrophic and eutrophic conditions ($p < 0.05$). The one-way analysis of variance (ANOVA) of the relative growth advantage see Fig. 3d, and the two-way ANOVA of the IRP by submerged macrophytes see Table 1 and Fig. 6.

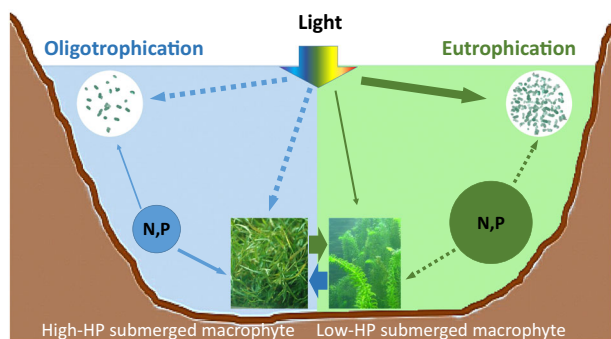


Fig. 8 Stoichiometric homeostasis mechanism by which eutrophication drives regime shift. Blue on the left indicates that oligotrophic conditions under which a submerged macrophyte assemblage is dominated by high-homeostasis-for-phosphorus (HP) species can strongly inhibit phytoplankton and maintain a clear-water state in shallow lakes. In contrast, green on the right indicates that eutrophic conditions under which a submerged macrophyte assemblage is dominated by low-HP species has weakened ability to inhibit phytoplankton, thus inducing excessive phytoplankton proliferation and a regime shift to a turbid state in shallow lakes. The solid arrows from light or N,P indicate exploitation competition of limited resources by submerged macrophytes and phytoplankton, whereas the dotted arrows indicate their utilization of unlimited resources. Thicker solid arrows indicate more competition between submerged macrophytes or phytoplankton for this resource.

water state, whereas those dominated by low-HP-submerged macrophyte communities tend to shift to a turbid state¹⁸. Furthermore, the hysteresis of regime shifts in response to changing nutrient levels could be caused by prolonged changes in submerged macrophyte assemblages between high-HP species-dominance and low-HP species-dominance³⁰.

Eutrophication-induced excessive phytoplankton proliferation and regime shifts in shallow lakes have become global environmental concerns^{5,7}. Therefore, reducing the nutrient load into lakes has garnered the attention of relevant stakeholders as a primary excessive phytoplankton proliferation-control strategy, which includes reducing external loading and mitigating internal

loading^{7,16}. However, the formation and maintenance of a clear-water state through single nutrient reduction is expensive and time-consuming, considering the stability of the turbid state^{40,41}. Thus, submerged macrophyte restoration is emphasized as an effective additional strategy for promoting the formation and maintenance of a clear-water state²⁷. Traditional submerged macrophyte restoration activities have generally focused on low-HP species that are fast growing and show high-nutrient absorption rates⁴². In the present study, we emphasized that high-HP species should be promoted as part of submerged macrophyte assemblage restoration, due to its strong inhibition effect on phytoplankton.

Although only two model species of varying HP were analyzed in this study, the increase of IRP with increased submerged macrophyte assemblage HPI was consistent with findings of previous field studies regarding the ability of complex submerged macrophyte assemblages to maintain a clear-water state^{18,20}. However, the submerged macrophyte HP was seldom considered in previous studies. Consequently, a potentially universal mechanism was revealed in this study, wherein eutrophication drives regime shift via changes in stoichiometric homeostasis-based-submerged macrophyte assemblages. Future research should focus on the interspecific relationships of more high- and low-HP species along nutrient gradients.

Our in situ mesocosm experiment revealed the species-specific responses of two submerged macrophytes to elevated TP levels. Although the low-HP species *H. verticillata* grew rapidly with eutrophication, it exhibited a lower phytoplankton inhibition effect. In contrast, the growth of the high-HP species, *P. maackianus*, was not significantly influenced by P levels on day 90; however, it exhibited a higher phytoplankton inhibition effect under eutrophication. The interspecific relationship between the two submerged macrophyte species tended to be negative or neutral under mesotrophication and eutrophication but changed to positive during oligotrophication. Differences in growth strategies led to the change in submerged macrophyte assemblages from high-HP species-dominance to low-HP species-dominance, thus may be the key mechanism underlying the shift from a clear-water state to a turbid state in eutrophic shallow lakes. We emphasize that future eutrophication management actions should focus on high-HP species in lake restoration after

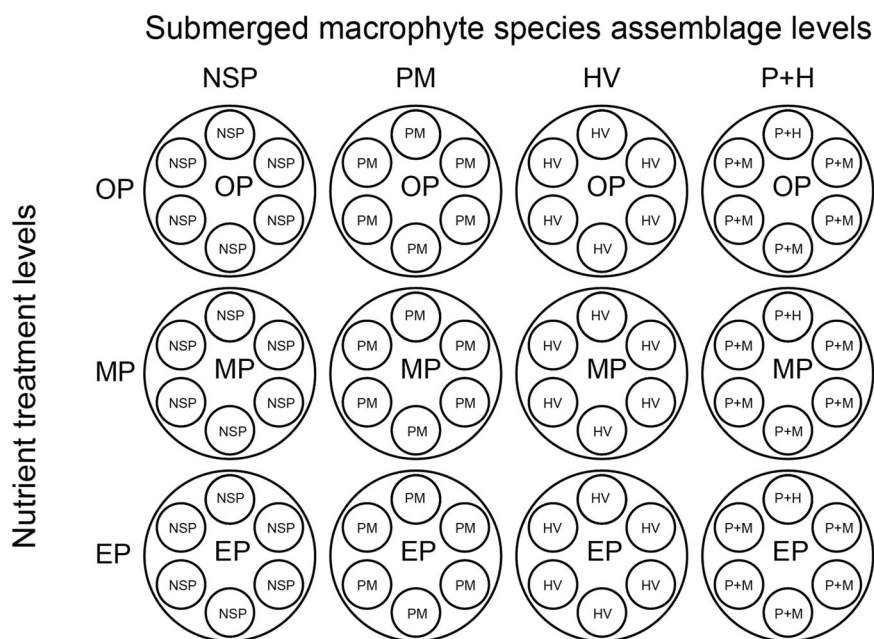


Fig. 9 Treatment combinations in this study. Each row represents a nutrient level, and each column represents a submerged macrophyte assemblage. The diagram represents one of the three replicates of each treatment combination. The big circles represent the tanks; the small circles represent the pots. Each of the pots was planted with two apical shoots of the studied species. Nitrogen (N) concentration in all tanks was 2 mg L^{-1} . OP, MP, EP indicated three phosphorus (P) levels of oligotrophic P (P concentration under detection limit and no P addition), mesotrophic P (0.05 mg L^{-1}), eutrophic P (0.10 mg L^{-1}), respectively. NSP, PM, HV, P + H indicated four levels of treatment for submerged macrophyte species assemblage of no submerged plant, monoculture *Potamogeton maackianus*, monoculture *Hydrilla verticillata*, a mixture of the two plants (1:1 plant individuals), respectively.

further in-depth experimental studies on additional submerged macrophyte species.

METHODS

Study species

P. maackianus, a high-HP-submerged macrophyte, and *H. verticillata*, a low-HP-submerged macrophyte, are both common species in lakes in the middle and lower reaches of the Yangtze River, China¹⁸. *P. maackianus* is a non-rhizomatous bottom-dwelling perennial with slender creeping stems at its base. It is generally found in shallow waters as its short shoots limit exposure to light. *H. verticillata* is an erect perennial with long shoots extended to the water surface to obtain abundant light; thus, it is generally found in relatively deep waters³. The *P. maackianus* and *H. verticillata* specimens used in this study were collected from Shanbo Lake, Hunan Province, China (29.42°N ; 112.00°E). Upon collection, the apical shoots were cleaned thoroughly and pre-incubated in tap water for seven days under natural conditions to exclude the influence of epiphyton on submerged macrophyte growth and interspecific interactions^{43,44}. Each apical shoot was of equal length (15 cm), as per previous studies^{26,45–47}. Twenty ramets of equal length per species were randomly selected and dried in an oven (60°C) until they reached a constant weight, which was determined using an analytical balance, to measure their respective initial biomass values³¹. The initial biomass values (mean \pm standard deviation) of *P. maackianus* and *H. verticillata* were $0.036 \pm 0.009 \text{ g}$ per ramet and $0.013 \pm 0.003 \text{ g}$ per ramet, respectively. This 3-fold difference in the initial biomass per ramet between *P. maackianus* and *H. verticillata* represents the different water contents in the plant tissue, i.e., low-HP macrophytes exhibit a fast-growing strategy and require high water contents in their plant tissue to maintain active metabolism⁴⁸.

Study design

Our in situ mesocosm experiment, consisting of 36 plastic tanks (top diameter 80 cm, bottom diameter 50 cm, height 70 cm, and volume 220 L), was performed from May 28 to August 26, 2020. The experiment was conducted in the center of a greenhouse located in the riparian zone of Shanbo Lake, in the middle reach of the Yangtze River. The greenhouse

(length \times width \times height = $30 \text{ m} \times 10 \text{ m} \times 2.5 \text{ m}$) was ventilated on all sides to expose the mesocosms to natural wind-induced water stir, and its canopy was built with transparent plastic film to expose the mesocosms to natural sunlight but not to precipitation. No artificial lights or water stirring were applied during the study. Therefore, natural meteorological and hydrological conditions were simulated but deposition caused by precipitation was eliminated. A fully factorial experimental design was employed, which included three levels of nutrient treatments and four levels of submerged macrophyte species assemblages in 12 treatment combinations, with three replicates per treatment combination. The tanks were randomly assigned to one of 12 experimental treatment combinations (Fig. 9).

As submerged macrophytes showed significant stoichiometric homeostasis for P but not for N¹⁸, the total N (TN) level in all treatments was kept constant at 2.0 mg L^{-1} . The following three levels of total P were employed: 0.00 mg L^{-1} (oligotrophic (OP)), P concentration under detection limit of 0.01 mg L^{-1} , and no P addition, i.e., the control), 0.05 mg L^{-1} (mesotrophic (MP)), and 0.10 mg L^{-1} (eutrophic (EP)). The nutritional levels were based on a previous study of lakes in the middle and lower reaches of the Yangtze River, where the TP thresholds for forward and backward shifts, i.e., from a clear-water state to a turbid-water state and vice versa, were identified as $0.08\text{--}0.12 \text{ mg L}^{-1}$ and $0.04\text{--}0.06 \text{ mg L}^{-1}$, respectively⁴⁹. The nutritional levels were not too high to damage the physiology of the submerged macrophytes^{3,50}. The TN to total P (TP) ratios in our treatment were 88.6 and 44.3 for MP and EP, respectively (the TN:TP ratio for OP could not be calculated); thus, P was the potential limiting nutrient for the growth of submerged macrophytes and phytoplankton. Previous incubation experiments conducted in Lake Taihu (another lake located in the middle-lower Yangtze River basin) showed that a TN:TP ratio of <21.5 indicates potential N limitation, whereas a ratio of >24.7 indicates potential P limitation; the remaining values indicate potential co-limitation⁵¹. The four levels of treatment for the submerged macrophyte species assemblage were as follows: no submerged plant species (NSP), monoculture *P. maackianus* (PM), monoculture *H. verticillata* (HV), and a mixture of the two plants (1:1 plant individuals; P + H) (Fig. 9).

At the beginning of the experiment (day 0, May 28), six plastic pots (top diameter 17 cm, bottom diameter 10 cm, height 14 cm) filled with washed sand were placed in each of the 36 tanks, generating $36 \times 6 = 216$ pots in

total (Fig. 9). The sand was the only substrate in the pots to exclude the effect of substrate nutrients on water nutritional levels, according to previous studies^{47,52}. Although most vascular plants generally take up P from sediment via their roots, studies have confirmed that water can also be an important source of nutrients for submerged macrophytes^{12,53}. Each of the pots was planted with two apical macrophyte shoots according to the experimental design. Each pot in the same tank was planted with the same submerged macrophyte species assemblage and was separated by 180 μm -pore plastic nets to avoid plant interactions between pots (Fig. 9). To remove herbivores (e.g., crustaceans, zooplankton), the water collected from Shanbo Lake was filtered through a 64 μm mesh. Following this, the filtered lake water was diluted 20 times with pure water to achieve initial TP, TN, and chlorophyll a (Chl a) levels below their detection limits (0.01 mg L⁻¹, 0.05 mg L⁻¹, and 2 μg L⁻¹ for TP, TN, and Chl a, respectively). Each tank was filled with 200 L of filtered and diluted lake water. The water depth was 65 cm in each tank to inundate all six pots, thereby maintaining consistent conditions among all pots. Subsequently, N [KNO₃ and (NH₄)₂SO₄] and P (KH₂PO₄) were added to all tanks except the OP treatment tanks according to the designated nutrient levels.

During the 90-day experimental period (from May 28 to August 26, 2020), the water level was maintained at a constant level in the tanks by resupplying pure water to compensate for evaporation loss⁵³. We mixed the water with N [KNO₃ and (NH₄)₂SO₄] and P (KH₂PO₄) every 10 days to maintain the designated N and P levels in the tanks.

Water temperature during the experiment was recorded at a frequency of 30 min using an automatic temperature recorder (DS1922E; Shanghai Bobang Technology Co., Ltd., Shanghai, China). On average, the daytime (from 08:00 to 18:00) water temperature on days 0–30, 30–60, and 60–90 was 25.58 \pm 1.65 °C, 26.22 \pm 1.00 °C, and 30.04 \pm 1.27 °C, respectively.

Sampling and measuring

The biomass of submerged macrophytes and Chl a of water were sampled and measured every 30 days (i.e., day 30, June 27; day 60, July 27; and day 90, August 26). On sampling days, two randomly selected pots from each tank were carefully removed, the plants in each pot were harvested and sorted into species to measure their respective biomass (the biomass of the whole plant, including aboveground and belowground, was harvested and measured), and the pots without plants were then placed back in the tank. The biomass of each plant species was measured after drying them in an oven (60 °C) until they reached a constant weight, as determined using an analytical balance⁵³. Water samples (300 mL water at half the depth below the water surface) were collected from each tank to measure the level of Chl a. After filtering the water sample through a Whatman GF/F filter, Chl a was extracted in 95% ethanol, after which the solution was measured using a spectrophotometer (UV-2800, Unic, China)⁵⁴.

At the end of the experiment (August 26, 2020), the TP content in the plant tissues was determined, in addition to measuring the biomass of submerged macrophytes and Chl a of water. To avoid the systematic error caused by the sampling date, dried plants harvested from the same tank on days 30, 60, and 90 were mixed into a single sample for TP content determination¹². The TP content in the plant tissues was determined using the ammonium molybdate ascorbic acid method, after digesting the dried plant samples in sulfuric acid/hydrogen peroxide¹⁸.

Data analysis

The relative yield (RY) of the submerged macrophyte species was used to evaluate whether species growth and development were facilitated or inhibited by the associated species. RY was calculated using the following equation:

$$RY_A = Y_{AB}/(P_A \times Y_A); RY_B = Y_{BA}/(P_B \times Y_B), \quad (1)$$

where, P_A is the proportion of species A in the mixture (%); P_B is the proportion of species B in the mixture (%); $P_A + P_B = 100$; Y_A is the biomass of species A in the monoculture (g); Y_B is the biomass of species B in the monoculture (g); Y_{AB} is the biomass of species A in the presence of species B (g); and Y_{BA} is the biomass of species B in the presence of A (g). When RY_A or $RY_B = 1$, the presence of associated species B/A has no significant influence on target species A/B; if RY_A or $RY_B > 1$, target species A/B benefits from the presence of associated species B/A; if RY_A or $RY_B < 1$, target species A/B is harmed by the presence of associated species B/A²⁶.

The relative yield total (RYT) was used to evaluate the facilitation or competition between submerged macrophyte species. RYT was calculated

using the following equation:

$$RYT_{AB} = (RY_A + RY_B)/2, \quad (2)$$

where, RY_A and RY_B are the RYs of submerged macrophyte species A and B, respectively. When $RYT = 1$, the interaction between species was neutral; if the value was >1 , the interaction between species was positive (facilitation); if the value was <1 , the interaction between species was negative (competition)²⁶.

The relative growth advantage (RGA) determines the growth advantage of one submerged macrophyte species over another, which reflects changes in the submerged macrophyte assemblages. The formula is as follows:

$$RGA_{AB} = \ln((B_{At2}/B_{At1})/(B_{Bt2}/B_{Bt1})), \quad (3)$$

where, B_{At1} , B_{At2} , B_{Bt1} , and B_{Bt2} are the biomass of species A at time t_1 (g), the biomass of species A at time t_2 (g), the biomass of species B at time t_1 (g), and the biomass of species B at time t_2 (g), respectively. If RGA_{AB} is >0 , species A will gain a growth advantage over species B during $t_1 - t_2$, indicating that the plant assemblage tends to change from a species B-dominance assemblage to a species A-dominance one. If RGA_{AB} is <0 , species B will gain a growth advantage over species A during $t_1 - t_2$, indicating that the plant assemblage tends to change from species A-dominance assemblage to a species B-dominance one. If RGA_{AB} is $=0$, the growth advantage between the two species is non-significant and the plant assemblage tends to be stable during $t_1 - t_2$.

The index of stoichiometric homeostasis for P (HPI) was used to quantify the submerged macrophyte species HP. The formula is as follows:

$$\log(y) = \log(c) + (1/HPI) \times \log(x), \quad (4)$$

where, y is the P level in the plant tissue (mg kg⁻¹), x is the P level in the water column (μg L⁻¹), and c is a constant. Submerged macrophytes can be divided into high- and low-HP species according to their HPI: high-HP species have $HPI > 2$ and low-HP species have $HPI < 2$ ⁵⁵.

The submerged macrophyte assemblage HPI was calculated using the following equation:

$$\text{Assemblage HPI} = B_A \times HPI_A / (B_A + B_B) + B_B \times HPI_B / (B_A + B_B), \quad (5)$$

where, B_A , B_B , HPI_A , and HPI_B are the biomass of species A, the biomass of species B, the HPI of species A, and the HPI of species B, respectively¹⁸.

The inhibition ratio of phytoplankton (IRP) was used to determine the biomass of phytoplankton inhibited by the submerged macrophyte assemblage per unit of biomass, which reflects the clear-water state maintenance of submerged macrophyte. The formula is as follows:

$$IRP = V \times (\text{Chl } a_c - \text{Chl } a_s) / B_s, \quad (6)$$

where, V , $\text{Chl } a_c$, $\text{Chl } a_s$, and B_s are the water volume in the tank (i.e., 0.2 m³), the Chl a level in the tank without submerged macrophytes (μg L⁻¹), the Chl a level in the tank with submerged macrophytes (μg L⁻¹), and the biomass of the submerged macrophyte assemblage (g), respectively⁵⁶.

A two-way repeated-measures analysis of variance (ANOVA) and Tukey post hoc test were used to test the effects of the P level and the submerged macrophyte species assemblage, as well as their interaction on *P. maackianus* biomass per plant, *H. verticillata* biomass per plant, Chl a, and IRP. A one-way repeated-measures ANOVA and Tukey post hoc test were used to explore differences in the submerged macrophyte assemblage HPI, RYT, RY of *P. maackianus*, RY of *H. verticillata*, and RGA of *H. verticillata* over *P. maackianus* between treatments, as these factors can only be calculated in treatment P + H. All analyses were conducted using SPSS 21 for Windows (SPSS Inc., Chicago, USA), with a set at <0.05 for all analyses.

DATA AVAILABILITY

All data needed to evaluate the conclusion in this paper are presented in the paper.

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AUTHOR CONTRIBUTIONS

C.Z., H.P.: conceptualization, methodology, and manuscript draft preparation; C.L.: data processing and analysis; P.L.: experiment conduction; C.L., G.L.: manuscript review and editing.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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