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Nitrogen Metabolism in *Acorus calamus* L. Leaves Induced Changes in Response to Microcystin–LR at Environmentally Relevant Concentrations

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Abstract

Acorus calamus L., a semiaquatic plant with a high capacity to remove nitrogen and phosphorus from polluted water, is a potential candidate plant for use in the restoration of eutrophic aquatic ecosystems. However, it is not clear how microcystins (MCs), commonly found in eutrophic water, influence plant growth since the effects of MCs are likely to be dose and species dependent. The present study aimed to investigate the regulation of nitrogen metabolism, a key metabolic process related to plant growth, in the leaves of *A. calamus* L. exposed to microcystin–leucine-arginine (MC–LR) (1.0–29.8 μg/L). Nitrate (NO_3^-) uptake, assimilation and transformation was stimulated in the leaves of *A. calamus* L. when exposed to 1.0 μg/L MC–LR through the elevation of nitrate reductase (NR), glutamine synthetase (GS), glutamate synthase (GOGAT), glutamic-pyruvic transaminase (GPT), and glutamic-oxaloacetic transaminase (GOT) activity. Conversely, MC–LR inhibited nitrogen metabolism by decreasing NO_3^- uptake and the activities of enzymes related to nitrogen metabolism following exposure to MC–LR (9.9–29.8 μg/L) for 30 days, while, ammonium nitrogen (NH_4^+) content and glutamate dehydrogenase (GDH) activity increased significantly ($p < 0.05$, LSD test), when compared with the control group. Chronic exposure to MC–LR (9.9–29.8 μg/L) negatively influenced nitrogen metabolism in *A. calamus* L. leaves, which suggested that it may not be a suitable candidate species for use in the restoration of eutrophic aquatic ecosystems containing MC–LR at concentrations ≥ 9.9 μg/L.

Keywords Microcystin-leucine-arginine · Environmentally relevant concentrations · *Acorus calamus* Linnaeus · Nitrogen metabolism

Aquatic plants are widely used to remove excess nutrients in eutrophic water (Zhang et al. 2018a). However, algal toxins, particularly microcystins (MCs), which are commonly found in eutrophic water, are one of the key biotic stresses to aquatic plants in eutrophic environments. MCs may alter plant physiological processes and negatively influence plant growth, which would in turn influence nutrient removal efficiency (Wang and Wang 2018). Consequently, whether a plant species can survive in MCs-contaminated water is a key factor to consider when selecting appropriate plants for use in water restoration activities. Nitrogen metabolism is an important metabolic process that is related to plant growth. The process is regulated by the activities of key enzymes, such as nitrate reductase (NR), glutamine synthetase (GS), glutamate synthase (GOGAT), glutamate dehydrogenase (GDH), glutamic-pyruvic transaminase (GPT) and glutamic-oxaloacetic transaminase (GOT) (Fu et al. 2018). In addition, nitrogen metabolism plays a major role in the biosynthesis of important compounds in plants, such as amino acids, proteins, nucleic acids and chlorophylls. The regulation of nitrogen metabolism in plants, therefore, could influence plant MCs tolerance. Consequently, studying nitrogen metabolism in plants exposed to MCs at environmentally relevant concentrations is essential in assessing their potential application in eutrophic aquatic ecosystem restoration activities.

At present, more than 90 MCs isoforms have been identified (Pantelic et al. 2013). Microcystin–leucine-arginine (MC–LR), the most toxic isoform, is widely distributed in eutrophic water (Amrani et al. 2014; Major et al. 2018; Hu et al. 2018). Some previous studies have reported that

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MC–LR (≤ 100 µg/L) could negatively influence chlorophyll content, net photosynthetic rate, germination, root and plant growth (Rojo et al. 2013; Pereira et al. 2009; Wang and Wang 2018), and alter protein folding and biosynthesis (Azevedo et al. 2014) or negatively affect activities of key enzymes linked to nitrogen assimilation in plants (Lahrouni et al. 2016). In addition, the studies reported that the effects of MC–LR on plants (promotion, inhibition, or insignificant effects) may vary based on exposure time, dosage, or plant species. In most cases, MC–LR concentrations in eutrophic water are below 30 µg/L (Major et al. 2018; Zhang et al. 2018b; Hu et al. 2018). In eutrophic water, where the MC–LR isoform generally persists for up to several months or even all year round (Amrani et al. 2014; Zhang et al. 2018b; Hu et al. 2018), chronic exposure is likely to occur, which calls for the investigation of the chronic effects of MC–LR on aquatic plants at low concentrations (< 30 µg/L).

Acorus calamus L., a semiaquatic plant, can effectively assimilate nitrogen and phosphorus from polluted water (Peng et al. 2012). Therefore, it is a potential candidate species for use in the restoration of eutrophic aquatic ecosystems. The present study investigated the effects of exposure of *A. calamus* L. leaves to MC–LR (< 30 µg/L) for 15 or 30 days on their nitrogen metabolism by determining chlorophyll-*a*, nitrate (NO_3^-), ammonium nitrogen (NH_4^+), and soluble protein (SP) contents, in addition to NR, GS, GOGAT, GDH, GPT and GOT activity. The results could provide insights into the physiological changes that occur in *A. calamus* L. following chronic exposure to MC–LR and *A. calamus* L. to MC-LR tolerance, which could facilitate the determination of its suitability for adoption in restoration activities in eutrophic ecosystems containing MCs.

Materials and Methods

Acorus calamus L. plants with 10–12 cm long apical shoots were purchased from a local nursery (Xiamen, Fujian, China). They were then washed clean with deionized water and flushed three times with sterile water. Finally, each plant was inserted into a hole in a foam board with one foam board bearing three *A. calamus* L. shoots. The boards were then separately placed in a glass cylinder with 2 L of 1/4 Hoagland nutritive solution (pH 6.5) and pre-cultured for 5 days. Subsequently, the nutritive solution was replaced with 1/4 Hoagland nutritive solution containing different concentrations of MC–LR (0 µg/L as the control group, and 1.0, 9.9, and 29.8 µg/L as treatment groups). The pure MC-LR (purity ≥ 96%) was purchased from Alexis Biochemicals (Läufelfingen, Switzerland). The MC–LR concentrations in the nutritive solution were determined using enzyme linked immunoassay kit (detection limit 20 ng/L) (ShangHai HengYuan Biological Technology Co., Ltd China). There were three replicates in each group. Therefore, there are nine shoots in each group. The glass cylinders were placed in an MGC-450BPY-2 intelligent light incubator. The plants were grown under a light:dark cycle of 14:10 h, with a photosynthetically active radiation of 50 µmol/(m^2s) (400–700 nm), and at a temperature of 25°C. The respective Hoagland nutrient solution in each of the groups were replaced every 3 days to maintain the nutrient and MC–LR concentrations at relatively stable concentrations. One plant was randomly selected from each glass cylinder for the collection of fresh leaves after exposure for 0, 15 and 30 days, respectively, with the aim of investigating the chronic effect of MC–LR on the nitrogen metabolism in *A. calamus* L.. The leaves were washed clean with deionized water and then blotted dry with clean tissue paper. Each sample was macerated in liquid nitrogen for 1 min and stored at − 80°C until analysis.

The content of NO_3^- was measured based on the absorbance values at 430 nm after the addition of salicylic acid and NaOH (Cataldo et al. 1975). The content of SP was estimated using Bradford G-250 reagent (Bradford 1976). The content of NH_4^+ was estimated using a colorimetric assay after boiling samples in a water bath for 15 min and measuring absorbance values at 580 nm after the addition of ninhydrin reagent and ascorbic acid. The content of chlorophyll-*a* was estimated based on the absorbance values of acetone extracts at 645 and 663 nm (Shanghai Institute of Plant Physiology, Chinese Academy of Sciences 1999).

The activity of NR was measured using a colorimetric assay following the appearance of NO_2^- at 540 nm after the addition of N-(1-naphthyl) ethylene diamine dihydrochloride and sulfanilamide. The activity of GS was determined by monitoring the production of γ-glutamylhydroxamate which was determined based on the absorbance at 540 nm. The activity of GDH was assayed by monitoring reduction of NAD^+, which was measured based on the absorbance at 340 nm. The activitives of GOT and GPT were measured according to changes in NADH based on the absorbance values at 340 nm (Shanghai Institute of Plant Physiology, Chinese Academy of Sciences 1999). In addition, the activity of GOGAT was estimated by monitoring NADH oxidation rates, which were determined based on the absorbance values at 340 nm (Chen and Cullimore 1988).

One-way analysis of variance (ANOVA) with a least-significant difference (LSD) test was used to assess differences between groups. Paried *t*-test was used to assess differences between parameters. Differences were considered as significant when $p < 0.05$. All the data were analyzed in SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA) and described as mean ± standard deviation (SD). The illustrations were created using SigmaPlot 10.0 (Systat Software, Inc., San Jose, CA, USA).

Results and Discussion

After 15 and 30 days of exposure to 1.0 µg/L MC-LR, NO_3^- contents were increased by 18.1%–19.2% ($p < 0.05$, LSD test) (Fig. 1), while GS, GOGAT, GPT and GOT activities were increased by 13.0%–17.1%, 16.7%–17.4%, 16.3%–19.7% and 20.5%–21.8% ($p < 0.05$, LSD test), respectively, comparing with the control group (Fig. 2), which indicated that exposure to 1.0 µg/L MC–LR would have a positive impact on the nitrogen metabolism in *A. calamus* L. leaves. Furthermore, 15 days exposure to MC–LR at 9.9 µg/L caused no significant change in the contents of NO_3^-, SP, and the activities of NR, GS, GOGAT, GPT and GOT ($p > 0.05$, LSD test), whereas, after 30 days exposure to the same concentration of MC–LR, SP content and NR, GS, GOT, GPT activities were reduced by 16.3%, 17.5%, 17.3%, 20.5% and 16.2% ($p < 0.05$, LSD test), respectively, as compared with the control group, which indicated that, exposure to MC-LR at 9.9 µg/L showed a negative effect on the nitrogen metabolism in *A. calamus* L. leaves with prolonging exposure time. Both 15 and 30 days of exposure to 29.8 µg/L MC–LR also inhibited the nitrogen metabolism with a significant decrease in the contents of NO_3^-, SP, and the activities of NR, GS, GOGAT, GOT ($p < 0.05$, LSD test), respectively, when compared with the control group (Fig. 2). Moreover, after 30 days of exposure to 9.9 and 29.8 µg/L MC–LR, NH_4^+ contents and GDH activities were increased by 27.1%–34.5% and 15.2%–44.8% ($p < 0.05$, LSD test), respectively, as compared with the control group. The above results indicated that the effect of MC–LR (1.0–29.8 µg/L) on plant nitrogen metabolism was dose and time dependent.

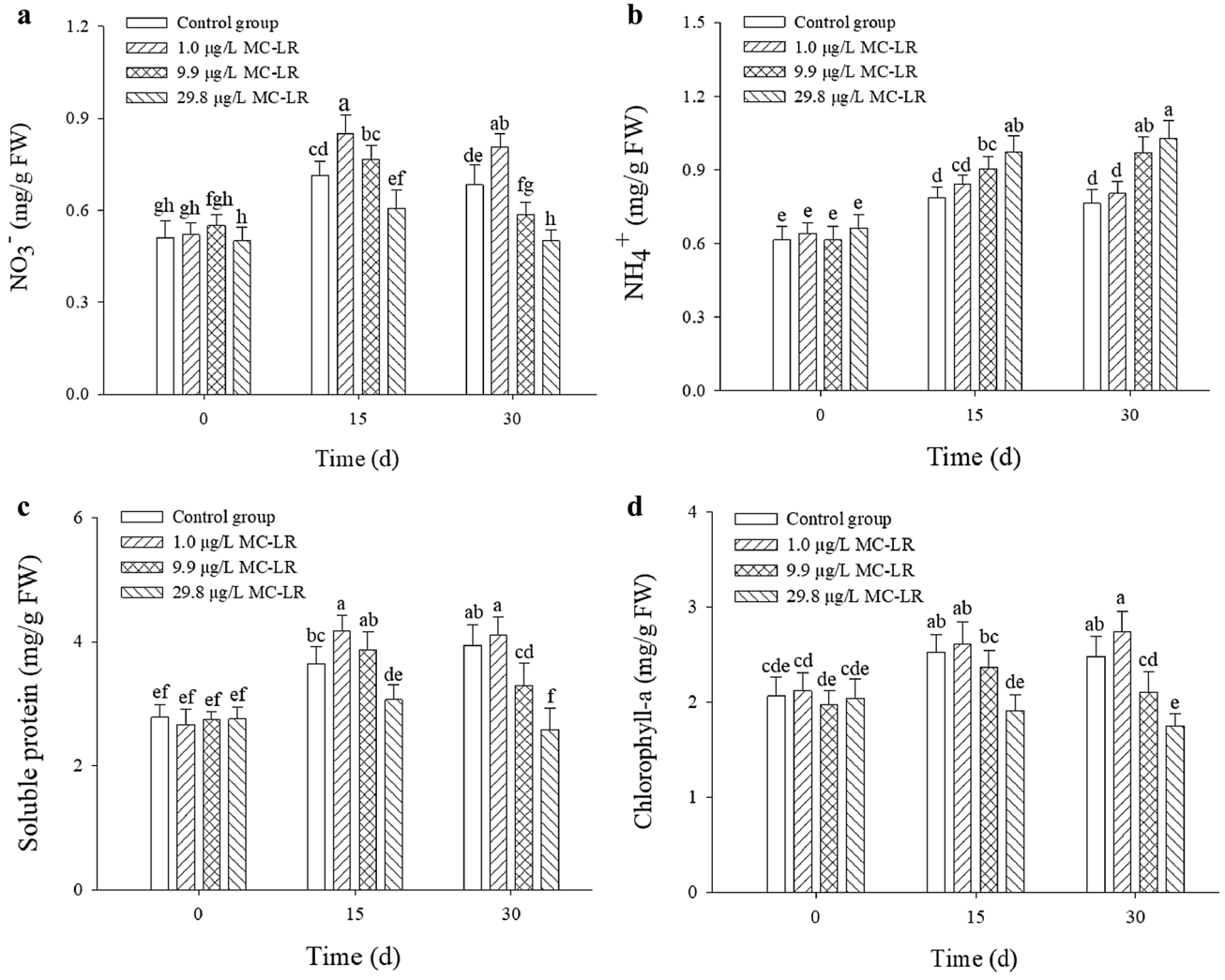

Fig. 1 The NO_3^- **a** NH_4^+ **b** soluble protein **c** and chlorophyll-a **d** contents in *A. calamus* L. leaves after 15 or 30 days of exposure to microcystin-leucine-arginine (MC–LR). Data are mean ± standard deviation (SD) of three replicates. Different lower case letters indicate significant differences ($p < 0.05$) between groups. FW means fresh weight

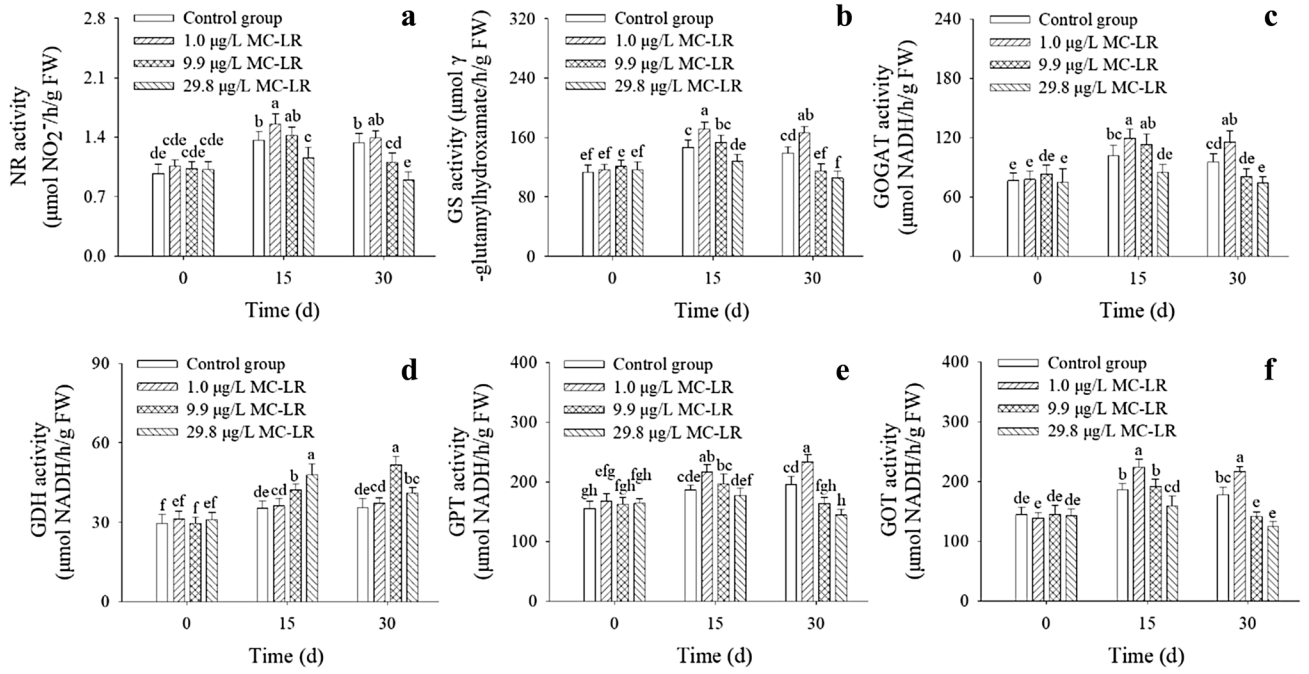

Fig. 2 The activities of nitrate reductase (NR) **a** glutamine synthetase (GS) **b** glutamate synthase (GOGAT) **c**, glutamate dehydrogenase (GDH) **d**, glutamic-pyruvic transaminase (GPT) **e**, and glutamic-oxaloacetic transaminase (GOT) **f** in *A. calamus* L. leaves after 15 or 30 days of exposure to microcystin-leucine-arginine (MC–LR). Data are mean ± standard deviation (SD) of three replicates. Different lower case letters indicate significant differences ($p < 0.05$) between groups. FW means fresh weight

In the present study, NO_3^- was the only nitrogen source supplied in the nutrient solution and it was absorbed by roots and then translocated to every part of the plant. The uptake of NO_3^- in plants depends on the efficiency of absorbing NO_3^- in plant roots (Parween et al. 2011). Our results showed that, compared with the control group, the length of the largest root of *A. calamus* L. and root biomass increased significantly ($p < 0.05$, LSD test) after 30 days of exposure to 1.0 μg/L MC–LR (data not shown). Chorus and Bartram (1999) also observed that, 1 μg/L MCs enhanced the rice growth at seedling stage. Plants would require more nutrients to facilitate growth after exposure to low level of MCs, which is consistent with the results in the present study, where 1.0 μg/L MC–LR stimulated NO_3^- uptake, which was beneficial to plant growth. However, Pereira et al. (2009) observed that aqueous extracts from *Microcystis aeruginosa* containing MC–LR (5.9–56.4 μg/L) inhibited the root growth in *Lactuca sativa* L. The result is consistent with our findings where 30 days of exposure to 9.9–29.8 μg/L MC–LR had a negative effect on root growth, particularly 29.8 μg/L MC–LR, which significantly ($p < 0.05$, LSD test) reduced the length of the largest root and root biomass of *A. calamus* L. (data not shown). The inhibition of root growth would affect NO_3^- absorption, which could also explain the significant reduction in NO_3^- uptake ($p < 0.05$, LSD test) after 30 days of exposure to MC–LR (9.9–29.8 μg/L), when compared with the control group. Nitrate reductase, a substrate-induced enzyme, is the rate-limiting enzyme in nitrogen assimilation in higher plants, and it in turn regulates nitrogen metabolism (Jawad et al. 2017). In the leaves of *A. calamus* L. exposed to 1.0 μg/L MC–LR, NO_3^- accumulation could be attributed to elevating in NR activity, and ultimately stimulated nitrogen assimilation and transformation. After 30 days of exposure to MC–LR (9.9–29.8 μg/L), the reduced NO_3^- content decreased NR activity ($p < 0.05$, LSD test), when compared with the control group. However, compared with the control group, NH_4^+, a key substrate of the GS/GOGAT cycle, was accumulated considerably ($p < 0.05$, LSD test) after 30 days of exposure to 29.8 μg/L MC–LR, although the GS/GOGAT cycle activity decreased ($p < 0.05$, LSD test), which indicated that the GS/GOGAT cycle could have been limited by other factors such as photosynthesis.

Photosynthesis, which provides energy (ATP) and carbonated skeletons (a-oxoglutarate) for NO_3^- reduction and amino acid synthesis (Lawlor 2002), is closely linked to nitrogen metabolism. Chlorophyll is the key pigment for photosynthesis, and its content is an important indicator of plant photosynthetic capacity (Liang and Wang 2015). Rojo et al. (2013) observed that, MC–LR (1–16 μg/L) significantly reduced chlorophyll a content and the net photosynthetic rate in five charophyte species. This result is inconsistent with our findings where compared with the control group, the chlorophyll-a content in *A. calamus* L. didn't change significantly ($p > 0.05$, LSD test) after exposure

to 1.0 µg/L MC–LR (Fig. 1d), which could be explained by species dependent MC–LR effects in plants (Rojo et al. 2013). However, compared with the control group, the chlorophyll-a content in *A. calamus* L. leaves decreased significantly ($p < 0.05$, LSD test) following 30 days of exposure to MC–LR (9.9–29.8 µg/L) (Fig. 1d). The result could be attributed to MC–LR induced oxidative stress (Pflugmacher et al. 2006). A reduction in chlorophyll a content would reduce the rate of photosynthesis, resulting in insufficient energy (ATP) and a-oxoglutarate, which are essential in the process of NO_3^- reduction and GS/GOGAT cycle (Lawlor 2002), in turn leading to a decrease in NR and GS/GOGAT cycle activity.

The GDH pathway, which plays a complementary role in glutamate metabolism, has been reported to facilitate resistance against various types of stress (Fu et al. 2018). Under saline–alkaline stress, NR, GS, GOGAT, GPT, and GOT activities decreased in two varieties of maize seedlings, while GDH activity and NH_4^+ contents increased (Fu et al. 2018). Under UV-B stress, NR, GS, GOGAT activities decreased in tomato seedlings, while GDH activity and NH_4^+ contents increased (Bashri et al. 2018). These results are consistent with our findings whereby GDH activity and NH_4^+ content in *A. calamus* L. leaves increased ($p < 0.05$, LSD test) after 15–30 days of exposure to MC–LR (9.9–29.8 µg/L), which indicated that, under MC–LR stress, *A. calamus* L. enhanced GDH activity to compensate for the reduction of ammonia assimilation, which was associated with the decrease in the GS/GOGAT cycle activity. However, GOGAT activity was still significantly ($p < 0.05$, Paired *t*-test) higher than GDH activity, which suggested that the GS/GOGAT cycle was still the dominant pathway for ammonia assimilation. GPT and GOT are the major enzymes associated with plant transamination (Fu et al. 2018). After 30 days of exposure to MC–LR (9.9–29.8 µg/L), a decrease in GPT and GOT activity in *A. calamus* L. leaves ($p < 0.05$, LSD test) compared with the control group could be due to the decline in NH_4^+ assimilation rates. Although GDH activity increased, GS/GOGAT cycle activity decreased significantly ($p < 0.05$, LSD test), resulting in insufficient glutamate substrate for GPT and GOT. The decreased GPT and GOT activities could result in reduced rates of synthesis of other amino acids, eventually resulting in reduced protein contents (Fig. 1c).

In summary, the present study demonstrated that exposure to 1.0 µg/L MC–LR stimulated NO_3^- uptake, assimilation, and transformation in *A. calamus* L. leaves, while, 30 days of exposure to MC–LR (9.9–29.8 µg/L) had adverse effects on nitrogen metabolism and protein synthesis, which would in turn have a negative impact on *A. calamus* L. growth. The findings highlight the need for studies investigating the chronic effects of different MCs concentrations in different species of plants or at different growth stages, which could facilitate the selection of suitable plants for use in the restoration of eutrophic water.

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