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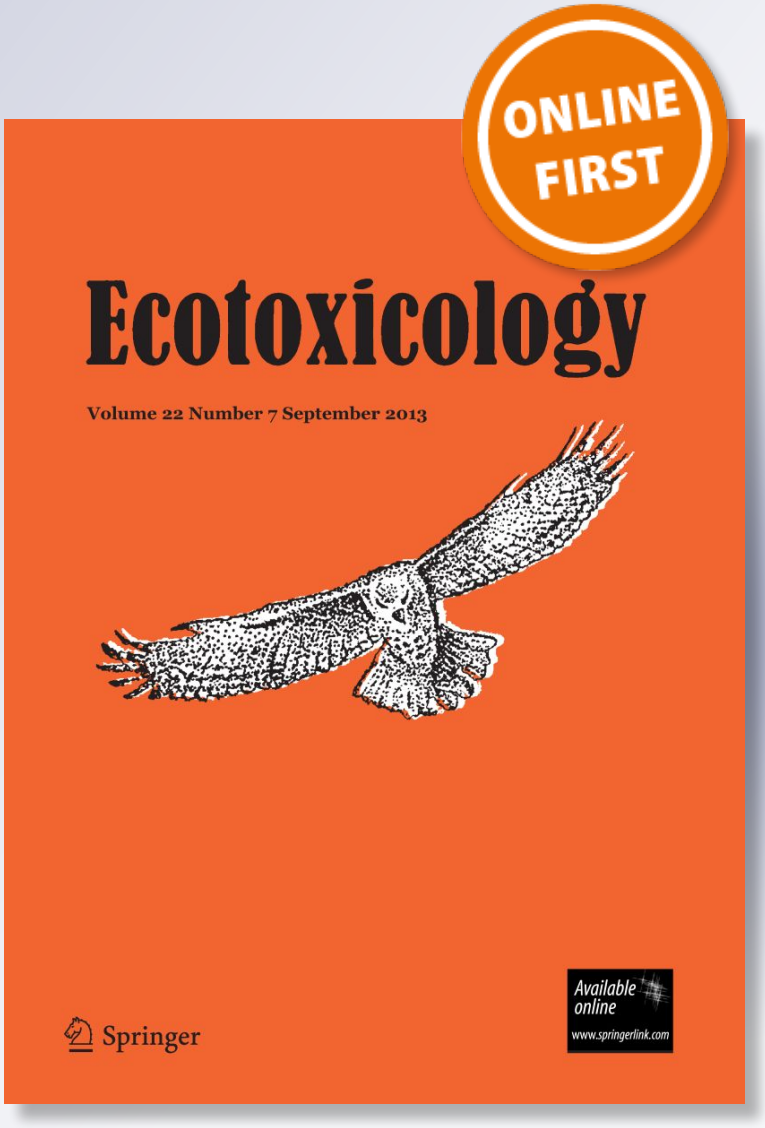

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Comparative ecotoxicity of single and binary mixtures exposures of nickel and zinc on growth and biomarkers of *Lemna gibba*

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Abstract

The aim of this study was to compare the ecotoxicity of nickel (Ni) and zinc (Zn) assayed as single and as binary mixture. In addition, how were affected the population growth rates and oxidative stress biomarkers, comparing single to binary exposures. The toxicity tests were performed on *Lemna gibba* using a 7-day test. All calculations were made using measured total dissolved metal concentrations. IC50-7d, based on growth rate calculated on frond number and fresh weight, were 2.47/3.89 mg/L, and 76.73/76.93 mg/L, for Ni and Zn, respectively. Single metals affected plant growth following a non-linear concentration–response relationship. LOEC values for each metal were obtained at 0.92 and 20.1 mg/L for Ni and Zn, respectively. Biomarkers of the antioxidant response like Catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APOX; EC 1.11.1.11) and guaiacol peroxidase (GPOX; EC 1.11.1.7) activities in single metals assays were higher than controls, but when similar concentrations were added as mixtures, that increase was reduced and inhibition with respect to the control was observed for GPOX. APOX showed the highest activity. The concentration addition (CA) approach was evaluated and resulted in a correct predictor of Ni-Zn mixture toxicity on *Lemna gibba*. This was made comparing the EC50 and LOEC, measured taking the growth rate as endpoint, with those expected values according to the CA model. However, the measured biomarkers indicating a positive response to free radicals did not fit to concentration addition model when assayed in the binary mixture. Also, the main activity response of these was observed within a range of concentrations below the LOEC values for the mixture.

Keywords Heavy metal · *Lemna gibba* · Antioxidant enzymes · Binary mixture · CA model

Introduction

Aquatic and terrestrial ecosystems are negatively influenced by the pollution of heavy metals, constituting a serious threat for biota (Nasri et al. 2015). The industrial development began several years ago leading to certain environmental problems. The presence of heavy metals in the environment is one of them. It becomes a relevant issue considering they present high toxicity, are persistent in nature, non-biodegradable and can bioaccumulate in living animals and plants. Mixtures of different heavy metals, produced by many anthropogenic activities, are components of wastewaters often discharged in the environment without the proper treatment resulting in a serious social-environmental problem around the world, as they are sources of contaminations of water bodies, affecting water quality and organisms in there (Fernández et al. 2018; Verma and Suthar 2015; Chaoui et al. 1997; Doganlar 2013; Dogan et al. 2018; Chorom et al. 2012). The widespread introduction of trace metals and metalloids into the environment are causing acute and diffuse contamination in soil and waters (Singh and Singh 2016). As persistence substances, they may achieve a truly global distribution due to atmospheric transport and deposition on soils and surface waters. Once in waters, rivers can transport contaminants far from the pollution source, reaching pristine biosystems and endangering its stability (Hoffman et al. 2003; Basile et al. 2015). Heavy metals are present in earth's geological structures and can be leached by heavy rains or flowing water, thus entering in water resources producing a natural background concentration.

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The presence of heavy metals in the environment becomes exacerbated due to some human activities such as former and present mining sites, foundries and smelters, combustion by-products and traffics, electroplating, timber preservation, tanning, industrial waste storage and treatment, sewage and storm water discharges (Khan et al. 2011). Fertilizers and biosolids are also sources of metal contamination, specially after continued fertilization, which could increase its concentration. These heavy metals have the potential to contaminate groundwater under certain conditions (Wuana and Okieimen 2011), which can mean a risk for surface water as they are commonly hydraulically connected (Winter et al. 1998). Besides, metal toxicity in soils can cause poor plant growth, decreasing coverage that subsequently can lead to metal mobilization in runoff water with deposition into nearby water bodies (Lasat 2000). As aquatic ecosystems are often final receivers of these elements, they are mostly at risk (Parnian et al. 2016). Pollutants loaded in aquatic ecosystems may have effects on the primary trophic level composed partly of aquatic vascular plants, also called macrophytes. These organisms play a critical role in this environment as they serve as a food source for invertebrates, fish and waterfowl as well as providing habitat and shelter for many of them. Furthermore, they are essential in the solar energy catchment, oxygen production, nutrient cycling and carbon assimilation (Hoffman et al. 2003). Hence, as they are a primary energy source and play critical and functional roles in aquatic ecosystem stability we consider imperative the assessments of potential risks of toxicants over macrophytes.

Lemna gibba, also referred to as duckweed, is a free-floating aquatic angiosperm member of the family *Lemnaceae*, also referred to as duckweed. Duckweeds have been by far the most commonly used vascular plants in toxicity test and are considered as model hydrophytes in ecotoxicology. *Lemna* is frequently used in ecotoxicology research as a representative of higher aquatic plants. The reason is that they present many advantageous features that make them suitable organisms. For example, their worldwide distribution; the fact they can be commonly found year-round in fresh waters in temperate climates; their small size, though fronds are sufficiently large to be easily counted with the unaided eye; that they are easy to culture and handle in laboratory conditions; present fast growth and have a short life span; and the fact that they are not rooted in the hydrosoil (Wang 1991; Prasad et al. 2011; Drost et al. 2007; Hou et al. 2007; Dirilgen 2011; Hoffman et al. 2003; Basile et al. 2015).

Many works determining the toxic effects of compounds discharged into aquatic systems have paid more attention to fish or invertebrates than on primary producers. In this paper we evaluated the phytotoxic effects of Ni and Zn, separately and in mixtures, on *Lemna gibba* in order to evaluate morphological and physiological changes in a concentration dependent manner. Despite environmental risks of chemicals are often assessed individually, contaminants in the actual environment usually exist in a mixed state, therefore the effects of mixtures are neglected many times (Feng et al. 2018; Balistrieri and Mebane 2014; Backhaus and Faust 2012). Although metal mixtures can result in larger effects when co-occur than each of the individual metals in it, current environmental risk assessment frameworks worldwide does not explicitly incorporate metal mixture toxicity (Nys et al. 2018). Even though mixtures toxicity tests have been conducted for some years, there is a need to understand how metals interact so as to predict their toxicity accurately (Meyer et al. 2014). We consider of relevant importance evaluating and predicting the effect of heavy metal mixtures, especially in aquatic environments since they are generally, as previously mentioned, the final receivers of contamination. Ni and Zn are two of the metals identified as being of major importance to the aquatic environment. Both are included within the essential elements. Zn is an important component of a large number of enzymes. It is associated with the carbohydrate metabolism, protein synthesis, gene expression and regulation. It forms complexes with DNA and RNA and affects their stabilities, ribosome's structural integrity and phosphate metabolism (Radić et al. 2009; Cherif et al. 2011). On the other hand, Ni is a cofactor for urease and many other Ni-containing enzymes (Doganlar et al. 2012). Although some metals are essential nutrients, when they are present in excess, they can cause physiological and biochemical changes such as oxidative stress and inhibition of enzymes in plant tissues (Drost et al. 2007). Effects of metal mixtures and studies of mixture interactions on biochemical parameters as antioxidant enzymes, are rather scarce (Sharma et al. 1999; Guo et al. 2007; Maleva et al. 2012). Here we determined the antioxidant enzymatic activity of Catalase (CAT), Ascorbate peroxidase (APOX) and Guaiacol peroxidase (GPOX) as they are an important defense mechanism against reactive oxygen species which are known to increase because of heavy metal toxicity (Doganlar et al. 2012). For this reason, toxicity of metal mixtures could be explained more accurately by biochemical parameters rather than by growth parameters, as they are coupled with internal defense mechanisms and metabolism. Field studies of industrial effluents downloaded into Luján river freshwater (Buenos Aires Province, Argentina) have allowed us to know environmental concentrations of both metals and define the assayed concentrations in this study (Di Marzio et al. 2005). The aim of this work was to evaluate and describe the interaction at environmental concentrations between Nickel and Zinc in binary mixture exposures, according to the concepts known as additivity, synergism and antagonism. Effects of single and binary

fixed ratio mixtures of both metals on growth and oxidative stress biomarkers of *Lemna gibba* were evaluated.

Materials and methods

Test organisms and chemicals

Lemna gibba plants were collected from a natural pond at the National University of Luján. Plants were maintained in laboratory conditions in stock cultures in the same medium as used for testing at least two weeks prior to use. Cultured and tested conditions followed, were according to OECD (2006) guidelines. Growth medium intended for testing was prepared 1–2 days before use to allow pH stabilization. Just before the start of the test, the pH of the medium was adjusted to 7.5 (±0.1) using 0.1N NaOH or HCl. pH was measured daily in the test chambers. A test was carried on in 250-mL Erlenmeyer flasks containing 100 mL of culture medium for 7 days; 24h-day photoperiod with a mean cool white fluorescent lighting intensity of 6000 lux. Flasks were placed in a thermostatic room at 24 ± 2 °C. Chemicals $ZnCl_2$ and $NiSO_4{\cdot}6H_2O$ of analytical grade were obtained from Sigma Chemical Co. (St. Louis, MO).

Experimental design

Single exposures of metals were done, in order to assess the toxicity of Ni and Zn by performing 7 day growth inhibition test. The initial biomass of 12 fronds consisting of colonies of 2–4 visible fronds each, were transferred from the stock cultures under aseptic conditions to test vessels. Number of fronds and colonies were the same in each treatment. Definitive toxicity test of both metals was done using a set of metal concentrations. Zn was added to the growth media in concentrations of 2.3–4.6–9.2–20.1–36.7–74.6–183–284–587 mg Zn/L, respectively, from a stock solution of $ZnCl_2$. Ni was added to the growth media in concentrations of 0.18–0.37–0.92–1.46–3.61–6–11.82 mg Ni/L, respectively from a stock solution of $NiSO_4{\cdot}6H_2O$. Selection of both metal test concentrations in definitive toxicity testing was done considering a previous range finding test. The range of test concentrations for definitive toxicity test was arranged in a geometric series using a separation factor of 0.5 between test concentrations. In addition, cultures of growth media without metal were included as control. Both controls and treatments were carried out by duplicate. Flasks were randomized in the growth chamber in order to minimize the light intensity or temperature differences, under experimental conditions mentioned above. All metal concentrations reported in the present paper refer to initial measured total dissolved metal concentrations. Metal analysis was conducted using graphite furnace atomic absorption or flame atomic absorption spectroscopy in a PerkinElmer spectrophotometer model analyst 200. The relative difference between measured and nominal concentration was in a ranged between 1–9%.

Binary mixture toxicity tests were performed under same experimental conditions as in single metal tests. With the aim of assess Ni/Zn toxicity mixtures, three different fixed ratios were chosen for mixture assays. The ratios were calculated based on the concept of toxic units (UT), that is defined as the ratio of the concentration of an individual component in the mixture to the concentration that is needed to a specified effect concentration in a single metal situation. UT ratios were 2/3 Ni–1/3 Zn (called hereafter Ratio 1); 1/2 Ni–1/2 Zn (called hereafter Ratio 2); 1/3 Ni–2/3 Zn (called hereafter Ratio 3). Five metal concentrations combinations were included in each ratio (Jonker et al. 2005). Concentrations of Ni and Zn in five concentrations tested for each ratio were: Ratio 1: 0.38–6.32; 0.77–12.65; 1.54–25.3; 3.08–50.6; 6.15–101.21; Ratio 2: 0.29–9.49; 0.58–18.97; 1.15–37.96; 2.31–75.9; 4.62–151.8, and Ratio 3: 0.19–12.71; 0.38–25.43; 0.76–50.85; 1.52–101.71; 3.05–203.42, respectively. For simplicity, in the Results and Discussion section, each of the just mentioned treatments was expressed as the sum of both metal concentrations.

Growth measurements

For single and mixture assays, plants were removed from the solutions at the end of the 7 days of exposure and two endpoints, frond number and fresh weight, were measured.

From these endpoints different response variables were calculated: Average specific growth rate (μ) and percent inhibition of growth rate (% I_r).

Specific growth rate (μ) was calculated as the average of two replicates in each flask

$$\mu\,\mathrm{i}-\mathrm{j}=\frac{\ln(\mathrm{Nj})-\ln(\mathrm{Ni})}{\mathrm{t}}$$

where N_j is measured variable in the test or control vessel at the end of the experiment, N_i is measured variable in the test or control vessel at the start of the experiment and t is the time period from i to j. The relative inhibition of average specific growth rate (%Ir) was calculated as a percentage inhibition of specific growth rate with respect to the control treatment.

$$\%\mathrm{Ir}=\frac{(\mu\mathrm{C}-\mu\mathrm{T})}{\mu\mathrm{C}}\times 100$$

where μC is the mean value for μ in the control flask and μT is the mean value for μ in the treatment group.

Both single and binary mixture toxicity tests, controls meet the required growth for the tests to be valid (OECD 2006), namely, a sevenfold increase in frond number in control flasks at the end of the test and an average specific

growth rate between 0.20 and 0.27 d^{-1}. Single metal test data were used to interpret binary metal mixture toxicity.

Biomarkers

Activity of antioxidant enzymes was analyzed at the two lowest concentrations of each single metal and at the three lowest of each mixture ratio. At the end of the tests, fresh weight was measured in controls and treatment plants. After that, extracts for determination of catalase (CAT), guaiacol peroxidase (GPOX) and ascorbate peroxidase (APOX) were performed from the total plant mass from each test vessel, under ice-cold conditions in 2 ml of sodium phosphate buffer (pH 7.4) at 4 °C with 0.1% w/v Triton X-100. The homogenates were centrifuged at $10000 \times g$ for 20 min controlling temperature at 4 °C. Supernatant fraction was held on the ice. Determinations for all enzymes were done in Spectrophotometer Shimadzu Model UV1800. CAT (EC 1.11.1.6) activity was determined spectrophotometrically by measuring the consumption of H_2O_2 at 240 nm (ε: 0.036 mM^{-1} cm^{-1}) in a reaction medium containing 50 mM sodium phosphate buffer (pH 7), 33 mM of H_2O_2 and enzymatic extract as proposed by Chance et al. (1979). Blank was carried out with H_2O_2 and a buffer. GPOX (EC 1.11.1.7) activity was determined by measuring the increase in absorption at 470 nm due to the formation of tetraguaiacol (ε: 26.6 mM^{-1} cm^{-1}) in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7), 10 mM guaiacol, 10 mM H_2O_2, and enzymatic extract according to Maehly and Chance (1954). Blank was carried out with H_2O_2 and a buffer. APOX (EC 1.11.1.11) activity was determined by oxidation of ascorbate, following the decrease in absorbance at 290 nm (ε: 2.8 mM^{-1} cm^{-1}) in a reaction medium containing 50 mM sodium phosphate (pH 7), 10 mM H_2O_2, 5 mM ascorbate and enzymatic extract according to Nakano and Asada (1981). Blank consisted in H_2O_2 and buffer. Total protein determination of controls and treated plants was evaluated by the method of Bradford (1976) using bovine serum albumin as standard.

Mixture toxicity assessment

Analysis of mixtures interactions was made by multiple regression analysis (MRA) using frond number inhibition (FNI) as response variable in order to fit the observed mixture toxicity data to metal concentration (Gopalapillai and Hale 2015; Zar 2010). The selected full model was:

$$\%\,FNI = \beta_0 + \beta_1[Ni] + \beta_2[Zn] + \beta_3[Ni] \times [Zn] + \varepsilon$$

where β values represent the regression coefficients and ε is the normal error. Standardized regression coefficients were also calculated. The p value was <0.05. MRA was performed using Statistica V8. Toxicity in mixtures can be predicted using the concentration addition (CA) reference model based on single-metal toxicity data (Jonker et al.), where the sum of toxic units of the individual components of the mixture is calculated. In this way, the concentration of the two metals in the mixture expected to result in 50% frond number inhibition (FNI) were calculated by solving the multiple regression for Ni while holding the concentrations of Zn constant. These combinations of concentrations were used to calculate the sum of toxic units for each mixture test case, as follows:

$$\sum \mathrm{TU} = \sum_{i=1}^{n} \frac{Ci}{EC50i}$$

where Ci denotes the concentration of the ith toxicant in the mixture causing 50% FNI and EC50i is the concentration of the ith toxicant causing 50% FNI in a single metal exposure. When $\sum \mathrm{TU} = 1$, CA occurred; Interactions are defined as deviations from additivity, so deviations from unity can occur, like when the sum is more than unity (indicating that the mixture effect is less-than additive) or when the sum is less than unity (indicating a more than additive). In this way, the interactions are synergistic when toxicant in the mixture is more toxic than individually and is defined as antagonistic when the toxicant in the mixture is less toxic than in a single form (Gopalapillai and Hale 2015).

Statistical analysis

Single-metal toxicity thresholds as IC10, IC25 and IC50 (e.g. the 10% inhibition concentration) for test values were calculated for both endpoints (frond number and fresh weight) for both metals by nonlinear regression analysis using the Solver function of Microsoft Excel. Enzymatic activity of plants exposed to single metal was compared using one-way ANOVA and Dunnet's Test ($\alpha < 0.05$) using ToxStat V3.5 (Zar 2010; Sparks 2000). For analysis of growth parameters and enzymatic activity in mixtures, data were compared by one-way analysis of variance (ANOVA) and Dunnett's multiple comparison test to determine significant differences among treatment and control groups (not treated) ($\alpha < 0.05$).

Results and discussion

Effects of metals on growth

Single metal toxicity

The effects of Ni and Zn on average specific growth rate (μ) based on frond number and on fresh weight on *L. gibba*

Table 1 Values of average specific growth rate (μ) and percent inhibition of growth rate (% Ir) for single metal tests calculated on basis of frond number and fresh weight

	μ (a)	% I_r (a)	μ (b)	% I_r (b)
Ni Cnt	0.200		0.200	
0.18 mg/L	0.207	3.13**	0.212	5.98**
0.37	0.187	6.61	0.196	1.76
0.92	0.113*	43.63*	0.123	38.62
1.46	0.090*	56.05*	0.146	27.07
3.61	0.058*	71.21*	0.097	51.47
6	0.041*	80.04*	0.123	38.37
11.82	0*	100*	0.037*	81.26*
Zn Cnt	0.202		0.186	
2.3	0.233	15.6**	0.184	1.48**
4.6	0.192	4.83	0.143	23.36
9.2	0.172	14.73	0.111	40.27
20.1	0.133*	33.94*	0.132	29.44
36.7	0.124*	38.74*	0.141	24.41
74.6	0.121*	39.99*	0.091*	50.9*
183	0.045*	77.45*	0.048*	74.35*
284	0.006*	97.11*	0.046*	75.53*
587	0*	100*	0*	100*

Cnt control

*Significant, ANOVA-Dunnet's Test $\alpha < 0.05$; **stimulatory; (a): based on frond number; (b): based on fresh weight

growth under different single concentrations are shown in Table 1. The results show that average specific growth rate (μ) decreased with increasing concentration of both metals. At the end of the exposition time, plants exposed to the highest concentrations of each metal, did not grow, since the number of fronds at this time were the same of at the beginning of the test, resulting in $\mu = 0$ and 100% inhibition of growth rate (% I_r) (Table 1). Considering the effects of Ni concentrations from 0.92 mg/L and higher, significant inhibition respect to the control of growth rate based on frond number was observed, while for fresh weight only the higher was significantly inhibited ($p < 0.05$). In the case of Zn, 20.1 mg/L and higher concentrations exerted significant inhibition respect to the control of growth rate based on frond number, while for fresh weight this inhibition was from 74.6 mg/L and higher ($p < 0.05$). For both metals, frond number was the most sensitive endpoint. At the highest concentrations, Ni inhibited plant growth by 80 and 81%, while Zn inhibited plant growth by 77 and 75%, considering frond number and fresh weight respectively (Table 1). At the end of the test, plants exposed to Ni did not show frond disconnection (detachment of fronds from the colonies) and they preserved a green color and a rather healthy appearance, conversely Zn exposed plants showed frond disconnection and a notorious chlorosis with an almost total progression from green to yellow-white color at the two highest concentrations. These are some of the visible symptoms indicating severe metal phytotoxicity. A concentration-dependent response of Ni and Zn stress was observed based on frond number and fresh weight. The highest inhibitions of the two endpoints, measured at the end of 7 days testing time, were observed at the highest concentrations of Ni and Zn. In accordance with these results, Radić et al. (2009) also observed a 35% decrease in relative growth rate of *Lemna minor* exposed to 20 mg Zn/L for 15 days (Table 1). Megateli et al. (2009) showed that concentrations of Zn between 4 and 50 mg/L inhibited growth of *Lemna gibba* (colony number) by 50–79% in plants exposed in modified Hoagland's medium with a photoperiod of 16 h of light (4000 Lux) and 8 h of darkness. On the other hand, Khellaf and Zerdaoui (2010) have shown that after 4 days of exposure to Ni, the growth of this species was decreased by 50% (based on total frond area) when medium contained 0.75 mg/L. The lowest concentrations, 0.18 and 2.3 mg/L of Ni and Zn respectively, had a stimulatory effect on growth when it was based both in frond number and fresh weight. This stimulatory effect could be a result of the increased availability of these two essential micronutrients, which may improve organism's physiology when concentrations do not reach toxic values.

Estimated toxicity threshold values for both metals in a single exposure assays can be seen in Table 3. IC50 values obtained based on frond number and fresh weight were 2.47/3.88 mg/L, and 76.63/76.83 mg/L, for Ni and Zn, respectively. It can be noticed that Ni was more toxic than Zn to *L. gibba*. For this metal, all three toxicity thresholds (IC10, IC20, IC50) were lower based on the frond number than when the fresh weight was considering.

Binary mixture toxicity

The effects of binary mixtures of Ni and Zn on average specific growth rate (μ) based on frond number and on fresh weight of *L. gibba* is shown in Table 2. The results show that average specific growth rate (μ) decreased with increasing concentration of both metals in the mixtures within each of the three ratios or proportions performed. In accordance with this, an increase of the percentages of inhibition of growth based both on frond number and fresh weight was observed (Table 2). The percentages of inhibition obtained from Ratio 1 ranged between 18–88 and 19–66% for frond number and fresh weight respectively. Instead, for Ratio 2 were 19–100 and 14–79%, respectively, whereas inhibition of frond number ranged from 31–100% and fresh weight from 38–100% in Ratio 3 mixtures.

At the end of the test, a 100% inhibition of growth was observed at the highest combined concentrations of Ratio 2 and 3, based only on the frond number in the first case

Table 2 Values of average specific growth rate (μ) and percent inhibition of growth rate (% Ir) for binary mixtures of Ni:Zn calculated on the basis of frond number and fresh weight

	μ (a)	% I_r(a)	μ (b)	% I_r(b)
Ratio Ni:Zn				
2/3Ni-1/3Zn Cnt	0.23		0.21	
6.7	0.18*	18.67	0.17	19.63
13.42	0.19*	15.64	0.17	19.94
26.84	0.12*	46.95*	0.11*	48.78*
53.68	0.09*	56.69*	0.095*	54.68*
107.36	0.03*	88.15*	0.07*	66.88*
0.5Ni-0.5Zn Cnt	0.23		0.21	
9.78	0.18*	19.17*	0.18*	14.19
19.55	0.13*	44.63*	0.108*	48.41*
39.11	0.08*	64.93*	0.104*	50.42*
78.21	0.06*	74.93*	0.01*	95.43*
156.42	0*	100*	0.043*	79.60*
1/3Ni-2/3Zn Cnt	0.186		0.159	
12.9	0.13*	31.03*	0.099*	38.05*
25.81	0.11*	41.40*	0.064*	59.57*
51.61	0.11*	37.79*	0.042*	73.37*
103.23	0.08*	56.92*	0.009*	94.64*
206.47	0*	100*	0*	100*

Cnt control

*Significant, ANOVA-Dunnet's Test $\alpha < 0.05$; (a): based on frond number; (b): based on fresh weight

(156.42) and in both endpoints in case of Ratio 3 (206.47) (Table 2). In most of the combinations, the effect on growth rates was more evident when considering the frond number than fresh weight, indicating a more sensitive endpoint. However, binary mixtures of ratio 3 exhibited a reduction in growth rates that was more evident when fresh weight was analyzed (Table 2). Stimulatory effects on growth rates were not noticed in neither combination of binary metal mixture assays.

As visible symptoms of the deleterious effects of binary mixtures, plants exposed to the highest concentrations of the three Ratios, showed frond disconnection (detachment of fronds from the colonies) affecting the release of daughter fronds, being remarkably worse at 206.47 mg/L of Ratio 3, where colonies were formed by only one frond. Toxicity threshold data from binary mixtures assays can be seen in Table 3. IC50 values obtained based on frond number were in a range between 25.48 and 68.43 whereas for fresh weight this range was 33.10 and 43.60 mg/L, respectively. Frond number seems to be a more sensitive endpoint. Considering all three toxicity thresholds (IC10, IC20, IC50) a difference can be indicated, as in Ratio 1 and 3 fresh weights derived values resulted much lower; nevertheless, for Ratio 2, frond number was the endpoint which yields lower results of these toxicity index.

In spite of the fact that Ni and Zn are essential micronutrients for plants, playing a physiological role that their complete absence causes severe damage for normal development, when their presence in the environment is above certain thresholds limit, indicate not only industrial pollution from diverse anthropogenic sources (Di Marzio et al. 2005) but cause deleterious effects on living plants. As a first response, they become inhibitory of growth development but afterwards they clearly state as toxic. Excessive amount of Ni inhibits germination, chlorophyll production and proteins, induce chlorophyll degradation and interfere photosystem activity, cause chlorosis and necrosis resulting from disturbed iron uptake and metabolism and inhibition of cell division of root meristems. High concentrations of Zn produce inhibition of growth by altering normal metabolism, as it plays very important role in enzyme activation and cofactor of several enzymes associated with nitrogen metabolism, cell multiplication and photosynthesis. Deleterious toxicity effects as a consequence of excessive amount of this metal are being noticed, related to nutritional deficiencies. I have been observed symptoms as chlorosis as a result from a Zn induced Fe or Mg deficiency, based on the fact that these metals have similar ion radii that Zn (Seregin and Kozhevnikova 2006; Gautam et al. 2017).

Effects of metals on antioxidant enzymes

GPOX, APOX and CAT are three of the most important indicators of metal toxicity and major scavengers of hydrogen peroxide which is produced under metal stress. Only monometallic responses have been mostly investigated and studies on responses against metal interaction are very rare. An example of those works is that of Israr et al. (2011), where a series of binary mixtures were evaluated on *Sesbania drummondii* seedling resulting Ni + Zn mixture the most toxic, presenting an increase in APOX activity. One of the goals of the present work was to evaluate, in single metal and mixture tests, the levels of antioxidant enzymes that are known to act as one of the main defense mechanisms and provide plants some extent of stress tolerance against ROS induced by heavy metals. This ROS production results from the interaction of heavy metals with electron transport activities and can disrupt the redox status of cells (Shahid et al. 2014). Despite ROS are constantly produced in normal cells on account of their metabolism, alterations in its levels must be kept under stringer control because severe or persistent oxidative stress eventually results in programmed cell death (Gechev et al. 2005). The activity of these antioxidant enzymes was affected not only in single applications, but also in mixtures assays (Fig. 1).

Table 3 Toxicity threshold values (mg/L) calculated on the basis of Frond number and Fresh weight after 7-days exposure to single and binary mixtures of Zn and Ni

	IC10	IC20	IC50
Frond number—Single			
Zn	2.12 (0.51–5.38)	9.38 (5.01–16.2)	76.73 (65.9–123)
Ni	0.05 (0–0.24)	0.24 (0.05–0.63)	2.47(1.32–4.63)
Fresh weight—Single			
Zn	1.31 (1.03–1.64)	6.66 (5.89–7.50)	76.93 (73.1–81.8)
Ni	0.07 (0.01–0.21)	0.36 (0.18–0.67)	3.89 (2.85–5.55)
Frond number—Mixtures			
Ratio 1	1.78 (0.25–4,8)	5.78 (2,76–10.59)	34.11 (24.57–48.48)
Ratio 2	0.66 (0.23–1,33)	2.84 (1,76–4.24)	25.48 (20.29–33.51)
Ratio 3	0.71 (0.07–2,83)	4.38 (1.69–10.1)	68.43 (44.39–110.2)
Fresh weight—Mixtures			
Ratio 1	0.84 (0.67–1.26)	4.06 (3.6–5.09)	43.60 (41.03–49.15)
Ratio 2	0.83 (0.2–2.24)	3.62 (1.95–6.5)	33.10 (24.8–46.06)
Ratio 3	0.28 (0.21–0.36)	1.95 (1.68–2.25)	36.78 (34.13–39.62)

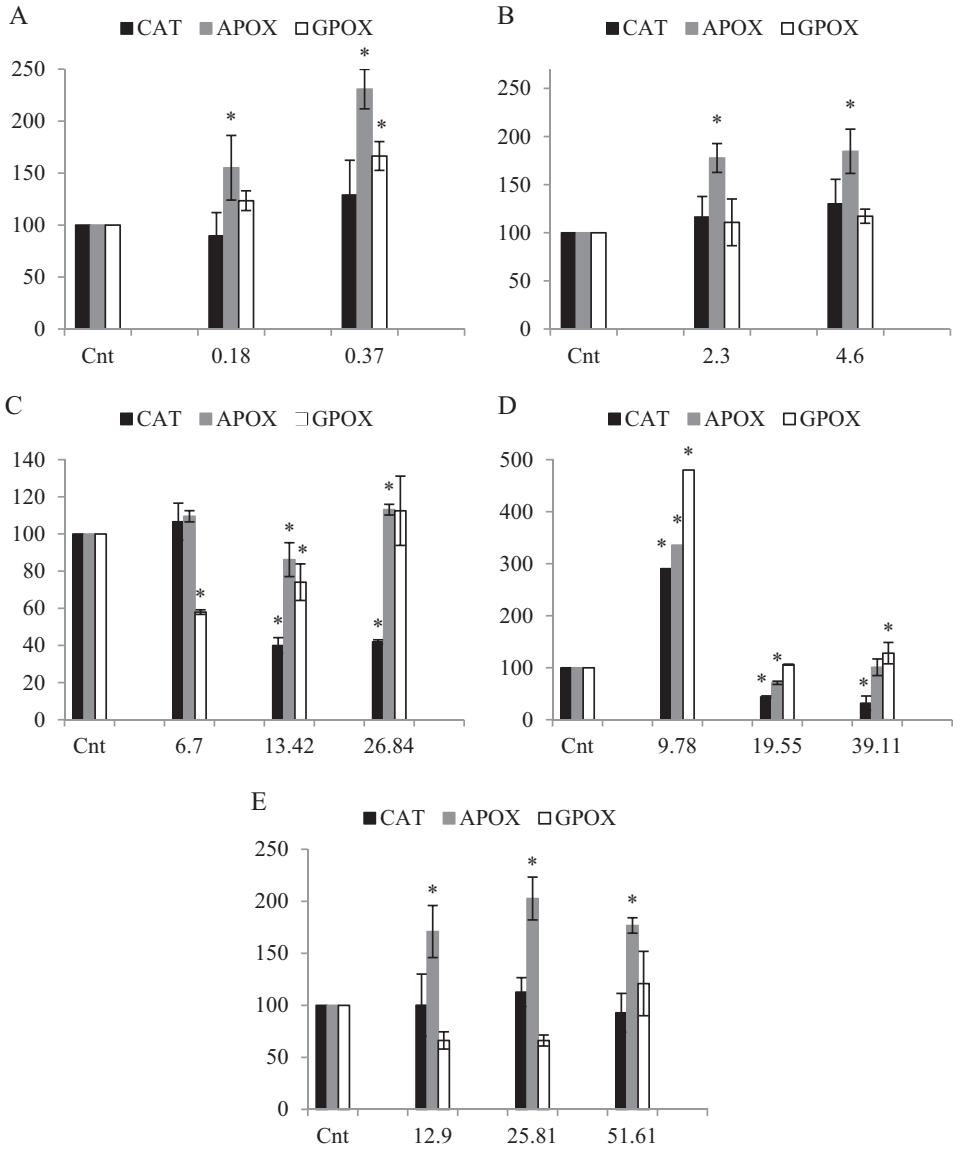

Fig. 1 Enzymatic activity (measured as enzymatic units) for: **a** Ni and **b** Zn, as single metal. **c–e** for the three metal mixture ratios: **c** 2/3 Ni–1/3 Zn; **d** 1/2 Ni–1/2 Zn; **e** 1/3 Ni–2/3 Zn. Values expressed as percentage respect the control. Asterisk indicates significant difference with control $\alpha < 0.05$

Single metal assays

A rise of activity of these enzymes showed that Ni and Zn cause increased amounts of hydrogen peroxide when applied individually (Fig. 1a, b). Plants exposed to Ni concentrations of 0.18 and 0.37 mg/L showed a significant increase of APOX activity respect to the control ones. This increase was about 231% at the highest Ni concentration tested of 0.37 mg/L. Catalase was the less activated enzyme for this metal (Fig. 1a). APOX was also significantly activated when plants were exposed to both Zn concentration of 2.3 and 4.6 mg/L. In this case, the highest value was recorded at 4.6 mg/L with an increase of 185% (Fig. 1b). In spite of this observation, Radić et al. (2009) reported that APOX activity did not increase significantly when plants of *Lemna minor* were exposed to Zn concentrations as high as 20 mg/L. In the same work, CAT activity markedly declined at exposure concentrations of 10 mg Zn/L. In the present work, although we worked with concentrations below this value, it can be observed an incipient increase in all enzyme activities when increasing concentration, even CAT enzyme. In the presence of tests Zn concentrations, guaiacol peroxidase was the enzyme less activated (Fig. 1b).

Protein content decreased in all tested concentrations compared to controls. Protein synthesis is one of the primary targets of ROS damage in plant. Metals can decrease protein content as a result of oxidative damage, gene expression modification, increasing ribonuclease activity, amino acid consumption in scavenging ROS and reduction of free amino acid by alteration in nitrogen metabolisms (Shahid et al. 2014). Doganlar et al. (2012) also reported a decline in total soluble protein in *Lemna gibba* exposed to 0.25, 1 and 16 mg Ni/L; but in this case after 48 h and said that it could be explained by the disruption of metabolism. Even though, another work, also with *Lemna gibba*, showed that the contents of total soluble protein and chlorophylls increased respect control in plants exposed to 10 and 20 mg Ni/L (Yilmaz and Parlak 2011).

Binary mixture assays

The effects of different Ratios of Ni and Zn in binary mixtures assays are shown in Fig. 1c–e. CAT levels in all mixtures were lower respect controls. The greatest increase in its activity was observed at the lower concentration from Ratio 2 (291%) (Fig. 1d). CAT activity of single Ni at 0.37 mg/L was 129% that of the control, however, when 6.32 or 25.43 mg Zn/L was added to the medium, it decreased to 107% and 113%, respectively (Fig. 1c–e). Individual Zn at 4.6 mg/L presented a CAT activity of 130%, but when a similar concentration was evaluated in the presence of Ni (lower of Ratio 1) the activity of this enzyme decreased close to control values. APOX activity at concentrations of Ratio 1 mixture remained close to control values, but a great increment (336%) was observed in the smaller concentration of Ratio 2 mixtures (Fig. 1d); also, all concentrations from Ratio 3 mixtures presented enhancement respect the control from 171 to 203 % (Fig. 1e). The APOX activity of single Ni at 0.18 mg/L was 155% that of the control and when 12.71 mg Zn/L was added (smaller concentration from Ratio 3 mixtures) it raised up to 171%. Individually, 0.37 mg Ni/L and 4.6 mg Zn/L showed 231 and 185% of activity respect the controls respectively. But when similar concentrations were evaluated together, it decreased up to 110% indicating that some mitigating action could be occurring between these two metals over APOX activation. The enhanced activity was recovered when that concentration of Ni was evaluated in the presence of higher Zn dose (25.43 mg/L) (sum = 25.81 mg/L from Ratio 3) showing 203% respect the control (Fig. 1e). The GPOX activity of 0.18 mg Ni/L showed an activity of 123% respect control, however, when 12.71 mg Zn/L was added to that concentration, the activity decreased to 66%. Similarly, individually 0.37 mg Ni/L and 4.6 mg Zn/L presented an activity of 166% and 117%, respectively that of the controls, but when those concentrations were evaluated in mixture the activity decreased to 58%. These data could be indicating some competitive interaction between metals over the activation of this enzyme. In mixtures, GPOX was enhanced respect the control mostly at the highest concentrations. In all cases, APOX presented a higher activity that CAT. This fact could be explained as these two enzymes have different affinities for H_2O_2, which is a reactive oxygen species produced in excess under heavy metal stress. APOX presents much more affinity than CAT (Gomes-Junior et al. 2006). Moreover, APOX is present in mitochondria, chloroplast, peroxisomes and cytosol whereas CAT is mostly localized in peroxisomes showing no or very low activities in other compartments (Mizuno et al. 1998). It has been reported in the same work that CAT activity in *Solanum* sp. exposed to low temperatures decreased first, until APOX tended to decline and then increased slowly to basal levels. Thus, when plants are under oxidative stress, APOX may be first strongly activated to protect detoxifying the H_2O_2 production.

In this work GPOX also presented, in most cases, higher activity than CAT. GPOX like APOX is a peroxidase. APOX is a key part of the ascorbate-glutatione cycle that catalyzes the conversion of H_2O_2 to H_2O using ascorbate as specific electron donor (Caverzan et al. 2012). In the case of GPOX, detoxification from H_2O_2 occurs directly through the use of glutathione as the reducing agent. Here, GPOX activity seems to behave similarly to APOX in many cases (except in individual Zn and Ratio 3 where it presented an activity very similar to CAT) in which CAT had been always lower. In concordance with that, it has been said that

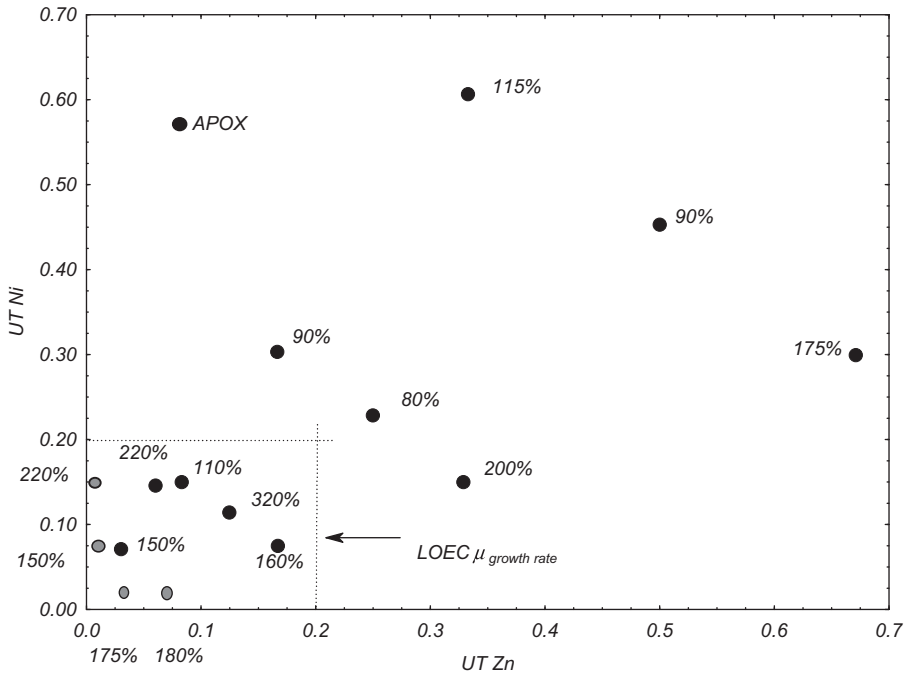

Fig. 2 Apox activity and toxic units in the binary mixture (black circle) and single metals (gray circle). Values in % are relative to control activity that indicates 100%. Dashed lines indicate the limit for LOEC values in the mixture as shown in Table 2

in the context of a balance of scavenging enzymes, APOX and GPOX are up-regulated when CAT activity is reduced in plants (Sofo et al. 2015). In this work, a GPOX activity in plants exposed to individual Zn was not enhanced up close to the levels of APOX. On the other hand, GPOX in Ratio 3 was also much lower respect to APOX. This Ratio presented Zn in a higher percentage than in the others. This could be showing that GPOX activity is not much activated by Zn treatment at assayed concentrations. Similarly, Aravind and Prasad (2003) showed that GPOX did not show a significant increase in hydrogen peroxide scavenging activity, in *Ceratophyllum demmersum* exposed to increasing concentration of Zn, indicating that it acts secondarily to APOX in these cases. According Caverzan et al. (2012) a major H_2O_2 detoxifying system in plant cells is the APOX enzymes and their isoforms presenting at different subcellular compartments, such as mitochondria, chloroplasts and cytosol. However, as was registered in this study (Fig. 2) other authors have observed that APOX activity was increased at lower metal concentrations (Ni, Cd) and was almost no detectable in cells or plants exposed to higher concentrations (Gomes et al. 2006; Sharma and Dubey 2007).

As all enzymes evaluated individually showed higher activities than those same concentrations evaluated in mixtures, it could be considered that a mitigating interaction might be occurring between these metals respect the activation of these enzymes when they are together.

Versieren et al. (2017) found that some biomarkers were sensitive to both Cu and Cd assayed as single metal but not to Cu–Cd mixtures. The root elongation explained mixture effects better than most of the 16 measured biomarkers (i.e., the biochemical effects). It is concluded that these biomarkers are not robust indicators for metal mixture toxicity, potentially because different metals have different parallel modes of action on growth that are insufficiently indexed by the biomarkers. Balen et al. (2011) reported also similar lower values of oxidative stress parameters in *Lemna minor* for combined Cd-Zn treatments compared with single Cd treatments. In according with Versieren et al. (2017) it is difficult to translate any of the biomarkers to the physiological effects, because these biomarkers might not be linearly linked to the response. Nevertheless, we found the highest values for APOX activity under the limit of LOEC concentrations for single as well as a binary mixture of Ni and Zn (Fig. 2), indicating that at least this enzyme could be a possible indicator of both metal mixture toxicity at low concentrations.

Metal toxicity interaction

In the evaluation of the concentration addition (CA) approach, when multiple regression was applied, the multiplicative interaction between metals ([Zn] × [Ni]) was not significant (p value = 0.19, >0.050), showing a β value of −0.064 (Table 4). The negative value could indicate an alleviation of toxicity or less than additive, however, its p value was >0.050. The first step in evaluating concentration addition approach was done by solving the multiple regression to calculate the concentrations expected to result in 50% frond number inhibition. It was solved for Ni while

Table 4 Multiple linear regression analysis of the effect of Ni and Zn versus the observed percentage of frond number inhibition of *Lemna gibba* based on the binary mixture exposures

	Intercept	b	β	p
	26.53			0.001*
Ni		0.98	10.78	0.025*
Zn		0.83	0.27	0.050*
Ni × Zn		−0.84	−0.064	0.19

Regression coefficients (β) as well as the standardized regression coefficients (b) for concentrations of [Ni] and [Zn] are presented. $R^2 = 0.86$, adjusted $R^2 = 0.85$

*Significant values <0.05

holding the [Zn] constant. Using these concentrations and individual EC50s for each individual metal, ΣTU were calculated to evaluate CA (Table 3). In this study we obtained an average sum of toxic units of 1.03 with a range between 0.90 and 1.16, which is very close to unity. This suggests that CA model estimates the ecotoxicity of Ni-Zn mixture on *Lemna gibba* resulting a correct predictor when frond number inhibition was considered.

If we take in consideration the LOEC values for single metals as 0.92 and 20.1 mg/L for Ni and Zn, respectively from Table 1, LOEC expected concentrations following CA approach in the mixture, would be 0.46 and 10.05 mg/L of Ni and Zn, giving a total toxic unit of 0.3. From the data that arise from those indicated in Table 2 for each assayed ratio, we obtained LOEC values when organisms are exposed to 0.28, 0.31 and 0.27 UT. That means that the CA model could be applied and there would be deviations due to the level or dose of metals (Di Marzio et al. 2018).

The interaction between the metals and living systems is directed by the properties of its ions forms, as acids (electron pair acceptors) or complex anions (electron pair donor). Reaction sites of metals can be oligosaccharides or proteins of biological membranes. Ni and Zn are acids, therefore, they belong to the same group and the ions of these atoms (Ni^{+2} and Zn^{+2}) can compete for the same reaction center, as Nitrogen or Oxygen atoms of proteins that acts as hard bases. Ni and Zn metal mixture used in this work are essential micronutrients required for plants for growing; this argument in addition with the affirmation that they belong to the same chemical group, as mentioned above, could result in a no preference uptake of one or another, which, in terms, would explain the additive interaction observed in this work. This same behavior was observed for Cu^{+2} and Ni^{+2}, that are also essential micronutrients, when plants of *Sirodela polyrrhiza* were exposed to their binary mixtures. (Montvydienė and Marčiulionienė 2007).

Conclusions

Ni and Zinc in single exposure caused deleterious effects on growth and provoked an increase in the activity of antioxidant enzymes, especially of APOX. The effect on growth rate of binary mixtures of metals was more evident when considering the frond number than fresh weight, indicating a more sensitive endpoint.

As all enzymes evaluated individually showed higher activities than those same concentrations evaluated in mixtures, it could be considered that a mitigating interaction might be occurring between these metals respect the activation of these enzymes when they are together. APOX showed the highest activity at binary metal mixture low concentrations.

Concentration addition (CA) approach was evaluated and resulted in a correct predictor of Ni-Zn mixture toxicity on *Lemna gibba* considering the growth rate as an endpoint, this was analyzed considering the expected EC50 as well as LOEC values according to CA. The measured biomarkers indicating a positive response to free radicals, did not fit to concentration addition model when assayed in the binary mixture. Also, the main activity response to this was observed within a range of concentrations below the LOEC values for the mixture. At least the biomarkers studied here, GPOX, APOX and CAT were not linearly related to the concentrations of Ni–Zn in the mixture and would not be good indicators of the effects correlated with the mixture of both metals.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

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