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Effects of salinity on removal of nitrogen and phosphorus from eutrophic saline water in planted *Lythrum salicaria* L. microcosm systems

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ABSTRACT

Treatment of eutrophic saline water is problematic due to high concentrations of nitrogen and phosphorus often combined with high salinity levels. In this paper, microcosm systems planted with *Lythrum salicaria* L. (purple loosestrlfe) were set up to evaluate the capacity of the systems to remove excessive nutrients from fresh water (0.05% salinity), and saline water (0.5% and 1.0% salinity). The average removal efficiencies were 30.6%–45.3% for total nitrogen (TN), 31.4%–55.4% for ammonia nitrogen (NH_4^+-N), 28.0%–45.9% for nitrate nitrogen (NO_3^--N), and 9.1–16.2% for total phosphorus (TP) at different salinities, respectively. It was observed that the treatment performance was significantly inhibited by increasing salinity. However, increasing salinity could promote the NO_3^--N removal in the systems. In addition, the elevated salinity of eutrophic saline water was found to induce a stress response that could be quantified by a series of metabolic assays measuring the chlorophyll (Chl), proline (Pro), and malondialdehyde (MAD). At different salinities, no significant differences were observed in pigments' content on the third day of the experiment. But on the seventh day of the experiment, 0.5 and 1.0% salinity resulted in sharp decrease in the levels of chl *b*, total chl, and total chl/carotenoids. Compared to 0.05% salinity, proline accumulation and MAD increased significantly with increasing salinity, but the content of MAD was still very low ($<0.1\,\mu mol\,g^{-1}$ FW). The results of this study indicate that the planted salt-tolerant aquatic macrophytes systems can be a low-cost ecological phytoremediation technology to treat eutrophic saline water, and the application of cellular stress assays can provide useful tools to monitor salt-induced responses in aquatic macrophytes.

Keywords: *Lythrum salicaria* L.; Nitrogen; Phosphorus; Stress responses; Eutrophic saline water; Salt stress

1. Introduction

Eutrophication as an urgent environmental problem has received increasing attention since it triggered unhealthy algal blooms, the spread of certain aquatic macrophytes, oxygen depletion, and the loss of key species. Thus, many freshwater ecosystems were deteriorated due to eutrophication [1]. Meanwhile, it was reported that aquatic plants have an important role in the nitrogen and phosphorus

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removal [2–4]. However, each plant species has a different nutrient uptake rate and a specific growth pattern in nature and constructed aquatic ecosystems, which had great influence on removing pollutants from various wastewater sources [5].

Tanner [6] has mentioned the general requirements for aquatic macrophytes in wastewater treatment systems as being tolerant of target pollutants, having a high pollutant removal capacity, adapting to local climate, and establishing and growing fast. The most commonly utilized aquatic macrophytes are fast-growing emergent plant species [7], such as *Typha latifolia* and *Phragmites australis* [8,9]. However, when dealing with a certain saline wastewater, the plant species selection must be valued. Because the salt-tolerant plant species could maintain a better performance if being capable of accommodation to the salinity of the influent. Calheiros et al. [10] have proposed that constructed wetlands may potentially be used to treat saline wastewater, but the selection of the plant species may be a key issue. Brown et al. [11] have determined the feasibility of *Suaeda esteroa*, *Salicornia bigelovii*, and *Atriplex barclayana* as biofilters for removing nutrients from saline aquaculture wastewater. In fact, there are a wide range of salt-tolerant aquatic plant species in nature. *L. salicaria* is a herbaceous perennial plant, the stems are reddish-purple or red to purple and square in cross-section. The flowers are reddish-purple, flowering lasts throughout the summer. However, the most important thing is that it is one of the common salt and brackish marsh plant species [12,13], and has been widely used in wetlands to treat wastewater [14,15]. However, few studies have paid attention on the interaction between *L. salicaria* and salinity, which might have a significant effect on eutrophic saline water.

High salinity disturbs ion homeostasis in vacuoles, reduces the water potential of the roots and makes it harder to extract water. It also destroys the photosynthetic systems by inhibiting chlorophyll synthesis, and restrains many enzyme processes, which ultimately cause the excessive generation of reactive oxygen species (ROS) that can damage a number of intracellular targets including proteins, lipids, and nucleic acids in the stressed plant cells [16,17]. But the plants develop a plethora of biochemical and molecular mechanisms to cope with and defend salt stress [18]. To accommodate the ionic balance in the vacuoles, cytoplasm accumulates low-molecular-mass compounds termed compatible solutes [19,20], including proline [21,22], which plays a significant role against ROS [23,24], and many plants accumulate proline as a nontoxic and protective osmolyte under saline condition [25]. Malondialdehyde is a cytotoxic product of lipid peroxidation and an indicator of free radical production and consequent tissue damage [26]. However, different species of plants inherently possess different measures and capacities of treating with high salinity, and salt stress responses and tolerance vary between species [27].

The aim of the present study was to assess the potential of using *L. salicaria* to remove nutrients from eutrophic saline water within the tolerant range of salinity in microcosm systems and characterize the stress responses of the *L. salicaria*. The treatment performances of these microcosm systems were monitored for total nitrogen (TN), ammonia nitrogen (NH_4^+-N), nitrate nitrogen (NO_3^--N), and total phosphorous (TP) in eutrophic water with different salinity. Meanwhile, chlorophyll (chl), proline (pro), and malondialdehyde (MAD) were determined as indicators of oxidative stress to eutrophic water with different salinity.

2. Material and methods

2.1. Experimental setup

2.1.1. Plants culture and exposure

Approximately the same length rooted cuttings of *L. salicaria* were transplanted into 7 L plastic buckets, containing 2 kg (dry weight) of quartz sand, at 4–5 plants per bucket. Before exposure to different salinities, the plants were cultured for 1 week with tap water, and then for one week in 1.5 L of $1/2 \times$ Hoagland solution [28] for acclimatization. The experimental treatments consisted of five levels of salinity (0.05, 0.5, 1.0%, 1.5, and 2.0%) prepared from Hoagland solution and reagent grade sodium chloride (NaCl). Freshwater concentration, which was represented by 0.05% concentration and served as the control. Each treatment was done in triplicate. The nutrient medium with different NaCl concentrations was replaced twice a week, followed by replenishment with fresh solution. After exposure to different salinity levels, the plants status was observed and recorded every day. Two weeks later, the salt-tolerance range of the *L. salicaria* was determined based on the survival threshold [29], which refers to the soil salt concentration that triggers the death of half of the plant species grown in soil.

2.1.2. Nutrient removal and stress response performance

The study was conducted in autumn (25 ± 1℃). Approximately the same length, the equal mass, and well-grown rooted cuttings of *L. salicaria* were

transplanted into 7 L plastic buckets, containing 5 L of simulated eutrophic water. The plants were fixed in perforated polyethylene sheets. The water quality of the simulated eutrophic water was summarized in Table 1. The experimental treatments for *L. salicaria* consisted of three levels of salinity (0.05, 0.5, and 1.0%) according to the survival threshold, and 0.05% concentration represented freshwater concentration and served as the control. The treatment solutions were prepared from simulated eutrophic water and reagent grade sodium chloride (NaCl). Different levels represented the different concentrations of eutrophic saline water. Three units unplanted, each treatment was done in triplicate. Water samples were collected and determined every three days, and the same parts of the plants tissue were collected and measured on the third day and seventh day of the experiment.

2.2. Physico-chemical and plant physiological indexes analysis

Salinity was monitored daily using an LC model DDBJ-350 portable conductivity meter (LC Shanghai, China) during the whole experiment. The water quality parameters (TN, NH_4^+-N, NO_3^--N, and TP) were analyzed based on Standard Methods [30].

The same parts of the plants tissue were collected, washed quickly with distilled water, frozen in liquid N_2, and stored at −70°C for physiological indexes (Chl, Pro, and MAD).

The content of Chl *a*, Chl *b*, total Chl, and total carotenoids (Xanthophylls and Carotenes) in the leaves of the *L. salicaria* were determined by UV–vis spectroscopy [31]. The freeze-dried leaves were cut into small pieces from which subsamples of 5–10 mg were extracted with 8 mL of 96% ethanol in the dark at room temperature for 24 h. The absorbance of extracts was measured at 470, 649, and 665 nm wavelengths, respectively.

Table 1
Eutrophic saline water quality (mean ± SD, $n = 3$)

Parameters	Salinity			Unit
	0.05%	0.5%	1.0%	
pH		8.10 ± 0.03		–
Temperature		23.7 ± 0.2		°C
TN	10.55 ± 0.01	10.55 ± 0.02	10.17 ± 0.02	mg L^{-1}
NH_4^+-N	7.98 ± 0.02	8.27 ± 0.02	8.37 ± 0.05	mg L^{-1}
NO_3^--N	2.09 ± 0.01	2.05 ± 0.01	2.06 ± 0.01	mg L^{-1}
TP	4.88 ± 0.01	5.06 ± 0.03	5.10 ± 0.04	mg L^{-1}

Proline analysis was performed according to Bates et al. [32] with some modification. About 0.2–0.5 g FW (fresh weight) of the frozen leaves were cut into small pieces, and put into test tubes with 5 mL of 3% sulfosalicylic acid, the extract was centrifuged at 4,000×*g* for 15 min to remove debris after boiling for 15 min. To 2 mL of supernatant, 2 mL of ninhydrin was added with 2 mL glacial acetic and was incubated at boiling temperature for 30 min. The mixture was extracted with toluene, and then proline was quantified spectrophotometrically at 520 nm from the organic phase.

Malondialdehyde content was measured according to Heath and Packer [33]. Approximately 0.3 g small pieces of leaves were homogenized in 2 mL of 5% trichloroacetic acid. The homogenate was centrifuged at 1,500×*g* for 10 min, and then the supernatant was diluted to 10 mL. The subsample of diluted extract with a volume of 2 mL was mixed with 2 mL of 0.6% 2-thiobarbtiuric acid. The mixture was incubated in boiled water for 30 min, and then centrifuged at 1,500×*g* for 10 min. Absorbencies of aqueous phase at 450, 532, and 600 nm wavelengths were measured, respectively.

2.3. Statistical analysis

All statistical analyses were performed with the statistical program SPSS 11.0 (SPSS Inc., Chicago, USA), including analysis of variance (ANOVA), Univariate, Bartlett's and levine's test for homogeneity of variance and normality, and Duncan´s multiple range test for differences between means. In all tests, differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Salt-tolerance range of the L. salicaria

L. salicaria grew and developed well at 0.05, 0.5, and 1.0% salinity and without symptoms of salt toxicity two weeks later. However, at 1.5% and 2.0% salinity, the leaves of *L. salicaria* started to turn yellowish, and some of the plants became wilted, or even died. Based on the survival threshold [29], the survival rate of *L.* salicaria exceeded 50% at 0.05, 0.5, and 1.0% salinity, up to 98, 95, and 86%, respectively. However, the survival rate reached 18 and 6% at 1.5 and 2.0% salinity, respectively.

3.2. Nutrients removal performance

The performance of nutrients removal in planted *L. salicaria* microcosm systems with different salinities are represented in Figs. 1 and 2. TN concentrations

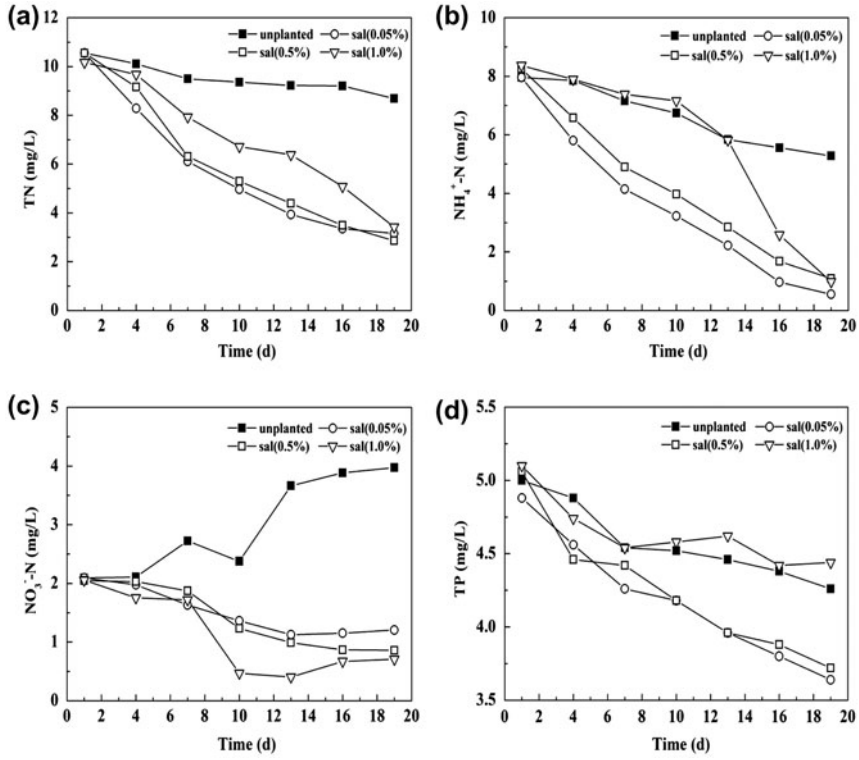

Fig. 1. The profile of TN(a), NH_4^+-N(b), NO_3^--N(c), and TP(d) in planted *L. salicaria* microcosm systems for the treatment of eutrophic water with different salinities during the experiment.

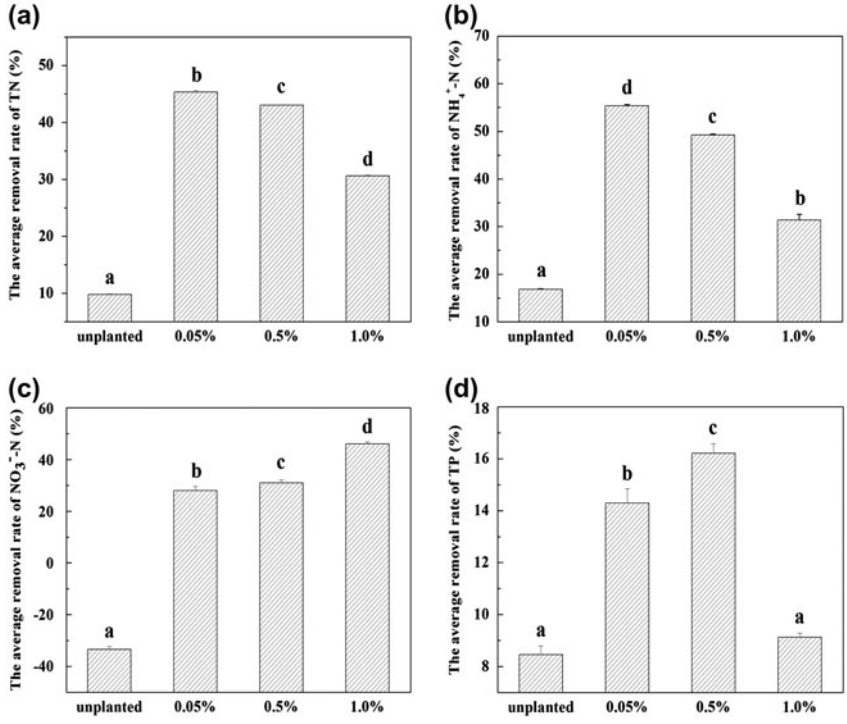

Fig. 2. The average removal efficiencies (mean ± SD, $n = 3$) of TN(a), NH_4^+-N(b), NO_3^--N(c), and TP(d) in planted *L. salicaria* microcosm systems for the treatment of eutrophic water with different salinities during the experiment. Different letters above the columns indicate significant differences between salinities ($p < 0.05$).

Table 2
Leaf pigments' contents in *L. salicaria* (mean ± SD, $n = 3$) grown at different concentrations of eutrophic saline water on the third day and seventh day of the experiment. Different letters between the columns indicate significant differences between salinities ($p < 0.05$)

Salinity	Chl *a*	Chl *b*	Total Chl	Chl *a*/*b*	Carotenoids	Total Chl/ Carotenoids
3d						
0.05%	2.166 ± 0.036a	1.004 ± 0.008a	3.170 ± 0.043a	2.158 ± 0.020a	0.348 ± 0.008a	9.111 ± 0.088a
0.5%	2.200 ± 0.053a	1.011 ± 0.018a	3.211 ± 0.056a	2.177 ± 0.065a	0.349 ± 0.032a	9.252 ± 0.695a
1.0%	2.194 ± 0.057a	1.006 ± 0.041a	3.200 ± 0.095a	2.180 ± 0.049a	0.368 ± 0.017a	8.708 ± 0.136a
7d						
0.05%	2.137 ± 0.028a	0.925 ± 0.003b	3.062 ± 0.026b	2.310 ± 0.035a	0.373 ± 0.004a	8.199 ± 0.025b
0.5%	2.048 ± 0.106a	0.765 ± 0.053a	2.813 ± 0.053a	2.691 ± 0.339a	0.425 ± 0.053ab	6.673 ± 0.667a
1.0%	2.020 ± 0.002a	0.761 ± 0.009a	2.781 ± 0.009a	2.653 ± 0.033a	0.464 ± 0.007b	5.991 ± 0.077a

decreased from 10.1–10.6 mg L^{-1} to 2.9–3.4 mg L^{-1} (Fig. 1(a)). At 0.05% salinity, the average removal rate (45.3%) was significantly better than that at 0.5 and 1.0% salinity (43.0 and 30.6%). The average removal efficiencies of the planted systems at 0.05, 0.5, and 1.0% salinities were significantly higher than those of the unplanted systems. ($p < 0.05$) (Fig. 2(a)). The NO_3^--N concentration decreased from 8.0–8.6 mg L^{-1} to 0.6–1.1 mg L^{-1} over time at 0.05, 0.5, and 1.0% salinities (Fig. 1(b)). The NH_4^+-N removal efficiencies significantly decreased with increasing salinity. However, the average removal rates of the planted systems at 0.05, 0.5, and 1.0% salinities were significantly higher than those of the unplanted systems, more than 38.6, 32.4, and 14.6%, respectively, ($p < 0.05$) (Fig. 2(b)). The NO_3^--N concentration decreased gradually during the operating period, except in the unplanted systems (Fig. 1(c)). However, the average NO_3^--N removal efficiencies, which significantly increased with the gradual increasing salinity, were 28.0, 30.9, and 45.9%, respectively. There are significant differences in NO_3^--N removal between planted systems at 0.05,0.5, and 1.0% salinities and unplanted systems ($p < 0.05$) (Fig. 2(c)). TP concentration throughout the experimental period ranged from 4.9–5.1 mg L^{-1} to 3.6–4.4 mg L^{-1} (Fig. 1(d)). *L. salicaria* exhibited relatively low TP removal efficiencies (9.1–16.2%) at 0.05, 0.5, and 1.0% salinities, which significantly decreased with increasing salinity. No significant differences were observed in TP removal between planted systems at 1.0% salinity and unplanted systems ($p < 0.05$) (Fig. 2(d)).

3.3. Leaf pigments' concentration responses to eutrophic water with different salinity

As shown in Table 2, in comparison with the 0.05% salinity, no significant differences were observed in chl *a*, chl *b*, total chl, chl *a*/*b*, carotenoids, and total chl/carotenoids at 0.5 and 1.0% salinity on the third day of the experiment. But on the seventh day of the experiment, 0.5 and 1.0% salinity resulted in significant decrease in the levels of chl *b*, total chl, and total chl/carotenoids, and significant increase in the levels of carotenoids, as compared with 0.05% salinity ($p < 0.05$).

3.4. Proline accumulation responses to eutrophic water with different salinity

The effects of salinity on the levels of proline in the leaves of *L. salicaria* are shown in Fig. 3. The constitutive proline contents were 1.37 and 5.04 μg g^{-1} FW at 0.05% salinity on the third day and seventh day of the experiment, respectively. Increasing salinity effectively induced proline accumulation in the leaves and maximal proline levels were 10.7- and 7.9-fold more than those at 0.05% salinity on the third day and seventh day of the experiment, respectively. The concentrations of proline on the seventh day were significantly higher than those on the third day, and higher than 267, 549,

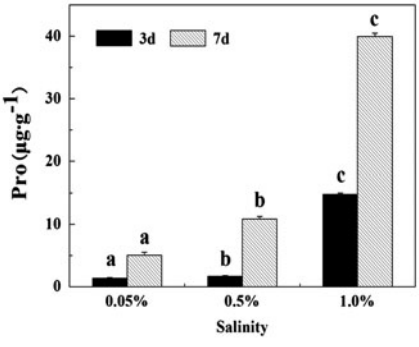

Fig. 3. Effects of different concentrations of eutrophic saline water on proline contents in the leaves of *L. salicaria* (mean ± SD, $n = 3$) on the third day and seventh day of the experiment. Different letters above the columns indicate significant differences between salinities ($p < 0.05$).

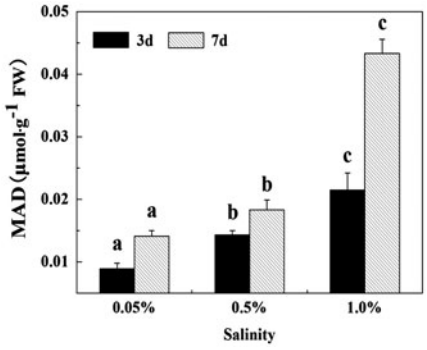

Fig. 4. Effects of different concentrations of eutrophic saline water on MAD contents in the leaves of *L. salicaria* (mean ± SD, $n = 3$) on the third day and seventh day of the experiment. Different letters above the columns indicate significant differences between salinities ($p < 0.05$).

and 170%, respectively. As a result, salinity, time and the interaction between them had significant effect on the content of proline ($p < 0.05$).

3.5. MAD responses to eutrophic water with different salinity

MAD level is routinely used as an index of lipid peroxidation and damage to the cell membrane system under stressful conditions. As shown in Fig. 4, the contents of MAD in the plants were significantly affected by salinity and increased from 0.009–0.014 μmol g^{-1} FW at 0.05% salinity to 0.014–0.018 μmol g^{-1} FW, and 0.022–0.042 μmol g^{-1} FW at 0.5 and 1.0% salinity on the third day and seventh day of the experiment, respectively. Exposure to eutrophic saline water for seven days resulted in a significant elevation in MAD level compared to three days at 0.05, 0.5, and 1.0% salinity, and higher than 58.5, 28.3, and 95.6%, respectively. In addition, the interaction between salinity and time had a significant effect on the content of MAD ($p < 0.05$).

4. Discussion

4.1. Nutrient removal

Removal of nitrogen from eutrophic water using aquatic plants may be mainly attributed to plant uptake and microbial processes around rhizosphere, i.e. nitrification and denitrification [34]. However, there are many abiotic factors influencing the processes, such as dissolved oxygen, pH, and temperature [35]. In the present study, the impact of salinity on nitrogen (TN and NH_4^+-N) removal was significant, and the average removal rates decreased with increasing salinity. This outcome could be attributed to salinity and it not only affects plant growth directly, but also acts as an abiotic inhibitor influencing the growth of plant roots, plant root exudates, which are the foundations of normal growth and metabolism of rhizosphere micro-organisms [35,36]. High nutrient had greater effects on carbon-nitrogen balance, previous studies have shown that in high nutrient water, carbohydrate consumption increased due to its transformation into carbon skeleton of free amino acids [37,38]. This mechanism was effective in reducing the accumulation of NH_4^+-N in macrophytic tissue due to luxury absorption [37–40]. Moreover, salinity is known to affect NH_4^+ uptake and the amount of rubisco [41]. The reduced contents of N in the plant tissues may have been the result of impeded uptake and assimilation of NH_4^+ as Na^+ is known to competitively inhibit NH_4^+ uptake [42]. This result is also in agreement with other literatures. Brown et al. [11] proposed that the removal of both total nitrogen and inorganic nitrogen by the plant-soil system was significantly inhibited by increasing salinity. Klomjek and Nitisoravut [43] emphasized high salt concentration was a major factor that caused unexpectedly poor treatment performance, because extreme salt concentrations normally affect the function biota, such as plants and micro-organisms. The statistical analysis showed there were significant differences between the unplanted and planted units, indicating the plants play a dominant role in nitrogen (TN and NH_4^+-N) removal. Weker et al. [44] and Ottova et al. [45] proposed that wetlands with robust aquatic macrophyte communities have richer microbial communities than those where macrophytes are absent, which lead to different treatment performance.

In the current study, the unplanted systems could effectively accumulate NO_3^--N over the experiment period. There are some possible reasons for this result, including no plant roots and rhizomes supporting denitrifier communities growth and metabolism, and no environmental conditions for denitrification, i.e. hypoxic microenvironment or some heterotrophic bacteria competing carbon sources with denitrifier communities, and this case is consistent with results reported by Lin et al. [46] and Sindilariu et al. [47]. While, in planted systems, the average removal rates increased with increasing salinity, this result might be due to the fact that the present salinity levels had limited impact on the denitrification process, or halotolerant bacteria dominated the denitrifier communities [48].

Removal of phosphorus in aquatic ecosystems is a manifold process [47], including uptake by plants and microbes, as well as sorption on the substrate [49–51]. In this study, The TP removal efficiencies were

relatively low (9.1–16.2%), these levels are lower than those reported by Tilley et al. [52] (31%) and Lee et al. [53] (33%). This phenomenon could be caused by the following reasons: (1) no substrate in the systems, the processes removing phosphorus by sorption, complexation, and precipitation are ignored; (2) *L. salicaria* might not be the right plant species for phosphorus removal, Hunter et al. [49] proposed that the differences in phosphorus removal rate could be attributed to various vegetation type and density. Khan and Shah [54] also indicated that planting type was perhaps one of the most important factors for phosphorus removal than the species richness; (3) phosphorus accumulated in plant tissues or microbial cells is rapidly returned into water through degradation when phosphorus saturation [55]. In addition, (4) retention time, loading rate, and temperature are the factors affecting phosphorus removal [49]. The results showed that the TP removal efficiencies significantly decreased with increasing salinity, and no significant differences were observed in TP removal between planted units at 1.0% salinity and unplanted units. This outcome might be attributed to the dominant role played by salinity, which affects the growth of plants and micro-organisms [35,36], thereby affecting the absorption of phosphorus.

4.2. Responses to eutrophic water with different salinity

High NaCl concentrations in the growth medium of plants generate primary and secondary effects that negatively affect plants growth and development. Primary effects are ionic toxicity and osmotic stress, and secondary effects of salt stress was inhibiton of K^+ uptake, membrane dysfunction, and generation of ROS in the cell [56,57].

Salt stress has been proved to influence chlorophyll biosynthesis. The data from literature indicated that salinity significantly caused the reduction in the chlorophyll content [58,59], other inhibitory processes are also involved including inhibition of electron flow, decreased photosystem function, diminished rubisco abundance and activity, and changes in chloroplast ultrastructure [60], which result in carbohydrate from photosynthesis is low and severe carbon-nitrogen unbalance. No significant changes in the photosynthetic pigments were observed on the third day of the experiment, but the photosynthetic pigments were enhanced at 0.5% salinity, and then decreased slightly at 1.0% salinity except carotenoids, which suggests a certain salt stimulation may promote the growth of some plant species [61]. The results also showed that long-time salt stress led to significant decreases in the levels of chl *b*, total chl, and total chl/carotenoids. This is in consistent with the study of Chang et al. [58], who suggested that chlorophyll *b* content was more sensitive to NaCl stress than chlorophyll *a*, and lower total chl/carotenoids at high salinity indicated stress and damage to the photosynthetic apparatus [59]. It is well established that carotenoids offer protection against photooxidation by helping dissipate the excessive energy of excitation [61]. In the present study, the increasing content of carotenoids at 0.05% and 1.0% salinity on the third day and seventh day of the experiment may act as a protective mechanism, and shield the leaves from the deleterious effects of oxidative damage resulting from medium salt stress.

Accumulation of free proline in response to salt stress seems to be wide-spread among plants [62,63]. In the present study, the content of proline increased with increasing salinity, the longer the exposure to the salt medium, the higher the content of proline. This result demonstrated that proline may play an important role in the protection of *L. salicaria* against salt stress, and *L. salicaria* has a capacity to sequester Na^+ and Cl^- in the vacuoles and synthesize proline as a compatible compound.

The content of MAD, a product of lipid peroxidation, has been considered as an indicator of oxidative damage [64,65]. In the present study, even though the content of MAD increased with increasing salinity, it was still very low ($<0.1\ \mu mol\ g^{-1}$ FW) indicating that serious plasma membrane lipid peroxidation of *L. salicaria* may not happen.

5. Conclusion

It was found that the microcosm systems planted with *L. salicaria* was an option for improving the treatment of the eutrophic water with certain salinity. The results showed that *L. salicaria* was more effective in removing TN, NH_4^+-N, NO_3^--N, and TP from both eutrophic fresh and saline water because of its strong salt tolerance. In addition, physiological biomarkers, such as pigment content, proline, and MAD, were also used simultaneously to rapidly assess the health of aquatic macrophytes. These techniques can be used to support further studies with longer duration that aim to monitor plants resilience and recovery time associated with prolonged or reoccurring stress events.

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