# DIVERSITY AND ECO-PHYSIOLOGICAL RESPONSES OF AQUATIC PLANTS

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PUBLISHED IN: Frontiers in Plant Science

frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88963-797-3
DOI 10.3389/978-2-88963-797-3

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# DIVERSITY AND ECO-PHYSIOLOGICAL RESPONSES OF AQUATIC PLANTS

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Aquatic plants refer to a diverse group of aquatic photosynthetic organisms large enough to be seem with the naked eye, and the vegetative parts of which actively grow either permanently or periodically (for at least several weeks each year) submerged below, floating on, or growing up through the water surface. These include aquatic vascular plants, aquatic mosses and some larger algae. Aquatic plants are grouped into life forms, each of which relates differently to limiting factors and has distinct ecological functions in aquatic ecosystems. Life form groups include emergent macrophytes (plants that are rooted in sediment or soils that are periodically inundated, with all other structures extending into the air), floating-leaved macrophytes (rooted plants with leaves that float on the water surface), submersed macrophytes (rooted plants growing completely submerged), free submerged macrophytes (which are not rooted but attached to other macrophytes or submerged structures) and free-floating macrophytes (plants that float on the water surface).

Aquatic plants play an important role in the structure and function of aquatic ecosystems by altering water movement regimes, providing shelter and refuge and serving as a food source. In addition, aquatic plants produce large standing crops which can also stabilize sediments, accumulate large amounts of nutrients thus improving water healthy. Thus, because of their ecological role, aquatic plants are an important component of aquatic ecosystems. Aquatic plants are very vulnerable to human activities and global changes, and many species of the plants had become endangered in the past several decades due to habitat loss, flooding, damming, over foraging, biological invasion and eutrophication, which might not be halted but enforced in the future when more extreme weathers coincide with enhanced human activities.

**Citation:** Liu, C., Thomaz, S. M., Li, Z., CAO, T., Kovalenko, K. E., eds. (2020). Diversity and Eco-Physiological Responses of Aquatic Plants. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88963-797-3

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frontiers in Plant Science

ORIGINAL RESEARCH
published: 16 April 2018
doi: 10.3389/fpls.2018.00512

# Responses to Sedimentation in Ramet Populations of the Clonal Plant *Carex brevicuspis*

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In aquatic ecosystems, sedimentation is an important factor that affects plant growth, mainly due to sediment depth. Clonal morphological plasticity is an effective strategy in clonal plants for acclimatization to sediment burial. To date, few studies have examined growth responses to sedimentation on the clonal plants at the ramet population level. This study aimed to explore the interactive effects of population size and burial depth on growth and clonal morphology of *Carex brevicuspis*. Three population sizes (2, 8, and 32 ramets) and 3 burial depths (0 cm, 5 cm, and 10 cm) were used in this experiment. Under shallow (5 cm) and deep (10 cm) burial conditions, biomass accumulation and relative growth rate (RGR) were lower than in the no burial treatment ($P < 0.05$). RGR of the small and medium populations was especially high compared to the large populations ($P < 0.05$). Biomass allocation was higher to belowground parts than aboveground parts, except for the small populations in the 5 cm burial treatments. Both shallow burial and smaller populations led to more biomass being allocated to aboveground parts. Deep burial elongated the first order spacer more than shallow burial, and sedimentation had negative effects on the second order spacer length. The number of new ramets did not decrease in the 5 or 10 cm burial treatments compared to the unburial treatment, and larger populations usually had more ramets than smaller ones; the proportion of clumping ramets was higher than the proportion of spreading ramets, and deeper burial and smaller populations led to higher proportions of spreading ramets. These results indicated that the growth of *C. brevicuspis* was limited by sediment burial at the ramet population level. Smaller populations enable *C. brevicuspis* to adjust its escape response to burial stress, may allow this species to effectively survive and widely distribute in Dongting Lake wetland.

**Keywords: clonal plant, biomass accumulation, ramet population, morphological plasticity, spacer length, growth form, sedimentation, Dongting Lake**

OPEN ACCESS

***Edited by:***
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***Specialty section:***
*This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science*

***Received:*** *08 February 2018*
***Accepted:*** *04 April 2018*
***Published:*** *16 April 2018*

***Citation:***
*Pan B-H, Xie Y-H, Li F, Zou Y-A and Deng Z-M (2018) Responses to Sedimentation in Ramet Populations of the Clonal Plant Carex brevicuspis. Front. Plant Sci. 9:512. doi: 10.3389/fpls.2018.00512*

## INTRODUCTION

In aquatic ecosystems, such as lake wetlands and river wetlands, frequent sedimentation directly affects growth and distribution of plants (Maun, 1998; Li et al., 2009, 2016; Zheng et al., 2009). Sedimentation has multiple effects on the growth of plants, mainly due to the depth of sediment burial (Maun, 1998; Shi et al., 2004). Under deep burial conditions, growth of plants, such as biomass accumulation, relative growth rates and number of plants, are usually inhibited (Yu et al., 2004; Li and Xie, 2009; Pan et al., 2012). These limitations caused by sediment burial are mainly caused by the physical burden on the buried parts of plants (Maun, 1998; Werner and Zedler, 2002; Koning, 2004) and fluctuations in abiotic conditions, such as decreases in the light availability (Madsen et al., 2001), changes in nutrient contents, particle composition, and temperature of the soil (Xiao et al., 2010; Pan et al., 2016). However, under shallow or medium burial conditions, growth of some plants can be promoted via phenotypic plasticity, such as elongation of petioles, stem internodes, and rhizomes, and adjusting the number of ramets and the biomass allocation to aboveground parts (Maun, 1998; Deng et al., 2008; Luo and Zhao, 2015).

Clonal plants, which are dominant in wetland ecosystems, can cope with complicated burial stresses by higher levels of phenotypic plasticity compared with non-clonal plants (Dong et al., 1997; Santamaria, 2002; Li and Xie, 2009; Chen et al., 2011). In clonal plants, studies on the responses to sediment burial mostly focus on the plasticity of clonal architecture and the change in clonal growth forms, such as the length of rhizomes and the number of clumping or spreading ramets (Klimes et al., 1993; Li and Xie, 2009; Chen et al., 2011, 2017). However, clonal integration among interconnected ramets, generally aids clonal plants to endure frequently occurring sediment burial (Yu et al., 2001, 2004; Chen et al., 2010; Luo and Zhao, 2015).

Integrations of clonal plants, such as genets or fragments, refers to the interconnection of clonal network, shares the resources and information among ramets of clonal plants (Liu et al., 2016); these integrations of clonal plants can be seen as populations of ramets (Herben et al., 1994; Wang et al., 2004). At the population level, there are many studies on the responses of the clonal plants to disturbances, such as sand burial (Yu et al., 2001, 2004), defoliation (Wang et al., 2004, 2017), wind erosion (Yu et al., 2008), grazing (Liu et al., 2009), and soil nutrient heterogeneity (Zhou et al., 2012). Further, the growth responses to burial stress in clonal plants with different ramet population sizes remain unclear. In the present study, a factorial experiment was conducted to elucidate the role of ramet population size, on the growth and clonal morphology of *Carex brevicuspis*, in response to the depth of burial by sediment.

In particular, we tested the following hypotheses: (1) plant growth of *C. brevicuspis* populations will be promoted under shallow burial conditions and be limited under deep burial conditions, and larger populations will have higher biomass accumulation and relative growth rate (RGR) compared to other sizes of populations; (2) In *C. brevicuspis* populations, elongation of spacers and increase in number of ramets, biomass allocation to aboveground parts, and proportion of spreading ramets, are effective strategies to escape burial stress, and these morphological changes will be smaller in larger populations under deeper burial, compared to other sizes of population.

## MATERIALS AND METHODS

### The Species

The genus *Carex*, which consists of approximately 2000 species, is important in wetland ecosystems (Bernard, 1990). The pseudoculm of *C. brevicuspis* is a series of overlapping leaf sheaths, 20–55 cm in height. This species is a perennial rhizomatous sedge and widely distributed in eastern mainland China and Taiwan (Dai et al., 2000). *C. brevicuspis* is one of the dominant species in the Dongting Lake wetland, where is the second largest freshwater lake and is connected to the Yangtze Rivers in China. This wetland is usually flooded from May to October, accrete 3–7 cm sediment annually during the flooding season (Zheng et al., 2009; Li et al., 2016). Recruitment of this plant in the Dongting Lake wetlands is mainly through vegetative ramets emerging from underground rhizomes, from which tiller clumps or tussocks of various sizes can be formed (Chen et al., 2014). Growth of *C. brevicuspis* is inhibited and more biomass is allocated to leaves under high water level (Pan et al., 2012). Two types of ramets, clumping and spreading, can be produced in response to sedimentation (Chen et al., 2011).

### Experimental Design

On June 15, 2014, mature populations of *C. brevicuspis* were transported from the sampling site (29°30′ N, 112°48′ E) of East Dongting Lake wetlands, China. They were transplanted into three outdoor concrete pools (200 cm in length and width, and 100 cm in height) at the Dongting Lake Station for Wetland Ecosystem Research, The Chinese Academy of Sciences. A two-way factorial design was used for the experiment, which combined 3 burial depths (0, 5, and 10 cm; which were referred to as control, shallow, and deep burial conditions) with three population sizes (small, medium, and large). Small, medium and large populations were consisted of 2, 8, and 32 tillers, which were constructed with clumps of 5, 10, and 20 cm. The initial biomass of plants in each population size was recorded (3.98 ± 0.29 g, 13.59 ± 1.17 g, 54.37 ± 1.26 g, respectively; mean ± SE). Each pool was divided equally into nine square blocks, using bricks (40 cm in height). Each block (63 cm in length, 40 cm in height) had 30 cm sediment placed in the bottom. Plants for each population size were then randomly transplanted in the center of each block.

On June 30, 2014, a one-time sediment addition (0, 5, or 10 cm) was made to blocks of each population size. The experiment therefore comprised, nine treatments (small, medium, and large populations, with 0, 5, and 10 cm burial), with three replicates (one in each concrete pool). Sediment used in this experiment was collected from the area with *Carex* vegetation in the east Dongting Lake (29°30′ N, 112°48′ E), containing 2.6% organic matter, 9.6 $\mu g\ g^{-1}$ total nitrogen, and 0.13 $\mu g\ g^{-1}$ total phosphorus). Well water (containing 30 $\mu g\ L^{-1}$ $NH_4^+$-N, 40 $\mu g\ L^{-1}$ $NO_3^-$-N, and 20 $\mu g\ L^{-1}$ $PO_4^{3+}$-P, pH = 7.59) was added every week to maintain a 30 cm water level, relative to the pool bottom.

### Harvest and Measurements

Plants were harvested on June 30, 2015, before the plants of the large populations in the 0 cm burial treatments reached the edge of the blocks. Whole plants in each block were carefully cleaned using well water, and the number of new ramets produced by original plant were counted. The numbers of clumping or spreading ramets were counted, according to the method by Chen et al. (2011). Spacer length (distance from each ramet to the original plants) were measured according to their order (Dong et al., 1997; Li and Xie, 2009); the first order spacer referred to the rhizome between the original plants and the first ramets, and the second order spacer referred to the rhizome between the first and the second ramets. Biomass of different plant components (aboveground: shoots; belowground: rhizomes and roots) was measured after drying at 85°C for 48 h in an oven. Relative growth rate (RGR) was calculated using the following equations: $RGR = (\ln w_2 - \ln w_1) / (t_2 - t_1)$, where $w_1$ was the initial biomass, $w_2$ was the biomass at harvest time, and $(t_2 - t_1)$ was the duration of the experiment.

## Data Analysis

The D'Agostino-Pearson omnibus test was used to analyze whether all factors were normally distributed. Data were square root or logarithm transformed, if necessary, to meet the assumptions of normality. Two-way ANOVAs, with the population size and burial depth as fixed factors, were performed to determine the main effects and interactions on biomass accumulation, relative growth rate, aboveground and belowground biomass allocation, length of the first and the second order spacers, number of new ramets, and number of clumping and spreading ramets. Multiple comparisons were applied by *post hoc* Tukey's test at the 0.05 significance level. All analyses were performed using the software SPSS 17.0 for Windows (SPSS Inc., Chicago, IL, United States).

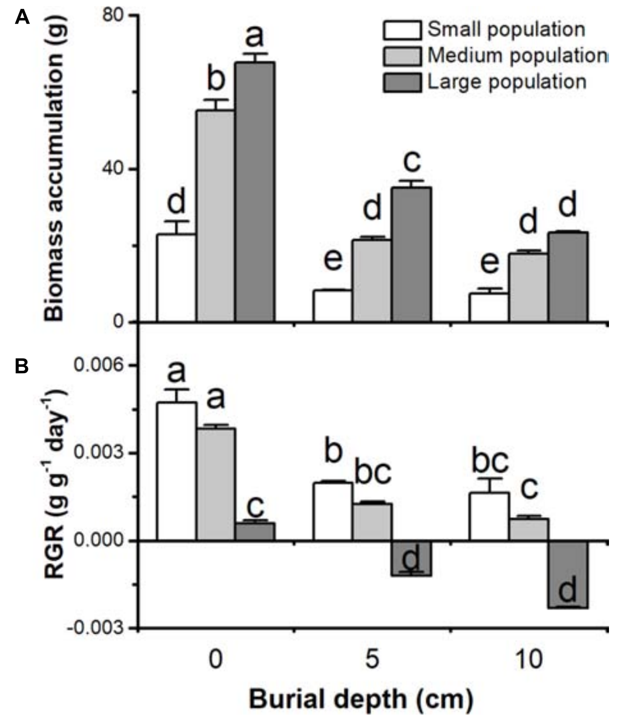


**FIGURE 1 | (A)** Biomass accumulation and **(B)** relative growth rate (RGR) of three population sizes of *Carex brevicuspis* growing in three burial depths. Error bars indicate the standard error of the mean. Different letters indicate significant differences among different treatments (Tukey's tests, $\alpha = 0.05$).

# RESULTS

## Biomass Accumulation and Relative Growth Rate

Biomass accumulation and relative growth rate (RGR) of *C. brevicuspis* populations were significantly affected by both burial depth and population size, with significant interactions ($P < 0.05$, **Figure 1** and **Table 1**). Biomass accumulation and RGR were higher in the 0 cm burial treatments than in the 5 and 10 cm burial treatments ($P < 0.05$, **Figure 1**). Biomass accumulation in larger populations was higher than that in small or medium populations ($P < 0.05$, **Figure 1A**). However, RGR of the small and medium populations was especially high compared to the large populations ($P < 0.05$, **Figure 1B**). RGR was negative only in large populations under 5 or 10 cm burial conditions ($-0.001$ to $-0.002$ g $g^{-1}day^{-1}$, $P < 0.05$, **Figure 1B**). It was clear that sedimentation had negative effects on plant growth, and had larger negative effects on larger populations than smaller ones.

## Biomass Allocation

Biomass allocation to aboveground or belowground parts in *C. brevicuspis* populations was significantly affected by both burial depth and population size, with significant interactions ($P < 0.05$, **Figure 2** and **Table 1**). Biomass allocation to belowground parts was higher than aboveground parts, except in small populations with 5 cm sediment treatments ($P < 0.05$, **Figure 2**). Under shallow or deep burial conditions, more biomass was allocated to belowground parts in larger populations than smaller ones ($P < 0.05$, **Figure 2**). Shallow or deep sediment burial led to more biomass being allocated to aboveground parts than in 0 cm treatments, and it was higher in 5 cm than 10 cm burial treatments ($P < 0.05$, **Figure 2**). It was clear that both smaller populations and shallower burial led to greater biomass allocation to aboveground parts.

**TABLE 1 |** Summary of two-way ANOVA analysis in three population sizes of *Carex brevicuspis* growing at three sedimentation depths.

| Dependent variable | Burial depth (B) | Population size (P) | B × P |
|---|---|---|---|
| Biomass accumulation (g) | 276.418*** | 200.808*** | 18.986*** |
| Relative growth rate (g $g^{-1}$ $day^{-1}$) | 140.901*** | 215.039*** | 1.319$^{ns}$ |
| Biomass allocation (%) | 509.026*** | 29.996*** | 23.869*** |
| Length of the first order spacer (cm) | 73.108*** | 29.003*** | 16.762*** |
| Length of the second order spacer (cm) | 147.467*** | 40.798*** | 21.066*** |
| Number of new ramets | 25.708*** | 135.424*** | 4.776** |
| Proportion of ramets (%) | 115.578*** | 12.788*** | 2.049$^{ns}$ |
| d. f. | 2 | 2 | 4 |

****P < 0.001, **P < 0.01, *P < 0.05,* $^{ns}P > 0.05$.

## Spacer Length

Length of the first and the second order spacers of *C. brevicuspis* populations were significantly affected by both burial depth and population size, with significant interactions ($P < 0.05$, **Figure 3**

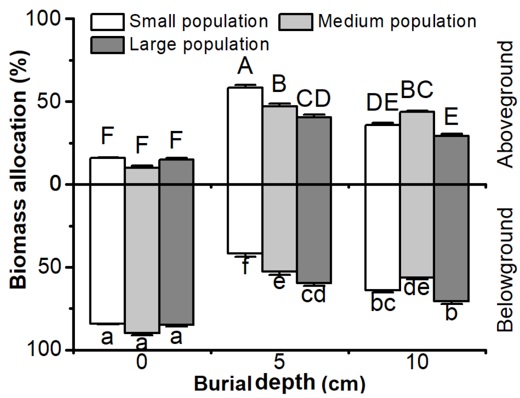


**FIGURE 2 |** Biomass allocation to above- and belowground parts of *Carex brevicuspis*, growing in three population size at three burial depths. Error bars indicate the standard error of the mean. Different uppercase and lowercase letters indicate significant differences among different treatments (Tukey's tests, α = 0.05).

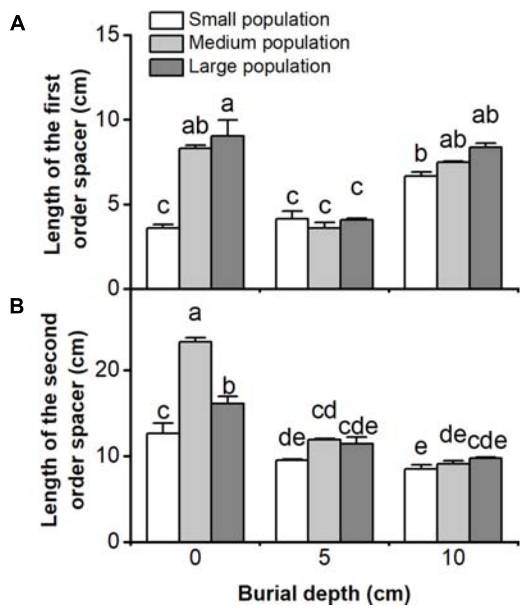


**FIGURE 3 |** Lengths of **(A)** the first order spacer, and **(B)** the second order spacer of three population sizes and three burial depths of *Carex brevicuspis*. Error bars indicate the standard error of the mean. Different letters indicate significant differences among different treatments (Tukey's tests, α = 0.05).

and **Table 1**). In 5 or 10 cm burial treatments, length of the first or the second order spacers did not differ according to population size ($P > 0.05$, **Figure 3**). The first order spacer was longer under deep burial than shallow burial, and it was longer in larger populations with 0 cm burial ($P < 0.05$, **Figure 3A**). Under shallow or deep burial conditions, length of the second order spacer was not significantly different and was lower than that in the 0 cm treatments; it was the longest in medium populations without sediment burial ($P < 0.05$, **Figure 3B**). It was clear that deep burial elongated the first order spacer more than shallow burial, and that sedimentation had negative effects on the growth of the second order spacers.

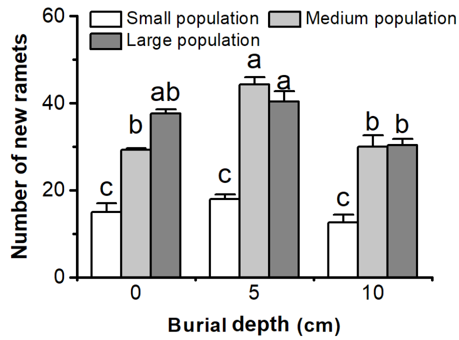


**FIGURE 4 |** Number of new ramets of three population sizes of *Carex brevicuspis* growing in three burial depths. Error bars indicate the standard error of the mean. Different letters indicate significant differences among different treatments (Tukey's tests, α = 0.05).

## Number of New Ramets

The number of new ramets of *C. brevicuspis* populations was significantly affected by both burial depth and population size (**Figure 4** and **Table 1**). The number of new ramets did not decrease with 5 or 10 cm burial depths, and larger populations usually had more new ramets than smaller ones ($P < 0.05$, **Figure 4**). The number of new ramets was the highest in the medium and large populations in the 5 cm burial treatments ($P < 0.05$, **Figure 4**). Sediment burial had no effect on the number of new ramets in small populations ($P > 0.05$, **Figure 4**).

## The Proportion of Clumping and Spreading Ramets

The proportion of clumping or spreading ramets in *C. brevicuspis* populations was significantly affected by both burial depth and population size ($P < 0.05$, **Figure 5** and **Table 1**). The proportion of clumping ramets was higher than that of spreading ramets overall, while increasing burial depth from 5 to 10 cm led to an increase in the proportion of spreading ramets ($P < 0.05$, **Figure 5**). Larger populations had a higher proportion of clumping ramets than smaller populations in the 5 cm burial treatments ($P < 0.05$, **Figure 5**). It was clear that deeper burial and smaller populations led to an increase in the proportion of spreading ramets within *C. brevicuspis* populations.

# DISCUSSION

In our treatments, space did not limit plant growth, since the plants did not reach the edge of the blocks before harvest. We evaluated the role of population size of *C. brevicuspis* in its response to sedimentation stress. Plant growth of *C. brevicuspis* populations were significantly affected by both sediment

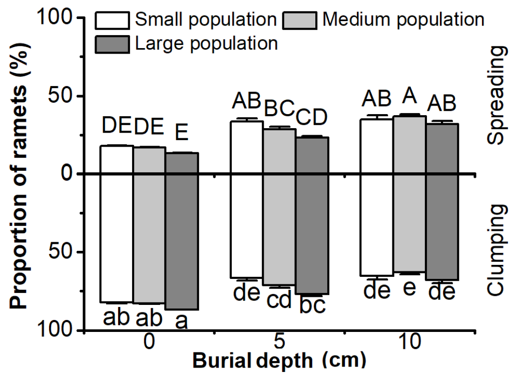


**FIGURE 5 |** Proportion of clumping and spreading ramets of *Carex brevicuspis* for three population sizes and three burial depths. Error bars indicate the standard error of the mean. Different uppercase and lowercase letters indicate significant differences among different treatments (Tukey's tests, α = 0.05).

burial and population size, and the biomass accumulation and RGR of *C. brevicuspis* populations decreased under sediment burial conditions. Under deep burial conditions, our observations were consistent with those of previous studies (Pan et al., 2012; Li et al., 2016; Chen et al., 2017), which found that *C. brevicuspis* cannot acclimatize to heavy sedimentation. In this experiment, we did not find the stimulatory effect of shallow burial on plant growth, which has been reported previously by other studies (Pan et al., 2012; Chen et al., 2017). However, those studies were performed with single plants, and the stimulatory effect might be insignificant on larger population sizes. Additionally, the stimulatory effect might also be limited by intraspecific resource competition (Schwinning and Weiner, 1998; van Kleunen et al., 2001). Therefore, the responses to sedimentation might be different between single plants and ramet populations.

It is well known that larger populations may contain more physiologically integrated ramets, which can help them to endure sedimentation stress more than smaller populations or single ramets (Yu et al., 2001, 2004). However, the RGR of the small and medium populations were especially high compared to the large populations. Additionally, larger populations had more negative effects on growth than the smaller ones. These results indicated that smaller populations might help the species to withstand sedimentation stress, and are partly consistent with our hypothesis 1, which predicts that larger populations will have a higher biomass accumulation and RGR compared to other sizes of population. The negative effects of growing population size are consistent with previous studies (Ekstam, 1995; Horvitz and Schemske, 2002), in which RGR was negatively correlated with plant size. Other factors, such as plant density, light availability, and nutrient limitation (Schwinning and Weiner, 1998; van Kleunen et al., 2001; Li et al., 2014), may also limit plant growth. In fact, shelf-shading might cause a lower RGR of larger populations, similar to the previously reported lower RGR of larger plants (Poorter and Remkes, 1990). It seems that large population do not have a high integration capacity based on RGR.

Deep burial elongated the first order spacers more than shallow burial, which is consistent with previous research (Li and Xie, 2009; Chen et al., 2011). The elongation of rhizomes or other perennating organs, helps to escape burial stress, for photosynthesis and future growth (Maun, 1998; Shi et al., 2004; Yu et al., 2004). However, negative effects were found on the growth of the second order spacers under burial conditions. These effects might be caused by the larger cost to form longer spacers under deep burial conditions (Sammul, 2011). Additionally, length of the second order spacers in the 5 and 10 cm burial treatments was not different for different population sizes. The number of new ramets was not lower in the 5 or 10 cm burial treatments, and sediment burial had no effect on the number of new ramets in small populations. These results suggest that sufficient resource can be provided at the ramet population level to escape sedimentation stress (Maun, 1998; Deng et al., 2008; Luo and Zhao, 2015), even in small populations. Therefore, the second ramets might be free to grow at the new shallower depths reached by the first ramets.

Both shallow burial and smaller populations led to increased biomass allocation to aboveground parts. Under these conditions, plant growth might be limited by competition for soil nutrients (Li et al., 2014; Chen et al., 2017); therefore, the high ratio of biomass allocation to aboveground parts can enable plants growing on the sediment surface to acquire the most limiting resources, such as light and oxygen (Brown, 1997; Dech and Maun, 2006). However, biomass allocation to belowground parts was higher in deep burial treatments and large populations, indicating that competition for nutrient resources among clones is intense (Brown, 1997; Maun, 1998; Deng et al., 2008). Therefore, biomass allocation responses of clonal plants of different population sizes to sediment burial could be attributed to resource limitation.

In our experiment, deeper burial and smaller populations led to a higher proportion of spreading ramets of *C. brevicuspis*, which is partly consistent with our hypothesis 2. In response to heavy sedimentation, *C. brevicuspis* can change its growth form with a shift from clumping to spreading ramets (Ye et al., 2006; Chen et al., 2011). In contrast to a previous study (Chen et al., 2011), the proportion of clumping ramets was higher in all treatments at the ramet population level similar to natural conditions (Chen et al., 2014). These results were similar to the biomass allocation, with resource limitation of smaller populations inducing them to produce more spreading ramets to escape sedimentation, or to forage for resources (van Kleunen et al., 2001). The findings of the present study indicated that smaller populations of *C. brevicuspis* enabled the plants to adjust their escape responses to sedimentation stress. However, besides the depth of sediment burial and population size, growth of *C. brevicuspis* populations is influenced by other properties of sediments and populations. Further studies are required to clarify the interactive effects between sediment heterogeneity, population structure, and the intra- or interspecific competition on clonal growth of aquatic macrophytes.

## AUTHOR CONTRIBUTIONS

B-HP and Y-HX wrote the manuscript text and executed the technical assays and statistical analysis. Y-HX designed the experiment and edited the manuscript text. B-HP, Y-HX, FL, Y-AZ, and Z-MD contributed to data collection and interpretation of the data. All authors reviewed the manuscript.

## FUNDING

This study was supported by the National Natural Science Foundation of China (31770471, 31570431, and 31600344), the National Key Technology Research and Development Program of China (2014BAC09B03), and the Youth Innovation Promotion Association of CAS (2014337).

## ACKNOWLEDGMENTS

We greatly appreciate the assistance of L. L. Zhu, C. Wu, and C. Zhang with the collection of materials and the plant harvest. We would like to thank Editage (http://www.editage.cn/) for English language editing.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 01 May 2018
doi: 10.3389/fpls.2018.00582

# Changes of Vegetation Distribution in the East Dongting Lake After the Operation of the Three Gorges Dam, China

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Water regime is regarded as the primary factor influencing the vegetation distribution in natural wetland ecosystems. However, the effect of water regime change induced by large-scale hydraulic engineering on vegetation distribution is still unclear. In this study, multi-temporal TM/ETM+/OLI images and hydrological data from 1995 to 2015 were used to elucidate how the change in water regime influenced the vegetation distribution in the East Dongting Lake (EDTL), especially after the operation of the Three Gorges Dam (TGD) in 2003. Using unsupervised and supervised classification methods, three types of land cover were identified in the study area: Water and Mudflat, Grass, and Reed and Forest. Results showed that the total vegetation area in EDTL increased by approximately 78 $km^2$ during 1995–2015. The areas of Reed and Forest and Grass exhibited a contrasting trend, dramatic increase in Reed and Forest but sharp decrease in Grass, particularly after the operation of TGD. The lowest distribution elevations of Grass and Reed and Forest decreased by 0.61 and 0.52 m, respectively. As a result of water level variation, submergence duration increased at 20–21 m and 28 m elevations (1–13 days), but significantly decreased at 22–27 m and 29–30 m elevations (−3 to −31 days). The submergence duration of Grass and Reed and Forest was 246 and 177 days, respectively. This study indicated that wetland vegetation pattern significantly changed after the operation of TGD, mainly as a result of changes in submergence condition. Submergence duration might be an effective indicator to predict the shift of vegetation distribution in EDTL, and which could provide scientific guidance for vegetation restoration and wetland management in this lake.

**Keywords: vegetation distribution, water regime, Three Gorges Dam, East Dongting Lake wetland, submergence duration, lowest distribution elevation**



***Edited by:***
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***Specialty section:***
*This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science*

***Received:*** *04 March 2018*
***Accepted:*** *13 April 2018*
***Published:*** *01 May 2018*

***Citation:***
*Hu J-Y, Xie Y-H, Tang Y, Li F and Zou Y-A (2018) Changes of Vegetation Distribution in the East Dongting Lake After the Operation of the Three Gorges Dam, China. Front. Plant Sci. 9:582. doi: 10.3389/fpls.2018.00582*

## INTRODUCTION

Wetland is a crucial component of the earth's landscape and is considered as one of the most diverse and productive ecosystems on the planet (Kingsford, 2000; Rosenberg et al., 2000; Tockner and Stanford, 2002). They can store large quantities of water in regular wet season and also provide a buffer zone and a place of energy exchange for the development of flora and fauna in dry season

(Sedell et al., 1990; Semlitsch and Bodie, 2003; Zedler and Kercher, 2005). However, wetland is a sensitive ecosystem. Variations in water regime, such as the frequency, duration, magnitude, and timing of flooding, can transform some key environmental factors for the development of downstream wetlands (Poff et al., 1997). These changes directly or indirectly impact wetland structure and community dynamics (Miller and Zedler, 2003; Zedler and Kercher, 2005; Fraser and Miletti, 2008; Todd et al., 2010), sometimes even resulted in the disappearance of flood-dependent vegetation (Auble et al., 1994; Taylor et al., 1996; Arthington and Pusey, 2003).

Water level is considered as a major factor influencing the development of wetlands and an intuitive indicator to assess the changes of vegetation distribution, since water level fluctuation strongly affects the growth and survival of vegetation (Casanova and Brock, 2000; Johansson and Nilsson, 2002; Coops et al., 2003). Zonation is a general response of wetland vegetation to regular water level fluctuation. In seasonal temporary wetlands, woody vegetation usually distributes at a higher elevation where there is less water level fluctuation and even no submergence. On the other hand, the herbaceous plant usually distributes at a lower elevation where there is usually under constant and regular submergence. In some particular regions, vegetation will shift to lowland types within a few years when the water level keeps at higher level. In other regions, the community types will be easily replaced by the perennial types with clonal reproduction when the water level keeps at lower water level (Chapin and Paige, 2013). Therefore, the pattern of vegetation distribution appears to be unclear when water level is variable.

Due to interference of human activities, such as hydroelectricity generation and flood control (e.g., dam building and river management) over 60% of the global river systems, the downstream wetlands have been affected by altered stream flows (Revenga et al., 2000). Dongting Lake wetland is a typical example, which receives water inflow from three channels (Songzi, Taiping, and Ouchi) directly connected to the Yangtze River and four rivers (Xiang, Zi, Yuan, and Li) in Hunan Province (**Figure 1**). This lake and its wetland have been threatened by unpredictable and dynamic changes in water regime, particularly after the operation of the Three Gorges Dam (TGD) in 2003, the largest hydrological project in the world. Several studies have confirmed that the TGD has changed the water regime of downstream (Zhang et al., 2012; Gao et al., 2013; Wang et al., 2013). Specifically, the TGD directly influenced the flooding patterns of wetlands, local vegetation and habitat of migratory birds in both Dongting Lake and Poyang Lake (Han et al., 2015; Mei et al., 2015; Feng et al., 2016; Wu and Liu, 2017). Even though these studies have elucidated the changes of eco-hydrological environment in the downstream lakes and wetlands after the operation of TGD, the relationship between vegetation distribution and water regime remains largely unclear, particularly in the Dongting Lake wetland.

In recent years, remote sensing and RS/GIS techniques provide the possibility to investigate vegetation distribution over large scales (Murkin et al., 1997; Munyati, 2000; Frazier et al., 2003; Accad and Neil, 2006; Rebelo et al., 2009). This study aimed to use these techniques to investigate the change of vegetation distribution in the East Dongting Lake (EDTL) in response to the water regime change. The specific objectives of the present study are as follows: (1) to characterize the change of vegetation distribution in the EDTL during 1995–2015, and (2) to explore the relationship between the dominant vegetation distribution and changes of water regime.

## MATERIALS AND METHODS

### Study Area

Dongting Lake (28°44′ and 29°35′N, 111°53′ and 113°05′E), the second largest freshwater lake (2,625 km$^2$) in China, is located in the northeast of Hunan Province (**Figure 1A**). This lake is one of the only two river-connected lakes in the Yangtze River Basin. Therefore, its water level is strongly influenced by the Yangtze River, especially after the operation of TGD (Sun et al., 2012). It consists of three sub-lakes: the East, West, and South Dongting Lakes and all of them are international important wetlands (Ramsar Sites).

The EDTL, covering approximately 1,321 km$^2$, accounts for nearly half of Dongting Lake's area. This wetland is characterized by a distinct plant zonation pattern along elevation gradients. For instance, the dominant plant communities *Polygonum hydropiper*, *Carex brevicuspis*, *Miscanthus sacchariflorus*, *Phragmites australis*, and *Populus nigra* are usually distributed along with increasing elevations (Li et al., 2013). The vegetation, especially grass (mostly Carex spp.), provides important habitat for winter migratory waterbirds (Yuan et al., 2014). The lake is usually inundated during the wet season (May–October) and exposed to air during the dry season (November–April). The annual fluctuation of water level is up to 10–12 m, the maximum and minimum water levels occur in July–August and January–February, respectively.

### Datasets and Processing

In this study, 12 time-series of dual-season Landsat TM/ETM+/OLI images of EDTL were chosen (**Table 1**). These images, obtained from the United States Geological Survey (USGS) archives, included winter and pre-winter scenes for each period. Winter images were used because vegetation characteristics are relatively stable and are easily identified in the winter. Meanwhile, pre-winter images were used to eliminate the disturbances of reed harvest in the winter. Winter images were chosen when the daily water level was $< 23$ m, and the potential vegetation area can be completely exposed to the air, based on the investigation of Dongting Lake Station for Wetland Ecosystem Research, CAS.

Because some pixels are missing in the ETM+ images after the 31 May 2003 due to scan line corrector (SLC) failure, these images were corrected using the gap-fill algorithm proposed by Scaramuzza et al. (2004). The imagery preparation and classification were performed using ENVI 4.8, while the map production and accuracy assessment were done using ArcMap 10.2.

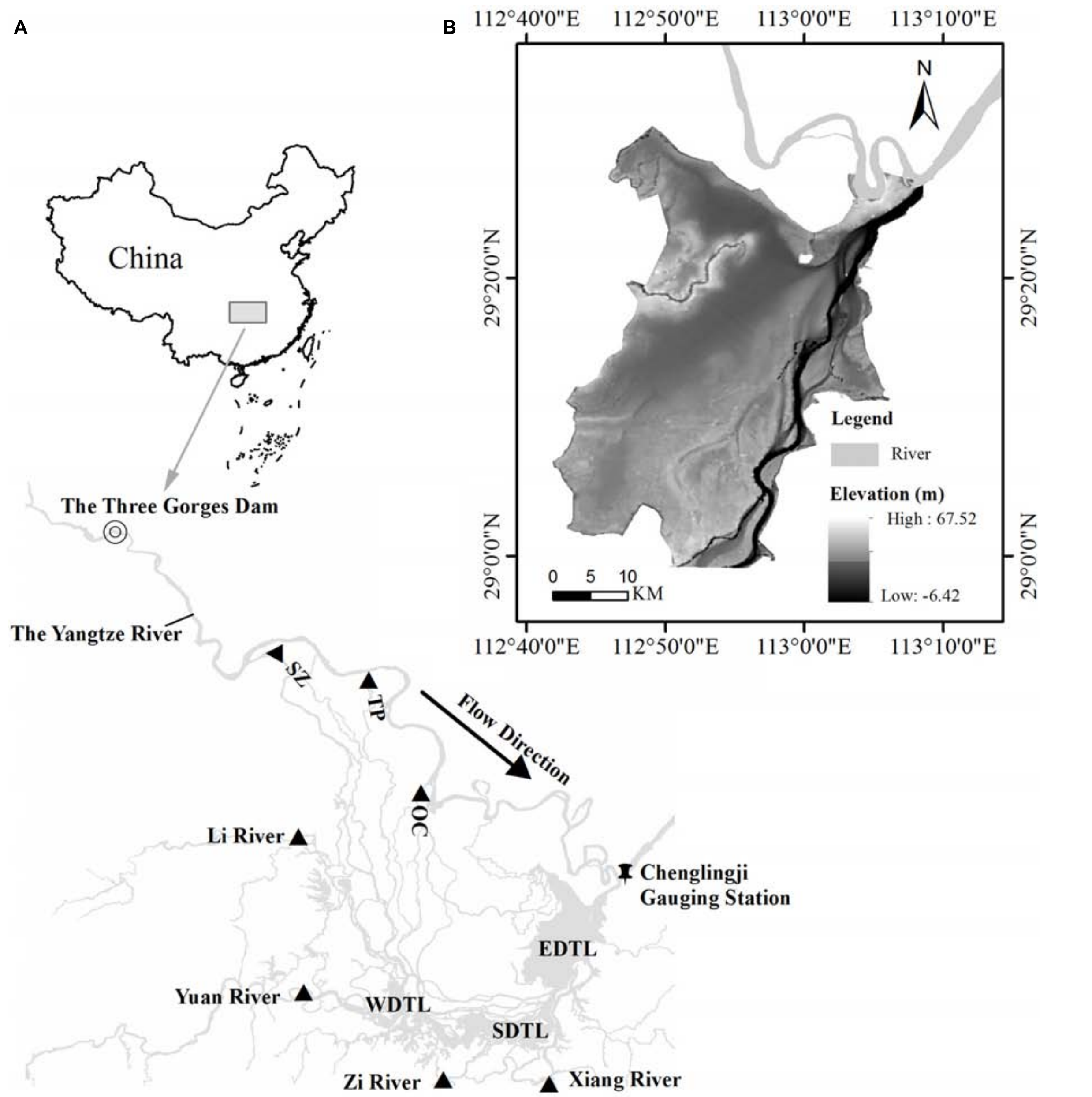


**FIGURE 1 |** Location of the East Dongting Lake. **(A)** Relationship between the TGD and the Dongting Lake. SZ–Songzi Channel; TP-Taiping Channel; OC-Ouchi Channel, **(B)** Digital elevation model of East Dongting Lake.

The field data on vegetation type were acquired from Dongting Lake Station for Wetland Ecosystem Research, CAS in 2014 (a total of 231 data). These data were used for the validation of classification results. The Digital Elevation Model (DEM, 1:10000) of Dongting Lake used in this study (**Figure 1B**), was provided by the Changjiang Water Resource Commission. Based on this DEM, the elevation data (integer) of EDTL was extracted and then converted to WGS84 coordinate system.

Data on daily water level at 8:00 AM at Chenglingji Hydrological Gauging Station during 1990–2015 were also provided by the Changjiang Water Resource Commission. These data are widely used to assess water regime in the EDTL (Hu et al., 2015; Xie et al., 2015; Yuan et al., 2015).

## Image Classification

Generally, only one image at a given time was usually applied for the Remote Sensing image interpretation. However, the conventional classification methods may not be suitable for some wetlands, especially for the seasonal wetlands with high intensity of human disturbance. Therefore, dual images were combined into one image to generate a complete wetland map, and unsupervised and supervised classification methods

**TABLE 1 |** The list of Landsat images.

| Season | Date | Landsat images | Water level (m) |
|---|---|---|---|
| Winter | 1995/12/05 | Landsat5 TM | 21.49 |
| Pre-winter | 1996/10/04 | Landsat5 TM | 26.24 |
| Winter | 2001/01/11 | Landsat7 ETM+ | 21.86 |
| Pre-winter | 2001/10/18 | Landsat5 TM | 26.40 |
| Winter | 2003/01/17 | Landsat7 ETM+ | 22.01 |
| Pre-winter | 2003/10/16 | Landsat7 ETM+ | 26.63 |
| Winter | 2006/01/09 | Landsat7 ETM+ | 20.79 |
| Pre-winter | 2004/10/02 | Landsat7 ETM+ | 27.40 |
| Winter | 2010/12/22 | Landsat7 ETM+ | 22.88 |
| Pre-winter | 2010/10/03 | Landsat7 ETM+ | 27.32 |
| Winter | 2015/03/31 | Landsat8 OLI | 22.87 |
| Pre-winter | 2014/10/22 | Landsat8 OLI | 24.10 |

were conducted using ISODATA (Iterative Self-Organizing Data Analysis Technique) and DTC (Decision Tree Classification) techniques according to the previous study (Xie et al., 2015). The winter images were classified into water, mudflat, and vegetation using ISODATA algorithm with five iterations and 0.95 threshold. After that, DTC algorithm was applied to extract Reed and Forest from pre-winter images using Band 4 and Band 7 (Xie et al., 2015). Finally, three types of land cover were differentiated for each map: Water and Mudflat, Grass, and Reed and Forest. The subtypes of Grass mainly included *C. brevicuspis* and *P. hydropiper,* while Reed and Forest included *M. sacchariflorus*, *P. australis, Salix babylonica* and *P. nigra.*

## Accuracy Assessment

Based on the field data, the accuracy of classification results is evaluated using the ENVI 4.8 confusion matrix tool. We randomly chose 1000 pixels in the study area and compared them with the 2014 field data. Kappa Index and overall accuracy were then calculated to assess the accuracy of vegetation classification.

## Area and Elevation of Vegetation Distribution

Six-period land cover maps were produced after classification. The areas of the different land cover types (Water and Mudflat, Grass, and Reed and Forest) were calculated by multiplying the pixel number with the spatial resolution (30*30 m). Total vegetation area included the areas of Grass and Reed and Forest.

Furthermore, the land-cover maps were overlaid with elevation data to analyze the elevation distribution of each land cover type. Bezier curve was used to fit the distribution and detect the intersection between land cover types according to the method of Xie et al. (2015). The intersection elevation of two curves was then defined as the distribution edge of two land cover types.

## Calculation of Monthly Water Level and Submergence Duration

Average monthly water level was calculated using the daily water level at Chenglingji Station. The submergence duration was calculated using the daily water level data at 20–30 m elevations with 1 m interval. When the water level was higher than the defined elevation, the wetland was considered as submergence. In order to compare the change of submergence duration and average monthly water level before and after the operation of TGD, daily water level data were divided into two periods: 1990–2002 and 2003–2014. The change of submergence duration was calculated by the submergence days before and after the operation of TGD at a given elevation.

Considering the lag time in the change of vegetation distribution induced by the changed water regime, the water level data of 1–5 years before a reference year were used to calculate submergence duration. For example, taking 1995 as the reference year, we calculated the average submergence duration for each of the following year/year range: 1994, 1993–1994, 1992–1994, 1991–1994, and 1990–1994. Pearson correlation analysis was used to determine the best lag time, then we calculated the submergence duration for each vegetation type using the average daily water level during 1990–2015. Finally, a linear regression model and ANOVA were used to identify the change trend in water level, corresponding to the submergence duration of each vegetation type during 1995–2015.

# RESULTS

## Assessment of Classification Accuracy

The overall accuracy and Kappa coefficient were 92.2% and 0.875, respectively. The products accuracy and user accuracy were highest in Water and Mudflat (94.69 and 98.57%, respectively), intermediate in Reed and Forest (94.66 and 89.21%, respectively), and lowest in Grass (83.91 and 82.48%, respectively). These results confirmed that the classification method was valid.

## Changes in Land Cover Area

The area of three types of land cover showed different pattern during 1995–2015 (**Figure 2**). The area of Water and Mudflat continually decreased from 497.7 $km^2$ in 1995 to 419.4 $km^2$ in 2015. The Grass area also decreased from 463.0 to 433.5 $km^2$ during 1995–2015. Specially, the Grass area actually experienced a marked increase from 2003 to 2005, after which it began to decrease. On the contrary, the area of Reed and Forest increased from 361.1 to 468.8 $km^2$ during the same period. Meanwhile, total vegetation area increased from 824.1 to 902.4 $km^2$ during 1995–2015.

After the operation of TGD, the total vegetation area extended more quickly (0.08 $km^2$/year vs. 6.47 $km^2$/year before and after 2003). The decline in Grass area was also more rapid after the operation of TGD ($-1.37$ $km^2$/year vs. $-1.66$ $km^2$/year before and after 2003). The area of Reed and Forest increased rapidly after the operation of TGD (1.45 $km^2$/year vs. 8.13 $km^2$/year before and after 2003) and covered 35.5% of the total vegetation area.

## Changes in Vegetation Distribution Elevation

The lowest distribution elevation of Grass continually decreased from 23.06 m in 1995 to 22.44 m in 2015 (**Figure 3**). The lowest

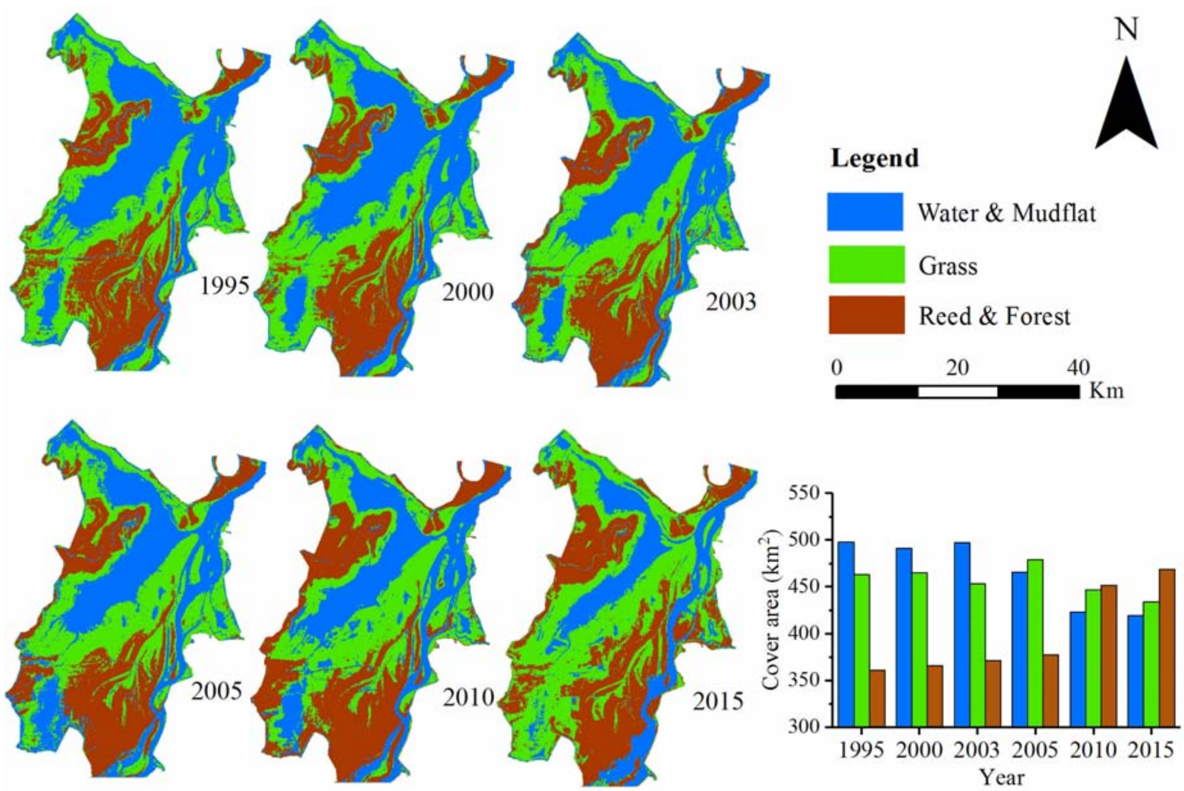


**FIGURE 2 |** Land cover change in the East Dongting Lake during 1995–2015.

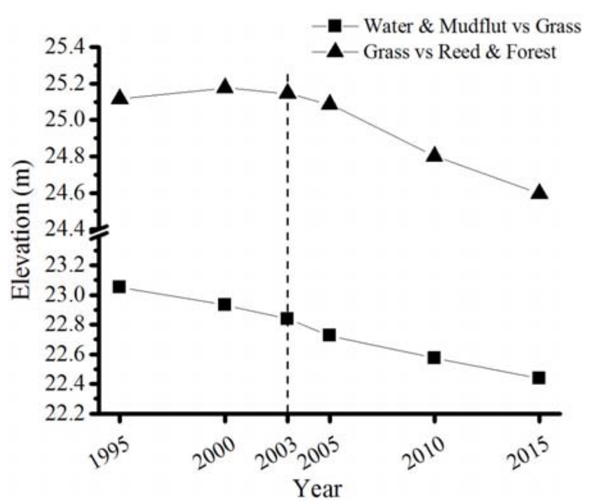


**FIGURE 3 |** The lowest distribution elevation of both vegetation types during 1995–2015.

distribution elevation of Reed and Forest also showed a declining trend, slightly decreasing during 1995–2003, but quickly declined from 25.08 m in 2005 to 24.59 m in 2015. Over the 20-year period, the lowest distribution elevation had moved down by about 0.61 m for Grass and about 0.52 m for Reed and Forest. In both vegetation types, the decline of the lowest distribution elevations was faster after the operation of TGD. The decline rate of the lowest distribution elevation was 2.4 cm/year vs. 3.1 cm/year in the Grass before and after 2003, and −0.3 cm/year vs. 4.2 cm/year for Reed and Forest.

## Changes in Monthly Water Level and Submergence Duration

Compared to the average monthly water level during 1990–2002, water level was 0.08–0.20 m higher from January to March, but 0.14–1.69 m lower from April to December after the operation of TGD. In July, August, and October, average monthly water level decreased by more than 1 m after the operation of TGD (**Figure 4A**). As a result of water level variation, the average submergence duration during 1990–2002 and 2003–2014 also changed at different elevations (**Figure 4B**). After the operation of TGD, submergence duration increased at 20, 21, and 28 m elevations (1–13 days), but significantly decreased at 22–27 m and 29–30 m elevations (−3 to −31 days), especially at 23–25 m elevations (<−27 days).

## Submergence Duration at the Lowest Distribution Elevation of Vegetation Distribution

The 5-year submergence duration was significantly correlated with the lowest elevation distribution in both vegetation types

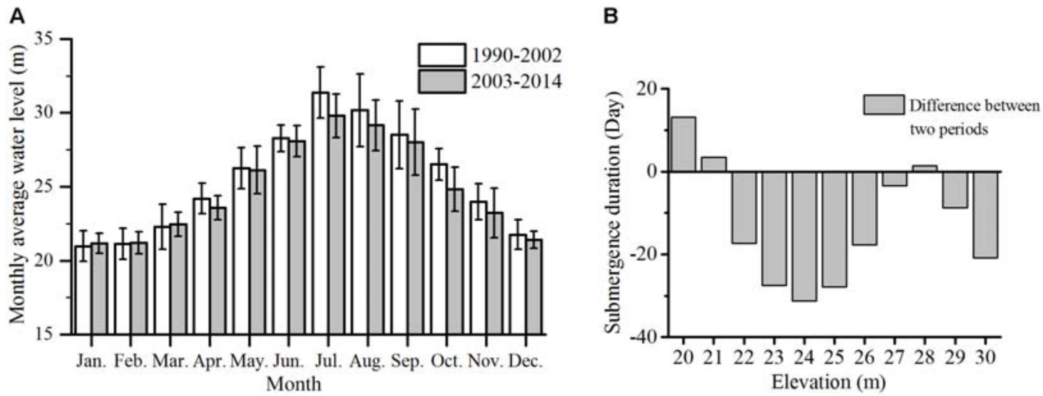


**FIGURE 4 | (A)** Changes in average monthly water level and submergence duration between 1995–2002 and 2003–2014. **(B)** Negative and positive values indicate decrease and increase in duration, respectively, after the operation of Three Gorges Dam.

($R^2$ = 0.64). The submergence duration was 233–257 days for Grass and 160–192 days for Reed and Forest during the six periods, respectively. The average submergence duration was 246 days for Grass and 177 days for Reed and Forest, respectively.

Based on the average submergence durations of Grass and Reed and Forest, we can get the corresponding elevation in any year using the daily water level data. Linear regression analyses were then conducted to elucidate the change trend of distribution elevation in both vegetation types during 1995 – 2015 (**Figure 5**). Both linear regression equations were statistically significant (for Grass, $F = 41.9$, $P < 0.01$; for Reed and Forest, $F = 93.7$, $P < 0.01$), suggesting that the distribution elevations of both vegetation types were consistently decreased during 1995–2015.

## DISCUSSION

During the past two decades, the eco-hydrological environments of the EDTL have considerably changed, particularly after the operation of TGD (Feng et al., 2013). The dam has been modified the probabilistic regularity of the flooding periods (Zhang et al., 2012; Yuan et al., 2015; Han et al., 2016). As a result of changed water regime, the area of Water and Mudflat decreased about 5.5% (78.2 km$^2$) of EDTL in winter season due to the rapid expansion of vegetation. Vegetation distribution has shown a new pattern. As a response to the decreased water level, the vegetation at high elevations tended to expand rapidly to the central part of the lake. Moreover, the vegetation replacement process has also been accelerated, especially after the operation of TGD, which is consistent with the findings of another study (Hu et al., 2015) and parallel to what happened in another lake in the middle reaches of the Yangtze River, the Poyang Lake, where one vegetation type has been completely converted to another (Li et al., 2016).

The total area and spatial distribution of the two vegetation types have also greatly changed. These results might be closely related to the topographic characteristics of EDTL (**Figure 6**). The land area at 21–27 m elevation covers 90% of total lake basin. In this study, the lowest distribution elevations for Grass and Reed and Forest were 23.06–22.45 m and 25.08–24.55 m, respectively. Therefore, rapid changes of water level at 22–25 m

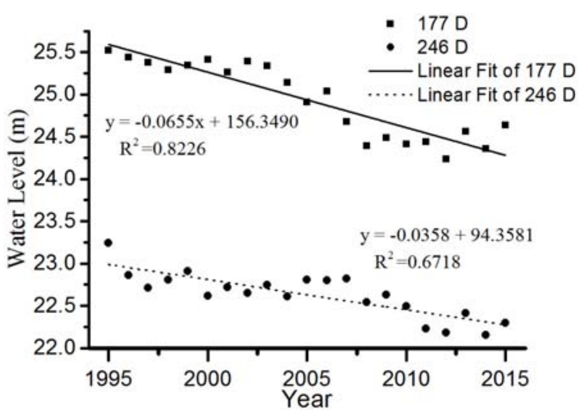


**FIGURE 5 |** Changes of water level corresponding to submergence duration 177 and 246 days during 1995–2015.

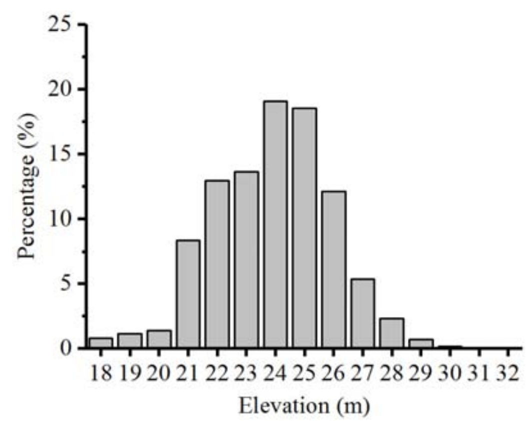


**FIGURE 6 |** Elevation-area relationship in East Dongting Lake.

elevations (accounting for 64.14% of the total area) would lead to significant change in total vegetation area and vegetation distribution pattern.

The alteration of vegetation distribution is usually considered as an adaptive mechanism to the changed water level. After the operation of TGD, the decline of monthly average water level occurs earlier in either the annual flood season or dry season, consequently exposing the lake-basin to air for a longer time (Huang et al., 2014), which provides favorable condition for plant growth and development. As a result, the vegetation also had a longer time to recover and accumulate more energy after flooding, which might facilitate vegetation expansion in either Reed and Forest or Grass. Additionally, changed water regimes altered the lowest distribution elevation of each vegetation type. The dynamic balance between Grass and Reed and Forest was also changed by the lower water level, leading to the vegetation at lower elevation rapidly replaced by higher-elevation one. This may explain why Reed and Forest had a faster expansion rate compared to Grass (4.9 cm/year vs. 3.3 cm/year). The expansion area was mainly distributed between the elevations of 22–24 m, where used to be occupied by Water and Mudflat. It was clear that the changed water regimes at this elevation range might promote the expansion of Grass to Water and Mudflat. Therefore, Reed and Forest occupied part of the space of Grass and Grass occupied part of the space of Water and Mudflat.

Most wetland plants have formed adaptive strategy to the regular change in water regime through a long-term evolution process (Baldwin et al., 2001). According to the variance of water level at different elevations, the submergence duration clearly increased at 20–21 m elevations but decreased at 22–27 m elevations, particularly at 24 m. The lowest distribution elevation of Grass continually decreased from 23.06 m in 1995 to 22.44 m in 2015, and from 25.08 m in 2005 to 24.59 m in 2015 for Reed and Forest. In this study, average submergence duration was 246 days for Grass and 177 days for Reed and Forest, respectively. Although the lowest distribution elevations for Grass and Reed and Forest were continually decreased during 1995–2015, the average submergence duration corresponding to the lowest distribution elevation was relatively constant in either Grass or Reed and Forest. Therefore, submergence duration may be a good indicator for predicting the change of vegetation distribution.

The characteristic of individual plant species can also account for the change of vegetation distribution. The Grass, mainly Carex species (Cyperaceae), has found to be a strong tolerance to long-time submergence (Li et al., 2013). Carex has two growing seasons in Dongting Lake wetlands, and can grow immediately when the flooding is receded. In contrast, the Reed and Forest has a higher tolerance to drought but lower tolerance to flooding (Li et al., 2013). Along with the declining water level, the advantage of high flood tolerance for Grass was weakened and it was easily replaced by the Reed and Forest, which can grow more favorably in areas with low water level (Deng et al., 2014).

As a member of Ramsar convention, Dongting Lake contains approximately 1420 plant species, 114 fish species and 217 bird species and has been listed as one of the 200 global conservation priority eco-regions proposed by the WWF. The Grass-dependent animals and migratory birds may be impacted by the change of habitat structure, as a result of degeneration of Grass. Change of water regime also accelerated human disturbance, which might be a risk for the wetland ecosystem (Zhao and Fang, 2004). For example, black poplar (*Populus nigra*) was introduced to plant in the Dongting Lake for economic profitability (Li et al., 2014). These activities have accelerated the change of vegetation dynamic and vegetation distribution, although some *Populus* trees have been cut down by local government. Therefore, how to maintain the ecosystem stability and structural integrity in Dongting lake wetlands is an important problem, especially after the operation of TGD. Further model research on the relationship between vegetation distribution and water regime should be conducted to better predict the change trend of vegetation distribution, which could provide scientific guidance for vegetation restoration and wetland management in this lake.

## AUTHOR CONTRIBUTIONS

J-YH analyzed the remote sensing data and wrote the manuscript. Y-HX designed the initial framework. YT provided a part of the water level data and the classification method. FL and Y-AZ contributed to revising the manuscript. The ideas of this paper were generated through discussions by all authors.

## FUNDING

This study was financially supported by the National Key Technology Research and Development Program of China (2014BAC09B03), the National Natural Science Foundation of China (31570431 and 31600344), the Youth Innovation Promotion Association of CAS (2014337), the Key Research Program of the Chinese Academy of Sciences (KFZD-SW-318), and Foundation of Hunan Province, China (2016JJ3130).

## ACKNOWLEDGMENTS

We thank Xinsheng Chen, Zhengmiao Deng, Zhiyong Hou, Xu Li, and Jing Zeng for their valuable work in the field investigation and data processing.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

ORIGINAL RESEARCH
published: 22 June 2018
doi: 10.3389/fpls.2018.00870

# Variations in Species-Level N:P Stoichiometry of Charophytes and Aquatic Angiosperms on the Tibetan Plateau

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The variations in nitrogen (N) and phosphorus (P) stoichiometry between species and along environmental gradients reflects plant growth and survival under certain conditions. Exploring the determinants of plant N and P stoichiometry at species level could help us understand the mechanisms of plant distribution. Temperature is considered a driving factor in forming the geographical patterns of plant N and P stoichiometry at the community level. Here we selected four common aquatic plants to explore the divergence of plant N and P stoichiometry between species and the species-level variations across large geographical gradients on the Tibetan Plateau. We found that plant N and P concentrations and N:P ratios were significantly different among the four species/groups. Charophytes had the lowest N and P concentrations, but the N:P ratio did not differ significantly from those of angiosperms. All four species/groups plant N concentrations were positively correlated with P concentrations. The temperature was also the primary explanatory variable, while the habitats properties showed weak and inconsistent effects on plant N and P stoichiometry. Plant N and P concentrations increased, but N:P ratios decreased, with decreasing temperature. Altitude, rather than latitude, determined the environmental patterns of plant N and P stoichiometry by affecting the temperature. These findings indicated that, after removing the influences of species replacement at the community level, temperature still plays a primary role in forming the geographical patterns of plant N and P stoichiometry at species level. Plants of each species could optimize their investment strategies of elements under different environmental conditions. The Tibetan Plateau is recognized as an area that is sensitive to global warming. Our results provided evidence, in terms of N and P stoichiometry, of potential variations among aquatic plants in nutrient absorption and element cycling under climatic warming.

**Keywords: alpine wetland, aquatic plants, freshwater ecosystems, stoichiometric homeostasis, temperature**

OPEN ACCESS

***Edited by:***
*Katya E. Kovalenko, University of Minnesota Duluth, United States*

***Reviewed by:***
*Emily Palm, Università degli Studi di Firenze, Italy*
*Martina Pollastrini, Università degli Studi di Firenze, Italy*

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***Specialty section:***
*This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science*

***Received:*** *12 January 2018*
***Accepted:*** *04 June 2018*
***Published:*** *22 June 2018*

***Citation:***
*Wang Z, Wu Z, Wang Y and Yu D (2018) Variations in Species-Level N:P Stoichiometry of Charophytes and Aquatic Angiosperms on the Tibetan Plateau. Front. Plant Sci. 9:870. doi: 10.3389/fpls.2018.00870*

**Abbreviations:** GST, growing season mean air temperature; STN, soil total nitrogen concentration; STP, soil total phosphorus concentration; WTN, water total nitrogen content; WTP, water total phosphorus content.

## INTRODUCTION

Aquatic plants are the primary producers in freshwater ecosystems and supply food to the primary consumers (Bornette and Puijalon, 2011). Plants, with their species-specific nutrient concentrations and ratios, determine the amount of nutrients that enter ecosystems, support specific consumers with certain nutrient concentrations, and control the stability of freshwater ecosystems, especially under global change regimes (Brothers et al., 2013). Nitrogen (N) and phosphorus (P) are two vital elements for plant growth and survival (Sterner and Elser, 2002; Ågren, 2008). Biologically, plant nutrient concentrations are species-specific functional traits (Duarte, 1992; Demars and Edwards, 2007). The N and P absorption capacities of plants are under genetic control, and maintain the species-specific tissue concentrations under fluctuating conditions of nutrient supply from substrates, called "stoichiometric homeostasis" (Sterner and Elser, 2002; Elser et al., 2010). This theory explains the inter-species variations in plants under the same environmental conditions. However, plants exhibit relatively greater intra-species stoichiometric variability than heterotrophs (Wang et al., 2012), and show significant variations under different environmental conditions (Reich and Oleksyn, 2004).

At large geographic scale, temperature determines the patterns of plant elemental stoichiometry, by directly controlling plant physiological processes, or indirectly via altering species composition in the local community (Reich and Oleksyn, 2004; He et al., 2008; Zhang et al., 2012; Wang et al., 2015). In cold regions, plants usually invest more N and P to biochemical processes to counterbalance the depressed efficiency of enzymatic reactions, which are restricted by low temperature (Reich and Oleksyn, 2004). Furthermore, low temperature can pick out cold-tolerant species to assemble local community in cold regions (Bornette and Puijalon, 2011), and then enhance the plant N and P concentrations at the community level (Demars and Edwards, 2008; Frost and Hicks, 2012; Xia et al., 2014; Wang et al., 2015). Most previous studies have been carried out at the community level, mixing the direct effects of environmental factors on plant physiological process as well as species replacement (Elser et al., 2010). To differentiate the direct and indirect influences of temperature, stoichiometric studies on widespread species are necessary. The air-water interface can buffer the fluctuations of temperature, causing aquatic plants to be more cosmopolitan than terrestrial plants (Santamaría, 2002). The widespread species of aquatic plants allow us to examine the adaption mechanisms of plants at species level across sufficient environmental gradients.

In this study, we focused on four common aquatic plants species/groups (*Stuckenia filiformis*, *Halerpestes tricuspis*, *Triglochin palustris* and charophytes) on the Tibetan Plateau. *S. filiformis* is one of the most widespread submerged species on the Tibetan Plateau, and forms an underwater community as constructive species (Guo et al., 2010b). Charophytes are macroalgae that are ascribed to green plants Viridiplantae, possess superiority in oligotrophic or brackish waters, and can form dense underwater meadows in favorable conditions (Kufel et al., 2016). *H. tricuspis* and *T. palustris* are two common species inhabiting in riparin zones (Wang and Michio, 2001; Guo et al., 2010a). Climatic change (included warming and the variations in precipitation patterns) in the Tibetan plateau threaten the survival of common aquatic plants. With strong topographic relief and the resulting alpine climatic gradients, the Tibetan Plateau provides an ideal platform to explore the mechanisms for plants adapting to drastically varied abiotic environments. Our aims were to (1) test the inter-specific differences of plant N and P stoichiometry to clarify the effects of taxonomy, and (2) examine the intra-specific variations in plant N and P stoichiometry along environmental gradients, including geographical and climatic variables and habitats properties, to explore the relative importance of the environmental factors at species level.

## MATERIALS AND METHODS

### Study Area

The field investigation was carried out on the Tibetan Plateau from July to August 2012. The Tibetan Plateau is known as "water tower" in Asia and is the source of many great rivers. Thus, the riparian habitats provide suitable environments for aquatic macrophytes. In addition, 1091 lakes ($\geq$ 1 km$^2$ in the area) were recorded in the Tibetan Plateau, and provide another type of aquatic habitat (Wang and Dou, 1998). Furthermore, vast expanses of marshes, numerous ponds and channels provide additional aquatic habitats for plants in the Tibetan Plateau.

Topographically, the elevation of the plateau rises from the southeast to northwest, creating variations in climatic variables and habitats properties. Because of the obstruction of the Himalayas, the warm and wet air current from the Indian Ocean travels to the plateau mainly via the canyon of the Yarlung Zangbo River, which lies in the southeastern part of the Tibetan Plateau. The mean multi-annual precipitation shows decreasing trend from about 800 mm in the southeast to about 20 mm in the northwest of the plateau. The mean air temperatures for the year, January, and July are $-5$ to 11$^\circ$C, $-18$ to $-6^\circ$C and 5 to 20$^\circ$C, respectively, which also show a decreasing trend from the southeast to the northwest. On the Tibetan Plateau, the highest temperature is coupled with the greatest water availability on the same period in summer. The growing season on the plateau is from May to September (Wang et al., 2013).

### Species

#### *Stuckenia filiformis* (Persoon) Börner

*Stuckenia filiformis* (Potamogetonaceae) is a perennial, cosmopolitan species and totally submerged in fresh or brackish water. The species mainly occurs on the Tibetan Plateau and adjacent regions in Asia and south and north America. On the Tibetan Plateau, *S. filiformis* is one of the most common species and frequently observed to dominate the aquatic community. The stems of the species are slender and the leaves are linear and sessile. The blossom and fruit period are from July to October (Guo et al., 2010b).

### *Halerpestes tricuspis* (Maximowicz) Handel-Mazzetti

*Halerpestes tricuspis* (Ranunculaceae) is a perennial and small herb species, and always grows in marshes, wet meadows or spreads to water surface with slender stolons. The species mainly occurs on the Tibetan Plateau and adjacent regions and Mongolia. The basal leaves have a petiole, and the leaf blades are always 3-lobed with an area of less than 3 cm × 3 cm. The florescence last from May to August (Wang and Michio, 2001).

### *Triglochin palustris* L.

*Triglochin palustris* (Juncaginaceae) is a perennial and slender herb species and always grows in marshes and wet meadows below 4500 m in elevation. The species is cosmopolitan in temperate regions. The basal leaves are linear with the shape of ca. 20 cm in length and ca. 1 mm in width. The blossom and fruit period are from June to October (Guo et al., 2010a).

#### *Charophytes*

Charophytes are cosmopolitan submerged cryptogams, especially in temperate regions, and prefer calcareous aquatic habitats (Forsberg, 1964; Wiik et al., 2015). Charophyte species have a height of 15–30 cm, and differentiate into rhizoid, stem (axis), and branchlet (Han and Li, 1994). Both stems and branchlets are photosynthetically active.

In this study, we did not identify the species of charophytes but treated all the species as a group and compare their stoichiometric characteristics with those of angiosperms.

## Field Sampling

All of the samples were collected in July and August 2012. For the submerged species, *S. filiformis* and charophytes, 30 segments of plant shoots (ca. 20 cm from the tips) were sampled randomly from each site. All of the leaves (*S. filiformis*) were picked off, while whole shoots samples of charophytes were collected, and put together for each site. For the other two species, *H. tricuspis* and *T. palustris*, we collected 30–50 fully expanded and intact leaves randomly in each site, respectively. All of the samples were oven-dried at 75°C for 48 h, and then finely ground by pulverizer and ball-mill. In total, we investigated 126 sites of aquatic habitats, of which 98, 73, 54 and 36 sites were sampled for *S. filiformis*, *H. tricuspis*, *T. palustris* and charophytes, respectively (**Figure 1**).

For the sediment samples, we dug three vertical and cylindrical cores (20 cm high × 3 cm diameter) randomly in each site. The samples were air-dried and sifted through an 80-mesh sieve. For water samples, we first measured the pH and salinity in the field using a handheld multi-parameter meter (PROPLUS, YSI, United States). A bottle of clean water was collected to measure WTN and WTP.

### Chemical Measurements

All plants and sediments samples were ground to powder. The C and N concentrations were determined using a CN elemental analyzer (vario MACRO cube, Elementra, Germany), and the P concentrations were measured using the molybdate/stannous chloride method (Kuo, 1996). The N:P ratios were mass-based ratios which were calculated via N concentrations (mg $g^{-1}$) divided by P concentrations (mg $g^{-1}$). For water samples, the WTN and WTP were determined with a photometer (Palintest 7500, Palintest, United Kingdom) within 12 h of collection.

## Data Analysis

The data of plant N and P concentrations and N:P ratios were first $\log_{10}$-transformed to normalize their distribution, and the relationships of plant N to P concentrations were regressed by power functions (Reich et al., 2010). Analysis of variance (ANOVA) and Bonferroni-adjusted significance values were employed to assess the differences in plant N and P concentrations and N:P ratios between the species. In this study, we had four groups of data for each variable (N, P and N:P) and performed six times pairwise comparisons in multiple testing of ANOVA. The significance values were adjusted to 0.0083 by dividing 0.05 by the six times of pairwise comparisons. We introduced one climatic variable (GST) and six habitats properties (water pH, salinity, WTN and WTP, and soil total nitrogen and phosphorus) to build general linear models (GLMs) for leaf N and P concentrations and N:P ratios. *F*-tests were used to perform ANOVAs of the GLMs. The percentage of the total sum of squares (%SS) were introduced to quantify the degree of each explanatory variable accounting for in the GLMs (He et al., 2008). In the GLMs, different orders of explanatory variables could not affect the total explaining degree of the model but could vary the degree of each explanatory variable. We entered the explanatory variables in different orders and offered the %SS for each variable when it was the first variable in the GLMs.

Using $\log_{10}$-transformed data of plant N and P concentrations and N:P ratios, we applied a family of simple regressions to test the effects of temperature, latitude and altitude on plant N and P concentrations and N:P ratios, respectively. Therefore, each family included 3 sequential tests applying to the same data (e.g., N concentration of each species). The Holm's Sequential Bonferroni Procedures were introduced to increase the power of the statistical tests (Abdi, 2010). According to the procedure, the original *p*-value was firstly obtained from each test, and then the tests were ordered from the one with the smallest *p*-value to the one with the largest *p*-value. The corrected *p*-value for the *i*th-test, denoted $p_{\text{Bonferroni}}, i/C$ was computed as:

$$p_{\text{Bonferroni}}, i/C = (C - i + 1) \times p$$

where *C* was the number of tests (3 in this study) and *p* was the original *p*-value of each test. If the original *p*-value of *i*th-test was smaller than the corrected *p*-value, we kept the original one reported in the results. If not, the test was non-significant.

The air temperatures were obtained by entering geographic coordinates into equations derived from data collected at meteorological stations across China. The GST were calculated by averaging the monthly mean temperatures from May to September. All statistical analyses were conducted with R 3.4.2 (R Development Core Team, 2007).

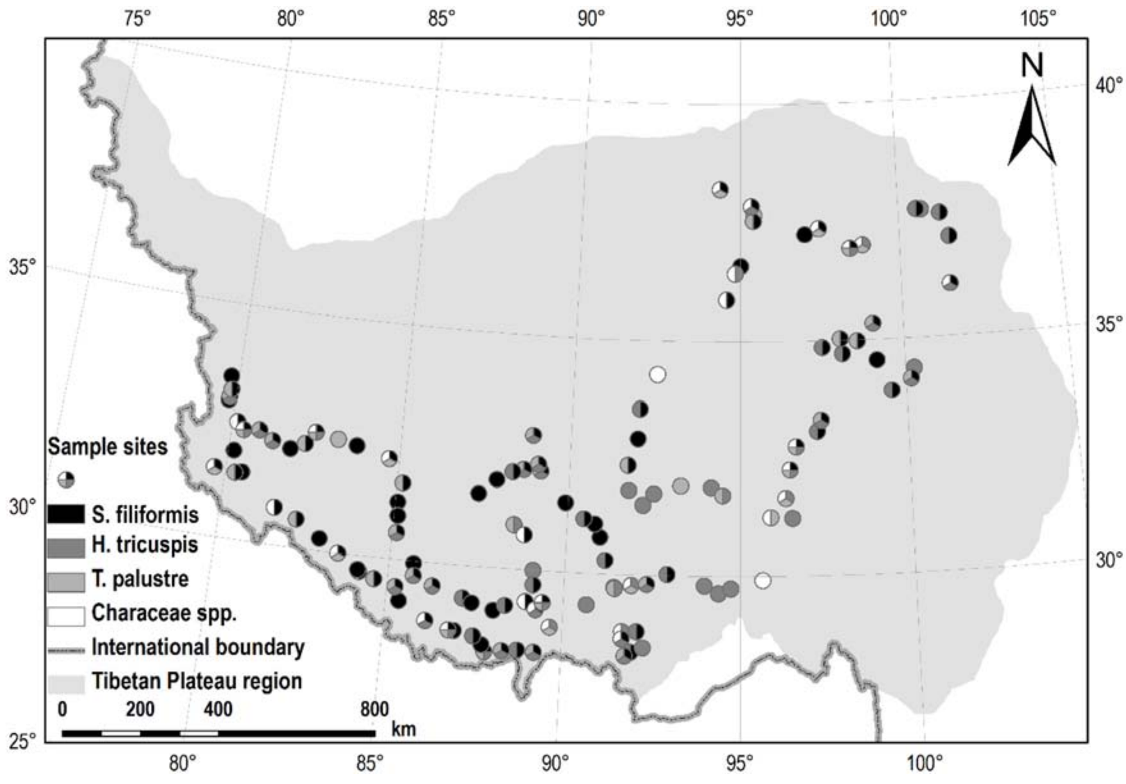


**FIGURE 1 |** Sample sites in this study. Of all the 126 sites, *S. filiformis*, *H. tricuspis*, *T. palustris* and charophytes were sampled from 98, 73, 54, and 36 sites, respectively.

# RESULTS

## Plant N, P Concentrations and N:P Ratios

Results of ANOVA showed that plant N and P concentrations and N:P ratios were significantly different among the four species/groups, and among the three angiosperm species (significance level $p < 0.001$). When pairwise comparisons were performed, the N and P concentrations of charophytes were significantly lower, but the N:P ratio did not differ from their angiosperm counterparts (**Table 1**). Within angiosperms, *S. filiformis* showed the lowest leaf N concentration and leaf N:P ratio, while *H. tricuspis* showed the highest leaf N and P concentrations (**Table 1**).

For all of the four species/groups, plant N concentrations positively correlated with P concentrations (**Figure 2**).

## Effects of Environmental Factors on Plant N and P Stoichiometry

The general linear models (GLM) which contained seven explanatory variables explained 16.85 to 42.67%, 20.78 to 47.45%, and 6.75 to 33.87% of the variations in plant N, P concentrations and N:P ratios, respectively (**Table 2**). Among the explanatory variables, GST was the primary variable, while habitat properties showed weak and inconsistent effects on the plant N and P stoichiometry.

Plant N and P concentrations of the four species/groups increased with decreasing GST (**Figures 3A–H**). The N:P

**TABLE 1 |** Plant N, P concentrations and N:P ratios of the four aquatic species/groups.

| Species | *n* | N | | | P | | | N:P | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Mean | *SD* | *CV* | Mean | *SD* | *CV* | Mean | *SD* | *CV* |
| *Stuckenia filiformis* | 98 | 26.13[b] | 6.54 | 0.25 | 2.97[b] | 0.98 | 0.33 | 9.51[b] | 3.08 | 0.32 |
| *Halerpestes tricuspis* | 73 | 33.78[a] | 7.75 | 0.23 | 3.66[a] | 1.23 | 0.34 | 9.92[ab] | 3.15 | 0.32 |
| *Triglochin palustris* | 54 | 31.13[a] | 7.27 | 0.23 | 2.85[b] | 1.03 | 0.36 | 11.83[a] | 3.61 | 0.31 |
| Charophytes | 36 | 10.64[c] | 4.92 | 0.46 | 1.30[c] | 0.85 | 0.65 | 9.73[ab] | 4.52 | 0.46 |

*Different letters indicated significant differences in plant N and P concentrations and N:P ratios between species, at the Bonferroni-adjusted significance level p < 0.0083. SD, standard deviation; CV, the coefficient of variation (SD/mean).*

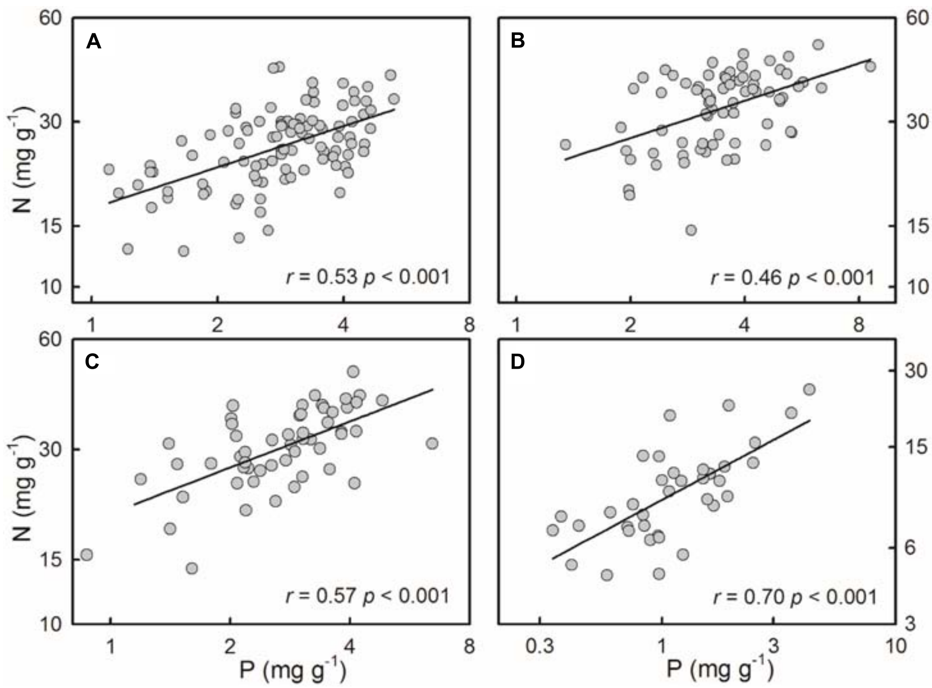


**FIGURE 2 |** Positive relationships between N and P concentrations of the four species/groups. The correlations were fitted in power functions. **(A)** *S. filiformis*, **(B)** *H. tricuspis*, **(C)** *T. palustris*, and **(D)** Charophytes.

**TABLE 2 |** The percentage of the total sum of square (%SS) of each explanatory variable in the general linear model.

| Variables | N | | | | P | | | | N:P | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *S. fil* | *H. tri* | *T. pal* | *Cha* | *S. fil* | *H. tri* | *T. pal* | *Cha* | *S. fil* | *H. tri* | *T. pal* | *Cha* |
| GST | 12.79*** | 18.63*** | 37.28*** | 9.16 | 11.00*** | 22.03*** | 41.12*** | 25.71*** | 0.87 | 2.46 | 9.35* | 16.47* |
| pH | 0.86 | 3.93 | 0.92 | 4.28 | 5.98* | 3.64 | 6.05* | 19.02*** | 4.48* | 0.21 | 5.53 | 10.51* |
| Salinity | 1.44 | 0.11 | 1.11 | 1.84 | 0.01 | 0.30 | 1.76 | 10.19* | 1.12 | 0.10 | 0.65 | 4.82 |
| WTN | 0.05 | 3.09 | 1.51 | 1.72 | 0.29 | 3.12 | 0.11 | 6.38 | 0.69 | 0.23 | 0.38 | 4.92 |
| WTP | 5.75** | 0.95 | 0.05 | 0.61 | 4.56* | 0.09 | 0.10 | 0.47 | 0.28 | 0.23 | 0.04 | 3.00 |
| STN | 0.34 | 0.53 | 3.47 | 1.44 | 2.77 | 6.44* | 7.93* | 1.15 | 2.21 | 4.66 | 4.16 | 0.09 |
| STP | 0.11 | <0.01 | 1.99 | 0.01 | 0.46 | 0.72 | 1.34 | 0.75 | 0.28 | 0.82 | 0.09 | 1.65 |
| Residuals | 74.62 | 76.91 | 57.33 | 83.15 | 79.22 | 72.32 | 52.55 | 53.69 | 89.49 | 93.25 | 82.11 | 66.13 |

*The %SS represents the explanatory degree of each explanatory accounted for. The explanatory variables include growing season mean temperature (GST), water pH value, salinity, water total nitrogen content (WTN), water total phosphorus content (WTP), soil total nitrogen concentration (STN), and soil total phosphorus concentration (STP). S. fil, Stuckenia filiformis; H. tri, Halerpestes tricuspis; T. pal, Triglochin palustris; Cha, Charophytes. *p < 0.05, **p < 0.01, ***p < 0.001.*

ratios of *T. palustris* and charophytes decreased with decreasing GST (**Figures 3K,L**), while those of the other two species showed no significant relationships with GST (**Figures 3I,J**).

## Geographic Patterns of Plant N and P Stoichiometry

Plant N and P concentrations and N:P ratios of the four species/groups did not show consistent trends along latitude gradients (**Figures 4A–L**). Regarding altitude gradients, plant N and P concentrations of the four species/groups increased toward high altitude (**Figures 5A–H**), except for N concentration of charophytes (**Figure 5D**). The N:P ratio of *T. palustris* and charophytes decreased, but those of the other two species showed no significant relationships with increasing altitude (**Figures 5I–L**).

# DISCUSSION

## Stoichiometric Homeostasis of Aquatic Plants on the Tibetan Plateau

Stoichiometric homeostasis refers that organisms maintain specific element composition by adjusting the organismic physiological processes when faced with variations in nutrient availability in their surroundings (Sterner and Elser, 2002; Elser

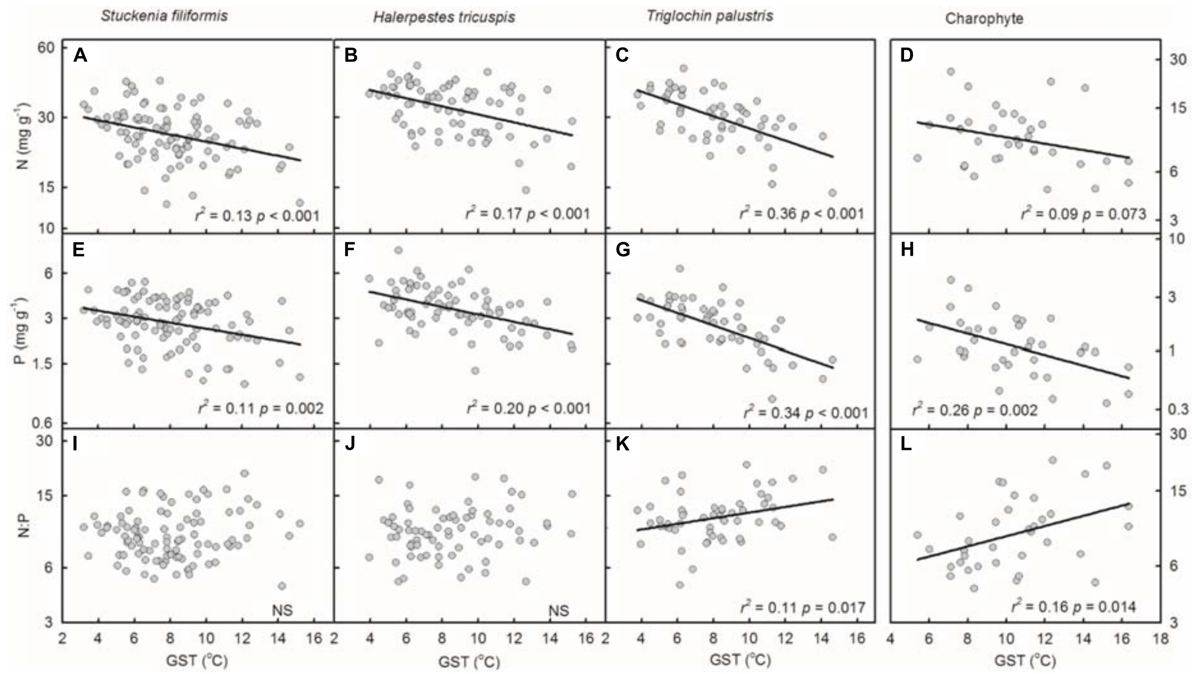


**FIGURE 3 |** Trends of plant N and P concentrations and N:P ratios along temperature gradients **(A–L)**. The growing season mean temperature (GST) was used to test the effects of temperature on plant N and P concentrations and N:P ratios. The *p*-value in each panel was the original one of each test if it was smaller than the corrected one obtained by Holm's Sequential Bonferroni Procedure. If not, the test was non-significant (NS).

et al., 2010; Yu et al., 2010). In this study, three patterns were observed. The first was that the plant nutrient concentrations were different among species, especially between angiosperms and charophytes. These results are consistent with Blindow (1992), who stated that charophytes species (*Chara tomentosa* and *Nitellopsis obtusa*) contain less N (10.5 – 14.0 mg $g^{-1}$) and P (0.63 – 0.88 mg $g^{-1}$) than their angiosperm counterparts (N, 24.2 – 24.5 mg $g^{-1}$; P, 2.11 – 2.51 mg $g^{-1}$ in *Myriophyllum spicatum* and *Potamogeton pectinatus*) from shallow lakes in Sweden. Furthermore, several independent studies demonstrated the same patterns when charophytes and angiosperms were taken into account simultaneously (reviewed in Kufel and Kufel, 2002). However, charophytes may not differ so much from angiosperm species as the data shows. Charophytes always build calcite encrustation, which is composed mainly of calcium carbonate, tightly bound to their thalli, and precipitate much more calcium than vascular aquatic plants (Kufel et al., 2016). The encrustation occupies a large proportion (may exceed 70% in extreme situations) of dry plant mass and explains the lower N and P concentrations of charophytes (Blindow, 1992; Kufel and Kufel, 2002; Kufel et al., 2016). In addition, the proportions of encrustation in dry plant mass of charophytes are species-specific (Kufel et al., 2016), inducing a higher value of the coefficient of variation (CV) when charophytes are treated as a group of species, as done in this study (**Table 1**). In further studies, identifying the species, removing the calcite encrustation, and calculating N and P concentrations on an ash-free dry weight basis of charophytes might lead to more accurate conclusions when comparing the nutrient concentrations of charophytes with those of vascular aquatic plants. In the case of the three angiosperm species in this study, mean nutrient concentrations differed from each other at the species levels. As the samples were collected from the same region, such variations were likely attributed to taxonomic differences, rather than the ambient environments (Demars and Edwards, 2007).

Although significant differences observed between the nutrient concentrations of charophytes and their angiosperm counterparts, the N:P ratios of the two plant groups remained within a similar range, and not differ from each other on species mean levels. This was the second principal trend detected in this study. The N:P ratios rather than N and P concentrations are considered to reflect the nutrient limitation of plants (Güsewell and Koerselman, 2002). The similarity of N:P ratios between charophytes and angiosperm species suggests that the lower nutrient concentrations of charophytes did not result from lower ambient nutrient availability since the sampling area for each species were overlapping. On the contrary, innately low nutrient concentrations of charophytes imbue the species with competitive superiority in oligotrophic habitats (Blindow et al., 2014).

The third principal pattern detected here was the positive N-P relationships, which were well documented in previous studies at the species level (Li et al., 2014; Wu et al., 2014) or on community level (Duarte, 1992; Demars and Edwards, 2007; He et al., 2008; Reich et al., 2010). In this study, both charophytes and angiosperms showed coincident positive N-P relationships. Functionally, P is primarily allocated to ribosomal RNA, which

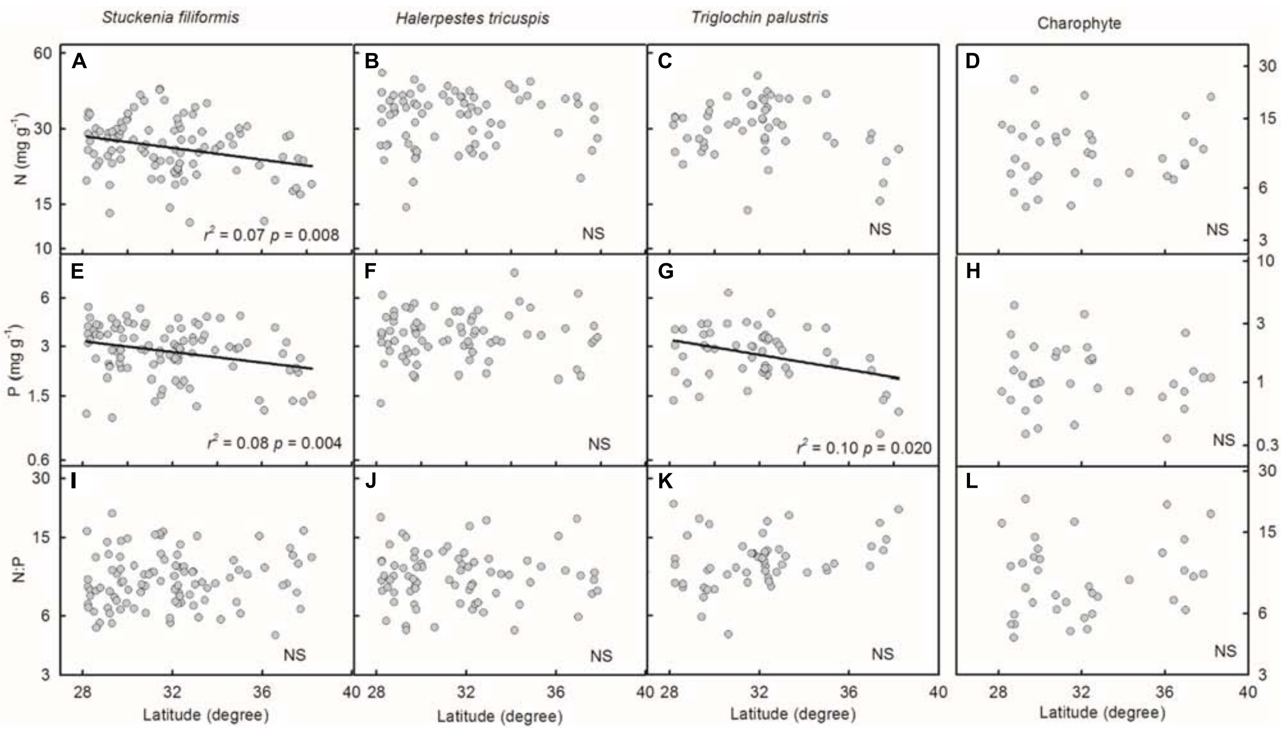


**FIGURE 4 |** Trends of plant N and P concentrations and N:P ratios along latitudinal gradients **(A–L)**. The *p*-value in each panel was the original one of each test if it was smaller than the corrected one obtained by Holm's Sequential Bonferroni Procedure. If not, the test was non-significant (NS).

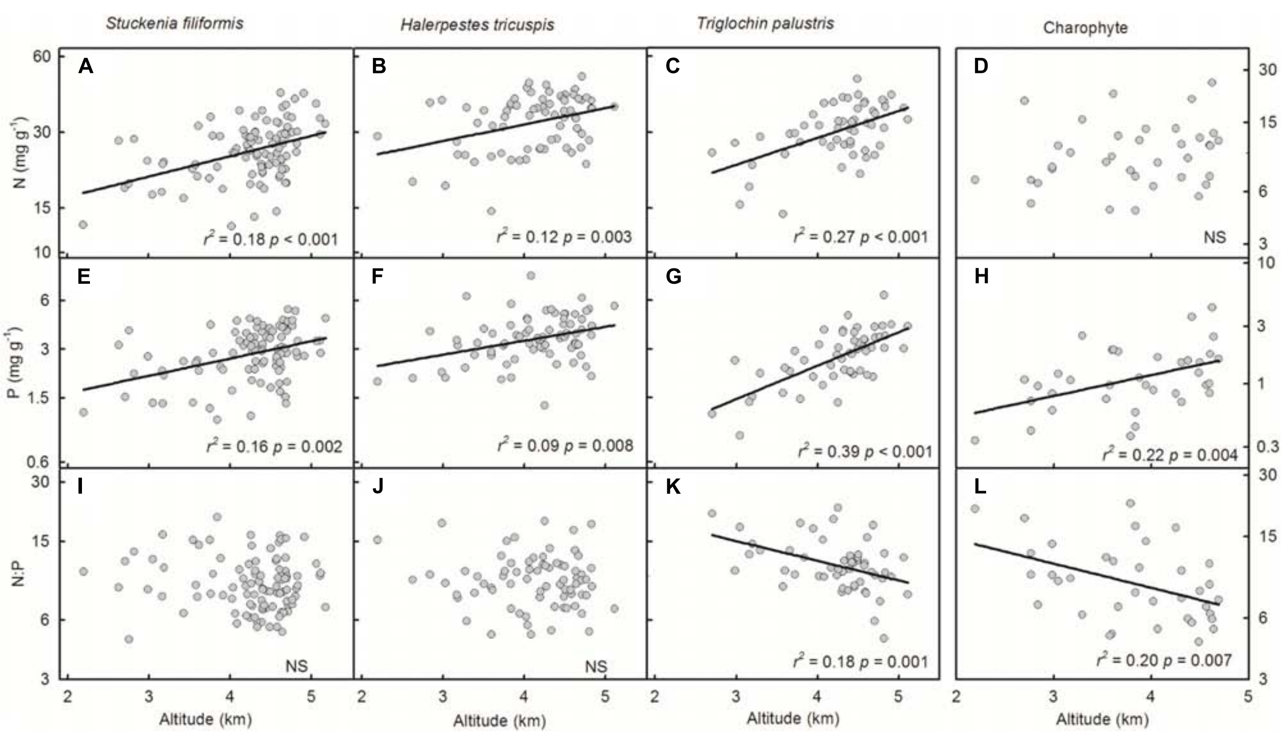


**FIGURE 5 |** Trends of plant N and P concentrations and N:P ratios along altitudinal gradients **(A–L)**. The *p*-value in each panel was the original one of each test if it was smaller than the corrected one obtained by Holm's Sequential Bonferroni Procedure. If not, the test was non-significant (NS).

is used to synthesize N-rich proteins, indicating that N and P concentrations might co-vary relative to each other to maintain optimal tissue N:P ratios (Sterner and Elser, 2002; Ågren, 2008).

Each species contained specific nutrient concentrations and maintained relatively stable ratios and correlations between elements. The stoichiometric nutrient composition was species-specific, and the plants could control stoichiometric homeostasis in fluctuating environments.

## Stoichiometric Variability of Aquatic Plants and the Effects of the Environment

Empirically, stoichiometric homeostasis is an approximation rather than a strict value, as the organisms always need to respond to the fluctuations of resources availability (Wang et al., 2012). This study revealed that the nutrient concentrations varied considerably within the same species. The N concentrations varied 4-fold and P varied 10-fold within species, indicating the significant influences of the ambient environments. Among all of the environmental factors, temperature played the primary role in determining the patterns of plant N and P concentrations along environmental gradients. Plant N and P concentrations increased with decreasing temperature. Such patterns supported the temperature-plant physiological hypothesis, which suggested that plants invested more N and P in enzyme systems to compensate the depressed efficiency in cold regions (Reich and Oleksyn, 2004). Habitat properties had weak effects on plant N and P stoichiometry (Sardans et al., 2012). Similar results were also reported at the species level for *Ranunculus natans* (Ranunculaceae) in the arid zone of northwest China (Li et al., 2015). Regarding life forms, emergent plants, *T. palustris* and *H. tricuspis*, which leaves were exposed to air directly, had a higher explanatory degree by GST than those of submerged species, *S. filiformis* and charophytes. These results indicate that water buffered the drastic variation of air temperature, and alleviated the stress of low temperature for submerged plants. In all, temperature determined the patterns of plant N and P stoichiometry at the community level (Wang et al., 2015), as well as species level (Li et al., 2015 and this study) across large environmental gradients.

Geographically, temperature decreases with increasing latitude and altitude. Latitudinal patterns of plant element stoichiometry at regional and global scales have been well documented (Reich and Oleksyn, 2004; Han et al., 2005; Xia et al., 2014), whereas altitudinal trends are often neglected. However, altitudinal gradients are known to dramatically alter environmental factors (e.g., temperature) in relatively small areas (Lacoul and Freedman, 2006; Wang et al., 2013; De Long et al., 2015). In this study, the latitude range extended approximately 10 degrees, from 28.18 to 38.21°N, but the altitude range spanned nearly 3000 m, from 2194 to 5176 m, which introduces more drastic variations in temperature than does the latitude range. Leaf N and P had no or weakly significant relationships with latitude, but significantly increased with increasing altitude. The results were consistent with Körner's (1989) finding that herbaceous plants from high elevation regions contained more nutrients. Therefore, on the Tibetan Plateau, we suggest that the patterns of plant N and P stoichiometry at the species level are determined by the stresses of low temperature, which were induced by altitude rather than latitude.

## AUTHOR CONTRIBUTIONS

ZWa and DY designed the study. ZWa and ZWu performed the field investigation. ZWa, ZWu, and YW analyzed the data and wrote the manuscript. All authors worked together to produce the final version of the text.

## FUNDING

ZWa was supported by the National Natural Science Foundation of China (Grants 31300296).

## ACKNOWLEDGMENTS

We thank the reviewers for comments on the manuscript. We thank Zhenjie Ding and Xiaolong Huang for help with the fieldwork. We also thank Dr. Heyun Wang for her valuable suggestions of revised manuscript.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

ORIGINAL RESEARCH
published: 02 August 2018
doi: 10.3389/fpls.2018.01129

# Carbon Gain Limitation Is the Primary Mechanism for the Elevational Distribution Limit of *Myriophyllum* in the High-Altitude Plateau

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**Specialty section:**
This article was submitted to
Functional Plant Ecology,
a section of the journal
Frontiers in Plant Science

**Received:** 28 December 2017
**Accepted:** 12 July 2018
**Published:** 02 August 2018

**Citation:**
Xie D, Wu Z, Chen HYH, Wang Z, Wang Q and Yu D (2018) Carbon Gain Limitation Is the Primary Mechanism for the Elevational Distribution Limit of Myriophyllum in the High-Altitude Plateau. Front. Plant Sci. 9:1129. doi: 10.3389/fpls.2018.01129

Temperature comprises a major driver for species distribution and physiological processes in alpine plants. For all terrestrial plant species tested to date, elevation associated decreases in temperature have been observed to influence the balance between carbon acquisition and usage; restricting the upper limit of most alpine trees (i.e., treeline). However, such a carbon source-sink balance has not been tested in any alpine aquatic plants, which is an important component of the alpine aquatic ecosystem. The *Myriophyllum* species inhabits a broad range of habitats across the high-altitude plateau. Three *Myriophyllum* species (*Myriophyllum spicatum*, *Myriophyllum verticillatum*, and *Myriophyllum sibiricum*) from 12 water bodies at elevational gradients between 2766 and 5111 m were collected in the Qinghai-Tibetan Plateau. The late growing seasonal concentrations of non-structural carbohydrates (NSC) in the leaves were measured to find how high-altitude conditions influence the carbon balance in aquatic plants. Regression tree analysis separated the 12 water bodies into two groups according to water turbidity (seven water bodies with high turbidity and five water bodies with low turbidity). Overall, leaf NSC concentrations (primarily starch) decreased significantly with increasing elevation in widely distributed *M. spicatum* and *M. verticillatum*. Regression tree analysis indicated that water turbidity (i.e., shady environment) was a strong determinant of leaf NSC. In the low turbidity group (<3.5 NTU), leaf NSC concentrations decreased with increasing elevation; however, in the high turbidity group (>3.5 NTU), leaf NSC concentrations were low and had no association with elevation. Unlike most recent studies in tree species, which show low temperatures limited growth at high-elevations, our results demonstrated that carbon gain limitation is the primary mechanism for the elevational distribution limit of *Myriophyllum* species in the Qinghai-Tibetan Plateau. Moreover, water turbidity moderated the effects of low temperature by masking the expected carbon limitation trend. Therefore, at least two environmental factors (i.e., temperature and light availability) induced photosynthesis decreases might explain the NSC responses for aquatic plants in response to elevation.

**Keywords: alpine submerged macrophytes, growth-limitation hypothesis, high-altitude plateau, low temperature, *Myriophyllum*, non-structural carbohydrates**

## INTRODUCTION

At high-elevations, plants often face severe environmental conditions, such as low temperature, ice/snow cover and high ultraviolet-B radiation, which restrict growth, reproduction, and many metabolic functions (Lacoul and Freedman, 2006a; Aichner et al., 2010; Loayza-Muro et al., 2013). Several studies on terrestrial plants have demonstrated the existence of low temperature-adapted patterns in phenology (Potvin, 1986), growth (Shi et al., 2008) and morphology (Alvarez-Uria and Körner, 2007). Although the aquatic environment may affect elevation gradient responses by buffering temperature fluctuations, the diversity of aquatic plant species have been associated with (water) temperature across elevation gradients, ranging from 77 to 4980 m in the Himalayas (Lacoul and Freedman, 2006b). Jones et al. (2003) also revealed that species richness declined with the increase of elevation in Cumbria, United Kingdom (elevation gradients range from 2 to 837 m). These findings are similar to those for terrestrial plants, where previous studies have attempted to utilize environmental factors, such as temperature and water availability, to explain their physiological mechanisms in response to high elevations (Körner, 1998; Hoch et al., 2002; Hoch and Körner, 2009; Fajardo et al., 2011); however, we have little knowledge of the elevational responses in aquatic plants.

Common plant species with extensive distribution may perform well under a broad range of environmental conditions (Joshi et al., 2001). Many aquatic plant species are widely dispersed, reproduce asexually, and often possess limited genetic variation (Santamaría, 2002). The successful propagation of aquatic plants under variable environmental conditions is often linked to their phenotypic plasticity. For instance, Ganie et al. (2014) observed that morphogenic plasticity caused the successful propagation of 10 *Potamogeton* species across habitats with different water flow types in the Kashmir Himalayas. Environmentally induced phenotypic plasticity may lead to rapid changes in plant phenotypic characteristics, which support the survival, reproduction, and dispersal of aquatic plant species across a broad range of habitats (Ganie et al., 2014).

Elevation associated temperature is regarded as one of the major drivers of plant distribution and individual physiological processes, not only for terrestrial plants (Hoch and Körner, 2009), but also for aquatic plants (Rooney and Kalff, 2000). Aquatic plants (particularly submerged macrophytes) are believed to be eurythermic and able to thrive under a wide range of temperatures (Madsen and Brix, 1997). A research from global dataset revealed that physiological acclimation of plants will lead increase of leaf nitrogen (N) and phosphorus (P) concentrations to offset the depressed biochemical efficiencies (e.g., N-rich enzymes and P-rich RNA) in colder, rather than warmer, climates (Reich and Oleksyn, 2004). The changes of leaf N and P concentrations will also regulate carbon (C) acquisition and use in plants (Reich and Oleksyn, 2004). When exposed to low temperatures/cold stress environments, perennial aquatic plant species typically exhibit physiological plasticity, in terms of photosynthesis, storage accumulation and nutrient elements absorption (Madsen and Brix, 1997; Klimeš et al., 1999; Olesen and Madsen, 2000). For instance, Wang et al. (2015) found increased concentrations of leaf N and P in aquatic plants in response to low temperatures in the Qinghai-Tibetan Plateau. Being different from plants in terrestrial habitats, aquatic plants in high elevational water bodies are not only subjected to extreme low-temperature environmental conditions, but also shade stresses (e.g., from high suspended organic and/or inorganic particle concentrations and filamentous algae) (Jackbsen, 2008). Although alpine water bodies are typically clear, some high mountain lakes show specific turbid stages related to the thermal budget of the lakes with cold turbid water inflow (Root et al., 2006). Even within very short distances, some streams may also exhibit very different mean levels and ranges of suspended solids (Jackbsen, 2008). Surprisingly, despite the relatively extensive literature dealing with aquatic plants in response to temperature or shade gradients, to our knowledge, few studies have considered the potentially simultaneous influences of temperature decreases and turbid stages on the distribution of submerged macrophytes at high elevations.

For both terrestrial and aquatic plants, non-structural carbohydrates (NSC) including soluble sugars and starches are common storage molecules, which serve to increase plant survival and recovery in habitats with frequent disturbances (Puijalon et al., 2008; Huber et al., 2012; Adams et al., 2013). Previous studies with terrestrial plants have shown that elevation-induced low temperatures trigger the increased storage of NSC in woody tissue, and growth restriction of multiple tree species in the treeline ecotone (Hoch et al., 2002; Shi et al., 2008; Fajardo et al., 2012). These results indicated that tree cell and tissue formation are initially limited by elevation associated decreases in temperature (i.e., growth-limitation hypothesis, GLH) (Körner, 1998), and not what was previously thought; that low temperatures limited photosynthetic decline, which limited plant growth at high-elevations (i.e., carbon-limitation hypothesis, CLH). A growing number of studies support the GLH in trees (e.g., Shi et al., 2008; Fajardo et al., 2012; Palacio et al., 2014; Hoch, 2015); however, several studies suggested that a direct connection between NSC accumulation and restrained growth was inconclusive (Wiley and Helliker, 2012; Fajardo and Piper, 2014). The most straightforward approach for the resolution of such a debate was the comparison of NSC concentrations of plant species along an elevational gradient (Fajardo et al., 2011). There has only been a single study that measured NSC concentrations in the rhizomes of emergent macrophyte *Phragmites australis* (Cav.) Trin. ex Steud. at two elevations (400 and 1350 m, respectively), and found that the NSC concentrations of rhizomes were higher in the high, rather than the low, elevation site (1350 m, 35.2% compared with 30.5%, respectively) (Klimeš et al., 1999). Hence, a better understanding is required in terms of how the energy storage of aquatic plants responds to a wide range of elevations.

The principal aim of this study was to test the C acquisition-demand balance in submerged alpine macrophytes in response to variable elevational temperature gradients, and the influence of water turbidity on the response of the C balance to elevation (**Figure 1**). We addressed the following questions: (1) do NSC concentrations of *Myriophyllum* species increase or decrease

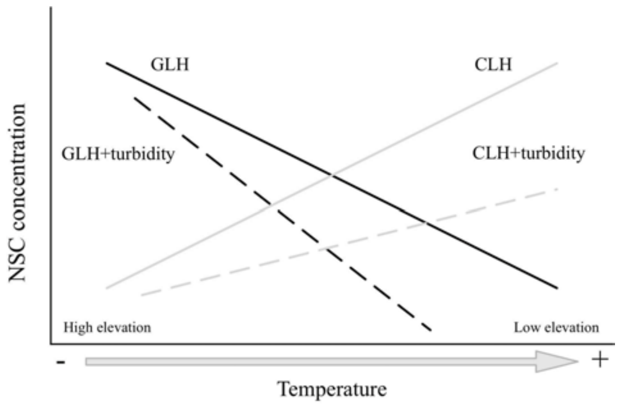


**FIGURE 1 |** Expected trends of non-structural carbohydrate concentrations (NSC) with elevational gradients (temperature effects) according to the growth-limitation hypothesis (GLH; black lines) and carbon-limitation hypothesis (CLH; gray lines), which considers the alternative effects of water turbidity. The effect of temperature on either the photosynthesis (CLH), or the growth (GLH) is displayed by continuous lines. The alternative impact of water turbidity is displayed by long-dashed lines. If supporting the GLH, a steeper NSC-elevation slope is predicted (black long-dashed line). In contrast, if supporting the CLH, higher NSC concentrations are anticipated to occur at lower elevations (with higher temperatures) (gray long-dashed line) (see text for further details on the expectations).

with elevation; and (2) besides temperature, how do water turbidity alter the C balance in plants in response to an elevational gradient? The genus *Myriophyllum* species were used because these species comprise submerged macrophytes that occupy an extensive range of habitats globally, including high-elevation regions (Cook, 1990; Jackbsen, 2008). The Qinghai-Tibetan Plateau, in China is a unique geographic unit that is subject to harsh environmental conditions (e.g., mean altitude over 4000 m; the average warmest month temperature is below 10°C in large areas). There are multiple shallow lakes, which are covered with aquatic macrophytes during the short growing season (5–6 months). This region provides an ideal platform for the investigation of the responses of plants along an elevational gradient. The genus *Myriophyllum*, primarily *Myriophyllum spicatum*, *Myriophyllum verticillatum*, and *Myriophyllum sibiricum*, represents one of the largest aquatic genera, and inhabits a broad range of habitats across the Qinghai-Tibetan Plateau (Aichner et al., 2010; Wang et al., 2015; Wu et al., 2016). Based on previous trends of NSC concentrations (in trees) with elevational gradients, we anticipated that if increasing elevation (decreased temperatures) reduced plant growth (C consumption) more than photosynthesis (C accumulation), the NSC will increase with elevation (acquisition > demand), supporting the GLH (**Figure 1**; decreasing continuous black line). Conversely, if photosynthesis becomes more limited than growth, in correspondence with increasing elevation, the NSC will decrease (acquisition < demand), which is supportive to the CLH (**Figure 1**; increasing continuous gray line). Moreover, water turbidity may limit C gains in submerged macrophytes (e.g., Lacoul and Freedman, 2006a; Puijalon et al., 2008; Huber et al., 2012); therefore, lower NSC concentrations may be associated with higher water turbidity (**Figure 1**, long-dashed lines). In addition, if temperature acts to limit plant more so than photosynthesis (GLH), a steeper NSC-elevation slope may be predicted, as water turbidity reduces the amount of light that is available for photosynthesis (**Figure 1**, black long-dashed line). In contrast, if temperature limits photosynthesis more than growth (CLH), a steeper NSC-elevation slope is also expected to occur in the opposite direction (**Figure 1**, gray long-dashed line), in that photosynthesis increases with temperature.

## MATERIALS AND METHODS

### Study Sites

In this study, we sampled 12 water bodies in the Qinghai-Tibetan Plateau, China, spanning a gradient of 1100 km, from east to west. The total elevation gradient spanned from between 2766 and 5111 m. As NSC levels are more stable at the end of the growing season (Madsen, 1997; Hoch and Körner, 2009), all samples were collected during July to August, 2012. The water bodies that we selected to study for *Myriophyllum* species were based on extensive field reconnaissance. We focused on near-pristine (no point sources of pollution, no obvious signs of human impact or grazing) water bodies. More information in regard to the investigation of these water bodies is provided in Supplementary Table 1.

### Field Sampling and Environmental Variables

At each water body, sampling points were established along a sampling line (200 m) every 30 m, where six individual plants (without leaf damage) were selected for sampling. From each sampled individual, upper shoot leaf tissues were collected from between 10:00 and 15:00 h. All plant samples were rinsed/cleaned with tap water, and then bagged, labeled, and sealed over silica gel in order to dry the samples.

To gain a better mechanistic understanding of the variation of NSC, we measured a number of physical characteristics, including conductivity ($\mu S\ cm^{-1}$), pH, salinity (%), total dissolved solids ($ml\ l^{-1}$), dissolved oxygen ($ml\ l^{-1}$), and turbidity (Nephelometric Turbidity Units, NTU) with a handheld multi-parameter meter (Proplus, YSI, Yellow Springs, OH, United States) at each sampling water body, from 10:00 to 15:00 h. At each designated water body, three water samples were extracted from a depth of 20 cm and initially filtered with a GF/F filter, and then employed to determine water chemical characteristics, including total nitrogen (TN, $ml\ l^{-1}$), total phosphorus (TP, $ml\ l^{-1}$), $NH_4^+$ ($ml\ l^{-1}$), and $NO_3^-$ ($ml\ l^{-1}$), using an ion chromatography system (ICS-1000, Dionex, Sunnyvale, CA, United States). We derived the growth season temperature (GST) of each water body by entering their geographic coordinates into equations from data collected at meteorological stations across China between the years of 1949 and 1999 (Fang et al., 2001; Wang et al., 2015). The GST is negatively correlated to the elevation (Supplementary Figure 1).

## Non-structural Carbohydrate Analysis

Prior to analysis, all leaf samples were dried to a constant weight at 80°C for 48 h and then ground into a fine powder. The NSC concentrations, including free low molecular weight soluble sugars (SS, including glucose, fructose, and sucrose) and starch, were analyzed using an Agilent 1290/6460 liquid chromatography system and tandem mass spectrometer (Agilent Technologies, Santa Clara, CA, United States) with a Waters XBridge$^{TM}$ BEH Amide 2.5 um 2.1 × 50 mm XP column (Waters, Milford, MA, United States). Approximately 20 mg of plant powder samples were extracted with 2 ml of ethanol (80%, v/v) at 80°C for 30 min., and then centrifuged (10,000 *g* for 10 min.). Subsequent to three extraction processes, the supernatant was utilized for the determination of SS, and the residue was dried with nitrogen for 24 h to dislodge any ethanol. The starch was initially hydrolyzed with diastase (Tokyo Chemical Industry, Japan) (60°C for 10 min.) and then analyzed using the same method as for the determination of SS. We added SS and starch concentrations to obtain NSC concentrations. All sugar and starch concentrations in these tissue samples were expressed as per unit of weight (mg $g^{-1}$).

## Statistical Analysis

To describe and quantify the environment of the individual water bodies, we used the nine characteristic variables (GST, pH, salinity, dissolved oxygen, turbidity, TN, TP, $NH_4^+$, $NO_3^-$) to create principal component analysis (PCA) that together explain nearly 87.35% of the variation present in the original nine variables (Supplementary Figure 2). The resulting components represent two combinations of the original environmental data, with the first one (Comp.1, 54.29%) mainly representing GST, dissolved oxygen, turbidity and total nitrogen, the second one (Comp.2, 33.06%) mainly representing GST, dissolved oxygen and turbidity. The *princomp* function in R was used for conducting the PCA analysis.

Regression tree analysis is well-suited for data that have complex ecological interactions among environmental variables, which forward selected variables (De'ath and Fabricius, 2000). Therefore, our data was initially divided into two water turbidity groups (high turbidity group vs. low turbidity group) using the regression tree, via the analysis of the untransformed NSC concentration data (high turbidity: > 3.97 NTU, six water bodies; low turbidity: < 3.97 NTU, six water bodies, Supplementary Figure 3). There were four water physical and chemical measurements from PCA1 (GST, dissolved oxygen, turbidity, and total nitrogen) that were employed for the regression tree analysis, which was conducted using the *rpart* and *partykit* packages in R. All of the experimental data were then transformed using log(x) or sqrt(x) functions to meet the assumptions of homogeneity in variance and normality.

To study whether the NSC concentration trends of *Myriophyllum* species increased or decreased with elevation, we used the model II regression to fit a linear relationship between the individual NSC concentration and elevational gradients in the high turbidity, and low turbidity groups, respectively (*lmodel2* R package) (Fajardo et al., 2011). However, in the high turbidity group, we found that the slopes of model II regressions did not differ from 0 ($P > 0.05$). We subsequently employed a linear mixed effect model (lme) to compare the mean values between sites, using the GST and turbidity as fixed factors, and the water bodies as the random factors (*nlme* R package). All statistical analyses were performed with R version 3.2.0[1].

## RESULTS

Overall, across the three *Myriophyllum* species, NSC concentrations in the leaves were observed to decrease significantly with elevation (**Figure 2A**). In most sampled water bodies, starch was the primary component of leaf NSC (mean 61.7%, **Figure 2**). Leaf starch concentrations also decreased with elevation (**Figure 2B**); however, leaf SS did not vary significantly with elevation ($F = 1.713$, $P = 0.144$) (**Figure 2C**). Among individual species, leaf NSC and starch concentrations of *M. spicatum* and *M. verticillatum* decreased with elevation (except for NSC concentrations in *M. verticillatum*, **Figures 2A,B** and Supplementary Table 2). For *M. sibiricum*; however, NSC and SS concentrations increased significantly with elevation, although there were only two water bodies that contained this species (**Figures 2A,C** and Supplementary Table 2).

With the water bodies classified into two water turbidity groups by regression tree analysis, the leaf NSC and starch concentrations were found to decrease with higher elevations in the low turbidity group (<3.97 NTU); however, in the high turbidity group (>3.97 NTU), no such significant trend was observed (**Table 1**). In contrast, SS concentrations did not reveal a significant association with elevation, in either the low turbidity or high turbidity groups. Similar trends occurred with GST (**Table 1**). For water bodies in the low turbidity group, both NSC and starch concentrations increased significantly with GST (**Figures 3A,B**); however, the relationship between the SS concentration and GST was not significant in the high turbidity group (**Figure 3C**). Nevertheless, for water bodies with high turbidity, the starch concentrations decreased with GST, the SS concentrations increased with GST (**Figures 3D–F**). On average, plants from the low turbidity group had higher NSC and starch concentrations than those from the high turbidity group (**Figure 3**).

The GST and water turbidity comprised the two critical factors that affected the leaf NSC and starch concentrations across all elevations (Supplementary Figure 4 and Supplementary Table 3). However, the interaction between GST and turbidity was only significant in the starch concentrations (Supplementary Table 3). The NSC and starch concentrations were positively correlated with temperature, but negatively correlated with the water turbidity (Supplementary Figure 4).

## DISCUSSION

The majority of our results found support for the CLH, rather than the GLH. Among the three *Myriophyllum* species, our data

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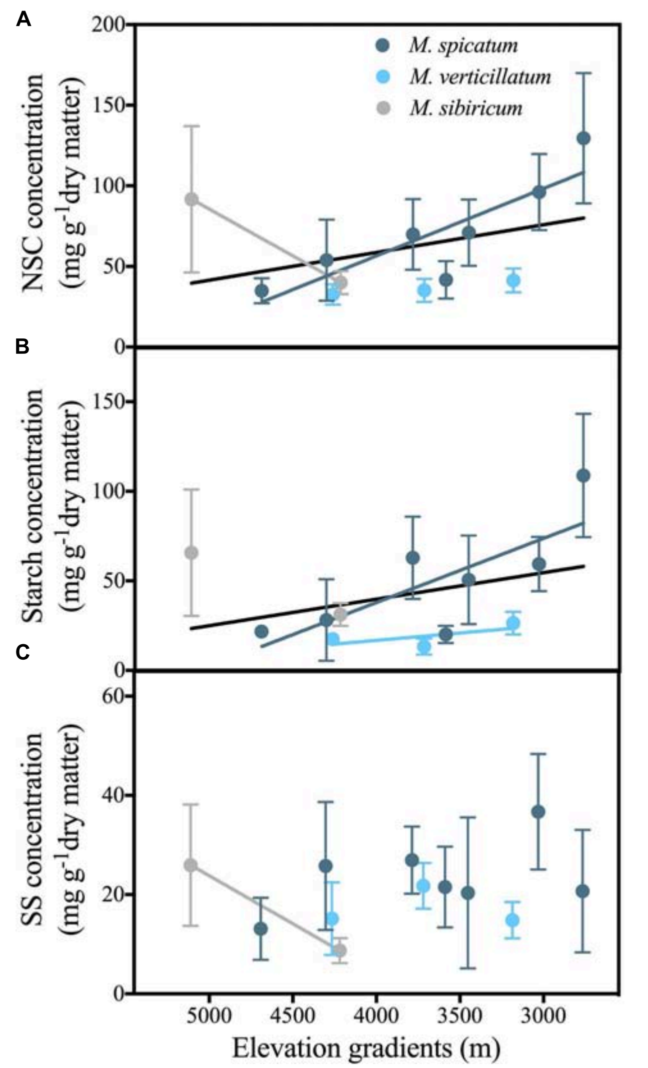


**FIGURE 2 |** Relationships of the non-structural carbohydrate (NSC) **(A)**, starch **(B)**, and SS (soluble sugars) of leaves **(C)** in response to elevation for three *Myriophyllum* species (*M. spicatum*, *M. verticillatum* and *M. sibiricum*) combined at the Qinghai-Tibetan Plateau, China. Black lines represent the combined regression lines of three *Myriophyllum* species. Only the significant relationships are presented. Please see Appendix S2 for statistical details. The data are presented with a mean ± 2 standard error. NSC = SS + starch.

revealed that leaf NSC concentrations decreased with elevation, although NSC concentrations in *M. sibiricum* demonstrated an increased trend with elevation (albeit there were only two water bodies involved). In contrast to our results, several previous studies from tree species reported an increasing trend of plant tissue NSC concentrations with elevation (e.g., Fajardo et al., 2012; Hoch, 2015). According to the GLH, the growth of trees is initially limited by the decreased temperatures that are inherent to higher elevations, particularly in the treeline area (Körner, 1998), because cell division and expansion are more sensitive to low temperature than photosynthesis (Palacio et al., 2014). However, aquatic environments involve more complexity than terrestrial environments (Lacoul and Freedman, 2005), in that they require additional heat to alter the water temperature, in comparison to changing the air temperature. Thus, terrestrial environments have much greater daily/seasonal changes in temperature than aquatic environments; hence, terrestrial organisms (e.g., trees) must have the capacity to tolerate a wider range of temperature ranges.

Although increased temperature has been observed to enhance the growth of several types of submerged macrophytes, this phenomenon is more significant in clear and deep lakes (Rooney and Kalff, 2000). However, in shallow/highly eutrophic lakes, the increased growth of submerged macrophytes under elevated water temperatures would be restrained due to high levels of periphyton/phytoplankton shading, unless this light-limitation is somehow overcome (Cao et al., 2014; Dalinsky et al., 2014). Additionally, submerged macrophyte responses to water temperature are also species-specific. For instance, Patrick et al. (2012) suggested that water temperature elevation, as the result of climate change, extends the growing season for *M. spicatum*, but not for *M. sibiricum*. They also found strong interactions between the *Myriophyllum* species and zooplankton abundance, which indirectly influenced shading effects due to periphyton abundance under warming water conditions (Patrick et al., 2012). All of these studies suggested that, aside from water temperature, subsurface light conditions may modify the growth of submerged macrophytes.

Our results also revealed that the turbidity of each sampled water body was the most significant factor that affected leaf NSC concentrations in all sampled *Myriophyllum* species, which also altered the relationship between NSC storage in response to GST. It is known that, at least in lowland areas, light availability (primarily influenced by periphyton/phytoplankton abundance) in the water column, controls the species richness and abundance of submerged macrophytes (Rooney and Kalff, 2000; Lauridsen et al., 2015).

In high-elevation lakes, however, water transparency was associated with suspended solids, not periphyton/phytoplankton abundance, due to low nutrient concentrations (Lacoul and Freedman, 2005). Lacoul and Freedman (2005) studied 34 lakes in the Himalayan Mountains, Nepal, and found that the lakes of the high Himalayan Mountain region (from 4200 to 5600 m in elevation) possessed much higher clarity than those at lower elevations. They found that the Secchi depth of most lakes in the high mountain (from 2900 to 3600 m in elevation) was less than 2.5 m (Lacoul and Freedman, 2005). We also found that the water bodies at above 4500 m had increased transparency, although we employed a different indicator (i.e., water turbidity). In our study, the water clarity tendency was matched with the NSC variation tendency.

Several studies on aquatic plants supported our results, and revealed that high light availability had a significant effect on the accumulation of NSC in shoots, which was positively correlated with increases in biomass (e.g., Huber et al., 2012). Indeed, at high-elevations, such as in the Hymalayan region, high-transparency aqueous habitats are highly suitable for the growth of submerged macrophytes (Lacoul and Freedman, 2006b). In addition, previous *in situ* experiments revealed that the

**TABLE 1 |** Equations and $R^2$ and *P* values for non-structural carbohydrate (NSC), starch and soluble sugar (SS) concentrations in relation to elevation (m) and growth season temperature (GST, °C) for the *Myriophyllum* species. Model II linear equations were introduced to fit the data.

| Variables | Source | Equation | $r^2$ | *P* (1-tailed) | *n* |
|---|---|---|---|---|---|
| NSC | Low turbidity | $\log NSC = -6.28 \times 10^{-5} \times elevation + 0.487$ | 0.18 | $4.51 \times 10^{-3}$ | 36 |
| | High turbidity | $\log NSC = -9.41 \times 10^{-5} \times elevation + 0.581$ | 0.01 | 0.32 | 36 |
| Starch | Low turbidity | $\log Starch = -3.34 \times 10^{-4} \times elevation + 2.847$ | 0.20 | $2.97 \times 10^{-3}$ | 36 |
| | High turbidity | $\log Starch = 5.21 \times 10^{-4} \times elevation - 0.879$ | 0.05 | 0.09 | 36 |
| SS | Low turbidity | $sqrtSS = -1.81 \times 10^{-3} \times elevation + 11.118$ | <0.01 | 0.34 | 36 |
| | High turbidity | $sqrtSS = -3.27 \times 10^{-3} \times elevation + 17.538$ | 0.09 | 0.04 | 36 |
| NSC | Low turbidity | $\log NSC = 1.16 \times 10^{-2} \times GST + 0.129$ | 0.45 | $3.62 \times 10^{-6}$ | 36 |
| | High turbidity | $\log NSC = 1.45 \times 10^{-2} \times GST + 0.070$ | 0.01 | 0.30 | 36 |
| Starch | Low turbidity | $\log Starch = 0.06 \times GST + 0.943$ | 0.40 | $2.00 \times 10^{-5}$ | 36 |
| | High turbidity | $\log Starch = -0.08 \times GST + 2.145$ | 0.10 | 0.03 | 36 |
| SS | Low turbidity | $sqrtSS = 0.33 \times GST + 0.781$ | 0.05 | 0.09 | 36 |
| | High turbidity | $sqrtSS = 0.51 \times GST - 0.242$ | 0.20 | $3.07 \times 10^{-3}$ | 36 |

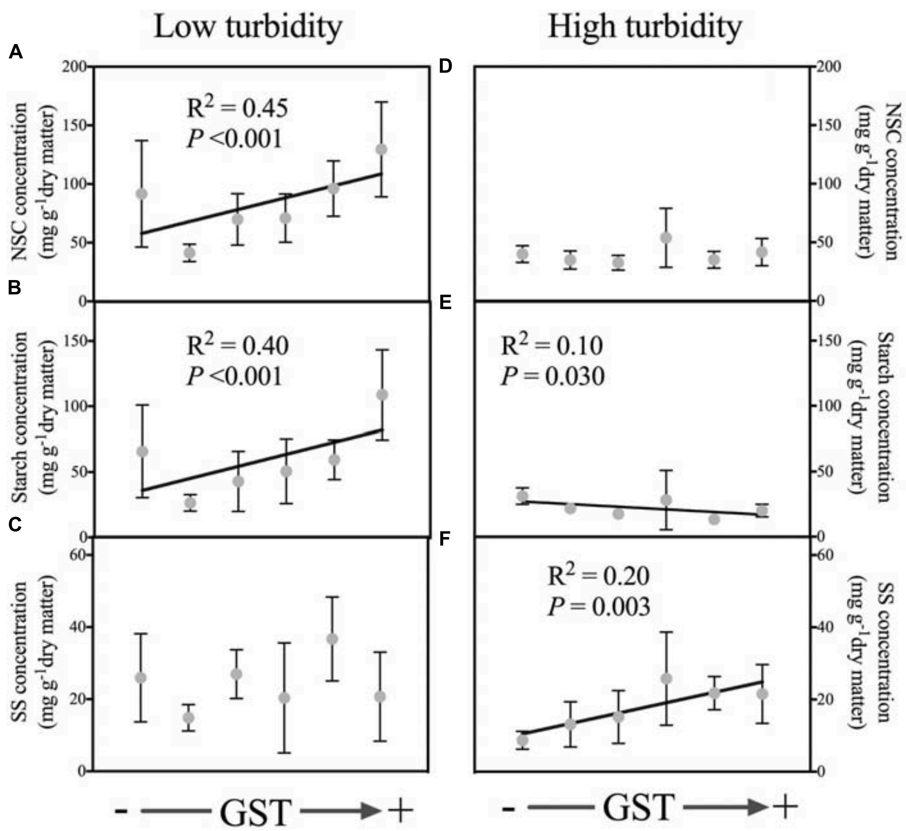


**FIGURE 3 |** Relationships of the non-structural carbohydrate (NSC), starch and SS (soluble sugars) of leaves to mean annual temperature (GST, °C) in low turbidity (<3.97 NTU, **A–C**, including five water bodies) and high turbidity (>3.97 NTU, **D–F**, including seven water bodies) sampled water bodies, respectively. In low turbidity water bodies, the GST ranged from 4.0 to 16.3°C; In high turbidity water bodies, the GST ranged from 5.3 to 12.7°C. The data are presented with a mean ± 2 standard error. Only the significant relationships are presented. Please see **Table 1** for statistical details.

relative growth rates (RGR) in submerged macrophytes were not significantly affected by temperature; however, photosynthetic rates were positively correlated to temperature and were similar between environments with comparable light availability (e.g., Riis et al., 2012). These data highlighted that additional NSC, and no increase in RGR, may be expected under high-temperature conditions, due to the increase of photosynthesis. Furthermore, similar trends of starch concentration in both low and high turbidities were observed. Both slopes between starch concentrations and elevation/GST in low turbidity were higher than slopes from high turbidity, which supported our hypothesis that *Myriophyllum* species were carbon limited due to the photosynthesis limitation by high water turbidity. However, starch cannot be utilized directly by the plant and must be transformed to soluble sugar for utilization (Hajirezaei et al., 2003). The SS is a successful resource supply in maintaining plant growth rather than starch. Indeed, the SS concentration is related to tissue growth and may regulate hormone concentrations to modify the plant's morphology (Huber et al., 2012; Deng et al., 2013). In our study, the increased SS concentrations in high turbidity group may reflect the growth of leaves to avoid shading in high GST condition. However, SS concentrations were in high levels in low turbidity conditions, which may serve to response to immediate environmental changes (e.g., low light or low temperature) and sustain cell growth (Huber et al., 2012). Therefore, light availability, in conjunction with water temperature, determined the distribution and growth of submerged macrophytes (at least for the *Myriophyllum* species) in high-elevation regions.

Palacio et al. (2014) argued that NSC storage in annual herbaceous *Arabidopsis* plants is different from that of trees, in that when C becomes limiting in these annual plants, it is most likely that all stores within the plant are mobilized and consumed. We support the argument that at low elevations, the NSC storage in *Myriophyllum* species is employed for growth and respiration, and the NSC is maintained at a low level during the growing season (Madsen, 1997). However, most of the data available to date, on NSC in response to elevation is derived from the treeline ecotone, which is far different from aquatic habitats (e.g., no changes in light conditions). Our study raises a number of questions (e.g., is such NSC response trend consistent in other species and larger scale?) in terms of explaining the tradeoffs between storage and growth in submerged macrophytes under high-elevation environments.

Additionally, inorganic C limited photosynthesis is much more common in aquatic, rather than terrestrial plants (Pedersen et al., 2013). In aquatic habitats, free $CO_2$ constitutes only a small proportion of C resources in the water column, as the diffusion velocity of free $CO_2$ comprises only 1/10000 in the water column in contrast to the ambient atmosphere (James and Larkum, 1996). Plants growing in such environments often experience low dissolved inorganic C availability conditions, as photosynthesis reduces $CO_2$ concentrations to a very low level (Jones et al., 2002; Bornette and Puijalon, 2011).

All submerged macrophytes utilize dissolved $CO_2$ directly, unless there is a considerable fluctuation in the pH value of the water (Schippers et al., 2004). Many submerged macrophytes have evolved alternative capacities to obtain C (i.e., bicarbonate usage) in order to acclimatize to low dissolved $CO_2$ conditions (Schippers et al., 2004; Cavalli et al., 2012). In our study, although bicarbonate concentrations in water bodies were not tested (because it is very difficult to bring the water samples back to the lab), most of the pH values from the sampled water bodies exceeded 8.22 (except one pH value 7.4). At this pH value range, the quantity of dissolved $CO_2$ is very low, where bicarbonate is the dominant source of C for the *Myriophyllum* species. Lacoul and Freedman (2006b) also reported that the water bicarbonate concentration is one of the major influences (e.g., temperature, lake surface area, suspended solids, bicarbonate and dissolved phosphorus) related to aquatic plant distribution and species richness in the Himalayas; however, compared with water transparency, pH value and bicarbonate concentrations only significantly influence aquatic plant distribution and species richness over larger geographical gradients. These results highlighted that the distribution of these plants in this region is likely associated with photosynthesis, which is mainly influenced by water transparency characteristics, such as water turbidity or Secchi depth.

We also compared stoichiometric data between the Qinghai-Tibetan Plateau and the middle and lower reaches of the Yangtze River, and found that the elemental C concentrations in *M. spicatum* were lower in the first region (Qinghai-Tibetan Plateau: 344.7 mg $g^{-1}$; middle and lower reaches of the Yangtze River: 359.9 mg $g^{-1}$) (Xing et al., 2013; Wang et al., 2015). This result at least partly reflected that there was support for carbon limitation (i.e., photosynthesis is limited by low temperature/low light condition) as an explanation in *M. spicatum*, in terms of how this species responded to higher elevations, which indicated high physiological plasticity in submerged macrophytes under different environmental conditions (Santamaría, 2002; Bornette and Puijalon, 2011).

## CONCLUSION

The leaf NSC concentrations in *Myriophyllum* species decreased with elevation. We suggest that this trend in NSC is attributable to the physiological plasticity of submerged macrophytes in response to light availability (e.g., water turbidity in our study). Our results implied no support for the GLH (i.e., harsh environmental conditions restricted cell division and expansion). At least two environmental factors (i.e., temperature and light availability/water turbidity) induced photosynthesis decreases might explain the NSC responses for submerged macrophytes in response to elevation at the Qinghai-Tibetan Plateau. More data is required in terms of multiple species to research how the aquatic plants responds to a wide range of elevations. The results will help the accurate prediction of plant responses to current and future climate changes worldwide.

## AUTHOR CONTRIBUTIONS

DX and DY conceived the ideas, DX, ZWu, ZWa, and QW performed the field sampling and lab analysis, DX, ZWu, and HC analyzed the data and drafted the paper. All authors strongly contributed to writing the paper.

## FUNDING

This research was supported by the National Science and Technology Major Project of the Ministry of Science and Technology of China (2013FY112300) and the National Natural Science Foundation of China (31370382), a project funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD), a start-up fund of Nanjing Forestry University (GXL035), and Forestry Science and Technology Innovation and Extension Program of Jiangsu [LYKJ(2017)10].

## ACKNOWLEDGMENTS

We thank Dr. Keyan Xiao, Dr. Wenhua You, Dr. Ligong Wang, and Mr. Xuewei Liao for their laboratory/field assistance and helpful discussions. We also greatly appreciate two reviewers for valuable comments on an early version of the manuscript.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01129/full#supplementary-material

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 14 August 2018
doi: 10.3389/fpls.2018.01207

# Submerged Macrophytes Exhibit Different Phosphorus Stoichiometric Homeostasis

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Phosphorus (P) is a limiting element in many aquatic ecosystems. Excessive P input often leads to cyanobacterial bloom, thus triggering ecological imbalances and a series of environmental problems. Submerged macrophytes have a strong ability to absorb P and play important roles in maintaining aquatic ecosystem functions. However, the degree to which submerged macrophytes maintain their tissue P contents in various nutrient levels and the corresponding influencing factors are still not very clear. In this study, the stoichiometric characteristics and stoichiometric homeostasis of P in the aboveground and belowground parts of three submerged macrophytes, *Vallisneria natans* (Lour.) Hara, *Hydrilla verticillata* (L.f.) Royle, and *Ceratophyllum demersum* (L.), with great differences in growth forms, were studied under different growth times and nutrient levels via laboratory experiments. The results showed that the water conductivity, turbidity, and chlorophyll content increased significantly with the increasing nutrient levels. The variation of species, organ, growth time, and nutrient level could significantly affect the P contents of submerged macrophytes. Among these factors, the variance contribution rates caused by the differences of nutrient levels in water column were the highest at more than 50%. The P stoichiometric homeostasis index ($H_P$) in the belowground parts of the three submerged macrophytes was higher than that of the aboveground parts. The $H_P$ decreased by the growth time; the $H_P$ of *V. natans* was significantly higher than those of *H. verticillata* and *C. demersum*. In summary, the P stoichiometric homeostasis in submerged macrophytes could reflect their responses to environmental changes, and the P content of submerged macrophytes was an indicator of the bioavailability of external P. *H. verticillata* exhibited a high growth rate and a high accumulation of P content, making it the most suitable species in this study for removing large amounts of P from water in a short term.

**Keywords: submersed macrophyte, phosphorus, ecological stoichiometry, interspecific difference, nutrient**

OPEN ACCESS

**Edited by:**
*Te Cao,*
*Institute of Hydrobiology (CAS), China*

**Reviewed by:**
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*Huazhong Agricultural University, China*
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**Specialty section:**
*This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science*

**Received:** *15 April 2018*
**Accepted:** *27 July 2018*
**Published:** *14 August 2018*

**Citation:**
*Li W, Li Y, Zhong J, Fu H, Tu J and Fan H (2018) Submerged Macrophytes Exhibit Different Phosphorus Stoichiometric Homeostasis. Front. Plant Sci. 9:1207. doi: 10.3389/fpls.2018.01207*

## INTRODUCTION

Ecological stoichiometric homeostasis refers to the ability of organisms to maintain the stability of their own element contents and ratios in a changing environment (Elser et al., 2000; Sterner and Elser, 2002; Yu et al., 2010; Yu et al., 2011). It is a basic theory of ecological stoichiometry and reflects the response of physiological and biochemical allocations within the organisms to the external environment (Yu et al., 2011; Leal et al., 2017). Yu et al. (2010) demonstrated that the stoichiometric homeostasis of Inner Mongolia grassland plants was positively correlated with its dominance and productivity in ecosystems, and plant communities with a high level of homeostasis also had a highly stable structure and function. The study by Gu et al. (2017) also showed that the phosphorus (P) and nitrogen (N): P stoichiometric homeostasis indices ($H_P$'s) of tundra plants were positively correlated with the biomass of their aboveground parts.

Submerged macrophytes are important primary producers in aquatic ecosystems and they play an important role in maintaining the biological diversity and functional stability of aquatic ecosystems (Carpenter and Lodge, 1986; Scheffer et al., 1992). Their habitat structure, reproductive system, and gene flow are quite different from those of terrestrial plants (Barrett et al., 1993; Van Zuidam and Peeters, 2015). Generally, morphological plasticity is important for submerged macrophytes to adapt to a changing water environment (Barrett et al., 1993; Strand and Weisner, 2001). Submerged macrophytes can adapt to fluctuations of the water level or low-light stress by changing their stem length, number of branches, aboveground and belowground biomass allocation, and leaf area index (Strand and Weisner, 2001; Zhu et al., 2012; Yuan et al., 2016). Morphological plasticity is closely related to the ecological stoichiometric characteristics and stoichiometric homeostasis of submerged macrophytes (Li et al., 2015; Leal et al., 2017). The ability of submerged macrophytes to adjust their own chemical elements and the proportions of the elements is of great importance for adapting to the changing environments (Sistla and Schimel, 2012).

P is a limiting element in many aquatic ecosystems, and excessive P input leads to cyanobacterial bloom, thus triggering ecological imbalances and a series of environmental problems (Elser and Bennett, 2011; Tong et al., 2017). Submerged macrophytes have a strong ability to absorb P (Zhang et al., 2011; Christiansen et al., 2016). Previous field investigations of Güsewell and Koerselman (2002), Su et al. (2016), and our group (Li et al., 2013, 2017) showed that the P content of aquatic plants was more affected by the nutrient levels in the environment than by interspecific variations, suggesting that the P content of aquatic plants may be closely related to plant survival strategies and environmental adaptability. In addition, according to the growth rate hypothesis (GRH) in the theory of ecological stoichiometry (Elser et al., 2000; Sterner and Elser, 2002), organisms with a high growth rate have a high P content and low carbon (C): P and N: P ratios in their tissues, because the P content of ribosomal RNA is high and ribosomal RNA is closely related to the growth rate. The GRH has been verified in many studies (Lovelock et al., 2007; Yu et al., 2012; Xing et al., 2016). However, the degree to which submerged macrophytes maintain their tissue P contents in various nutrient levels (P stoichiometric homeostasis) and the corresponding influencing factors are still not very clear.

In this study, the stoichiometric characteristics and homeostasis of P in the aboveground and belowground parts of three submerged macrophytes with great differences in growth form were studied under different growth times and nutrient levels via laboratory experiments. The following questions were explored: (1) the characteristics of the P stoichiometric homeostasis of submerged macrophytes and the corresponding interspecific difference; and (2) the main factors affecting the P stoichiometric homeostasis of submerged macrophytes. This study is of great significance for understanding the P stoichiometric characteristics in submerged macrophytes, which provides a scientific basis for screening the pioneer species in the ecological restoration of polluted water.

## MATERIALS AND METHODS

### Species Selection and Pretreatment

*Vallisneria natans* (Lour.) Hara, *Hydrilla verticillata* (L. f.) Royle and *Ceratophyllum demersum* (L.), which are very common in the waters of China, were the studied macrophyte species. The growth forms and biomass allocation strategies of these three submerged macrophytes are very different. *C. demersum* is a canopy-type submerged macrophyte, which can concentrate most of its biomass on the surface of the water to obtain maximum light. Because *C. demersum* does not have roots, it relies on the lower part of the stems and leaves buried in the sediment to attach the macrophyte to the substrate. Asexual reproduction often occurs by breaking off stems (Best, 1980; Gross et al., 2003). *V. natans* is a rosette-type submerged macrophyte, with long striped leaves, well-developed roots, and short upright stems (Xie et al., 2005; Fu et al., 2012). *H. verticillata* is an erect-type submerged macrophyte, whose biomass allocations to leaves, stems, and roots are relatively even (Langeland, 1996). The biomass ratios of the aboveground and belowground parts of these three macrophytes are in the order of *C. demersum* > *H. verticillata* > *V. natans*, and the macrophytes may have different nutrient absorption and metabolism strategies. Although different in many ways, the three studied species often coexist in many submerged macrophyte communities.

The three studied species of submerged macrophytes were all collected in Poyang Lake. *C. demersum* and *H. verticillata* were grown by apical shoots, and *V. natans* was grown by seedlings with roots according their growth characteristics. To get a uniform initial growth condition, the apical shoots or seedlings were selected to have the same size, with normal growth, and no branches. The length of the apical shoot was 15 cm, and the macrophyte height and root length of the *V. natans* seedlings were 12 and 3 cm, respectively. In order to simulate natural growth condition, all the three macrophyte species are grown together.

### Experimental Design

This experiment was carried out in 12 medium-sized glass tanks (0.6 m × 0.5 m × 1.0 m) at the Poyang Lake Model Test Research Base (115° 50′ 14.98″ N, 29° 13′ 19.57″ E). The glass tanks were put in a large greenhouse (180 m in length, 110 m in width, and 21 m in height) whose roof was made of steel frame with large panes of glass, which was very helpful to control the light intensity.

Ceramic sands with diameter of 1 ± 0.5 mm were bought and used as the substrate to fix the submerged macrophytes in this study. The pretreated apical shoots or seedlings of the three selected submerged macrophytes were planted in plastic square trays (19.5 cm × 13.5 cm × 5 cm) containing a 4-cm thick layer of substrate. Each tray was used to plant four individuals of one species, and each glass tank contained six trays, consisting of two trays each of the three species. These submerged macrophytes were subjected to the experimental treatment after 7 days of adaptive growth. Air aerated tap water was added to the glass tank to a depth of 0.8 m and a total volume of 240 L. The concentrations of total dissolved N and total dissolved P in the tap water were 0.02 ± 0.01 and 0.01 ± 0.00 mg/L, respectively. Different amounts of ammonium sulfate solution and monopotassium phosphate solution were added to each glass tank, and each treatment was performed in triplicate. The nutrient enrichment treatments were CK, for which no extra nutrient was added into the glass tank, T1, for which 48 mg N and 24 mg P were added, making 0.2 mg/L N content and 0.1 mg/L P contents in the water column in the beginning of the experiment, T2, for which 96 mg N and 48 mg P were added, making 0.4 mg/L N content and 0.2 mg/L P contents in the water column in the beginning of the experiment, and T3, for which 192 mg N and 96 mg P were added, making 0.8 mg/L N content and 0.4 mg/L P contents in the water column in the beginning of the experiment. The nutrient solutions were added once a week and the physicochemical indicators of water were determined. Tap water was added into the glass tanks to maintain all of them 240 L of water.

## Sample Processing and Parameter Measuring

The experiment was carried out for 40 days, and macrophyte sampling was performed on the 20th and 40th days, respectively. On macrophyte sampling days, one tray of each species in each glass tank was taken out and all macrophyte biomass in the tray was divided into aboveground and belowground parts. The samples were washed carefully with deionized water and processed immediately. They were repeatedly dried with water-absorbing paper until no water dropped by hard shaking. The fresh weight of the submerged macrophytes was determined by electronic balance. After weighing, the macrophyte samples were desiccated at 105°C for 1 h then dried at 70°C to constant weight, and ground to a uniform fine powder. The P content of the macrophyte was determined using a sulfuric acid-hydrogen peroxide digestion and ammonium molybdate – antimony potassium tartrate – ascorbic acid spectrophotometric method (Kuo, 1996).

During the experiment, water temperature (T), dissolved oxygen (DO), conductivity (COND), total dissolved solids (TDS), oxidation-reduction potential (ORP), and chlorophyll content (Chl) were measured using a handheld multi-parameter water quality meter (HQ40D, Hach Inc., United States), and total dissolved N (TDN) and total dissolved P (TDP) were measured using a standard method (Huang, 2000).

## Statistical Analysis

The relative growth rate (RGR) of the submerged macrophytes was calculated using the equation RGR = $\ln(M_2/M_1)/\mathrm{d}t$, where $M_1$ is the initial fresh weight of the submerged macrophyte, $M_2$ is the fresh weight of the submerged macrophyte after sampling, and d$t$ is the growth days of the submerged macrophyte.

According to the principle of ecological stoichiometry (Sterner and Elser, 2002), the stoichiometric homeostasis of an element in a plant refers to the capacity of the element to be stable in a changing environment. It can be expressed by the stoichiometric homeostasis index $H$ calculated by the formula $y = cx^{1/H}$, where $y$ is the content of an element or the element stoichiometric ratio in the plant, $x$ is the content of the element or the stoichiometric ratio in the external environment, and $c$ is a constant. The formula can be converted to $\log y = \log c + 1/H \log x$, and $H$ can be obtained according to the regression relationship between log $x$ and log y. According to Persson et al. (2010), the stoichiometric homeostasis of a species can be classified as follows: plastic ($0 < H < 1.33$), weakly plastic ($1.33 < 1/H < 2$), weakly homeostatic ($2 < H < 4$), and homeostatic ($H > 4$).

Data processing, analysis and plotting were completed using SPSS software (SPSS V16.0, SPSS Inc., Chicago, IL, United States). The differences between treatments on the physicochemical parameters, macrophyte morphologies and physiological parameters were analyzed by the one-way analysis of variance (ANOVA) method. The effects of growth parts (aboveground parts, belowground parts), growth length (20 days, 40 days), and nutrient levels (CK, T1, T2, and T3) on P contents of submerged macrophytes were analyzed by three-way ANOVA.

We used variance partitioning based on the sum of squares (SS) of three-way ANOVA with growth parts (P), growth length (L), and nutrient levels (N) as factors to indicate their contribution to the variance in the P concentrations (Güsewell and Koerselman, 2002; Li et al., 2013). The total SS of the ANOVA was decomposed as: $SS_{total} = SS_P + SS_T + SS_N + SS_{Error}$. Variance contribution of each factor was then expressed as percentage of total SS (SS%).

# RESULTS

## Characteristics of Water Quality Parameters

During the experiment, the temperature of the water body was about 32°C. In the treatment groups (T1–T3), the increasing level of nutrients led to different degrees of growth of the algae in the water column, thus causing an increase in chlorophyll content. The DO in the water column was in a supersaturated state (over 100%). With the increasing amount of added nutrients, TN, TP, COND, TDS, DO, and Chl contents

**TABLE 1 |** Physic-chemical characteristics of the water at different treatments.

| | CK | T1 | T2 | T3 |
|---|---|---|---|---|
| T(°C) | 31.53(0.14)[a] | 31.67(0.30)[a] | 31.60(0.11)[a] | 31.79(0.22)[a] |
| COND (μS/cm) | 96.30(1.02)[a] | 119.95(16.67)[b] | 133.63(19.14)[bc] | 146.53(12.43)[c] |
| TDS (mg/l) | 55.50(0.58)[a] | 69.25(9.54)[b] | 77.00(10.92)[bc] | 84.25(6.95)[c] |
| DO (% sat) | 121.70(2.89)[a] | 192.05(27.21)[b] | 209.45(16.21)[b] | 235.28(10.58)[c] |
| ORP (mV) | 95.48(2.13)[a] | 132.08(7.31)[b] | 139.23(10.35)[bc] | 145.33(4.72)[c] |
| Chl (μg/L) | 0.12(0.07)[a] | 33.39(28.74)[ab] | 51.10(23.65)[b] | 145.31(48.20)[c] |
| TDN (mg/L) | 0.068(0.023)[a] | 0.390(0.022)[b] | 0.748(0.025)[c] | 1.423(0.048)[d] |
| TDP (mg/L) | 0.029(0.004)[a] | 0.129(0.006)[b] | 0.232(0.008)[c] | 0.423(0.010)[d] |

*Values are shown as mean (SD). Different letters on the top right of the values indicate significant difference at the 0.05 level.*

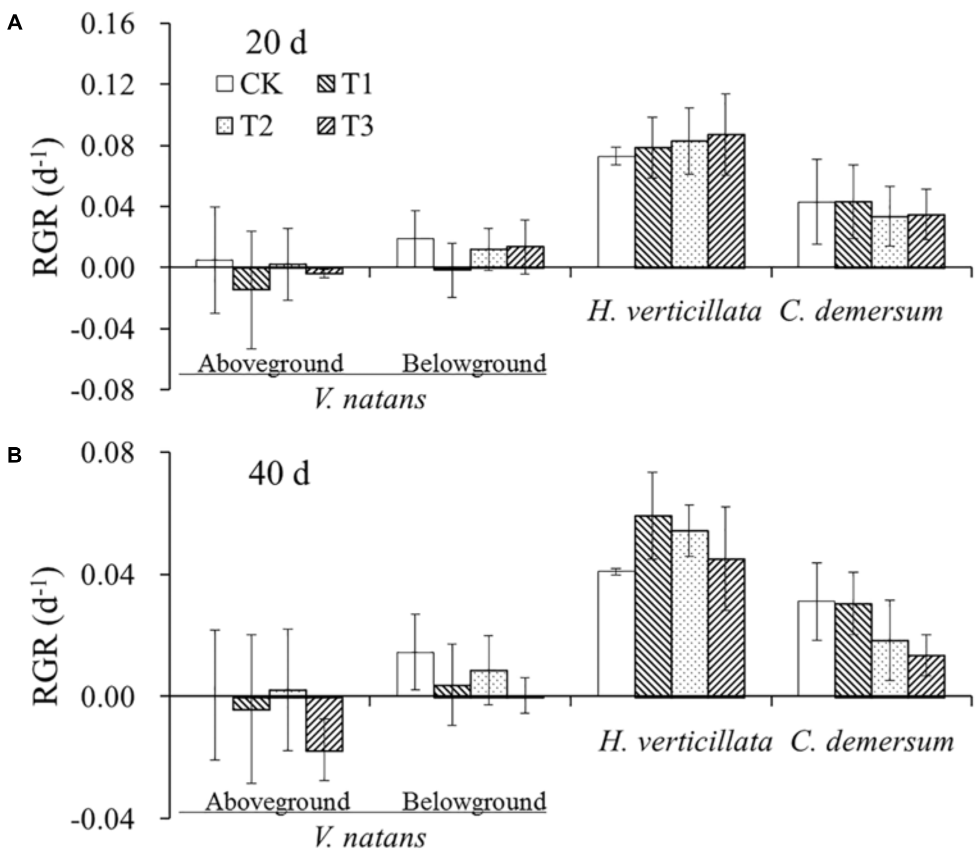


**FIGURE 1 |** The change of relative growth rates (RGR) of the three studied submerged macrophytes in **(A)** 20 days and **(B)** 40 days in response to different nutrient treatments.

increased significantly, while ORP showed an upward trend (**Table 1**).

## Growth Characteristics of Submerged Macrophytes

The average initial weights of the aboveground and belowground parts of *V. natans* were 0.79 ± 0.17 g and 0.23 ± 0.04 g, respectively. The growth of *V. natans* was slow, and the average growth rates were close to zero in 20 and 40 days. The initial weight of the aboveground part of *H. verticillata* was 0.32 ± 0.06 g. The average growth rates of the aboveground part were 0.08 ± 0.02 and 0.05 ± 0.01 $d^{-1}$, respectively, in 20 and 40 days. The initial weight of the aboveground part of *C. demersum* was 0.59 ± 0.19 g. The average growth rates of the aboveground part were 0.04 ± 0.02 and 0.02 ± 0.01 $d^{-1}$, respectively, in 20 and 40 days. The growth rate of *C. demersum* was faster than that of *V. natans* and slower than that of *H. verticillata*. There were also no significant differences in the growth rate of the aboveground and belowground parts among the three treatments and CK groups in the two growth stages for all the three species (**Figure 1**).

### P Contents and Stoichiometric Homeostasis of Submerged Macrophytes

The initial P content in the aboveground part of *V. natans* was 2.60 ± 0.25 mg/g, and the P contents in the aboveground part of the treatment groups and CK groups on the 20th day were higher than the initial levels. In the aboveground part on the 40th day, except for the CK groups whose P content was lower than the initial level, all of the treatment groups showed an increase in the P contents. The initial P content of the belowground part of *V. natans* was 2.41 ± 0.10 mg/g, the P contents of the CK groups on the 20th and 40th days were lower than the initial levels, but the P contents of the treatment groups were all higher than the initial levels. The P contents of the aboveground and belowground parts of *V. natans* increased significantly with the increasing P content in the water body (**Figure 2**).

The initial P content in the aboveground part of *H. verticillata* was 5.14 ± 0.20 mg/g, and the P contents in the CK and T1 groups decreased on the 20th and 40th days, while the P contents of the T2 and T3 groups increased. The P contents of the aboveground and belowground parts of *H. verticillata* increased significantly with the increasing P content in the water body (**Figure 2**).

The initial P content in the aboveground part of *C. demersum* was 4.63 ± 0.56 mg/g, the P contents of the CK and T1 groups on the 20th day were lower than the initial levels, and the P contents of the T2 and T3 groups were higher than the initial levels. The P content of the aboveground part of *C. demersum* was significantly increased with the increasing P content in the water body, and the P content of the belowground part increased slightly on the 20$^{th}$ day but increased substantially on the 40th day (**Figure 2**).

For the P stoichiometric homeostasis index ($H_P$), the values of the aboveground parts of the three submerged macrophytes were smaller than those of the belowground parts, the values on the 20th day were higher than those on the 40th day, the value of *V. natans* was higher than those of *H. verticillata* and *C. demersum*, and no significant difference ($p > 0.05$) was found in this value between *H. verticillata* and *C. demersum* (**Figure 3**). All of the aboveground parts of the three submerged macrophytes exhibited plastic or weakly plastic P stoichiometric homeostasis except for *V. natans*. However, all of the belowground parts of the three submerged macrophytes exhibited weakly homeostatic or homeostatic P stoichiometric homeostasis in 20 days growth (**Figure 3**).

### Factors Affecting P Contents in the Submerged Macrophytes

The organ and nutrient level had a significant effect on the P contents of *V. natans*, *H. verticillata*, and *C. demersum*, while the growth length only had a significant effect on the P content in *C. demersum* (**Table 2**). Among the three factors, the nutrient level had the greatest effect on the P content in the submerged macrophytes, and the variance contribution rate was over 50%. The organ had the second greatest impact on the P content, and the variance contribution rate was between 10 and 20%. The growth length had no significant effect on the P contents of *V. natans* and *H. verticillata*, but its variance contribution rate to the P content of *C. demersum* was 9.16%.

## DISCUSSION

In this study, the characteristics of P content in three submerged macrophytes with different growth forms, different growth rates, and different characteristics of biomass allocation were studied. The results showed that the responses of the three submerged macrophytes to the increasing nutrient levels in the water were consistent. That is, the P content for all of the macrophytes increased with the increasing nutrient contents in the water body. However, the three submerged macrophytes exhibited significant differences on the P stoichiometric homeostasis. The $H_P$ of *V. natans* was significantly higher than those of *H. verticillata* and *C. demersum*, and no significant difference was found between *H. verticillata* and *C. demersum*. The study by Xing et al. (2016) found that the N:$H_P$ of *V. natans* was significantly higher than those of *C. demersum* and *Myriophyllum spicatum*, suggesting that the high level of stoichiometric homeostasis might be an essential feature of *V. natans*. The study by Yu et al. (2010) on the vascular plants in the grassland of Inner Mongolia showed that the plants with a high level of stoichiometric homeostasis had a relatively high productivity and community stability. This is because plants with a high level of stoichiometric homeostasis are more conservative on nutrient absorption and utilization, so they are dominant in low-nutrient ecosystems. In the ecosystems with a high nutrient level, the submerged macrophytes are under high nutrient stress. The submerged macrophytes with a low level of stoichiometric homeostasis can absorb and store more nutrients, but consume more hydrocarbons at the same time (Cao et al., 2011; Yuan

**TABLE 2 |** Effects of growth parts (aboveground parts, belowground parts), growth length (20 days, 40 days), and nutrient levels (CK, T1, T2, and T3) on P contents of submerged macrophytes.

| Source | SS | df | F | Sig. | SS% |
|---|---|---|---|---|---|
| *V. natans* | | | | | |
| Growth part | 19.67 | 1 | 15.53 | 0.000 | 10.09 |
| Growth length | 3.16 | 1 | 2.50 | 0.120 | 1.62 |
| Nutrient level | 98.67 | 3 | 25.97 | 0.000 | 50.61 |
| Error | 73.45 | 58 | | | |
| *H. verticillata* | | | | | |
| Growth part | 128.03 | 1 | 54.23 | 0.000 | 17.09 |
| Growth length | 0.00 | 1 | 0.00 | 0.979 | 0.00 |
| Nutrient level | 484.06 | 3 | 68.34 | 0.000 | 64.62 |
| Error | 136.95 | 58 | | | |
| *C. demersum* | | | | | |
| Growth part | 165.97 | 1 | 50.73 | 0.000 | 13.70 |
| Growth length | 110.94 | 1 | 33.91 | 0.000 | 9.16 |
| Nutrient level | 744.86 | 3 | 75.90 | 0.000 | 61.48 |
| Error | 189.74 | 58 | | | |

*SS: Type III Sum of Squares; SS%: the relative variance rate; df: degree of freedom.*

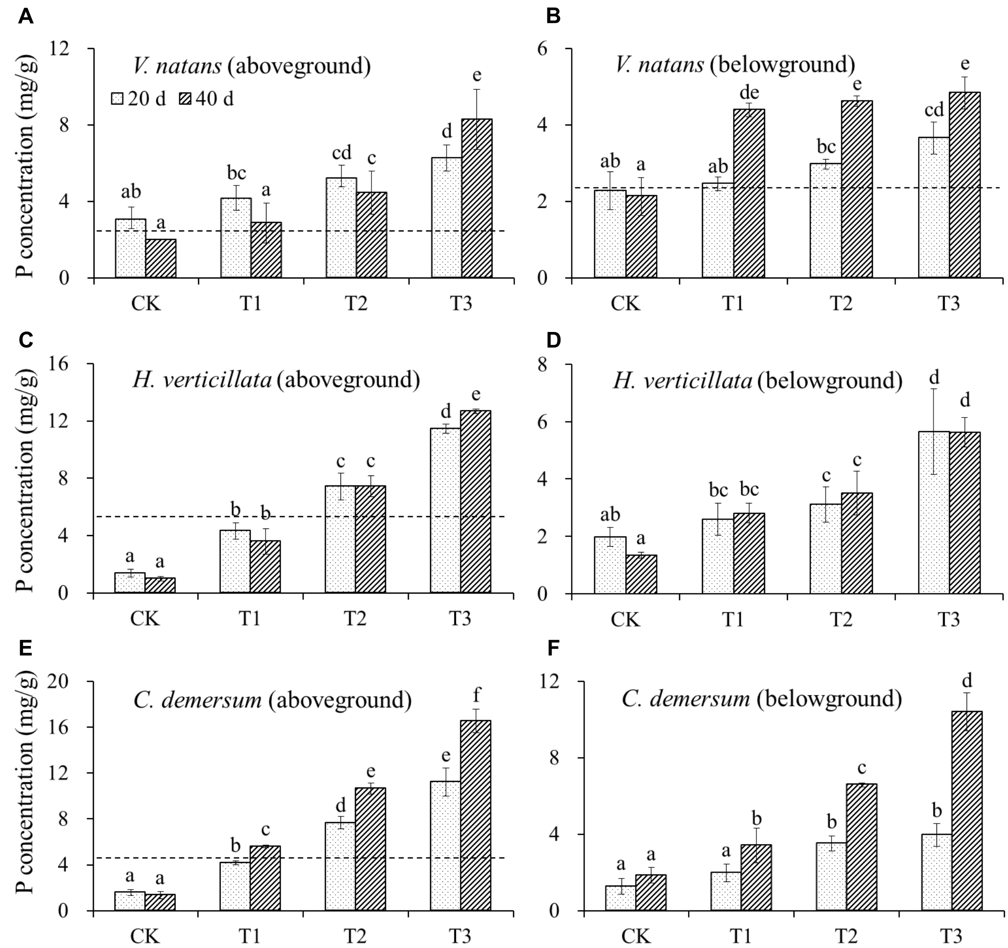


**FIGURE 2 |** The change of P contents in **(A)** aboveground of *V. natans*, **(B)** belowground of *V. natans*, **(C)** aboveground of *H. verticillata*, **(D)** belowground of *H. verticillata*, **(E)** aboveground of *C. demersum*, and **(F)** belowground of *C. demersum* in response of growth time and nutrient treatments. Dash lines represent initial P contents of the studied macrophytes before planted. Different letters indicate significant difference at the 0.05 level.

et al., 2013), which may be unfavorable for the reproduction of submerged macrophytes and resistance to environmental interference.

This study indicated that the species, organs, growth length, and nutrient level could significantly affect the P content of the submerged macrophytes, and the nutrient level had the greatest impact, followed by the organs, while the impact of species and growth length were relatively small. This finding confirmed the author's previous field study that the P content of submerged macrophytes was more affected by the environment than by interspecific variations (Li et al., 2017) and it was consistent with other wetland plants (Güsewell and Koerselman, 2002), indicating that the P content in the plants could effectively indicate the availability of P in the environment. Additionally, the results of this study showed that submerged macrophytes with higher P contents exhibited a lower level of P stoichiometric homeostasis, which indicated that the characteristics of P stoichiometric homeostasis of submerged macrophytes were consistent with that of terrestrial plants (Yu et al., 2011). There is a phenomenon of luxury consumption for submerged macrophytes in a high-P environment. Furthermore, the P contents and growth rates of *H. verticillata* and *C. demersum* were higher than that of *V. natans* in this study, suggesting that the P content of submerged macrophytes was consistent with the change in their growth rate, which is in accordance with the GRH.

The average water temperature in this study was over 30°C. Therefore, with the increasing nutrient content in water body, the growth rate of algae in the water body also increased, which was manifested by significant increases in the conductivity, turbidity, and chlorophyll content of the water. Submerged macrophytes grown in high levels of nutrients may be subjected to severe low-light stress, which might be one of the reasons why no significant differences were found in the biomass and growth rate of the submerged macrophytes among treatments. However, the P contents of the aboveground and belowground parts of the three submerged macrophytes increased with the increasing nutrient concentration in the water body, and the P content of *H. verticillata* and *C. demersum* in the T3 treatment group was seven- to eightfold higher than that of the CK group. The

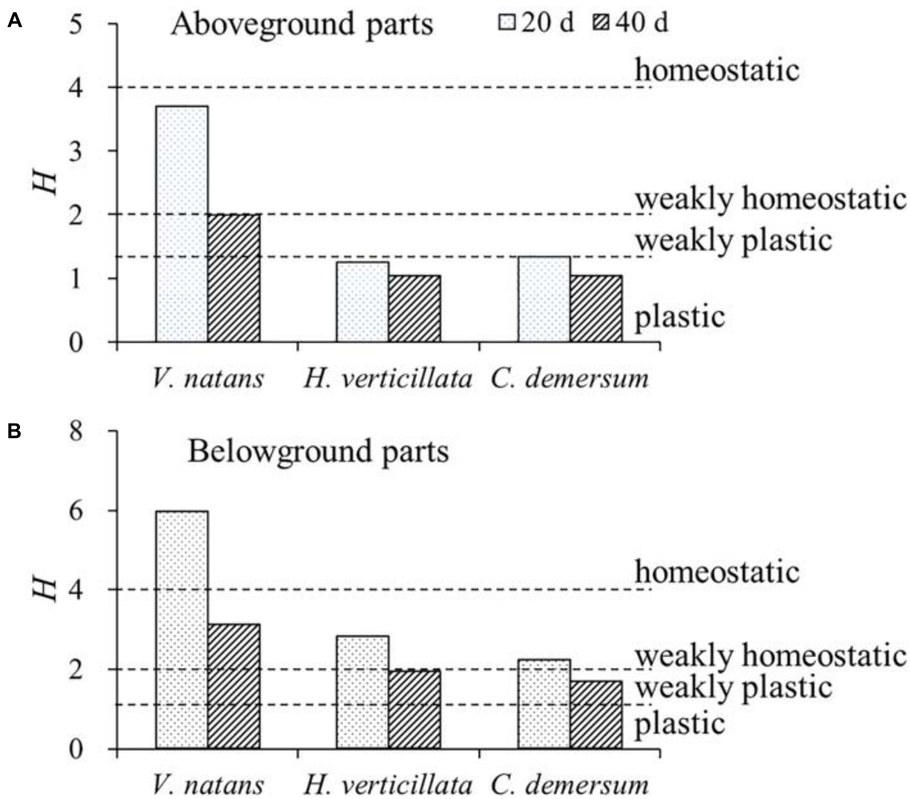


**FIGURE 3 |** Stoichiometric homeostasis index (*H*) of **(A)** aboveground and **(B)** belowground parts of the three submerged macrophytes in 20 and 40 days. The *H*-values were calculated from the equation of linear regression between LN-transformed phosphorus contents in water and in submerged macrophytes. Dash lines represent threshold values of *H* classified as follows: plastic ($0 < H < 1.33$), weakly plastic ($1.33 < 1/H < 2$), weakly homeostatic ($2 < H < 4$), and homeostatic ($H > 4$), according to Persson et al. (2010).

above results suggested that although the high nutrient stress affected the growth rate of the submerged macrophytes, it had a minor impact on P absorption by the submerged macrophytes, especially for *H. verticillata* and *C. demersum*. In addition, the results in our study which conducted in the laboratory are not very consistent with results in field conditions. For example, our previous field study (Li et al., 2017) indicated that P contents of water in Dianchi Lake were four times as much as those in Erhai Lake, while P contents of submerged macrophytes in Dianchi Lake were not significantly different from those in Erhai Lake. These differences may be mainly resulted from the length of growth time for submerged macrophytes. Greenhouse experiments often conducted in short period which often last from several hours to less than one year, while in field long-term observation the second-year growth of plants was usually affected by their nutrient storage in previous year (Güsewell, 2005; Li et al., 2015).

Submerged macrophytes can absorb the N and P not only in the sediment through their belowground parts, but also in the water body through their aboveground parts (Rattray et al., 1991). When the N and P concentrations in the water are higher than the requirement of the submerged macrophytes, they can absorb excessive N and P and store them in tissues (Xing et al., 2016). Therefore, the N and P contents of submerged macrophytes are highly plastic, reflecting the N and P nutritional status of the aquatic environment in which they are present, and they can serve as the quality indicators for the water environment. Previous studies (Li et al., 2015, 2017) showed that the variation coefficient of the P content in submerged macrophytes is greater than that of N, and that P is a limiting element for many water bodies. An increase in P content is one of the important reasons for cyanobacterial bloom (Tong et al., 2017). Therefore, monitoring the P content of submerged macrophytes is crucial for understanding the bioavailability of P. In this study, the levels of P stoichiometric homeostasis of *H. verticillata* and *C. demersum* were significantly lower than that of *V. natans*, so *H. verticillata* and *C. demersum* were ideal indicator organisms for the bioavailability of P in the water body. *H. verticillata* showed the highest growth rate among the three submerged macrophytes and a higher accumulation of P, which might be the most suitable species for removing P from the water body in a short period of time.

## AUTHOR CONTRIBUTIONS

WL, YL, and HF contributed to the conception and design of the study. WL, YL, JZ, HF, JT, and HbF performed the experiments and the statistical analysis. WL wrote the manuscript. All the authors contributed to the manuscript revision, read and approved the submitted version.

## FUNDING

This study was co-funded by the Science and Technology Project of Jiangxi Provincial Department of Education (GJJ171004), the Open Foundation of Jiangxi Engineering Research Center of Water Engineering Safety and Resources Efficient Utilization (OF201611), the Open Foundation of Chinese Ministry of Water Resources Research Center of Poyang Lake Water Resources and Water Environment (ZXKT201708 and ZXKT201511), the Science and Technology Program Project of Jiangxi province, China (20161BBG70048 and 20161BAB216109), and 2017 National Students' Platform for Innovation and Entrepreneurship Training Program (201711319006).

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
published: 21 September 2018
doi: 10.3389/fpls.2018.01398

# Spatial Patterns of Leaf Carbon, Nitrogen, and Phosphorus Stoichiometry of Aquatic Macrophytes in the Arid Zone of Northwestern China

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**Specialty section:**
*This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science*

**Received:** *16 March 2018*
**Accepted:** *03 September 2018*
**Published:** *21 September 2018*

**Citation:**
*Gong X, Xu Z, Lu W, Tian Y, Liu Y, Wang Z, Dai C, Zhao J and Li Z (2018) Spatial Patterns of Leaf Carbon, Nitrogen, and Phosphorus Stoichiometry of Aquatic Macrophytes in the Arid Zone of Northwestern China. Front. Plant Sci. 9:1398. doi: 10.3389/fpls.2018.01398*

Ecological stoichiometry is a powerful indicator for understanding the adaptation of plants to environment. However, understanding of stoichiometric characteristics of leaf carbon (C%), nitrogen (N%), and phosphorus (P%) for aquatic macrophytes remains limited. In this study, 707 samples from 146 sites were collected to study the variations in leaf C%, N%, and P%, and tried to explore how different environmental conditions affect leaf C, N, and P stoichiometry. Results showed that the mean values of leaf C%, N%, P%, and N:P ratios were 39.95%, 2.12%, 0.14%, and 16.60% of macrophytes across the arid zone of northwestern China, respectively. And the mean values of leaf P% were lower than those from the Tibetan Plateau and eastern China, which maybe due to an adaptation strategy of the plants to the unique conditions in the arid zone in the long-term evolutionary process. The higher N:P ratios suggested that P was established as the limiting factor of the macrophytes communities in the arid zone of northwestern China. There were significant differences in leaf C%, N%, P%, and their ratios among different life forms. Our results also showed strong relationships between leaf N% and N:P ratios and longitude, leaf N%, P%, and N:P ratios and latitude, and leaf N% and P% and altitude, respectively. In addition, the results showed that pH can significantly influence leaf C%. Our results supported the temperature-plant physiology hypothesis owing to a negative relationship between leaf N% and P% of macrophytes and mean annual temperature in the arid zone of northwestern China. The different patterns of leaf stoichiometry between the arid zone of northwestern China and eastern China indicated that there were different physiological and ecological adaptability of macrophytes to environmental gradients in different climatic zones.

**Keywords: aquatic macrophytes, arid zone, ecological stoichiometry, environmental factors, temperature-plant physiology hypothesis**

## INTRODUCTION

Ecological stoichiometry is a fundamental discipline that studies the balance between energy and various chemical elements in biological interactions and nutrient cycling in ecosystems (Elser et al., 2000, 2003, 2010). Such research not only helps to distinguish between different regional ecological stoichiometry patterns (Han et al., 2005), but also better determines the relationship between the pattern and environmental variables (Han et al., 2011). In addition, this research can help to characterize the biogeochemical cycles (Hedin, 2004; Reich and Oleksyn, 2004; Portillo-Estrada et al., 2013).

In the last twenty years, many studies are focused on large-scale plant leaf stoichiometry and demonstrated that leaf N% and P% increased with increasing latitude (Mc Groddy et al., 2004; Reich and Oleksyn, 2004; Han et al., 2005; Wang et al., 2011) and altitude (Körner, 1989). However, other studies indicated that the leaf N% and P% decreased with altitude (Soethe et al., 2008; Zhao et al., 2014). These inconsistencies suggest that many crucial research questions of the leaf stoichiometry patterns and determinants have not been thoroughly elucidated to date. Moreover, most studies of plant leaf stoichiometry have focused on terrestrial ecosystems, and considerably less attention has been devoted to freshwater systems. Until recently, only a small number of studies have examined regional geographical patterns of leaf stoichiometry in some freshwater macrophytes (Xia et al., 2014; Li et al., 2015; Wang et al., 2015), and the results of these studies indicated that variability in foliar N%, P%, and N:P stoichiometry across diverse habitats showed considerable differences (Xia et al., 2014; Li et al., 2015; Wang et al., 2015). These different results suggested that geographical patterns of leaf stoichiometry of macrophytes may change with regard to the spatial extent of the study and geographical location of the study area and highlighted that further studies are needed to understand geographical patterns of leaf stoichiometry at different spatial scales and study areas.

Leaf nutrient contents and stoichiometry are sensitive to such factors as spatial scale, habitats, and plant types (Li et al., 2010; Van de Waal et al., 2010; Wu et al., 2012). Previous studies showed that the leaf N:P ratio changed with soil age gradient (Reich and Oleksyn, 2004), precipitation, and temperature (He et al., 2006, 2008). Further studies showed that the main factors shaping leaf nutrient stoichiometry varied with spatial scale and study area (He et al., 2006, 2008). To date, many hypotheses have been proposed (Thompson et al., 1997; Tjoelker et al., 1999; Weih and Karlsson, 2001; Sterner and Elser, 2002; Elser et al., 2003). Among the hypotheses, Temperature-Biogeochemistry Hypothesis (TBH) and Temperature-Plant Physiology Hypothesis (TPPH) are generally accepted by biogeographers and ecologists. TBH proposed that lower temperatures lead to the low activity of microbes, which reduces soil N and P, resulting in the insufficient leaf N and P of plants (Aerts and Chapin, 1999). TPPH assumed that plants from habitat of low temperature and high latitude may have a higher N and P content in their leaves to counterbalance the depressed biochemical efficiency caused by low temperatures (Oleksyn et al., 1998; Weih and Karlsson, 2001). However, these studies have largely been conducted on terrestrial plants, hindering further examination of the mechanistic basis of large-scale patterns of leaf stoichiometry in freshwater systems (Su et al., 2016). Thus, further research on leaf nutrient contents and stoichiometry of aquatic macrophytes is necessary (Li et al., 2015).

There were three climate zones in China, including a humid monsoon climate zone in eastern China, a cold area of the Tibetan plateau, and an arid zone in northwestern China (Feng et al., 1989; Tang et al., 1992). Recently, the leaf stoichiometry pattern of aquatic macrophytes was studied in eastern China (Xia et al., 2014) and the Tibetan plateau (Wang et al., 2015), which indicated that the variability in foliar N%, P%, and N:P stoichiometry and the main factor affecting the leaf stoichiometry pattern in each climate zone showed considerable differences. In the arid zone of northwestern China, extreme aridity gradients exist over relatively short distances in both the west-east and north–south directions (Feng et al., 1989; Tang et al., 1992). Over such environmental transects, plants encounter a variety of microclimates differing in temperature, precipitation and vapor pressure gradients, each of which may influence the variation of plant nutrient stoichiometry. In this study, we collected 707 aquatic plant samples from 146 sampling sites across the arid area of northwestern China, including 92 species (56 species of emergent plants, 7 species of floating-leaved plants, 29 species of submerged plants) from 37 genera and 24 families. The leaf ecological stoichiometry patterns of aquatic macrophytes were analyzed to investigate the relationships between environmental factors and plant leaf element contents. Two major questions were stressed: (1) what is the leaf stoichiometry pattern of the aquatic macrophytes and their general causes in the arid zone of northwestern China? (2) what are the differences of leaf stoichiometry patterns of the aquatic macrophytes and their main environmental factors among the three climatic zones in China?

## MATERIALS AND METHODS

### Study Area

The arid zone (35°–49°N, 73°–106°E) is a land-locked region located in northwestern China (**Figure 1**) and is surrounded by the Qinghai-Tibet Plateau and many high mountains. The climate is generally water limited, and steppe biomes are prevalent. The annual rainfall in the arid zone is less than 250 mm, with certain areas receiving less than 100 mm annually, but the annual evaporative capability is above 2000 mm. The mean annual temperature is 2–6°C, with the maximum monthly mean temperature being above 28°C, and the minimum monthly mean temperature is below −16°C; and the daily temperature fluctuates significantly (Feng et al., 1989).

### Sampling and Measurements

The aquatic macrophyte collections were conducted in the arid zone from July to October in 2011. The field sites in this study covered almost entire the arid zone of northwestern China, which ranged from 36° to 55°N, 80° to 103°E, and the altitude varied from 79 m to 3,185 m above sea level (**Figure 1**). At each

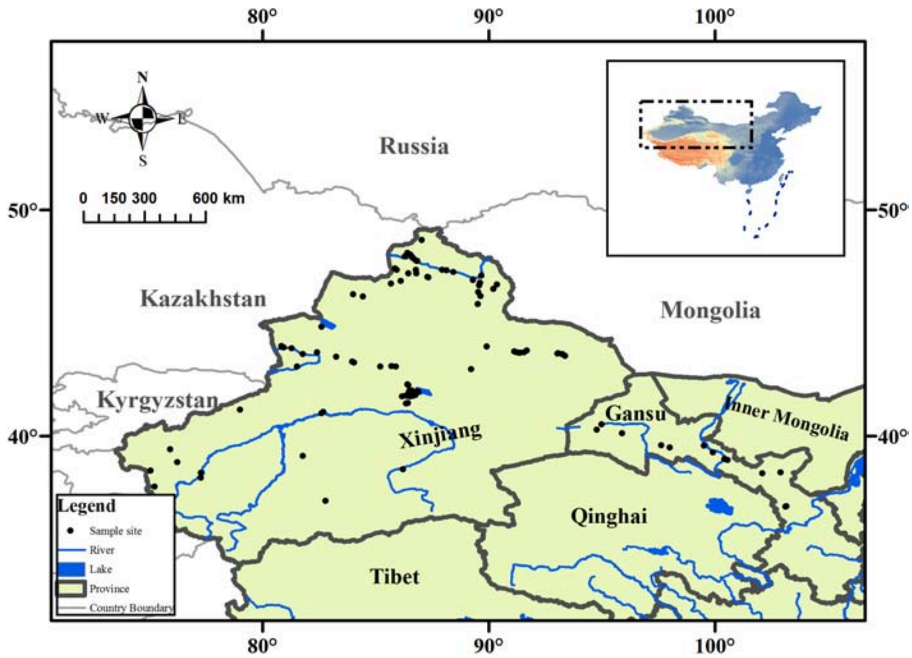


**FIGURE 1 |** Map showing the collection sites of aquatic plant, and its location in the arid zone of northwestern China.

sampling site, latitude, longitude, and altitude were recorded using the global positioning system (GPS). We also measured the pH of each water body using a portable pH meter. Mean annual temperature (MAT) and mean annual precipitation (MAP) were obtained by entering geographic coordinates into equations derived from data collected at meteorological stations across China (Fang et al., 2001). The model formula is given by

$$\mathrm{MMT(MP)} = \mathrm{a} \times \mathrm{Latitude} + \mathrm{b} \times \mathrm{Longitude} + \mathrm{c} \times \mathrm{Altitude} + \mathrm{d}$$

where a, b, c, and d are the regression coefficients.

According to estimated monthly mean temperature and monthly precipitation, MAT and MAP of each site were calculated.

Plants collected at each site were placed in paper envelopes and dried in the sun. These samples were dried to a constant mass at 60° for 72 h in the oven upon returning to the laboratory. All dried leaf samples of each plant were ground to a fine powder with a mortar in the laboratory. The total C and N contents were determined with an elemental analyser (NA2500, Carlo Erba Reagenti, Milan, Italy). The total P was measured using a sulfuric acid/hydrogen peroxide digest and the ammonium molybdate ascorbic acid method (Sparks et al., 1996). The detailed data is shown in **Appendix S1**.

## Data Analysis

SPSS Statistics 19 was used for statistical analysis. We calculated the mean and standard deviation (SD) of leaf C%, N%, P%, C:N, C:P, and N:P ratios for all species and each life form. Analysis of variance was applied to determine the statistical significance of the differences of leaf C%, N%, P%, C:N, C:P, and N:P ratios from different life forms ($p < 0.05$). Pearson correlations among the leaf C%, N%, P%, C:N, C:P, and N:P ratios were also calculated for all observations. Before performing one-way ANOVA, all data were tested for normality and homogeneity. Non-normal data were transformed (log10) to obtain normality. We used leaf C%, N%, P%, C:N, C:P, and N:P ratios to perform linear regressions with longitude, latitude and altitude. We included the effects of fixed (MAT, mean annual temperature; MAP, mean annual precipitation, pH and Life forms) and random (Species nested in Plot) on leaf C%, N%, P%, C:N, C:P, and N:P ratios to build linear mixed effect model. Linear mixed effect model was also made for each life forms. Redundancy analyses (RDA) were also performed with CANOCO for windows (version 5) to elucidate the relationship between plant C:N:P signatures and the environmental parameters (**Appendix S2**).

# RESULTS

## Pattern and Variation of Leaf C%, N%, P% Contents and Their Ratios in the Arid Zone of Northwestern China

For all species and sites pooled (observations $n = 707$), foliar C%, N%, P%, and C:N, C:P, N:P of aquatic macrophytes across the study areas varied widely. The mean of leaf C%, N%, and P% of aquatic macrophytes across the arid zone of northwestern China was 39.95%, 2.12%, and 0.14%, respectively, which varied from 19.67% to 47.79%, 0.22% to 5.45%, and 0.03% to 1.15%. The mean of C:N, C:P, N:P ratios was 23.55, 346.91, and 16.60, respectively, with a range of 7.29 to 187.41 for the C:N ratio, 37.32 to 1256.81 for the C:P ratio, and 2.55 to 55.09 for the N:P ratio (**Table 1**).

In the arid zone of northwestern China, submerged plants had the lowest leaf C%, and sharply lower than that of emergent plants and floating-leaved plants. For the leaf N% and P%, floating-leaved plants presented significantly higher than the other two life forms. Our results showed there were significant differences in the leaf C:N, C:P, and N:P ratios among the three life forms, and emergent plants presented the highest leaf C:N, C:P, and N:P ratios (**Table 1**).

## Relationships Between the Leaf C%, N%, P%, and Their Ratios

Correlation analyses indicated that the leaf C%, N%, P%, and the C:N C:P and N:P ratios were correlated with each other (**Table 2**). The leaf C% showed strong positive correlations with the leaf N% and the C:N, C:P, N:P ratios, but it was not related to the leaf P%. Leaf N% was strongly negatively correlated with C:N and C:P ratios and strongly positively correlated with leaf P% and N:P ratio. The leaf P% presented strong negative correlations with C:N, C:P, and N:P ratios. The C:N ratio displayed strong negative correlations with N:P ratio, and positive correlations with C:P ratios. Additionally, strong positive correlations were observed between C:P and N:P ratios.

## Relationships Between the Leaf C%, N%, P%, and Their Ratios and Environmental Factors

Linear regression indicated that leaf N% was positively associated with altitude and longitude but negatively associated with latitude (**Figures 2D–F**). Leaf P% exhibited strongly positive correlations with altitude and latitude (**Figures 2H,I**). The leaf C:N ratio was negatively related to altitude and longitude (**Figures 3A,C**). The leaf C:P ratio was negatively correlated with altitude and latitude (**Figures 3E,F**). The leaf N:P ratio related positively to longitude but negatively to latitude (**Figures 3G,H**). The results showed no significant relationship between leaf C% and longitude, latitude and altitude, leaf P% and longitude, C:N and latitude, C:P and longitude and N:P and altitude, respectively (**Figures 2A–C,G**, **3B,D,I**).

The results of a linear mixed effect model analysis indicated that differences in MAT were the major environmental factors determining the variations in leaf N%, P%, C:N, C:P, and N:P ratios for all species, and MAT exhibited strongly negative correlations with leaf N% and P%, but positive correlations with their ratios. pH and MAP affected leaf C% and C:N ratio of all plants from different aspects, respectively. Life forms, species and plot also had significant effects on element contents of all aquatic macrophytes. For submerged plants, MAT was negatively associated with leaf N%, P% but positively associated with C:N and C:P ratios. We also observed that MAP was the most significant environmental factor for floating-leaved plants, which was negatively correlated with leaf N% and N:P ratio, whereas it was positively associated with C:N ratio. Moreover, MAT had a significant positive effect on leaf C% in floating-leaved plants. For emergent plants, MAT displayed strong negative correlations with leaf N%, but it presented strong positive correlations with leaf C%, C:P, and N:P ratios. Furthermore, the effects of Species and Plot on leaf ecological stoichiometry characteristic were reduced in each life forms (**Table 3**). Pearson correlation among environmental factors is shown in **Appendix S3**.

**TABLE 2 |** Pearson correlation among leaf element contents.

| | C% | N% | P% | C:N | C:P | N:P |
|---|---|---|---|---|---|---|
| C% | 1.00 | 0.107** | 0.003 | 0.101** | 0.255** | 0.155** |
| N% | | 1.00 | 0.500** | −0.962** | −0.436** | 0.488** |
| P% | | | 1.00 | −0.500** | −0.967** | −0.493** |
| C:N | | | | 1.00 | 0.500** | −0.468** |
| C:P | | | | | 1.00 | 0.524** |
| N:P | | | | | | 1.00 |

***p < 0.01, *p < 0.05.*

# DISCUSSION

## Patterns of Leaf C%, N%, P%, and Their Ratios of Aquatic Macrophytes in the Arid Zone of Northwestern China

Our results showed the mean leaf C% of aquatic plant in the arid zone of northwestern China was 39.95%, approximately identical to the value of aquatic macrophyte in eastern China (36.97%, Xia et al., 2014) and Tibetan Plateau (37.83%, Wang et al., 2015).

**TABLE 1 |** Leaf C%, N%, P%, and their ratios for overall observations and different life forms. Different letters indicated significant differences in leaf C%, N%, P%, and their ratios between different life forms.

| Plant group | Overall | Life form | | | Sig. |
|---|---|---|---|---|---|
| | | Submerged | Floating-leaved | Emergent | |
| n | 707 | 220 | 29 | 458 | |
| C% | 39.95 ± 4.60 | 36.48 ± 5.48b | 40.11 ± 4.81a | 41.61 ± 2.90a | <0.001 |
| N% | 2.12 ± 0.91 | 2.05 ± 0.79b | 2.66 ± 0.93a | 2.12 ± 0.92b | 0.004 |
| P% | 0.14 ± 0.08 | 0.15 ± 0.07b | 0.20 ± 0.15a | 0.14 ± 0.08b | 0.001 |
| C:N | 23.55 ± 15.96 | 20.54 ± 8.58b | 16.84 ± 5.89b | 25.43 ± 18.58a | <0.001 |
| C:P | 346.91 ± 176.29 | 296.08 ± 134.53b | 252.03 ± 115.95b | 377.34 ± 189.13a | <0.001 |
| N:P | 16.60 ± 7.85 | 14.99 ± 5.16b | 15.93 ± 7.06ab | 17.41 ± 7.85a | <0.001 |

*The number of samples (n) and mean values (%) (±SD) are shown. Different letters indicate significant differences at p < 0.05. Sig, significance level.*

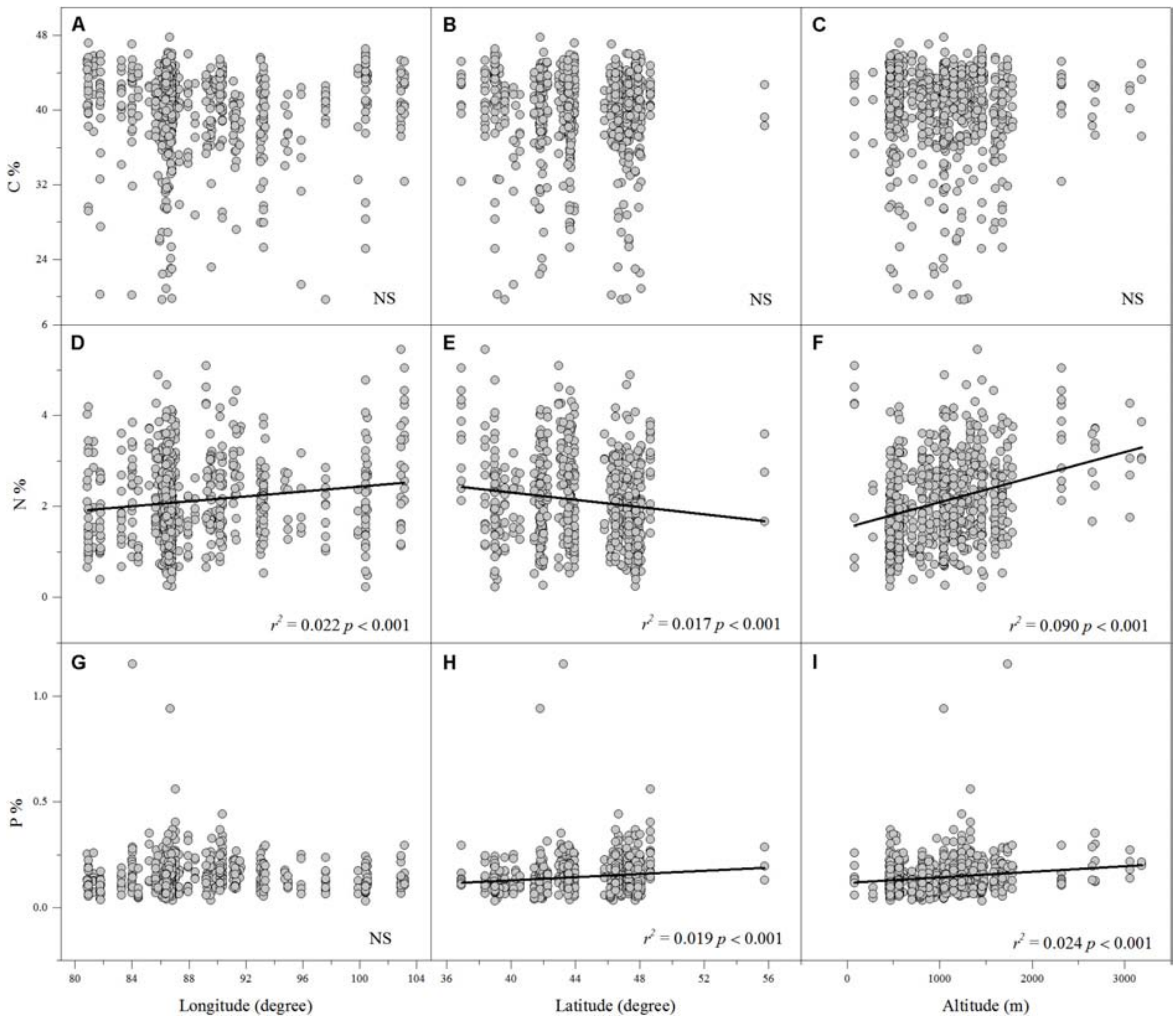


**FIGURE 2 |** The relationships between leaf C%, N% and P% and longitude, latitude and altitude **(A–I)**. (Sig. $p < 0.05$).

Our findings with the results of the two abovementioned studies showed that the mean leaf C% of aquatic plant was drastically lower than the value reported by McGroddy et al. (47%, 2004) for forest plants and by He et al. (43.8%, 2006) for Chinese grassland. One possible explanation for this finding is that the low leaf C% results are due to relatively low contents of lignin and cellulose in aquatic plants (Santamaría et al., 2003), because water buoyancy can provide support for aquatic macrophytes, especially for submerged plants.

For leaf N%, our research indicated that mean nitrogen content was 2.12% and closed to 2.02% for terrestrial plants in China (Han et al., 2005). Regarding the leaf P% of aquatic plants in the arid zone of China, the mean value was nearly equal to the mean value for 753 terrestrial plant species in China (0.15%, Han et al., 2005) and 343 aquatic macrophytes species in Spanish shallow lakes (0.13%, Fernández-Aláez et al., 1999). However, compared to two other climate zones in China, the leaf N% and P% of aquatic plant in the arid zone, especially for leaf P%, were substantially below these average values in humid monsoon eastern China (Xia et al., 2014) and the cold area in Tibetan Plateau (Wang et al., 2015). Several previous studies have shown that the mean annual precipitation and temperature can significantly influence leaf nitrogen and phosphorus content (Reich and Oleksyn, 2004; Han et al., 2005; Wang et al., 2015). Thus, the mean values of leaf N% and P% were lower than those from the Tibetan Plateau and eastern China, which may be due to an adaptation strategy of the plants to severe drought. If so, this finding suggests a remarkable climatic characteristic in the arid zone in the long-term evolutionary process. In agreement with other studies (Xia et al., 2014), our study also showed that floating-leaved plants presented significantly higher leaf N% and P% than the other two life forms of plants, which can be explained by that floating-leaved plants can absorb more dissolved N or P from the water and the sediment through their adventitious roots produced from their leaves or stems (Greenway, 1997).

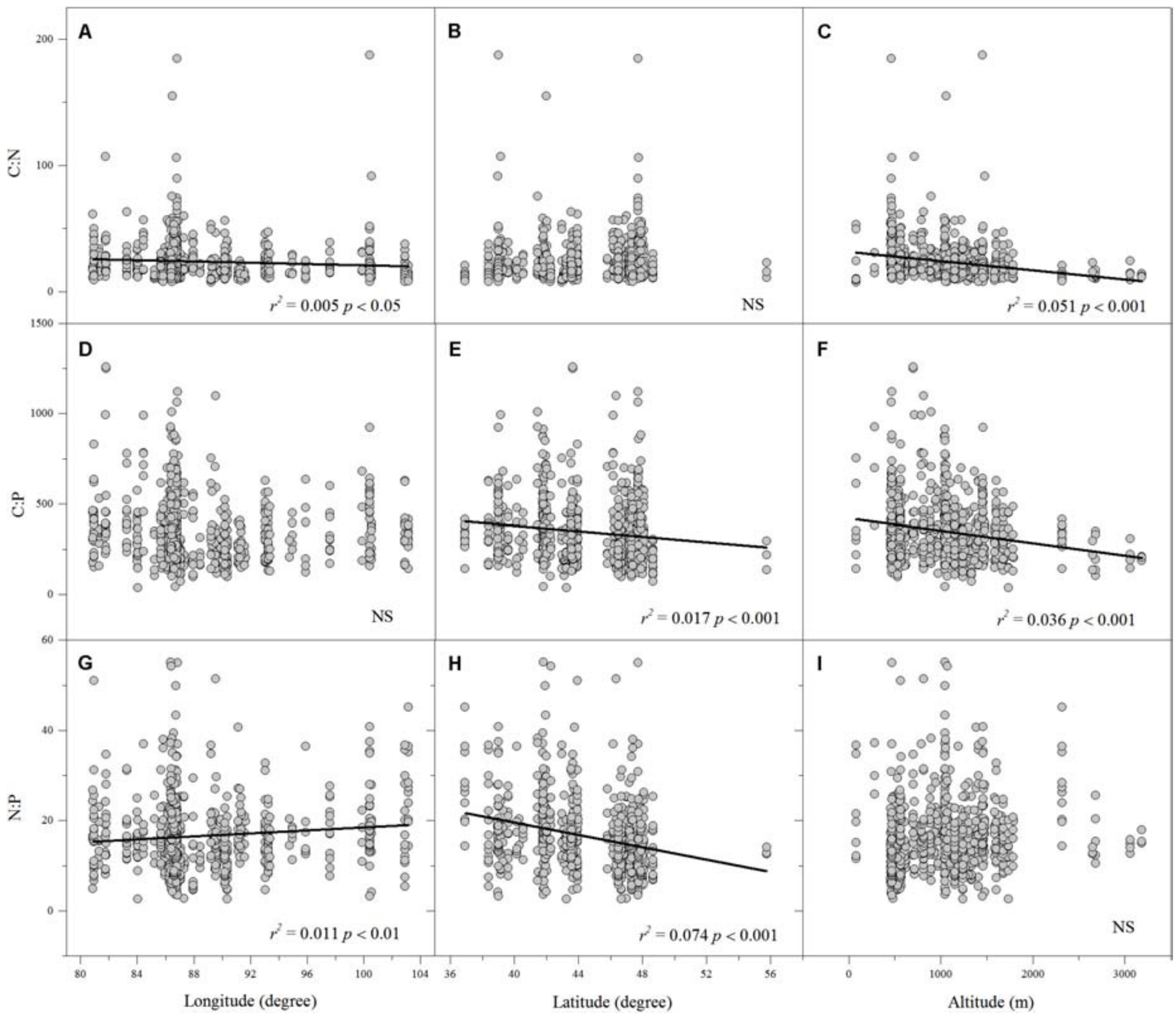


**FIGURE 3 |** The relationships between leaf C:N, C:P and N:P ratios and longitude, latitude and altitude **(A–I)**. (Sig. $p < 0.05$).

Similarly, our findings also showed that the leaf N:P ratios (16.60) of aquatic plants in the study area were higher than the counterpart values of aquatic macrophytes in eastern China (9.5, Xia et al., 2014) and Tibetan Plateau in China (11.3, Wang et al., 2015). The N:P ratios are always used as one of the important indicators for current restricted nutrient judgement. Several studies showed that plant growth is limited by P contents if the leaf N:P > 16 (Koerselman and Meuleman, 1996). The obtained leaf N:P value in this study suggested that the productivity of aquatic macrophytes in the arid zone of northwestern China may be limited by surrounding P contents.

## Effect of Environmental Factors on the Leaf C%, N%, and P% Stoichiometry

Environmental factors, such as light, temperature, precipitation, and soil nutrients, can significantly affect leaf ecological stoichiometry characteristics (Striebel et al., 2008; Cross et al., 2015; Li et al., 2015; Wang et al., 2015). Temperature plays an important role in ecological stoichiometry characteristics of plants due to the direct effects on plant physiological processes (photosynthesis and respiration) (Weih and Karlsson, 2001; Lacoul and Freedman, 2006). Previous research has found that temperature was the greatest environmental factor affecting leaf stoichiometry of aquatic macrophytes in two other climate zones in China (**Appendix S4**). These findings showed that the leaf N% increased as the temperature decreased in the Tibetan Plateau (Wang et al., 2015), while another study showed the opposite result (Xia et al., 2014). These conflicting results could provide evidence to support TPPH or TBH, respectively. In this study, the leaf N% and P% of all plants recorded increased with decreasing MAT, which supported the TPPH. The higher leaf N% and P% can increase plant metabolic activity to counteract the effect of low temperatures on enzyme activity in plants, in other words, the low temperature promotes the domestication and adaptation of plants (Oleksyn et al., 1998; Tjoelker et al., 1999). We also found that MAT showed positive correlations with the N:P ratios of all plants, which is in agreement with

**TABLE 3 |** Summary statistics of linear mixed effect model, which are for the effects of fixed (MAT, mean annual temperature; MAP, mean annual precipitation, pH and Life forms) and random (Species nested in Plot) on leaf C%, N%, P%, and their ratios.

| | Variables | d.f. | C% Estimate | N% Estimate | P% Estimate | C:N Estimate | C:P Estimate | N:P Estimate |
|---|---|---|---|---|---|---|---|---|
| **All species** | | | | | | | | |
| Fixed | MAT | 1 | 0.093 | −0.043** | −0.008*** | 1.077*** | 17.971*** | 0.477*** |
| | MAP | 1 | 0.005 | −0.001 | $-7.085 \times 10^{-5}$ | 0.028* | 0.178 | 0.002 |
| | pH | 1 | −0.513* | −0.018 | −0.006 | 0.376 | −1.838 | −0.084 |
| | Life forms | 2 | 2.487*** | 0.027 | −0.005 | 2.517*** | 43.151*** | 1.269*** |
| Random | Plot(Species) | | 5.867* | 0.464*** | $-1.880 \times 10^{-6}$* | 208.100*** | 8399.000 | 31.223* |
| **Submerged** | | | | | | | | |
| Fixed | MAT | 1 | −0.301 | −0.011*** | −0.008*** | 1.057*** | 13.530** | 0.068 |
| | MAP | 1 | −0.002 | −0.002 | $-2.534 \times 10^{-5}$ | 0.030* | 0.050 | −0.012 |
| | pH | 1 | −1.107 | −0.072 | −0.001 | 0.564 | −5.480 | −0.761 |
| Random | Plot(Species) | | 18.040** | 0.242 | $3.260 \times 10^{-4}$ | 45.421** | 3267.000 | 3.206 |
| **Floating-leaved** | | | | | | | | |
| Fixed | MAT | 1 | 1.071** | −0.010 | 0.002 | 0.711 | 7.207 | −0.040 |
| | MAP | 1 | 0.005 | −0.011* | $-1.610 \times 10^{-4}$ | 0.097*** | −0.364 | −0.089** |
| | pH | 1 | −0.641 | −0.194 | −0.073 | 1.301 | 42.323 | 2.282 |
| Random | Plot(Species) | | 5.647 | 0.226 | 0.008 | 8.056 | 4456.000 | 11.572 |
| **Emergent** | | | | | | | | |
| Fixed | MAT | 1 | 0.204*** | −0.131 | −0.008*** | 1.064* | 20.017*** | 0.686*** |
| | MAP | 1 | 0.007* | 0.001 | $-9.020 \times 10^{-5}$ | 0.022 | 0.230 | 0.012 |
| | pH | 1 | −0.401 | 0.009 | −0.004 | 0.068 | −5.999 | −0.006 |
| Random | Plot(Species) | | 0.435 | 0.538*** | 0.004 | 296.540*** | 12367.000 | 46.440** |

*Linear mixed effect model were also made for each life forms. d.f, degrees of freedom. ***p < 0.001, **p < 0.01, *p < 0.05.*

the studies in eastern China (Xia et al., 2014) and the Tibetan Plateau (Wang et al., 2015). A possible explanation is that under lower mean annual temperature conditions, plant with lower leaf N:P ratios can match higher growth rates to resist shorter growth cycles (Güsewell, 2004; Reich and Oleksyn, 2004; Kerkhoff et al., 2006). In addition, MAT had a significant positive effect on leaf C% in floating-leaved plants and emergent plants. This finding may be observed because plants grown at a warmer temperature produced more leaf area and had higher daytime rates of net ecosystem exchange or $CO_2$ uptake (Park and Day, 2007). Furthermore, floating-leaved plants and emergent plants need to produce more mechanical tissue of leaves than submerged plants during growth promotion caused by temperature rise.

Precipitation is considered to be another crucial factor that influences the contents of plant leaf elements because drought can affect the cell concentration to enhance the protection of water (Field and Mooney, 1986; Osmond et al., 1987; Seligman and Sinclair, 1995). Several studies showed that the leaf ecological stoichiometry characteristics of terrestrial plants were extremely limited by precipitation (He et al., 2006; He et al., 2008). We found that the C:N ratios of all aquatic macrophytes were positively related to MAP. This result could be due to abundant rainfall increasing the supply of water to reduce the adverse effects of drought, which can lead to a significant addition in plant C:N ratios. Furthermore, our results demonstrate that the leaf C% of emergent plants increased along with MAP, which maybe due to the rapid increase of plant biomass associated with increasing rainfall in dry area (Liu et al., 2012). MAP was found to be the major influencing factor for floating plants, which was negatively correlated with leaf N%. Such allocation pattern is considered to be a functional trade off between roots and leaves (Bloom et al., 1985; Barko et al., 1991), that is, plants have to allocate more internal resources to the organ growth responsible for obtaining external resources that are in lacking supply. This is an adaptability strategy of floating-leaved plants to dynamic nutrient contents caused by water level.

Water physicochemical factors can affect directly or indirectly the elemental distribution of aquatic macrophytes (Kirk, 1983; Cronk and Fennessy, 2001; Lacoul and Freedman, 2006). pH is an important factor influencing ecological stoichiometry characteristics of plants in the arid zone of northwestern China because the water in this region is quite saline and alkaline. We found that there was a significant negative influence of pH on the leaf C% of all aquatic macrophytes. This finding may be observed because under higher pH condition, the decreased concentration of $HCO_3^-$ and dissolved $CO_2$ limits the supply of carbon sources in water for aquatic macrophytes (Adams et al., 1978).

Altitude, longitude and latitude are always considered to be geographical factors that affect the large-scale geographic distribution patterns of leaf ecological stoichiometry (Körner, 1989; Reich and Oleksyn, 2004; Han et al., 2005; Li et al., 2015). Our results showed that in the arid zone of northwestern China, the leaf N% of aquatic macrophtes decreased with increasing latitude, whereas the leaf P% increased. One possible explanation for this is the effect of the rate of soils development which depends on rainfall and temperature

(Lambers et al., 2008). Moreover, in this study, we found the leaf N% and P% appeared to have strong positive correlations with altitude but strong negative correlations with MAT. This finding is not surprising, since altitude changes in temperature are known to affect leaf N% and P% (Körner, 1989; Soethe et al., 2008; Fisher et al., 2013; Zhao et al., 2014), which may be observed because changes in foliar N% and P% are functional responses to water availability, as altitude is closely linked to potential evapotranspiration and precipitation (Li and Yu, 2009). Thus, the pattern of ecological stoichiometry characteristics of aquatic macrophytes in the study area may be a result of different combinations and interactions of local environmental factors and geographical factors (altitude, longitude, and latitude), which can influence temperature, precipitation, and potential evapotranspiration.

## CONCLUSION

In this study, we found that the mean values of leaf P% were lower than those in the Tibetan Plateau and eastern China, and P may be the limiting factor of the macrophyte communities in the arid zone of northwestern China. Our results supported the temperature-plant physiology hypothesis owing to significant, negative relationships between leaf N% and P% of macrophytes and mean annual temperature. Our results also showed strong relationships between leaf N% and N:P ratios and longitude, leaf N%, P%, and N:P ratios and latitude, and leaf N% and P% and altitude, respectively. In addition, the results showed that pH can significantly influence leaf C%. The different patterns of leaf stoichiometry between the arid zone of northwestern China and eastern China indicated that there were different physiological and ecological adaptability of macrophytes to environmental gradients in different climatic zones. Compared to prior studies, our study suggests the determinants that influence leaf carbon, nitrogen, and phosphorus stoichiometry of aquatic macrophytes differed among different climatic zones. Thus, further research is warranted to investigate leaf stoichiometry patterns and the primary influencing factors on larger spatial scales. Also, we only studied the effect of three environmental factors (mean annual temperature, mean annual precipitation, pH) on the leaf C, N, and P stoichiometry. It will be of interest in the future to analyse in detail the effects of other factors, such as soil physicochemical characteristics and water physicochemical characteristics.

## AUTHOR CONTRIBUTIONS

ZL and JZ designed the research. XG, ZX, WL, YT, and YL collected and analyzed samples. ZL, JZ, XG, ZW, and CD analyzed and discussed the data. XG and ZL wrote the manuscript.

## FUNDING

This study was jointly supported by the National Natural Science Foundation of China (Nos. 31270261 and 30870260) and Outstanding Young and Middle-aged Science and Technology Innovation Team Plan of Hubei Provincial Department of Education (NO. T201701 Global change and ecological protection).

## ACKNOWLEDGMENTS

We thank the reviewers for comments on the manuscript. We also thank AJE (https://www.aje.com/) for English language editing.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01398/full#supplementary-material

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 16 October 2018
doi: 10.3389/fpls.2018.01514

# Turbidity, Waterfowl Herbivory, and Propagule Banks Shape Submerged Aquatic Vegetation in Ponds

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The aquatic vegetation in nutrient-rich shallow lakes and ponds is structured by the interplay of multiple biotic and abiotic drivers. We tested the contribution of the macrophyte propagule bank and the delayed as well as direct impact of waterbirds on submerged aquatic vegetation in a peri-urban pond system. To clarify the functional hierarchy of predictor variables, effects of herbivorous waterfowl and propagule bank potential were ranked relative to environmental, phytoplankton, and zooplankton indicators. Two aspects of the aquatic vegetation – community composition and total pond-scale cover – were discriminated. Within vegetation communities, phytoplankton biovolume and waterfowl herbivory during summer were linked to low macrophyte abundance, whereas propagule density of angiosperms was positively associated with specific assemblages of submerged macrophytes. High algal biovolume and summer waterfowl grazing seemed to affect maximal pond-scale cover of submerged aquatic vegetation. The presence of waterfowl in cold and spring periods was unrelated to vegetation structure in the consecutive main growth season. In addition, availability of propagules in the sediment did not automatically prompt pond-wide vegetation cover (especially when overruled by high waterfowl densities), nor did it guarantee a position in the submerged macrophyte community. Nonetheless, propagule bank potential was related to the waterbody's general ecological status, since turbid ponds exhibited impoverished propagule reserves compared to ponds residing in a clear, macrophyte-dominated state. Inadequate recruitment therefore represents a plausible bottleneck for macrophyte establishment. We conclude that phytoplankton-caused turbidity and high waterfowl biomass densities greatly restrict submerged macrophyte abundance. Propagule banks also participate in structuring submerged aquatic vegetation, though a stronger role is reserved for herbivorous waterfowl.

**Keywords: submerged macrophytes, phytoplankton, avifauna, Anatidae, seeds, oospores, biomanipulation**



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***Specialty section:***
*This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science*

***Received:*** *18 July 2017*
***Accepted:*** *26 September 2018*
***Published:*** *16 October 2018*

***Citation:***
*Van Onsem S and Triest L (2018) Turbidity, Waterfowl Herbivory, and Propagule Banks Shape Submerged Aquatic Vegetation in Ponds. Front. Plant Sci. 9:1514. doi: 10.3389/fpls.2018.01514*

## INTRODUCTION

The aquatic ecosystem of shallow lakes and ponds is molded by a complex set of interacting biotic and abiotic components (Jeppesen et al., 1997; Søndergaard et al., 2003; Scheffer, 2004). Submerged macrophytes, containing plants and macroalgae adapted to underwater life, play a decisive role in shallow waterbodies by promoting high water clarity (Scheffer et al., 1993) and increased biodiversity in a number of vulnerable faunal groups (De Meester et al., 2006). In nutrient enriched standing waters, submerged aquatic vegetation (SAV) risks collapse when confronted with high phytoplankton-caused turbidity or stress associated with increased productivity of other autotrophic competitors, including periphyton and free-floating plants (Jones and Sayer, 2003; Scheffer et al., 2003; Hidding et al., 2016). The occurrence and persistence of phytoplankton blooms – often the result of high fish densities – can lead to an alternative stable state as opposed to the macrophyte-dominated equilibrium (Scheffer et al., 1993).

Because light availability is such an essential requirement for SAV development, the effect of phytoplankton abundance and zooplankton filtering has been studied extensively (Burns, 1969; Knoechel and Holtby, 1986; Sayer et al., 2010). Nonetheless, other features of the freshwater ecosystem directly or indirectly influencing macrophyte growth have been identified. For one, aquatic vegetation tends to be more sensitive to herbivory compared to terrestrial plants, due to the relatively high palatability of submerged macrophyte species (Lodge, 1991; Bakker et al., 2016). The intensity of waterfowl grazing inversely relates to the standing crop of wetland vegetation, provided that bird counts are converted into biomass density (kg/ha; Wood et al., 2012).

The potential impact of waterfowl has inspired research focusing on SAV dynamics (Irfanullah and Moss, 2004; Hilt, 2006; Gayet et al., 2011; Gyimesi et al., 2011; van Altena et al., 2016). Nevertheless, birds are easily overlooked during routine assessment of macrophyte dynamics because of their mobility and migratory habits, especially outside major wetland areas or in regions not experiencing striking seasonal peaks in waterfowl numbers. As a consequence, studies on avifauna-SAV interactions in pond systems are currently scarce compared to those in large wetlands (Hangelbroek et al., 2002; Hidding et al., 2010a).

A further element plausible to affect the composition of SAV is the macrophytic propagule bank (Bakker et al., 2013), the collection of sexually or vegetatively produced dispersal and survival units accumulated over time in the sediment. In charophycean macroalgae, sexually generated oospores usually form the dominant propagation mode (Bonis and Grillas, 2002). Larger variation exists within angiosperm macrophytes, with the production of seeds, turions, tubers, and rhizomes as well as less-specialized fragments (Grace, 1993; Barrat-Segretain, 1996).

Although the composition of the submerged propagule bank does not necessarily match the standing vegetation (Arthaud et al., 2012), it forms the foundation of new macrophyte emergence after an unfavorable season (Liu et al., 2006) or a major disturbance (Combroux et al., 2001), including water drawdown during biomanipulation (Peretyatko et al., 2012). Therefore, spontaneous recovery of SAV will depend at least partly on the presence of propagules. It remains an open question, however, to what extent a rich, dense propagule bank could guide the ecosystem toward the desired clear, macrophyte-dominated equilibrium under nutrient-rich conditions (Hilt, 2015), and how strongly individual species rely on input from propagules to gain a foothold within the vegetation.

In view of the broad spectrum of drivers affecting cover and assemblage of SAV, it is recommended to simultaneously explore their significance. Acknowledging the relevance of, as well as the interaction among, predictor variables will greatly substantiate and streamline conservation efforts directed at restoring aquatic vegetation (Madsen et al., 2001; Case and Madsen, 2004; Irfanullah and Moss, 2004; Liess and Hillebrand, 2004; Gulati et al., 2008; Hidding et al., 2016).

In this study, we ordered an elaborate set of candidate parameters involved in SAV development in a temperate, nutrient-rich, peri-urban pond system. Data on herbivorous waterfowl and macrophyte propagule banks – two freshwater components rarely credited in smaller waterbodies – were incorporated to test their role in proportion to physical–chemical, nutrient, phytoplankton as well as zooplankton indicators. We hypothesized that (1) phytoplankton abundance would be particularly pertinent, and that (2) waterfowl negatively, and (3) propagule banks positively influence submerged macrophytes. The impact mechanism of bird populations was predicted to vary periodically (4), due to postponed effects triggered by consumption outside of the growth season peak. The occurrence of macrophyte species within the vegetation (5), as well as the ponds' general ecological status (6), was expected to echo the composition and richness of the propagule bank. Finally, we hypothesized that (7) the relevance of predictor variables is species-specific and, additionally, depends on the distinction between the vegetation community (macrophyte assemblage aspect) and SAV extent at the scale of the waterbody (total macrophyte cover aspect).

## MATERIALS AND METHODS

### Study Area and Conditions

The study was conducted in 2009 and 2010 in 16 shallow man-made ponds located at the outskirts of the Brussels-Capital Region, Belgium (**Figure 1**). Historically, all ponds have been excavated along stream courses, and generally a flow-through hydrological regime is maintained. Thirteen ponds are situated in the Woluwe catchment, a small river largely fed by forested headwaters. Eleven ponds are situated within parks with broad grass lawns, while the remaining five have perimeters that are predominantly forested. Pond area ranges from 0.11 to 3.57 ha (average 1.10 ha), with depths spanning from 0.47 to 1.30 m (average 0.90 m).

As a result of past anthropogenic eutrophication largely sustained through internal loading, nutrient levels in ponds of the Brussels-Capital Region typically correspond to hypereutrophic conditions (OECD, 1982). To battle algal and cyanobacterial blooms and increase ecological value, all ponds had been biomanipulated within the past decade prior to the study. Biomanipulation in the region typically encompasses water drawdown with subsequent fish removal (Peretyatko et al., 2012). The short-term success of this management procedure has proven to be almost complete (De Backer et al., 2012), so that most ponds at least during the initial phase following biomanipulation reside in a clear-water state and develop SAV.

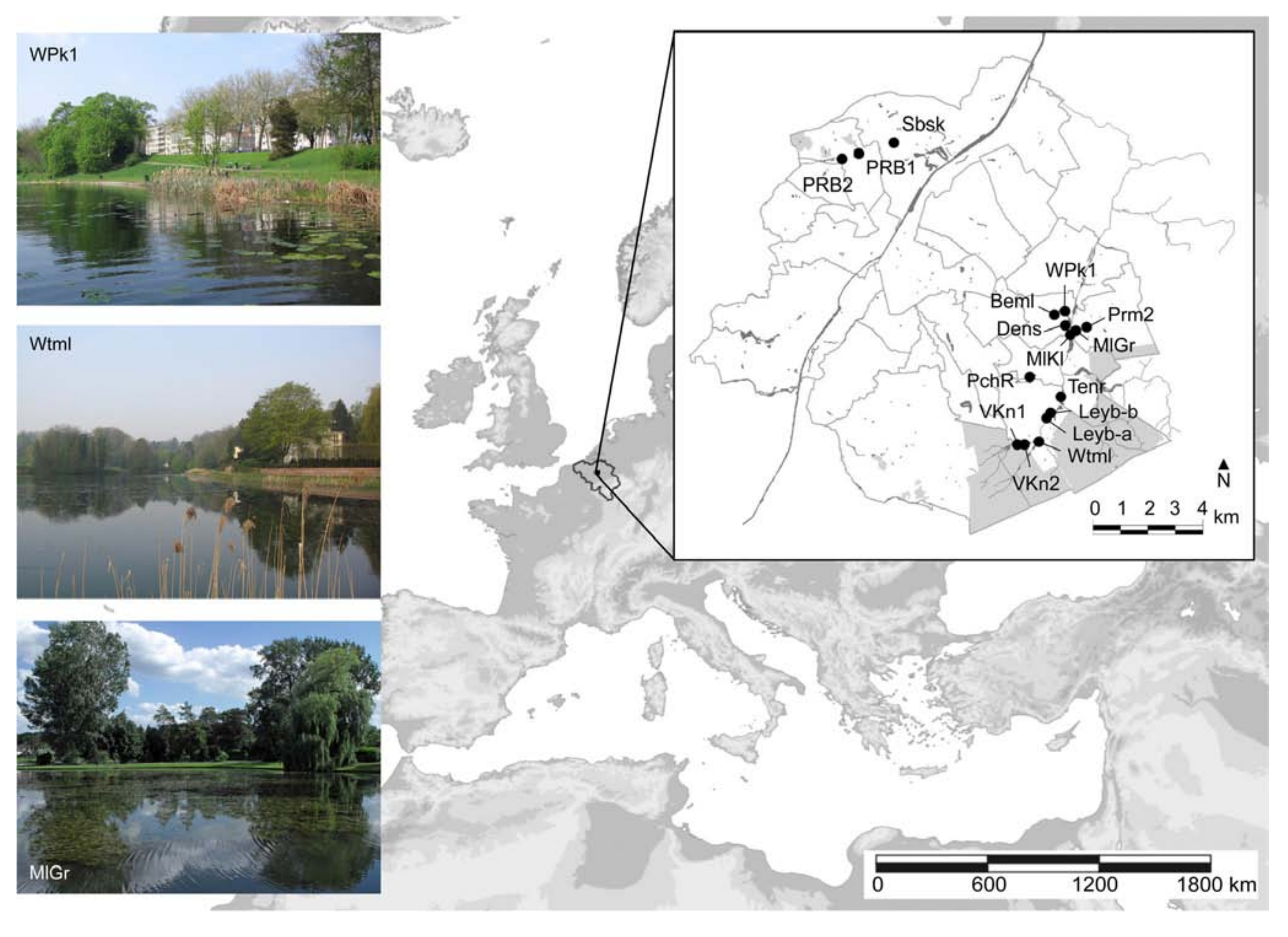


**FIGURE 1 |** Location of the Brussels-Capital Region (gray area: forest) with 16 studied ponds. Left insets: examples of ponds.

In one pond, however (PchR), active biological interference was limited to stocking with juvenile Northern pike (*Esox Lucius* L.), without success. Three ponds (MlGr, MlKl, and Prm2) were emptied early 2009, refilled after the growth season and only sampled in the summer of the next year. At the time of sampling, at least two upstream ponds were presumably fishless (Beml, Dens; De Backer et al., 2012), while in several others large benthivorous fish and/or schools of zooplanktivores were observed (Leyb-a, Leyb-b, PchR, PRB1, PRB2, Prm2, Tenr, VKn2, and WPk1).

## Propagule Bank

### Sediment Collection

Sediment was collected in spring (April) 2009 with a core sampler (Eijkelkamp multisampler; diameter 40 mm) in three non-overlapping transects per pond. Length, direction and spacing of transects varied in function of pond geometry (size and morphology), and were chosen to maximize representation of the main non-littoral zones of the waterbody. Along each transect and at equal intervals, we stopped at five stations and took three subsamples per stop (15 subsamples in total). Sediment cores were divided into an upper layer of 5 cm (supposed to be the most ecologically active; Dugdale et al., 2001; Bonis and Grillas, 2002; Spencer and Ksander, 2002) and a lower layer from a depth of 5–10 cm (Csontos, 2007) used for comparability and potentially acting as a refuge (Ozimek, 2006). This way, the sediment from five stops per transect was pooled into two samples, representing either the top or the lower sediment layer (abbreviated ‘top’ and ‘sub’). In total, 90 samples were collected in 16 ponds. The sediment was stored for 2.5 months at 4°C, to extend the cold stratification period and increase the likelihood of germination (Hay et al., 2008).

### Germination Experiment

The seedling-emergence method described by Ter Heerdt et al. (1996) was adopted to germinate viable macrophyte propagules in a greenhouse. Sediment samples were gently homogenized. Fine mineral and organic particles of a known volume (generally 0.7 L) were removed using a sieve tower with smallest mesh size of 0.212 mm, suited to retain most charophycean oospores (Haas, 1994). The remaining fractions were pooled and spread out as a thin layer in plastic trays (surface area: 34 × 21 cm, height: 8 cm) filled with 1 cm of sand on top of a 1.5-cm-thick nourishing layer (mixture of sand and potting soil in a 4:1 proportion). All substrate material had been sterilized prior to the germination trials. Trays containing the samples were perforated and kept in larger, outer trays, to facilitate addition of water without sediment disturbance.

Samples were immediately submerged in a mix of equal proportions distilled and tap water. The water level was maintained at ca. 5 cm during the growth experiment. We chose to inundate our samples instead of keeping them waterlogged, to avoid excessive germination of emergent taxa and to increase germination success in submerged species (Boedeltje et al., 2002; Xiao et al., 2010). *Daphnia* were allowed to emerge from ephippia and redistributed over the samples if necessary. The next 6 months, macrophyte seedlings were picked from the sediment, identified and counted on regular basis. Counts of non-identifiable *Chara* germlings were redistributed based on relative proportions of confirmed identifications within the sample.

At the end of the experiment, the sample layer was recovered and scanned for ungerminated, non-oospore propagules (mainly angiosperm seeds). As a number of thus retrieved fruits (mainly achenes belonging to *Potamogeton* spp.) still spontaneously germinated afterward, all seeds were considered viable but dormant and added to counts obtained from the germination trials. Final propagule densities were converted to numbers per liter sediment (equivalent to 1/50th of a $m^2$). Shannon indices (H') were calculated as a measure of diversity within the top and sub layers.

In statistical analyses, angiosperm and charophycean propagule densities were considered separately, since reproductive strategies of submerged species of Angiospermae generally involve investing in fewer, heavier propagules compared to Charophyceae (Haas, 1994; Bonis and Grillas, 2002; Kleyer et al., 2008).

## Vegetation Survey

We evaluated two ecological aspects of the aquatic vegetation: (1) the community composition, and (2) total pond-scale cover of SAV. Firstly, macrophytes growing along the three sediment sampling transects (including five stops/transect) were monitored twice, once in July 2009 and again in July 2010. From a boat, a floating PVC frame (1 $m^2$) was placed on top of the macrophyte assemblage followed by species cover estimation. Low-growing vegetation, as well as the floor of turbid ponds, was sampled using a rake. Vertical stratification of vegetation was taken into account, allowing the combined cover to exceed 100%.

Secondly, in both years waterbody-scale relative cover of submerged macrophytes was visually surveyed by traversing the pond in several directions, on three occasions: end of May, beginning of July and mid August. The maximal SAV extent during the growth season was used in the statistical analyses.

## Environmental Variables, Phytoplankton, and Zooplankton

During two campaigns per year (end of May and at the time of vegetation community analysis in July), we measured a number of environmental variables (**Table 1**): water depth, Secchi depth, physical–chemical variables (temperature, pH, conductivity, and oxygen concentration) and nutrients ($NO_x$, sum of nitrate and nitrite; $NH_4{}^+$, ammonium; SRP, soluble reactive phosphorus; TP, total phosphorus). When the Secchi disk was still detectable at the bottom, a correction was added in function of disk visibility (either 0.5, 0.3, or 0.1 m). Per pond, a water volume of 10 L was collected with a vertical tube sampler for measurements of physical–chemical variables (recorded on the field with a portable WTW Multi 340i multimeter) and nutrient analysis. Nutrient subsamples were frozen upon return and concentrations were analyzed using spectrophotometry (APHA, 1995).

Additionally, we integrated results of phytoplankton and zooplankton analyses cumulated in an internal database (**Table 1**). Phytoplankton and zooplankton samples had been collected end of May and beginning of July using a tube sampler and a water volume of 10 L. Two measures of phytoplankton abundance were extracted: pigment concentrations (Chl $a$ + phaeophytin) and biovolume.

For zooplankton, we used identifications and length measurements of large Cladocera (mainly *Daphnia*; functional group as defined by Moss et al., 2003) to calculate both density (LCD, Large Cladocera Density) and mean length (LCL, Large Cladocera Length) per pond and growth season. Both measures of zooplankton grazing efficiency have been tested in Brussels peri-urban ponds, with LCL generally displaying the strongest relation with pond turbidity level (Peretyatko et al., 2009; De Backer et al., 2014). Also, LCL can be considered to be a surrogate for zooplanktivorous fish predation, because of down-regulation of cladoceran size (Brooks and Dodson, 1965).

To further improve potential predictive power of zooplankton on SAV structure, we combined information on density and body size in a metric predicting the filtering rate of the total population of large Cladocera (LCFR, Large Cladocera Filtering Rate; Reynolds, 2006). Calculating individual filtering rate for 20 specimens of known size (incorporating temperature data; Burns, 1969) and multiplying this rate with LCD provided an indication of the grazing potential, expressed as the total volume of water filtered per day by large zooplankters present in 1 L of pond water (Reynolds, 2006).

For environmental, phytoplankton, and zooplankton variables, the warm-period average (May and July) for each pond was used in statistical analyses.

## Waterfowl

Effect of fully or partially herbivorous avifauna species on macrophytes was tested using records obtained from a regional database compiled with year-round input from various sources, including citizen science (Leefmilieu Brussel[1]). These data are reliable since a large fraction is validated by experts on the basis of attached images or originates from experienced bird-watchers. Coordinates were used to assign bird observations to particular ponds. A 50 m buffer extended the zone of interest to surrounding green areas potentially containing

[1]bru.observations.be

**TABLE 1 |** Variables considered in multivariate community analysis and multiple regression of pond-scale SAV cover predictors.

| Type | Variable abbreviation | Variable full | Unit | Proxy[a] | SAV community (RDA) | SAV cover (multiple regression) |
|---|---|---|---|---|---|---|
| Abiotic | | | | | | |
| Physical–chemical | Cond | Conductivity | μS/cm | Cond | X* | X |
| | $O_2$ | Oxygen concentration | mg $O_2$/L | pH | | |
| | pH | pH | | pH | X* | X* |
| Nutrients | DIN | Dissolved inorganic nitrogen | mg N/L | DIN | X | X |
| | $NH_4{}^+$ | Ammonium | mg N/L | DIN | | |
| | $NO_x$ | Nitrate + nitrite | mg N/L | DIN | | |
| | SRP | Soluble reactive phosphorus | mg P/L | TP | | |
| | TP | Total phosphorus | mg P/L | TP | X | X |
| Physical | D | Depth | m | D | X | X |
| | SD | Secchi depth | m | SD/D | | |
| | SD/D | Secchi depth/depth | | SD/D | X | X |
| Pond characteristics | TSB | Time since biomanipulation | years | TSB | X | X |
| Biotic | | | | | | |
| Phytoplankton | Chl *a* + phaeo | Chl *a* + phaeopigments | μg/L | Biovol | | |
| | Biovol | Biovolume | $mm^3$/L | Biovol | X* | X* |
| Zooplankton | LCL | Large Cladocera length | mm | LCFR | | |
| | LCD | Large Cladocera density | n/L | LCFR | | |
| | LCFR | Large Cladocera filtering rate | mL/L/day | LCFR | X | X |
| Propagule bank | T_Charo | Top layer Charophyceae prop. density | n/L | T_Charo | X | X |
| | S_Charo | Sub layer Charophyceae prop. density | n/L | T_Charo | | |
| | T_Angio | Top layer Angiospermae prop. density | n/L | T_Angio | X* | X |
| | S_Angio | Sub layer Angiospermae prop. density | n/L | T_Angio | | |
| | T_Shannon | Top layer Shannon H' | H' | T_Angio | | |
| | S_Shannon | Sub layer Shannon H' | H' | T_Angio | | |
| Macrophytes | MF_FM | Cover free-floating + floating-leaved macrophytes | % | MF_FM | | X |
| Waterfowl | WF_Cold | Waterfowl cold period biomass density | kg/ha | WF_Cold | X | X |
| | WF_Spring | Waterfowl spring period biomass density | kg/ha | WF_Spring | X | X |
| | WF_Warm | Waterfowl warm period biomass density | kg/ha | WF_Warm | X* | X* |

[a]*Proxy: strongly correlated ($r_s$ > 0.7) variable selected for multivariate and regression analyses. Original list has been reduced according to covariate structure, retaining key variables of interest (X). Asterisk, significant or relevant.*

resting, roosting or grazing waterfowl. If a clear obstacle was present between the observation and a particular pond, or if distance to a nearby pond was shorter, the count was not retained. Only waterfowl species that potentially include macrophytes in their diet were selected (Wood et al., 2012).

Since the type and magnitude of waterfowl herbivory differs between macrophyte growth stages, avifauna observations were partitioned over three time frames per year, roughly corresponding to meteorological seasons and the pattern of bird migration. This allowed separating effects of belowground herbivory in autumn and winter from primarily direct grazing in the sprouting phase and the main growth period. During vegetation senescence and throughout the major part of autumn and winter, conjointly termed the *cold* period (WF_Cold; September 1st – February 28th), herbivory would mainly impact the macrophyte propagule bank (Perrow et al., 1997; Hidding et al., 2010b). On the other hand, the *spring* period or recruitment phase (WF_Spring; March 1st – May 15th) is known to be a delicate window of opportunity for macrophyte establishment (Marklund et al., 2002). Finally, during the *warm* period following initial SAV expansion (WF_Warm; May 16th – August 31st, including the peak of summer), herbivory likely affects both standing crop biomass and propagule production (Hidding et al., 2010b; Gayet et al., 2011; Marco-Méndez et al., 2015). In total, bird observations spanned September 1st 2008 till August 31st 2010.

Bird counts were converted to a measure of effective *herbivorous* biomass, by multiplying them with species-specific average biomass and percentage plant material (including seeds) in diet, provided by Wood et al. (2012). Because of supposedly substantial amounts of multiplicates and false negatives in the observation database, we used the maximum herbivorous biomass per period and pond as a proxy for the influence of a species in that particular period, and summed the species maxima to obtain the total herbivorous biomass of the avifauna community. Finally, total herbivorous biomass was divided by pond size. The resulting biomass density (kg/ha) represents a potential peak in waterfowl-induced stress (Wood et al., 2012). Features of the aquatic vegetation in July were related to waterfowl herbivorous biomass density in the cold and spring periods immediately prior to, and the warm period during, the summer of macrophyte survey.

## Data Analysis

### Propagule Bank, Waterfowl, and SAV Species

Species-specific patterns in abundance of submerged macrophyte species in relation to propagule density and waterfowl pressure were graphically explored by means of descriptive statistics. The potential of the propagule bank and of SAV in predicting species occurrence in vegetation of subsequent summer season(s) is presented using the overlap in species incidence between physical compartments (propagules in sediment versus standing vegetation).

### Propagule Bank and Pond Ecological Status

In order to determine the relationship of the propagule bank with the general condition of the ecosystem, ponds were categorized in function of the pond-scale SAV cover and their turbidity level. Thresholds were 30% maximal pond-scale SAV cover (marking the lower boundary of ecosystems stabilized by submerged macrophytes; Jeppesen et al., 1990; De Backer et al., 2012) and 20 $mm^3$/L averaged phytoplankton biovolume (indicating a high turbidity; Peretyatko et al., 2007). Three classes were created, representing different levels of ecological status: clear-water, highly vegetated (macrophyte-dominated) ponds (*Clear H*; $\geq$30% SAV cover and <20 $mm^3$/L phytoplankton biovolume; $n$ = 17 over the study period), clear-water ponds with low vegetation cover (*Clear L*; <30% SAV and <20 $mm^3$/L biovolume; $n$ = 7) and turbid ponds without aquatic vegetation (*Turbid*; <30% SAV and $\geq$20 $mm^3$/L biovolume; $n$ = 5).

The relationship of propagule densities with the pond ecological state was tested using zero-inflated generalized linear mixed models (ZIGLMM; Bolker et al., 2009; Zuur et al., 2009) with pond status as a fixed factor and pond-year nested within status as a random factor. Pond in a given year was treated as blocking factor since ponds contained three transects and are reset during winter, which allowed them to switch status between years. Within the framework of the study, the propagule bank was considered to be a relatively invariable element of pond identity. Therefore, results from the germination experiment performed in the first year were coupled to pond status as observed in the first as well as the second year. This assumption is backed by the high similarity between top and sub layers in terms of propagule contents (see the Section "Results"), an indication of propagule bank continuity at short temporal scale.

Patterns in angiosperm and charophycean propagule densities as well as top and sub sediment layers were separately analyzed. Furthermore, we calculated Chao-Sørensen abundance-based similarity (Chao et al., 2005, 2006) among propagule bank and developed vegetation. The Chao-Sørensen index ranges from 0 (no shared species) to 1 (complete overlap) and integrates information on species incidence and their relative abundance. We excluded emergent taxa, included free-floating and floating-leaved species and ignored the non-vegetated portion of survey plots. If both propagule bank and the corresponding vegetation transect lacked macrophytes, similarity values were fixed at 1.

ZIGLMM analysis was performed with the *glmmTMB* package in R3.2.0 (Magnusson et al., 2017). Models for propagule densities were fitted using the negative binomial distribution, because of overdispersion in the count data ($k$ < 10; Bolker, 2007). Beta regression was selected for Chao-Sørensen similarities (extremes 0 and 1 were slightly adjusted for this purpose). The significance threshold was set at 0.05.

### Predictors of Macrophyte Community

For multivariate analysis, transect data were averaged per pond. Prior to ordination, macrophyte cover was Hellinger transformed (Legendre and Gallagher, 2001). The non-vegetated portion (i.e., bare sediment) within a quadrat was entered as an additional species variable. As a direct gradient analysis, we used Redundancy Analysis (RDA). The original list of environmental variables was a mix of biotic ($n$ = 14) and abiotic ($n$ = 12) variables potentially interacting with macrophyte growth (**Table 1**). Additionally, the time since last biomanipulation (TSB, number of years) was included, given the tendency for gradual deterioration following successful fish removal (Søndergaard et al., 2008; see **Supplementary Material**).

Explanatory variables were log-transformed. Spearman's rank-order correlations were used to eliminate multicollinearity: one key variable was selected for each group of strongly related parameters ($p$ < 0.05 and $r_s$ > 0.7; **Table 1**). The resulting 14 abiotic and biotic variables avoided multicollinearity and were used in the final RDA. Significance was tested using forward selection, with Monte Carlo permutations (999 repetitions) under restricted model. A PCA with supplementary variables was used to illustrate the relationship between macrophyte species and the environment. Ordination was performed in CANOCO 4.5 (ter Braak and Šmilauer, 2002).

### Predictors of SAV Pond-Scale Cover

The maximal pond-scale cover of submerged vegetation in summer (May–August) was used as a response variable in multiple regression. Predictor variables in this analysis were taken from the reduced set of variables given in **Table 1**. A further reduction was achieved using the output from simple regressions between dependent and single independent variables. All models were fitted using beta distributions (*betareg* package; Cribari-Neto and Zeileis, 2010), because of the proportional nature of the data (ranging from 0, SAV absent, to 1, complete cover). A small correction was applied to all 0 and 1 values.

After acquiring the shortlist of potentially relevant variables, multimodel inference (*AICcmodavg* package; Mazerolle, 2016) was applied on all possible models, to estimate the contribution of these variables in predicting SAV cover (Burnham and Anderson, 2002; Grueber et al., 2011). An AICc-based (Akaike Information Criterion for finite sample size) threshold to retain only top-ranked models was not deemed necessary because of the relatively small set of predictors. The explanatory power of predictors was evaluated using the weighted estimates and their 95% confidence intervals (CI; Grueber et al., 2011).

# RESULTS

## Abiotic Variables, Phytoplankton, and Zooplankton

Summer values of abiotic and biotic variables are given in **Supplementary Material**. Conductivity was high (>800 μS/cm) in Beml, PRB1, Prm2, and WPk1, and low (<500 μS/cm) in ponds supplied by forest streams (VKn1, VKn2, Wtml). A few ponds experienced hypoxic conditions, due to high lemnid or floating-leaved plant cover. Most ponds were characterized by low acidity values (average pH of 8.1 ± 0.5 SD). DIN varied widely, with an average of 0.226 ± 0.294 SD mg N/L. Total phosphorus status of the ponds also fluctuated strongly, but was overall high (average of 0.370 ± 0.374 SD mg P/L). Mean water depth was 0.9 ± 0.2 SD m, while Secchi depth was 1.2 ± 0.5 SD m on average – suggesting light penetration generally permitted SAV colonization.

Transparency levels ranged from very clear (minimum phytoplankton pigment concentration and biovolume were 3.1 μg/L and 0.2 $mm^3$/L, respectively) to extremely turbid in ponds experiencing phytoplankton blooms (maximum values were 257.0 μg/L Chl *a* + phaeophytin and 36.6 $mm^3$/L biovolume).

In zooplankton communities, *Daphnia* was the dominant large cladoceran genus. A number of ponds contained marginal numbers of *Eurycercus* and *Simocephalus*. Individuals were very small in some ponds (minimum LCL of 0.20 mm in Leyb-b in 2010), or completely absent (LCD = 0 n/L in Leyb-a in 2010). On multiple occasions (especially in fishless ponds), LCL and/or LCD were high, resulting in potential clearance of the complete basins' volume each day (LCFR > 1 L). LCFR exceeded 3,800 mL/L/day in Sbsk in 2010. Generally, zooplankton parameters varied widely, indicating a broad range of potential filtering capacities (see **Supplementary Material**). LCFR was very low in turbid ponds.

## Waterfowl

In total, 515 waterfowl entries were extracted from the dataset (around 6,300 individuals belonging to 19 herbivorous or omnivorous taxa; see **Supplementary Material**). These included 16 species of Anatidae (ducks, geese, swans, sheldgeese, and Egyptian goose) as well as three Rallidae species (rails). Five common waterfowl species dominated the avifauna in terms of computed total biomass, two of which are non-native to Belgium. In decreasing order of importance, these were *Cygnus olor* Gmelin (Mute swan; 34% of herbivory-related biomass), *Branta canadensis* L. (Canada goose, non-native; 21%), *Alopochen aegyptiacus* L. (Egyptian goose, non-native; 16%), *Anas platyrhynchos* L. (Mallard; 14%) and *Fulica atra* L. (Eurasian coot; 9%).

The presence of waterfowl was unevenly distributed, both spatially and temporarily (**Figure 2**). Cold period peaks (WF_Cold) matched with Beml and Sbsk (616 and 644 kg/ha), while spring values (WF_Spring) were generally low. Peaks observed during the warm season (WF_Warm) were even more prominent, mainly in Beml and Dens (703 and 723 kg/ha, respectively). Within these ponds' perimeter, extensive flocks of larger Anatidae species aggregated in summer. With a surface area of 0.43 ha, Beml endured the company of 60 *A. aegyptiacus* and 17 *C. olor* in the warm season of 2009. In Dens (surface area 0.33 ha), 70 *B. canadensis* and three *Chloephaga picta* Gmelin (Magellan goose, non-native) were noticed simultaneously. Average biomass densities for the six consecutive periods were 84 ± 16 SD, 37 ± 52 SD, 110 ± 237 SD, 164 ± 189 SD, 32 ± 49 SD, and 65 ± 97 SD kg/ha.

## Propagule Bank

A total of 11,015 propagules from eight submerged macrophyte species germinated during the study period. Most (95.07%) belonged to three species of Charophyceae: oospores of *Chara vulgaris* L., *C. globularis* Thuillier and *Nitella mucronata* (Braun) Miquel, respectively, accounted for 65.60, 23.09, and 6.38% of all propagules. Angiosperm submerged macrophytes were represented mainly by *Zannichellia palustris* L. (3.07%) and *Potamogeton pusillus* L. (1.71%), accompanied by *P. pectinatus* L. (0.11%), *Callitriche obtusangula* Le Gall ex Hegelm. (0.04%) and *P. crispus* L. (0.01%).

Large differences in presence and densities of submerged species were observed between ponds. Top and sub layers contained on average 290.5 ± 388.2 SD and 107.7 ± 153.2 SD oospores/L, respectively. Mean angiosperm propagule densities were 15.8 ± 23.4 SD n/L in the top sediment layer and 7.9 ± 12.9 SD n/L in the underlying layer. Top and sub layers were correlated in terms of density (Charophyceae: $r_s = 0.86$, $p < 0.0001$; Angiospermae: $r_s = 0.90$, $p < 0.0001$) and on average showed relatively high Chao-Sørensen similarity (0.66 ± 0.45 SD). Overall, Shannon diversity within the propagule pool of both sediment layers was low, as a result of charophycean dominance and low species richness (H' top: 0.31 ± 0.35 SD; H' sub: 0.35 ± 0.36 SD).

A number of ponds were characterized by relatively diverse and/or dense submerged macrophyte propagule banks (Dens, Leyb-a, Leyb-b, MlGr, VKn1 and Wtml, for instance); others displayed poor recruitment potential. In one turbid pond, no propagules sprouted from the collected sediment (PchR). Five ponds lacked angiosperm propagules (PchR, PRB1, PRB2, Prm2, and VKn2), while germinating oospores were entirely absent from only one site (PchR).

## Standing Vegetation

The submerged macrophyte community in July was composed of nine species in total (**Figures 3–5**). A number of ponds completely lacked SAV during one (Beml and Dens in 2009) or both monitoring periods (PchR and PRB2). Others were sparsely colonized with submerged species (≤5% cover; Beml, Dens, Prm2 and VKn1, all in 2010). At macrophyte-rich sites, *P. pectinatus*, *P. pusillus* and *C. globularis* were particularly common, often (co-)dominating the aquatic vegetation. Other submerged species sporadically reaching high cover levels were *Ceratophyllum demersum* L., *C. vulgaris*, *Elodea nuttallii* (Planch.) H. St. John and *Z. palustris*. VKn2 was completely filled with *C. demersum* throughout the study period. Submerged species richness was relatively high in MlGr (five species in 2010), MlKl

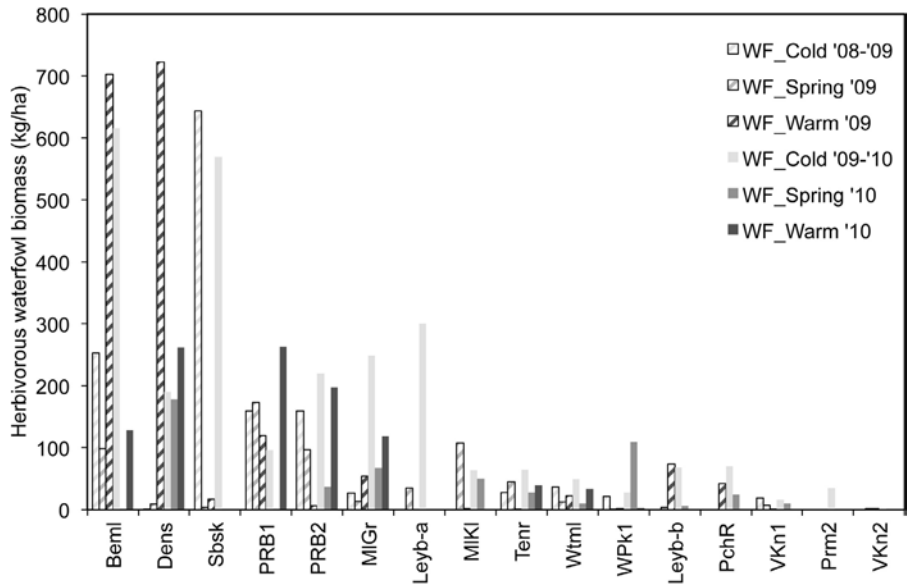


**FIGURE 2 |** Herbivory-related biomass density of waterfowl in the studied ponds during six seasons. WF_Cold, Waterfowl observed in cold periods; WF_Spring, Waterfowl observed in spring periods; WF_Warm, Waterfowl observed in warm periods. Ponds are arranged according to decreasing average waterfowl density.

(five species in 2010), Tenr (six species in both years), and Wtml (four species in 2009 and seven in 2010).

Floating-leaved macrophytes [*Nuphar lutea* (L.) Sm. and *Nymphaea alba* L.] were locally abundant in Beml, WPk1, and Wtml, along the fringes or in smaller patches. During summer, two ponds (Sbsk and VKn1) became gradually covered by lemnids [including *Lemna trisulca* L., *Lemna minor* L., and *Spirodela polyrhiza* (L.) Schleid.], while WPk1 developed extensive detached algal mats [composed of filamentous algae and *Enteromorpha intestinalis* (L.) Nees]. Finally, *Riccia fluitans* L., *Sagittaria sagittifolia* L., and *Veronica anagallis-aquatica* L. were recorded within vegetation transects of a single pond each.

## Macrophyte Species Occurrence in Relation to Propagule Bank and Waterfowl

Macrophyte species exhibited variable relationships with propagule densities and herbivory by avifauna (average of spring, warm, and cold periods), although in this context the influence of both predictor variables on summer vegetation abundance seemed rather weak (**Figures 3**, **4**). Maximum species-specific macrophyte cover values concurred with intermediate propagule densities for *C. globularis*, *C. vulgaris*, *P. pectinatus*, *P. pusillus*, and *Z. palustris*. Nevertheless, for three common angiosperm species (*P. pectinatus*, *P. pusillus*, and *Z. palustris*), maximum cover values were attained at sites with relatively rich propagule banks compared to the pattern observed for charophycean algae. Additionally, *C. demersum*, *E. nuttallii*, *P. pectinatus*, and *P. pusillus* were also able to develop medium or high summer cover along some vegetation transects despite absence of propagules in sediment. *C. obtusangula* was only found within the propagule bank.

For common macrophytes, maximal waterfowl densities – as retrieved for ponds Beml and Dens,– were systematically linked with (near) vegetation absence in summer (**Figures 3**, **4**). In other ponds, the relationship between potential waterfowl herbivory and macrophyte occurrence revealed a fairly chaotic pattern. Some ponds developed high macrophyte cover unhindered by abundant waterfowl residing in or traveling through the area, while in others, species were unable to establish even at low bird biomass densities.

In terms of species incidence, the predictive power of propagule bank and of vegetation in the first year differed among macrophytes (**Figure 5**). *C. obtusangula*, *C. vulgaris*, and *Z. palustris* showed comprehensive unrealized potential, since few viable seeds and oospores managed a transition toward standing populations (**Figure 5**, left). Other species (*C. globularis*, *P. pectinatus,* and *P. pusillus*) expressed their recruiting abilities in the majority of transects. On the other hand, for all species presence within the macrophyte community was not exclusively explained by propagule availability in spring.

Within the vegetation, continuity over consecutive years was high for *C. demersum*, *E. nuttallii* and two common *Potamogeton* species (especially *P. pusillus*), but less for charophycean macroalgae and *Z. palustris* (**Figure 5**, right).

## Relationship Between Propagule Bank and Pond Ecological Status

In 2009, two ponds were turbid (Turbid), three were clear but lacked abundant SAV (Clear L), while eight resided in a macrophyte-dominated state (Clear H; see **Supplementary Material**). In addition, in the first year three newly biomanipulated ponds were completely drawn down for

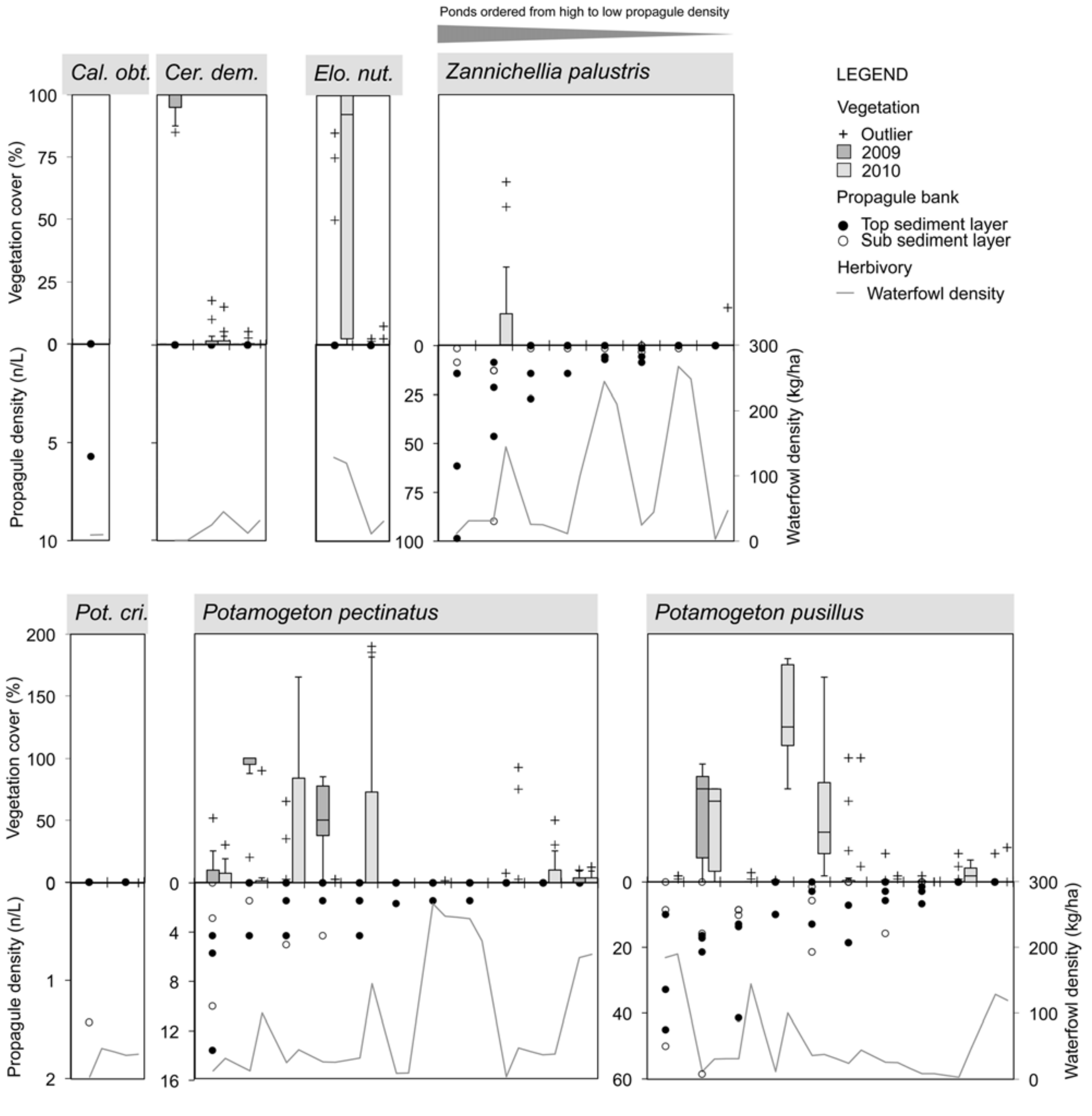


**FIGURE 3 |** Species-specific relationship for Angiospermae between spring sediment propagule density (lower inverted *Y*-axis on left side; data points separately shown: $n = 3$ for top and sub layer each), herbivorous waterfowl biomass (lower *Y*-axis on right side; average of cold, spring, and warm periods) and established species cover in two consecutive summer seasons (upper *Y*-axis; box = 25th percentile-median-75th percentile, whiskers = non-outlier range, crosses = outliers and extremes, $n = 15$). Ponds (*X*-axis) are ordered from high to low propagule densities, excluding those in which the macrophyte species is absent. *Cal. obt.*, *Callitriche obtusangula*; *Cer. dem.*, *Ceratophyllum demersum*; *Elo. nut.*, *Elodea nuttallii*; *Pot. cri.*, *Potamogeton crispus*.

the duration of the growth season. The ensuing year, the three re-inundated ponds showed high water transparency, yet only two were extensively colonized by submerged macrophytes (MlGr and MlKl). In 2010, three ponds were turbid, four had an intermediate ecological state (Clear L) and nine were abundantly vegetated (Clear H). In between years, three ponds switched categories: Leyb-b (from Clear H to Turbid), VKn1 (from Clear H to Clear L) and WPk1 (from Clear L to Clear H).

Compared to macrophyte-dominated waterbodies, turbid ponds featured significantly lower propagule densities in the ecologically accessible top sediment layer (Angiospermae: $p = 0.015$; Charophyceae: $p = 0.022$; **Figure 6**, left and middle; **Table 2**). In clear, vegetation-poor ponds the same layer was

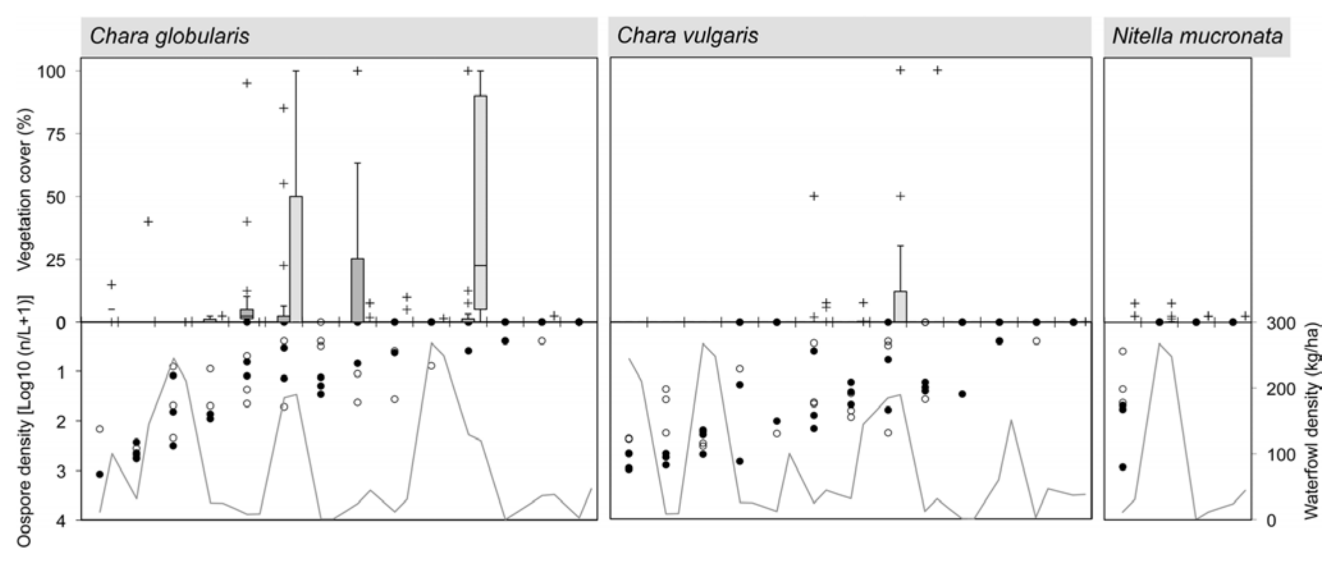


**FIGURE 4 |** Species-specific relationship for Charophyceae between spring sediment propagule density (lower inverted *Y*-axis on left side; data points separately shown: *n* = 3 for top and sub layer each), herbivorous waterfowl biomass (lower *Y*-axis on right side; average of cold, spring, and warm periods) and established species cover in two consecutive summer seasons (upper *Y*-axis; box = 25th percentile-median-75th percentile, whiskers = non-outlier range, crosses = outliers and extremes, *n* = 15). Ponds (*X*-axis) are ordered from high to low propagule densities, excluding those in which the macrophyte species is absent. For legend, see **Figure 3**. Note logarithmic scale for oospore densities.

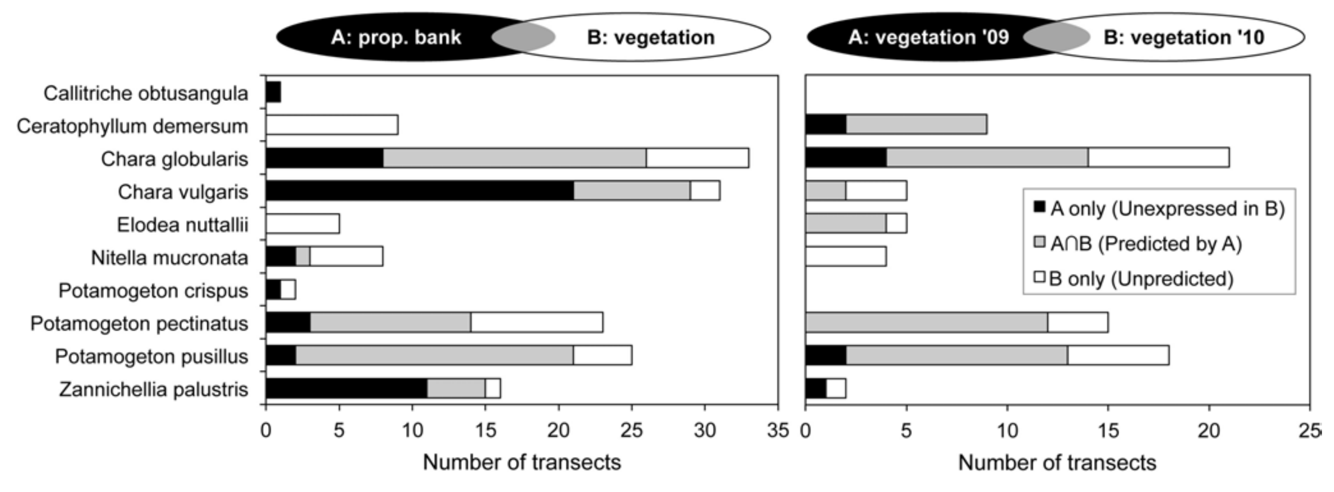


**FIGURE 5 |** Occurrence of submerged macrophytes and similarity between compartments (A vs. B). Gray areas visualize the predictive potential in terms of overlapping species presence. Left: occurrence as propagules in spring propagule bank (A) and as expressed vegetation in summer (B); right: occurrence within vegetation of 2009 (A) and of the following year (B), excluding ponds that were empty in the first year.

characterized by intermediate angiosperm propagule densities, but significantly richer oospore reservoirs in comparison to turbid ponds ($p$ = 0.031). In the deeper layer, the situation was similar for Charophyceae (Clear H vs. Turbid: $p$ = 0.018; Clear L vs. Turbid: $p$ = 0.007), while for Angiospermae there was a significant difference between Clear H and Clear L ponds ($p < 0.001$), with Turbid ponds positioned in-between. Chao-Sørensen similarity between top or sub layer contents and vegetation was highly variable in Clear H and Turbid ponds, although the former group had a tendency for low overlap between compartments (**Figure 6**, right and **Table 2**). Similarity was extremely low in Clear L ponds and significantly weaker compared to Turbid ponds.

## Predictors of Macrophyte Community

The first two axes of the RDA explained 37.8% of variance in the species data and 58.1% of variance in the species-environment relationship (see **Supplementary Material**). In the PCA (**Figure 7**), Clear H ponds were directed toward the clear end of the spectrum (corresponding to higher SD/D values) and clustered in two subgroups, the first being species-rich

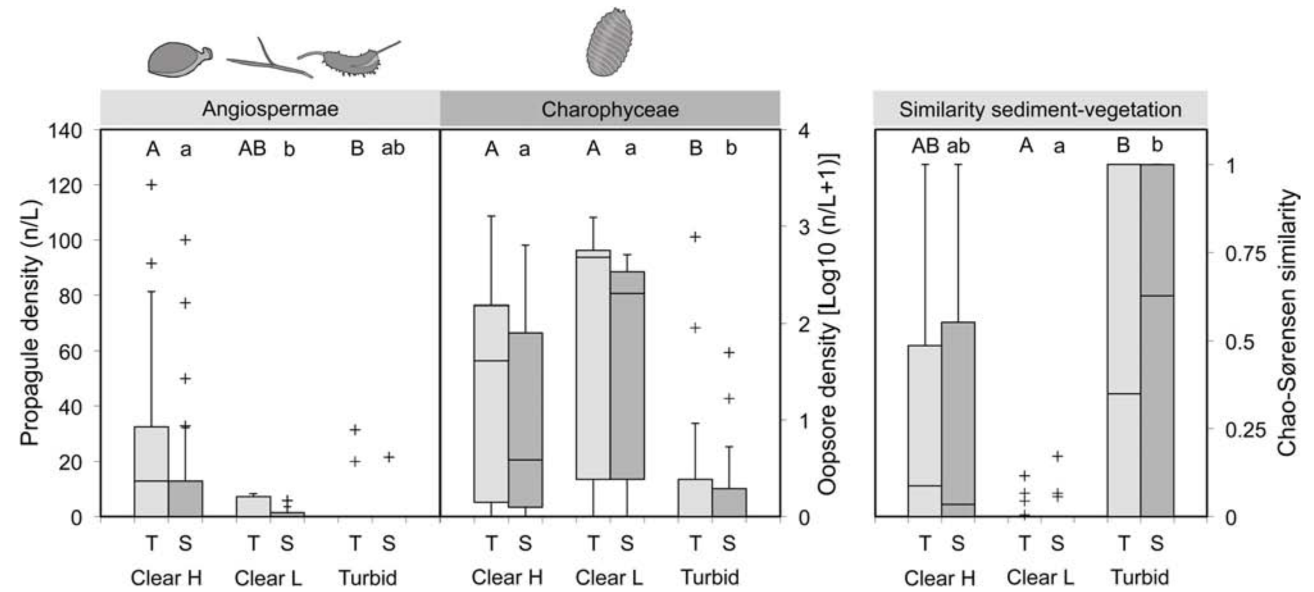


**FIGURE 6 |** Differences in propagule density (left: Angiospermae – turions, tubers, seeds; middle: Charophyceae – oospores) and abundance-based similarity (right) between ponds grouped according to ecological status (Clear H: clear ponds with high SAV cover; Clear L: clear ponds with low SAV cover; Turbid: turbid ponds devoid of SAV). A distinction is made between top (T) and sub (S) sediment layers. box = 25th percentile-median-75th percentile, whiskers = non-outlier range, crosses = outliers and extremes. Significant differences within a specific sediment layer (ZIGLMM output) are marked with contrasting letters (T: capital letters; S: lower case).

and dominated by *P. pectinatus* and *P. pusillus* (associated with higher values of T_Angio and pH), the second related to high *C. globularis*, *C. demersum* and lemnid cover. With a few exceptions, Clear L sites grouped together with Turbid ponds, sharing high values of bare ground. Typical vegetation attributes (i.e., low abundance of macrophytes) of these two ecological categories were linked with high phytoplankton biovolume (Biovol), conductivity (Cond), and waterfowl herbivory in the warm period (WF_Warm).

Forward selection retained five significant contributors to macrophyte community composition (conditional effects; **Table 3**), with decreasing effect size: phytoplankton biovolume (Biovol; $\lambda = 0.10$; $p = 0.004$), angiosperm top-layer propagule density (T_Angio; $\lambda = 0.09$; $p = 0.019$), waterfowl herbivorous biomass density in the warm period (WF_Warm; $\lambda = 0.08$; $p = 0.024$), pH ($\lambda = 0.07$; $p = 0.031$) and conductivity (Cond; $\lambda = 0.06$; $p = 0.026$). Nutrients and cladoceran filtering rate (LCFR) behaved neutral with respect to macrophyte assemblages.

## Predictors of SAV Pond-Scale Cover

We obtained a shortlist of four candidate variables displaying significant singular relationships with SAV cover: phytoplankton biovolume (Biovol), pH, angiosperm propagule density in top layer (T_Angio) and waterfowl herbivory in the warm period (WF_Warm). The maximal summer extent of submerged macrophytes in ponds showed a positive trend with pH (CI range: unidirectionally positive) and a negative relationship with phytoplankton biovolume and waterfowl herbivory in the warm period (CI range: unidirectionally negative; **Table 4** and **Figure 8**). The evidence for an effect of density of angiosperm propagules in the upper sediment layer is less convincing (i.e., CI range bidirectional relative to zero value; **Table 4**).

# DISCUSSION

## Influence of Abiotic Variables and Time Since Biomanipulation

Apart from conductivity and pH, none of the abiotic variables included in the analyses were associated with characteristics of macrophyte composition and abundance. Acidity values were lower in ponds dominated by SAV, especially coinciding with high abundance of tall *Potamogeton pectinatus* (in agreement with Capers et al., 2010) and *P. pusillus*. Nevertheless, pH reached high levels in turbid ponds as well and correlated significantly with oxygen concentrations, indicating decreasing acidity should be interpreted as a by-product of carbon consumption in the process of primary production.

Conductivity was generally relatively low in macrophyte-dominated ponds and high in clear, vegetationless ponds, though not consistently. Possibly, conductivity values were a reflection of a combined effect of photosynthetic activity (Pedersen et al., 2013) and the source of water supply to the waterbody. Although higher values potentially indicate reception of contaminated runoff (Hatt et al., 2004), conductivity remained equivalent to freshwater conditions, and most likely did not profoundly influence macrophyte species filtering or growth.

**TABLE 2 |** Properties of propagule banks and similarity with vegetation in ponds with different ecological status (Clear H, clear ponds with high SAV cover; Clear L, clear ponds with low SAV cover; Turbid, turbid ponds).

| Layer | DV Reference category | Descriptives | | | Contrasted category | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Clear H | | | Clear L | | | Turbid | | |
| | | Min | Median | Max | Est. | *SE* | p | Est. | SE | p | Est. | *SE* | p |
| **Top** | *Angiospermae* | | | | | | | | | | | | |
| | *ClearH* | 0.0 | 12.9 | 120.0 | – | – | – | –2.01 | 1.44 | 0.164 | –4.70 | 1.93 | 0.015* |
| | *ClearL* | 0.0 | 0.0 | 8.3 | 2.01 | 1.44 | 0.164 | – | – | – | –2.70 | 2.15 | 0.209 |
| | *Turbid* | 0.0 | 0.0 | 31.4 | 4.70 | 1.93 | 0.015* | 2.70 | 2.15 | 0.209 | – | – | – |
| | *Charophyceae* (*log*$_{10}$) | | | | | | | | | | | | |
| | *ClearH* | 0.0 | 1.6 | 3.1 | – | – | – | 0.28 | 1.54 | 0.854 | –4.32 | 1.89 | 0.022* |
| | *ClearL* | 0.0 | 2.7 | 3.1 | –0.28 | 1.54 | 0.854 | – | – | – | –4.61 | 2.13 | 0.031* |
| | *Turbid* | 0.0 | 0.0 | 2.9 | 4.32 | 1.89 | 0.022* | 4.61 | 2.13 | 0.031* | – | – | – |
| | *Chao-Sørensen similarity* | | | | | | | | | | | | |
| | *ClearH* | 0.00 | 0.09 | 1.00 | – | – | – | –1.19 | 0.61 | 0.051 | 1.07 | 0.67 | 0.112 |
| | *ClearL* | 0.00 | 0.00 | 0.12 | 1.19 | 0.61 | 0.051 | – | – | – | 2.26 | 0.80 | 0.005** |
| | *Turbid* | 0.00 | 0.35 | 1.00 | –1.07 | 0.67 | 0.112 | –2.26 | 0.80 | 0.005** | – | – | – |
| **Sub** | *Angiospermae* | | | | | | | | | | | | |
| | *ClearH* | 0.0 | 0.0 | 100.0 | – | – | – | –3.04 | 0.65 | 2.9E-6*** | –0.24 | 1.37 | 0.861 |
| | *ClearL* | 0.0 | 0.0 | 5.7 | 3.04 | 0.65 | 2.9E-6*** | – | – | – | 2.80 | 1.46 | 0.056 |
| | *Turbid* | 0.0 | 0.0 | 21.4 | 0.24 | 1.37 | 0.861 | –2.80 | 1.46 | 0.056 | – | – | – |
| | *Charophyceae* (*log*$_{10}$) | | | | | | | | | | | | |
| | *ClearH* | 0.0 | 0.6 | 2.8 | – | – | – | 1.17 | 1.35 | 0.387 | –4.04 | 1.71 | 0.018* |
| | *ClearL* | 0.0 | 2.3 | 2.7 | –1.17 | 1.35 | 0.387 | – | – | – | –5.21 | 1.91 | 0.007** |
| | *Turbid* | 0.0 | 0.0 | 1.7 | 4.04 | 1.71 | 0.018* | 5.21 | 1.91 | 0.007** | – | – | – |
| | *Chao-Sørensen similarity* | | | | | | | | | | | | |
| | *ClearH* | 0.00 | 0.03 | 1.00 | – | – | – | –0.91 | 0.51 | 0.073 | 1.05 | 0.56 | 0.061 |
| | *ClearL* | 0.00 | 0.00 | 0.17 | 0.91 | 0.51 | 0.073 | – | – | – | 1.96 | 0.67 | 0.004** |
| | *Turbid* | 0.00 | 0.63 | 1.00 | –1.05 | 0.56 | 0.061 | –1.96 | 0.67 | 0.004** | – | – | – |

*Significance codes *p < 0.05; **p < 0.01; ***p < 0.001. ZIGLMM results are shown under 'Contrasted category,' as crossed with the reference category. DV, dependent variable; Est., estimate; SE, standard error; p, significance value. Significant differences are indicated with an asterisk.*

The only sign of a potential structuring effect of accumulated (de-icing or other) salts within the study area was the co-occurrence of high conductivity in WPk1 and proliferation of *Enteromorpha intestinalis*, a euryhaline, yet predominantly brackish-water macroalgal species (Martins et al., 1999; Gallego et al., 2015).

Nutrients presumably were not relevant since phosphorus is the primary limiting element in many freshwater ecosystems (Correll, 1998), yet readily available in the strongly eutrophic ponds within the study area (Peretyatko et al., 2012; Teissier et al., 2012). The elapsed time since TSB (more specifically, water drawdown combined with fish removal) did not affect macrophyte dynamics. Søndergaard et al. (2008) pointed out that successful biomanipulation outcomes in shallow lakes tend to last between 2 and 6 years, followed by gradual deterioration after recovery of fish. The ponds included in our study were mostly biomanipulated within 4 years prior to the analyses, and therefore did not show much relationship with the timing of biomanipulation. With only a partial fish reduction that was executed eight years before the onset of the study, the macrophyte-dominated Tenr pond seemed to be exceptionally resilient to degradation, most likely because of a balanced fish community including a strong piscivorous guild (Leefmilieu Brussel, pers. comm.).

## Influence of Phytoplankton and Zooplankton

As expected, high phytoplankton biovolume was the principal driver explaining submerged macrophyte community composition and pond-scale cover. This is in agreement with relative importance of turbidity in shallow lakes in the Netherlands (Van den Berg et al., 2003; van de Haterd and Ter Heerdt, 2007), and emphasizes the mutually exclusive nature of phytoplankton and submerged macrophyte dominance (Scheffer et al., 1993). In the absence of top-down control of phytoplankton, submerged macrophytes are readily out-competed and will disappear (Scheffer et al., 1993). The results stress the need for continuing management efforts to reduce external and internal loading affecting ponds in the region (Søndergaard et al., 2003; Jeppesen et al., 2005; Hilt et al., 2006).

Although zooplankton plays a paramount role in the control of phytoplankton (Jeppesen et al., 1997, 2005), the estimated filtering rate of large Cladocera (LCFR) did not directly relate to any SAV characteristic. Likely this results from the fact that large *Daphnia* in a number of fishless ponds (Beml and Dens)

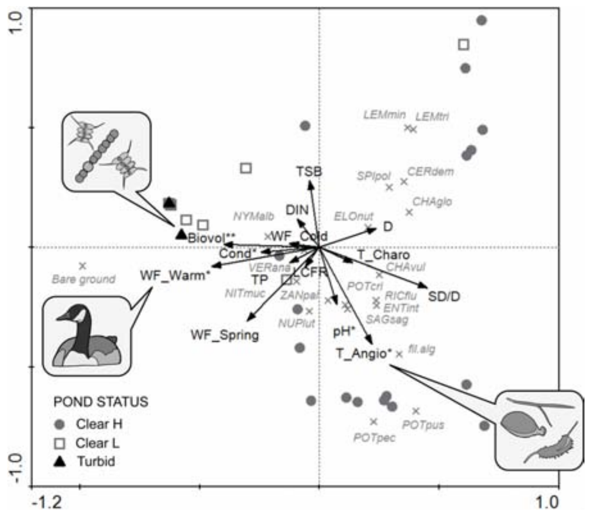


**FIGURE 7 |** PCA triplot of aquatic vegetation communities including supplementary variables (arrows), species (crosses) and sites (symbols). Variables with a significant conditional contribution after RDA forward selection are marked with an asterisk (*$p < 0.05$; **$p < 0.01$). See **Table 1** for codes of environmental variables. Species abbreviations: CERdem, *Ceratophyllum demersum*; CHAglo, *Chara globularis*; CHAvul, *C. vulgaris*; ELOnut, *Elodea nuttallii*; ENTint, *Enteromorpha intestinalis*; fil. alg, filamentous algae; LEMmin, *Lemna minor*; LEMtri, *L. trisulca*; NITmuc, *Nitella mucronata*; NUPlut, *Nuphar lutea*; NYMalb, *Nymphaea alba*; POTcri, *Potamogeton crispus*; POTpec, *P. pectinatus*; POTpus, *P. pusillus*; RICflu, *Riccia fluitans*; SAGsag, *Sagittaria sagittifolia*; SPIpol, *Spirodela polyrhiza*; VERana, *Veronica anagallis-aquatica*; ZANpal, *Zannichellia palustris*.

maintained high water clarity, while SAV did not successfully develop. Similarly, the filtering capacity of zooplankton in vegetated ponds can easily be misjudged if large Cladocera exhibit diel vertical (e.g., to and from *Chara* meadows) or horizontal migration (Burks et al., 2002), or if the contribution of macrophyte-associated cladocerans to total phytoplankton clearance potential is significant (Balayla and Moss, 2004).

**TABLE 3 |** RDA results for marginal (unique) and conditional (within the constructed model) effects of environmental variables measured in ponds.

| Marginal effects | | Conditional effects | | |
|---|---|---|---|---|
| **Variable** | **Lambda1** | **Variable** | **LambdaA** | **P** |
| T_Angio | 0.09 | Biovol | 0.10 | 0.004** |
| SD/D | 0.09 | T_Angio | 0.09 | 0.019* |
| WF_Warm | 0.08 | WF_Warm | 0.08 | 0.024* |
| Biovol | 0.07 | pH | 0.07 | 0.031* |
| Cond | 0.06 | Cond | 0.06 | 0.026* |
| WF_Spring | 0.06 | LCFR | 0.04 | 0.095 |
| D | 0.05 | T_Charo | 0.04 | 0.161 |
| pH | 0.05 | WF_Spring | 0.04 | 0.171 |
| WF_Cold | 0.05 | SD/D | 0.04 | 0.243 |
| T_Charo | 0.04 | WF_Cold | 0.03 | 0.237 |
| TSB | 0.03 | TP | 0.02 | 0.317 |
| LCFR | 0.03 | D | 0.02 | 0.663 |
| DIN | 0.02 | DIN | 0.01 | 0.605 |
| TP | 0.02 | TSB | 0.01 | 0.810 |

*Asterisks highlight significant variables (*$p < 0.05$; **$p < 0.01$). For variable abbreviations, see* ***Table 1****.*

**TABLE 4 |** Summary of multimodel inference after multiple regression with SAV pond-scale cover as dependent variable ($n = 29$).

| Parameter | Averaged estimate | Unconditional SE | Unconditional CI | Relative importance |
|---|---|---|---|---|
| Intercept | −0.41 | 0.21 | (−0.82, 0.01) | |
| Biovol | −1.08 | 0.24 | (−1.56, −0.60)* | 1.000 |
| pH | 0.92 | 0.24 | (0.45, 1.39)* | 0.990 |
| T_Angio | 0.27 | 0.23 | (−0.18, 0.71) | 0.274 |
| WF_Warm | −0.70 | 0.21 | (−1.11, −0.28)* | 0.971 |

*Confidence intervals (CI) marked with * indicate a unidirectional relationship with the predictor variable, suggesting a true effect on SAV cover. Relative importance based on summed AICc (Akaike Information Criterion for finite sample size) weights of models containing the variable.*

## Propagule Banks

Propagule banks in the peri-urban ponds were rarely species-rich and displayed low taxon evenness, because of a dominance of charophycean oospores. Oospores are ubiquitous in many freshwater sediments (de Winton et al., 2000; Bonis and Grillas, 2002; Kalin and Smith, 2007), and high densities might be required for establishment and colonization (Van den Berg et al., 2001). Angiosperm propagules were far less abundant, although any direct comparison with oospore densities would be biased given the small size of oospores. Correcting for propagule dimensions could enable to offset variations in storage capacity, providing a method to evaluate the relative contribution of each species to the propagule bank potential.

Since propagule densities in top- and sub-layers of the sediment were correlated, we focused on the upper 5 cm, supposed to be in close contact with ecological processes in the water column (Dugdale et al., 2001; Bonis and Grillas, 2002; Spencer and Ksander, 2002). However, the extent to which bioturbation by burrowing unionid bivalves, foraging benthivorous fish or waterfowl could mix layers and homogenize strata should not be underestimated (McCall et al., 1995; Vaughn and Hakenkamp, 2001; Huser et al., 2016).

Several elements indicated that the composition and density of the propagule bank plays a significant, albeit modest role in the structuring of SAV in nutrient-enriched ponds. Overall, propagules mainly influenced the assemblage of the macrophyte community (with wealthy stocks of angiosperm propagules accompanying development of small-leaved *Potamogeton* communities), rather than secured SAV dominance.

At the level of individual submerged macrophyte species, the relationship between recruitment reserves and cover within the aquatic vegetation could be summarized as ambiguous. The status of the propagule bank matched maximal vegetation abundance of the most common Angiospermae (*P. pectinatus*, *P. pusillus*, and *Zannichellia palustris*) slightly better than was the case for Charophyceae (indicating some positive influence of high propagule density on vegetation cover), but for each species there

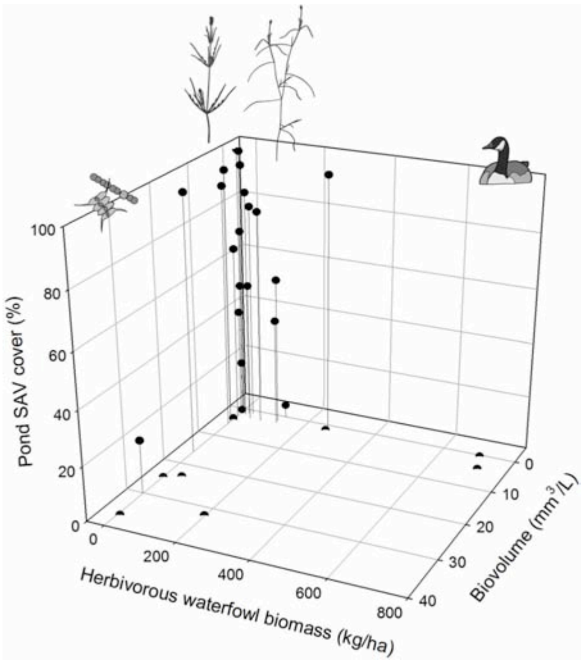


**FIGURE 8 |** Maximal pond-scale SAV cover in relation to herbivory-related waterfowl density in the warm period (WF_Warm) and phytoplankton biovolume.

were clear dissimilarities depending on the pond. Propagules of *Chara vulgaris* and *Z. palustris* poorly contributed to standing vegetation. Recruitment failure of certain macrophyte species suggests that environmental conditions in the waterbody are not beneficial for germination or growth (the impact of which is mitigated by dormancy of the propagules; Bewley, 1997; Sederias and Colman, 2007; Hay et al., 2008; Nonogaki, 2014).

Local occurrence of submerged macrophytes was not exclusively predicted by presence of propagules, likely because most are capable of vegetative, either non-specialized (e.g., *Ceratophyllum demersum* and *Elodea nuttallii*) or rhizomatous (e.g., *P. pectinatus* and *Z. palustris*), spread and survival (Van Vierssen, 1982; Barrat-Segretain, 1996; Boedeltje et al., 2004; Vári, 2013; Vári and Tóth, 2017). For Charophyceae, establishment without prior availability of oospores would have been less plausible, suggesting that prevailing germination conditions did not always break dormancy (Sederias and Colman, 2007).

Importantly, turbid ponds contained less angiosperm and charophycean propagules in the top sediment layer compared to macrophyte-dominated, clear ponds. Turbid ponds in the Brussels-Capital Region possess high densities of zooplanktivorous and benthivorous fish, resulting in persistent phytoplankton dominance over multiple years and a need for biomanipulation to force a shift. Following a crash of macrophyte domination and winter resetting of autotrophic production, moderate recruitment in spring might start depleting the propagule bank. In case dispersal input does not compensate for the loss, opportunities for submerged macrophyte establishment finally will be lost, leaving the ruined propagule bank as a critical bottleneck for ecosystem recovery. On the contrary, in the shallow Lake Terra Nova (Netherlands), van de Haterd and Ter Heerdt (2007) did not identify propagule limitation after a long period of turbidity. Here, (within-lake) dispersal or less intense phytoplankton blooming could have allowed sparse macrophyte reproduction under turbid conditions. Alternatively, Lake Terra Nova might experience less intense organic matter accumulation on top of propagule-rich sediment when compared to the sapropelium-rich turbid ponds in our study.

Interestingly, propagule densities in clear but sparsely vegetated ponds typically seemed sufficient, though not fully translated as a result of waterfowl herbivory (Beml and Dens in 2009), lemnid cover (VKn1 in 2010), or some other factor affecting SAV. This results in significantly lower similarity between sediment content and vegetation composition when compared to turbid ponds, where submerged macrophytes remain underrepresented in both compartments.

In terms of overall ecological status, the emerging trend seems to be that: (1) for clear, macrophyte-dominated ponds the propagule potential is realized; (2) clear ponds with low SAV cover are unable to utilize their potential; and (3) in turbid ponds recruitment opportunities are inadequate. This is different from patterns observed in interconnected fishing ponds in France, where Arthaud et al. (2012) found no relationship between density of propagules and Chl *a* concentrations in the water, supposedly because propagule banks in the French pond system are regularly replenished – either through dispersal or during recurrent clear-water phases. If propagule banks are known to be impoverished, reproductive success and natural dispersal functioning could be assessed before proceeding to active assistance of macrophyte establishment (Smart et al., 1998; Hilt et al., 2006; Van Onsem et al., 2018).

## Waterfowl Herbivory

During the warm period of the growth season, peaks of plant-consuming waterfowl coincided with negligible SAV abundance, even in the presence of high propagule densities and in the absence of fish or turbidity stress. This confirms the need to incorporate macrophyte herbivory in aquatic research (Bakker et al., 2016; van Altena et al., 2016; Wood et al., 2017). The ranking of waterbird herbivory as second most important biotic predictor following phytoplankton-induced turbidity is consistent with van de Haterd and Ter Heerdt (2007).

It is difficult to situate the avifauna biomass densities obtained for Brussels ponds with respect to other regions, because of the use of peak densities instead of the periodic averages provided by Wood et al. (2012). Nonetheless, maximum densities found in our study seem considerable at times, potentially provoking loss of macrophytes on short notice. The swarming of swans, geese, shelducks, and ducks becomes especially problematic when population sizes overstretch the carrying capacity of the waterbody. In urbanized areas, the pond perimeter can offer a copious supply of staple or alternative food in the form of park lawns and thrown-away bread, thereby attracting and potentially bonding birds to a particular location. Near small, shallow ponds, such an aggregation of opportunistic herbivores lowers the likelihood of large-scale and sustainable SAV establishment.

Nevertheless, the impact of waterfowl seemed to be restricted to direct grazing or disturbance in summer. Although autumn foraging on seeds and tubers or spring grazing on freshly emerged shoots could have had a time-lagged effect (Marklund et al., 2002; Hidding et al., 2010b), high waterfowl biomass density during the spring or the cold season did not weigh on macrophyte performance at the height of the growth season. With an average depth of 0.90 m, the studied ponds are rarely sufficiently deep to prevent herbivory on belowground or freshly emerging biomass. Presumably, waterfowl occurring in the area of ponds was less inclined to forage directly on the water during colder periods. Limited disturbance by waterfowl during the spring establishment phase is in accordance with coot effects found in British broads (Perrow et al., 1997) and a Turkish lake (Sandsten et al., 2005).

Overall, the results illustrate that ecological pond management in densely populated regions should comprise some form of waterfowl control, especially during summer. This can be achieved by prohibiting bird feeding, in combination with regulation of resident, invasive alien waterfowl (specifically *A. aegyptiacus* and *B. canadensis*).

## CONCLUSION

Given the unambiguous ecological services provided by submerged macrophytes, it is important to understand the relative role of various factors influencing their composition and abundance. We demonstrate that SAV structure depended on three biotic drivers that were tested, namely phytoplankton-induced turbidity, summer waterfowl and propagule banks – in that order of importance. Both turbidity and high summer abundance of large-sized Anatidae species negatively affected submerged macrophyte occurrence. No time-lagged effects of high waterfowl biomass density in cold or spring periods were observed. Propagule banks, on the other hand, displayed poor potential in turbid ponds, indicating an obstacle for establishment. In the studied pond system, rich propagule potential did not guarantee successful colonization, though represented a typical trait of macrophyte-dominated waterbodies and seemed to stimulate small-leaved *Potamogeton* communities. Overall, waterfowl and macrophyte propagule banks are relevant ingredients of pond ecosystem functioning, even within peri-urban areas.

## AUTHOR CONTRIBUTIONS

The study was conceived and designed by LT and SVO. SVO collected and processed samples, performed statistical analyses and redacted a draft paper, guided, finalized and approved by LT.

## FUNDING

Financial support for this study was obtained from the Vrije Universiteit Brussel (BAS42) and Leefmilieu Brussel.

## ACKNOWLEDGMENTS

We thank Leefmilieu Brussel for allowing access to ponds in the Brussels-Capital Region, and are grateful to Jardin Botanique Jean Massart (ULB) for the provision of greenhouse space. We thank all those involved in fieldwork, data gathering, and lab processing: S. De Backer, A. De Meirlier, S. Labiese, A. Peretyatko, T. Sierens, I. Stiers, S. Teissier, Y. Troucheau, L. Van Onsem, and X. Vermeersch.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01514/full#supplementary-material

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 05 November 2018
doi: 10.3389/fpls.2018.01608

# Influence of Differ P Enrichment Frequency on Plant Growth and Plant C:N:P in a P-Limited Subtropical Lake Wetland, China

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OPEN ACCESS

**Edited by:**
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**Reviewed by:**
Fuzhong Wu,
Sichuan Agricultural University, China
Xinhou Zhang,
Northeast Institute of Geography and Agroecology (CAS), China

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**Specialty section:**
This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science

**Received:** 31 March 2018
**Accepted:** 17 October 2018
**Published:** 05 November 2018

**Citation:**
Li F, Hu C, Xie Y, Liu W, Chen X, Deng Z and Hou Z (2018) Influence of Differ P Enrichment Frequency on Plant Growth and Plant C:N:P in a P-Limited Subtropical Lake Wetland, China. Front. Plant Sci. 9:1608. doi: 10.3389/fpls.2018.01608

Phosphorus (P) enrichment as a result of anthropogenic activities can potentially alter plant C:N:P stoichiometry. However, the influence of different P enrichment frequencies on plant C:N:P stoichiometry in P-limited ecosystems is still unclear. In this study, we conducted a P-addition experiment to elucidate the effect of various P enrichment frequencies on the plant C:N:P stoichiometry of *Carex brevicuspis* in a freshwater wetland at Dongting Lake, China. We used four P enrichment frequencies (treatment A: no P addition; treatment B: three 0.1 g $kg^{-1}$ additions at 10-day intervals; treatment C: two 0.15 g $kg^{-1}$ additions at 15-day intervals; and treatment D: one 0.3 g $kg^{-1}$ addition during the experimental period) in a factorial design with an experimental duration of 30 days. Biomass accumulation was lowest in the treatment A and highest in the treatment C, and increased with decreasing P addition frequency. The shoot:root ratio did not differ significantly between the four treatments. Both foliar and root C concentrations were not significantly different between the treatments. Foliar N concentration was significantly lower in the treatment D than in the other three treatments, while root N concentration did not differ significantly between the treatments. Both foliar and root P concentrations, and foliar C:N were much higher in the treatment B than in the treatment A. However, root C:N did not differ significantly between treatments. Both foliar and root C:P and N:P of *C. brevicuspis* were lower in the treatment B than in the treatment A. These results indicated that different frequencies of P addition significantly influenced plant growth. Moreover, P enrichment, rather than frequency, significantly influenced plant C:N:P stoichiometry. Our results improve our understanding of the influence of different P enrichment frequencies on plant C:N:P stoichiometry and nutrient cycling in freshwater wetlands.

**Keywords: *Carex brevicuspis*, plant C:N:P stoichiometry, P enrichment frequency, biomass accumulation, biomass allocation**

## INTRODUCTION

Phosphorus (P) plays an important role in determining plant growth and community structure in both terrestrial and aquatic ecosystems (Elser et al., 2007; He and Dijkstra, 2015; Mao et al., 2016). Recently, anthropogenic discharges of P have doubled the natural P loading due to the intensity of activities such as aquaculture and agriculture (Mao et al., 2015). These large quantities of P are discharged into wetland ecosystems, leading to a series of ecological and environmental problems, such as eutrophication and biodiversity reduction (Rashid and Romshoo, 2013; Bu et al., 2016).

Relative abundances of carbon (C), nitrogen (N), and P, and C:N:P stoichiometry in plants are powerful indicators of ecological processes, e.g., community organization, nutrient limitation, food webs, and decomposition (Elser et al., 2000; Güsewell et al., 2003; Xia et al., 2014). Therefore, studies on plant ecological stoichiometry enhance our understanding of the growth and nutrient-use strategies of plants, and their responses to various environmental stresses. For example, plant C:N:P stoichiometry is significantly affected by external nutrient availability (Elser et al., 2007; Mao et al., 2016). To date, the influence of increased P loading on plant C:N:P stoichiometry has been studied in various wetland ecosystems (Rejmánková et al., 2008; Mao et al., 2016). Most of these studies confirmed that increasing P loading enhanced plant P concentration and decreased C:P, while results regarding the influence of increasing P loading on plant N concentration, C:N, and N:P were inconsistent across studies (Mao et al., 2016). In P-limited ecosystems, P enrichment would promote plant growth and reduce plant N concentrations and N:P ratios, mainly due to the dilution effect (Feller et al., 2007; Mao et al., 2016). However, in N-limited ecosystems, the response of plant N concentration, C:N ratio and N:P ratio might be determined by the plant's nutrient use strategies (Yuan and Chen, 2015; Mao et al., 2016). Therefore, more studies are needed to investigate the general influence of P enrichment on plant C:N:P stoichiometry.

Nutrient concentrations can increase at different rates over different spatiotemporal scales, which influences plant growth performance (Xie et al., 2004; Zhang et al., 2018). For instance, biomass allocation patterns and P allocation ratios in *Eichhornia crassipes* differed significantly with different modes of nutrient increase (Xie et al., 2004). Different nutrient enrichment rates would alter soil nitrogen-phosphorus imbalances, affecting the structure, function, and diversity of ecosystems and organisms (Peñuelas et al., 2013). Sun et al. (2018) also confirmed that different N and P input ratios significantly changed the levels of N, P, and other elements in plants, and that the influence differed among different plant organs. While many studies have investigated the influence of P enrichment on plant C:N:P stoichiometry in different types of wetlands (Newman et al., 2004; Feller et al., 2007; Mao et al., 2016), the effects of different P enrichment frequencies on plant stoichiometry remain unclear.

This study investigated the effects of different P enrichment frequencies on wetland plant stoichiometry at Dongting Lake wetland, China. This lake is the second largest freshwater lake in China and has the largest water exchange capacity with the Yangtze River (Xie and Chen, 2008). Our previous studies confirmed that plants were limited by P in this lake (Li et al., 2017, 2018), but it has received increasing inputs of P mainly due to the use of agricultural fertilizer in the local area. The quantity of P input into the lake is about $4.1 \times 10^4$ t annually (He et al., 2009). Here, we report the changes in plant C:N:P stoichiometry and growth performance of *Carex brevicuspis* after four different P enrichment frequency treatments. Based on the above statement, we hypothesized that (1) P enrichment would promote the growth of *C. brevicuspis* and the influence differ with different P enrichment treatments; (2) P enrichment would increase plant P concentration and C:N ratio, but reduce plant N concentration, C:P and N:P ratios. Moreover, these influences would differ with different P enrichment frequencies.

## MATERIALS AND METHODS

### Study Site

Dongting Lake (28°30′–30°20′ N, 111°40′–113°10′ E) is located on the south bank of the mid-section of the Yangtze River (Xie and Chen, 2008). The lake receives inflow from four rivers (Xiang, Zi, Yuan, and Li) in Hunan Province and four channels (Songzikou, Taipingkou, Ouchikou, and Tiaoxiankou) connect it to the Yangtze River (**Figure 1**). The wetlands are characterized by large seasonal fluctuations in water level and are usually completely flooded from May to October and susceptible to drought from November to April. The mean annual temperature is 16.8°C, with hot summers (June to August, 27.3°C) and cold winters (December to February, 5.8°C). Annual precipitation is 1382 mm, with more than 60% falling from April to August (Deng et al., 2015).

### Study Species

*Carex brevicuspis* is widely distributed in Taiwan and eastern mainland China (Dai et al., 2010). At Dongting Lake, this species can form mono-dominant communities or co-exist with other species e.g., *Miscanthus sacchariflorus* and *Polygonum hydropiper*. *C. brevicuspis* usually has two growing phases which are related to changes in the water rhythm in Dongting Lake, usually flowering and fruiting in April or May before the flooding, and remaining completely submerged during the flooding season. After flooding, the shoots emerge immediately (November) and grow until January. In January, the above-ground plant parts wither. Subsequently, new ramets emerge and grow rapidly in February and/or March (Deng et al., 2015). In the Dongting Lake, the *C. brevicuspis* community plays an important role in biodiversity maintenance owing to its multiple ecological functions, for example as a food resource for migratory birds and a spawning ground for migratory fish.

Plants were collected in May 2017 from Junshan county (29°22′ N, 112°59′ E), East Dongting Lake wetland. Small blocks (25 × 25 cm) of *C. brevicuspis* vegetation were cut and transported to an experimental field at the Dongting Lake Station for Wetland Ecosystem Research, Chinese Academy of Sciences. The blocks were placed in plastic buckets (87 × 65 × 62 cm), which contained 20 cm soil (7.07 mg $g^{-1}$ organic matter, 0.87 mg $g^{-1}$ total N,

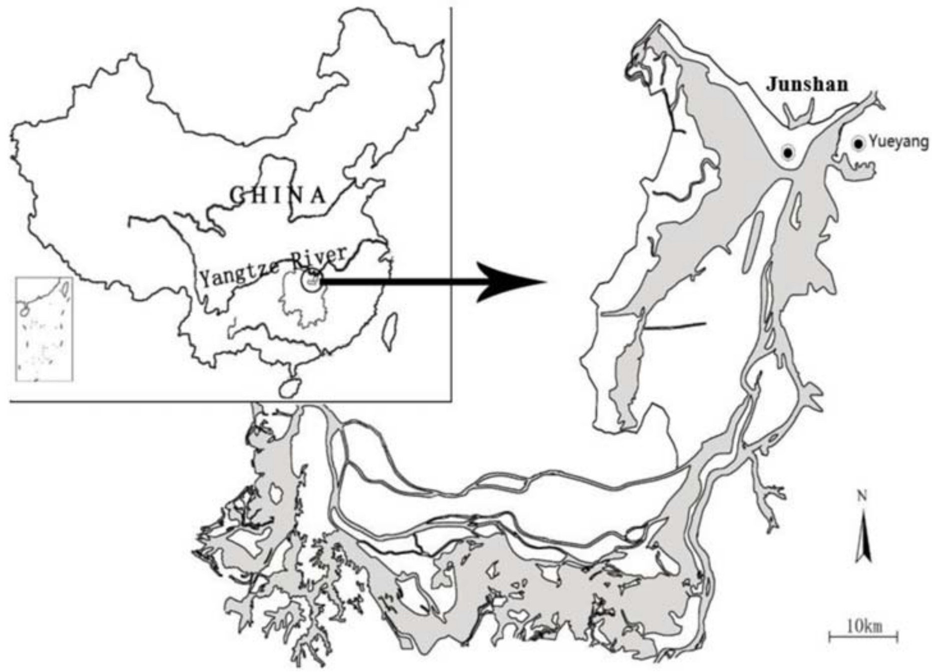


**FIGURE 1 |** Dongting Lake, showing the location of the study site. The shaded areas represent the wetlands.

and 0.73 mg $g^{-1}$ total P; **Table 1**) to germinate new ramets. Soil was also collected from the area (0–20 cm depth) in which the *C. brevicuspis* plants were collected. The plants were watered once weekly with tap water (0.511 μg $L^{-1}$ $NH_4^+$−N, 1.760 μg $L^{-1}$ $NO_3$−N, 0.527 μg $L^{-1}$ $PO_4^{3+}$−P, pH = 7.2).

## Experimental Design

Four P enrichment treatments were applied: treatment A – no P addition; treatment B – three 0.1 g $kg^{-1}$ P additions at 10-day intervals; treatment C – two 0.15 g $kg^{-1}$ P additions at 15-day intervals; and treatment D – one 0.3 g $kg^{-1}$ P addition during the experimental period.

On July 11, 2017, 840 seedlings of similar size (3–4 leaves, 20 ± 3 cm in height) were transplanted into 56 pots (30 cm height, 23 cm diameter, and 15 seedlings per pot), which were filled with 7 kg soil (same soil as for seedling cultivation). All pots were placed into 1 of 7 cement ponds (100 × 100 × 100 cm, two pots per treatment per pond) in a random block design. Experimental treatments began on July 18, 2017. Phosphorus was added as $NaH_2PO_4$. Firstly, the required mass of $NaH_2PO_4$ was dissolved in 150 ml tap water and then sprayed uniformly into the pot. For each P addition treatment, the pots that did not receive $NaH_2PO_4$ were leached using 150 ml tap water. Each pot was supplied 500 mL tap water once weekly and exposed to natural sunlight.

## Harvest

The plants were harvested 30 days after the first P treatment, before they flowered. Plant roots were carefully dug out by hand and rinsed using tap water to remove sediment. Then, plant parts were separated into leaves and roots (root and rhizome), due to the rhizome is difficult to separated. All parts were oven dried at 80°C for 48 h and weighed. Biomass accumulation was calculated as the collective mass of all tissues. Shoot:root ratio was defined as the ratio of leaf mass to root mass. After plant harvest, we collected soil samples at each pot for soil analysis.

**TABLE 1 |** Soil characteristics (mean ± SE) after different P addition frequency treatments (treatments A–D represent: no P addition treatment; three – time P addition treatment; two-time P addition treatment; and one-time P addition treatment, respectively).

| Treatments | Total nitrogen content (mg $g^{-1}$) | Total phosphorus content (mg $g^{-1}$) | Organic carbon content (mg $g^{-1}$) | C:N | C:P | N:P |
|---|---|---|---|---|---|---|
| A | $0.87 \pm 0.02^{a}$ | $0.73 \pm 0.01^{b}$ | $7.07 \pm 0.14$ | $8.20 \pm 0.14^{b}$ | $9.69 \pm 0.15$ | $1.18 \pm 0.02^{a}$ |
| B | $0.82 \pm 0.02^{ab}$ | $0.77 \pm 0.01^{ab}$ | $7.23 \pm 0.15$ | $8.87 \pm 0.14^{ab}$ | $9.42 \pm 0.17$ | $1.07 \pm 0.03^{ab}$ |
| C | $0.79 \pm 0.05^{ab}$ | $0.79 \pm 0.02^{a}$ | $7.14 \pm 0.25$ | $9.25 \pm 0.42^{a}$ | $9.11 \pm 0.34$ | $1.00 \pm 0.06^{bc}$ |
| D | $0.72 \pm 0.04^{b}$ | $0.77 \pm 0.02^{ab}$ | $6.87 \pm 0.24$ | $9.64 \pm 0.25^{a}$ | $8.92 \pm 0.33$ | $0.94 \pm 0.05^{c}$ |

*Different letters indicate significant differences among treatments at the 0.05 significance level.*

## Laboratory Analysis

Dry foliar and root samples were ground for further analysis. Total N and C concentrations were measured using an elemental analyzer (Vario MAX CN, Elementar, Germany), and total P concentration was measured using the molybdenum blue colorimetric method after digesting the samples in a solution of $H_2SO_4$ and $H_2O_2$ (Zhang et al., 2015).

Soil samples were air-dried and sieved through a 0.15 mm sieve before analysis. Soil organic C content was measured using wet oxidation of organic matter with a solution of $KCr_2O_7$ and $H_2SO_4$, followed by back-titration using $FeSO_4$. Soil N concentration was measured using the Kjeldahl method and soil P concentration was measured using acid digestion with a solution of $H_2SO_4$ and $HClO_4$ (Zhang et al., 2015).

## Data Analysis

One-way analysis of variance (ANOVA) was performed in conjunction with Duncan's test to determine the effect of P addition frequency on biomass accumulation, shoot:root ratio, and plant stoichiometry of *C. brevicuspis*, as well as soil characteristics (total N, total P, organic carbon content, C:N, C:P, and N : P). Tukey's *post hoc* tests were used for multiple comparisons. Data were log10-transformed where necessary to reduce the heterogeneity of variance. Liljefor's test and Levene's test were used to test the normality and homogeneity of data, respectively. All statistical analyses were conducted using SPSS ver. 15.0 (SPSS Inc., Chicago, IL, United States).

# RESULTS

## Biomass Accumulation and Shoot:Root Ratio

The P enrichment frequency had a significant influence on the biomass accumulation of *C. brevicuspis* ($F = 9.562$; df = 6; and $P < 0.001$; **Figure 2A**), which was lowest in the treatment A and highest in the treatment C. Moreover, biomass accumulation increased with decreasing P enrichment frequency. In contrast, shoot:root ratio did not differ significantly between the four frequency treatments ($F = 0.103$; df = 6; and $P > 0.05$; **Figure 2B**).

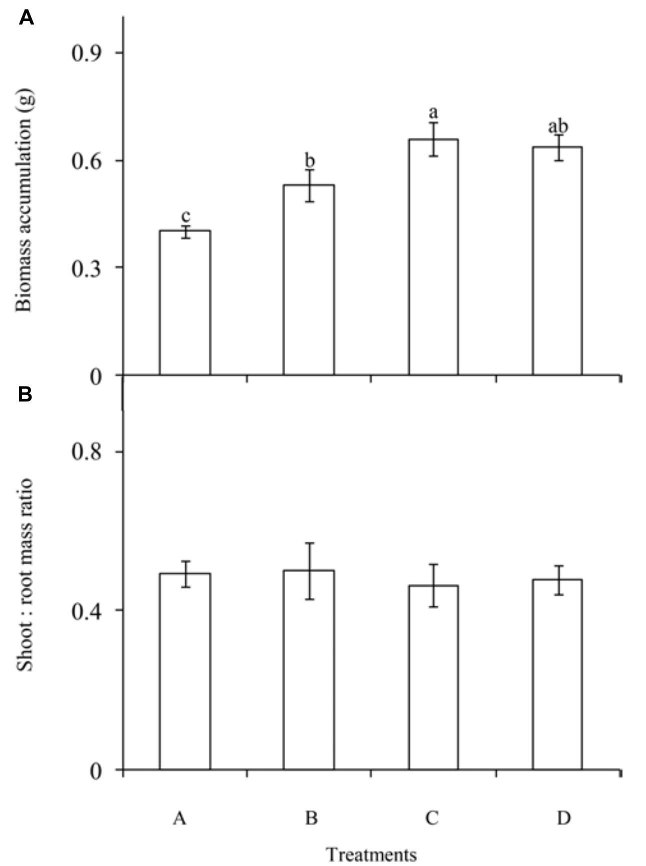


**FIGURE 2 |** Biomass accumulation **(A)** and allocation **(B)** of *Carex brevicuspis* (means ± standard errors, $n = 7$) under different P addition frequencies (treatments A–D represent: no P addition treatment; three - time P addition treatment; two-time P addition treatment; and one-time P addition treatment, respectively). Different letters indicate significant differences between treatments at the 0.05 significance level.

## Foliar Stoichiometry

The frequency of P addition had no significant influence on the foliar total C concentration of *C. brevicuspis* ($F = 2.041$; df = 6; and $P > 0.05$; **Figure 3A**), although it was lower in the treatment D than in the other treatments. Foliar total N was significantly affected by P addition frequency ($F = 3.465$; df = 6; and $P < 0.05$; **Figure 3B**), which was much lower in the treatment D than in the other three treatments. Foliar total P concentration was much higher in the three P addition treatments than in the control ($F = 30.219$; df = 6; and $P < 0.05$; **Figure 3C**), but did not differ significantly between the three P addition treatments.

Foliar C:N showed a similar trend as foliar P concentration, which was much higher in the three addition treatments than in the control ($F = 4.009$; df = 6; and $P < 0.05$; **Figure 3D**), but did not differ significantly among the three P addition treatments. Both foliar C:P ($F = 47.787$; df = 6; and $P < 0.05$; **Figure 3E**) and foliar N:P ($F = 61.053$; df = 6; and $P < 0.05$; **Figure 3F**) showed similar trends, and were much lower in the three P addition treatments than in the control.

## Root Stoichiometry

Root total C ($F = 2.223$; df = 6; and $P > 0.05$; **Figure 4A**) and total N ($F = 1.299$; df = 6; and $P > 0.05$; **Figure 4B**) were not significantly different between the treatments. Different P addition treatments significantly influenced the root P content ($F = 15.931$; df = 6; and $P < 0.05$; **Figure 4C**), which was much higher in the three P addition treatments than in the control, and the highest root P content occurred in the treatment C.

Root C:N did not differ significantly between the treatments ($F = 0.541$; df = 6; and $P > 0.05$; **Figure 4D**). Both root C:P ($F = 31.954$; df = 6; and $P < 0.05$; **Figure 4E**) and N:P ($F = 12.001$; df = 6; and $P < 0.05$; **Figure 4F**) were significantly lower in the three P addition treatments than in the control, but were not significantly different between the three P addition treatments.

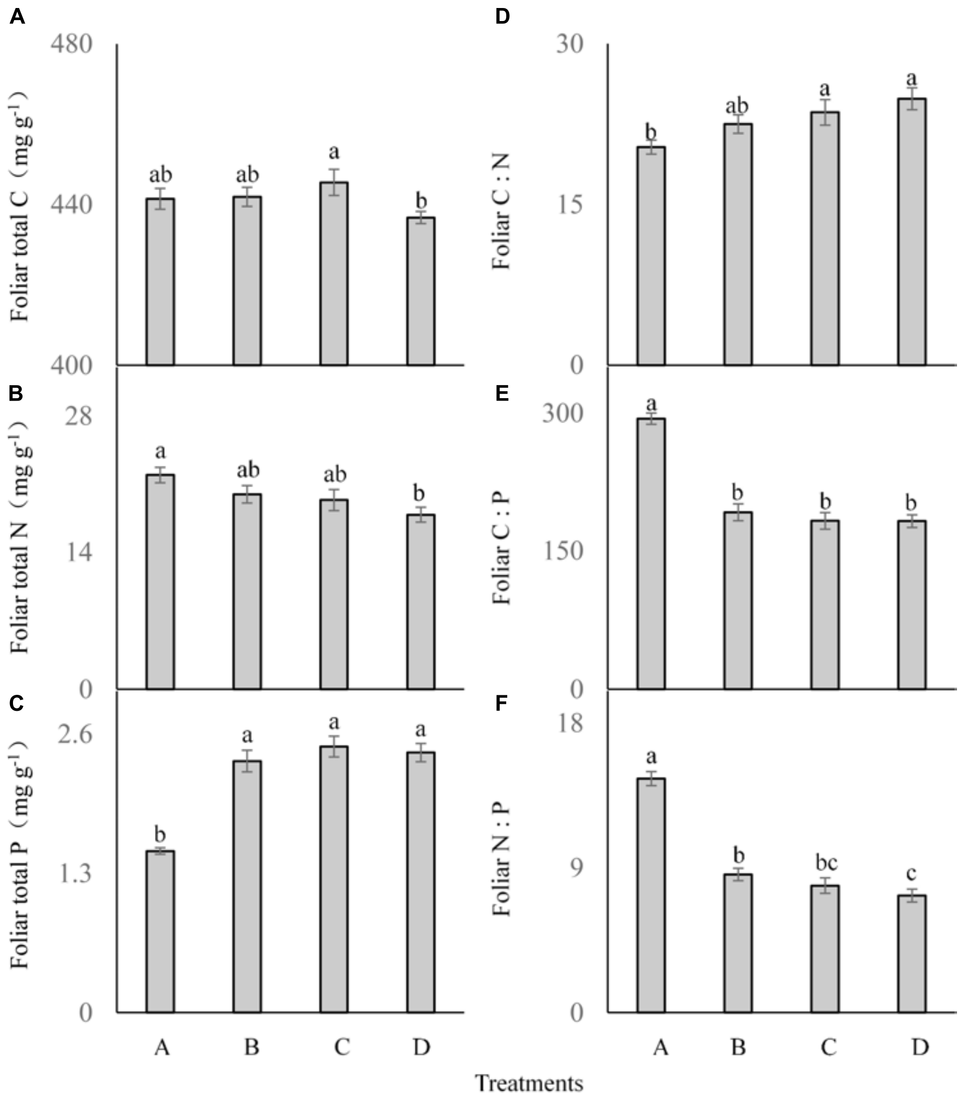


**FIGURE 3 |** Foliar ecological stoichiometry **(A–F)** of *Carex brevicuspis* (means ± standard errors, $n = 7$) under different P addition frequencies (treatments A–D represent: no P addition treatment; three - time P addition treatment; two-time P addition treatment; and one-time P addition treatment, respectively). Different letters indicate significant differences between treatments at the 0.05 significance level.

## DISCUSSION

Our study confirmed that biomass accumulation in *C. brevicuspis* was much higher in the three P addition treatments than in the control, and much higher in the two-time P addition treatment than in the three-time P addition treatment. These results were consistent with hypothesis 1 and suggested that both P addition and addition frequency significantly influenced growth of *C. brevicuspis*. Moreover, our results confirmed that P enrichment frequency did not significantly influence the biomass allocation of *C. brevicuspis*, indicating that this was not an effective way for plants to acclimate to different P enrichment frequencies.

The stimulation of plant growth by additional P has been widely reported in other studies (Chiang et al., 2000; McCormick et al., 2001; Mao et al., 2016). For instance, addition of P resulted in a two-fold increase in the biomass of sawgrass and mixed sawgrass-cattail communities in the Everglades Wetland, United States (Chiang et al., 2000). Studies have also confirmed that water lily leaf size was enhanced in response to P enrichment (McCormick et al., 2001; Newman et al., 2004). However, our results contradict those of some other studies (Song et al., 2011; Gao et al., 2016). For instance, Song et al. (2011) found that increased P input had no effect on aboveground biomass of *Calamagrostis angustifolia* in the Sanjiang Plain Wetland, China, which might have been because plants were adapted to earlier soil P conditions and responded slowly to the addition of P (Macek and Rejmánková, 2007). Another possibility is that P was not a limiting nutrient in the Sanjiang Plain Wetland (Mao et al., 2016). However, our previous study showed that *C. brevicuspis*

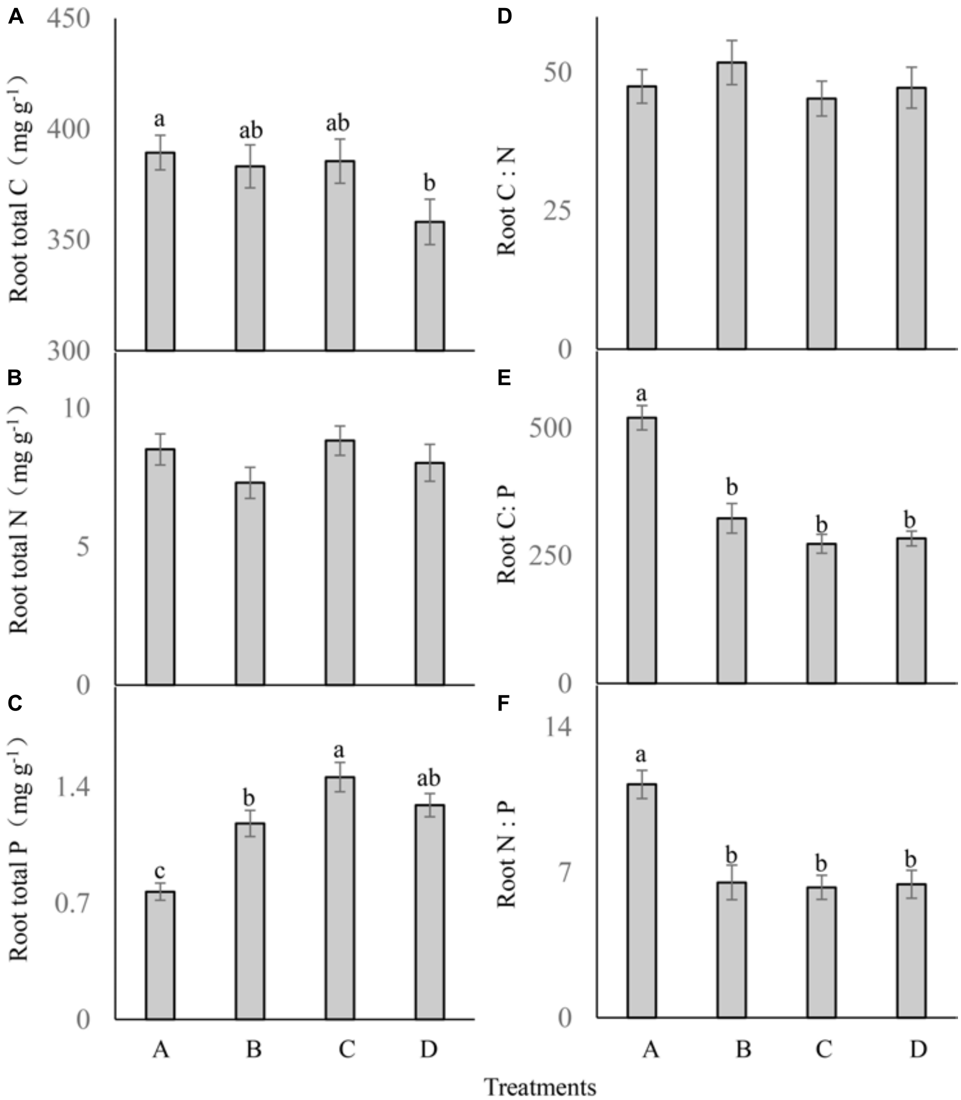


**FIGURE 4 |** Root ecological stoichiometry **(A–F)** of *Carex brevicuspis* (means ± standard errors, $n = 7$) under different P addition frequencies (treatments A–D represent: no P addition treatment; three - time P addition treatment; two-time P addition treatment; and one-time P addition treatment, respectively). Different letters indicate significant differences between treatments at the 0.05 significance level.

was limited by P at Dongting Lake (Li et al., 2017, 2018); this might explain why the addition of P promoted *C. brevicuspis* growth.

Both foliar and root P concentrations in *C. brevicuspis* were significantly increased in the three P addition treatments, which might explain the decrease in C:P and N:P in these treatments. These results were partially consistent with our hypothesis 2. Increased plant P concentration following P enrichment has been widely reported (Ostertag, 2010; Song et al., 2011; Mao et al., 2016). However, study of *Eleocharis* spp. showed that leaf tissue P content decreased with the addition of P, and caused higher C:P and N:P in enriched plots (Daoust and Childers, 2004). These results indicate that plant P concentration responses to P enrichment are species-specific and might be related to plant absorption efficiency and nutrient use strategies, as well as soil microorganism and enzyme activity (Rejmánková et al., 2008; Song et al., 2011; Yuan and Chen, 2015). Moreover, our study confirmed that both foliar and root P concentrations, C:P, and N:P in *C. brevicuspis* were not significantly different between the three P addition treatments. However, foliar N:P and root P concentration were significantly different, indicating that the influence of P addition frequency on plant stoichiometry was relatively weak. These results contradict our hypothesis 2 and the findings of other studies (Sun et al., 2018). A possible reason is that the amount of P in the three–phase frequency addition treatment was sufficient to support the growth of *C. brevicuspis*. Another possible reason is that *C. brevicuspis* has a relatively high stoichiometric homeostasis, which may have maintained stoichiometric stability under different P addition treatments (Han et al., 2014).

Our results suggest that P enrichment decreased foliar N content and increased C:N, which is consistent with the results of other studies (Feller et al., 2007; Yuan and Chen, 2015). The main reason for this finding might be that increased P availability generally stimulates plant growth in P-limited ecosystems, leading to a decline in plant N concentration owing to the dilution effect (Yuan and Chen, 2015; Mao et al., 2016). Another possible reason might be the decrease in soil total N content in the one–time P addition treatment (**Table 1**). He and Dijkstra (2015) also confirmed that P addition can result in considerable losses of gaseous N from P-poor soils, most likely via direct stimulation of nitrification and denitrification. However, our results differ from those of some other studies (Newman et al., 2004; Mao et al., 2016). In a northern Everglades slough wetland, N concentrations in water lily generally increased in response to increased P loads (Newman et al., 2004), which might have been due to increased soil pore water $NH_4$—N concentrations, mainly from the increased decomposition of resultant nutrient regeneration (Newman et al., 2001). These results suggested that the influence of P enrichment on plant stoichiometry may vary with wetland type and is possibly related to nutrient-limited conditions (Mao et al., 2016).

In conclusion, our results showed that P enrichment promoted plant growth and that this effect increased with decreasing addition frequency. Moreover, we also confirmed that P enrichment, irrespective of the frequency, increased plant C:N and P concentration, but decreased plant N concentration, C:N, and N:P. In recent years, increasing amounts of P have been discharged into Dongting Lake, due to the high intensity of anthropogenic activities. Our results improve our understanding of the influence of P enrichment on wetland nutrient cycling in this lake. However, P input usually occurs with other nutrient elements e.g., N and K. Therefore, the influences of other elements, as well as their interactive effects on plant stoichiometry should be studied to better understand the influence of exogenous nutrient input on nutrient cycling in Dongting Lake.

## AUTHOR CONTRIBUTIONS

FL and CH wrote the manuscript and conducted the technical assays and statistical analysis. YX and WL designed the experiment and edited the manuscript. FL, CH, XC, ZD, and ZH contributed to data collection and interpretation. All authors reviewed the manuscript.

## FUNDING

This study was supported by the National Natural Science Foundation of China (31570431, 41601106), the Open Funding Project of the Key Laboratory of Aquatic Botany and Watershed Ecology, Chinese Academy of Sciences (Y752701s02), the Key Research Program of the Chinese Academy of Sciences (KFZD-SW-318), the Hunan Provincial Natural Science Foundation of China (2017JJ3374), and the Youth Innovation Promotion Association of CAS (2014337).

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
published: 21 November 2018
doi: 10.3389/fpls.2018.01592

# Seed Germination Indicates Adaptive Transgenerational Plasticity in a Submerged Macrophyte

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Adaptive transgenerational plasticity is an important evolutionary strategy in plants. We investigated the resource allocation strategy in sexual reproduction and performed an *in situ* seed germination experiment of *Potamogeton maackianus* to reveal their responses to different water depths. Later, we discussed the biased adaptability to the maternal habitat in this species. We found a positive correlation between sexual and asexual reproduction in water depths from 1.0 m to 3.0 m, such a correlation failed to occur in 4.0 m water depth. These results indicate that the trade-off between sexual and asexual reproduction should only be expected in a stressful habitat, where resource acquisition is limited. For trade-off between quantity and quality of sexual units in different water depths, *P. maackianus* tends to produce more but lower quality sexual reproductive units in shallow water, and fewer but higher quality sexual units are found in deep water. The total germination percentage of seeds of *P. maackianus* was relatively poor, less than 46.65% in all of the treatments. The maximum germination percentage of seeds from 1.0 m, 2.0 m, 3.0 m, and 4.0 m water depths are 14.4%, 17.75%, 25.51%, and 46.65%, respectively. Seeds with higher germination percentage were from deeper water depths. The most interesting result was that the maximum final germination percentage occurred only when treatment water depth was the same as collection water depth. Our result showed that the variations in germination characters of the studied species appear to be based partly on the effects of maternal environmental factors. Our findings proved the adaptive transgenerational plasticity in *P. maackianus*, which will play an important role in evolutionary response to the selection of water depths.

**Keywords: transgenerational plasticity, submerged macrophyte, seed germination, water depth, adaptive characters, trade-offs**

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***Specialty section:***
*This article was submitted to
Functional Plant Ecology,
a section of the journal
Frontiers in Plant Science*

***Received:*** *13 June 2018*
***Accepted:*** *15 October 2018*
***Published:*** *21 November 2018*

***Citation:***
*Su H, Zhu T, Bai X, Ni L, Xie P and Zhang X (2018) Seed Germination Indicates Adaptive Transgenerational Plasticity in a Submerged Macrophyte. Front. Plant Sci. 9:1592. doi: 10.3389/fpls.2018.01592*

## INTRODUCTION

Acquisition and maintenance of adaptability is essential for the evolution of plants. Some of the adaptive variations in response to particular environmental stresses could be inherited by the offspring from their maternal individuals, and these variations could enhance offspring fitness under the same environmental stress (Herman and Sultan, 2011). Such adaptive transgenerational

**Abbreviations:** CWD, collection water depth; PMGD, potential maximum germination depth; TWD, treatment water depth.

plasticity due to maternal environments is common in terrestrial plants (Galloway, 1995, 2001a,b, 2002, 2005; Galloway and Etterson, 2007). Recently, adaptive transgenerational plasticity has been re-considered as a potential source of ecologically and evolutionarily meaningful variations (Roach and Wulff, 1987; Sultan, 1996; Donohue and Schmitt, 1998). The importance of adaptive transgenerational plasticity was considered to be related to population maintenance, evolutionary process, and species invasion (Herman and Sultan, 2011). This heritable plastic response to the environment may play a central role in the process of evolution in plants.

However, most of the case studies and reviews of adaptive transgenerational plasticity are from terrestrial plants (Galloway, 1995, 2001a,b, 2002, 2005; Latzel and Klimešová, 2010; Herman and Sultan, 2011), although aquatic plants (especially submerged macrophytes) play a very important role in freshwater ecosystems (Carpenter and Lodge, 1986; Scheffer, 1990; Jeppesen et al., 1998). Until now, adaptive transgenerational plasticity and maternal effects have been rarely reported in true aquatic plants. Several studies indicated local adaptation in some aquatic plants (Purohit and Singh, 1987; Hangelbroek et al., 2003; Iida et al., 2007; Richards et al., 2011; Xie et al., 2015), but their experimental design and results failed to directly prove adaptive transgenerational plasticity. Among the former studies, Li et al. (2015) indicated the potential relationship between seed behavior and maternal environments of *Potamogeton pectinatus*, but their study lacked *in situ* experimental support.

The rare report of adaptive transgenerational plasticity in aquatic plants could be attributed to two main reasons. The first reason is the longtime overlooking of sexual reproduction in aquatic plants. Although asexual reproduction (vegetative reproduction) is often assumed to be the dominant mode of reproduction in aquatic plants (Sculthorpe, 1967; Hutchinson, 1975), sexual reproduction continues to play a central role in the population biology of these plants (Philbrick and Les, 1996). Based on recent studies (Hangelbroek et al., 2002, 2003; Li et al., 2004; Chen et al., 2006; Triest and Fenart, 2014), the frequency of sexual reproduction in aquatic plants has been overlooked for a long time. The second reason is the common conclusion that the water habitat is more stable than the terrestrial habitat, which is based on the following facts: water exhibits greater chemical and thermal stabilities than air and buffers against many types of catastrophic disturbances; and the convergent evolution in aquatic plants (Sculthorpe, 1967; Philbrick and Les, 1996). However, the water habitat is never more stable than the terrestrial one but the unstability of water habitat displays in some other aspects. Unlike the terrestrial habitat, temperature and moisture are relatively constant in a water body, but other factors change rapidly along with water depth, such as underwater light intensity, current velocity, and dissolved oxygen. There are many heterogeneous environmental niches within the same water body and such heterogeneities are predictable for aquatic plants. Consequently, adaptive transgenerational plasticity should also be expected in aquatic plants.

Among the many environmental factors in water habitats, water depth is the most important one because it affects underwater light intensity and $O_2$ availability, shapes characters of individual plants and population, affects assembly of community, and also affects reproductive allocation (Spence, 1982; Maberly, 1993; Wantzen et al., 2008; Fu et al., 2012; Li et al., 2017). Submerged macrophytes are directly under the selective pressure of water depth. The adaptation to water depth is vital to their survival. However, most submerged macrophytes are not restricted to a fixed water depth but usually spread widely along water depth gradient and assemble various structured communities. The understanding of how they adapt to different water depths and how these adaptive characters can be inherited by their offspring is important in evolutionary research of aquatic plants.

*Potamogeton maackianus* is a typical submerged macrophyte widely distributed in East and Southeast Asia (Wiegleb and Kaplan, 1998; Flora of China, Vol. 23) especially in the watershed of the Yangtze River, before eutrophication. It is considered as an indicator species for water quality (Ni, 2001; Fu et al., 2013; He et al., 2015). In many freshwater shallow lakes, *P. maackianus* colonizes large areas and forms dense populations. This is because of its relatively wide ecological niche and high plasticity. According to our former studies, *P. maackianus* has dramatic plastic variation in morphology and biomass allocation (such as stem length, leaf length, branching pattern, specific leaf area, root-shoot biomass ratio, and growth rate) in response to changes in water depth (Fu et al., 2013, 2014). Because there are no specialized turions in this species (Wiegleb and Kaplan, 1998), rhizomes and seeds are the main means of population maintenance and expansion. The main pollination type of this species is anemophily (Jin and Guo, 2001; Zhang et al., 2009). With bisexual flowers, outcrossing and selfing can both be expected. Seeds represent the link between maternal parent and offspring, and seed germination is the first stage of the plant life cycle where natural selection can operate. Therefore, we chose *P. maackianus* as the material and seed germination speed and proportion as principal variables to study potential adaptive transgenerational plasticity.

To find the relationship between water depth (selective pressure) and seed germination (responses) and further discuss the potential transgenerational plasticity, we investigated reproductive allocation and designed an *in situ* germination experiment in response to different water depths to test three hypotheses: (1) Water depth will affect reproductive allocation by means of seed quality, and such affects could be represented by seed germination; (2) Since water depth is an environmental pressure in the germination of seeds, the lowest germination percentage could be found in the deepest water habitat; and (3) The responses of seed germination to different water depths could be related to the habitat in which the seeds mature.

## MATERIALS AND METHODS

### Studied Species and Experimental Site

*Potamogeton maackianus* is always submerged, and its flowering time is from May to August while blooming time is in July in South China. Three small bisexual flower whorls arranged oppositely on the spica, at the tip of each branch, are found in

*P. maackianus*. In each bisexual flower, there are two carpels. Although the pollination type of *P. maackianus* is anemophilous, the stigma can be self-pollinated by pollen grains moving through the air bubble around the inflorescence if the flower is underwater when blooming (Jin and Guo, 2001; Zhang et al., 2009).

Erhai Lake (25°52′N, 100°06′E) is a freshwater lake located in Yunnan Province, Southwest China. The lake is characterized by a surface area of 250 $km^2$ (when water level is 1974 m above sea level), with a maximum water depth of 21 m and an average depth of 11 m. Most of the macrophytes inhabit and dominate the shallow water area (0–3.0 m depth), and only a few species such as *P. maackianus* and *Vallisneria natans* colonize deeper water areas. The population of *P. maackianus* presents a zonation from shoreline to about a 5-m depth in this lake. Haichaohe bay is the biggest bay in the northern part of the lake with an area of 10 $km^2$. In this bay, the population of *P. maackianus* has maximum density and is at its deepest distributed limitation (about 5 m). Haichaohe bay was thus used for material collection and for *in situ* germination experiments. The location of Erhai Lake and Haichaohe bay are shown in **Figure 1**.

## Material Collection

Three kinds of materials were collected from July to September 2016: mature inflorescences with undehisced anthers for pollen counting; ripe seeds for germination experiments; and individuals with ripe seeds for the investigation of resource allocation in sexual reproduction. In this study, we defined a complete shoot with only one internode of stolon as an individual. All the three kinds of materials were collected along the gradient of water depth in Haichaohe bay. Four water depths (1.0 m, 2.0 m, 3.0 m, and 4.0 m) were defined as collection water depths (CWDs) CWD1, CWD2, CWD3, and CWD4, respectively.

In each CWD, 15 individuals with mature inflorescence were randomly collected in July for pollen counting. At least 15 whole individuals with ripe seeds were randomly collected from each CWD in August and September for the study of sexual reproduction allocation traits. The distance between adjacent individuals was maintained as at least 10 m to avoid collecting from the same genet. Ripe seeds, as many as possible, were randomly collected from all CWDs for the germination experiments.

## Sexual Reproduction Traits and Current Velocity Measurement

The following traits were recorded: pollen amount per inflorescence; inflorescence number; seed amount per individual; single seed weight; seed biomass per individual; seed set; and shoot biomass. Because *P. maackianus* never forms specialized asexual organisms such as tubers and turions, we treated the biomass of vegetative parts (shoot biomass) as asexual reproductive allocation. We also calculated seeds/shoot biomass ratio as sexual reproductive resource allocation proportion. In total, 60 inflorescence (15 inflorescence for each CWD) for

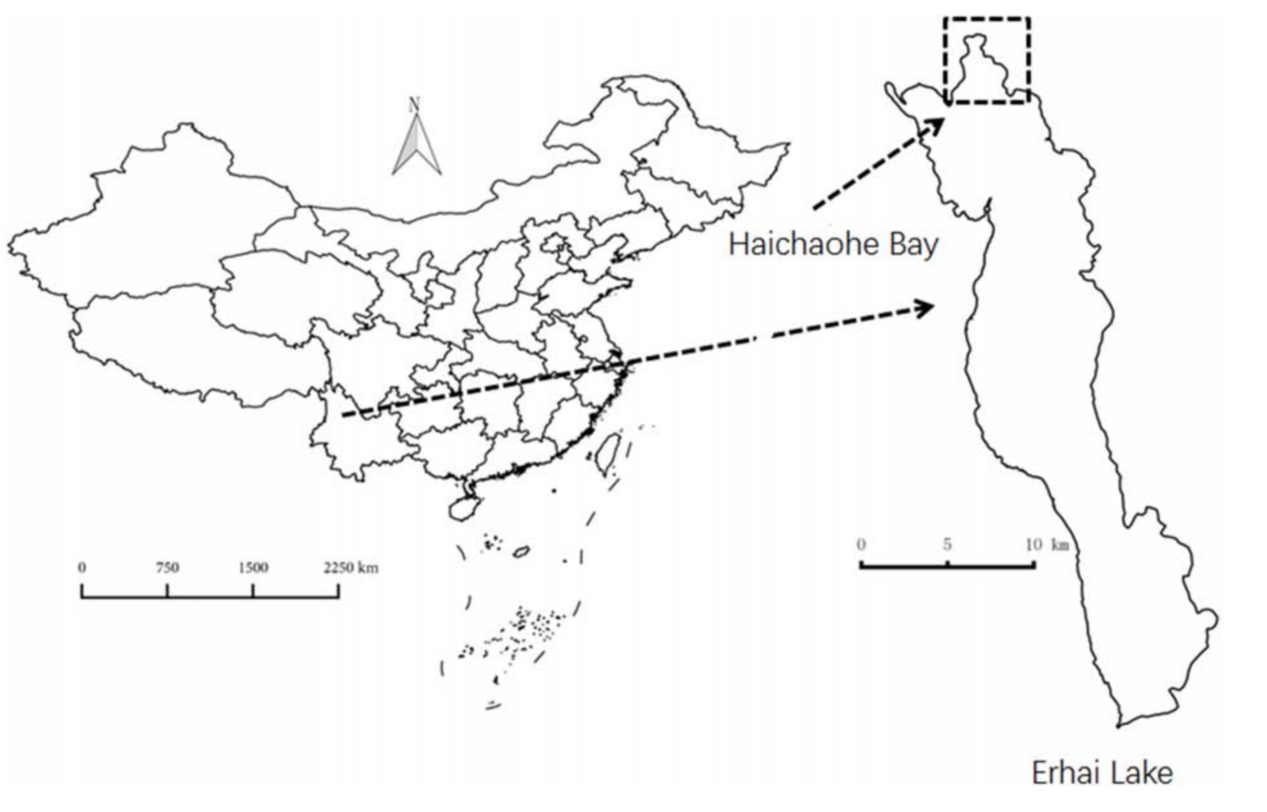


**FIGURE 1 |** The map shows the location of Erhai Lake and experimental site.

pollen counting were fixed in FAA fixative solution [constituted of formalin (37–40%), acetic acid, and alcohol (50%) at a ratio of 5: 5: 90 by volume] after collection. All the 12 anthers (there are four anthers in every single flower and three flowers in one inflorescence) were removed from each inflorescence under a dissecting microscope and prepared to count pollen grains. The methods of manual counting of pollen grains followed the method described by Kearns and Inouye (1993). A total 60 individuals (from four CWDs) for the investigation of reproduction allocation traits were washed using tap water to remove the attached algae. All the individuals were separated into vegetative parts (shoots) and seeds. Only ripe seeds were used for the investigation. The number of seeds of each individual was counted. After that, seeds and shoots were dried in an oven at 80°C for 48 h to constant weight. Later, seed biomass and shoot biomass were measured using an analytical balance.

Because seed dispersal distance is closely related to lake current velocity, we used River Surveyor M9 (SonTek) to measure the current velocity of an entire section in Haichaohe bay in October (seed dispersal period).

## *In situ* Germination Experiment

About 2000 ripe seeds were collected randomly from the four CWDs for the germination experiment. After collection, the seeds were washed roughly using tap water to remove algae and then stored at 4°C in darkness until the beginning of the experiments in March 2017 to break the dormancy of the seeds (Hay et al., 2008).

The *in situ* germination experiment was conducted in the area where water depth is 4 m in Haichaohe bay. The treatment water depth (TWD) for the *in situ* experiment was defined as five water depths (0.0 m, 1.0 m, 2.0 m, 3.0 m, and 4.0 m). Three bamboo poles (6 m in length) were used as supports for the *in situ* experiment. Firstly, we made five marks according to the five TWDs on the pole, and the intervals were kept as 1-m. Later, according to the four CWDs, four bags (7 cm × 9 cm) made of transparent tulle were tied at each mark, and each bag contained 30 seeds from each CWD. The CWD of seeds was signed outside the bags. A square board was fixed on the pole just below the lowest mark (TWD4). Subsequently, we inserted the pole into the sediment until the square board was just on the mud surface and made sure the top mark (TWD0) was just on the water surface. The design of the germination device is shown in **Figure 2**. Three replications were made in this germination experiment. We checked the bags every 3 days to count the newly germinated seeds. After counting, germinated seeds were removed from the bags. A seed was considered as germinated if the radicle that extended from the seed coat was as long as the seed diameter. The experiment was continued until no more seeds germinated over 10 consecutive days.

The *in situ* experiment started on March 15, 2017 and lasted 45 days. During the experiment, Secchi depth (SD), water temperature (T), pH, dissolved oxygen concentration (DO), and underwater photosynthetic active radiation (PAR) were measured near the poles. The SD was measured by a 30-cm diameter Secchi-disk. The parameters of T, DO, and pH were measured using a multifunctional YSI meter (Yellow Springs Instruments, OH, United States). The PAR was measured using an underwater radiation sensor (UWQ-8342) connected to a data logger (Li-1400; LI-COR Company, Lincoln, NE, United States). The extinction coefficient of water was calculated using PAR in different water depths. All the environmental parameters were measured every 5 days over the experimental period.

## Data Analysis

One-way analysis of variance (ANOVA) was used to evaluate the variation of sexual reproduction traits among different CWDs. Pearson's correlation analysis was performed among the eight reproduction characters to test the covariation of trade-offs. Two-way ANOVA was used to examine the effects of CWD and TWD on seed germination in the *in situ* germination experiment. The final germination percentage was square root transform and treated as dependent variable, while TWD and CWD were treated as independent variables. Post hoc comparisons for all analyses were made with Tukey's HSD test. Significant differences were determined when $p < 0.05$ or $p < 0.01$.

In many related studies, the time required for half final germination ($T_{50}$) was considered as a good estimator to describe the germination speed because $T_{50}$ is less affected by a small number of seeds having very long germination times (Thornley, 1986; Li et al., 2000; Jian et al., 2003). It can be derived from a logistic equation:

$$G = \frac{k}{1 + a \exp(-rT)} \tag{1}$$

where $G$ is the percentage of germination at time $T$; $k$ is the maximum germination capacity (observed final germination percentage); and $a$ and $r$ are estimated parameters. From the fitted equations, the time required for half final germination ($T_{50}$) in each treatment can be calculated by the following equation:

$$T_{50} = \frac{\ln a}{r} \tag{2}$$

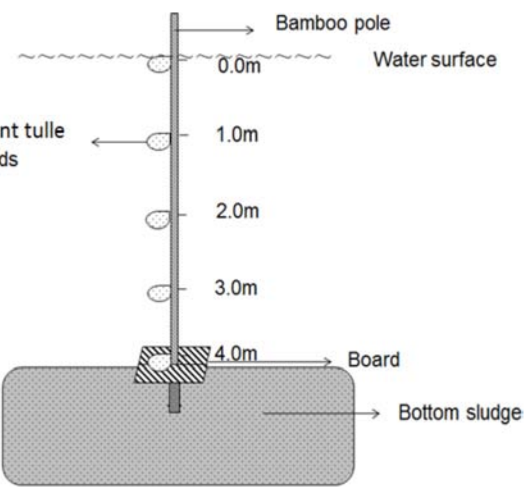


**FIGURE 2 |** Design of the germination experiment device.

**TABLE 1 |** Reproductive allocation characters among different water depths (means ± standard error, in each CWD $n$ = 15).

| Water depth | Seed set (%) | Shoot biomass (g) | Pollen amount per inflorescence | Inflorescence number | Seed biomass per individual (g) | Seed amount per individual | Single seed weight (mg) | Seeds/shoot ratio (%) |
|---|---|---|---|---|---|---|---|---|
| CWD1 | 39.72 ± 3.95 | 0.81 ± 0.08 | 63253.33 ± 1001.82 | 8.53 ± 0.29 | 0.16 ± 0.01 | 11.87 ± 1.07 | 13.64 ± 0.30 | 3.93 ± 0.27 |
| CWD2 | 28.89 ± 3.24 | 1.70 ± 0.25 | 68949.33 ± 2762.78 | 5.4 ± 0.19 | 0.26 ± 0.04 | 20.93 ± 3.34 | 12.63 ± 0.30 | 3.67 ± 0.33 |
| CWD3 | 27.5 ± 4.44 | 1.89 ± 0.33 | 91810.13 ± 3257.66 | 2.93 ± 0.18 | 0.36 ± 0.05 | 26.2 ± 4.20 | 13.95 ± 0.40 | 3.09 ± 0.30 |
| CWD4 | 35.56 ± 3.61 | 2.06 ± 0.17 | 93888 ± 3314.80 | 2.0 ± 0.53 | 0.27 ± 0.03 | 13.93 ± 1.62 | 20.22 ± 0.90 | 2.68 ± 0.29 |

After calculating $T_{50}$, a linear regression analysis was performed between $1/T_{50}$ and water depth to get the potential maximum germination depth (PMGD) in this lake.

All statistical analyses were carried out with SPSS version 22.0.

# RESULTS

## Sexual and Asexual Reproduction Allocation

The reproductive allocation characters of the individuals from different CWDs were shown in **Table 1**. Pollen amount per individual was positively correlated with single seed weight (**Table 2**), and these two characters increased significantly with water depth (**Figures 3A,B**). However, single seed weight was negatively correlated with seed amount, which was positively correlated with seed biomass per individual (**Table 2**). The inflorescence number reduced significantly along with increase of water depth (**Figure 3G**). This is because the individuals in deep water have fewer branches where the inflorescence is formed. Both single seed weight and seed amount determined seed biomass, which showed an inverse variation trend of shoot biomass (**Figures 3F,H**). There were significant variations in most sexual characters among different CWDs except seed set (**Figure 3**). To investigate the relationship between sexual and asexual reproduction, seeds and shoot biomass per individual were analyzed by correlation analysis and linear regression (**Figure 4**). We found that there were positive correlations between seeds and shoot biomass in CWD1, CDW2, and CDW3, but such correlations disappeared in CWD4 ($r$ = 0.18, $p$ > 0.05).

## *In situ* Germination Experiment

### Environmental Factors

According to our results of the four environmental factors measured, there is no stratification in the water column of the experimental area. Environmental factors during the experimental period are shown in **Figure 5**. The SD varied from 1.50 m to 1.75 m during the experiment with an average of 1.63 m. Water temperature increased from 17.40°C to 21.00°C at the end. Dissolved oxygen in water column changed a little greatly, and the mean value is 5.95 mg/L. The extinction coefficient of water was related to SD. Most of the environmental factors were relatively stable during the experiment.

The current velocity of the transaction and shoreline area are shown in the **Appendix**. The average speed in shoreline area (water depth between 1 m and 6 m) was 0.20 m/s, and there was no significant variation in current velocity in this area.

### Germination Results

The highest final germination (46.65%) was found in the treatment CWD4×TWD4, while the lowest germination (0%) was found in CWD1× TWD3 (**Table 3**, **Figure 6**). In general,

**TABLE 2 |** Pearson correlation analysis among the eight characters.

| | Seed set | Pollen amount per inflorescence | Seed biomass per individual | Single seed weight | Seeds/shoot radio | Inflorescence number | Seed amount per individual |
|---|---|---|---|---|---|---|---|
| Pollen amount per inflorescence | $r$ = −0.154 | | | | | | |
| Seed biomass per individual | $r$ = −0.055 | $r$ = 0.223 | | | | | |
| Single seed weight | $r$ = −0.189 | $r$ = 0.465** | $r$ = −0.069 | | | | |
| Seeds/shoot radio | $r$ = 0.013 | $r$ = −0.141 | $r$ = 0.218 | $r$ = −0.295* | | | |
| inflorescence number | $r$ = 0.239 | $r$ = −0.708** | $r$ = −0.360** | $r$ = −0.505** | $r$ = 0.358** | | |
| Seed amount per individual | $r$ = −0.012 | $r$ = 0.073 | $r$ = 0.964** | $r$ = −0.293* | $r$ = 0.260* | $r$ = −0.207 | |
| Shoot biomass | $r$ = −0.049 | $r$ = 0.178 | $r$ = 0.715** | $r$ = 0.112 | $r$ = −0.408** | $r$ = −0.471** | $r$ = 0.654** |

*$p$ < 0.05; **$p$ < 0.01; $n$ = 60.*

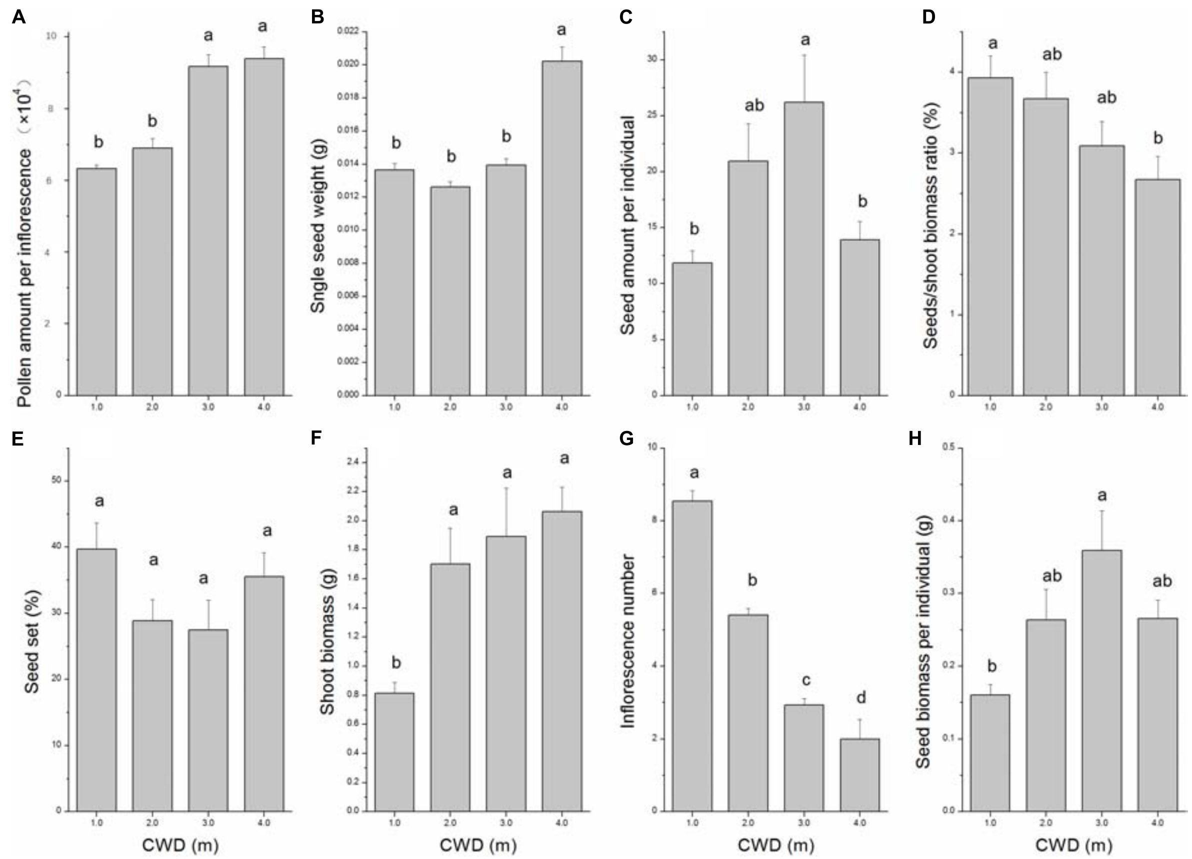


**FIGURE 3 |** Variation in eight characters depending on water depth analyzed by one-way ANOVA. **(A)** Pollen amount per inflorescence, **(B)** Single seed weight, **(C)** Seed amount per individual, **(D)** Seeds/shoot biomass ratio, **(E)** Seed set, **(F)** Shoot biomass, **(G)** Inflorescence number, and **(H)** Seed biomass per individual. Error bars mean standard error. Turkey's HSD test was used for *post hoc* comparisons. Different letters indicate significant differences ($p < 0.05$).

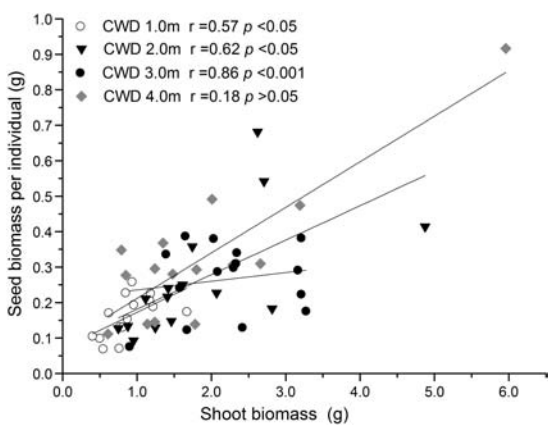


**FIGURE 4 |** Linear correlation analysis between seed biomass and shoot biomass in different water depth environments. The significant positive correlation in 1 to 3 m depth disappeared in the 4-m depth.

CWD4 seeds present the highest germination percentage in every TWD. Both CWD and TWD dramatically affect the final germination percentage (**Table 4**).

The maximum germination percentages of seeds from CWD1, CDW2, CDW3, and CDW4 are 14.4%, 17.75%, 25.51%, and 46.65%, respectively. In the same CWD, there is a significant differentiation in final germination percentage according to different TWDs. Seeds exhibited maximum germination percentage only when TWD was consistent with CWD (**Figure 6**). For instance, CWD4 seeds have the highest germination percentage (46.65%) only in TWD4 condition. Similarly, CWD3 seeds exhibit the highest germination percentage (25.51%) only in TWD3 condition. In other words, the most suitable germination depth is the same as its growing depth.

In each TWD, seeds from the deepest water habitat (CWD4) always represent highest germination percentage (14.44%, 31.09%, 33.32%, 34.44%, and 46.65% for TWD-0, TWD1, TWD2, TWD3, and TWD4, respectively). Another interesting result was that in every TWD, the seeds from deeper water showed higher final germination percentage than those from shallower water. When we separated the data by different CWDs, the average final germination percentage increased with CWD. This means that the seeds coming from deeper habitats have higher germination ability, which could be treated as seed quality. When we considered the variation of seed amount (quantity) in different

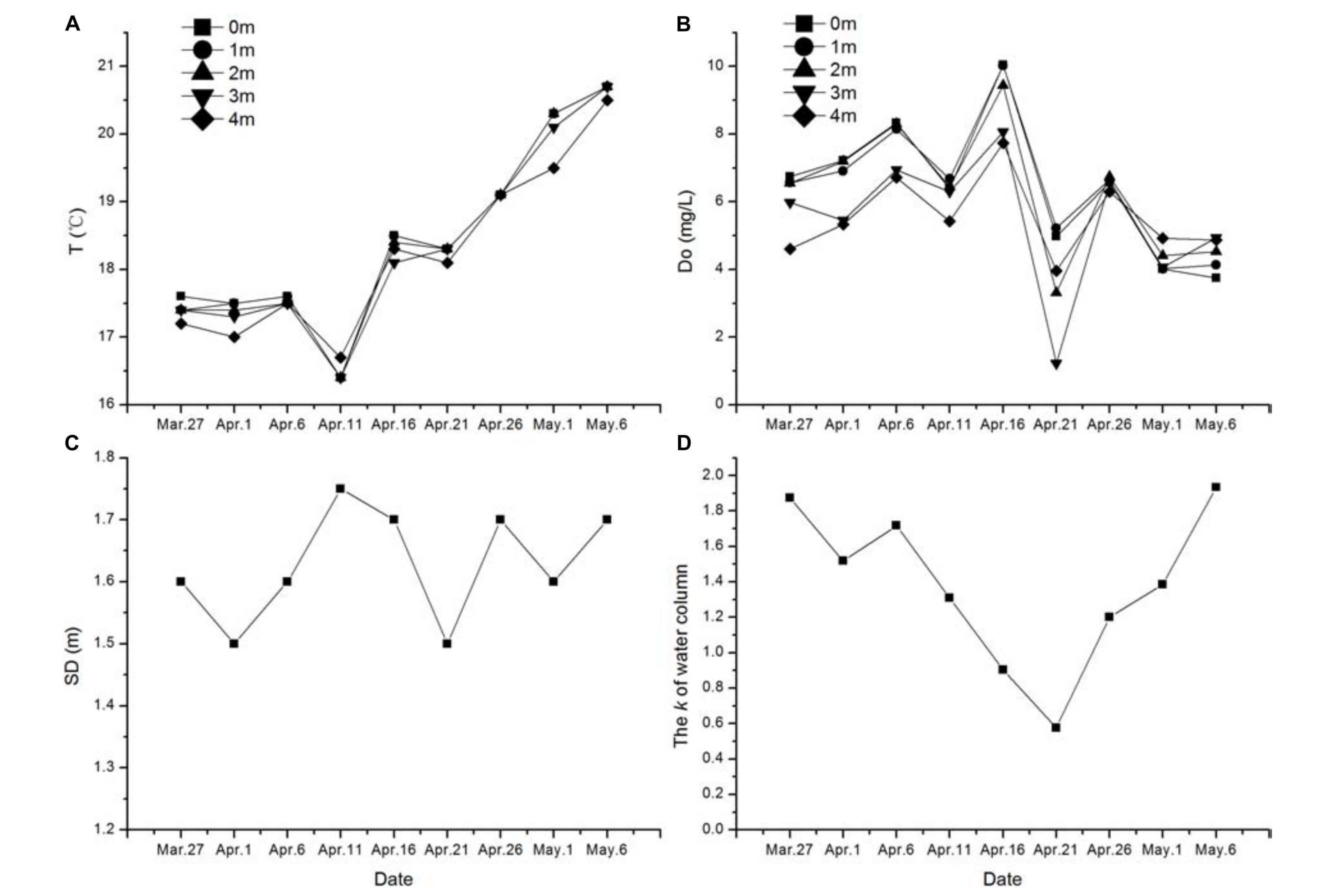


**FIGURE 5 |** The variation of environmental characters during the germination experiment. **(A)** Temperature of water, **(B)** Dissolved oxygen, **(C)** Secchi depth, and **(D)** Extinction coefficient of water.

**TABLE 3 |** Final germination percentage of the *in situ* germination experiment (means ± standard error, *n* = 3).

| | | CWD | | | |
|---|---|---|---|---|---|
| | | **1.0 m** | **2.0 m** | **3.0 m** | **4.0 m** |
| TWD | 0.0 m | 7.78 ± 1.11 bc | 4.44 ± 1.11 b | 7.78 ± 1.11 c | 14.44 ± 1.11 c |
| | 1.0 m | 14.4 ± 1.1 a | 8.88 ± 1.92 b | 13.32 ± 1.11 bc | 31.09 ± 1.02 b |
| | 2.0 m | 9.99 ± 1.92 b | 17.75 ± 1.11 a | 16.64 ± 1.92 b | 33.32 ± 1.93 b |
| | 3.0 m | 0 ± 0 d | 7.77 ± 1.92 b | 25.51 ± 1.09 a | 34.44 ± 1.1 b |
| | 4.0 m | 4.44 ± 1.1 bd | 6.66 ± 1.09 b | 11.11 ± 1.11 bc | 46.65 ± 1.92 a |

*Different letters indicate significant variation in each CWD group (p < 0.05).*

water depths, such a tendency could be treated as a trade-off between seed quantity and quality depending on water depth.

## Half Final Germination Time and Potential Germination Depth

Most of the seeds completed germination within 20 days from sowing, but germination speed varied among the CWDs. The average $T_{50}$ of the four CWD seeds was 14.77 days (**Table 5**). The highest (26.00 days) was found in the CWD1 seeds germinated in TWD4, while the lowest was 8.85 days, found in CWD2 seeds germinated in TWD0. Based on $1/T_{50}$ and TWD, a linear analysis was performed (**Figure 7**). From the intersection with the TWD axis, we got the PMGD for seeds from each CWD and also for total seeds.

# DISCUSSION

## Trade-Offs in Different Aspects

Both sexual and asexual reproduction are important to aquatic plants, and the resource allocation strategy between sexual and asexual reproduction has been discussed for a long time

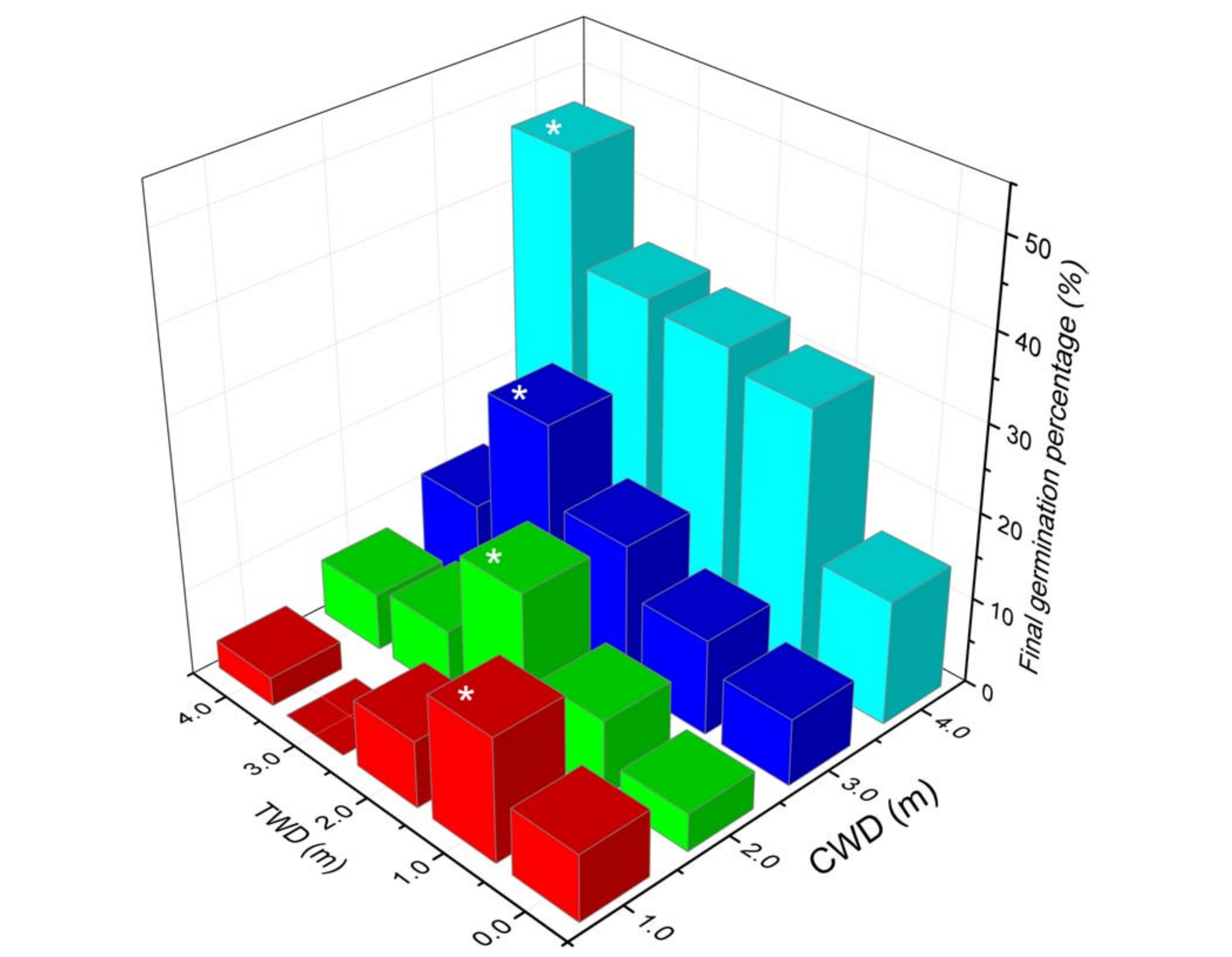


**FIGURE 6 |** The final germination percentage of the *in situ* germination experiment. Different colors present different CWD groups. The asterisk indicates highest germination percentage of each CWD group ($p < 0.001$).

**TABLE 4 |** Two-way ANOVA results of the final germination percentage.

| Source of variation | *df* | SS | MS | *F* | *P* |
|---|---|---|---|---|---|
| TWD | 4 | 766.596 | 191.649 | 35.683 | <0.001 |
| CWD | 3 | 5882.704 | 1960.901 | 365.096 | <0.001 |
| TWD × CWD | 12 | 2083.865 | 173.655 | 32.332 | <0.001 |
| Error | 40 | 214.837 | 5.371 | | |
| Total | 60 | 23466.822 | | | |

(Philbrick and Les, 1996) and evaluated in many aquatic plants (Geber et al., 1992; Prati and Schmid, 2000; van Kleunen et al., 2002; Thompson and Eckert, 2004; Liu et al., 2009; van Drunen and Dorken, 2012; Eckert et al., 2016). However, almost no related findings were reported in submerged aquatic plants until now. Our results indicated trade-offs not only between sexual and asexual reproduction but also between different sexual characters, and the existence of trade-offs is related to habitat difference.

Trade-offs are based on the theory that there is a limitation in the resources allocated to different strategies. Although trade-offs between sexual and asexual reproduction in plants are widely expected, they are less consistently found. One of the reasons is that trade-offs are usually obscured by variation in resource acquisition among individuals, which usually cause positive covariation (Eckert et al., 2016). At the species scale, we found a strong positive covariation between seeds and shoot biomass (**Table 2**). However, when we consider different CWDs,

**TABLE 5 |** Half final germination time ($T_{50}$) of the *in situ* germination experiment (days).

| | CWD1 | CWD2 | CWD3 | CWD4 |
|---|---|---|---|---|
| TWD0 | 11.00 | 8.85 | 11.00 | 11.14 |
| TWD1 | 15.29 | 10.59 | 11.14 | 13.82 |
| TWD2 | 16.13 | 13.23 | 11.83 | 15.47 |
| TWD3 | | 13.72 | 17.20 | 16.70 |
| TWD4 | 26.00 | 19.69 | 18.64 | 19.16 |

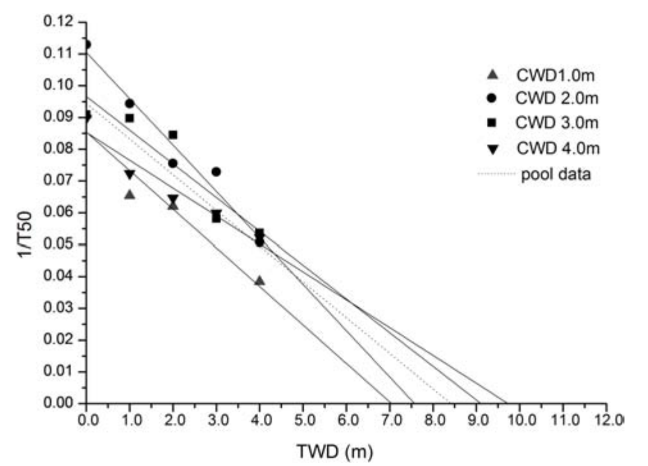


**FIGURE 7 |** Linear regression analysis between TWD and $1/T_{50}$.

the relationship changed. The positive correlation was presented in CWD1 to CDW3, but there was no correlation in CWD4 (**Figure 4**). This implies that resource acquisition could be limited in this stressful environment and induces the breakdown of the positive correlation. A transition from positive to negative covariation is to be expected in deeper, more stressful water.

According to our results, water depth will significantly affect almost all the characters of this submerged species except seed set (**Figure 3**) and shape the whole population into different groups (**Figure 4**). We found that seeds/shoot ratio decreased dramatically with water depth (**Figure 3D**). This result means that allocation in sexual reproduction is going to be reduced when water depth stress increases. Based on our observations, the old shoots sink to the bottom of the lake at the end of the growing season, and new roots and shoots will sprout from each node of the old shoots at the beginning of the next growing season. So, the biomass of the shoot (without sexual organs) can be used as an indicator of allocation to asexual reproduction. Our results are consistent with the former conclusion that along the water depth gradient, sexual reproduction proportion will be reduced (Hutchinson, 1975; Philbrick and Les, 1996; Li, 2014).

Furthermore, when we consider detailed aspects of sexual reproduction, we found that both single inflorescence pollen amount and single seed weight increased dramatically from CWD1 to CWD4, indicating that resources allocated to a single sexual unit is increased. This seems to be in conflict with our former conclusion. But considering the decrease of sexual unit numbers (inflorescence number and seed amount per individual) in deeper CWD (**Figure 3G**), the total resource allocation in sexual reproduction is still reduced. These results illustrated a trade-off between quality and quantity of sexual reproductive units. This species tends to produce fewer but higher quality sexual units when it is facing more stressful water depth, while it tends to form more but lower quality sexual units in relatively less stressful conditions.

## Adaptive Transgenerational Plasticity in *P. maackianus*

Germination was significantly affected by water depth. But our results cannot support the second hypothesis that the lowest germination percentage is expected to be found in the deepest water habitat. However, we found a novel pattern in seed germination under water depth stress of this species (**Figure 6**). The maximum final germination percentage occurred only when the TWD was the same as the CWD. In other words, the water depth where seeds matured was the most suitable germination condition. Such results have never been reported before in aquatic plants. The most reliable reason for this phenomenon is adaptive transgenerational plasticity. The adaptive transgenerational plasticity was initially considered as a noise of evolution, while three decades ago it was reconceptualized as a potential source of ecologically and evolutionarily meaningful variations (Roach and Wulff, 1987; Schmitt et al., 1992; Sultan, 1996; Donohue and Schmitt, 1998). The central meaning of adaptive transgenerational plasticity was summarized as follows: parent individuals alter specific developmental traits in progeny in response to particular environmental stresses to enhance offspring fitness under the same stresses, and it can be expected to evolve if parental habitats reliably predict their offspring habitats (Agrawal et al., 2001; Galloway, 2005; Herman and Sultan, 2011).

Water depth stress is an important environmental challenge that aquatic plants (especially submerged plants) must confront. In deeper habitats, submerged plants need to change resource allocation strategy, which will not only represent in plant phenotypes but also population genetic structures and will lead to community succession (Barko and Smart, 1981; Strand and Weisner, 2001; Li et al., 2004; Yang et al., 2004; Fu et al., 2012). If the water depth of the offspring's habitat could be predicted, it is crucial for parent individuals to maximize the fitness of their offspring in the likely habitat. As we observed, most seeds of *P. maackianus* will sink to the bottom with their maternal individuals. Considering the relative slow current velocity in the vegetation area of the lake (**Appendix**), we can expect that the dispersal distance of seeds of this species is very limited. Other research on population spatial genetic analysis of a related species, *P. pectinatus*, revealed that the seedling recruitment distance is less than 5 m (Triest and Fenart, 2014). Based on the underwater topographic map of Erhai Lake, the lake bed depth varies slowly on the west side, but the east side is cliffy. This species is distributed mainly in the western part of the lake. The distances between every two adjacent CWDs in the bays dominated by this species are 10.80 m to 298.00 m, 13.60 m to 265.00 m, and 24.00 m to 589.30 m for CWD12, CWD23, and CWD34, respectively. This means that the dispersal distance of seeds is smaller than the scale of environmental heterogeneity within a population of this species. In this condition, adaptive plasticity between generations could be evolved to enhance offspring fitness in the predictable similar environment (Galloway, 2005). Our results clearly illustrate this opinion.

This research is the first attempt to directly prove the adaptive transgenerational plasticity in a typical submerged macrophyte. We can speculate that the original population of *P. maackianus* will gradually separate into different groups according to water depth. Considering our results, in each group, seed fitness is highest in the parental habitat. This will accelerate the fixation of specific adaptive characters and result in a lot of change in population genetic structure, which will play an important role in the evolutionary dynamics of populations (Rasanen and Kruuk, 2007).

The mechanism for these remarkably specific effects of parental environment on seedling growth patterns may be found in the fact that content and balance of growth hormones in seeds is affected by many aspects of the parental environment, including drought, mineral nutrient supply, light quality and duration, and temperature (Gray and Thomas, 1982; Gutterman, 1982). But more evidence from different aspects (physiological, ecological, genetic, etc.) is needed to reveal the mechanisms of this phenomenon and its evolutionary meaning at ecological timescales.

## Potential Maximum Germination Depth

According to our results, the seeds from CWD2 and CWD3 represent higher germination speed but were affected by germination water depth significantly. While seeds from CWD4 failed to show a high germination speed, they represented some resilience to water depth. Based on the linear regression between TWD and $1/T_{50}$, we can determine the threshold water depth over which germination of seeds almost does not take place, and this water depth is defined as PMGD. In practice, this model can be used to predict the germination dynamics of seeds simply from the mean water depth of a given lake, as long as the light attenuation characteristics are constant. In our research, we can get the PMGD for *P. maackianus* seeds in this lake, when we use all the data for calculation.

The aquatic vegetation in many freshwater lakes, including Erhai Lake, being affected by eutrophication, was reduced dramatically. From the 1990s, the aquatic vegetation coverage in this lake dropped from 40% to 10% (Fu et al., 2013). Considering the important ecosystem functions of submerged macrophytes, submerged vegetation restoration is arising as an important task for many eutrophicated lakes. Since the 1990s, *P. maackianus* has become the dominant species in this lake, and there was once a large population of this species in the south central part of the lake before 1998 (Fu et al., 2013). According to historical records, the maximum water depth in this large population was about 7–8 m. This is very close to our PMGD result. Based on our results, a supply of ripe seeds of *P. maackianus* into this area could be helpful in vegetative restoration. Ripe seeds collected from 4-m deep or deeper habitats are more recommended. Our research provided a solid support to the usage of seeds for aquatic vegetation restoration.

## AUTHOR CONTRIBUTIONS

XZ and HS conceived the study. TZ and XB assisted the experiments. HS and XZ analyzed the data. HS, LN, PX, and XZ wrote and revised the paper.

## ACKNOWLEDGMENTS

This research was supported by the National Key Research and Development Program of China (2017YFA0605203) and the National Natural Science Foundation of China (Grant No. 31200356).

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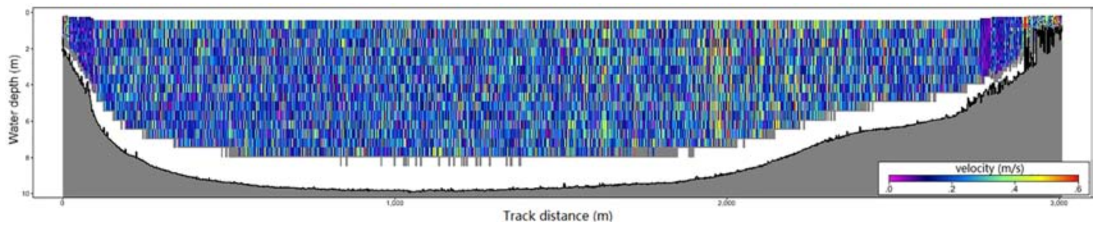
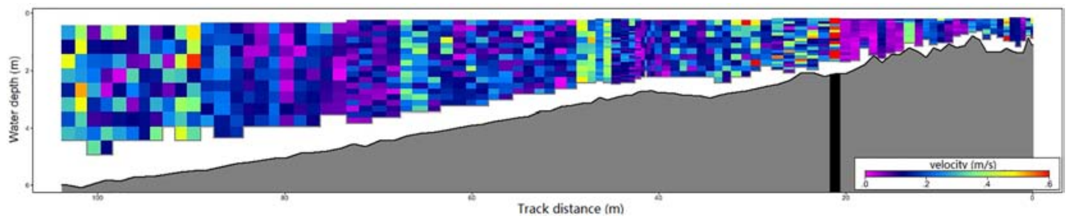
**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

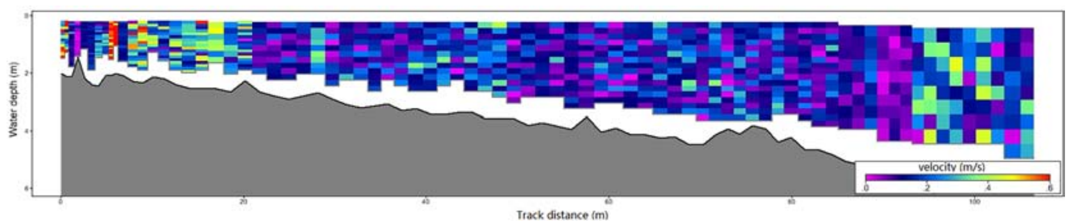
# APPENDIX




**APPENDIX A1 |** The current velocity of a transection in Haichaohe bay.




**APPENDIX A2 |** The current velocity in the west bank of the transection.




**APPENDIX A3 |** The current velocity in the east bank of the transection.

ORIGINAL RESEARCH
published: 18 December 2018
doi: 10.3389/fpls.2018.01835

# Allelopathic Effect of the Invasive *Ludwigia hexapetala* on Growth of Three Macrophyte Species

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The release of allelochemicals by plants can affect the performance of other organisms positively or negatively. We tested the effects of aqueous extracts and leachates derived from the leaves and roots of the invasive water primrose (*Ludwigia hexapetala*) on one submerged native species – *Ceratophyllum demersum*, and two exotic species – the submerged *Egeria densa* and the emergent growth form of *Myriophyllum aquaticum*. The effect of the aqueous extracts and leachates of *L. hexapetala* on photosynthetic yield, growth (i.e., relative growth rate, leaf area), root length, and length of the lateral shoots of each species were analyzed in spring and in autumn. In autumn, an allelopathic effect was established on the traits of the three macrophytes species. The root extracts stimulated leaf area and the photosynthetic yield of *C. demersum* and of *E. densa*, whereas leaf treatments (leachates and extracts) and root leachate reduced the leaf area of *M. aquaticum*. The autumnal root leachate of *L. hexapetala* decreased the relative growth rate of *C. demersum*, whereas it had no effect on the two others plants. The root extract increased the length of lateral branches of *M. aquaticum* in autumn, suggesting a positive effect of *L. hexapetala* on the lateral growth of *M. aquaticum.* Three main allelochemicals were identified in leaves: quercitrin, prunin, myricitrin. The concentrations of these allelochemicals were greater in the leaf extract taken from *L. hexapetala* in autumn than in spring, and those found in the leaf leachates for both seasons. This assessment of autumnal allelopathy could help to explain the patterns of plant community succession in invaded areas.

**Keywords: allelopathy, macrophytes, native, exotic species, functional traits**



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***Specialty section:***
*This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science*

***Received:*** *18 July 2018*
***Accepted:*** *27 November 2018*
***Published:*** *18 December 2018*

***Citation:***
*Thiébaut G, Thouvenot L and Rodríguez-Pérez H (2018) Allelopathic Effect of the Invasive Ludwigia hexapetala on Growth of Three Macrophyte Species. Front. Plant Sci. 9:1835. doi: 10.3389/fpls.2018.01835*

## INTRODUCTION

The allelochemicals released by organisms into the environment, also called "allelopathy" (Rice, 1984; Elakovich and Wooten, 1989) have beneficial or detrimental effects on neighboring organisms (e.g., phytotoxicity, soil sickness). Allelochemicals are released directly from plants through different mechanisms, such as root exudation, leaching of aerial parts, and volatilization, and also passively through plant decomposition. The role of allelopathy on the structure and composition of biological communities is relatively unexplored in freshwaters (Kulshretha and Gopal, 1983; Agami and Waisel, 1985; Elakovich and Wooten, 1989; Gross, 2003; Dandelot et al., 2008). Nevertheless, some studies showed that several aquatic macrophytes (i.e., *Elodea nuttallii*, *Myriophyllum spicatum*) can impact the phytoplankton (Gross, 2003) and inhibit germination and/or seedling growth (Gopal and Goel, 1993; Dandelot et al., 2008) *via* the release of allelochemicals. Bioassays, using plant extracts (i.e., leachates, exudates), are one of the most common methods used to assess the allelopathic effects of plants. Overall, they have generally only tested the allelopathic potential of plants at one point in time, even though the synthesis of allelochemicals and their concentrations in the plant fluctuate throughout the year (Helmig et al., 2013; Santonja et al., 2018). Indeed, the seasonal variation in allelochemicals could be explained by the fluctuations of abiotic and biotic parameters, i.e., climatic conditions (Petrussa et al., 2013), the presence of herbivores and/or pathogens (Gatti et al., 2014; Silva et al., 2014) and stage in the life-history of the plant (Lombardo et al., 2013; Santonja et al., 2018). The seasonal dependence of plant allelopathic interactions is still understudied, although it could help to explain exotic plant establishment, their spread and plant community succession in invaded areas. Indeed, the potential allelopathy of exotic plants could favor their establishment and their spread into their introduced range (Callaway and Ridenour, 2004). Many exotic plants could synthesize unknown allelochemicals and release them into the native community (c.f. "Novel Weapons Hypothesis," Callaway and Ridenour, 2004). These novel allelochemicals could inhibit the growth of native plants and thus improve the growth of the invasive species (Callaway and Ridenour, 2004; Kim and Lee, 2011). In this way, as stipulated in the Invasional Meltdown Hypothesis (Simberloff and Von Holle, 1999), the introduction of one species may favor the introduction and spread of one or more other exotic species. However, they can also affect other invasive species negatively, if they do not come from the same biogeographical area.

This paper is focused on the potential allelopathic effect of the invasive water primrose *Ludwigia hexapetala* (Hook. and Arn.) Zardini, H. Y. Gu and P. H. Raven. (syn. *L. grandiflora* subsp. *hexapetala*) on three macrophytes species. The amphibious *L. hexapetala*, native to South America, was introduced to Southern France in 1830 (Thouvenot et al., 2013a). Once established, it formed dense mats in freshwaters, on riverbanks and in meadows (Thouvenot et al., 2013a). Its invasive success could be partially explained by the release of secondary metabolites into the recipient community (Dandelot et al., 2008; Santonja et al., 2018) which could limit the growth of native plants. Indeed, the presence of *L. hexapetala* reduces both the plant richness and the abundance of the native species such as the submerged *Ceratophyllum demersum* or some emergent species (*Alisma plantago-aquatica*, *Lycopus europaeus*; Stiers et al., 2011). The water primrose *L. hexapetala* exhibits a horizontal growth stage over water with small round leaves and a growth stage with erect elongated leaves. We used root and leaf leachates and aqueous extracts of *L. hexapetala* from individuals collected in spring and in autumn to analyze the impact of these solutions on the traits of one native species [*C. demersum* L. (Ceratophyllaceae)], and on two exotic species [*Myriophyllum aquaticum* (Vell.) Verdc. (Haloragaceae)], and [*Egeria densa* Planch. (Hydrocharitaceae)]. Our aim was to gain a better understanding of the responses of other macrophytes species to the invasive species. We hypothesized (1) that leaf and root treatments would induce a decrease in the growth of the three target species and (2) that the effects of *L. hexapetala* on the target plants would change according to the season.

## MATERIALS AND METHODS

### Plant Materials

The amphibious Parrot's Feather, *M. aquaticum*, native to South America was introduced as ornamental plant into France in approximately 1880 (Sheppard et al., 2006). The species can cause severe problems in Southern Europe (Les and Merhoff, 1999), in the southern states of the United States, in South America (Fernandez et al., 1993), in New-Zealand and Australia. Once introduced into a new region it spreads rapidly, primarily by vegetative stem fragmentation. It is often found in eutrophic water bodies (small streams, ponds, slow-running waters and irrigation channels). Stems of *M. aquaticum* float out over the water surface to form dense mats from which emergent shoots arise (Hussner, 2009). This species has demonstrated a potential inhibitory effect on neighboring plants (Elakovich and Wooten, 1989).

The Brazilian water-weed, *E. densa*, is a native, submerged macrophyte coming from Argentina, Brazil, and Uruguay. Historically, this species was introduced outside its native range by the aquarium trade. It has been in cultivation in France at least since 1919. It was observed in the field in France in 1960 (Cook and Urmi-König, 1984) and is considered as a nuisance in Central and North America, in Europe and in Australasia (Cook and Urmi-König, 1984). *E. densa* reproduces vegetatively from plant fragments. It has a massive build-up of biomass, allowing it to become highly invasive. Its dense mats reduce recreational activities and crowd out native species, as well as altering the hydrology. Several authors (Nakai et al., 1999; Vanderstukken et al., 2011; Espinosa-Rodríguez et al., 2016) have found allelochemicals which affect phytoplankton negatively.

The European Coontail (*C. demersum*) is a rootless submerged plant found in freshwaters with moderate to high nutrient levels. This plant drifts in the water without being attached to the sediment and the species is usually well equipped to capture high to very high nutrient levels from the surrounding water (Denny, 1972). According to several authors (Kleiven and Szczepańska, 1988; Elakovich and Wooten, 1989). *C. demersum* contains allelopathic compounds. Aqueous extracts showed inhibitory effects on seed development of *Lepidium sativum* (Kleiven and Szczepańska, 1988) and on seedling radicle growth of lettuce (Elakovich and Wooten, 1989).

All the target species are macrophytes with an allelopathic potential. To avoid a history of interactions between the target species and the water primrose, the target species *E. densa*, *M. aquaticum*, and *C. demersum* were bought in a garden center, whereas *L. hexapetala* was collected in the field. In this way, the target plants were considered "naïve" to the water primrose.

### Methods

#### Preparation of the Treatments

For this study, 100 g of small round leaves and 100 g of sediment roots of *L. hexapetala* were collected from a pond at

Apigné in Brittany, France (01°44′ 25.2″ O, 48°05′ 41.4″ N), in spring at mid-March and in autumn at the end of September. The leaves and roots of *L. hexapetala* were washed to remove zooplankton and epiphytes. Aqueous extracts of live leaves and sediment roots were prepared in tap water by crushing 100 g of fresh leaves or fresh roots in 2000 mL of tap water, and the pulpy mixture was stored for 72 h at 4°C. The mixture was filtered through filter paper (Whatman #1) to remove smaller particulate matter (Elakovich and Wooten, 1989). Then it was centrifuged for 15 min at 9,000 rpm. The supernatant thus obtained constituted the aqueous extract. The leaf and root leachates of *L. hexapetala* were prepared by soaking 100 g (fresh leaves or fresh roots) in 2000 mL of tap water for 72 h in the dark at 4°C. They were then filtered through filter paper (Whatman #1). Each leachate/aqueous extract was divided into two: one part (1500 mL) was used to test the potential allelopathic effect of *L. hexapetala* on the three macrophytes species and the second (500 mL) was used to identify allelochemicals.

### Experimental Design

The individuals of *E. densa*, *M. aquaticum*, and *C. demersum* were bought in a garden center 15 days before the beginning of the experiment in spring and in autumn (respectively, at the beginning of March and in mid-September) and acclimatized in tap water at the ambient temperature for 2 weeks. The tap water was slightly basic with a moderate nutrient concentration (mean annual value according to French Water Agency data: conductivity = 462 µS $cm^{-1}$; pH = 7.95; [$NO_3^-$ N] = 6.95 mg $L^{-1}$; [$NH_4^+$ N] = 0.03 mg $L^{-1}$; [$PO_4^{3-}$ P] = 0.043 mg $L^{-1}$). The amphibious *M. aquaticum* had both submerged leaves and aerial leaves which emerged above the surface of the water. After the acclimatization period, the three target plants – *C. demersum*, *M. aquaticum*, and *E. densa* – were rinsed with distilled water and their shoots cut to a length of 5 cm. All the selected shoots had an intact apex, no roots, and no trace of necrosis, buds, or lateral stems. One shoot of each plant species (*E. densa*, *C. demersum*, and *M. aquaticum*) was placed in a cylindrical plastic tube (100 mL, height: 10 cm) filled with 50 mL of the solution (i.e., leaf or root leachates, or leaf or root extracts, or tap water). The water level in each plastic tube was maintained by adding tap water, to avoid increasing allelochemical concentrations, plant desiccation and nutrient deficiencies and to offset losses from evaporation. Ten replicates were used per plant species and treatment. Plants were placed in one growth chamber (Photon Flux Density 80 µmol $s^{-1}$ $m^2$, 12 h light/12 h dark cycle, and at 16°C) for 28 days, and their position in the chamber was completely randomized. The incubation temperature of 16°C was the maximal temperature observed in spring and autumn.

### Measured Variables

Photosynthetic yield was monitored to assess the impact of allelochemical stress responses on the photosynthesis of the target plants. To evaluate the allelopathic effect on the photosynthetic yield, a pulse amplitude modulation (PAM) chlorophyll fluorometer was used to measure photosynthetic activity. PAM is a convenient and sensitive method for monitoring photosynthetic activities. The fluorescence yield was measured on the apex leaves using a Diving-PAM underwater fluorometer (Walz) after a 30-min period of dark adaptation in the afternoon of the first day and then every week for 28 days. The initial fluorescence – Fo – and maximal fluorescence – Fm – were recorded by turning on the weak measuring light, and Fm was given after the saturation flash. The maximum quantum yield (Fv/Fm) was calculated as Fv/Fm = (Fm−Fo)/Fm with Fv variable Fluorescence.

Four morphological traits (plant stem length, roots and lateral branches length, and leaf area) of each plant were measured after 28 days of exposure to the aqueous extracts or leachates solutions in the laboratory. One picture was taken of one leaf per plant at the beginning and at the end of the experiment. The leaf area was measured using Image J software. The relative growth rate (RGR; cm $d^{-1}$) was calculated following Hunt's (1990) formulation: RGR = [ln(L2)-ln(L1)]/(T2-T1), in which L1 and L2 refer to stem length at time points T1 and T2. The same experimental design was applied in spring and in autumn.

## Chemical Composition of the Leaf/Root Aqueous Extracts and Leachates of *L. hexapetala*

The leaf leachates and leaf aqueous extracts of *L. hexapetala* that were not used in the allelopathy experiment, were lyophilized and ground into a powder prior to chemical analysis. The leaf leachates and aqueous extracts of *L. hexapetala* in spring and in autumn were analyzed using liquid chromatography and high resolution mass spectrometry (LCMS) according to the method described by Santonja et al. (2018). There was not enough root material after lyophilisation to conduct the analyses.

## Statistical Analysis

Photosynthetic yields were analyzed on a repeated measures basis using a non-parametric test (Naguchi et al., 2012), since the data did not meet homoscedasticity and normality requirements for parametric tests. Whenever treatment effect or the interaction between treatment and time was significant, a pairwise comparison of treatment levels and treatment levels within a given sampling time was performed using a Mann–Whitney–Wilcox test, and a Benjamini-Hochberg False Discovery Rate (10% acceptance level) correction was subsequently applied to multiple test series (Benjamini and Yekutieli, 2001). Non-parametric repeated measures analyses were performed with R software (R Core Team, 2016) and a nparLD package (Naguchi et al., 2012).

The abilities of each plant species to grow and produce roots and lateral branches under different treatments depending on the season were analyzed using a two-way linear model. The leaf area growth of *C. demersum*, as well as the length of the lateral shoots and roots of each species were log-transformed to check their residual homoscedasticity and normality. When the number of data available per trait and combination of season treatments was strictly lower than three, the combination was not included in the statistical analysis dataset. This was particularly the case for the lateral shoots and root length, as the species did not produce lateral shoots or roots in every treatment. Consequently, some treatments do not appear in the results section or in the Figures and Tables in this paper. The adequate distribution of model residuals was verified for each trait by checking the model plots. Tukey's HSD tests were applied to observe differences between treatments. Untransformed means and standard errors were used in the Figures to facilitate interpretation. Statistical analyses were performed with R software (R Core Team, 2016).

## RESULTS

There were no significant effects of spring aqueous extracts and leachates on the photosynthetic yield or on the morphological traits of *M. aquaticum*, *E. densa*, and *C. demersum* (**Tables 1**, **2** and **Figures 1–4**). Moreover, aqueous extracts and leachates had no significant effect on the photosynthetic yield of *M. aquaticum* in autumn (**Table 1** and **Figures 1A,B**). The RGR of *M. aquaticum* was higher in autumn than in spring ($F = 195.51$; $p < 0.0001$, **Figure 2A**), although it was not impacted by the treatments (**Table 2**). Leaf area growth was affected negatively in autumn by the leaf treatments and by the root leachates ($F = 3.17$; $p = 0.018$, **Figure 2B**). The lengths of lateral shoots of *M. aquaticum* were longer after exposure to the root extracts ($F = 3.89$; $p = 0.027$, **Figure 2C**) than after exposure to leaf treatments.

The autumnal root treatments and the leaf leachates stimulated the photosynthetic yield of *E. densa* after 7 and 28 days in the plants exposed to the root extracts (interaction treatment × sampling date, **Table 1** and **Figures 3C,D**). RGR and leaf area growth of *E. densa* were affected by the interactions between the treatment and the season (**Table 2**), but the length of the roots only depended on the season (**Table 2**). The autumnal root extract stimulated the growth of the *E. densa* leaves (interaction season × treatment: $F = 6.02$; $p = 0.0003$, **Figure 3B**). The lengths of the roots ($F = 150.27$; $p < 0.0001$, **Figure 3D**) were higher in autumn than in spring (**Table 2**).

The photosynthetic yield of *C. demersum* was stimulated by the root extracts in autumn (**Table 1** and **Figure 1F**). Relative

**TABLE 1** | Effects of leachates and extracts of *Ludwigia hexapetala* on the photosynthetic yield of target species in spring and in autumn.

| | | Autumn | | | Spring | | |
|---|---|---|---|---|---|---|---|
| | | **ATS** | **df** | ***p*** | **ATS** | **df** | ***p*** |
| *Myriophyllum aquaticum* | Treatment | 0.73 | 3.23 | 0.6 | 0.96 | 2.37 | 0.4 |
| | Time | 39.67 | 3.02 | **<0.0001** | 39.18 | 3.30 | **<0.0001** |
| | Treatment × time | 1.14 | 8.75 | 0.3 | 1.65 | 8.03 | 0.1 |
| *Egeria densa* | Treatment | 16.11 | 3.85 | **<0.0001** | 0.19 | 3.88 | 0.9 |
| | Time | 40.56 | 3.58 | **<0.0001** | 25.21 | 2.86 | **<0.0001** |
| | Treatment × time | 2.68 | 10.66 | **0.002** | 1.24 | 7.81 | 0.3 |
| *Ceratophyllum demersum* | Treatment | 5.09 | 2.66 | **0.003** | 1.94 | 3.31 | 0.1 |
| | Time | 6.67 | 1.69 | **0.002** | 11.93 | 2.43 | **<0.0001** |
| | Treatment × time | 1.20 | 2.01 | 0.3 | 1.4 | 3.5 | 0.2 |

*The significant differences are indicated in bold type; ATS denotes ANOVA Type Statistic.*

**TABLE 2** | Summary of the two-factor linear model, with season and treatment as factors, on the different morphological traits measured for each species (*C. demersum*, *E. densa*, and *M. aquaticum*) in the laboratory experiment (with the degrees of freedom (Df), the range of the number of replicates per modalities for each factor (n), *F*-values for each factor per trait, and the significance level (p).

| | | Relative growth rate | | | Leaf area growth | | | Length of lateral shoots | | | Length of roots | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Df** | ***n*** | ***F*** | ***p*** | ***n*** | ***F*** | ***p*** | ***n*** | ***F*** | ***p*** | ***n*** | ***F*** | ***p*** |
| ***C. demersum*** | | | | | | | | | | | | | |
| Season | 1 | 31–50 | 0.55 | 0.46 | 29–50 | 2.00 | 0.16 | / | / | / | / | / | / |
| Treatment | 4 | 10–20 | 2.30 | *0.07* | 10-20 | 1.19 | 0.32 | / | / | / | / | / | / |
| Season × treatment | 3 | 6–10 | 3.54 | **0.02** | 4–10 | 2.72 | *0.051* | / | / | / | / | / | / |
| ***E. densa*** | | | | | | | | | | | | | |
| Season | 1 | 46–50 | 55.92 | **<0.0001** | 44–50 | 12.31 | **<0.001** | 11–34 | 3.03 | *0.09* | 31–35 | 150.27 | **<0.0001** |
| Treatment | 4 | 16–20 | 4.57 | **0.002** | 16–20 | 3.26 | **0.015** | 6–12 | 1.35 | 0.27 | 6–19 | 2.51 | *0.052* |
| Season × treatment | 4 | 6–10 | 4.84 | **0.001** | 6–10 | 6.02 | **<0.001** | 3–8 | 0.48 | 0.62 | 3–10 | 1.98 | 0.13 |
| ***M. aquaticum*** | | | | | | | | | | | | | |
| Season | 1 | 42–48 | 195.51 | **<0.0001** | 40–49 | 35.08 | **<0.0001** | / | / | / | 23–27 | 1.43 | 0.24 |
| Treatment* | 4 | 15–20 | 1.39 | 0.25 | 16–20 | 3.30 | **0.015** | 4–7 | 3.70 | **0.03** | 7–14 | 0.87 | 0.49 |
| Season × treatment | 4 | 6–10 | 0.26 | 0.90 | 6–10 | 3.17 | **0.02** | / | / | / | 3–9 | 0.62 | 0.65 |

*Significant results are in bold type; tendencies are in italic. *Df = 3 for the length of lateral shoots.*

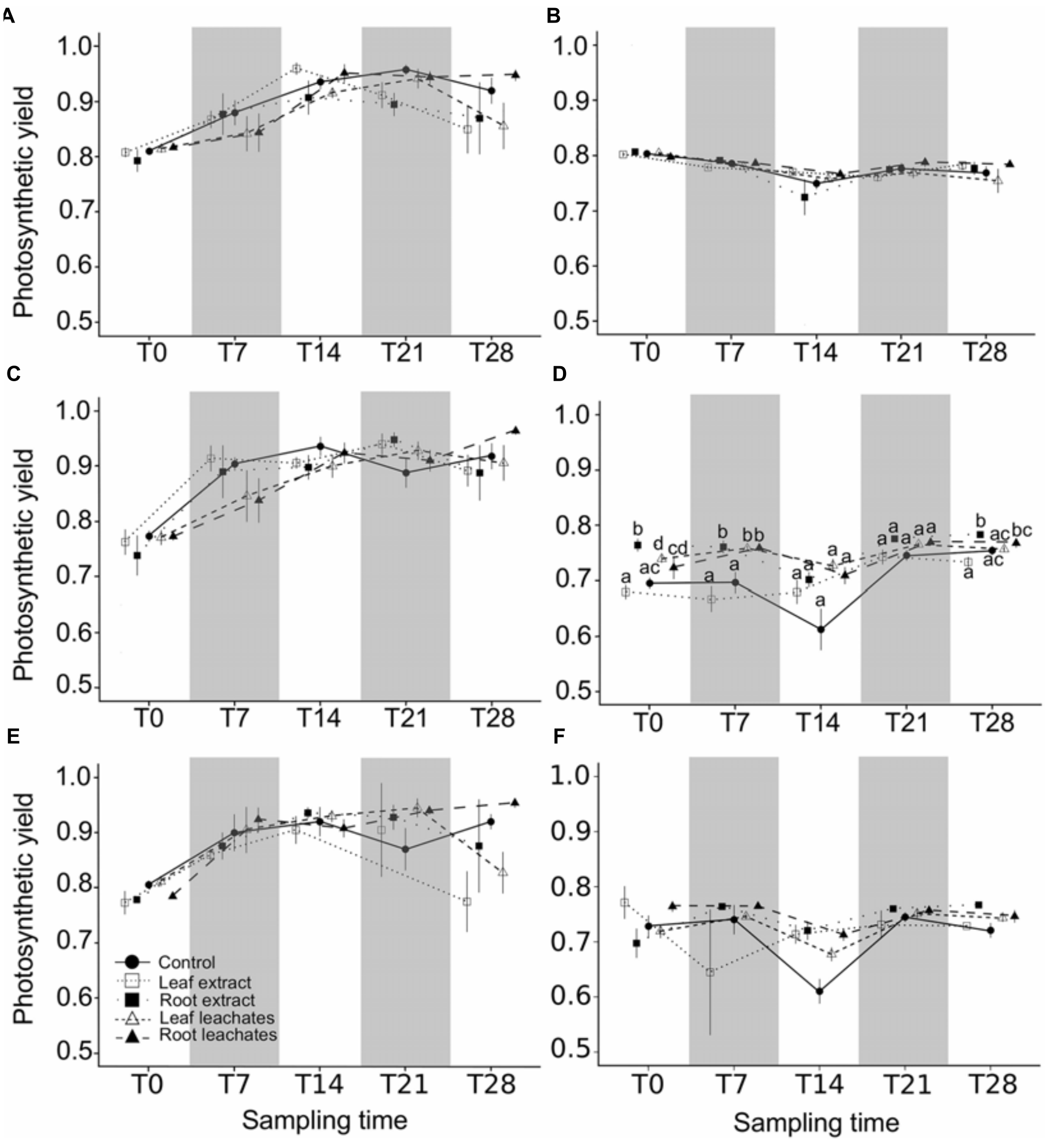


**FIGURE 1 |** Mean photosynthetic yield plus standard error for the three species in spring and autumn: *Myriophyllum aquaticum* (**A** in spring and **B** in autumn), *Egeria densa* (**C** in spring and **D** in autumn), and *Ceratophyllum demersum* (**E** in spring and **F** in autumn). Treatments are denoted by white squares for leaf extract treatments, black squares for root extract treatments, white triangles for leaf leachate treatments, black triangles for root leachate treatments, and black circles for control series. Symbols marked with the same letter are not significantly different for treatment factors within the same period ($p > 0.05$), according to Mann–Whitney–Wilcoxon pairwise comparisons significance test adjusted by the Benjamini-Yekutieli correction.

growth rate (RGR) of *C. demersum* was particularly affected by the interaction season × treatment ($F = 3.54$; $p = 0.019$, **Table 2**). Indeed, the root leachates of *L. hexapetala* decreased the RGR of *C. demersum* in autumn (**Figure 4A**). In contrast, the growth of the leaf area tended to be higher when *C. demersum* was exposed to the root treatments than the control treatment in autumn (interaction season × treatment: $F = 2.72$; $p = 0.051$, **Figure 4B**). The Coontail (*C. demersum*) produced no roots and very few lateral branches both in spring and autumn (**Table 2**).

Three main allelochemicals were identified in the leaf leachates and extracts of *L. hexapetala*: quercitrin, prunin, myricitrin. Concentrations of these allelochemicals were greater in the leaf extracts than in the leaf leachates (**Table 3**). They were higher in autumn than in spring (**Table 3**).

# DISCUSSION

## Potential Allelopathic Effects of *L. hexapetala* on the Growth of Other Macrophyte Species

This study aimed to assess whether the aqueous leaf/root extracts or the leaf/root leachates of the *L. hexapetala* had a positive

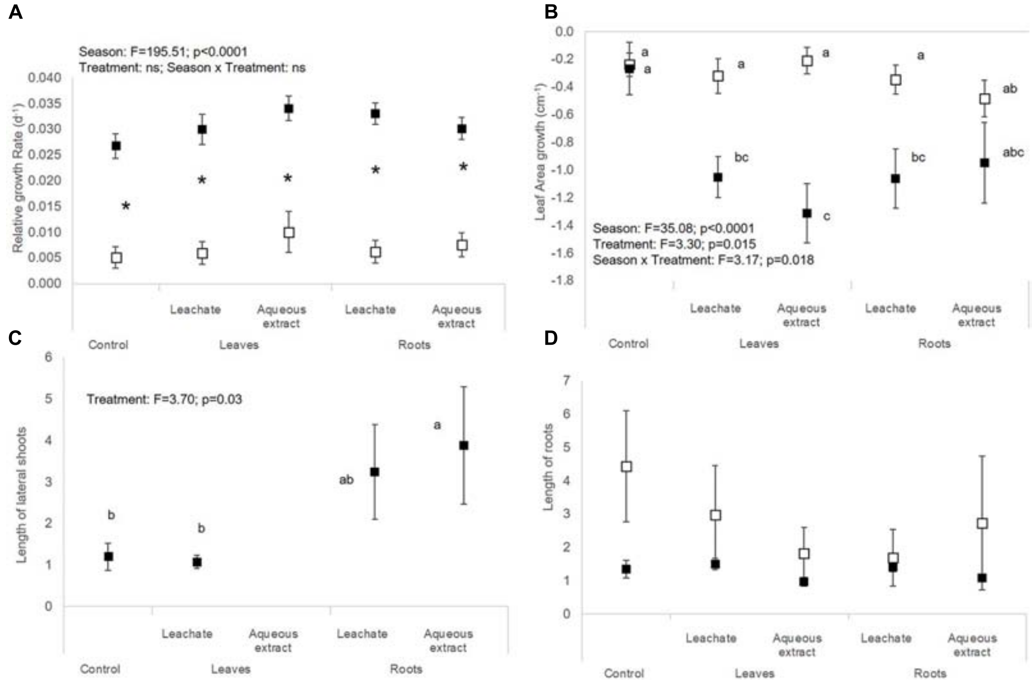


**FIGURE 2 | (A)** Relative growth rate, **(B)** leaf area growth, **(C)** length of lateral shoots, and **(D)** length of roots of *M. aquaticum* (mean ± SE) in spring (white symbols) and autumn (black symbols) depending on the treatment (i.e., control, leaf leachates, leaf aqueous extracts, root leachates, root aqueous extracts) after a 28-day experiment in the laboratory. Different small letters indicate significant differences between the interaction season × treatment. Stars show significant differences between seasons.

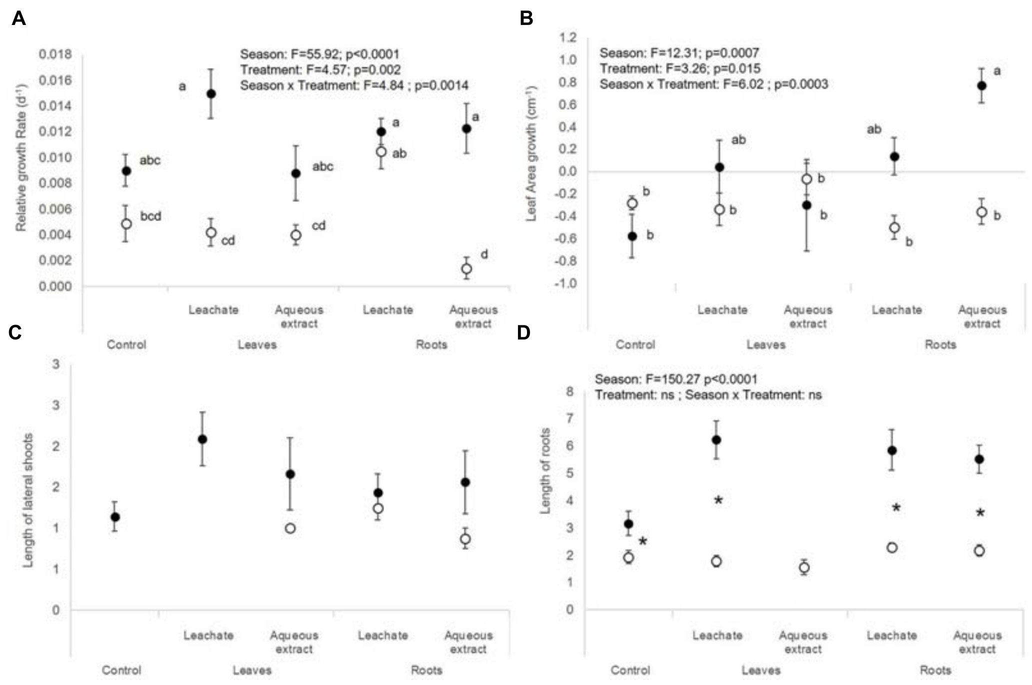


**FIGURE 3 | (A)** Relative growth rate, **(B)** leaf area growth, **(C)** length of lateral shoots, and **(D)** length of roots of *E. densa* (mean ± SE) in spring (white symbols) and autumn (black symbols) depending on the treatment (i.e., control, leaf leachates, leaf aqueous extracts, root leachates, roots aqueous extracts) after a 28-day experiment in the laboratory. Different small letters indicate significant differences between the interaction season × treatment. Stars show significant differences between seasons.

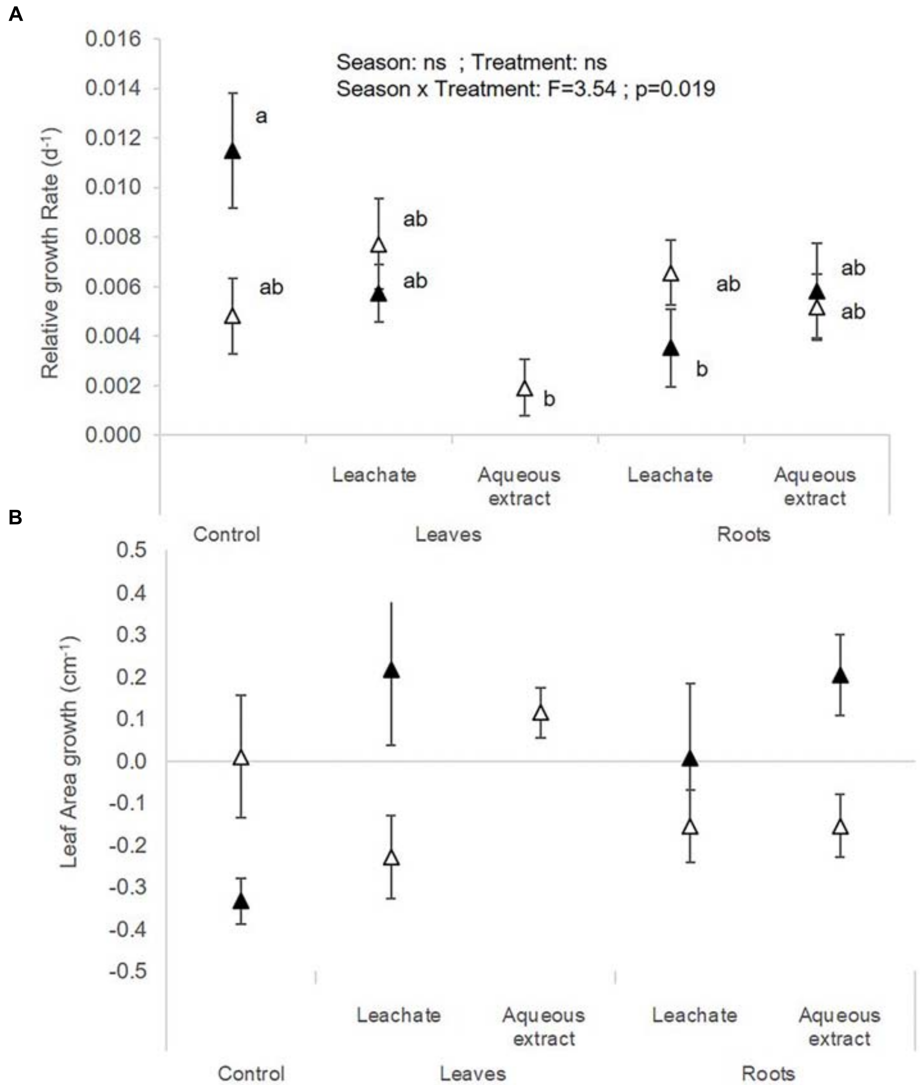


**FIGURE 4 | (A)** Relative growth rate and **(B)** leaf area growth of *C. demersum* (mean ± SE) in spring (white symbols) and autumn (black symbols) depending on the treatment (i.e. control, leaf leachates, leaf aqueous extracts, root leachates, root aqueous extracts) after a 28-day experiment in the laboratory. Different small letters indicate significant differences between the interaction season × treatment.

or negative effect on the development of other macrophyte species. Our results showed that the root extracts stimulated the lateral branches of *M. aquaticum* and the leaf area of the two submerged species *C. demersum* and *E. densa*. Conversely, the leaf treatments and root leachates inhibited the leaf area growth of *M. aquaticum*. The latter result could be explained by similar biological type and niche overlap: *L. hexapetala* and *M. aquaticum* are both exotic species with similar growth forms (amphibious) native from the same geographical range. Although the two species co-exist in the field, their patches are spatially separated. In a previous study, we found that *L. hexapetala* stimulated the root production and the growth of *M. aquaticum* at low densities (Thouvenot et al., 2013b). The allelochemicals released by roots of *L. hexapetala* could directly favor the lateral growth of *M. aquaticum* (length of lateral shoots) or could also indirectly affect its development by modifying the chemical and physical properties of the soil/sediment and by regulating the soil microbial community (Walker et al., 2003). Parrot's Feather (*M. aquaticum*) may have the capacity to protect itself from allelopathy by metabolizing certain phenolic compounds with allelopathic activity (Elakovich and Wooten, 1989). This ability of Parrot's Feather to synthesize phenols gives it an advantage in allelopathic interactions and may favor its competitiveness. Further studies testing the effect of *M. aquaticum* root/leaf leachates on *L. hexapetala* are required.

However, we observed that the effects of the root and leaf treatments differ according to the biological growth forms of the exotic species. The biological type of plants affects levels of secondary compounds. For example, emergent species in wetlands contain more phenolics than submerged plant species

**TABLE 3 |** Mean concentrations (± SE; $n = 3$; $\mu g\ L^{-1}$) of chemical compounds found in the leaf aqueous extracts and leachates of *Ludwigia hexapetala* in spring and in autumn.

| | Spring | Autumn |
|---|---|---|
| **Leaf leachates** | | |
| Myricitrin | 88.0 +/− 9.1[a] | 334.4 +/− 9.2[b] |
| Prunin | 56.6 +/− 0.6[a] | 121.5 +/− 3.4[b] |
| Quercitrin | 97.7 +/− 1.7[a] | 141.9 +/− 4.1[b] |
| **Leaf aqueous extract** | | |
| Myricitrin | 2538[a] | 8899[b] |
| Prunin | 2185[a] | 3187[b] |
| Quercitrin | 2877[a] | 3981[b] |

*One-way ANOVAs were performed for differences between seasons for each chemical compound. F-values and associated P-values are indicated. Different letters denote significant differences between the two seasons with a < b (post hoc Tukey tests results after one-way ANOVA); d.f., degree of freedom.*

(Smolders et al., 2000). In our experiment, there was no effect of treatments on the apical growth (RGR) of *E. densa*, a species from the same geographical area as the water primrose. Previously, we had established that there was no competition between *E. densa* and *L. hexapetala,* but that there was facilitation (Thouvenot et al., 2013b). The secondary compounds produced by the roots of the water primrose stimulated leaf area growth and consequently had a positive impact on the photosynthetic yield. Our results suggested positive interactions between the water primrose and the submerged *E. densa*. In contrast, the growth of the native submerged plant *C. demersum* was inhibited by root leachates. This result is congruent with the "Novel Weapons Hypothesis" (Callaway and Ridenour, 2004) and with the literature (Sakpere et al., 2010). Previously, Kulshretha and Gopal (1983) established that *C. demersum* and *C. muricatum* were negatively affected by the exotic submerged *Hydrilla verticillata*. Moreover, Sakpere et al. (2010) observed that exudates of *Ludwigia decurrens* and *Ludwigia adscendens* reduced the stem length of *Corchorus olitorius* seedlings in early growth.

## Seasonal Effect of *L. hexapetala* on the Other Macrophyte Species

The autumnal treatments had significant effects on the photosynthetic yield and on the morphological traits of the two submerged species and the exotic amphiphyte, whereas no spring effects were established. The variation in significance of the effects of *L. hexapetala,* depending on the time at which the roots and the leaves were collected, confirmed the hypothesis that the plants change seasonally. Previous studies have also reported a seasonal pattern of allelopathic interactions (Bauer et al., 2009; Silva et al., 2014). For example, Bauer et al. (2009) showed an optimal inhibitory effect of *Myriophyllum verticillatum* on the cyanobacteria *Anabaena variabilis* in spring. Indeed, the secondary compound composition in *L. hexapetala* could be different in spring and autumn (Dandelot et al., 2008; Santonja et al., 2018) due to variations in the environmental conditions of the Apigné ponds (i.e., nutrients, light intensity or water depth), of climatic parameters (Chaves and Escudero, 1999) and of the plant phenological stage. The presence of allelochemicals produced by short leaves is dependent on the season (Dandelot et al., 2008). The seasonal fluctuations could alter the allelopathic activity of the secondary compounds (Santonja et al., 2018). Our results should be used with caution because the different impact of aqueous extracts and leachates according to the season could be explained both by seasonal fluctuations in the physiology of the target species (*C. demersum*, *E. densa*, *M. aquaticum*) and of the donor species *L. hexapetala*. However, our target species are clonal individuals, cultivated under glasshouse, conditions which should reduce the fluctuations due to plant phenology. Furthermore, the absence of a seasonal fluctuation in the RGR of *E. densa* and of *C. demersum* in the control suggested that the seasonal fluctuation of donor species *L. hexapetala* is the basis of potential seasonal effects of the allelopathy. The growth rate of *M. aquaticum* was not affected by allelopathy but only by season. The ability of *M. aquaticum* to metabolize phenolic compounds could counteract the impact of high concentrations of allelochemicals produced by the water primrose in autumn.

## Chemical Composition of the Leaf/Root Aqueous Extracts and Leachates of *L. hexapetala*

Three main flavonoids belonging to the polyphenol family were identified in the leaf treatments in spring and in autumn: quercitrin, prunin, myricitrin. The allelochemical composition is phylogenetically determined (Grutters et al., 2017). Numerous compounds are produced by *Ludwigia* sp.: saponins, tannins, polyphenols, alkaloids, linoleic acids, and flavonoids (Averett et al., 1990). However, phenolics are the compounds most frequently involved in allelopathy in freshwaters (Gross, 2003; Iason et al., 2013) or between aquatic plant species (Dandelot et al., 2008). According to an analysis of root extract conducted by Marcellin-Gros (2015), the most abundant secondary compounds in *L. hexapetala* are two tannins (pedunculagin and an ellagic acid) and the flavonoid quercetin. The result found by Marcellin-Gros (2015) suggested that composition of the root extracts differed from that of the leaf treatments, except for quercitrin. Quercitrin had a positive effect on the photosynthetic yield of phytoplankton (Santonja et al., 2018), and was higher in the root extracts of *L. hexapetala* (Marcellin-Gros, 2015) than in leaf extracts. This secondary compound may have been released into the water by the roots of the water primrose and could have stimulated the photosynthetic yield and the leaf area of the two submerged species *E. densa* and *C. demersum*. Previous work has suggested that the tannin pedunculagin is characterized by its antioxidant properties and its positive effects on human health (Biswas et al., 2014), whereas ellagic acid is a rooting inhibitor (Viéitez and Ballester, 1986; Qin et al., 2006). However, there was no root inhibition detected for *E. densa* and *M. aquaticum*. Thus, the roles of these two tannins are unknown and need to be investigated further.

The concentrations of the allelochemicals in the leaves were higher in autumn, which may be related to the seasonal fluctuations of environmental parameters and trade-offs between primary and secondary metabolisms. In autumn, plants are exposed to a decrease in temperatures and solar radiation, which has been reported to have an inverse relationship with secondary metabolite accumulation in plant tissues (Silva et al., 2014).

## CONCLUSION

The first hypothesis of this study was partially validated for *M. aquaticum* and for *C. demersum*, indicating an autumnal phytotoxic effect of leaf treatments on the leaf area growth of the exotic Parrot's Feather and of root leachates on the growth of the native species. However, *L. hexapetala* favors the growth of lateral shoots of *M. aquaticum* and the leaf area and photosynthetic yield of *E. densa*; suggesting a facilitation effect of the root treatment on the two other exotic species. In contrast, *L. hexapetala* strongly reduced the growth of the native plant. However, many plants release allelochemicals into the environment with little impact on the performance of native plants, due to a long coevolutionary history (Thorpe et al., 2009). Indeed, there are several biotic and abiotic factors (interactions between plants and herbivores/pathogens, climatic conditions) that are able to change allelopathic impact on the recipient community (Inderjit et al., 2011).

Our second hypothesis, that the impact of leaf and root treatments on the growth of the three target species showed seasonal fluctuations, was validated. There was no impact of the treatment in spring. Plant growth is optimal in spring. There are trade-offs between the primary biological functions of plants, such as growth, and resource allocation for chemical defense (Herms and Mattson, 1992). The variation in the effects of *L. hexapetala* demonstrated in this study highlights the importance of conducting allelopathy research during different seasons; if this variation is not considered, the results may not reflect the potential effect of a plant species correctly (Silva et al., 2014). Moreover, the seasonal dependence of biotic interactions has not been studied in depth and it is necessary to take this into account in order to gain a better understanding of the interactions between native and exotic macrophyte species and between different exotic species.

## AUTHOR CONTRIBUTIONS

GT and LT designed and conducted the experiments. LT and HR-P analyzed the data. GT wrote the manuscript with contributions from LT and HR-P.

## ACKNOWLEDGMENTS

The researchers would especially like to thank Camille Puech and Laurent Martinez for their assistance during laboratory experiments, Mathieu Santonja for chemical analysis, and Nathalie Marnet for assistance during the chemical analyses. Chemical analyses were performed at the Plateau de Profilage Métabolique et Métabolomique (P2M2, INRA, Le Rheu, France).

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 07 January 2019
doi: 10.3389/fpls.2018.01938

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# The Analysis of Leaf Traits of Eight *Ottelia* Populations and Their Potential Ecosystem Functions in Karst Freshwaters in China

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Submerged macrophytes play a structuring role in the shallow freshwater ecosystem by increasing the heterogeneous state in freshwaters. The macrophytes in genus *Ottelia* were featured for their broad leaves, which might consequently produce specialized functions that differed from other submerged species. To explore the potential ecological role of *Ottelia*, a field investigation was conducted on leaf traits in eight populations of *Ottelia* ranging from the southwestern Yunnan–Guizhou plateau to the southern Hainan island in China covering a distance of >1,700 km. The eight populations included all the extant *Ottelia* species and varieties in China except the well-documented *O. alismoides*. Carbon-related traits [bicarbonate usage, photosynthetic characteristics, capability of Crassulacean acid metabolism (CAM)], pigment content and parameters of chlorophyll fluorescence, morphology and mass of the leaves were determined. The different populations showed distinct functional traits of mature leaves; *O. acuminata* var. *songmingensis* had the thickest and longest leaf with $CaCO_3$ precipitation on the both sides of the leaf, and *O. cordata* showed putative CAM activity with the highest diel acidity changes 12.5 μequiv $g^{-1}$ FW. Our results indicated an important role of *Ottelia* populations in carbon cycling as the dominant species in karst freshwaters in China.

**Keywords: *Ottelia*, Crassulacean acid metabolism, bicarbonate usage, leaf traits, shallow freshwaters**

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***Edited by:***
*ZhongQiang Li, Hubei University, China*

***Reviewed by:***
*Lina Fusaro, Sapienza University of Rome, Italy*
*Enhua Li, Institute of Geodesy and Geophysics (CAS), China*

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***Specialty section:***
*This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science*

***Received:*** *26 June 2018*
***Accepted:*** *12 December 2018*
***Published:*** *07 January 2019*

***Citation:***
*Cao Y, Liu Y, Ndirangu L, Li W, Xian L and Jiang HS (2019) The Analysis of Leaf Traits of Eight Ottelia Populations and Their Potential Ecosystem Functions in Karst Freshwaters in China. Front. Plant Sci. 9:1938. doi: 10.3389/fpls.2018.01938*

## INTRODUCTION

Submerged macrophytes are considered as one of the most important primary producers in shallow oligotrophic freshwaters and strongly affect the nutrient turnover for freshwater ecosystem (Wetzel, 1964; Epstein et al., 2012; Olsen et al., 2017). In addition, submerged macrophytes can interact with other organisms, e.g., protecting the zooplankton from fish grazing or providing substrate for periphyton growth (Jeppesen et al., 1998; Cao et al., 2014, 2017). Plant functional traits, as a series of core properties describing the growth, survival and reproduction of plants, are useful tools to explore the ecological function of submerged macrophytes in freshwater systems (Grime, 1974). Most studies related with plant functional traits are focusing on terrestrial forest or grass (Kraft et al., 2008; Klimešová et al., 2016). For example, Kraft et al. (2015) stated that using the combination of functional traits (e.g., the growth form), but not simplistic usage of single functional trait, was important to infer community assembly processes in grassland. For submerged macrophytes, there are only few recent studies related with functional traits, which have investigated the functional traits at the community level along water depth gradients in natural lakes (Fu et al., 2014, 2018; Liu and Wang, 2018).

Leaf is the most important photosynthetic organ, and leaf traits are one of central plant functional traits (Petter et al., 2016; Damián et al., 2018). Especially the concept of 'leaf economic spectrum' (LES) has revealed the importance of leaf traits; a typical trade-off between leaf functional traits of >2,000 species has been found, which shows that leaves with the higher photosynthesis rate are featured with shorter life span and lower leaf mass per area (MPA; Wright et al., 2004). In addition, Cornwell et al. (2010) linked the decomposition rate of terrestrial plant litter with the position of the species in the LES, indicating a close relationship between leaf traits and ecosystem function. The submerged macrophytes also have contrasting decomposition rates (*Potamogeton crispus* vs. *P. macckianus*), which can significantly affect the carbon cycling in shallow lakes (Wang et al., 2016). However, none of submerged species has been included in LES. Compared with other submerged species, leaves of *Ottelia* are usually much broader with long petiole, and meantime these leaves can play an extra role in ecosystem carbon cycling through $CaCO_3$ precipitation on the leaf surface compared with those of terrestrial plants (Prins et al., 1982; Yin et al., 2017).

The genus *Ottelia* widely distributes from tropical to temperate areas and consists of ca. 21 species in the globe[1]. Among these species, *O. alismoides* has been under extensive investigation. The species used to widely spread in China and presently under threat due to habitat fragmentation (Chen et al., 2008). As an annual species, the seed germination was featured with density dependence (Yin et al., 2009, 2013). In addition, *O. alismoides* was found with three carbon concentrating mechanisms, i.e., bicarbonate usage, Crassulacean acid metabolism (CAM) and C4 (Zhang et al., 2014; Shao et al., 2017), which showed potentially strong influence on carbon cycling in freshwater ecosystems dominated by the species (Maberly and Gontero, 2017; Shao et al., 2017). While other species in genus *Ottelia* were less investigated, and the main focus was about the phylogenetic relationship among these species based on the characteristic of isozyme, flower, seed and qualitative description of the species (Kaul, 1969; Cook et al., 1984; He, 1991). For instance, Chen et al. (2017) has studied five recorded varieties of *O. acuminata,* an endemic species in China, based on molecular proofs, and the authors stated that most of collected varieties reflected genetically differentiated group, and the genetic divergences could be linked with the past tectonic movements. Other than *O. alismoides,* the *Ottelia* species or varieties grew in a localized and relatively stable karst freshwater (Chen et al., 2017). Consistently, Li et al. (2018) assumed a fast speciation process of *O. acuminata* due to geographic features in these areas.

[1]http://foc.eflora.cn/content.aspx?TaxonId=123432, accessed on 06-28-2018.

As the dominant submerged species in pristine karst freshwaters, populations of *O. acuminata* are potentially important factors of carbon source/sink in the ecosystem (Wang et al., 2017). Therefore, an *in-situ* investigation of leaf traits could give the indispensable information of ecological functions of the *Ottelia* populations in the freshwater ecosystem.

In this study, we sampled eight *Ottelia* populations across the distance of 1,700 km in karst freshwaters in China, and we hypothesized that leaf traits of the *Ottelia* populations can correlate with phylogenetic relationship, and a detailed analysis of leaf traits facilitates to reveal the role of *Ottelia* populations in the carbon cycling in karst freshwaters.

## MATERIALS AND METHODS

Eight populations in Luguhu (LG), Heqing (HQ), Jianchuan (JC), Eryuan (EY), Guiyang (GY), Songming (SM), Jingxi (JX), and Haikou (HN) were distributed in the provinces of Yunnan, Guizhou, Guangxi and Hainan, covering a distance of >1,700 km. Most of the collected species grew in localized karst freshwaters, and the geographic information of the sampling sites was listed in **Table 1**.

The physico-chemical variables in each site including atmospheric pressure (AP), dissolved oxygen (DO), conductivity (C), total dissolved solid (TDS), pH, and oxidation–reduction potential (ORP) were measured *in situ* by a YSI Pro Plus multiparameter meter (Xylem, United States). Photosynthetically active radiation (PAR) was determined at the water depth of 0 cm and 40 cm by a LI-1400 Data Logger and a LI-192 underwater quantum sensor (LI-COR, United States), and the light attenuation was calculated assuming an exponential decay of PAR in the water column (Kirk, 1977). Two or more liters of water samples were collected by a plastic tube sampler and separated into aliquot for the determination of total nitrogen (TN), total phosphorus (TP), alkalinity (Alk), and phytoplankton chlorophyll a (PhyChla). Samples for TN and TP were frozen at −20°C, transferred into lab and determined by the $K_2S_2O_8$ digestion (Huang et al., 1999). Alkalinity (Alk) was determined using the Gran titration of 0.1 M HCl. At least 1 l of water was filtered through the GF/C filter for the determination of PhyChla, and the filter was extracted by 95% ethanol and determined by a spectrophotometer (Jespersen and Christoffersen, 1987).

Based on Kraft et al. (2015), we used the combination of functional traits (not a single trait) to evaluate the ecological functions of the leaves of *Ottelia*. We classified the leaf traits into three categories: leaf morphology and mass, pigment content and parameters of chlorophyll fluorescence and carbon-related traits (bicarbonate usage, capability of CAM and photosynthetic characteristics).

The individuals of *Ottelia* were harvested gently from the ponds or rivers. Twenty intact leaves were randomly chosen for the determination of leaf morphology and fresh weight. The maximum width and the maximum length were measured by a ruler, and the thickness was determined by a vernier caliper. The leaf was then placed into a standard plate and photographed for the determination of the leaf area (Shao et al., 2017). Afterward,

**TABLE 1 |** The geographic information of the sampling sites.

| Taxon | Population code | Location | Latitude (N) | Longitude (E) | Habitat |
|---|---|---|---|---|---|
| *O. acuminata* var. *crispa* | LG | Luguhu, Yunnan | 27.67° | 100.76° | Lake |
| *O. acuminata* var. *acuminata* | HQ | Heqing, Yunnan | 26.55° | 100.17° | Pond |
| | JC | Jianchuan, Yunnan | 26.53° | 99.96° | Pond and stream |
| | EY | Eryuan, Yunnan | 26.16° | 99.93° | Lake |
| *O. balansae* | GY | Guiyang, Guizhou | 26.43° | 106.67° | Pond |
| *O. acuminata* var. *songmingensis* | SM | Songming, Yunnan | 25.27° | 102.88° | Pond and stream |
| *O. acuminata* var. *jingxiensis* | JX | Jingxi, Guangxi | 24.84° | 103.45° | River |
| *O. cordata* | HN | Haikou, Hainan | 19.94° | 110.40° | Stream |

**TABLE 2 |** The physic-chemical variables of the sampling sites.

| Sampling sites | Temp (°C) | AP mmHg | DO mg $L^{-1}$ | C us $cm^{-1}$ | TDS mg $L^{-1}$ | pH | ORP mV | Kd $m^{-1}$ | Alk mmol $L^{-1}$ | PhyChla mg $L^{-1}$ | TN mg $L^{-1}$ | TP $\mu$g $L^{-1}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LG | 21.1 | 550.9 | 6.63 | 219.2 | 153.4 | 8.94 | −0.3 | 0.13 | 1.81 | 1.0 | 0.28 | 37 |
| HQ | 17.1 | 585.1 | 8.59 | 216.4 | 165.1 | 8.84 | 17.4 | 0.16 | 2.36 | 0.9 | 0.67 | 26 |
| JC | 16.3 | 584.8 | 14.7 | 288.5 | 213.6 | 7.98 | 167.2 | 0.58 | 3.15 | 2.5 | 0.69 | 29 |
| EY | 23.4 | 566.5 | 6.33 | 213.0 | 143.2 | 8.87 | −27 | 0.72 | 1.43 | 5.0 | 1.21 | 59 |
| GY | 23.1 | 666.9 | 7.65 | 469.6 | 317.2 | 7.74 | 183.9 | 0.47 | 3.10 | 1.5 | 2.47 | 11 |
| SM | 20.3 | 607.9 | 7.25 | 281.5 | 204.1 | 8.39 | 114.6 | –[a] | 2.80 | 0.8 | 1.77 | 22 |
| JX | 22.2 | 685.8 | 6.52 | 430.6 | 297.3 | 7.79 | 51.2 | 0.31 | 3.95 | 0.5 | 2.31 | 29 |
| HN | 30.8 | 755.5 | 2.83 | 273.7 | 165.8 | 7.33 | 137.3 | 0.88 | 1.52 | 0.3 | 2.62 | 69 |

[a] *$K_d$ is not determined in the site SM.*

the leaf was blotted by an absorbent paper and submerged into a half-filled measuring cylinder, and the volume changes of the water in the cylinder was estimated as the leaf volume.

Leaf pigment content including chlorophyll a (Chla), chlorophyll b (Chlb), and carotenoids (Car) was measured by the extraction of 95% ethanol (Huang et al., 1999). The parameters of leaf chlorophyll fluorescence were determined by chlorophyll fluorometer MONITORING-PAM (Walz, Germany) following the methods in Kramer et al. (2004) and Jiang et al. (2017). $\Phi_{PSII}$ is derived from a pure 'lake' or 'puddle' model referring to the fraction of the energy absorbed by photosystem (PS) II that is used in photochemistry. $\Phi_{NPQ}$ and $\Phi_{NO}$ are used to estimate the flux of excitation energy into the non-photochemical pathways, and $\Phi_{NPQ}$ refers to the yield induced by downregulatory processes, $\Phi_{NO}$ for the yield of other energy losses. $\Phi_{PSII}$, $\Phi_{NPQ}$, and $\Phi_{NO}$ were obtained by an induction curve by setting the PAR of the active light at 127 $\mu$mol $E^{-1}$ $s^{-2}$. $rETR_{max}$ and $I_k$ were acquired by a rapid light curve using the 12 steps of PAR gradients from ca. 2 to 1500 $\mu$mol $E^{-1}$ $s^{-2}$.

The end-point pH in 1 mM Na/$KHCO_3$ solution for the leaves of each population was determined by a pH-drift method (Zhang et al., 2014). CAM capability was determined according to the diel change of acidity in leaves using the titration of 0.01M NaOH to pH 8.3 (Shao et al., 2017). Photosynthesis rates were determined by measuring the changes of DO in the 50 ml glass bottles prior to and 30 min after adding the leaves into the bottle at the concentrations of 0, 1, 2, 4, 6, 8, and 10 mM dissolved inorganic carbon (DIC, represented as Na/$KHCO_3$), respectively. As a commonly used method, the DIC in the water was stable during the determination (Zhang et al., 2014; Clement et al., 2016). The light was provided by LED light bulb (ca. 100 $\mu$mol $E^{-1}$ $s^{-2}$), and the dark respiration rates were determined in the brown bottles prior to and 30 min after adding the leaves into the bottle at the concentrations of 0 and 10 mM DIC. The net photosynthesis rates at different concentrations of DIC was fitted to a slightly modified Michaelis–Menten equation that considered the compensation point for DIC (Clement et al., 2016). The equation is:

$$\text{Net photosynthesis rate} = \frac{V_{\max} * (\text{DIC} - \text{CP})}{K_{half} + (\text{DIC} - \text{CP})}$$

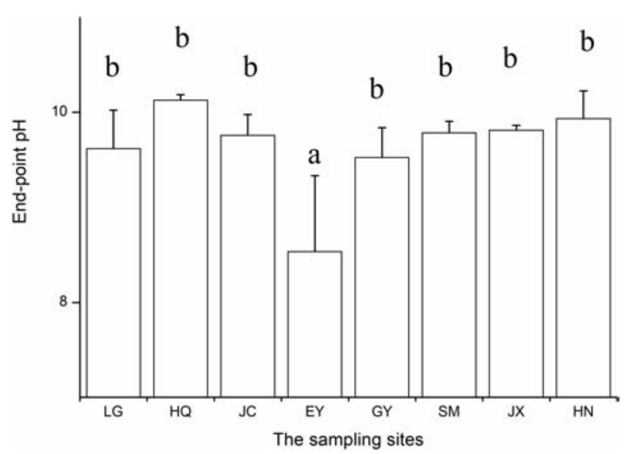


**FIGURE 1 |** The end-point pH of the pH-drift experiments in the eight populations.

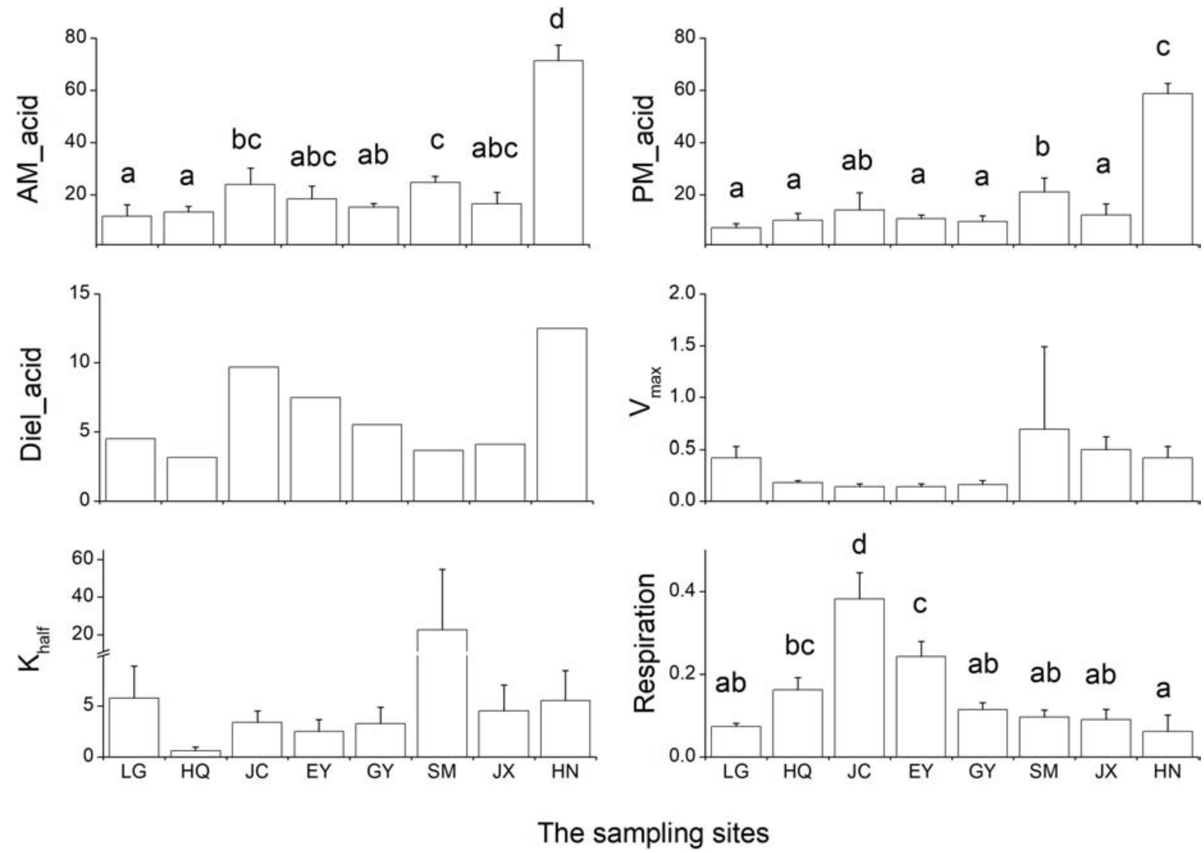


**FIGURE 2 |** The six carbon-related leaf traits in the eight *Ottelia* populations. AM_acid and PM_acid is the acidity of the leaves at ca. 7 am and 7 pm (unit: μ equiv $g^{-1}$ FW). Diel_acid is the changes between mean AM_acid and mean PM_acid (unit: μ equiv $g^{-1}$ FW). $V_{max}$ is the maximum rate of net photosynthesis (unit: mg $O_2$ $g^{-1}$ FW $h^{-1}$), and $K_{half}$ is the concentration of dissolved inorganic carbon producing half-maximal rates of net photosynthesis calculated from the Michaelis–Menten equation (unit: mM). Respiration is the respiration rate in the dark (unit: mg $O_2$ $g^{-1}$ FW $h^{-1}$). Due to the method of calculation, Diel_acid, $V_{max}$ and $K_{half}$ are not analyzed by ANOVA and therefore no statistical results for *post hoc* test. The different letters in the figure indicate the significant difference among the eight population.

where (rate as mg $O_2$ $g^{-1}$ FW $h^{-1}$ and concentration as mM) $V_{max}$ is the maximum rate of net photosynthesis; CP is the DIC compensation concentration; $K_{half}$ is the concentration of DIC producing half-maximal rates of net photosynthesis.

**TABLE 3 |** The Pearson correlation between six carbon-related leaf traits in the eight populations.

| | AM_acid | PM_acid | $V_{max}$ | Respiration | $K_{half}$ |
|---|---|---|---|---|---|
| Diel_acid | 0.804 | 0.729 | −0.027 | 0.240 | 0.182 |
| AM_acid | | 0.993* | 0.307 | −0.251 | 0.393 |
| PM_acid | | | 0.358 | −0.336 | 0.416 |
| $V_{max}$ | | | | −0.718 | 0.693 |
| Respiration | | | | | −0.525 |

*AM_acid and PM_acid is the acidity of the leaves at ca. 7 am and 7 pm (unit: μ equiv $g^{-1}$ FW). Diel_acid is the changes between mean AM_acid and mean PM_acid (unit: μ equiv $g^{-1}$ FW). $V_{max}$ is the maximum rate of net photosynthesis (unit: mg $O_2$ $g^{-1}$ FW $h^{-1}$). Respiration is the respiration rate in the dark (unit: mg $O_2$ $g^{-1}$ FW $h^{-1}$). $K_{half}$ is the concentration of dissolved inorganic carbon producing half-maximal rates of net photosynthesis calculated from the Michaelis–Menten equation (unit: mM). The star (*) indicates a correlation coefficient > 0.9.*

Five replicates were measured for the CAM capability, and three replicates were measured for other indicators of macrophyte leaves.

## Statistical Analysis

For most indicators of leaf traits, one-way ANOVA was used to analyze the difference among the eight populations. *Post hoc* test was conducted using Tukey method at the significance level of 0.05. The data was log-transformed to achieve variance homogeneity prior to the statistical analyses, if needed. Because the diel change of leaf acidity was determined by the difference of mean leaf acidity at dusk and at dawn and thus had no replicates, it was not quantitatively analyzed by ANOVA. $V_{max}$ and $K_{half}$ were estimated by fitting the Michaelis–Menten equation with standard errors (as stated above). The traits were classified into three categories: leaf morphology and mass, pigment content and parameters of chlorophyll fluorescence and carbon-related traits (bicarbonate usage, capability of CAM and photosynthetic characteristics). Pearson correlation and the cluster analysis were analyzed within each aspect. In this study the correlation coefficient that is >0.9 was defined as a strong relationship

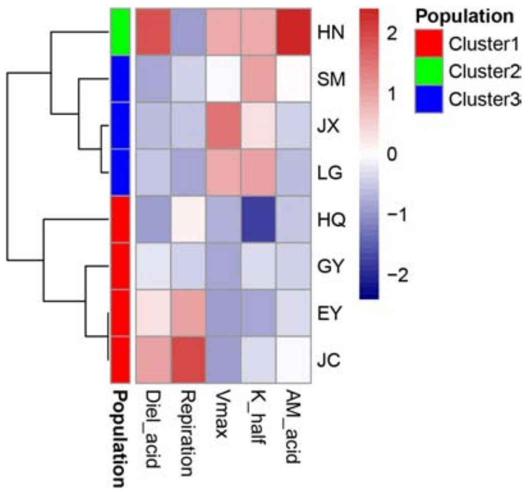


**FIGURE 3 |** The cluster analysis of carbon-related leaf traits in the eight *Ottelia* populations. AM_acid is the acidity of the leaves at ca. 7 am (unit: μ equiv $g^{-1}$ FW). Diel_acid is the changes of leaf acidity between ca. 7 am (dusk) and 7 pm (dawn) (unit: μ equiv $g^{-1}$ FW). $V_{max}$ is the maximum rate of net photosynthesis (unit: mg $O_2$ $g^{-1}$ FW $h^{-1}$), and $K_{half}$ is the concentration of dissolved inorganic carbon producing half-maximal rates of net photosynthesis calculated from the Michaelis–Menten equation (unit: mM). Respiration is the respiration rate in the dark (unit: mg $O_2$ $g^{-1}$ FW $h^{-1}$).

between the leaf traits, and only one trait was included in the further clustering analysis to reduce the statistical bias. The clustering analysis was conducted by package 'mclust' using the 'euclidean distance' after 'scale' the data. All the statistical analyses were determined in R 3.4.0. Data is presented in mean ± SD if not explicitly stated.

## RESULTS

### Physic-Chemical Conditions in the Eight Sampling Sites

As shown in **Table 2**, most sites had low nutrient levels, low phytoplankton biomass, low light attenuation, and slight alkaline with relative high alkalinity, indicating a pristine status with clear water.

### pH-Drift

At the end of the pH-drift experiment pH was lowest in the EY population (**Figure 1**). After excluding the EY population, the end-point pH did not differ among the rest seven populations (ANOVA, $F = 1.98$, $p > 0.05$). Only the end-point pH in the HQ population is >10 (10.13 ± 0.06). Consistently, we observed that $CaCO_3$ precipitation on the surface of the mature leaves

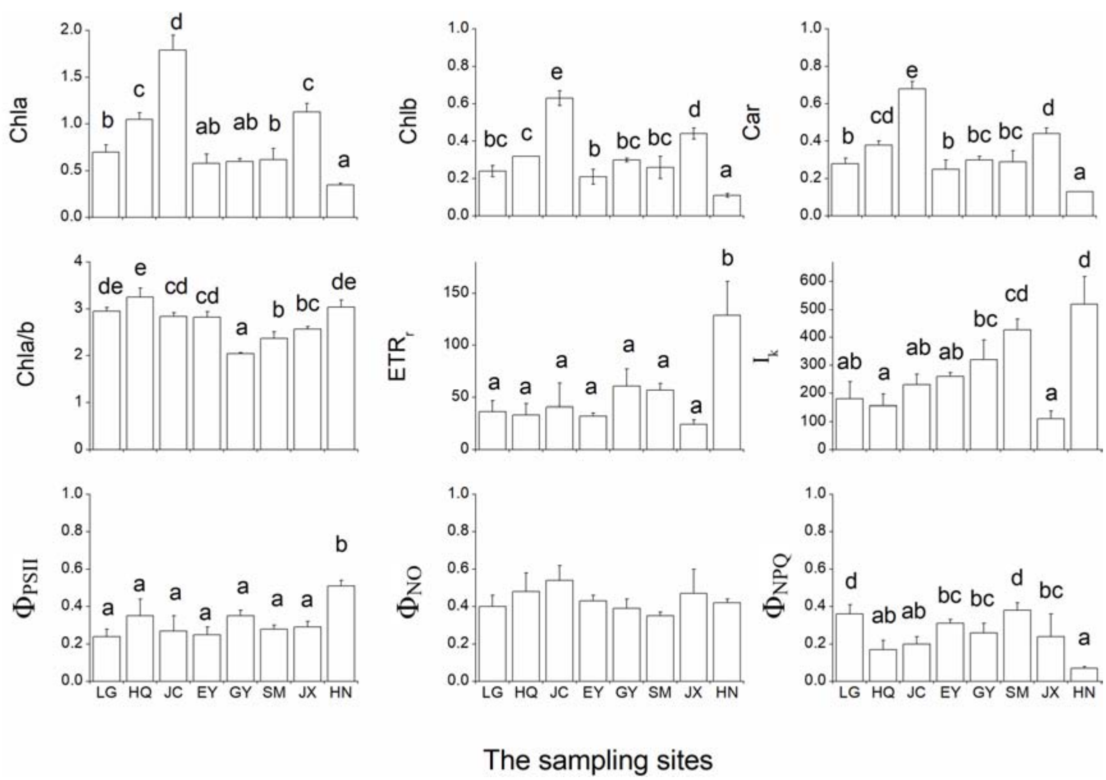


**FIGURE 4 |** The nine pigment-related leaf traits in the eight populations. Chla, Chlb, Car, and Chla/b refer to the leaf chlorophyll a (unit: mg $g^{-1}$ FW), chlorophyll b (unit: mg $g^{-1}$ FW), carotenoids (unit: mg $g^{-1}$ FW) and the ratio of Chla and Chlb, respectively. $rETR_{max}$ (unit: μmol electron $m^{-2}$ $s^{-1}$), $I_k$ (μmol photon $m^{-2}$ $s^{-1}$), $\Phi_{PSII}$, $\Phi_{NPQ}$ and $\Phi_{NO}$ are five leaf chlorophyll fluorescence parameters in the inductive curve and rapid light curve. The different letters in the figure indicate the significant difference among the eight population.

**TABLE 4 |** The Pearson correlation between nine pigment-related leaf traits in the eight populations.

| | Chlb | Chla/b | Car | $rETR_{max}$ | $I_k$ | $\Phi_{PSII}$ | $\Phi_{NO}$ | $\Phi_{NPQ}$ |
|---|---|---|---|---|---|---|---|---|
| Chla | 0.968* | 0.167 | 0.987* | −0.524 | −0.583 | −0.415 | 0.833 | −0.117 |
| Chlb | | −0.083 | 0.991* | −0.528 | −0.542 | −0.448 | 0.732 | −0.031 |
| Chla/b | | | 0.016 | 0.047 | −0.188 | 0.180 | 0.474 | −0.426 |
| Car | | | | −0.556 | −0.563 | −0.475 | 0.761 | −0.024 |
| $rETR_{max}$ | | | | | 0.880 | 0.872 | −0.311 | 0.576 |
| $I_k$ | | | | | | 0.62 | −0.534 | −0.231 |
| $\Phi_{PSII}$ | | | | | | | −0.075 | −0.822 |
| $\Phi_{NO}$ | | | | | | | | −0.507 |

*Chla, Chlb, Car, and Chla/b refer to the leaf chlorophyll a (unit: mg $g^{-1}$ FW), chlorophyll b (unit: mg $g^{-1}$ FW), carotenoids (unit: mg $g^{-1}$ FW) and the ratio of Chla and Chlb, respectively. $rETR_{max}$ (unit: μmol electron $m^{-2}$ $s^{-1}$), $I_k$ (μmol photon $m^{-2}$ $s^{-1}$), $\Phi_{PSII}$, $\Phi_{NPQ}$ and $\Phi_{NO}$ are five leaf chlorophyll fluorescence parameters in the inductive curve and rapid light curve. The star (*) indicates a correlation coefficient > 0.9.*

in all eight populations, especially on two sides of leaves of *O. acuminata* var. *songmingensis*.

## Carbon-Related Leaf Traits

The leaf acidity at dawn and dusk was highest in the HN population (**Figure 2** and **Supplementary Table S1**). The largest diel acidity change was also detected in the HN population, arriving at 12.5 μequiv $g^{-1}$ FW. $V_{max}$ and $K_{half}$ were both high in the SM population but with large variation among the replicates. The respiration rate in the dark was highest in the JC population, intermediate in the EY population, and lowest in the HN population. Since there was strong correlation between the leaf acidity at dusk and at dawn (**Table 3**), the further cluster analysis only included the leaf acidity at dusk. The hierarchical clustering revealed three clusters in the eight populations (**Figure 3**). The HQ, GY, EY, and JC populations were grouped into one cluster, and the HN population were one cluster, with the rest three as one cluster.

## Leaf Pigment Content and Chlorophyll Fluorescence

The leaf chlorophyll a (Chla), chlorophyll b (Chlb) and carotenoids were highest in the JC population, intermediate in the JX population and lowest in the HN population (**Figure 4**). In contrast, the ratio of Chla and Chlb (Chla/b) was lowest in the GY population.

Both $rETR_{max}$ and $\Phi_{PSII}$ were significantly higher in the HN than in the other populations (**Figure 4**). $I_k$ was low in the HQ and JX populations and high in the SM and HN populations. $\Phi_{NO}$ did not differ among the eight populations while $\Phi_{NPQ}$ was highest in LG and SM populations.

The strong correlation was found among Chla, Chlb, and carotenoids (**Table 4**). The cluster analysis discovers only one cluster for the eight populations (**Figure 5**).

## Leaf Traits of Morphology and Mass

The SM population had the largest leaf length (reaching ca. 100 cm), length/width ratio (Len/width), thickness, area, volume, fresh weight, MPA compared with the rest seven populations (**Figure 6**). While for leaf width, the EY population had the highest values, the LG population the lowest.

There was strong correlation among several traits of leaf morphology and mass (**Table 5**), and only four traits (leaf length, width, area and thickness) were included in the further hierarchical clustering analysis (**Figure 7**). Seven clusters were identified with the HQ and JC populations as one cluster, and other populations are distinct from each other.

# DISCUSSION

Based on 24 leaf traits, we have provided a quantitative profile of the eight *Ottelia* populations in field. After grouped into three aspects (traits of morphology and mass, carbon-related traits and leaf pigment and chlorophyll florescence parameters), the

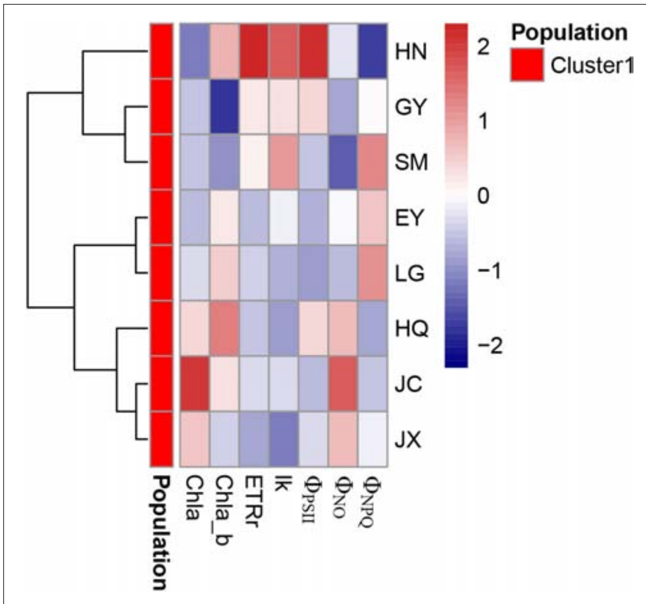


**FIGURE 5 |** The cluster analysis of pigment-related leaf traits in the eight populations. Chla, Chlb, Car, and Chla/b refer to the leaf chlorophyll a (unit: mg $g^{-1}$ FW), chlorophyll b (unit: mg $g^{-1}$ FW), carotenoids (unit: mg $g^{-1}$ FW) and the ratio of Chla and Chlb, respectively. $rETR_{max}$ (unit: μmol electron $m^{-2}$ $s^{-1}$), $I_k$ (μmol photon $m^{-2}$ $s^{-1}$), $\Phi_{PSII}$, $\Phi_{NPQ}$, and $\Phi_{NO}$ are five leaf chlorophyll fluorescence parameters in the inductive curve and rapid light curve.

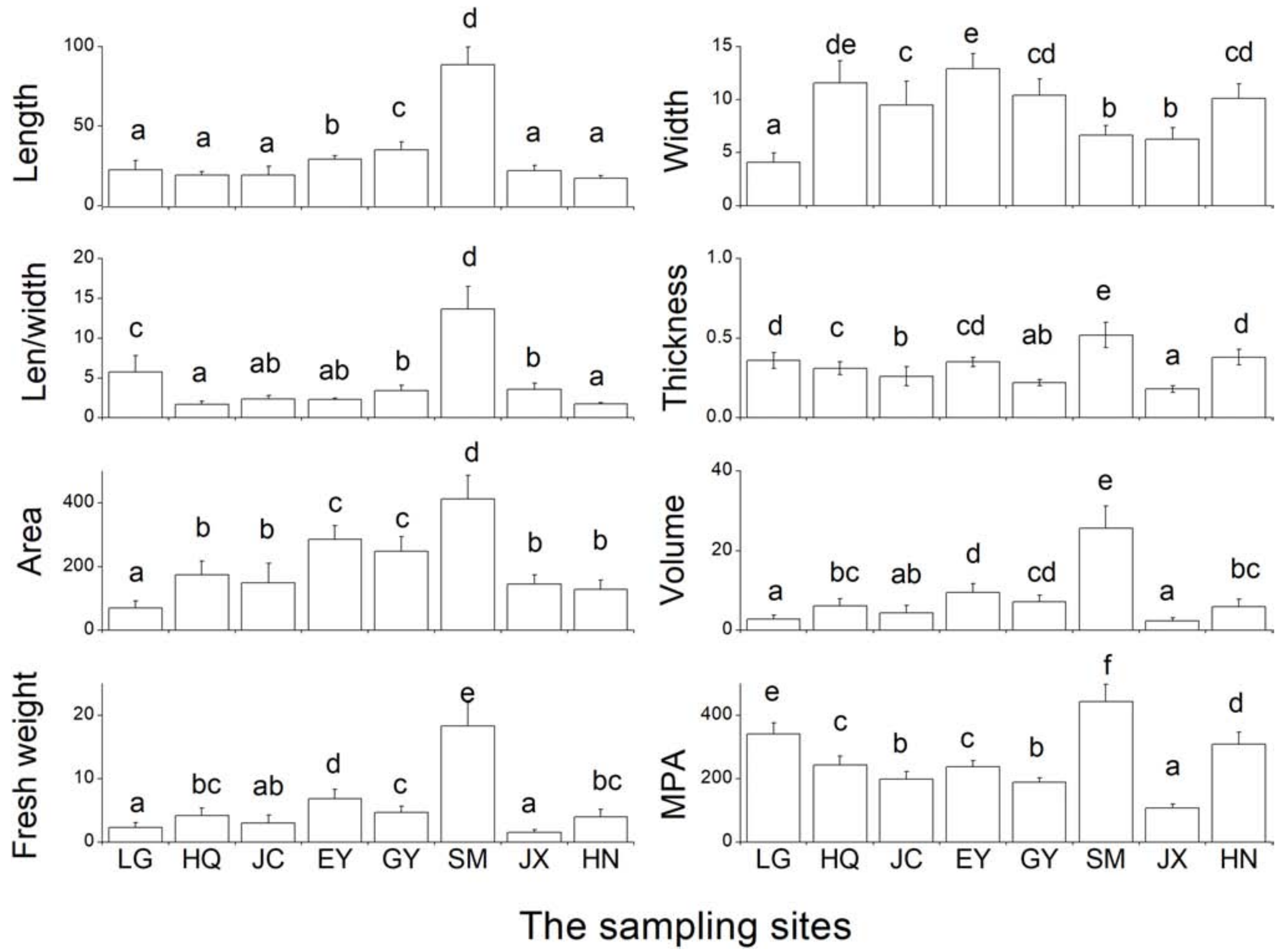


**FIGURE 6 |** The eight leaf traits of morphology and biomass in the eight populations. Len/width refers to the ratio of leaf length and leaf width. MPA refers to the biomass per area of the determined leaf (unit: g $m^{-2}$). Leaf length and width is with the unit of cm, thickness with the unit of mm, area with the unit of $cm^2$, and volume with the unit of $cm^3$, fresh weight with the unit of g. The different letters in the figure indicate the significant difference among the eight population.

**TABLE 5 |** The Pearson correlation between eight leaf traits of morphology and biomass in the eight populations.

| | Width | Len/width | Area | Thickness | Volume | FW | MPA |
|---|---|---|---|---|---|---|---|
| Length | −0.243 | 0.933* | 0.870 | 0.680 | 0.960* | 0.961* | 0.649 |
| Width | | −0.560 | 0.231 | −0.151 | −0.033 | −0.051 | −0.289 |
| Len/width | | | 0.646 | 0.694 | 0.840 | 0.850 | 0.717 |
| Area | | | | 0.510 | 0.908* | 0.901* | 0.396 |
| Thickness | | | | | 0.780 | 0.795 | 0.964* |
| Volume | | | | | | 0.999* | 0.706 |
| FW | | | | | | | 0.722 |

*Len/width refers to the ratio of leaf length and leaf width. FW refers to the fresh weight (unit: g). MPA refers to the biomass per area of the determined leaf (unit: g $m^{-2}$). Leaf width is with the unit of cm, thickness with the unit of mm, area with the unit of $cm^2$, and volume with the unit of $cm^3$. The star (*) indicates a correlation coefficient > 0.9.*

leaf traits showed different divergences in these populations. The physiological traits (such as diel acidity changes, leaf florescence parameters, and etc.) were usually measured in the studies of macrophytes exposed to toxicity or under other stress due to the sensitive responses (Zhang et al., 2014; Shao et al., 2017). While in this study, the physiological traits were less sensitively divergent than morphological ones (seven clusters) among the eight populations, probably reflecting the physiological adaptation to the clear water conditions benign for the growth of *Ottelia*. The quantitatively determined traits showed to some extent

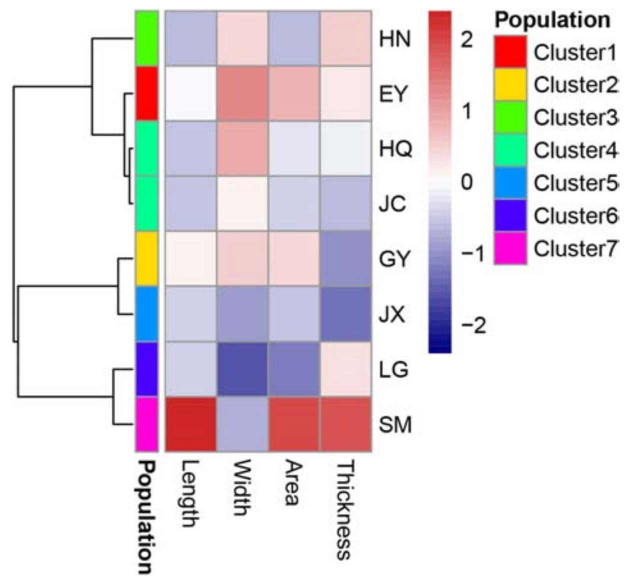


**FIGURE 7 |** The cluster analysis of leaf traits of morphology and biomass in the eight populations. Leaf length and width is with the unit of cm, area with the unit of $cm^2$ and thickness with the unit of mm.

correlation with the phylogenetic relationship, implying that variation or speciation of *Ottelia* in these isolated water systems have produces various traits in each population. For example, the morphological traits were clustered into seven groups (the two populations from the same *O. acuminata* var. *acuminata* were merged into one cluster), and the HN population was singled out base on the carbon-related traits. This is consistent with the results of He (1991) using several qualitative or semi-quantitative traits such as flower, seed and other structures. We also found special features within two populations. The SM population (*O. acuminata* var. *songmingensis*) had thickest and longest leaves in all the population and interestingly with calcium precipitation on both sides of the leaves, an interesting phenomenon that requires further study. The HN population (*O. cordata*) had high diel acidity changes, only similar with *O. alismoides* but not with other species in the genus *Ottelia* in China, which provided a clue for future phylogenetic research.

The leaf functional traits have been well investigated in terrestrial plants, and the photosynthesis rate, leaf weight per area and leaf life span of >2,000 species of plants have been recorded (Wright et al., 2004). The authors proposed a LES and claimed that there was a typical trade-off between leaf functional traits that leaves with high photosynthesis rate are featured low life span and low leaf MPA (Wright et al., 2004). To our knowledge, this study is the first attempt to systematically analyze the leaf functional traits in submerged freshwater macrophytes. The low leaf MPA and high photosynthesis rate of the *Ottelia* leaves fit well with LES and placed in the spectrum of the quick return on nutrient and biomass investment. Accordingly, the leaf life span of *Ottelia* was expected to be short as other submerged species (Hemminga et al., 1999; Yamamoto et al., 1999; Kamermans et al., 2001). In addition, Cornwell et al. (2010) revealed the predominant influence of plant functional traits on decomposition rates at a global scale. Based on our findings on functional traits of *Ottelia* populations, the decomposition rate and carbon turnover of these populations should be very fast, similar to *Potamogeton crispus* (Wang et al., 2016), and thus a fast carbon cycling was expected for the *Ottelia*-dominated karst freshwaters. However, our results, together with Yin et al. (2017), indicated that all the species or varieties of the genus *Ottelia* in China could use bicarbonate as inorganic carbon supply though there was variation among the populations. Similar like *Potamogeton lucens* (Prins et al., 1982), the *Ottelia* leaves were expected with polarity of pH between the adaxial and abaxial side, but with much broader leaves probably stronger effects on the polarity. Therefore, the calcium precipitation (as $CaCO_3$) on the surfaces of *Ottelia* leaves could bring abundant carbon burial when the macrophytes were dominant producers and thus strongly affect the carbon cycling. Thirdly, high diel acidity changes $>10$ μequiv $g^{-1}$ FW were found even at high inorganic carbon supply, which is potentially inducible CAM feature similar with that *O. alismoides* showed in Shao et al. (2017). A dense macrophyte bed with strong CAM capacity could also affect the pH of water column at night (Keeley, 1983). Consequently, the changes of pH have a strong effect on the inorganic carbon species in the water column and other primary producers, e.g., periphyton on the leaves (Maberly, 1996; Hao et al., 2017). In summary, the role of *Ottelia* populations on the carbon cycles in the karst freshwaters warrants further studies.

There is also small variation of plant traits among the three populations (HQ, EY, and JC) belonging to the same variety *O. acuminata* var. *acuminata*. The lower end-point pH in EY population concurred with habitat fragmentation and destruction in Eryuan County. During our field survey, we found that the natural populations of *O. acuminata* var. *lunanen* and *O. emersa* disappeared due to habitat destruction. The extinct of the *Ottelia* populations should be highlighted in the future projects of macrophyte conservation.

## CONCLUSION

Our trait analyses profiled the eight populations of genus *Ottelia* in details. With the unique growth form Otteliids and limited distribution in the localized area, the investigated species or varieties may form special effects on the growing freshwaters distinct from other submerged species. Our results indicated an important ecological role of submerged macrophyte *Ottelia* spp. in the karst freshwaters. More studies about whether direct uptake of bicarbonate or relying on extracellular carbonic anhydrase in *Ottelia* leaves would provide more accurate knowledge about the effects of the species on other primary producers and the carbon cycling in the karst freshwaters.

## AUTHOR CONTRIBUTIONS

YC, WL, and HJ designed the experiments. YC and LX determined the physic-chemical variables. YL determined the leave morphology. LN determined the photosynthesis rate.

HJ determined the acidity of leaves. YC and HJ wrote the first edition of the manuscript, and other co-authors contributed to the modification of the manuscript.

## FUNDING

This research was supported by the Strategic Priority Research Program of Chinese Academy of Sciences, Grant No. XDB31010000 and the National Natural Science Foundation of China (31870345 and 31670368).

## ACKNOWLEDGMENTS

We are grateful to Zhizhong Li, Yu Cheng, Ying Zhang, Haisu Wang, and Quan Jin during the field sampling.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01938/full#supplementary-material

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

ORIGINAL RESEARCH
published: 09 January 2019
doi: 10.3389/fpls.2018.01980

# Effects of Water Quality Adjusted by Submerged Macrophytes on the Richness of the Epiphytic Algal Community

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Submerged macrophytes and epiphytic algae play significant roles in the functioning of aquatic ecosystems. Submerged macrophytes can influence the epiphytic algal community by directly or indirectly modifying environmental conditions (nutrients, light, etc.). From December to June of the following year, we investigated the dynamics of the dominant winter species *Potamogeton crispus*, its epiphytic algae, and water quality parameters in the shallow Liangzi Lake in China. The richness of epiphytic algae had a trend similar to that of *P. crispus* coverage, which increased in the first four months and then decreased in the following three months. The structural equation model (SEM) showed that *P. crispus* affected the richness of epiphytic algae by reducing nutrient concentrations (reduction in total organic carbon, total nitrogen and chemical oxygen demand) and enhancing water transparency (reduction in turbidity and total suspend solids) to enhance the richness of epiphytic algae. The results indicated that high amounts of submerged macrophyte cover can increase the richness of the epiphytic algal community by changing water quality.

**Keywords: submerged macrophytes, coverage, epiphytic algae, richness, water quality**



**Edited by:**
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**Specialty section:**
*This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science*

**Received:** *06 June 2018*
**Accepted:** *20 December 2018*
**Published:** *09 January 2019*

**Citation:**
*Lv T, He Q, Hong Y, Liu C and Yu D (2019) Effects of Water Quality Adjusted by Submerged Macrophytes on the Richness of the Epiphytic Algal Community. Front. Plant Sci. 9:1980. doi: 10.3389/fpls.2018.01980*

## INTRODUCTION

A least-disturbed shallow ecosystem should have high water quality and biodiversity (Mcnaughton, 1988; Downing et al., 2014). Submerged macrophytes play a significant role in maintaining good water quality and high biodiversity in shallow ecosystems (Jeppesen et al., 1998; Kuiper et al., 2017). Submerged macrophytes inhibit algal blooms through the reduction of nutrients, allelopathy and shading (Nakai et al., 2000; Engelhardt and Ritchie, 2001; Casartelli and Ferragut, 2018). Epiphytic algae play a significant role in the functioning of shallow ecosystems, contributing to material circulation, energy flow and the maintenance of food webs (Rodusky et al., 2001; Vadeboncoeur and Steinman, 2002; Song et al., 2017). The community structure of epiphytic algae in shallow ecosystems is influenced by a number of physical (Vadeboncoeur et al., 2003; Tóth and Palmer, 2016), chemical (Rodusky et al., 2001; Trochine et al., 2014) and biological factors (Jones and Sayer, 2003; Tunca et al., 2014; Hao et al., 2017).

It is widely acknowledged that the relationship between epiphytic algae and macrophytes plays an important role in maintaining the function and stability of the shallow ecosystems (Liboriussen and Jeppesen, 2006; Scheffer and Nes, 2007). The epiphytic algal community can be strongly influenced by macrophytes, especially with a high coverage of macrophytes, which has been frequently reported by researchers (Chambers et al., 2008; Santos et al., 2013; Souza et al., 2015). The macrophytes can directly or indirectly modify the environmental conditions for epiphytic algae, which complicates the relationship between them. Macrophytes participate in the nutrient cycling process through nutrition absorption, precipitation, mobilization, decomposition (Carignan and Kalff, 1980; Barko and James, 1998), processes that can change nutrient and light conditions for epiphytic algae. Macrophytes can provide surfaces for epiphytic algae development, but they may also decrease epiphytic algae growth through reduced light availability due to shading and allelochemical production (Erhard and Gross, 2006; Meerhoff et al., 2007). Therefore, it is reasonable to assume that macrophytes may be a determinant of the community structure of epiphytic algae.

However, few studies have identified a model for variation in the epiphytic algal community, especially considering the effects of water quality changes by aquatic macrophytes on the epiphytic algal community. Therefore, we surveyed the interrelationship between the epiphytic algal community, macrophyte coverage and water quality variables to determine the direct and indirect effects on the epiphytic algal community.

## MATERIALS AND METHODS

### Study Area

Liangzi Lake, Hubei Province, China (30°05′ ∼ 30°18′N, 114°21′ ∼ 114°39′E) is a typical Macrophyte-dominated mesotrophic shallow lake [average annual diaphaneity is 1.2 m, average annual pH is 8.0, average annual salinity is 0.07 ppt, average annual total suspended solids is 19.0 mg/L, average annual total nitrogen is 0.53 mg/L, average annual total phosphorus is 0.023 mg/L and average annual chemical oxygen demand (COD) is 3.68 mg/L] in the central of Yangtze River Basin with good water quality and high biodiversity (Xie et al., 2015). It has a surface area of 304.3 $km^2$ and the mean depth varies from 2.5 to 6 m (Fan et al., 2015). It is a dimictic lake, water retention is 0.53 year, about $1.48 \times 10^9$ $m^3$ water take part in the water replacement each year because of seasonal precipitation and draining into the Yangtze river. Liangzi Lake features a subtropical monsoon climate, and the weather is relatively moderate with an annual average temperature of 17.3°C, the mean freezing period is 15 days. This lake was divided into five regions based on different macrophyte community composition and hydrologic conditions (**Figure 1**) (Xu et al., 2018). *P. crispus* is an annual submerged macrophyte and a dominant winter species in Liangzi Lake (Qian et al., 2014). It germinated in the autumn (September to November) and slowly grew throughout the winter (December to the coming February, there is no ice, average temp is 7.9°C), increased its biomass rapidly from March to April and declined in June (Rogers and Breen, 1980; Kunii, 1982; Chen, 1985).

### *P. crispus* and Epiphytic Algae Samples

Due to enclosure of other two regions, five fixed sampling sites were distributed in three regions of Liangzi lake (Qianjiangdahu, Manjianghu, and Gaotanghu) (**Figure 1**). From December 2016 to June 2017, each site was surveyed on the 15th–17th day each month (total of seven times samples). Five quadrats (1 m × 1 m, quadrats were placed without overlapping, randomly) with a *P. crispus* monodominant community were investigated at each site, and the plant coverage of each sample was surveyed by ocular estimate (Fang et al., 2009). The coverage of *P. crispus* (macrophyte cover) at each site was calculated as the mean of the five samples. Ten pieces of *P. crispus* leaves approximately 50 cm from the top were carefully selected from those five quadrats to ensure uniformity in the growth state (young leaf) and size to ensure the minimum sampling error in sample size. Then, each leaf was placed into a wide-mouth plastic bottle with 200 ml of distilled water at each site. The area of the selected leaves was measured by area meter (LI-3100C, LI-COR, United States). Epiphytic algae were removed by a banister brush in water (Foerster and Schlichting, 1965) and preserved in a well-labeled plastic container, with 2 ml Lugol's solution to fix the epiphytic algal sample. Epiphytic algae were identified to species and quantified with a microscope using the blood count plate method (Hu and Wei, 2006; Effiong and Inyang, 2015; Qian et al., 2015). The total abundance of each month was the mean of the five fixed sites. The richness of epiphytic algae was the summation of species at each site per month.

### Water Samples

Eighteen physical and chemical water parameters were measured at a depth of 1.5 m underwater. Water temperature (T), dissolved oxygen (DO), conductivity (Cond) and pH of water samples were measured with a portable water quality monitor (PROPLUS, YSI, United States), and chlorophyll a (Chla) was measured with a handheld probe (HYDROLAB DS5, HACH, United States). Turbidity (Turb) and total suspended solids (SS) were measured with a turbidity meter (2100Q, HACH, United States) and a portable spectrophotometer (DR900, HACH, United States) in the field tests. Additionally, water samples were collected from each site and stored on ice. Total nitrogen (TN) and total phosphorus (TP) were analyzed by a flow injection analyzer (QC8500, LACHAT, United States), total organic carbon (TOC) was analyzed by a total organic carbon analyzer (TOC-L, SHIMADZU, Japan), the cations and anions ($Na^+$, $K^+$, $Mg^{2+}$, $Ca^{2+}$, $F^-$, $Cl^-$, and $SO_4{}^{2-}$) were determined by a ion chromatograph (ICS-1000, DIONEX, United States) and COD was analyzed with a digestion solution for COD and landscape photometry (DR900, HACH, United States).

### Data Analyses

To ensure that the data conform to a normal distribution, all water parameters were $\log_{10}$-transformed before performing regressions and SEM (Zuur et al., 2010), whereas macrophyte cover and epiphytic richness were not $\log_{10}$-transformed (O'Hara and Kotze, 2010). Macrophyte cover and epiphytic algal richness in different months were compared using repeated-measures ANOVA by *post hoc* Bonferroni tests for multiple comparisons (Thompson et al., 2001). The linear regressions were used to test the patterns of epiphytic algal richness along significant

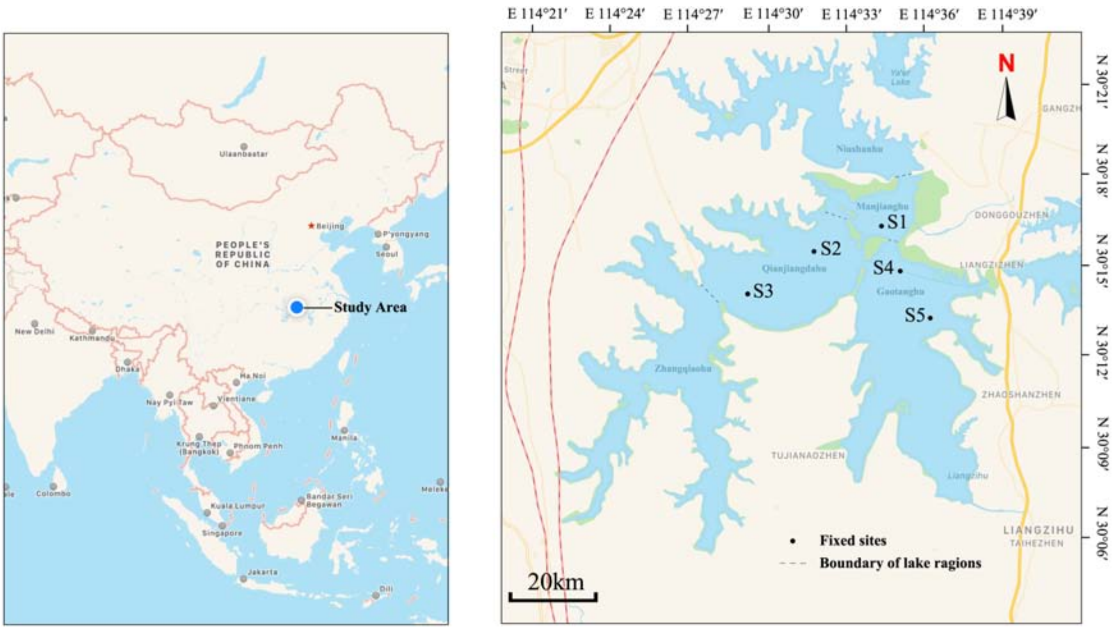


**FIGURE 1 |** Map of Liangzi Lake showing sampling sites. Five fixed sites were distributed in three lake regions: S1 in Manjianghu, S2 and S3 in Qianjiangdahu, S4 and S5 in Gaotanghu.

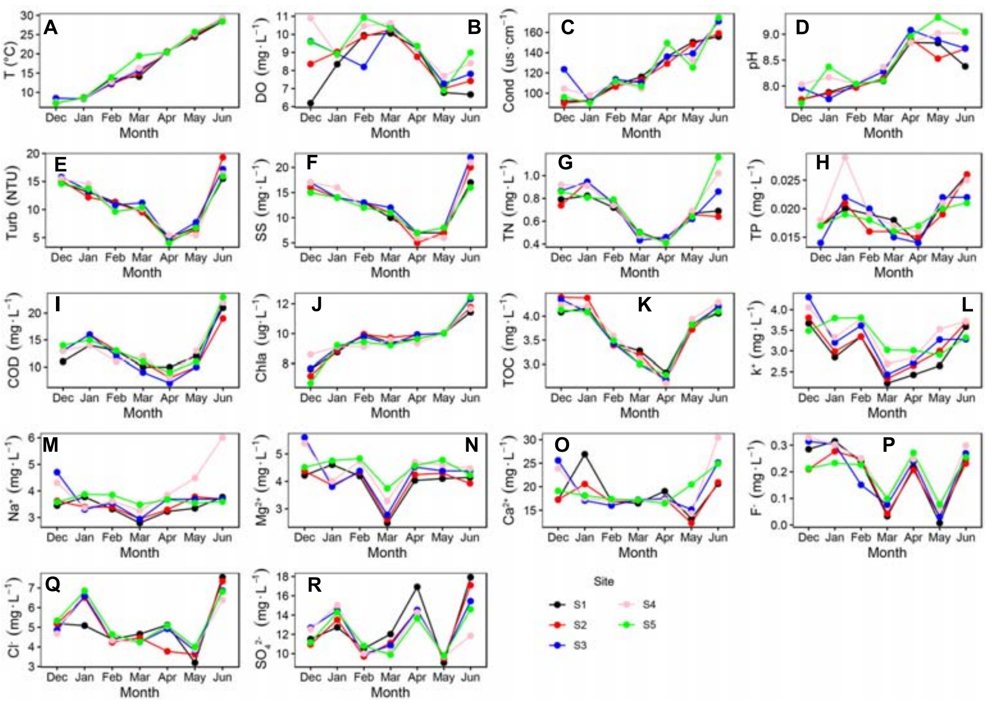


**FIGURE 2 |** Variations in some physical and chemical parameters of Liangzi Lake (December 2016–June 2017). **(A)** water temperature, **(B)** dissolved oxygen, **(C)** conductivity, **(D)** pH, **(E)** Turbidity, **(F)** total suspended solids, **(G)** Total nitrogen, **(H)** total phosphorus, **(I)** chemical oxygen demand (COD), **(J)** chlorophyll a, **(K)** total organic carbon, **(L)** $K^+$ **(M)** $Na^+$, **(N)** $Mg^{2+}$, **(O)** $Ca^{2+}$, **(P)** $F^-$, **(Q)** $Cl^-$ and **(R)** $SO_4^{2-}$ ($n$ = 35).

environmental gradients and the regressions coefficients squared were corrected for multiple tests. To determine the relative importance of direct vs. indirect effects of *P. crispus* community dynamics driving epiphytic algal richness, we built a structural equation model (SEM; Oberski et al., 2014) including macrophyte cover, nutrient environmental factors (i.e., TN, TP, COD, and

TOC), and light-related environmental factors (i.e., Turb and SS), with richness of epiphytic algae. Statistics were performed using R version 3.5.1 (R Development Core Team, 2011) and the packages agricolae (Mendiburu, 2009) and lavaan (Rosseel et al., 2011).

# RESULTS

## Physical and Chemical Parameters

The T ($P < 0.001$), Cond ($P < 0.001$), pH ($P < 0.001$) and Chla ($P < 0.001$) showed an increasing trend during the survey periods (**Figures 2A,C,D,J**). The DO Turb, SS, TN, TP, TOC, COD, $Na^+$, $K^+$, $Mg^{2+}$, $Ca^{2+}$, $F^-$, $Cl^-$, and $SO_4{}^{2-}$ were shown a non-liner trend during the survey periods (**Figures 2B,E–I,K–R**). Turb, SS, TN, TP, TOC, COD decreased in the first four months of the observation period, followed by increases in the remaining observation period (**Figures 2E–I,K**). The six values (i.e., Turb, SS, TN, TP, TOC, and COD) in April were smaller than those in other months, which indicated that the water column was cleaner in April than in other months.

## Coverage of *P. crispus* and Epiphytic Algae

Macrophyte cover ($F_{(2.07,8.29)} = 72.67$, $P < 0.001$) and epiphytic algal richness ($F_{(2.60,10.40)} = 96.53$, $P < 0.001$) were significantly difference in month. macrophyte cover increased in the first four months and then decreased in the last three months (**Figure 3**). macrophyte cover reached a peak of approximately 75–80% in mid-April (**Figure 3**). The mean macrophyte cover increased from 28% in December to 38% in January of following the year, showing that *P. crispus* slowly grew throughout the winter. The mean macrophyte cover increased from 38% in January to 78% in April, showing that *P. crispus* increased its biomass rapidly in the spring (**Figure 3**). The mean macrophyte cover decreased from 78% in April to 36% in June, showing that *P. crispus* declined in the early summer (**Figure 3**).

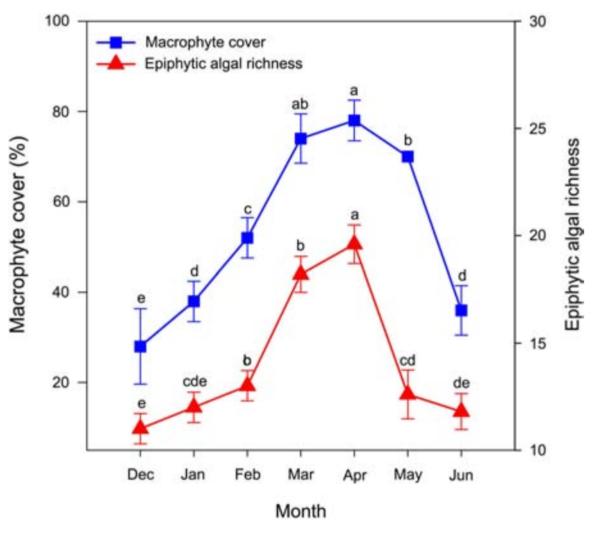


**FIGURE 3 |** Community dynamics of *P. crispus* and epiphytic algae. The data are presented as the mean ± SE of 5 fixed sites each month ($n$ = 35). Means with the different letters are significantly different at $P < 0.05$ in different months (Bonferroni test).

The richness of epiphytic algae had a trend similar to that of *P. crispus* coverage dynamics, first increasing during the first four months and then decreasing during the last three months (**Figure 3**). The richness of epiphytic algae reached a peak at approximately 20 species in the mid-April (**Figure 3**). A total of 33 epiphytic algae species belonging to 6 phyla were identified on *P. crispus* in Liangzi Lake (**Supplementary Table S1**). Fifteen genera of diatoms, 10 genera of green algae, 6 genera of blue green algae, 1 genus of cryptomonad, 1 genus of euglenoid and 1 genus of golden algae were identified (**Supplementary Table S1**). Diatoms were the dominant group of epiphytic algae in richness and reached a peak of approximately 10.6 species in mid-March (**Figure 4**). Green algae had the highest richness in the April with approximately 6.8 species and the lowest richness in December with approximately 1 species (**Figure 4**). The richness of blue green algae increased over the last three months, reaching a peak of approximately 3 species in mid-June (**Figure 4**). Only 1 species of euglenoid appeared from March to May (**Figure 4**). Only 1 species of cryptomonad and golden algae appeared in January and June, respectively (**Figure 4**).

## Effects of Biotic and Abiotic Environmental Factors on Epiphytic Algal Richness

The epiphytic algal richness was positively correlated with macrophyte cover, DO and pH (**Figures 5A–C**). The richness was negatively correlated with Turb, SS, TN, TP, COD, TOC, Na+, $K^+$, $Ca^{2+}$, $Mg^{2+}$, $F^-$, and $Cl^-$ (**Figures 5D–O**). The epiphytic algal richness was no significantly correlated with T ($R^2$ = 0.00, $P$ = 0.176), Cond ($R^2$ = 0.00, $P$ = 0.392), Chla ($R^2$ = 0.00, $P$ = 0.252) and $SO_4{}^{2-}$ ($R^2$ = 0.00, $P$ = 0.987).

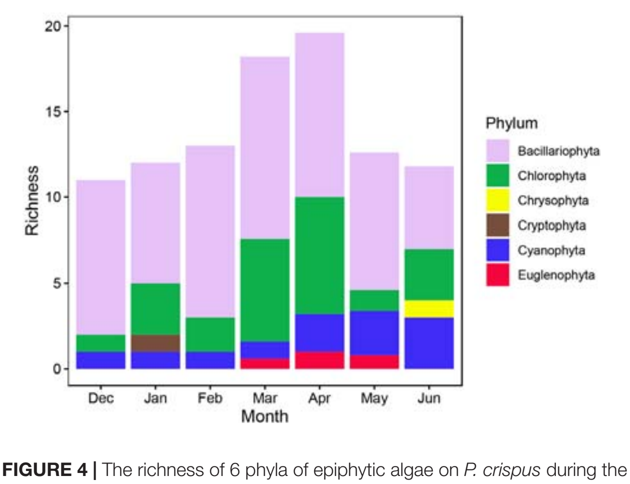


**FIGURE 4 |** The richness of 6 phyla of epiphytic algae on *P. crispus* during the study period. The richness of each phylum was the mean of 5 fixed sites ($n$ = 35).

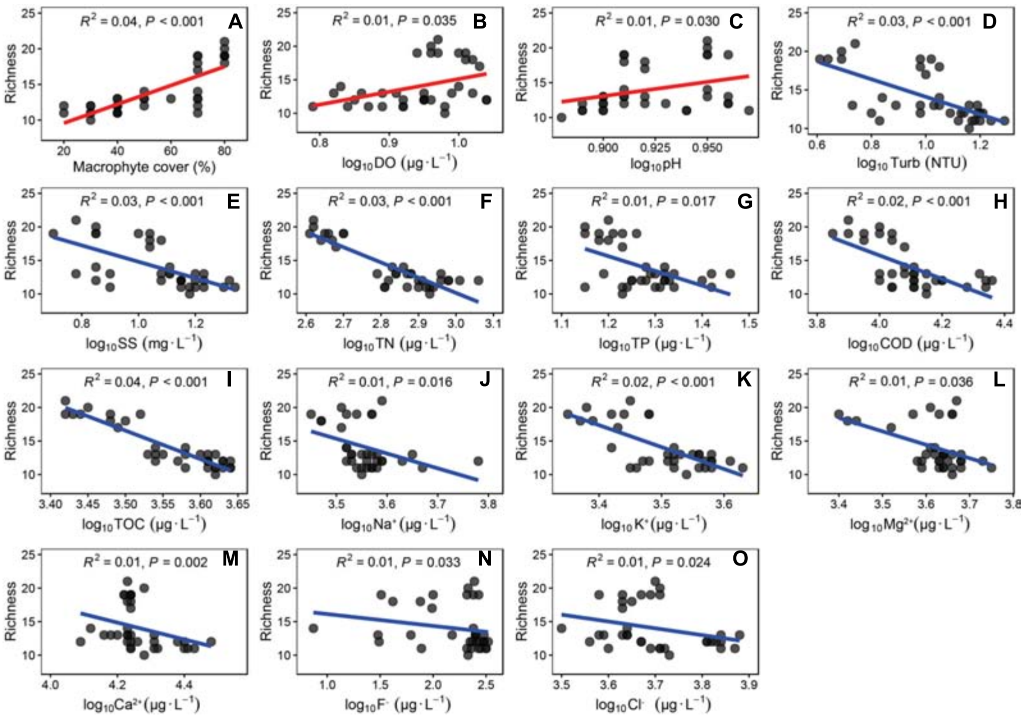


**FIGURE 5 |** The linear regressions between richness of epiphytic algae and the coverage of *P. crispus* and water quality parameters. Environmental factors were correlated with the richness of epiphytic algae. **(A)** coverage of *P. crispus*, **(B)** dissolved oxygen, **(C)** pH, **(D)** Turbidity, **(E)** total suspended solids, **(F)** Total nitrogen, **(G)** total phosphorus, **(H)** COD, **(I)** total organic carbon, **(J)** $Na^+$, **(K)** $K^+$, **(L)** $Mg^{2+}$, **(M)** $Ca^{2+}$, **(N)** $F^-$ and **(O)** $Cl^-$. The regressions coefficients squared and $P$-values are given for the regression by correction for multiple tests ($n$ = 35).

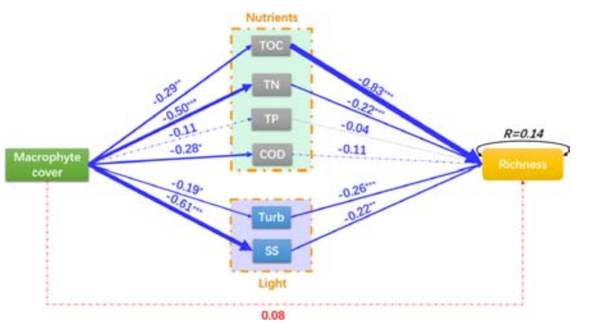


**FIGURE 6 |** A structural equation model of macrophyte cover effects on the richness of epiphytic algae. Red and blue arrows represent significant positive and negative pathways, respectively. Arrow width is proportional to the strength of the relationship, and solid and dotted lines represent significant and non-significant pathways, respectively. Numbers indicate the standard path coefficients ($C$) $\chi^2$ = 242.68, $P$ < 0.001; RMSEA = 0.66, $P$ < 0.001; AIC = 1216.45. Significance levels are indicated by asterisks: ***$P$ < 0.001, **$P$ < 0.01, *$P$ < 0.05.

The macrophyte cover had a significant negative effect on TOC ($C$ = –0.29, $P$ = 0.003), TN ($C$ = –0.50, $P$ < 0.001), COD ($C$ = –0.28, $P$ = 0.003), Turb ($C$ = –0.19, $P$ = 0.020) and SS ($C$ = –0.61, $P$ < 0.001) (**Figure 6**). TOC ($C$ = –0.83, $P$ < 0.001), TN ($C$ = –0.22, $P$ < 0.001), Turb ($C$ = –0.26, $P$ < 0.001), and SS ($C$ = –0.22, $P$ = 0.001) had a negative effect on the richness of epiphytic algae (**Figure 6**). The macrophyte cover had a non-significant negative effect on the richness of epiphytic algae ($C$ = 0.08, $P$ = 0.276, **Figure 6**). The model shows that *P. crispus* effects the diversity of epiphytic algae by reducing nutrient concentration (TOC and TN decreases) and increasing the clarity of the water (Turb and SS decreases) to improve the richness of epiphytic algae.

## DISCUSSION

Changes in the macrophyte community can be an important cause of changes in the epiphytic algal community structure (Souza et al., 2015). Increasing macrophyte coverage could increase the species richness of epiphytic algae by providing more diverse and heterogeneous habitats for epiphytic algae (Cattaneo et al., 1998; Toporowska et al., 2008; Celewicz-Gołdyn and Kuczyńska-Kippen, 2017). Liangzi Lake had a high abundance of aquatic macrophytes, especially *P. crispus* during winter to early summer, and the abundance varied over this period (Qian et al., 2014). Our results showed that the total richness of epiphytic algae had a similar trend to that of *P. crispus* coverage (**Figure 3**), which suggested that higher coverage of *P. crispus* might provide more habitats and spatial niches for epiphytic algae. Therefore, within a range of coverage and conditions examined, a greater *P. crispus* coverage could accommodate more species of epiphytic algae.

On the other hand, when the coverage of *P. crispus* increases, total organic carbon, total nitrogen, COD, turbidity and total suspended solids in the water column are decreased. These results may suggest that *P. crispus* improved the water quality at the growing season in terms of improving transparency, decreasing nutrients are represented by the first part of the SEM. A large amount of nutrients and suspended solids in the water column were absorbed for macrophyte growth and reproduction that have been widely confirmed by many studies (Scheffer, 1999; Cao et al., 2018). The water quality was improving, which usually manifested as high transparency, low nutrient concentrations and high biodiversity in a shallow ecosystem (Karr and Dudley, 1981; Gandhi, 2012; Cao et al., 2018). The diversity of epiphytic algae was positively correlated with water with high transparency and few suspended solids (Kollar et al., 2015). Increased radiation and spectrum would support a more heterogeneous environment for the epiphytic algal community that would accommodate more species of epiphytic algae (Algarte et al., 2017). The enhanced transparency improved the richness of epiphytic algae shown on the light pathway in the SEM; thus, the total richness of epiphytic algae increased with the increasing *P. crispus* coverage. Eutrophication has been confirmed as one of the main drivers of biodiversity loss in recent decades (Hillebrand et al., 2007; Isbell et al., 2013; Newbold et al., 2015; Wang et al., 2016). Increasing *P. crispus* coverage was correlated with reduced the nutrients of the water column ($C$ = –0.50, $P < 0.001$, TN; $C$ = –0.11, $P$ = 0.13, TP; $C$ = –0.28, $P$ = 0.003, COD; $C$ = –0.29, $P$ = 0.003, TOC) and improved the richness of epiphytic algae, as shown by the nutrient pathway in the SEM. The nutrient increase can lead to cyanobacterial dominance (Dokulil and Teubner, 2000), community structure simplification and biodiversity loss especially in the mesotrophic and eutrophic lakes (Qin et al., 2013). In the decline phase of *P. crispus*, plant decomposition caused nutrients to be released into the water column that led to the overgrowth and dominance of several species epiphytic algae (such as: *G. subclavatum*, *A. exigua*, *C. vulgaris*, *A. flos-aquae* (Lyngb.) and *O. fraca*; **Supplementary Table S1**); many epiphytic algae were excluded due to the competition for nutrients and space. Moreover, the TOC was the strongest factor effected on the epiphytic algal richness. While, most algae couldn't utilize organic matter (Lee, 2008), but the bacteria decomposed organic matter into inorganic carbon which could be utilized by epiphytic algae (Jones et al., 2002; Rier and Stevenson, 2002). The increasing inorganic carbon might led to the overgrowth and dominance of several species epiphytic algae which might excluded many epiphytic algae. On the other hand, the effect of organic matters attenuated light in water column (Babin et al., 2003) which might decrease the epiphytic algal richness.

The pathway form the coverage of *P. crispus* to epiphytic algal richness shown a non-significant effects, which indicated that the changes of *P. crispus* coverage cannot direct explain the variation of epiphytic algal richness. The path coefficient which from the coverage of *P. crispus* to epiphytic algal richness via nutrients ($C$ = 0.38) was greater than which via light ($C$ = 0.18) and direct effect of *P. crispus* coverage ($C$ = 0.08). As the result of the above comparison, the indirect effects (adjusted nutrients concentrations and transparency of water column) of the *P. crispus* coverage on epiphytic algal richness was stronger than that direct effect. The SEM clarified the mechanism by which *P. crispus* improved the epiphytic algal richness by absorbing nutrients and increasing the transmittance of water.

We concluded that *P. crispus* affected the richness of epiphytic algae by reducing nutrients concentrations (TOC, TN, and COD decreased) and increasing transparency (Turb and SS decreased). This result suggests that high submerged macrophyte cover can improve the richness of the epiphytic algae community indirectly by changing water qualities.

## AUTHOR CONTRIBUTIONS

DY and CL designed and executed the research project. TL, QH, and YH collected the data. TL led the reflectance data analysis and drafted the manuscript with the assistance of CL. All the co-authors commented on and approved the final manuscript.

## FUNDING

This research was supported by the Major Science and Technology Program for Water Pollution Control and Treatment (2015ZX07503-005), and the Special Foundation of National Science and Technology Basic Research (2013FY112300).

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01980/full#supplementary-material

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling Editor is currently organizing a Research Topic with one of the authors CL, and confirms the absence of any other collaboration.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 10 January 2019
doi: 10.3389/fpls.2018.01981

# Response of Macrophyte Traits to Herbivory and Neighboring Species: Integration of the Functional Trait Framework in the Context of Ecological Invasions

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**Specialty section:**
This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science

**Received:** 31 July 2018
**Accepted:** 20 December 2018
**Published:** 10 January 2019

**Citation:**
Thouvenot L, Gauzens B, Haury J and Thiébaut G (2019) Response of Macrophyte Traits to Herbivory and Neighboring Species: Integration of the Functional Trait Framework in the Context of Ecological Invasions. Front. Plant Sci. 9:1981. doi: 10.3389/fpls.2018.01981

With the increase in the number of introduced species each year, biological invasions are considered as one of the most important environmental problems for native biodiversity. In invaded habitats, the establishment of exotic plant species depends on the abiotic and biotic environment. Herbivores and neighboring plants (native or exotic) comprise an important part of the latter. Herbivores cause trophic and non-trophic damage to focal plants, which respond to herbivory by varying their different traits quantitatively (e.g., growth rate and biomass changes) and qualitatively (e.g., variation in morphological and chemical defenses strategies affecting plant palatability). Neighboring plant species also affect functional traits and the fitness of focal plant species, thus herbivore effects on a focal plant could also depend indirectly on the palatability and defensive traits of the neighboring species inside the community. Here, in a first step toward the integration of associational susceptibility/resistance theories in the field of ecological invasion, we performed a microcosm experiment to consider the effects of an exotic crayfish on the growth rate, morphological traits and damage level of three macrophytes (two exotic, one native) growing in pairwise combinations. We found that (i) the response to herbivore presence and to neighboring species identity seemed to be species specific, and (ii) crayfish enhance the fragmentation rate of the two exotic macrophytes *Ludwigia grandiflora* and *Egeria densa* in the presence of the native macrophyte *Myriophyllum spicatum*, which could indirectly facilitate their invasion success. Indeed, fragmentation can increase dispersal abilities of the exotic macrophytes considered in this study as they are able to generate new plants from their fragments. However, our results showed that the interaction herbivore-neighbor species was hardly significant. Our paper presents some first results on associational resistance/susceptibility and lays the foundation for developing a general framework that combines plant community ecology and biological invasion ecology to explain invasive species success.

**Keywords: associational susceptibility/resistance, biological invasion, community assemblage, ecological strategy, functional traits, herbivory**

## INTRODUCTION

Biological invasions are one of the most important environmental threats for native biodiversity (Sala et al., 2000; Murphy and Romanuk, 2014), with the number of introduced species continuously increasing at a global scale (Seebens et al., 2016). In invaded habitats, exotic species tend to interact more often with each other, potentially reciprocally affecting their colonization success (Green et al., 2011). Indeed, in these new ranges, the establishment of exotic plant species depends on the abiotic and biotic environment (Shea and Chesson, 2002), and thus is modulated by surrounding herbivores and neighboring plants that can be native, exotic or both.

Herbivores cause trophic damage (Bakker et al., 2016; Wood et al., 2016), and/or non-trophic, non-consumptive indirect damage on plants like uprooting or propagule production *via* stem fragmentation/cutting (Gherardi and Acquistapace, 2007). Plants species respond to herbivory by varying different traits (Karban and Myers, 1989; Baldwin and Preston, 1999; Karban et al., 1999; Tiffin, 2000; Gong and Zhang, 2014), quantitatively (e.g., growth rate and biomass changes) and qualitatively (e.g., variation of morphological and chemical defense traits affecting plant palatability). In consequence, herbivores modify plant biomass allocation and thus plant competitive abilities against exotic and native neighboring plant species (Crawley, 1989; Duffy and Hay, 2000). However, the plant neighborhood also affects these traits (Callaway et al., 2003) and some functional plant traits vary according to the distance from the neighboring plants (Bittebiere and Mony, 2015). For example, in terrestrial ecosystems, the presence of neighboring plant species at a distance ranging from 5 to 25 cm from a focal plant species could explain 70% of the variation of leaf dry matter content (LDMC) (Bittebiere and Mony, 2015), a functional trait which is particularly involved in plant palatability. Thus, neighboring plant species can directly affect functional traits and fitness of focal plant species, but herbivore effects on a focal plant could also indirectly depend on the palatability and defensive traits of the neighboring species inside the community (Barbosa et al., 2009).

Indeed, the associations of different plant species and their effects on plant–herbivore interactions in terrestrial ecosystems has received lot of attention during the last decades (Barbosa et al., 2009; Ruttan and Lortie, 2014; Underwood et al., 2014), but less is known in freshwater ecosystems. The association between an unpalatable (or highly defended) plant and a palatable (or less defended) plant species can have two outcomes for each species: either a resistance or a susceptibility to herbivory. Several theories are derived from these two main outcomes (Atsatt and O'Dowd, 1976; Wahl and Hay, 1995; Ruttan and Lortie, 2014). Among them, the Associational susceptibility hypothesis (Wahl and Hay, 1995; White and Whitham, 2000; Emerson et al., 2012) stipulates an overconsumption of the palatable plant by herbivores in the presence of an unpalatable plant species, which in return will be eaten less by herbivores. Furthermore, the damage to unpalatable species by herbivores could be reduced in the presence of palatable species ("Attractant-decoy hypothesis"; Atsatt and O'Dowd, 1976; Ruttan and Lortie, 2014), or increased due to the presence of highly palatable species in the surrounding area, thus increasing patch attractiveness, as stipulated by the "Shared doom hypothesis" (Wahl and Hay, 1995). So, the outcome of the plant–plant–herbivore interaction relates simultaneously to the palatability and defense degree of the plant species involved, but also depends on the selectivity of the herbivore.

In the broader context of biological invasions, theories related to associational resistance/susceptibility could help to explain the establishment success of invasive species and potentially invasional meltdowns (Simberloff and Von Holle, 1999). The simultaneous introduction of an exotic herbivore and exotic plants could also explain the dispersal and colonization abilities of invasive plant species. For example, Parker et al. (2006) have shown that the percentage of total plant cover or biomass of exotic plants was 52% higher in communities grazed by exotic herbivores than in communities grazed by native herbivores. Exotic herbivores generally promote co-adapted exotic plants from the same native range (Parker et al., 2006) by negatively affecting native plants (*via* these consumptive and non-consumptive effects) (Wood et al., 2016) and by positively affecting the colonization abilities of exotic plants by stem fragmentation and propagule dispersal (Thouvenot et al., 2017), for example. However, the interactive effects of the introduction of an exotic herbivore and an exotic plant, and their outcomes on native plant communities are still understudied.

To take the first step toward the integration of associational susceptibility/resistance theories in the field of ecological invasions, we performed a microcosm experiment to consider the effects of an exotic crayfish on the growth rate, morphological traits and damage levels of three macrophytes (two exotic, one native) growing in pairwise combinations. The Red Swamp Crayfish, *Procambarus clarkii* (Girard, 1852), native to south-central United States (Louisiana) and north-eastern Mexico, has been successfully introduced worldwide for commercial purposes. *P. clarkii* occupies a key position in invaded ecosystems, with dramatic impacts on ecosystem structure and functions (Gutiérrez-Yurrita et al., 1998; Alcorlo et al., 2004). This species is an omnivore with plants as the first food source (88.45% of occurrence in the stomachs of the crayfish studied) followed by animals (59.16%) and detritus (31.19%) (Gutiérrez-Yurrita et al., 1998).

Overall, we tested (1) whether plant response depends on the neighboring plant species in the presence of an exotic crayfish (associational susceptibility vs. associational resistance) and (2) whether there is a facilitative effect of exotic crayfish on exotic plant dispersal.

## MATERIALS AND METHODS

### Biological Material

*Procambarus clarkii* was collected in the Brière marshes (02° 18′ 53.3″ O, 47° 23′ 39.5″ N) in the Loire area, France. This species was chosen because of its abundance in marshes and ponds in north-western France and its high impact on the macrophyte community (Chambers et al., 1990; Gherardi and Acquistapace, 2007; Souty-Grosset et al., 2016). Only adult males with lengths ranging from 60 to 99 mm were selected to avoid sexual and maturity biases. Crayfish were starved in tap water for 1 week at ambient temperature.

Three macrophytes species differing in their morphology, origin and palatability to herbivores were chosen (**Table 1**): the native species with low palatability *Myriophyllum spicatum,* the exotic and highly palatable species *Egeria densa* and the exotic *Ludwigia grandiflora* with low palatably. The submerged shoots of each macrophyte species were collected in the field in Brittany either from ponds at Apigné (01° 44′ 25.2′′ O, 48° 05′ 41.4′′ N) or from the Gannedel marshlands (01° 56′ 09.6′′ O, 47° 41′ 49.7′′ N). These sites were selected as no crayfish species had been recorded, in order to avoid co-evolutionary adaptations. Shoots of plants species were stored for acclimatization in tap water for 1 week at ambient temperature.

## Experimental Design

To test our hypotheses, we set-up a greenhouse microcosm experiment in July 2011, with two crayfish treatments, with and without (control) crayfish, assigned to each of the different pairwise combinations of plants, in order to measure the effects of the exotic crayfish on the development of these macrophytes and the outcome of the plant–plant–crayfish interactions. For this purpose 24 containers were divided into experimental units ($L \times W \times H$: 33 cm × 40 cm × 35 cm) with an opaque plastic barrier that was impermeable to water. Each experimental unit was filled to 15 cm with water and contained 5 cm of sand as well as two patches of two different species: either *L. grandiflora*/*E. densa* or *L. grandiflora*/*M. spicatum* or *M. spicatum*/*E. densa*. Each plant patch was planted in each half of each experimental unit and corresponded to three shoots of one plant species (with a total fresh biomass per species ranging from 2.3 to 3.3 g). Each shoot corresponded to a stem fragment (length ranging from 6 to 12.5 cm) with an apical apex and without roots or lateral buds. Only shoots with green leaves without any sign of grazing or necrosis were used for the experiment. In addition to the control treatment (without crayfish), a crayfish treatment (with one crayfish) was set-up in order to evaluate both the trophic and non-trophic damage to plants. The density of crayfish per aquaria (corresponding to $\approx$ 7.6 ind.m$^{-2}$) was chosen to be in the range of the densities recorded in invaded natural areas. For example, around 3.8 ind.m$^{-2}$ were trapped in a Spanish wetland (Angeler et al., 2001) while 14 ind.m$^{-2}$ were recorded in a Mediterranean wetland (Scalici and Gherardi, 2007). Each treatment with each plant pair was replicated eight times. The six combinations were randomly assigned to the different containers. The chemical composition of the tap water was analyzed at the beginning of the experiment using spectrophotometric techniques (WTW kit and Photolab S12) (mean value: pH = 8.47; conductivity: 618 μS/cm; $[O_2]$ = 10.47 ± 0.24 mg.L$^{-1}$; $[NO_2^-]$ = 0.13 ± 0.02 mg.L$^{-1}$; $[NO_3^-]$ = 14.82 ± 0.51 mg.L$^{-1}$; $[NH_4^+]$ = 0.11 ± 0.02 mg.L$^{-1}$; $[PO_4^{3-}]$ = 0.15 ± 0.02 mg.L$^{-1}$). During the experiment, the water was not aerated, no nutrients were added, and the water levels in the containers were maintained regularly with tap water. The light intensity was natural and the temperature was measured every half minute with three sensors (HOBO TidbiT Water Temperature Data Logger). The water temperature in the glasshouse (means ± SD: 20.96 ± 0.05°C) was similar to those found in the summer in the channels of the Brière marshes. The experiment was stopped after 3 days, when the biomass of one macrophyte species was reduced by half, allowing us to measure traits and make comparisons with the literature (Cronin et al., 2002; Anastacio et al., 2005; Carreira et al., 2014). All plant fragments were removed, and plant traits were measured at the end of the experiment.

## Plant Traits Analyses

Six morphological traits were measured on plants. Plant growth, an indicator of tolerance to herbivory (Coley et al., 1985; Agrawal, 2011), was evaluated by the Relative Growth Rate (RGR) adapted from Hunt (1990): *RGR = (ln B2 – ln B1)/(T1-T2)*, where *B1* and *B2* refer to the total fresh biomass of the three fragments at time 1 and time 2 (biomasses only considered the growth of the original fragments and excluding cut shoots that appeared during the experiment). We quantified the damage to plants induced by crayfish by measuring the mean percentage of damaged leaves per shoot (leaves with scars or holes) and the free leaf biomass. Free leaf biomass consisted of only the leaves cut by the crayfish and found in the water column or floating at the water surface in the aquarium. When a shoot fragment had disappeared from the aquarium, we included a number of 100% of damaged leaves for this shoot in the calculation. As plants fragments of *E. densa*, *M. spicatum*, and *L. grandiflora* are able to regenerate new plants (Hussner, 2009; Riis et al., 2009; Heidbüchel et al., 2016), we assumed that crayfish could have a positive impact on the invasive success of exotic plants by enhancing their dispersal due to the increase in shoots cut by crayfish. We quantified this impact on plant dispersal by using the mean number of additional fragments, which was calculated as the number of shoots at the end of the experiment, less this number at the beginning. To evaluate the palatability of the plant species, we measured the dry matter content (DMC) and LDMC. A low water content (i.e., high DMC) and high concentrations of proteins and nitrogen in plants are associated with a high nutritive value (Cronin et al., 2002). The LDMC is used as a proxy to predict variations in macrophyte palatability (Elger and Willby, 2003): it is related to the average density of leaf tissues (Cornelissen et al., 2003) and leaf constituents such as lignin, fiber, and silica contents which contribute to leaf toughness (Elger and Willby, 2003) and to the morphological defenses of plants. Leaves and shoots were collected, weighed (fresh mass), dried (for 1 week at 70°C) and then reweighed (dry mass). Prior to drying, fresh leaves of the invasive species *L. grandiflora* and *E. densa* were scanned and leaf area was measured using Scion Image software in order to calculate the specific leaf area (SLA). Leaf area of *M. spicatum* was not calculated as its leaves are small, thin and highly dissected. DMC (g.g$^{-1}$) was assessed for each pool of three shoots per species, using the dry mass of the pool divided by the fresh mass of the pool of individuals. The LDMC (mg.g$^{-1}$), calculated as dry mass of the leaf divided by its fresh mass, was measured on three leaves attached to each planted shoot (upper part of the plant shoot). SLA (mm$^2$.mg$^{-1}$) which is correlated to relative growth rate and investment in structural tissue was calculated as the ratio

**TABLE 1 |** Characteristics (common name, family, biological type, status, habitat, and morphology) of the three aquatic plant species used in mixed cultures: *Egeria densa*, *Ludwigia grandiflora,* and *Myriophyllum spicatum*.

| | *Egeria densa* | *Ludwigia grandiflora* subsp. *hexapetala* | *Myriophyllum spicatum* |
|---|---|---|---|
| Common name | Brazilian waterweed | Water primrose | Eurasian watermilfoil |
| Family | Hydrocharitaceae | Onagraceae | Haloragaceae |
| Biological type | Submerged freshwater plant | Amphibious freshwater plant | Submerged freshwater plant |
| Status/native area | Exotic in some European countries, Australia, New Zealand, Turkey/Native to South America[1] . | | Native to Europe, Asia, and Northern Africa/ Exotic in the United-States, Australia, South Africa, India[6] |
| Habitat | Still and flowing waters, lakes, ponds, pools and quiet streams. | Marshes, ponds, slow-running rivers, as well as wet meadows[3]. | Slow moving or still eutrophic water[7]. |
| Morphology | Dense monospecific stands Very bushy plant with dense whorls of robust leaves Four leaves per whorl and each leaf is at least 2 cm long. Palatable aquatic macrophyte[2] | Creeping submerged stems (glabrous to sparsely pubescent) and aerial shoots. Alternate, polymorphic[4] leaves Reported as an unpalatable plant, whereas cases of grazing have been observed[5] | Stems grow to water surface and frequently form dense mats. Mature leaves: typically arranged in whorls of four leaves. Leaf has 12 or more leaflet pairs. Low palatable plant[8]: production of polyphenols[9]. |

[1]Cook and Urmi-König, 1985; Dutartre et al., 2007; Hussner, 2009; Thouvenot et al., 2013, [2]Parker et al., 2006, [3]Lambert et al., 2010, [4]European and Mediterranean Plant Protection Organization, 2011, [5]Pine and Anderson, 1991; Grillas et al., 1992; Legrand, 2002; Lambert et al., 2009, [6]Weyl and Coetzee, 2014, [7]Aiken et al., 1979, [8]Li et al., 2004, [9]Gross, 2000.

of fresh leaf area to leaf dry mass ($mm^2$ $mg^{-1}$). Data of SLA and LDMC were averaged per stem.

## Statistical Analysis

We tested the associational resistance and susceptibility hypotheses by performing a two-factor Bayesian ANOVA per species. ANOVA was defined as a linear model with the identity of the neighboring species (two categories in each analysis), the crayfish (presence or absence) and their interaction as factors. To account for heteroscedasticity, we used an additional parameter to estimate within factor variability. When several measurements were performed on the same individual (here, traits related to leaves: SLA and LDMC), we built a mixed effect model, with a specific error term to consider individuals as a random factor. Logarithmic transformations were used for SLA and LDMC.

As there was no variability in the control treatment for the free leaf biomass, the proportion of damaged leaves and the number of cut shoots due to the absence of crayfish, the damage induced by the presence of the crayfish was tested for each species by comparing posterior distributions of the mean to zero. Then, the influence of neighboring species was analyzed only for treatments with crayfish. We tested the diet preference of crayfish by doing pairwise comparisons of posterior distributions of the herbivory effect on the RGR for each plant species.

Posterior effect size distributions for simple effects were computed as contrast (a difference in posterior mean distributions for the two levels of one factor). Effect sizes for pairwise comparisons were computed as the difference in posterior distributions of the considered treatments. Significance of effects was defined as the probability of effect sizes (posterior distributions for the interaction term) being lower or greater than zero. We used a threshold of significance equal to 0.05 (thereafter we speak of tendencies when we used a threshold of 0.1). We chose non-informative priors for each of the model parameters. Model parameters were estimated by Markov chain Monte Carlo sampling (MCMC) with the rags 4.3.0 library in R 3.4.4. We ran four independent chains of 50,000 iterations with a burn-in period of 20,000. Quality of model fit was assessed using Gelman–Rubin diagnostics. Untransformed means and standard errors were used in the figures to facilitate interpretation. Several points appeared to have a significant impact on the conclusions of the statistical analyses. Outliers detected using an approach based on the Cook distance were removed from analyses (see **Supplementary Materials** for more details on the procedure). When data points were removed, we present the output of our models with and without these data points in the Results section. All analyses were performed with R software (R Core Team, 2017). Codes used for data analyses are accessible on GitHub at https://github.com/gauzens/traits_and_invasions.git.

# RESULTS

## Crayfish Selectivity

By comparing differences in RGR induced by herbivory, we found that *E. densa* was consumed more than *L. grandiflora* and *M. spicatum*. Indeed, posterior distribution of the herbivory effect on *E. densa* was significantly smaller than the one found for *L. grandiflora* ($p$ = 0.002) and *M. spicatum* ($p$ = 0.0195). We did not find a significant difference in selectivity between *L. grandiflora* and *M. spicatum* ($p$ = 0.271).

## Effect of Neighborhood and Herbivory on the More Palatable Exotic Plant *E. densa*

The functional traits of the exotic *E. densa* mainly depended on the presence of crayfish, and were marginally affected by the neighboring species (**Tables 2**, **3**). Its proportion of damaged leaves increased significantly because of herbivory in presence of *M. spicatum* ($p$ = 0.037) and *L. grandiflora* ($p < 0.001$, **Table 3** and **Figure 1**). *E. densa* tended to lose more leaf biomass ($p$ = 0.077) and was more fragmented ($p$ = 0.001) due to severing, especially in the presence of *M. spicatum* as a neighbor species, while these effects were not detected in the presence of *L. grandiflora* (**Table 3**

**TABLE 2 |** Summary of two-factor Bayesian ANOVAs performed for each species and each measured trait: relative growth rate (RGR), dry matter content (DMC), leaf dry matter content (LDMC), and specific leaf area (SLA).

| | RGR ($d^{-1}$) | | DMC ($g.g^{-1}$) | | LDMC ($mg.g^{-1}$) | | SLA ($mm^2.mg^{-1}$) | |
|---|---|---|---|---|---|---|---|---|
| *E. densa* | | | | | | | | |
| Neighboring species (N) | 0.032 | (0.288) | *0.792* | *(0.092)* | 0.055 | (0.234) | 0.067 | (0.267) |
| Crayfish treatment (C) | **0.266** | **(<0.001)** | 0.156 | (0.399) | 0.095 | (0.107) | 0.116 | (0.128) |
| Interaction (N) × (C) | 0.069 | (0.268) | 0.917 | (0.400) | 0.230 | *(0.068)* | **0.356** | **(0.040)** |
| *L. grandiflora* | | | | | | | | |
| Neighboring species (N) | 0.009 | (0.320) | 0.135 | (0.377) | *0.041* | *(0.074)* | **0.084** | **(0.001)** |
| Crayfish treatment (C) | **0.049** | **(0.022)** | 0.107 | (0.398) | 0.016 | (0.280) | 0.012 | (0.319) |
| Interaction (N) × (C) | 0.041 | (0.173) | 0.022 | (0.496) | *0.088* | *(0.063)* | 0.014 | (0.393) |
| *M. spicatum* | | | | | | | | |
| Neighboring species (N) | 0.005 | (0.465) | 1.261 | (0.146) | **0.099** | **(0.048)** | / | |
| Crayfish treatment (C) | *0.088* | *(0.067)* | 0.730 | (0.271) | 0.056 | (0.196) | / | |
| Interaction (N) × (C) | *0.156* | *(0.088)* | 0.857 | (0.352) | 0.072 | (0.299) | **/** | |

*Values presented are effect sizes of the different factors estimated from the posterior distributions and associated p-values within brackets. Significant results are in bold type, and tendencies are in italics.*

**TABLE 3 |** Summary of two-factor Bayesian ANOVAs performed for each species and each type of damage induced by crayfish: percentage of damaged leaves, free leaf biomass and number of cut shoots.

| **Species** | ***E. densa*** | | ***L. grandiflora*** | | ***M. spicatum*** | |
|---|---|---|---|---|---|---|
| **Neighboring species** | ***M. spicatum*** | ***L. grandiflora*** | ***E. densa*** | ***M. spicatum*** | ***E. densa*** | ***L. grandiflora*** |
| **Damaged leaves (%)** | | | | | | |
| Neighboring species (N) | 0.254 (0.123) | | 0.014 (0.458) | | 0.004 (0.490) | |
| Crayfish treatment (C) | **0.442 (0.037)** | **0.6959 (<0.001)** | **0.496 (<0.001)** | **0.481 (0.002)** | **0.265 (0.009)** | **0.260 (0.002)** |
| **Free leaf biomass (g)** | | | | | | |
| Neighboring species (N) | 0.002 (0.409) | | 0.113 (0.125) | | 0.071 (0.195) | |
| Crayfish treatment (C) | *0.007 (0.077)* | 0.005 (0.134) | **0.122 (0.011)** | **0.235 (0.010)** | *0.108 (0.092)* | *0.037(0.082)* |
| **Number of cut shoots** | | | | | | |
| Neighboring species (N) | *0.877 (0.081)* | | *1.139 (0.071)* | | 0.359 (0.297) | |
| Crayfish treatment (C) | **1.382 (0.001)** | 0.504 (0.158) | **0.746 (0.038)** | **1.88 (0.008)** | 0.614 (0.136) | 0.255 (0.237) |

*Values presented are effect sizes of the different factors estimated from the posterior distributions and associated p-values within brackets. Significant results are in bold type, and tendencies are in italics.*

and **Figure 1**). However, we only detected a marginal difference in fragmentation between the two neighboring species ($p = 0.081$).

The RGR of *E. densa* was significantly reduced by herbivory from crayfish ($p < 0.001$), but it was not affected by the identity of the neighboring species ($p = 0.288$). No significant interaction between these two factors was found ($p = 0.268$, **Figure 2** and **Tables 2**, **4**). For the DMC of *E. densa*, we found that one data point was quite influential (five times larger than the mean value of its group) and led to the absence of any effects. Once removed, the DMC of *E. densa* tended to be higher in the presence of *M spicatum* than in the presence of *L. grandiflora* ($p = 0.092$, **Table 2** and **Figure 2**), and mainly in the crayfish treatment as shown by the pairwise comparison between treatment crayfish-*L. grandiflora* and crayfish-*M. spicatum* (**Table 4**), while the DMC of *E. densa* was similar in the control treatment, whatever the neighboring species. The LDMC was marginally affected by the interaction crayfish-neighboring species with values tending to be higher ($p = 0.068$, **Table 2** and **Figure 3**) in the presence of *M. spicatum*. This is most likely to be explained by the significant decrease of LDMC due to crayfish in presence of *L. grandiflora* ($p = 0.024$, **Table 4** and **Figure 3**), while we did not detect any effect of crayfish in the presence of *M. spicatum* on LDMC. We observed a significant interaction between the crayfish treatment and the identity of the neighboring species for the SLA ($p = 0.040$, **Table 2** and **Figure 3**), explained by a significantly higher SLA in the presence of *L. grandiflora* in comparison with values observed in the presence of *M. spicatum* for crayfish treatments ($p = 0.050$, **Table 4**), while the SLA of *E. densa* was similar in control treatments whatever the identity of the neighboring species ($p = 0.209$, **Table 4**).

## Effect of Neighborhood and Herbivory on the Less Palatable Exotic Plant *L. grandiflora*

In contrast to the palatable exotic species and despite the different types of damage induced by crayfish, the RGR of *L. grandiflora* remained positive under crayfish pressure. Whatever the neighboring species, the free leaf biomass

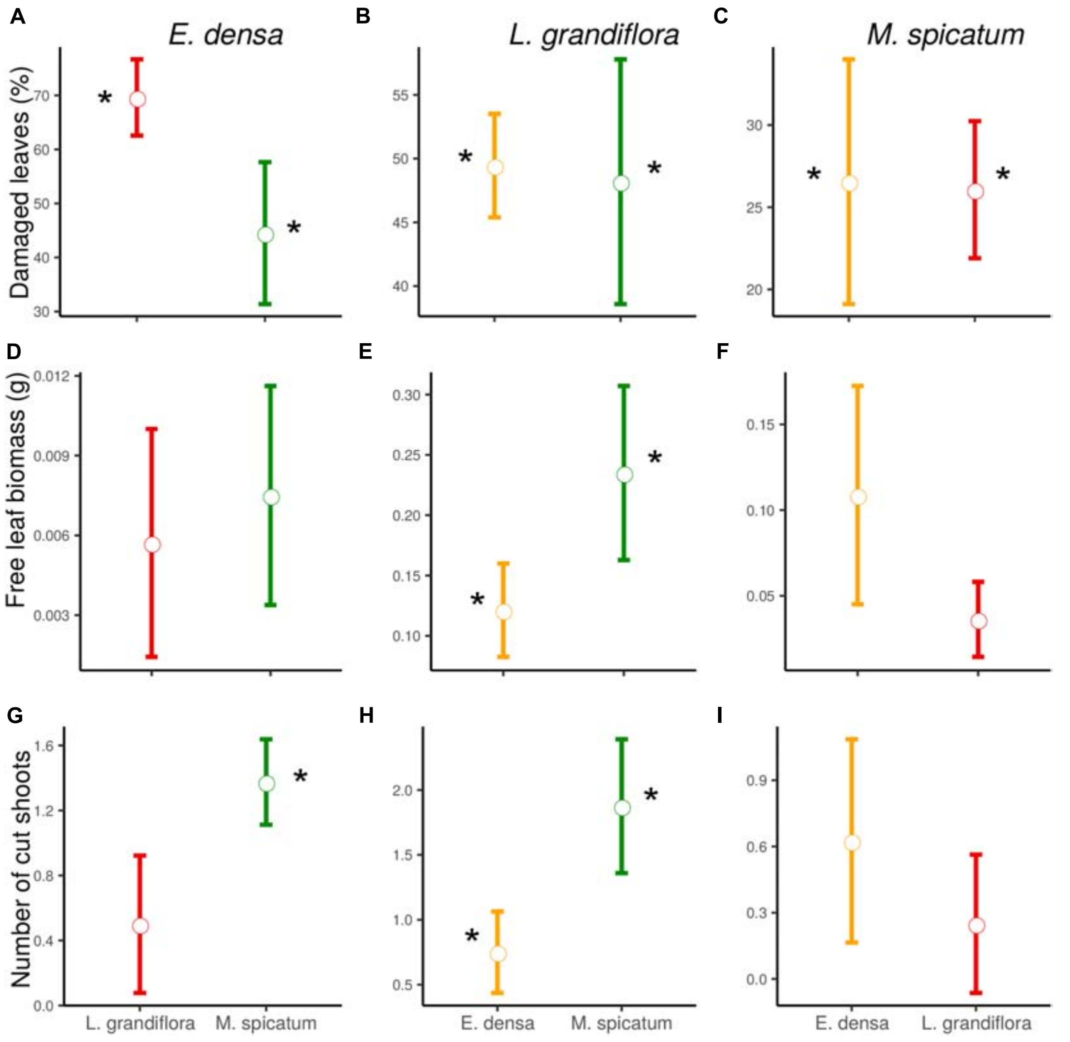


**FIGURE 1 |** Effect of neighboring species and herbivory on the damaged leaves **(A–C)**, free leaf biomass **(D–F)**, and number of cut shoots **(G–I)** of *E. densa*, *L. grandiflora* and *M. spicatum* (Means ± SE). Colors code the identity of the neighboring species: orange for *E. densa*, red for *L. grandiflora* and green for *M. spicatum*. Stars show the significant herbivory effects (significance threshold of 0.05).

of *L. grandiflora* found in the water column significantly increased in the presence of crayfish (in association with *M. spicatum*: $p$ = 0.010, *E. densa:* $p$ = 0.011) and its leaves were highly damaged (in the presence of *M. spicatum*: $p$ = 0.002, *E. densa*: $p < 0.001$, **Table 3** and **Figure 1**). Similarly, the presence of crayfish induced an increase in stem fragmentation of *L. grandiflora* (in the presence of *M. spicatum*: $p$ = 0.008, *E. densa*: $p$ = 0.038, **Table 3** and **Figure 1**), which tended to be higher in the presence of the native species *M. spicatum* ($p$ = 0.071) in comparison with the association with *E. densa*.

Furthermore, the RGR of *L. grandiflora* depended on crayfish presence but not on the neighboring species. Crayfish presence reduced the RGR of *L. grandiflora* ($p$ = 0.022, **Figure 2** and **Table 2**), particularly when this species grew up with the exotic species *E. densa* ($p$ = 0.021, **Table 4**). This effect became marginal in the presence of *M. spicatum* ($p$ = 0.053, **Table 4**) due to a stronger size effect, corresponding to a large variability of RGR values observed for treatments with crayfish and *M. spicatum*. SLA was significantly affected by the identity of the neighboring species ($p$ = 0.001, **Table 2** and **Figure 3**), values always being significantly higher in the presence of *M. spicatum*. We did not observe any effect of crayfish presence ($p$ = 0.319) nor interaction ($p$ = 0.393, **Table 2**) on SLA of *L. grandiflora*. When we performed analyses including the three data points from the experimental unit removed, we only found a marginal interaction effect ($p$ = 0.080), as the mean SLA value for the control treatment in the presence of *E. densa* increased drastically from 29.11 $mm^2.mg^{-1}$ (the smallest observed mean within all treatments) to 50.12 $mm^2.mg^{-1}$ (the largest one), thus obscuring the crayfish effect. The DMC of *L. grandiflora* was not impacted by the different treatments nor their interaction (**Table 2**), but we found a marginal interactive effect on LDMC ($p$ = 0.063, **Table 2** and **Figure 3**), because of a significant

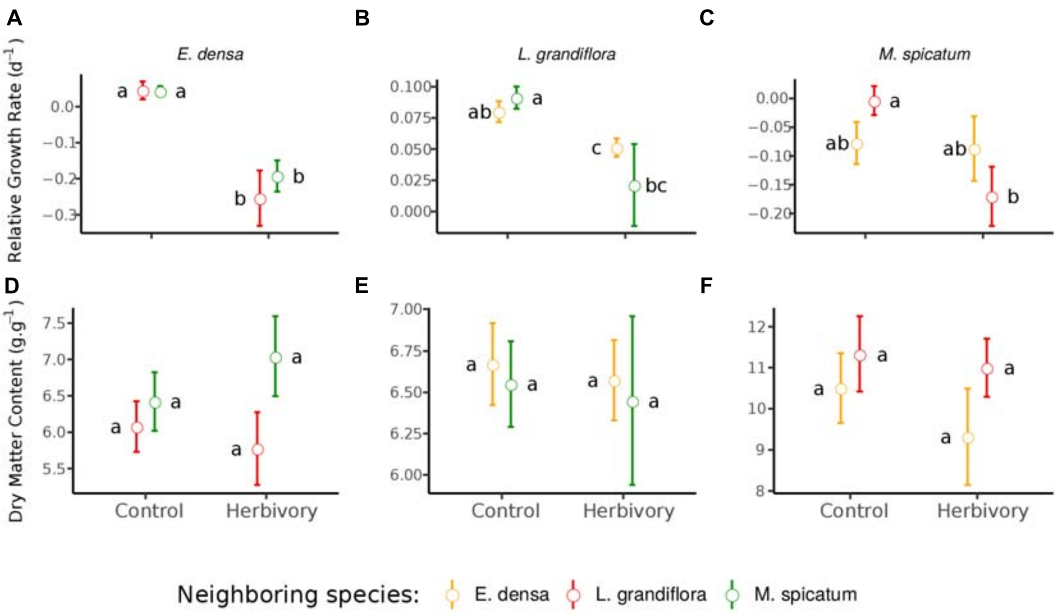


**FIGURE 2 |** Effect of neighboring species and herbivory on the relative growth rate **(A–C)** and dry matter content **(D–F)** of *E. densa*, *L. grandiflora* and *M. spicatum* (Means ± SE). Colors code the identity of the neighboring species: orange for *E. densa*, red for *L. grandiflora* and green for *M. spicatum*. Letters indicate the significance of pairwise comparisons (significance threshold of 0.05).

**TABLE 4 |** Summary of the pairwise comparisons performed for each species and each measured trait: relative growth rate (RGR), dry matter content (DMC), leaf dry matter content (LDMC), and specific leaf area (SLA).

| | RGR (d$^{-1}$) | | DMC (g.g$^{-1}$) | | LDMC (mg.g$^{-1}$) | | SLA (mm$^2$.mg$^{-1}$) | |
|---|---|---|---|---|---|---|---|---|
| *E. densa* | | | | | | | | |
| Crayfish, *L. grandiflora* – Control, *L. grandiflora* | **−0.300** | **(0.006)** | −0.303 | (0.344) | **−0.210** | **(0.024)** | **0.241** | **(0.029)** |
| Crayfish, *L. grandiflora* – Crayfish, *M. spicatum* | −0.066 | (0.263) | *−1.251* | *(0.094)* | *−0.171* | *(0.068)* | **0.241** | **(0.050)** |
| Crayfish, *L. grandiflora* – Control, *M. spicatum* | **−0.300** | **(0.006)** | −0.637 | (0.206) | *−0.150* | *(0.086)* | 0.179 | (0.114) |
| Control, *L. grandiflora* – Crayfish, *M. spicatum* | **0.235** | **(0.003)** | −0.948 | (0.129) | 0.039 | (0.361) | 0.0003 | (0.360) |
| Control, *L. grandiflora* – Control, *M. spicatum* | 0.002 | (0.474) | −0.334 | (0.298) | 0.060 | (0.282) | −0.062 | (0.209) |
| Control, *M. spicatum* – Crayfish, *M. spicatum* | **−0.230** | **(0.001)** | 0.614 | (0.226) | 0.020 | (0.418) | −0.062 | (0.312) |
| *L. grandiflora* | | | | | | | | |
| Crayfish, *E. densa* – Control, *E. densa* | **−0.029** | **(0.021)** | −0.096 | (0.411) | *−0.061* | *(0.071)* | 0.004 | (0.442) |
| Crayfish, *E. densa* – Crayfish, *M. spicatum* | 0.030 | (0.223) | 0.146 | (0.413) | −0.003 | (0.474) | **−0.092** | **(0.007)** |
| Crayfish, *E. densa* – Control, *M. spicatum* | **−0.040** | **(0.005)** | 0.028 | (0.473) | 0.025 | (0.249) | **−0.073** | **(0.015)** |
| Control, *E. densa* – Crayfish, *M. spicatum* | *0.059* | *(0.082)* | 0.242 | (0.366) | *0.058* | *(0.090)* | **−0.096** | **(0.006)** |
| Control, *E. densa* – Control, *M. spicatum* | −0.011 | (0.220) | 0.123 | (0.402) | **0.086** | **(0.022)** | **−0.077** | **(0.008)** |
| Control, *M. spicatum* – Crayfish, *M. spicatum* | *−0.070* | *(0.053)* | −0.118 | (0.433) | 0.028 | (0.247) | 0.019 | (0.311) |
| *M. spicatum* | | | | | | | | |
| Crayfish, *E. densa* – Control, *E. densa* | −0.008 | (0.466) | −1.158 | (0.256) | −0.008 | (0.467) | / | |
| Crayfish, *E. densa* – Crayfish, *L. grandiflora* | 0.082 | (0.196) | −1.690 | (0.158) | 0.061 | (0.255) | / | |
| Crayfish, *E. densa* – Control, *L. grandiflora* | −0.082 | (0.147) | −1.991 | (0.142) | *0.156* | *(0.053)* | / | |
| Control, *E. densa* – Crayfish, *L. grandiflora* | 0.090 | (0.130) | −0.532 | (0.342) | 0.069 | (0.215) | / | |
| Control, *E. densa* – Control, *L. grandiflora* | *−0.074* | *(0.089)* | −0.833 | (0.294) | **0.163** | **(0.040)** | / | |
| Control, *L. grandiflora* – Crayfish, *L. grandiflora* | **−0.165** | **(0.017)** | −0.301 | (0.411) | 0.094 | (0.164) | / | |

*Values presented are effect sizes of the different factors estimated from the posterior distributions and associated p-values within brackets. Significant results are in bold type, and tendencies are in italics.*

impact of neighboring species in the control (larger values when associated to *E. densa*, $p = 0.022$, **Table 4**) becoming not significant in crayfish treatments ($p = 0.474$, **Table 4**). When we performed the analyses including the data points of the experimental unit initially removed, all the results became not significant.

### Effect of the Exotic Neighboring Plant and the Exotic Herbivore on the Less Palatable Native Plant *M. spicatum*

The native *M. spicatum* was barely affected by the crayfish and neighboring species, although these factors or their interaction tended to affect some of its traits. We did not observe any effect of the neighboring species on the damage to *M. spicatum*; the only significant differences were related to the presence of crayfish (**Table 3** and **Figure 1**). The number of cut shoots was not affected by crayfish presence or by the neighboring species. The percentage of damaged leaves increased in the presence of crayfish ($p = 0.002$ when associated with *L. grandiflora*, $p = 0.009$ when associated to *E. densa*; **Table 3**), but we did not find a significant (only a marginal) effect of crayfish on the free leaf biomass lost by *M. spicatum* ($p = 0.082$ in presence of *L. grandiflora* and $p = 0.092$ in presence of *E. densa,* **Table 3**).

The RGR of *M. spicatum* tended to decrease in the presence of crayfish ($p = 0.067$, **Table 2** and **Figure 2**), this effect being significant in the presence of *L. grandiflora* ($p = 0.017$, **Table 4**), but disappearing in the presence of *E. densa* ($p = 0.466$, **Table 4**) explaining a marginal interaction ($p = 0.088$, **Table 2**). When the outlier was included in our analysis, the only qualitative change was for the interaction term ($p = 0.427$). The DMC of *M. spicatum* was not affected by the neighboring species, crayfish presence or their interaction (**Table 2** and **Figure 2**). We observed a significant effect of neighboring species on the LDMC ($p = 0.048$, **Table 2**): the values of the LDMC of *M. spicatum* were higher in the presence of *E. densa* than in the presence of *L. grandiflora* in control treatments but not in the presence of crayfish ($p = 0.040$, **Table 4**).

## DISCUSSION AND PERSPECTIVES

### Plant Response to the Neighboring Plant Species and the Herbivore

Crayfish caused significant damage to the plant species, as shown by the systematic decrease of RGR and the increase of damaged leaves, and as reported by many authors (Flint and Goldman, 1975; Feminella and Resh, 1989; Sánchez and Angeler, 2006). However, the absence of significant crayfish effects on floating free leaf biomass and number of cut shoots (floating plus rooted) observed for *M. spicatum* and to a lesser extend for *E. densa* could be explained by the ingestion of these floating plant organs by the crayfish, which were consequently not collected in the water column at the end of the experiment. This hypothesis could be verified in further short-term experiments by checking crayfish feeding behavior using video tracking.

In this experiment, we studied plant traits in the context of the interactive effect of neighboring species and herbivore pressure that had rarely been considered before. Overall, the effects of the crayfish treatment and of the neighboring species seemed to be species specific. We did not find general patterns of plant responses (SLA, LDMC, etc.) or of herbivory damage (number of cut shoots, percentage of damaged leaves, free leaf biomass) to neighboring species and herbivory, suggesting that the three plants considered follow different strategies regarding crayfish presence and the identity of the neighboring species. Indeed, the response of plant traits (**Figure 4**) and especially plant palatability (regarding SLA and LDMC) were only modestly affected by the interaction between neighboring species and the herbivore, but rather by the identity of neighboring species or the herbivore effect alone. SLA is a function of LDMC and leaf thickness (Cornelissen et al., 2003; Pérez-Harguindeguy et al., 2013) and consequently it would explain the trends observed regarding the neighboring effect on the LDMC of *L. grandiflora,* and the interaction effect of neighboring species and crayfish on the LDMC of *E. densa* (**Table 4**).

The SLA values of *E. densa* depended on the interaction neighboring species - crayfish treatment. In the presence of crayfish, high SLA values were observed when the neighboring species was *L. grandiflora,* while no variation was observed in the presence of *M. spicatum*. This variation observed under herbivore pressure for *E. densa* could have two explanations. Firstly, it might be a direct effect of the consumption of younger leaves (with high SLA and low LDMC) by herbivores. Secondly, this difference can be explained by a morpho-physiological response of *E. densa*, suggesting that macrophytes species could show a rapid adaptation in response to herbivory. As we observed changes of the SLA of *E. densa* only in presence of *L. grandiflora* (despite a significant herbivory with the two neighboring species), the first explanation is rather unlikely. Moreover, several examples showing that macrophytes can exhibit morphological responses to stressors are documented, arguing for our second hypothesis. For example, *Rumex palustris* can increase its leaf laminae in response to submergence within 3 days (Voesenek and Blom, 1989). Morphological responses were also observed in 3 days for alga *Padina jamaicensis* in response to a reduction of grazing intensity (Lewis et al., 1987). Fast changes in leaf orientation and coloration can be observed after 24 h of herbivory on *M. spicatum* (Fornoff and Gross, 2014) and this species increased its DMC in its apices after 5 days under herbivore pressure (Fornoff and Gross, 2014). The DMC of *Elodea canadensis* and *Elodea nuttallii* were also higher in the presence of the herbivore after 6 days of experiment (Thiébaut et al., 2017). In the specific case of *E. densa*, it has been shown that this species has high growth abilities, with a RGR of up to 37–40 mg $g^{-1}$ $day^{-1}$ (Tanner et al., 1993), or from 0.03 to 0.05 g g $day^{-1}$ of dry mass (Pistori et al., 2004). This strong growth rate suggests that *E. densa* could have a high ability to respond to damage through fast leaf/stem elongation. With the increase in SLA of *E. densa* (and thus indirectly to photosynthetic activity), we observed more allocation of energy to growth and not to structural tissues (which would imply an increase of LDMC) in the presence of *L. grandiflora*, potentially leading to more brittle and appetizing plant individuals of *E. densa*. This result suggests that *E. densa* allocated its resource to growth in order to compensate herbivore damage (Strauss and Agrawal, 1999). Furthermore, Lemoine et al. (2009) have shown that *M. spicatum* and *Elodea canadensis* reduced their DMC in response to herbivores. The reduction of DMC is associated with a reduction of the cell wall resistance (Gatehouse, 2002; Lemoine et al., 2009) which is positively correlated to the

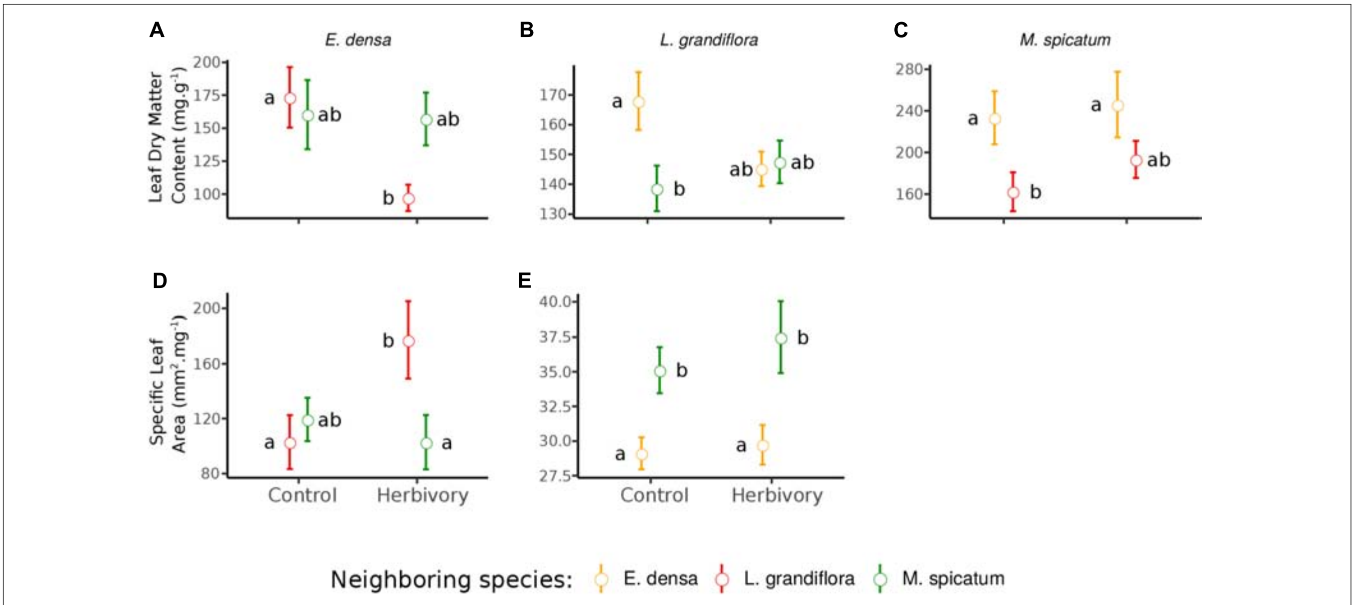


**FIGURE 3 |** Effect of neighboring species and herbivory on the leaf dry matter content **(A–C)** and specific leaf area **(D,E)** of *E. densa*, *L. grandiflora*, and *M. spicatum* (Means ± SE). Colors code the identity of the neighboring species: orange for *E. densa*, red for *L. grandiflora* and green for *M. spicatum*. Letters indicate the significance of pairwise comparisons (significance threshold of 0.05).

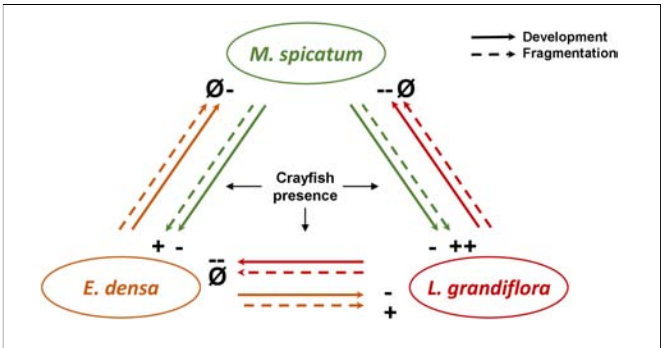


**FIGURE 4 |** Schematic diagram of the effects of interactions between the different plant species (the native species *M. spicatum,* and the exotic species *E. densa* and *L. grandiflora*) under herbivore pressure on the development (RGR, LDMC) and fragmentation (number of cut shoots) of the plant species involved in the experiment. The effects are positive (+), negative (–) and neutral (i.e., without effect, Ø).

fragmentation ability of plants (Gatehouse, 2002) and could be considered as an escape strategy against herbivory. *E. densa,* which is a species phylogenetically close to *E. canadensis,* had a higher SLA and seemed to have a lower DMC and LDMC in the presence of *L. grandiflora* and crayfish (**Figure 2** and **Tables 2**, **4**) than in the presence of *M. spicatum*, while no variations were observed in the control treatments. Thus, this strategy suggests that under herbivore pressure, *E. densa* favors its fragmentation and dispersal abilities in the presence of the unpalatable plant species *L. grandiflora*.

In the case of *M. spicatum* and *L. grandiflora*, SLA and LDMC values were different in the control treatments, with higher LDMC values and lower SLA values (SLA only for *L. grandiflora*) being found when they were grown in association with the palatable species *E. densa*. These differences in LDMC completely disappeared in the presence of crayfish for *L. grandiflora*, and tended to decrease for *M. spicatum*. In the meantime, the responses of their RGR values were rather similar: the RGR of both species tended to decrease in the presence of an unpalatable neighbor, while the impact of crayfish was much lower in the presence of the palatable species *E. densa* (nearly no herbivory effect for *M. spicatum* with *E. densa*). Plants with high LDMC and low SLA generally possess strong quantitative leaf defenses and deter herbivores (Cornelissen et al., 2003; Kirk et al., 2012), while a high SLA and low LDMC are correlated with a high relative growth rate, but low resistance to herbivory (Cornelissen et al., 2003). Thus these two species seem to have similar responses to the presence of a neighbor species (high investment in defense in the presence of a palatable species), but this strategy is blurred in the presence of herbivores. The disappearance of the impact of a neighbor effect in the presence of crayfish would suggest that in our experiment the stress induced by herbivory was much stronger than the interaction with neighbors. As both *M. spicatum* and *L. grandiflora* are able to produce defensive compounds (Gross, 2000; Dandelot et al., 2008; Bauer et al., 2009; Lemoine et al., 2009), it is possible that the ecological strategy of these two species is toward other defense mechanisms to herbivory that were not considered here (i.e., secondary compounds involved in chemical defenses).

Considered together, our results highlight that the variation of SLA and LDMC could reflect anti-herbivore (*E. densa*) and/or anti-neighbor strategy (*L. grandiflora*, *M. spicatum*) during the time of our experiment (3 days). However, we still do not know how fast the plant traits response to herbivory or neighboring species could be. This calls for further experiments with time-series sampling to reinforce or question our conclusions. In addition, our results also showed that plant traits were affected by herbivory but also by an interaction with neighboring species, suggesting that these two ecological processes interact, and call for further investigations on underlying mechanisms and ecological consequences.

## Consequences of Plant Strategy on the Outcomes of the Plant–Plant–Herbivore Relationship

Plant traits influence plant performance and explain their growth and survival across different ecosystems. The set of the different trait values and their combinations represents alternative ecological strategies of the plant species in response to variations of environment. Considering the overall responses of all the traits of each species to its neighbors and under crayfish pressure would make it possible to assess the outcomes of these plant–plant–herbivore interactions. Below, we summarize the different effects observed on the traits of each species in order to deduce the potential outcomes (**Figure 4**).

### *E. densa*

The reduction of *E. densa* growth due to herbivory was always stronger than that observed for the two other species. This is coherent with the high palatability of the species. The effect size of the herbivory effect was rather similar, whatever the associated neighboring plant was. However, in presence of *L. grandiflora*, the SLA of *E. densa* highly increased, potentially due to the reallocation of biomass to the apical growth, or due to the lower consumption of young parts of the plant. Thus, our results suggest that the youngest parts of *E. densa* would be less consumed in presence of *L. grandiflora* than in presence of *M. spicatum* and/or that *L. grandiflora* stimulated the growth of *E. densa* to compensate the loss due to crayfish. It would be interesting to test whether over a longer time period this compensation would attenuate the crayfish effect on the growth of *E. densa*.

### *L. grandiflora*

Despite having a high number of cut shoots and a low SLA, *L. grandiflora* exhibited the lowest reduction of RGR under herbivore pressure. Thus, this species might be able to continue to acquire resources in order to tolerate crayfish consumption and to compensate the damage by its high growth rate. The effect of the herbivore was stronger in presence of *M. spicatum* despite the fact that the traits related to palatability remained similar whatever the identity of the neighboring species: this suggests more a dilution effect of crayfish impacts in the presence of *E. densa* than a higher susceptibility to crayfish damage in the presence of *M. spicatum*.

### *M. spicatum*

Similarly, the growth of the native *M. spicatum* decreased under crayfish treatment whatever the identity of the neighboring species, with a higher effect in the presence of *L. grandiflora* than *E. densa*, also suggesting a dilution effect of herbivory on *M. spicatum* in the presence of the more palatable *E. densa*. *E. densa* could be an attractant-decoy plant, and consequently would decrease the crayfish damage on the neighboring species *M. spicatum*.

## Is There a Facilitative Effect of an Exotic Herbivore on Exotic Plant Development and Propagation in the Presence of a Native Plant?

Exotic crayfish promoted the propagation and the dispersal capacities of both exotic plants in the presence of the native plant during our experiment. Indeed, we observed that crayfish broke up each species, increasing the number of cut shoots.

The presence of the native *M. spicatum* in crayfish presence had an indirect positive effect on the invasive species *L. grandiflora* and *E. densa* by increasing their fragmentation rate. We can also suppose that the fragmentation of *E. densa* was increased in presence of *L. grandiflora*, explaining the high decrease of its RGR, but that the cut shoots were consumed by crayfish due to their higher appetence/palatability. As previously developed, fragmentation could be considered as an escape strategy (Lemoine et al., 2009) and could lead to plant propagation. Thus, this direct negative impact of crayfish in the presence of the native species could influence the rate at which an invasion occurs, with a positive effect on the dispersal and propagation of the exotic plant species in ecosystems.

Although the survival and anchorage rate of these cut floating shoots and their abilities to generate new plants are crucial points that remain to be investigated, the literature shows that most aquatic plants can regenerate new plants from their fragments (Pieczynska, 2003). Stem fragments of *L. grandiflora*, with nodes, and with or without leaves showed a high potential for regeneration (Hussner, 2009) and had a higher anchorage rate in the presence of the invasive species *E. canadensis* (Thiébaut and Martinez, 2015). Similarly, stem fragments of *E. densa* (1 cm in length) (Cook and Urmi-König, 1984; Getsinger and Dillon, 1984; Riis et al., 2009) and stem fragments (2 cm in length) of *M. spicatum* have a high regeneration capacity and can develop into new shoots, even without the presence of an apical bud (Riis et al., 2009; Kuntz et al., 2014; Heidbüchel et al., 2016).

## Associational Susceptibility/Resistance Theories in the Biological Invasions Context

A lot of attention has been paid to the defense strategies of plants against herbivory, however, little is known on how it will affect plant coexistence in the face of a combination of several biotic factors (competition with invasive species and herbivory in our paper). Our study showed that the effect of exotic crayfish and neighboring exotic plants influence each other. Therefore, these two factors could be important for the establishment of exotic species in new areas and for the structure and composition of new communities. As traits of neighboring species can affect the response of plants to herbivory, we could expect that exotic palatable species could modify the establishment and growth of other less palatable exotic species, and favor the disappearance of native palatable plants in an invaded area. Unfortunately, associational resistance and associational susceptibility were, to our knowledge, not tested in the context of biological invasions. This experiment is the first step to investigate this topic. By using one native species and two exotic species, we were not able to determine a general framework on the protective effects of exotic plants on native plants, as well as the protective effects of exotic plants on other exotic plants (or native on native plants), leading to a potential invasional meltdown (Simberloff and Von Holle, 1999) between exotic species. However, the different effects highlighted between exotic and native species in our study provide the first examples of such effects and call for further investigation, using different plants (target and neighboring species from native and exotic ranges) and herbivore species (native and exotic) to consider the generality of our findings. The foraging behavior and origin of the herbivore species also deserve some attention, as the control of exotic plants can depend on herbivore origin (Parker et al., 2006). Plant selectivity by herbivores is a multi-factorial choice and might combine several plant traits (morphotype, stoichiometric ratio, DMC, anatomical or structural traits, chemical defense compounds, toxins, deterrents, digestibility reducers, etc.), other than the one that we used (related to LDMC). Thus, the relative importance of each trait to palatability might differ depending on external conditions and/or the herbivore species. Unfortunately, only one or few herbivore species are compared in most of the studies on herbivory, while several herbivore species should be tested to measure response of the plant to herbivory in a general framework. Finally, an important step will be to test such a framework at a larger scale, allowing the herbivore to choose between different patches. Indeed, the positive effect of palatable species on the growth of less palatable species (associational susceptibility) can be blurred by higher patch attractiveness in the presence of palatable species.

## CONCLUSION

The main result of our study is that plant neighboring species and herbivores modestly interact in affecting plant traits involved in their dispersal strategies and establishment success. Furthermore, we found that (i) the response to crayfish presence and to the identity of neighboring species seemed to be species specific, and (ii) crayfish enhance the fragmentation rate (putatively related to plant regeneration/propagation) of the two exotic macrophytes *L. grandiflora* and *E. densa* in the presence of the native *M. spicatum*. Thus, the exotic crayfish could indirectly facilitate the invasion success of these exotic macrophytes.

To conclude, our paper presents some of the first results on associational resistance/susceptibility and lays the foundation for developing a general framework that combines plant community ecology and biological invasion ecology which could explain invasive species success. We showed that an important future step in the field of biological invasion is a better understanding of the response of plant traits to a set of different environmental constraints considered simultaneously. The new exotic–exotic interactions and associational resistance/susceptibility to herbivory should be taken into account to better understand exotic species establishment in the native recipient communities and their consequences.

## ETHICS STATEMENT

The experiment followed the French law (Directive 2010/63/UE) for animal manipulation.

## AUTHOR CONTRIBUTIONS

GT and JH initiated the research project. LT and GT defined the research question and designed the experiments. LT conducted the experiments. BG analyzed the data. LT and BG interpreted the results and wrote the paper with contributions from all the authors. All authors have approved the manuscript.

## FUNDING

We acknowledge support from the German Research Foundation (DFG), Leipzig University within the program of Open Access Publishing. LT and BG gratefully acknowledge the support of the German Centre for Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig funded by the German Research Foundation (FZT 118).

## ACKNOWLEDGMENTS

We thank the staff of the Unité Éxpérimentale d'Écologie et d'Écotoxicologie Aquatique (Institut National de la Recherche Agronomique, Rennes, France), namely Didier Azam, Martine Ollitrault, Alphonse Quéméneur, Maïra Coke, Antoine Gallard, Bernard Joseph, Cédric Lacoste, and Julien Tremblay for preparing the experimental design and their assistance during the experiments. We thank Anne Tréguier for field assistance, as well as Nolwenn Renaud and Laurent Martinez for technical assistance. We are grateful to Ulrich Brose for providing a review of the paper and Benjamin Rosenbaum for advice on Bayesian modelization. We also thank the reviewers for their helpful comments on the manuscript.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01981/full#supplementary-material

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 11 January 2019
doi: 10.3389/fpls.2018.01987

# Effects of Temporal Heterogeneity of Water Supply and Spatial Heterogeneity of Soil Nutrients on the Growth and Intraspecific Competition of *Bolboschoenus yagara* Depend on Plant Density

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OPEN ACCESS

**Edited by:**
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**Specialty section:**
*This article was submitted to*
*Functional Plant Ecology,*
*a section of the journal*
*Frontiers in Plant Science*

**Received:** *12 May 2018*
**Accepted:** *20 December 2018*
**Published:** *11 January 2019*

**Citation:**
*Yu H, Shen N, Yu D and Liu C (2019) Effects of Temporal Heterogeneity of Water Supply and Spatial Heterogeneity of Soil Nutrients on the Growth and Intraspecific Competition of Bolboschoenus yagara Depend on Plant Density.*
*Front. Plant Sci. 9:1987.*
*doi: 10.3389/fpls.2018.01987*

Clonal plants may face various types of resource heterogeneity in their natural habitats; as such, spatial or temporal resource heterogeneity can affect the growth of clonal plants. Clonal plants can concentrate their organs in a smaller area where resources are high would cause heterogeneity to increase competition between plants. Most studies on resource heterogeneity have investigated the response of plants under a single density or by manipulating a single resource. Few studies have tested the effects of the heterogeneous distribution of two covariable resources on plant growth and intraspecific competition. A greenhouse experiment was therefore conducted to study plant responses to the spatial and temporal heterogeneity of the soil and water supply under a variety of plant densities (one, two, four, or six plants per container). The perennial clonal herb *Bolboschoenus yagara* was grown under different combinations of water supply patterns, soil nutrient distribution types and plant densities while maintaining the total water and soil nutrient availability per container constant. Compared with that at a relatively high plant density, soil nutrient heterogeneity resulted in significantly less total plant biomass and less-modified morphological traits when the plant density is relative low. At the highest plant density, compared with the homogeneous soil treatments, the heterogeneous soil treatments significantly increased the total biomass and R/S ratio. Water supply patterns also clearly affected plant morphological traits at the highest plant density. Furthermore, soil heterogeneity significantly increased intraspecific competition intensity at low plant densities, but did not significantly affect intraspecific competition intensity at higher plant densities. Water heterogeneity had little impact on intraspecific competition. These results suggest that the growth performance and intraspecific competition of *B. yagara* are more strongly affected by soil nutrient distribution rather than by water supply patterns and that competition for soil nutrients may increase plant sensitivity to soil heterogeneity.

**Keywords: resource heterogeneity, intraspecific competition, growth performance, clonal plant, population density**

## INTRODUCTION

Resources (light, water, and nutrients) exhibit spatial and temporal heterogeneity which is ubiquitous within natural habitats (Jackson and Caldwell, 1993; Gross et al., 1995; Ryel et al., 1996; Farley and Fitter, 1999). Owing to the plasticity of their various plant traits, clonal plants can adapt to changing environments (Sultan, 1987; Hutchings and de Kroon, 1994). For example, plastic foraging, by organism searches or rootlets in areas where nutrient levels are higher than those in low-nutrient quality areas, can lead to more efficient use of heterogeneously distributed resources (Wijesinghe et al., 2001; Hutchings and John, 2004; de Kroon et al., 2005; Guo et al., 2011). In addition, as a physiological response to heterogeneity, organs of foraging plants (such as rhizomes, stolons, and corms) can take up nutrients at greater rates in nutrient-rich areas (Jackson and Caldwell, 1996; Hodge, 2004). The clonal plants can transport resources through connecting spacers among connected ramets in homogeneous or heterogeneous resources habitats (Alpert, 1999; He et al., 2011).

Besides resources heterogeneity, clonal plants are exposed to many other environmental stressors such as plant density (Fransen et al., 2001; Li et al., 2017). Plant density has a significant effect on the growth and reproduction performance of individual, population structure, and competitive relationship (Grace and Tilman, 1990; Stevens and Carson, 1999). The effect of intraspecific competition depends on the plant density (Antonovics and Levin, 1980). In addition, intraspecific competition may be affected by resource heterogeneity (Day et al., 2003b; Wang Y.J. et al., 2016). Several studies reported that the effects of resource heterogeneity on intraspecific competition led to changes in competitive intensity under heterogeneous distribution of a single resource (Day et al., 2003b; Hagiwara et al., 2010). For example, light heterogeneity significantly increased the intraspecific competition intensity of *Duchesnea indica* (Wang et al., 2012).

The relative nutrient concentrations in different soil areas (hereafter referred to as "soil nutrient heterogeneity") can determine the extent to which plants concentrate more nutrient-absorbing organs in areas where nutrients are high; in addition, the efficient forage for nutrients in high-nutrient quality areas may lead to increased biomass, ramets and root production in heterogeneous environments compared to homogeneous environments that have the same amount of nutrient supply (Gersani et al., 1998; Fransen et al., 2001; Day et al., 2003b). Therefore, soil nutrient heterogeneity can influence interspecific and intraspecific competition (Day et al., 2003b; van der Waal et al., 2011; Mommer et al., 2012). Because plants prioritize the investment of relatively greater amounts of biomass in areas where nutrients are high in heterogeneous environments, competition between ramets and roots of neighboring plants may increase in intensity in smaller soil areas (Fransen et al., 2001; Day et al., 2003b). However, the results of other? experiments involving *Festuca ovina* (Day et al., 2003b), *Hydrocotyle vulgaris* (Dong et al., 2015) and *Alternanthera philoxeroides* (Zhou et al., 2012) have indicated that competition between plants is not influenced by soil nutrient heterogeneity or that this effect is temporary.

The temporal and spatial heterogeneity of water supplies (hereafter referred to as "heterogeneity of water supply") clearly affects plant biomass allocation (Fay et al., 2003; Hagiwara et al., 2008), further altering community structure and composition (Maestre and Reynolds, 2007). For the same amount of water input, a stable water supply (hereafter referred to as "homogeneity of water supply") can promote plant root systems to absorb water more efficiently and thus grow larger (Novoplansky and Goldberg, 2001; Hagiwara et al., 2012). In contrast, many plants exhibit negative biomass growth under conditions of heterogeneous water supply because those plants compensate for periodic water shortages by greater investment in roots, thus they have less to invest in other parts (Novoplansky and Goldberg, 2001; Fay et al., 2003; Hagiwara et al., 2010, 2012). In addition, spatial heterogeneity or temporal variation in water availability can alter intraspecific competition of *Perilla frutescens* (Hagiwara et al., 2010) and *Iris japonica* (Wang Y.J. et al., 2016).

Most studies on resource heterogeneity have investigated the response of only one species or the entire community by manipulating a single resource, e.g., nutrients, water, or light (Fransen et al., 2001; Day et al., 2003b; Fay et al., 2003; Moore and Franklin, 2012; Dong et al., 2015). Few studies have tested the heterogeneity of two resources affects intraspecific competition among clonal plants (Wang et al., 2012; Wang Y.J. et al., 2016), as the effects of resource heterogeneity on the relationships between plants may be altered by the supply patterns of other resources (Maestre and Reynolds, 2007).

Thus, we investigated the effects of heterogeneity in soil nutrients and water supply on the growth of both individual plants and the entire population under a variety of plant densities, as a single plant or a population at different densities usually experience both types of resource heterogeneity in their natural habitats. To test the responses of clonal plants to soil heterogeneity and water heterogeneity at different plant densities, we conducted a greenhouse experiment involving clonal plants of the rhizomatous species *B. yagara* (Ohwi).

Specifically, we addressed the following questions:

(a) Does the soil nutrient heterogeneity and heterogeneity of water supply affect the biomass accumulation in *B. yagara*?
(b) How do morphological traits of *B. yagara* respond to resource heterogeneity?
(c) Is the intensity of intraspecific competition of *B. yagara* affected by resource heterogeneity?

## MATERIALS AND METHODS

### The Species

*Bolboschoenus yagara* (Ohwi) is a perennial clonal herb in the Cyperaceae family; this species develops underground rhizomes that terminate in a globose tuber (Board, 2010; Hroudová et al., 2014). Plants of this species occur in wet habitats such as swamps and wetlands and are distributed mainly in the northeastern, northwestern and southwestern regions of China (Board, 2010).

## Experimental Design

On January 5, 2015, corms of *B. yagara* were obtained from mono-populations in a riparian area of Liangzi Lake, Hubei Province, China (30°05′–30°18′N, 114°21′–114°39′E). The corms were sprouted in sandy clay before the experiment setup. On April 1, 2015, 312 morphologically identical plants (without branches, height: approximately 12 cm; corm diameter: 0.91 ± 0.02 cm) were selected for the experiment described below, and 30 plants were randomly selected to measure their initial dry biomass (initial biomass: mean ± *SE*, 0.41 ± 0.02 g; corm biomass: 0.29 ± 0.02 g). The experiment involved a three-way factorial design. The first factor involved the pattern of water supply: homogeneous (800 ml of water daily) or heterogeneous (4 L of water every 5 days) water was supplied to each container, and the total amount of water provided was kept constant throughout the experimental period. Eight hundred milliliters equated to soil saturation, as measured by a soil moisture probe (SIN-TN8, Hangzhou, Liance Instrument, China). The environmental parameters of the water were as follows: total nitrogen (TN) concentration = 0.63 ± 0.009 $mg.L^{-1}$; total phosphorus (TP) concentration = 0.04 ± 0.002 $mg.L^{-1}$; pH = 8.55 ± 0.013; and salinity (SAL) = 0.09 ± 0.002 ppt [mean ± *SE*, measured by a YSI Professional Plus water quality meter (YSI Inc., Yellow Springs, OH, United States)]. The second factor involved the following four plant density treatments: one, two, four, or six plants per container. The third factor involved the substrate type. The first substrate represented the heterogeneous soil treatment. For this treatment, containers (70 cm long × 50 cm wide × 47 cm deep) were divided into four areas (35 cm long × 25 cm wide) (**Figure 1**): two areas were filled with clay (TN = 3.05 ± 0.05 $mg.g^{-1}$; TP = 1.33 ± 0.03 $mg.g^{-1}$; organic matter content = 60.67 ± 1.01 $mg.g^{-1}$), and other two were completely filled with sand (TN = 0.02 ± 0.002 $mg.g^{-1}$; TP = 0.25 ± 0.011 $mg.g^{-1}$; organic matter content = 0.75 ± 0.02 $mg.g^{-1}$) [mean ± *SE*, measured by a Flash 2000 Organic Elemental Analyzer (Thermo Fisher Scientific Inc., United States), IL500 TP Automatic Analyzer (Hach Corp., Loveland, CO, United States), and a Multiwave 3000 device (Anton Paar Corp., Austria)]. The second substrate represented the homogeneous soil treatment. For this treatment, containers were filled with the same soil type (comprised of equal volumes of clay and sand), after which the soils were completely homogenized. The total concentration of soil nutrients was the same in all treatments. Therefore, 16 treatment combinations (two water supply patterns × two soil nutrition distribution types × four plant densities) existed, and each combination was replicated 8 times. The mean temperature and mean humidity in the greenhouse were 25.34 ± 2.55°C and 64.67 ± 5.02% (mean ± *SE*), respectively. The experiment lasted for 70 days (duration of the pattern of water supply)—from April 2nd to June 10th 2015.

## Harvest and Measurements

The soil moisture (volumetric water content) and temperature were recorded with a soil moisture probe (SIN-TN8, Hangzhou, Liance Instrument, China) during the experimental period. The measurements were carried out daily before watering. To test for differences in the temporal heterogeneity of soil moisture between the two watering heterogeneity treatments, the soil moisture ($M_m$) values, soil moisture minimum ($M_{min}$) values

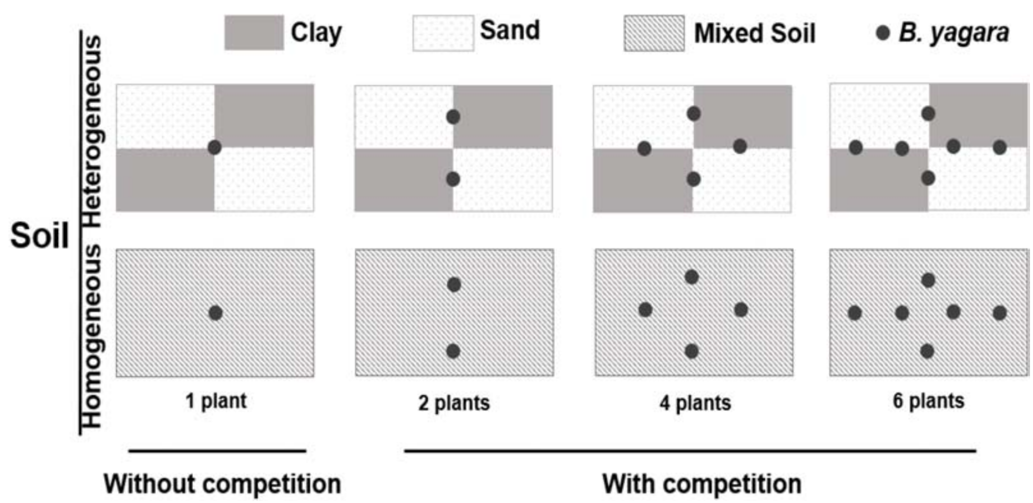


**FIGURE 1 |** Experimental design. The experiment consisted of three factors. The first factor involved two patterns of water supply: a homogeneous (800 ml of water daily) and a heterogeneous (4 L of water every 5 days) water supply, with the total amount of water provided to each container kept constant throughout the experimental period. The second factor involved the intraspecific competition treatments: without competition (one plant per container) and with competition (two, four, or six plants per container). The third factor involved the substrate type. The first substrate represented the heterogeneous soil treatment, in which the containers were divided into four areas: two areas were filled with clay, and the other two were filled with true sand. The second substrate represented the homogeneous soil treatment, in which the containers were filled with the same completely homogeneous soil type. The total amount of soil nutrients was the same in all treatments.

and soil moisture maximum ($M_{max}$) values were measured and recorded, and the temporal mean value of the relative soil moisture content was calculated (James et al., 2003; Hagiwara et al., 2010).

$$\text{Relative soil moisture} = (M_m - M_{min})/(M_{max} - M_{min})$$

We used one-way ANOVA to test the effects of the water heterogeneity treatment on both the temporal variability in the relative soil moisture content and the mean variance during the 5-day cycle in different water treatments. The mean value of the relative soil moisture was not affected by water temporal heterogeneity ($F$ = 1.215, $P$ = 0.275); however, the variance in the relative soil moisture during the 5-day cycle was significantly different between the water homogeneity treatments and the water heterogeneity treatments ($F$ = 38.626, $P < 0.001$).

At harvest, plant height, fresh weight, rhizome length, ramet number, corm number and corm diameter were measured and recorded. The *B. yagara* material was subsequently divided into aboveground (leaves and stems above the soil surface) and underground parts (roots, corms and rhizomes). All the separated parts were oven-dried at 70°C for at least 3 days to obtain dry weights. To further calculate the performance of *B. yagara* at different densities, the root-to-shoot (R/S) ratios were calculated (Perez-Harguindeguy et al., 2013) as:

$$\frac{R}{S}\,\text{ratio}(g.g^{-1}) = \frac{\text{underground (root mass + corm mass + rhizome mass)}}{\text{aboveground (leaf mass + stem mass)}}$$

The effects of soil nutrient distribution and watering regimen on the intensity of intraspecific competition were calculated by the log response ratio (LnRR) of biomass (Armas et al., 2004).

$$\text{LnRR} = \ln\left(\frac{\text{Bmono}}{\text{Bmix}}\right)$$

$B_{mono}$ represents the total biomass in the absence of competition (i.e., solitary plant density treatment), and $B_{mix}$ represents the average biomass of a plant per container in the presence of competition (i.e., multidensity treatment). The LnRR for each plant density treatment (2, 4, and 6) are calculated separately. The LnRR values are symmetrical around zero, and no ceiling is imposed on the maximum possible competition intensity (Goldberg et al., 1999; Weigelt and Jolliffe, 2003).

## Data Analysis

We measured biomass and morphological traits and calculated the R/S ratio and LnRR on a per-initial-plant basis for each container. All data was transformed by $\log_{10}$ prior to analysis to meet the requirements for homoscedasticity and normality. The treatment effects on plant height, corm number, corm diameter, rhizome length, ramet number, total mass, the R/S ratio and the LnRR were analyzed via a three-way ANOVA. One-way ANOVA in conjunction with Duncan's ($P < 0.05$) test for *post hoc* comparisons was used to investigate the differences in biomass and morphological traits as well as in the R/S ratio, and the LnRR between the soil nutrient heterogeneity and the heterogeneity of water supply combinations at each plant density. To investigate the treatment effects on the intensity of competition, the LnRR was analyzed via a three-way ANOVA at each density. All of the analyses were conducted using SPSS 22.0 (SPSS, Chicago, IL, United States).

# RESULTS

## Biomass and Biomass Allocation

Both soil nutrient treatment ($P$ = <0.001) and plant density ($P$ = <0.001) significantly affected biomass, whereas water supply treatment ($P$ = 0.351) did not (**Table 1**). The interactive effects between soil nutrient treatment and plant density ($P$ = <0.001), and between water supply treatment, soil nutrient treatment and plant density significantly ($P$ = <0.001) affected biomass (**Table 1**). The biomass was 37.5–55% larger under the homogeneous soil nutrient distribution than under the heterogeneous soil nutrient distribution in the one-, two- and four-plant density treatments, while the six-plant density treatment exhibited opposite results (**Figure 2**). The R/S ratio was significantly affected only by the density treatment (**Table 1**). The biomass allocation was not affected by soil

**TABLE 1** | Three-way ANOVAs of the effects of water heterogeneity (W), soil heterogeneity (S), and plant density (D) and their interaction on biomass, the R/S ratio, corm number and corm diameter of *B. yagara*.

| | Biomass (g) | | | R/S (g.g$^{-1}$) | | | Corm number | | | Corm diameter (cm) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **d.f.** | ***F*** | ***P*** | **d.f.** | ***F*** | ***P*** | **d.f.** | ***F*** | ***P*** | **d.f.** | ***F*** | ***P*** |
| Water | 1.80 | 0.881 | 0.351 | 1.80 | 0.105 | 0.747 | 1.80 | 0.551 | 0.460 | 1.80 | 5.334 | **0.023** |
| Soil | 1.80 | 36.000 | **<0.001** | 1.80 | 0.632 | 0.429 | 1.80 | 7.834 | **0.006** | 1.80 | 0.915 | 0.342 |
| Density | 3.80 | 1059.788 | **<0.001** | 3.80 | 32.325 | **<0.001** | 3.80 | 159.783 | **<0.001** | 3.80 | 196.861 | **<0.001** |
| W × S | 1.80 | 1.883 | 0.174 | 1.80 | 0.998 | 0.321 | 1.80 | 0.303 | 0.583 | 1.80 | 0.040 | 0.841 |
| W × D | 3.80 | 2.222 | 0.092 | 3.80 | 0.725 | 0.540 | 3.80 | 3.113 | **0.031** | 3.80 | 13.202 | **<0.001** |
| D × S | 3.80 | 29.590 | **<0.001** | 3.80 | 4.242 | **0.008** | 3.80 | 7.755 | **<0.001** | 3.80 | 6.071 | **0.001** |
| W × S × D | 3.80 | 9.462 | **<0.001** | 3.80 | 1.675 | 0.179 | 3.80 | 1.091 | 0.358 | 3.80 | 2.740 | **0.049** |

*Significant P-values are presented in bold.*

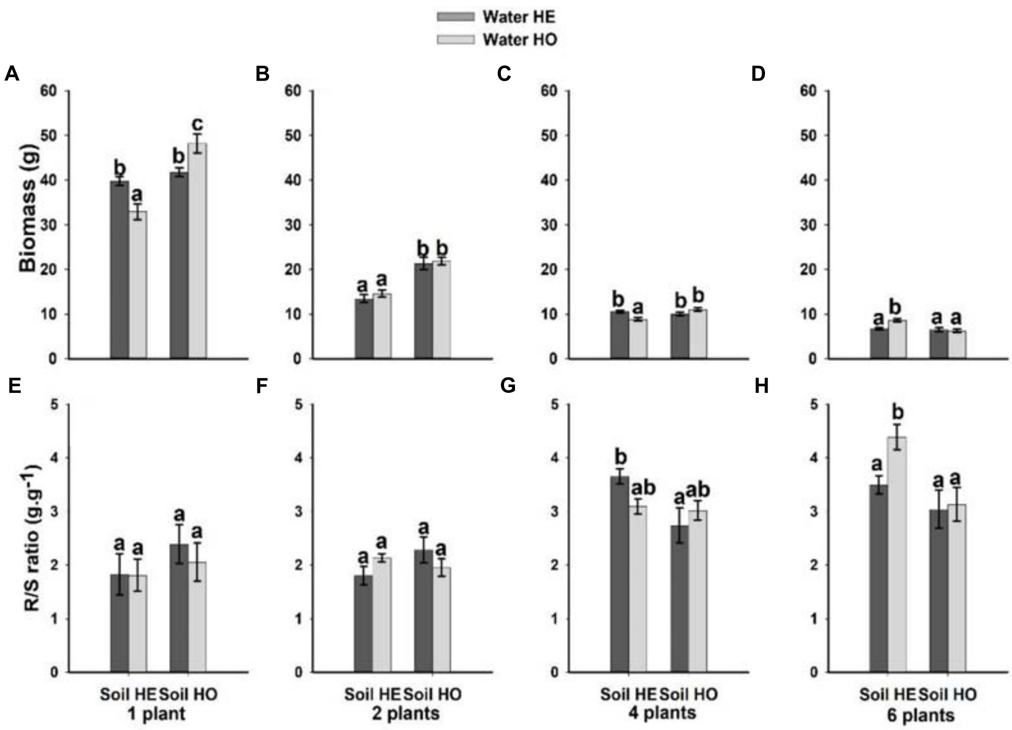


**FIGURE 2 |** Effects of the heterogeneity of water supply and soil nutrients on the biomass **(A–D)** and R/S ratio **(E–H)** (±SE) of *Bolboschoenus yagara* at each plant density.

nutrient heterogeneity at low plant densities (**Figures 2E,F**). However, the R/S ratios in the six-plant density treatments were greater under soil nutrient heterogeneity than under the soil nutrient homogeneity (**Figures 2G,H**). Also, there was no significant effect of water supply heterogeneity on plant biomass (**Table 1**).

## Morphological Traits

Compared with the water supply treatment the soil nutrient treatment significantly affected the morphological traits of *B. yagara* at low plant densities, although plant height and rhizome length were unaffected (**Table 2** and **Figures 3**, **4**). There were significant interactive effects between water supply treatment, soil nutrient treatment and plant density on corm diameter (**Table 1**). For example, compared with the soil nutrient heterogeneity treatment, the soil nutrient homogeneity treatment significantly increased the corm number, corm diameter and ramet number at low plant densities (**Figures 3A,B,F**, **4E,F**). However, compared with the soil nutrient treatment, the water supply treatment significantly affected the morphological traits of *B. yagara* at high plant densities (**Tables 1**, **2**). For example, with the exception of plant height, compared with the homogeneous water supply treatment, the heterogeneous water supply treatment significantly increased the corm

**TABLE 2 |** Three-way ANOVAs of the effects of water heterogeneity (W), soil heterogeneity (S), and plant density (D) and their interaction on rhizome length, plant height, ramet number, and LnRR of *B. yagara*.

| | Rhizome length (cm) | | | Plant height (cm) | | | Ramet number | | | LnRR | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | d.f. | *F* | *P* | d.f. | *F* | *P* | d.f. | *F* | *P* | d.f. | *F* | *P* |
| Water | 1.80 | 2.399 | 0.125 | 1.80 | 3.806 | 0.055 | 1.80 | 5.593 | **0.020** | 1.80 | 1.820 | 0.182 |
| Soil | 1.80 | 0.780 | 0.380 | 1.80 | 4.827 | **0.031** | 1.80 | 13.119 | **0.001** | 1.80 | 16.429 | **<0.001** |
| Density | 3.80 | 520.744 | **<0.001** | 3.80 | 509.556 | **<0.001** | 3.80 | 219.411 | **<0.001** | 3.80 | 346.382 | **<0.001** |
| W × S | 1.80 | 0.176 | 0.676 | 1.80 | 3.052 | 0.084 | 1.80 | 0.560 | 0.456 | 1.80 | 0.202 | 0.655 |
| W × D | 3.80 | 10.309 | **<0.001** | 3.80 | 1.922 | 0.133 | 3.80 | 5.566 | **0.002** | 3.80 | 2.335 | 0.106 |
| D × S | 3.80 | 2.419 | 0.072 | 3.80 | 0.828 | 0.482 | 3.80 | 6.142 | **0.001** | 3.80 | 38.437 | **<0.001** |
| W × S × D | 3.80 | 0.791 | 0.502 | 3.80 | 0.656 | 0.582 | 3.80 | 0.848 | 0.472 | 3.80 | 7.808 | **0.001** |

*Significant P-values are presented in bold.*

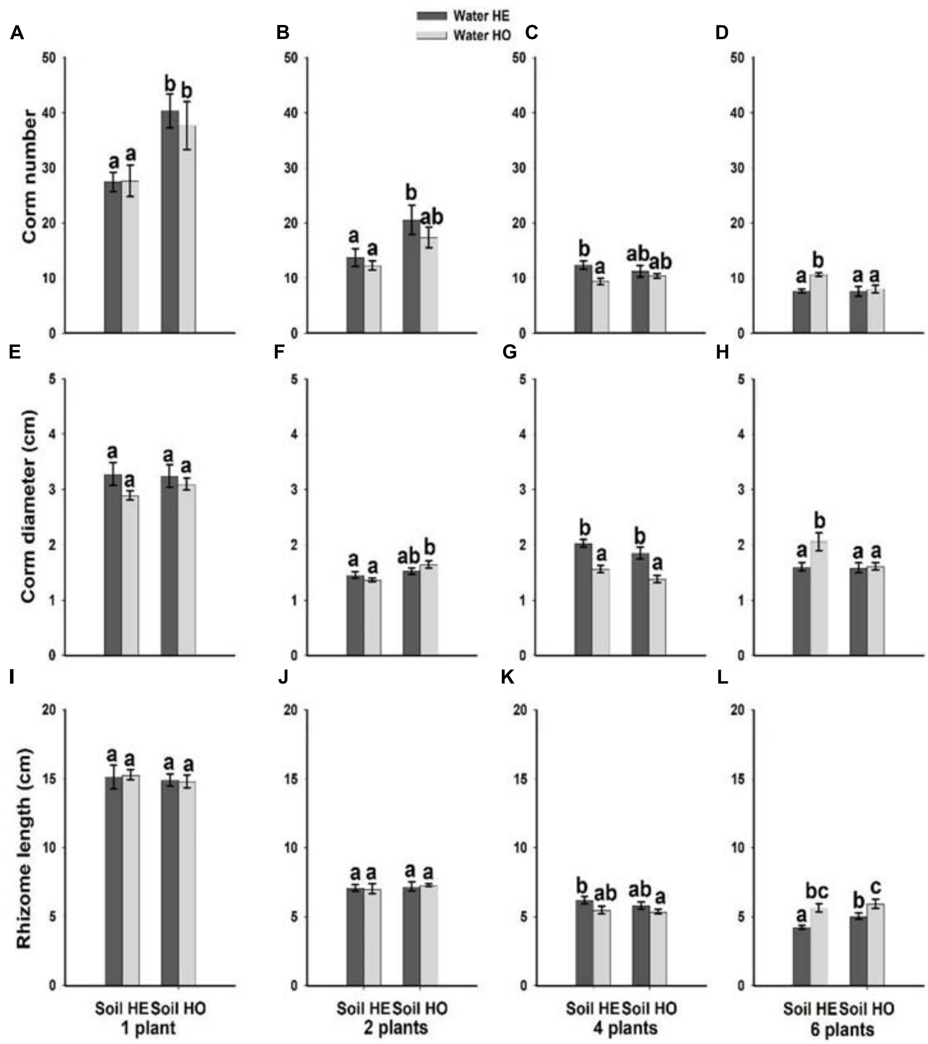


**FIGURE 3 |** Effects of the heterogeneity of water supply and soil nutrients on the corm number **(A–D)**, corm diameter **(E–H)** and rhizome length **(I–L)** (±SE) of *B. yagara* at each plant density.

number, corm diameter, rhizome length and ramet number of *B. yagara* at the four-plant density; however, compared with the heterogeneous water supply treatment, the homogeneous water supply treatment significantly increased the corm number, corm diameter, rhizome length, plant height, and ramet number of *B. yagara* at the six-plant density (**Figures 3C,D,G,H,I**, **4D,G,H**).

## Intensity of Competition

Compared with the water supply treatment, the soil nutrient treatment significantly affected the LnRR (**Table 2**). The interactive effects between water supply treatment, the soil nutrient treatment and plant density significantly affected the LnRR (**Table 2**). Compared with the soil nutrient homogeneity treatment, the soil nutrient heterogeneity treatment significantly increased the LnRR of the biomass at the two- and four-plant densities (**Figures 5A,B**). However, the opposite results occurred at the highest, six-plant density treatment (**Figure 5C**). These results mean that competition was more severe as plant density increased and was significantly and more strongly affected by the soil substrate heterogeneity than by the water supply heterogeneity.

# DISCUSSION

## Biomass and Biomass Allocation

The results of several previous experiments have shown that some plant species accumulate greater biomass under heterogeneous conditions than under homogeneous conditions, given the same total concentration of available nutrients (Hutchings and Wijesinghe, 2008; García-Palacios et al., 2011). However, in the present experiment, plant biomass was greater in the homogeneous soil nutrient treatment than in the heterogeneous soil nutrient treatment (**Figures 2A–H** and **Table 1**). These

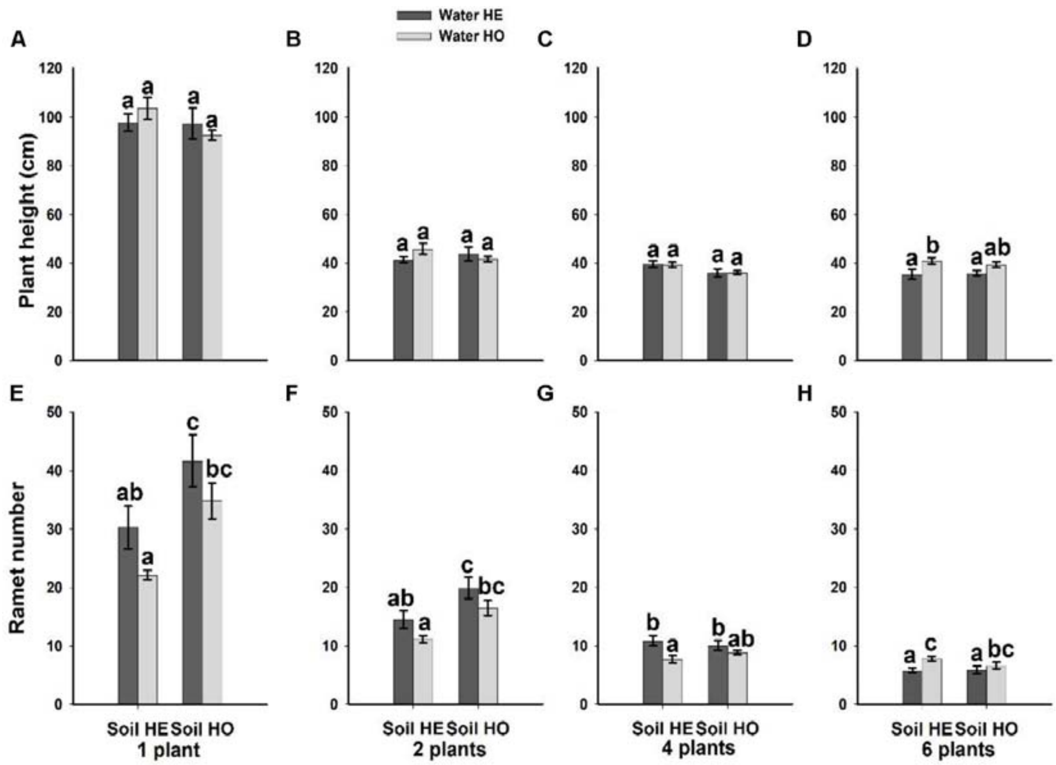


**FIGURE 4 |** Effects of the heterogeneity of water supply and soil nutrients on the plant height **(A–D)** and ramet number **(E–H)** (±SE) of *B. yagara* at each plant density.

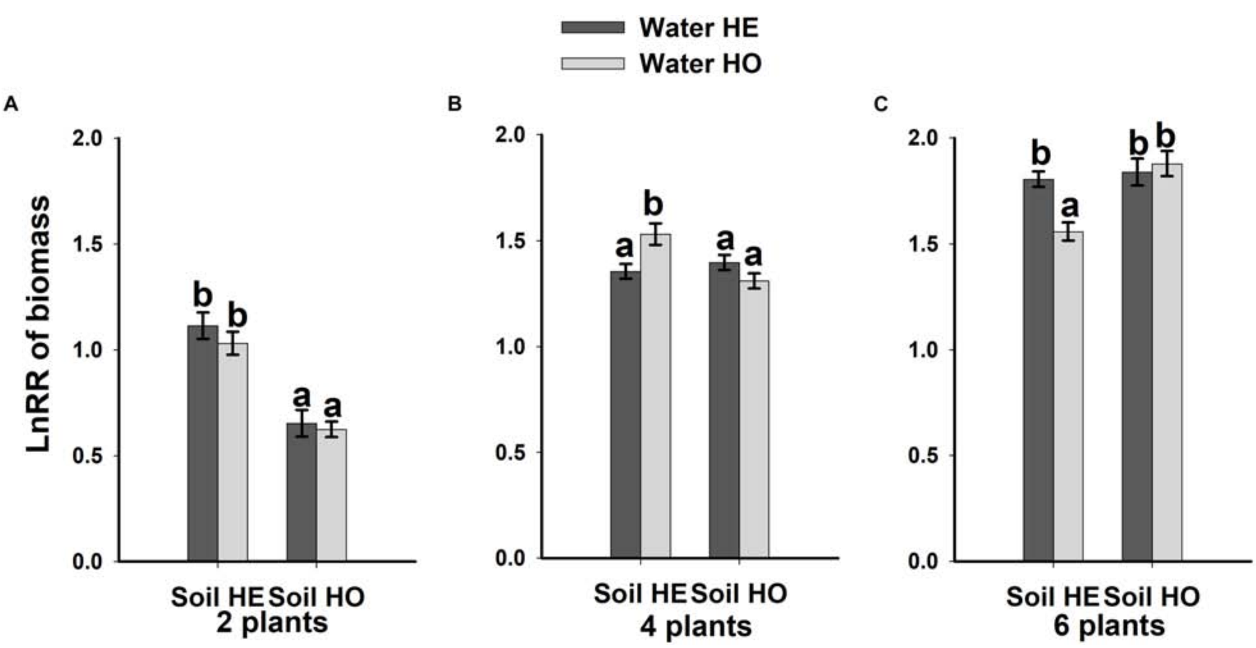


**FIGURE 5 |** Competitive intensity as measured by the log response ratio (LnRR) of the biomass **(A–C)** (±SE) of *B. yagara* in response to heterogeneity of water supply and soil nutrients.

patterns may result from the homogeneous conditions in which the nutrients are evenly distributed, which is therefore more conducive to plant growth (Maestre and Reynolds, 2007; Dong et al., 2012; Hagiwara et al., 2012), and partly due to physiological integration that allows ramets to share resources with other ramets (Dong et al., 2015). Plant density significantly affected the growth of *B. yagara*, as the average biomass of the plants gradually decreased as the density increased (**Figures 2A–D** and **Table 1**). These findings indicated that both the existence of intraspecific competition among plants and the competition intensity increased as the plant density increased, because of both the density-dependent effect attributable to increased numbers of competitors and the increased effect of competition on individuals in single-species plant populations (Antonovics and Levin, 1980; Watkinson, 1980).

Plants grow larger under a more homogeneous water supply than under a more heterogeneous water supply because they can take up water more consistently under homogeneous conditions that the low variability in resources availability, thereby allowing the plants to increase their growth performance continuously (Novoplansky and Goldberg, 2001; Hagiwara et al., 2010, 2012). In the present study, compared with the heterogeneous conditions, the homogeneous of water supply clearly led to more plant biomass when the plants grew in isolation (**Figure 2A**). In addition, we found that the heterogeneous of water supply did not affect *B. yagara* biomass accumulation, which may be because *B. yagara* is more sensitive to soil nutrients treatment than to water supply treatment.

Previous experiments have shown that some plant species employ morphological specialization and physiological responses to heterogeneity to place more nutrient-absorbing organs (e.g., roots or ramets) in nutrient-rich areas to forage efficiently for heterogeneously distributed nutrients (de Kroon et al., 2005; Gao et al., 2012). However, the R/S ratio of *B. yagara* increased only at higher plant densities under soil nutrient heterogeneity treatment (**Figures 2G,H** and **Table 1**). As the density increased, the plants were more likely to encounter resources deficits. Thus plants invested more in underground part rather than aboveground part to acquire resources (Day et al., 2003b; Hagiwara et al., 2010, 2012; van der Waal et al., 2011).

## Morphological Traits

Soil space decreased as the planting density increased, which caused the effect of soil nutrient heterogeneity to gradually diminish. Thus, *B. yagara* is more sensitive to water deficit at high plant density, and plants can alter their morphological characteristics according to the external environment. For example, plants have been shown to alter the length and angle of their spacers (includes stolon, rhizomes and corms, etc.), and their number and distribution of ramets (Hutchings et al., 2003; de Kroon et al., 2005; Gao et al., 2012).

Owing to high morphological plasticity, clonal plants generally respond positively to resource heterogeneity (Eilts et al., 2011; Zhou et al., 2012; Liu et al., 2017). For example, except at the 4-plant density, the *B. yagara* plants in the present study responded positively to resource homogeneity. That's probably because the low variability in resource availability under the homogeneous conditions allowed the plants to absorb resources steadily, effectively improving their growth performance (Saeed and El-Nadi, 1998; Novoplansky and Goldberg, 2001; Maestre and Reynolds, 2007; Dong et al., 2012; Hagiwara et al., 2012). Overall, these results are consistent with other experiments that positive foraging responses to resource heterogeneity may not always be adaptive (Roiloa and Retuerto, 2006; Dong et al., 2015) and may be temporary (Day et al., 2003a,b).

## Intensity of Competition

Our results demonstrated that, compared with the heterogeneity of water supply, soil nutrient heterogeneity significantly affected the intraspecific competition of *B. yagara*. For example, soil nutrient heterogeneity increased the intraspecific competition at the two- and four-plant densities (**Figures 5A,B**). One explanation is that, to efficiently take up heterogeneously distributed resources, clonal plants place more nutrient-absorbing organs in nutrient-rich areas in heterogeneous environments. Also, the roots of neighboring plants would proliferate in nutrient-rich areas (de Kroon et al., 2005; Gao et al., 2012), thus competition becomes more severe under heterogeneous soil nutrient conditions than under homogeneous ones (Fransen et al., 2001; Day et al., 2003b). However, in the present study, heterogeneous soil nutrient conditions had no effect on the intensity of intraspecific competition at the six-plant density under a heterogeneous water supply. Other experiments have also shown that soil nutrient heterogeneity does not alter intraspecific competition at the container level for *Poa pratensis* (Maestre et al., 2006), *Achillea millefolium* (Rajaniemi, 2011), *A. philoxeroides* (Zhou et al., 2012) or *H. vulgaris* (Dong et al., 2015). These results may have been observed because resource heterogeneity can significantly affect plant competition when individuals are not genetically identical (Day et al., 2003b; Zhou et al., 2012). Other may be due to high resource depletion rate in the high density population, the nutrient-rich patches might gradually decline to the same level of suitability as the nutrient-poor patches, and then lead to high density population less sensitive response to soil nutrient heterogeneity (Roiloa and Retuerto, 2006; Dong et al., 2015). Thus, heterogeneity in soil nutrient availability has different effects on the intensity of intraspecific competition of *B. yagara* at different densities.

# CONCLUSION

We found that plants respond differently to environmental heterogeneity with respect to the supply of two covariable resources at different plant densities. The soil nutrient treatment significantly influenced the biomass and intraspecific competition of *B. yagara*. However, only the water supply treatment influenced the morphological traits of *B. yagara* at high plant densities, and heterogeneity of water supply had little impact on intraspecific competition. In addition, the interactive effect of soil nutrient heterogeneity and heterogeneity of water supply had no significant effect on the growth performance and competition relationship of *B. yagara*. Therefore, *B. yagara* was more sensitive to soil nutrient heterogeneity than to heterogeneity of water supply. Spatial or temporal heterogeneity in soil nutrient distribution and water supply patterns may be highly important with respect to the growth performance and population structure of clonal plants (Hutchings et al., 2003; Wang T. et al., 2016; Wang Y.J. et al., 2016; You et al., 2016). The ecological effects of resource heterogeneity should be investigated further due to various ecological factors (temperature, light, and humidity) that affect the growth performance of clonal plants. In addition, we should investigate how pulses of resource availability influence growth performance at individual, population, and community levels, because resource pulses provides opportunities to understand the dynamics of natural systems.

## AUTHOR CONTRIBUTIONS

CL and DY designed the experiment and edited the manuscript text. HY and NS performed the experiment. HY and NS wrote the manuscript text and executed the technical assays and statistical analysis. All authors reviewed the manuscript.

## FUNDING

The authors gratefully acknowledge funding support from the Special Foundation of National Science and Technology Basic Research (2013FY112300) and the Major Science and Technology Program for Water Pollution Control and Treatment (2015ZX07503-005).

## ACKNOWLEDGMENTS

We greatly appreciate the assistance of Y. Wang and L. G. Wang for the valuable work in the field and the laboratory.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling Editor is currently co-organizing a Research Topic with one of the authors CL and confirms the absence of any other collaboration.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 20 February 2019
doi: 10.3389/fpls.2019.00169

# Littoral Slope, Water Depth and Alternative Response Strategies to Light Attenuation Shape the Distribution of Submerged Macrophytes in a Mesotrophic Lake

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**Specialty section:**
This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science

**Received:** 29 June 2018
**Accepted:** 31 January 2019
**Published:** 20 February 2019

**Citation:**
He L, Zhu T, Wu Y, Li W, Zhang H, Zhang X, Cao T, Ni L and Hilt S (2019) Littoral Slope, Water Depth and Alternative Response Strategies to Light Attenuation Shape the Distribution of Submerged Macrophytes in a Mesotrophic Lake. Front. Plant Sci. 10:169. doi: 10.3389/fpls.2019.00169

Light is a major limiting resource in aquatic ecosystems and numerous studies have investigated the response of submerged macrophytes to low light conditions. However, few studies have tested whether different light response strategies can also have consequences for macrophyte distribution along different littoral slopes in lakes, which are known to affect macrophyte biomass due to differences in drag forces and sediment characteristic. In this study, we tested (1) whether two macrophyte species of different growth forms (canopy-forming: *Potamogeton maackianus,* rosette-type: *Vallisneria natans*) differ in their response strategies to low light conditions and (2) how these responses influence their distribution along different basin slopes in the mesotrophic Lake Erhai, China. We hypothesized that the canopy-forming species responds to low light conditions at deeper sites by stem elongation while the rosette-type species increases its shoot chlorophyll content. As a consequence, *P. maackianus* should have a higher susceptibility to drag forces and thus prevail at sites with lower slopes. Sites with higher slopes should offer a niche for rosette-type species like *V. natans* that can better withstand drag forces. We surveyed the distribution and abundance of the two macrophyte species at 527 sampling points along 97 transects in Lake Erhai and measured their height, leaf and stem/rhizome biomass, and leaf chlorophyll *a* content at different water depths. Our results confirmed stem elongation as a strategy to low light conditions by the canopy-forming species *P. maackianus*, while *V. natans* produced more chlorophyll *a* per shoot biomass at deeper sites to tolerate shading. As hypothesized, these alternative response strategies to low light conditions resulted in a trade-off regarding the plants ability to grow at different basin slopes. *P. maackianus* was dominant at sites with low-moderate slope (0–4%) and low-moderate water depth (2–4 m), while sites with high basin slope (4–7%) combined with moderate-high water depth (3–5 m) were dominantly colonized by *V. natans*. The latter habitat thus represents a potential refuge for rosette-type macrophyte species that are often outcompeted when shading increases during eutrophication.

**Keywords: submerged macrophyte, littoral slope, water depth, response strategies, species distribution**

## INTRODUCTION

Light limitation has driven the evolution of highly plastic adaptive strategies in plants to either tolerance or avoidance of shading by neighboring vegetation (Franklin, 2008). In freshwater ecosystems, roughly 10% of the global radiation is reflected at the air-water interface and a significant portion is attenuated with depth resulting in low light conditions in most deep aquatic habitats (Spence, 1981). Consequently, numerous aquatic plants have evolved a high plasticity in morphological and physiological traits in response to low light conditions (Maberly, 1993; Olesen et al., 2002). Previous studies showed that the response strategy of submerged macrophytes to low light conditions largely depends on their growth form (Chambers and Kalff, 1987; Fu et al., 2012). Canopy-forming species, such as *Myriophyllum spicatum* and *Potamogeton wrightii*, tend to allocate more biomass to stems, grow taller and form dense canopies to counter light attenuation in the water column (Strand and Weisner, 2001; Fu et al., 2012). In contrast, rosette-type macrophytes such as *Vallisneria* species can increase their plant height in deeper sites as well (Fu et al., 2012), but mainly tolerate shading through a lower light compensation point and a higher leaf mass ratio of total plant mass compared to canopy-forming macrophytes (Su et al., 2004; Chen et al., 2016).

Increasing eutrophication of lakes leads to increased shading of submerged macrophytes by phytoplankton and periphyton. As a consequence, small species such as charophytes and rosette-type angiosperms disappear and tall, canopy-forming macrophytes that can escape low light conditions become dominant (Sand-Jensen et al., 2008; He et al., 2015; Hilt et al., 2018). However, other factors such as morphometric characteristics of the littoral area additionally may influence the biomass and community structure of submerged macrophytes in lakes (Kolada, 2014). Littoral slope for example was suggested as a good predictor of the maximum biomass of submerged macrophyte communities (Duarte and Kalff, 1986). Low slope areas usually have fine, nutrient-rich sediments and little water movement, while coarse, nutrient-poor sediments and stronger water movement characterize high slope areas in temperate lakes (Håkanson, 1977; Duarte and Kalff, 1986). In aquatic environments, hydrodynamic forces caused by water movement can be many times the drag forces produced by wind on land (Puijalon et al., 2011). Currents and waves can cause strong damage to or even uproot submerged plants (Bornette and Puijalon, 2011). However, the degree of damage depends on the sediment characteristic (Barko and Smart, 1986; Schutten et al., 2005; Spierenburg et al., 2013) and the macrophyte's ability to resist breakage and uprooting, which is closely related to its size and shape (Schutten and Davy, 2000; Schutten et al., 2004). Generally, short rosettes with linear soft leaves are better at resisting drag forces compared to tall canopy-forming submerged macrophytes at a given water velocity (Puijalon et al., 2011). Hence, short rosette-type species may be better adapted to disturbance by water movement. In addition, canopy-forming submerged macrophytes need more nutrients to support their high growth rates, while short rosette-type species grow slowly and can survive under lower nutrient supply (Chambers, 1987). Therefore, the canopy-forming species may dominate in low slope areas of mesotrophic lakes, while rosette-type species can better tolerate the stress of higher slopes.

Although numerous studies have investigated the response of macrophytes to low light conditions, only a few of them have tested whether these response strategies to shading conditions affect the distribution of macrophytes along other environmental gradients such as sediment characteristic and drag forces (Spierenburg et al., 2013). In this study, we measured different morphological and physiological parameters and the abundance of two dominant macrophyte species, the canopy-forming *P. maackianus* and the rosette-type species *V. natans* at different water depths (determining light availability) and littoral slopes (determining sediment characteristic and drag forces) in the mesotrophic Lake Erhai, China. We tested (1) whether the two species show the response strategies to low light conditions typical for canopy- and rosette-type macrophytes and (2) whether these responses influence their distribution along different basin slopes. We hypothesized that the canopy-forming species *P. maackianus* responds to low light conditions in deeper water by stem elongation while *V. natans* increases its shoot chlorophyll content. As a consequence, *P. maackianus* should have a higher susceptibility to drag forces and thus prevail at sites with lower slopes. In contrast, the rosette-type species *V. natans* should tolerate higher drag forces and thus be prevalent at sites with higher slopes.

## MATERIALS AND METHODS

### Study Location and Macrophyte Species

The study was carried out in the mesotrophic Lake Erhai (25°52′N, 100°06′E), located in the Yunnan province of China (**Figure 1**). The lake has a total area of 249 $km^2$, a moderate water depth (maximum depth 20.5 m, mean depth 10.5 m) and a large variation in littoral slopes. Macrophytes show a zonation along the water depth gradient, with most of the 12

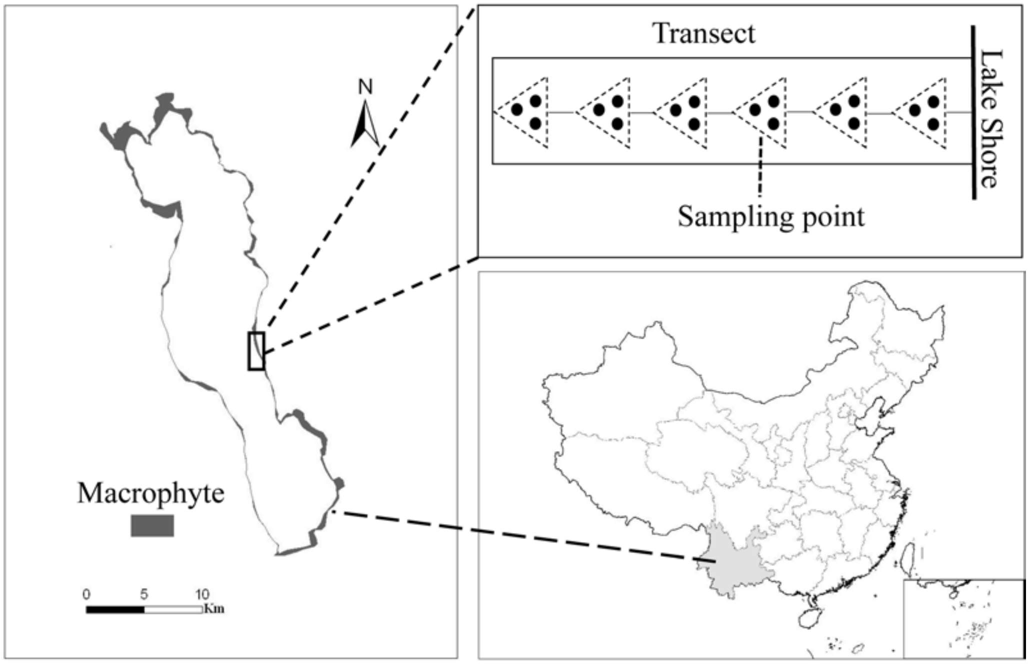


**FIGURE 1 |** Macrophyte coverage in Lake Erhai (left), location of the lake in China (below) and sampling scheme at each transect (above). The small rectangle at the bottom right shows the South China Sea Islands.

submerged species inhabiting shallow water (0–3.0 m depth), and only a few species extending to deeper water (Fu et al., 2014). Maximum colonization depth of submerged macrophytes was around 5.5 m and about 20 $km^2$ of the lake littoral area was covered by submerged macrophytes (**Figure 1**) and the community was dominated by canopy-forming *P. maackianus* and rosette-type *V. natans* in 2013. *P. maackianus* is a clonal and perennial submerged macrophyte widely distributed in East Asia (Sun, 1992), and forms dense canopies and monocultures in many mesotrophic shallow lakes (Ni, 2001). *V. natans* is a common rosette-type submerged macrophyte species in China. It mainly spreads by clonal reproduction and its aboveground part can overwinter in Lake Erhai.

## Submerged Macrophytes Sampling and Trait Measurements

Submerged macrophyte biomass was collected at 97 transects uniformly distributed perpendicular to the shoreline around Lake Erhai from 16 July to 6 August 2013. Samples were taken at intervals of 1 m water depths along each transect from the shore to the deepest site of macrophyte occurrence (i.e., from around 0.5 m to 5.5 m water depth) (**Figure 1**). Depending on the macrophyte colonization depth, this resulted in 5 to 6 samples per transect, and in total 527 sampling points. A reaping hook covering a bottom area of 0.2 $m^2$ was used to collect submerged macrophytes, with 3 replicates at each sampling point (**Figure 1**). Plants were washed, sorted by species, and weighed as fresh biomass (FW). GPS coordinates of each sampling point were recorded by a GPS receiver (GPS map 60CSx).

We randomly sampled around 20 full-grown healthy individuals of *P. maackianus* or *V. natans* at each sampling point when the community was dominated by one of these two species and all plant samples were kept in dark containers with ice bags during field work. Three individuals from each sampling point were immediately put into a freezer at −20°C when returning to the laboratory for measuring leaf chlorophyll (chl) *a* content. Part of the mixed freeze-dried leaves from each individual were ground in a mortar and extracted in 96% ethanol for 24 h at 4°C in the dark. The solution was then centrifuged at 4000 × *g* for 10 min, and the chl *a* content determined spectrophotometrically at 665 nm and 649 nm. Plant heights of the remaining individuals were measured and plants were separated into leaves, stems (for *P. maackianus*) or rhizomes (for *V. natans*) and dried at 80°C for 48 h to determine the dry weight (DW).

## Data Analysis

Shoot (leaf+stem/rhizome) chl *a* content is an important indicator for photosynthetic capacity of submerged macrophytes (Nielsen and Sand-Jensen, 1989). We determined leaf chl *a* contents and then related it to the entire shoot biomass by calculating (leaf chl *a* content) × (leaf dry weight)/(leaf dry weight + stem or rhizome dry weight). We used *t*-tests to compare the trait values between the two species and linear regression analysis to evaluate the response of different plant traits to water depth. Plant trait values at each sampling point were represented by the average of all the individuals in that sampling point (i.e., 20 individuals for shoot mass, leaf mass, stem mass, shoot height and 3 individuals for leaf chl *a* and shoot chl *a* content). When plant traits were significantly correlated with water depth for both species, analysis of covariance (ANCOVA) was used to test for differences in the slope of the regression line between the two species. For traits that were correlated with plant size (i.e., leaf biomass, stem biomass, and shoot height), we additionally analyzed biomass-corrected values. To remove differences in size, we conducted a linear regression of each log-transformed trait on log-transformed shoot biomass. The residual values from this regression were saved because they represent size-independent measures of normalized traits (McCoy et al., 2006). We then used linear regression analysis to evaluate the relationship between the residual values and water depth. In this case, the individual trait values were used for the analysis.

GPS coordinates of each sampling point were used to calculate the distance from the shallowest sampling point to the other sampling points in each transect. Then, the littoral slope of each transect was calculated as follows:

$$\mathrm{D} - \mathrm{D1} = \mathrm{a} \times \mathrm{L} \quad (1)$$

$$\mathrm{Slope} = \mathrm{a} \times 100\% \quad (2)$$

Where D is the water depth of the sampling point (except for the shallowest sampling point); D1 is the water depth of the shallowest sampling point; L is the distance from the sampling point to shallowest sampling point in each transect and a is the coefficient of Equation 1. The proportion of the two target species in the total macrophyte biomass (sum of all species) was calculated for each sampling point. In order to compare our results with an earlier study by Duarte and Kalff (1986) on the influence of littoral slopes on biomass of submerged macrophyte communities, we averaged the total macrophyte biomass of all the sampling points in each transect and then examined the relationship between the averaged biomass and the littoral slope. We used the GAM (Generalized Additive Model) to fit the relationship between slope, water depth and total macrophyte biomass as well as proportion of the target species in total macrophyte biomass. GAM is a semi-parametric extension of generalized linear models that enables the user to fit complex non-linear relationships and handle different types of error distributions (Wood, 2006). The models were built with function "gam" in package "mgcv" using penalized regression splines as the smoothing function, Gaussian error distribution, and automatic calculation of smoothing parameters. The main effects and the interaction of the slope and water depth were included in the models. All analyses were performed in R (R Core Team, 2017).

## RESULTS

### Comparison of Plant Traits Between *P. maackianus* and *V. natans* in Lake Erhai

Most of the plant traits measured were significantly different between *P. maackianus* and *V. natans* (**Table 1**). Shoot biomass of *P. maackianus* (0.54 ± 0.22 g) was lower than that of *V. natans* (0.73 ± 0.36 g), while plant height of *P. macckianus* (187 ± 52 cm) was much higher than that of *V. natans* (115 ± 28 cm). Leaf biomass of *P. maackianus* (0.20 ± 0.14 g) was much lower than that of *V. natans* (0.63 ± 0.2 g). Inversely, the stem biomass of the *P. maackianus* (0.34 ± 0.12 g) was much higher than rhizome biomass of *V. natans* (0.09 g). The leaf chl *a* content of the two species was similar (*V. natans* 7.1 ± 2.0 mg $g^{-1}$, *P. maackianus* 7.2 ± 2.2 mg $g^{-1}$); however, the chl *a* content per shoot dry weight of *P. maackianus* (2.5 ± 0.9 mg $g^{-1}$) was much lower than that of *V. natans* (6.3 ± 2.0 mg $g^{-1}$) (**Table 1**).

### Relationship Between Trait Values and Water Depth of *P. maackianus* and *V. natans*

Plant height of *P. maackianus* were higher than that of *V. natans* at a given water depth (**Figure 2**). Plant height of both species increased linearly with water depth, but the slope of the regression line of *P. maackianus* was steeper than that of *V. natans* ($t = -2.94$, $p < 0.01$). Leaf chl *a* contents were significantly positively correlated with water depth for both *P. maackianus* and *V. natans* (**Table 2**). Shoot chl *a* content of *P. maackianus* was stable with increasing water depth, while shoot chl *a* content of *V. natans* increased strongly. *P. maackianus* allocated less biomass into leaves with increasing water depth while the opposite was true for *V. natans* (**Table 2**).

**TABLE 1** | Comparison between traits values (means ± SD) of *Potamogeton maackianus* and *Vallisneria natans* from Lake Erhai.

| Variable | *P. maackianus* | | *V. natans* | | Significance |
|---|---|---|---|---|---|
| | *n* | Mean ± SD | *n* | Mean ± SD | |
| Shoot biomass (g DW) | 278 | 0.55 ± 0.31 | 519 | 0.73 ± 0.36 | *** |
| Leaf biomass (g DW) | 278 | 0.20 ± 0.19 | 519 | 0.64 ± 0.33 | *** |
| Stem/rhizome biomass (g DW) | 278 | 0.35 ± 0.17 | 519 | 0.09 ± 0.07 | *** |
| Leaf/stem or rhizome biomass ratio | 278 | 0.59 ± 0.40 | 519 | 9.47 ± 5.53 | *** |
| Plant height (cm) | 278 | 187.2 ± 57.1 | 519 | 114.7 ± 33.8 | *** |
| Leaf chlorophyll *a* content (mg $g^{-1}$ DW) | 48 | 7.21 ± 2.20 | 87 | 7.08 ± 1.99 | NS |
| Shoot chlorophyll *a* content (mg $g^{-1}$ DW) | 48 | 2.53 ± 0.90 | 87 | 6.26 ± 1.95 | *** |

*Significance of differences was tested using t-test. DW: dry weight. Significance: *P < 0.05, ** P < 0.01, *** P < 0.001, NS = not significant, n = sample size.*

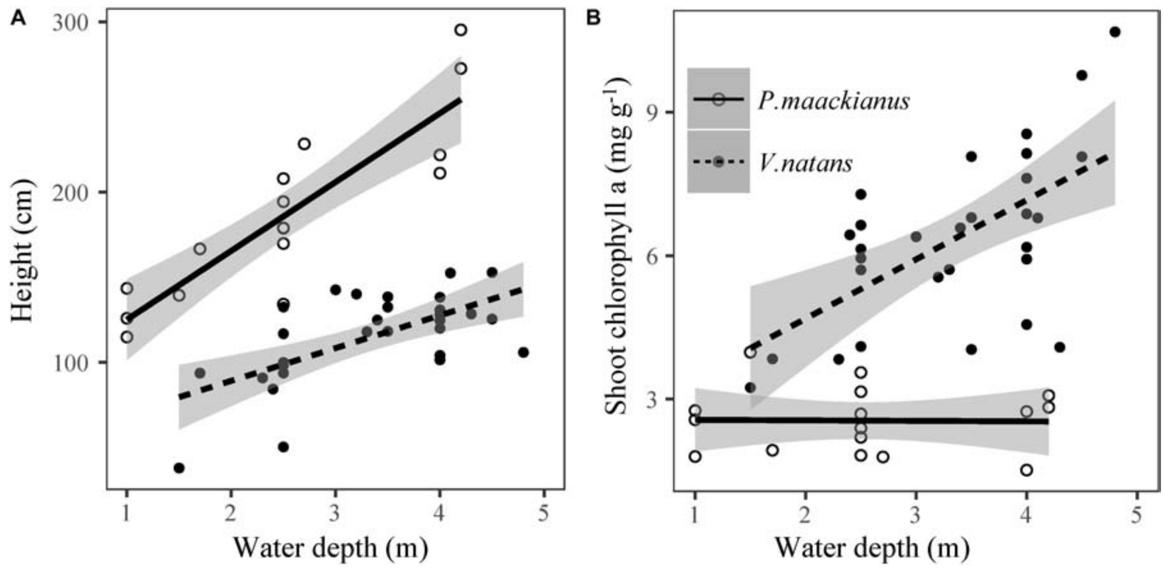


**FIGURE 2 |** Relationship between plant height **(A)** and shoot chlorophyll *a* content **(B)** of *Potamogeton maackianus* and *Vallisneria natans* and water depth in Lake Erhai. The lines are predicted by linear models.

The corrected stem biomass and plant height of *P. maackianus* increased with water depth and the reverse was found for corrected leaf biomass (**Table 2**). While corrected rhizome biomass of *V. natans* decreased with water depth, the reverse was found for leaf mass and height (**Table 2**).

**TABLE 2 |** Relationship between plant traits of *Potamogeton maackianus* and *Vallisneria natans* and water depth in Lake Erhai based on linear models.

| Trait | *P. maackianus* | | | *V. natans* | | |
|---|---|---|---|---|---|---|
| | *n* | $r^2$ | Regression coefficient | *n* | $r^2$ | Regression coefficient |
| Shoot biomass (g DW) | 16 | 0.037 | $-0.035^{NS}$ | 29 | 0.002 | $-0.012^{NS}$ |
| Leaf biomass (g DW) | 16 | 0.146 | $-0.051^{NS}$ | 29 | 0.005 | $0.015^{NS}$ |
| Stem biomass (g DW) | 16 | 0.025 | $0.017^{NS}$ | 29 | 0.240 | −0.027** |
| Plant height (cm) | 16 | 0.751 | 40.391*** | 29 | 0.375 | 19.206*** |
| Corrected leaf biomass | 278 | 0.129 | −0.143*** | 519 | 0.232 | 0.045*** |
| Corrected stem/rhizome biomass | 278 | 0.202 | 0.085*** | 519 | 0.232 | −0.290*** |
| Corrected plant height | 278 | 0.700 | 0.223*** | 519 | 0.380 | 0.213*** |
| Leaf chlorophyll *a* content (mg $g^{-1}$ DW) | 16 | 0.135 | 1.015* | 29 | 0.319 | 1.729*** |
| Shoot chlorophyll *a* content (mg $g^{-1}$ DW) | 16 | 0.000 | $-0.011^{NS}$ | 29 | 0.347 | 1.240*** |

*Significance: *P < 0.05, **P < 0.01, ***P < 0.001, NS = not significant, n = sample size,* $r^2$ *= coefficient of determination, DW = dry weight.*

**TABLE 3 |** Results of GAMs.

| Model parameter | gam(ATB)∼s(S) | gam(TB)∼s(S)+s(W)+s(S,W) | gam(PP)∼s(S)+s(W)+s(S,W) | gam(PV)∼s(S)+s(W)+s(S,W) |
|---|---|---|---|---|
| Deviance explained % | 52.9 | 40.2 | 20.7 | 26.8 |
| $R^2$ adj. | 0.51 | 0.38 | 0.17 | 0.25 |
| N | 97 | 527 | 527 | 527 |
| Smooth terms F/edf | | | | |
| s(S) | 19.24/3.96 | 3.33/6.51 | 1.05/7.56 | 2.34/1.00 |
| s(W) | - | 5.91/2.67 | 0.00/1.00 | 2.78/1.00 |
| s(S,W) | - | 0.52/5.76 | 2.67/13.84 | 1.60/14.31 |

*ATB, averaged total macrophyte biomass of transect; TB, total macrophyte biomass of sampling point; PP, proportion of Potamogeton maackianus; PV, proportion of Vallisneria natans; S, slope; W, water depth; edf, estimated degrees of freedom of smoothing function.*

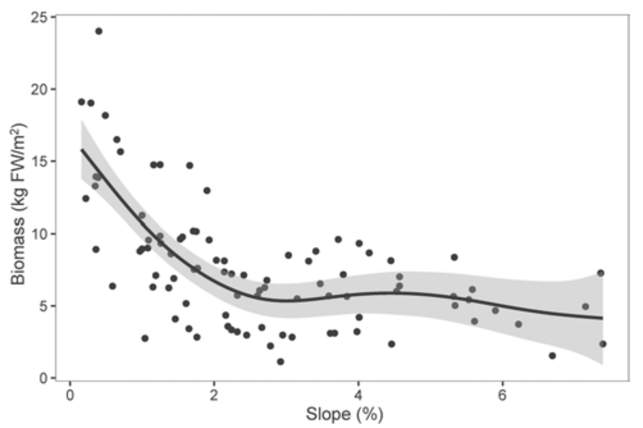


**FIGURE 3 |** Total biomass of submerged macrophytes (FW: fresh weight) along littoral slopes of Lake Erhai. The line is predicted by Generalized Additive Model ($n$ = 97).

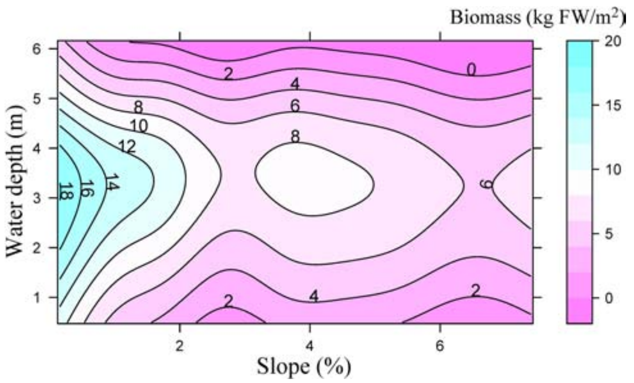


**FIGURE 4 |** Total biomass of submerged macrophytes (kg fresh weight $m^{-2}$) depending on water depth and littoral slope of Lake Erhai based on a Generalized Additive Model ($n$ = 527).

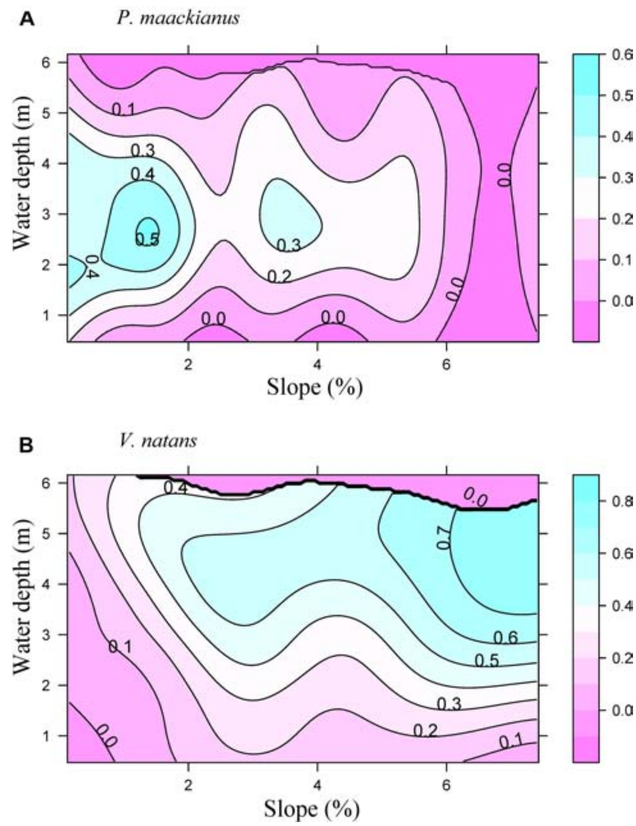


**FIGURE 5 |** Proportion of the submerged macrophyte species *P. maackianus* **(A)** and *V. natans* **(B)** in the total macrophyte biomass along the water depth and littoral slope of Lake Erhai based on a Generalized Additive Model ($n$ = 527).

## Distribution and Abundance of *P. maackianus* and *V. natans* in the Littoral of Lake Erhai

Littoral slope explained about 53% of the variation of the averaged total macrophyte biomass of transect in Lake Erhai (**Table 3**). The averaged total macrophyte biomass of transect decreased with basin slope when slopes were lower than 2% (**Figure 3**), but was relatively stable with increasing slopes above 2%. The combination of littoral slope, water depth and their interaction explained 40% of the variation of the total macrophyte biomass of sampling point (**Table 3**). The highest biomass of submerged macrophytes appeared at a low slope (0–1%) and moderate water depth (2–4 m) (**Figure 4**). The GAM model explained 21% and 27% of the variation of the proportion of *P. maackianus* and *V. natans* in the total macrophyte biomass, respectively (**Table 3**). The proportion of *P. maackianus* in the total macrophyte biomass decreased with increasing littoral slope (**Figure 5A**), whereas the proportion of *V. natans* increased with increasing littoral slope (**Figure 5B**).

## DISCUSSION

Our data on morphological and physiological traits and distribution of two abundant macrophyte species with different growth forms in Lake Erhai confirmed both initial hypotheses. Both tested species show response strategies to low light conditions typical for canopy-forming (*P. maackianus*) and rosette-type (*V. natans*) macrophyte species (**Table 4**) and these response strategies significantly influenced their distribution along different water depth and basin slopes.

## Response Strategies of Submerged Macrophytes to Low Light Conditions

Stem elongation was found as a major response strategy to low light conditions by the canopy-forming species *P. maackianus*, while the rosette-type species *V. natans* produced more chl *a*

**TABLE 4 |** Response strategies of canopy-forming and rosette-type submerged macrophytes to low light conditions.

| Species | Growth form | Response strategies | Study location | Study type | Reference |
|---|---|---|---|---|---|
| *Potamogetonm aackianus* | Canopy-forming | stem elongation; increasing specific leaf area; allocating less biomass to leaves and roots | Lake Erhai, China | Controlled experiment | Fu et al., 2012 |
| *Potamogetonma ackianus* | Canopy-forming | stem elongation; increasing leaf chlorophyll content | Lake Donghu, China | Controlled experiment | Ni, 2001 |
| *Potamogeton maackianus* | Canopy-forming | stem elongation; decreasing branch numbers, minimum saturating irradiance and maximum relative electron transport rate | Lake Donghu, China | Controlled experiment | Chen et al., 2016 |
| *Potamogeto nwrightii* | Canopy-forming | stem elongation; increasing specific leaf area; allocating less biomass to leaves and roots | Lake Erhai, China | Controlled experiment | Fu et al., 2012 |
| *Myriophyllum spicatum* | Canopy-forming | stem elongation; decreasing branch numbers; allocating less biomass to belowground parts | Lake Krankesjon, Sweden | Controlled experiment | Strand and Weisner, 2001 |
| *Vallisneria natans* | Rosette-type | increasing shoot height; decreasing minimum saturating irradiance and maximum relative electron transport rate | Lake Donghu, China | Controlled experiment | Chen et al., 2016 |
| *Vallisneria natans* | Rosette-type | increasing shoot height; allocating more biomass to leaves; allocating less biomass to roots | Lake Erhai, China | Controlled experiment | Fu et al., 2012 |
| *Vallisneria americana* | Rosette-type | increasing shoot height, leaf chlorophyll content and photosynthetic efficiency | Gloucester Point, Virginia | Controlled experiment | French and Moore, 2003 |

per shoot biomass at deeper sites to tolerate light attenuation. These findings are consistent with a similar study by Chen et al. (2016) assessing the response of *P. maackianus* and *V. natans* to low light conditions in Lake Donghu, China. In our study, *V. natans* increased leaf chl *a* content and allocated more biomass to leaves at deep sampling points, resulting in a strong increase of chl *a* content per shoot biomass. Such strategy combined with a low light compensation point for photosynthesis (Chen et al., 2016), allows *V. natans* to successfully survive at sites with low light conditions in Lake Erhai and colonize deep areas with high slopes. Other rosette-type species such as *V. americana* have shown a similar morphological and physiological response to low light conditions (French and Moore, 2003). This indicates that rosette-type species may mainly adopt a tolerance strategy towards stress in low light conditions (**Table 4**). In contrast, *P. maackianus* responds to low light conditions mainly through allocating more shoot biomass into stems to concentrate leaves closer to the water surface. In our study, leaf chl *a* content of *P. maackianus* slightly increased with water depth, but the chl *a* content per shoot biomass did not increase with water depth as less leaf biomass formed at deeper sites. This shade avoidance strategy has also been found in terrestrial plants (Franklin, 2008) and seems common among canopy-forming submerged species (**Table 4**).

## Response to Low Light Conditions Affects Macrophyte Distribution Along Basin Slopes and Water Depth

The alternative response strategies to low light conditions of *P. maackianus* and *V. natans* affected their ability to grow at different basin slopes and water depths. The canopy-forming species *P. maackianus* was dominant at sites with low-moderate water depth (2–4 m) and low-moderate basin slopes (0–4%). In contrast, the rosette-type *V. natans* prevailed at deeper sites (3–5 m) with higher slopes (4%–7%). The potential mechanism allowing *V. natans* growing at deeper waters than *P. maackianus* is that photosynthetic adjustments would become more important in determining plant abundance in deep water due to the lower carbon requirements compared with shoot elongation (Chen et al., 2016).

Previous studies suggested the plant communities may be governed by a dominance–tolerance trade-off, where most species perform best in benign, productive sites (i.e., undisturbed sites with a high availability of resources); however, there is often a trade-off between the ability to dominate at productive sites or to sequester high-quality resources and the ability to persist on low-quality resources or to tolerate harsh conditions (Wisheu and Keddy, 1992; McGill et al., 2006). Lake eutrophication enhances the availability of nutrients both in sediment and water column which initially increases the competition for light between the submerged macrophytes. As a result, short-growing species (e.g., charophytes and rosette-type angiosperms) are commonly replaced by canopy-forming species (Hilt et al., 2018), which can form dense monocultures in mesotrophic lakes (He et al., 2015). However, high slope areas in mesotrophic lakes may provide a refuge for short-growing species due to a trade-off between the ability to compete for light and the ability to tolerate harsh conditions like strong drag forces and nutrient-poor sediment at high slope areas. As a consequence of the different response strategies to low light conditions, *P. maackianus* plants are taller than *V. natans* in a given water depth and therefore have an advantage in competing for light. However, this strategy and their thin stems result in a lower resistance of *P. maackianus* to drag forces by currents and waves (Puijalon et al., 2011; Fu et al., 2014). In contrast, *V. natans* has ribbon-like leaves growing close to the bottom, which are more resistant to drag than *P. maackianus* in a given flow velocity (Puijalon et al., 2011). The nutrient and organic matter content in sediment in low slope areas are not assumed to limit the growth of *V. natans* since this species has been shown to grow on a wide range of sediment types in this lake (He et al., 2017). For instance, the total nitrogen, total phosphorus and organic matter contents in sediments were around 3, 0.7, and 100 mg $g^{-1}$, respectively in Haichao bay of Lake Erhai (unpublished data), all of which are suitable for growth of *V. natans* (Xiao et al., 2007). However, the macrophyte community was dominated by *P. maackianus* rather than *V. natans* in this area, which indicated the *V. natans* might be excluded by competition other than sediment nutrient content in low slope areas. Consequently, this trade-off between the response strategy to low light conditions and hydrodynamic disturbance resistance likely determines the distribution and abundance of the two major macrophyte species with different growth forms in Lake Erhai. Still, other mechanisms such as wind or other sediment characteristics may contribute to the observed distribution of the two species (Schutten et al., 2004, 2005).

Our finding of a significantly decreased total macrophyte biomass with increasing basin slope is consistent with previous observations made in temperate lakes by Duarte and Kalff (1986). Total macrophyte biomass in Lake Erhai decreased significantly at littoral slopes above 2%. Our data suggest that the different response of macrophyte species to low light conditions may contributes to this pattern. In the 1970s and 1980s, submerged macrophytes covered around 40% of Lake Erhai, and the dominant species were *Hydrilla verticillata* and *V. natans.* Macrophyte coverage decreased from 40% to 8% from 1980s to 2012 due to eutrophication. During this period, most of the short species such as charophytes and *V. natans* were lost in deeper areas and *H. verticillata* was replaced by *P. maackianus* (Fu et al., 2013). However, *V. natans* still occupies large areas of the littoral zone in this lake, especially in high slope areas, confirming our finding that these habitat conditions may provide a niche for survival of this species. Similar displacements of rosette species have been found in temperate lakes, where *Isoetes* tend to grow deep under oligotrophic conditions, but are similarly displaced as *V. natans* to the littoral zone with strong disturbance during eutrophication (Arts, 2002).

We conclude that the different response strategies of submerged macrophytes with different growth forms (rosette-type versus canopy-forming) to low light conditions might significantly affect their distribution and abundance in lakes along gradients of other stressors such as physical forces. Morphological heterogeneity of lakes may thus contribute to the maintenance of a high diversity of submerged macrophytes, especially under mesotrophic conditions where competition for light between macrophytes is particularly relevant (Salgado et al., 2017).

## AUTHOR CONTRIBUTIONS

TC and LN conceived the idea and proposed the method. LH, TZ, YW, WL, HZ, and XZ contributed to conduct the sampling and traits measurements. LH, TC, XZ, and SH wrote the manuscript. All authors read and approved the final manuscript.

## FUNDING

This work was supported by State Key Laboratory of Freshwater Ecology and Biotechnology (Grant No. 2016FBZ08), National Science Foundation of China (Grant Nos. 31700402 and 31870446), and Natural Science Foundation of Jiangxi Province (Grant No. 2015ZBBF6008).

## ACKNOWLEDGMENTS

We thank Spencer Froelick for checking the language.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling Editor is currently organizing a Research Topic with one of the authors TC and confirms the absence of any other collaboration.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 22 February 2019
doi: 10.3389/fpls.2019.00161

# Spatially Structured Environmental Variation Plays a Prominent Role on the Biodiversity of Freshwater Macrophytes Across China

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***Specialty section:***
*This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science*

***Received:*** *12 July 2018*
***Accepted:*** *29 January 2019*
***Published:*** *22 February 2019*

***Citation:***
*Zhang M, García Molinos J, Su G, Zhang H and Xu J (2019) Spatially Structured Environmental Variation Plays a Prominent Role on the Biodiversity of Freshwater Macrophytes Across China. Front. Plant Sci. 10:161. doi: 10.3389/fpls.2019.00161*

Different non-mutually exclusive mechanisms interactively shape large-scale diversity patterns. However, our understanding of multi-faceted diversity and their determinants in aquatic ecosystems is far from complete compared to terrestrial ones. Here, we use variation partitioning based on redundancy analysis to analyze the relative contribution of environmental and spatial variables to the patterns of phylogenetic, taxonomic, and functional diversity in macrophyte assemblages across 214 Chinese watersheds. We found extremely high spatial congruence among most aspects of biodiversity, with some important exceptions. We then used variation partitioning to estimate the proportions of variation in macrophyte biodiversity explained by environmental and spatial variables. All diversity facets were optimally explained by spatially structured environmental variables, not the pure environment effect, implying that macrophyte are taxonomically, phylogenetically, and functionally clustered in space, which might be the result of the interaction of environmental and/or evolutionary drives. We demonstrate that macrophytes might face extensive dispersal limitations across watersheds such as topography and habitat fragmentation and availability.

**Keywords: freshwater macrophyte, functional diversity, spatial congruence, species richness, taxonomic distinctness, phylogenetic diversity**

## INTRODUCTION

Macrophytes support many structural and functional aspects of freshwater ecosystems and their ecological properties have been extensively studied over (Westlake, 1975; Jeppesen et al., 1998; Wiegleb et al., 2015). Highly productive, macrophyte communities have a key role in carbon and nutrient fluxes, serving as sinks for organic material and sources of nutrients to the water (Jeppesen et al., 1998; Chambers et al., 2008; Hansson et al., 2010). They provide structurally complex habitat for a large diversity of organisms such as macroinvertebrate, fish and birds (Westlake, 1975; Jeppesen et al., 1998; Hansson et al., 2010). Consequently, a significant change in the structure and composition of macrophyte communities can have important knock on effects on freshwater ecosystems with important management conservation implications (Hellsten and Riihimäki, 1996; Hansson et al., 2010; Wiegleb et al., 2016; Zhang et al., 2019). The development of local macrophyte assemblages strongly depends on a variety of abiotic and biotic factors, e.g., nutrient concentrations, flow velocity, light condition, and trophic interaction (Westlake, 1975; Dodkins et al., 2005; Zhang et al., 2019). However, consensus regarding the generality of large-scale processes driving spatial variation in biodiversity of macrophytes remains elusive (Chappuis et al., 2014; Wiegleb et al., 2015).

Most macrophyte species are regarded as cosmopolitan, their broad distribution traditionally explained by common life history traits such as long-distance hydrochory, anemochory, and zoochory seed dispersal (Santamaria, 2002; Chambers et al., 2008). Such strong dispersal capacity has facilitated the evolution of their plastic response and ecological tolerances to local environmental change. For example, many macrophytes are very resilient because of their fast asexual reproduction abilities such as clonal growth and abundant propagules (Chambers et al., 2008). Consequently, under natural conditions, regional-scale taxonomic richness is generally high and relatively stable across space (Alahuhta et al., 2018). However, high environmental heterogeneity may promote species selection (environmental filtering) and persistence (niche diversification), increasing the spatial turnover of species at landscape scales (Xu et al., 2015; Alahuhta et al., 2018). Therefore, regional spatial diversity patterns in aquatic taxa may be governed by the interplay between their dispersal capacity and the spatiotemporal heterogeneity (Shmida and Wilson, 1985; Shurin, 2007; Gianuca et al., 2017; Cai et al., in press).

Three aspects of species diversity patterns (i.e., taxonomic, phylogenetic, and functional diversity) are the main focus of macroecological studies. Taxonomic diversity, the most straightforward and commonly used measurement of biodiversity in broad-scale studies (Tisseuil et al., 2013; Heino et al., 2015; Cai et al., 2018), treats all species as functionally equivalent and phylogenetically independent. However, phylogenetic diversity and functional trait variation can exert a much stronger control on biodiversity effects on ecosystem functions, such as production or nutrient cycling, than taxonomic diversity (Cadotte et al., 2011; Flynn et al., 2011). Phylogenetic diversity, incorporating evolutionary relationships between species, provides also promising way of interpreting the role of biogeographic history in community ecology (Webb et al., 2002). Therefore, studies considering all these complementary facets can provide a more complete understanding of the mechanistic links between ecological processes and evolutionary history in shaping biodiversity patterns and the provision of ecosystem services (Devictor et al., 2010; Tucker and Cadotte, 2013; Pardo et al., 2017). Here, we assess the relative contribution of spatial and environmental variables to multifaceted biodiversity patterns in macrophyte assemblages across 214 tributary drainage basins (hereafter watersheds) covering the whole China. While the existence of spatial congruence among patterns in diversity facets is an open debate, our previous work in the Yangtze River has suggested high spatial congruence of macrophyte taxonomic and functional diversity at the catchment level (Zhang et al., 2018). Whereas these patterns hold at larger, regional scales across catchments remains to be tested. On the other hand, environmental variables are themselves spatially structured (Legendre and Legendre, 2012), making it possible for spatially structured environmental variation to drive the observed variability in spatial patterns within and among diversity facets.

# MATERIALS AND METHODS

## Data Acquisition and Key Definitions

Data on freshwater macrophyte species compiled from published sources (see below) was grouped at the watershed-scale (214 watersheds) across China (National Remote Sensing Center of China, as delimited by the National Council of China under the National Water Resources Strategic Plan[1]. These watersheds represent subdivisions of main river basins based on different ecohydrological criteria (e.g., river order, landscape, climate), and provide a spatial basis for the development, utilization, conservation, and management of hydrological resources in China (Cai et al., 2018). Pooling data sets into meaningful, large spatial working units such as watersheds (Pool et al., 2014) or ecoregions (Veech and Crist, 2007) is a practical, compromise solution frequently used for the analysis of macroscale diversity patterns with spatially sparse data sets (Cai et al., in press).

Although different definitions of "macrophyte" are available in the literature, we follow Cook (1974) by considering any aquatic plant that is visible to the naked eye including all higher aquatic plants, vascular cryptogams, bryophytes, and groups of algae that can be seen to be dominated by a single species. Based on this definition, we made a detailed literature review of macrophyte species in China from published (1960–2010) records related to lakes, rivers, and seasonal agricultural ponds. Documented sources included research articles and monographs together with the Scientific Database of China Plant Species[2], the Database of Invasive Alien species in China[3], Chinese Species Information System[4], and gray research reports. This exhaustive literature review provided information for a total of 992 aquatic plant species. We then prepared a data matrix covering taxonomic information and functional traits of the species. To guarantee consistency across the data set we used five quality-control rules: (1) non-macrophyte species were filtered according to the Cook's definition for macrophytes (Cook, 1974) and the records in Flora of China, (2) scientific names were standardized and synonyms were removed on the basis of the Chinese Virtual Herbarium[4], (3) varieties were treated as a single species, (4) the distribution traits of the species were corrected according to the Flora of China, and (5) non-freshwater species were excluded. The

[1] http://www.nrscc.gov.cn/nrscc/
[2] http://db.kib.ac.cn/eflora/Default.aspx
[3] http://www.chinaias.cn/wjPart/index.aspx
[4] http://db.kib.ac.cn/

application of these rules resulted in a total of 469 species from 214 watersheds retained for analysis, including 93 submerged species, 40 floating-leaved species, 25 free-floating species, and 311 emergent species.

## Measurements of Multiple Facets of Biodiversity

We defined taxonomic richness (TRic) as the number of species recorded in each watershed over the study period. Given the lack of sufficient and consistent phylogeny information about all included macrophytes in our data set, phylogeny diversity was assessed using taxonomic hierarchies as a proxy for phylogenetic relationships (Schweiger et al., 2008; Heino et al., 2015). Taxonomic distinctness (TDis) measures the mean taxonomic (i.e., phylogenetic) distances between species (Clarke and Warwick, 1998), which was calculated by giving equal branch lengths and six supra-species taxonomic levels (i.e., genus, family, order, subclass, class, and phylum). Hence, watershed with low TDis value indicates low phylogenetic diversity, and vice versa. TDis was calculated using the functions *"taxondive"* and *"taxa2dist"* in the R package *"vegan"* (Oksanen et al., 2016).

To calculate functional distance between macrophyte species, we gathered information on four categorical functional traits from the Flora of China, namely life form (i.e., free-floating, emergent, floating-leaved, submerged), life cycle (i.e., annual, perennial), morphology (i.e., turion, stem, rosette, leafy), sexual propagation (monoecism, dioecy), and species' mean adult weight (Zhang et al., 2018). Morphology was defined qualitatively based on the plant description. Leafy plants typically have more lamina, often the parts concentrating the majority of photosynthesis; stem plants are those with stem and easy to propagate due to broken branches; rosette plants have a shortened stem axis and relatively large projection area that facilitates light competition; turion plants produce winter/overwintering buds as dormant storage organs in response to unfavorable ecological conditions (Cook, 1974; Adamec, 2018). Although we acknowledge that quantitative morphological traits such as shoot height, stem diameter, specific leaf area, or leaf dry mass content, available from some local studies (Fu et al., 2014, 2018), would provide a more precise assessment, these were not available given the nature of our data set and the large scale of our study area.

Four multidimensional functional diversity indices (i.e., functional richness, functional evenness, functional divergence, and functional dispersion) were computed for each assemblage. Functional richness (FRic) describes the convex hull volume filled by a community in the multidimensional functional trait space and used as a measure of the functional richness. Functional evenness (FEve) describes the evenness of the distribution of species in a community over the functional trait space by using the minimum spanning tree. Feve quantifies the regularity with which the functional space is filled by species. Functional divergence (FDiv) describes how species distribute within the volume of functional trait space. For presence/absence data, FDiv is the highest when all the species are on the convex hull and at equal distance to its center of gravity (i.e., if the center of gravity of the convex hull is also a center of symmetry of the functional space). Finally, functional dispersion (FDis) describes the mean distance of each individual species to the centroid of all other species in the assemblage (Anderson et al., 2006), which was calculated using the function *"dbFD"* in the R package *"FD"* (Laliberté et al., 2014).

## Environmental Factors

We include several major environmental factors (**Supplementary Table S1**) used in previous similar macroecological studies (Whittaker et al., 2001; Tisseuil et al., 2013; Cai et al., 2018), namely mean annual precipitation (MAP), mean annual temperature (MAT), solar radiation (SOLAR), and total annual runoff (RUN), as surrogates for the energy input in each watershed, together with total surface area of each watershed (AREA), and spatial variation of altitude, MAT, MAP, SOLAR, and the Shannon diversity index based on the proportions of land cover classes (forest, grass, farm, urban, water, and desert) (i.e., ALTVAR, MATVAR, MAPVAR, SOLARVAR, and LANDVAR) within each watershed representing important factors shaping biodiversity through increasing habitat diversity and availability (Whittaker et al., 2001; Tisseuil et al., 2013). ALTVAR is used as a proxy for topographic heterogeneity, calculated as the range between the maximum and minimum altitude for each watershed. The selected factors should cover the environmental drivers of macrogeographic distributions of species at the watershed scale.

All environmental data were extracted from open-access databases such as Data Sharing Infrastructure of Earth System Science, National Science and Technology Infrastructure Center[5] and the Data Center of Institute of Geographic Sciences and Natural Resources Research, Chinese Academy of Sciences. All parameters were calculated as the average of all cell values with centroids falling within each watershed at a spatial resolution of $0.5° \times 0.5°$ over the study period (1960–2010). Prior to the analyses, variables were logarithm or square root-transformed to improve normalization when necessary and standardized to have a mean of 0 and a variance of 1. We also computed the values of variance inflation factor (VIF) for each predictor variable before analyses to assess collinearity (Cai et al., 2018).

## Spatial Structure

Spatial structure in natural communities can be simultaneously generated by spatial autocorrelation in species assemblages and forcing (explanatory) variables, such as environmental and biotic controls or life history events (Borcard et al., 2004). In order to account for the contribution of the spatial structure of watershed configuration to observed variability in diversity facets, we used multiscale principal coordinates of neighbor matrices (PCNM; Borcard and Legendre, 2002) applied to a geographic distance

[5] http://zgswz.lifescience.com.cn/

matrix computed from the watershed centroid coordinates. In essence, distance between watersheds was first represented as a Euclidean distance matrix calculated from the watershed centroid coordinates. Principal coordinates analysis (PCoA) was then conducted on this matrix after truncation using a threshold distance equivalent to the minimum spanning tree of the distance matrix, defining what is considered to be "large" distances.

The resulting eigenvectors with positive eigenvalues (distance-based Moran's Eigenvector Maps; dbMEMs), provide a spectral decomposition of any possible spatial relationships between the watersheds (Borcard and Legendre, 2002). That is, each eigenvector captures the dominant spatial structures, i.e., low-order eigenvectors (dbMEM1-3 in **Supplementary Figure S1**), associated with large eigenvalues, represent country-scale groupings whereas high-order eigenvectors (dbMEM69-71 in **Supplementary Figure S1**), associated with small eigenvalues, represent more regional-scale groupings. Following a constrained analysis, those positive eigenvectors found to significantly explain variation in macrophyte diversity were selected as independent explanatory variables for further analysis. dbMEMs were calculated using the function *"eigenmap"* with default values in the package *"codep"* in R (Dray et al., 2012).

## Data Analysis

Spatial congruence of watersheds with high diversity was assessed as the amount of concordance between the top 10% of watersheds (i.e., highest diversity values) for all six diversity indices (Pool et al., 2014). That is, the 21 watersheds with the highest FRic values (i.e., the top 10%) were compared to the 21 watersheds with the highest FEve values to calculate how many watersheds were shared within both groups. Subsequently, spatial congruence of pairwise diversity was assessed at the successive 10% intervals. A randomization procedure was performed (999 iterations) for all six diversity indices to determine whether watershed congruence was greater than the random expectations.

We used variation partitioning based on redundancy analysis (RDA) models, with significance assessed using 999 Monte Carlo permutations, to reveal the partial effects of environmental variables and spatial structure on each diversity index (Peres-Neto et al., 2006). This procedure decomposes the total variation in the response dataset into a pure spatial component (S|E), a pure environmental component (E|S), a component of the spatial structured environmental variation (E∩S) and the unexplained variation. Only significant predictor variables were used for variation partitioning as identified from multiple linear regression models by using forward selection procedure and two stopping rules: either exceeding the critical *p*-value ($P = 0.05$) or the adjusted $R^2$ value of the reduced model against the global model based on 999 random permutations (Blanchet et al., 2008). We ran the variation partitioning analyses by using function *"varpart"* in the R package *"vegan"* (Oksanen et al., 2016). We then computed the Moran's *I* correlograms to evaluate the degree of spatial autocorrelation of the diversity indices and the residuals from the linear models (Diniz-Filho and Bini, 2005) by using function *"correlog"* in the R package *"pgirmess"* (Giraudoux, 2016).

Given the nature of our data set, sampling bias might be expected among the 214 studied watersheds. For example, watersheds that are easy to access or largely populated may be expected to be more intensively sampled. However, Pearson correlation analysis between the number of literature sources (NOL, ranging from 4 to 23; **Supplementary Figures S2a,b**) compiled for each watershed, used as proxy for the total sampling effort, and species richness showed no significant correlation (**Supplementary Figure S2c**). Furthermore, results from a sensitivity analysis conducted by repeating the analysis only on watersheds with NOL > 8 (25th quantile; $n = 160$) remain largely invariant to those obtained from the complete data set (**Supplementary Figure S2d**). Therefore, we report results for all watersheds.

# RESULTS

Higher values of taxonomic richness and most functional indices concentrated in watersheds from central-southern China (**Figures 1A–F**). In contrast, higher values of macrophyte taxonomic distinctness and functional evenness appeared in watersheds from western China (**Figures 1B–D**). Among all six diversity indices, taxonomic, and functional richness presented the highest range of variation across watersheds (**Figures 1A–C**), with mean values representing, respectively, 62 and 70% of the total pool of 469 documented species and functional richness across China. At the other extreme, taxonomic distinctness of individual watersheds was very homogeneous across China (**Figure 1B**), but accounted on average for 96% of the total distinctness in the study region.

Spatial congruence for the top 20% of each type of diversity indices presented two distinct groups with high (>40%) and low (<20%) pairwise congruence (**Figure 2**). The highest and lowest congruence occurred, respectively, for FDiv and FDis (58.6%) and FRic and FEve (0%). At this level, all high-congruence pairs were significantly more congruent than random expectations (999 iterations; $p < 0.001$), and showed a significant strong positive correlation (**Figure 3**). On the contrary, incongruent pairs showed mainly weak or strong negative (e.g., FEve – TRic and FEve – FRic) correlations (**Figure 3**).

Based on the selected linear regression model interpreting the variation of the diversity indices (**Table 1**), the percentage of variation explained varied from 40% for FEve to 81% for FDis and FDiv (**Figure 4**). Within each diversity index, the proportion of variation explained by shared fractions (spatially structured environmental gradients) was significantly higher compared to the proportion by unique fractions (pure effects), although the pure spatial component was responsible for relatively high amounts of variation in TRic, FRic, FEve, and FDiv (**Figure 4**). Geographic variations of the six diversity indices were strongly spatially autocorrelated along with a steady decreasing of Moran's I coefficient across distances (**Supplementary Figure S3**). Most of the residuals of the models showed weak spatial patterns, with the exception of the significantly positive autocorrelation at short distances, indicating that our models captured well the

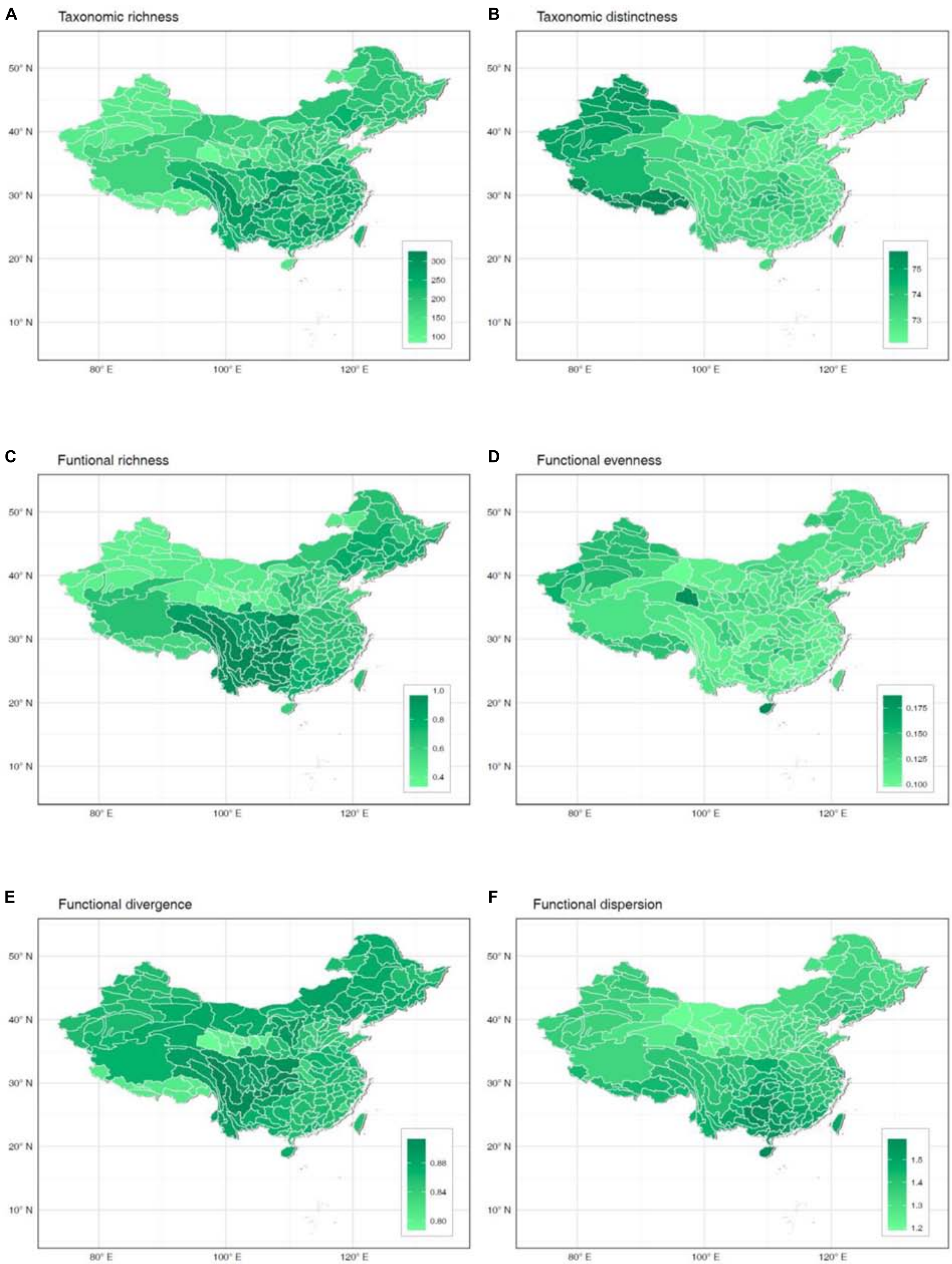


**FIGURE 1 |** Spatial patterns of taxonomic richness **(A)**, taxonomic distinctness **(B)**, functional richness **(C)**, functional evenness **(D)**, functional divergence **(E)**, and functional dispersion **(F)** of freshwater macrophyte assemblages across Chinese watersheds.

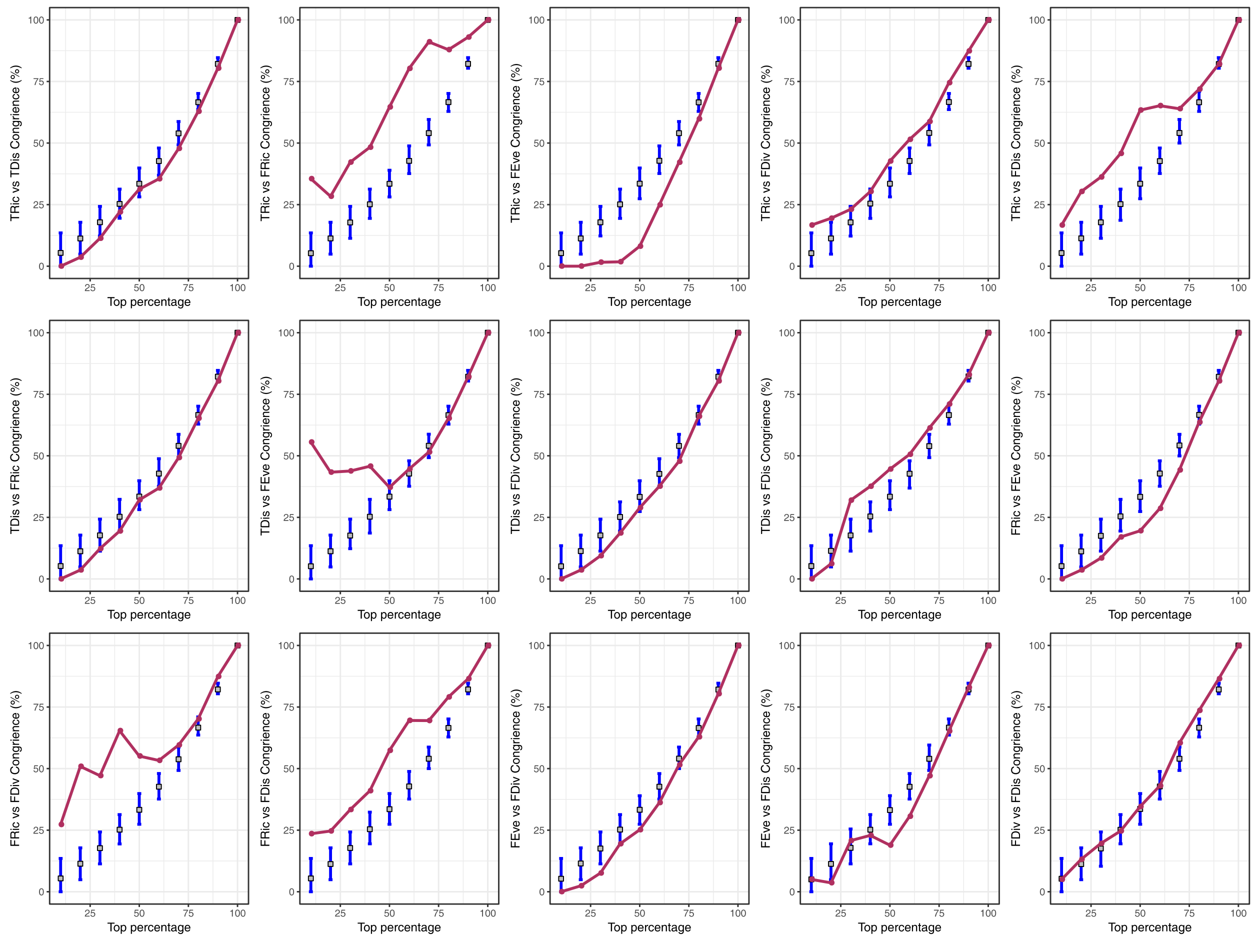


**FIGURE 2 |** Pairwise congruence between taxonomic richness (TRic), taxonomic distinctness (TDis), functional richness (FRic), functional evenness (FEve), functional divergence (FDiv), and functional dispersion (FDis) of freshwater macrophyte assemblages across Chinese watersheds. Congruence was assessed by comparing the spatial concordance for each pair of biodiversity facets across watersheds grouped by percentiles (10% intervals).

major ecological factors underlying geographic gradients of the diversity facets.

## DISCUSSION

We found a relatively high spatial congruence between many, but not all, biodiversity facets (i.e., TRic and FRic, FRic and FDis, TDis and FEve, FDiv and FDis, and FRic and FDiv). Given the strong effects of spatially structured environmental factors on shaping the biodiversity patterns (**Figure 1**), this high spatial congruence can presumably be explained by topography-related dispersal limitation affecting specific functional groups and, consequently, species. Our results corroborate previous evidence on strong correlations between different components of diversity. For instance, Heino et al. (2008) found that species richness was highly correlated with functional richness in stream macroinvertebrate assemblages. Likewise, Strecker et al. (2011) and Pool et al. (2014) reported high spatial congruence among three aspects of species diversity patterns of freshwater fish assemblages. Meynard et al. (2011) found evidence that hypotheses generated for local and regional taxonomic diversity were equally applicable to both phylogenetic diversity and functional diversity. These findings suggest the possibility of using a single diversity measurement as a surrogate for other facets to optimize conservation planning. Given resources are often limited, pinpointing conservation priorities and simultaneously protecting multiple diversity facets is highly desirable (Devictor et al., 2010).

On the other hand, the extremely low spatial congruence found between FEve and both TRic and FRic (**Figure 2**), and the low spatial congruence between TDis and both TRic and FRic may be related to the definition of the measurements, whereby increases in taxonomic diversity and functional diversity can only cause small changes in phylogenetic diversity (Schweiger et al., 2008; Pool et al., 2014; Cai et al., 2018). The scatter of the negative correlation indicates that taxonomic and functional richness increase with decreasing phylogenetic and functional diversity (**Figure 3**). This suggests a stronger effect of species identity relative to that of taxonomic

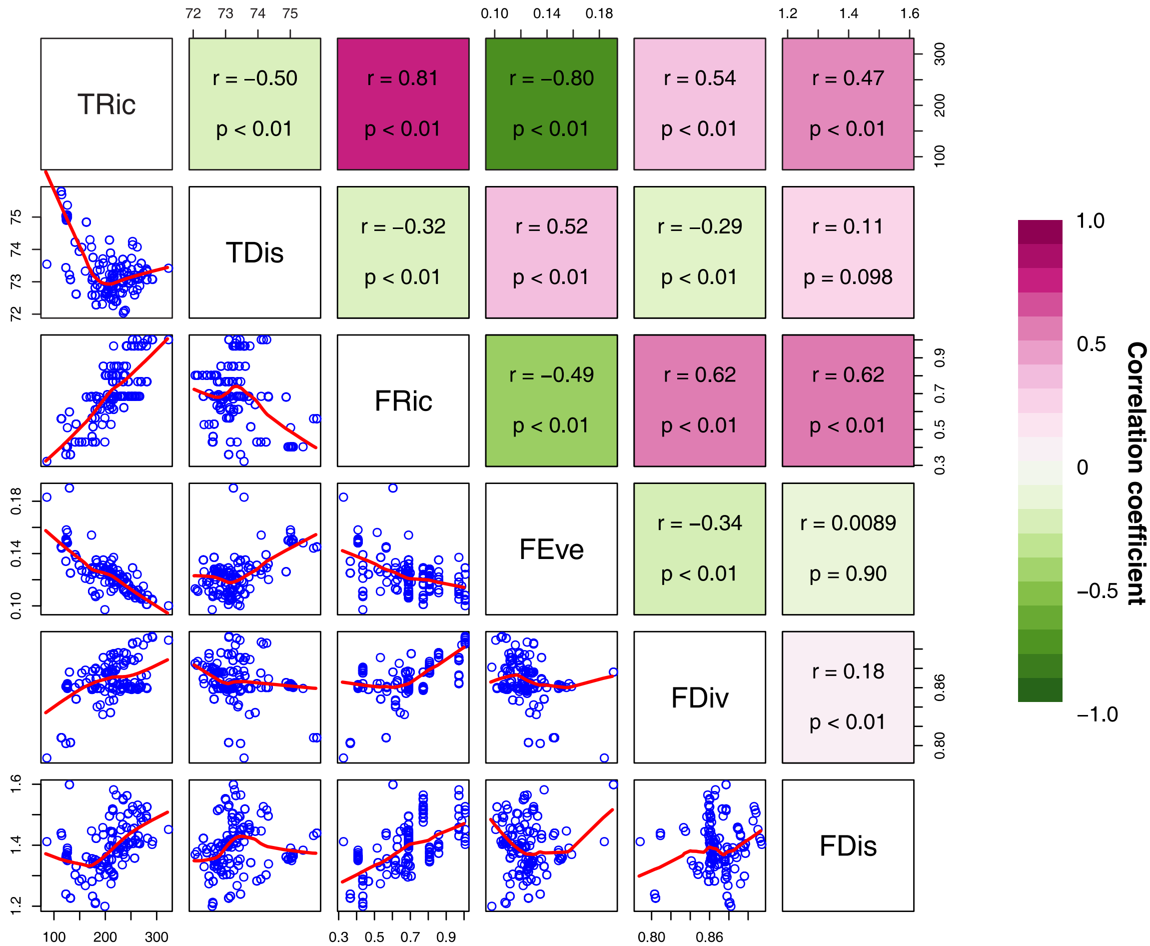


**FIGURE 3 |** Paired scatterplots and Pearson correlations between diversity indices of freshwater macrophyte assemblages across Chinese watersheds (Abbreviations as in **Figure 2**). Red and blue represents positive and negative correlations, respectively.

and functional richness. For instance, watersheds from central-southern China show highest TRic and FRic but relatively low FEve and TDis, whereas watersheds from western China show relatively high FEve and TDis but low TRic and FRic. Macrophytes from watersheds in central-southern China were phylogenetically and biologically more closely related, whereas those in western China were more distantly related to each other. An increasing number of studies reveal spatial mismatches among the three aspects of species diversity patterns (Heino et al., 2005; de Carvalho and Tejerina-Garro, 2015), suggesting that species occurring locally may originate from regional species pools with distinct biogeographic and evolutionary processes, respectively (Losos, 2008; Devictor et al., 2010). In other cases, species in the same region might respond differently to environmental variables affecting spatial TDis patterns and result in mismatches among different facets of diversity (Tucker and Cadotte, 2013). Thus, it is not surprising that great longitudinal gradients in China from west to east are associated with distinct environmental filtering conditions and dispersal limitations affecting functional traits and phylogenetic diversity of macrophytes.

Our results showed that both environmental and spatial factors influence the different facets of macrophyte biodiversity. In particular, spatially structured environmental gradients, rather than pure environmental effects, shaped the different facets of macrophyte biodiversity in watersheds across China (**Figure 4**). Pure spatial factors had a significant role in shaping several facets of the macrophyte biodiversity patterns suggesting that dispersal limitations exert a strong effect on macrophyte assemblage structure across the different diversity facets.

We found significant spatial autocorrelations among all six diversity metrics, whereas the selected spatially structured environmental variables optimally explained the spatial structure of all diversity facets. This results highlights the role of spatially structured environmental gradients, over and above the effect of environmental factors *per se*, as a major driver of biodiversity, which feeds into the debate about the effects of environmental heterogeneity and dispersal limitations on species distributions (Field et al., 2009). Our results also highlight the dominant role of climatic gradients in driving spatially structured patterns of all facets of diversity across watersheds. Climatic gradients at large spatial scales can influence biodiversity patterns through

**TABLE 1 |** Variables retained, adjusted $R^2$, and significance values from the forward-selected multiple regression models examining the effect of environmental and spatial factors on six aspects of macrophyte biodiversity.

| Functional diversity index | Factors | Selected variables | $R^2_{adj}$ | *F* | *P* |
|---|---|---|---|---|---|
| Taxonomic richness | Environment | MAP + MAPVAR − ALTVAR + SOLARVAR + AREA | 0.494 | 42.66 | <0.001 |
| | Space | dbMEM4 − dbMEM1 + dbMEM2 − dbMEM34 + dbMEM6 − dbMEM5 − dbMEM15 + dbMEM23 + dbMEM49 − dbMEM3 − dbMEM46 + dbMEM10 + dbMEM38 | 0.618 | 27.56 | <0.001 |
| Taxonomic distinctness | Environment | ALTVAR + MATVAR − MAPVAR | 0.321 | 34.62 | <0.001 |
| | Space | −dbMEM4 + dbMEM2 + dbMEM1 + dbMEM5 + dbMEM6 − dbMEM3 + dbMEM7 + dbMEM15 + dbMEM21 + dbMEM9 | 0.661 | 42.56 | <0.001 |
| Functional richness | Environment | MAP + MAPVAR + SOLAR | 0.612 | 110.4 | <0.001 |
| | Space | dbMEM2 + dbMEM4 − dbMEM1 − dbMEM3 + dbMEM6 + dbMEM15 | 0.695 | 81.88 | <0.001 |
| Functional evenness | Environment | MAP + ALTVAR + SOLARVAR + MAPVAR | 0.331 | 34.62 | <0.001 |
| | Space | −dbMEM4 − dbMEM3 + dbMEM1 + dbMEM34 − dbMEM38 + dbMEM56 − dbMEM44 − dbMEM49 + dbMEM5 + dbMEM59 − dbMEM69 | 0.381 | 12.94 | <0.001 |
| Functional divergence | Environment | MAP + SOLARVAR + MAT | 0.567 | 94.14 | <0.001 |
| | Space | dbMEM2 − dbMEM1 + dbMEM6 − dbMEM3 + dbMEM9 − dbMEM13 | 0.794 | 137.4 | <0.001 |
| Functional dispersion | Environment | MAP + MAT + SOLARVAR + SOLAR | 0.901 | 150.3 | <0.001 |
| | Space | −dbMEM1 + dbMEM2 + dbMEM6 − dbMEM3 − dbMEM5 + dbMEM4 + dbMEM17 − dbMEM21 − dbMEM13 − dbMEM40 + dbMEM39 + dbMEM9 | 0.614 | 85.9 | <0.001 |

*For each factor, forward selection was applied to identify which variables best described variation in functional diversity index using an inclusion threshold of alpha = 0.05.*

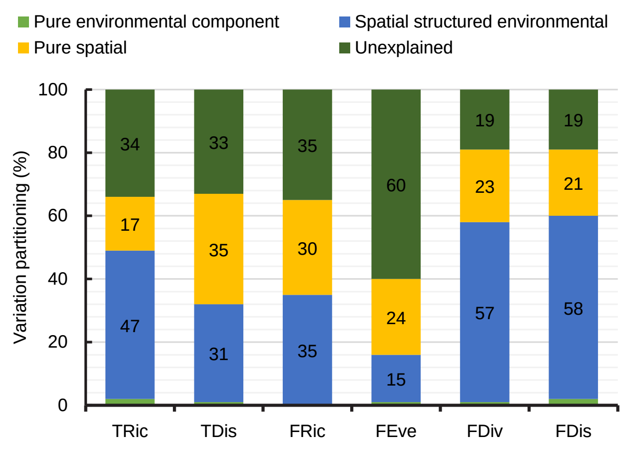


**FIGURE 4 |** Variation partitioning for diversity indices of freshwater macrophyte assemblages across Chinese watersheds (Abbreviations as in **Figure 2**).

multiple mechanisms related to the physiology, energetic demand and dispersal limitations of species (Fu et al., 2018; Li et al., 2018; Zhang et al., 2019). Spatial structure in the diversity facets of TRic, FRic, FEve, and FDiv was significantly explained by broad-scale dbMEMs (**Supplementary Figure S1**). Macrophytes seem to generally confront dispersal limitations although they are often recognized as good dispersers (Santamaria, 2002; Chambers et al., 2008). Our findings suggest that, given the presence of mountain ranges, habitat variability, and other obstacles across the studied watersheds, macrophytes assemblages across China for most diversity aspects are strongly structured by dispersal limitation. Such a pattern of species distributions is consistent with a number of previous studies for other aquatic plant assemblages at regional scales (Capers et al., 2010; Mikulyuk et al., 2011; Alahuhta and Heino, 2013; Viana et al., 2014).

## CONCLUSION

Our study on variation-partitioning analysis demonstrates that macrophyte diversity patterns in watersheds across China are not always congruent and mainly driven by spatially structured environmental determinism. This finding implies that macrophyte are taxonomically, phylogenetically, and functionally clustered in space, which might be the result of environmental and/or evolutionary forces.

## AUTHOR CONTRIBUTIONS

JX and MZ conceived the ideas and compiled the data. MZ, GS, and JX analyzed the data. MZ, JGM, GS, HZ, and JX wrote the manuscript. All authors contributed to the final manuscript.

## FUNDING

This research was supported by the National Key R&D Program of China (2018YFD0900904), the National Natural Science

Foundations of China (Grant Nos. 31872687 and 31370473), and the Water Pollution Control and Management Project of China (Grant No. 2018ZX07208005). JGM was supported by the "Tenure−Track System Promotion Program" of the Japanese Ministry of Education, Culture, Sports Science and Technology (MEXT). GS was funded by the China Scholarship Council. HZ was supported by the Hundred-Talent Program of the Chinese Academy of Sciences. There are no actual or potential competing financial interests. The funders didn't involve in the study design, data collection, and analysis, decision to publish, or preparation of the manuscript.

## ACKNOWLEDGMENTS

We are grateful to Yingying Chen and Yi Yang at the Huazhong Aquaculture University and Xiaohu Huang at Hubei University for help in data compiling and sampling.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00161/full#supplementary-material

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

ORIGINAL RESEARCH
published: 25 February 2019
doi: 10.3389/fpls.2019.00144

# Greater Performance of Exotic *Elodea nuttallii* in Response to Water Level May Make It a Better Invader Than Exotic *Egeria densa* During Winter and Spring

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***Specialty section:***
*This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science*

***Received:*** *31 July 2018*
***Accepted:*** *28 January 2019*
***Published:*** *25 February 2019*

***Citation:***
*Wang Y, Chen X, Liu J, Hong Y, He Q, Yu D, Liu C and Dingshanbayi H (2019) Greater Performance of Exotic Elodea nuttallii in Response to Water Level May Make It a Better Invader Than Exotic Egeria densa During Winter and Spring. Front. Plant Sci. 10:144. doi: 10.3389/fpls.2019.00144*

The strategy of producing rapid initial growth and establishing early in the growing season is important, and it is employed by invasive macrophytes. *Elodea nuttallii* and *Egeria densa*, two Hydrocharitaceae species, became weeds after invading many countries in recent years. Comparative studies on their invasive traits in relation to native species during winter and spring are limited. In the present study, we compared the growth performance of these two exotic species with a perennial native species, *Potamogeton maackianus*, in different water depths (1, 2, and 3 m) during winter (January and February) and spring (March and April). Three morphological traits (shoot number, root number and shoot length), total biomass, relative growth rate (RGR) and two physiological photosynthetic traits (total chlorophyll content and the maximum quantum yield of PSII [*F*v/*F*m]) were measured for each macrophyte. All three species could overwinter as entirely leafy plants. Biomass, RGR, morphological traits and physiological traits were all different among species. However, water depths had a significant effect only on morphological traits. At all water depths, *E. nuttallii* had significantly higher values for morphological traits, total biomass and RGR than *P. maackianus*, while *E. densa* had significantly fewer roots and a lower total chlorophyll content than *P. maackianus*. Except for *F*v/*F*m at a 3 m water depth, morphological and physiological photosynthetic traits, biomass and RGR of *E. nuttallii* were significantly higher than those of *E. densa*. In addition, a large number of adventitious roots developed from *E. nuttallii* but not from the other two species. These results indicate that the advantages of *E. nuttallii* to grow in winter and spring may make it more prone to expansion than *E. densa* in China.

**Keywords: invasive, submerged, water depth, overwinter, morphological, photosynthesis**

## INTRODUCTION

Currently, with the process of economic globalization and trade liberalization, many species are imported in places that are out of their indigenous geographical ranges, and some of them develop into invasive species (Richardson et al., 2000). With its negative effects, biological invasion of exotic species has become a universal ecological problem (Mack et al., 2000). Compared to controlling outbreaks of invaders, prevention is more feasible and economical (Waage and Reaser, 2001). Therefore, it is important to predict the potential invasiveness of species (Rejmánek and Richardson, 1996; Barrat-segretain et al., 2002), which can help us set priorities for the control of introduced invasive species (Rejmánek and Richardson, 1996).

In freshwater ecosystems, invasive plants are alien species that possess advantages for proliferation (produce large numbers of sexual and/or vegetative propagules), dispersal (multiple means and long-distance dispersal) and rapid colonization (Richardson et al., 2000; Kercher and Zedler, 2004). Numerous studies have considered invasive aquatic plants with regards to their growth, regeneration capacity, photosynthesis traits, genetic, reproductive, overwintering strategies and management (Lui et al., 2005; Hussner, 2009; Zhang et al., 2010; Wersal and Madsen, 2011; You et al., 2014; Liu et al., 2016; Hussner et al., 2017). Water depth could affect morphological traits, photosynthetic traits, reproduction and distribution of native or exotic aquatic macrophytes (Chambers and Kaiff, 1985; Strand and Weisner, 2001; Xiao et al., 2010; Fan et al., 2015; Liu et al., 2016; Xu et al., 2016; Han et al., 2018; Li et al., 2018; Zhao et al., 2018). On the other hand, overwintering is important for the invasion of exotic species. The strategy of invasive macrophytes to produce rapid initial growth and establish early in the growing season may increase their competitive ability and allow them to successfully replace native species (Nichols and Shaw, 1986). In addition, the response of submerged macrophytes to the environment might differ depending on the timing of environmental changes and the growth phases of plants (Cao et al., 2015). However, the influence of water depth on the growth of invasive submerged species during the early growth season has not been well elucidated.

*Elodea nuttallii* (Planch.) H. St John is a submerged macrophyte and is native to temperate North America (Cook and Urmi-König, 1985). It has been defined as an invasive species and is now receiving increasing attention for its rapid and lasting invasion of many freshwater habitats throughout Europe, Asia and Australia (Kunii, 1981, 1982, 1984; Barrat-segretain et al., 2002; Zehnsdorf et al., 2015). Another submerged macrophyte, *Egeria densa* (Planch.), which is native of South America, became a weed after it was introduced to North America, Europe and New Zealand (Cook and Urmi-König, 1984; Champion and Tanner, 2000; Gassmann et al., 2006). It can form monodominant and dense stands which have negative effects on native water ecosystems (Yarrow et al., 2009; Fujiwara et al., 2016). *E. nuttallii* and *E. densa* are both species that can tolerate and survive cold temperature, even surviving under ice cover (Kunii, 1984; Yarrow et al., 2009). Moreover, both species can tolerate a wide range of water depths (Vöge, 1994; Carrillo et al., 2006). *E. nuttallii* was introduced to China approximately 30 years ago (Xu et al., 2007). Previous studies considered the influence of *E. nuttallii* on the native ecosystems of China (Zhang et al., 1999) and found that this species had begun to invade the local aquatic community of the East Taihu Lake (Gu et al., 2005) and expanded in other sites in recent years (Yu et al., 2018). *E. densa* was introduced in China as an ornamental plant in recent years, but it has escaped and naturalized in fields, with a limited scope (Yu et al., 2018).

The objective of this study was to compare the growth under various water depths (1, 2, and 3 m) of these two species during winter and spring to that of native species *Potamogeton maackianus*, which is a primary submerged macrophyte in the Yangtze Plain of China that can grow in the winter (Ni, 2001; Su et al., 2019). We hypothesized that (1) not both of exotic species may have advantages in growth traits over native species and (2) water depths would influence the growth of all three macrophytes during winter and spring, and the effects of water depths were expected to be different for native and exotic species.

## MATERIALS AND METHODS

### Plant Materials and Study Sites

This study was conducted at The National Field Station of Freshwater Ecosystem of Liangzi Lake, Wuhan University, China (30°5′–30°18′N, 114°21′–114°39′E). In early November 2013, all samples of two exotic species (*E. nuttallii* and *E. densa*) and one native species, *P. maackianus* were collected from the field station. We chose 37 apical shoots of each plant for the experiment, which were collected from parent individuals with an equal length of 15 cm. For each species, we recorded the fresh weight of all shoots, and we used the fresh and dry (after drying at 70°C for 72 h) weights of 16 apical shoots for the transformation. The mean dry weights of the shoots of *E. nuttallii, E. densa*, and *P. maackianus* were $0.024 \pm 0.001$, $0.116 \pm 0.009$, and $0.066 \pm 0.005$ g (mean ± SD), respectively.

### Experimental Design

We set three water depths (1, 2, and 3 m) in this experiment because the actual mean water depth is 3.0 m in Liangzi Lake (Xu et al., 2018). The experiment was conducted in outdoor cement pools (4 m × 4 m × 4 m; length × width × height) filled with lake water [TN $0.71 \pm 0.01$ mg $L^{-1}$,TP $0.04 \pm 0.01$ mg $L^{-1}$, measured with a YSI Professional Plus water quality meter (YSI Inc., Yellow Springs, OH, United States)]. Seven cement pools were used and one pool represented a replicate. We placed three steel tubes (>4 m; length) on each cement pool at the same intervals and hung three rubber buckets (25 cm × 15 cm; diameter × depth) on each steel tube with the same intervals (**Figure 1**). One apical shoot was planted in a rubber bucket filled with homogeneous mud sediment [TN 0.74 mg $g^{-1}$, TP 0.03 mg $g^{-1}$, measured with IL500 Automatic Analyzer (Hach Corp., Loveland, CO, United States)] from Liangzi Lake and placed at the water level of 0.5 m for a month to root and acclimatize. A month later, we used ropes to control three water depths (1, 2, and 3 m) (**Figure 1**).

A total of 21 individuals of each species were used. During the experiment, lake water was added to cement pools twice a week to compensate for evaporation loss. The water temperature and

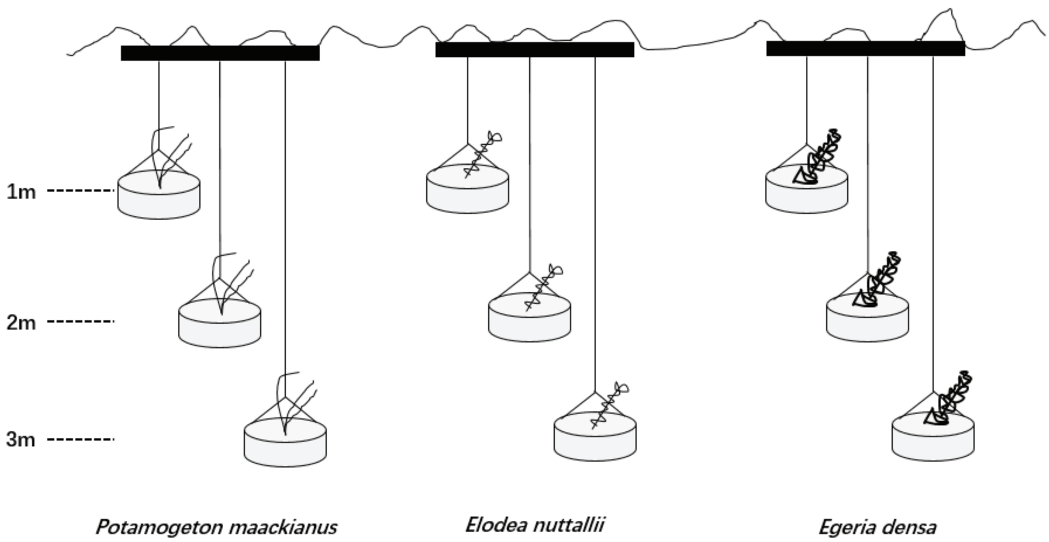


**FIGURE 1 |** Design of the experiments showing three of the 9 containers in which the three species were planted.

light intensity were recorded twice a week, and the average daily temperature of each month from January to April was 7.22 ± 0.5, 7.12 ± 1.96, 10.57 ± 1.9, and 16.74 ± 2.9°C (mean ± 1SE); photosynthetically active radiation (PAR) at noon at different water depths (1, 2, and 3 m) was 108.6 ± 90.1, 65.8 ± 57.0, and 14.8 ± 13.4 $\mu mol\ m^{-2}\ s^{-1}$, respectively (means ± 1SE). The experiment lasted 5 months (from 22 November 2013 to 22 April 2014, including a month for acclimation).

## Trait Measurement

The shoot number, root number (rooted in sediment), shoot length and two physiological photosynthetic traits (total chlorophyll content, maximum quantum yield of PSII [$F_v/F_m$]) were measured for each macrophyte. Adventitious roots (water roots) number of *E. nuttallii* were also recorded. All biomass was obtained after drying plants in an oven at 70°C for 72 h. Relative growth rate (RGR) was calculated as $RGR = (\ln w_2 - \ln w_1)/(t_2 - t_1)$, where $w_1$ and $w_2$ stand for initial and final dry weight, respectively, and $(t_2 - t_1)$ represents the experimental duration of each species from 22 November 2013 to 22 April 2014. A portion of 0.1 g of leaves (fresh weight) obtained from each plant was used for the measurement of chlorophyll content. Plant material was ground to a fine powder, and then extracted with 95% ethanol. The total chlorophyll content (the summed values of chlorophyll a and b) was determined by spectrophotometer, according to Lichtenthaler (1987). Chlorophyll fluorescence on the fully developed, healthy leaves from the top was measured after harvesting. Before measurements, leaves were covered for at least 20 min to allow for dark adaptation, then the maximum ($F_m$) and the minimum ($F_o$) fluorescence yields were measured with the saturation pulse method (Schreiber et al., 2000), with a portable chlorophyll fluorometer (DIVING-PAM, Walz, Effeltrich, Germany). Variable fluorescence ($F_v$) was calculated as $F_m$–$F_o$; consequently, the maximum quantum yield of PSII ($F_v/F_m$) was obtained.

## Statistical Analysis

Differences in growth traits were analyzed first by analysis of covariance (ANCOVA) with initial biomass as the covariate. Initial biomass did not significantly affect any growth traits (all $P > 0.05$). Then, two-way ANOVA with water depths and species as the main factors was performed to determine the main effects and interactive effects on all growth traits. If a significant treatment effect was detected, *post hoc* pairwise comparisons of means were made to examine differences between treatments using studentized Tukey's HSD for multiple comparisons. One-way ANOVA was performed to determine the effects of water depth on the water root number of *E. nuttallii*. Data were log-transformed (shoot and root number, total biomass and RGR) or square root-transformed (shoot length) to ensure normality of residuals and homogeneity of variances, and homogeneity was tested using Levene's test. All data analyses were conducted using SPSS 18.0 (SPSS, Chicago, IL, United States).

# RESULTS

All morphological traits were significantly different between species and water treatments (**Table 1**). All morphological traits (shoot number, shoot length and root number) of *E. nuttallii* were significantly higher than those of *E. densa* and *P. maackianus* at all water depths (**Figures 2A–C**). The shoot number of *P. maackianus* at 2 and 3 m depths and the shoot length of *P. maackianus* at the 2 m depth were significantly higher than those of *E. densa* (**Figures 2A,B**). The root number of *E. densa*

**TABLE 1 |** *F* values and *P* values for species and water depth treatments for shoot number, root number, shoot length, total biomass, RGR, total chlorophyll content and *Fv*/*Fm* by two-way ANOVA ($n = 7$).

| Traits | Species (S) | | Water depth (W) | | S × W | |
|---|---|---|---|---|---|---|
| | *F* | *P* | *F* | *P* | *F* | *P* |
| Shoot number[a] | 197.671 | **0.000** | 5.874 | **0.005** | 0.682 | 0.608 |
| Root number[a] | 100.195 | **0.000** | 6.703 | **0.003** | 1.154 | 0.341 |
| Shoot length[s] | 129.582 | **0.000** | 5.136 | **0.009** | 6.457 | **0.000** |
| Total biomass[a] | 56.852 | **0.000** | 2.190 | 0.122 | 0.038 | 0.997 |
| RGR[a] | 55.168 | **0.000** | 2.615 | 0.083 | 0.143 | 0.965 |
| Total chlorophyll content | 24.578 | **0.000** | 0.327 | 0.723 | 1.475 | 0.223 |
| *Fv*/*Fm* | 17.789 | **0.000** | 1.866 | 0.165 | 0.532 | 0.713 |

*[a]Log transformed, [s]Square root transformed. Bold type is used for significant differences ($P < 0.05$).*

was significantly lower than that of *P. maackianus* at all depths (**Figure 2C**). With increasing water depth, the shoot number and shoot length of *E. nuttallii* decreased and increased, respectively, while depth had no significant effect on the shoot traits of *E. densa* and *P. maackianus* (**Figures 2A,B**). Water depth had no significant effect on the root number of *E. nuttallii* and *E. densa,* while the root number of *P. maackianus* at the 3 m depth was significantly lower than that of plants at the 1 and 2 m depths (**Figure 2C**). Depth had no significant effect on the water root number ($F = 0.192$, $P = 0.827$) of *E. nuttallii*, and the mean number of water roots was $54.71 \pm 9.85$ per plant among all three water depths.

Total biomass and RGR were significantly different among species but not among different water depths (**Table 1**). The total biomass and RGR of *E. nuttallii* were significantly higher than those of *E. densa* and *P. maackianus* at all water depths (**Figures 3A,B**). There were no significant differences in total biomass and RGR between *E. densa* and *P. maackianus* (**Figures 3A,B**).

Total Chl and *Fv*/*Fm* were significantly different among species but not among different water depth treatments (**Table 1**). At all three water depths, the total Chl of *E. nuttallii* was not significantly different from that of *P. maackianus,* while the total Chl of *E. densa* was significantly lower than that of the other two species (**Figure 4A**). At the 1 m depth, both *Fv*/*Fm* of *E. nuttallii* and *E. densa* were similar to *P. maackianus*, while *Fv*/*Fm* of *E. nuttallii* was significantly higher than that of *E. densa* (**Figure 4B**). At the 2 m depth, *E. densa* and *P. maackianus* had no difference in *Fv*/*Fm*, while both values were significantly lower than that of *E. nuttallii* (**Figure 4B**). The difference in *Fv*/*Fm* among the three species disappeared when plants were grown at a depth of 3 m (**Figure 4B**).

## DISCUSSION

Previous studies revealed that some exotic aquatic plants survival increased with increasing winter temperatures (Hussner and Lösch, 2005; You et al., 2014; Liu et al., 2016), which may be responsible for facilitating their invasion. Our results revealed that both exotic macrophytes could overwinter as entire plants. Compared with invasive species that cannot overwinter, overwintering ability may aid these species in invasion.

The shoot number, shoot length, root number, total biomass and RGR of *E. nuttallii* were significantly higher than those of *E. densa* and *P. maackianus* at all water depths (**Figures 2A–C**, **3A,B**). This suggests that the recovery of *E. nuttallii* happens earlier than the other two species, consistent with the findings of *E. nuttallii* compared with *E. canadensis* (Ozimek et al., 1993). This may be due to the extension of the growth season of *E. nuttallii,* beginning in winter with the cumulative daily water temperatures over 4°C (Kunii, 1981); the critical temperature for its active growth lies between 8.2 and 12.0°C (Kunii, 1982), which is lower than the temperature for active growth of many other submerged plants (Ozimek et al., 1993). It was also found that *E. nuttallii* grows well and has a competitive advantage over native species at low water temperatures in Japan (Kunii, 1981, 1982, 1984). The strategy adopted by invasive macrophytes to produce rapid initial growth and establish early in the growing season is a much less conservative life strategy than that of the species that grow and reproduce until midsummer (Nichols and Shaw, 1986); thus, our results suggested that the early development of *E. nuttallii* might be another important means to promote its colonization. A similar phenomenon of the winter growth of *E. nuttallii* has been reported in its related species *E. canadensis* (Haag, 1979). *E. densa* did not show advanced winter growth, compared to *E. nuttallii* and the indigenous *P. maackianus*, in the present study. The winter growth of *E. densa* is slow and this species tends to store starch for subsequent spring growth (Yarrow et al., 2009). It can recover from winter senescence and quickly reinvade water bodies through energy stored in basal stems and the root crown (Pennington and Sytsma, 2009; Cabrera et al., 2013), but the optimal temperature range for its active growth is 14 to 25°C (Pennington, 2008). However, the water temperature was only up to 14°C in late April, which could explain why *E. densa* grew slowly and did not recover during the experimental period.

Once the invasion of exotic macrophytes has taken place, subsequent colony growth proceeds primarily by stem fragmentation (Hofstra et al., 1999), owing to clonal fragmentation being one of the essential means of duplication and dispersal of aquatic plants (Barrat-segretain, 1996). The three macrophytes in our study mainly reproduce vegetatively

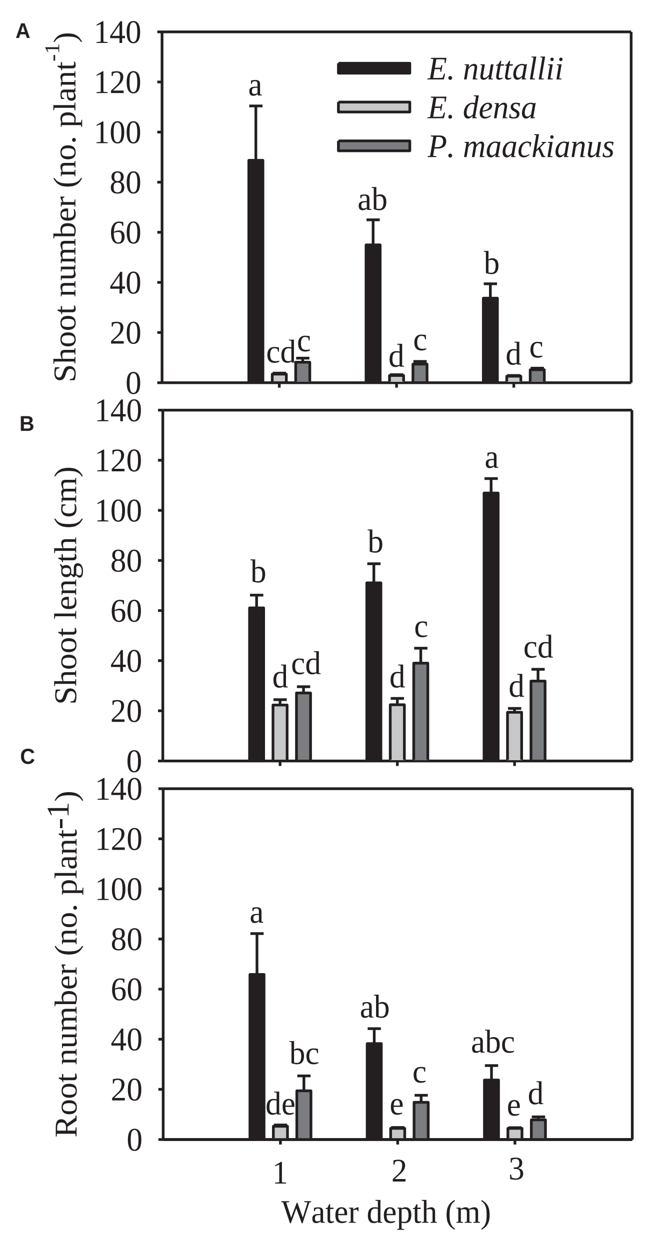


**FIGURE 2 |** Changes in the morphological traits (shoot number **A**, shoot length **B**, and root number **C**) of the three macrophytes, data are presented as the means ± 1SE ($n$ = 7). Means with different small letters are significantly different at $P$ < 0.05 among different treatments.

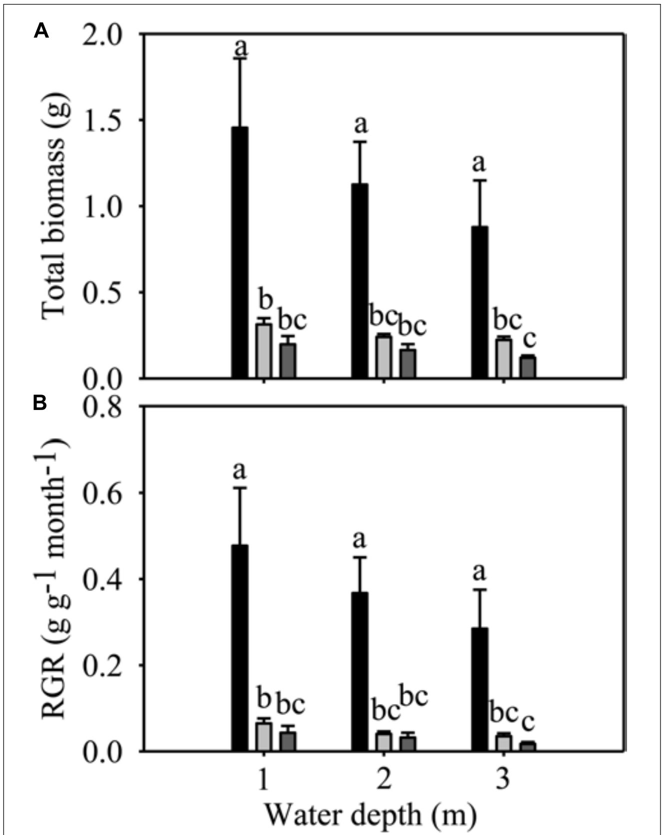


**FIGURE 3 |** Changes in the total biomass **(A)** and RGR **(B)** of three macrophytes. The data are presented as the mean ± 1SE ($n$ = 7). Means with different small letters are significantly different at $P$ < 0.05 among different treatments.

through clonal growth of stolons or fragments (Cook and Urmi-König, 1984; Li et al., 2004; Nagasaka, 2004). Therefore, the number of propagules could be reflected by their shoot number and shoot length. In our study, the shoot number and shoot length of *E. nuttallii* were significantly higher than that of *P. maackianus*, whereas the reverse was true for *E. densa* under most treatment conditions (**Figures 2A,B**), which might aid *E. nuttallii* and inhibit *E. densa* in occupying niches early in the spring. The number of invaders (propagule pressure) is one of the traits that influence the ability of species to invade new communities (Lonsdale, 1999; Xie et al., 2013). Furthermore, *E. nuttallii* may produce a canopy shading out and inhibiting other species due to its shoot length (Barrat-segretain and Elger, 2004); a similar study suggested that *E. nuttallii* invests more in shoot elongation than *E. canadensis* and thus has a greater advantage for light capture (Szabó et al., 2019). We suggested that the earlier the start of the vegetative period, the higher the capability for vegetative reproduction, and a greater shoot length might contribute to *E. nuttallii* being more invasive than *E. densa* during winter and spring.

The roots of submerged plants could maintain many of the functions of terrestrial roots, such as the absorption and transportation of nutrients and water and the supply of shoots with anchorage and phytohormones (Agami and Waisel, 1986; Hussner et al., 2009). The root number of *E. densa* was significantly lower than that of the other two macrophytes in all treatments, while that of *E. nuttallii* was the highest among the three species at all water depths (**Figure 2C**). Since this species can absorb and accumulate large quantities of nitrogen through its roots (Ozimek et al., 1993), the greater amount of roots of *E. nuttallii* may result in an

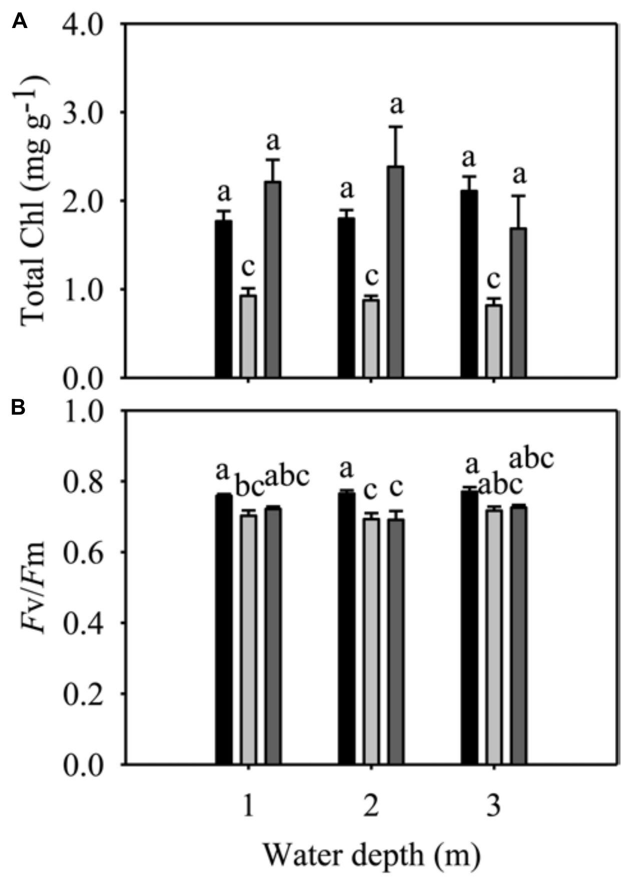


**FIGURE 4 |** Changes in the total chlorophyll content **(A)** and *F*v/*F*m **(B)** of three macrophytes. The data presented are the mean ± 1SE ($n$ = 7). Means with different small letters are significantly different at $P < 0.05$ among different treatments.

advantage in nitrogen absorption over the other two species. A previous study also found that another invasive species, *E. canadensis*, allocated more biomass to roots during adverse environmental conditions (heat wave) to absorb more nutrients (Cao et al., 2015). Furthermore, adventitious roots were of greater importance in P-storage under high resource availabilities (Garbey et al., 2004). The development of adventitious roots may aid the survival through the uptake of water column nutrients, which may be a mechanism for survival during adverse conditions, a means of long distance dispersal of fragments, or it may offer a competitive advantage over species that rely on sediment nutrients (Wersal and Madsen, 2011). Moreover, the development of adventitious roots may contribute to the ability to spread and colonize in local habitats or in new habitats once fragments occur, because shoots are very often broken and fragmented by animals and water flow (Barrat-segretain, 1996), in plants that possess higher survival rates (Barrat-segretain et al., 2002). Adventitious roots (water roots) developed from shoots of *E. nuttallii* in our experiment, as observed by previous studies, were located at nodes of lateral branches (Ozimek et al., 1993; Barrat-segretain, 2005). Therefore, the development of more roots and adventitious roots in *E. nuttallii* may contribute to its rapid growth and distribution.

Since the conditions of light and substrate availability change and waves may occur with increasing water depth (Duarte and Kalff, 1990), and light intensity underwater decreases significantly (Zhu et al., 2012), water depth has an important influence on the growth, reproduction and distribution of aquatic macrophytes (Chambers and Kaiff, 1985; Xiao et al., 2010; Zhu et al., 2012; Fan et al., 2015; Li et al., 2018; Zhao et al., 2018). Contrary to our hypothesis, our results showed that water depth had less influence on most traits of *E. nuttallii* and *P. maackianus*, except that deeper water (3 m) decreased the shoot number of *E. nuttallii* and root number of *P. maackianus*, and increased the shoot length of *E. nuttallii*. Similar results were reported for other species, also including *P. maackianus* (Strand and Weisner, 2001; Yang et al., 2004; Zhu et al., 2012; Fan et al., 2015). In addition, in this study, water depth had no influence on any traits of *E. densa* (**Figures 2–4**). Previous studies found that these three species could tolerate a wide range of water depths and adapt well under low light conditions (Barko and Smart, 1981; Vöge, 1994; Ni, 2001; Carrillo et al., 2006), which probably accounts for the indistinct differences in their growth among different water depths in our study. In addition, plant growth was not essentially sensitive to light intensity at cool temperatures (Rajan et al., 1973). The *F*v/*F*m ratio is normally within the range of 0.75–0.85, and the decline in this ratio indicates photoinhibitory damage caused by environmental stresses (Bolhár-Nordenkampf et al., 1989). For example, water depth (≥3 m) severely reduced the *F*v/*F*m of the submerged plants *Vallisneria natans,* indicating that flooding treatments imposed severe stress on the fitness of this species (Han et al., 2018), or shallow water decreased *F*v/*F*m of the submerged plants *Ottelia acuminata* because of the strong light (Zhao et al., 2018). However, water depth had no significant effects on the *F*v/*F*m of the three macrophytes in our study (**Figure 4B**). Similarly, there was no significant change in the total Chl content of the three species among different water depth treatments (**Figure 4A**). The maintenance of the photosynthetic pigment concentration (Chl concentration) explains the stability of *F*v/*F*m (Meneguelli-Souza et al., 2016). These results suggested that 2 m or 3 m water depths did not cause damage to these three macrophytes, because they can tolerate a wide range of water depths (Vöge, 1994; Carrillo et al., 2006; Zhu et al., 2012; Li et al., 2013). Contrary to our second hypothesis, the growth of these three macrophytes was less influenced by water depth.

## CONCLUSION

All three species could overwinter as whole leafy plants. The growth and photosynthesis of the three species were less influenced by water depths. The results verified our first hypothesis that not both exotic species showed advantages over the native species. *E. nuttallii* presented superior advantages over others observed in its considerable growth and shoot increase (such as the larger RGR, more shoots and roots, and higher shoot length) in late winter and spring, which might help it to establish in its niche early and express its invasive traits, thus favoring its expansion and potential outbreak. By contrast, *E. densa* was disadvantaged compared with the other two macrophytes because of its weak growth and photosynthetic rate in winter and early spring.

## AUTHOR CONTRIBUTIONS

YW, DY, and CL designed the research and executed the research project. YW, XC, JL, YH and QH collected the field data. YW and CH analyzed the data and wrote the manuscript. HD revised the paper critically.

## FUNDING

This work was supported by the Major Science and Technology Program for Water Pollution Control and Treatment (2015ZX07503-005) and the Special Foundation of National Science and Technology Basic Research (2013FY112300).

## ACKNOWLEDGMENTS

The authors gratefully acknowledge the support Ligong Wang provided for the laboratory and field work, and the useful suggestions provided by Shufeng Fan, Chengxing Xia, Tong Wang, Xiaolong Huang, and Haihao Yu.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling Editor is currently organizing a Research Topic with one of the authors CL, and confirms the absence of any other collaboration.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 11 April 2019
doi: 10.3389/fpls.2019.00442

# Leaf Soluble Carbohydrates, Free Amino Acids, Starch, Total Phenolics, Carbon and Nitrogen Stoichiometry of 24 Aquatic Macrophyte Species Along Climate Gradients in China

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**Edited by:**
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**Specialty section:**
*This article was submitted to*
*Functional Plant Ecology,*
*a section of the journal*
*Frontiers in Plant Science*

**Received:** *13 April 2018*
**Accepted:** *22 March 2019*
**Published:** *11 April 2019*

**Citation:**
*Chou Q, Cao T, Ni L, Xie P and Jeppesen E (2019) Leaf Soluble Carbohydrates, Free Amino Acids, Starch, Total Phenolics, Carbon and Nitrogen Stoichiometry of 24 Aquatic Macrophyte Species Along Climate Gradients in China.*
*Front. Plant Sci. 10:442.*
*doi: 10.3389/fpls.2019.00442*

Leaf soluble carbohydrates (SC), free amino acids (FAA), starch, total phenolics (TOPH), carbon (C), and nitrogen (N) stoichiometry of 24 aquatic macrophyte species were studied at 52 selected sites in eastern, 31 sites in southwestern and 6 sites in western China, including 12 submerged, 6 floating-leaved, 4 emergent and 2 free-floating macrophytes. The leaf stoichiometric characteristics differed significantly among the plant species of the four different life forms, the lowest C content occurring in submerged macrophytes and the highest N content in free-floating macrophytes. Overall, though the variance explained by the linear regression models was low, the C and N contents decreased toward the northern latitudes, the C content and the C:N ratios increased with increasing altitude. Multiple regressions revealed that the stoichiometric characteristics of submerged macrophytes varied significantly across the large spatial and climatic gradients and among the species studied. For floating-leaved and emergent macrophytes, no correlation between climate factors and SC, FAA, starch, TOPH, C, and N contents and C:N ratio was observed. For free-floating macrophytes, the TOPH content was markedly positively correlated with latitude and altitude. We conclude that the C and N contents related more closely to latitude, altitude or mean annual air temperature than did the C and N metabolic indicators for the submerged macrophytes, while the relationships with the metabolic indicators turned out to be insignificant for most species of the other life forms. The results helped us to identify species with significant physiological plasticity across geographic and climatic gradients in China, and such information is useful when conducting restoration of lost aquatic plants in different climate regions.

**Keywords: aquatic macrophytes, life form, stoichiometry, biogeographical, climate gradients**

## INTRODUCTION

Generally, plant nutrient status has been evaluated by examining nutrient contents in plant tissues (Duarte, 1992; Gerloff and Krombholz, 1966). Ecological stoichiometry provides an integrative approach to explore the relationships between plants and their environment using parameters such as species composition and distribution, population dynamics, food web and biogeochemistry at various spatial scales (Sterner et al., 1992; Elser et al., 2000, 2010; Sterner and Elser, 2002). In freshwater and terrestrial ecosystems, the contents of mineral elements in organisms have been frequently studied (Jackson et al., 1991; Duarte, 1992; Mcjannet et al., 1995; Aerts and Chapin, 1999), particularly the major elements such as carbon (C), nitrogen (N), and phosphorous (P), which are the key nutrients supporting the life of organisms and vital to the ecological functions of ecosystems (Reich and Oleksyn, 2004; Han et al., 2005, 2011; He et al., 2006). Temperature is an important factor affecting the biological activity and nutrient metabolism of both individual organisms and ecosystems, and stoichiometric patterns in plant tissues have been observed to differ across large geographical and climatic scales, reflecting variation in biological activity and the biogeochemical cycling of essential elements such as N and P (Sterner and Elser, 2002; Reich and Oleksyn, 2004; Han et al., 2005, 2011).

Macrophytes are fundamental components affecting food webs and functions in many shallow aquatic ecosystems (Jeppesen et al., 1998; Frost and Hicks, 2012). Spatial heterogeneity in the sediment N content caused by eutrophication may affect the metabolic activity of the plants as well as intermediate metabolites such as soluble carbohydrate (SC), starch, free amino acids (FAA), phenolic compounds (TOPH) and the N-related stoichiometries (Sterner and Elser, 2002; Cronin and Lodge, 2003; Cao et al., 2008; Li et al., 2015). SC and starch serve as a storage of energy, as C reservoir and as structural components (Coolidge-Stolz et al., 2002). Carbohydrates provide carbon skeleton and energy for the synthesis of amino acids that play a central role in the metabolism of C and N by acting as a nitrogen transporter and reservoir and as precursors for proteins and many secondary metabolites (Cao et al., 2008). The FAA content in aquatic plants can be affected by the balance between light and N availability and generally increases when plants are exposed to environmental stressors causing reduced growth (Lea and Forde, 1994; Lam et al., 1998; Foyer et al., 2003). The FAA content is therefore used as a physiological indicator in plants. Phenolic compounds

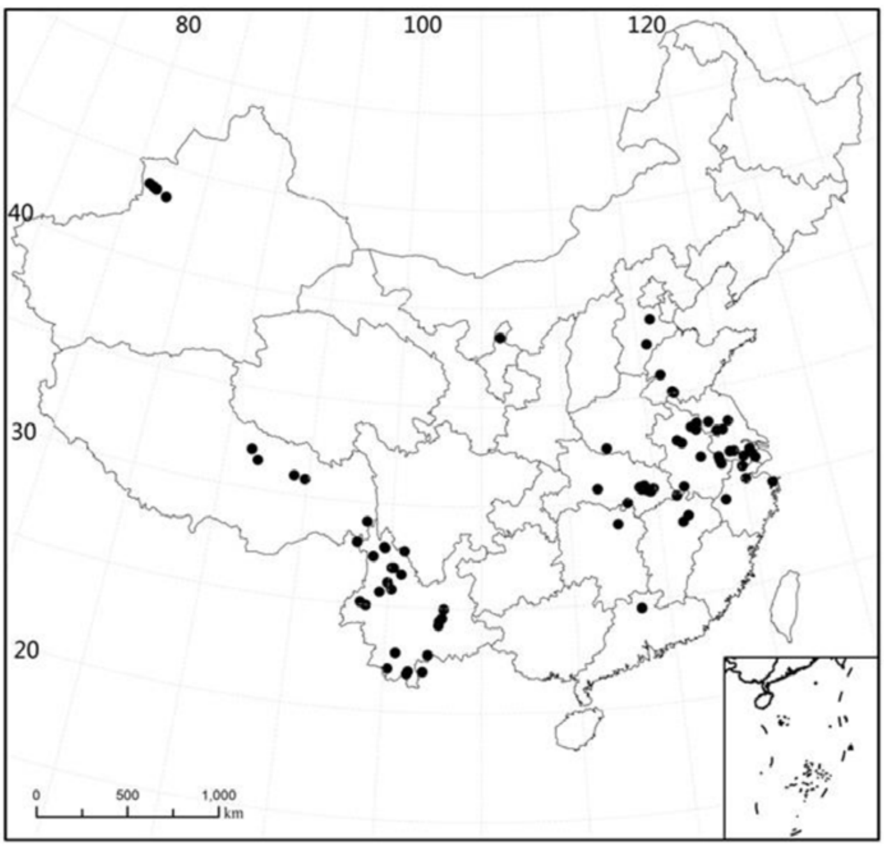


**FIGURE 1 |** Sampling location of all study sites. Provincial boundaries are shown.

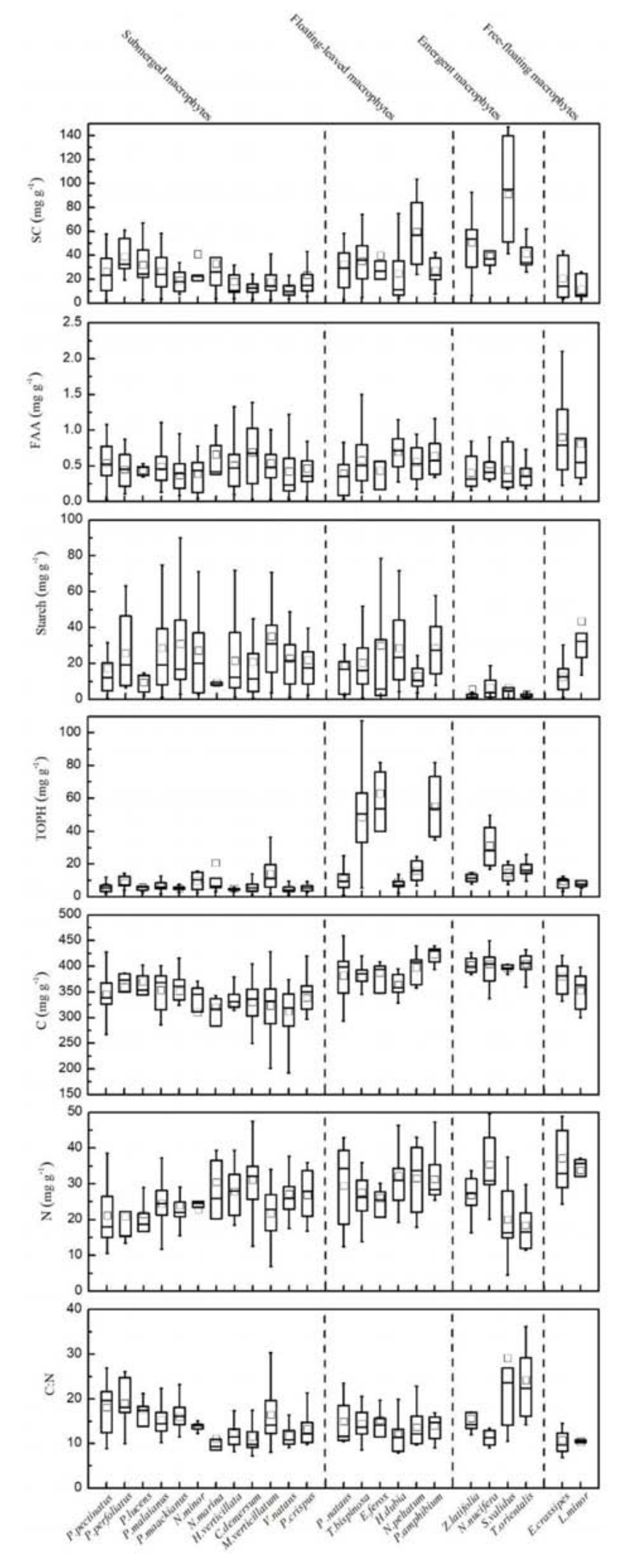


**FIGURE 2 |** Leaf stoichiometric characteristics for all 24 aquatic plant species. Leaf soluble carbohydrates (mg $g^{-1}$), free amino acids (mg $g^{-1}$), starch (mg $g^{-1}$), total phenolics (mg $g^{-1}$), carbon (mg $g^{-1}$), nitrogen (mg $g^{-1}$) and the C:N ratio, mean ± SD.

are important secondary metabolites of plants and act as precursors for lignin synthesis and antioxidants (Hong et al., 2016; Adbelrahman et al., 2017). High light and carbon dioxide and low nitrogen availabilities, as well as plant damage caused by herbivorous fish, generally increase the content of phenolic compounds in plant tissues (Dudt and Shure, 1994; Cronin and Lodge, 2003). Thus, in addition to elementary stoichiometry, examination of the contents of SC, FAA, starch, and TOPH may help gain insight into the C and N metabolic strength of macrophytes.

Compared with the great attention paid to terrestrial plants few studies have focused on the stoichiometric characteristics of aquatic macrophytes that occur over large geographic scales (Elser et al., 2000; Reich and Oleksyn, 2004; Han et al., 2005, 2011; He et al., 2006) and thus are exposed to significant differences in climate and human activities (Proctor, 1982; Wu et al., 1999; Lacoul and Freedman, 2006; Frost and Hicks, 2012; Sardans et al., 2012). However, some experiments and field investigations on aquatic plants have attempted to define the range and variation of their physiological state by exploring various metabolic indicators. The emphasis of these studies has been placed on the stoichiometry differences between various species (Fernández-Aláez et al., 1999; Li et al., 2015), the relationship between the contents of various elements (Frost and Hicks, 2012; Li et al., 2014; Xia et al., 2014) and the influence of lake sediment and water column nutrient gradients on plant stoichiometry (Qiu et al., 2013; Xing et al., 2013; Su et al., 2016). So far, though, no studies have dealt with the differences in stoichiometry of different life forms of aquatic plants over large geographic and climatic gradients. In this investigation, we examined the contents of SC, FAA, starch, TOPH, C, and N in the leaves of 24 aquatic macrophytes across large geographic and climatic gradients in China with the aim to elucidate plant C and N stoichiometry. We hypothesized that (1) plant biochemical and stoichiometric parameters would vary noticeably across latitude and altitude and with mean annual air temperature (MAT), and that (2) latitude, altitude and MAT would affect the C to N stoichiometry more consistently in submerged than in emergent, floating-leaved and free-floating macrophytes due to the fact that submerged macrophytes live below the water surface where temperature is less variable than at the water-air interface and above, and water availability is not of importance.

## MATERIALS AND METHODS

### Study Area

The plant samples were collected at 89 sites in China (21°26′ – 43°57′N, 80°39′ −121°39′E) during the period 2003 to 2009. The sites were located in 15 major provinces, with 26 sites in Yunnan, 1 site in Guangdong, 1 site in Hunan, 2 sites in Jiangxi, 4 sites in Zhejiang, 12 sites in Hubei, 1 site in Henan, 2 sites in Hebei, 6 sites in Shandong, 9 sites in Anhui, 11 sites in Jiangsu, 6 sites in Xinjiang, 5 sites in Tibet, 1 site in Ningxia, and 1 site in Shanghai (**Figure 1**). For all sampling sites, the MAT range was −3 to 22.4°C (mean: 14.8°C, median:

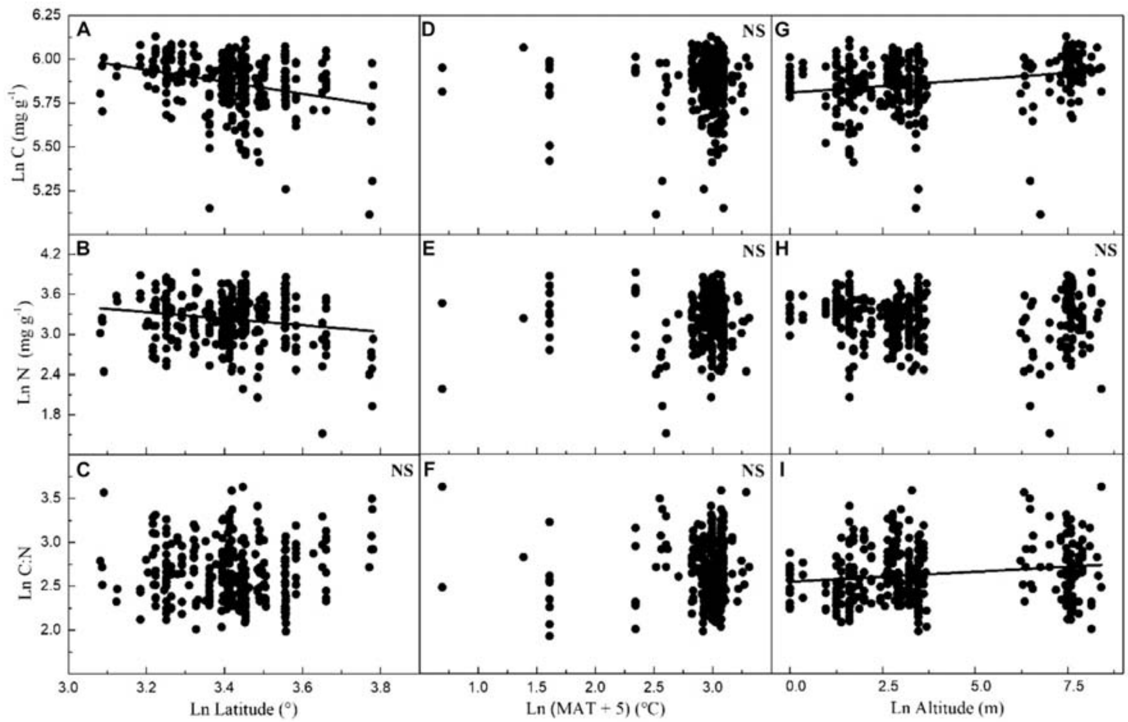


**FIGURE 3 |** Relationships between leaf carbon and nitrogen contents and the C:N ratio of plants, latitude, mean annual temperature and altitude in China. Each data point represents a value of all observations of carbon and nitrogen at each sampling site (**Figure 1**). Linear regressions are shown for **(A)** latitude and leaf carbon ($r^2$ = 0.080, $n$ = 339, $P$ < 0.001); **(B)** latitude and leaf nitrogen ($r^2$ = 0.027, $n$ = 339, $P$ = 0.001); **(C)** latitude and C:N ratio ($r^2$ = –0.002, $n$ = 338, $P$ = 0.634); **(D)** MAT and leaf carbon ($r^2$ = –0.002, $n$ = 347, $P$ = 0.669); **(E)** MAT and leaf nitrogen ($r^2$ = –0.002, $n$ = 347, $P$ = 0.336); **(F)** MAT and C:N ratio ($r^2$ = –0.002, $n$ = 346, $P$ = 0.595); **(G)** altitude and leaf carbon ($r^2$ = 0.059, $n$ = 339, $P$ < 0.001); **(H)** altitude and leaf nitrogen ($r^2$ = 0.003, $n$ = 339, $P$ = 0.172); **(I)** altitude and C:N ratio ($r^2$ = 0.027, $n$ = 338, $P$ = 0.001). All dates were log-transformed (base $e$). Bonferroni correction significance level threshold value, $P$ = 0.05/3.

15.5°C) and the span of altitude was 1.0 to 4477 m (mean: 695.7 m, median: 25 m).

## Aquatic Plant Sampling and Biochemical Analysis

We collected all species present at the sites during the same season (summer) of the year to avoid the impact of inter-seasonal differences in climate. At each sampling site, latitude and altitude were recorded using a portable GPS (Garmin 60csx), and MAT was derived from the website of the meteorological department[1]. Unfortunately we have no nutient data for lakes as the plants used were sampled with the purpose of describing species distribution.

We took macrophyte samples using a reaping hook in quadrat areas sized 0.2 m$^2$. Three replicates were taken at each site and

[1]https://www.wunderground.com/

**TABLE 1 |** Linear regression models explaining the average leaf soluble carbohydrates, free amino acids, starch, total phenolics, carbon and nitrogen contents and the C:N ratio of all species included in the study.

| Dependent variable | *df* | *P* | $r^2_{adj}$ | Linear model |
|---|---|---|---|---|
| Ln SC | (1, 381) | 0.002 | 0.023 | 4.599*** – 0.540** ln MAT+5 |
| Ln C | (1, 337) | <0.001 | 0.079 | 7.037*** – 0.343*** ln latitude |
| | (2, 336) | <0.001 | 0.091 | 6.712*** – 0.257*** ln latitude + 0.008* ln altitude |
| Ln N | (1, 337) | 0.001 | 0.027 | 4.912*** – 0.493** ln latitude |
| | (2, 336) | <0.001 | 0.060 | 6.105*** – 0.808*** ln latitude – 0.031*** ln altitude |
| Ln C:N | (1, 336) | 0.001 | 0.028 | 2.547*** + 0.023** ln altitude |
| | (2, 335) | <0.001 | 0.044 | 1.083 + 0.034*** ln altitude + 0.417* ln latitude |

*The models result from a forward stepwise selection procedure using the independent variables latitude, altitude, and mean annual temperature. All significant (P < 0.05/3) regression models are shown. All dates were log-transformed (base e), the significance of the regression cofficients is indicated by *P < 0.05, **P < 0.01, ***P < 0.001, without asterisk P > 0.05. Bonferroni correction significance level threshold value, P = 0.05/3.*

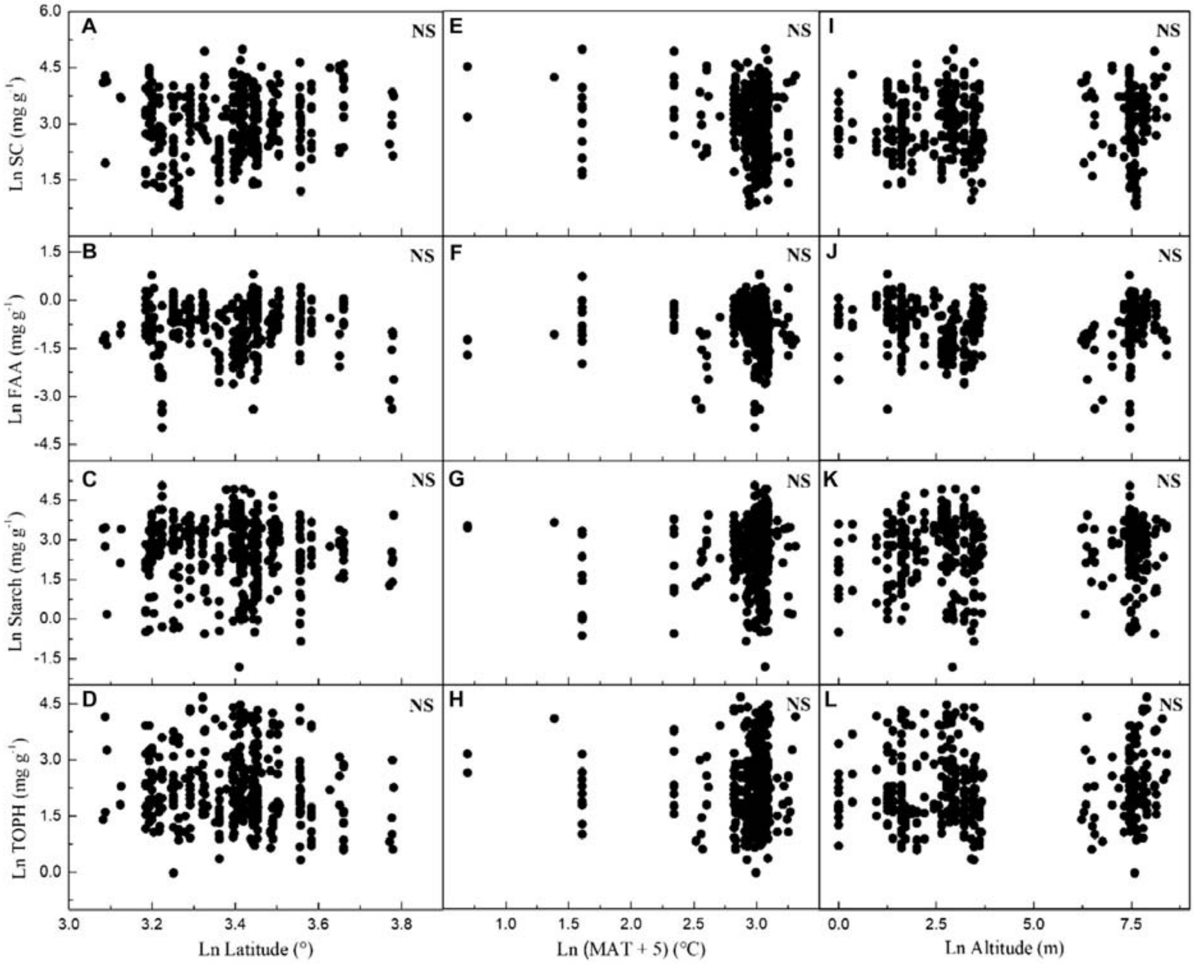


**FIGURE 4 |** Relationships between leaf soluble carbohydrates, free amino acids, starch and total phenolics contents of plants, latitude, mean annual temperature and altitude in China. Each data point represents a value of all observations of soluble carbohydrates, free amino acids, starch and total phenolics at each sampling site (**Figure 1**). Linear regressions are shown for **(A)** latitude and leaf soluble carbohydrates ($r^2$ = 0.008, $n$ = 383, $P$ = 0.043); **(B)** latitude and leaf free amino acids ($r^2$ = –0.002, $n$ = 371, $P$ = 0.670); **(C)** latitude and leaf starch ($r^2$ = –0.002, $n$ = 376, $P$ = 0.814); **(D)** latitude and leaf total phenolics ($r^2$ = 0.005, $n$ = 383, $P$ = 0.098); **(E)** MAT and leaf soluble carbohydrates ($r^2$ = 0.011, $n$ = 392, $P$ = 0.020); **(F)** MAT and leaf free amino acids ($r^2$ = –0.002, $n$ = 380, $P$ = 0.784); **(G)** MAT and leaf starch ($r^2$ = 0.009, $n$ = 385, $P$ = 0.031); **(H)** MAT and leaf total phenolics ($r^2$ = –0.002, $n$ = 392, $P$ = 0.653); **(I)** altitude and leaf soluble carbohydrates ($r^2$ = –0.003, $n$ = 383, $P$ = 0.828); **(J)** altitude and leaf free amino acids ($r^2$ = 0.002, $n$ = 371, $P$ = 0.180); **(K)** altitude and leaf starch ($r^2$ = –0.002, $n$ = 376, $P$ = 0.736); **(L)** altitude and leaf total phenolics ($r^2$ = –0.002, $n$ = 383, $P$ = 0.298). All dates were log-transformed (base $e$). Bonferroni correction significance level threshold value, $P$ = 0.05/3.

sorted into species. Not all sites hosted all species. The aquatic macrophytes sampled in lakes and rivers included four life forms: 12 submerged macrophytes (*Potamogeton pectinatus*, *P. perfoliatus*, *P. lucens*, *P. malaianus*, *P. maackianus*, *P. crispus*, *Najas minor*, *Najas marina*, *Hydrilla verticillata*, *Ceratophyllum demersum*, *Myriophyllum verticillatum*, and *Vallisneria natans*), 6 floating-leaved macrophytes (*Potamogeton natans*, *Trapa bispinosa*, *Euryale ferox*, *Hydrocharis dubia*, *Nymphoides peltatum*, and *Polygonum amphibium*), 4 emergent macrophytes (*Zizania latifolia*, *Nelumbo nucifera*, *Scirpus validus*, and *Typha orientalis*) and 2 free-floating macrophytes (*Eichhornia crassipes* and *Lemna minor*). The macrophytes were collected, sorted into species, washed gently and brought to the laboratory where they were oven-dried at 80°C for 72 h to constant weight for further analysis (Su et al., 2016). A total of 392 samples were taken– 241, 90, 16, and 45 samples of submerged, floating-leaved, free-floating and emergent macrophytes, respectively. The dry samples of leaves were grounded into fine powder using a pestle and mortar for the analysis of SC, FAA, starch, TOPH, C, and N. Of each powder, about 100 mg was extracted with 10 mL 80% ethanol at 80°C for 20 min and then centrifuged for 15 min at 5,000 g (Cao et al., 2008, 2009). After centrifugation, the supernatant was used for determination of SC and FAA contents after reacting with anthrone and ninhydnn, respectively (Yemm and Willis, 1954; Yemm and Cocking, 1955) using alanine and glucose as standards. The supernatant was used for measurement of TOPH following the method described by Mole and Waterman (1987). Tannic acid (Sigma Chemical Company) was used as standard. The residue was used for the analysis of starch content following the method of Dirk et al. (1999). The C and N contents of all samples were determined applying an elemental analyzer (Flash EA 1112 series, CE Instruments, Italy).

## Data Analysis

We compared the statistical differences in leaf SC, FAA, starch, TOPH, C, and N and C:N (mass:mass) ratio for all the species and when divided into the four different life forms. To characterize the biogeographical gradient patterns of leaf stoichiometry, we first log-transformed (base *e*) the data of latitude, altitude, MAT [ln(MAT+5)], leaf SC, FAA, starch, TOPH, C, and N content and the C:N ratio for each species at each sampling site and then performed linear regressions for all the species and stepwise regressions for all the species, different life forms and specific species. Bonferroni correction was made as we tested three independent hypotheses on the same set of data. We also explored distribution patterns in the plant characteristica mentioned above.

The analyses were conducted with IBM SPSS Statistics 22. A sampling site map was produced using ArcMap 10.2. All figures were plotted using OriginPro 9.0.

# RESULTS

## The Contents of SC, FAA, Starch, TOPH, C, and N and the C:N Ratios of Aquatic Plants Across All Species

The contents of SC, FAA, starch, TOPH, C, and N and the C:N ratio in the leaves of the aquatic macrophytes varied greatly (**Figure 2**, **Supplementary Figure S1**, and **Supplementary Table S1**). The contents of SC, FAA, TOPH, C, and N and the C:N ratios fitted log-normal distributions, while an exponential model was most reliable for starch (**Supplementary Figure S1**). The coefficients of variation (CV) for the contents of SC, FAA, starch, TOPH, C, and N and the C:N ratios were 0.83, 0.64, 1.05, 1.17, 0.14, 0.32, and 0.44, respectively (**Supplementary Figure S1** and **Supplementary Table S2**).

Considering all macrophytes together, latitude correlated negatively with the C and N contents ($p < 0.05/3$; **Figure 3**, **Table 1**, and **Supplementary Table S3**), whereas no such correlation was found for the SC, FAA, starch, and TOPH contents (**Figure 4** and **Table 1**). Altitude correlated positively with the C content and the C:N ratio ($p < 0.05/3$ for both; **Figure 4**, **Table 1**, and **Supplementary Table S3**), but no correlation between altitude and N, SC, FAA, starch, and TOPH contents was observed (**Figure 4** and **Table 1**). However, there was no significant relationship between MAT and the stoichiometric characteristics (**Figures 3**, **4** and **Table 1**).

## The Contents of SC, FAA, Starch, TOPH, C, and N and the C:N Ratios of Aquatic Macrophytes With Different Life Forms and Species

The contents of SC, FAA, starch, TOPH, C, and N and the C:N ratios differed significantly among the macrophytes with different

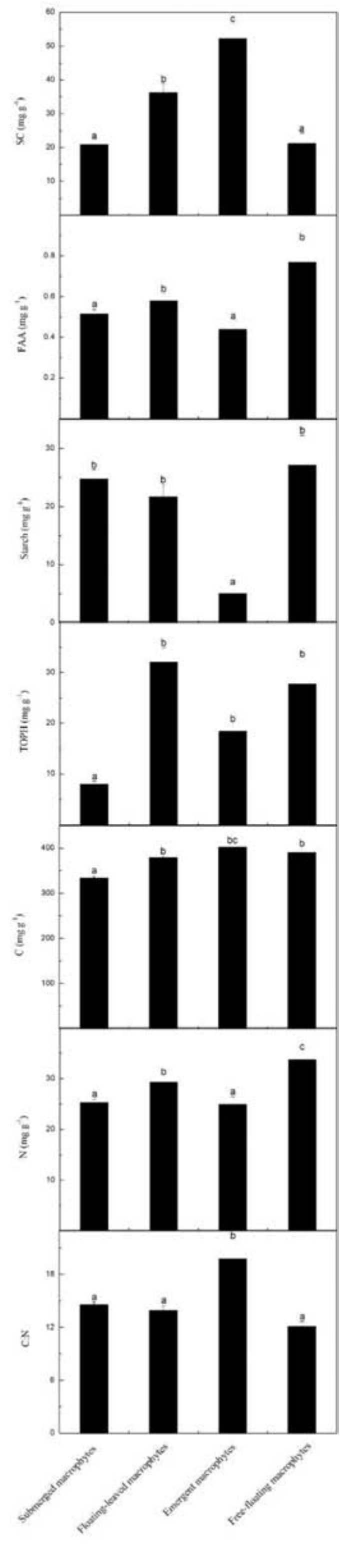


**FIGURE 5 |** Leaf stoichiometric characteristics across four different macrophyte life forms. Leaf soluble carbohydrates (mg $g^{-1}$), free amino acids (mg $g^{-1}$), starch (mg $g^{-1}$), total phenolics (mg $g^{-1}$), carbon (mg $g^{-1}$), nitrogen (mg $g^{-1}$) and the C:N ratio, mean ± SE. Different letters indicate significant differences.

life forms (**Figures 2**, **5**). Among the four life forms, emergent macrophytes had the highest SC and the lowest starch contents (**Figure 5**). The FAA content of floating-leaved and free-floating macrophytes was significantly higher than that of submerged and emergent macrophytes (**Figure 5**). Submerged macrophytes had the lowest TOPH and C contents, free-floating macrophytes had the highest N content and emergent macrophytes the highest C:N ratio (**Figure 5**). Of all the species, *S. validus* had the highest SC content and the highest C:N ratio, *E. crassipes* had the highest FAA content, *T. orientalis* and *V. natans* had the lowest starch and TOPH contents, respectively, and *N. minor* and *L. minor* had the highest C and N contents, respectively (**Figure 2**).

Overall, multiple regressions revealed that the stoichiometric characteristic of the submerged macrophytes varied significantly across the large spatial and climatic gradients studied (**Tables 2**, **3**). For submerged macrophytes, the stepwise regression revealed that the contents of SC and TOPH correlated negatively with MAT ($p < 0.05/3$; **Table 2**) and that the C, N, and TOPH contents had a marked negative correlation with latitude ($p < 0.05/3$ for both; **Table 2** and **Supplementary Table S4**), while the C content and the C:N ratios correlated positively with altitude and the N content negatively with altitude ($p < 0.01$ for all; **Table 2** and **Supplementary Table S4**). Significant linear trends between the C and N contents and the C:N ratios of three submerged species (*P. maackianus*, *P. crispus,* and *M. verticillatum*) and latitude, MAT and/or altitude were found ($p < 0.05/3$; **Table 3**). For floating-leaved and emergent macrophytes, no correlation between climate factors and SC, FAA, starch, TOPH, C, and N contents and C:N ratio was observed, but when refining to species level, the FAA, C, and N contents of *T. bispinosa*, *H. dubia* and *N. peltatum* demonstrated linear relationship with latitude, MAT and/or altitude ($p < 0.05/3$ for all; **Table 3**), the SC, TOPH and N contents and the C:N ratios of *T. orientalis* exhibited marked linear trends with altitude ($p < 0.05/3$ for both; **Table 3**). For free-floating macrophytes, the TOPH content had a marked positive correlation with latitude and altitude ($p < 0.05/3$ for both; **Table 2**), while the TOPH content and C:N ratio of *L. minor* increased with increasing latitude and MAT, respectively ($p < 0.05/3$ for both; **Table 3**).

## DISCUSSION

We found that the C and N stoichiometry of 24 aquatic macrophytes varied greatly across geographic and climatic gradients in China, indicating physiological plasticity. Considering all species, the C and N contents decreased significantly from low to high latitudes, which is in agreement with the results in the study of 753 terrestrial plants from across China undertaken by Han et al. (2005) and of 122 aquatic macrophytes in the eastern part of China (Xia et al., 2014), though the variance explained by the linear regression models in our study was low. In China, climatic factors and human activities vary greatly across the country's large geographic scales; thus, southern (lower latitude) areas experience higher temperatures and precipitation, less cloudiness and frost and more severe eutrophication than northern areas (Wu et al., 1999). This benefits the growth and assimilation of inorganic carbon and nutrients of aquatic plants, which might have contributed to the finding of enhanced C and N contents. In the present study, the C content of several aquatic species tended to increase with increasing altitude (though low variance was explained, which may be due to lack of nutrient data), possibly reflecting the fact that plants require higher C storage at the low $CO_2$ pressure in the Tibetan plateau (He et al., 2006; Yang et al., 2014). Although significant temperature changes occur across the large geographic scale applied in our study, it is surprising that the C and N contents did not correlate with MAT, implying that physiological processes might depend more on C and N availability (i.e., stem openness and surface area of roots, Cramer et al., 2001) than on MAT-related adjustment of enzyme activity (Reich and Oleksyn, 2004). SC, FAA, starch, and TOPH contents did not correlate with latitude, altitude and MAT. SC, FAA,

**TABLE 2 |** Linear regression models explaining the average leaf soluble carbohydrates, free amino acids, starch, total phenolics, carbon and nitrogen contents and the C:N ratio of different life forms.

| Life form | Dependent variable | *df* | *P* | $r^2_{adj}$ | Linear model |
|---|---|---|---|---|---|
| Submerged macrophytes | Ln SC | (1, 235) | 0.013 | 0.022 | 4.349*** – 0.532* ln MAT+5 |
| | Ln TOPH | (1, 235) | <0.01 | 0.052 | 5.791*** - 1.172*** ln latitude |
| | | (2, 234) | <0.001 | 0.079 | 8.152*** – 1.390*** ln latitude – 0.546** ln MAT+5 |
| | Ln C | (1, 201) | <0.001 | 0.151 | 7.465*** – 0.486*** ln latitude |
| | | (2, 100) | <0.001 | 0.170 | 7.073*** – 0.383*** ln latitude + 0.011* ln altitude |
| | Ln N | (1, 201) | 0.004 | 0.035 | 3.279*** – 0.028** ln altitude |
| | | (2, 200) | <0.001 | 0.089 | 6.018*** – 0.046*** ln altitude – 0.780*** ln Latitude |
| | Ln C:N | (1, 201) | <0.001 | 0.110 | 2.448*** + 0.047*** ln altitude |
| Free-floating macrophytes | Ln TOPH | (1, 24) | 0.009 | 0.224 | −10.583* + 4.076** ln latitude |
| | | (2, 23) | 0.004 | 0.332 | −19.143** + 6.302** ln latitude + 0.190* ln altitude |

*The models result from a forward stepwise selection procedure using the independent variables latitude, altitude and mean annual temperature. All significant (P < 0.05/3) regression models are shown. All dates were log-transformed (base e), the significance of the regression cofficients is indicated by *P < 0.05, **P < 0.01, ***P < 0.001, without asterisk P > 0.05. Bonferroni correction significance level threshold value, P = 0.05/3.*

starch, and TOPH are intermediates of C and N metabolim and account for a small proportion of the C and N contents, and they may therefore respond rapidly and flexibly to local habitat alterations. This contrasts with the contents of C and N that are largely structural compounds in plant tissue and may be indicative of changes in the regional environment (Elser et al., 2000; Reich and Oleksyn, 2004; Han et al., 2005, 2011; He et al., 2006).

We found significant differences in the contents of SC, FAA, starch, TOPH, C, and N and in C:N ratios among the macrophytes with different life forms. As $CO_2$ diffuses 10,000 times less in water than in air and light availability attenuates

**TABLE 3 |** Linear regression models explaining the average leaf soluble carbohydrates, free amino acids, starch, total phenolics, carbon and nitrogen contents and the C:N ratio of different species.

| Life form | Genus | Dependent variable | *df* | *P* | $r^2_{adj}$ | Linear model |
|---|---|---|---|---|---|---|
| Submerged macrophytes | *P. pectinatus* | Ln C | (1, 22) | 0.003 | 0.310 | 5.721*** + 0.024** ln altitude |
| | *P. perfoliatus* | Ln C | (1, 4) | 0.009 | 0.814 | 7.675*** – 0.534** ln latitude |
| | | Ln C:N | (1, 4) | 0.017 | 0.747 | −0.339 + 1.173* ln MAT+5 |
| | | | (2, 3) | 0.006 | 0.947 | 1.614 – 0.257* ln altitude + 1.170** ln MAT+5 |
| | *P. malaianus* | Ln TOPH | (1, 20) | 0.012 | 0.240 | 8.207** – 1.891* ln latitude |
| | | Ln C | (1, 16) | 0.003 | 0.398 | 7.737*** – 0.548** ln latitude |
| | *P. maackianus* | Ln starch | (1, 14) | 0.006 | 0.380 | −7.315* + 3.489** ln MAT+5 |
| | | | (2, 13) | 0.003 | 0.543 | −20.374** + 4.300* ln latitude + 3.039** ln MAT+5 |
| | | | (3, 12) | 0.001 | 0.670 | −63.164** + 14.426** ln latitude + 0.399* ln altitude + 5.392** ln MAT+5 |
| | | Ln N | (1, 11) | 0.006 | 0.463 | 6.708*** – 1.212** ln Altitude |
| | | Ln C:N | (1, 11) | 0.008 | 0.446 | −0.253 + 1.010** ln MAT+5 |
| | *P. crispus* | Ln TOPH | (1, 21) | <0.001 | 0.491 | 1.143*** + 0.165*** ln Altitude |
| | | Ln N | (1, 20) | 0.003 | 0.324 | −2.103 + 1.779** ln MAT+5 |
| | | Ln C:N | (1, 20) | 0.005 | 0.304 | 7.639*** – 1.687** ln MAT+5 |
| | | | (2, 19) | 0.002 | 0.421 | 7.924*** + 0.051* ln altitude – 1.835** ln MAT+5 |
| | *N. minor* | Ln C | (1, 3) | 0.002 | 0.958 | 12.060*** – 1.838** ln latitude |
| | | Ln N | (1, 3) | <0.001 | 0.996 | 10.643*** – 2.186*** ln latitude |
| | *C. demersum* | Ln N | (1, 25) | 0.010 | 0.206 | −2.356 + 1.681* ln latitude |
| | | Ln C:N | (1, 25) | 0.002 | 0.287 | 8.478*** – 1.780** ln latitude |
| | *M. verticillatum* | Ln TOPH | (1, 42) | 0.001 | 0.230 | 13.481*** – 3.267** ln latitude |
| | | Ln C | (1, 35) | 0.001 | 0.271 | 8.345*** – 0.749** ln latitude |
| | | Ln N | (1, 35) | 0.007 | 0.167 | 1.840*** + 0.408** ln MAT+5 |
| | | Ln C:N | (1, 35) | 0.002 | 0.212 | 3.956*** - 0.415** ln MAT+5 |
| | *V. natans* | Ln C:N | (1, 23) | 0.003 | 0.305 | 6.542*** – 1.196** ln latitude |
| Floating-leaved macrophytes | *T. bispinosa* | Ln FAA | (1, 35) | 0.002 | 0.227 | 10.493** – 3.710** ln MAT+5 |
| | | Ln C | (1, 35) | 0.005 | 0.180 | 5.895*** + 0.017** ln altitude |
| | *H. dubia* | Ln C | (1, 13) | 0.017 | 0.314 | 6.828*** – 0.269* ln latitude |
| | *N. peltatum* | Ln N | (1, 9) | 0.017 | 0.428 | 9.085 – 1.640* ln Latitude |
| Emergent macrophytes | *N. nucifera* | Ln TOPH | (1, 7) | 0.001 | 0.787 | 11.411*** – 2.367** ln latitude |
| | *S. validus* | Ln C | (1, 4) | 0.016 | 0.751 | 5.829*** + 0.054* ln MAT+5 |
| | *T. orientalis* | Ln SC | (1, 11) | 0.004 | 0.497 | 3.266*** + 0.117** ln altitude |
| | | Ln TOPH | (1, 11) | 0.010 | 0.423 | 2.455*** + 0.102* ln altitude |
| | | Ln N | (1, 10) | 0.011 | 0.439 | 3.277*** – 0.132* ln altitude |
| | | Ln C:N | (1, 10) | 0.007 | 0.481 | 2.718*** + 0.134** ln altitude |
| Free-floating macrophytes | *L. minor* | Ln C:N | (1, 5) | 0.005 | 0.786 | 0.626 + 0.575** ln MAT+5 |
| | | Ln TOPH | (1, 5) | 0.001 | 0.872 | −4.274** + 1.921** ln latitude |

*The models result from a forward stepwise selection procedure using the independent variables latitude, altitude and mean annual temperature. All significant (P < 0.05/3) regression models are shown. All dates were log-transformed (base e), the significance of the regression cofficients is indicated by *P < 0.05, **P < 0.01, ***P < 0.001, without asterisk P > 0.05. Bonferroni correction significance level threshold value, P = 0.05/3.*

exponentially through the water column, inorganic C and light availability vary extensively from the shore to the deep water (Cao et al., 2008). Expectedly, submerged macrophytes experience much lower $CO_2$ and light availability than emergent and free-floating macrophytes, while floating-leaved macrophytes experience intermediate $CO_2$ and light availability in fluctuating water (Cao et al., 2008), and these factors may contribute to the differences in C contents. Aquatic macrophytes can take up nutrients from both sediment and water (Rattray et al., 1991; Madsen and Cedergreen, 2010; Cao et al., 2011; Li et al., 2013). Rooted macrophytes absorb nutrients mainly from the sediment, while free-floating macrophytes primarily absorb nutrients from the water column (Barko and Smart, 1986; Fernández-Aláez et al., 1999). We found that the N content was significantly higher in the free-floating plants than in the other life forms, which is in line with the results reported by Xia et al. (2014), possibly reflecting the simplified mechanical support structure required by this life form compared with the other forms studied (Bonser and Geber, 2005; Weijschedé et al., 2006).

The C metabolic pathway of all aquatic macrophytes in the current study were C3. At life forms level, a significant correlation was found between stoichiometric characteristics such as C, N or C:N with one or more climatic variables (latitude, altitude, and MAT) for submerged macrophytes (*P. pectinatus*, *P. perfoliatus*, *P. malaianus*, *P. maackianus*, *P. crispus*, *N. minor*, *C. demersum*, *M. verticillatum*, and *V. natans*), while the other life forms characteristics were not better related to the climate variables. Whether this difference reflects differences in the access to C (lower for submerged macrophytes) and N with implications for the metabolites remains to be elucidated. However, the correlations of leaf stoichiometric characteristics with the three climate variables were more significant for generalist species (e.g., *P. maackianus*, *P. crispus*, and *M. verticillatum*), than for specialized species. This indicates a more obvious physiological plasticity of generalist species across the geographic and climatic gradients in China, which is useful information for lake managers in the restoration of lost aquatic plants in lakes different climate regions.

Our study allows us to draw the following conclusions: (1) the C and N stoichiometry of aquatic macrophytes varied greatly across the large geographic and climatic study gradient; (2) among the different life forms, the C and N contents related more closely than the C and N metabolic indicators to latitude, altitude, or MAT for the submerged macrophyte life form, while the relationships with metabolic indicators turned out to be insignificant for most species of the other life forms. As we have no nutrient data from these sites we cannot fully rule out that some of the relationships we found are not affected, in part, by systematic variation in nutrient concentrations along the climate gradients used (latitude, altitude, and MAT).

## AUTHOR CONTRIBUTIONS

QC was responsible for sample processing, data analysis, and draft completion. TC was responsible for the collection of samples. LN was responsible for samples collection. PX gaves guidance during sample processing and data analysis. EJ gave his opinions during the data analysis process and gave a lot of comments during the revision of the draft.

## FUNDING

This study was supported by the State Key Laboratory of Freshwater Ecology and Biotechnology (Grant No. 2016FBZ08), the Natural Science Foundation of Jiangxi Province (Grant No. 2015ZBBF6008) and the Major Science and Technology Program for Water Pollution Control and Treatment (Grant No. 2012ZX07105-004). The work was also partly supported by a scholarship awarded to QC from the China Scholarship Council (Grant No. 201704910729) enabling a visit to Aarhus University. EJ was supported by the AU Centre for Water Technology (watec.au.dk).

## ACKNOWLEDGMENTS

We are grateful to A. M. Poulsen for manuscript editing and A. Nielsen and D. Trolle for providing the comfortable working environment and giving the comments during the revision of the manuscript.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00442/full#supplementary-material

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling Editor is currently co-organizing a Research Topic with one of the authors TC, and confirms the absence of any other collaboration.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 16 April 2019
doi: 10.3389/fpls.2019.00485

# Developmentally Programmed Division of Labor in the Aquatic Invader *Alternanthera philoxeroides* Under Homogeneous Soil Nutrients

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Clonal traits can contribute to plant invasiveness, but little is known about the roles of division of labor (a key clonal trait) in homogeneous habitats. The hypothesis tested is that clonal integration allows division of labor and increases the overall performance of an invasive clonal plant, especially under higher soil nutrients. Clonal fragment pairs of aquatic invader *Alternanthera philoxeroides* (each with four ramets and a stolon apex) were grown in two homogenous habitats with high or low soil nutrient supply, and with stolon connections being either severed (clonal integration prevented) or kept intact (clonal integration allowed). Results showed that stolon connection allowed the division of labor within the clonal fragment, with basal ramets specializing in acquisition of belowground resources and apical ramets specializing in acquisition of aboveground expansion. Moreover, the capacity for division of labor was greater, which brought the clonal fragments of *A. philoxeroides* stronger clonal propagation and better performance in high nutrient habitats than in low nutrient habitats. The results supported our hypotheses that the developmentally programmed division of labor may facilitate the clonal expansion of this aggressive invader in some homogeneous habitats with high resource availability.

**Keywords: alligator weed, biomass allocation, chlorophyll fluorescence, clonal growth, physiological integration**

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***Edited by:***
*Chunhua Liu,*
*Wuhan University, China*

***Reviewed by:***
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*Taizhou University, China*
*Yao-Bin Song,*
*Hangzhou Normal University, China*

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***Specialty section:***
*This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science*

***Received:*** *20 August 2018*
***Accepted:*** *29 March 2019*
***Published:*** *16 April 2019*

***Citation:***
*Xi D-G, You W-H, Hu A-A, Huang P and Du D-L (2019) Developmentally Programmed Division of Labor in the Aquatic Invader Alternanthera philoxeroides Under Homogeneous Soil Nutrients. Front. Plant Sci. 10:485. doi: 10.3389/fpls.2019.00485*

## INTRODUCTION

Invasive plants have greatly threatened biodiversity, the environment and economic development worldwide (Mack et al., 2000; Vila et al., 2011; van Kleunen et al., 2015). While the mechanisms that contribute to the successful invasion of exotic plants remain unsolved (Alpert et al., 2000; Levine et al., 2003; Vila et al., 2011), an emerging pattern in plant invasion is that a great number of aggressive invaders are clonal plants (Kolar and Lodge, 2001; Liu et al., 2006; Xu et al., 2010; You et al., 2013; Keser et al., 2014; Roiloa et al., 2016). Recently, an increasing number of studies have pointed out that the clonal traits such as clonal integration and division of labor may contribute to the invasiveness of these invaders (Aguilera et al., 2010; Roiloa et al., 2010; Song et al., 2013; You et al., 2014a, 2016a; Roiloa et al., 2016; Wang Y.J. et al., 2017). However, so far, the knowledge of contribution of clonal traits on invasion success of these clonal invaders is still limited (Song et al., 2013; Keser et al., 2014; Wang Y.J. et al., 2017).

A key clonal trait is the capacity for division of labor (Hutchings and Wijesinghe, 1997; Stuefer, 1998), which is mediated by physiological integration and driven by the source–sink relationship (Roiloa and Retuerto, 2006; You et al., 2013). Due to physiological integration, when two essential resources are heterogeneously distributed (negatively correlated), the connected ramets can specialize to acquire the resource that is relatively more abundant within the clone to enhance the overall performance of the clone (Hutchings and Wijesinghe, 1997; Stuefer, 1998; Roiloa et al., 2007). Such specialization is called "division of labor," which is environmentally induced (Stuefer, 1998; Roiloa et al., 2016). In comparison, when the clone consists of the ramets that are in different developmental stages, or differing in ability to acquire resources, the connected ramets within the clone may get different amounts of resources even with the same external resource supply (Dong et al., 2015). In this case, division of labor and physiological integration between ramets may still increase plant performance even in the homogeneous environments, with relatively older ramets specializing in taking up belowground resources and relatively younger ramets specializing in aboveground resources and spread (Stuefer, 1998; Roiloa et al., 2013). Ramet specialization in such homogeneous environments is termed "developmentally programmed division of labor" (Stuefer, 1998; Roiloa et al., 2013), which is inherent in clonal plants (Roiloa et al., 2013). Although division of labor is beneficial to clonal plants, the importance of ramet specialization for the invasiveness of clonal plants is still far from clear (Roiloa et al., 2016).

The essential resources for plants in many habitats are commonly heterogeneously distributed (Caldwell and Pearce, 2012), however, other natural and some anthropogenic habitats are relatively homogeneous (Dutilleul, 2011; Dong et al., 2015). For example, some shallow rivers and wetlands where water movement can homogenize the habitats, and many anthropogenic habitats such as irrigation ditches and crop lands could be homogeneous (Dong et al., 2015). These homogeneous habitats are expected to be easier to invade by exotic plants because of their relatively lower biodiversity than other natural habitats (Levine and D'Antonio, 1999; You et al., 2016a). However, very few studies have investigated the role of the division of labor in shaping the invasion success of plant invaders in homogeneous habitats (Roiloa et al., 2013; Wang P. et al., 2017). Recently, through a conceptual model, Dong et al. (2015) discovered that physiological integration may also increase the performance of a notorious plant invader when its connected ramets differ in ability to acquire resources in homogeneous environments with high resource supply. Based on this conceptual model, it can be predicted that clonal plants containing ramets that are in different developmental stages or differing in ability to acquire resources may have a greater capacity for the division of labor, and thereby benefit from physiological integration more in homogenous habitats with high resource supply than with low resource supply. Unfortunately, no previous study has tested this prediction.

Using clonal fragments of an amphibious clonal invader *Alternanthera philoxeroides*, we conducted a greenhouse experiment to test the above prediction. We grew ramet pairs of *A. philoxeroides* in two homogenous habitats, either with high soil nutrient supply or with low soil nutrient supply, with stolon connections either severed (clonal integration prevented) or kept intact (clonal integration allowed). Here, we tested the following hypotheses: (1) Stolon connection (clonal integration) allows division of labor between ramets of *A. philoxeroides* clonal fragments in homogenous habitats. We test if the division of labor happens for *A. philoxeroides* in homogeneous habitats in term of the biomass allocation (i.e., root mass to shoot mass ratio). Considering that the connected ramets of *A. philoxeroides* are in different developmental stages (i.e., basal ramets are relatively older and apical ramets are relatively younger), we expect that stolon connection will increase biomass allocation to roots of basal (older) ramets (specialization in the uptake of belowground resources). Meanwhile, we expect that stolon connection will increase biomass to shoots and enhance the photochemical efficiency (as indicators of the energy allocated to harvest aboveground resources) of apical (younger) ramets. (2) Clonal integration increases the overall performance of the clonal fragments. We predict higher photochemical efficiency, clonal propagation and biomass production, resulting in higher growth capacity and invasiveness in connected clonal fragments than severed ones. (3) The capacity for division of labor is stronger and its benefits to *A. philoxeroides* clonal fragment is greater when grown in high soil nutrient than in low soil nutrients. Based on the conceptual model presented by Dong et al. (2015), we expect a greater division of labor (i.e., basal ramets specializing in taking up belowground resources and apical ramets specializing in the uptake of aboveground resources), resulting in higher performance of clonal fragments grown in high soil nutrient than in low soil nutrients.

## MATERIALS AND METHODS

### Plant Species

*Alternanthera philoxeroides* (Mart.) Griseb. (Amaranthaceae), commonly called "alligator weed," is an amphibious, perennial clonal plant native to South America (Julien et al., 1995). It has caused serious environmental and economic problems both locally and globally (Julien et al., 1995; Gunasekera and Bonila, 2001). *A. philoxeroides* rarely produces seeds and propagates vegetatively by stems and root buds (Julien et al., 1995; Schooler, 2012). In its introduced regions, *A. philoxeroides* has invaded widely from aquatic to terrestrial habitats, such as irrigation ditches and crop lands where are relatively homogenous due to anthropogenic activities (Geng et al., 2007; Dong et al., 2015; You et al., 2016a). In China, the genetic diversity of *A. philoxeroides* is extremely low (Xu et al., 2003; Wang et al., 2005), and an increasing number of studies have demonstrated that physiological integration and the division of labor may determine its growth and spread (Wang et al., 2008; Yu et al., 2009; You et al., 2016b, 2018).

### Experimental Design

In mid-April 2016, we collected plant material of *A. philoxeroides* from the surrounding wetlands of Gonghu Bay in the Taihu Lake, Jiangsu province of China (N 31°25′–31°28′, E 120°15′–120°21′) and then propagated them in a greenhouse. In this experiment, we used 32 clonal fragments of *A. philoxeroides* in similar size (tip cuttings, 14.32 ± 0.16 cm in length, 0.37 ± 0.07 g in dry mass; means ± SE) as experimental material. Each clonal fragment contained four ramets with a stolon apex, which were divided into two parts: a "basal part" with two relatively old ramets (distal to the tip), an "apical part" with two relatively young ramets (close to the tip) and a stolon apex (see **Figure 1**). Within each clonal fragment, the basal part was placed in the basal pot, and the apical part was placed in the apical pot (**Figure 1**).

The experiment was conducted in a greenhouse in the Field Station of Jiangsu University, by a full factorial design with stolon connection (stolon connections were intact or severed) and soil nutrient (high nutrient or low nutrient) as fixed factors. For the stolon connection treatment, the connection between basal and apical ramets in each clonal fragment was either severed (clonal integration prevented) or kept intact (clonal integration allowed). After the two original ramets of both apical and basal part rooted, the stolon connections were cut halfway between the basal and apical ramets. There was no negative effect of the severing treatment observed during the experiment (immediate death or disease). New ramets for both basal and apical part produced during the experiment were not allowed to root. All the experimental pots (12 cm in upper diameter, 8.8 cm in bottom diameter and 10.8 cm tall) were filled with the mixture of clean river sand and green zeolite (water retention) at a volume of 3: 1. For high and low soil nutrient treatments, the experimental pots were mixed evenly with 2 g and 0.4 g of slow-release fertilizer (Osmocote$^R$, N–P–K: 16–9–12) powder, respectively. Each treatment combination was replicated eight times ($n$ = 8). During the experimental period, the mean light intensity was 1200–1500 μmol m$^{-2}$ s$^{-1}$ at noon and the mean air temperature was 25–28°C in the greenhouse. To mimic a natural wetland habitat condition, plants were watered regularly to keep the soil with an overlying water of 2 cm deep. The experiment was conducted for 9 weeks and ended on June 28, 2016.

## Measurements

The chlorophyll fluorescence was measured 3 days before the final harvest. According to the saturation pulse method (Schreiber et al., 1998; Maxwell and Johnson, 2000), after a more than 20 min' dark adaptation, the minimum ($F_0$) and the maximum ($F_m$) fluorescence yield were measured on a healthy mature leaf of the second-youngest ramet by a portable chlorophyll fluorometer (PAM-2100, Walz, Effeltrich, Germany). $(F_m - F_0)/F_m$ was defined as the maximum quantum yield of PSII ($F_v/F_m$). Similarly, after an actinic light pulse of 120 μmol m$^{-2}$ s$^{-1}$ for 10 s, $F'_m$ is measured as the maximal fluorescence yield reached in a pulse of saturating light, and $F_t$ is the fluorescence yield of the leaf at that photosynthetic photon flux density. Then the effective quantum yield of PS II (Yield) was calculated as $(F'_m - F_t)/F'_m$ (Roiloa and Retuerto, 2006; You et al., 2013). The chlorophyll content index (the relative chlorophyll content) was also measured by a portable chlorophyll meter (TYS-A, TOP, Zhejiang, China). The leaves used for measuring the relative chlorophyll content were opposite to the leaves used for determination of $F_v/F_m$ and Yield.

Nine weeks after the beginning of the experiment, all the clonal fragments were harvested. The number of ramets (i.e., number of nodes) were counted, and the total stolon length (e.g., the sum of main stolon length and branch stolon length) were measured for both apical and basal ramets. Then, all the *A. philoxeroides* plants were separated into leaves, stolons, and roots, and each part was weighed after drying to constant weight at 70°C for 72 h.

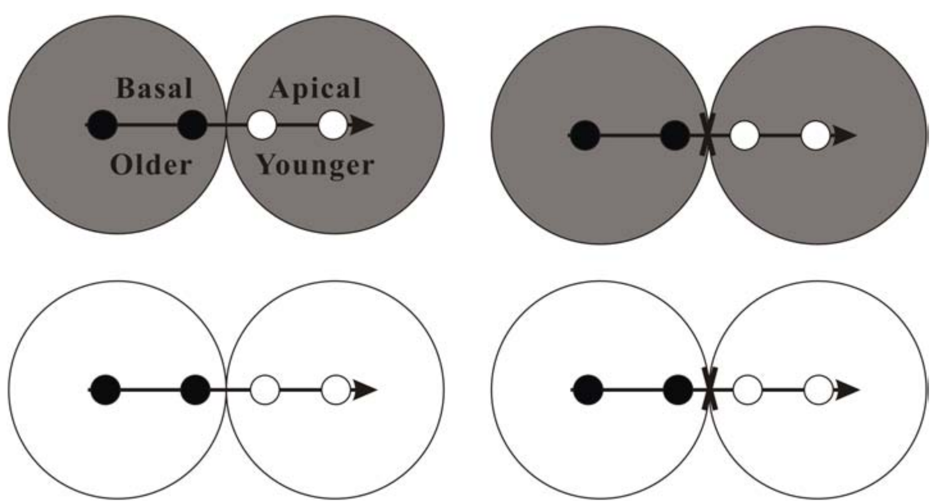


**FIGURE 1 |** Schematic representation of the experimental design. There were four experimental treatments involving stolon connection and soil nutrient availability. Clonal fragments of the invasive plant *A. philoxeroides*, each consisting of two basal ramets (black circles) and two apical ramets (white circles) with a stolon apex (horizontal arrow), were grown either under high soil nutrient availability (gray) or under low soil nutrient availability (white), and with the stolon connections between basal and apical ramets were either intact or severed (fork). See text for additional explanation.

## Statistical Analysis

Before the analysis, to meet the assumptions of normality and homoscedasticity, the data were log-transformed (the proportions were angular transformed) if necessary. The growth measurements (final biomass, total stolon length, and ramet number) for the apical part, the basal part and the whole clonal fragment, the physiological measurements ($F_v/F_m$, Yield and chlorophyll content index) and root mass/shoot mass ratios (hereafter denoted by R/S ratio) for the apical part and the basal part were analyzed by Two-way ANOVAs, using stolon connection and soil nutrient level as fixed factors. To examine differences between the treatments, we used Studentized Tukey's HSD for multiple comparisons. Statistical significance was assigned at $P < 0.05$. All data analyses were performed by SPSS 18.0 (SPSS, Chicago, IL, United States).

# RESULTS

## Biomass Allocation and Growth

Soil nutrient, stolon connection and their interaction significantly affected biomass allocated to roots of both basal (older) and apical (younger) ramets, as determined by the R/S ratio (**Table 1**). Stolon connection greatly increased the proportion of biomass allocated to roots in basal ramets (**Figure 2A**) whereas decreased it in apical ramets (the effect of stolon connection on R/S ratio for apical ramets in low nutrient treatment was marginally significant, $P = 0.09$) (**Figure 2B**). Such effects of stolon connection on basal and apical ramets were significantly stronger when *A. philoxeroides* was grown in high soil nutrients than in low soil nutrients, as demonstrated by the significant effects of nutrient × stolon connection (**Figure 2** and **Table 1**).

**TABLE 1 |** Two-way ANOVA analyses for the effects of soil nutrient and stolon connection on the growth (final biomass, total stolon length, and total node number) and root mass to shoot mass ratio (R/S ratio) of *Alternanthera philoxeroides* for the basal part, apical part, and whole clonal fragment.

| Dependent variable | Nutrient (N) | Connection (C) | N × C |
|---|---|---|---|
| **Basal** | | | |
| Final biomass | 197.41*** | 14.21** | 4.12* |
| Total stolon length | 84.08*** | 22.74*** | 0.10 |
| Total node number | 65.98*** | 13.30** | 0.15 |
| R/S ratio | 8.37** | 31.74** | 4.91* |
| **Apical** | | | |
| Final biomass | 121.03*** | 25.62*** | 1.77 |
| Total stolon length | 92.96*** | 31.36*** | 4.94* |
| Total node number | 57.05*** | 30.49*** | 4.80* |
| R/S ratio | 27.63*** | 18.81*** | 7.88** |
| **Whole fragment** | | | |
| Final biomass | 242.66*** | 5.20* | 3.98* |
| Total stolon length | 220.48* | 7.02* | 4.80* |
| Total node number | 166.19*** | 8.55** | 4.01* |
| d.f. | 1.28 | 1.28 | 1.28 |

*Values give F; significant P-values are indicated by *. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.*

All the growth measures (e.g., final biomass, ramet number, and total stolon length) of basal and apical ramets were significantly affected by soil nutrient and stolon connection (**Table 1**). The final biomass of basal ramets, total solon length and ramet number of apical ramets were also significantly affected by soil nutrient × stolon connection (**Table 1**). High nutrient treatment greatly promoted the growth measures of both basal and apical ramets (**Figure 3**). Stolon connection significantly decreased the growth of basal ramets (except the final biomass of *A. philoxeroides* grown in high soil nutrient) (**Figures 3A,C,E**), whereas the growth of apical ramets were greatly improved by stolon connection, and such positive effects on apical ramets were stronger when *A. philoxeroides* was grown in high soil nutrient than in low soil nutrient, as demonstrated by the significant effects of nutrient × stolon connection for total stolon length and ramet number (**Figures 3B,D,F** and **Table 1**).

The growth of whole clonal fragments (basal + apical ramets) were significantly influenced by soil nutrient, stolon connection and soil nutrient × stolon connection (**Table 2**). The growth of whole fragments were greatly enhanced by high nutrient supply (**Figure 4**). When grown in low soil nutrient, stolon connection had no significant effect on the growth of clonal fragments (**Figure 4**), however, stolon connection significantly increased plant growth when clonal fragments were grown with high soil nutrient supply (**Figure 4**).

## Chlorophyll Fluorescence and Content

The chlorophyll fluorescence measurements (Yield and $F_v/F_m$) and chlorophyll content index of basal ramets were only significantly affected by nutrient treatment (nutrient × stolon connection effect on the chlorophyll content index was also significant), whereas these values of apical ramets were significantly influenced by nutrient and stolon connection (**Table 2**). High nutrient treatment greatly improved plant chlorophyll fluorescence and content for both basal and apical ramets (**Figure 5**). However, stolon connection and its interaction with nutrient treatment had no significant effect on chlorophyll performance of basal ramets (**Figures 5A,C,E**). For the apical ramets, stolon connection had no significant effect on chlorophyll fluorescence and content in low soil nutrient, whereas it greatly enhanced the Yield of *A. philoxeroides* grown in high soil nutrient (**Figures 5B,D,F**).

# DISCUSSION

As hypothesized, the specialization of basal ramets in root production and apical ramets in shoot production and photosynthetic performance (as estimated by the Yield) were observed within the clonal fragment, supporting the occurrence of the division of labor for *A. philoxeroides* in homogeneous habitats at both morphological and physiological levels. Moreover, we found that the connected apical younger ramets invested more biomass to aboveground structures, resulting in a stronger clonal propagation (estimated by total stolon length and ramet number) and thus greater lateral expansion in both high and low nutrient conditions (Roiloa et al., 2013;

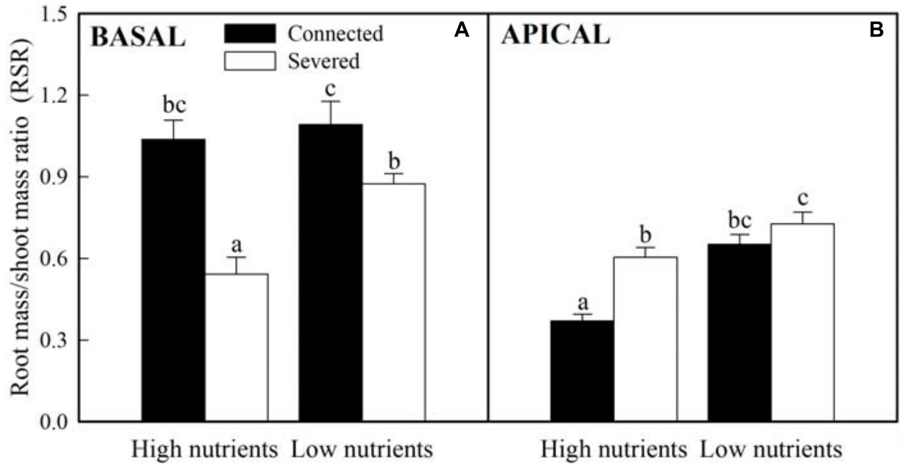


**FIGURE 2 |** Effects of experimental treatments on the root to shoot mass ratio of basal **(A)** and apical **(B)** ramets of *A. philoxeroide*. The data indicate the means + SE ($n$ = 8). The bars sharing the same letter are not significantly different at $P$ = 0.05.

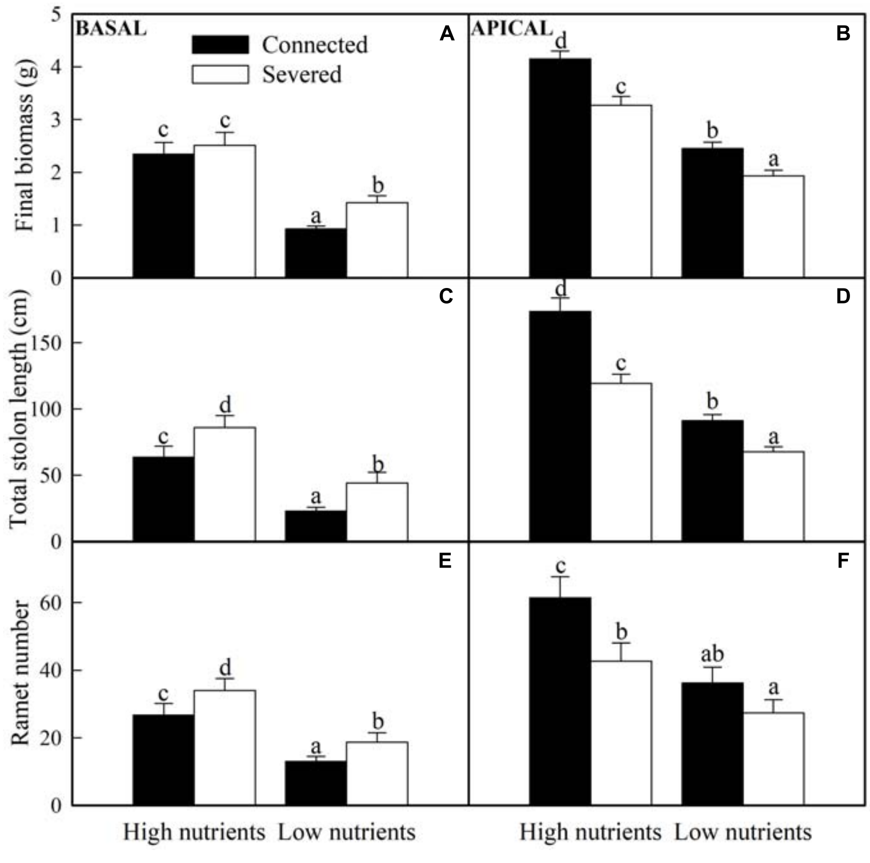


**FIGURE 3 |** Effects of experimental treatments on the final biomass, total stolon length and ramet number of basal **(A,C,E)** and apical **(B,D,F)** ramets of *A. philoxeroide*. The data indicate the means + SE ($n$ = 8). The bars sharing the same letter are not significantly different at $P$ = 0.05.

You et al., 2016a). These findings agree with those obtained in previous studies on several invasive clonal plants, including *A. philoxeroides* (Wang et al., 2008; You et al., 2014b, 2016a, 2018; Wang P. et al., 2017), *Myriophyllum aquaticum* (You et al., 2013), and *Eichhornia crassipes* (Lyu et al., 2016), which demonstrated that clonal integration can improve clonal propagation and performance of younger ramets and thus allow them to occupy surrounding new space in natural habitats.

**TABLE 2 |** Two-way ANOVA analyses for the effects of soil nutrient and stolon connection on the chlorophyll content index and chlorophyll fluorescence (yield and $F_v/F_m$) of *Alternanthera philoxeroides* for the basal part, apical part, and whole clonal fragment.

| Dependent variable | Nutrient (N) | Connection (C) | N × C |
|---|---|---|---|
| **Basal** | | | |
| Chlorophyll content index | 14.72** | 2.49 | 1.53* |
| Yield | 35.83*** | 2.08 | 0.10 |
| $F_v/F_m$ | 87.00*** | 0.45 | 0.09 |
| **Apical** | | | |
| Chlorophyll content index | 16.82*** | 5.45* | 0.30 |
| Yield | 65.14*** | 27.60*** | 4.58* |
| $F_v/F_m$ | 131.47*** | 5.73* | 0.41 |
| d.f. | 1.28 | 1.28 | 1.28 |

*Values give F; significant P-values are indicated by *. *P < 0.05, **P < 0.01, and ***P < 0.001.*

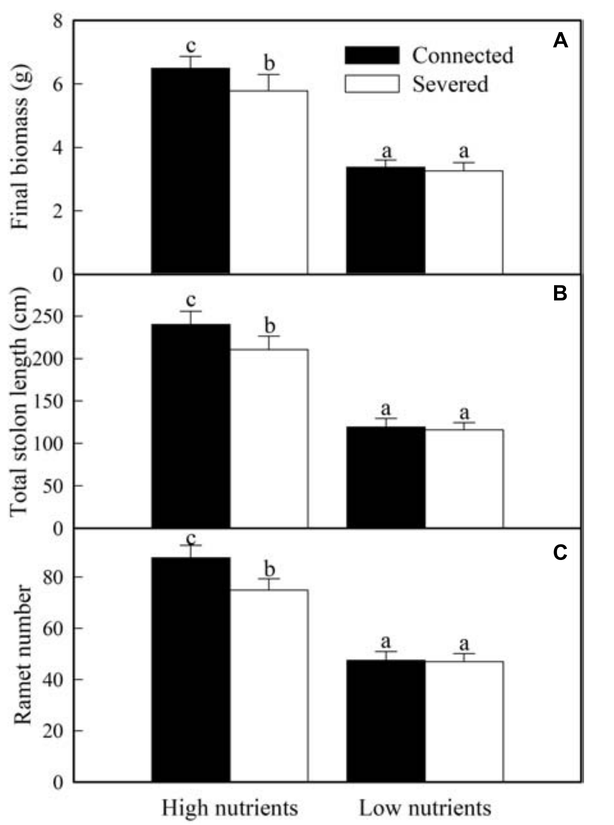


**FIGURE 4 |** Effects of experimental treatments on the final biomass **(A)**, total stolon length **(B)**, and ramet number **(C)** of the whole clonal fragment of *A. philoxeroide*. The data indicate the means + SE ($n$ = 8). The bars sharing the same letter are not significantly different at $P$ = 0.05.

As predicted, the division of labor for *A. philoxeroides* was greater in the high nutrient habitat than in the low nutrient habitat (stolon connection increased R/S ratio of the basal ramets more, whereas decreased R/S ratio of apical ramets more, under high soil nutrient conditions than under low soil nutrient conditions), which brought higher clonal propagation (in terms of total stolon length and ramet number) of younger ramets grown in high soil nutrient. This is probably because there was little resource to share for the connected ramets under low nutrient availability, and the division of labor may be relatively weak in such low nutrient conditions (You et al., 2016b). We even detected that the effect of stolon connection on R/S ratio of apical ramets in low nutrient treatment was only marginally significant, suggesting that the factor most limiting the growth of the connected clonal fragments under low soil nutrient conditions might be soil nutrients but not light or lateral expansion (You et al., 2016b). When grown with high nutrient supply, considering that the connected ramets within the fragment are in different developmental stages and thus with different resource storage in the stolons and internodes (Song et al., 2014), and differing in ability to acquire resources (e.g., basal ramets are relatively older with more abundant established roots, whereas apical ramets are relatively younger with less root production), basal older ramets specializing in the acquisition of soil-based resources (such as nutrient and water) in the high-resource condition were considered to be more economical and effective, and apical younger ramets specializing in acquisition of aboveground expansion can facilitate the invasion of clonal invader (Roiloa et al., 2013; You et al., 2016a). Similarly, Wang P. et al. (2017) also detected that clonal integration can increase the new node production and growth of younger ramets of *A. philoxeroides* in homogeneous habitats, especially with higher nitrogen supply, suggesting that clonal integration (division of labor allowed) should be crucial for lateral expansion of this plant invader in some homogeneous habitats (You et al., 2016a).

Although stolon connection allowing the division of labor had positive effects on the performance of apical ramets in both high and low nutrient treatments, such positive effects were generally at the expense of the performance decrease for basal ramets (only the final biomass of basal ramets under high nutrients was not significantly decreased by stolon connection). As a result, stolon connection had different effects on the growth performance of the whole clonal fragments in different soil nutrient conditions. In the low nutrient condition, clonal integration had no significant effect on the growth performance of clonal fragments. This result agreed with Wang P. et al. (2017), who found that clonal integration did not affect clonal performance of *A. philoxeroides* at clonal fragment level in N-limited homogeneous environments. However, clonal integration greatly increased the growth and clonal propagation of clonal fragments in the high nutrient condition. These results occurred most likely because, the capacity for the division of labor of *A. philoxeroides* was greater, which brought it higher benefits at the clonal fragment level in the high nutrient condition than in the low nutrient condition (Dong et al., 2015; You et al., 2016b). Our findings provide strong evidence to support the conceptual model proposed by Dong et al. (2015), which demonstrated that physiological integration (division of labor allowed) may also have a positive effect on the performance of *A. philoxeroides* in homogeneous environments with high resource supply, suggesting that the division of labor may be more important for the invasion of *A. philoxeroides* in homogeneous

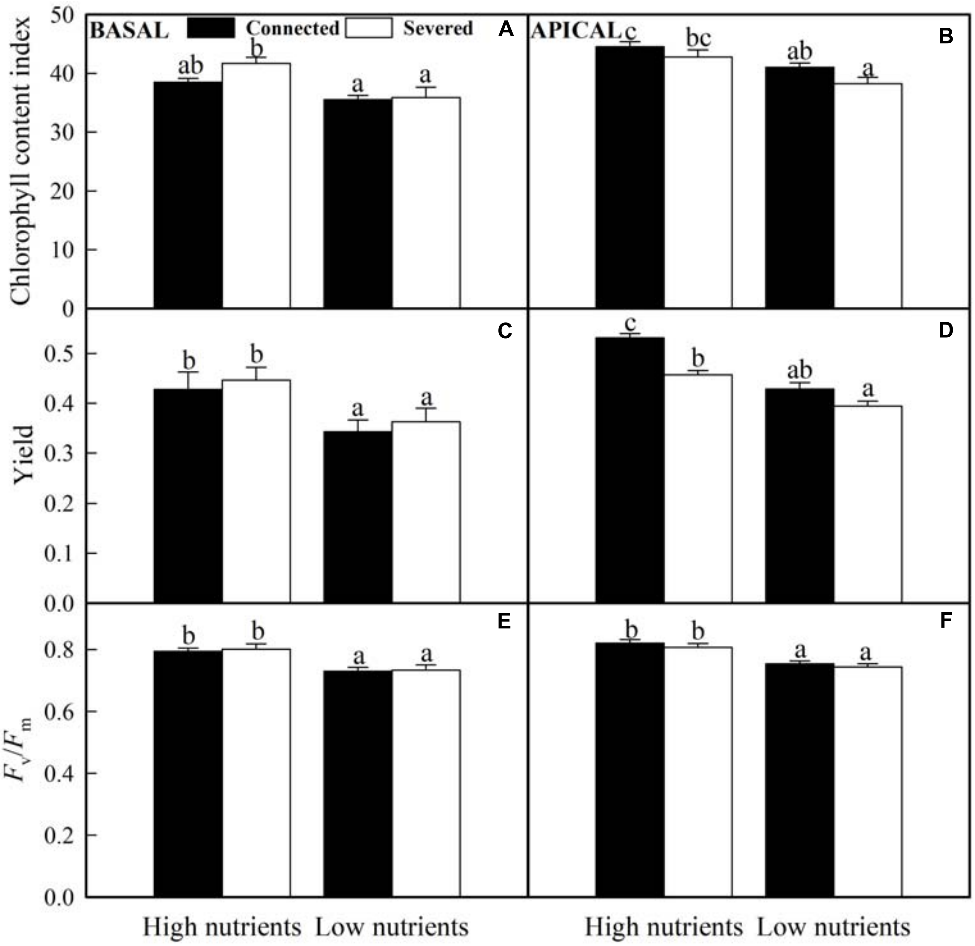


**FIGURE 5 |** Effects of experimental treatments on the chlorophyll content index, the effective quantum yield of PS II (Yield) and the maximum quantum yield of PSII ($F_v/F_m$) of basal **(A,C,E)** and apical **(B,D,F)** ramets of *A. philoxeroide*. The data indicate the means + SE ($n$ = 8). The bars sharing the same letter are not significantly different at $P$ = 0.05.

habitats with high resource supply than with low resource availability (Dong et al., 2015; You et al., 2016b). This may explain in part why *A. philoxeroides* spreads so fast in high-nutrient environments. Therefore, developmentally programmed division of labor may play an important role in determining the invasion of this plant invader to some relatively homogeneous habitats (such as anthropogenic habitats and aquatic ecosystems) (You et al., 2016b).

In heterogeneous habitats, the benefits of the division of labor have been extensively investigated in clonal plants (including some invasive plants) (Stuefer, 1998; Pennings and Callaway, 2000; Wang et al., 2008; You et al., 2013; Roiloa et al., 2016; Lin et al., 2018). However, the present study reported the division of labor for an invasive clonal plant in the homogeneous environment, with all basal and apical ramets subjected to the same external resources. Similarly, Roiloa et al. (2013) also found functional and structural specialization in a stoloniferous clonal plant invader, which benefited from developmentally programmed division of labor in terms of increasing aboveground growth of apical ramets and effective colonization of the surrounding area. The present study provided another strong evidence that the invasive clonal plant can develop the capacity for the division of labor, facilitating its expansion and thus invasiveness under homogeneous conditions. This finding is of great importance, because it proves that the benefits of the division of labor are not only widely existing in heterogeneous environments (Pennings and Callaway, 2000; Saitoh et al., 2002; Wang et al., 2008, 2011; You et al., 2013; Roiloa et al., 2016), but also relevant in homogeneous habitats (Stuefer, 1998; Roiloa et al., 2013). This developmentally programmed division of labor is inherent in clonal plants and irrelevant to environmental heterogeneity (Roiloa et al., 2013). Therefore, besides the relatively low biodiversity, developmentally programmed division of labor may be an alternative mechanism accounted for high invasibility by exotic clonal plants in some homogenized fine-scale conditions such as shallow wetlands and some anthropogenic habitats (You et al., 2016a).

Interestingly, we found that apical ramets always grew better than basal ramets. Such size uniformity might facilitate the division of labor. One possibility may be that clonal integration usually tended to increase the biomass more for the younger, apical ramets to facilitate the lateral expansion, as we mentioned above (Wang et al., 2008; You et al., 2016a; Wang P. et al., 2017). Another possibility may be that the apical ramets had the stolon apexes, which may potentially produce new ramets (Julien et al., 1995). We excluded the possibility that the apical dominance induced the size uniformity, because the original ramets were relatively independent new ramets (the two original ramets of both apical and basal part already rooted before the severing treatment) and probably unlikely affected by apical dominance. However, severance of the apex may affect the growth performance and clonal propagation of the youngest apical ramets, thus additional researches are needed to test this.

More interestingly, besides the benefits of the division of labor detected in the growth and clonal propagation of apical ramets, our results showed that photosynthetic efficiency (estimated by the Yield) of apical ramets was significantly increased by stolon connection, especially in high soil nutrient habitats. This result proved that the division of labor can also be detected at physiological level (Roiloa et al., 2007; Liu et al., 2008; Xu et al., 2010; You et al., 2016b). Recently, we also found a similar benefit of physiological integration (division of labor allowed) in photosynthetic efficiency of *A. philoxeroides* in homogeneous habitats with high water supply (You et al., 2016b). In the present study, the benefits of the division of labor in photosynthetic efficiency were transferred into improved growth and clonal propagation. Owing to such benefits at both physiological and morphological levels, the division of labor may facilitate the colonization capacity and invasion ability, especially in resource-rich habitats (Roiloa et al., 2013; You et al., 2016b).

In short, using a control experiment, we showed that *A. philoxeroides* can develop developmentally programmed division of labor at both physiological and morphological level, thus enhancing its photosynthetic efficiency and growth performance in high soil nutrient conditions. Such benefits of the division of labor could be important for *A. philoxeroides* to colonize new space and spread (You et al., 2016b). In some homogenized fine-scale conditions such as shallow wetlands and anthropogenic habitats, developmentally programmed division of labor may facilitate the invasiveness of exotic clonal plants, which may be an alternative mechanism accounted for notorious plant invasion in these habitats. Furthermore, this study supports the proposal that key clonal traits such as physiological integration and the division of labor can lead to invasion of exotic clonal plants (Song et al., 2013; You et al., 2013). Therefore, prior to introducing alien clonal plants, clonal traits could be important components that could be used to assess their potential invasiveness (Gordon et al., 2012; Song et al., 2013). However, to allow for a comprehensive extrapolation, future in-depth researches are urgently needed on the capacity for the division of labor between invasive and related native plants, or between invasive and non-invasive alien plants (van Kleunen et al., 2010; Wang Y.J. et al., 2017).

## AUTHOR CONTRIBUTIONS

W-HY conceived and designed the experiments. D-GX, A-AH, and W-HY performed the experiments. D-GX, PH, and W-HY analyzed the data. W-HY and D-LD contributed reagents, materials, and analysis tools. D-GX and W-HY wrote the manuscript.

## FUNDING

We are very thankful for the financial support by the National Natural Science Foundation of China (31770394, 31400312, and 31770446), the Jiangsu University Research Foundation (15JDG030), the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD), the Jiangsu Collaborative Innovation Center of Technology and Material of Water Treatment, and the Research Innovation Project for College Graduates of Jiangsu Province (SJLX16_0435).

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 25 April 2019
doi: 10.3389/fpls.2019.00514

# Association Between the Success of an Invasive Macrophyte, Environmental Variables and Abundance of a Competing Native Macrophyte

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**Reviewed by:**
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**Specialty section:**
*This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science*

**Received:** *30 July 2018*
**Accepted:** *03 April 2019*
**Published:** *25 April 2019*

**Citation:**
*Pulzatto MM, Cunha ER, Dainez-Filho MS and Thomaz SM (2019) Association Between the Success of an Invasive Macrophyte, Environmental Variables and Abundance of a Competing Native Macrophyte. Front. Plant Sci. 10:514. doi: 10.3389/fpls.2019.00514*

The success of invasive species depends on the overcoming of abiotic and biotic filters. Abiotic variables likely have greater relative importance over invasion at broad spatial scales, while biotic interactions are more important at fine spatial scales. In this study, we tested the hypotheses that (i) the abundance of the invasive *Hydrilla verticillata* is more correlated with abiotic factors than with competing native species at broad spatial grain; and that (ii) *H. verticillata* abundance is more correlated with competing native species than with abiotic factors at fine spatial grain. Here, we considered spatial scale as the grain size (*i.e.*, the extent of sampling unit) assuming broad spatial scales as a large area encompassing the entire patches of macrophytes, and fine spatial scales as a small area inside one macrophyte patch. We collected the abundance of hydrilla and the competing native species along with environmental variables in a large subtropical reservoir. To evaluate how the relative importance of the abiotic factors and the competing native species vary between spatial grains we used Bayesian Generalized Linear Models. At broad grain, the abundance of the competing native species, maximum fetch (positive correlation), turbidity and conductivity (negative correlation) were the most important factors to explain the hydrilla abundance. At fine grain, alkalinity, total organic matter of the sediment and the abundance of a competitive native species (all negative correlations) were the most important variables. Our results indicate a greater importance of abiotic factors at broader grains while competitive interactions seem to be important only in the finer spatial grains. Environmental heterogeneity may explain the positive correlation between native and invasive abundances at broad grain, while the negative correlation at fine grain suggests the effect of competition. In synthesis, we show that the abiotic factors that explain the invasion success of a submerged invasive macrophyte are the same in two spatial grains, but the importance of biotic interactions changed with grain. Thus, our data suggest that models that attempt to explain the success of invasive plants, should consider spatial scales.

**Keywords: invasive species, competition, modeling statistics, standing water, fresh water**

## INTRODUCTION

The success of invasive species in their introduced range depends first on the overcoming of dispersion and establishment barriers (Davis, 2009). These processes are determined by assembly rules, which specify which species of a regional pool can potentially establish in a local pool (Keddy, 1992; Moyle and Light, 1996). Thus, multiple mechanisms are involved at different spatial scales, which are related to the invading potential of the species (traits) and to the susceptibility of invasion of native communities and ecosystems (environmental aspects) (Davis, 2009).

In order to colonize and spread successfully in new areas, invasive species have to tolerate and surpass filters as they pass through the different invasion stages. During the dispersion stage, for example, the potential invader must cross the biogeographic barriers, which occurs through human action (Rahel, 2002). Then, at the establishment stage, the physical and chemical aspects act as physiological filters regulating the invasion process (Rahel, 2002). These filters, which include in aquatic ecosystems the underwater radiation (Barko and Smart, 1981), the chemical characteristics of the sediment (Barko and Smart, 1983; Barko et al., 1991; Wang et al., 2017) and water (Vestergaard and Sand-Jensen, 2000a; Lee et al., 2004) and wave disturbances (Doyle, 2001; Strand and Weisner, 2001), are especially important in the colonization phase of many organisms (Theoharides and Dukes, 2007), especially for invasive aquatic plants. Finally, biotic interactions regulate the invasion success by acting as a biotic filter (Rahel, 2002; Theoharides and Dukes, 2007). The main mechanism associated with the biotic filter is the biotic resistance (Elton, 1958), *i.e.*, the resistance that native species provide to the invasive species, mainly through competition (Gurevitch, 2011; Petruzzella et al., 2018) parasitism and other biotic interactions (Levine et al., 2004).

The roles of abiotic and biotic filters above mentioned vary at different spatial scales (Weiher and Keddy, 1995). The interactions between native and exotic species are considered local processes, and therefore tend to be more apparent and important at fine spatial scale (microcosms or plots of few square meters). Biotic interactions at fine spatial scales have been demonstrated both experimentally (Dukes, 2001) and in observational studies (Case, 1990). In contrast, at broad spatial scales which include sufficient heterogeneity in environmental conditions that do not reflect local processes (e.g. species interactions), the response of invaders tends to be more influenced by abiotic factors (Huston, 1999).

In addition, these environmental filters may also be altered by other impacts resulting from human action, which can make ecosystems more susceptible to invasions (Alpert et al., 2000). This scenario has been widely reported for aquatic environments, where dam construction acts as a modifier of environmental filters (Moyle and Light, 1996; Malmqvist and Rundle, 2002; Rahel, 2002; Johnson et al., 2008). For example, reservoirs alter natural conditions over wide areas, acting as stepping stones for species invasion (Moyle and Light, 1996; Havel et al., 2005; Pitelli et al., 2014). Another typical change relates to sediment trapping, what in turn increases the underwater light availability (Malmqvist and Rundle, 2002; Roberto et al., 2009) and improves habitat suitability for submerged macrophytes. In addition to changing these abiotic filters, the construction of dams can also reduce species diversity and increase instability on biotic interactions (Malmqvist and Rundle, 2002; Havel et al., 2005), decreasing the efficiency of biotic resistance. Owning to these environmental changes, reservoirs favor the establishment of invasive species, including submerged macrophytes.

*Hydrilla verticillata* (L.f.) Royle (Hydrocharitaceae), native to Asia (Zhu et al., 2015) is considered one of the worst aquatic invasive plants worldwide (Cook and Lüönd, 1982; Langeland, 1996; Madeira et al., 2007). It was first recorded outside its native area in 1960 in Florida, United States (Allen, 1976). From then on, *H. verticillata* expanded its distribution to almost all continents, with the exception of Antarctica (Cook and Lüönd, 1982). In 2005, *H. verticillata* was first recorded in the Paraná River basin in Brazil (Sousa, 2011). Since then, its distribution has expanded and it reached several reservoirs (Thomaz et al., 2009; Pitelli et al., 2014). The increase in the water transparency promoted by reservoirs in the Paraná River basin and the propagule pressure from the upstream reservoirs facilitated the invasion in several habitats (Thomaz et al., 2009).

Considering the potential ecological, economic and social impacts of *H. verticillata* and its wide global distribution (Cook and Lüönd, 1982; Langeland, 1996; Sousa, 2011), evaluating the importance of environmental and biotic factors that influence its success becomes a task that interest invasion biologists and environmental managers. In this work, we quantified the relative importance of abiotic variables (abiotic filter) and of a competing native species (biotic filter) on the performance of *H. verticillata* in a subtropical large reservoir. Considering the importance of spatial scales to determine the role of abiotic and biotic variables on species performance (Duarte and Kalff, 1990), we used two databases, one representing broad spatial grain (entire patches of macrophytes) and another at fine spatial grain (small area inside one macrophyte patch), to address our questions.

We tested the hypotheses that (i) *H. verticillata* abundance is more correlated with abiotic factors than with competing native species at broad spatial grain; and that (ii) *H. verticillata* abundance is more correlated with competing native species than with abiotic factors at fine spatial grain. These hypotheses are based on the assumption that abiotic variables tend to be more important for invader establishment in aquatic environments (Moyle and Light, 1996) and that abiotic variables are more important at broad spatial grains while interspecific interactions (biotic filters) are more important at fine spatial grains (Huston, 1999; Fridley et al., 2007). Thus, we expect that the native species *Egeria najas* Planchon (Hydrocharitaceae), which is a strong and potential *H. verticillata* competitor, may hamper the invasive species success at the fine grains. We chose this native species because it belongs to the same family of *H. verticillata* and they are morphologically similar (Sousa, 2011), which may enhance their interactions. *Egeria najas* is also the most important submerged species in terms of occurrence and abundance at the Itaipu Reservoir (S.M. Thomaz, unpublished), what makes it potentially the most important competitor. In addition, the literature shows that a single competitor plant, as the submerged macrophyte *Vallisneria americana* Michx., also of the family

Hydrocharitaceae, is capable of effectively reduce establishment of hydrilla fragments (Owens et al., 2008).

## MATERIALS AND METHODS

The Itaipu Reservoir (24° 05′- 25° 33′ S, 54° 00′- 54° 37′ W), located between Brazil and Paraguay, was formed by damming the Paraná River in October 1982 (**Figure 1**). It has an area of 1350 km$^2$, an average depth of 22.5 m, a length of 170 km and an average width of 7 km. It has a residence time of about 40 days, being shorter in the main axis of the reservoir (about 29 days). Its hydrometric level is relatively stable (oscillations of less than 1 m per year). The seven arms evaluated in this study differ in relation to the physical and chemical characteristics (Thomaz et al., 2009), varying from oligotrophic to eutrophic conditions.

The large margin development and the shallow areas in the arms favor the growth of aquatic macrophytes in Itaipu. In addition, damming caused the reduction of water velocity, the loss of periodicity and the amount of upstream flood pulses, and the increase of underwater radiation in the entire reservoir due to sediment deposition (Malmqvist and Rundle, 2002). These factors favored the colonization and growth of submerged aquatic macrophytes. Among the 14 submerged species recorded for the area, the most important in terms of occurrence and abundance are those belonging to the family Hydrocharitaceae. Among them, the most frequent ones are the native *E. najas*, found in most arms with high biomass and frequency, and the invasive *H. verticillata*, first recorded in 2007 in Itaipu Reservoir (Thomaz et al., 2009). Other submerged species may occur, but they are historically very rare in the reservoir. According to the monitoring program in Itaipu, *E. najas* and *H. verticillata* reached 89.7% of the total sample effort of all the submerged macrophytes (10 species in total) (authors' personal data – data collected in 2015–2017). Given the low significance of the other submerged macrophytes as possible competitors for *H. verticillata* and considering their low occurrence in the entire reservoir, we selected only *E. najas* as an indicator of competition.

In terms of spatial scale, we particularly refer to the grain size, which is related to extent of sampling unit (*sensu* Wiens, 1989). We defined the grain at the broad scale as a large area encompassing the entire patches of macrophytes or even more than one patch (a transect c.a. 100 m long). At this grain, the macrophyte patches could be either monospecific or with mixed species. On the other hand, we defined the grain at the fine scale as a small area inside one macrophyte patch (a square with 0.5 m side length). Studies investigating biomass variation at small spatial scales have successfully used similar or even smaller sampled areas (Thomaz et al., 2006; Olsen et al., 2019). In addition, we used a fine spatial grain because we wanted to observe the characteristics of the microhabitat of the macrophytes, i.e., in the case of submerged macrophytes, where they are rooted. Considering that these two species are herbaceous small plants, an area of 0.25 m$^2$ is enough to encompass even hundreds of ramets, and then is enough to represent their local abundance. Moreover, such grain extent

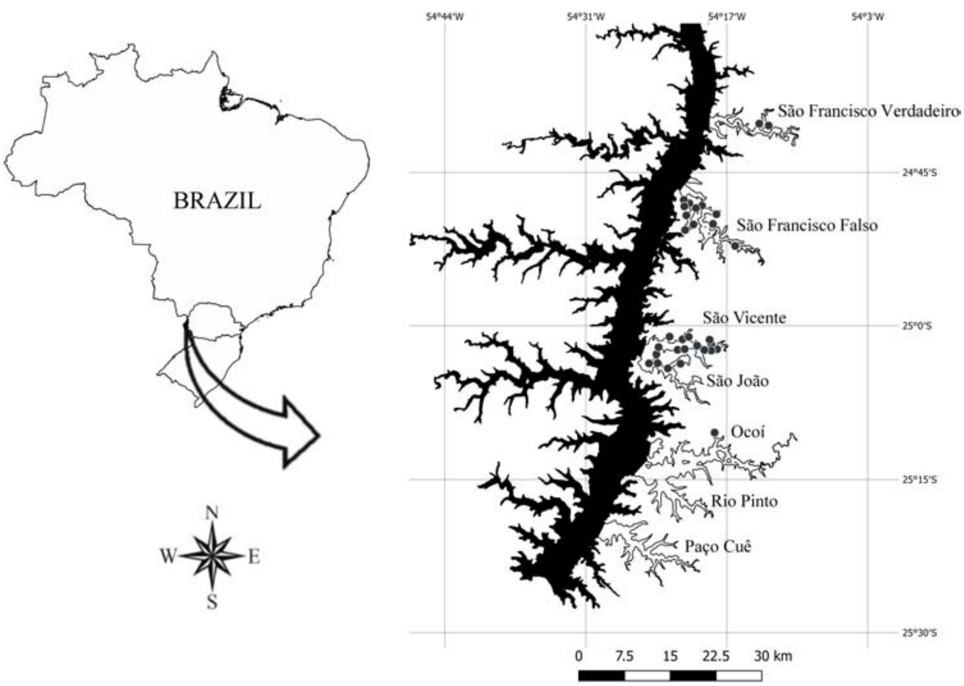


**FIGURE 1 |** Map of the Itaipu Reservoir, Brazil (Geographic Coordinate System – EPSG: 4326). The sites at broad spatial grain (*n* = 195) are distributed in the lateral arms: São Francisco Verdadeiro (*n* = 28), São Francisco Falso (*n* = 29), São Vicente (*n* = 27), São João (*n* = 29), Ocoi (*n* = 27), Rio Pinto (*n* = 26) and Paço Cuê (*n* = 29). The sites at fine grain (*n* = 31) are represented by grey points on the lateral arms: São Francisco Verdadeiro (*n* = 2), São Francisco Falso (*n* = 11), São João (*n* = 4), São Vicente (*n* = 13) and Ocoi (*n* = 1). The sites sampled are located along the Brazilian margin of the reservoir.

allowed sampling sites where only a single species occurred and also where both species occurred together (where they potentially compete).

## Samplings at Broad Spatial Grain

In April 2017, abundance data of *H. verticillata* and *E. najas* and environmental variables were collected at 195 geo-referenced sites (Geographic Coordinate System – EPSG: 4326) (**Figure 1**). These sites were distributed in seven arms of the Itaipu Reservoir (**Figure 1**), aiming to cover an extensive area with wide variation of environmental conditions. Among the 195 sampling points, *H. verticillata* and *E. najas* co-occurred in 82 points, *H. verticillata* occurred alone in 8 points and *E. najas* occurred alone in 70 points; in the others 35 points, none of them occurred, providing information about potential limiting environmental factors.

In order to determine the relative abundance of each species in each site, a sinuous transect were sampled parallel to the shores of the reservoir (about 80 to 100 m in length, over an area of approximately 3000 to 5000 $m^2$). We performed these surveys from a boat at constant low speed. We carried out the samplings up to ca. 4 m depth, which is, in general, the maximum depth of occurrence of the submerged macrophytes in Itaipu (unpublished data). At each transect, we dragged 12 approximately equidistant points with a rake attached to a pipe (4.2 m). In each of these trawls, we measured the abundance rating of each species of macrophyte, which ranged from 0 (absence) to 5 (maximum biomass, with canopies reaching the surface). We estimated the relative abundance for each species of macrophyte per site by adding up all the abundance rating values measured in each transect, to obtain the additive abundance rating. Thus, macrophytes' additive abundance rating varied potentially from 0 (absence) to 60 (12 points with abundances values of 5) (based on Yin and Kreiling, 2011). This method is highly efficient to represent the relative abundance of submerged macrophytes (Yin and Kreiling, 2011).

At each site, besides the biotic variable (additive abundance rating of *E. najas*), we measured four additional variables representing the abiotic conditions potentially related to the additive abundance rating of *H. verticillata* at broad grain (**Table 1**). In order to obtain the littoral slope, which is an indication of the extent of the potential area colonized by macrophytes, we used Equation 1:

$$slope(\%) = \frac{ad}{hd} \times 100 \quad (1)$$

Where: *ad* = altitude difference and *hd* = horizontal distance between the altitudes 215-220 m a.s.l. These altitudes correspond to approximate depths where macrophytes colonize the reservoir (data of this work). We obtained the altitudes used to estimate slopes, using satellite imagery.

The maximum fetch represents the maximum open water distance (without crossing the reservoir shore or islands) over which the wind is able to run in a specific direction (Håkansson, 1981; Burrows et al., 2008). Its values were obtained with ArcGIS software (version 9.1), using a reservoir map (Geographic Coordinate System – EPSG: 5880) to measure the maximum distances from the sampling site to the next shore or island. We did not correct the fetch measure for wind velocity, due to the scarcity of wind measurement stations for the large surface area of the Itaipu Reservoir. Even though, we can consider our fetch measure a surrogate of disturbances in macrophyte patches via wave action, as shown previously for the same sampling points by using the same protocol (Thomaz et al., 2003, 2009). We measured the turbidity and the conductivity with a multi-parameter HORIBA sensor. We highlight that the conductivity and alkalinity are highly and positively correlated in the Upper Paraná waters (Roberto et al., 2009) and thus, the former variable can be considered an indicator of inorganic carbon availability, in addition to total ions concentration. Both variables were measured close to macrophyte stands to minimize their effects on local limnological conditions (Carpenter and Lodge, 1986; Duarte and Kalff, 1990).

**TABLE 1 |** Indicators of biotic interaction, disturbances and resource and their respective variables for broad (a) and fine (b) spatial grains.

| Indicator | Variable |
|---|---|
| Competition | *Egeria najas* additive abundance rating[a]<br>*E. najas* biomass[b] |
| Wind disturbance | Maximum fetch[ab]<br>Littoral slope[ab] †<br>Depth[b] † |
| Light availability | Turbidity[ab]<br>Depth[b] † |
| Carbon availability | Conductivity[a]<br>Alkalinity[b] |
| Sediment quality | Littoral slope[ab] †<br>Available nitrogen[b]<br>Available phosphorus[b]<br>Organic matter[b] |

† *The same variable can represent more than one type of indicator.*

## Samplings at Fine Spatial Grain

In May 2017, we obtained the relative abundance of each species in each site by measuring the biomass of the two submerged macrophyte species and the environmental variables in 31 geo-referenced sites (Geographic Coordinate System – EPSG: 4326) distributed heterogeneously in five arms of the Itaipu Reservoir (**Figure 1**). We were looking for a biomass gradient among patches, from the absence of plants to well-established patches of both submerged macrophyte species, and also an environmental gradient. Therefore, considering that *H. verticillata* is widely distributed in the whole reservoir, we rather distributed sampling sites along the environmental gradient than distributing sampling sites homogenously along the reservoir. For this, we haphazardly selected the sampling sites across the arms of the reservoir from a boat, to capture a gradient for all explanatory variables considered. In order to ensured sample independence, we maintained long distances among sampling sites (**Figure 1**). Among the 31 sampling points, *H. verticillata* and *E. najas* co-occurred in 25 points, while *H. verticillata* occurred alone in one point, *E. najas* occurred alone in three points, and there were only two points where neither occurred.

In 16 sampling points, we collected the biomass of each species by delimiting an area of 0.25 $m^2$ with a steel box (1.5 m high × 0.5 m × 0.5 m – quadrat of 0.25 $m^2$) and twisting a rake in a 360° turn. After, we collected all the remaining material inside the quadrat with the aid of the rake to remove the plants from the sediment. We carefully washed the plants in tap water to remove excess sediment and attached material and dried them in an oven at 70°C to constant weight. For these quadrats, we obtained the total biomass by adding the biomass of the rake with the biomass remaining in the box. For the other 15 sites, the biomass was obtained only with the rake (without the presence of the box), to facilitate and streamline our samplings. In this case, we predicted the values of biomass per $m^2$ through one simple linear regression between the two biomasses obtained at the previous sampling points (from the rake and from the steel box) for each species. The data were transformed ($X^{0.28}$, that corresponds to a transformation between cube and fourth root that best linearized data) for both species in order to reach the regression assumptions.

At each site, we collected eight environmental variables potentially related to the biomass of *H. verticillata*, representing the environmental conditions at fine spatial grain (**Table 1**). All the variables, except those related to sediment, slope and depth, were measured before sampling the plants, near the border of the macrophyte patches, in order to minimize the effects of the plants on the limnological conditions (Carpenter and Lodge, 1986; Duarte and Kalff, 1990). We measured the slope using Equation 1, with *ad* as the depth difference between two measures taken and *hd* as 4.6 m that represents the distance between both ends of the boat where we took the measures. We obtained slope and depth near to the quadrat. We quantified maximum fetch following the same protocol described previously at the broad grain. We quantified the turbidity with a multi-parameter HORIBA sensor. The total alkalinity (a surrogate of inorganic carbon availability) was determined through Gran titration (Carmouze, 1994). We measured both variables close to macrophyte patches by the same reasons presented above (See "*Samplings at broad grain*"). Sediment was collected inside the patches where plants were collected; we used a Petersen grab and frozen the samples for further analyses of available N (Bremmer, 1965) and available P (Stainton et al., 1977). The percentage of sediment organic matter (OM) was obtained by the difference of sediment weight before and after burning sediment samples in a muffle at 550°C for 4 h (Teixeira et al., 1965).

## Data Analysis

In order to assess for independent contribution of environmental variables on the additive abundance rating (at broad grain) and biomass (at fine grain) of the invasive macrophyte *H. verticillata*, we used Bayesian Generalized Linear Models. For obtaining standardized coefficients, we scaled prior distributions of parameters using zero mean priors divided by two standard deviations. For the broad grain, as the additive abundance rating are discrete measures and showed high over-dispersion of the data, the response variable was modeled following a Negative Binomial distribution; the *theta* value for describing the shape of the Negative Binomial distribution was obtained by using maximum likelihood techniques. Before analysis, we transformed the explanatory variables *E. najas* additive abundance rating, turbidity, and maximum fetch into *ln* (x + 1) to linearize the relations. We used non-informative *a priori* distributions for estimating parameters, which means that sampling data mostly influenced the resulting *a posteriori* distribution.

For the fine grain, we adjusted the models using a Gamma distribution that accounts for continuous measurements and positive skewness of residual distributions. We added a small constant (0.01) to the response variables in order to deal with zero values that the Gamma distribution did not handle. Before the analysis, the explanatory variables *E. najas* biomass, turbidity, maximum fetch and sediment OM were transformed into ln (x + 1), to linearize the relations. Similar to previous analysis, we used non-informative a *priori* distributions for estimating parameters.

We employed a model selection procedure using the Akaike Information Criterion (AIC) for both spatial grains. The competing explanatory models that followed the conservative selection criterion ($\Delta$AIC < 2) (Burnham and Anderson, 2002) were obtained from all possible subsets of models (all subsets approach). If more than three competing explanatory models were selected, we used Akaike's weighted average model to infer the effects of the variables. To compare the relative importance between abiotic factors and the competing native species for each spatial grain, we divided the variables contained in the explanatory models selected using AIC into the abiotic group and competing native species abundance. For each of these two groups, we used the sum of the relative weights (*wi*) of each model containing at least one variable present in the group.

Owning to technical problems, we missed one alkalinity result and, in this case, we applied the missForest non-parametric method to insert missing data, following the protocols of (Stekhoven and Bühlmann, 2012). All analyzes were performed in the R software (R Core Team, 2017), using the MASS, car, arm, missForest, MuMIn and bbmle packages (Venables and Ripley, 2002; Fox and Weisberg, 2011; Stekhoven, 2013; Gelman and Su, 2016; Barton, 2017; Bolker and Development Core Team, 2017).

# RESULTS

## Environmental Features

In general, sampling at both grains ensured a wide gradient for abundance of the competing native species *E. najas* (**Tables 2**, **3**). However, this species did not reach maximum additive abundance rating (60) at any sampling point at the broad spatial grain (**Table 2**). In the two grains, most of the sites had low littoral slopes ( < 10%) and the maximum fetch values presented great variations among sites (ca. 0.2 – 19 km), indicating a broad gradient of wind disturbance. The sampling depths, measured only at the fine grain, were also relatively low (most sites < 2m) (**Table 3**). Most of the sites had high water transparency, based on the observed low values of turbidity (**Tables 2**, **3**). The conductivity and alkalinity indicated low inorganic C availability at most sites (**Tables 2**, **3**). In the sediment samples (collected

**TABLE 2 |** Descriptive statistics of the abiotic variables and the competing native species collected in seven arms of the Itaipu Reservoir, at broad spatial grain.

| Variables (units) | Minimum | Q1 | Median | Q3 | Maximum | Mean (SD) |
|---|---|---|---|---|---|---|
| *Egeria najas* additive abundance rating | 0 | 1.5 | 11.0 | 24.0 | 48.0 | 13.5 (12.4) |
| Littoral slope (%) | 1.17 | 3.46 | 5.25 | 7.42 | 51.92 | 6.24 (4.87) |
| Maximum fetch (km) | 0.26 | 1.37 | 2.14 | 3.48 | 18.68 | 3.10 (3.22) |
| Turbidity (NTU) | 0 | 0 | 0.40 | 0.85 | 26.30 | 1.95 (5.09) |
| Conductivity ($\mu$S $cm^{-1}$) | 43 | 56 | 58 | 61 | 148 | 59 (8.6) |

*Q1: first quartile, which means that about 25% of the numbers in the data set lie below Q1; SD: standard deviation; Q3: third quartile, which means that about 75% of the numbers lie below Q3.*

**TABLE 3 |** Descriptive statistics of the abiotic variables and the competing native species collected in five arms of the Itaipu Reservoir at the fine spatial grain.

| Variable (units) | Minimum | Q1 | Median | Q3 | Maximum | Mean (SD) |
|---|---|---|---|---|---|---|
| *Egeria najas* biomass (g DW $m^{-2}$) | 0 | 0.3 | 8.4 | 97.5 | 1012.6 | 85.6 (193.5) |
| Littoral slope (%) | 0 | 2.2 | 6.3 | 8.7 | 14.1 | 5.9 (3.9) |
| Depth (m) | 0.2 | 1.1 | 1.8 | 2.4 | 4.0 | 1.8 (0.9) |
| Maximum fetch (km) | 0.26 | 2.15 | 4.16 | 9.99 | 17.70 | 5.70 (4.72) |
| Turbidity (NTU) | 0 | 0.20 | 1.20 | 1.85 | 7.00 | 1.31 (1.41) |
| Alkalinity (mEq $L^{-1}$) | 318 | 411 | 445 | 464 | 734 | 454 (78) |
| Sediment nitrogen ($\mu$g $g^{-1}$) | 0.26 | 11.06 | 17.60 | 27.80 | 65.53 | 21.54 (15.05) |
| Sediment phosphorus ($\mu$g $g^{-1}$) | 13 | 26 | 83 | 119 | 155 | 76 (48) |
| Sediment organic matter (%) | 4.1 | 6.8 | 8.4 | 9.6 | 16.2 | 8.7 (2.7) |

*Q1: first quartile, which means that about 25% of the numbers in the data set lie below Q1; SD: standard deviation; Q3: third quartile, which means that about 75% of the numbers lie below Q3.*

only at the fine spatial grain), the nitrogen attained lower values than phosphorus and OM covered a relatively broad gradient, although most values remained below 10% OM (**Table 3**).

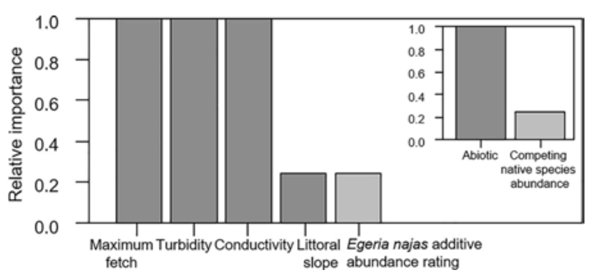


**FIGURE 2 |** Relative importance of the explanatory variables and of the abiotic and the competing native species *Egeria najas*, based on the weighting of the explanatory models obtained to explain the variability in the additive abundance rating of *Hydrilla verticillata* in the Itaipu Reservoir at broad spatial grain (macrophyte patches).

## Response of *H. verticillata* at the Broad Spatial Grain

For the broad spatial grain, despite the low explanatory power of the competing models selected using AIC ($R^2{}_1$ = 0.176, $R^2{}_2$ = 0.179, $R^2{}_3$ = 0.179), consistent statistical effects were observed for all the explanatory variables. In general, the group of abiotic variables presented greater relative importance in relation to the native competing species abundance to explain independent portions of the variability of *H. verticillata* abundance (**Figure 2**). The maximum fetch, turbidity and conductivity were the most important variables within the abiotic group of variables (**Figure 2**). Maximum fetch correlated positively with the additive abundance rating of the invasive macrophyte (**Figure 3**). In contrast, turbidity and conductivity correlated negatively with the additive abundance rating of *H. verticillata* (**Figure 3**). Only the second and third models selected the slope and the additive abundance rating of the native competitor species *E. najas*, respectively (**Figure 3**).

## Response of *H. verticillata* at the Fine Spatial Grain

For the fine spatial grain, the weighted model obtained from four selected models had a moderately low explanatory power (R-weighted = 0.38) but presented consistent statistical effects of six of the nine explanatory variables evaluated at this grain. In general, both the group of abiotic variables and the competing native species abundance were equally important to explain independent portions of the abundance variability of the invasive macrophyte *H. verticillata* at fine grain (**Figure 4**). *Egeria najas* biomass, alkalinity and sediment OM were the variables with the highest relative importance (**Figure 4**) and they correlated negatively with *H. verticillata* biomass (**Figure 5**). Alkalinity correlated negatively with *H. verticillata* biomass, paralleling the relation found for conductivity at the broad grain. For the sediment OM, *H. verticillata* tended to occur in sites with low percentage of organic matter, which apparently becomes limiting to macrophyte growth at about 10% (inspection in our graphical analysis; data not shown).

The depth, turbidity, and maximum fetch were less important to explain *H. verticillata* biomass, although they also contributed

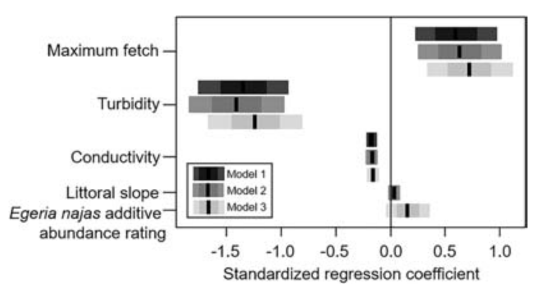


**FIGURE 3 |** Competitive explanatory models obtained to explain the variability in the additive abundance rating of *Hydrilla verticillata* in the Itaipu Reservoir, at broad spatial grain (macrophyte patches). The black line, the darkest bars and the lightest bars correspond to the mean, the standard deviation and the confidence interval (95%), respectively, for regression coefficients of each of the three competing models.

to explaining independent portions of the invasive biomass variability at the fine spatial grain (**Figure 4**). The depth correlated positively with the invasive biomass (**Figure 5**). Both turbidity and maximum fetch paralleled the relationships found for the broad spatial grain, being negatively (the former) and positively (the latter) related with *H. verticillata* biomass (**Figures 3**, **5**). However, at the fine spatial grain, both variables had less relative importance to explain *H. verticillata* biomass than they had at the broad spatial grain to explain *H. verticillata* additive abundance rating (**Figures 2**, **4**).

## DISCUSSION

Our main findings show that at broad spatial grain (i.e., transects), abiotic variables are important to explain the invasive *H. verticillata* abundance, providing support for our first hypothesis. In contrast, at fine spatial grain (quadrats), our findings demonstrate that both the abiotic variables and the native competing species abundance are equally important, which rejects our second hypothesis. These results provide additional support to the idea that environmental factors are more relevant to explain invasive success at broad grain and, at a fine grain, competition may also play an important role (invasion paradox; Fridley et al., 2007).

### Response of *H. verticillata* at the Broad Spatial Grain

Our study is consistent with others that have suggested the greater importance of abiotic factors in relation to biotic factors at broad spatial scales for the success of invasive species (e.g., Stohlgren et al., 2006; Thomaz et al., 2009). Although the definition of scale extension is somewhat subjective (in our study measured as the grain size, sensu Wiens, 1989), broad scales can be understood as those that include environmental heterogeneity high enough so that no single species is able to inhabit the entire spatial grain (Fridley et al., 2007). Indeed, in the wide grain chosen in our work (ca. 3000 to 5000 $m^2$), both the invasive *H. verticillata* and the native *E. najas* did not occur in all the sampling points, and they showed a great variation of abundance where they occurred. It shows the importance of the abiotic factors regulating the presence and the abundance of these species in the study area. In additon, broad scales can be understood as those where many individuals can inhabit but with little direct interaction (e.g., competition) among individuals within their neighborhoods (Fridley et al., 2007). Thus, the strength of local competitive interactions decreases while the variation of environmental conditions increases with the scale (Fridley et al., 2007). Therefore, extrinsic factors, rather than biotic interactions, tend to determine the structure of communities at broad scales (Davies et al., 2005), which is consistent with our results.

In this study, *H. verticillata* abundance positively correlated with maximum fetch and negatively correlated with turbidity and conductivity at broad spatial grain. Although the presence and abundance of most macrophyte species tend to be negatively related to fetch, a surrogate of wind disturbance (Doyle, 2001; Thomaz et al., 2003; Azza et al., 2007), some submerged species might be positively affected by waves, when the disturbance is mild (e.g., Doyle, 2001). The positive relationship we found for the abundance of the invasive *H. verticillata* and fetch are in line with previous studies performed in the same study area (Thomaz et al., 2009). The successful performance of *H. verticillata* in sites more exposed to wind is likely related

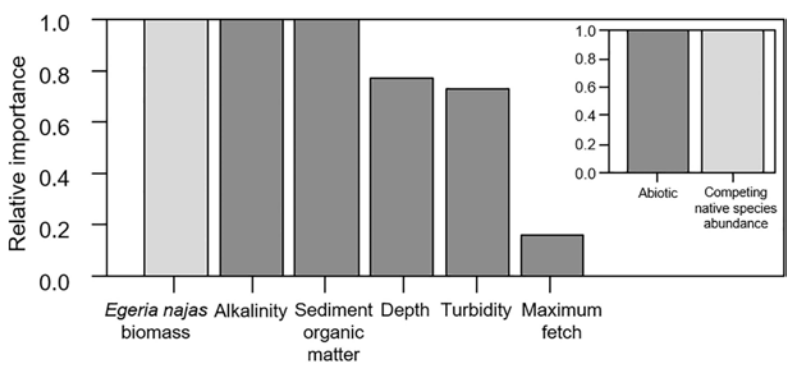


**FIGURE 4 |** Relative importance of the explanatory variables and of the abiotic and competing native species *Egeria najas*, based on the weighting of the explanatory models obtained to explain the variability in the biomass of *Hydrilla verticillata* in the Itaipu reservoir, at fine spatial grain (quadrat).

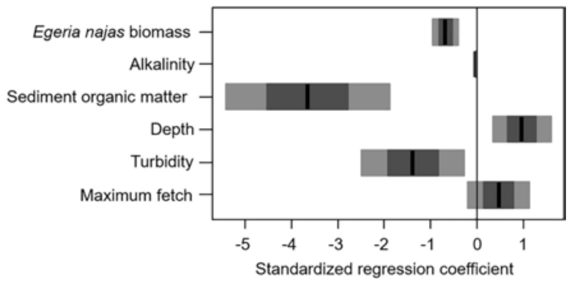


**FIGURE 5 |** Weighted model for the explanatory models obtained to explain the variability in the biomass of *Hydrilla verticillata* in the Itaipu Reservoir, at fine spatial grain (quadrat). The black line, the darkest bars and the lightest bars correspond to the mean, standard deviation and confidence interval (95%), respectively.

to the presence of well-developed root systems and resistance organs (tubers and turions) that allow a rapid regeneration after disturbances (Langeland, 1996; Bianchini et al., 2010; Sousa, 2011). In addition, wind disturbances can break plant stems and generate propagules, enhancing the propagule pressure. Due to the rapid regeneration of macrophyte fragments (Kuntz et al., 2014; Bickel, 2017), *H. verticillata* is able to rapidly colonize new sites, benefiting from dispersing capabilities enhanced by waves in places with long fetch values.

Light availability has been considered one of the main predictors of submerged macrophyte abundances (Duarte et al., 1986), including *H. verticillata* (Sousa et al., 2009), which explains the negative response of *H. verticillata* to increasing turbidity. We also found a negative relation between abundance of *H. verticillata* and conductivity, which can be likely explained by the effect of ion absorption (including inorganic carbon) by submerged macrophytes. In this respect, it is worth mentioning that macrophytes belonging to the elodeids group (including *H. verticillata*) are able to use $HCO_3^-$ as a carbon source (Madsen and Sand-Jensen, 1991; Vestergaard and Sand-Jensen, 2000b) and may be capable of reducing this ion from the water column. In experiments, for example, the high photosynthetic rates of *H. verticillata* can reduce inorganic C to near zero (Van et al., 1976; S.M. Thomaz, unpublished data from Itaipu Reservoir). The effects of submerged macrophytes on carbon concentration might be enhanced where inorganic carbon is less available, like in Itaipu (alkalinities values usually $< 500$ m Eq $L^{-1}$; see **Table 3**). Thus, even that we conducted the sampling near the macrophyte patches, the water mixture probably kept the electrical conductivity low near macrophytes, which likely explains the negative relation between *H. verticillata* abundance and conductivity observed here.

Although others have reported a negative correlation between the littoral slope and macrophyte biomass (Duarte and Kalff, 1986) and macrophyte diversity (Thomaz et al., 2003), we found a positive relationship between littoral slope and *H. verticillata* abundance. It is important to highlight that only one model selected the slope, resulting in a weaker and less consistent relation with the abundance of *H. verticillata*, and so, interpretations should be made with caution. The conflicting result we found here might be associated with several non-mutually exclusive potential causes. For example, the abundance of *H. verticillata* is likely to be smaller in sites with gentle slopes, which are more sensitive to water level fluctuations, which also negatively affects submerged macrophytes through desiccation (Beklioglu et al., 2006; Carmignani and Roy, 2017).

## Response of *H. verticillata* at the Fine Spatial Grain

The effects of environmental heterogeneity and dispersion on invasive success are minimized at fine spatial scales where individuals interact directly (Fridley et al., 2007). Our study pointed an increase of the importance of the native *E. najas* competing species to explain *H. verticillata* abundance at the fine spatial grain. However, different from our expectations, our results also suggested an important role of abiotic factors at the fine grain. This finding is, in some degree, congruent with other studies (e.g., June-Wells et al., 2016). Species competitiveness may change according to environmental variations (Moyle and Light, 1996), highlighting the importance of both factors at fine spatial scales, as we found here.

In this study, the *H. verticillata* abundance correlated negatively with alkalinity and sediment OM at the fine spatial grain. The negative correlation between the invasive macrophyte abundance and alkalinity paralleled the one found between the invasive abundance and conductivity at broad spatial grain, which is not surprising. For the negative relationship found between *H. verticillata* and sediment OM, this outcome may reflect accumulation of phytotoxic compounds from aerobic decomposition that is commonly observed in organic sediments (Barko and Smart, 1983; Wu et al., 2009). In fact, similar patterns were previously observed in other ecosystems in the Upper Paraná River (Sousa et al., 2009).

Depth and maximum fetch correlated positively with *H. verticillata* abundance at the fine spatial grain, although to a lesser relative importance than found at the broad spatial grain. This species shows a particular ability to colonize deep areas (maximum 4 m in this study, 4.0−7.3 m - (Thomaz et al., 2009), where the native species cannot survive ( $< 2$ m for *E. najas*; Thomaz et al., 2003). Colonizing different zones of the aquatic environment allows this invader macrophyte to avoid the high biotic resistance imposed by the higher density of native species at lower depths (Thomaz et al., 2003, 2009), reducing competition between them. In addition, the high abundance of other macrophytes in shallower areas increases the concentration of sediment OM (Godshalk and Wetzel, 1978), which should also indirectly explain why *H. verticillata* was favored in deeper areas. In relation to maximum fetch, the same rationale employed to explain the findings at broad spatial scale may apply at fine spatial scale. In addition, moderate wave action increases water flow and reduces the boundary layer around the leaves, enhancing $CO_2$ uptake (Madsen et al., 2001), and reducing periphyton growth (Istvánovics et al., 2008). Both processes increase the photosynthetic rates of submerged macrophytes and probably explain why *H. verticillata* attains higher abundances where fetch is longer.

The negative correlation between turbidity and *H. verticillata* abundance was also similar to results we found at the broad spatial grain. However, the relative importance of this variable was smaller at the fine than at the broad spatial grain. The greater variability of turbidity between the different arms obtained at the broad spatial grain can explain this finding (**Tables 2**, **3**). Thus, light availability may have been more limiting, and therefore its explanatory importance was greater at the broad spatial grain.

### Spatial Grain Dependency of the Importance of Native Competitor on the Invasive Performance

The abundance of the competing native species *E. najas* correlated positively with the invasive *H. verticillata* abundance at broad spatial grain, but the abundance of both species correlated negatively at fine spatial grain. Opposing correlations between indicators of the success of invasive and native species (usually expressed by the diversity of both groups) at different spatial scales have been reported by others (Herben et al., 2004; Davies et al., 2005; Fridley et al., 2007). As the spatial scale (or grain) increases, the variation of resources among local habitats (abiotic heterogeneity), rather than the average of these resources, enhances the native and exotic cumulative diversity, favoring the co-occurrence among them (Davies et al., 2005; in this specific study, the spatial scale varied from a 1 $m^2$ quadrat to a 23 716 $m^2$ block in a grassland). Then, high co-occurrences between the invasive and the native species would exist at large spatial scales in case of a great spatial heterogeneity (Fridley et al., 2007), which is observed in the arms of the Itaipu Reservoir (Thomaz et al., 2003). A great environmental heterogeneity allows more species (including invasive ones) to co-occur, because there is a great variability of available resources and abiotic conditions to be explored (Davis et al., 2000; Tilman, 2004). Thus, at broad spatial scales, one expects to find positive relations between the performance of invasive species and native ones, as observed for the two species evaluated in this study.

The negative native-invasive relationship we found at fine spatial grain should be more cautiously interpreted, since the abundance of *E. najas* presented less influence on abundance of *H. verticillata*. Even though, this result may open some questions about the mechanisms involved in the interaction between both species. We can suggest that this relationship may be related to factors associated with the niche theory, considering that both species are phylogenetically close and functionally similar (Sousa, 2011). We observed that when both plants were neighbors, the abundance of *H. verticillata* usually decreased with rising *E. najas* abundance. It suggests competition between species may occur, or they differ in terms of environmental optima due to the different physiological tolerances (Fridley et al., 2007). Both observational studies (Sousa et al., 2009) and experimental studies (Thomaz et al., 2012; Silveira et al., 2016) have been describing an indication of inter-specific competition between *H. verticillata* and *E. najas* and differences between their environmental optima. However, stronger conclusions require more studies investigating mechanisms driving this relationship.

## CONCLUDING REMARKS

In summary, we found that similar abiotic factors (mainly the fetch and underwater light) might play an important role regulating the success of an invasive submerged macrophyte at both large and fine spatial grains. Particularly at fine grains, other abiotic factors related to sediment fertility and stress, like organic matter, become important determinants of the invasive success. Moreover, in addition to mechanisms driven by abiotic constraints, competing interactions may relate to invasion inhibition only at these fine spatial grains. It is important to note, however, that the explanatory power of the selected models was not strong, and thus the generalizations should be considered with caution. It is worth noting also that the result obtained here are limited to restricted spatial scales in a large reservoir and, so, extrapolation to different spatial scales and other ecosystems (e.g., streams or rivers) deserves additional investigation. Despite of limitations, our major finding suggests that models that attempt to explain the success of invasive plants, should consider spatial scales. Our findings may guide future investigations addressing mechanisms that are more specific regulating invasion success by *H. verticillata* at different spatial scales.

## AUTHOR CONTRIBUTIONS

MP, MD-F, and ST conceived the ideas and the sampling design. MP and MD-F conducted the field samples. MP and EC conducted the statistical analysis and their interpretations. MP and ST lead the manuscript writing. All authors contributed crucially to the final version of the manuscript and agree with it.

## FUNDING

The Coordination for the Improvement of Higher Education Personnel (CAPES), an organ of the Brazilian Government, provided MSc scholarship for MP. The Brazilian Council of Research (CNPq) provided the MD-F's Ph.D. scholarship. The Fundação Araucária, an organization of the Parana state government (Brazil), provided the EC's Ph.D. scholarship. ST is Productivity Researcher from the CNPq and acknowledges this agency for constant funding. The authors are grateful to Itaipu Binacional, which also funded partially this work.

## ACKNOWLEDGMENTS

We thank Lucas Assumpção Lolis (Aquatic Macrophytes Laboratory) for helping with field samplings. We also acknowledge Raquel Freitas Duarte (Itaipu Binacional) for providing data about slopes at broad spatial grain.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling Editor is currently organizing a Research Topic with one of the authors ST, and confirms the absence of any other collaboration.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 03 May 2019
doi: 10.3389/fpls.2019.00572

# Light Availability and Patterns of Allocation to Reproductive and Vegetative Biomass in the Sexes of the Dioecious Macrophyte *Vallisneria spinulosa*

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***Edited by:***
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***Specialty section:***
*This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science*

***Received:*** *20 January 2019*
***Accepted:*** *16 April 2019*
***Published:*** *03 May 2019*

***Citation:***
*Li L, Ding M, Lan Z, Zhao Y and Chen J (2019) Light Availability and Patterns of Allocation to Reproductive and Vegetative Biomass in the Sexes of the Dioecious Macrophyte Vallisneria spinulosa. Front. Plant Sci. 10:572. doi: 10.3389/fpls.2019.00572*

Environmental changes, e.g., eutrophication, in aquatic ecosystems can greatly alter light available to submerged macrophytes. In dioecious plants, given potential for sex-specific differences in resource requirements (i.e., high-carbon for seeds vs. high-nitrogen for pollen), females and males are expected to divergently adjust allocations toward resource acquisition structures when resources are limited during growth. Here, *Vallisneria spinulosa* was used as a representative dioecious submerged macrophyte to detect sex-specific responses to light limitation and assess whether sexual dimorphism varied with resource availability. Plants were grown under varying levels of light availability in nine outdoor mesocosms for 14 weeks. Late in the reproductive season, allocations to vegetative and reproductive traits for both sexes were determined and relative allocation to reproduction vs. vegetative growth was analyzed. Female and male reproductive plants differed in adjustments of resource allocation in response to light availability. Under low light, females showed a smaller reduction in allocation of resources to vegetative tissues and greater leaf area than males, suggesting female plasticity to increase carbon capture. Under low light, males showed a smaller reduction in reproductive allocation than females (flowers and inflorescences in males vs. fruits in females), suggesting that carbon limitation has greater impacts on sexual reproduction by females than males. Our study provides evidence of differences in reproductive costs and currencies for female vs. male reproduction in aquatic macrophytes, as *V. spinulosa* responded plastically to reduced light, with sexually dimorphic allocation strategies. Sex-related resource currencies are potentially important drivers for sex-specific variations in allocation patterns, with females safeguarding their vegetative carbon-rich biomass to satisfy future fruit and seed production.

**Keywords: carbon limitation, dioecious plant, light intensity, plasticity, resource allocation, sexual dimorphism, submersed macrophyte**

## INTRODUCTION

Most flowering plants are hermaphroditic, with dioecy in only 5 to 6% of angiosperm species (Renner, 2014). Separate sexes have evolved repeatedly from hermaphroditic ancestors, in different lineages, probably in response to selection either for inbreeding avoidance (Charlesworth and Charlesworth, 1978) or sexual specialization (Charnov et al., 1976; Charlesworth, 1999). Transitions from hermaphroditism to dioecy are often related to evolution of sexual dimorphism. Once plants have evolved separated sexes, males and females have distinct roles; therefore, selection may favor divergence in life-history traits to optimize seed and pollen production (Barrett and Hough, 2013), presumably enabling sex-specific strategies of resource acquisition and reallocation.

Sexual dimorphism occurs in many dioecious terrestrial plants, with sexual morphs differing in: number, size and morphology of flowers and inflorescences; rates of growth, time to reproduction and timing of senescence; physiological traits, e.g., rates of photosynthesis and water uptake; and allocation toward life-history traits and anti-herbivore defenses (reviewed by Ågren et al., 1999; Dawson and Geber, 1999; Delph, 1999; Eckhart, 1999). Degree of sexual dimorphism varies among plant and animal species (Harris and Pannell, 2010; Laiolo et al., 2013; Tonnabel et al., 2014) and among populations within a species (Teder and Tammaru, 2005; Tonnabel et al., 2017).

Whereas sexual selection resulting from variation among individuals in mating success is the most likely cause of sexual dimorphism in animals (Ågren et al., 1999), evolution of sexually dimorphic characters in plants probably occurs due to differential resource requirements for reproduction. Female function (i.e., seed and fruit production) generally requires much carbon (Antos and Allen, 1990; McDowell et al., 2000), whereas male function probably has larger nitrogen requirements for pollen production (Harris and Pannell, 2008; Van Drunen and Dorken, 2012). Given female and male reproduction are differently limited by contrasting resources or "currencies," selection on resource-harvesting traits likely differs between sexes, potentially eliciting sex-specific differences in "somatic cost of reproduction" for such different types of currencies (Obeso, 2002).

The differential plasticity hypothesis proposed that males and females differ in plastic responses to environmental factors, causing variation in degree of sexual dimorphism across environmental gradients (Delph and Bell, 2008). Divergent resource currency requirements for female vs. male reproduction may result in evolution of sex-specific plasticity in resource acquisition traits in response to environmental changes in resource availability (i.e., carbon and nitrogen). Previous studies examining the differential plasticity hypothesis focused on effects of nitrogen limitation from the perspective of relative resource investment in functionally different organs. For example, under low-nitrogen conditions, males likely benefit more than females from increasing allocation to belowground structures, as they harvest nitrogen needed for pollen production (Harris and Pannell, 2008; Teitel et al., 2016). However, some studies have also found that under dune soils, characterized by low-nitrogen contents, female plants showed greater belowground biomass than males (Sanchez-Vilas et al., 2012). If reproductive effort in females is limited more by carbon than nitrogen, females may exhibit plasticity in growth when exposed to carbon limitation to secure more carbon than males to ensure future carbon-rich reproduction. Females may also deploy sufficient resources to aboveground vegetative organs before involving carbon allocation toward flowering, as a way to maximize carbon acquisition. However, sex-specific variations in reproductive effort in response to a carbon limitation, which potentially limits female function, is not well understood (Tonnabel et al., 2017).

Submerged macrophytes play an important role in maintaining healthy aquatic ecosystems. Eutrophication and degraded underwater light climate is one of the fundamental reasons causing worldwide disappearance of submerged macrophytes (Schelske et al., 2010; Zhang et al., 2017). Variations in light reception influence the potential of a plant to capture carbon, which may limit plant growth and photosynthesis. Substantial phenotypic plasticity for resource capture is important for submerged macrophytes (Barrett et al., 1993; Going et al., 2008; Hyldgaard and Brix, 2012), enabling them to be more competitive (Chambers and Kalff, 1987). For instance, submerged species are highly responsive to light shortage and can have morphological and structural adaptations over the period of growth to optimize light utilization by increasing shoot length, decreasing branches, producing longer, wider and thinner leaves and changing resource allocation to various plant structures (Riis et al., 2012; Schneider et al., 2015; Chen et al., 2016). Numerous studies have been conducted to address the responses of submerged macrophytes to decreased light, albeit mostly from the perspective of growth conditions. To our knowledge, whether male and female plants are optimized by different resource allocation strategies in response to altered light availability is not well known. Thus, we herein address the responses of submerged macrophytes to limited light conditions from the perspective of sexual dimorphism.

In shallow freshwaters, substantial changes in light availability in populations of submerged macrophytes could be induced by eutrophication (Cao et al., 2011; Zhang et al., 2016). Plants generally respond plastically to changed resource availability during growth, even after reproduction commences. However, whether such a response is sexually dimorphic and reflects expectations based on potential existence of sex-specific resource currency requirements, remains to be investigated. Here, we used *Vallisneria spinulosa* as a representative dioecious submerged macrophyte to investigate potential responses in vegetative and reproductive traits to light availability and to evaluate patterns of sexual dimorphism, based on the differential plasticity hypothesis. In an aquatic mesocosm experiment that varied light availability of plants during their growing season, we tested three hypotheses: (1) females respond to reduced light by decreasing allocation toward aboveground growth less than males to satisfy carbon demands for future reproduction; (2) with increasing light limitation, females decrease allocation toward sexual reproduction more than males to ensure future capacity for carbon acquisition; and (3) sexual dimorphism in resource allocation is enhanced under stressful environmental conditions (e.g., limited light). Our results demonstrated that variations in light availability differentially affected males and females in their ability to perform photosynthesis, with implications for further understanding the effects of eutrophication on submerged macrophytes and also evolution of aquatic plants to varied environments.

# MATERIALS AND METHODS

## Study Species

*Vallisneria spinulosa* S. Z. Yan (Hydrocharitaceae) is a dioecious, rosette-forming clonal submerged macrophyte with a hydrophilous pollination system. Light interception and utilization in *V. spinulosa* are determined by its long, strap-shaped leaves rather than by its short basal meristem stem; therefore, leaf growth impacts survival and reproduction. This plant commonly occurs in ponds, streams and lakes in mid-lower reaches of the Yangtze River in China (Wang et al., 2010) and grows well in waters with medium turbidity (Chambers and Kalff, 1987). It provides food for waterfowl, nursery habitats for fish and a substrate for invertebrates, and may modulate water quality (Wang et al., 2010).

In common with other *Vallisneria* species (Sculthorpe, 1967; Cox, 1988), female flowers develop underwater and are brought to the surface singly on elongated slender peduncles and at anthesis float on the surface. Male inflorescences (spathes) are located among bases of leaves with hundreds of packed male flowers. At maturity, male flowers are released to the water surface and aggregate around female flowers. Pollinated flowers are retracted underwater via a helix of peduncles of female flowers, where fleshy fruit develop. Clonal growth results from production of rosette-like ramet along elongating stolons and in the autumn, ramets produce several tubers that overwinter in sediment and germinate the following spring.

## Tuber Collection

Numerous *V. spinulosa* tubers were randomly collected (February 2017) from a large dioecious population at Bang Lake (115°55′–116°06′E, 29°11′–29°18′N; average water depth, 3.0 m) in Poyang Lake National Nature Reserve (Jiangxi Province, China, 115°55′–116°03′E, 29°05′–29°15′N). The tubers could not be sexed when they were collected. Tubers were cleaned, transported to the laboratory and kept in a water-filled plastic container maintained in darkness at 4°C for approximately 4 months.

## Experimental Design

To investigate sex-specific patterns of phenotypic plasticity in response to light availability and how sexual dimorphism in resource allocation varied across light gradients, an outdoor experiment was conducted in nine concrete mesocosms (2 × 2m, with water 1.6 m deep) at the Poyang Lake Laboratory for Wetland Ecosystem Research, Chinese Academy of Sciences (116°03′E, 29°26′N) located in Lushan City, China. Initially, 234 mature *V. spinulosa* tubers of uniform size (mean fresh mass ± SE = 0.906 ± 0.146 g; range of tuber mass = 0.722–1.335 g) were individually planted into plastic pots (23 cm diameter and 17 cm deep) containing a 4:1 mixture of Poyang Lake sediment and fine sand. Sediment was dried, ground and filtered through a sieve with a 0.5-cm mesh. Water (TN: 1.50 mg $l^{-1}$; TP: 0.05 mg $l^{-1}$; Chla: 6.6 μg $l^{-1}$) was pumped from Poyang Lake and filtered (64-μm diameter net). There were three levels of light availability in a randomized block design, with each of the three blocks containing one replicate of each treatment. Six mesocosms were covered with black neutral shade nets to achieve the following light levels as percent of incoming light: 20% and 60% ambient light, representing low and medium light intensity, respectively. The other three mesocosms were unshaded and thus received 100% of ambient light, representing high light intensity. There were 234 pots randomly assigned to nine concrete mesocosms. For each light treatment, three replicate mesocosms were used and each mesocosm contained 26 pots ($n$ = 78 pots/treatment). Each pot was tied with a nylon rope to a galvanized metal tube, suspended at 60 cm water depth. To renew water and maintain a consistent level, two or three times weekly, water from a common header tank was added to each mesocosm. Epiphyton on the leaves of *V. spinulosa* was removed with a soft brush every month to exclude shading of epiphyton on macrophyte. Plants grew in mesocosms for 14 weeks during summer and autumn (4 June to 10 September 2017). Sexual dimorphism is inconspicuous in pre-reproductive juveniles and plants can only be accurately sexed in *V. spinulosa* a few days after onset of flowering, based on spathe morphology. Therefore, because we used randomly chosen plants once tubers geminated, sex ratios of flowering plants in each treatment showed a strong male bias (average sex ratio = 2.87).

Photosynthetically active radiation (PAR) was measured with a Li-COR UWQ-192SA sensor coupled with a Li-1400 data logger (Li-Cor, Lincoln, NE, United States) at 10:00–12:00 under clear skies on 16 July 2017. PAR at water surface in low to high light regimes was 436.82 ± 6.45, 889.17 ± 16.45 and 1787.89 ± 18.75 μmol $m^{-2}$ $s^{-1}$ (mean ± SE, $n$ = 9), respectively. For sediment in pots, contents of total N, total P and organic matter were 2.45 mg $g^{-1}$, 0.73 mg $g^{-1}$ and 6.61% ($n$ = 3 pots).

## Allocation Measurements

At fruit maturation (98 days after planting), above- and below-ground portions of well-developed, intact plants of both sexes in each mesocosm were harvested. Final numbers of harvested male plants were 59, 44, and 35 for low, medium, and high light treatments, respectively, and those of harvested female plants were 19, 22, and 10. Because some stunted plants grew very poorly and did not flower and others were grazed by aquatic insects, only plants that grew vigorously and flowered were harvested. After removal from sediments, plants were carefully washed with tap water.

To assess male and female resource allocations, traits for both growth and reproduction were measured. Plant height, ramet number, shoot biomass (i.e., leaf biomass), leaf number and leaf area are key traits related to plant size and competitive interactions for light. Plant height was measured as length of the longest leaf. Total number of leaves for 10–15 plants per treatment were recorded and entire leaf surface area for each plant determined with an AM-350 Leaf Area Meter (ADC Bioscientific, Hoddesdon, United Kingdom). To determine percentage of flowering ramets, total and flowering ramet numbers for each plant were counted. Number of tubers and for males, number of spathes (including intact spathes and pedicles of spathes that had fallen off) and for females, number of fruits, were counted. Every plant was separated into three components: vegetative biomass (including leaves, roots, and stolons); sexual reproductive biomass (spathes or peduncles and fruits); and clonal reproductive biomass (tubers). To determine dry mass for each component, samples were dried at 75°C for 3 days and then weighed on a SI-114 four-decimal place gram balance (Denver Instrument, Denver, CO, United States). Because dispersal of pollen from some male spathes occurred when plants were harvested, we randomly sampled 10 non-dehiscent, mature male spathes from five male plants within each light regime and weighed each spathe individually after drying. Data from these 10 male spathes obtained in each light regime were averaged and multiplied by the total number of spathes produced per male to estimate biomass of sexual reproductive tissues of male plants for each light regime. In this study, dry mass of leaves, roots, and stolons (i.e., non-reproductive parts) was combined as vegetative investment (i.e., vegetative growth). Sexual reproductive effort was calculated by dividing total mass of male spathes or female peduncles and fruits by resources allocated to the entire plant. Allocation to clonal reproduction was determined by dividing tuber biomass by total biomass.

## Statistical Analyses

To investigate if sexual dimorphism in resource allocation varied with light availability, a Restricted Maximum Likelihood (REML) linear mixed model was used. For this model, sex and light treatment were fixed factors and block was random factor, with each life-history trait analyzed independently. For all traits, the interaction between sex and light pointed to significant effect of light on sexual dimorphism. *Post hoc* comparisons among groups used a Tukey honestly significant difference (HSD) test procedure ($\alpha < 0.05$). To investigate our differential plasticity hypothesis, for each sex, relative allocation to vegetative and sexual reproductive biomass was calculated as percentage change in biomass under low light compared to mean allocation under high light. Tests of equality of means between sexes were done with a Student's $t$-test ($\alpha = 0.05$). All analyses used SPSS software, version 19.0 (SPSS Inc., Chicago, IL, United States).

# RESULTS

## Sexual Dimorphism in Aboveground Growth and Reproductive Traits

Of traits representing allocation to growth, all but one, "plant height," had significant sexual dimorphism (**Table 1**). Under high or medium light, all vegetative traits showed a non-significant tendency to be greater in females compared to males (**Figure 1**). Under low light, females exhibited significantly greater investment in leaf and vegetative biomass, ramet number, leaf number, and leaf area than males (**Figures 1B–F**).

All measured reproductive traits, except those reflecting allocation toward clonal propagation (tuber number, clonal reproductive effort) displayed significant sexual dimorphism (**Table 1**). Males had a higher percentage of flowering ramets than females, but only in medium or low light treatments (**Figure 2B**). Males produced more flowering ramets and inflorescences than females under high or medium light (**Figures 2A,C**), but there was no significant difference between sexes under low light. In contrast, females invested proportionally more resources in sexual reproductive structures than males across all light treatments (**Figure 2E**).

## Sex-Specific Plasticity in Response to Variable Light

Light availability differentially affected males and females both in leaf biomass (sex × light interaction: $P = 0.019$; **Table 1** and **Figure 1E**) and vegetative biomass (sex × light interaction: $P = 0.023$; **Table 1** and **Figure 1F**). Males and females reduced leaf and vegetative biomass with decreasing light intensity; however, males did so more than females (**Figures 1E,F**). Leaf area also exhibited sex-differential plasticity in response to light availability (sex × light interaction: $P = 0.044$; **Table 1** and **Figure 1D**). Whereas female leaf area was independent of light availability, males decreased their leaf area under low light (**Figure 1D**). Males and females both increased plant height with decreasing light (**Figure 1A**), whereas ramet number and leaf number had an opposite pattern (**Figures 1B,C**). Light availability did not affect sexual dimorphism in plant height (sex × light interaction: $P = 0.813$; **Table 1** and **Figure 1A**), ramet number (sex × light interaction: $P = 0.100$; **Table 1** and **Figure 1B**), and leaf number (sex × light interaction: $P = 0.789$; **Table 1** and **Figure 1C**).

In both males and females, flowering ramet and inflorescence number were significantly reduced in low light (**Figures 2A,C**), but males had steeper decreases in flowering ramets (sex × light interaction: $P = 0.013$; **Table 1** and **Figure 2A**) and inflorescences (sex × light interaction: $P = 0.001$; **Table 1** and **Figure 2C**) than females, as light intensity decreased. Number of tubers in both sexes was reduced similarly with decreasing light (sex × light interaction: $P = 0.957$; **Table 1** and **Figure 2D**).

## Growth vs. Reproductive Allocation

The degree of sexual dimorphism in sexual reproductive effort (proportion of biomass allocated to sexual reproductive structures) varied with light treatment (sex × light interaction: $P = 0.004$; **Table 1** and **Figure 2E**). Both males and females displayed plastic responses in their relative allocation to sexual reproductive vs. vegetative tissues; this ratio was significantly lower in low light for both sexes (Male: *post hoc* comparison: $P = 0.007$; Female: *post hoc* comparison: $P = 0.047$; **Figure 2E**). However, decreased light caused females to reduce relative

**TABLE 1 |** Results of the Restricted Maximum Likelihood (REML) linear mixed model analyses examining effects of sex and light treatment on traits of *V. spinulosa*.

| Traits | Results | Source of Variation | | |
|---|---|---|---|---|
| | | **Sex** (*df* = 1) | **Light** (*df* = 2) | **Sex × Light** (*df* = 2) |
| **Vegetative traits** | | | | |
| Plant height | *F* | 2.575 | 96.924 | 0.207 |
| | *P* | 0.110 | **<0.001** | 0.813 |
| Ramet number | *F* | 19.331 | 174.326 | 2.337 |
| | *P* | **<0.001** | **<0.001** | 0.100 |
| Leaf number | *F* | 6.691 | 176.379 | 0.237 |
| | *P* | **0.010** | **<0.001** | 0.789 |
| Leaf area | *F* | 4.262 | 15.660 | 0.546 |
| | *P* | **0.041** | **<0.001** | **0.044** |
| Leaf biomass | *F* | 18.980 | 28.092 | 0.792 |
| | *P* | **<0.001** | **<0.001** | **0.019** |
| Vegetative biomass | *F* | 15.194 | 40.126 | 0.879 |
| | *P* | **<0.001** | **<0.001** | **0.023** |
| **Reproductive traits** | | | | |
| Flowering ramet number | *F* | 29.442 | 48.428 | 4.465 |
| | *P* | **<0.001** | **<0.001** | **0.013** |
| Percentage of flowering ramet | *F* | 104.251 | 5.924 | 7.965 |
| | *P* | **<0.001** | **0.003** | **<0.001** |
| Spathe/fruit number | *F* | 55.715 | 20.717 | 7.863 |
| | *P* | **<0.001** | **<0.001** | **0.001** |
| Tuber number | *F* | 0.791 | 62.656 | 0.044 |
| | *P* | 0.375 | **<0.001** | 0.957 |
| Sexual reproductive effort | *F* | 68.400 | 10.920 | 5.682 |
| | *P* | **<0.001** | **<0.001** | **0.004** |
| Clonal reproductive effort | *F* | 1.124 | 1.781 | 4.391 |
| | *P* | 0.290 | 0.171 | **0.014** |

*Bold values are significant at $P < 0.05$.*

allocation to sexual reproduction more than males (**Figure 2E**). Vegetative biomass tended to decrease less in females than in males under lower light levels (**Figure 3A**), whereas allocation toward sexual reproductive biomass under lower light levels tended to decrease more in females than in males (**Figure 3B**). Whereas females reduced clonal reproductive effort under limited light (*post hoc* comparison: $P = 0.028$; **Figure 2F**), males maintained the same ratio among all light conditions (*post hoc* comparison: $P = 0.564$; **Figure 2F**).

## DISCUSSION

Effects of altered light availability on performance of submerged macrophytes are well known (e.g., Xie et al., 2007; Riis et al., 2012; Yuan et al., 2012; Eller et al., 2015), but sex-specific plasticity in sexual dimorphism in response to varied light levels over an entire growing season has not been well characterized. The present study provides evidence supporting the differential plasticity hypothesis in aquatic environment, particularly in the context of sex-specific resource requirements, with insights into evolution of sexual dimorphism in dioecious macrophytes. Reproductive males and females differentially adjusted resource allocation to reproduction and aboveground vegetative growth in response to decreased underwater light availability. Whereas females showed a greater allocation of resources to vegetative tissues and leaf area than males under limited light, males showed a smaller reduction of resource allocation to sexual reproduction than females. Because carbon acquisition is more critical for female reproduction, under conditions of light limitation females are expected to decrease their reproductive allocation more than males, as a way to conserve resources for vegetative organs to ensure their carbon needs for future reproduction. Relevant results can help further understanding of the effects of eutrophication on submerged macrophytes and evolution of aquatic plants to varied environments.

### Plastic Resource Allocation With Varied Light

In our experiment, resource allocation in *V. spinulosa* was highly responsive to a change in light availability. Although various macrophyte species may respond differently to light deficiency (Chambers, 1987), decreased light in water often reduces coverage and decreases growth of macrophytes in the littoral zone and vice versa (*Vallisneria americana*, French and Moore, 2003;

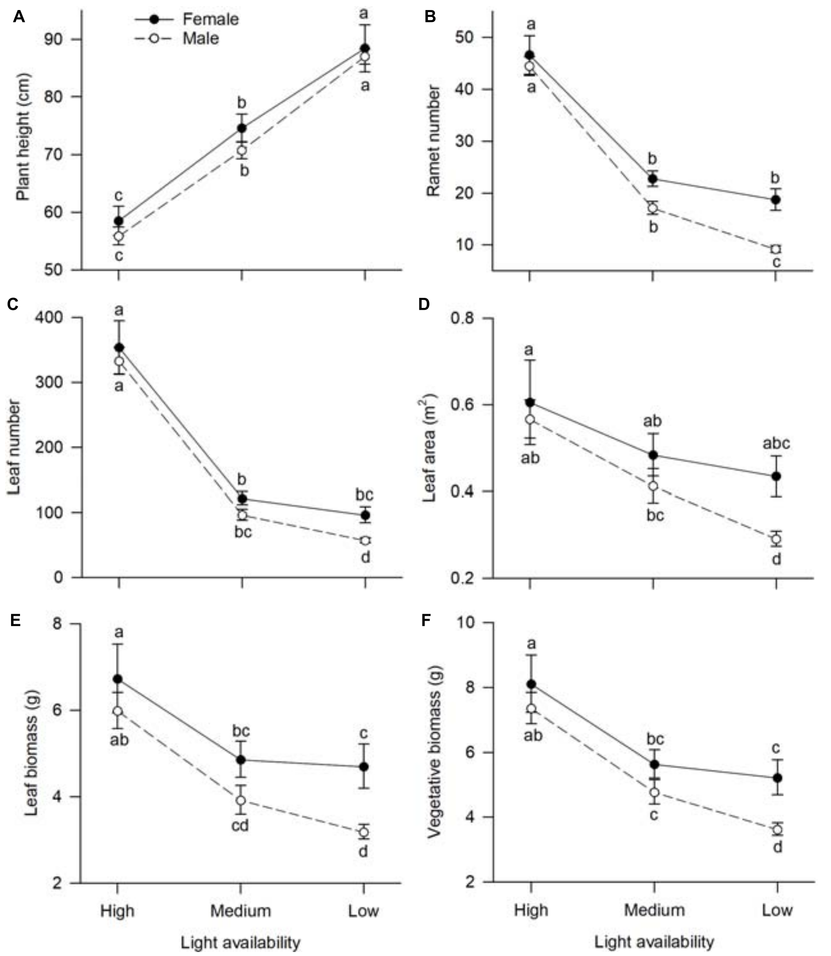


**FIGURE 1 |** Effects of light availability on allocation to vegetative tissues for both males and females of *V. spinulosa* at reproductive maturity: **(A)** plant height, **(B)** ramet number, **(C)** leaf number, **(D)** leaf area, **(E)** leaf biomass, and **(F)** vegetative biomass. Letters denote significant difference ($P < 0.05$), using Tukey HSD comparisons. Error bars show ± SE. Curves are usually used to display differences between sexes under different environmental conditions (Harris and Pannell, 2008; Hesse and Pannell, 2011).

*Vallisneria natans*, Chen et al., 2016). In our experiment, both vegetative and reproductive traits tended to decrease as light decreased from 100% to 20% incident light. Unlike canopy formers (e.g., *Potamogeton maackianus*), *V. spinulosa*, being rosette-forming, cannot rapidly elongate to reach the water surface where light availability is high. Thus, aboveground vegetative growth (leaf biomass) of *V. spinulosa* declined in limited light condition, as also reported in *V. americana* (French and Moore, 2003) and *V. natans* (Fox et al., 2013) in adaptation experiments to shading.

A plastic response to decreased light in *V. spinulosa* involved linear increases in plant height through leaf elongation, decreased ramet number and leaf number and thereby decreased leaf area. Thus, reproductively mature plants showed less lateral growth and more elongated growth with decreasing light gradients, resulting in a shift of leaf area into better illuminated areas (Valladares and Niinemets, 2008). Such a plastic response has the same effect as foraging concept in most herbaceous terrestrial plants with erect stems, which hypothesizes that plants develop longer stems and less branches in low-light conditions to improve resource acquisition and reduce respiratory cost (de Kroon and Hutchings, 1995). Greater resource allocation to vertical growth also involved a trade-off, causing decreased resource allocation to reproduction, as reported (Harper, 1977), but decreasing total plant biomass. In the present study, plants also responded plastically to reduced light by altering resource allocation to reproductive traits. A negative effect on sexual reproduction is likely to influence seed dispersal, re-establishment after disappearance and may reduce resistance to other environmental stressors, due to lower genetic diversity (Boedeltje et al., 2008).

## Evidence of Differential Plasticity Hypothesis in Macrophytes

Our study highlighted flexibility of plants in resource allocation and how reproductive male and female plants responded plastically to light stress with resource allocation that maximized fitness. The differential plasticity hypothesis has been tested

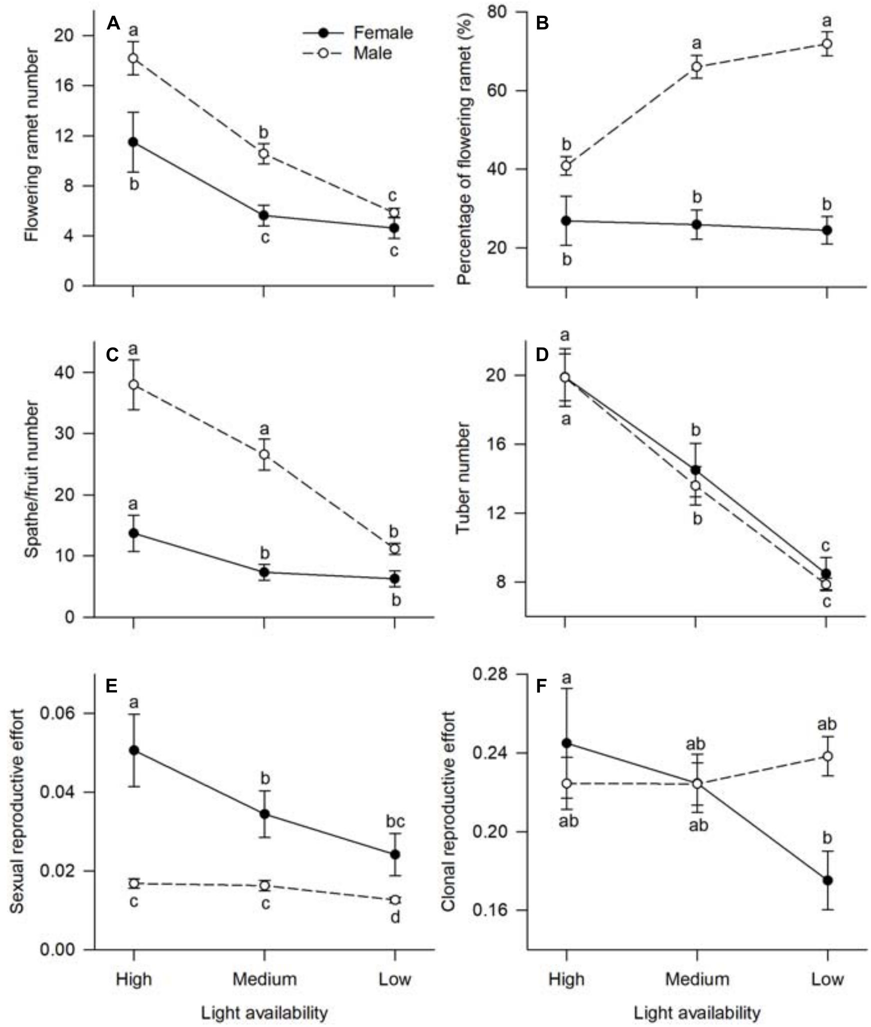


**FIGURE 2 |** Effects of light availability on allocation to reproductive tissues for males and females of *V. spinulosa* at reproductive maturity: **(A)** flowering ramet number, **(B)** percentage of flowering ramet, **(C)** spathe/fruit number, **(D)** tuber number, **(E)** sexual reproductive effort, and **(F)** clonal reproductive effort. For each individual sexual and clonal reproductive efforts were calculated as the ratio between dry mass of the respective tissues (male: spathes; female: fruits) and total biomass. Letters denote significant difference ($P < 0.05$), using Tukey HSD comparisons. Error bars show ± SE. Curves are usually used to display differences between sexes under different environmental conditions (Harris and Pannell, 2008; Hesse and Pannell, 2011).

in several terrestrial plants (e.g., *Silene latifolia*, Lovett Doust et al., 1987; *Mercurialis annua*, Harris and Pannell, 2008; *Rumex hastatulus*, Teitel et al., 2016), with mixed results. Regarding the differential plasticity hypothesis, we emphasized plant flexibility in responding to local environments, particularly in varying allocation to growth vs. reproduction. Greater sexual dimorphism in vegetative tissues and smaller sexual dimorphism in resource allocation to sexual reproduction for dioecious *V. spinulosa* under low light was consistent with the differential plasticity hypothesis.

Flexible reproductive strategies are likely an adaptive advantage in aquatic environments, which often have strong spatio-temporal heterogeneity (Eckert et al., 2016). In aquatic ecosystems, environmental heterogeneity, e.g., eutrophication, can strongly affect access to light for submerged macrophytes, similar to that we manipulated in our study. We inferred plasticity in sexual dimorphism is advantageous to aquatic plants in the face of adverse environmental conditions caused by eutrophication, such as high turbidity and low irradiance penetration. In accordance with the differential plasticity hypothesis, males and females of *V. spinulosa* differentially adjusted their relative allocation of resources among vegetative and reproductive tissues with decreasing underwater light availability, reflecting sex-specific adaptation. Divergent plastic responses of male and female plants in vegetative and reproductive traits implied a potential reason for intraspecific variation in sexual dimorphism in resource allocation under light limitation (i.e., more stressful environment).

More specifically, decreased light for a submerged plant during its life cycle, as in our study, could be induced

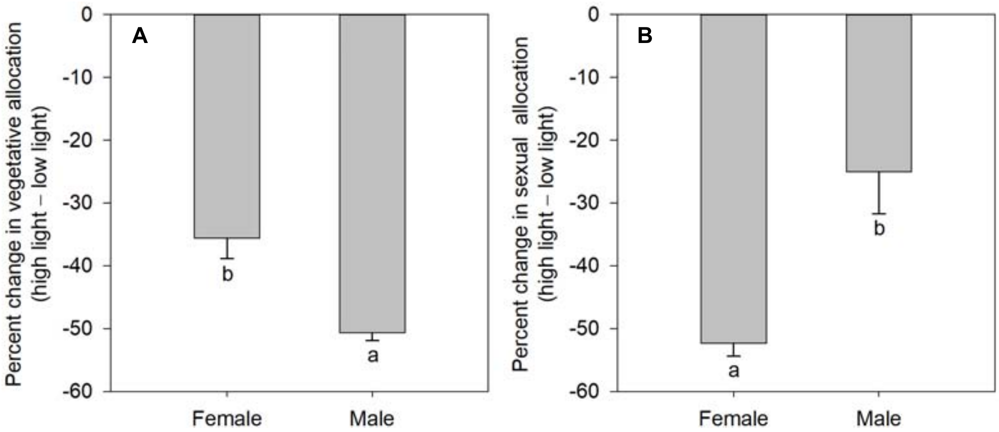


**FIGURE 3 |** The mean percentage change in vegetative and sexual reproductive tissues under low light regime relative to their average allocation under high light regime; values are means ± SE, for males and females of *V. spinulosa*. **(A)** Vegetative and **(B)** sexual reproductive allocations were calculated as percentage change in biomass of the respective tissues compared to the mean allocation to these tissues under high light regime for each sex. Values with different letters denote statistically significant differences between sexes ($P < 0.05$), calculating using likelihood ratio test.

by re-suspension of sediment, variations in periphyton and phytoplankton abundance, or by concentrations of dissolved materials due to nutrient enrichment. If resource limitation elicits sex-specific plastic responses, spatial variation in this resource will cause spatial variation in degree of sexual dimorphism (Barrett and Hough, 2013). There is also ample evidence that higher reproductive expenditure and/or greater sensitivity to stress in females should result in more male-biased sex ratios among populations of a species along gradients of resource availability (e.g., Pickering and Hill, 2002; Li et al., 2007). Our results reflect environmental variation between sexes in their allocation to vegetative tissues and reproduction and provide support for differential plasticity contributing to variations in sexual dimorphism in aquatic macrophytes. A growing evidence from studies of sexual dimorphism under varying resource levels supported the differential plasticity hypothesis (Harris and Pannell, 2008; Hesse and Pannell, 2011; Teitel et al., 2016; Li et al., 2019). However, details of how resource currencies are differently reallocated from sources to sinks and how patterns of variation in sexual dimorphism are driven by different resource currencies, remain largely unknown.

## Sex-Specific Strategies of Resource Allocation

Our results illustrated that high levels of plasticity in response to the environment affected traits that can be sexually dimorphic in dioecious plants; therefore, indices of dimorphism themselves become plastic. In *V. spinulosa*, how resource currencies were reallocated from sources to sinks was altered in response to light limitation, consistent with responses in dioecious *M. annua* (Tonnabel et al., 2017). Sex-specific allocation patterns in *M. annua* vary temporally and also respond to environmental heterogeneity (Harris and Pannell, 2008). Males and females used disparate resource allocation strategies, exhibiting sex-specific resource trade-offs, which can involve disparate resource currencies in females and males. In many dioecious plants, female reproductive costs may exceed those of males, due to high investments in carbon-rich fruits and seeds (Barrett and Hough, 2013). In particular, flowering in many submerged macrophytes requires considerable investment in reproductive accessory structures, e.g., production of peduncles in female *V. spinulosa*, to bear flowers at or above the water surface. Reproductive costs result in physiological trade-offs in resource distributions, which can influence future vegetative growth and reproduction. Therefore, females of *V. spinulosa* are expected to have stronger trade-offs with other life-history traits, due to their typically higher investment in sexual reproduction.

Our results conformed to expectations arising from the differential plasticity hypothesis; namely, that females maximize allocation to aboveground growth under conditions of light limitation more than males, as carbon acquisition is more critical for female reproduction. This pattern was due to a stronger reduction of allocation to reproductive tissues in females than in males and a smaller reduction of allocation to vegetative tissues in females than in males when light availability decreased. This points to existence of a greater trade-off in females than males between current and future reproduction in terms of carbon; with limited light, females decreased sexual reproductive effort more than males. This response may be due to a difference in resource currencies that limit female vs. male functions, e.g., carbon may limit reproduction by females more than that by males (Antos and Allen, 1990; McDowell et al., 2000). Perhaps carbon limitation causes a smaller decrease in allocation to carbon-harvesting organs (i.e., shoot and leaves) in females than in males, as females probably safeguard capacity to acquire carbon for future seed and fruit production.

Consistent with our results, similar evidence on sex-specific resource (carbon) budgets and differential allocation strategies in response to low light have been reported. For example, females reduced their allocation to vegetative growth in response to competition for light less than males (Lovett Doust et al., 1987;

Tonnabel et al., 2017), perhaps due to a higher total carbon cost for female reproduction. In contrast, in some studies, females experienced a steeper or similar decrease in resource allocation to aboveground biomass in response to reduced access to light (Hesse and Pannell, 2011; Labouche and Pannell, 2016). These observations were contrary to the expectation, on the basis of a differential somatic cost of carbon between sexes, that females reduce aboveground biomass less than males when carbon becomes limiting. Therefore, our study on submerged macrophyte added to growing evidence providing support for a stronger somatic cost of carbon in females, potentially causing a sex-specific plastic response to limited carbon.

## CONCLUSION

In conclusion, our results highlighted that males and females have different strategies of resource allocation in response to carbon limitation. A decrease in light availability led to a smaller decrease in allocation to carbon harvesting organs in females than in males for the sake of maintaining a capacity for carbon acquisition for later seed and fruit production. Limited light condition caused a smaller reduction of reproductive allocation in males than in females, suggesting that the production of reproductive organs in females is more carbon-limited than in males (fruits in females vs. flowers and inflorescences in males). Eutrophication and degraded underwater light climate is a fundamental cause of worldwide declines in submerged macrophytes (Zhang et al., 2017). Therefore, investigating sex-specific strategies of resource allocation in response to varying light availability is essential for better understanding the adaptation and evolution of submerged macrophytes in freshwater habitats.

## AUTHOR CONTRIBUTIONS

LL designed the study, executed the experiments and wrote the first draft of the manuscript. LL and MD conducted the statistical analyses. JC, ZL, and YZ provided the scientific advice.

## FUNDING

This work was co-supported by the National Key R&D Program of China (2018YFD0900904), the National Natural Science Foundation of China (31400403), and the Natural Science Foundation of Jiangxi Province (20161BAB214157).

## ACKNOWLEDGMENTS

The authors thank Siyuan Xu and Fangyan Liu for help during data collection.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 04 October 2019
doi: 10.3389/fpls.2019.01261

# Effects of Eutrophication and Different Water Levels on Overwintering of *Eichhornia crassipes* at the Northern Margin of Its Distribution in China

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OPEN ACCESS

**Edited by:**
*Katya E. Kovalenko, University of Minnesota Duluth, United States*

**Reviewed by:**
*Thaísa Sala Michelan, Federal University of Pará, Brazil*
*Wei Xing, Chinese Academy of Sciences, China*

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**Specialty section:**
*This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science*

**Received:** *29 July 2018*
**Accepted:** *11 September 2019*
**Published:** *04 October 2019*

**Citation:**
*Yu H, Dong X, Yu D, Liu C and Fan S (2019) Effects of Eutrophication and Different Water Levels on Overwintering of Eichhornia crassipes at the Northern Margin of Its Distribution in China. Front. Plant Sci. 10:1261. doi: 10.3389/fpls.2019.01261*

When exotic species are introduced into new areas, establishment is a vital step in their invasion process. Therefore, overwintering strategies determine whether an exotic species from low latitudes can successfully invade middle- and high-latitude areas. In this study, we investigated the effects of nutrient and water drawdown on overwintering in an exotic aquatic plant from the tropical zone, *Eichhornia crassipes*, at the northern margin of its distribution in China. The population density, size of individuals, and the size and nitrogen concentration of overwintering organs (stem base) of *E. crassipes* that grew in high-nutrition water were greater than those that grew in low-nutrient water before winter. The overwinter survival rate of *E. crassipes* was significantly affected by the water level and nutrient. The thick and dense floating mat of *E. crassipes* can increase the temperature of water bodies**;** therefore, the overwinter survival rate of *E. crassipes* was higher in constant-water-level and high-nutrient treatment. In contrast, due to the loss of heat preservation provided by the floating mats and the low nitrogen concentration in the stem base, all individuals of *E. crassipes* died in constant-water-level and low-nutrient treatment. In the water-drawdown treatments, the stem base of *E. crassipes* was directly exposed to low-temperature air; therefore, the overwinter survival rate of *E. crassipes* was lower. Our results reveal that eutrophication can not only improve the competitiveness of *E. crassipes* but can also improve the survival rate of overwintering plants in temperate regions. Our study also suggests that removing nutrients from the water and regulating the water level can limit the invasion of *E. crassipes* in temperate and subtropical regions.

**Keywords: eutrophication, water drawdown, invasion, overwintering, *Eichhornia crassipes***

## INTRODUCTION

When exotic species are introduced to new ranges, only a few species can establish populations and become invasive. Propagule pressure and the self-sustaining ability of species in adverse environments mainly influenced the establishment (Williamson and Fitter, 1996a; Williamson and Fitter, 1996b). In high-altitude and high-latitude regions, overwintering abilities and strategies are critical for the establishment, distribution, and spread of exotic species, especially for species that originated from habitats in warmer regions. Studies of the overwintering ability of species and factors that affect overwintering allow us to clarify the mechanisms of invasion and better control them.

Overwintering is the process by which some organisms pass through or wait out the winter season. During this period, organisms experience numerous kinds of abiotic (cold or sub-zero temperatures, frost, frost heave, ice, snow, low precipitation, drought) and biotic (limited food supplies, low-temperature fungi, and bacteria) stresses, making normal activity or even survival difficult or near impossible (Bertrand and Castonguay, 2003). Temperature is a limiting factor for survival, growth, and reproduction in plants and many animals (Woodward, 1987; Charnov and Gillooly, 2003). Low-temperature and freezing events in winter result in the invasion failure of many introduced exotic species or restrict the distribution ranges of species that have successfully invaded (Owens et al., 2004; Walther et al., 2009; MacIsaac et al., 2016). Although climate warming causes exotic species to invade regions within which they could not survive before, extends their temporal and spatial distribution, and increases their performance (Walther et al., 2002; Hellmann et al., 2008; Walther et al., 2009; Chown et al., 2012; Concilio et al., 2013; Sorte et al., 2013), the ranges of some exotic species may also be reduced (Merow et al., 2017). In some ranges, precipitation was also low in winter, which leads to a decline in the water levels of lakes and rivers. Water level is an important factor affecting the growth and reproduction of aquatic plants in freshwater ecosystems (Chambers and Kalff, 1985; Ishii and Kadono, 2004; Deegan et al., 2006; Smith and Brock, 2007; Xiao et al., 2007; Xie et al., 2008). Moreover, water is usually considered to be a temperature buffer to prevent aquatic plants from direct damage by freezing in winter (You et al., 2013). Therefore, water level changes can also influence the overwintering of exotic species.

Nutrients are also another important abiotic factor affecting species growth, propagation, or colonization, and as one of the results of global changes that is driven by human activities and rapid economic growth, eutrophication has become increasing common and severe in water systems (Ryther and Dunstan, 1971; Smith et al., 1999). Previous studies found that the relative growth rates, reproductive rates, photosynthetic rates, leaf nitrogen contents, and photosynthetic nitrogen-use efficiencies of aquatic exotic plants increase more intensively with increasing nutrient availability than those of native species (Xie et al., 2010; Fan et al., 2013). However, few studies have examined the effects of eutrophication on the overwintering of exotic plants. With the intensification of global change, water level fluctuation (e.g., floods and droughts) will become more intense and frequent, and eutrophication will also be more severe and widespread in freshwater ecosystems (Arnell and Reynard, 1996; Allen et al., 2001; Schindler, 2006). Studies the effects of water level changes and eutrophication on the overwintering of exotic aquatic plants can help to predict and manage exotic aquatic plants.

One of the world's most prevalent invasive aquatic plants, *Eichhornia crassipes* (water hyacinth), is a free-floating and mat-forming aquatic plant that originates in tropical South America. Now, *E. crassipes* has invaded over 50 countries on five continents (Villamagna and Murphy, 2010). It occurs in various freshwater ecosystems (estuaries, rivers, lakes, ponds, reservoirs, and canals); forms thick, extensive mats; and causes severe ecological and socio-economic changes in where it has invaded (Mitchell, 1985; Center, 1994). *E. crassipes* reproduces both sexually and asexually. In invaded regions, it increases in population size mainly through vegetative reproduction, forming new ramets from axillary buds on stolons produced through the elongation of internodes (Center and Spencer, 1981). Sexual reproduction rarely occurs, owing to the lack of suitable pollinators and appropriate sites for germination and seedling establishment in invaded regions (Barrett, 1980). Temperature and water nutrient levels are key factors that affect the invasion of *E. crassipes* (Wilson et al., 2005). The optimal growth temperatures for water hyacinth are 28–30°C; growth stops if the water temperature falls below 10°C or rises above 40°C (François, 1969; Gopal, 1987), and the plant dies when it experiences prolonged cold temperatures below 5°C (Gopal, 1987; Owens and Madsen, 1995). The edge of the distribution of *E. crassipes* occurs where the mean temperature in January is 1°C, the mean annual temperature is 13°C, and the average lowest temperature during the year is −3°C (Ueki et al., 1976). The distribution of *E. crassipes* is considered to be limited to tropical or subtropical regions because it cannot overwinter in environments with extreme cold temperatures or ice cover (Aurand, 1982; Tyndall, 1982; Madsen et al., 1993). However, some researchers have predicted that its distribution may expand into higher latitudes as temperatures rise (Rodríguez-Gallego et al., 2004; Hellmann et al., 2008; Rahel and Olden, 2008). The growth and reproduction of *E. crassipes* are closely related to the nutrient level of water bodies (Reddy et al., 1989; Reddy et al., 1990; Xie et al., 2004). Some studies found that a high-nutrient supply can improve the photosynthetic capacity, resource-use efficiency, and competitiveness of *E. crassipes* (Ripley et al., 2006; Fan et al., 2013). In addition, both the depth of the water and changes in water level are important in the growth of *E. crassipes* (Téllez et al., 2008). For example, the results of study of Oki and Ueki (1984) indicated the leaf area and growth rate of *E. crassipes* in shallow water were higher than those in deep water, while more roots were found in the latter. Moreover, some researchers have found fluctuations in water level promoted the invasion of *E. crassipes* (Freidel et al., 1978; Téllez et al., 2008).

*Eichhornia crassipes* was introduced into China as an ornamental plant in the early 1900s and is now widely distributed in 17 provinces or cities and causes severe damage in more than 10 provinces. In tropical China, water hyacinth can grow all year round. In subtropical regions, the plant dies back in winter and sprouts new plants from axillary buds on the stem base the following year (Gao, 2005). In the middle and lower reaches of the Yangtze River, the overwintering survival rate of water hyacinth is very low (Gao, 2005). However, we knew little about the overwintering mechanisms of water hyacinth (You et al., 2013; Liu et al., 2016). In order to test whether eutrophication and water level changes can facilitate the overwintering of *E. crassipes* as well as climate warming and found the reasons, we investigated the effects of nutrients and water drawdown on the overwintering of *E. crassipes*, at the northern margin of its distribution in China. We attempted to address the following questions: (i) Do high nutrient levels affect the performance of *E. crassipes*? (ii) Do different treatments lead to different overwintering temperatures of *E. crassipes*? (iii) Can high nutrient levels or water cover increase the overwintering survival rate of *E. crassipes*?

## MATERIALS AND METHODS

### Study Site

The experiment was conducted at The National Field Station of Freshwater Ecosystem of Liangzi Lake, Wuhan University, China (30°05′–30°18′N, 114°21′–114°39′E). Liangzi Lake is a shallow lake with an area of 304.3 km$^2$ in the central reaches of the Yangtze River basin. The climate of this area is a typical subtropical climate. The average temperature in winter ranges from 3 to 7°C, which is the critical temperature for the overwintering of *E. crassipes*; therefore, Liangzi Lake is located at the northern margin of the *E. crassipes* distribution in China (Li and Xie, 2002).

### Experimental Design

In early July 2015, *E. crassipes* plants were collected from the bay of Liangzi Lake and cultivated in a 4 × 4 × 4-m concrete pool. Fifteen days later, 240 healthy plants of similar size (mean fresh biomass 63.56 ± 5 g, mean height 14.28 ± 3 cm, mean leaf number 12 ± 3) were transferred to 24 concrete pools (length: 2 m, width: 2 m, depth: 1.5 m) filled with approximately 10 cm of lake sediment (from Liangzi Lake, N:P = 2.35:0.014 mg/L) and 120 cm of water (from Liangzi Lake, N:P = 0.6:0.05 mg/L); each pool was planted with 10 plants. The whole experiment lasted about 35 weeks, from July 20, 2015, to April 12, 2016, and was divided into two stages (**Figure 1**). During the first stage (growing stage, from the beginning of the experiment to December 1, 2015), the pools were divided into two treatments: low nutrients and high nutrients, and each nutrient level had 12 replicates (n = 12). During the second stage (overwintering stage, from December 2, 2015, to the end of the experiment), a water level factor was added to half of the pools in those two treatments until the experiment finished; there were therefore four treatments during this stage: 1-1: low nutrients and water drawdown; 1-2: low nutrients and a constant water level; 2-1: high nutrients and water drawdown; and 2-2: high nutrients and a constant water level. Each treatment had six replicates (**Figure 1**).

During the growing stage, in the high-nutrient treatment, we increased the total nitrogen (TN) and total phosphorus (TP) concentrations to 20 mg/L N and 1.0 mg/L P by adding $KH_2PO_4$, $NH_4NO_3$, and 10% Hoagland solutions to the lake water. In the low-nutrient treatment, only 10% Hoagland solution was added to the lake water. To maintain a relatively constant concentration of the culture solution, an appropriate amount of nutrients was added into relevant pools every half month after the nutrient level was measured. At the end of the growing stage, the temperature dropped with the onset of the yellowing of leaves, causing the plants to stop growing. Then, the experiment entered the second stage (overwintering stage). In the water-drawdown treatments, the water was drained (no water over sediment) by pump. In constant water level treatments, the water level was kept in line with that of the first stage. And the water levels of each treatment after rain were maintained by pumping. No nitrogen phosphorus fertilizer was added to each treatment during the second stages, because the plants have stopped growing. To study question (ii), do different treatments lead to different overwintering temperatures of *E. crassipes*? the temperature of the microenvironment in which the plants were located was monitored at 30-min intervals with four automatic thermometers during the overwintering stage. These thermometers were placed in four pools selected randomly from the four different treatments. In the water-drawdown treatments, the thermometer probes were placed closed to the stem base of the water hyacinth. In the treatments with a constant water level, the thermometer probes were placed in the surface layer of water (5 cm from the surface of the water) where the stem base of the water hyacinth was occurred.

### Data Collection

To answer question (i), do high nutrient levels affect the performance of *E. crassipes*? we counted the plants in each pool and harvested three plants randomly from each pool to

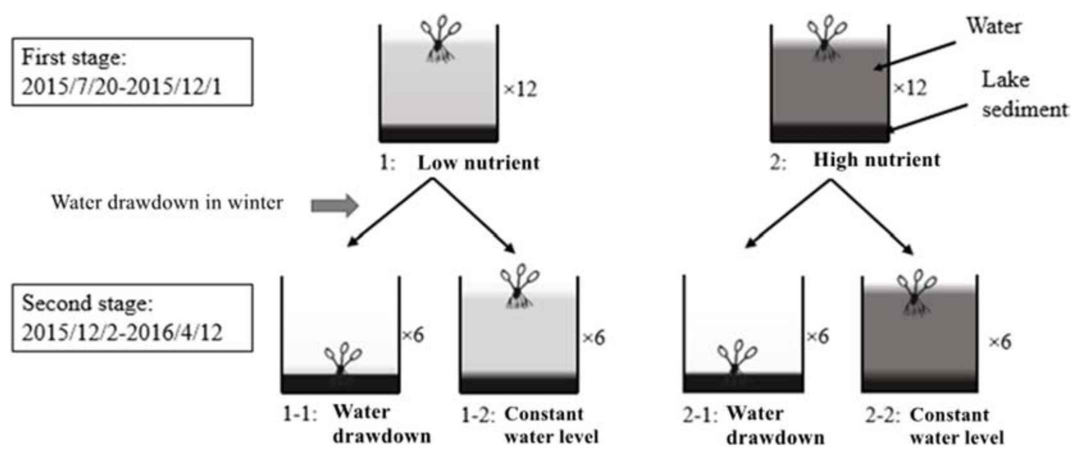


**FIGURE 1 |** Diagram of the experimental design (the number following the "×" indicates the number of replicates. One plant in diagram represents 10).

measure the traits before winter at the end of the growing stage. The length and diameter of the stem base were measured with a Vernier caliper. The biomass of the plants and the stem base were obtained after drying plants in an oven at 70°C for 72 h to a constant weight. The soluble sugar and starch concentrations of the stem base were measured by the sulfuric acid anthrone colorimetric method described by Hansen and Moller (1975). The nitrogen and carbon concentrations of the stem base were analyzed by a FLASH 2000 Organic Elemental Analyzer (Thermo Fisher Scientific Inc., USA).

To answer question (iii), can high nutrient levels or water cover increase the overwintering survival rate of *E. crassipes*? we counted the number of surviving plants (with the original plant and its new ramets counted as one plant) approximately every 10 days from early March 2016 (when some plants began to regrow) to April 12, 2016 (when the weather became warm enough to ensure that all plants with survival potential survived successfully). In addition, we identified the plants with new green leaves as those that had survived. The final survival rate was calculated as the number of plants that had survived divided by the number of plants at the end of the growing stage.

## Data Analysis

To answer question (i), do high nutrient levels affect the performance of *E. crassipes*? One-way ANOVA was performed to examine the effects of nutrients on plant number, plant biomass, stem base biomass, stem base length, stem base diameter, and the soluble sugar concentration, starch concentration, carbon concentration, and nitrogen concentration in the stem base. To test question (ii), do different treatments lead to different overwintering temperatures of *E. crassipes*? the differences in the mean temperatures of the microenvironment in which the stem bases of the plants were located among the different treatments were also tested by one-way ANOVA. To answer question (iii), can high nutrient levels or water cover increase the overwintering survival rate of *E. crassipes*? number of plants after the winter was tested with a general linear model (Poisson distribution), which indicated that both the nutrient level and water level significantly impacted the number of surviving plants. Then, two-way ANOVA was used to test the impacts of nutrient level and water level in winter and their interaction effect on the survival rate and number of surviving plants after winter. The survival rate, length and weight of the stem base, number of plants before winter, and soluble sugar concentration of the stem base were transformed using the SQRT function to ensure the homogeneity of the variance or a normal distribution of the residuals before the analysis. All data were analyzed with SPSS 19.0 software (SPSS, Chicago, IL, USA).

# RESULTS

## Growth Traits During the Growing Stage

Nutrient addition significantly and positively affects the populations of *E. crassipes* and the performance of the overwintering stem base (question i). The number of *E. crassipes* plants in the high-nutrient treatments was higher than those in the low-nutrient treatments (**Figure 2A**, F=219.227, P < 0.001). Biomass of single plants in the high-nutrient treatments was also higher than those in the low-nutrient treatments (**Figure 2B**, F = 19.625, P < 0.001). The population of *E. crassipes* in the high-nutrient treatments covered the whole surface of the water and formed dense, interlocking mats; the mean density reached 152 ind/m$^2$. However, in the low-nutrient treatments, weaker plants were scattered on the surface of the water, and the mean density of *E. crassipes* was 21.23 ind/m$^2$. The biomass, length, and diameter of the stem base in the high-nutrient treatments were all higher than those in the low-nutrient treatments (**Figures 2C–E**; $F_{biomass}$ = 36.722, $F_{length =}$ 38.491, $F_{diameter =}$ 51.417; $P_{biomass}$ < 0.001, $P_{length}$ < 0.001, $P_{diameter}$ < 0.001). The high nutrient level also significantly increased the N concentration in the stem base (**Figure 3A**, F = 13.562, P < 0.01). But the C concentration, C/N ratio, and soluble sugar concentration in the low-nutrient treatments were higher than those in the high-nutrient treatments (**Figures 3B–D**; $F_{C =}$ 19.353, $F_{C/N =}$ 21.358, $F_{sugar}$ = 26.571; $P_C$ < 0.001, $P_{C/N}$ < 0.001, $P_{sugar <}$0.001). There was no difference in the starch concentration between the high- and low-nutrient treatments (**Figure 3E**, F = 0.002, P > 0.05).

## Microenvironment Temperature and Survival Traits During the Overwintering Stage

In winter, the temperatures of the microenvironments in which the stem bases of the plants located were different (question ii). The mean temperature and minimum temperature of the water around plants in the constant-water-level treatments were higher than the mean temperature and minimum temperature of the air near the plants in the water-drawdown treatments (**Table 1**). In contrast, the maximum temperatures were higher in the latter treatments. In the constant-water-level treatments, the mean temperature and minimum temperature in the high-nutrient treatment were higher than those in the low-nutrient treatment (**Table 1**). In water-drawdown treatments, the mean temperature and minimum temperature under high nutrient level were lower than those under low nutrient level (**Table 1**).

The final survival rate and total number of plants after overwintering were significantly affected by affected by nutrient level, water level, and their interaction (**Table 2**) (question iii). The high-nutrient treatments increased the survival rate and number of *E. crassipes* plants after overwintering (**Table 2**, **Figures 4A**, **B**). Under the high-nutrient conditions, water drawdown in winter significantly decreased the survival rate and total number of *E. crassipes* plants after overwintering (**Figures 4A**, **B**). *E. crassipes* in the high-nutrient and constant-water-level treatment exhibited the highest survival rate (4.71 ± 3.69%) and number of surviving plants (31 ± 25.84), which were markedly much higher than those in the other three treatments, and there were no significant differences in survival rate among the other three treatments. It is worth noting that all the plants died in the low-nutrient and constant-water-level treatment (**Figures 4A**, **B**).

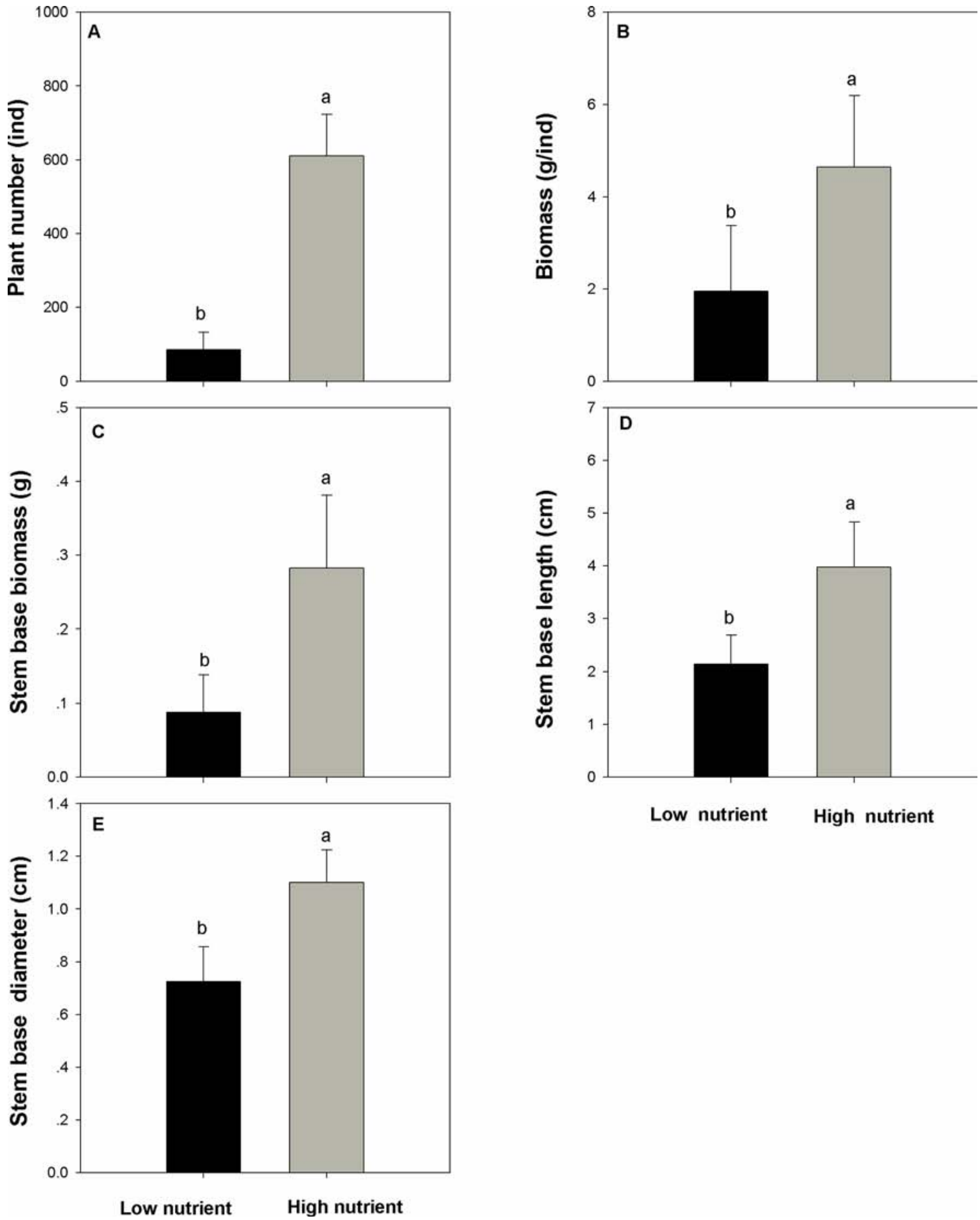


**FIGURE 2 |** Number **(A)**, biomass **(B)**, stem base biomass **(C)**, stem base length **(D)**, and stem base diameter **(E)** (mean ± SD) of *Eichhornia crassipes* under different nutrient treatments before winter. Significant differences among the treatments are indicated by different letters at $P < 0.05$.

## DISCUSSION

In our study, both nutrients in the water and water level affected the survival rate of *E. crassipes*. *E. crassipes* originated in the tropics, and the low temperature in winter limits its distribution in introduced regions. Previous studies have shown that survival, growth, and clonal integration in *E. crassipes* are limited by low temperatures (Li et al., 1995, Wilson et al., 2005); therefore, increasing the temperature in winter can increase the survival rate of *E. crassipes* (You et al., 2013; Liu et al., 2016). In China, the

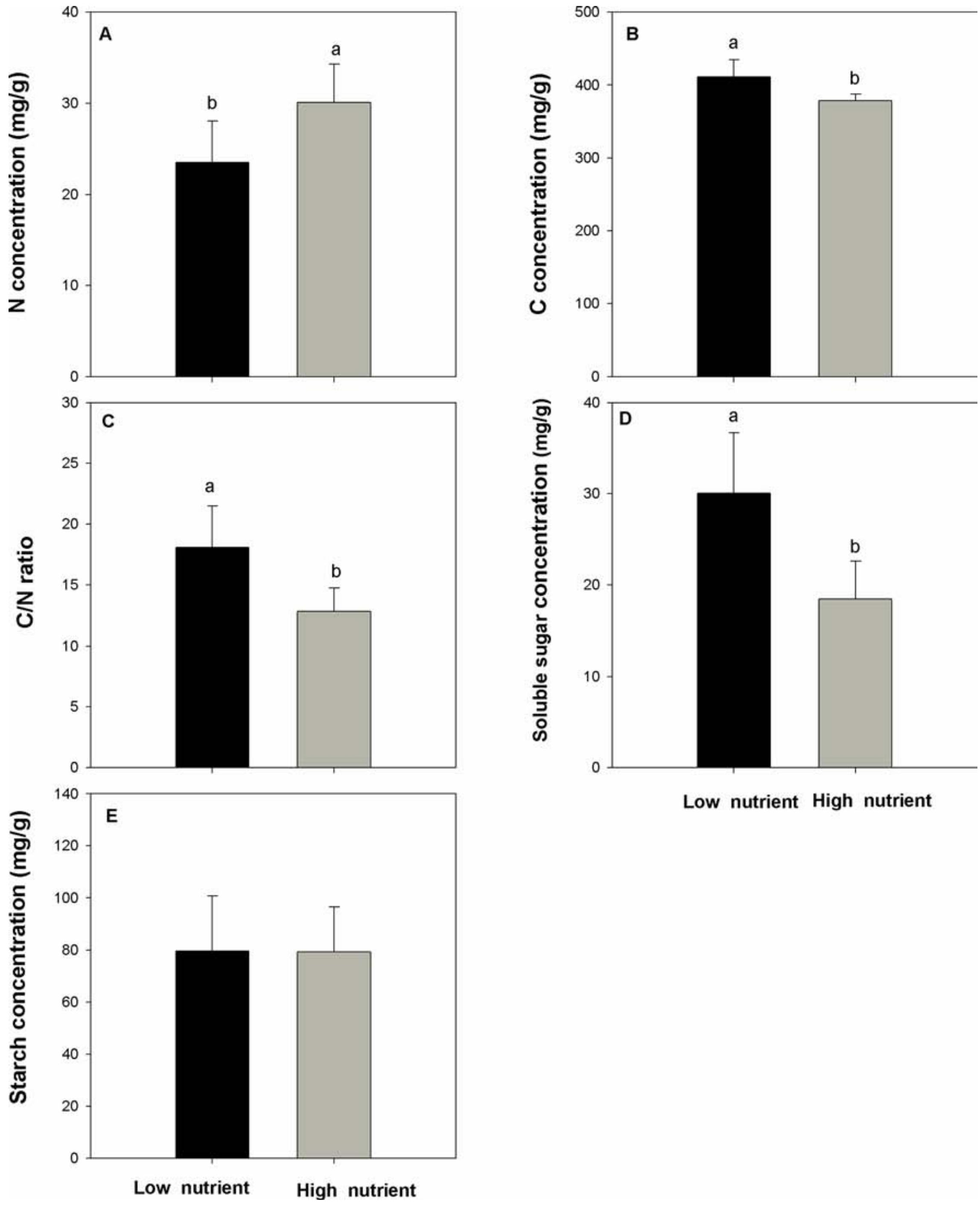


**FIGURE 3 |** N concentration **(A)**, C concentration **(B)**, C/N ratio **(C)**, soluble sugar concentration **(D)**, and starch concentration **(E)** (mean ± SD) in the stem base of *Eichhornia crassipes* under different nutrient treatments before winter. Significant differences among the treatments are indicated by different letters at P < 0.05.

Yangtze River basin occurs at the northern margin (32°N) of the distribution of *E. crassipes*. Although *E. crassipes* can grow and blossom in summer at higher latitudes, it cannot naturally survive in winter in China (Li and Xie, 2002). Similarly, *E. crassipes* also cannot overwinter successfully in the Laurentian Great Lakes, although it can grow and produce seed in the summer in this area (MacIsaac et al., 2016). The different treatments caused the stem bases of *E. crassipes* (overwintering organ) was exposed to different media, resulting them being subjected to different temperatures in winter. In our study, the covering of the stem bases with water can prevent direct damage from freezing and improve the overwintering temperature of the propagule.

**TABLE 1** | Microenvironment temperature in winter. Significant differences among the treatments are indicated by different letters at $P < 0.05$.

| Treatments | Temperature of air near plants (ºC) | | | Temperature of water around plants (ºC) | | |
|---|---|---|---|---|---|---|
| | Mean | Max. | Min. | Mean | Max. | Min. |
| **Low nutrient + water drawdown** | 6.41c | 16.1 | −2.8 | | | |
| **Low nutrient + constant water level** | | | | 8.09b | 14.1 | −1.2 |
| **High nutrient + water drawdown** | 6.33d | 17.1 | −4.0 | | | |
| **High nutrient + constant water level** | | | | 8.10a | 11.1 | 5.2 |

**TABLE 2** | Effects of nutrient and water level in winter on survival rate and number of surviving plants of *Eichhornia crassipes* after winter (two-way ANOVA).

| Trait | Nutrients | | Water level | | Nutrients × water level | |
|---|---|---|---|---|---|---|
| | F | P | F | P | F | P |
| Survival rate | 7.365 | 0.013* | 3.567 | 0.074 | 14.280 | 0.001*** |
| Number of surviving plants | 13.560 | 0.001*** | 7.327 | 0.014* | 15.242 | 0.001*** |

**P < 0.05; ***P < 0.001*

Therefore, the survival rate of *E. crassipes* in constant-water-level treatments was significantly higher. Previous studies also showed that water cover or sediment burial of stem bases facilitated the overwintering of *E. crassipes* (Owens and Madsen, 1995; You et al., 2013).

Similar to water cover, the litter layer can also buffer the cold temperatures and protected organisms under litter from freezing damage during winter and can therefore improve the survival rate of the organisms (Facelli and Pickett, 1991). Litter may also protect seedlings from being killed by frost in early spring (Heady, 1956). Previous studies have found that litter cover can improve the survival rates of animals and plants (Watt, 1970; Lahiri et al., 2015; Miura et al., 2017). In high-nutrient environments, *E. crassipes* plants are taller and larger and can form dense, interlocking mats. In winter, the large number of withered leaves can form a thick litter layer in high-nutrient treatments. In contrast, in the low-nutrient treatments, the litter layer was absent because of the low population density and the small and sparse leaves of *E. crassipes*. The water temperature was higher in the high-nutrient treatments than in the low-nutrient treatments because of the protection of the litter layer in winter. Moreover, the litter cover can also prevent the axillary buds away from the freezing damage.

The *E. crassipes* in the high-nutrient environment also developed a high-quality overwintering organ. In our study, the biomass, length, and diameter of the stem base in the high-nutrient treatments were all higher than those in the low-nutrient treatments. Meanwhile, the N concentration of the stem base was also higher in high-nutrient treatments than in the low-nutrient treatments. More storage leads to more protective substances to survive stressful environments. Plants with more vegetative storage proteins have been shown to increase in response to short days and low temperatures, improving their winter survival rates (Bertrand and Castonguay, 2003; Avice et al., 2003). Biomass and stem base size can also affect overwintering in *E. crassipes*. You et al. (2013) found that the overwintering survival rate of *E. crassipes* with large stem bases was much higher than that of plants with small stem bases. Although the starch concentrations of the stem base were similar in the high- and low-nutrient environments, the stem base in the high-nutrient environment still stored more starch, as the size of the stem base was larger. More carbohydrate reserves that make large stem bases are beneficial to the regrowth of new plants in the spring. Therefore, our study suggests that high levels of nutrients can improve the overwintering survival rate of *E. crassipes* in two ways: through the production of a thicker litter layer and the storage of more protective substances stored in the stem base.

The high nutrient level not only improved the overwintering survival rate of *E. crassipes* but also increased the performance of *E. crassipes*. In our experiments, the population density of *E. crassipes* in the eutrophic water increased to more than six times than that in the low-nutrient water. The individual biomass in the eutrophic water was 2.4 times that in the low-nutrient water. Our previous study found that, only at a high nutrient level, *E. crassipes* had a higher resource-use efficiency than the confamilial native aquatic plant *Monochoria vaginalis* (Fan et al., 2013). Zhao et al. (2006) also found that eutrophication further boosts the competitive advantages of water hyacinth over native plants and thus facilitates the invasion of this weed into water bodies. During the past 30 years in China, rapid urbanization, gross domestic product (GDP) increases, vast population growth, and living standard improvements have all produced domestic and industrial wastewater. Moreover, due to insufficient sewage treatment capacities, some of this wastewater is discharged, untreated, directly into rivers and lakes (Shao et al., 2006; Yang and Pang, 2006; Le et al., 2010), which causes organic pollution and eutrophication in many water bodies and the replacement of grass-dominated ecosystems with algae-dominated ecosystems (Jin et al., 2005). Seventy-three percent of the major lakes in China have undergone severe eutrophication, and the area of eutrophication amounts to 11,632 $km^2$ (Li, 2006). Therefore, our results suggest that *E. crassipes* will spread to a wider area

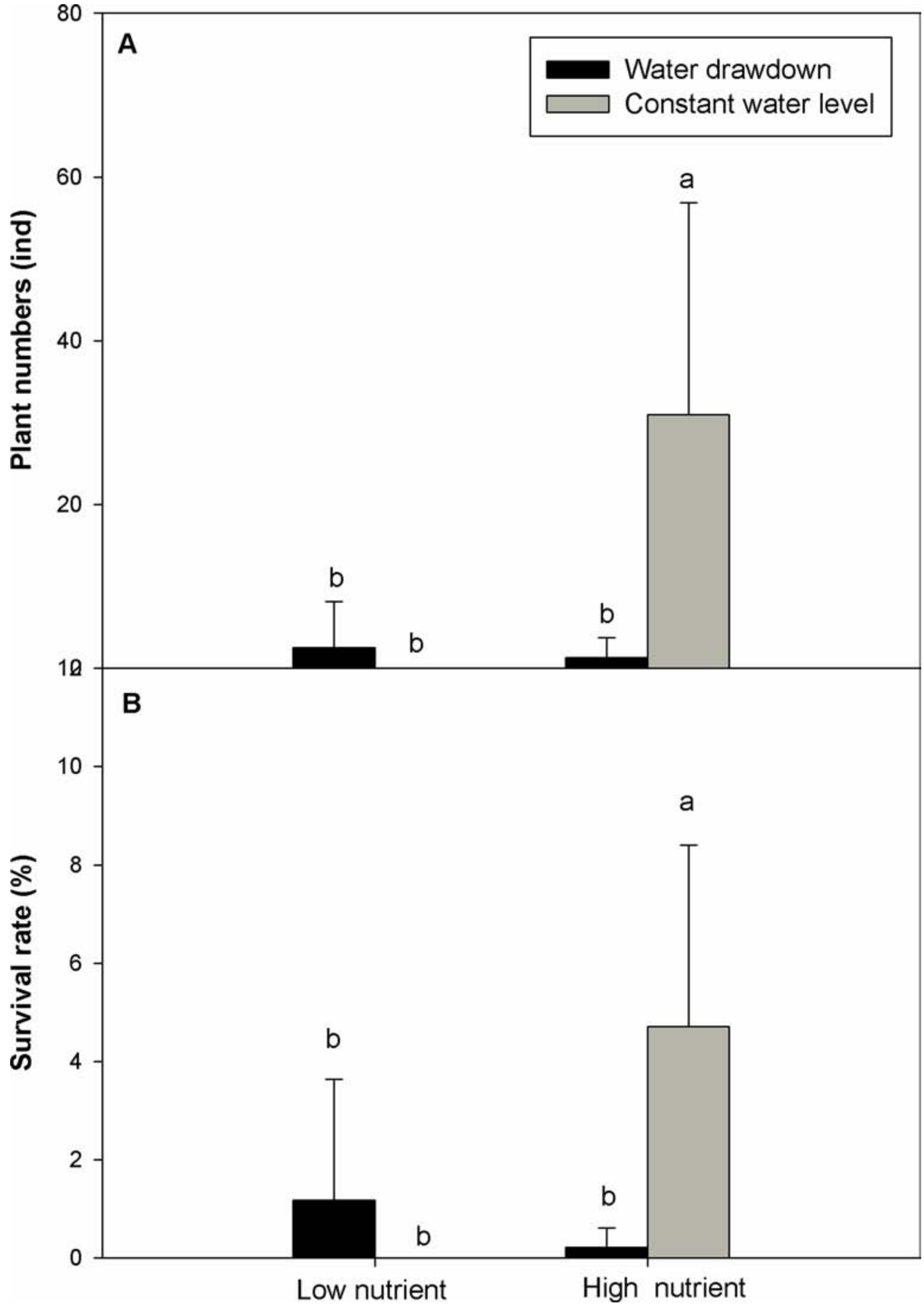


**FIGURE 4 |** Plant number **(A)** and survival rate **(B)** (mean ± SD) of *Eichhornia crassipes* in different treatments after winter. Significant differences among the treatments are indicated by different letters at P < 0.05.

and cause worse effects under the background of intensified eutrophication and climate warming.

Many techniques have been used to control and eliminate the ecological and socio-economic impacts of *E. crassipes* (Villamagna and Murphy, 2010). *Neochetina eichhorniae* and *Neochetina bruchi* are two commonly used weevil species from the plant's native range (Deloach and Cordo, 1976; Julien and Griffiths, 1999; Sosa et al., 2007). *N. eichhorniae* was introduced into China to control *E. crassipes* in 1995 (Ding et al., 2001). However, the population of *N. eichhorniae* was limited due to low temperatures in the Yangtze River basin. Our study indicated that eliminating eutrophication and regulating the water level can control or even eradicate *E. crassipes* in the Yangtze River basin. The overwintering survival rate of *E. crassipes* in the low-nutrient treatment was less than 5%, even all plants died in the low-nutrient and constant-water-level treatment. Musil and Breen (1977) also considered broad-scale nutrient reduction plans as the most sustainable solution for controlling *E. crassipes* outside of its native range. Even in water bodies with high nutrient levels, the overwintering survival rate of *E. crassipes* can be reduced by the water-drawdown treatment. Previous studies also found that drawdown can be used to manage aquatic vegetation (Nichols, 1975; Cooke, 1980).

In conclusion, our study found that high levels of nutrient not only increased the performance of *E. crassipes* but also improved the overwintering survival rate of *E. crassipes* by producing a thicker litter layer and more protective substances stored in the stem base in at the middle and lower reaches of the Yangtze River basin, which suggests that *E. crassipes* can invade higher latitudes under the trend of climate warming and water eutrophication, whereas exposing the stem base of *E. crassipes* to air of lower temperature by lowering the water level, which can reduce the survival rate of *E. crassipes* in winter. In addition, it also indicated that eliminating eutrophication and regulating the water level can control *E. crassipes* effectively in temperate regions and some subtropical regions.

## AUTHOR CONTRIBUTIONS

DY, SF, and CL designed the research and executed the research project. HY and XD collected the field data. SF and XD analyzed data. XD and HY wrote the paper.

## FUNDING

This study was supported by the National Natural Science Foundation of China (31500295 and 31170339), the Major Science and Technology Program for Water Pollution Control and Treatment (2015ZX07503-005), and the Special Foundation of National Science and Technology Basic Research (2013FY112300).

## ACKNOWLEDGMENTS

We thank Jing Li, Yuanyuan Gao, Ligong Wang, Shihe Li, Baobo Zhao, Yaozhou Fan, Shiliang Li, Lifu Cui and Changjun Cai for their valuable work in the experimental procedures and data collection.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor is currently organizing a Research Topic with one of the authors CL, and confirms the absence of any other collaboration.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 25 November 2019
doi: 10.3389/fpls.2019.01504

# Submerged Vegetation and Water Quality Degeneration From Serious Flooding in Liangzi Lake, China

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In shallow lake ecosystems, flooding is a key disturbance factor of aquatic vegetation. Aquatic plants, especially submerged plants, play key roles in water ecosystems. Liangzi Lake experienced severe flooding in July 2010, and the elevated water levels lasted for 3 months. In this study, 10 transects with 120 monitoring points were set up for monthly monitoring during the 3-year period, encompassing the period before and after the flooding (2009–2011). The numbers, biomass, and diversity of the submerged plants, as well as the physical and chemical characteristics of the lake water, were surveyed. There were 12 species belonging to 7 families and 7 genera in Liangzi Lake. Eleven of the submerged plant species were found in 2009, but, after the flood, that number decreased to five in 2011. The total biomass differed significantly over the three years ($P < 0.05$), with the largest biomass in 2009 and smallest in 2011. In 2009 and 2010, *Potamogeton maackianus* was the dominant species, but its dominant position weakened in 2011. After the flood, water transparency decreased, and the water depth, turbidity, total nitrogen, and total phosphorus increased. A redundancy analysis between the submerged plants and environmental factors found that the water transparency, turbidity, and water depth were the key environmental factors affecting the plants. These results suggest that the long-lasting severe flooding of Liangzi Lake in 2010 led to the degradation of both the submerged plant community and water quality.

**Keywords: flooding, submerged vegetation, dominant species, water quality, diversity**

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***Edited by:***
*Te Cao,*
*Chinese Academy of Sciences, China*

***Reviewed by:***
*Wei Xing,*
*Chinese Academy of Sciences, China*
*Hui Fu,*
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***Specialty section:***
*This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science*

***Received:*** *29 March 2019*
***Accepted:*** *29 October 2019*
***Published:*** *25 November 2019*

***Citation:***
*Wang L, Han Y, Yu H, Fan S and Liu C (2019) Submerged Vegetation and Water Quality Degeneration From Serious Flooding in Liangzi Lake, China. Front. Plant Sci. 10:1504. doi: 10.3389/fpls.2019.01504*

## INTRODUCTION

Submerged macrophyte vegetation plays a central role in the functioning of shallow lake ecosystems (Coops and Doef, 1996; Jeppesen et al., 1997; Meerhoff et al., 2003). It can provide feeding and spawning habitats for fish, provide sanctuary for zooplankton, and generally help improve the biodiversity and stability of lake ecosystems (Jeppesen et al., 1998; Wetzel, 2001; Heikkinen et al., 2009; Tamire and Mengistou, 2013; Yu et al., 2016). Among lakes, the factors influencing the submerged macrophyte distribution, diversity, and abundance include light availability (Middelboe and Markager, 1997; Phillips et al., 2016; Zhang et al., 2016; Verhofstad et al., 2017), water temperature (Scheffer et al., 1992; Short et al., 2016), nutrient enrichment (Sand-Jensen et al., 2008), bottom substrate (Andersson, 2001), herbivory (Marklund et al., 2002; Sponberg and Lodge, 2005), and the water level (Wilcox and Meeker, 1991). In shallow lake ecosystems, water level fluctuations are the main factor affecting the biomass and spatial distribution of aquatic plants and are an important ecological factor affecting their growth and reproduction (Gafny and Gasith, 1999;

Strand and Weisner, 2001; Ishii and Kadono, 2004; Deegan et al., 2007; Schneider et al., 2018).

Flood is one of the important factors leading to fluctuation of water levels (Wantzen et al., 2008). In addition to a rise in the water level, the surface runoff caused by floods carries large amounts of potentially labile nitrogen and phosphorus into the lake, and the original endogenous nutrients used by aquatic plants are also released into the water (Carpenter, 2008; Keitel et al., 2016). Floods also resuspend the sediment, increasing the concentrations of suspended solid particles, nitrogen, and phosphorus in the water (Newman and Reddy, 1992; Tong et al., 2017). The phosphorus released by the resuspension of precipitates is 20–30 times higher than when they are undisturbed (Søndergaard et al., 1992). In addition, floods also restrict the availability of oxygen, inhibiting the growth of emergent and floating-leaved plants (Drew, 1997; Deegan et al., 2007; Lemke et al., 2014) and the germination of some species in the seed bank (Casanova and Brock, 2000; Johansson and Nilsson, 2002; Hölzel and Otte, 2004; Cui et al., 2017), thereby reducing the diversity of aquatic plant species (Jeppesen et al., 2015). Correspondingly, aquatic animal habitat and food sources also disappear, reducing species diversity, and thus the entire ecosystem becomes very vulnerable (Junk and Robertson, 1997; Dorn and Cook, 2015). In addition, climate change manifested through increasing temperatures and more variable precipitations impacted water quality, biodiversity, and ecological status of the world's lakes (Solheim et al., 2010; O'Reilly et al., 2015). Climate change is predicted to lead to earlier, stronger, and more frequent flooding (Fowler and Hennessy, 1995; Trenberth, 2011; Cai et al., 2015; Lehmann et al., 2015). For example, floods have become more frequent in the central United States (Hirsch and Archfield, 2015), and global warming has been linked to a substantial increase in flood risk in most countries in Central and Western Europe (Alfieri et al., 2018). The high frequency of future floods may have a more serious impact on water ecosystems (Watts et al., 2015; Castello and Macedo, 2016).

Flooding is a key disturbance factor of aquatic vegetation composition and community diversity in floodplain lakes (Tockner et al., 2000; Maltchik et al., 2005; Van Geest et al., 2005; Chaparro et al., 2014). The growth of emergent floating-leaved plants is not limited by low light penetration in the lake (Qiu et al., 2001a). Spate floods affected small to intermediate-sized submerged plant species, and long-term inundating floods affected tall submerged plant species (Bornette and Puijalon, 2011). The effects of water levels on submerged plants by simulating water level fluctuations for individual plants have been studied in depth (Armstrong et al., 1994; Vartapetian and Jackson, 1997; Vermaat et al., 2000; Lenssen et al., 2004; Wang et al., 2016a). For example, water depths greater than 3 meters severely reduced the survival of *Vallisneria natans* (Han et al., 2018). *Potamogeton maackianus* disappeared at an average depth of 6 meters in Erhai Lake (Fu et al., 2018a). *Myriophyllum spicatum*, *Ceratophyllum demersum*, and *Potamogeton malaianus* were more tolerant of deep water and flood intensity than *P.maackianus* and *Hydrilla verticillata*, as indicated by their larger biomass, plant height, stem tensile properties, and root anchorage strength (Zhu et al., 2012; Ye et al., 2018). The response of submerged plants to floods is species specific. Therefore, flooding with extreme water levels may cause shifts towards a macrophyte-dominated state (Coops et al., 2003).

In recent decades, floods have become more frequent in the middle reaches of the Yangtze River in China, and the rise in water levels has been greater than before (Li et al., 2015; Wang and Yuan, 2018). Lake Liangzi is located in the middle and lower reaches of the Yangtze River. From 2007 to 2016, two major floods occurred in Lake Liangzi, one in 2010 and one in 2016 (Xu et al., 2018). Ten transects with 120 monitoring points were set up for monthly monitoring during the 3-year period between 2009 and 2011 in order to compare the submerged vegetation and water characters before and after the flood in 2010. Specifically, we analyzed the relationship between submerged aquatic communities and water quality. Finally, we evaluate the consequences of flood regulation on the dominant submerged species.

## MATERIALS AND METHODS

### Study Area and Flood

Liangzi Lake (30°04′55″–30°20′26″ N, 114°31′19″–114°42′52″ E) is located south of the Yangtze River in the southeast of Hubei Province, China. It is a typical grass-type lake, a type common in East China, with high vegetation coverage. The lake covers an area of 304.3 $km^2$ and has a water storage capacity of about $14 \times 10^8$ tons.

The Liangzi Lake Basin suffered heavy rain, and Liangzi Lake was seriously flooded in July 2010, with the water level rapidly rising from 4.25m to 6.2m. In addition, the high water levels continued for three months. The survey was conducted for monthly monitoring from January 2009 to December 2011. Monitoring plots were established in three regions of Liangzi Lake (named as Qianjiangdahu Lake, Manjianghu Lake, and Gaotanghu Lake) (**Figure 1**). Altogether, there were ten transects, with 120 monitoring points for the sampling set up at 400-meter intervals (**Figure 1**).

### Collection of Plant Samples and Water Parameter Measurements

During each survey, submerged plant samples were collected on-site using a boat positioned by GPS (GARMIN eTrex Summit; Garmin, Inc., Olathe, KS, USA) navigation. At each monitoring site, the submerged plants were randomly sampled twice with a Peterson's mud filter (0.2m × 0.3m). The collected samples were packed in plastic bags (0.03 L) and brought to the National Field Station for Freshwater Ecosystem at Liangzi Lake (hereinafter referred to as the Liangzi Lake National Station). The plants from the monitoring sites were first classified, the number of each species was counted, and they were then drained of surface water and weighed using an electronic scale (0.01g) to obtain the wet weight. Some plant samples were dried at 80°C for 72 h and were then weighed to obtain dry-weight biomass, which was later converted to a submerged plant dry weight per unit area (1$m^2$).

Water parameters were monitored at the time of the submerged plant sampling. The water pH, dissolved oxygen, and temperature were measured using a Pro Plus water quality monitor (YSI Inc., Yellow Springs, OH, USA); the water turbidity

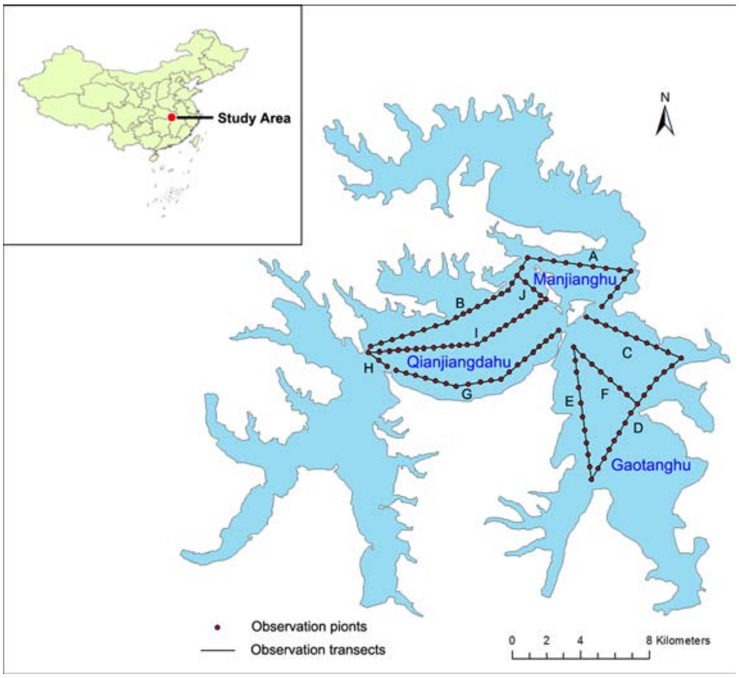


**FIGURE 1 |** Map of Liangzi lake showing sampling sites observation points. Ten transects: A. B. C. D. E. F. G. H. I. J.

was measured in nephelometric turbidity units (NTUs) using a HACH 2100P turbidity meter (HACH Co., Loveland, CO, USA); and the water depth and transparency were measured by Secchi depth monitoring. A Plexiglass water sampler was used to collect water samples; ten sites were sampled once a month from 2009 to 2011, and these were returned to the instrument room of the Liangzi Lake National Station where the total phosphorus and total nitrogen were determined with HACH IL500P and IL500N analyzers (HACH Co., USA). We thus had a total of 4,320 samples (3 years × 12 months × 120 sites), 360 total phosphorus, and total nitrogen samples (3 years × 12 months × 10 sites).

## Data Analysis

The submerged plant diversity index was analyzed using the Shannon index formula:

$$H= -\sum(Pi)(\log_2 Pi)$$ (MaGuarran, 1988).

Only six submerged species were common in Liangiz Lake. Thus, the dominance analysis and redundancy analysis (RDA) were analyzed with the data for these six species. The dominance of the submerged plant species was calculated with the equation:

$$\text{Dominance} = [(\text{relative frequency} + \text{relative weight})/2] \times 100\%$$ (Chen, 1980).

The data analyses were conducted using SPSS 22.0 software. To ensure that all data met the normal distribution requirements, data that were not normally distributed underwent a logarithmic transformation, but, to the data that were not normally distributed, non-parametric statistics were applied. We conducted a Kruskal–Wallis test to determine the differences in the water quality. A one-way ANOVA with Duncan's ($P < 0.05$) test for *post hoc* comparison was used to analyze the differences in species number, total biomass per area, total biomass of dominant species among 2009, 2010, and 2011, or during the same month over different years. A redundancy analysis (RDA) based on the biomass was conducted for the major water environmental factors affecting the submerged plant communities using Canoco for Windows 5.0 software.

# RESULTS

## Species Number, Total Biomass and Diversity Index

Twelve submerged plant species were monitored in Liangzi Lake from 2009 to 2011, which belonged to seven genera in seven families (**Table 1**). There was a significant change in the number of submerged plant species over the three years (**Figure 2A**), the number of species in 2009 being significantly higher than that in 2010 and 2011 (**Figure 2A**). A comparison of the number of species before and after flooding found no significant differences from March to July in 2009 and 2010, while the number present from August to December in 2009 was significantly higher than that in 2010 (**Figure 2C**). There were significant differences in the number of submerged plants with each month within the three years (**Figure 2C**).

There were significant differences in total biomass over the three years($F = 504.227$, $P < 0.001$, the largest biomass identified

**TABLE 1 |** Submerged plant species in Lake Liangzi.

| Family | Species |
|---|---|
| Characeae | *Chara vulgaris* |
| Ceratophyllaceae | *Ceratophyllum demersum* |
| Haloragaceae | *M. spicatum* |
| Hydrocharitaceae | *H. verticillata* |
| | *Elodea nuttallii* |
| | *Vallisneria spiralis* |
| Lentibulariaceae | *Utricularia aurea* |
| Najadaceae | *Najas marina* |
| | *N.minor* |
| Potamogetonaceae | *Potamogeton crispus* |
| | *P. maackianus A. Bennett* |
| | *P.malaianus* |

in 2009 and the smallest in 2011 (**Figures 2B**, **D**). According to monthly data, the three years also had significant differences in the total biomass during each month (All $P < 0.05$). The total biomass of the submerged plants differed significantly from each other in February, April, June, July, and August (**Figure 2D**). In January, March, and May, the total biomass of the submerged plants in 2009 showed no significant difference to that in 2010, whereas both them were significantly higher than in 2011 (**Figure 2D**).

Flooding decreased the Shannon diversity index (**Figure 3**). The Shannon index was highest in November 2009, while only one species was found in September, October, and December 2011, resulting in the lowest diversity index (**Figure 3**).

## Changes in the Dominant Species

In the three years sampled, the dominance of *P. maackianus* was above 60%, while the dominance was less significant among *C. demersum*, *M. spicatum*, and *P. crispus*. *P. maackianus* was the dominant species during whole year both in 2009 and 2010. However, the dominant species was *P. crispus* in March, April, May, November, and December of 2011, and only *P. maackianus* was present from June to October 2011 (**Figure 4**).

## Water Environmental Parameters

There were significant difference in water depth ($\chi^2 = 871.013$, $P < 0.001$), transparency ($\chi^2 = 1667.673$, $P < 0.001$), turbidity ($\chi^2 = 1649.164$, $P < 0.001$), dissolved oxygen ($\chi^2 = 218.637$, $P < 0.001$), pH($\chi^2 = 804.817$, $P < 0.001$), total nitrogen ($\chi^2 = 1165.63$, $P <$

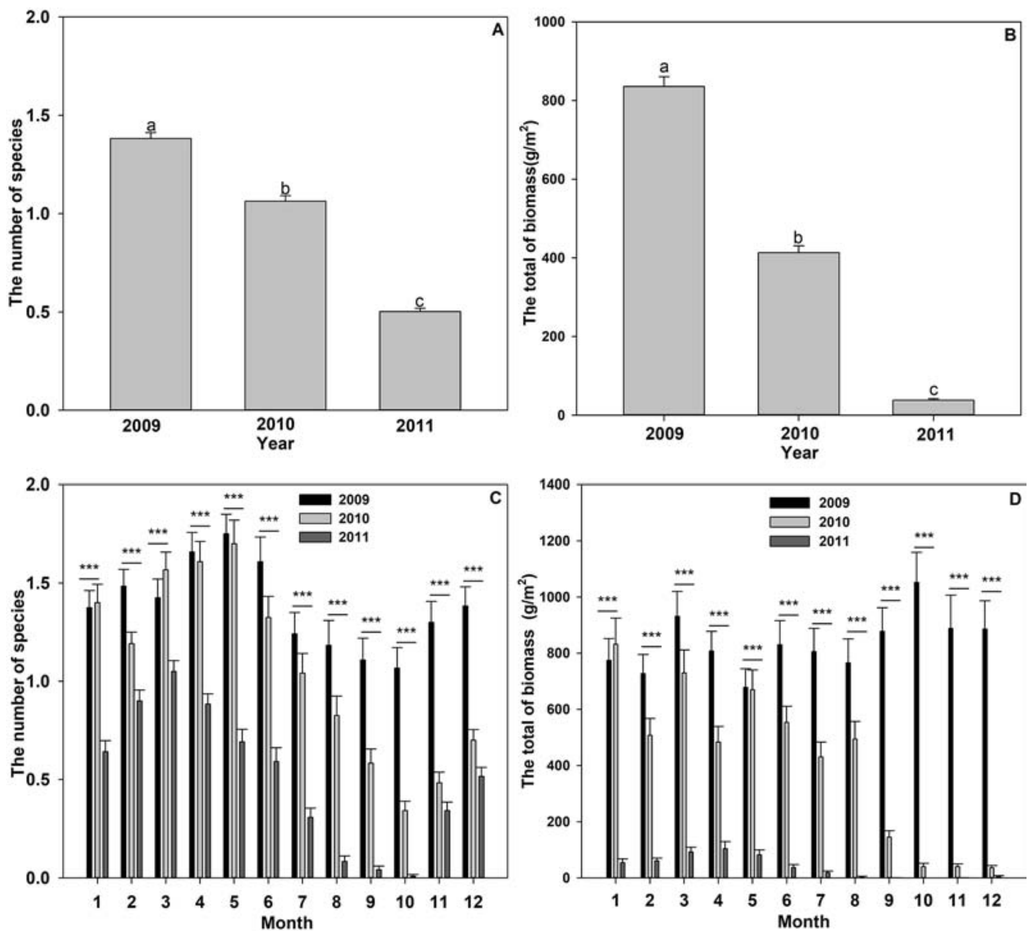


**FIGURE 2 |** Submerged species number **(A**, **C)** and the total biomass **(B**, **D)** in different years or different months. Values shown are means ± S.E. Bars with different lowercase letters above are significantly different. Significant differences: ***$P < 0.001$.

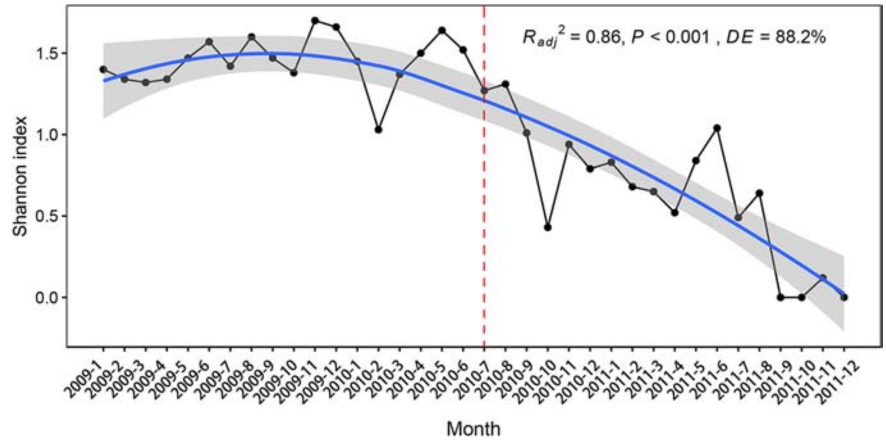


**FIGURE 3 |** The Shannon diversity species index of the submerged plants in 2009, 2010, and 2011.

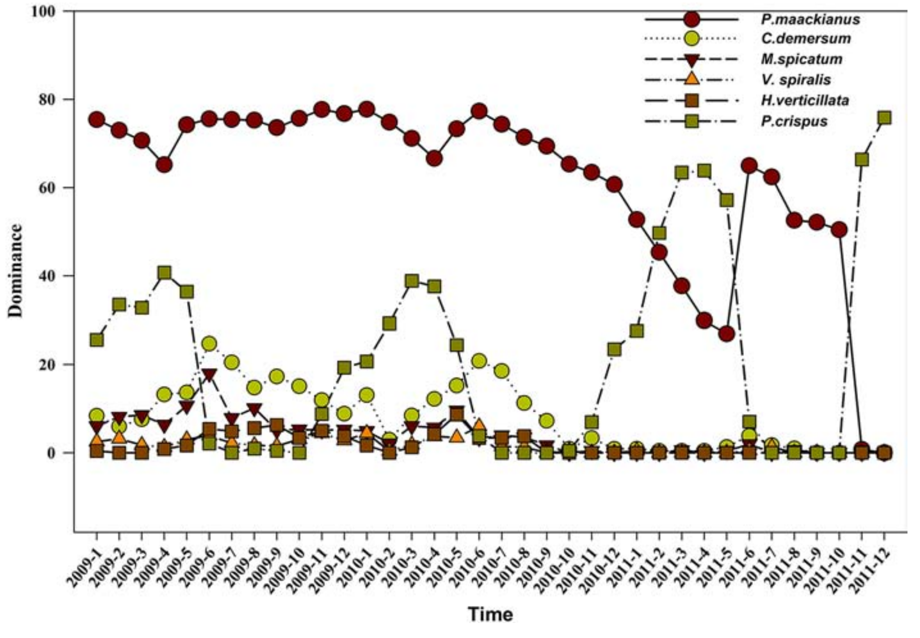


**FIGURE 4 |** The species dominance of the submerged plants in 2009, 2010, and 2011.

0.001), and total phosphorus ($\chi^2$ = 1704.382 $P$ < 0.001) over the three years, There was no significant difference in temperature ($\chi^2$ = 2.4722, $P$ = 0.291).

The mean water depth of Lake Liangzi from January to October was greater in 2010 than in 2009 and 2011 (**Figure 5A**). On the other hand, water transparency was significantly lower in 2011 than that in 2009 and 2010, and the maximum transparency was found in August 2009 (**Figure 5B**). Water turbidity was significantly higher in 2011 than that in 2009 and 2010, reaching its highest value in March 2011 (**Figure 5C**). The highest water temperature values were reached in August, with an average maximum temperature of 34°C, and lowest values were present in January, when the average minimum temperature was 3°C (**Figure 5D**). In contrast to the temperature, dissolved oxygen had an inverse trend, decreasing in the summer and increasing in the winter (**Figure 5E**). The lowest dissolved oxygen concentration values were present in July, with a mean of 6.34 ± 0.793mg·$L^{-1}$ (**Figure 5E**). The pH was significantly higher in 2011 than in 2009 (**Figure 5F**). Total nitrogen (TN) was lower in 2009 (0.310 ± 0.01mg·$L^{-1}$) than in 2010 (0.411 ± 0.011mg·$L^{-1}$) and 2011(0.429 ± 0.109 mg·$L^{-1}$). (**Figure 5G**). Total phosphorus (TP) fluctuated slightly in 2009, whereas it fluctuated widely in 2010 and 2011 (**Figure 5H**). The mean value was 0.007 ± 0.0005mg·$L^{-1}$in 2009, and it was significantly less than in 2010 (0.020 ± 0.001 mg·$L^{-1}$) and 2011 (0.020 ± 0.0008 mg·$L^{-1}$) (**Figure 5H**).

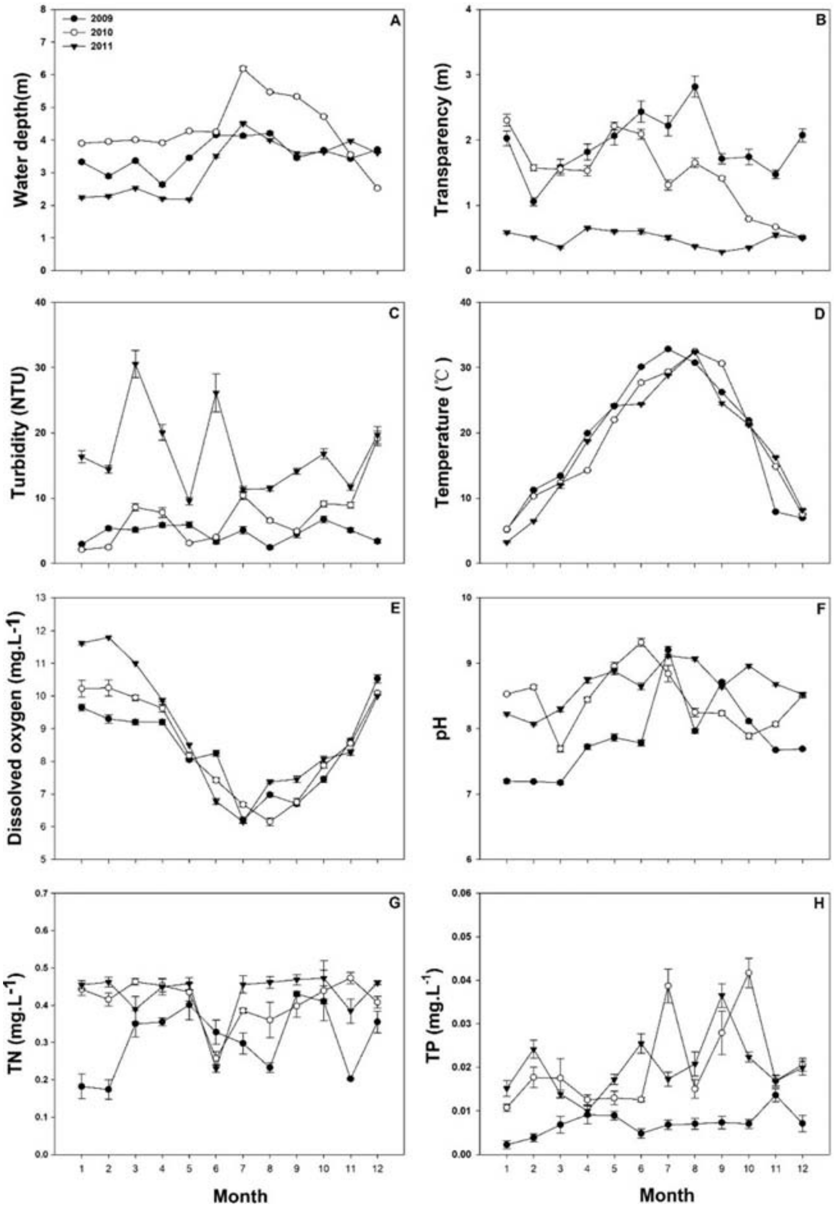


**FIGURE 5 |** The water parameters of Lake Liangzi. **(A)** water depth, **(B)** transparency, **(C)** turbidity, **(D)** temperature, **(E)** dissolved oxygen, **(F)** pH, **(G)** total nitrogen (TN), **(H)** total phosphorus (TP).

## Redundancy Analysis (RDA) of Submerged Macrophyte Communities and Water Environmental Cactors

The eight environmental factors cumulatively accounted for 41.17% of the species change information in the two axes. A Monte Carlo displacement test showed that the eight environmental factors were significant ($P = 0.002$), indicating that the transparency (which explained 36.8% of the variability with a correlation of 72.05% with the presence of submerged macrophytes), turbidity (which explained 11.9% of the variability), and water depth (which explained 8.3% of the variability) were factors affecting the structure of the submerged plant communities and were, therefore, key environmental water factors.

The gradient of the first axis from left to right shows that as the transparency increases and turbidity decreases, and the submerged macrophytes (except *P. crispus*) are distributed in the areas of high transparency (i.e., the positive direction of the first axis) (**Figure 6**). *P. maackianus* was related to the first axis and significantly positively correlated with transparency ($P < 0.001$); dissolved oxygen is significantly related to the second axis and negatively correlated with temperature ($P < 0.001$) (**Figure 6**).

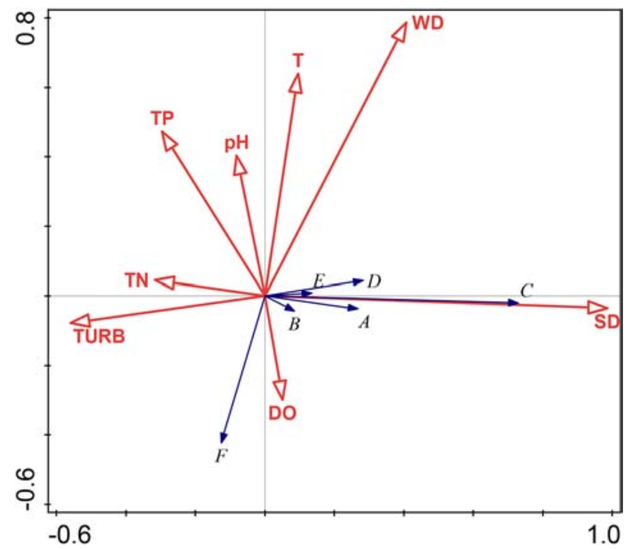


**FIGURE 6 |** Redundancy analysis ordination diagram of the submerged plant and environmental factors. Species codes: A, *Myriophyllum spicatum*; B, *Vallisneria spiralis*; C, *Potamogeton maackianus A. Bennett*; D, *Ceratophyllum demersum*; E, *Hydrilla verticillata*; F, *Potamogeton crispus*. Environmental codes: DO, Dissolved oxygen; pH, pH of water; SD; Transparency; T, Temperature; TN, Total nitrogen; TP, Total phosphorus; TURB, Turbidity; WD, Water depth.

# DISCUSSION

## Dynamic Changes in the Submerged Plant Communities

In shallow lake ecosystems, the water level is the main factor affecting aquatic plant biomass (Wallsten and Forsgren, 1989; Zhang et al., 2016), and the natural water level is a dynamic factor (Rea and Ganf, 1994). High water levels caused by extreme flooding are known to reduce the diversity of aquatic plant species (Jackson, 1984; Arias et al., 2018). For example, flooding increased the water level and decreased diversity and biomass of the aquatic vegetation; the most serious effect observed was in submerged plants in Poyang Lake in China (Cui et al., 2000). In our survey, after the flood in 2010, the total number of species in Lake Liangzi decreased from ten species in 2009 to five in 2011, and the average number of species per site decreased from 1.38 ± 0.03 per $m^2$ in 2009 to 0.5 ± 0.017 per $m^2$ in 2011. The diversity and biomass of submerged plants were all significantly decreased by flooding in 2010. Those decreases were mainly due to a medium-term 3 m rise in the water level within several days, and this reached more than 6 m for a brief time; consequently, such an increased water depth significantly inhibited the growth of many submerged plants (Wang et al., 2016b). In addition, there was a negative relationship between submerged macrophyte dominance and the long-term annual duration of inundation (Van Geest et al., 2003). Thus, the long duration of higher water levels (a greater than 4 m increase in water level that persisted for four months from July to October) caused by the flooding of Lake Liangzi in 2010 resulted in the dying-off of a large number of the submerged plants, and the dry biomass thus decreased significantly. On the other hand, a certain period of time is required for plants to adapt to different water levels (Bornette and Puijalon, 2011). The lack of significant differences in the number of species in July and August of 2009 and 2010 suggests that the submerged plants in Lake Liangzi had some tolerance for the short-term changes in water levels during flooding.

The RDA analysis showed that water transparency, turbidity, and water depth were the key water environmental factors affecting the submerged plants (**Figure 6**). The increase in water level leads to reduced light availability in shallow lakes, thereby limiting the growth of submerged plants (Van Geest et al., 2007). Decreasing water transparency significantly decreased the communities in terms of biomass, and it also decreased the submerged plants species' richness (Vestergaard and Sand-Jensen, 2000; Wang et al., 2016b). The key factor determining whether submerged plants can regenerate is the underwater light conditions during the germination period of the plants' vegetative propagules (Lu et al., 2012). Weak underwater light intensity prevents germination, thus the number of species decreases (Madsen et al., 2001). Thus, when sediment is disturbed by flooding, it causes the water transparency to decrease and the turbidity to increase, and this lack of underwater light affects the growth and reproduction of the submerged plants. In the present study, the water turbidity increased, the transparency of the water decreased after flooding, and these factors inhibited the growth and regeneration of submerged plants.

*P. maackianus* is the dominant species in the submerged vegetation of many lakes in the middle and lower reaches of the Yangtze River (Li et al., 2004). It is a constructive species in submerged plant communities, and the distribution area of the *P. maackianus* community once accounted for 50% of the total area of submerged plants in Lake Liangzi (Zhan et al., 2001). We also found that it was the dominant species in Lake Liangzi (dominance > 60%) in 2009 and 2010. However, after the 2010 flooding, the dominant species was *P. crispus* in February, March, April, May, November, and December of 2011. P. *maackianus* was the dominant species only from June to October of 2011 (**Figure 4**), which was mainly because the summer buds (dormant buds) of *P. crispus* germinate in the autumn and then grow over the winter. It was thus able to become the dominant species from February to May in 2011. Although *P. maackianus* can grow in winter, the flooding caused turbidity to increase, water transparency to decrease, and light intensity to weaken, resulting in the *P. maackianus* gradually dying. The tolerance of the summer buds (dormant buds) of *P.* crispus is strong. For example, higher water turbidity (90NTU) had no effect on the germination rate and growth of summer buds (Li, 2012), whereas four meters of water depth significantly affected the growth of *P. maackianus* (Zhu et al., 2012; Li et al., 2013), In addition, previous studies have found that *P. crispus* can successfully recover, while it has been difficult to successfully restore *P. maackianus* because *P. maackianus* are K-selected plants (Qiu et al., 2001b; Zhu et al., 2012; Fu et al., 2018b) and *P.* crispus are r-selected plants (Pierce et al., 2012).

## Dynamic Changes in the Water Environmental Factors

The submerged macrophytes improve their own light climate by enhancing the water transparency (Van den Berg et al., 1998). There is a significant positive relationship between water transparency and the maximum colonization depth of aquatic plants (Canfield et al., 1985; Sondergaard et al., 2013). These two parameters of the water before the flooding in 2009 were stable due to the high species numbers and biomass of submerged vegetation. Floods have an important effect on water clarity (Xu et al., 2018), and extreme water levels may cause shifts between the turbid and the clear, and the macrophyte-dominated state may change to a without-vegetation turbid state (Coops et al., 2003; Scheffer and Carpenter, 2003). The flooding of Lake Liangzi in 2010 caused the turbidity to increase and, consequently, the water transparency to decrease. In addition, large areas of aquatic vegetation disappeared in Lake Liangzi after the flood. Submerged plants were only found at five monitoring points and one monitoring point in September and October 2011.

Nutrient input, mainly of N and P, is derived from the eutrophic main channels during floods (Van den Brink et al., 1994). A large amount of suspended sediment and, consequently, a higher concentration of nutrients into Lake Liangzi is caused by flooding that increases the content of nitrogen and phosphorus in water. During the growth phase, the water column is depleted in nutrient concentrations, whereas, during the decay period, there is a significant increase in water column nutrients (Shilla et al., 2006). Furthermore, the decomposition of submerged macrophytes is influenced by several factors, though water temperature has been cited as an important environmental factor (Carpenter and Adams, 1979; Carvalho et al., 2005). After the flooding in Lake Liangzi it was still a hot season, the higher temperature accelerating the decomposition of dead aquatic plants caused by the flood in 2010. Higher turbidity, higher total nitrogen and phosphorus, and lower transparency after the flooding in 2010 all contributed to the downward trend in water quality.

# CONCLUSION

The serious flooding of 2010 in Lake Liangzi decreased species diversity and the biomass of submerged aquatic plants and resulted in declining water quality. *P. maackianus* was the dominant submerged species during the whole year before flooding, while this dominant position weakened after the flooding. The results suggest that heavy flooding may change the submerged community succession.

# DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the manuscript and the supplementary files.

# AUTHOR CONTRIBUTIONS

CL designed the study. LW, YH, and SF performed the field monitoring. LW, YH and HY analyzed the data. LW drafted the manuscript with the assistance of CL. All the co-authors commented on and approved the final manuscript.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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