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Improvement of Cr phytoremediation by *Pistia stratiotes* in presence of nutrients

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Abstract

The effects of different concentrations of P and N, added separately or combined, on the Cr(III) accumulation capacity of *P. stratiotes* were studied. Plants and pond water with the addition of contaminant(s) were placed in plastic aquaria. Cr concentration was 5 mg L^{-1}, while P and N concentrations were 5 mg L^{-1} or 10 mg L^{-1}. Nutrient addition significantly favoured Cr removal and enhanced Cr translocation to leaves. In Cr treatments a high detritus formation from loss of root biomass was observed probably due to its toxicity. Cr was mainly accumulated in the detrital fraction, whereas P and N were retained fundamentally in leaves. A toxic effect was observed in the Cr+P10 and Cr+N10 treatments. These results could be applied to enhance Cr removal efficiency of constructed wetlands using *P. stratiotes,* where nutrient enrichment could be attained by treating sewage together with the industrial effluents.

Keywords: metal, free-floating macrophyte, toxicity, uptake efficiency, wetlands

1. Introduction

Nutrients and metals play an important role in the growth and metabolism of plants. However, they produce toxic effects at high concentrations (Kabata-Pendias, 2011). In their natural habitats, plants are usually exposed to low concentrations of metals and nutrients whereas the conditions for plants growing in

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wetlands constructed for industrial wastewater treatment are completely different: metal and nutrient concentrations are usually high.

Several studies focused on determining the contaminant removal efficiency of constructed wetlands planted with different plant species (Vymazal and Krópfelová, 2005; Calheiros *et al*., 2007; Kadlec and Wallace, 2009; Maine *et al*., 2009). These authors demonstrated the high efficiency of different macrophyte species in the removal of metals, nutrients, organic matter, etc., from many types of effluents.

The interactions between metal accumulation in tissues and the nutrient concentrations in surrounding water have also been studied. Lee and Wang (2001) reported that an increase in water nitrate concentration resulted in a significant increase in the Cd accumulation rate in *Ulva fasciata* Delile, whereas the accumulation rate of Cr(VI) and Zn was not affected. Ammonium concentrations did not affect metal accumulation. Cd and Zn accumulation was inversely correlated with the water phosphate concentration, while Cr(VI) showed the opposite trend, suggesting that the influence of major nutrients on metal accumulation was metal-specific. Göthberg *et al*. (2004) reported higher metal accumulation in *Ipomoea aquatica* Forssk. at lower nutrient concentrations. Sundaramoorthy *et al*. (2010) studied the correlation between Cr accumulation and the uptake of nutrients using *Oryza sativa* L. and found that the uptake of macronutrients (N, P and K) and micronutrients (Mn, Cu, Zn and Fe) decreased gradually as Cr concentration increased. The interaction between metals and nutrients uptake is controversial. Apparently this interaction is not only metal-specific but also species-specific.

A wetland was constructed to treat the effluents of a metallurgical industry located in Santo Tomé city, Santa Fe (Argentina). The effluents contained Cr, Ni and Zn (Maine *et al*., 2009). Locally common macrophytes were transplanted into the wetland. *P. stratiotes* was dominant among the floating macrophytes. It was hypothesized that nutrient enrichment enhances the metal tolerance of macrophytes and would therefore enable the development of floating vegetation in constructed wetlands at metal concentrations which would otherwise inhibit plant growth. Increased nutrient concentrations might be attained by treating the sewage of the factory with the industrial wastewater once the appropriate primary treatment has been carried out.

P. stratiotes is commonly used in constructed wetlands due to its high efficiency in the nutrients and metals uptake (Aoi and Hayashi, 1996; Lu *et al*., 2010; Maine *et al*., 2004; Odjegba and Fasidi, 2004). The aim of this study was to evaluate the effect of different concentrations of P and N, added separately or combined, on the Cr(III) accumulation capacity of *P. stratiotes*.

2. Material and methods

2.1. Experimental design

Water and *P. stratiotes* plants were collected from an unpolluted pond from the Middle Paraná River floodplain. The collected plants were healthy. After collection, they were washed and acclimatized in a greenhouse. For experimental purposes, plants of a uniform size (number of leaves per plant = 11 ± 3; root length = 12.5 ± 2.5 cm) and weight (12.2 ± 2.4 g fresh weight) were selected. In each aquarium, 40 g of plant fresh biomass and 4 L of treatment solution were disposed. Cr studied concentration was 5 mg L^{-1} Cr (added as $CrCl_3.6H_2O$). Nutrient concentrations were 5 mg L^{-1} or 10 mg L^{-1} P and N (added as H_2KPO_4 and NH_4Cl, respectively) designed as P5, P10, N5 and N10. N was added as ammonium due to the preference of *P. stratiotes* for ammonium over nitrate previously reported (Aoi and Hayashi, 1996; Reddy *et al*., 1989). Solutions were prepared with pond water. Water pH was maintained between 5.4--5.8 to avoid metal precipitation. The treatments were arranged as follows:

Treatment 1: Cr

Treatment 2: Cr + P5

Treatment 3: Cr + P10

Treatment 4: Cr + N5

Treatment 5: Cr + N10

Treatment 6: Cr + P5 + N5

Treatment 7: Cr + P10 + N10

Treatment 8: P5

Treatment 9: P10

Treatment 10: N5

Treatment 11: N10

Treatment 12: P5 + N5

Treatment 13: P10 + N10

Treatment 14: control (without additions)

Each treatment was conducted in duplicate. Plastic aquaria were placed outdoors under a semi-transparent plastic roof receiving natural light. During the experimental period (spring) temperature ranged from 24 to 28ºC. The experiment lasted 16 days. Pond water was added on a daily basis to

compensate water losses through plant transpiration and evaporation, maintaining the initial volume of 4 L.

Water was sampled initially and at 2, 8 and 24 h and at 3, 7, 10 and 16 d. In each sampling, Cr, soluble reactive phosphorus (SRP) and NH_4^+ concentrations were measured. At the beginning and at the end of the experiment total Kjeldahl nitrogen (TKN) and Total phosphorus (TP) were measured in water samples.

At the beginning and at the end of the experiment plants were separated into leaves and roots and dried at 105°C until constant weight was reached (APHA, 1998; Westlake, 1974). The relative growth rate was calculated according to Hunt's equation (1978):

$RGR = \ln W_2 -- \ln W_1 / T_2 -- T_1$

where RGR is the Relative Growth Rate (g g^{-1} day^{-1}), W_1 and W_2 are the initial and final dry weight, respectively, and ($T_2 -- T_1$) is the experimental period (days).

Plant tissue Cr, TP and TKN, and leaf chlorophyll *a* concentrations were determined at the beginning and at the end of the experiment.

2.2. Chemical analysis

The physicochemical characterization of pond water used in the experiment was done according to APHA (1998). Its chemical composition was (mean ± standard deviation): conductivity = 104 ± 1 µS cm^{-1}; dissolved oxygen (DO) = 7.6 ± 0.10 mg L^{-1}; SRP = 0.015 ± 0.002 mg L^{-1}; N-NH_4^+ = 0.150 ± 0.019 mg L^{-1}; N-NO_3^- = 2.05 ± 0.012 mg L^{-1}; N-NO_2^- = 0.018 ± 0.002 mg L^{-1}; Ca^{2+} = 10.3 ± 0.8 mg L^{-1}; Mg^{2+} = 5.8 ± 0.5 mg L^{-1}; Na^+ = 15.7 ± 1.0 mg L^{-1}; K^+ = 3.50 ± 0.5 mg L^{-1}; Cl^- = 10.6 ± 1.3 mg L^{-1}; SO_4^{2-} = 8.0 ± 1.8 mg L^{-1}; Total alkalinity = 45.2 ± 1.2 mg L^{-1}; Fe < 5 µg L^{-1}; Cr = non detected (Detection limit = 5 µg L^{-1}).

Dried plant tissues (leaves and roots) were ground and digested with a $HClO_4$:HNO_3:HCl (7:5:2) mixture (Maine *et al*., 2004). Cr concentrations in water samples and digests of plant tissue were determined by atomic absorption spectrometry (Perkin Elmer 5000; APHA, 1998). SRP was determined in water by the colorimetric molybdenum blue method (Murphy and Riley, 1962; UV--VIS Perkin Elmer Lambda 20). Ammonium concentrations in water were determined colorimetrically following APHA

(1998). TP concentration was measured in the plant tissue digests as SRP. Tissues and water TKN was determined by the Macro-Kjeldahl method according to APHA (1998). TP in water samples was determined as SRP, after an acid digestion following APHA (1998).

The Bioconcentration Factor (BCF) was calculated as the ratio of Cr, P and N concentrations Roots/water. In addition, the Translocation Factor (TF) was expressed by the ratio of the concentrations Leaves/roots to show translocation properties from roots to aerial parts (Stoltz and Greger, 2002).

In the mass balance, Cr, P and N amounts (mg) were estimated by multiplying Cr, TP or NTK concentration in plant tissues or in water (mg g^{-1} dry weight or mg L^{-1}) by biomass or volume (g dry weight or L).

Chlorophyll was extracted with acetone for 48 h in cold darkness (3--5°C) (APHA 1998). The percentage of transmittance of the extracts at 645 and 665 nm was recorded with a spectrophotometer UV-Vis (Westlake, 1974).

2.3. Statistical analysis

One-way analysis of variance was carried out to determine whether significant differences among treatments existed in contaminant water removal, RGR, chlorophyll *a* concentration, root biomass, BCF and TF. Two-way analysis of variance was used to determine whether significant differences in contaminant accumulation among treatments and contaminant accumulation compartments existed. The normality of residuals was tested graphically, and the homoscedasticity of variances was checked applying Bartlett's test. Duncan's test was used to differentiate means where appropriate. A level of $p<0.05$ was used in all comparisons.

2.4. QA/QC

All glassware were pre-cleaned and washed with 2N HNO_3 prior to each use. All reagents were of analytical grade. Certified standard solutions were used. The blanks were run all the time. Replicate analyses (at least ten times) of the samples showed a precision of typically less than 4% (coefficient of variation). The sample preparation methods used were also checked against the spiked sample which is the certified solution standards; mean recoveries were in the range 95.89--98.13%. The Cr detection limits were 2 µg l^{-1} for water and 5 µg g^{-1} for macrophyte.

3. Results and discussion

3.1. Plant tolerance

All treatments with the addition of Cr showed a significantly lower RGR than that obtained in the control (Fig. 1). Cr is involved in plant metabolism, including gene control, oxygen transport and active centres in enzymes (Bonilla, 2008). However, when metal concentration reaches a threshold value, metal becomes first inhibitory and afterwards toxic. Maine *et al*. (2004) reported that RGR of *P. stratiotes* presented a negative correlation with increasing Cr concentrations. The RGR measured in Cr+P5+N5, Cr+P10+N10, Cr+P5 and Cr+N5 treatments were significantly lower than that of the control but they were positive, while the Cr, Cr+P10 and Cr+N10 treatments were toxic to the plants showing negative RGR. Growth inhibition by Cr exposure observed in the present study has previously been reported and represents a sensitive indicator of Cr toxicity (Shanker *et al*., 2005). Reductions in RGR due to Cr exposure were attenuated by nutrient enrichment at 5 mg L^{-1} of P or N and in the treatments with both nutrients at both concentrations studied, suggesting an improving effect of nutrient enrichment on the Cr tolerance of *P. stratiotes*. Even though Cr+P10 and Cr+N10 treatments were toxic, Cr+P10+N10 treatment presented only an inhibitory effect, due to the fact that plants need both nutrients for their growth. The diminution of metal toxic effects in the presence of nutrients was previously reported by Hadad *et al*. (2007). Nutrient enrichment enhances not only macrophyte production but also the overall biological activity, leading to a higher metal uptake by the macrophyte.

Chlorophyll concentrations in all treatments were not significantly different from the control, except Cr+P10 and N10 treatments (Fig. 2). Since no effect on chlorophyll concentration was observed, it can be proposed that Cr toxicity may not be mediated through the photosynthetic metabolism in *P. stratiotes*. Hadad *et al*. (2007) and Kolotov *et al*. (2004) proposed that chlorophyll concentration in plants is a good toxicity indicator for different metals. However, the plant responses depend on the contaminant and the macrophyte species. Delgado *et al*. (1993) observed chlorosis in *E. crassipes* during exposure to several increasing concentrations of Cr, Cd and Zn, being more intense in the case of Cd. Hadad *et al*. (2011) used a concentration of 1 mg L^{-1} Cr and observed that this metal exhibited a toxic effect on

chlorophyll concentration in *E. crassipes*. Maine *et al*. (2004) recorded a decrease in chlorophyll when *P. stratiotes* was exposed to 6 mg L^{-1} Cr, whereas *S. herzogii* did not show a decrease in this pigment.

The mechanisms regulating metal tolerance in macrophytes are not completely identified and they could consist of different mechanisms operating simultaneously (Odjegba and Fasidi, 2004). The Cr+P10 treatment showed the lowest RGR and chlorophyll concentration at the end of the experiment, being the most toxic treatment. The N10 treatment showed a RGR significantly lower than that obtained in the control and the highest final chlorophyll concentration. N is an element that improves chlorophyll synthesis because it is accumulated mainly in leaves (Bonilla, 2008), but high concentrations in tissues affect plant growth.

3.2. Contaminant removal from water

Fig. 3 shows the removal percentage of Cr, P and N from water along time. In the first 24 h Cr removal was 52.2 - 58.7% in the different treatments (Fig. 3a). At the end of the experiment a 73.9--90.0% removal was observed in the different treatments. Statistically significant differences among treatments were found in Cr removal at the end of the experiment. The highest Cr removal was found in Cr+P5+N5 and Cr+P10+N10 treatments. These treatments also presented the highest RGR among the treatments with Cr addition (Fig. 1). The treatment of Cr without the addition of nutrients showed the lowest Cr removal, demonstrating that nutrient enrichment enhances the metal uptake not only by increasing the macrophyte biomass but also by enhancing the overall biological activity. Maine *et al*. (2004) exposed *P. stratiotes* to concentrations of 1, 2, 4 and 6 mg L^{-1} Cr, obtaining water removal efficiencies of 98--99% after 30 days of experimentation regardless the initial concentration. These authors proposed that the obtained removal was due to the fact that the sorption of Cr(III) is probably a competitive-consecutive mechanism of reversible reaction steps. Cr removal rate was similar to previous works, being Cr accumulated fundamentally during the first minutes of contact (Maine *et al*., 2004). The rapidity of Cr uptake from water suggests that adsorption to the cell walls of roots was the main mechanism of Cr accumulation in tissues. The efficiency of the metal adsorption processes was also corroborated using non-living roots (Schneider and Rubio, 1999).

In the first 24 h, P removal was 21.4--73.1% in the different treatments (Fig. 3b). During all the experiment, the P5+N5 treatment showed a significantly higher removal percentage in comparison with the other treatments. At the end of the experiment a 51.2--93.5% removal was observed in the different treatments. Statistically significant differences among treatments were found in P removal at the end of the experiment. The highest P removal was found in P5+N5 and Cr+P5+N5 treatments. On the other hand, in the presence or absence of Cr, the addition of 10 mg L^{-1} P results in the lowest P removal. The Cr+P10 treatment was the treatment with the lowest P removal, showing a toxic effect represented by the lowest RGR (Fig. 1).

In the first 24 h, N removal was 28.7--98.9% in the different treatments (Fig. 3c). At the end of the experiment, a 92.1--99.8% removal was observed in the different treatments. Statistically significant differences were found in the removal of N at the end of the experiment. The highest N removal was found in N5 treatment. On the other hand, the Cr+P5+N5 treatment was the treatment with the lowest N removal.

N showed higher removal percentages than P at the end of the experiment. Ammonium is a source of nitrogen that is easily transported by metabolic systems located at the macrophyte plasmalemmas (Bishop and Eighmy, 1989), while protein membrane carriers mobilize P as fast as they can. However, when there is an excess of phosphate, the transport velocity is limited by the saturation of their capabilities of transport (Bonilla, 2008).

3.3. Contaminant distribution

The contaminants were accumulated in leaves, roots and detritus (from the loss of root biomass). Table 1 shows the BCF and TF of Cr, P and N. As it can be seen in TF, Cr concentration was significantly higher in roots than in leaves at the end of the experiment in all treatments in agreement with previously reported research (Göthberg *et al*., 2004; Hadad *et al*., 2007, 2011). A higher tolerance of roots than leaves together with a trend to decrease translocation with increasing metal concentration in the roots represents a common feature of the different metals and plants studied. Binding positively charged toxic metal ions to negative charges in the cell walls of the roots, metal-phosphate and metal-phytate formation, and chelation to phytochelatins followed by accumulation in vacuoles have been invoked as

mechanisms to reduce metal transport and increase metal tolerance (Göthberg *et al*., 2004). Contrarily, there was higher P and N concentration in leaves than in roots, similarly to that observed in other studies of *P. stratiotes* growing in natural wetlands (Hadad and Maine, 2007) and in constructed wetlands (Maine *et al*., 2009). Macrophytes possess efficient apoplastic nutrient transfer routes to leaf photosynthetic cells (Bonilla, 2008).

A mass balance was performed in the different treatments, taking into account water, roots, leaves and detritus. Cr, N and P amounts are shown in Fig. 4. Cr accumulated mainly in the detrital fraction (27--65%) and in roots (9--44%) (Fig. 4a). In all cases, nutrient addition increased Cr removal from water, significantly. If only leaves and roots are taken into consideration, the higher Cr accumulation occurred in Cr+P5+N5 and Cr+P10+N10 treatments. The addition of 10 mg L^{-1} of P (Cr+P10 treatment) affected Cr accumulation in plant tissues, being significantly lower than that of the treatment with Cr without nutrient addition. In this treatment, a toxic effect produced senescence with a remarkable root loss, with a consequent higher Cr accumulation in detritus. A decrease in root biomass in the treatments with Cr addition confirms the generation of detritus from roots (Table 2). In all cases, nutrient addition favoured the translocation of Cr to leaves (Table 1). The highest metal accumulation in leaves was found in Cr+N5 treatment.

P and N amounts were significantly higher in leaves than in the other compartments (Figs. 4b and 4c). P root accumulation was not significantly different among treatments but the accumulation in leaves was affected by the different additions. A higher accumulation in leaves was observed in treatments added with 5 mg L^{-1} P, in coincidence with a lower P amount in water. In the treatments with the addition of 10 mg L^{-1} P, a higher amount of P remained in water. These facts suggest that P water uptake was limited by a decrease in the transport capacity of protein membrane carriers due to an excess of phosphate (Bonilla, 2008).

In all cases, the addition of Cr affected the accumulation of N by leaves and roots negatively. In the treatments with the addition of Cr, N accumulation was significantly higher in the detrital fraction than in the other treatments (Fig. 4c), probably due to the toxic effect of Cr, which caused a high detritus mass. Nitrogen content in *O. sativa* decreased significantly with the increase of Cr concentration

(Sundaramoorthy *et al*., 2010). Similar inhibition of N metabolism caused by metals was also observed by Mayz and Cartwright (1984) in wheat. These authors observed that Cr treatment affects N uptake and assimilation, which is evident from the decline in the concentration of N in leaves.

These results could be applied to wastewater treatment. Many industrial processes produce wastewaters containing metals that could be efficiently treated in a constructed wetland using *P. stratiotes*. The process efficiency could be enhanced by treating the factory sewage (that contains N and P) with the industrial wastewater after environmental safety issues are addressed.

4. Conclusions

The simultaneous addition of P and N, and the addition of 5 mg L^{-1} P or $N\text{-}NH_4^+$ favoured Cr tolerance of *P. stratiotes* whereas the addition of 10 mg L^{-1} P or $N\text{-}NH_4^+$ increased Cr toxicity.

Nutrients enhanced Cr accumulation in roots and increased Cr translocation. Cr was mainly accumulated by the detrital fraction, which was generated by the loss of root biomass.

Industrial wastewaters containing metals could be efficiently treated in a constructed wetland using *P. stratiotes*. The process performance could be enhanced by treating the sewage of the factory together with the industrial wastewater.

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Table 1. Translocation (TF) and Bioconcentration factors (BCF) of Cr, P and N (Mean ± S.D.) obtained in each treatment.

Treatments	TF (Leaves/root)	BCF (Roots/water)
Cr		
Cr	0.057 ± 0.003	4.882 ± 0.335
Cr+P5	0.071 ± 0.001	8.751 ± 0.210
Cr+P10	0.123 ± 0.001	5.788 ± 0.081
Cr+N5	0.182 ± 0.003	7.559 ± 0.008
Cr+N10	0.135 ± 0.002	7.840 ± 0.132
Cr+P5+N5	0.067 ± 0.001	16.55 ± 0.298
Cr+P10+N10	0.083 ± 0.003	9.979 ± 0.355
P		
Cr+P5	1.347 ± 0.138	2.368 ± 0.254
Cr+P10	1.261 ± 0.261	1.122 ± 0.287
Cr+P5+N5	1.504 ± 0.139	4.237 ± 0.344
Cr+P10+N10	1.416 ± 0.222	1.862 ± 0.212
P5	2.417 ± 0.020	1.456 ± 0.009
P10	1.513 ± 0.010	0.829 ± 0.011
P5+N5	1.978 ± 0.011	8.810 ± 0.090
P10+N10	1.706 ± 0.042	1.312 ± 0.055
N		
Cr+N5	1.589 ± 0.079	18.72 ± 2.293
Cr+N10	1.773 ± 0.023	17.99 ± 0.473
Cr+P5+N5	0.807 ± 0.028	27.38 ± 2.415

Cr+P10+N10	1.411 ± 0.052	12.24 ± 0.419
N5	1.820 ± 0.049	31.39 ± 2.445
N10	1.356 ± 0.046	28.21 ± 3.421
P5+N5	1.629 ± 0.100	14.92 ± 0.039
P10+N10	1.190 ± 0.033	32.96 ± 1.376

Table 2. Mean ± S.D. of leaves, root and detritus mass (g d.w.) obtained in each treatment. Different letters among treatments represent statistical significant differences.

Treatments	Leaves	Roots	Detritus
Initial			
	1.52 ± 0.12 a	1.27 ± 0.10 a	-
Final			
Cr	1.72 ± 0.13 a	0.91 ± 0.08 b	0.950 ± 0.086 a
Cr+P5	1.99 ± 0.21 b	0.75 ± 0.08 b	0.886 ± 0.074 a
Cr+P10	1.57 ± 0.19 a	0.44 ± 0.05 c	1.4515 ± 0.120 b
Cr+N5	2.03 ± 0.20 b	0.80 ± 0.07 b	0.7828 ± 0.065 c
Cr+N10	1.64 ± 0.14 a	0.91 ± 0.10 b	0.6414 ± 0.059 c
Cr+P5+N5	2.24 ± 0.29 b	0.88 ± 0.09 b	0.5402 ± 0.081 c
Cr+P10+N10	2.34 ± 0.25 b	1.03 ± 0.12 b	0.5862 ± 0.053 c
P5	2.13 ± 0.20 b	1.71 ± 0.20 d	0.231 ± 0.015 e
P10	2.55 ± 0.32 c	1.61 ± 0.15 d	0.250 ± 0.010 e
N5	3.02 ± 0.25 d	1.52 ± 0.14 d	0.242 ± 0.018 e
N10	1.99 ± 0.23 b	1.27 ± 0.13 a	0.263 ± 0.010 e
P5+N5	2.62 ± 0.29 c	1.41 ± 0.15 a	0.294 ± 0.015 e
P10+N10	3.20 ± 0.35 d	1.09 ± 0.10 a	0.301 ± 0.013 e

Figure captions

Fig. 1. Relative growth rate (g g^{-1} d^{-1}) of *P. stratiotes* obtained at the different treatments. Bars represent standard deviations. Different letters represent statistical significant differences.

Fig. 2. Chlorophyll concentrations (mg g^{-1} d.w.) of *P. stratiotes* obtained at the different treatments. Bars represent standard deviations. Different letters represent statistical significant differences.

Fig. 3. Cr, P and N removal from water along time in each treatment.

Fig 4. Cr, P and N total amounts (expressed in%) in water, roots, leaves and detritus of *P. stratiotes* obtained in each treatment.

Fig. 1.

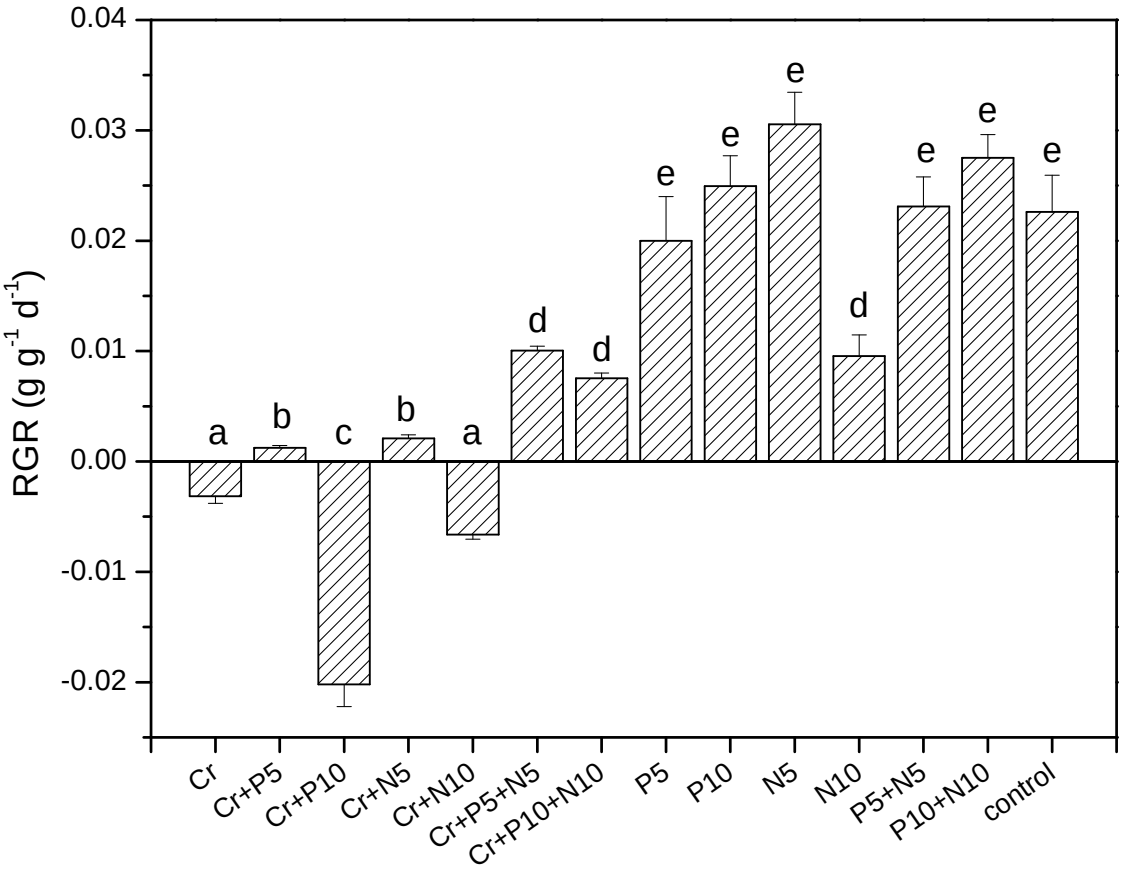

Fig. 2.

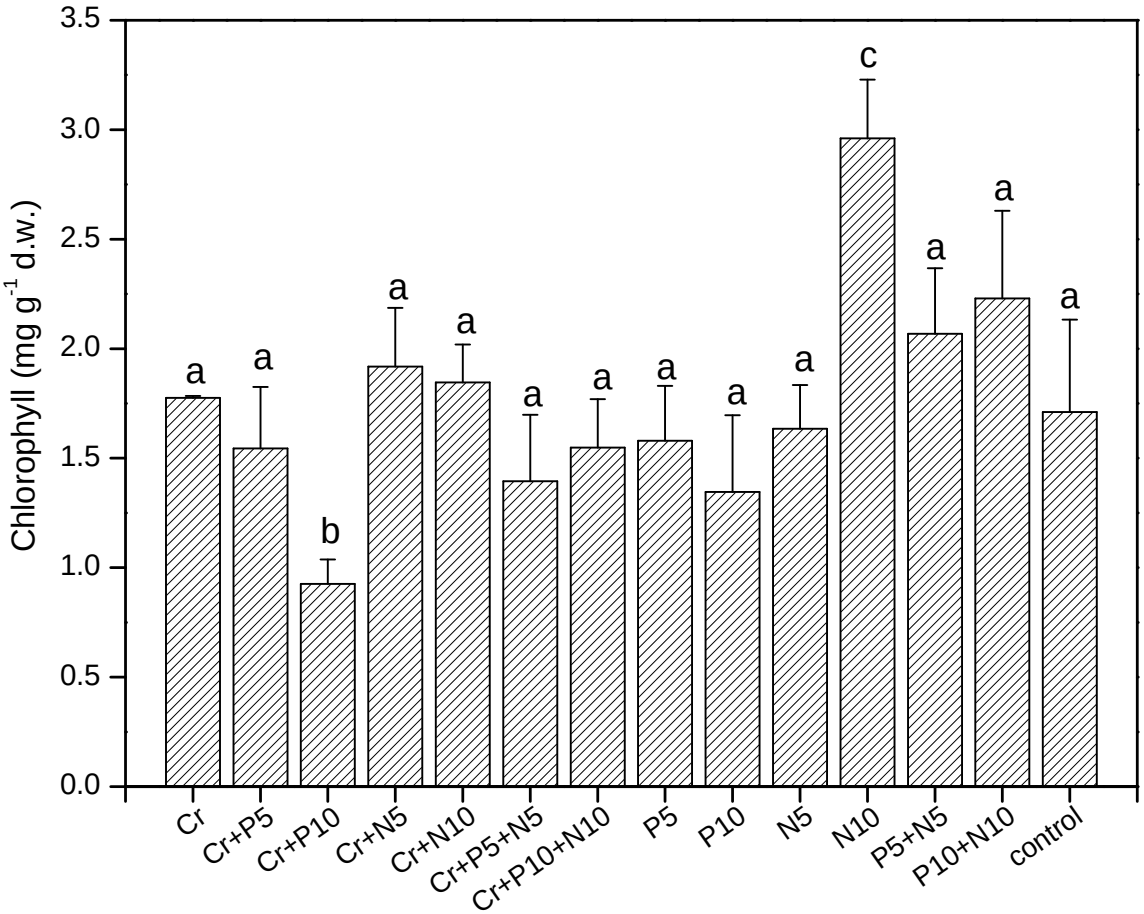

Fig. 3.

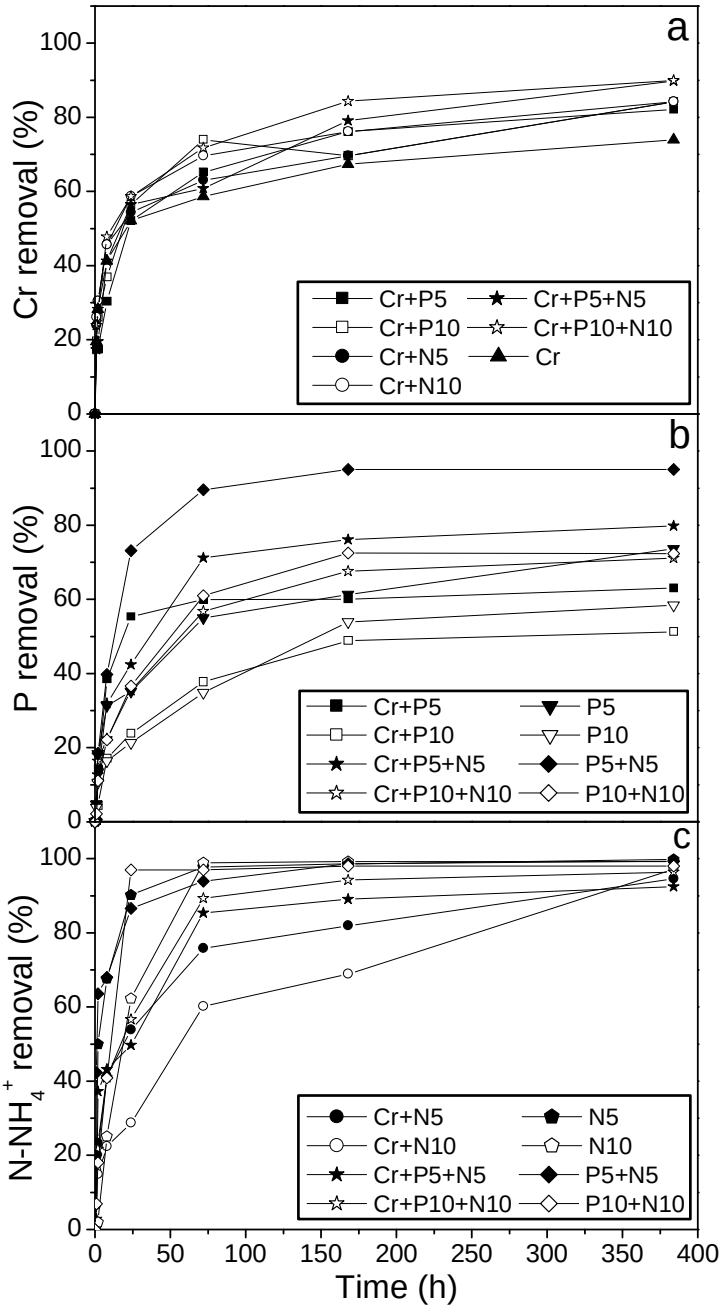

Fig. 4.