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Effects of flooding depth on metal(loid) absorption and physiological characteristics of *Phragmites australis* in acid mine drainage phytoremediation ☆

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ABSTRACT

To solve the problem of determining which flooding depth is suitable for the process of phytoremediation of acid mining drainages (AMDs), we compared the responses of reed (*Phragmites australis*), an amphibious plant widely used in phytoremediation, to different flooding depths. In the greenhouse, synthetic AMD was set to four flooding depths (5, 15, 35 and 55 cm); the culture medium consisted of non-acidified tailings; and the growth, trace element contents in different organs and physiological indexes of the reeds were measured 90 days after planting. The results showed that compared with the control group without reeds, the reeds increased the pH value of AMD. More than 5 cm of flooding inhibited the activity of roots and promoted adventitious roots. The plant height and tiller number were significantly increased by 35 cm of flooding. In addition, the levels of trace element absorption of different organs were different at various depths. In general, deeper flooding promoted the absorption of trace elements (except As), and most of these trace elements accumulated in the roots. Through the calculation of the translocation index (TI), it was found that Mn had the highest value and increased with depth. The physiological effects of flooding on different organs were often diverse at various depths, but the leaf blades maintained high enzyme activity and proline content, while the leaf sheaths contained the greatest amount of soluble protein. Therefore, the 35 cm depth of flooding is reasonable for the phytoremediation of AMD to avoid effects on plant growth from the water depth and/or low pH.

1. Introduction

Flooding stress is one of the threats that plants often face in the process of plant growth. The effects of flooding on plants vary and include disruption of the normal physiological activities of plants at all levels. Many field studies of these effects have been carried out for a long time. Some studies have observed plant changes from short-term flooding experiments: for example, 7 days after corn flooding, the decomposition of chlorophyll increased along with the degree of lipid peroxidation and the contents of O_2^- and H_2O_2 (Yan et al., 1996). The stomatal conductance and quantum yield of

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rabbiteye blueberry decreased 5 days after submergence (Davies and Flore, 1986). Wample and Davis (1983) found that the starch content in the chloroplasts and leaves of sunflower increased significantly after 4 days of flooding. The net photosynthesis and leaf conductance of apricot plants decreased after 3 days of flooding (Domingo et al., 2002). Iwanaga and Yamamoto (2008) found that the plant height and biomass of *Alnus japonica* were significantly lower under 30 cm of flooding than under 1 cm of flooding, while the number of adventitious roots increased significantly. The development of adventitious roots is a response of plants to waterlogging. This condition has been reported (Pimentel et al., 2014) to be induced by root hypoxia in Prunus. The height of seedlings of red alder was also affected by flooding (20 d), and a reduction in the leaf area was observed (Harrington, 1987). More important than the depth of flooding is the duration of flooding, which can even affect the development of communities and the way plants reproduce. Researchers have suggested that the plant physiological response to flooding was mainly reflected in metabolism, hormones, development, and physiology. Such responses include ethylene accumulation, stomatal regulation, biomass reduction and adventitious root formation (Domingo et al., 2002).

Of course, plants have developed their own survival strategies under flooding stress. There are two such survival strategies (Bailey-Serres and Voesenek, 2008): static growth restrictions to conserve energy and stimulation of growth to escape the flooded conditions. Stomatal regulation appears in the early stage of flooding, preventing tissue dehydration and decreasing photosynthesis to increase stress resistance (Domingo et al., 2002). A large number of gene expression changes also occur after flooding (Hsu and Shih, 2013). Many plants can develop tissues of the stems or roots that are capable of ventilation, and some also exhibit hypertrophy of the lower stem (Striker et al., 2005; Harrington, 1987). Flood resistance can be achieved through a variety of adaptive mechanisms, and the regulation of antioxidant systems can become a part of the whole system. One such response may be the increased expression of the ascorbic acid peroxidase (APX) gene to protect against oxidative stress. APX is a hydrogen peroxide scavenging enzyme that is supposed to protect cells from the accumulation of hydrogen peroxide, especially under non-stress conditions. Excessive production of reactive oxygen species (ROS), such as superoxide radicals, hydrogen peroxide, singlet oxygen and hydroxyl radicals, can cause oxidative damage to cell components. ROS participation in many biotic and abiotic stresses has been fully demonstrated (Shigeoka et al., 2002; Li et al., 2013). is the first enzyme in the detoxification process. It can convert superoxide anion radicals (O_2^-) to hydrogen peroxide (H_2O_2). APX uses ascorbic acid as a specific electron donor to reduce H_2O_2 to water (Lee et al., 2007).

In addition, acid mining drainages (AMDs) present a difficult problem in the remediation of environmental pollution. AMDs are highly acidic, metal-rich solutions formed during the processes of coal and mineral exploitation. Once produced, their acidity, metal toxicity, sedimentation and other harmful characteristics can severely impact the surrounding ecosystem. Phytoremediation is a new passive treatment technique, and reed (*Phragmites australis*) is one of the plant species commonly used for this technique. Reeds have a widespread distribution and can be found in wetlands, rivers and ponds, or on dry lands worldwide. However, many plants lack the aeration adaptations of reeds, which can carry out gas transport smoothly in anoxic soil. Reeds exhibit strong adaptability, and their growing environments even include deep-water lake crossings and dry tailings ponds. The large proportion of aerenchyma tissue in stems and rhizomes and the high stomatal density of leaves make reed more effective than any other wetland plant species in obtaining oxygen for aerobic respiration, carbon dioxide for photosynthesis, and mineral nutrients for growth and development. Owing to their number and arrangement of mechanical tissues and the utilization of rhizomes, the abundance and density of reeds are greater than those of all wetland plants (Ailstock, 2000). Because of all of these qualities, reeds are widely used in the phytoremediation of constructed wetlands. AMD exerts substantial stress and pressure on the growth of plants in the process of restoration because of its acidity, salinity and complex ion components. Aquatic plants are not immune to flooding stress, but previous studies on flood control only included anatomical and morphological studies of stems or were related to cash crops; at the same time, the absorption and physiological parameters of trace elements in some phytoremediation plants and their different tolerances to flooding remain unknown. To this end, we want to explore several problems via experimentation: first, whether the flooding depth has an impact on the growth of reeds; second, whether different flooding depths impact metal(loid) accumulation in different organs of reeds; third, what changes occur in the physiological and biochemical activities of reeds under different flooding depths and what differences exist in the physiological reactions of different organs; fourth, the flooding depths most suitable for AMD restoration using reeds.

2. Materials and methods

2.1. Preparation of experimental materials

The substrate used in this experiment was non-acidified tailings from Shuimuchong tailings ponds (30°56′11″N, 117°51′35″E), Tongling city, Anhui Province. The physical and chemical properties of the initial tailings were as follows: pH 7.4, organic matter (OM) 1.02%, total nitrogen (TN) 6.61 mg L^{-1}, total phosphorus (TP) 487.66 mg L^{-1}, and available phosphorus (AP) 2.78 mg L^{-1}. The forms and contents of the trace elements are shown in **Table S1**.

The concentrations of AMD ions used in this study are common in the surrounding environments of some mines. However, the composition of wastewater in these environments is complex, making it difficult to obtain measurable results in short-term experiments. Therefore, synthetic AMD with known ion concentrations was provided, which was helpful for understanding how plants respond to metal(loid) toxicity. The AMD used in this experiment was prepared manually according to the results in our previous study of Tongling. The ion concentrations of the prepared synthetic AMD are shown in Table 1. The preparation process was carried out in a plastic bucket with a cover, and the solution was stable for 3 days before use.

Table 1
Ion concentrations of synthetic acid mine drainage (mg L^{-1}).

Ion	Concentration	Ion	Concentration
Ca^{2+}	186.50	Mn^{2+}	3.47
Fe^{3+}	151.80	Pb^{2+}	1.64
H^{+}	33.52	Cd^{2+}	1.56
Al^{3+}	23.66	K^{+}	1.05
Mg^{2+}	17.25	SO_4^{2-}	940.49
Na^{+}	14.73	NO_3^{-}	15.55
Zn^{2+}	10.94	Cl^{-}	9.61
Cu^{2+}	6.44	PO_4^{3-}	8.76
NH_4^{+}	4.23		

2.2. Experimental setup

The experiment was performed in a greenhouse under natural light and temperature (between 5 °C and 35 °C) conditions and consisted of two phases. The first phase was carried out in February 2019. *P. australis* rhizomes with buds were dug from the Anhui University campus (N 31°46′29″, E 117°11′32″), brought back to the greenhouses, planted in plastic containers filled with sand and watered daily, keeping the water level no lower than 2 cm above the surface of the sand. When the height of seedlings reached approximately 100 cm, plants with uniform growth were selected for the experiment.

The second phase lasted 90 days. At the beginning, four treatments (5, 15, 35, 55 cm) with different flooding depths were established in April. Each treatment was divided into two groups for comparison purpose: the test group with reed planting and the control group without reed planting. Four replicates were used for each group. The test group included the *P. australis* seedlings selected from the first phase in a container (plastic bucket, 65 cm in diameter at the top, 55 cm in diameter at the bottom, 80 cm in height, with scale on the side of the bucket) with 20 cm of tailings, a small amount of AMD and 4 *P. australis* seedlings. After planting, the AMD that was prepared in advance was added to the corresponding scale. The other treatments in the control group were the same as those in the test group except for the plants. All containers were replenished with tap water every other day to maintain the water levels. Nitrogen fertilizer was applied every 15 days by adding 2 g of urea to each container. The pH value of AMD in each bucket was determined by a portable pH meter (HQd, HACH, Colorado, USA) on days 3, 40, 55, 70 and 90, respectively.

2.3. Sample collection

Before the end of the experiment, the number of tillers and the heights of the four reeds planted in each container were recorded. Photographs of adventitious roots of *P. australis* were taken at 15 and 35 cm (no adventitious roots were found at 5 cm, and 55 cm was too deep for photographs to be taken). After that, plants were removed from the containers and washed with deionized water. Part of the fresh tissue of *P. australis* was frozen and stored at −70 °C for physiological analysis, and the other part was dried at 40 °C for 72 h and ground for elemental analysis.

2.4. Determination of physiological parameters

Root activity was determined by the triphenyltetrazolium chloride (TTC) reduction method (Comas et al., 2000). The content of soluble protein was analyzed according to the method of Bradford (1976).

The determination of CSase activity was performed according to the methods of Warrilow and Hawkesford (1998, 2000), with modifications. Part of a fresh sample (0.5 g) was combined with 3.5 mL of enzyme extract and quartz sand and then fully ground. The enzyme extract consisted of 50 mM Tris–HCl (pH 8.0), 5 mM EDTA-Na, 10 μM pyridoxal phosphate (PLP), 0.1% (V/V) Triton X-100, 0.1% (W/V) dithiothreitol (DTT) and 0.2% (W/V) ascorbic acid. The supernatant was centrifuged for 15 min at 12,000 rpm. The total volume of the reaction solution was 500 μL, which consisted of 20 μL of supernatant, 230 μL of 100 mM phosphate buffer (pH 8.0), 125 μL of 10 mM O-acetylserine (OAS), and 125 μL of 4 mM Na_2S. After 10 min of reaction at 30 °C in a water bath, 1.5 mL of ninhydrin chromogenic solution (0.5 g of ninhydrin dissolved in 48 mL of glacial acetic acid and 12 mL of concentrated hydrochloric acid) was added, and the mixture was boiled for 10 min. After cooling, 2 mL of 95% ethanol was added, and the absorbance was measured spectrophotometrically at 560 nm.

Leaf blades, leaf sheaths, stems and roots (0.5 g of fresh weight each) were homogenized in 5 mL of cold phosphate buffer (50 mM, pH 7.8) containing 1 mM EDTA and 1% polyvinylpyrrolidone. The homogenate was centrifuged at 10,500 rpm for 20 min at 4 °C. The supernatant was considered the crude enzyme extract.

The APX activity was determined according to Nakano and Asada (1981) by the decrease in the absorbance of ascorbate at 290 nm. The SOD activity was measured by the method of Beauchamp and Fridovich (1971). The POD activity was measured by the method of Civello et al. (1995). The MDA content was measured according to the method of Rajinder et al. (1981).

The proline content was measured according to the method of Bates et al. (1973). In total, 0.5 g of plant sample was homogenized in 5 mL of 3% sulfosalicylic acid and then incubated in boiling water for 15 min. A 2 mL filtrate sample, 2 mL of acid ninhydrin and 2 mL of glacial acetic acid were mixed together and subsequently immersed in boiling water for 10 min. After the mixture cooled, 5 mL of toluene was added to the mixture. The test tube was then shaken for 30 s and incubated in darkness for 2–3 h. The top layer fluid was obtained and subjected to absorbance measurements at 520 nm.

2.5. Determination of trace element concentrations

The method of trace element determination was to add 7 mL HNO_3 and 1 ml H_2O_2 to a 0.2 g plant sample for microwave digestion (Esmaeilzadeh et al., 2017). The total contents of Mn, Fe, Cu, Zn, Pb, Cd and As were determined by ICP-MS (iCAP Q, Thermo, Massachusetts, USA). The analytical procedure was validated using standard reference materials, including citrus leaves (GSW10020) and celery (GSW10048). The recovery rates were approximately 90%–110%.

2.6. Data analysis

The translocation index (TI) was used to evaluate the ability of plants to accumulate trace elements in the shoots:

$$TI\% = C_{shoot} \big/ (C_{shoot} + C_{root}) \times 100 \quad \text{(i)}$$

where C_{shoot} and C_{root} represent the values of trace element concentrations in aerial parts and roots, respectively (Hladun et al., 2015).

2.7. Statistical analysis

Mean and standard deviation (SD) were calculated from the data collected. The data analyzes were performed with SPSS 22 (IBM, Armonk, NY, USA). The level of significance was set at $p < 0.05$.

3. Results

3.1. Changes in the pH of synthetic AMD

The pH value of the AMD varied with the different treatments (Fig. 1). For different depths, deeper flooding depth correlated with lower initial pH value. Whether reeds were planted influenced pH. Regardless of the depth, the pH value of the planted group was higher than that of the control group. Generally, the pH value of AMD in the reed planting group was increased. When the water depth of the control group was shallow (such as 5 and 15 cm), the pH value of AMD slightly decreased, while when the water depth was deep, the pH value increased by approximately 1. When the depth was 35 cm, the difference between the two groups was the largest. Compared with the initial pH value, when the water depth was 35 cm, the pH value of the experimental group increased the most.

3.2. Plant growth

During the experiment, many tillers were observed in the reeds. The tiller numbers of reeds in the four groups were 13±3, 11±1, 15±3 and 12 ± 1, respectively. Obviously, the maximum number of tillers was obtained at the 35 cm depth. The same results were found for plant height, with reeds at the 35 cm depth being the tallest (Fig. 2). However, the highest root activity was found in the treatment with the lowest water depth of 5 cm (Fig. 3). Many adventitious roots emerged from the submerged stems (Fig. 4).

3.3. Physiological parameters of Phragmites australis

Table 2 shows the physiological and biochemical indexes of various organs of the reeds in this study. CSase activity was highest in the roots and varied with depth in other organs. Different parts showed different activity at different depths. For example, the POD activity was the highest in leaves (except 5 cm) and the lowest in stems. Generally, the activity decreased with increasing depth. The activity of APX followed the trend of leaf > sheath > root > stem, except that it was slightly different at 55 cm (leaf > stem > sheath > root). In the stems and roots, the activity was inversely proportional to the depth, but that trend was almost opposite in the leaves and sheaths. At any depth, the activity/content of SOD, MDA and proline in all organs showed almost the exact same trend: leaf > sheath > stem > root. However, each organ reacted differently at different depths. Some contents were almost unaffected by depth (such as MDA), some contents decreased significantly with increasing depth (such as proline), and some activities were only reflected in some organs (such as SOD in leaves: 35 > 15 > 5 > 55 cm). The TSP content exhibited a trend of sheath > leaf > stem > root at different depths.

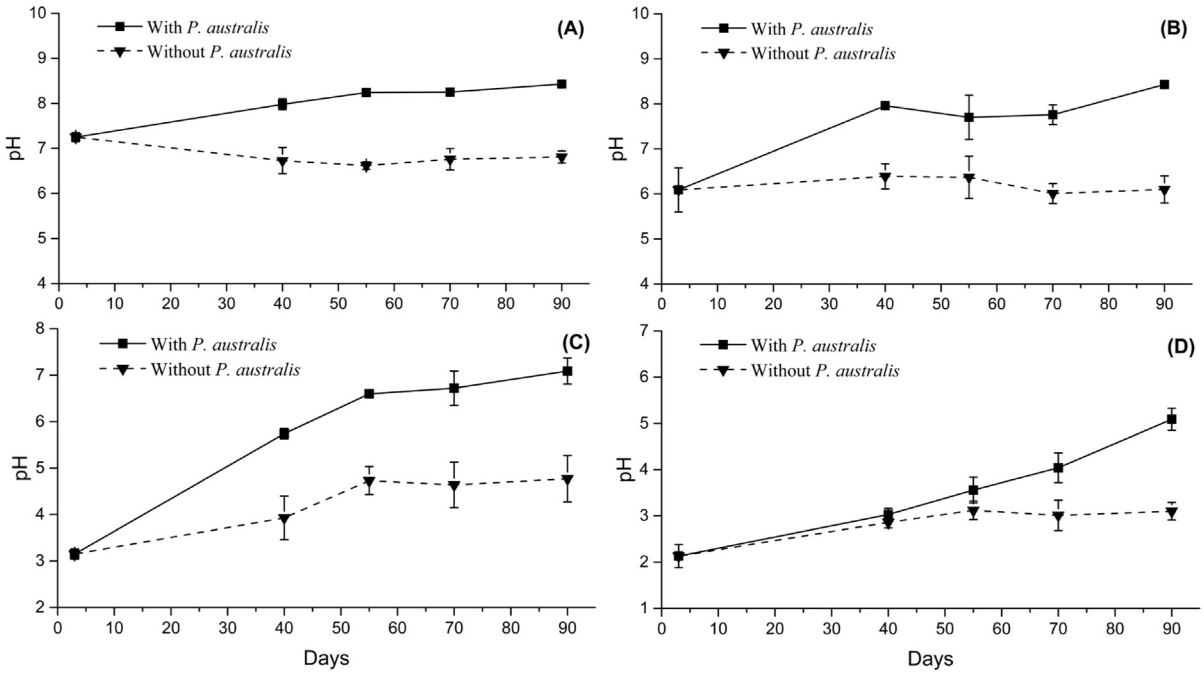

Fig. 1. The changes in the pH value of synthetic AMD with various flooding depths. The solid line represents the experimental group with *Phragmites australis*, and the dotted line represents the control group without *P. australis* (A: 5 cm; B: 15 cm; C: 35 cm; D: 55 cm; Error bars represent SD, n = 4).

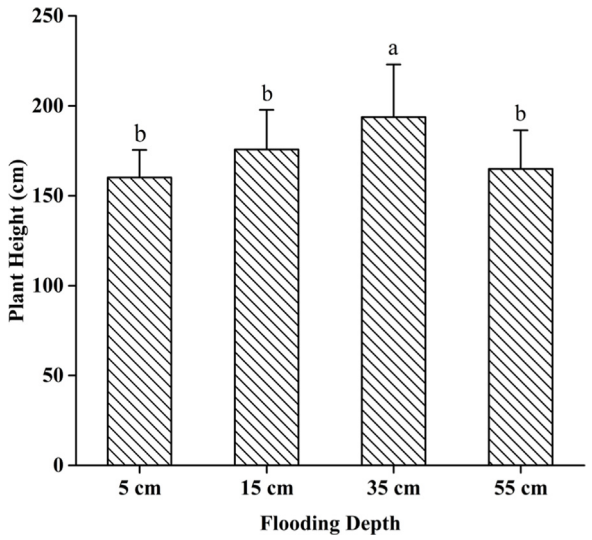

Fig. 2. Plant height of *Phragmites australis* under different flooding depths (Error bars represent SD, n = 4; Values followed by different letters are significantly different at $p < 0.05$ according to Duncan's test).

3.4. Distribution of trace elements in plant tissues

Table 3 shows that the concentrations of different trace elements in different organs and flooding depths were variable. In general, the greatest accumulation of trace elements occurred in the roots, whereas the least accumulation occurred in the stems. The accumulation of Mn in the aboveground parts was greatest, and the content of each organ was the lowest under the deepest submergence condition (55 cm). Unlike that which occurred for Mn, increasing the submergence depth properly promoted the absorption of Fe, Cu, Zn, CD, Pb in the reeds. The accumulation of Zn and Cd at the 35 cm submergence depth was significantly higher than that at the other depths, as observed in the aboveground reed organs.

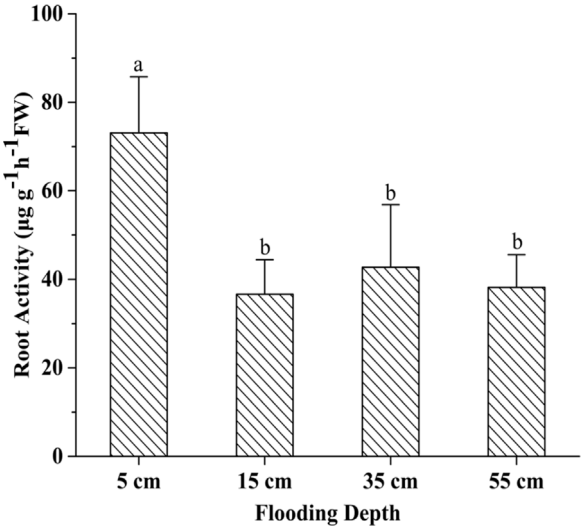

Fig. 3. Root activity of *Phragmites australis* under different flooding depths (Error bars represent SD, n = 4; Values followed by different letters are significantly different at $p < 0.05$ according to Duncan's test).

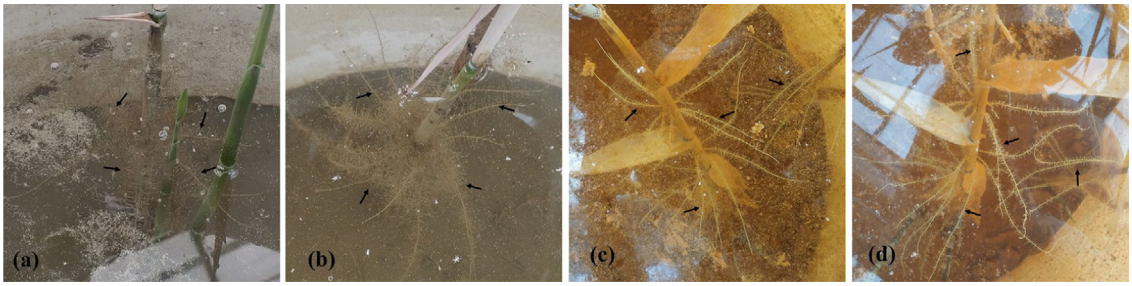

Fig. 4. The adventitious roots of *Phragmites australis* at 15 cm (a, b) and 35 cm (c, d) flooding depth (Arrow indicates adventitious roots).

3.5. Trace elements transferred from roots to shoots

The mobility of Mn was the lowest when the flooding depth was 5 cm and 15 cm, respectively (Fig. 5), indicating that the increase in water depth was conducive to the transport of Mn to aboveground parts. The mobility trends of Cu and Zn were similar, i.e., the mobility of these trace elements was significantly higher at 5 cm and 15 cm than when submerged at 35 cm and 55 cm, which indicates that the water depth limits the transport of Cu and Zn from the roots of plants to the aboveground parts. The TI value of As at 5 cm of flooding was significantly higher than that at other water depths, which represents the inhibition of its mobility with increasing water depth. In contrast, the results for Cd, whose TI values were higher at 15 cm and 35 cm, indicated that flooding depths that were too high or too low affected the mobility of Cd in reeds. For Pb, no significant mobility difference was found, suggesting that the flooding depth does not affect the upward transport of Pb in reed roots. In addition to the influence of flooding depth, it was easy to determine that Mn had the largest TI value, followed by Zn; in contrast, the TI values of Fe and As were relatively low, so it can be suggested that reed restricts the upward transport of Fe and As in the presence of AMD flooding, and that the transport was not affected by depth.

4. Discussion

Compared with the control group, the presence of reeds in this study significantly improved the pH value of AMD. The large number of hydrogen ions in AMD easily damages plants (Islam et al., 1980). Keeping a high pH value in the rhizosphere of the root tip is important for normal growth of plants on acid substrates. The increase in acidity in the rhizosphere may lead to the substitution of Ca^{2+} by H^{+}, which increases the permeability of the protoplasm membrane (Raven and Smith, 1976). Therefore, only by maintaining normal metabolism can excess H^{+} be eliminated and can the loss of the pH regulating ability of root cells be avoided. This also represents one of the adaptive mechanisms that plants formed to adapt to various stressful environments, especially nutrient deficiency or toxicity, over the long-term process

Table 2
Physiological parameters of *Phragmites australis* in various organs.

Physiological parameter	Depth (cm)	Leaf blade	Leaf sheath	Stem	Root
POD ($\mu g\ g^{-1}$ FW min^{-1})	5	1521.38 ± 131.87*bB**	1675.57 ± 246.51aAB	200.18 ± 61.87aC	2035.45 ± 488.73aA
	15	1675.31 ± 48.14bA	1419.67 ± 97.58bB	202.92 ± 41.33aD	893.34 ± 81.34bC
	35	1658.49 ± 266.01bA	1153.55 ± 140.02cB	125.95 ± 25.17bD	802.17 ± 67.30bC
	55	1096.68 ± 162.68aA	539.26 ± 95.74 dB	155.73 ± 29.33abC	625.08 ± 48.89bB
CSase ($\mu g\ g^{-1}$ FW min^{-1})	5	9.37 ± 0.83abB	1.97 ± 0.00cC	4.95 ± 0.50bC	17.51 ± 4.63cA
	15	11.21 ± 1.41aB	9.48 ± 0.73aB	8.65 ± 1.52aB	23.77 ± 2.81abA
	35	7.95 ± 1.29bcB	7.34 ± 0.49bB	7.64 ± 1.00aB	19.04 ± 3.25bA
	55	6.60 ± 1.34cB	7.93 ± 1.64bB	9.08 ± 0.82aB	26.31 ± 3.27aA
APX (mmol VC g^{-1} FW h^{-1})	5	576.42 ± 107.55aA	167.14 ± 60.30aB	28.93 ± 4.97cC	41.78 ± 24.34bC
	15	453.22 ± 36.93bA	173.57 ± 49.80aB	51.43 ± 21.00bcC	91.07 ± 16.55aC
	35	413.86 ± 67.99bA	200.00 ± 67.09aB	73.93 ± 28.50bC	99.64 ± 16.18aC
	55	381.07 ± 51.02bA	115.72 ± 37.85aB	135.00 ± 38.57aB	106.78 ± 25.75aB
SOD (U g^{-1} FW h^{-1})	5	2118.98 ± 102.60aA	1005.82 ± 74.83aB	895.76 ± 24.55aB	673.00 ± 195.38aC
	15	2158.94 ± 87.50aA	1088.90 ± 12.80aB	874.54 ± 45.44aC	672.38 ± 201.68aD
	35	2200.45 ± 49.94aA	1050.00 ± 81.16aB	827.14 ± 66.18aC	409.09 ± 156.14aD
	55	2079.24 ± 53.10aA	1007.01 ± 67.17aB	866.82 ± 66.59aC	652.98 ± 120.94aD
TSP ($\mu g\ g^{-1}$ FW)	5	1.85 ± 0.34aB	2.86 ± 0.80aA	0.75 ± 0.31bC	0.45 ± 0.26abC
	15	1.18 ± 0.20cB	4.06 ± 1.85aA	1.54 ± 0.34aB	0.21 ± 0.14bB
	35	1.29 ± 0.50bcB	3.93 ± 1.89aA	1.28 ± 0.44aB	0.32 ± 0.04bB
	55	1.82 ± 0.25abB	5.51 ± 1.77aA	1.41 ± 0.18aB	0.69 ± 0.35aB
MDA ($\mu mol\ g^{-1}$ FW)	5	2.58 ± 0.45aA	2.18 ± 0.39aA	1.47 ± 0.19bB	1.33 ± 0.33abB
	15	1.87 ± 0.13bAB	1.89 ± 0.45aAB	1.99 ± 0.41aA	1.44 ± 0.12aB
	35	2.03 ± 0.20bA	1.95 ± 0.46aA	1.62 ± 0.37abA	1.01 ± 0.19bB
	55	1.99 ± 0.15bA	1.93 ± 0.28aA	1.87 ± 0.15abA	1.15 ± 0.24abB
pro ($\mu g\ g^{-1}$ FW)	5	194.39 ± 36.15aA	65.54 ± 2.55aB	35.05 ± 5.23aC	25.14 ± 12.72aC
	15	143.21 ± 35.12bA	57.57 ± 7.10bB	32.29 ± 3.50aBC	6.13 ± 7.35bC
	35	117.20 ± 35.79bA	48.81 ± 8.90bB	31.83 ± 7.71aB	1.12 ± 1.29bC
	55	106.45 ± 19.93bA	68.23 ± 7.23aB	32.29 ± 3.74aC	0.00 ± 0.00bD

Notes: *Mean ± SD; **Means within the same column with the same lowercase letter are not significantly different ($p < 0.05$); those within the same row with the same capital letter are not significantly different ($p < 0.05$).

Table 3
Accumulation of trace elements in various organs of *Phragmites australis* at different flooding depths.

Element (mg kg^{-1} FW)	Depth (cm)	Leaf blade	Leaf sheath	Stem	Root
Mn	5	766.41 ± 175.38bB	560.89 ± 99.62aB	94.58 ± 4.06aB	2657.87 ± 1328.70aA
	15	1002.66 ± 149.92aB	604.60 ± 84.27aC	94.87 ± 13.99aD	2553.14 ± 451.19aA
	35	828.92 ± 66.67abB	516.72 ± 102.21aC	103.05 ± 17.25aD	1738.36 ± 224.26abA
	55	434.25 ± 35.20cB	375.65 ± 66.59bB	67.34 ± 2.79bC	815.06 ± 341.15bA
Fe	5	250.72 ± 142.77aB	130.54 ± 63.88abB	87.62 ± 46.93bB	28072.03 ± 4093.65aA
	15	127.59 ± 18.76aB	46.74 ± 23.89bB	34.86 ± 14.06bB	33463.37 ± 4642.42aA
	35	153.06 ± 46.96aB	120.17 ± 30.38abB	49.38 ± 8.44bB	28352.12 ± 4752.25aA
	55	171.06 ± 30.62aB	227.65 ± 114.07aB	154.64 ± 68.02aB	33760.60 ± 17721.19aA
Cu	5	7.66 ± 0.97aB	7.96 ± 1.83aB	6.71 ± 1.71aB	183.43 ± 34.88bA
	15	7.13 ± 0.21aB	7.58 ± 0.77aB	6.97 ± 1.04aB	260.08 ± 66.39abA
	35	7.69 ± 1.22aB	8.35 ± 1.35aB	7.40 ± 2.13aB	314.25 ± 146.63abA
	55	7.52 ± 0.49aB	8.27 ± 1.24aB	7.59 ± 0.49aB	394.02 ± 60.81aA
Zn	5	20.61 ± 3.09aC	38.69 ± 7.41aC	91.55 ± 12.79aB	357.56 ± 43.59bA
	15	17.84 ± 1.57aC	41.47 ± 7.96aC	127.52 ± 21.27aB	504.64 ± 59.57abA
	35	20.05 ± 3.08aB	51.93 ± 30.99aB	130.53 ± 101.50aB	770.36 ± 338.06aA
	55	17.29 ± 3.73aB	24.59 ± 3.69aB	61.06 ± 1.84aB	648.55 ± 228.89abA
As	5	0.56 ± 0.22aB	0.30 ± 0.12aB	0.35 ± 0.09aB	69.30 ± 16.56aA
	15	0.27 ± 0.05bB	0.19 ± 0.05abB	0.14 ± 0.00bB	80.11 ± 24.41aA
	35	0.27 ± 0.06bB	0.18 ± 0.03bB	0.13 ± 0.02bB	59.33 ± 18.07aA
	55	0.34 ± 0.05bB	0.21 ± 0.07abB	0.19 ± 0.05bB	69.15 ± 18.13aA
Cd	5	0.11 ± 0.08bB	0.39 ± 0.24bB	0.33 ± 0.14bB	10.20 ± 3.80bA
	15	0.63 ± 0.16bB	3.44 ± 1.17bB	2.58 ± 0.67bB	64.49 ± 23.92bA
	35	2.14 ± 0.92aB	9.64 ± 6.72aB	7.56 ± 5.04aB	149.75 ± 79.27aA
	55	0.43 ± 0.06bB	1.77 ± 0.38bB	2.08 ± 0.41bB	165.11 ± 57.21aA
Pb	5	0.24 ± 0.10aB	0.16 ± 0.04bB	0.09 ± 0.03bB	23.50 ± 2.85bA
	15	0.53 ± 0.71aB	0.13 ± 0.02bB	0.04 ± 0.02bB	32.57 ± 6.25abA
	35	0.26 ± 0.10aB	0.29 ± 0.05bB	0.11 ± 0.02bB	36.65 ± 11.94abA
	55	1.06 ± 1.24aB	0.62 ± 0.21aB	0.53 ± 0.01aB	60.72 ± 35.87aA

Notes: *Mean ± SD; **Means within the same column with the same lowercase letter are not significantly different ($p < 0.05$); those within the same row with the same capital letter are not significantly different ($p < 0.05$).

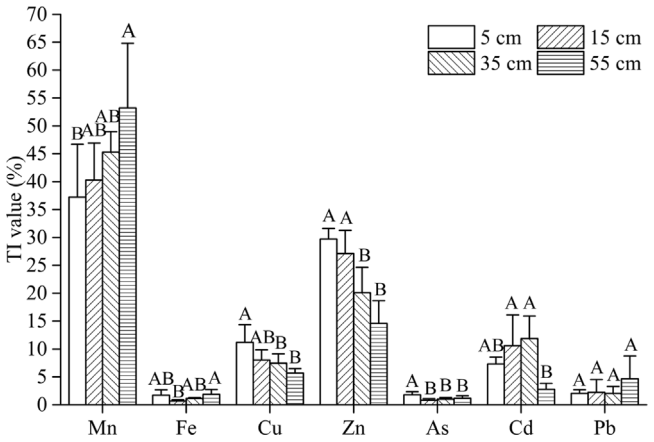

Fig. 5. The TI values of trace elements in *Phragmites australis* growing at different flooding depths (Error bars represent SD, n = 4; Values followed by different letters are significantly different at $p < 0.05$ according to Duncan's test).

of evolution. Therefore, in phytoremediation of AMD constructed wetlands, the pH of the medium can be significantly improved depending on the role of the plants.

The effect of AMD on the growth of the reeds was first reflected in the plant phenotype. The height of aquatic plants is often affected by the depth of water, which promotes the growth of plants to obtain more oxygen and sunlight. However, in deep water, energy conservation via growth limitation is also a kind of survival strategy employed by plants. In this study, the depth of 35 cm resulted in the greatest plant height, not the greatest depth of 55 cm, which indicated that the plant stress caused by excessively deep flooding water or low pH was too great, and low pH limits the growth of plants (Islam et al., 1980). However, due to the limitation of gas diffusion in the underwater parts of plants such as roots, the accumulation of endogenous gases such as ethylene also has a toxic effect (Voesenek et al., 2003). Therefore, we found that the activity of reed roots decreased when the flooding depth was greater than 5 cm. At the same time, we observed the formation of adventitious roots at water depths greater than 5 cm. These reactions at the anatomical level promoted oxygen capture in the submerged tissues, alleviated the hypoxic conditions (Colmer, 2003; Suralta and Yamauchi, 2008), and aided plant survival. Not only roots but also new shoots are affected in deep water. Under deep water (more than 40 cm), hydrostatic pressure increases the resistance of new bud elongation and inhibits asexual propagation (Chen et al., 2010). This will affect the shoot density in wetland bioremediation.

The consequences of oxidative stress caused by hypoxia and other factors depend on tissue tolerance, membrane properties, the endogenous antioxidant content and the ability of plants to induce the response of the antioxidant system. A highly efficient antioxidant enzyme system is very important for survival under waterlogged conditions. Antioxidants play a synergistic role through a series of redox reactions. Flooding increases ROS and causes antioxidant reactions in plants. Yan et al. (1996) determined that the production of MDA was induced by O_2^- and that the accumulation of MDA was caused by a decrease in SOD activity. This was not the case in our study. SOD activity is also high in organs with high lipid peroxidation. The increase in antioxidant activity directly represents the enhancement of anti-stress ability (Pimentel et al., 2014). However, in some cases, overexpression of a single antioxidant enzyme does not prevent stress, suggesting that overexpression of an enzyme does not control the function of the entire antioxidant pathway (Lee et al., 2007). Plants have an antioxidant system to prevent the formation of ROS: low molecular weight antioxidants (ascorbic acid, glutathione), reducing antioxidant enzymes, and enzymes that interact with ROS, such as SOD, POD and APX (Olga et al., 2003; Chiang et al., 2014). The successful process of resistance to stress cannot lack the participation of non-enzyme antioxidants. Some osmoregulants also play an important role in cells. For example, proline accumulation is a common phenomenon in plants under adverse conditions, and stress-resistant plants can accumulate more proline than sensitive varieties (Li et al., 2013). Proline, as an osmotic protective substance, can reduce the oxygen damage caused by osmotic stress. Proline levels in leaves and roots of *P. australis* increased significantly with increasing depth. It is generally believed that the higher the proline level, the stronger the antioxidant capacity (Arbona et al., 2008; Barnawal et al., 2012; Yang et al., 2011). The same resistance to osmotic stress is reflected in the content of soluble protein, which not only plays an important role in maintaining osmotic pressure but also reflects the level of metabolism. In contrast to proline, the content of soluble protein in the leaf sheath was higher than that in the leaves. The reason for this finding needs to be further explored in the future. Under stress, the structure, function, position and hypoxia of each organ lead to different antioxidant defenses. It is generally believed that leaves are more sensitive to flooding than other organs, especially roots (Yang et al., 2015). The regionalization and antioxidant localization of ROS, the synthesis and transport of antioxidants, the ability to induce antioxidant defense and the coordination (and/or compensation) between different antioxidant systems are the factors that determine the antioxidant system capacity (Olga et al., 2003). These antioxidant systems act in different organs, and they are related to each other and work together to resist external stress.

In most cases, the concentrations of trace elements in plants reflect the abundance of the trace elements in the growth media (e.g., soil, nutrient solution, water). Roots are the plant organs that come into the most direct contact with pollutants, and they are also the first barrier to the absorption of substances. Plant roots also have a strong capability to take up less-mobile forms of trace elements because they release various exudates that change the pH of the rhizosphere soil solution and chelate elements (Kabata-Pendias and Mukherjee, 2007). Some trace elements, particularly trace metals such as Cu, Fe, Mn and Zn, play key roles in plant metabolism and are cofactors of several enzymes. The absorption of trace elements by plants can be classified as active or passive absorption. When the amount of metal(loid)s absorbed through passive absorption exceeds a certain tolerable value, plants are considered to be under metal(loid) stress, and the toxic effects of the metal(loid)s will affect the normal physiological activities of the plants. For example, according to Påhlsson (1989), when the concentration of Cd reaches 3–10 mg kg^{-1} plant dry weight, the plants will experience Cd toxicity. To connect this to our findings, when the water depth in our study was 15 to 35 cm, the Cd concentrations in all organs except leaves reached this toxic value; in particular, in the roots, Cd concentrations dozens of times higher than this toxic value occurred. According to (Kabata-Pendias and Mukherjee, 2007), the plant species, root activity and rooting patterns may affect the accumulation of Cd, which can result in elevated Cd accumulation in the leaves. Usually, the Cd concentration is the highest in roots and decreases with increasing plant part height (Welch and Norvell, 1999). Leaves are the organs that are farthest from the roots, but leaves often accumulate the second-highest metal(loid) concentrations, after the roots. One of the most important processes in the adaptation to or avoidance of stress factors is binding the stress factors by higher plants to the cell walls. The behavior of cadmium in plants is closely related to that of zinc because both metals have an affinity for sulfur, especially sulfhydryl groups (Garrett, 1996). Metal(loid) accumulation in stems is generally the lowest because of stem structure and function; stems clearly play a major role in metal(loid) transport and distribution. In addition, the water depth exerted different cumulative effects on the accumulation of different metal(loid)s. Fe, Zn, Cd and Pb accumulated in organs more easily in the deep water treatment, but Mn accumulated more easily in the shallow water treatment. The high concentration of H^+ in the deep water may have affected the solubility of heavy metals in the tailings (Bailey-Serres et al., 2012). Moreover, the interactions of trace elements affect the extent to which they are absorbed and transferred to plant tissues (Tavarez et al., 2015). For example, in rice (Liu et al., 2003) and tomato (Lopez-Millan et al., 2009), heavy metals had synergistic effects on the absorption of Cd or micronutrients by roots.

The TI value reflects the proportion of metal(loid)s transferred from roots to shoots. Mn had the highest TI value, suggesting that reeds not only readily accumulate Mn in their roots but also translocate Mn more easily than other elements to the stem, to the leaf sheaths and ultimately to the leaves. These findings are similar to those of other studies on different plant species (Hladun et al., 2015); moreover, Pb is sequestered mainly in the reed root system, where it binds to the root surface and cell walls (Påhlsson, 1989). Copper is only slightly mobile in plants, as it is strongly bound by nitrogen and proteins (Kabata-Pendias and Mukherjee, 2007). The high Fe contents in the reed roots and the low TI value for Fe may be attributed to the fact that Fe is the main component of the iron plaque on the root surface, and to the limited amount of Fe that enters the root interior. According to Yoon et al. (2006), plants with high TI values are suitable for phytoextraction. In this study, the TI value (Mn) of *P. australis* increased with increasing depth and was higher than at a water depth of 55 cm, suggesting that *P. australis* can be used for phytoextraction in these conditions. Calculating the TI is beneficial for evaluating the projected plant harvest from future heavy metal removal phytoremediation projects in constructed wetlands.

5. Conclusion

Due to the universality of *Phragmites australis* in plant restoration applications, its growth process was observed under various stresses. Flooding and low pH are two important abiotic stresses of plants worldwide that may have synergistic effects on plants, affecting plant height, density and physiological activity. It was found that the presence of *P. australis* significantly improved the pH value of AMD, and some heavy metals (especially Mn) could be removed by harvesting the aboveground parts. We found that *P. australis* develops different survival strategies in the process of resisting stress. In addition, ROS accumulation in reeds resulted in lipid peroxidation and proline accumulation, and the response of the antioxidant defense system to waterlogging was tissue specific. This may suggest that each organ cooperates separately in the process of stress resistance. Generally, the optimal submergence depth of AMD was 35 cm, which was more suitable for the growth and reproduction of *P. australis* than the other depths. In this paper, the growth and physiological response of *P. australis* to different depths of AMD were studied to provide a basis for the evaluation of the appropriate submergence depth for the complex process of plant restoration of AMD.

CRediT authorship contribution statement

Ziwei Ding: Experimental processing, Data interpretation, Wrote the paper. **Qingye Sun:** Planned the study, Finalized the final draft of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found online at https://doi.org/10.1016/j.eti.2021.101512.

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