Modifying effects of leaf litter extracts from invasive versus native tree species on copper-induced responses in *Lemna minor*

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Invasive plant species tend to migrate from their native habitats under favourable climatic conditions; therefore, trophic and other relationships in ecosystems are changing. To investigate the effect of natural organic matter derived from native *Alnus glutinosa* tree species and from invasive in Lithuania *Acer negundo* tree species on copper toxicity in *Lemna minor*, we analysed the dynamics of Cu binding in aqueous leaf litter extracts (LLE) and plant accumulation, morphophysiological parameters, and antioxidative response. The results revealed that *A. glutinosa* LLE contained polyphenols (49 mg pyrogallol acid equivalent (PAE)/g DM) and tannins (7.5 mg PAE/g DM), while *A. negundo* LLE contained only polyphenols (23 mg PAE/g DM). The ability of LLE to bind Cu increased rapidly over 1.5–3 h to 61% and 49% of the total Cu concentration (6.0 ± 0.9 mg/L), respectively for *A. glutinosa* (AG) and *A. negundo* (AN), then remained relatively stable until 48 h. At the same time, *L. minor* accumulated 384, 241 or 188 µg Cu/g FW when plants were exposed to Cu (100 µM CuSO₄), Cu with 100 mg/L dissolved organic carbon (DOC) from either AG LLE or AN LLE, accordingly. Catalase (CAT) and guaiacol peroxidase (POD) played a dominant role in hydrogen peroxide scavenging when plants were exposed to Cu and 10 or 100 mg/L DOC<sub>AG</sub> mixtures in both the first (up to 6 h) and the second (6–48 h) response phases. Due to functioning of oxidative stress enzymes, the levels of the lipid peroxidation product malondialdehyde (MDA) reduced in concentration-dependent manner, compared to Cu treatment. When combining Cu and DOC<sub>AN</sub> treatments, the most sensitive enzymes were POD, ascorbate peroxidase and glutathione reductase. Their activities collectively with CAT were sufficient to reduce MDA levels to Cu-induced in the initial, but not the second response phase. These data suggest that leaf litter extracts of different phenolic compositions elicited different antioxidant response profiles resulting in different
reductions of Cu stress, thus effecting *L.minor* frond and root development observed after seven days. The complex data from this study may be useful in modelling the response of the aquatic ecosystem to a changing environment.
Modifying effects of leaf litter extracts from invasive versus native tree species on copper-induced responses in *Lemna minor*

A running head title: Tree leaf litter DOM moderate Cu toxicity to *L. minor*

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Abstract

Invasive plant species tend to migrate from their native habitats under favourable climatic conditions; therefore, trophic and other relationships in ecosystems are changing. To investigate the effect of natural organic matter derived from native *Alnus glutinosa* tree species and from invasive in Lithuania *Acer negundo* tree species on copper toxicity in *Lemna minor*, we analysed the dynamics of Cu binding in aqueous leaf litter extracts (LLE) and plant accumulation, morphophysiological parameters, and antioxidative response. The results revealed that *A. glutinosa* LLE contained polyphenols (49 mg pyrogallol acid equivalent (PAE)/g DM) and tannins (7.5 mg PAE/g DM), while *A. negundo* LLE contained only polyphenols (23 mg PAE/g DM). The ability of LLE to bind Cu increased rapidly over 1.5–3 h to 61% and 49% of the total Cu concentration (6.0 ± 0.9 mg/L), respectively for *A. glutinosa* (AG) and *A. negundo* (AN), then remained relatively stable until 48 h. At the same time, *L. minor* accumulated 384, 241 or 188 µg Cu/g FW when plants were exposed to Cu (100 µM CuSO₄), Cu with 100 mg/L dissolved organic carbon (DOC) from either AG LLE or AN LLE, accordingly. Catalase (CAT) and guaiacol peroxidase (POD) played a dominant role in hydrogen peroxide scavenging when plants were exposed to Cu and 10 or 100 mg/L DOC<sub>AG</sub> mixtures in both the first (up to 6 h) and the second (6–48 h) response phases. Due to functioning of oxidative stress enzymes, the levels of the lipid peroxidation product malondialdehyde (MDA) reduced in concentration-dependent manner, compared to Cu treatment. When combining Cu and DOC<sub>AN</sub> treatments, the most sensitive enzymes were POD, ascorbate peroxidase and glutathione reductase. Their activities collectively with CAT were sufficient to reduce MDA levels to Cu-induced in the initial, but not the second response phase. These data suggest that leaf litter extracts of different phenolic compositions elicited different antioxidant response profiles resulting in different reductions of Cu stress, thus effecting *L. minor* frond and root development observed after seven days. The complex data from this study may be useful in modelling the response of the aquatic ecosystem to a changing environment.

*Keywords:* copper toxicity; invasive species; leaf litter extracts; *Lemna minor*; lipid peroxidation; oxidative stress enzymes.
Introduction

Copper is needed for normal plant growth and development and is a cofactor for physiological processes such as photosynthesis, mitochondrial respiration, superoxide scavenging, ethylene sensing and lignification (Maksymiec, 1997). However, copper released into the environment in surplus concentrations is toxic to plants (Naumann et al., 2007). Transition metals, including Cu, stimulate the formation of hydroxyl radicals (\(\cdot\)OH) from the non-enzymatic chemical reaction between superoxide (\(O_2^\cdot\)) and \(H_2O_2\) (Haber-Weiss reaction). Excess Cu can induce negative effects including the production of reactive oxygen species (ROS) via Fenton reaction (Halliwell and Gutteridge, 1984). ROS, in turn, can oxidize lipids (De Vos et al., 1991), disrupt protein functions due to binding to sulphydryl groups (Weckx and Clijsters, 1996) and inhibit photosynthesis and electron transport (Thomas et al., 2013; Xia and Tian, 2009). Plant growth can be inhibited as a result.

The presence of oxygen in intracellular environments due to aerobic metabolism poses a constant oxidative threat to cellular structures and processes. ROS affect the metabolism, growth and development of plant cells. ROS formation and consumption are tiny balanced and coherent in cells. At any condition in which cellular redox homeostasis is disrupted, ROS production becomes far greater than the capacity of the tissues to scavenge them, thus can be defined as oxidative stress (Alschcer et al., 1997). The environmental stressors can increase the synthesis of non-enzymatic antioxidants such as thiol tripeptides, glutathione and ascorbate and \(\alpha\)-tocopherol, as well as the modification of the activity of antioxidant enzymes including superoxide dismutase, catalase, glutathione peroxidase, ascorbate peroxidase and glutathione reductase (Foyer et al., 1997; Schützendübel and Polle, 2002). Accordingly, excess of Cu in plants can cause oxidative stress, and therefore change antioxidative pathways (Babu et al., 2003; De Vos et al., 1992; Gupta et al., 1999; Teisseire and Guy, 2000; Wang et al., 2004).

The solubility, adsorption, transport and toxicity of metals in natural surface waters are strongly influenced by complexation with dissolved organic matter (DOM) (Kim et al., 1999; Koukal et al., 2003; Manceau and Matynia, 2010; Marx and Heumann, 1999). Natural waters contain different concentrations of DOM, which, depending on the geographical area are ranging between < 1 and 100 mgC/L (Wetzel, 2001). Higher DOM concentrations (up to 300 mgC/L) have also been reported in some Canadian wetlands (Blodau et al., 2004). DOM is a complex and polymorphous mixture, which includes proteins, carbohydrates, polyphenols, and other vital compounds that originate chiefly from the degradation of plant and animal matter (Stevenson, 1994). A major fraction of DOM in waters comprises humic substances representing more than 60–80% of the total dissolved organic carbon (DOC), which consists mainly of humic and fulvic acids (Steinberg, 2003).

Leaf litter is a readily available allochthonous source of DOM and plays an important role in freshwater ecosystems serving as a key source of nutrients (Tank et al., 2010). The enrichment with DOM relates to litter quality, which, in turn, is leaf species-dependent and
furthermore can depend whether they are of native or non-native origin (Casas et al., 2013). In addition, leaf litter emits phenolic compounds including tannins, a significant component of plant secondary metabolites (Lachman et al., 2011; Lin et al., 2006). Tannins may provide a nutrient conservation mechanism by reducing decomposition rates of litter and decreasing nitrogen leaching potential (Lin et al., 2010). It has been found that DOM might cause oxidative stress in freshwater organisms (Nimptsch and Pflugmacher, 2008; Steinberg et al., 2003), and leaf litter leachates obtained from various tree species such as white pine Pinus strobus and red oak Quercus robur (Earl et al., 2012) can be toxic to aquatic organisms. Moreover, it has been suggested that black alder Alnus glutinosa, native in Lithuania species, and boxelder maple Acer negundo, invasive in Lithuania species, impacted the same aquatic organisms in different ways (Krevš et al., 2013; Manusadžianas et al., 2014). A. negundo became widespread in Lithuania after its escape from cultivation in the mid-twentieth century (Gudžinskas, 1998). It colonized coastal zones of lakes and rivers that are dominated by autochthonous A. glutinosa (Prieditis, 1997). In this context, it might be interesting to reveal the potential of the DOM obtained from diverse species to modify metal effects on aquatic plants.

Alongside the natural sources, the increase of DOM in water bodies depends on rural and municipal activities. Similar anthropogenic sources of copper appearance were emphasized, i.e. agriculture and industrial wastes (Hou et al., 2007; Panagos et al., 2018), and combating massive growth of cyanobacteria (Huh and Ahn, 2017). To investigate possible phytotoxicity effects, we used duckweed Lemna minor, a well-known bioindicator of eutrophic water bodies (Environment Canada, 2007; US Environmental Protection Agency, 2012). This plant is considered to be a suitable model for physiological and ecotoxicological studies due to its small size, fast growth rate, vegetative reproduction, ease of culture and sensitivity to numerous pollutants. Lemna has been used for antioxidative response studies (Forni et al., 2012; Radić and Pevalek-Kozlina, 2010; Razinger et al., 2007; Teisseire et al., 1998; Teisseire and Guy, 2000; Zezulka et al., 2013). However, information on the involvement of oxidative stress under the combined treatment of Cu and DOM obtained from leaf litter extracts is lacking in L. minor.

The main objective of this study was to investigate the effect of natural organic matter derived from native Alnus glutinosa tree species and from invasive in Lithuania Acer negundo tree species on copper toxicity in L. minor. We focused on time-dependent alterations of (1) Cu binding to DOM in media and accumulation in the plant, (2) morphophysiological parameters (frond area and root length), and (3) oxidative stress characteristics such as lipid peroxidation, hydrogen peroxide content and the activities of antioxidant enzymes, i.e. catalase, guaiacol and ascorbate peroxidases, and glutathione reductase. We limited our observations of oxidative stress characteristics to 48 hours; thus, relatively high concentrations of 100 µM CuSO₄ and up to 100 mg/L DOC were used.
Materials and Methods

Plant material

We have been collecting duckweed (*Lemna minor* L.) plants from a local freshwater pond (54°75′ N, 25°29′ E; Verkiai Regional Park, Vilnius) and cultured fronds under controlled conditions for several years. Species specificity of experimental clone morphologically similar to *L. minor* was proved by sequencing the chloroplast DNA fragment (611 bp) at the Laboratory of Molecular Ecology of the Nature Research Centre (Vilnius). The highest chloroplast DNA sequence identity (99%) to *L. minor* strain RDSC 7210 (Sequence ID KX212888.1), which included ATPase subunit I gene, partial cds; atpF-atpH intergenic spacer, complete sequence; and ATPase subunit II gene, partial cds, indicated that plant clones used for the experiments in the current study should be attributed to *L. minor* species.

A stock culture was cultivated in 500-mL plastic containers 60x85x100 (mm, WxLxH) in a modified Steinberg growth medium containing 3.46 mM KNO$_3$, 1.25 mM Ca(NO$_3$)$_2$, 0.66 mM KH$_2$PO$_4$, 0.072 mM K$_2$HPO$_4$, 0.41 mM MgSO$_4$, 0.63 µM ZnSO$_4$, 1.94 µM H$_2$BO$_3$, 0.18 µM Na$_2$MoO$_4$, 0.91 µM MnCl$_2$*", 2.81 µM FeCl$_3$*", and 4.03 µM Na$_2$EDTA*" (chemicals were from Roth, Acros Organics* and Fisher**). The pH was adjusted to 6.0 with 1 M NaOH.

Stock cultures and treated plants were kept in growth chambers at 24 ± 2°C under constant illumination with cool white fluorescent light at a photosynthetic photon flux density of 160 µmol m$^{-2}$ s$^{-1}$.

Preparation of aqueous extracts

Fallen leaves of *A. negundo* and *A. glutinosa* were collected in Rudnia (54°05′ N, 24°40′ E; Varėna district, Lithuania) in autumn. After collection, tree leaves were dried for 10 days in the shade at room temperature. The dried materials (without petiole and central vein) were mechanically ground to obtain a homogenous powder. One gram powder was extracted in 100 mL of deionised water (dH$_2$O) for 3 h at 65°C followed by rapid filtration through a Whatman #3 disk in order to obtain a clear crude extract solution, and then re-filtered through a nitro-cellulose paper filter (0.2 µm) to reduce the risk of interference by microorganisms.

Experimental design

The experimental scheme comprised control (growth medium) and four treatments (growth medium supplemented with Cu (100 µM CuSO$_4$ or 6.4 mg Cu/L); 100 mg/L DOC; Cu + 10 mg/L DOC and Cu + 100 mg/L DOC. Plants were incubated in 500-mL plastic containers (60x85x100) having 200 mL of the corresponding medium. Media were adjusted to pH 6.

For growth experiments, healthy colonies with 2–3 fronds from stock cultures were transferred to the containers with the corresponding exposure medium. The frond area
(zones without the signs of chlorosis) and root length were measured at 0-day and after seven days by using image control system (Software MOTIC 2.0). The growth rate per day was calculated with the following equation 
\[ r = \frac{\ln x_{t2} - \ln x_{t1}}{t_{2}-t_{1}}, \]
where \( x_{t1} \) and \( x_{t2} \) are the values of observation parameter at \( t_{1} \) and \( t_{2} \) day, respectively. Two independent experiments in quadruplicate were conducted.

To study \( \text{H}_2\text{O}_2 \) kinetics, lipid peroxidation and antioxidant enzyme activities, the cultures were started by transferring healthy colonies with 3–4 fronds from stock cultures into four containers (0.8–0.9 g in each container) for each corresponding exposure medium. Plant samples were collected 0.75, 1.5, 3, 6, 12, 24 and 48 h after the onset of the exposure. For each exposure time, we prepared new exposure medium. Two independent experiments in quadruplicate for each parameter at each exposure time were conducted. Then samples of \( L. \text{minor} \) (0.2 g each) were processed after Hildebrand et al. (1986). Cold potassium phosphate buffer (0.1 M, pH 7.0) containing 1% (w:v) polyvinylpyrrolidone and 1% (v:v) Triton X-100 was added to chilled (4°C) mortar and pestle containing the sample. Each sample was macerated with 1 mL of extracting buffer and was further ground with another 1 mL of the buffer. A 1.5 mL aliquot of homogenate was centrifuged at 15 000 g for 15 min at 4°C (Hou et al., 2007). The supernatant was frozen immediately for future total protein content and enzyme assays. Total protein content was determined using bovine serum albumin (BSA) as standard (Bradford, 1976). Spectrophotometric measurements were carried out on a Libra S32 PC UV-VIS (Biochrom, UK).

Chemicals used for the determination of the total protein content and oxidative enzyme activities were purchased from Sigma-Aldrich (ascorbic acid, BSA, \( \text{Na}_2\text{EDTA} \), Folin-Ciocalteau’s phenol reagent, hide powder, \( \text{H}_2\text{O}_2 \), pyrogallol, polyvinylpyrrolidone, sodium carbonate decahydrate, Triton X-100, thiobarbituric acid (TBA), trichloroacetic acid (TCA)) and from Roth (GSSG, \( \text{KH}_2\text{PO}_4 \), \( \text{K}_2\text{HPO}_4 \), NADPH, TRIS).

**Determination of Cu**

Before determining the metal content, the plants from control and Cu, Cu + 10 mg/L DOC and Cu + 100 mg/L DOC treatments (2 experiments with four replicates at each exposure time) were washed triple with deionised water. All liquids on the surface of plant materials were blotted with paper towels. 0.2 g of fresh weight of plant materials was transferred to a ceramic crucible (Haldenwanger, Waldkraiburg, Germany) to destroy the combustible (organic) portion of the sample by thermal decomposition in a muffle furnace (SNOL-1,6.2.5, Borispol, Ukraine) at 450–550°C for 2–3 h. After the sample was digested in 0.5 mL pure \( \text{HNO}_3 \) (Roth, Karlsruhe, Germany) and heated until the acid evaporates up to a half volume and made up to final volume of 5 mL with \( \text{dH}_2\text{O} \).

Cu fractionation in treatment solutions (without plants) followed Adam et al. (2014) procedure. The fraction with dissolved Cu was obtained after ultrafiltration for 1 h in Microsep™ Advance Centrifugal Devices (Pall Corporation, Ann Arbor, MI, USA) containing polyethersulfone membranes with a cut-off of 1 kDa, at 5000 g (5430R, Eppendorf,
Hamburg, Germany). Cu content was determined in duplicate at 0.75, 1.5, 3, 6, 12, 24 and 48 h before and after ultrafiltration by Perkin Elmer Optima 7000 Dual View ICP Optical Emission Spectrometer (Waltham, MA, USA) with standard method and calculated according to the standard curve. Cu concentration was expressed as mg/L or µg/g fresh weight (FW).

CuSO$_4$ • 5H$_2$O was from Sigma-Aldrich (purum p.a.).

Catalase (CAT) EC1.11.1.6

CAT was determined according to Aebi (1984). The assay medium contained 50 mM potassium phosphate buffer (pH 7.4, 25°C), 12.5 mM H$_2$O$_2$, 50 µl supernatant containing enzyme extract and dH$_2$O to make up the volume to 3 mL. The reaction was initiated by adding H$_2$O$_2$. The decrease in absorbance of H$_2$O$_2$ was recorded at 240 nm for 60 s with 50 mM potassium phosphate buffer used as the blank. The enzyme activity was calculated from the initial rate of the reaction using extinction coefficient $\varepsilon = 0.04$ mM$^{-1}$ cm$^{-1}$ for H$_2$O$_2$.

Ascorbate peroxidase (APX) EC 1.11.1.11

APX activity was measured according to Nakano and Asada (1981). The 3 mL reaction medium was composed of 50 mM potassium phosphate buffer (pH 7.0, 25°C), 0.5 mM ascorbic acid, 0.1 mM Na$_2$EDTA, 0.1 mM H$_2$O$_2$ and 100 µL supernatant containing enzyme extract. The reaction was initiated by adding H$_2$O$_2$. The decrease in the optical density to ascorbic acid was recorded at 290 nm for 30 s. The enzyme activity was calculated from the initial rate of the reaction using $\varepsilon = 2.8$ mM$^{-1}$ cm$^{-1}$ for ascorbate.

Guaiacol peroxidase (POD) EC 1.11.1.7

POD activity was determined according to Upadhyaya et al. (1985). The assay medium contained 2.5 mL of 50 mM potassium phosphate buffer (pH 6.1, 25°C), 1 mL 1% H$_2$O$_2$, 1 mL 1% guaiacol and 50 µL supernatant containing enzyme extract. The reaction was initiated by adding supernatant containing enzyme extract. The change in the optical density was recorded at 420 nm for 1 min. The enzyme activity was calculated using $\varepsilon = 26.6$ mM$^{-1}$ cm$^{-1}$ for oxidized tetraguaiacol polymer.

Glutathione reductase (GR) EC 1.6.4.2

GR activity was determined according to Mannervik (2001). The assay medium contained 0.2 M potassium phosphate buffer (pH 7.0)/2 mM Na$_2$EDTA, 20 mM GSSG, 2 mM NADPH and 100 µL supernatant containing enzyme extract and dH$_2$O to make up the volume to 1 mL. The reaction mixture was equilibrated at 30°C. The decrease in the NADPH concentration was recorded at 340 nm for 1 min against the assay solution. Corrections were made for the non-enzymatic oxidation of NADPH by recording the decrease at 340 nm.
without adding GSSG to the assay medium. The enzyme activity was calculated from the
initial rate of the reaction after subtracting the non-enzymatic oxidation using \( \varepsilon = 6.2 \text{ mM}^{-1} \text{ cm}^{-1} \) for NADPH.

**Determination of hydrogen peroxide**

The level of H\(_2\)O\(_2\) in plant was determined according to Jana and Choudhuri (1981) with
slight modification (Chen et al., 2000). H\(_2\)O\(_2\) was extracted by homogenizing 0.2 g plant
with 2 ml of phosphate buffer (50 mM, pH 7.0). The homogenate was centrifuged at 6000 g
for 25 min at 4\(^\circ\)C. 900 \(\mu\)L of the supernatant was mixed with 300 \(\mu\)L of 0.1% titanium
chloride in 20\% (v/v) H\(_2\)SO\(_4\) and mixture was then centrifuged at 6000 g for 15 min. The
intensity of yellow colour of the supernatant was measured at 410 nm. H\(_2\)O\(_2\) level was
calculated using the extinction coefficient 0.28 \(\mu\)mol\(^{-1}\)cm\(^{-1}\).

**Determination of lipid peroxidation**

The level of lipid peroxidation in plant was assessed by thiobarbituric acid (TBA) reactive
metabolites chiefly malondialdehyde (MDA) as described by Heath and Packer (1968).
Plant tissues (0.2 g) were extracted in 2 mL of 0.25\% TBA made in 10\% TCA. Extract was
heated at 95\(^\circ\)C for 30 min and then quickly cooled on ice. After centrifugation at 10 000 g
for 10 min, the absorbance of the supernatant was measured at 532 nm. Correction of non-
specific turbidity was made by subtracting the absorbance value taken at 600 nm. The level
of lipid peroxidation was expressed as MDA concentration formed using \( \varepsilon = 155 \text{ mM}^{-1} \text{ cm}^{-1} \).

**Total phenol and tannin contents**

The total phenol content of the dry leaves was measured spectrophotometrically after
reaction with Folin-Ciocalteau phenol reagent, according to the manual method described
by Singleton et al. (1968) with little modifications (Amorim et al., 2008; Atanassova et al.,
2011). A 1 mL aliquot of extracts or a standard solution of pyrogallol acid was added to a
25 mL volumetric flask containing 9 mL of dH\(_2\)O. 1 mL of the Folin-Ciocalteau phenol
reagent was added to the mixture and shaken. After 5 min, 10 mL of 7\% Na\(_2\)CO\(_3\) solution
was added to the mixture. The solution was diluted to 25 mL with dH\(_2\)O and incubated for
30 min at room temperature. Absorbance was measured at 760 nm against a blank
prepared with dH\(_2\)O.

For determination of tannin content, aqueous extracts of leaves were shaken with
hide powder for 60 min in ultrasonic bath. The non-tannin phenolics in the clear
supernatant were determined in the way similar to this of total phenol content. Tannin
content was calculated as a difference between total phenolic and non-tannin phenolic
contents in the extract. Pyrogallol acid in deionised water was used for making a standard
curve. Total phenol and tannin values are expressed as pyrogallol acid equivalents (PAE) in
mg/g DM.
Dissolved organic carbon

DOC concentration in DOM extracts was determined according to ISO 8245:1999 in a certified analytical laboratory (JSC Water Investigations, Vilnius, Lithuania). Dissolved organic carbon in DOM extracts was determined according to ISO 15705 in a certified analytical laboratory (JSC Water Investigations, Vilnius, Lithuania).

Statistical analysis

The statistical analysis was carried out using the software PASW Statistics 18.0 (Predictive Analytics Software, IBM).

To validate an aggregation of the replicates from two experiments on frond and root growth rates, the two-way MANOVA were used. The factors in the analysis were a two-level experiment factor (experiment 1 and experiment 2) and a seven-level treatment factor. To validate an aggregation of the replicates from two experiments on specific enzyme activity, MDA or H$_2$O$_2$ concentrations, the two-way ANOVA were used. The factors in the analysis were a two-level experiment factor (experiment 1 and experiment 2) and a five-level treatment factor. In both analyses, there was no significant difference between two levels of experiment factor and interaction between the factors (p > 0.05), therefore, the replicates were pooled to yield n = 8.

After checking for normality (the Shapiro-Wilk test) and homogeneity of variances (the Levene test) the differences of treatments from control within each leaf species were analysed by the one-way ANOVA and the Dunnet test ($\alpha = 0.05$). Additionally, the Tukey post-hoc test was used for differences among treatments ($\alpha = 0.05$).

Results

Frond and root growth rate

The duckweed frond and root growth rates (FGR and RGR, respectively) were significantly affected by all treatments, with an exception of FGR in the treatment with 100 mg/L DOC derived from $A.~negundo$ (100-DOC$_{AN}$) (Fig. 1). Irrespective of the kind of leaf litter extract (LLE) and the type of treatment, the reduction in root length was higher than in the frond area.

As for the effect on fronds, Cu (100 $\mu$M or 6.4 mg/L) slowed the development of frond area by 97% after seven-day exposure (Fig. 1A). Due to the development of chlorosis and thus the decrease of a photosynthetically active square, the FGR was negative (-0.3 mm$^2$ d$^{-1}$). The 100-DOC$_{AN}$ diminished insignificantly the development of the frond area. Due to chlorosis, the FGR was -0.02 mm$^2$ d$^{-1}$ in Cu + 10-DOC$_{AG}$ treatment, and 5.0 times lower than the controls in Cu + 100-DOC$_{AG}$. The mixtures of Cu with $A.~negundo$ LLE of 10 and 100 mg/L DOC both stopped the growth of fronds.
As for the effect on roots, the growth was not observed over seven days in *L. minor* treated by Cu (Fig. 1B). 100 mg/L DOC derived from *A. glutinosa* (100-DOC<sub>AG</sub>) significantly diminished the development of roots (root growth rate (RGR) decreased 2.1 times compared to controls). The 100-DOC<sub>AN</sub> significantly decreased the RGR by 1.6 times. The mixture of Cu + 10-DOC<sub>AG</sub> inhibited growth of the roots totally, while the effect of Cu + 100-DOC<sub>AG</sub> was lower (RGR decreased 5.1 times compared to controls). The mixtures of Cu with *A. negundo* LLE of 10 and 100 mg/L DOC both diminished RGR by 8.8 and 2.1 times compared to controls, respectively.

**Fig. 1.** Frond (A) and root (B) growth rates of *L. minor* in control (C) and exposed for seven days with 100 μM CuSO<sub>4</sub> (Cu), mixtures of Cu and *A. glutinosa* leaf litter extracts (LLE) of 10 and 100 mg/L DOC (Cu + 10-DOC<sub>AG</sub> and Cu + 100-DOC<sub>AG</sub>), mixtures of Cu and *A. negundo* LLE of 10 and 100 mg/L DOC (Cu + 10-DOC<sub>AN</sub> and Cu + 100-DOC<sub>AN</sub>) as well as with 100 mg/L DOC of *A. glutinosa* (100-DOC<sub>AG</sub>) and *A. negundo* (100-DOC<sub>AN</sub>) LLE. Data represent mean ± SD (n = 8). Asterisks indicate significant difference from the control, different letters indicate significant difference among the means (α = 0.05).

Total phenols and tannins

Polyphenol and tannin concentrations in the DOM of *A. glutinosa* leaf litter were quite high (49 and 7.5 mg PAE/g DM, respectively), meanwhile DOM of *A. negundo* leaf litter had as much as twice lower polyphenol concentration (23 mg PAE/g DM) and no tannins.

Cu accumulation

The ability of LLE to bind Cu increased rapidly. Lower concentrations of dissolved Cu measured after ultrafiltration in the mixtures of Cu + 100-DOC<sub>AG</sub> and Cu + 100-DOC<sub>AN</sub>, made up 61.9% and 63.8%, respectively, of the total Cu (6.0 ± 0.9 mg/L, n = 26) already after 45 min. Lower concentrations of dissolved Cu were measured (after ultrafiltration) in the mixtures of Cu + 100-DOC<sub>AG</sub> and Cu + 100-DOC<sub>AN</sub> already after 45 min, making up 61.9% and 63.8% of the total Cu (6.0 ± 0.9 mg/L, n = 26), respectively. The percent of dissolved Cu decreased further until 1.5 h in the case of 100-DOC<sub>AG</sub> and then remained relatively stable (39.3% of the total Cu) up to the end of exposure at 48 h, while it diminished until 3 h in the case of 100-DOC<sub>AN</sub> and then remained relatively stable up to the end of exposure (51.4% of the total Cu).

The Cu concentration in *L. minor* increased with time in all treatments (Fig. 2). The plant accumulated 99.6 ± 4.6 and 384 ± 20.4 μg Cu/g FW in the treatment with Cu, respectively at 45 min and 48 h. Similar Cu uptake was found in plants treated with Cu + 10-DOC<sub>AG</sub> or Cu + 10-DOC<sub>AN</sub>. However, 100 mg/L DOC obtained from each of the leaf extracts diminished Cu uptake compared to Cu-treatment throughout all the exposure
periods (Fig. 2). Accumulated Cu content was 55.6 ± 2.7 and 241 ± 6.1 µg Cu/g FW, respectively at 45 min and 48 h for *A. glutinosa*, and 60.8 ± 4.0 and 188 ± 7.8 µg Cu/g FW, respectively at 45 min and 48 h for *A. negundo* treatments.

**Fig. 2.** Cu concentration in *L. minor* treated by Cu and Cu + DOC of leaf litter extracts (µg g\(^{-1}\) FW). Plants were incubated in control medium (■), 100 µM Cu (●), 100 µM Cu + 10 mg/L DOC (▼), 100 µM Cu + 100 mg/L DOC (▲) from *A. glutinosa* or *A. negundo*. Each value represents mean ± SD (n = 8). Asterisks indicate significant difference from the control at \(\alpha = 0.05\).

**Hydrogen peroxide**

The highest changes in hydrogen peroxide levels in plants were induced at early stages of the treatments. The \(\text{H}_2\text{O}_2\) content increased significantly after 3 h of exposure to Cu, its combinations with either *A. glutinosa* or *A. negundo* extracts, and to 100-DOC\(_{AG}\) or 100-DOC\(_{AN}\) alone (Fig. 3). At this exposure time, it was approximately by 2.2 times higher in Cu, Cu+10-DOC\(_{AG}\), Cu+100-DOC\(_{AG}\) and 100-DOC\(_{AG}\) treatments than in control (0.27 ± 0.10 mM, n = 56), and there were no significant differences among these treatments. However, treatments that included *A. negundo* extracts induced DOC-dependent \(\text{H}_2\text{O}_2\) levels in *L. minor*. The \(\text{H}_2\text{O}_2\) levels in Cu+10-DOC\(_{AN}\) and Cu+100-DOC\(_{AN}\) treatments exceeded that of Cu treatment by 21% and 30%, respectively, and reached maximum 40% value in the treatment of 100 mg/L DOC\(_{AN}\).

At 12 h exposure, irrespective to the kind of leaf extract, the level of hydrogen peroxide in the treatments of Cu and its combinations with the extracts did not exceed that of control, but it was by 60% higher in the treatments of the leaf extracts alone (Fig. 3). Subsequently, within the 24–48 h exposure period, the level of \(\text{H}_2\text{O}_2\) in *L. minor* treated with 100-DOC\(_{AG}\) or 100-DOC\(_{AN}\) decreased to that of control, whereas Cu and its combinations with leaf extracts induced augmentation of hydrogen peroxide up to 1.5–1.7 times.

**Fig. 3.** Concentration of \(\text{H}_2\text{O}_2\) in *L. minor* treated by Cu, DOC or combinations of Cu and DOC of corresponding leaf litter extracts. Plants were incubated in control medium (■), 100 µM Cu (●), 100 mg/L DOC from *A. glutinosa* or *A. negundo* (○), and Cu + 10 mg/L DOC (▼) or Cu + 100 mg/L DOC (▲). Each value represents mean ± SD (n = 8). Asterisks indicate significant difference from the control at \(\alpha = 0.05\).
Lipid peroxidation

After 1.5 h of exposure, a significant increase of MDA concentration in L. minor over the control levels (Fig. 4, 10.1 ± 2.13 nM/g FW and 9.58 ± 2.22 nM/g FW, left and right graphs, respectively) was observed in all treatments, and irrespective to the leaf species (Fig. 4). This 20–45% increase in MDA content was led by its decrease that continued up to 3–6 h. Then, MDA augmentation was observed up to 48 h in the treatments with Cu and the mixtures of Cu and the extracts of both leaf species, however in different strength. Specifically, in the case of A. glutinosa, the addition of 100 mg/L DOC suppressed Cu-induced augmentation of MDA more strongly than the addition of 10 mg/L DOC (Fig. 4). At 48 h, no significant difference in MDA was observed between Cu and the combined treatment of Cu + 10-DOC<sub>AG</sub>. Contrary, in the case of A. negundo, the addition of neither 10 nor 100 mg/L DOC were able to change significantly the course of Cu-induced augmentation of MDA (Fig. 4), the level of which, after 48 h, reached as almost twice higher as the control level.

After the initial approximately 20%-peak in MDA content at 1.5 h, the effect of 100 mg/L DOC extracts obtained from the NOM of both leaf species was weak, especially in the case of A. glutinosa, and showed no more than 15% deviation from the control level of MDA in exposures longer than six hours (Fig. 4).

CAT activity

After 45 min exposure of L. minor plants, significant increases of CAT activities were observed in the treatments of Cu + 100-DOC<sub>AG</sub>, Cu + 100-DOC<sub>AN</sub> and 100-DOC<sub>AN</sub> (Fig. 5). After 1.5 h, the plants responded by significant increases of CAT activities to Cu, Cu + 10-DOC<sub>AG</sub> and Cu + 100-DOC<sub>AG</sub> treatments in case of A. glutinosa, while the reaction in case of A. negundo was weaker. Then, after the initial increases in CAT activity, the reaction, in general, slowed down towards the control level (682 ± 188 nkat/mg protein and 800 ± 121 nkat/mg protein for treatments of A. glutinosa and A. negundo, respectively; mean ± SD, n = 56) from 3rd hour and up to the end of exposure at 48 h, with irregular deviations. The exceptions comprised the decreasing tendency in CAT activities in the treatments of 100 mg/L DOC of both leaf species and with Cu + 100-DOC<sub>AN</sub> reaching significantly lower levels from those of controls by 20–30% at the end of exposure as well as the increasing tendency in CAT activities in the treatment of Cu + 10-DOC<sub>AG</sub> reaching significantly higher level from the controls by 20% at the end of exposure (Fig. 5).
POD activity

Guaiacol peroxidase activities were significantly enhanced after treatments of Cu and its mixture with 10 and 100 mg/L DOC from A. glutinosa, but not with the 100-DOC<sub>AG</sub> during the 45 min–48 h period reaching a 2–2.5-fold increase at 24 and 48 h (Fig. 5). In case of A. negundo, the increases of POD activity over control (316 ± 122 nkat/mg protein and 406 ± 112 nkat/mg protein for treatments of A. glutinosa and A. negundo, respectively; mean ± SD, n = 56) were significant later, throughout the 12–48 h period, in the same treatments as above, and again with the exception of 100-DOC<sub>AN</sub> (Fig. 5).

Fig. 5. Activities of catalase (CAT), guaiacol peroxidase (POD), ascorbate peroxidase (APX) and glutathione reductase (GR) in L. minor treated by Cu, DOC of leaf litter extracts from A. glutinosa or A. negundo, and combinations of Cu and the DOC of respective extracts. Plants were incubated in control medium (■), 100 µM Cu (●), 100 mg/L DOC (●), Cu + 10 mg/L DOC (▲) or Cu + 100 mg/L DOC (▲). Each value represents mean ± SD (n = 8). Asterisks indicate significant difference from the control at α = 0.05.

APX activity

No ascorbate peroxidase activity alterations were observed at 45<sup>th</sup> min, meanwhile, at 1.5 h, the 15–20% decreases were evident in the treatments of Cu + 10-DOC<sub>AG</sub> and Cu + 100-DOC<sub>AG</sub>, and Cu + 10-DOC<sub>AN</sub> and Cu + 100-DOC<sub>AN</sub> (Fig. 5). Then, at the 6–24 h period, the significant suppression of APX activities was observed in the treatments of Cu, Cu + 10-DOC<sub>AG</sub> and Cu + 100-DOC<sub>AG</sub> and Cu + 100-DOC<sub>AN</sub> and at 6<sup>th</sup> hour, in the treatment of Cu + 10-DOC<sub>AN</sub> (Fig. 5). At the end of exposure at 48-h, the activities of all treatments did not differ from those of controls (32.6 ± 6.99 nkat/mg protein and 15.2 ± 2.47 nkat/mg protein for treatments of A. glutinosa and A. negundo, respectively; mean ± SD, n = 56).

GR activity

The glutathione reductase activity changes in L. minor were irregular during the 12 hours of exposure in all treatments with the DOM of A. glutinosa and Cu alone, then GR activity decreased by approximately 20% below control level (409 ± 46.6 nkat/mg protein and 430 ± 72.6 nkat/mg protein for treatments of A. glutinosa and A. negundo, respectively; mean ± SD, n = 56) in the treatments of Cu and Cu + 10-DOC<sub>AG</sub> (Fig. 5). At 24 and 48 h, GR activity increased up to approximately 20% in the treatment of Cu + 100-DOC<sub>AG</sub>. In the case of A. negundo, the activity of GR activity was slightly, yet significantly, suppressed in the treatments of Cu + 10-DOC<sub>AN</sub> and Cu + 100-DOC<sub>AN</sub>, during the 1.5–3-h period. Then GR activity tended to recover to the control level at 24 h and to exceed it at the end of 48 h exposure period, in the treatment of Cu + 10-DOC<sub>AN</sub>, while a significant increase in 20–30% above control level was seen in the treatment of Cu + 100-DOC<sub>AN</sub> during the period of 12–48 h (Fig. 5). No significant alterations of GR activities were found in the LLE of 100 mg/L DOC of either species.
Discussion

Our results showed a high accumulation of copper in *L. minor*, which increased with time over 48 h. Similar accumulation properties of duckweed for this metal have already been documented for various exposure durations (Drost et al., 2007; Kanoun-Boulé et al., 2009; Razinger et al., 2007). The accumulation of metals in aquatic plants is often accompanied by a variety of morphological and physiological changes, some of which directly contribute to the tolerance capacity of plants (Prasad et al., 2001; Xing et al., 2010). *L. minor* exposed to Cu, the extracts from *A. glutinosa* or *A. negundo* leaf litter, and to the combination of Cu with the extracts indicated inhibition of the growth parameters, enhanced levels of the lipid peroxidation and altered enzyme activities. To explore oxidative damage, we applied certainly toxic concentration of Cu (100 µM) within up to 48-hour exposure durations as compared to growth inhibition values of 7-d EC50, i.e. 2.7 and 9.7 µM reported by Nauman et al. (2007) and Drost et al. (2007), respectively. The time span longer than about one day can already be considered as long-term since the amount of *L. minor* biomass doubles every two days (Environment Canada, 2007) and the doubling time for fronds ranges from 1.3 to 2.8 days (Wang, 1990) under optimal nutrient, light and temperature conditions. According to our findings, the reduction in root growth rate was higher than in the frond growth rate, irrespective of the kind of treatment. It is known that root length inhibition is a more sensitive endpoint than that of the frond area (Gopalapillai et al., 2014). However, the concentration-dependent antagonistic action of Cu and *A. glutinosa* extract mixture was evident on fronds and roots, while in the case of Cu and *A. negundo* extract mixture this was evident on roots only, suggesting that the origins of the diminishing influence of *A. glutinosa* and *A. negundo* extracts on Cu-induced toxicity effects should not be the same.

The complex interactions between physical-chemical and biological factors in the aquatic medium may change metal bioavailability (Cuss and Guéguen, 2012; Koukal et al., 2003). The data obtained in Cu measurements in *L. minor* within 45 min–48 h period revealed that the accumulation rate in the combined treatments of Cu and *A. glutinosa* or *A. negundo* leaf extracts (100 mg/L DOC) was respectively two or three times lower than that in the CuSO₄ treatment (~6 µg g⁻¹ FW h⁻¹). The Cu accumulation dynamics at a constant rate did not correspond with the free Cu ions concentration dynamics in media, wherein it reached relatively constant level after 1.5 h or 3 h, respectively for mixtures of Cu and *A. glutinosa* or *A. negundo* leaf extracts, and the Cu²⁺ levels were respectively 39% and 51% of that of nominal Cu concentration. Fact that Cu²⁺ concentration in the medium with NOM from *A. negundo* leaves remained higher than that from *A. glutinosa*, but a higher amount of Cu accumulated in *L. minor* in the case of NOM from *A. glutinosa* leaves suggests that part of Cu could enter the cell being bound on NOM or could be bound in the cell wall, and this part was higher in the case of *A. glutinosa* leaf extract. This assumption is supported by the comparative analysis of effects on *L. minor* root growth rate (RGR). As large as twice the
increase of inhibition of RGR (= (RGR_{Cu+100-DOC} - RGR_{100-DOC})/RGR_{100-DOC}) was found for the mixture of Cu and A. glutinosa leaf extract as for Cu and A. negundo (Fig. 1). Otherwise, a stronger effect on L. minor exposed to the mixture of Cu and A. negundo leaf extract should be observed; at least, due to a higher concentration of Cu ions measured in the medium.

The mechanisms of copper ions translocation from the medium into plant cell comprise, at least, transition metal transporters (González-Guerrero et al., 2016; Palmgren and Nissen, 2011) including low-molecular-weight organic molecules (Sinclair and Krämer, 2012) and binding in the cell wall. It was estimated that 74% of Cu accumulated in the cell wall of macrophytic alga Nitellopsis obtusa, when its internodal cells were treated with 50 µM of CuSO₄ (Manusadžianas et al., 2017).

Plants exposed to long-term stress pass through different physiological states, from resistant to exhaustive (Lichtenthaler, 1996); thus, different kinetics of primary and successive plant responses might be expected. We found two phases of responses: namely, the first (up to 6 h) and the second one (within 6–48 h) could be identified for lipid peroxidation and hydrogen peroxide, and also, in part, enzyme activity kinetics. In addition, frond abscission/disintegration delimited these response phases at the physiological level. A process of chlorosis to necrosis leading to frond disintegration is a recognized symptom of toxicity in fronds of Lemna exposed to pollutants (Khellaf and Zerdaoui, 2009). Release of daughter fronds from the metal stressed mother frond would increase the chance of the daughter fronds survival (Li and Xiong, 2004).

The high content of Cu (100 µgCu/g FW) accumulated in L. minor over initial 45 minutes caused a half-fold increase in the amount of MDA at 1.5 h and temporal increase of H₂O₂ with the peak at 3 h. It is widely accepted that malondialdehyde, the end product of lipid peroxidation, can be used as an indicator of membrane damage (Heath and Packer, 1968) and oxidative stress (Zezulka et al., 2013). At the end of the first response phase (plant resistance state), we observed the frond abscission. The oxidative stress progressed during the second response phase (plant exhaustive state) in terms of up to a 2-fold increase in MDA content and elevated H₂O₂ over control level at 48 h. A similar picture, in general, could be seen when plants were exposed to Cu and its combinations with leaf litter extracts; however, in the case of Cu and A. glutinosa leaf extract mixture, MDA content in L. minor was inversely dependent on DOC concentration in the extract (Fig. 4).

The response patterns of the plants exposed to each of the leaf litter extracts singly were mainly the same as exposed to Cu or Cu combined with the extracts, during the first response phase (up to 6 h). However, the levels of H₂O₂ and MDA relaxed to that of control during the second response phase. Therefore, it can be suggested that plants treated with extracts alone underwent oxidative stress in terms of H₂O₂ production and MDA increase, however, due to enzymatic activity they were able to resist this initial stress and consequently avoid detrimental effects in terms of growth and development at the final 7-day exposure, yet partially slowed down (Figs. 1, 3 and 4).
In the first response phase, alterations of oxidative stress enzymes in *L. minor* were observed as early as after 45-min, the shortest exposure period. Specifically, Cu or its combinations with *A. glutinosa* extracts induced CAT activity increases that were not led by significant overrun of the control levels of H$_2$O$_2$ and MDA. This should show that the initial response of the enzymes prevented membrane damage due to ROS generation. Later on, however, concentrations of H$_2$O$_2$ and MDA significantly increased over the control reaching their peak values within 1.5–3 h, and this coincided with the maximum CAT activity. Bayliak et al. (2006) and Martins and English (2014) have demonstrated that increasing H$_2$O$_2$ levels (up to 1mM) in yeast cells stimulate CAT. We measured H$_2$O$_2$ concentrations of 0.6–0.7 mM to be the highest ones in treated *L. minor*. Similar fast mobilization of CAT was considered as a cellular adaptation in primary leaves of *Phaseolus vulgaris* to cope with H$_2$O$_2$ overproduction generated by Cu$^{2+}$ (Weckx and Clijsters, 1996). Interestingly, the increase in CAT activity during the initial 1.5 h was followed by attenuation of APX activity (Fig. 5). This might be linked with the decreased GR activity since GR converts GSSH into GSH by consuming NADPH and so maintains AsA level. One of the characteristic properties of APX that distinguishes it from POD, cytochrome c peroxidase and GR, is the rapid inactivation of the enzyme under conditions where an electron donor is absent (Miyake and Asada, 1996). The lower reduction state of AsA could be due to both the insufficient supply of electrons from GSH via direct interaction between GSH and AsA, and inadequate activities of MDHAR and DHAR (Asada and Takahashi, 1987; Foyer and Halliwell, 1976). In our experiments, GR activity began to increase after the initial decrease at 45 min, restoring the control level after three hours of exposure to Cu and Cu+DOC$_{AG}$ (Fig. 5). This increase of GR activity created, indirectly, favourable conditions for augmentation of ascorbic acid content, which allowed the APX activity to increase toward the control level during 1.5–3 h. Results of Xiang and Oliver (1998) have shown that the liquid culture of *Arabidopsis* tissues exposed to 100 µM Cu$^{2+}$ respond fairly rapidly by increasing the transcript levels of the genes encoding the GSH-synthesizing enzymes (GSH1, GSH2) and the GSSG-reducing enzyme (GR1). Elevated transcript levels were evident one hour after the exposure to Cu$^{2+}$, plateaued at six hours, and remained high for 18 h, and the high levels of transcripts continued for a few days under this condition. Another factor that prevented H$_2$O$_2$ accumulation in *L. minor* treated by Cu and Cu+DOC$_{AG}$ could be elevated POD activity within a 3–6-h period, which compensated gradual decrease of CAT activity toward the control level (Fig. 5). Similar concomitant activation of POD and inactivation of CAT has been found in various plants as an oxidative stress response to pathogen within 30 hours (Madhusudhan et al., 2009).

The observed alterations in enzyme activities are likely related to oxidative reactions due to increased H$_2$O$_2$ level, which may eventually yield increased lipid peroxidation. Indeed, at the end of the first response phase, the highest MDA content in *L. minor* was found in the treatment with Cu (Fig. 2), which, however, was diminished by the DOC$_{AN}$, but not by the DOC$_{AG}$, in a concentration-dependent manner. The latter could be due to higher
contents of polyphenols in the DOC of *A. glutinosa* than that of *A. negundo*, respectively 49
and 23 mg PAE/g DM and, in addition, tannins (7.5 mg PAE/g DM) that were lacking in the
DOC of *A. negundo*. It has been found that tannins exhibit strong antioxidant properties in
comparison to low molecular weight phenolic compounds. The presence of catechol or
galloyl groups in tannin structure are essential to complex formation with transition metals
(Andjelković et al., 2006) including copper (Brown and Kelly, 2007; Miller et al, 1996).
Phenolic compounds with additional hydroxy groups on aromatic ring bind Cu\(^{2+}\) more
efficiently (Brown and Kelly, 2007). The lack of tannins and low polyphenol content, and
thus weak Cu binding ability in *A. negundo* LLE may explain, at least in part, negligible
impact of *A. negundo*-derived DOM on the moderation of Cu-induced MDA levels. However,
it is also known that phenolics can display prooxidant activities in the presence of metal
ions in plants (Decker, 1997; Azam et al., 2004).

In the second response phase, Cu and its combination with *A. glutinosa* extracts
induced a continuous increase of MDA concentration up to the end of 48-h exposure. This
could be related to inactivation of APX, despite that the CAT and POD activity remained
above the control level. It is known that inhibition of APX results in an increased level of
H\(_2\)O\(_2\) that contributes to defence gene activation and acts as a substrate for POD involved in
defence responses such as lignification and crosslinking of cell wall proteins (Bradley et al.,
1992). DOC of *A. glutinosa* is characterized by higher contents of polyphenols and tannins
that are known to support the primary detoxification system (Yamasaki et al., 1997). Our
results also showed that *A. glutinosa* leaf extract stimulated POD when it acted individually.
In the Cu+DOC\(_{AG}\) treatments, exogenous polyphenols and tannins and continuous increase
of POD activity, exceeding that of Cu-induced at the end of the 48-h period, suggests that
*L. minor* avoids H\(_2\)O\(_2\) overproduction through a phenolic-dependent resistance mechanism.
This possibility is further supported by the observation that MDA content was diminished
by the DOC\(_{AG}\) in a concentration-dependent manner, which was also true for the first
response phase.

Combined treatments of Cu and *A. negundo* extracts, within initial 3 h, induced
different responses of certain oxidative stress enzymes in relation to those of Cu and
*A. glutinosa* extracts. Contrary to the case of *A. glutinosa*, when CAT enzyme activity
decreased monotonically toward controls (the tendency observed up to 6 h), a rapid
decrease of CAT activity to control level at 1.5 h indicated that the ability to scavenge H\(_2\)O\(_2\)
in plants was weakened. Indeed, *A. negundo* extract (100 mg DOC/L) even acting
individually induces fast inactivation of CAT. During 3–6 h period, the relatively higher
activity of POD in the relation of CAT, APX and GR activities in the treatments of Cu and
*A. negundo* extracts indicated that actual level of lipid peroxidation could be mainly
associated with the POD scavenging of H\(_2\)O\(_2\). Inactivation of both CAT and the AsA-GSH
pathway may prevent the cell from depleting NAD(P)H reserves (Heineke et al., 1991).
Overall, within three hours of exposure, the treatments of Cu in combination with DOC
from either *A. negundo* or *A. glutinosa* extract yielded lower MDA levels than in the
treatment of Cu, and this was supported by various responses of ROS enzymes. However, after six hours of exposure, plants were unable to cope with H$_2$O$_2$ excess in the treatments of Cu+DOC$_{AN}$ when lipid peroxidation augmented up to the level observed in Cu-treatment, in contrast to Cu+DOC$_{AG}$ when lipid peroxidation was lower (Fig. 4). This distinction between the influences of various leaf species extracts at the background of Cu action could be caused by the differences in contents of polyphenolic compounds (see above).

Extended exposure of L. minor for 6–48 h in the mixture of Cu+100-DOC$_{AN}$ induced higher activities of APX and GR. This finding indicates that consumption of H$_2$O$_2$ can be associated not only with a POD (see below), but with the enzymatic activities in the AsA-GSH cycle, as well. Alteration of these enzymes was led by the inactivation of CAT. The first reason for the latter could be an excess level of AsA in the presence of Cu. Davison et al. (1986) have shown that AsA alone is not very damaging and that AsA’s inhibitory action can be released by O$_2$, H$_2$O$_2$ or Cu$^{2+}$, i.e. when AsA is oxidized to semidehydroascorbate (or ascorbyl radical). Ascorbate toxicity depends on the presence of copper (or iron) and oxygen, but oxygen is not required in the presence of H$_2$O$_2$ (Samuni et al., 1983). Another reason for progressive inactivation of CAT observed in our study could be the presence of phenolic compounds in leaf litter extracts. Although the quantity and composition of the extracts differed, the effect on CAT activity decrease was evident in 100 mg/L DOC of either extract, especially at the end of 48-h exposure. The similar inhibitive action of certain phenolic compounds on CAT within 24-h exposure has been found for thermophilic fungi (Yüzügüllü et al., 2011).

POD activity did not differ between the treatments of Cu and either Cu+DOC$_{AN}$ or Cu+DOC$_{AG}$ at 24$^{th}$ hour of exposure. However, the extract type probably determined the opposing POD response at 48$^{th}$ hour of exposure. At this time point, the highest POD activity (exceeding that of the Cu-induced) was observed in the case of Cu+DOC$_{AG}$. The lack of the differences in MDA levels in the second phase of L. minor response to Cu or its combinations with DOC$_{AN}$ suggests that, under the influence of A. negundo extracts, CAT, POD and AsA-GSH cycle enzymes were unable to minimize the oxidative damage induced by Cu. This is the opposite to the action of A. glutinosa extracts.

Invasive plant species tend to migrate from their native habitats under favourable climatic conditions; therefore, trophic and other relationships in ecosystems are changing. It has been suggested that black alder Alnus glutinosa, native species in Lithuania, and boxelder maple Acer negundo, invasive species in Lithuania, impact the same aquatic organisms in different ways (Krevš et al., 2013; Manusadžianas et al., 2014). In this context, we revealed the potential of diverse species DOM to modify Cu toxicity effects on Lemna minor. Both types of leaf litter extracts protected L. minor from deleterious effects of lipid peroxidation products during the first response phase (up to 6 h) when plants activated stress-coping mechanisms. Throughout the second response phase (6–48 h), cellular defence mechanisms were impaired and the vitality of L. minor steadily decreased. Overall, the analyses of Cu accumulation in L. minor and binding on DOM, and the dynamics of MDA...
content that represents integrative biochemical response, suggest that the reason of beneficial action of *A. glutinosa* extracts compared to that of *A. negundo* is based on the higher contents of polyphenols and tannins.

**Conclusions**

We revealed that leaf litter extracts of black alder *Alnus glutinosa*, native species in Lithuania, and boxelder maple *Acer negundo*, invasive species in Lithuania have various potential to modify Cu toxicity effects on *Lemna minor*. Analyses of duckweed responses, dynamics of Cu accumulation in the plant and its binding on the DOM in media allowed to conclude that both types of leaf litter extracts protected *L. minor* from deleterious effects of lipid peroxidation products during the first response phase within 6 h, however, cellular defence mechanisms were impaired during the prolonged exposure within 6–48 h. The differences in antioxidant enzyme activity profiles ascertained in *L. minor* treated by mixtures of Cu and various leaf extracts over two days were considered to condition dissimilar effects on the development of plant fronds and roots observed after seven days. The complex data obtained in the current study could be useful for modelling of aquatic ecosystem responses to the changing environment.

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**References**

Adam N, Leroux F, Knapen D, Bals S, Blust R. 2014. The uptake of ZnO and CuO nanoparticles in the water-flea *Daphnia magna* under acute exposure scenarios. *Environmental Pollution* **194**:130–137 [DOI 10.1016/j.envpol.2014.06.037].

Aebi H. 1984. Oxygen radicals in biological systems. *Methods in Enzymology* **105**:121–126 [DOI 10.1016/S0076-6879(84)05016-3].

Alscher RG, Donahue JL, Cramer CL. 1997. Reactive oxygen species and antioxidants: relationships in green cells. *Physiologia Plantarum* **100**:224–233 [DOI 10.1111/j.1399-3054.1997.tb04778.x].

Amorim ELC, Nascimento JE, Monteiro JM, Sobrinho TJS, Araújo TAS, Albuquerque UP. 2008. A simple and accurate procedure for the determination of tannin and flavonoid levels and some applications in ethnobotany and ethnopharmacology. *Functional Ecosystems and Communities* **2**:88–94.

Andjelković M, Van Camp J, De Meulenaer B, Depaemelaere G, Socaciu C, Verloo M, Verhe R. 2006. Iron-chelation properties of phenolic acids bearing catechol and galloyl groups. *Food Chemistry* **98**:23–31 [DOI 10.1016/j.foodchem.2005.05.044].

Asada K, Takahashi M. 1987. Production and scavenging of active oxygen in photosynthesis. In: Kyle DJ, Osmond CB, Arntzen CJ, eds. *Photoinhibition (Topics in Photosynthesis)*. Amsterdam: Elsevier, 227–287.
Atanassova M, Georgieva S, Ivancheva K. 2011. Total phenolic and total flavonoid contents, antioxidant capacity and biological contaminants in medicinal herbs. *Journal of the University of Chemical Technology and Metallurgy* **46**:81–88.

Azam , Hadi N, Khan NU, Hadi SM. Prooxidant property of green tea polyphenols epicatechin and epigallocatechin-3-gallate: implications for anticancer properties. *Toxicoogy in vitro* **18**:555–561 DOI 10.1016/j.tiv.2003.12.012.

Babu TS, Akhtar TA, Lampi MA, Tripuranthakam S, Dixon DG, Greenberg BM. 2003. Similar stress responses are elicited by copper and ultraviolet radiation in the aquatic plant *Lemna gibba*: implication of reactive oxygen species as common signals. *Plant and Cell Physiology* **44**:1320–1329 DOI 10.1093/pcp/pcg160.

Bayliak M, Semchyshyn H, Lushchak V. 2006. Effect of hydrogen peroxide on antioxidant enzyme activities in *Saccharomyces cerevisiae* is strain specific. *Biochemistry* **71**:1013–1020 DOI 10.1134/S0006297906090100.

Blodau C, Basiliko N, Moore TR. 2004. Carbon turnover in peatland mesocosms exposed to different water table levels. *Biogeochemistry* **67**:331–351 DOI 10.1023/B:BIOG.0000015788.30164.e2.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**:248–254 DOI 10.1016/0003-2697(76)90527-3.

Bradley DJ, Kjellbom P, Lamb CJ. 1992. Elicitor- and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein: a novel, rapid defense response. *Cell* **70**:21–30 DOI 10.1016/0092-8674(92)90530-P.

Brown JE, Kelly MF. 2007. Inhibition of lipid peroxidation by anthocyanins, anthocyanidins and their phenolic degradation products. *European Journal of Lipid Science and Technology* **109**:66–71 DOI 10.1002/ejlt.200600166.

Casas JJ, Larrañaga A, Menéndez M, Pozo J, Basaguren A, Martínez A, Pérez J, González JM, Mollá S, Casado C, Descals E, Roblas N, López-González JA, Luis Valenzuela J. 2013. Leaf litter decomposition of native and introduced tree species of contrasting quality in headwater streams: how does the regional setting matter? *Science of the Total Environment* **458–460**:197–208 DOI 10.1016/j.scitotenv.2013.04.004.

Chen LM, Lin CC, Kao CH. 2000. Copper toxicity in rice seedlings: changes in antioxidative enzyme activities, $H_2O_2$ level, and cell wall peroxidase activity in roots. *Botanical Bulletin of the Academia Sinica* **41**:99–103.

Cuss CW, Guéguen C. 2012. Impacts of microbial activity on the optical and copper-binding properties of leaf-litter leachate. *Frontiers in Microbiology* **3**:1–10 DOI 10.3389/fmicb.2012.00166.

Davison AJ, Kettle AJ, Fatur DJ. 1986. Mechanism of the inhibition of catalase by ascorbate. *The Journal of Biological Chemistry* **261**:1193–1200.

De Vos CH, Vonk MJ, Vooijs R, Schat H. 1992. Glutathione depletion due to copper-induced phytochelatin synthesis causes oxidative stress in *Silene cucubalus*. *Plant Physiology* **98**:853–858 DOI 10.1104/pp.98.3.853.

De Vos CHR, Schat H, De Waal MAM, Vooijs R, Ernst WHO. 1991. Increased resistance to copper-induced damage of the root cell plasmalemma in copper tolerant *Silene cucubalus*. *Plant Physiology* **82**:523–528 DOI 10.1111/j.1399-3054.1991.tb02942.x.

Decker EA. 1997. Phenolics: prooxidants or antioxidants? *Nutrition Reviews* **55**:396–398.

Drost W, Matzke M, Backhaus T. 2007. Heavy metal toxicity to *Lemna minor*: studies on
the time dependence of growth inhibition and the recovery after exposure.

Chemosphere 67:36–43 DOI 10.1016/j.chemosphere.2006.10.018.

Earl JE, Cohagen KE, Semlitsch RD. 2012. Effects of leachate from tree leaves and grass litter on tadpoles. Environmental Toxicology and Chemistry 31:1511–1517 DOI 10.1002/etc.1829.

Environment Canada. 2007. Biological test method. Test for measuring the inhibition of growth using the freshwater macrophyte, Lemna minor. Report EPS 1/RM/37 DOI 10.1017/CBO9781107415324.004.

Forni C, Braglia R, Harren FJM, Cristescu SM. 2012. Stress responses of duckweed (Lemna minor L.) and water velvet (Azolla filiculoides Lam.) to anionic surfactant sodium-dodecyl-sulphate (SDS). Aquatic Toxicology 110–111:107–113 DOI 10.1016/j.aquatox.2011.12.017.

Foyer CH, Halliwell B. 1976. The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. Planta 133:21–25 DOI 10.1007/BF00386001.

Foyer CH, Lopez-Delgado H, Dat JF, Scott IM. 1997. Hydrogen peroxide- and glutathione-associated mechanisms of acclimatory stress tolerance and signalling. Physiologia Plantarum 100:241–254 DOI 10.1034/j.1399-3054.1997.1000205.x.

González-Guerrero M, Escudero V, Saéz Á, Tejada-Jiménez M. 2016. Transition metal transport in plants and associated endosymbionts: arbuscular mycorrhizal fungi and rhizobia. Frontiers in Plant Science 7:1088 DOI 10.3389/fpls.2016.01088.

Gopalapillai Y, Vigneault B, Hale BA. 2014. Root length of aquatic plant, Lemna minor L., as an optimal toxicity endpoint for biomonitoring of mining effluents. Integrated Environmental Assessment and Management 10:493–497 DOI 10.1002/ieam.1558.

Gudžinskas Z. 1998. Conspectus of alien plant species of Lithuania. 8. Aceraceae, Balsaminaceae, Elaeagnaceae, Geraniaceae, Hippocastanaceae, Linaceae, Lythraceae, Onagraceae, Oxalidaceae, Rutaceae, and Vitaceae. Botanica Lithuanica 4:363–377.

Gupta H, Cuypers A, Vangronsveld J, Clijsters H. 1999. Copper affects the enzymes of the ascorbate-glutathione cycle and its related metabolites in the roots of Phaseolus vulgaris. Physiologia Plantarum 106:262–267 DOI 10.1034/j.1399-3054.1999.106302.x.

Halliwell B, Gutteridge JMC. 1984. Oxygen toxicity, oxygen radicals, transition metals and disease. Biochemical Journal 219:1–14 DOI 10.1016/j.siny.2010.04.003.

Heath RL, Packer L. 1968. Photoperoxidation in isolated chloroplasts. Archives of Biochemistry and Biophysics 125:189–198 DOI 10.1016/0003-9861(68)90654-1.

Heineke D, Riens B, Grosse H, Hoferichter P, Peter U, Flugge U-I, Heldt HW. 1991. Redox transfer across the inner chloroplast envelope membrane. Plant Physiology 95:1131–1137 DOI 10.1104/pp.95.4.1131.

Hildebrandt DF, Rodriguez JG, Brown GC, Luu KT, Volden CS. 1986. Peroxidative responses of leaves in 2 soybean genotypes injured by 2-spotted spider-mites (Acari, Tetanychidae). Journal of Economic Entomology 79:1459–1465.

Hou W, Chen X, Song G, Wang Q, Chang CC. 2007. Effects of copper and cadmium on heavy metal polluted waterbody restoration by duckweed (Lemna minor). Plant Physiology and Biochemistry 45:62–69 DOI 10.1016/j.plaphy.2006.12.005.

Huh JH and Ahn JW. 2017. A perspective of chemical treatment for cyanobacteria control toward sustainable freshwater development. Environmental Engineering Research
784 22:1–11. DOI 10.4491/eer.2016.155.
785 Jana S, Choudhuri MA. 1981. Glycolate metabolism of three submerged aquatic
786 angiosperms during aging. Aquatic Botany 12:234–354.
787 Kanoun-Boulé M, Vicente JAF, Nabais C, Prasad MNV, Freitas H. 2009. Ecophysiological
788 tolerance of duckweeds exposed to copper. Aquatic Toxicology 91:1–9
789 DOI 10.1016/j.aquatox.2008.09.009.
790 Kim SD, Ma H, Allen HE, Cha DK. 1999. Influence of dissolved organic matter on the
791 toxicity of copper to Ceriodaphnia dubia: effect of complexation kinetics.
792 Environmental Toxicology and Chemistry 18:2433–2437.
793 Khellaf N, Zerdaoui M. 2009. Phytoaccumulation of zinc by the aquatic plant, Lemna gibba
794 L. Bioresource Technology 100:6137–6140 DOI 10.1016/j.biortech.2009.06.043.
795 Koukal B, Guéguen C, Pardos M, Dominik J. 2003. Influence of humic substances on the
796 toxic effects of cadmium and zinc to the green alga Pseudokirchneriella subcapitata.
797 Chemosphere 53:953–961 DOI 10.1016/S0045-6535(03)00720-3.
798 Krevš A, Darginavičienė J, Gylytė B, Grigutytė R, Jurkonienė S, Karitonas R,
799 Kučinskienė A, Pakalnis R, Manusadžianas L. 2013. Ecotoxicological effects evoked in hydrophytes by leachates of invasive Acer negundo
800 and autochthonous Alnus glutinosa fallen off leaves during their microbial
801 decomposition. Environmental Pollution 173:75–84
802 DOI 10.1016/j.envpol.2012.09.016.
803 Lachman J, Miholová D, Pivec V, Jírů K, Janovská D. 2011. Content of phenolic
804 antioxidants and selenium in grain of einkorn (Triticum monococcum), emmer
805 (Triticum dicoccum) and spring wheat (Triticum aestivum) varieties. Plant, Soil and
806 Environment 57:235–243 DOI 10.1007/s11104-009-0026-x.
807 Li T, Xiong Z. 2004. A novel response of wild-type duckweed (Lemna paucicostata
808 Hegelm.) to heavy metals. Environmental Toxicology 19:95–102
809 DOI 10.1002/tox.20000.
810 Lichtenthaler HK. 1996. Vegetation stress: an introduction to the stress concept in plants.
811 Plant Physiology 148:4–14 DOI 10.1016/S0176-1617(96)80287-2.
812 Lin YM, Liu JW, Xiang P, Lin P, Ye GF, Da Sternberg LSL. 2006. Tannin dynamics of
813 propagules and leaves of Kandelia candel and Bruguiera gymnorrhiza in the Jiulong
814 River Estuary, Fujian, China. Biogeochemistry 78:343–359 DOI 10.1007/s10533-005-
815 4427-5.
816 Lin YM, Liu XW, Zhang H, Fan HQ, Lin GH. 2010. Nutrient conservation strategies of a
817 mangrove species Rhizophora stylosa under nutrient limitation. Plant Soil 326:469–
818 479 DOI 10.1007/s11104-009-0026-x.
819 Madhusudhan KN, Srikanta BM, Shylaja MD, Prakash HS, Shetty HS. 2009. Changes in
820 antioxidant enzymes, hydrogen peroxide, salicylic acid and oxidative stress in
821 compatible and incompatible host-tobamovirus interaction. Journal of Plant
822 Interactions 4:157–166 DOI 10.1080/17429140802419516.
823 Maksymiec W. 1997. Effects of copper on cellular processes in higher plants.
824 Photosynthetica 34:321–342.
825 Manceau A, Matynia A. 2010. The nature of Cu bonding to natural organic matter.
826 Geochimica et Cosmochimica Acta 74:2556–2580 DOI 10.1016/j.gca.2010.01.027.
827 Mannervik B. 1999. Measurement of glutathione reductase. In: Maines MD, Costa LG,
828 Hodgson E, Reed DJ, Sipes IG, eds. Current Protocols in Toxicology. Hoboken, NJ: John
Masudzianas L, Darginavicienė J, Grylė B, Jurkonienė S, Krevš A, Kučinskienė A, Mačkinaitė R, Pakalnis R, Sadauskas K, Sendžikaitė J, Vitkus R. 2014. Ecotoxicity effects triggered in aquatic organisms by invasive Acer negundo and native Alnus glutinosa leaf leachates obtained in the process of aerobic decomposition. Science of the Total Environment 496:35–44 DOI 10.1016/j.scitotenv.2014.07.005.

Manusadžianas L, Grylė B, Grigutytė R, Karitonas R, Sadauskas K, Vitkus R, Šiliauskas L. 2017. Accumulation of copper in the cell compartments of charophyte Nitellopsis obtusa after its exposure to copper oxide nanoparticle suspension. Environmental Science and Pollution Research 24:27653–27661 DOI 10.1007/s11356-016-8023-0.

Martins D, English AM. 2014. Redox biology catalase activity is stimulated by H₂O₂ in rich culture medium and is required for H₂O₂ resistance and adaptation in yeast. Redox Biology 2:308–313 DOI 10.1016/j.redox.2013.12.019.

Marx G, Heumann KG. 1999. Mass spectrometric investigations of the kinetic stability of chromium and copper complexes with humic substances by isotope-labelling experiments. Fresenius Journal of Analytical Chemistry 364:489–494.

Miller NJ, Castelluccio C, Tijburg L, Rice-Evans C. 1996. The antioxidant properties of theaflavins and their gallate esters – radical scavengers or metal chelators? FEBS Letters 392:40–44 DOI 10.1016/0014-5793(96)00780-6.

Miyake C, Asada K. 1996. Inactivation mechanism of ascorbate peroxidase at low concentrations of ascorbate; Hydrogen peroxide decomposes compound I of ascorbate peroxidase. Plant and Cell Physiology 37:423–430 DOI 10.1093/oxfordjournals.pcp.a028963.

Nakano Y, Asada K. 1981. Hydrogen-peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant and Cell Physiology 22:867–880.

Naumann B, Eberius M, Appenroth KJ. 2007. Growth rate based dose-response relationships and EC-values of ten heavy metals using the duckweed growth inhibition test (ISO 20079) with Lemna minor L. clone St. Journal of Plant Physiology 164:1656–1664 DOI 10.1016/j.jplph.2006.10.011.

Nimptsch J, Pflugmacher S. 2008. Decomposing leaf litter: the effect of allochthonous degradation products on the antioxidant fitness and photosynthesis of Vesicularia dubyana. Ecotoxicology and Environmental Safety 69:541–545 DOI 10.1016/j.ecoenv.2007.09.003.

Palmgren MG, Nissen P. 2011. P-type ATPases. Annual Review of Biophysics 40:243–266 DOI 10.1146/annurev.biophys.093008.131331.

Panagos P, Ballabio C, Lugato E, Jones A, Borrelli P, Scarpa S, Orgiazzi A, Montanarella L. 2018. Potential sources of anthropogenic copper inputs to European agricultural soils. Sustainability 10:2380 DOI 10.3390/su10072380.

Prasad M, Malec P, Waloszek A, Bojko M, Strzałka K. 2001. Physiological responses of Lemna trisulca L.(duckweed) to cadmium and copper bioaccumulation. Plant Science 161:881–889.

Prieditis N. 1997. Alnus glutinosa – dominated wetland forests of the Baltic Region: community structure, syntaxonomy and conservation. Plant Ecology 129:49–94.

Radić, S, Pevalek-Kozlina B. 2010. Effects of osmotic stress on antioxidative system of duckweed (Lemna minor L). Periodicum Biologorum 112:293–299.
Razinger J, Dermastia M, Drinovec L, Drobne D, Zrimec A, Koce JD. 2007. Antioxidative responses of duckweed (Lemna minor L.) to short-term copper exposure. *Environmental Science and Pollution Research International* 14:194–201. DOI 10.1065/espr2006.11.364.

Samuni A, Aronovitch J, Godinger D, Chevion M, Czapski G. 1983. On the cytotoxicity of vitamin C and metal ions: a site-specific Fenton mechanism. *European Journal of Biochemistry* 137:119–124. DOI 10.1111/j.1432-1033.1983.tb07804.x.

Schützendübel A, Polle A. 2002. Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization. *Journal of Experimental Botany* 53:1351–1365. DOI 10.1016/S0981-9428(02)01411-0.

Sinclair SA, Krämer U. 2012. The zinc homeostasis netork of land plants. *Biochimica et Biophysica Acta* 1823:1553–1567. DOI 10.1016/j.bbamcr.2012.05.016.

Singleton VL, Orthofer R, Lamuela-Raventós RM. 1998. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods in Enzymology* 299:152–178. DOI 10.1016/S0076-6879(99)99017-1.

Steinberg CEW. 2003. Ecology of humic substances in freshwaters: determinants from geochemistry to ecological niches. Berlin, New York: Springer.

Steinberg CEW, Paul A, Pflugmacher S, Meinelt T, Klöcking R, Wiegand C. 2003. Pure humic substances have the potential to act as xenobiotic chemicals – a review. *Fresenius Environmental Bulletin* 12:391–401.

Stevenson FJ. 1994. Humus chemistry – genesis, composition, reactions, New York: John Wiley, 2nd ed.

Tank JIL, Rosi-Marshall EJE, Griffiths NA, Entrekin SA, Stephen ML. 2010. A review of allochthonous organic matter dynamics and metabolism in streams. *Journal of the North American Benthological Society* 29:118–146. DOI 10.1899/08-170.1.

Teisseire H, Couderchet M, Vernet G. 1998. Toxic responses and catalase activity of Lemna minor L. exposed to folpet, copper, and their combination. *Ecotoxicology and Environmental Safety* 40:194–200. DOI 10.1006/eesa.1998.1682.

Teisseire H, Guy V. 2000. Copper-induced changes in antioxidant enzymes activities in fronds of duckweed (Lemna minor). *Plant Science* 153:65–72. DOI 10.1016/S0168-9452(99)00257-5.

Thomas G, Stärk HJ, Wellenreuther G, Dickinson BC, Küpper H. 2013. Effects of nanomolar copper on water plants – comparison of biochemical and biophysical mechanisms of deficiency and sublethal toxicity under environmentally relevant conditions. *Aquatic Toxicology* 140–141:27–36. DOI 10.1016/j.aquatox.2013.05.008.

Upadhyaya A, Sankhla D, Davis TD, Sankhla N, Smith BN. 1985. Effect of paclobutrazol on the activities of some enzymes of activated oxygen metabolism and lipid peroxidation in senescing soybean leaves. *Journal of Plant Physiology* 121:453–461.

US Environmental Protection Agency. 2012. Ecological effects test guidelines. Aquatic plant toxicity test using Lemna spp. Report OCSP 850.4400.

Wang H, Shan X, Wen B, Zhang S, Wang Z. 2004. Responses of antioxidative enzymes to accumulation of copper in a copper hyperaccumulator of Commoelina communis. *Archives of Environmental Contamination and Toxicology* 47:185–192. DOI 10.1007/s00244-004-2222-2.

Wang W. 1990. Literature review on duckweed toxicity testing. *Environmental Research* 52:7–22. DOI 10.1016/S0013-9351(05)80147-1.
Weckx JEJ, Clijsters HMM. 1996. Oxidative damage and defense mechanisms in primary leaves of *Phaseolus vulgaris* as a result of root assimilation of toxic amounts of copper. *Physiologia Plantarum* 96:506–512 DOI 10.1034/j.1399-3054.1996.960322.x.

Wetzel RG. 2001. Limnology: lake and river ecosystems. San Diego, California: Academic Press, 3rd ed.

Xia J and Tian Q. 2009. Early stage toxicity of excess copper to photosystem II of *Chlorella pyrenoidosa* – OJIP chlorophyll a fluorescence analysis. *Journal of Environmental Sciences* 21:1569–1574 DOI 10.1016/S1001-0742(08)62457-2.

Xiang C, Oliver DJ. 1998. Glutathione metabolic genes coordinately respond to heavy metals and jasmonic acid in *Arabidopsis*. *Plant Cell* 10:1539–50 DOI 10.1105/tpc.10.9.1539.

Xing W, Huang W, Liu G. 2010. Effect of excess iron and copper on physiology of aquatic plant *Spirodela polyrrhiza* (L.) schleid. *Environmental Toxicology* 25:103–112 DOI 10.1002/tox.20480.

Yamasaki H, Sakihama Y, Ikehara N. 1997. Flavonoid-peroxidase reaction as a detoxification mechanism of plant cells against H$_2$O$_2$. *Plant Physiology* 115:1405–1412 DOI 10.1104/pp.115.4.1405.

Yüzügüllü Y, Ögel ZB, Bölükbaşı UB, Çoruh N, Karakaş G. 2011. Production of a novel bifunctional catalase-phenol oxidase of *Scytalidium thermophilum* in the presence of phenolic compounds. *Turkish Journal of Biology* 35:697–704 DOI 10.3906/biy-1009-105.

Zezulka Š, Kummerová M, Babula P, Váňová L. 2013. *Lemna minor* exposed to fluoranthene: growth, biochemical, physiological and histochemical changes. *Aquatic Toxicology* 140–141:37–47 DOI 10.1016/j.aquatox.2013.05.011.
Figure 1

Frond and root growth of *L. minor* exposed to copper, leaf litter extracts (LLE) of *Alnus glutinosa* or *Acer negundo*, and combinations of Cu and LLE.

Frond (A) and root (B) growth rates of *L. minor* in control (C) and exposed for seven days with 100μM CuSO$_4$ (Cu), mixtures of Cu and *A. glutinosa* leaf litter extracts (LLE) of 10 and 100mg/L DOC (Cu+10-DOC$_{AG}$ and Cu+100-DOC$_{AG}$), mixtures of Cu and *A. negundo* LLE of 10 and 100mg/L DOC (Cu+10-DOC$_{AN}$ and Cu+100-DOC$_{AN}$) as well as with 100mg/L DOC of *A. glutinosa* (100-DOC$_{AG}$) and *A. negundo* (100-DOC$_{AN}$) LLE. Data represent mean±SD (n = 8). Asterisks indicate significant difference from the control, different letters indicate significant difference among the means (α = 0.05).
Figure 2

Cu accumulation in *L. minor* treated by Cu, and combinations of Cu and leaf litter extracts from *A. glutinosa* or *A. negundo*

Cu concentration in *L. minor* treated by Cu and Cu+DOC of leaf litter extracts (μg g⁻¹ FW). Plants were incubated in control medium (■), 100 μM Cu (●), 100 μM Cu+10 mg/L DOC (▼), 100 μM Cu + 100 mg/L DOC (▲) from *A. glutinosa* or *A. negundo*. Each value represents mean ± SD (n = 8). Asterisks indicate significant difference from the control at α = 0.05.
Figure 3

Concentration of hydrogen peroxide in *L. minor* treated by Cu, leaf litter extracts and Cu + leaf litter extracts.

Concentration of H$_2$O$_2$ in *L.minor* treated by Cu, DOC or combinations of Cu and DOC of corresponding leaf litter extracts. Plants were incubated in control medium (■), 100 µM Cu (●), 100 mg/L DOC from *A. glutinosa* or *A.negundo* (○), and Cu+10 mg/L DOC (▼) or Cu + 100 mg/L DOC (▲). Each value represents mean ± SD (n = 8). Asterisks indicate significant difference from the control at α = 0.05.
Figure 4

Lipid peroxidation expressed as MDA concentration in *L. minor* treated by Cu, leaf litter extract of *A. glutinosa* or *A. negundo*, and combinations of Cu and corresponding extracts.

Lipid peroxidation expressed as MDA concentration in *L. minor* treated by Cu, DOC or combinations of Cu and DOC from *A. glutinosa* or *A. negundo* leaf litter extracts. Plants were incubated in control medium (■), 100 µM Cu (●), 100 mg/L DOC (○), and Cu + 10 mg/L DOC (▼) or Cu + 100 mg/L DOC (▲). Each value represents mean ± SD (n = 8). Asterisks indicate significant difference from the control at α = 0.05.
Figure 5

Oxidative stress enzyme activities in *L. minor* treated by Cu, leaf litter extract from *A. glutinosa* or *A. negundo*, and combinations of Cu and respective extracts

Activities of catalase (CAT), guaiacol peroxidase (POD), ascorbate peroxidase (APX) and glutathione reductase (GR) in *L. minor* treated by Cu, DOC of leaf litter extracts from *A. glutinosa* or *A. negundo*, and combinations of Cu and the DOC of respective extract. Plants were incubated in control medium (■), 100 µM Cu (●), 100 mg/L DOC (○), Cu+10 mg/L DOC (▼) or Cu + 100 mg/L DOC (▲). Each value represents mean ± SD (n = 8). Asterisks indicate significant difference from the control at α = 0.05.
Manuscript to be reviewed