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Plant Growth and Nutrient Composition of Shrub and Arbor Willows Grown in Cu-Contaminated Flooded Soil

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Abstract: Flooding can adversely worsen metal-contaminated soil and influence phytoremediation efficiency; thus, it is crucial to explore the eco-physiological responses of plants to the combined stress of metals and flooding. Here, the plant growth, photosynthesis, and nutrient composition in the arbor willow (*Salix jiangsuensis* 'J172') and shrub willow (*Salix integra* 'Yizhibi') were studied using a pot experiment with Cu-contaminated soil (239.5 mg$\cdot$kg^{-1}) under flooded versus non-flooded conditions. *S. integra* showed a larger bioconcentration factor (BCF) than *S. jiangsuensis* in both treatments. Flooding markedly decreased the BCFs while obviously increasing the translocation factor in the two willows ($p < 0.05$). Flooding enhanced the leaf C:P and N:P ratios while significantly decreased root C:P and N:P ratios, compared to the non-flooded condition. The shrub willow exhibited better tolerance to flooding, with little alteration in biomass and photosynthetic rate, and showed greater potential Cu accumulation capacity, even though its total biomass was significantly lower than that of the arbor willow. Our study also helps further understanding of nutrient balance and stoichiometry of willows in response to flooding and Cu contamination, promoting the management of Cu-contaminated flooded soils.

Keywords: willow; phytoremediation; nutrients; photosynthesis; flooding

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1. Introduction

Heavy metal contamination in soil primarily results from anthropogenic sources such as mining, industrial sludge, farming, and disposal of waste [1], and has become one of the most serious issues worldwide. Copper (Cu) is an essential micronutrient required by important physiological and biochemical processes in most organisms [2]. Industrial and agricultural activities (Cu-based fungicides, bactericides and pesticides, waste water discharge, sewage sludge application, etc.) have led to soil Cu contamination [3,4]. Excessive Cu levels in soil could cause toxicity to organism by interfering with their normal functions and subsequently induce severe problems for natural ecosystems and human health [5]. To minimize the impacts of Cu contamination on ecosystems, it is urgent to remediate Cu-contaminated soils. Phytoremediation, as an *in situ*, green alternative, cost-effective, and environmentally friendly approach, has been widely used for soil remediation in recent decades [1,6]. Previously, much interest concentrated on identifying hyperaccumulator plants, whose above-ground tissues can accumulate heavy metals at higher concentrations [7]. However, limitations of herbaceous hyperaccumulator plants have also appeared; therefore, the potential use of fast-growing woody species for phytoremediation has drawn attention [8]. For example, the leaves of *S. dasyclado* accumulated as high as 230–315 mg$\cdot$kg^{-1} Cd dry biomass [9] and *S. integra* accumulated 90–288 mg$\cdot$kg^{-1} Cd in leaves under hydroponic conditions [10]. Fast-growing woody plants are confirmed to be superior to hyperaccumulators for phytoremediation of metal-contaminated soils [11,12]. Using willows in phytoremediation has high potential to reduce the environmental risks of metal contaminants.

In addition to metal contamination, flooding is another severe issue worldwide caused by climate change, and it markedly affects plants in the riparian zone [13,14]. Diffusion of air in water is greatly reduced by flooding, causing a decrease in oxygen availability for the root zone. Furthermore, flooding could lead to root dysfunction and decline in photosynthesis, respiration, nutrient uptake, and translocation [15,16]. To cope with anoxic environment yielded by flooding, plants develop adventitious roots/aerenchyma tissues to avoid tissue anoxia [17]. In recent decades, many floodplain soils have been heavily contaminated by heavy metals [18,19], and the mobility of metals in soil is greatly affected by frequent flooding events that trigger significant changes [20,21]. Importantly, willow (*S.* spp.) species are becoming promising candidates to minimize the adverse effects from global climate change, and most willows are well adapted to flooding and have a high capacity for phytoremediation [22,23]. Although numerous studies have investigated metal or flooding stress, most of them focused on only single stress factor, and the joint effect (co-exposure of heavy metal and flooding) on woody plants has not been sufficiently studied. Thus, it is essential to study the performance of willows in metal-contaminated soil as affected by flooding.

Plants need both macro- and micro-elements to maintain their normal growth and development [24,25]. Among these essential elements, carbon (C), nitrogen (N), and phosphorus (P) are three major elements affecting plant growth, and their stoichiometric ratios could indicate the nutrient dynamics in response to the changing environment [26–28]. Under flooded conditions, plants exhibit different strategies for nutrient uptake and utilization, and this alteration could lead to differences in elemental stoichiometry [29,30]. Previous studies mainly focused on the nutrient composition and stoichiometric response to abiotic stresses such as water, drought, and heavy metal stress [14,29], whereas investigation on variations in nutrient stoichiometric ratios of organ comparisons in response to flooding in metal-contaminated soil still lags behind.

As reported previously, we observed that the shrub willow (*S. integra* 'Yizhibi' Thunb.) and arbor willow (*S. jiangsuensis* 'J172') exhibited great potential in Cu-contaminated soil and were tolerant to flooding [31,32]. The Cu accumulation and transportation of the two willow species were also studied [31,32], but what the differences between the shrub willow and arbor willow were still unclear. Thus, we further studied and compared the responses of *S. jiangsuensis* and *S. integra* as affected by flooding in Cu-contaminated soil, and assessed the suitability of these two willows for phytoremediation of Cu-contaminated wetlands. We examined the growth and photosynthetic efficiency of the two willows upon exposure to Cu and flooding; explored the effects of combined stresses on nutrient alteration and stoichiometric characteristics; and compared the phytoremediation capacity of the two willows for Cu under flooded versus non-flooded conditions. We hypothesized that the Cu accumulation by both willow species could be decreased under flooded conditions compared to non-flooded conditions, and arbor willow might exhibit greater potential for Cu phytoremediation in wetlands due to its high biomass and higher Cu accumulation capacity than that of the shrub willow. Our findings will provide valuable information for the phytomanagement of Cu-contaminated wetlands using fast-growing willows.

2. Materials and Methods

2.1. Soil Preparation and Plant Cultivation

The Cu-contaminated soil was collected from the surface horizon (0–20 cm) of a local field near Hangzhou City, Zhejiang Province. The physicochemical properties of the soil are presented in Table S1, with a relatively high total Cu concentration of 239.5 mg·kg^{-1}, which was markedly higher than the risk screening value (100 mg·kg^{-1}) according to the national soil quality standard (GB15618-2018, China). Air-dried soil (1.5 kg) was weighed into polyvinylchloride (PVC) tubes that were 11 cm in inner diameter and 25 cm in height.

Two willow species, *S. jiangsuensis*, an arbor hybrid willow with broad leaves, and *S. integra*, a shrub willow with narrow and long leaves, were selected in the current study. One-year-old willow branches approximately 0.8 cm in diameter were cut to 15 cm length, and then planted in each PVC tube as described above. The PVC tubes were randomly placed in greenhouse, each treatment consisted of four replicates, and each replicate contained three seedlings. The plants were first cultivated for 4 months under greenhouse condition (temperature: 23–28 °C; relative humidity: 60%–65%) in the Research Institute of Subtropical Forestry, Chinese Academy of Forestry. Soil moisture content was maintained at 70% of water-holding capacity by adding tap water every day. After 4 months' growth, non-flooded seedlings were still daily watered. For flooded treatment, plants were grown in a depth of 10 cm water level from the soil surface, lasting for 3 months. The whole experiment lasted for 7 months before harvest.

2.2. Measurement of the Leaf Gas Exchange

Leaf gas exchange was measured on days 7, 14, 28, 56, and 90 after the flooded treatment started. Three expanded mature leaves of each plant were selected for leaf gas exchange measurement. Photosynthesis parameters, including net photosynthetic rate (P_n), stomatal conductance (g_s), intercellular CO_2 concentration (C_i), and transpiration rate (T_r) were determined by a portable photosynthesis system (LiCor 6400; Lincoln, NE, USA) as described in [22]. The instrumental parameters were set to an intensity of 1000 μmol photon $m^{-2}s^{-1}$ from 9:00 to 14:00; the air flow through the sample chamber was set at 500 μmol s^{-1}, and the CO_2 concentration in the sample chamber was 400 μmol mol^{-1}.

2.3. Determination of Cu and Other Nutrients

After the last time point of the photosynthesis reading (90 d of flooding), the roots and above-ground tissues (cuttings, stems, and leaves) of each plant were sampled and washed thoroughly with deionized water. Plant tissues were dried in an oven at 75 °C for 72 h. Sample dry weights were recorded and the oven-dried tissues were ground into a fine powder using a ball miller (propeller mill, IKA, Staufen, Germany) for further analysis. Fresh roots (1 g) across all the treatments were extracted using the cold dithionite-citrate-bicarbonate (DCB) method [22] to determine the Cu, iron (Fe), manganese (Mn), and sulfur (S) contents in the Fe plaque on the root surfaces.

The fine powder (about 50 mg) of the different tissues was digested in a mixture (4 mL concentrated HNO_3 and 1 mL concentrated $HClO_4$) at 200 °C for 120 min in a hot block system (ED36, Lab Tech, Beijing, China). The digests were cooled down to the ambient temperature and were increased up to 25 mL with 2% (v/v) diluted HNO_3 solution. Subsequently, Cu and other nutrients such as P, S, potassium (K), calcium (Ca) and magnesium (Mg) were determined using inductively coupled plasma atomic emission spectrometry (ICP-AES; PerkinElmer Optima 8000, Waltham, MA, USA). To ensure the quality of the analyses, a certified reference material of poplar leaves (GBW 07604, National Research Center for Certified Reference Materials, Beijing, China) was utilized throughout the process of plant digestion and element analysis. Good agreement was obtained between our method and certified values (Table S2). C content was determined using an elemental analyzer (Vario Macro cube, Elementar, Germany) after sample combustion in an oxygen atmosphere. For N content, plant tissues were digested using the H_2SO_4-H_2O_2 method according to [33], and the contents of the digests were determined by an automated Kjeldahl analyzer (Kjeltec 8400, FOSS, Copenhagen, Denmark).

2.4. Calculations and Statistical Analysis

The bioconcentration factor (BCF) indicates the efficiency of metal accumulation from the surrounding environment in plant tissues, and is calculated as the ratio of target metal content in plant tissues to that in the soils [34]. The translocation factor (TF) indicates the efficiency of the plants in translocating the metals from the roots to the shoots, and is calculated as the ratio of target metal content in above-ground tissues to that in plant roots [34].

All statistical analyses were performed with Data Processing System Version software (DPS13.01, Zhejiang University, Hangzhou, China). Mean and standard deviation (SD) values of the four replicates were calculated. Two-way analysis of variance (ANOVAs) with the flooded treatment and willow species as the factors were employed to test any difference among the flooded and non-flooded conditions, different willow species, and their interactions. Differences were considered significant when the p value of the analysis of variance F-test was < 0.05. Pearson's correlation analysis and principal component analysis (PCA) were performed in R software, version 4.1.2 (R Development Core Team 2018, Vienna, Austria). Figures were plotted using Origin 2018 (Origin Lab, Northampton, MA, USA).

3. Results

3.1. Plant Growth and Biomass Production

Regardless of non-flooded or flooded treatment, the phenotypes of both *S. integra* and *S. jiangsuensis* were healthy, without any visual damage observed (e.g., leaf necrosis and abscission), and visible hypertrophied lenticels adventitiously occurred on the submerged portions of cuttings under flooded conditions. Flooding markedly decreased the root biomass by 29.9% and 24.7% in *S. integra* and *S. jiangsuensis*, respectively (Table 1). The decrease degree of the root biomass was notably greater than that of the leaf and cutting biomass. Notably, the leaf biomass of *S. jiangsuensis* was dramatically higher than that of *S. integra* under flooded condition. Flooding slightly decreased the leaf biomass of *S. jiangsuensis* by 3.8% and the reduction degree was markedly lower than for *S. integra*. Additionally, flooding also reduced the total biomass (7.8% and 10.7%) of both willow species compared to their respective non-flooded treatments.

Table 1. Biomass of *S. integra* and *S. jiangsuensis* exposed to Cu-contaminated soil as affected by flooding for 90 d.

Willow Species	Treatments	Leaf g, DW	Stem g, DW	Cutting g, DW	Root g, DW	Total g, DW	Plant Height cm	Leaf Area cm^2
S. integra	Non-flooded	4.82 ± 0.18 b	4.11 ± 0.51 b	2.63 ± 0.34 ab	4.35 ± 0.35 a	15.9 ± 1.1 b	110.0 ± 2.9 a	8.16 ± 0.2 b
	Flooded	3.88 ± 0.34 c	4.88 ± 0.62 b	2.41 ± 0.45 b	3.05 ± 0.46 c	14.2 ± 1.6 b	97.5 ± 4.9 bc	7.84 ± 0.4 b
S. jiangsuensis	Non-flooded	6.67 ± 0.42 a	6.62 ± 0.47 a	3.28 ± 0.51 a	4.85 ± 0.19 a	21.4 ± 1.3 a	102.1 ± 6.45 b	13.8 ± 0.7 a
	Flooded	6.42 ± 0.41 a	6.56 ± 0.36 a	3.12 ± 0.65 ab	3.65 ± 0.33 b	19.8 ± 0.8 a	91.9 ± 3.9 c	13.9 ± 0.3 a
Significance	Treatments	ns	ns	ns	****	****	****	ns
	Species	****	****	*	***	*	**	****
	Treatments × Species	ns	ns	ns	ns	ns	ns	ns

Each value represents the mean of four replicates ± standard deviation. Different letters indicate significant difference among the 4 treatments (2 willow species under non-flooded and flooded conditions) at the 0.05 level by Fisher's LSD test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; ns, not significant.

3.2. Leaf Gas Exchange

Flooding significantly decreased the P_n of *S. integra* and *S. jiangsuensis* ($p < 0.05$) compared to the non-flooded condition, and the decreases at different flooding stages in *S. integra* were generally higher than *S. jiangsuensis* (Figure 1). Furthermore, the P_n first elevated as the duration of flooding increased to 28 d in both willow species, and then decreased after 56 d exposure of flooding under non-flooded/flooded conditions. Similarly, the highest g_s and T_r were both observed 28 d after flooding under both conditions. However, the C_i was obviously high 56 d after flooding, but the interaction of flooding treatment and flooding time had no significant impact on *S. jiangsuensis* ($p > 0.05$). After 28 d of flooding, the g_s and C_i values were decreased by 28.9% and 10.0% in *S. jiangsuensis*, respectively, but these two parameters of *S. integra* increased (13.1% and 11.1% for g_s and C_i) in response to flooding compared to the respective non-flooded conditions. The largest reductions in T_r caused by flooding, which were 36.2% in *S. jiangsuensis* and 25.9% in *S. integra*, were observed at 90 d after flooding.

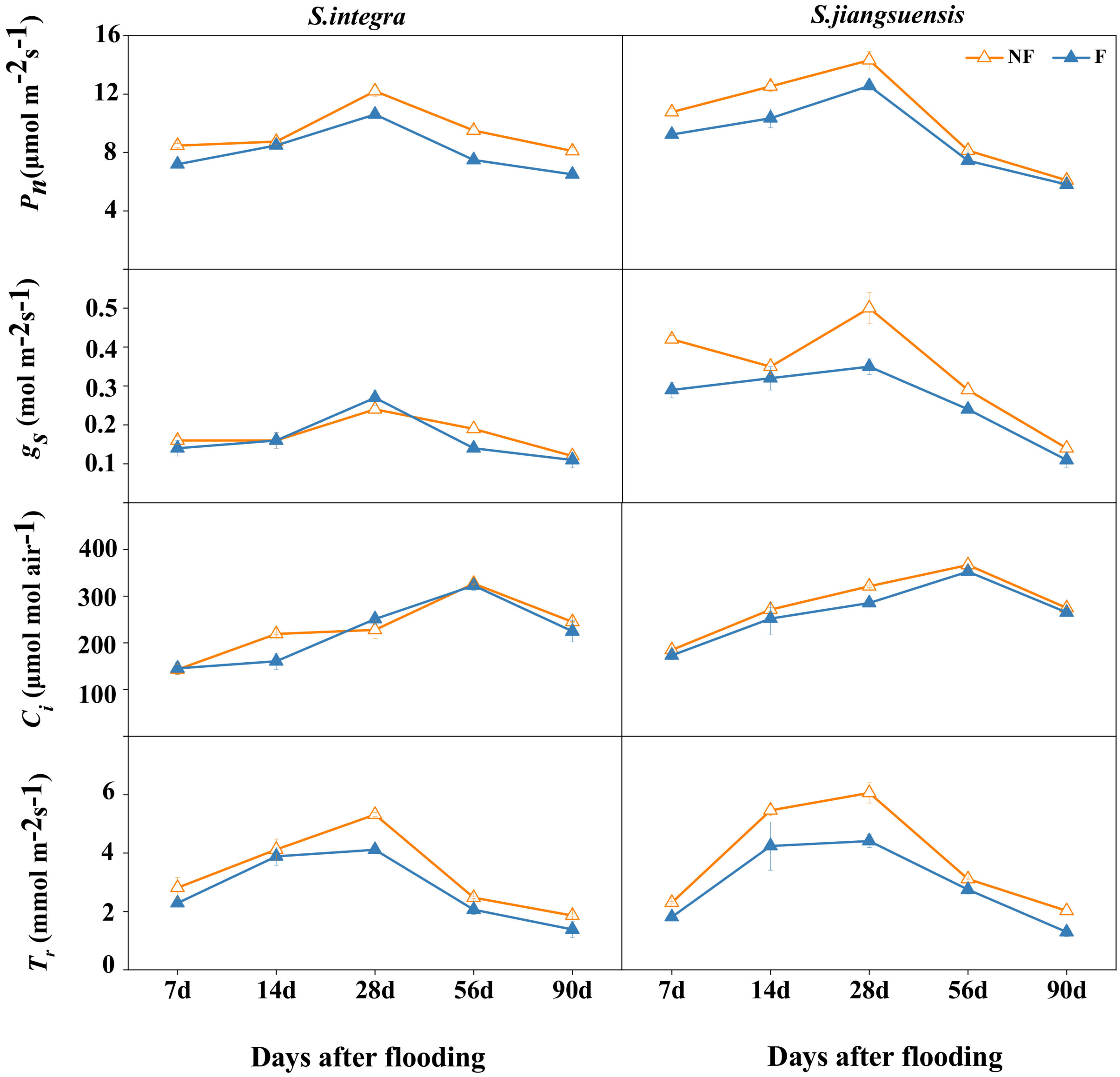

Figure 1. Temporal changes in photosynthetic rate (P_n, μmol.m^{-2}s^{-1}), stomatal conductance (g_s, mol H_2O m^{-2}s^{-1}), intercellular CO_2 concentration (C_i, μmol CO_2 mol air^{-1}), and transpiration rate (T_r, mmol H_2O m^{-2}s^{-1}) in leaves of *S. integra* and *S. jiangsuensis* exposed to Cu-contaminated soil under non-flooded (NF) and flooded (F) conditions for 90 d.

3.3. Accumulation and Distribution of Cu in Willows

The highest Cu concentration was found in the roots regardless of treatment or willow species (Figure 2). Generally, the Cu accumulation among different tissues was displayed in a descending order of root > cutting > stem > leaf. *S. integra* accumulated more Cu in roots (418.1 and 648.9 mg·kg^{-1}) than *S. jiangsuensis* (159.3 and 563.6 mg·kg^{-1}) in the flooded and non-flooded treatment, respectively. Moreover, the decrease (71.73%) in the root Cu concentration of *S. jiangsuensis* was greater than *S. integra* (35.5%), as affected by flooding.

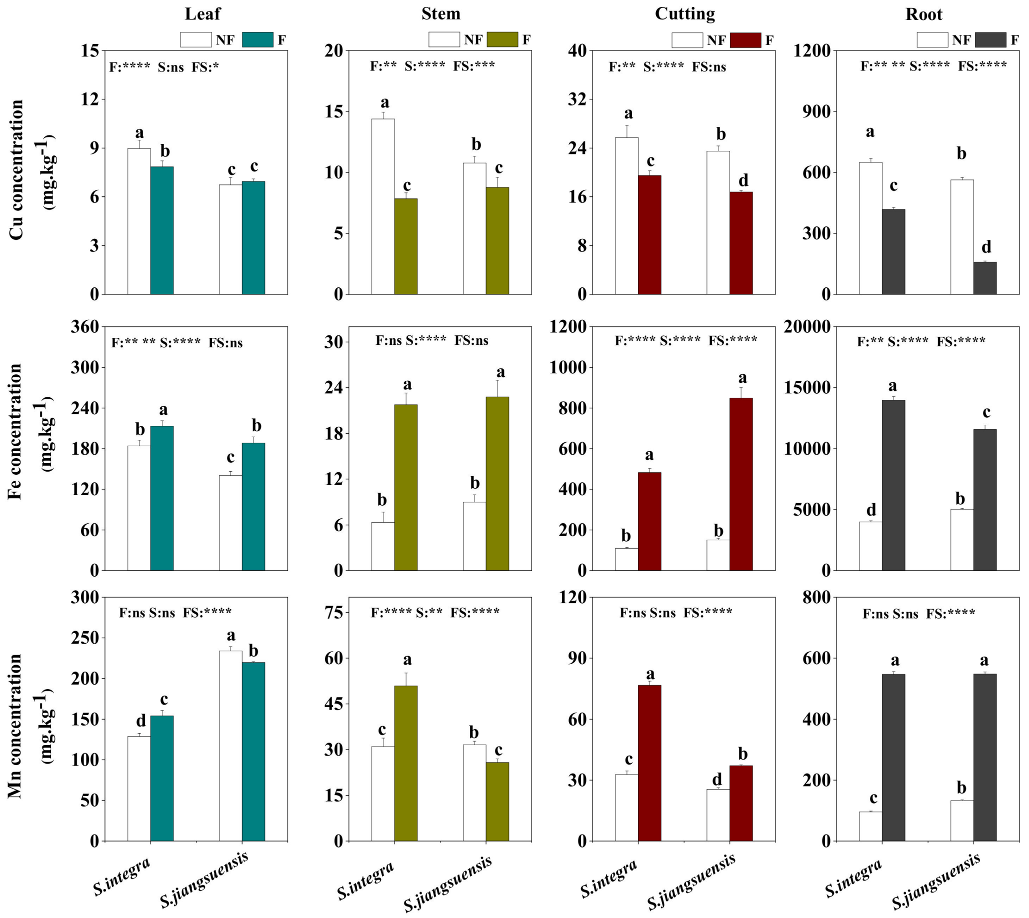

Figure 2. Trace elements (Cu, Fe, Mn in mg·kg^{-1} DW) in plant tissues of *S. integra* and *S. jiangsuensis* exposed to Cu-contaminated soil under non-flooded and flooded conditions for 90 d. (The data indicate the means ± SD (n = 4). Different letters indicate significant difference among the 4 treatments (2 willow species under non-flooded (NF) and flooded (F) conditions) at the 0.05 level by Fisher's. The p values of the ANOVA of willow species (S), flooding (F), and their interactions (FS) are also shown. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$, ns: not significant).

Additionally, the root BCF values of *S. integra* were higher than one (Table 2) and were also markedly higher than *S. jiangsuensis* under flooded and non-flooded conditions. The BCF values of the roots in the two willow species were all significantly higher than the above-ground BCFs under non-flooded and flooded conditions. Moreover, soil flooding decreased the BCF of the roots (35.6% and 71.9%) and above-ground tissues (29.5% and 21.6%) in *S. integra* and *S. jiangsuensis*, respectively. Conversely, flooding increased the TF values, and the increment of the TF in *S. jiangsuensis* was 172.73%, which was pronouncedly higher than for *S. integra* (8.7%). Additionally, the concentrations of Fe and Mn in both roots and the Fe plaque on the root surfaces were significantly higher under the flooded condition in comparison with the non-flooded condition (Table 3). Markedly negative correlations were found between DCB-Cu and DCB-Fe/DCB-Mn in the root surfaces of both willow species, which are consistent with the correlations between root Cu, Fe, and Mn contents (Figure 3 and Table S3).

Table 2. Bioconcentration factor (BCF) and translocation factor (TF) in *S. integra* and *S. jiangsuensis* after 90 d of non-flooded/flooded treatments.

	S. integra		***S. jiangsuensis***		**Significance**		
	Non-Flooded	**Flooded**	**Non-Flooded**	**Flooded**	**Treatments**	**Species**	**Treatments × Species**
BCF—root	2.70 ± 0.08 a	1.74 ± 0.04 c	2.35 ± 0.05 b	0.66 ± 0.02 d	****	****	****
BCF—aboveground tissues	0.061 ± 0.001 a	0.043 ± 0.001 c	0.051 ± 0.001 b	0.040 ± 0.002 d	****	****	***
TF	0.023 ± 0.000 bc	0.025 ± 0.000 b	0.022 ± 0.000 c	0.060 ± 0.003 a	****	****	****

Each value represents the mean of four replicates ± standard deviation. Different letters indicate significant difference among the 4 treatments (2 willow species under non-flooded and flooded conditions) at the 0.05 level by Fisher's. LSD test. *** $p < 0.001$; **** $p < 0.0001$.

Table 3. Fe, Mn, Cu, and S concentration (DW) in the plaque on the root surfaces of *S. integra* and *S. jiangsuensis* exposed to Cu-contaminated soil under non-flooded/flooding conditions for 90 d.

Willow Species	**Treatments**	**DCB Fe (mg· kg^{-1})**	**DCB Mn (mg· kg^{-1})**	**DCB Cu (mg· kg^{-1})**	**DCB S (g· kg^{-1})**
S. integra	Non-flooded	32.5 ± 2.8 d	6.67 ± 0.2 d	16.2 ± 1.3 a	28.2 ± 1.3 b
	Flooded	1286.9 ± 74.8 a	46.5 ± 2.3 a	3.08 ± 0.3 d	19.0 ± 0.4 c
S. jiangsuensis	Non-flooded	563.8 ± 33.1 c	16.2 ± 0.72 c	12.5 ± 0.8 b	7.8 ± 0.2 c
	Flooded	1065.1 ± 199 b	28.7 ± 2.3 b	32.1 ± 1.1 a	15.0 ± 1.1 d
Significance	Treatments	****	ns	****	****
	Species	****	ns	ns	ns
	Treatments × Species	****	****	****	***

Each value represents the mean of four replicates ± standard deviation. Different letters indicate significant difference among the 4 treatments (2 willow species under non-flooded and flooded conditions) at the 0.05 level by Fisher's. LSD test. *** $p < 0.001$; **** $p < 0.0001$. ns: not significant.

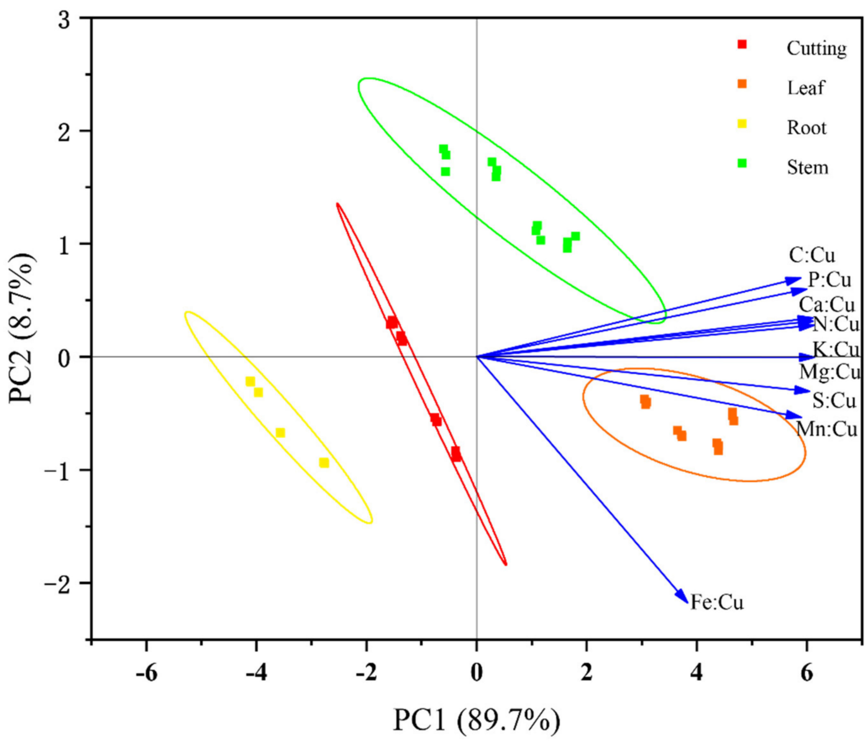

Figure 3. Principal component analysis (PCA) of different element:Cu ratios of the two willows in response to non-flooded and flooded treatments with Cu contamination. The first two principal component scores are indicated.

3.4. Plant C, N, and P and Corresponding Stoichiometry

The plant C concentration was slightly altered, and no significant difference was observed between the non-flooded and flooded conditions ($p > 0.05$). Flooding significantly decreased the root N concentration by 11.4% in *S. jiangsuensis* compared to the non-flooded treatment. For leaves and stems, the P concentrations of both willow species sharply decreased by 21.3–40.5% in response to flooding, whereas the root P concentrations significantly increased under the flooded condition compared to the non-flooded condition (Table 4). As a result, the C:P and N:P ratios in leaves and stems were markedly enhanced by flooding, while the root C:P and N:P ratios were decreased by flooding compared to the non-flooded condition. Significant differences in C, N, and P stoichiometry among different tissues were also observed, regardless of the presence of flooding (Table 4). The lowest C:N ratio (32.7–43.2) was observed in the leaves, but the lowest C:P (75.3–111.0) and N:P (1.1–1.3) ratios were both found in the roots.

Table 4. Statistics for plant C, N, and P ($g \cdot kg^{-1}$) and ratios of C:N, C:P, and N:P in plant tissues of *S. integra* and *S. jiangsuensis* exposed to Cu-contaminated soil as affected by soil flooding for 90 d.

Element and Ratios	Plant Species	Plant Tissues							
		Leaf		Stem		Cutting		Root	
		Non-Flooded	Flooded	Non-Flooded	Flooded	Non-Flooded	Flooded	Non-Flooded	Flooded
C	*S. integra*	419.0 ± 6.9 a	416.8 ± 9.4 a	501.7 ± 9.2 a	505.6 ± 5.5 a	491.9 ± 8.6 a	480.6 ± 9.4 b	450.1 ± 15.9 a	462.2 ± 18.0 a
	S. jiangsuensis	437.9 ± 16.0 a	425.6 ± 20.5 a	479.1 ± 10.2 b	497.7 ± 9.5 a	485.9 ± 8.1 ab	473.7 ± 10.4 ab	456.6 ± 8.3 a	466.5 ± 12.9 a
N	*S. integra*	12.8 ± 0.2 a	12.1 ± 0.1 b	3.9 ± 0.18 a	3.9 ± 0.21 a	3.8 ± 0.05 a	3.6 ± 0.08 b	6.8 ± 0.07 a	6.9 ± 0.17 a
	S. jiangsuensis	10.2 ± 0.2	9.9 ± 0.2 d	3.2 ± 0.04 b	3.3 ± 0.08 b	2.9 ± 0.06 c	3.5 ± 0.05 b	7.0 ± 0.08 a	6.2 ± 0.01 b
P	*S. integra*	2.6 ± 0.05 a	1.5 ± 0.04 c	1.6 ± 0.01 b	1.3 ± 0.11 c	1.5 ± 0.11 b	1.5 ± 0.04 ab	4.3 ± 0.15 c	6.1 ± 0.24 a
	S. jiangsuensis	2.2 ± 0.1 b	1.5 ± 0.03 c	2.0 ± 0.07 a	1.6 ± 0.05 b	1.6 ± 0.04 a	1.4 ± 0.01 c	4.1 ± 0.02	4.7 ± 0.18 b
C:N	*S. integra*	32.7 ± 0.3 b	34.4 ± 1.1 b	130.2 ± 6.8 b	129.5 ± 5.7 b	129.1 ± 1.2 b	133.1 ± 5.6 b	66.3 ± 2.9 b	66.9 ± 4.2 b
	S. jiangsuensis	42.8 ± 1.9 a	43.2 ± 1.4 a	148.6 ± 4.9 a	148.9 ± 5.3 a	167.6 ± 5.6 a	134.8 ± 2.5 b	65.7 ± 1.5 b	75.6 ± 2.2 a
C:P	*S. integra*	163.3 ± 1.4 c	277.0 ± 13.1 a	313.6 ± 6.8 b	403.4 ± 29.7 a	336.9 ± 31.2 ab	351.2 ± 14.3 a	105.62 ± 6.3 ab	75.3 ± 4.1 c
	S. jiangsuensis	203.2 ± 7.9 b	293.5 ± 9.4 a	238.8 ± 12.5 c	316.8 ± 16.0 b	306.0 ± 10.0 b	322.1 ± 10.6 ab	111.0 ± 1.6 a	99.5 ± 6.3 b
N:P	*S. integra*	5.0 ± 0.01 c	8.1 ± 0.2 a	2.4 ± 0.10 b	3.1 ± 0.11 a	2.6 ± 0.2 a	2.4 ± 0.05 a	1.6 ± 0.06 b	1.1 ± 0.05 d
	S. jiangsuensis	4.8 ± 0.2 d	6.8 ± 0.08 b	1.6 ± 0.04 d	2.1 ± 0.04 c	1.8 ± 0.06 b	2.6 ± 0.06 a	1.7 ± 0.03 a	1.3 ± 0.05 c

Each value represents the mean of four replicates ± standard deviation. Different letters indicate significant difference among the 4 treatments (2 willow species under non-flooded and flooded conditions) at the 0.05 level by Fisher's. Results of a two-way ANOVA are presented in Table S6.

3.5. Other Nutrients and Multi-Element:Cu Stoichiometry

The concentrations of Fe and Mn in both willow roots were significantly increased under the flooded condition compared to the non-flooded condition, and were 2.3–3.5-and 4.1–5.7-fold higher than their corresponding non-flooded condition, respectively (Figure 2). In the stems, the P and K concentrations exhibited a decreasing trend, and were significantly reduced by 16.7%–21.9% (Table S4) in response to flooding. Interestingly, the concentrations of Ca, Mg, and S all increased in the roots of both willow species as affected by flooding, but generally reduced in the leaves, stems, and cuttings. Both willow species had higher mineral element: Cu ratios in leaves than in other tissues, regardless of the presence of flooding. Additionally, flooding had significant effects on the multi-element: Cu stoichiometry in different tissues ($p < 0.05$, Table 5). For *S. integra*, there were significant elevations for the flooded condition in all element: Cu ratios of the leaves, except for the P: Cu ratio. Moreover, other element: Cu ratios were decreased by flooding in *S. jiangsuensis*, but the Fe: Cu ratio increased.

Table 5. Different element: Cu ratios of the two willows in response to non-flooded/flooded treatment with Cu contamination.

Log Element:Log Cu Ratio	Plant Species	Plant Tissues							
		Leaf		Stem		Cutting		Root	
		Non-Flooded	Flooded	Non-Flooded	Flooded	Non-Flooded	Flooded	Non-Flooded	Flooded
logC:logCu	*S. integra*	5.90 ± 0.01 d	6.28 ± 0.01 c	4.92 ± 0.01 d	6.38 ± 0.01 a	4.04 ± 0.01 d	4.40 ± 0.01 b	2.01 ± 0.01 d	2.16 ± 0.01 b
	S. jiangsuensis	6.81 ± 0.02 a	6.69 ± 0.02 b	5.50 ± 0.01 c	6.04 ± 0.01 b	4.15 ± 0.01 c	4.63 ± 0.01 a	2.06 ± 0.00 c	2.57 ± 0.01 a
logN:logCu	*S. integra*	4.31 ± 0.01 d	4.56 ± 0.01 c	3.10 ± 0.02 d	4.02 ± 0.03 a	2.54 ± 0.00 c	2.76 ± 0.01 b	1.36 ± 0.00 d	1.46 ± 0.00 b
	S. jiangsuensis	4.84 ± 0.01 a	4.75 ± 0.01 b	3.40 ± 0.01 c	3.74 ± 0.01 b	2.53 ± 0.01 d	2.89 ± 0.00 a	1.40 ± 0.00 c	1.72 ± 0.00 a
logP:logCu	*S. integra*	3.58 ± 0.01 c	3.55 ± 0.01 c	2.77 ± 0.00 d	3.47 ± 0.04 a	2.25 ± 0.02 d	2.46 ± 0.01 b	1.29 ± 0.01 d	1.45 ± 0.01 b
	S. jiangsuensis	4.02 ± 0.02 a	3.76 ± 0.01 b	3.20 ± 0.01 c	3.39 ± 0.02 b	2.33 ± 0.01 c	2.56 ± 0.00 a	1.31 ± 0.00 c	1.67 ± 0.01 a
logK:logCu	*S. integra*	4.36 ± 0.00 d	4.72 ± 0.02 c	3.10 ± 0.01 d	3.89 ± 0.04 a	2.53 ± 0.01 d	2.72 ± 0.01 b	1.38 ± 0.01 d	1.50 ± 0.00 b
	S. jiangsuensis	5.05 ± 0.00 a	4.81 ± 0.00 b	3.56 ± 0.02 c	3.82 ± 0.01 b	2.66 ± 0.01 c	2.92 ± 0.01 a	1.42 ± 0.00 c	1.85 ± 0.00 a
logCa:logCu	*S. integra*	4.87 ± 0.01 d	5.08 ± 0.02 c	3.50 ± 0.00 d	4.37 ± 0.05 a	2.76 ± 0.01 d	2.93 ± 0.01 b	1.35 ± 0.00 d	1.47 ± 0.00 b
	S. jiangsuensis	5.37 ± 0.01 a	5.25 ± 0.00 b	3.71 ± 0.02 c	3.97 ± 0.02 b	2.86 ± 0.01 c	3.01 ± 0.02 a	1.38 ± 0.00 c	1.73 ± 0.00 a
logMg:logCu	*S. integra*	3.33 ± 0.01 d	3.53 ± 0.02 c	2.12 ± 0.00 d	2.67 ± 0.04 b	1.76 ± 0.01 c	1.87 ± 0.01 b	1.04 ± 0.01 d	1.13 ± 0.00 b
	S. jiangsuensis	3.92 ± 0.01 a	3.87 ± 0.00 b	2.48 ± 0.02 c	2.72 ± 0.02 a	1.88 ± 0.01 b	2.08 ± 0.00 a	1.08 ± 0.00 c	1.39 ± 0.00 a
logS:logCu	*S. integra*	4.38 ± 0.01 c	4.60 ± 0.02 b	2.43 ± 0.01 d	3.09 ± 0.04 a	2.07 ± 0.01 c	2.13 ± 0.01 b	1.20 ± 0.00 d	1.33 ± 0.01 b
	S. jiangsuensis	4.84 ± 0.01 a	4.83 ± 0.00 a	2.59 ± 0.02 c	2.83 ± 0.02 b	2.06 ± 0.01 c	2.34 ± 0.00 a	1.25 ± 0.00 c	1.67 ± 0.00 a
logFe:logCu	*S. integra*	2.38 ± 0.02 c	2.60 ± 0.02 b	0.69 ± 0.08 c	1.49 ± 0.03 a	1.45 ± 0.01 d	2.08 ± 0.01 b	1.28 ± 0.00 d	1.58 ± 0.00 b
	S. jiangsuensis	2.59 ± 0.02 b	2.70 ± 0.02 a	0.92 ± 0.05 b	1.44 ± 0.04 a	1.59 ± 0.02 c	2.39 ± 0.02 a	1.35 ± 0.00 c	1.85 ± 0.01 a
logMn:logCu	*S. integra*	2.21 ± 0.01 d	2.44 ± 0.02 c	1.29 ± 0.03 c	1.91 ± 0.04 a	1.07 ± 0.02 c	1.46 ± 0.01 a	0.70 ± 0.00 d	1.04 ± 0.00 b
	S. jiangsuensis	2.86 ± 0.01 a	2.78 ± 0.00 b	1.45 ± 0.02 b	1.50 ± 0.02 b	1.03 ± 0.01 d	1.28 ± 0.00 b	0.77 ± 0.00 c	1.24 ± 0.00 a

Each value represents the mean of four replicates ± standard deviation. Different letters indicate significant difference among the 4 treatments (2 willow species under non-flooded and flooded conditions) at the 0.05 level by Fisher's. Results of a two-way ANOVA are presented in Table S7.

4. Discussion

4.1. Effects of Flooding on Willow Growth and Photosynthesis

Flooding induced water saturation/insufficient oxygen supply, and had adverse impacts on plants, including growth inhibition and photosynthesis decrease [35]. In the current study, *S. integra* and *S. jiangsuensis* exhibited sharp reductions in root biomass as affected by flooding, indicating that the roots were sensitive to flooding stress. Other studies also reported that Cu-induced morphological alterations could cause growth inhibition and reduce root biomass [2,36]. Flooding could cause poor soil aeration and rhizosphere hypoxia, and root growth was stunted, which adversely influenced the uptake of water and mineral elements [37]. In accordance with our earlier study [22], co-exposure of high Cu contamination and flooding affected the root morphology, resulting in the decrease in root biomass. Additionally, the biomass of *S. jiangsuensis* was significantly higher than *S. integra* under both non-flooded and flooded conditions, but the plant height of *S. jiangsuensis* was slightly lower than *S. integra* during the experimental period (Table 1). The reduction degree of the biomass in response to Cu stress was largely determined by plant species or clones [38]. The total Cu content of the tested soil obviously exceeded the phytotoxic range of 60–125 mg·kg^{-1} [39], which could have retarded the plant growth. Although the extent of inhibition to plant biomass varied, both willow species could survive and grow well in a co-existing scenario of high Cu level and flooding for 90 d. Previous study reported that certain plants withstanding flooding stress for more than 50 d were proposed to be relatively high-flooding-tolerance species [40]. Therefore, both the shrub and arbor willows could be tolerant to flooding and Cu.

Flooding stress generally caused alteration in leaf photosynthesis, which contributed to the difference in flood tolerance of the woody species [35]. The photosynthetic responses of *S. jiangsuensis* and *S. integra* were consistent with previous studies that showing that flooding induced decreases in P_n and g_s in flood-tolerant woody species [40,41]. Additionally, photosynthesis was markedly decreased within a few days after flooding (Figure 1), and the P_n slowly elevated as a result of the gradually reopened stomata with the prolonged flooding duration [42,43]. Here, both willow species exhibited a significant decrease in P_n and T_r on different days during the flooding ($p < 0.05$). After flooding for 90 d, all photosynthesis parameters were further decreased in both willow species. They exhibited significantly positive correlations among P_n, g_s and T_r ($p < 0.05$). The early decrease in P_n in flooded plants might be associated with stomatal closure, leading to a reduced CO_2 uptake by leaves [41,44]. Furthermore, the decrease in g_s could cause the reduction in C_i concentration and photosynthetic substrate, leading to the decline in P_n [42,45].

4.2. Effect of Flooding on Cu Accumulation and Distribution

Willows exhibited good potential for Cu phytoremediation under suitable environmental conditions [22]. The remediation efficiency relies on a variety of factors, such as soil properties, metal types, plant species, and environmental conditions [46,47]. The arbor willow (*S. jiangsuensis*) and shrub willow (*S. integra*) showed significant variations in Cu accumulation and distribution under flooded condition versus non-flooded condition. Large amounts of Cu accumulated in the roots of both willow species (Figure 2), and the Cu BCFs of the roots were significantly higher than those of the above-ground tissues (Table 2). Similar results were also evident in other wetland plants; that the shoot tissues had lower capacity to accumulate heavy metals [17,48], suggesting the detoxification strategy of restricting most of the heavy metals to the roots [49] rather than in the shoots. In the current study, flooding significantly decreased the Cu concentrations in the roots of the two willow species ($p < 0.05$). This result is similar to that of a previous study finding that flooded soil decreased the Cd accumulation of *S. cinereal* [50]. However, Kissoon et al. [51] showed that flooding treatment of *Typha angustifolia* accumulated more metals (Al, Fe, Mn and Zn) in the roots compared to non-flooded treatment. We speculated that the discrepancy might be due to the variation in the uptake capacity of metals among different wetland plant species, metal types, and total levels of metals in soils.

Although the total biomass of the arbor willow (*S. jiangsuensis*) was markedly higher than the shrub willow (*S. integra*), the Cu uptake capacity of *S. jiangsuensis* (2.94 and 0.74 mg per plant) was much lower than for *S. integra* (2.99 and 1.39 mg per plant) under non-flooded and flooded conditions, respectively. These results were accompanied with the higher BCF values in the roots (2.74 and 1.74) and root/shoot ratio (0.38 and 0.27) of *S. integra* compared to *S. jiangsuensis* under non-flooded and flooded conditions. Therefore, the metal tolerance and accumulation capacity were not only due to the total biomass production, but the root/shoot ratio could also modulate metal absorption from the soil [52]. In terms of Cu accumulation capacity and plant biomass, the shrub willow (*S. integra*) exhibited potential for Cu phytostabilization with relatively high Cu content in the roots regardless of flooded or non-flooded conditions. It is also worth pointing out that the markedly high biomass of the arbor willow (*S. jiangsuensis*) could make the process of phytoextraction quite effective.

Here, the Cu accumulation capacity in both willow species, expressed as content per plant, was markedly decreased by flooding ($p < 0.05$). Additionally, flooding significantly enhanced the Fe and Mn accumulation in the roots or root surfaces as compared to the non-flooded condition, but significantly decreased the root Cu content (Table 3). Furthermore, markedly negative correlations were also observed between DCB-Cu and DCB-Fe, DCB-Cu and DCB-Mn on the roots of both willow species (Table S3). Similar results were reported by Du Laing et al. [18]; the metal mobility could be influenced by Fe/Mn oxide reduction/oxidation. For example, the formation of an Fe plaque in rice resulted in the decline of As, Cd, and Pb uptake and translocation [53]. Several studies also reported that Fe plaques formed on the root surface decreased metal accumulation and further influenced the metal sequestration and translocation [47,54]. Consequently, the lower Cu accumulation in the roots may be attributed to the presence of the Fe/Mn plaque. Furthermore, the DCB-Cu was positively correlated with DCB-S ($r = 0.74$, $p < 0.01$) (Table S3), but the root Cu concentration was negatively correlated with the root S concentration ($r = -0.97$, $p < 0.01$) (Figure 4). Another explanation for the decreased Cu concentrations in roots could be related to sulfide formation. Flooding could lead to oxygen depletion in the rhizosphere, and further cause soil aerobic conditions [42]. Previous studies also found the reduction process of Cu (II) to Cu (I), subsequently resulting in Cu_2S precipitation [18,55].

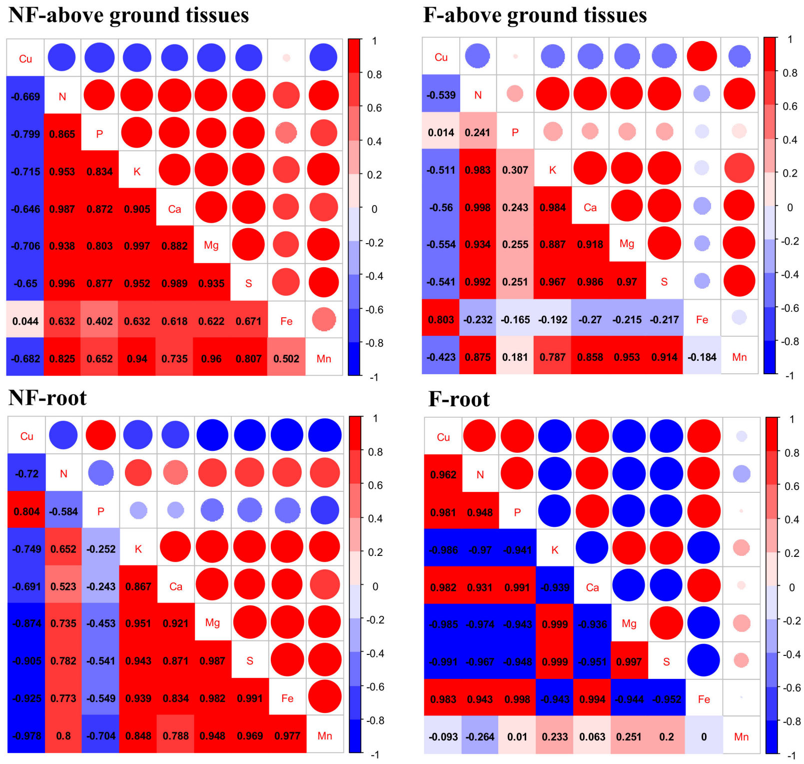

Figure 4. Pearson's correlation analysis performed on element concentrations measured in the above-ground parts and roots in of *S. integra* and *S. jiangsuensis* exposed to Cu-contaminated soil under non-flooded and flooded conditions for 90 d. (Positive correlations are displayed in red and negative correlations in blue color. Color intensity and circle size are proportional to the correlation coefficients. Significant levels of correlation test which were less than 0.05 are shown in the Figure).

4.3. Effects of Flooding on the Stoichiometry Patterns of Plant C, N, and P

The variations in the nutrient elements of wetland plants might reflect their available content in waterlogged soils. In this study, flooding induced alterations in the concentrations of C, N, and P and their corresponding stoichiometry in *S. integra* and *S. jiangsuensis* (Table 4). Here, the plant C contents were less variable than the nutrient stoichiometry traits, and exhibited no significant changes under the flooded condition compared to the non-flooded condition ($p > 0.05$). C in plants is not directly involved in the activities of plant production, instead mainly providing the structural basis as a relatively stable plant skeleton [56,57]. It was also suggested that C contents were stable and not affected by water supply/water depth in the shrub *Zygophyllum xanthoxylum* and macrophyte species such as *Potamogeton malaianus*, *Potamogeton maackianus*, *Myriophyllum spicatum*, *Ceratophyllum demersum*, and *Hydrilla verticillate* [29,58].

Both willow species exhibited lower N and P concentrations in leaves under the flooded condition than for the non-flooded condition, and the patterns of the N and P response to flooding were similar to *Zea mays* [59], *Lepidium latifolium* [44], and *Triticum aestivum* [60]. According to our analysis, significantly positive correlations between leaf N and leaf P concentrations were observed (Figure 3), regardless of flooding. N and P are both foundational elements and participate in multiple physiological processes, including plant growth [61], nutrient availability [62], and environmental adaption [3]. Thus, changes in leaf N are usually consistent with leaf P under the same environmental conditions [28]. Furthermore, flooding significantly enhanced the ratio of C:P in the leaves of *S. integra* (69.7%) and *S. jiangsuensis* (44.4%) compared to the non-flooded condition, indicating that flooding led to an elevated demand for assimilating more C to maintain normal metabolism in plants [28,63]. Flooding significantly reduced the ratio of C:P in the roots of both willow species, and the variation in *S. integra* (28.7%) was higher than for *S. jiangsuensis* (10.4%)

(Table 4). In line with previous observations in wetland plants, the C:P ratio was markedly declined by waterlogging [29,64]. Under the flooded condition, pronounced increases in root P were observed in *S. integra* and *S. jiangsuensis*, but root C in both willows was relative stable, which might be a possible reason for the reduced root C:P ratio. In agreement with other studies, significantly higher N contents and N:P ratios were observed in the leaves than in other tissues (stems, cuttings, and roots) [27,29,65]. The N and P contents and their corresponding stoichiometry differed significantly among the willow tissues because of the structures and functions/activities of different organs [27]. Generally, leaves contain many chloroplasts which are highly active in metabolic and photosynthetic processes, such that higher N contents and N:P ratios than other organs are required [66].

4.4. Effects of Flooding on Other Nutrients and Their Correlation with Cu

The nutrient concentrations among the tissues displayed significant differences (Figure 2). Generally, the leaves accumulated higher concentrations of macro-elements (N, P, K and Mg) than those in the roots of *S. integra* in both treatments. The differences possibly resulted from the mobility of nutrient elements in different tissues [67]. For instance, elements related to photosynthesis, encompassing N, P, and K, were translocated and accumulated in leaves to promote plant growth [28]. However, trace elements such as Fe and Mn accumulated more in the roots than in the leaves [22], and could have been tightly bound within the root cells, contributing to greater accumulation in the belowground tissues [67]. Indeed, the elements required at high concentrations are considered less sensitive and less variable to environmental variations during plant growth and development [24].

Cu toxicity could influence nutrient uptake and distribution within plant tissues due to the disruption of water homeostasis, the cellular permeability barrier, and changes of physiological function [22,68]. The macro- and micro-elements were significantly affected by co-exposure of flooding and Cu stress, and they exhibited different correlations with the Cu levels. Among all element: Cu ratios, the Fe:Cu, Mn:Cu, and K:Cu ratios were the top three contributors for the different tissues of the two willow species under the two treatments (Figure 3 and Table S5), probably due to the physiological roles of these trace elements in plants and their correlations with Cu in ecological processes under flooded and non-flooded conditions. Excessive Cu levels in soils could cause phytotoxicity, and further alter the uptake, transport, and utilization of mineral elements (e.g., Fe, Ca, and Mg) [22,69]. Additionally, elemental composition and nutrient stoichiometry for homoeostatic regulation are vital physiological mechanisms to maintain normal growth for plants suffering from environmental stresses [61,70,71]. Karimi and Folt [72] revealed that homeostatic capacities were highest for macronutrients, intermediate for essential trace elements, and lowest for non-essential metals. Relevant study also suggested that macronutrients such as N, P, and Ca were strongly stable, whereas trace elements were weak in equilibrium capacity [67].

The decreased P and Ca in the leaves were likely due to the enhanced Fe and Mn contents in the roots, which could inhibit their uptake and immobilize them in the roots, further interfering with their translocation to the shoots [54,73]. This is partially ascribed to the Fe/Mn plaque formation on root surfaces under soil flooding [22]. Another possible explanation is that these elements could compete with Cu in transport pathways because most metal transporters could work with various metal ions [22,74]. The enhanced accumulation of Ca, Mg, and S in roots under flooded condition could be explained by the rhizosphere oxidation processes, which might further stimulate nutrient uptake, affect nutrient sequestration, and create concentration gradients of nutrients that promote nutrient element movement in roots [51,75]. It was demonstrated that more metabolically active organs could likely accumulate more nutrients (e.g., N, Ca, K, P) to maintain high photosynthesis in leaves and uptake capacity in roots [76]. Therefore, these results indicated that increased uptake of nutrients is helpful for improving photosynthetic capacity and are also crucial to willow survival during flooding.

5. Conclusions

Both willow species were able to grow well and showed relatively high tolerance to the combined stresses of Cu contamination and flooding. Flooding slightly decreased the total biomass (7.8%–10.7%) of both willow species compared to the non-flooded condition. Under the flooded condition, *S. integra* showed greater potential Cu accumulation capacity (1.39 mg per plant), even though its total biomass was significantly lower than for *S. jiangsuensis* (arbor willow). Additionally, higher BCF values in the roots (1.74) and root/shoot ratios (0.27) of *S. integra* were also observed in the flooded treatment than those of *S. jiangsuensis*. Thus, *S. integra* was noted to be suitable for Cu phytostabilization, with relatively high Cu accumulation in its roots regardless of flooding. Although the characteristics of Cu accumulation differed between two willows, both species presented suitable tolerance mechanisms (regulating photosynthesis, nutrient uptake) to cope with flooding stress. Flooding markedly increased the C:P ratio in the leaves of *S. integra* (69.7%) and *S. jiangsuensis* (44.4%), suggesting that flooding led to an elevated demand for assimilating more C to maintain normal metabolism in the plants. Our study also helps further understanding the nutrient balance and stoichiometry of willows in Cu-contaminated soil and their responses to soil flooding.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f13070989/s1, Table S1: Physical and chemical characteristics of the selected soil. Table S2: The recovery data for the elemental analysis using inductively coupled plasma atomic emission spectrometry (ICP-ASE). Table S3: Correlations between DCB-Cu, DCB-Fe, DCB-Mn, and DCB-S. Table S4: Macroelements (K, Ca, Mg, S $g \cdot kg^{-1}$) in the dry weight plant tissues of *S. integra* 'Yizhbi' and *S. jiangsuensis* 'J172' exposed to Cu-contaminated soil as affected by soil flooding for 90 d. Table S5: Eigenvalues and variance of Principal Component Analysis for elements. Table S6: Summary of the two-way ANOVA for C, N, and P and their corresponding stoichiometric ratios in different plant tissues as affected by species and flooding. Table S7: Summary of the two-way ANOVA for element:Cu ratios in different plant tissues as affected by species and flooding.

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