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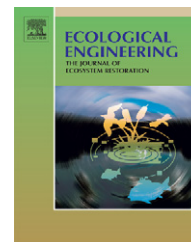**ScienceDirect**

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The effect of nutrient addition on metal tolerance in *Salvinia herzogii*

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ABSTRACT

Disappearance of floating macrophytes was observed in two successively constructed wetlands for the treatment of wastewater that contained Zn, Ni and Cr at a tool factory in Argentina. Experimental work was developed to test the hypothesis that nutrient enrichment enhances the metal tolerance of floating macrophytes. Relative growth rates, root biomass and chlorophyll concentration in *Salvinia herzogii* (water fern) were measured in greenhouse incubations exposed to different Zn, Ni and Cr concentrations, and compared with simultaneous treatments enriched with nitrogen and phosphorus. Relative growth rates were negatively correlated with metal exposures. Nutrient addition suppressed such effect. Root biomass was also negatively correlated with metal concentrations. Nutrient enrichment either attenuated (Cr and Zn) or suppressed (Ni) root biomass decrease in response to metal exposure. Chlorophyll concentration was negatively correlated with Ni exposures. Nutrient addition attenuated chlorophyll decrease in response to Ni exposure. Growth rate represented an early indicator of Cr and Zn toxicity, while growth rate and chlorophyll concentration represented suitable indicators of Ni toxicity. Metal concentration in leaves was correlated with metal exposure concentration. The effect of Zn exposure on Zn leaf concentration was attenuated by nutrient addition, apparently through P and Zn immobilization in the roots. Metal and nutrient concentration in water decreased throughout the experiments. Chromium removal from water was faster than that of Zn and Ni. Zinc and Ni were mainly sorbed by *S. herzogii* biomass, while Cr was also retained in the detrital fraction.

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1. Introduction

The construction of artificial wetlands for wastewater treatment has been developing quickly over the last decades and currently it represents an accepted and increasingly common treatment alternative (Gómez Cerezo et al., 2001; Ansola et al., 2003; Song et al., 2006). Nevertheless, current experience in Argentina remains largely unreported. In principle, conditions are favorable since the central and northern parts of the country have mild winters, allowing extended growth periods

for the vegetation. Low population density and a large availability of marginal land favour the application of constructed wetlands for wastewater treatment.

Bahco Argentina S. A. constructed a small-scale experimental wetland to assess the feasibility of treating wastewater at its tool factory in Santo Tomé (Argentina). The effluent had high pH (8.0–12) and conductivity (3.3–8.5 mS cm^{-1}); and high Cr (5.0–589 μg l^{-1}), Ni (3.0–750 μg l^{-1}) and Zn (40–210 μg l^{-1}) concentrations. While regionally abundant macrophyte species are adapted to the local climatic and edaphic conditions, their performance under the operational conditions imposed by the characteristics of the wastewater was unknown. An initial assemblage of three floating and eight emergent species representative of the commonly abundant macrophytes in the nearby Paraná River floodplain was transplanted. *Eichhornia crassipes*, *Salvinia herzogii* and *Pistia stratiotes* started active growth, attaining roughly 60% cover in 3 months, but later decreased progressively until their disappearance from the wetland by the 6th month. After 1 year of operation only a single emergent macrophyte, *Typha domingensis* (cattail), remained in the small-scale constructed wetland attaining profuse growth (Hadad et al., 2006). Metals, nutrients, biological and chemical oxygen demand decreased substantially as they reached the wetland outlet (Maine et al., 2005). The success in improving wastewater quality led this industry to develop a large-scale constructed wetland to treat the wastewater of the whole factory. A 40×50 m^2 wetland was built and it has been in operation for 3 years (Maine et al., 2006). Again, an assemblage of locally common macrophytes was transplanted. Floating macrophytes covered most of the surface for almost 1 year, followed by a receding stage that took another 6 months. Since then, only *T. domingensis* attained dense stands. The large-scale constructed wetland was also very efficient in metal retention (Maine et al., 2006). In both small- and large-scale constructed wetlands, the metals retained were stored mainly in the macrophyte biomass when floating macrophytes were dominant, while they were stored mainly in sediment when emergent macrophytes became dominant. Metal accumulation was faster in floating than emergent macrophytes, suggesting that metal exposure contributed to the earlier disappearance of the former. High concentrations could be attained because roots are immersed in the wastewater while the emergent macrophytes develop their roots in the reduced soil matrix where metals are largely immobilized.

We hypothesized that nutrient enrichment enhances the metal tolerance of floating macrophytes and would, therefore, enable the development of floating vegetation in constructed wetlands at metal concentrations that would otherwise inhibit plant growth. Increased nutrient concentrations might be attained by mixing the sewage of the factory with the industrial wastewater once the appropriate primary treatment has been undertaken.

Lee and Wang (2001) reported that an increase in ambient nitrate concentration resulted in a significant increase in the Cd accumulation rate in *Ulva fasciata*, whereas the rate of accumulation of Cr(VI) and Zn was not affected and ammonium concentrations did not affect the accumulation of Cr(VI), Cd and Zn. Metal accumulation was inversely correlated with the ambient 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* at lower nutrient concentrations. Apparently the interaction between metals and nutrients uptake is not only metal-specific but also species-specific.

This paper compares the tolerance of the floating macrophyte *S. herzogii* in greenhouse experiments at different Cr, Ni and Zn exposures with the same treatments enriched with nutrients.

2. Materials and methods

2.1. Sampling

Floating species disappeared from the constructed wetlands at Bahco Argentina S. A. after showing an active growth and a high efficiency in the retention of P and metals (Maine et al., 2005, 2006; Hadad et al., 2006). *S. herzogii* was employed for this study because it is a common and conspicuous representative species of the local macrophyte assemblages.

S. herzogii plants and water were brought from the Paraná River floodplain, an environment in which *S. herzogii* develops conspicuously. The chemical composition of the river water was (mean ± standard deviation): pH = 6.5 ± 0.2; conductivity = $118 \pm 12\ \mu$S cm^{-1}; dissolved oxygen (DO) = 8.2 ± 0.30 mg l^{-1}; soluble reactive phosphorous (SRP) = 0.056 ± 0.006 mg l^{-1}; NH$_4^+$–N = 0.486 ± 0.099 mg l^{-1}; NO$_3^-$–N = 0.101 ± 0.062 mg l^{-1}; NO$_2^-$–N = 0.009 ± 0.002 mg l^{-1}; chemical oxygen demand (COD) = 2.1 ± 0.30 mg l^{-1}; Ca^{2+} = 7.0 ± 1.8 mg l^{-1}; Mg^{2+} = 3.6 ± 0.5 mg l^{-1}; Na$^+$ = 26.5 ± 2.0 mg l^{-1}; K$^+$ = 2.15 ± 0.4 mg l^{-1}; Cl$^-$ = 26.6 ± 1.3 mg l^{-1}; SO$_4^{2-}$ = 8.6 ± 1.8 mg l^{-1}; HCO$_3^-$ = 58.0 ± 1.2 mg l^{-1}. Cr, Ni and Zn concentrations were below the detection limits of the method (Cr < 1 μg l^{-1}; Ni < 3 μg l^{-1}; Zn < 25 μg l^{-1}).

2.2. Plant material and experimental design

The plants showing the best condition were sampled in the field, considering their uniformity. The plants were acclimatized in a greenhouse. Experiments were carried out in spring in a greenhouse receiving natural light. Temperatures ranged from 24 to 28 °C. Plastic aquaria of 10 l capacity were used. Fresh weight of plants (50 g) and water (5 l) from the sampled natural environment were placed in every aquarium. Water pH was kept at a value of 5.5–6.5 by adding diluted HCl. Metals were added as CrCl$_3$·6H$_2$O, NiCl$_2$·6H$_2$O and ZnCl$_2$·6H$_2$O. All treatments were carried out by triplicate. The experiment lasted 11 days, when the plants exposed to the highest metal concentrations developed chlorosis and necrosis.

S. herzogii relative growth rate was calculated in each treatment according to Hunt's equation (1978):

$$R = \frac{\ln W_2 - \ln W_1}{T_2 - T_1}$$

where R is the relative growth rate (g g^{-1} d^{-1}), W_1 and W_2 is the initial and final dry weight, respectively, and (T_2–T_1) is the experimental period. Dry weight was estimated by drying the plants at 105 °C until constant weight was reached (APHA, 1998).

2.3. Range-finding experiments

In three range-finding experiments the relative growth rate of *S. herzogii* exposed to concentrations of Zn, Ni and Cr of 4, 6, 8 and 10 mg l^{-1} was measured (APHA, 1998). The aim was to establish approximate toxicity thresholds for each metal in order to carry out the subsequent definitive toxicity experiments. A control with water from the sampling site without the addition of metals was used.

2.4. Definitive experiments

Plant growth rates in response to metal exposures were compared with exposures enriched with 5 mg l^{-1} of P (as H_2KPO_4), 5 mg l^{-1} of NO_3^-–N (as KNO_3) and 5 mg l^{-1} NH_4^+–N (as NH_4Cl). Three concentrations for each metal were studied as follows:

- Treatments 1, 2 and 3: exposure to metal concentrations 1, 2 and 3.
- Treatment 4: control without metal addition.
- Treatments 5, 6 and 7: exposure to metal concentrations 1, 2 and 3 and simultaneous nutrient enrichment.

- Treatment 8: treatment with nutrient enrichment without metal exposition.

Metal concentrations 1, 2 and 3 were chosen according to the results previously obtained in the range-finding experiments and were different for each metal.

Chlorophyll, total phosphorous (TP), total Kjeldahl nitrogen (TKN) and total concentration of the metal used in each experiment were determined in roots and leaves at the beginning and at the end of each experiment. SRP, nitrate, ammonium and metal concentrations in water were determined throughout the experiments.

2.5. Analytical determinations

Conductivity was assessed with an YSI 33 model conductimeter, pH with an Orion pH meter and DO with a Horiba OM-14 portable meter. Water samples were filtered through Millipore membrane filters (0.45 μm) for nutrient determinations. Chemical analysis was performed following APHA (1998). Soluble reactive phosphorous was determined by the colorimetric molybdenum blue method (Murphy and Riley,
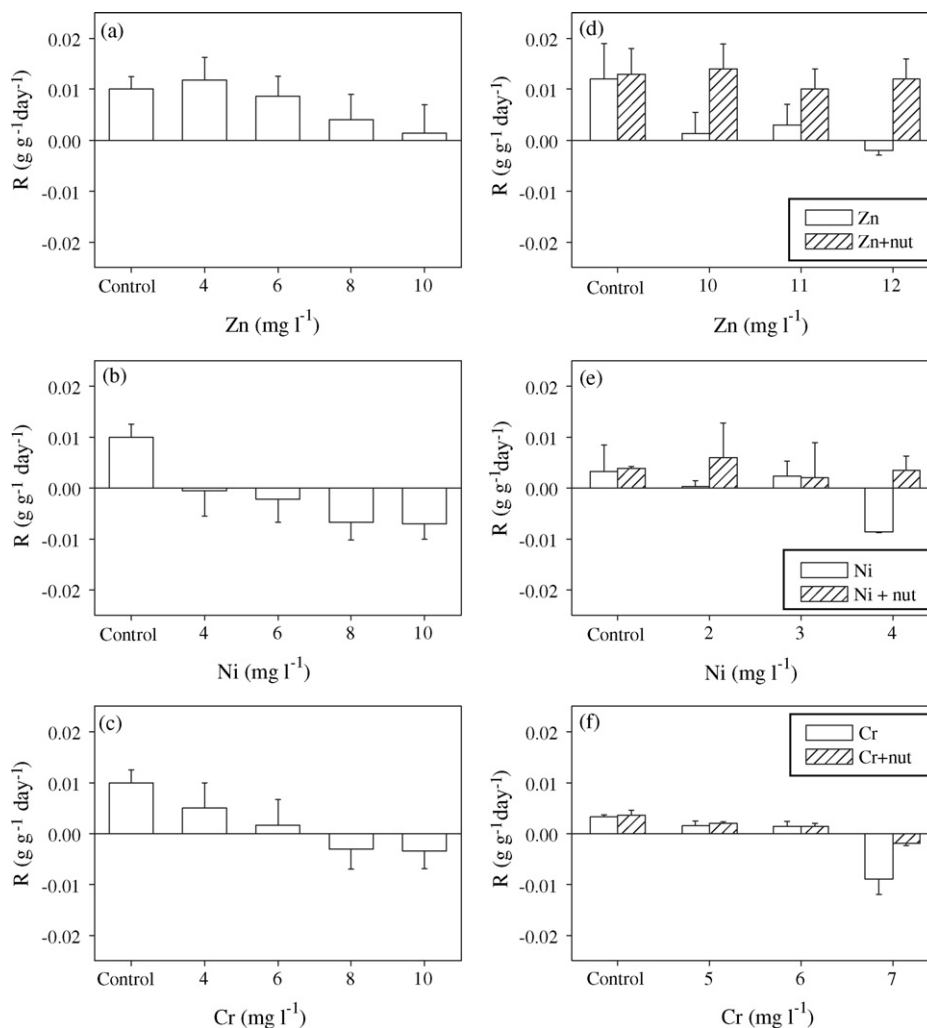

Fig. 1 – Relative growth rates (R) obtained in the Zn, Ni and Cr range-finding (a, b and c, respectively) and definitive metal exposure experiments (d, e and f, respectively). The bars represent standard deviation.

1962) (UV–Vis Perkin Elmer Lambda 20). Ammonium and nitrate were determined by potentiometry (Orion ion selective electrodes, sensitivity: $0.01\,mg\,l^{-1}$ of N, reproducibility: ±2%). Nitrite was determined by coupling diazotation followed by a colorimetric technique, COD was determined by the open reflux method. Ca^{2+} and Mg^{2+} were determined by EDTA titration. Na^+ and K^+ were determined by flame emission photometry. Bicarbonate was measured by HCl titration. Chlorine was determined by the argentometric method. $SO_4{}^{2-}$ was assessed by turbidimetry (APHA, 1998). Chromium, Ni and Zn concentrations were determined in water samples by atomic absorption spectrometry (Perkin Elmer 5000) (APHA, 1998).

Total phosporous in plants was determined after acid digestion with $HClO_4$:HNO_3:HCl (7:5:2) mixture and SRP concentration was measured in the digested samples (Murphy and Riley, 1962). Chromium, Ni and Zn were determined in the same digestions by atomic absorption spectrometry (Perkin-Elmer 5000). Total Kjeldahl nitrogen was determined by the Macro-Kjeldahl method according to APHA (1998). Chlorophyll a was determined at the beginning and end of each experiment. Chlorophyll was extracted with acetone for 48 h in cold darkness (3–5 °C). The percentage of transmittance of the extracts at wavelengths of 645 and 665 nm was recorded with a spectrophotometer UV–Vis (Westlake, 1974).

2.6. Statistical analysis

Linear regression and correlation analyses were used to examine relationships among the variables measured (relative growth rate, root biomass, chlorophyll, metal concentrations, TP and TKN concentrations in leaves and roots) and metal exposures (Zn, Cr and Ni concentrations). Regression (a and b) and correlation (r) coefficients were calculated. Significance of the regression and correlation coefficients were tested following Zar (1999). The linear regression equation of each response variable for each metal exposure was compared with the same exposure enriched with nutrients, following Zar (1999).

Metal concentration in water throughout each experimental dose exposition was compared with the same dose enriched with nutrients by means of a Student t-test for paired samples, following Zar (1999), by means of the XL-STAT software, version 7.5.3. The same test was used to compare nutrient concentration in the treatments enriched with nutrients against each metal exposure. All tests were performed using a significance level of $\alpha = 0.05$.

3. Results

3.1. Metal toxicity

The range-finding experiments showed that relative growth rates of S. herzogii were negatively correlated with metal concentration for Ni, Cr and Zn at the assayed concentrations, being the linear regression coefficients significantly different from zero (Fig. 1, Table 1). Relative growth rates of S. herzogii were also inversely correlated with metal concentrations in the definitive experiments (Fig. 1, Table 1). On the contrary, the treatments enriched with nutrients did not show significant correlation. Therefore, nutrient addition eliminated the observed decrease of the relative growth rate in response to metal exposition.

Table 1 – Linear regression analyses used to examine relationships among the measured parameters and metal exposures

Parameters	Zn	Zn + nutrients	Ni	Ni + nutrients	Cr	Cr + nutrients
Relative growth rate (range-finding experiments)	−0.822 (0.0035) −0.0009	–	−0.967 (<0.0001) −0.002	–	−0.977 (<0.0001) −0.002	–
Relative growth rate (definitive experiments)	−0.932 (0.0007) −0.001	–	−0.757 (0.030) −0.002	–	−0.734 (0.038) −0.0009	–
Root biomass	−0.936 (0.0006) −0.036	−0.929 (0.0009) −0.020	−0.756 (0.030) −0.040	–	−0.860 (0.006) −0.033	−0.876 (0.0044) −0.011
Chlorophyll concentrations	–	−0.966 (<0.0001) −0.309	−0.873 (0.005) −0.430	−0.855 (0.007) −0.258	–	–
Leaf metal concentrations	0.989 (<0.0001) 0.658	0.983 (<0.0001) 0.467	0.973 (<0.0001) 0.598	0.956 (<0.0002) 0.615	0.985 (<0.0001) 0.357	0.986 (<0.0001) 0.351
Root metal concentrations	0.983 (<0.0001) 1.390	0.971 (<0.0001) 1.473	0.963 (0.0001) 1.149	0.923 (0.0011) 1.111	0.987 (<0.0001) 1.961	0.992 (<0.0001) 2.041

Correlation coefficients, p values in parenthesis, and slopes, are given at the top, middle and bottom, respectively. Non-significant correlation are not presented.
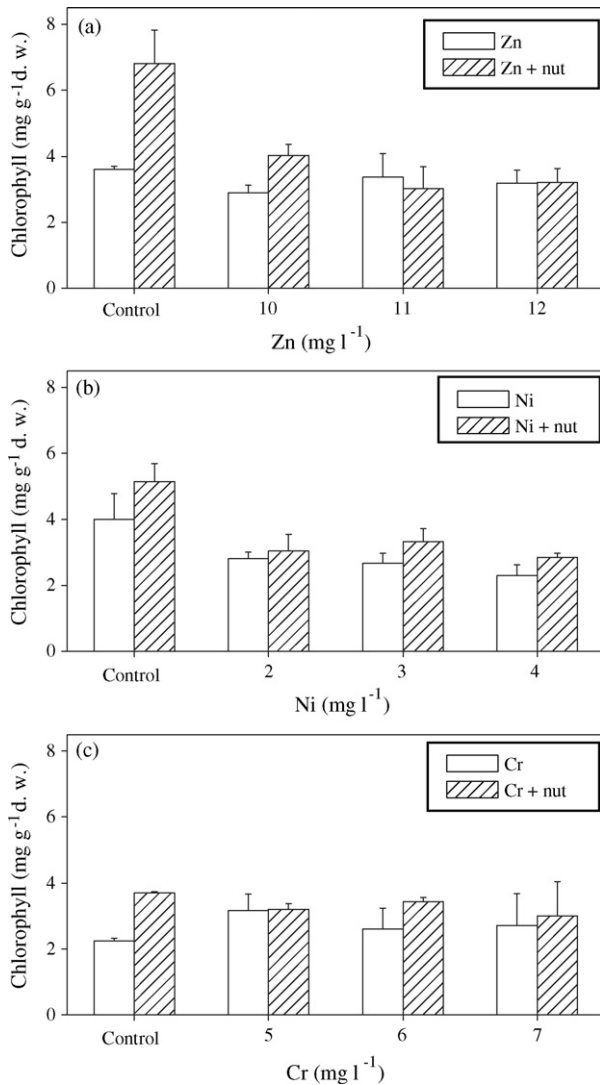
Fig. 2 – Chlorophyll concentrations obtained in the (a) Zn, (b) Ni and (c) Cr definitive experiments. The bars represent standard deviation.

Root biomass was inversely correlated with metal concentrations (Tables 1 and 2), being the linear regression coefficients significantly different from zero. Ni exposures with added nutrients did not show significant correlation, while Cr and Zn exposures resulted in significantly higher lin-

ear regression coefficients. Thus, nutrient enrichment either attenuated (Cr and Zn) or suppressed (Ni) root biomass decrease in response to metal exposure.

Chlorophyll concentration in S. herzogii was negatively correlated with Ni exposures (Fig. 2, Table 1). Nutrient addition significantly increased the regression coefficients. Thus, nutrient enrichment attenuated the observed decrease in chlorophyll concentration by Ni exposure. Chlorophyll did not correlate with Zn and Cr exposures. However, chlorophyll concentration showed a significant negative correlation in the Zn exposures enriched with nutrients.

3.2. Metal and nutrient concentrations in plants

Metal concentrations in plant tissue were higher in roots than in leaves (Table 3). Metal concentrations in leaves were positively correlated with metal concentration in water (Table 1). Nutrient enrichment significantly decreased the linear regression coefficient for Zn in leaves. Thus, nutrients attenuated the observed trend to increase Zn concentration in leaves with increasing exposures (Table 1).

Metal concentrations in roots were correlated with water concentration in each exposure (Table 1). Nutrient addition did not cause any significant change in the observed trend. Metals concentrations in plant tissues were different for the metals studied. Plant tissue metal concentration exhibited the following order of metal concentration in roots: $Zn > Cr > Ni$, and leaves: $Zn >> Cr \approx Ni$.

3.3. Metal and nutrient concentrations in water

Zinc concentrations in water decreased with time, reaching a plateau after about 4 days of exposure (Fig. 3a). Zinc concentrations in water were significantly lower in the treatments enriched with nutrients than in the treatments without enrichment (Fig. 3b). Nickel concentration in water decreased with time (Fig. 3c), the higher the initial concentration, the higher the final concentrations. Nutrient enrichment did not result in significant changes in Ni concentrations in water (Fig. 3d). A fast decrease in Cr concentration in water was observed without differences between the metal and nutrient enriched treatments (Fig. 3e and f). Roughly 80% of the added Cr was removed from the solution after the first day of incubation.

Soluble reactive phosphorous and ammonium concentrations in water decreased with time in the nutrient enriched

Table 2 – Mean ± S.D. of root biomass (g d.w.) in each treatment					
Zn experiment		Ni experiment		Cr experiment	
Initial	0.533 ± 0.021	Initial	0.813 ± 0.097	Initial	0.721 ± 0.072
10 mg l⁻¹	0.510 ± 0.029	2 mg l⁻¹	0.657 ± 0.008	5 mg l⁻¹	0.354 ± 0.031
11 mg l⁻¹	0.497 ± 0.057	3 mg l⁻¹	0.687 ± 0.054	6 mg l⁻¹	0.378 ± 0.039
12 mg l⁻¹	0.509 ± 0.036	4 mg l⁻¹	0.693 ± 0.004	7 mg l⁻¹	0.423 ± 0.025
Control	0.687 ± 0.018	Control	0.848 ± 0.030	Control	0.609 ± 0.013
10 mg l⁻¹ + nutrients	0.617 ± 0.024	2 mg l⁻¹ + nutrients	0.694 ± 0.023	5 mg l⁻¹ + nutrients	0.552 ± 0.014
11 mg l⁻¹ + nutrients	0.610 ± 0.012	3 mg l⁻¹ + nutrients	0.706 ± 0.136	6 mg l⁻¹ + nutrients	0.554 ± 0.034
12 mg l⁻¹ + nutrients	0.629 ± 0.014	4 mg l⁻¹ + nutrients	0.737 ± 0.107	7 mg l⁻¹ + nutrients	0.520 ± 0.028
Control + nutrients	0.850 ± 0.029	Control + nutrients	0.801 ± 0.097	Control + nutrients	0.611 ± 0.020

Table 3 – Mean (S.D.) of metals, TP and TKN concentrations (mg g^{-1} d.w.) in leaves and roots of S. *herzogii* obtained in the Zn, Ni and Cr definitive experiments

Experiment	Metal		TP		TKN	
	Leaves	Roots	Leaves	Roots	Leaves	Roots
Zn						
Initial	0.025(0.010)	0.119(0.013)	2.58(0.10)	1.67(0.10)	21.7(2.0)	13.8(2.0)
10 mg l^{-1}	6.15(0.302)	12.7(2.0)	2.11(0.033)	1.37(0.052)	21.5(0.410)	12.7(2.50)
11 mg l^{-1}	6.76(0.158)	15.1(1.84)	2.34(0.357)	1.33(0.006)	21.2(1.0)	11.0(2.0)
12 mg l^{-1}	8.51(0.41)	17.6(0.024)	2.05(0.060)	1.34(0.101)	22.2(2.77)	10.3(1.50)
Control	0.023(0.010)	0.10(0.015)	2.34(0.357)	1.47(0.066)	20.9(3.54)	12.7(1.65)
10 mg l^{-1} + nut	4.91(0.189)	15.9(4.30)	3.97(0.149)	4.55(0.902)	29.7(3.0)	14.6(2.17)
11 mg l^{-1} + nut	4.67(0.154)	16.8(1.27)	4.07(0.283)	4.97(0.318)	26.6(0.779)	11.3(1.39)
12 mg l^{-1} + nut	5.89(0.797)	16.9(0.796)	4.16(0.019)	4.96(0.089)	25.8(2.0)	10.2(2.0)
Control + nut	0.020(0.005)	0.09(0.005)	4.97(0.069)	5.19(0.367)	26.5(2.0)	13.9(0.40)
Ni						
Initial	0.01(0.001)	0.030(0.010)	3.16(0.10)	2.23(0.10)	22.7(1.50)	12.2(1.0)
2 mg l^{-1}	1.43(0.063)	2.93(0.056)	2.59(0.128)	1.54(0.007)	22.7(6.40)	14.4(2.90)
3 mg l^{-1}	1.94(0.083)	3.9(0.073)	2.65(0.079)	1.55(0.037)	23.8(1.0)	12.2(0.070)
4 mg l^{-1}	2.28(0.43)	4.52(1.0)	3.05(0.50)	1.67(0.10)	20.4(5.80)	12.8(0.920)
Control	0.015(0.009)	0.020(0.002)	3.34(0.218)	1.59(0.073)	21.2(1.0)	10.2(2.0)
2 mg l^{-1} + nut	1.24(0.066)	3.13(0.226)	4.48(0.061)	4.45(0.054)	25.1(1.0)	14.0(0.170)
3 mg l^{-1} + nut	2.09(0.70)	3.85(1.0)	3.86(1.10)	4.57(0.20)	25.6(1.4)	13.1(1.0)
4 mg l^{-1} + nut	2.38(0.16)	4.53(0.832)	4.40(0.041)	4.18(0.271)	27.9(2.33)	14.1(3.90)
Control + nut	0.011(0.003)	0.024(0.005)	5.00(0.37)	4.05(0.444)	23.1(1.0)	13.0(1.30)
Cr						
Initial	0.005(0.001)	0.020(0.001)	2.6(0.1)	1.84(0.07)	19.9(0.20)	12.8(3.50)
5 mg l^{-1}	1.84(0.325)	11.2(0.219)	2.62(0.240)	1.59(0.184)	20.4(0.440)	14.0(0.90)
6 mg l^{-1}	1.91(0.184)	12.6(0.361)	2.22(0.262)	1.46(0.325)	20.2(0.350)	12.0(0.30)
7 mg l^{-1}	2.63(0.212)	13.4(0.46)	2.55(0.318)	1.4(0.007)	20.7(0.610)	11.4(1.20)
Control	0.005(0.001)	0.015(0.002)	2.21(0.071)	1.42(0.057)	19.3(0.710)	10.4(0.819)
5 mg l^{-1} + nut	1.96(0.177)	10.6(1.0)	4.03(0.219)	3.77(0.071)	26.4(1.0)	15.3(1.40)
6 mg l^{-1} + nut	2.25(0.10)	12.4(1.10)	4.27(0.190)	4.47(0.360)	27.4(2.0)	12.2(0.35)
7 mg l^{-1} + nut	2.33(0.198)	14.1(1.20)	4.19(0.057)	4.45(0.580)	30.2(1.60)	11.9(0.70)
Control + nut	0.007(0.001)	0.020(0.010)	4.3(0.156)	3.12(0.021)	26.6(1.25)	14.8(0.422)

Table 4 – Metal balance for each treatment. The values in parentheses correspond to the percentage of the total metal removed.

| Treatments	Removal (%)	Metal in biomass (mg)	Metal in detritus (mg)
Zn			
10 mg l^{-1}	35	15.7 (90)	1.8 (10)
11 mg l^{-1}	41	20.0 (89)	2.6 (11)
12 mg l^{-1}	42	24.3 (97)	0.7 (3)
10 mg l^{-1} + nutrients	57	21.5 (75)	7.0 (25)
11 mg l^{-1} + nutrients	50	18.8 (72)	7.2 (28)
12 mg l^{-1} + nutrients	53	25.2 (79)	6.8 (21)
Ni			
2 mg l^{-1}	47	5.4 (98)	0.1 (2)
3 mg l^{-1}	52	7.1 (95)	0.4 (5)
4 mg l^{-1}	46	8.7 (97)	0.3 (3)
2 mg l^{-1} + nutrients	50	4.7 (92)	0.4 (8)
3 mg l^{-1} + nutrients	43	5.5 (92)	0.5 (8)
4 mg l^{-1} + nutrients	52	8.3 (87)	1.2 (13)
Cr			
5 mg l^{-1}	99	10.7 (43)	14.0 (57)
6 mg l^{-1}	100	10.8 (38)	18.0 (62)
7 mg l^{-1}	99	16.1 (50)	16.0 (50)
5 mg l^{-1} + nutrients	100	11.9 (46)	14.0 (54)
6 mg l^{-1} + nutrients	100	15.0 (52)	14.0 (48)
7 mg l^{-1} + nutrients	100	18.0 (53)	15.8 (47)
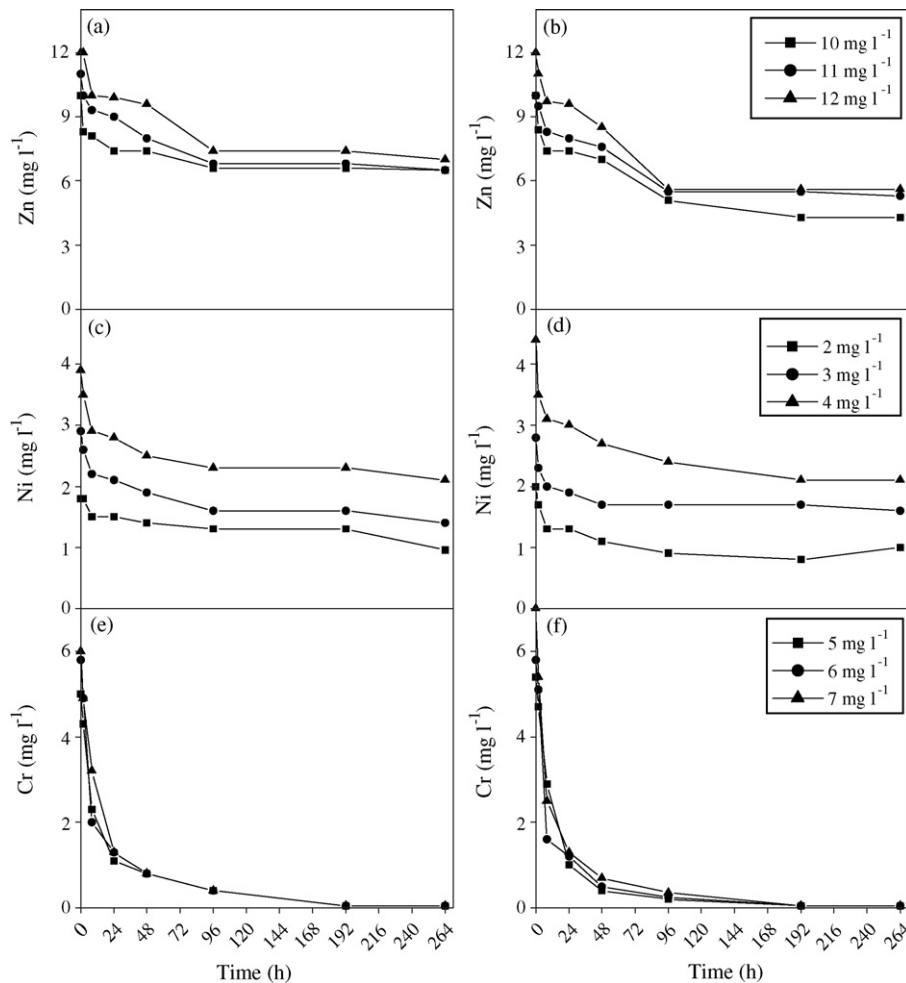
Fig. 3 – Zn, Ni and Cr water concentrations along time obtained in the (a) Zn, (b) Zn + nutrients, (c) Ni, (d) Ni + nutrients, (e) Cr and (f) Cr + nutrients exposures.

treatments (Fig. 4a–c). Lower SRP concentrations were attained in the aquarium with nutrient enrichment than in the Zn exposures. Lower nitrate concentrations were attained in the aquarium with nutrient enrichment than in the Zn and Cr exposures.

3.4. Metal mass balance

Table 4 shows the metal balance for each treatment. The amount removed was estimated as the difference between the initial and final water concentrations. The difference between the amount removed from the solution and the metal amount in the plant tissue was considered to be present in the detrital fraction. Such procedure might contain a considerable error. Results are reported in order to provide a first sight on the metal mass balance and show apparently different allocation patterns for the studied metals.

Zinc removal was improved from 35–42% in the metal exposures to 50–57% in the exposures enriched with nutrients. The higher retention was mostly due to an increase in the detrital fraction in the enriched treatments. Nickel removal represented roughly half of the initial concentration. Nickel was found mainly in the plant biomass without significant differ-

ences between metal exposures and the exposures enriched with nutrients. Chromium removal was 99–100% in all treatments. The amount of Cr retained in the detrital pool was larger than that of Zn and Ni.

4. Discussion

Nutrient enrichment enabled *S. herzogii* growth at Zn and Ni exposures that impaired growth in treatments without nutrient addition. The trend to increase Zn concentrations in leaves with increasing metal exposure was reduced by nutrient enrichment. Chaney (1993) reported that plants exhibiting Zn toxicity had lower P levels in shoots, and argued that it might result either from inhibited root growth or from insoluble Zn-phosphate formation and immobilization in the roots. Loneragan and Webb (1993) reported that P depresses either Zn absorption by roots or tanslocation from roots to shoots and commented that under conditions of high Zn supply, immobilization in the roots through the formation of Zn-phytate has been shown to occur. Simultaneous P and Zn immobilization in the roots is likely the mechanism for the increased tolerance of *S. herzogii* to Zn exposure.
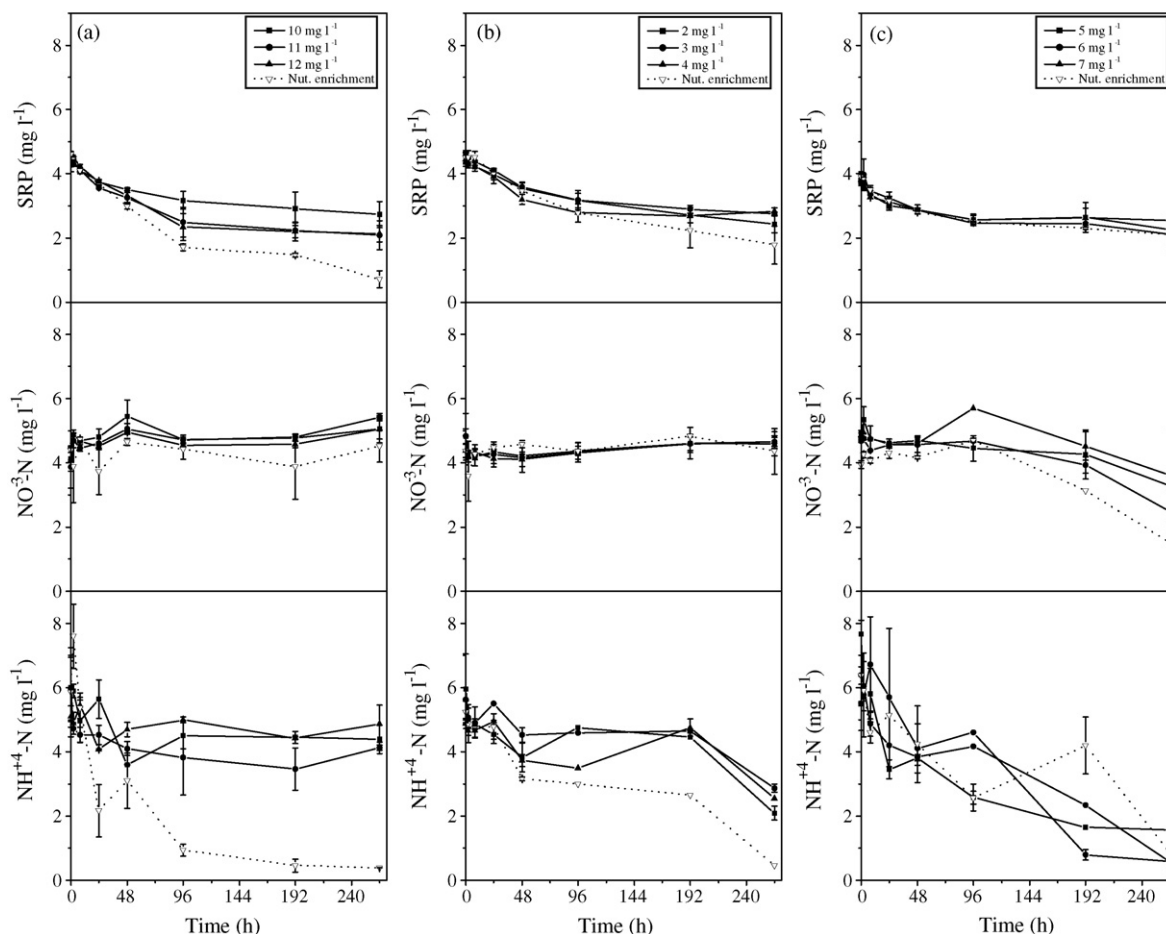
Fig. 4 – Soluble reactive phosphorous (SRP), nitrate and ammonium water concentrations along time obtained under nutrient enrichment in the (a) Zn, (b) Ni and (c) Cr definitive experiments. Bars represent standard deviation.

The inverse correlation between chlorophyll concentration and Zn exposure in the nutrient enriched treatments is consistent with the observation that excess Zn interferes with chlorophyll synthesis (Chaney, 1993). However, growth ceased at Zn concentrations in which chlorophyll did not decrease in the exposures not enriched with nutrients (Figs. 1d and 2a, Table 1), suggesting that other metabolic pathways are also impaired, and that growth rate is an earlier and more sensitive indicator of Zn toxicity than chlorophyll concentration.

Nickel exposure decreased relative growth rate and chlorophyll concentration simultaneously, suggesting that both variables represent equally suitable indicators of Ni toxicity. Monni et al. (2000) reported growth inhibition in *Empetrum nigrum*, an ericaceous shrub, with increasing Ni concentration in solution. The clearest responses to metal exposure were registered in the dry weights of shoots and roots. The authors suggested that growth suppression is the cost of tolerance because although growth was affected, plant survival did not decrease during the experiments.

Root 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; Scoccianti et al., 2006). Reductions in root biomass due to Cr exposure were attenuated by nutrient enrichment, suggesting

an improving effect of nutrient enrichment on the Cr tolerance of *S. herzogii* (Table 1). Since no effect on chlorophyll concentration was observed, Cr toxicity may not be mediated through the photosynthetic metabolism for *S. herzogii* under these experimental conditions. Maine et al. (2004) reported that the chlorophyll concentration of *P. stratiotes* decreased at Cr concentrations in water of over $1\,mg\,l^{-1}$, while *S. herzogii* did not show significant changes in chlorophyll concentrations up to $6\,mg\,Cr\,l^{-1}$.

Göthberg et al. (2004) found high metal concentrations in *I. aquatica* cultivated for human consumption in freshwater courses near Bangkok receiving variable amounts of cultural nutrient loads. The authors proposed fertilization as a means to attenuate metal accumulation. Their experimental work, in agreement with our findings, showed that nutrient enrichment increased *I. aquatica* tolerance to Cd, Pb and Hg. Different patterns of metal concentration in leaves of *I. aquatica* by nutrient addition were reported. Cadmium concentrations did not change with nutrient enrichment, as was observed with Ni and Cr in the present study, while Hg and Pb concentrations decreased with increasing nutrient concentrations, as was observed with Zn in the present study. Göthberg et al. (2004) suggested the competition between metals and nutrients in the root uptake in connection with the translocation to the

shoots as the probable mechanism governing metal concentrations in leaves and roots. Higher metal concentrations in roots than in leaves in macrophytes exposed to metals have been observed in the present study as has been shown in other previously reported research (Sen and Bhattacharyya, 1994; Banerjee and Sarker, 1997; Manios et al., 2003, Göthberg et al., 2004; Paris et al., 2005). Therefore, a higher tolerance of roots than shoots together with a trend to decrease translocation with increasing metal concentration in the roots represents a common feature for 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 (Chaney, 1993; Loneragan and Webb, 1993; Göthberg et al., 2004).

Metal concentrations in water decreased with time in all the experiments. This read was faster for Cr, as earlier observed by Maine et al. (2004) at different Cr(III) concentrations. Chromium(III) is readily adsorbed to particles and colloids (Masscheleyn et al., 1992). The total Cr balance (Table 4) suggests that faster removal from the solution than Zn and Ni is more related to the physicochemical chelation and adsorption to the detrital fraction than with the plant uptake. Chromium was mainly adsorbed to suspended matter or uptaken by the accumulated organic debris, derived from decomposing root fragments. The presence of waterborne suspended matter in the river water enhanced Cr adsorption followed by precipitation. Suspended matter in water ranged 60–110 mg l^{-1} in the Paraná River (Drago and Amsler, 1988). Chromium removal in the experiments was 99–100% at different metal exposures, consistent with previous results from laboratory experiments (Delgado et al., 1993; Maine et al., 2004), but higher than the removal efficiencies obtained in the small and large scale constructed wetlands at the Bahco tool factory in Santo Tomé (81% and 86%, respectively; Maine et al., 2005, 2006). On the other hand, Zn and Ni removals from the solution were similar to the removal efficiencies estimated in the small and large scale constructed wetlands (Maine et al., 2005, 2006). Zinc and Ni removal resulted mainly from S. herzogii uptake. Nutrient enrichment increased the removal efficiency with Zn exposure, by increasing the metal retention in the detrital fraction (Table 4).

Lower Zn concentrations in water and higher retention in the detrital fraction in the Zn exposures enriched with nutrients contributed to increase the growth capacity of S. herzogii, while its sensitivity might not actually be affected. On the other hand, decreased Zn concentration in leaves in response to nutrient addition strongly suggests a physiological mechanism of increased tolerance. Both processes operate simultaneously, and the relative importance of each one alone is difficult to ascertain within the experimental framework assayed. Further work is needed on this subject.

Ammonium and SRP were depleted in the treatments enriched with nutrients. The preference of S. herzogii for ammonium over nitrate has already been reported by Panigatti and Maine (2003). Ammonium is a source of nitrogen that is readily transported by metabolic systems located at the macrophyte plasmalemmas, while nitrate requires reduction

energy in order to be incorporated in the protein synthesis (Bishop and Eighmy, 1989). Zinc exposure reduced nutrient removal from water (Fig. 4, Table 3), emphasizing the role of competition between metals and nutrients for active sites in the uptake by roots and in the plant translocation system as suggested by Göthberg et al. (2004).

5.   Conclusions

Nutrient enrichment increased the tolerance of S. herzogii to metals. This effect has important implications for the use of constructed wetlands for industrial wastewater treatment. Many industrial processes in the metallurgic industry produce wastewaters containing high metal concentrations. Increased tolerance may determine the feasibility of using constructed wetlands for wastewater treatment by allowing macrophyte growth at metal concentrations that would otherwise impair macrophyte development.

Nutrient enrichment will improve metal removal by increasing macrophyte production, leading to a higher metal uptake by the macrophyte biomass, and also by enhancing the overall biological activity, attaining a higher retention in the detrital fractions.

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