Growth response of *Lemna gibba* L. (duckweed) to copper and nickel phytoaccumulation

Effet de l'accumulation de Cu et Ni sur la croissance de *Lemna gibba* L. (lentilles d'eau)

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RÉSUMÉ

Pour déterminer la tolérance et la capacité de phytoaccumulation du cuivre (Cu) et du nickel (Ni) par une espèce de lentilles d'eau, *Lemna gibba* L., les plantes sont exposées à différentes concentrations de Cu et Ni (0,1 à 2,0 mg/L) dans une solution de Coïc et Lesaint dilué à 1/4. Le pH est maintenu constant à 6,0 (± 0,1) et le flux de lumière est de 12 h/jour. Le cuivre et le nickel sont tolérés par *L. gibba* à des concentrations ≤ 0,3 mg/L et ≤ 0,5 mg/L, respectivement. Cependant, la croissance des plantes diminue de 50% (I_{50}) quand le milieu de culture contient 0,45 mg/L de Cu ou 0,75 mg/L de Ni. La plus faible concentration causant une inhibition complète (LCI) est de 0,5 et 1,0 mg/L respectivement en présence de Cu et Ni. Les résultats de l'analyse du métal dans les tissus des plantes révèlent une grande accumulation de Cu et une faible accumulation de Ni dans les tissus végétaux (pour la concentration ne causant aucune inhibition dans la croissance). Une diminution de la concentration de métal dans l'eau est également observée. On peut conclure que *L. gibba* peut être un bon candidat pour l'épuration des eaux contaminées par le cuivre.

ABSTRACT

To assess the tolerance and phytoaccumulation ability of the duckweed *Lemna gibba* L. to copper (Cu) and nickel (Ni), the aquatic plants were exposed to different concentrations of Cu and Ni (0.1 – 2.0 mg/L) in quarter Coïc and Lesaint solution at pH = 6.0 (± 0.1) and under a daily regime of 12 h light. Copper and nickel were tolerated by *L. gibba* at concentrations ≤ 0.3 mg/L and ≤ 0.5 mg/L, respectively. However, plant growth decreased by 50% (I_{50}) when the medium contained 0.45 mg/L of Cu or 0.75 mg/L of Ni. The observed LCI (lowest concentration causing complete inhibition) were 0.5 and 1.0 mg/L respectively in the presence of Cu and Ni. Results from metal analysis in plant tissues revealed a high accumulation of copper and a low accumulation of nickel within the plant (for concentrations causing no growth inhibition) and a corresponding decrease of metals in the water. The duckweed *L. gibba* L. could be a good candidate for the removal of low concentrations of copper from polluted water.

KEYWORDS

Copper; Growth; *L. gibba* L.; Nickel; Phytoremediation; Tolerance

1 INTRODUCTION

Certain plants are able to accumulate in their tissues several metals without showing any signs of toxicity. This natural accumulation is related with the resistance which represents response of plants to metal stress conditions. According to Papazoglou et al. (2005), metal resistance can be based on either avoidance or tolerance mechanisms. Avoidance reflects the cell protection against the metal whereas tolerance is the cell capability to protect themselves against injury by the metal (Sabreen & sugiyama, 2008). Kanoun-Boulé et al. (2008) reported in the tolerance of duckweed to copper that the release of organic anions might be involved in the protection of plants by chelating the metal ions in the rhizosphere to form non toxic complexes. However, according to Sabreen and Sugiyama (2008), tolerant populations can be characterized by a lower metal accumulation than the sensitive one.

Duckweed commonly refers to a group of floating, flowering plants of the family Lemnaceae. The different species (Lemna, Spirodela, Wolffia and Wolfiella) are worldwide distributed in freshwater and wetlands, ponds and some effluents are the most common sites to find duckweed. The plants are fast growing and adapt easily to various aquatic conditions. They are able to grow across a wide range of pH, from pH 3.5 to10.5 but survive best between pH 4.5 to 8.3 (Environnement Canada, 1999; Cayuela et al., 2007). The plants are found in temperate climates and serve as an important food source for various water birds and fish (Drost et al., 2007).

Some studies indicate that duckweed plants are sensitive to toxicity. Other studies however, report that duckweed plants are tolerant to environmental toxicity (Wang, 1990). To assess the tolerance of the species *L. gibba* to heavy metals, plants were exposed to concentrations of copper and nickel higher than those used in medium cultures. Toxic effect of pollutant on duckweed is generally evaluated by phytotoxicity tests based on growth inhibition (Geoffroy et al., 2004). Copper and nickel were chosen as the metals for this study for a number of reasons. Their presence above trace levels in the environment is an indicator of industrial pollution. On the other hand, they are essential micronutrients for plants; copper is a structural and catalytic component of many proteins and enzymes involved in metabolic pathways (Teisseire & Vernet, 2000) and nickel has an important role in the urease and hydrogenase metabolism (Harish et al., 2008). However, when the concentration reaches a threshold value, these essential metals become first inhibitory and afterwards toxic. Copper is responsible for many alterations of the plant cell (respiration, photosynthesis, pigment synthesis and enzyme activity) (Teisseire & Vernet, 2000; Kanoun-Boulé et al., 2009). Nickel inhibits germination, chlorophyll production and proteins (Zhou et al., 2009) in plants; several animal experimental studies have shown an increased cancer incidence associated with chronic exposure to nickel.

Each plant species has different resistance and tolerance levels to different contaminants (Kamal et al., 2004). Therefore, several studies have been performed to elucidate heavy metal toxicity to plants. In earlier study, we demonstrated that the duckweed *L. gibba* L. native to the north-east of Algeria tolerated Zn up to 18 mg/L and was effective in removing 60% of the metal pollutant from the nutrient medium (Khellaf & Zerdaoui, 2009). The present study investigates copper and nickel toxicity to duckweed to determine tolerance of this aquatic species to Cu and Ni. The study also investigates the potential accumulation of these two metals by the duckweed *L. gibba* L. The goal was to assess the possibility to use *L. gibba* L. for phytoremediation of Cu and Ni contamination in water.

2 MATERIALS AND METHODS

2.1 Plant material and culture medium

Lemna gibba L. native to the north-east region of Algeria was collected from a natural pond in the spring 2008 and placed in plastic aquaria containing quarter Coïc and Lesaint solution at pH = 6.0 (± 0.1). This culture medium consisted of (in g/L): $Ca(NO_3)_2.4H_2O$, 59.05; MES (2-morpholinoethanesulfonic acid) , 43.40; KNO_3, 30.33; $MgSO_4.7H_2O$, 18.49; NH_4NO_3, 16.01; KH_2PO_4, 10.21; H_3BO_3, 1.85; NaCl, 1.17; EDTA, 0.87; $MnSO_4.H_2O$, 0.84; $FeSO_4.7H_2O$, 0.83; K_2HPO_4, 0.52; $ZnSO_4.7H_2O$, 0.28; $CuSO_4.5H_2O$, 0.06; $Na_2MoO_4.2\ H_2O$, 0.06; $NiCl_2.6H_2O$, 0.06 (Coïc & Lesaint, 1973). A continuous aeration system provides oxygen for the fronds and prevents root fungal diseases (Kamal et al., 2004). Aquatic macrophytes were cultured at 21 ± °C with a 12 h photoperiod.

2.2 Toxicity test

The test protocols were derived from the standard for a 4-day growth inhibition developed by the Organisation for Economic Co-operation and development (OECD, 2002). Duckweed growth was measured after four days of exposure to different concentrations of each metal. The metals used for

this study were copper and nickel supplied as $CuSO_4.5H_2O$ and $NiCl_2.6H_2O$. Nine to twelve *Lemna* fronds were gently placed in crystallising cups (7 cm high, 5 cm in diameter) containing 100 ml of metal solution diluted in the nutrient medium. Metal concentration selected for this study varied from 0.1 to 2 mg/L. The dose- response tests were performed in conditions similar to those of the plant cultures. Control treatment (plants without metal) was necessary to compare the results and to demonstrate the effect of metal ions on duckweed growth. For the two metals, each test was performed in triplicate.

2.3 Accumulation experiment

Approximately 1 ± 0.05 g (fresh weight) of *L. gibba* fronds were exposed individually to 0.1 - 2 mg/L of Cu or Ni in 1 litre plastic vessels ((18 cm x 11 cm x 6 cm) containing quarter Coïc and Lesaint solution. The plants were placed under conditions similar to those of the cultures. The control was used to demonstrate the effect of the aquatic plants in metal removal. After four days of treatment, the plants were dried at 70 °C until constant weight, digested in 69% HNO_3 and weighted for the dry weight (Khellaf & Zerdaoui, 2009). The aquatic plants were analyzed for metal ion concentration. Copper and nickel were measured by an Atomic Absorption Spectrophotometer (Shimadzu AA 6601 F). Three replicates were made per bioassay.

2.4 Data analysis

Lemna gibba fronds were observed daily for toxicity symptoms (chlorosis, necrosis and frond disconnection). Total frond area was determined on days 0 and 4. The biomass based on the total frond area was determined by image analysis. A numeric video camera captured each cup and then the image was analysed by the software scion image (www.scioncorp.com, 2004) to determine the total frond area. A plant growth index was calculated as follows:

$$\textit{Growth index} = \frac{Biomass\,(t = 4\,days)}{Biomass\,(t = 0)} \quad (1)$$

The doubling time of frond number, T_d, was calculated according to the following equation:

$$T_d = \frac{\ln 2}{\mu} \quad (2)$$

Where μ [day^{-1}] is the average growth rate in the control, $\mu_{ij} = \frac{\ln Nj - \ln Ni}{tj - ti}$ (3)

Where i = 0, j = 4, N the frond number and t the time.

The bioconcentration factor (BCF) provides an index of the ability of the plants to accumulate metal element with respect to the element concentration in water. It was calculated as follows (Zayed et al., 1998):

$$BCF = \frac{Metal\,concentration\,in\,plant\,(mg/Kg\,DW)}{Metal\,concentration\,in\,solution\,(mg/L)} \quad (4)$$

2.5 Statistical analysis

Metal concentrations in plant tissues were reported in mg/g DW and are means of three replicates. Regression analysis was performed by using the statistical functions included with Microsoft Excel, Office 2003. In the case of the total frond area, the standard deviation was 0.14 and 0.11 on average respectively for Cu and Ni. For the metal concentration in plant tissues, the standard deviation was 0.018 mg/g DW on average for the two elements. Concentration values were considered significantly different when the 95% confidence intervals did not overlap.

3 RESULTS

3.1 Visible symptoms of toxicity

Copper and nickel caused visible damage to duckweed at concentration of 0.5 and 1 mg/L, respectively. Chlorosis (a progression of green to yellow colour on the frond) and frond disconnection (detachment of fronds from colonies) were toxicity signs observed at the start of exposing *L. gibba* to copper and nickel. These signs progressed to necrosis at the end of the treatment. Visibly, copper was toxic for *L. gibba* at concentrations ≥ 0.5 mg/L; fronds were chlorotic and some fronds separated from the others (necrosis was observed after 24 h of exposure plants to ≥ 0.5 mg/L of Cu). Nickel was less toxic for the plants; 2 mg/L of Ni in the culture medium caused visible damage (chlorosis and frond disconnection) 1 day after the treatment.

3.2 Concentration-growth relation

The concentration-growth index curves are presented in Figure 1 and Figure 2. Copper stimulated the growth for concentrations between 0.003 mg/L (control) and 0.3 mg/L. Over these values, the growth index decreased until a minimal value (indicated by broken line on Figure 1) corresponding to 0.5 mg/L. For concentrations ≥ 0.5 mg/L, treatments were not statistically different from each other. Nickel was tolerated by duckweed plants for concentrations ranged between 0.003 mg/L (control) and 0.5 mg/L. Frond biomass was significantly reduced as more than 0.5 mg/L was added to the nutrient medium. This effect indicates that excessive level of nickel suppresses the growth of *L. gibba*.

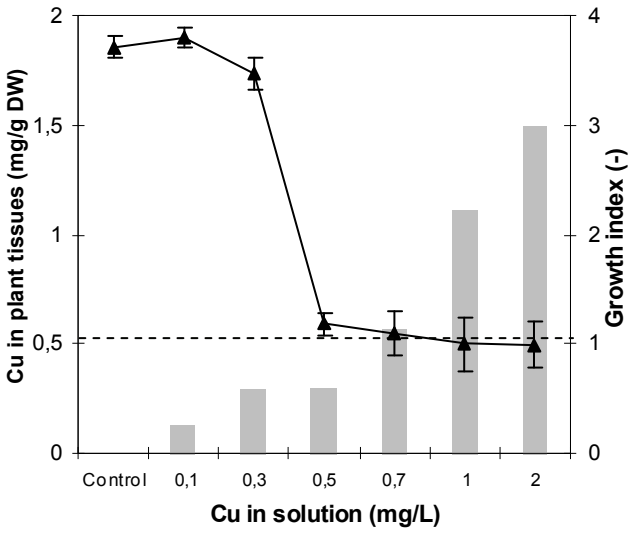

Figure 1 Growth (▲) and metal accumulation in fronds (■) *L. gibba* with increasing concentrations of copper. Vertical bars indicate standard deviation, n = 3. Broken line indicates minimal growth index

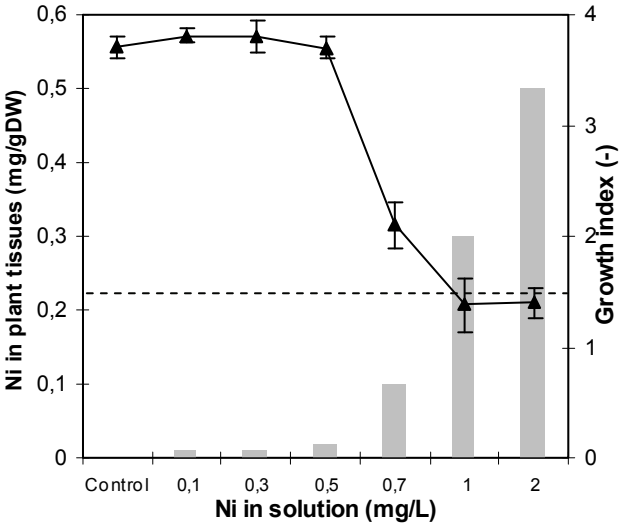

Figure 2 Growth (▲) and metal accumulation in fronds (■) of *L. gibba* with increasing concentrations of nickel. Vertical bars indicate standard deviation, n = 3. Broken line indicates minimal growth index

Copper and nickel had similar effects on the growth of *L. gibba*. Dose-response curves may be presented by the general allure given in Figure 3. When the concentration of copper and nickel reaches a threshold value (0.3 mg/L of Cu and 0.5 mg/L of Ni), they become first inhibitory (high concentration) and afterwards toxic (excessive concentration). At low concentration, the metals are tolerated by the plant.

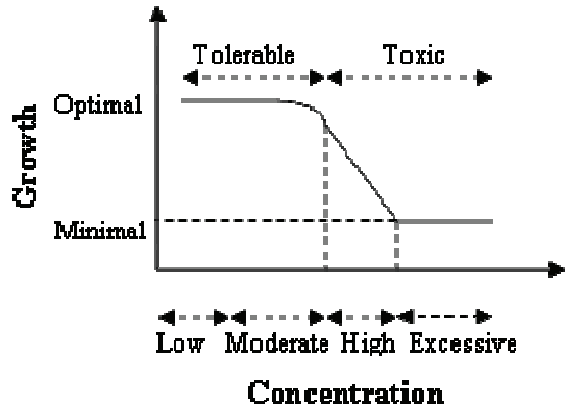

Figure 3 Growth versus metal concentration- General allure

3.3 Growth inhibition parameters

For metal toxicity testing to be valid, the doubling time of the frond number in the control, T_d, must be less than 2.5 days (OECD 2002). The observed T_d under experimental conditions was 1.9 days. The calculated concentration that results in a 50 % reduction in the growth of *L. gibba* (I_{50}) in the presence of copper and nickel were interpolated from linear regression of growth index as a function of concentration. Copper and nickel decreased the fronds growth index by 50 % when the medium contained respectively 0.45 and 0.75 mg/L (R^2 = 0.96). The highest metal concentration causing no growth inhibition (HNI) and the lowest concentration causing complete inhibition (LCI) in the presence of copper and nickel are also estimated (Remon et al. 2007). The parameters values are shown in Table 1.

Table 1 Inhibition parameters of *L. gibba* growth in the presence of copper and nickel

Metal	HNI (mg/L)	I_{50} (mg/L)	R^2	LCI (mg/L)
Copper	0.3	0.45	0.96	0.5
Nickel	0.5	0.75	0.96	1.0

HNI: highest metal concentration causing no growth inhibition; I_{50}: concentration causing 50% inhibition; LCI: lowest concentration causing complete inhibition; R^2: coefficient of determination.

3.4 Metal phytoaccumulation

The amounts of copper and nickel accumulated in *L. gibba* fronds under various metal concentrations are shown in Figures 1 and 2. The tissue concentration of copper and nickel ranged from 0.13 to 1.5 mg/g and 0.01 to 0.5 mg/g (dry weight), respectively. The BCF values at metal concentrations tolerated by the plants were ~ 1000 for Cu and ≤ 100 for Ni. Metal analysis in water showed a removal percentage of 60-80% of Cu and 10-15% of Ni (Table 2). Above a concentration of 0.29 and 0.02 mg/g (dry weight) accumulated in tissues, copper and nickel exhibited deleterious effect on *L. gibba*. The functions in Microsoft Excel were used to fit linear, logarithmic, polynomial and exponential regressions to the relation between metal concentration in the medium and the metal accumulated in *Lemna* tissues. Among these forms, the quadratic polynomial model has the least residual error. Table 3 shows the results of the regression equations where Y, X and R^2 represent the metal accumulated in duckweed (mg/g DW), the metal concentration in the medium (mg/L) and the correlation coefficient, respectively. Metal accumulated in *Lemna* tissues is highly correlated with the initial metal concentration in the nutrient medium (R^2 = 0.953 and 0.933 for Cu and Ni, respectively).

Table 2 Removal percentage and Bioconcentration factor of copper and nickel at metal concentrations tolerated by *L. gibba*

Metal	Concentration (mg/L)	Removal (%)	FBC
Copper	0.1	80	1300
	0.2	60	966.7
Nickel	0.1	15	100
	0.3	10	33.3
	0.5	10	40

Table 3 Quadratic polynomial models: metal accumulated in *L.* gibba tissues versus metal concentrations in the medium

Metal	Regressions equations	R^2
Copper	$y = - 0.163 X^2 + 1.107 X – 0.035$	0.953
Nickel	$y = - 0.029 X^2 + 0.214 X – 0.031$	0.933

Y, X and R^2 represent the metal accumulated in duckweed (mg/g DW), the metal concentration in the medium (mg/L) and the correlation coefficient, respectively.

4 DISCUSSION

Copper and nickel are essential in low concentrations to the metabolism of animals and plants being present as constituent of numerous proteins, enzymes and cofactors. However, when present in excess, they may cause deleterious effect (lethal or sublethal stress). In this study, we demonstrated that copper when present in the nutrient solution at concentrations ≤ 0.3 mg/L was tolerated by *L.*

gibba. At a concentration higher than 0.3 mg/L, copper decreased considerably the biomass (70%) and caused the photosystem alteration by reducing electron transport. This effect was manifest by a rapid development of chlorosis; after exposing *Lemna* fronds to the cupric ions (2 or 3 hours), the fronds colour changed from green to yellow and some fronds were separated from the colonies. These results are very different from those reported by Zayed et al. (1998). The authors used *L. minor* for the phytoaccumulation of copper in quarter-strength Hoagland's solution at pH = 6; the lowest copper concentration causing > 10% growth reduction was 5 mg/L. The difference with our results seems to be the cultivation medium.

Lemna gibba tolerated nickel up to 0.5 mg/L. Concentrations higher than 0.5 mg/L were inhibitory for the plant growth. The inhibition consisted of the reduction of the biomass and frond production; at concentrations ≥ 1 mg/L, nickel caused reduced growth, separation of the colonies and changes in fronds colour. The results also demonstrated that a concentration of 0.75 mg/L of nickel in the growth medium reduced the growth index by 50%. Nickel analysis in duckweed showed a low accumulation within tissues for concentrations causing no growth inhibition (≤ 0.02 mg/g DW). Zayed et al. demonstrated that *L. minor* fronds accumulated low amounts of Ni in their tissues (1.79 g/Kg in a medium containing 10 mg Ni/L). However, Axtell et al. reported the absorption of lead and nickel by *L. minor*; duckweed showed a preference to remove nickel (the removal rate was equivalent to 82% in an aqueous solution containing 5 mg Ni/L and 10 mg Pb/L).

Results from metal analysis in duckweed confirmed the accumulation of copper and nickel within the plant and a corresponding decrease of metals in the water. At concentration of 0.3 mg Cu/L and 0.5 mg Ni/L, *L. gibba* accumulated high concentration of Cu (0.29 mg/g DW) and low concentration of Ni (0.02 mg/g DW) without the production of any toxicity or reduction in growth. Tolerance to Cu is probably associated with the release of organic anions (e.g. citrate and oxalate) which sequester metal ions to form non toxic compounds (Kanoun-Boulé et al., 2008). On the other hand, tolerance to Ni (for concentration ≤ 0.5 mg/L) may be explained by the low metal accumulation in *L. gibba* tissues (Sabreen & Sugiyama, 2008). The bioconcentration factor is more significant than the amount accumulated in plants since it indicates the plant's ability to accumulate trace elements relative to their concentration in the external nutrient solution (Del-Campo Marin & Oron, 2007). At copper concentration tolerated by duckweed, the BCF values were approximately 1000 and the removal percentage was high; however the BCF values were very low for Ni. Based on these results, we can conclude that *L. gibba* could be a good candidate for the phytoremediation of low concentrations of copper from polluted water.

5 CONCLUSION

In this work, the tolerance of *L. gibba* L. to copper and nickel and the potential accumulation of these two metals by this genius have been investigated. The metals Cu and Ni were tolerated by *L. gibba* at 0.3 and 0.5 mg/L respectively. These results revealed high tolerance of this aquatic plant to the heavy metals copper and nickel. On the other hand, the high accumulation capacity of *L. gibba* for copper (290 mg/Kg DW) makes this genius a good candidate for the removal of low concentrations of this heavy metal from contaminated water (municipal effluents in which Cu content is relatively low). However, the low accumulation capacity of *L. gibba* for nickel (at concentration ≤ 0.5 mg/L) makes this aquatic species not very suitable for the phytoremediation of Ni-contaminated waters.

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