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Nitrogen/Phosphorus Ratio Affected the Growth of an Invasive Plant *Alternanthera philoxeroides* under Cadmium Stress

Rui Zhang¹, Le Ren¹, Zhihang Liu¹, Xinyue Gu¹, Shanshan Du¹, Xiao Chen¹, Bingran Xi¹, Bingnan Zhao^{1,2} and Chao Si^{1,*}

¹School of Life Science and Engineering, Handan University, Handan, 056005, China

²College of Life Sciences, Hebei University, Baoding, 071002, China

*Corresponding Author: Chao Si. Email: sc20200077@hdc.edu.cn

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ABSTRACT: Plant invasion poses a serious environmental challenge to freshwater ecosystems. Heavy metal pollution and nutrient enrichment, particularly nitrogen (N) and phosphorus (P), are two additional critical environmental issues known to influence the growth of invasive plants. Notably, the N/P ratio in the environments is often inconstant; however, whether these variations can cause differential responses in invasive plants remains underexplored, despite its crucial importance for understanding plant invasiveness. In this study, we examined the effects of different N/P ratios (4:1, 16:1, 32:1, 64:1) on the growth of a widespread invasive plant *Alternanthera philoxeroides* in cadmium (Cd) polluted water, while maintaining either a constant N concentration (2 mmol L⁻¹) or a constant P concentration (0.5 mmol L⁻¹). Two control treatments were also included: one without Cd, N, or P, and another with Cd but without N or P. The addition of N and P did not mitigate the inhibitory effects of Cd on *A. philoxeroides* growth nor did it enhance its tolerance. However, under conditions of constant N concentration, the N/P ratio of 4:1 significantly increased both the main stem length and the number of nodes in *A. philoxeroides* under Cd stress, potentially enhancing its expansion and clonal growth. Our results suggest that *A. philoxeroides* exhibits greater sensitivity to variations in the N/P ratio when N concentration is held constant, compared to when P concentration is constant in Cd polluted water. This study investigated the effects of varying N/P ratios on invasive plants under Cd pollution, which has been underexplored in previous research. The findings provide an experimental basis for controlling and preventing the spread of invasive plants in freshwater systems polluted with Cd and experiencing eutrophication.

KEYWORDS: *Alternanthera philoxeroides*; cadmium pollution; invasive plant; nitrogen/phosphorus ratio; nutrients

1 Introduction

Heavy metal pollution and eutrophication are both serious environmental issues in freshwater habitats, primarily caused by anthropogenic activities [1–4]. Industrial processes generate wastewater, waste gas, and waste residues that are discharged into the environment, thereby introducing heavy metals [5,6]. Notably, some heavy metals such as cadmium (Cd), mercury (Hg), and lead (Pb) are non-essential elements and even with toxic effects on living organisms [7,8]. Additionally, inefficient fertilizer use in agricultural practices can contribute to elevated levels of nutrients like nitrogen (N) and phosphorus (P) in the soil [9–12]. Furthermore, daily human activities including sewage discharge and waste disposal also release heavy metals as well as excessive amounts of N and P into the environment [5,12]. Ultimately, these pollutants enter freshwater ecosystems through mechanisms such as direct discharge, atmospheric deposition, and runoff causing significant harm to the ecosystem health [13–16].



The invasion of introduced plant species poses a formidable challenge to freshwater ecosystems [17–19]. Once established in an area, these invasive species can rapidly exploit the ecological niches of native species, leading to their displacement and eventual dominance [20,21]. In habitats already compromised by pollution and eutrophication, the behavior of invasive plants may exacerbate existing issues [22]. Previous studies have investigated the combined effects of heavy metals and nutrients, suggesting that elevated nutrient levels within a certain range could alleviate the inhibitory impacts of heavy metals on invasive plants [23–25]. Furthermore, due to the intricate and variable nature of heavy metals and nutrients in freshwater systems, some research has explored not only nutrient concentrations but also the interactions between different nitrogen sources and heavy metals [26–28]. The N/P ratio is also a crucial factor in nutrient dynamics that may influence the effects of heavy metals; however, its impact on heavy metal effects has received limited attention thus far.

An appropriate N/P ratio not only promotes plant growth and development, but also enhances photosynthesis efficiency, nutrient utilization efficiency, and adaptability to environmental changes and stresses [29–32]. Previous studies have indicated that certain plant species, such as alpine grasses, achieve optimal growth at an N/P ratio of 135:1 (through either nitrogen addition or phosphorus reduction) [33]. Other plant species may thrive at different N/P ratios, such as *Acer mono* Maxim. demonstrated superior growth performance at a N/P ratio of 5:4 [34]. Increasing N and P levels can enhance root neck diameter, chlorophyll content, and root development to improve the aboveground performance of plants [35,36]. For instance, N/P ratio of 5:4 can effectively stimulate root growth and development, contributing to overall plant health and productivity of *A. mono* [34]. Conversely, an imbalance in the N/P ratio can lead to suboptimal leaf growth with reduced photosynthetic efficiency ultimately inhibiting aboveground structure growth and development [33]. The importance of N/P ratios is particularly evident in helping plants adapt to environmental stresses such as flooding [37] and environmental pollution (including Cd, aluminum and ammonia) [38–40]. Currently, limited research has explored the responses of invasive plants to Cd pollution under varying N/P ratios in freshwater systems. Based on previous studies, it is hypothesized that different N:P ratios influence the growth and physiological responses of invasive plants exposed to Cd. Clarifying these impacts is crucial for assessing the invasion potential of specific species, predicting their spread rates, and establishing a robust theoretical foundation for managing and controlling invasive plants in freshwater ecosystems. Consequently, further research in this area is essential.

To investigate the potential impact of N/P ratio on the growth response of invasive plants under Cd stress, we conducted a greenhouse experiment using *Alternanthera philoxeroides*, a common invasive species. We cultivated single fragments of *A. philoxeroides* in containers filled with a cadmium solution, maintaining either a constant nitrogen concentration or a constant phosphorus concentration while varying the N/P ratios. As controls, we also grew single fragments in containers without Cd and NP, as well as in containers with Cd but without N and P. Specifically, we aimed to test the following hypotheses: (1) the response of *A. philoxeroides* to Cd is affected by N/P ratio; (2) the effect of N/P ratio on *A. philoxeroides* depends on whether N or P concentration remains constant.

2 Material and Methods

2.1 Plant Species

Alternanthera philoxeroides (Mart.) Griseb., is a perennial clonal plant that was introduced into China as a forage crop from South America in the 1930s [41,42]. The stolon of *A. philoxeroides* exhibits the ability to produce opposite leaves, side branches, and roots at each node, thereby functioning as ramets capable of establishing and clonal growth [25]. Due to its quickly asexual reproduction, high phenotypic plasticity,

and strong stress resistance, this species has emerged as one of the most problematic invasive plants in both aquatic and terrestrial habitats in China [43–47].

The plant materials used in this study were collected by cutting from naturally growing *A. philoxeroides* plants on the Handan University campus, located in Hebei province, China. Prior to the experiment, the plant materials were cultivated in plastic boxes filled with tap water in a greenhouse (36°34'N, 114°29'E) at Handan University.

2.2 Experimental Design

On 15 August 2023, more than one hundred fragments were cut from the aforementioned greenhouse-cultivated stock plant. Each fragment consisted of one node, two fully expanded opposite leaves, and a nascent bud in each axil on both sides. The fragments were subsequently placed in plastic containers filled with tap water for hydroponic cultivation in the greenhouse at Handan University, as part of this experimental procedure (as shown in Fig. 1).

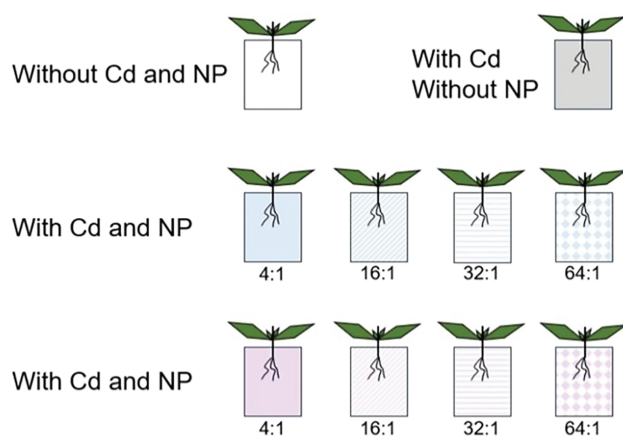


Figure 1: Schematic representation of the experimental design

To test the effect of N/P ratio, we conducted an experimental design involving two constant concentration treatments (maintaining either N or P concentration at a constant level) that were fully crossed with four N/P ratio treatments (4:1, 16:1, 32:1, and 64:1). Additionally, two control treatments were included, one without any N and P nutrients or Cd presence and another with Cd presence but lacking both N and P nutrients.

When maintaining a constant N concentration, it was set at 2 mmol L⁻¹, while for a constant P concentration, it was set at 0.1 mmol L⁻¹. N and P were supplied by Ca (NO₃)₂·4H₂O (Calcium nitrate, the purity is more than 99.0 percent, Tianjin Yongda Chemical Reagent Co., Ltd., Tianjin, China) and KH₂PO₄ (Potassium dihydrogen phosphate, the purity is more than 99.5 %, Xilong Scientific Co., Ltd., Shantou, China), respectively. Cd was provided by 3CdSO₄·8H₂O (Cadmium sulfate, the purity is more than 99.0%, Shanghai Chemical Reagent Co., Shanghai, China) at a concentration of 1 mg L⁻¹ Cd²⁺. Each treatment was replicated ten times, resulting in a total of 100 containers.

On 21 August 2023, a total of 100 fragments with the similar initial size (each measuring 13.2 cm in length and containing five pairs of leaves, n = 20) were selected from the cultured materials for experimental purposes. These fragments were individually inserted into designated apertures on floating plates within a container (with a diameter of 10 cm and height of 9 cm), which was filled with an appropriate treatment

solution volume of 800 mL. The roots were fully submerged while the leaves and stems grew above the water surface level. All containers were randomly positioned on a bench within the same greenhouse for material cultivation.

The experiment ran for a total of 20 days, concluding on 09 September 2023. By the end of the experiment, the heights of plants in many containers had reached levels comparable to those typically observed in the natural habitat of this species. Throughout the experimental period, the average temperature within the greenhouse was 25.0°C, while the average humidity was 58.2%. To compensate for water loss resulting from transpiration and evaporation processes, tap water was periodically replenished every 1–2 days during the course of the experiment. The solution in the container will be replaced on 03 September 2023.

2.3 Measurements and Data Analysis

At harvest, we measured the length of main stem and counted the number of nodes of *A. philoxeroides* in each container. The plants were divided into shoot (leaf and stem) and root parts, and subsequently dried at 70°C until a constant weight was achieved. The samples were weighed multiple times using an electronic balance (JT3003D, precision: 0.001 g, Lichen Technology Co., Changsha, China) to ensure that the measured values stabilized and no further changes were observed during the drying process. Final weights were then recorded. Additionally, we calculated the root-shoot ratio [root mass/(leaf mass + stem mass)] as well as tolerance index (TI) of both shoots and roots (which was calculated by dividing the shoot or root biomass of plants in each Cd treatment by that in treatments without Cd and NP) [48].

We employed two-way analysis of variance (ANOVAs) to investigate the effects of constant concentration treatment, N/P ratio, and their interactions on total mass, shoot mass, root mass, main stem length, number of nodes, root-shoot ratio and TI of shoot and root of *A. philoxeroides*. Differences among all treatments were assessed using the Duncan test. Prior to analysis, data were checked for normality and homoscedasticity and transformed as necessary to enhance homoscedasticity. All analyses were performed using SPSS 22.0 (IBM, Inc., Armonk, NY, USA).

3 Results

3.1 Effects of Constant Concentration Treatment and N/P Ratios on the Plant Growth

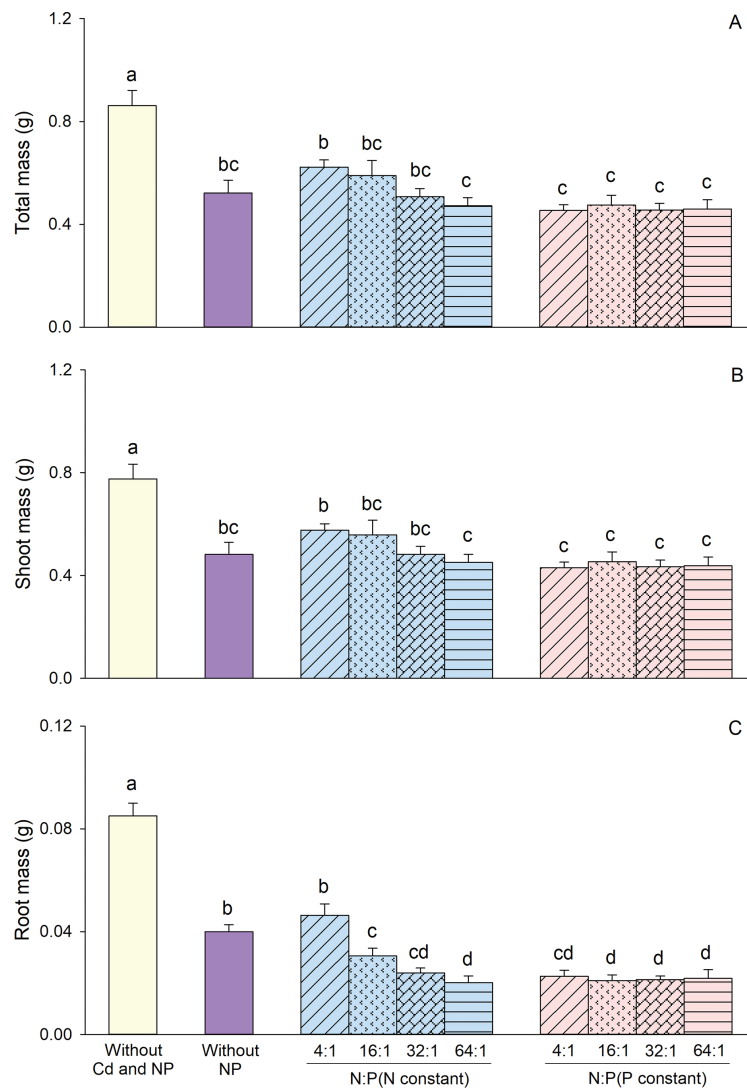
Based on the results of the two-way ANOVA, constant concentration treatment significantly affected biomass (total biomass, shoot biomass, and root biomass) (Table 1). Both N/P ratio and the interaction between constant concentration treatment and N/P ratio significantly affected root biomass (Table 1). In comparison to maintaining the constant P concentration, the greater biomass was observed when maintaining the constant N concentration (Fig. 2). Under conditions of constant N concentration, root mass decreased with increasing N/P ratio (Fig. 2C). However, no significant differences in root mass were observed among the four different N/P ratios when maintaining the constant P concentration (Fig. 2C).

Based on the Duncan test results, biomass in all treatments with both Cd, and N and P was significantly reduced compared to the treatment without both Cd and N and P, irrespective of constant N concentration or constant P concentration, as well as the N/P ratio (Fig. 2). Addition of nutrients did not have a significant impact on total mass or shoot mass when compared to the treatment with Cd but without N and P (Fig. 2A,B). However, under Cd stress, *A. philoxeroides* exhibited a significant reduction in root mass at an N/P ratio of 16:1 to 64:1 when N concentration was constant and at all N/P ratios when P concentration was constant (Fig. 2C).

Table 1: Results of analyses of variance conducted on total mass, shoot mass, and root mass in *Alternanthera philoxeroides* under different constant concentration treatments and N/P ratios

Variable	Concentration constant (C)		N/P ratio (R)		C × R	
	F _{1,72}	p	F _{3,72}	p	F _{3,72}	p
Total mass ^a	10.846	0.002	1.968	0.126	1.583	0.201
Shoot mass ^a	9.846	0.002	1.601	0.197	1.263	0.294
Root mass ^b	16.398	<0.001	7.697	<0.001	5.807	0.001

Note: ^a Nature log transformation. ^b Square root transformation. Values of F and p, and degree of freedom (subscript for “F”) are given. Values of F and p are in bold when p < 0.05.

**Figure 2:** Effects of different constant concentration treatments and N/P ratios on total mass (A), shoot mass (B), and root mass (C) of *Alternanthera philoxeroides*. Bars and vertical lines are means and SE. Different small letters above the bars indicate significant differences among the treatments

3.2 Effects of Constant Concentration Treatment and N/P Ratios on the Plant Morphology

Based on the results of the ANOVA, constant concentration treatment, N/P ratio and their interaction significantly affected main stem length, number of nodes and root-shoot ratio (Table 2). Main stem length, number of nodes and root-shoot ratio were overall greater when concentration was constant than that when P concentration was constant (Fig. 3). When N concentration was maintained constant, main stem length, number of nodes and root-shoot ratio reached their highest values at a N/P ratio of 4:1 (Fig. 3). However, when P concentration was constant, these measurements did not differ significantly among the four N/P ratios (Fig. 3).

Table 2: Results of analyses of variance conducted on main stem length, and root tolerance index in *Alternanthera philoxeroides* under different constant concentration treatments and N/P ratios

Variable	Concentration constant (C)		N/P ratio (R)		C × R	
	F _{1,72}	p	F _{3,72}	p	F _{3,72}	p
Main stem length ^a	5.893	0.018	9.274	<0.001	10.555	<0.001
Number of nodes ^a	4.500	0.037	6.903	<0.001	10.587	<0.001
Root-shoot ratio ^b	4.646	0.034	5.368	0.002	3.182	0.029

Note: ^a Nature log transformation. ^b Square root transformation. Values of F and p, and degree of freedom (subscript for “F”) are given. Values of F and p are in bold when $p < 0.05$.

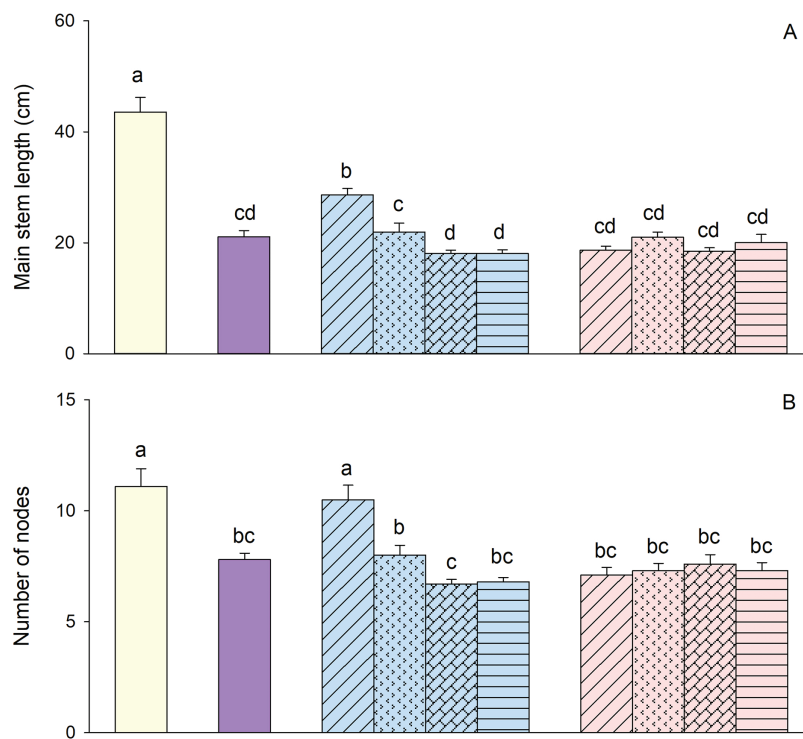


Figure 3: (Continued)

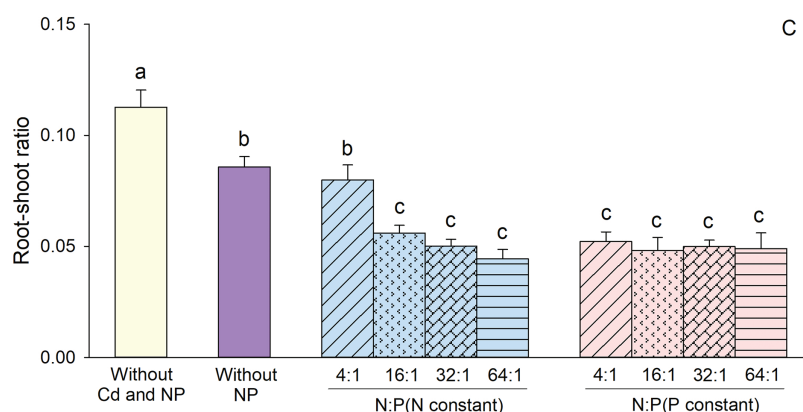


Figure 3: Effects of different constant concentration treatments and N/P ratios on main stem length (A), number of nodes (B), and root-shoot ratio (C) of *Alternanthera philoxeroides*. Bars and vertical lines are means and SE. Different small letters above the bars indicate significant differences among the treatments

According to the Duncan test, compared to the treatment without Cd, N and P, the treatments with Cd showed significant reductions in main stem length, number of nodes, and root-shoot ratio (Fig. 3), except for the N/P ratio of 4:1 when N concentration was constant which did not significantly affect the number of nodes (Fig. 3B). In comparison to the treatment with Cd but lacking N and P, an increase in main stem length and number of nodes was observed at a 4:1 N/P ratio when N concentration was constant; however, no significant changes were observed at ratios of 16:1, 32:1, and 64:1 when N was constant or any N/P ratios when P was constant (Fig. 3A,B). In addition, root-shoot ratio in all treatments with Cd and NP, except for the 4:1 N/P ratio under constant N conditions, exhibited lower values compared to the treatment with Cd alone (Fig. 3C).

3.3 Effects of Constant Concentration Treatment and N/P Ratios on the Tolerance Index

The results of the two-way ANOVA showed that constant concentration treatment and N/P ratio significantly affected the both shoot TI and root TI (Table 3). When N concentration was constant, both shoot TI and root TI were higher compared to when P concentration was constant (Fig. 4). The interaction between constant concentration treatment and N/P ratio significantly affected root TI (Table 3). Specifically, when N concentration was constant, both shoot TI and root TI were tended to decrease with increasing N/P ratio (Fig. 4). However, no differences in shoot TI or root TI were observed among the four N/P ratios when P concentration was maintained constant (Fig. 4).

Table 3: Results of analyses of variance conducted on shoot tolerance index and root tolerance index in *Alternanthera philoxeroides* under different constant concentration treatments and N/P ratios

Variable	Concentration constant (C)		N/P ratio (R)		C × R	
	F _{1,72}	p	F _{3,72}	p	F _{3,72}	p
Shoot tolerance index ^a	9.846	0.002	1.601	0.197	1.263	0.294
Root tolerance index ^b	16.398	<0.001	7.697	<0.001	5.807	0.001

Note: ^a Nature log transformation. ^b Square root transformation. Values of F and p, and degree of freedom (subscript for “F”) are given. Values of F and p are in bold when $p < 0.05$.

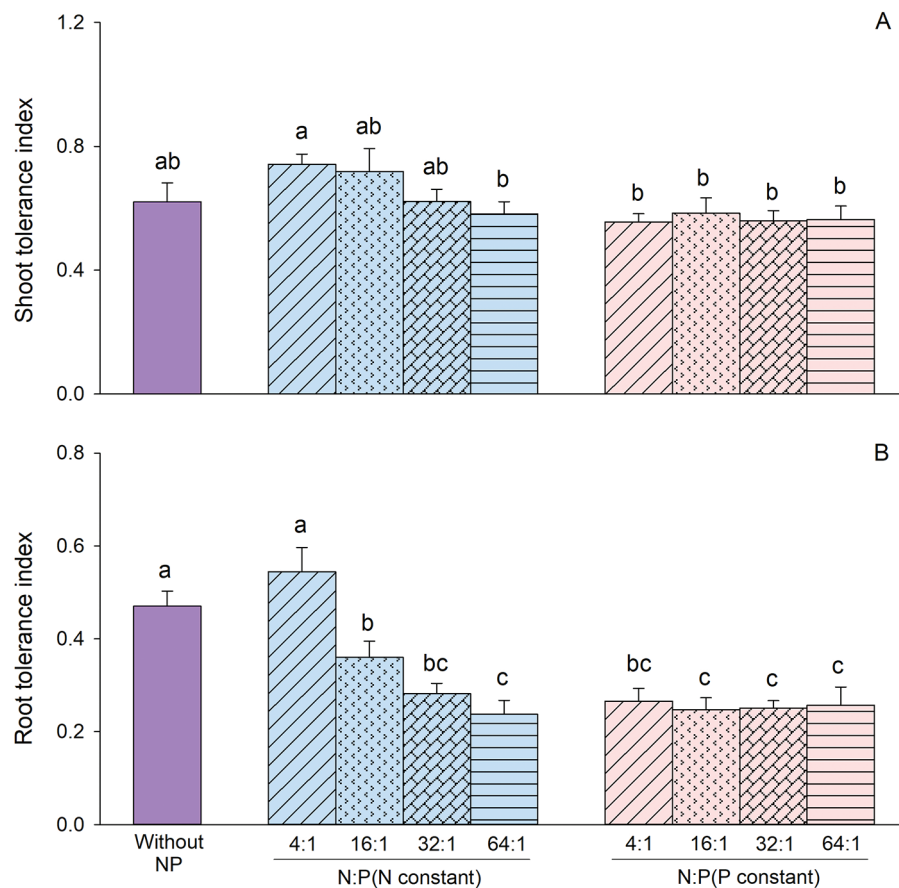


Figure 4: Effects of different constant concentration treatments and N/P ratios on shoot tolerance index (A) and root tolerance index (B) of *Alternanthera philoxeroides*. Bars and vertical lines are means and SE. Different small letters above the bars indicate significant differences among the treatments

The results of the Duncan test revealed no significant difference in shoot TI between treatments with Cd and various N/P ratios compared to the treatment with Cd alone (Fig. 4A). However, the root TI was significantly lower in treatments containing N and P (except for the treatment at 4:1 N/P ratio when N concentration was constant) compared to the treatment without N and P (Fig. 4B).

4 Discussion

4.1 Response of Plants Growth to Constant Concentration Treatment and N/P Ratios

The presence of Cd inhibited the overall growth of *A. philoxeroides* due to reduced absorption and transport of nutrients and water, increased oxidative damage, disrupted plant metabolism, and impaired plant morphology and physiology [49–51]. Previous studies have reported inconsistent effects of nutrients on plant growth under Cd stress [23–26,52]. For instance, some research has shown that higher nutrient levels alleviate the inhibitory effect of Cd on plant growth [23–24,52]. However, Zhang et al. [25] found no significant effect on plant growth under Cd stress with high nutrient levels. Furthermore, Li et al. [26] reported that increased nutrient availability exacerbated the inhibitory effects of Cd on plant growth. In this study, the addition of N and P did not significantly alleviate the inhibitory effects of Cd, regardless of whether the concentration of N or P was constant or their ratios. Although total nutrient concentrations were similar in these studies, variations in specific experimental conditions suggest potential explanations for divergent

results. Additionally, the complexity of environmental changes faced by natural aquatic ecosystems poses further challenges in estimating these effects. This serves as a reminder that although the results of existing greenhouse simulation experiments can offer some guidance for controlling and managing invasive plants in heavy metal polluted and eutrophic water bodies, additional experimental findings with diverse designs are still necessary as a basis.

4.2 Response of Plants Morphology to Constant Concentration Treatment and N/P Ratios

The presence of Cd significantly reduced the main stem length and number of nodes, consistent with previous studies indicating the inhibitory effect of Cd on *A. philoxeroides* elongation and asexual reproductive capacity [23–26]. The root-shoot ratio was significantly decreased in the presence of Cd, suggesting an escape strategy to alleviate its toxicity [24,25]. Under Cd stress, the morphological performance was related to either N concentration or P concentration was constant. Compared to the treatment of P concentration constant, the morphology of *A. philoxeroides* exhibits greater sensitivity to different N/P ratios when N concentration was constant. Specifically, within the concentration range set in this experiment, the morphology of *A. philoxeroides* was more susceptible to variations in P concentration compared to N concentration under Cd stress. Additionally, in contrast to the Cd treatment without the addition of N and P, the main stem length and number of nodes significantly increased under Cd stress with N and P addition, particularly when maintaining a constant N concentration at an N/P ratio of 4:1. Moreover, the root-shoot ratio remained consistent with that observed under conditions of N and P addition with presence of Cd exposure. Thus, this specific combination of N and P concentrations appears to promote clonal offspring expansion and generation in *A. philoxeroides* inhabiting Cd polluted water while simultaneously mitigating Cd induced inhibition on root growth.

4.3 Response of Plants Tolerance Index to Constant Concentration Treatment and N/P Ratios

The shoot TI and root TI exhibited similar responses to constant concentration and N/P ratio, with both measurements showing relatively higher values at a constant N concentration of 2 mmol L⁻¹ and tending to decrease as the N/P ratio increased under constant N concentration. Compared to a constant phosphorus concentration, the root TI displayed a stronger response to the N/P ratio when the N concentration was remained constant. Furthermore, there were no significant changes observed in shoot TI to Cd when comparing treatments with or without N and P under constant N concentration, constant P concentration, or varying N/P ratios. However, for root TI, significant negative effects were observed at N/P ratios of 16:1–64:1 under constant N concentration and all tested N/P ratios under constant P concentration. Root TI just matched that of the treatment without N and P when using an N/P ratio of 4:1 under constant N conditions; thus, adding N and P could not enhance *A. philoxeroides*' tolerance to Cd. In fact, providing nutrients at specific N/P ratios may actually diminish root tolerance [53]. This phenomenon could be attributed to exceeding a certain threshold of the N/P ratio becoming harmful for plants [31,54]. It is worth to note that this study represents a preliminary investigation into the effect of the N/P ratios. We focused on examining the effects within the range of 4:1 to 64:1 and under certain concentration conditions. However, the natural environment is considerably more complex, which may introduce certain limitations in the generalizability of our findings. Nevertheless, this study demonstrates the significant influence of the N/P ratio on the growth of *A. philoxeroides* in Cd polluted water. This provides a valuable scientific basis for managing and predicting the spread of this invasive species in similar environments.

5 Conclusion

Although the effects of N and P nutrients on invasive plants in polluted freshwater have been well studied, whether their effects are influenced by the N/P ratio remains underexplored. This study addresses this research gap, and the results showed that the addition of N and P in any treatment did not alleviate the inhibitory effect of Cd on the growth of *A. philoxeroides*. Moreover, neither shoot TI nor root TI improves with the addition of N and P; instead, the root TI even decreases (except for the N/P ratio of 4:1 under N concentration constant condition). However, it is worth noting that compared to Cd treatment without N and P, the N/P ratio of 4:1 under a constant N concentration condition significantly enhances both the main stem length and the number of nodes in *A. philoxeroides* under Cd stress, potentially facilitating its expansion and clonal growth. These findings establish a theoretical foundation for the management and control of *A. philoxeroides*, an invasive plant species, in freshwater ecosystems characterized by Cd pollution and eutrophic water conditions. Furthermore, they highlight the complexity of nutrient impacts on invasive plant responses to Cd stress. Considering the limited range of nutrient concentrations and N/P ratios examined in this experiment, the application of the research findings may be constrained. Therefore, future experimental designs should include a broader spectrum of conditions and account for additional environmental factors to provide more accurate guidance for managing and controlling invasive plants in freshwater ecosystems.

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Availability of Data and Materials: The data that support the findings of this study are available from the corresponding author, Chao Si, upon reasonable request.

Ethics Approval: Not applicable.

Conflicts of Interest: The authors declare no conflicts of interests to report regarding the present study.

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