

Research Article

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Risk expansion of Cr through amphibious clonal plant from polluted aquatic to terrestrial habitats

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Abstract: Resource sharing between the connected ramets of clonal plants through physiological integration can increase the tolerance of plants to environmental stress. However, the role of physiological integration in the translocation of heavy-metal pollutants between different habitats receives little attention, especially in the aquatic-terrestrial ecotones. An amphibious clonal plant *Alternanthera philoxeroides* was used to simulate plant expansion from unpolluted soil to a chromium (Cr)-polluted water environment. Basal older ramets growing in unpolluted soil were connected or disconnected with apical younger ramets of the same fragments in polluted environments at different Cr concentrations. Harvested basal ramets were also used for decomposition tests for the loss of residual mass and release of Cr to soil. With increasing Cr concentration there was reduction in biomass of the apical ramets, especially those separated from the basal parts. Cr was detected in the basal ramets with connection to apical parts. The decomposition of plant litter from the basal ramets connected with polluted apical parts might release retained Cr to unpolluted soil. The amount and chemical forms of Cr in the plant litter changed over time. It is concluded that Cr could be transferred from polluted aquatic to unpolluted terrestrial habitats through amphibious clonal plants.

Keywords: *Alternanthera philoxeroides*, chromium, decomposition, physiological integration, aquatic-terrestrial habitat

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1 Introduction

Within a clonal system, ramets physiologically connected by rhizomes, stolons or horizontal roots can share resources such as carbohydrates, water and nutrients, especially in spatially heterogeneous environments [1-5]. Support from the ramets in favorable environments can increase the performance of the connected ramets establishing themselves in less-favorable environments as well as of the whole clonal network [6, 7]. In aquatic-terrestrial ecotones, many amphibious plants can expand their populations from aquatic to terrestrial habitats and *vice versa* through clonal growth characteristics [3, 4, 7, 8]. Physiological integration has been shown in many studies to facilitate the survival, growth and expansion of asexual individuals subjected to various stresses in both aqueous and terrestrial environments [2, 9]. On the other hand, disease and some toxic pollutants (e.g., heavy metals) could also be spread or transmitted between connected ramets, imposing extra cost on the supporters and even on the whole clonal network [10-12].

Due to increasing anthropogenic activity, the ecological health and function of surface waters are severely threatened by various pollutants, such as heavy metals, hormones, pharmaceuticals, pesticides, nutrients, and so on [13-15]. With long-term and non-degradable characteristics, heavy metals have resulted in great damage to the function of aquatic and terrestrial ecosystems [10, 16], and have threatened human and planetary health throughout the food chain [11, 17]. In particular, chromium (Cr) is widely used in industrial manufacturing, such as alloy smelting, electroplating, tanning, dyeing, and production of medicine and fuels, and discharged into aquatic ecosystems along with waste water [13, 18]. Additionally, Cr in industrial residues abandoned in terrestrial ecosystems could also enter aquatic environments through precipitation, surface runoff and/or wind. In heavily Cr-polluted aquatic ecosystems, direct establishment of aquatic or amphibious plants is almost impossible [3, 10]. However, amphibious plants with clonal growth characteristics are able to

distribute young ramets into aquatic habitats polluted by Cr, with supports from established ramets. Along with the transmission of resources, it is also speculated that heavy metals are likely to be transported within the clonal network in aquatic-terrestrial ecotones, leading to the potential diffusion of contaminants. Though the effects of physiological integration in clonal plants on heavy metal translocation have been examined previously [10, 11, 19], amphibious clonal plants establishing themselves in aquatic-terrestrial ecotones are relatively less-studied [2].

It has been reported that plants are capable of storing plenty of nutrients, and release these nutrients into the ecosystem during decomposition of dead plant residues, contributing to nutrient cycling in the natural ecosystem [20-22]. Apart from nutrients, uptake of pollutants (e.g., heavy metals) by vegetation occurs commonly in contaminated sites [3, 10]. With the decomposition of plant residues, along with the release of nutrients, the retained heavy metals in plants could possibly be returned to the environment, becoming another source of pollution in aquatic and terrestrial ecosystems. Hence, it is important to take into consideration the return of heavy metals back into the ecotope following plant senescence in order to estimate the actual cycling of the pollutants. The risk of toxic stress may be shared among the clones through physiological integration [3, 10, 11, 19], resulting in the diffusion of heavy metals with the plant's decomposition. In this study, we simulated the expansion of *Alternanthera philoxeroides* from terrestrial to Cr-contaminated aquatic habitats, and the subsequent decomposition process in a terrestrial habitat. Specially, we tested the hypothesis that the transmission of heavy metals among the clonal systems growing in different habitats may lead to the translocation of pollution to originally uncontaminated environments.

2 Materials and methods

2.1 Plant species

Alternanthera philoxeroides (Mart.) Griseb (Amaranthaceae) is an amphibious, stoloniferous, perennial grass originally coming from South America [23]. It has invaded many countries in tropic and/or temperate regions worldwide, and is widely distributed in terrestrial (e.g., crop lands, roadside) and aquatic (e.g., still water, river flow) habitats in China [24, 25]. The creeping stem can grow erect and produce new shoots, opposite leaves and adventitious or perennial roots at any nodes. All the ramets integrated by stolon runners form a whole clone system in which

resource-sharing plays an important role in adaptation and invasion across physiological integration [4, 24-27]. The vegetative propagation with stems or perennial roots can help the plant to expand quickly to occupy both terrestrial and aquatic areas [24, 25].

2.2 The experiment and measurements

In 2014, plants of *A. philoxeroides* were collected along the bank of Moshui River in Qingdao, Shandong Province, China and then propagated in the greenhouse located at Qingdao Agricultural University, China (36°19'7.62"N, 120°23'57.40"E). In May 2016, we selected small fragments of *A. philoxeroides* at the same developmental stage each consisting of a perennial root, a single creeping stem and an apex. These clonal fragments were cut off from the clone population and transplanted into pots (40 cm×16 cm×13.8 cm, length × width × height; Fig. 1). Each pot was filled with a 1:1 mixture of soil and sand, and four grams of solid slow-release fertilizer (16N-11P₂O₅-11K₂O-3MgO + trace elements, 3-4 months, Osmocote Exact, Scotts International B.V, Heerlen, Netherlands) simulating terrestrial habitats. The rooting position of the ramets in pots was about one fourth of the pot length to leave enough space for growth and stolon extension. The pots simulating aquatic habitats, filled with tap water, were put next to the pots with soil mixture (Fig. 1A and B). For each clonal fragment, the ramets in soil were termed the 'basal part', and the ramets with their apex in water were the 'apical part'. After 2 weeks of recovery (on 1 June 2016), we selected 126 clonal fragments at similar size for the experiment.

To test the toxic effect of Cr on the basal and the apical ramets and associated transmission between the connected ramets, a factorial design was used with three levels of Cr pollution, i.e., no pollution (0 μM), low- (100 μM) and high-level (300 μM), and two levels of stolon connection treatments, i.e., connected or disconnected, resulting in a total of six treatments. The selected 42 fragments were randomly assigned to each of the six treatments. The remaining 84 ramets, prepared for the decomposition experiment, were kept intact and randomly distributed to the three pollution treatments. For the treatments of pollution in aquatic environments, Cr was added as potassium dichromate (K₂Cr₂O₇) into the tap water in a unique dose to simulate Cr pollution. During the experiment, fragments in terrestrial environments were watered regularly. The water surface elevations in aquatic environments were maintained at the nearly same height. For the stolon connection treatments, the

stolon between the basal and apical ramets was severed or remained intact.

After 60 days' growth, all the fragments in terrestrial and aquatic habitats were severed and harvested separately. The plants were separated into leaves, roots and stems with sharp scissors. The roots were carefully washed. The 42 plants originally assigned to the six treatments were used for the measurements of dry biomass and Cr concentrations. The dry biomass was determined after the leaves, roots and stems had been dried in a stove at 70°C for 48 h. After the measurement of dry biomass, Cr contents in different organisms were analyzed. Samples of leaves, roots and stems were ground using a ball mill (DECO-PBM-V-4L, Changsha, Hunan Province, China) and dried to constant weight. The homogenized samples were put into Teflon crucibles (effective volume 100 ml) with 25 ml H₂O₂ / HNO₃ at a ratio of 1:4 (v / v) for eight hours. Crucibles were sealed with steel lids and put into the stove for one hour at 80°C. After hydrolyzation, crucibles were heated on an electrical heating panel (MWJ-3020, Wuxi, Jiangsu Province, China) at 120°C for two hours to remove excess acid from the solution. The Cr contents of the extracts were analyzed by inductively coupled plasma-optical emission spectrometry (Optima 8000, Perkin Elmer, Massachusetts, the USA).

To test the Cr release from basal ramets in terrestrial habitats, a decomposition experiment was carried out with the remaining 84 connected basal ramets from August to November. For these intact fragments under different levels of Cr pollution, the cleaned roots, stems and leaves of the basal ramets were separated and cut into pieces about 1~3 cm and mixed. We randomly selected 14 fragments from each pollution level (in total 42 fragments) and dried the plant litter to a constant weight. To determine decomposition rate, a batch of dried litter of 1 g was weighed and placed into 15 cm × 10 cm nylon bags of 0.5 mm mesh size. These bags were placed in the pots simulating terrestrial habitats, covered with soil, and watered regularly (Fig. 1C). There were 15 replicates from each pollution level. Three bags of each decomposition treatment were randomly collected on day 0, 20, 40, 60 and 100 for the measurement of mass decomposition and total Cr concentration in the residual tissues using the same method. The whole experiment lasted for 100 days. All the litter was carefully washed after collection. The remaining 42 fresh plant litter samples were used for the measurement of the different forms of Cr. A batch of fresh litter of 3 g was treated in the same way. After collection, the fresh litter was combined with 50 ml extracting solution in a beaker for 12 hours. Then, the litter was extracted for two hours using the same solution twice. All the extracting solutions were gathered together for the test of Cr concentration. The solutions and sequences of extraction were 80% ethanol, deionized water, 1 mol L⁻¹ sodium chloride solution, 2% acetic acid solution and 0.6 mol L⁻¹ hydrochloric acid solution. The chemical forms of Cr were nitrate, chlorate, ammonium (F1), acylate, dihydric phosphate (F2), pectate, protein conjugates (F3), diphosphate, phosphate (F4), oxalate (F5) and residual form (F6).

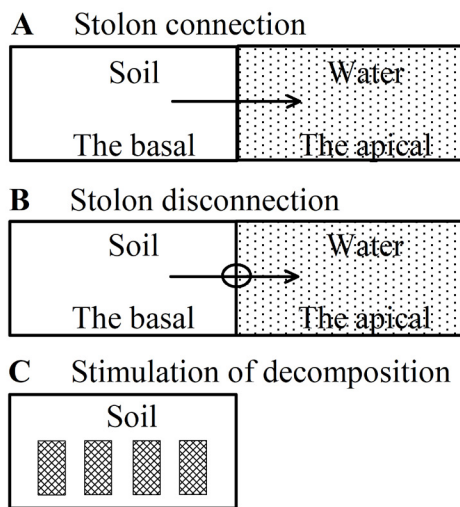


Figure 1. Schematic illustration describes the experimental design. One pot was filled with 1:1 mixture (composed of soil and sand) to simulate the terrestrial habitat and one pot was filled with tap water to simulate the aquatic habitat. The basal ramets were rooted in the soil and the apical ramets were placed in the water. Potassium dichromate was added into water to simulate different concentrations of Cr pollution. The basal and the apical ramets were either kept connection (A) or disconnection by severing the stolon where circle was (B). The decomposition experiment was conducted in the pots simulating the terrestrial habitats (C).

2.3 Data processing and analysis

The residual mass and Cr content of the dry plant litter and associated decomposition coefficient were used to describe the decomposition rate. The decomposition variation was fitted to the simple exponential model:

$$X_t = X_0 e^{-kt}$$

where X_t is the residual mass or Cr content of the dried plant litter, X_0 is the initial mass or Cr content of the dried plant litter, e is the natural log constant, k is the decomposition rate constant (day⁻¹, d⁻¹ in short hereafter), and t is the decomposition time (day, d in short hereafter).

Two-way ANOVA was used to test the effects of physical connection and different Cr concentrations on growth and Cr accumulation traits. Before analysis, data were checked for equality of variance with Levene's test and for normality with the Shapiro–Wilk test. One-way ANOVA with Tukey's post hoc test was used to test the variations in different traits in decomposition. SPSS 21 (IBM Inc., Armonk, New York, USA) was used for all of the statistical analyses. $P < 0.05$ was used as the significance level.

3 Results

3.1 Growth and Cr accumulation in the apical and the basal ramets

Physical connection between the basal and the apical ramets, different levels of Cr pollution, and their interaction had significant effects on all the growth variables of the apical parts of *A. philoxeroides* (Table 1). Under both low and high Cr concentrations, the biomass decreased markedly (Table 1; Fig. 2). Without Cr pollution, there were no significant differences in the variables between the connected and the disconnected ramets except leaf mass (Table 1; Fig. 2). However, when the plants in aquatic habitats suffered from Cr pollution, the connected ramets had distinctly higher values than these disconnected ramets (Table 1; Fig. 2). As for the basal part, Cr pollution significantly affected the biomass of different organs except for leaf (Table 1; Fig. 2). The low level of Cr pollution slightly increased root mass, but the high level decreased the root mass (Table 1; Fig. 2). The disconnected ramets of the basal part had higher total mass than those connected with the apical ramets in polluted water (Table 1; Fig. 2).

In general, the Cr concentrations that accumulated in the plants were relatively higher in roots but relatively lower in aboveground sections (Fig. 3). In the apical part, increasing Cr pollution level significantly increased Cr concentrations in root, stems and leaves (Table 1; Fig. 3), but physical connection significantly decreased Cr concentration in the roots (Table 1; Fig. 3). As a result of physical integration via the stolon, Cr was detected in the basal ramets connected with the apical ramets under Cr pollution (Table 1; Fig. 3). Without stolon connection, pollutant Cr was not found in the basal parts (Table 1; Fig. 3).

3.2 Decomposition of the basal ramets

On the whole, the residual mass of the plant litter decreased over time (Table 1; Fig. 4A). The average decomposition rate constants (d^{-1}) of the basal ramets originally connected with apical ramets under 0, 100 and 300 μM Cr pollution were 0.0049, 0.0067 and 0.0054 d^{-1} , separately (Table 1; Fig. 4A). In the first sixty days, the residual mass of the plant litter under low-level pollution was significantly smaller than those under 0 and high-level pollution (Fig. 4A). Then the decomposition of residual biomass entered a slow stage. In the end of the experiment, the mean residual mass ratios of the plant litter under different levels of pollution were 60.88%, 51.37% and 58.87% of the initial mass respectively (Fig. 4A).

There were significant differences in the total Cr concentrations between the different decay phases of the plant litter suffering from low- ($P=0.030$) and high-level ($P=0.016$) Cr pollution (Fig. 4B). The concentrations of Cr preserved in the plant litter under low- and high-level Cr pollution slightly increased in the first twenty days, and

Table 1. Results of two-way analyses of variance (ANOVA) for effects of physical connection (C), different Cr pollution (P), and their interactions (C×P) on growth and Cr accumulation characteristics of the basal and the apical ramets in terrestrial and aquatic habitats.

Effect	Root mass	Stem mass	Leaf mass	Total mass	Cr in root	Cr in stem	Cr in leaf
Basal part							
C	4.13*	0.04 ^{ns}	0.82 ^{ns}	1.24 ^{ns}	661.42***	279.57***	349.86***
P	5.43**	4.22*	2.08 ^{ns}	9.59***	181.91***	76.49***	94.01***
C×P	2.34 ^{ns}	1.53 ^{ns}	1.02 ^{ns}	3.67*	181.91***	76.49***	94.01***
Apical part							
C	0.00 ^{ns}	7.14*	22.69***	11.10**	12.82**	0.10 ^{ns}	0.03 ^{ns}
P	89.29***	32.26***	6.12**	35.59***	269.56***	186.46***	92.32***
C×P	3.38*	4.64*	0.28 ^{ns}	4.22*	3.53*	1.12 ^{ns}	1.32 ^{ns}

F values and significance levels are given (*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, ^{ns} $P > 0.05$).

then decreased (Fig. 4B). The final Cr concentrations in the ramets of both originally low- ($P=0.023$) and high-level ($P=0.017$) Cr treatments were significantly lower than the initial values (Fig. 4B). On the other hand, the total Cr concentrations of the plant litter under originally low-level Cr pollution were smaller than the high-level at all stages (Fig. 4B).

With the decay of the plant litter, Cr retained in the litter was released back into the environment gradually (Fig. 4C). Little Cr was released with decomposition in the first twenty days, and the release speed of Cr then accelerated in the next forty days. After sixty days, the release speed of Cr slowed again with the decrease in mass decomposition (Fig. 4C). In the end, about 66.07% and 60.84% of Cr in the plant litter under low- and high-level pollution were released into the environment (Fig. 4C). The projected liberation periods of 99% Cr under low- and high-level Cr levels were approximately 794 and 927 days respectively.

In the original fresh plant litter under different Cr pollution levels, the chemical forms 1, 4 and 5 accounted for the major proportion (Fig. 5) of the Cr. As the decomposition of plant litter proceeded, the proportions of the chemical forms 1, 2, 3 and 4 decreased overall, while the forms 5 and 6 increased gradually (Fig. 5). There were no significant differences in the forms 2, 3, 4 and 5 between the plant litter under different Cr pollution levels (Fig. 5). The amount of form 6 in fresh plant litter under low-level pollution was higher than that of high-level in the end (Fig. 5). Finally, the percentages of the forms with relatively low activity (F4, F5 and F6) became higher than the those with relatively high activity (F1, F2 and F3; Fig. 5).

4 Discussion

The results of this study demonstrated that the performances of the clonal fragments were affected by the existence of a physical connection and by the environment in which the connected ramets lived. The Cr pollution resulted in significant reduction in the biomass of the apical parts of *A. philoxeroides* separated from the basal parts. When the physical connection was kept intact, support from the basal ramets was able to alleviate the stress of Cr pollution exerted on the apical ramets. This agrees with many previous studies finding that physiological integration benefits ramets of other amphibious clonal species suffering from various environmental stresses, e.g., shading, flooding, lack of nutrients, heavy metal pollution, and so on [3, 5, 7,

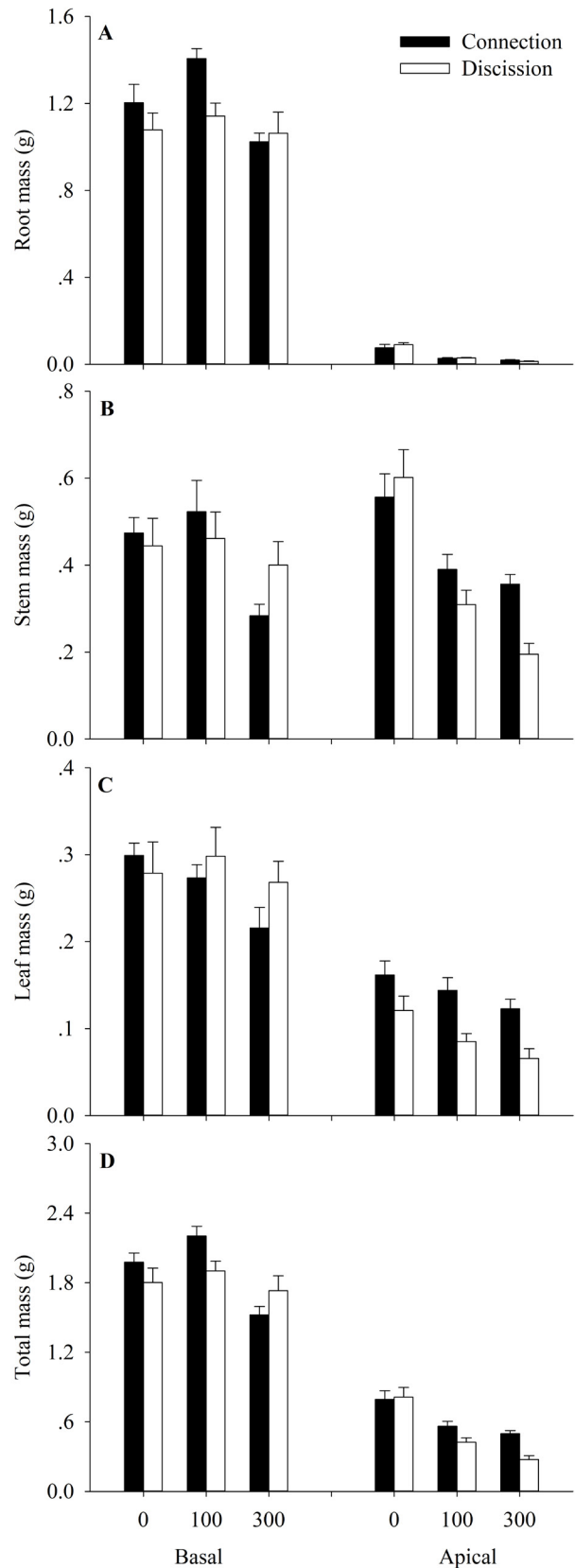


Figure 2. Dry biomass of root (A), stem (B), leaf (C) and total plants (D) of the basal and the apical ramets under different levels of Cr pollution. Data are mean \pm SE.

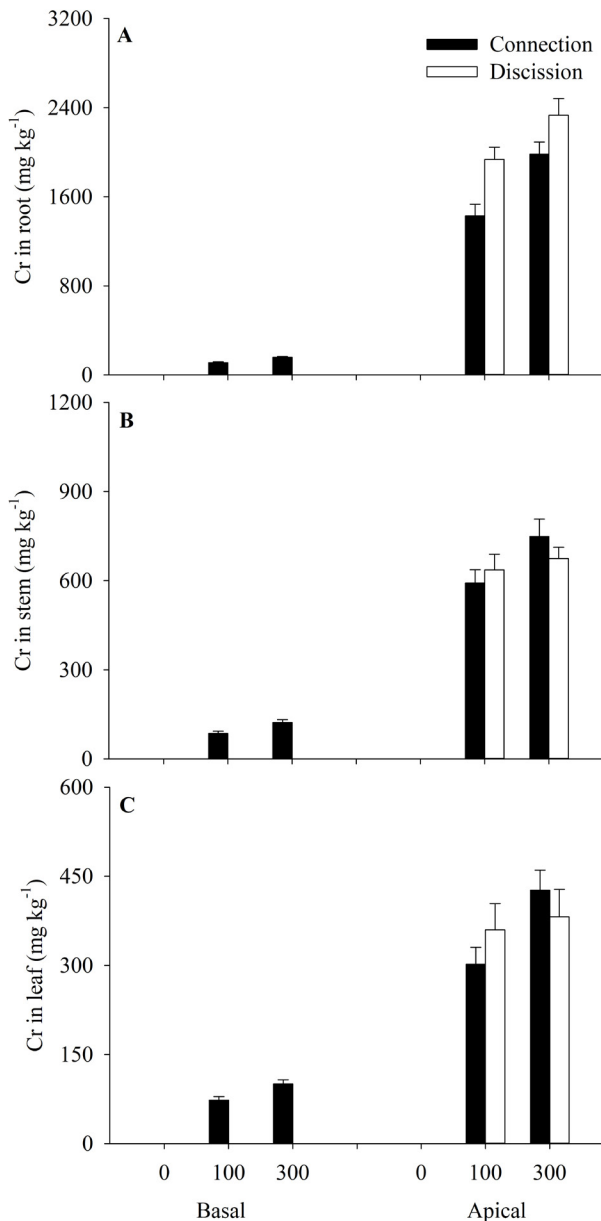


Figure 3. Cr concentrations accumulated in roots (A), stem (B) and leaf (C) of the basal and the apical ramets under different levels of Cr pollution. Data are mean \pm SE.

28]. In the natural ecosystem, the resources or stresses are constantly in patchy distribution. However, the distribution of pollutants in the water is generally uniform within certain areas, so it is not easy for the plants to escape from a polluted aquatic environment. Therefore, physiological integration may help the amphibious clonal species *A. philoxeroides* to tolerate pollution in the water not only at the early establishment stage, but also in the subsequent growth and spreading stages.

In some cases physiological integration is of great benefit to the ramets in stressful habitats at the cost of

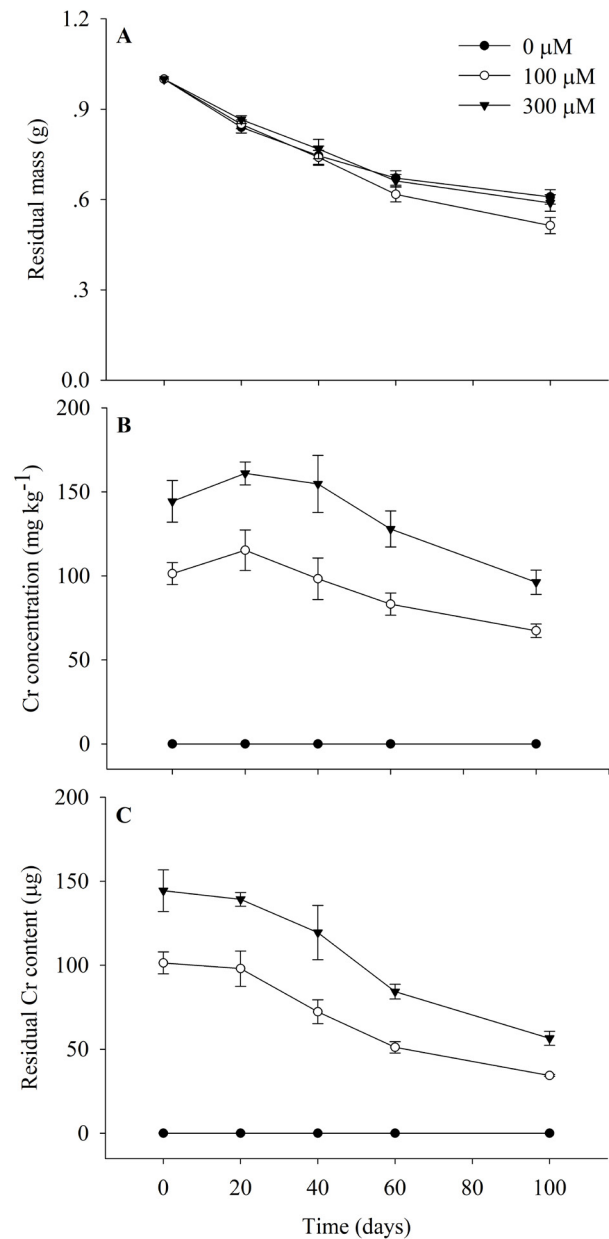


Figure 4. Changes in dry residual mass (A), Cr concentrations (B) and Cr content retained in the plant litter (C) of the basal ramets under different levels of Cr pollution during the decomposition period. Data are mean \pm SE.

the donor ramets most likely by the transfer of resources [7, 29]. However, physiological integration may not always impose such a cost, and may even be a positive stimulus for the photosynthesis and growth of the donors [1, 3, 29], perhaps due to the bidirectional transmission of surplus resources between donor and recipient ramets [1]. As a result, physiological connection between ramets is not only likely to allow transmission of resources, e.g., water, nutrients, photosynthates [1, 4, 5], but also heavy metals [2, 10, 19], from the apical ramets under pollution to the unpolluted basal parts. In a polluted aqueous environment,

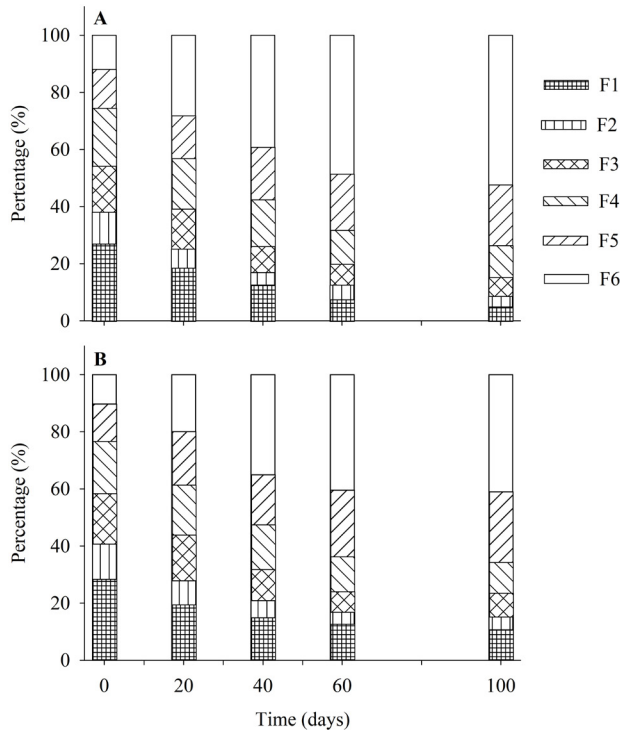


Figure 5. Percentages of different Cr chemical forms of the basal ramets under low-level (A) and high-level (B) of Cr pollution during the decomposition period.

Cr was firstly taken up by the adventitious roots and then horizontally moved to the stems and leaves. The pollutant Cr retained in the apical ramets was partly transferred to the basal ramets via connected stolons and then redistributed basipetally to belowground parts and acropetally to leaves and stems. The toxicity effect of heavy metals might appear when accumulation reaches certain levels [30]. With the stimulation of the low-level Cr pollution, the growth characteristics of the plants that had stolon connection increased, suggesting that the transmission of low-dose pollutants instead of surplus resources might be able to play a positive role in growth. If the concentrations of the heavy metal are increased, accumulation of the pollutant through physiological integration may make the ramets that are exposed to pollution indirectly lose the beneficial or neutral effects, and even reduce the fitness of the entire clonal fragments, depending on the plant species and variations of the stress [11]. Thus, with excessive accumulation of heavy metals transferred from the apical parts exceeding the threshold, the basal ramets may be poisoned (e.g., causing oxidative damage, mechanical damage) [2, 31], resulting in great growth reduction, defoliation, withering and even death of the plants.

By means of clonal amphibious plants living in aquatic-terrestrial ecotones, heavy metal in polluted

aquatic habitats is likely to spread to unpolluted terrestrial habitats. Decomposition of senescent vegetation plays an important role in element cycling, leading to release of retained elements into the surroundings. However, while most research has paid close attention to the release of nutrients after plant senescence, few have focused on the leaching of heavy metals along with the nutrients [32]. It is imperative to take the liberation of fixed Cr into account when considering the amount and transfer of pollutants. Generally the vegetation will start decaying naturally by the end of summer or autumn, and will degrade in the rest of the year [20, 33]. Plants that become withered or senescent as a result of a less-favorable environment also add to the decomposition supply. The plants' decomposition process and the associated release process to the ecosystem were characterized by obvious stages [21, 22], and affected by a number of environmental factors [22, 34]. In the first, the decay rate in low-level pollution was faster than those in control and high-level Cr concentrations (Table 1; Fig. 1), implying that low Cr intensity may have a promotive effect on the growth of microorganic communities, but the communities' performance parameters including activity, abundance and community composition, were inhibited under high intensity stress. On the whole, the pH of the decomposition environment decreased over time, possibly resulting from the accumulation of Cr in water and the increase in microorganisms.

A low level of heavy metal stress may stimulate the activity of microorganisms involved in decomposition, but high concentrations or prolonged low-level stress may negatively affect microorganism growth and metabolism, even deplete their biodegradation function [35]. The decomposition rate of the residual mass decreased over time, but Cr release remained in line with that of mass. With the release of Cr preserved in the plant litter, the decomposition rates of residual mass and the retained Cr decreased gradually after sixty days, suggesting that the low level of Cr was likely to have a positive effect, but the high level might inhibit the performance of the microorganic communities. On the other hand, the toxicity of Cr was also directly correlated with chemical forms [35]. Generally, Cr combined with nutrients and proteins were relatively more easily released than the residual forms. The biological activity and solubility of F1 and F2 are relatively high in the chromium rank, while the characteristics of F4 and F6 are relatively low. The decreased percentages of F1 and F2 suggested that they were easily freed from the plant litter. The water-insoluble Cr was mostly retained in the plant litter during the study period. The adverse effects gradually appeared with the enrichment of Cr in active forms. Theoretically, the whole

decomposition period may last for a long time, even a few years, releasing all the Cr stored in the substance. If such translocation between polluted and unpolluted ramets and release of pollutants occur widely, then there will be a potential risk for the spreading of heavy metals from polluted aquatic habitats to unpolluted terrestrial habitats, and slow accumulation therein [3, 11].

In conclusion, the present study found that physiological integration between the basal and apical ramets of *A. philoxeroides* could enhance the performance of the ramets established in stressful environments. Furthermore, pollutant Cr could be spread among the clonal fragments rooting in heterogeneously terrestrial and aquatic habitats through horizontal stolons. With decomposition of plant litter in the unpolluted terrestrial habitats, the Cr translocated from the apical ramets growing in polluted aquatic habitats was released and accumulated in the surrounding soils, contributing to the transfer of pollutants from polluted aquatic ecosystems to unpolluted terrestrial environments. The observation in this study adds to our understanding of how the transfer of heavy metal pollution between aquatic and terrestrial habitats could be conducted through amphibious clonal plants.

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