Separate and combined effects of cadmium (Cd) and nonylphenol (NP) on growth and antioxidative enzymes in *Hydrocharis dubia* (Bl.) Backer

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Abstract
Cadmium (Cd) is considered a priority pollutant, and nonylphenol (NP) is a common organic pollutant in water environments. However, the ecological risks of combined Cd and NP pollution have not been fully elucidated. In this study, the effects of Cd, NP, and Cd-NP on the growth and physiology of *Hydrocharis dubia* (Bl.) Backer were studied. The results indicated that Cd-NP joint toxicity is concentration-dependent. The joint toxicity of Cd and NP on *H. dubia* was antagonistic when the concentrations of Cd + NP were 0.01 + 0.1/1 mg/L. At 0.5 + 0.1/1 mg/L, Cd and NP had a strong synergistic effect on *H. dubia*. In addition, plant growth was significantly inhibited, and the chlorophyll contents were significantly reduced under Cd, NP, or Cd-NP exposure. The plant’s antioxidative enzyme system was destroyed. The activities of superoxide dismutase (SOD) and catalase (CAT) were significantly decreased under NP-only exposure. The activity of SOD was significantly decreased under Cd-only and under joint exposure. Compound pollution exceeded the oxidative defense capacity of the plants, so the *H₂O₂* content increased significantly. Our results indicated that the ecotoxicity of NP combined with Cd may be exacerbated in aquatic environments and cause obvious damage to *H. dubia*.

Keywords Nonylphenol · Cadmium · *Hydrocharis dubia* (Bl.) Backer · Compound pollution

Introduction
Cadmium (Cd) is a typical environmental pollutant with high toxicity. It can be biohazardous even if the concentration is in the range of 0.001 to 0.1 mg/L (Chellaiah, 2018). As a large industrial country, Chinese Cd mineral resources rank first in the world, accounting for 23% of the world’s total. The total amount of Cd discharged into the environment by industrial “three wastes” is more than 680 tons every year (Rehman et al., 2018). With the development of heavy metal smelting, mining, and other human activities, serious Cd pollution is occurring in local rivers, soil, and groundwater areas (Kersten et al., 2017), leading to an increase in Cd concentration in the water supply. It has been reported that the concentration of Cd was estimated to be significantly higher than that of chromium (Cr), nickel (Ni), copper (Cu), and zinc (Zn) in the Pearl River Estuary (Ye et al., 2020). The dissolved Cd values in many coastal waters around the world have varied from 1.5 ng/L to 50 μg/L in recent years (Achary et al., 2017; Juma and Al-Madany, 2008). Cd is harmful to plants and can be absorbed and accumulated through the food chain in plant tissues, affecting normal plant growth and development (Rizwan et al., 2016; Yan et al., 2021). The effects of Cd on aquatic plants have received increasing attention. It has been reported that growth inhibition, damage to the photosynthetic system, and oxidative damage of *Eichhornia crassipes* and *Lemna minor* L. all showed phototoxicity under Cd stress (Chaudhary and Sharma, 2019; Melignani et al., 2019).

Nonylphenol (NP) is a toxic heteromorphic compound, and it is one of the most important intermediates in the petrochemical and organic synthesis industries, being mainly used to produce antioxidants, lubricant additives, and nonionic surfactants (Deng et al., 2019; Sun et al., 2021). The annual use of NP in China accounts for 10% of the world’s use of NP (Gao...
et al., 2014). In recent years, the detection frequency of NP has been very high and it has even been detected in drinking water (Catav et al., 2020; Zhou et al., 2015). The concentrations of NP varied from 30.1 to 288.8 μg/L in various rivers and reservoirs in China (Jin et al., 2014). NP accumulates in algae, fish, and other aquatic organisms due to its high lipophilicity and persistent damage to organisms (Zhou et al., 2019). The basic toxicity of NP is mainly related to changes in cell membrane permeability and oxidative stress (Pretorius et al., 2016). NP exerts a strong inhibitory effect on cell growth and photosynthesis, and it contributes to oxidative stress in Chlorella pyrenoidosa and Scenedesmus obliquus (Yang et al., 2021). However, the effects of NP on aquatic plants are unknown.

In today’s environment, Cd and NP have been found simultaneously in lakes, rivers, and oceans, and Cd and NP will cause compound pollution to the water environment. However, most studies on the combined toxicity of Cd-NP have focused on animals. Ni et al. (2021) found that Cd and NP cocontamination severely inhibited the growth of ryegrass (Pseudomonas aeruginosa). Zheng et al. (2019) studied the interactions between Cd and NP on male Sebastiscus marmoratus and found that the concentrations of cotreatment resulted in an increase in brain aromatase activity and increased plasma 17 β-estradiol (E2) and VTG. However, data on the joint toxicity of Cd and NP to plants, especially aquatic plants, are scarce.

Hydrocharis dubia (Bl.) Backer is a perennial floating aquatic plant that grows in fresh or salty water. Its wide distribution range and large biomass as well as its ability to purify contaminated water make it an excellent aquatic plant for water monitoring and remediation (Liu et al., 2020). The present work aimed to (1) investigate the effects of Cd and NP and their joint stress on the growth and chlorophyll of H. dubia, and explore the mode of action of their combined ecotoxicological effects, and (2) study the responses of Cd, NP, and Cd-NP treatments on H. dubia’s antioxidant systems. Our research will offer a reference for the pollution control of heavy metals and organic pollutants and provide a basis for ecological toxicity effects and ecological safety assessments of combined pollution on aquatic plants.

Materials and methods

Cultivation and treatment of H. dubia

H. dubia plants were collected from a freshwater lake-Donghu (30°33.41′N, 114°22.59′E), Wuhan, Hubei Province. Before the experiment began, H. dubia plants were cultivated in a greenhouse. After 2 weeks of cultivation, each plant (3–5 leaves without ramets) was thoroughly cleaned and acclimated in a climate chamber for 3 days. All plants were grown in 10% Hoagland’s solution. The environmental conditions were regulated at 26 ± 1 °C with a 16/8 light/dark cycle and light intensity of 10,000 l×. (Zhong et al., 2018).

Toxicants and experimental design

The toxicants were CdCl2·2.5H2O and nonylphenol (C15H24O). The CdCl2·2.5H2O was purchased from Sinopharm Chemical Reagent Co., Ltd., Shanghai, China. The nonylphenol (with 99% purity) was obtained from Shanghai Macklin Biochemical Co., Ltd., Shanghai, China.

Depending on the environmental concentrations of two toxicants (Chen et al., 2016; De Bruin et al., 2017), Cd was set at 0.01, 0.1, 0.5, 1.0, and 5 mg/L, NP was set at 0.1, 0.5, 1.0, 5, and 10 mg/L, Cd+NP mixtures were set at 0.01 + 0.1, 0.01 + 1.0, 0.5 + 0.1, 0.5 + 1.0, 5 + 0.1, and 5 + 1.0 mg/L, respectively. The blank control (healthy control) was treated with 10% Hoagland culture solution of the same volume (5 L). All treatments of NP were assisted by 100 μL ethanol to make sure that the ethanol content was not more than 0.002%, which had negligible effects on H. dubia (Toyama et al., 2018). All treatments were set to three repetitions for 14 days. The experimental conditions were the same as described above. Solutions changed every 48 h to keep up approximately constant concentrations of toxicants and nutrients. After 14 days of exposure, harvested plants and determined various parameters.

Estimation of H. dubia growth

After 14 days, plants were rinsed with distilled water. After being dried on filter paper, every plant was measured individually and weighed. Morphological indices of the H. dubia included whole plant fresh weight (g), ramet fresh weight (g), maximum leaf width (cm), maximum leaf length (cm), ramet number, root length (cm), and leaf number, all of which were measured with a scale (accuracy 0.1 cm).

Photosynthetic pigments measurements

Photosynthetic pigments were determined using a method described by Jampeetong and Brix (2009) with some changes. Plant leaves (0.05 g, FW) were cut into many pieces, ground, and placed in 10-mL flasks. Then, the extraction of chlorophyll is done in 10 mL 95% (v/v) ethanol. Flasks were placed for 24 h and light-free. The chlorophyll content in the plant leaves was determined with a spectrophotometer at 470, 649, and 665 nm for chlorophyll a, chlorophyll b, and carotenoids, respectively. All spectrophotometric analyses were conducted in a final volume of 3 mL by a MAPADA UV-1200 spectrophotometer (Shanghai Meipuda Instrument Co. Ltd., Shanghai, China).
Measurement of thiobarbituric acid reactive substances (TBARS) content

TBARS content in leaves can be used to evaluate the level of lipid peroxidation in plants. The trichloroacetic acid (TBA) method (Cang and Zhao, 2013) was used to measure TBARS content. Plant leaves (0.05 g, FW) ground with 5 mL 10% trichloroacetic acid (TCA). The homogenate was centrifuged at 5000 g for 10 min at 4 °C. A total of 1.5 mL supernatant mixed with 1.5 mL 0.6% TBA solution, shaken and reacted in a boiling water bath for 30 min. Then cooled rapidly in the ice bath and centrifuged at 5000 g for 15 min at 4 °C. The supernatant was taken and measured the light absorption values at 450 nm, 532 nm, and 600 nm. The TBARS content in the leaves was calculated by the formula.

Measurement of and hydrogen peroxide (H₂O₂) content and enzyme activities

Plant leaves (0.1 g, FW) mixed with precooled phosphoric acid buffer (50 mM, pH 7.8, containing 1% PVP) in a pre-cooled mortar. The mixture was centrifuged at 10,000 g for 10 min at 4 °C. The supernatant was used to determine the activities of superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) and the contents of H₂O₂ and soluble protein.

The H₂O₂ content was examined according to Cang and Zhao (2013). A total of 1.4 mL supernatant mixed with 1.4 mL 5% (W/V) titanium sulfate for 10 min. Then the mixture was centrifuged at 5000 g for 10 min at 4 °C. The supernatant was taken to measure the absorbance at 410 nm by an ultraviolet photometer. The H₂O₂ content in plant tissues was calculated with the standard curve of absorbance value.

The method of nitrogen blue tetrazolium (NBT) photoreduction method was used to test the SOD activity (Cang and Zhao, 2013). Fifty percent inhibition of NBT photochemical reduction was defined as an enzyme activity unit (U/g·min FW). The reaction mixture contained enzyme extraction, methionine (Met) solution (130 mM), 50 mM phosphate buffer (pH 7.8), NBT solution (750 μM), EDTA-Na₂ (100 μM), riboflavin (20 μM), and distilled water. After the reaction stopped, the absorbance value of the reaction solution was tested at 560 nm immediately and calculated the SOD activity.

The CAT activity was measured according to the method described by Cang and Zhao (2013). The CAT enzymatic activity unit (U) was defined as a decrease of 0.1 in absorbance at 240 nm per minute. The reaction mixture included enzyme extraction from plant tissues, 20 mM phosphate buffer (pH 7.8, containing 1%PVP), distilled water, and H₂O₂ solution (0.1 M).

The activity of POD was determined following the guaiacol method (Cang and Zhao, 2013). An enzyme activity unit (U/g·min FW) of POD was defined as a 0.01 increase in absorbance of the reaction mixture at 470 nm per minute. The reaction mixture contained enzyme extraction, phosphoric acid buffer (100 mM, PH 6.0), guaiacol solution, and H₂O₂ solution (30%).

Measurement of soluble protein content

The soluble protein content was determined by spectrophotometry following the method described by Bradford (1976). A total of 0.1 mL extraction mixed with 5 mL Coomassie Bright Blue G-250 protein reagent in the test tube for 2 min. The absorbance was measured at 595 nm with blank as control. The protein content was calculated by the standard curve.

Joint toxicity assessment method

Abbott’s formula (Gatidou and Thomaidis, 2007) was used to evaluate the combined toxic effects of Cd and NP. The widely used model was used to compare the expected and observed inhibitory effects. In this model, the expected inhibition of combined pollutants expressed as the percentage of Cexp, calculated as follows:

\[ C_{exp} = A + B - AB/100 \]

where A and B are the inhibition levels caused by a single toxicant. Inhibition rates (RI) for combined pollutants were calculated as follows:

\[ RI = \frac{\text{observed inhibition}}{C_{exp}} \]

Interaction effects were evaluated by comparing RI with 1, where \( RI > 1 \) represents synergism, \( RI = 1 \) shows simple additivity, and \( RI < 1 \) indicates antagonism between the two toxicants. However, ecosystems are often exposed to complex pollution caused by multiple chemical pollutants. Therefore, the complex effect is not only a simple additivity, antagonism, and synergism but also involves independence, competition, protection, and so on. Therefore, this evaluation method still has limitations and is only suitable for a preliminary evaluation of the interaction effect of two pollutants.

Statistical analyses

All the data were expressed as mean ± standard deviation. The homogeneity of variance and normality of experimental values was checked by Levene’s test and Shapiro-Wilk test, respectively. SPSS 21.0 statistical analysis software was used to conduct one-way analysis of variance (ANOVA) and
Duncan’s test at $P = 0.05$ confidence level. SigmaPlot 12.5 software was used for drawing.

**Results**

**Morphology and growth indices**

After 14 days of exposure, *H. dubia* showed obvious damaging effects (Table 1). Under NP-only stress, when the concentration of NP was ≥ 1 mg/L, the whole plant fresh weight, maximum leaf width, maximum leaf length, and ramet number of *H. dubia* were all significantly lower than those of the control group ($P < 0.05$). When the concentration of NP was ≥ 5 mg/L, the ramet fresh weight and root length of *H. dubia* were significantly decreased compared to those of the control group ($P < 0.05$).

In the Cd-only groups, at higher concentrations (≥ 0.5 mg/L), the whole plant fresh weight, ramet fresh weight, and root length of *H. dubia* were significantly decreased compared to those of the control group ($P < 0.05$). At concentrations (≥ 0.1 mg/L), the maximum leaf width and maximum leaf length of *H. dubia* were significantly decreased ($P < 0.05$). At 1 mg/L, the ramet number and leaf number of *H. dubia* were significantly decreased compared with those of the control group ($P < 0.05$). The roots of *H. dubia* treated with a high concentration (1 mg/L) were seriously decayed and the root length could not be measured. The plants died at the highest concentration (5 mg/L).

Under combined stress, at Cd + NP ≥ 0.5 + 0.1 mg/L, *H. dubia*’s whole plant fresh weight, ramet fresh weight, maximum leaf width, maximum leaf length, ramet number, and leaf number decreased significantly compared with those of the control group ($P < 0.05$). The plants died at the highest concentrations, and the growth indices could not be measured (Table 1). The root length of *H. dubia* treated with Cd + NP (0.01 + 0.1 mg/L and 0.01 + 1 mg/L) was significantly lower than that of the control group ($P < 0.05$).

**Photosynthetic pigments content**

The photosynthetic pigment (chlorophyll a, chlorophyll b, and total chlorophyll) contents of *H. dubia* showed similar responses to NP, Cd, and Cd-NP exposure. At NP concentrations of 0.1 and 0.5 mg/L, the chlorophyll a content increased significantly compared with the control group ($P < 0.05$) (Fig. 1a). At NP concentrations of 5 and 10 mg/L, the photosynthetic pigment and carotenoid contents decreased significantly ($P < 0.05$) (Fig. 1e and g). Under Cd-only stress, the photosynthetic pigment contents

| Treatment   | Whole plant fresh weight (g) | Ramet fresh weight (g) | Maximum leaf-width (cm) | Maximum leaf-length (cm) | Ramet number | Root length (cm) | Leaf number |
|-------------|-----------------------------|------------------------|-------------------------|--------------------------|--------------|-----------------|-------------|
| 0           | 14.83 ± 10.36a               | 9.65 ± 8.90a           | 7.13 ± 0.35a            | 6.90 ± 0.20a             | 15.67 ± 10.79ab | 13.13 ± 4.97ab  | 33.00 ± 17.78abc |
| N0.1        | 16.60 ± 3.43a                | 9.25 ± 2.46a           | 6.23 ± 1.06a            | 5.90 ± 1.49a             | 26.00 ± 10.82a | 14.33 ± 5.96a   | 60.67 ± 23.54a  |
| N0.5        | 8.51 ± 1.87b                 | 3.81 ± 1.06b           | 5.40 ± 0.46b            | 5.00 ± 0.62b             | 15.67 ± 10.2ab | 7.63 ± 1.31bc   | 40.33 ± 17.16ab |
| N1          | 5.67 ± 1.35b                 | 3.35 ± 0.96ab          | 3.13 ± 3.10bc           | 3.03 ± 2.71bc            | 8.33 ± 6.66b   | 10.57 ± 2.84abc | 22.00 ± 13.45bcd |
| N5          | 2.36 ± 0.36b                 | 1.02 ± 0.38b           | 0.67 ± 0.70cd           | 0.77 ± 0.75cd            | 5.00 ± 3.61b   | 4.93 ± 1.16 cd  | 9.33 ± 5.13 cd  |
| N10         | 1.13 ± 1.06b                 | 0.18 ± 0.16b           | 0.23 ± 0.40d            | 0.30 ± 0.52d             | 0.67 ± 0.58b   | 1.00 ± 0.92d    | 1.00 ± 1.00d   |
| C0.01       | 11.98 ± 6.71ab               | 7.82 ± 4.66ab          | 6.43 ± 1.68a            | 5.63 ± 1.05a             | 10.00 ± 3.60ab | 8.93 ± 5.42ab   | 28.33 ± 9.50a  |
| C0.1        | 5.63 ± 1.74ab                | 3.76 ± 1.68ab          | 3.47 ± 1.10b            | 3.30 ± 1.15b             | 13.33 ± 3.06a  | 4.47 ± 1.42bc   | 28.33 ± 8.02a  |
| C0.5        | 2.17 ± 0.89b                 | 0.77 ± 0.57b           | 3.33 ± 0.066            | 2.90 ± 0.17b             | 7.33 ± 6.11ab  | 1.00 ± 1.25c    | 12.33 ± 13.05ab |
| C1          | 1.78 ± 0.08bc                | 0.40 ± 0.39b           | 2.73 ± 0.46f            | 2.50 ± 0.53b             | 1.67 ± 1.53b   | 0.00 ± 0.00     | 5.33 ± 2.08b   |
| C5          | /                            | /                      | /                       | /                        | /             | /               | /            |
| M0.01 + 0.1 | 7.35 ± 4.30ab                | 4.62 ± 1.81ab          | 4.27 ± 3.80ab           | 3.90 ± 3.57ab            | 9.00 ± 1.00ab  | 4.23 ± 2.87c    | 20.33 ± 4.04ab |
| M0.01 + 1   | 9.08 ± 0.32ab                | 5.55 ± 0.82ab          | 4.87 ± 0.47a            | 5.17 ± 1.24a             | 9.00 ± 3.61ab  | 7.70 ± 2.02bc   | 19.33 ± 7.23ab |
| M0.5 + 0.1  | 0.39 ± 0.68b                 | 0.22 ± 0.38b           | 0.53 ± 0.92c            | 0.93 ± 1.62b             | 1.33 ± 2.31b   | /               | 1.67 ± 2.89c   |
| M0.5 + 1    | 1.23 ± 0.57b                 | 0.67 ± 0.10b           | 0.97 ± 1.67bc           | 0.87 ± 1.50b             | 1.67 ± 0.58b   | /               | 4.33 ± 1.53bc  |
| M5 + 0      | /                            | /                      | /                       | /                        | /             | /               | /            |
| M5 + 1      | /                            | /                      | /                       | /                        | /             | /               | /            |

Values are mean ± SD for 3 replicates. Different letters show significant differences between treatment groups ($P < 0.05$, LSD test)
in the leaves of all treatments were significantly lower than those in the control group \((P < 0.05, \text{Fig. 1a and c})\). Under joint stress, photosynthetic pigment contents decreased significantly at \(\text{Cd} + \text{NP} \geq 0.01 + 1 \text{ mg/L} \) \((P < 0.05)\) (Fig. 1b and d). At 0.01 + 0.1 mg/L, the carotenoid content increased significantly; at 0.5 + 1 mg/L, it decreased significantly compared with the control group \((P < 0.05)\) (Fig. 1h).

**TBARS content**

After 14 days of exposure, at 0.1 mg/L, the TBARS content of the NP-only stress groups was slightly higher than that of the control group, but it did not reach a significant level (Fig. 2a). At the highest concentration (10 mg/L), because of serious leaf damage, the materials were not sufficient to determine their contents. Under Cd-only and combined stress, the TBARS content was not significantly different compared with that of the control group (Fig. 2a and b).

**H\textsubscript{2}O\textsubscript{2} content and antioxidant enzyme activity**

**H\textsubscript{2}O\textsubscript{2} content**

After 14 days of exposure, under NP-only stress, the \(\text{H}_2\text{O}_2\) content showed a tendency to increase first and then decrease. At 5 mg/L treatment, the \(\text{H}_2\text{O}_2\) content decreased significantly compared with that of the control group \((P < 0.05)\) (Fig. 3a). Under Cd-only stress, the \(\text{H}_2\text{O}_2\) content increased slightly, but did not reach a significant level. Under the combined stress, at 0.01 + 1 mg/L \(\text{Cd} + \text{NP}\), the \(\text{H}_2\text{O}_2\) content had a maximum, which was significantly higher than that of the control group \((P < 0.05)\) (Fig. 3b).

**SOD, CAT, and POD activity**

Under NP-only stress and combined stress, the SOD activity of \(H.\ dubia\) in all treatment groups decreased significantly compared with that in the control group \((P < 0.05)\) (Fig. 4a and b). When the Cd concentration was \(\leq 0.5 \text{ mg/L}\), the SOD activity also decreased significantly (Fig. 4a). The NP concentration was 5 mg/L, and the activity of CAT decreased significantly compared with that of the control group \((P < 0.05)\) (Fig. 4c). Under Cd-only and combined stress, there was no significant difference in CAT activity compared with the control group. Under NP-only, Cd-only and combined stress, with an increasing treatment concentration, the POD activity slightly increased (Fig. 4e and f). However, there was no significant difference in POD activity between any of the treatment groups and the control group.

**Soluble protein content**

Under NP-only and Cd-only stress, there was no significant difference in soluble protein content between the treatment groups and the control group (Fig. 5a). Under combined stress, at 0.5 + 1 mg/L \(\text{Cd} + \text{NP}\), the soluble protein content decreased significantly compared with that of the control group \((P < 0.05)\) (Fig. 5b).

**Evaluation of joint toxicity of cadmium and nonylphenol**

In this study, the inhibition rate of the total chlorophyll content was used to evaluate the combined toxicity of Cd and NP (Table 2). When \(\text{Cd} + \text{NP}\) was 0.01 + 0.1 mg/L and 0.01 + 1 mg/L, the RI value was significantly < 1, and the results showed that the two pollutants at these concentrations had antagonistic effects on \(H.\ dubia\). The RI value was significantly > 1, at higher concentrations of \(\text{Cd} + \text{NP}\) (0.5 + 0.1 mg/L and 0.5 + 1 mg/L). These results showed that the joint effect of the two toxicants on \(H.\ dubia\) was synergistic. At the highest concentrations (5 + 0.1 mg/L and 5 + 1 mg/L), the plants just died.

**Discussion**

In this experiment, we found that NP and Cd were highly toxic to \(H.\ dubia\). After 14 days of exposure, the roots and leaves of \(H.\ dubia\) were seriously damaged, and the root length and leaf number were significantly inhibited at higher NP concentrations. Similarly, Yang et al. (2020) studied the effects of NP on microalgae \(Chlorella pyrenoidosa\) and found that the cell density of \(C.\ pyrenoidosa\) was lower than that of the control group in NP toxicity testing. De Bruin et al. (2016) observed that NP (\(\geq 800 \mu\text{g/L}\) significantly impaired the growth of \(Lactuca sativa\) roots. However, there were no significant toxic effects on the plants and even some promoting effects at lower NP concentrations. This is a typical hormesis phenomenon (Calabrese, 2005). Under Cd stress alone, the fresh weight, leaf number, and root length of \(H.\ dubia\) decreased significantly compared with those of the control group at \(\geq 0.5 \text{ mg/L}\). At 5 mg/L, the plants were seriously poisoned and died. This result indicated that Cd seriously affected the normal growth of \(H.\ dubia\). Similarly, Ci et al. (2010) found that wheat seedling growth and gas exchange were generally depressed by Cd (50 \(\mu\text{M}\)) stress. Under the Cd-NP combined stress, plant growth indices decreased significantly compared with the control group. The results suggested that high Cd-NP stress inhibited plant growth.

An important cause of Cd poisoning is its high chemical similarity to functional active ions (especially Zn, but
also Ca and Fe) at active sites of enzymes and signal components, leading to their displacement from proteins (Gallego et al., 2012). Thus, it affects the absorption of many nutrients and micronutrients and inhibits plant growth. As a hydrophobic organic compound, NP easily penetrates the cell membrane and acts on the cell’s internal structure, thus stunting cell growth (Bhandari et al., 2021). The aggravation of plant compound pollution toxicity may be due to Cd changing the hydrophobicity of the cell membrane and increasing the absorption of NP. The experimental results showed that when exposed to the lowest concentration of Cd or NP, the growth of *H. dubia* was not significantly inhibited. In the concentration series, the lowest Cd concentration

![Fig. 1](image1.png)

**Fig. 1** The effects of Cd, NP, and Cd+NP on photosynthetic pigments of *H. dubia* after 14 days of exposure. Different letters in the bar show significant differences between treatment groups (*P* < 0.05, LSD test)

![Fig. 2](image2.png)

**Fig. 2** The effects of Cd, NP, and Cd+NP on TBARS content of *H. dubia* after 14 days of exposure. Different letters in the bar show significant differences between treatment groups (*P* < 0.05, LSD test)

![Fig. 3](image3.png)

**Fig. 3** The effects of Cd, NP, and Cd+NP on H₂O₂ content of *H. dubia* after 14 days of exposure. Different letters in the bar show significant differences between treatment groups (*P* < 0.05, LSD test)
(0.01 mg/L) was close to the corresponding environmentally related concentration (0.0015–0.05 mg/L) (Achary et al., 2017; Juma and Al-Madany, 2008), while the lowest NP concentration (0.1 mg/L) was close to the environmentally related concentration (0.0301–0.2888 mg/L) (Jin et al., 2014). Compared with the control, the plant growth inhibition after exposure to environmentally relevant concentrations of Cd or NP was negligible. However, after coexposed to environmentally relevant concentrations of Cd and NP, the plant root length was significantly damaged ($P < 0.05$). This suggests that even at fairly low concentrations, a combined exposure to Cd and NP will have ecotoxicological effects on aquatic plants, indicating we need to pay more attention to the joint effects of pollutants.

Chlorophyll is the material basis of photosynthesis, and its content can be used as a physiological index to measure the stress resistance of plants under environmental stress (Du et al., 2018). After 14 days of exposure, the chlorophyll a and chlorophyll b contents of H. dubia increased under NP (≤0.5 mg/L). This is consistent with the previous result that plant growth was promoted at low concentrations. However, the photosynthetic pigment contents of H. dubia decreased significantly under NP (≥1 mg/L), suggesting that H. dubia was seriously stressed. Similar to our results, Gao and Tam (2011) found that NP downregulated the chlorophyll a content and maximal photochemistry (Fv/Fm) of Chlorella vulgaris and Selenastrum capricornutum. Kim et al. (2019) also showed a decrease in chlorophyll content due to damage to the membrane barrier with an increase in cell permeability caused by NP. Under Cd-only stress, the photosynthetic pigment (chlorophyll a and b) and total chlorophyll contents all decreased. The carotenoid content decreased significantly compared with that of the control group, only when the Cd concentration was 5 mg/L. In addition, Han et al. (2020) found that the chlorophyll a concentration, oxygen evolution rate, and oxygen consumption rate of Sarcodia suiae decreased under Cd stress.

In this experiment, when H. dubia was subjected to lower combined concentrations (0.01 + 0.1 mg/L, 0.01 + 1 mg/L), the toxicity of Cd and NP had an antagonistic effect. When Cd + NP was 0.5 + 0.1 and 0.5 + 1 mg/L, the synergistic effect of the two pollutants on H. dubia was obvious. This may be due to (1) the hydroxyl and benzene rings of NP easily combining with Cd$^{2+}$ to form insoluble metal-NP complex compounds, which obviously affect the toxicity of NP and Cd on plants. (2) The complexation of NP and Cd becomes saturated at certain concentrations. Joint toxicity of Cd-NP enhances the permeability of cell membranes, enabling plants to absorb more pollutants, and thus they experience increased biological toxicity. Wang et al. (2018) studied the combined effect of Cd and 4-n-NP on Chlorella sorokiniana and observed a synergistic effect of Cd-NP on algal growth inhibition at 48 h and 72 h, and an additive effect was observed at 96 h.

Under stress or adverse environments, plants produce reactive oxygen species (ROS) such as superoxide free radicals (O$_2^-$), hydroxyl free radicals (OH$^-$) and H$_2$O$_2$. ROS can cause oxidative damage to lipids, proteins, and DNA in cells and induce oxidative stress (Liu et al., 2019). To remove excessive ROS, plants have formed a set of effective oxidation defense mechanisms and highly complex detoxification mechanisms, namely enzymatic (CAT, POD, and SOD) and nonenzymatic antioxidant protection systems (glutathione, acetylsalicylic acid, etc.). When the plant’s defense system cannot eliminate excessive ROS, it will cause damage to the cell and produce a large amount of TBARS (MDA). MDA is a marker of cell lipid peroxidation and it can indirectly reflect the level of ROS and the degree of damage to the plants (Malik et al., 2021).

The production and removal of H$_2$O$_2$ in plant tissues under adverse conditions can indirectly reflect the degree of plant damage caused by toxicants and the resistance of the plants. In this experiment, when the NP concentration was 5 mg/L, the H$_2$O$_2$ content was significantly reduced. Under Cd-only treatment, the H$_2$O$_2$ and TBARS contents were not significantly different compared with those of the control group. Under the combined treatment of single NP and Cd-NP, the content of TBARS in the plant did not change significantly. These results may be due to the significant decrease in SOD activity and the timely clearance of ROS by CAT and POD. In contrast to the results of this study, Jiang et al. (2020) found that the H$_2$O$_2$ and TBARS contents in the leaves and roots of Solanum lycopersicum L. were significantly increased under treatment with 200 mg/kg NP. Khan et al. (2007) reported that under Cd exposure, the contents of H$_2$O$_2$ and TBARS in wheat increased significantly, but under the combined Cd-NP (0.01 + 1 mg/L) stress, the H$_2$O$_2$ content in the plants increased significantly. This indicated that the plants experienced significant oxidative stress under combined pollutant exposure.

CAT, POD, and SOD are three important defense enzymes in plant antioxidant systems (Shakir et al., 2018). SOD is the first line of defense for plant cells to eliminate ROS and catalyze O$_2^-$ to form H$_2$O$_2$ and O$_2$. In this study, under single and combined Cd and NP stress, the SOD activity of plants was found to decrease significantly, indicating that SOD is an antioxidative stress protective enzyme of H. dubia. Xiao et al. (2007) observed a decrease in antioxidant enzyme activities in Paralichthys olivaceus cells when exposed to NP. In contrast, Guo et al. (2019) and Afzal...
et al. (2019) found that the SOD activity of wheat and rice increased significantly under Cd treatment.

During the process of removing excess H$_2$O$_2$ from cells, CAT and POD play an important role. CAT is a heme-containing protease that catalyzes H$_2$O$_2$ production during photorespiration, mitochondrial electron transport, and fatty acid oxidation, and it converts H$_2$O$_2$ to H$_2$O and O$_2$. In this study, CAT activity decreased markedly only when the NP concentration was 5 mg/L, but there was no significant change under the Cd-only and combined treatment. The ability of CAT to remove H$_2$O$_2$ is limited. The decrease in CAT activity was probably due to the consumption of CAT as an NP and Cd scavenger or the inactivation of enzymes after exposure to high concentrations of NP. Similarly, Zhang et al. (2016) studied the toxicity of NP on wheat seedlings using hydroponic experiments and found that CAT activity decreased with an increasing treatment concentration. Soluble proteins in cells are mostly enzymes involved in metabolism and they are an important indicator of metabolic intensity in plants (Qing et al., 2018). In this study, at 0.5 + 1 mg/L Cd + NP, the soluble protein content decreased significantly compared with the control group. The decrease in soluble protein content in plant tissues indicated that the degree of stress was enhanced under combined pollutant exposure.

**Conclusion**

We conclude that, (1) under NP-only exposure, the minimum concentration (0.1 mg/L) of NP could promote the growth of *H. dubia*, but with increasing concentrations, the toxicity of NP on *H. dubia* gradually increased. (2) Under Cd-only exposure, *H. dubia* showed a dose-dependent effect. With increasing Cd concentrations, the damage degree of the growth indices of *H. dubia* increased, and the chlorophyll content decreased significantly. (3) Under combined Cd-NP exposure, the growth indices of *H. dubia* showed exaggerated responses. The combined pollution had a strong toxic effect on the roots of *H. dubia*. (4) At 0.01 + 0.1 mg/L and...
0.01 + 1 mg/L, the combined toxicity of Cd and NP was antagonistic to *H. dubia*. At 0.5 + 0.1 mg/L and 0.5 + 1 mg/L, Cd and NP had a strong synergistic effect on *H. dubia*. In the treatment groups, the lowest Cd and NP concentrations were close to the corresponding ambient concentrations of 0.0015–0.05 mg/L and 0.0301–0.2888 mg/L, respectively. Our study shows that combined Cd and NP exposure can cause growth inhibition and ecotoxicological effects on aquatic plants even at environment-related concentrations. Therefore, more attention should be given to the combined effects of various pollutants.

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**Data availability** Not applicable.

**Declarations**

**Ethics approval** All the authors declare that this study does not contain any studies with human participants or animals performed.

**Consent to participate** Not applicable.

**Consent for publication** Not applicable.

**Competing interests** The authors declare no competing interests.

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