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Response of *Lemna minor* L. to short-term cobalt exposure: The effect on photosynthetic electron transport chain and induction of oxidative damage

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ABSTRACT

The effect of two concentrations of cobalt (Co^{2+}) on photosynthetic activity and antioxidative response in *Lemna minor* L. were assessed 24, 48 and 72 h after the start of the exposure. Higher concentration of cobalt (1 mM) induced growth inhibition while lower concentration (0.01 mM) increased photosynthetic pigments content. Analysis of chlorophyll *a* fluorescence transients revealed high sensitivity of photosystem II primary photochemistry to excess of Co^{2+} especially at the higher concentration where decreased electron transport beyond primary quinone acceptor Q_A^- and impaired function of oxygen evolving complex (OEC) was observed. Due to impairment of OEC, oxygen production was decreased at higher Co^{2+} concentration. Activity of superoxide dismutase was mainly inhibited while lipid peroxidation increased, at both concentrations, indicating that cobalt-induced oxidative damage after short exposure and moreover, susceptibility of the membranes in the cell to cobalt toxicity. Results obtained in this study suggest possible application of used parameters as tools in assessment of early damage caused by metals.

1. Introduction

Excess uptake of metals, regardless of plant requirements, results in toxic effects. According to the report from the 2012 (EBRC and ARCHE, 2012) emission of cobalt in Europe to surface water was 90895.9 kg Co/year. Cobalt pollution can originate from anthropogenic sources through wastewater from industry, manufacture, processing, use and disposal of cobalt-containing products thus increasing environmental concentration constantly. The mean cobalt concentrations in rivers are ranging from 0.1 to 1.1 $\mu g\,L^{-1}$ (0.0017 to 0.019 $\mu mol\,L^{-1}$) while samples collected from surface water and groundwater near mining in USA contained cobalt concentrations from <1 to 625 000 $\mu g\,L^{-1}$ (<0.017 to 0.01 $\mu mol\,L^{-1}$) (ATSDR, 2004).

Cobalt, as one of the major micronutrients in plants, is involved in the growth and metabolism (Palit et al., 1994). Mechanism of uptake and distribution of Co^{2+} in plants is species-dependent and differently regulated (Bakkaus et al., 2005). Toxic effect of cobalt was investigated on *Lemna minor* L. (Ince et al., 1999; Sree et al., 2015) as well as other plant species such as barley, oilseed rape and tomato (Nagajyoti et al., 2010). Results showed that Co^{2+} influences growth and biomass, chlorophyll content, starch accumulation, decreases photosynthetic efficiency, water potential and transpiration rate. Cobalt can displace Fe, Mn, Zn and Cu from physiologically important binding sites and thus might decrease uptake and translocation of essential micronutrients (Chatterjee and Chatterjee, 2000) and disable photochemical activity of the metal-containing pigments (Drzewiecka-Matuszek et al., 2005).

The photosystem II (PSII) and its component, the oxygen evolving complex (OEC), are the most sensitive to stress factors and are frequently used for evaluation of stress response in plants (Plekhanov and Chemeris, 2003). Capability of plants for adequate response when exposed to metal toxicity can be assessed by

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variable chlorophyll *a* fluorescence (Appenroth et al., 2001; Joshi and Mohanty, 2004). Mohanty et al. (1989) proposed mechanism of cobalt action on Q_B function leading to the impairment of PSII activity at the Q_B site. Recent reports showed that beside the possibility to impair PSII functionality and electron transport, metals can also influence OEC functionality (Belatik et al., 2013; Hasni et al., 2013).

As a response to metal toxicity, plants activate their defense mechanisms to cope with enhanced reactive oxygen species (ROS) production which enables them to prevent or diminish further damage. Plants have an antioxidant defense system which includes numerous enzymatic and non-enzymatic components. Superoxide dismutase (SOD) plays important role in antioxidative defense catalyzing dismutation of superoxide (O_2^-) to hydrogen peroxide (H_2O_2) and oxygen (O_2), therefore eliminating negative impact of ROS on the cell components and tissues. Metals are known to induce damage to cell membranes by lipid peroxidation, changing their structure and function. Lipid peroxidation is considered a reflection of oxidative stress in organism (Prasad, 2004).

Lemna minor L. can be found in numerous aquatic habitats and therefore represents a good biological indicator for detection of metal pollution. It is also frequently used as a model plant for studying physiological response to metal toxicity (Naumann et al., 2007; Panda, 2008; Razinger et al., 2008; Vidaković-Cifrek et al., 2015).

Previous studies have suggested that cobalt induces damage to the components of the photosynthetic apparatus hence influencing overall photosynthetic performance of the plant as well as activates SOD and elevates lipid peroxidation levels as a result of oxidative stress.

The aim of the present study was to investigate the early targets of cobalt toxicity by exploring concentration-dependent effect and changes induced in photosynthetic apparatus in *L. minor* along with possible induction of the oxidative stress. We used chlorophyll *a* fluorescence and oxygen evolution measurement to assess photosynthetic performance, measured activity of superoxide dismutase (SOD) and level of thiobarbituric acid reactive substances (TBARS) as a marker of lipid peroxidation.

2. Materials and methods

2.1. Plant material and cobalt treatment

Lemna minor L. plants were originally collected in the Botanical Garden of the Department of Biology, Faculty of Science, University of Zagreb in 1996 and sterilized according to Krajnčič and Devidé (1980). Stock cultures were grown under axenic conditions on the modified Pirson–Seidel's nutrient solution containing 3.95 mmol L^{-1} KNO_3, 5.46 mmol L^{-1} $CaCl2 \times 2H_2O$, 1.47 mmol L^{-1} KH_2PO_4, 1.21 mmol L^{-1} $MgSO4 \times 7H_2O$, 49 μmol L^{-1} Na_2EDTA x $2H_2O$, 20 μmol L^{-1} Fe-citrate, 1.5 μmol L–1 $MnCl2 \times 4H_2O$, 8.1 μmol L^{-1} H_3BO_3, 29.2 mmol L^{-1} sucrose and 0.66 mmol L^{-1} asparagine at pH 4.55 (Pirson and Seidel, 1950). Stock cultures were generous gift from professor Vidaković-Cifrek (Faculty of Science, University of Zagreb, Croatia) and are maintained since 2006 in our laboratory.

For cobalt toxicity assessment, 8–11 colonies were inoculated in Erlenmeyer flasks filled with 70 mL of nutrient solution supplemented with $CoCl_2$ in the final concentration of 0.01 mM and 1 mM. Control plants were grown on modified Pirson–Seidel's nutrient solution. Plants, both control and treated, were maintained in growth chamber at 16/8 light/dark regime under white fluorescent lamps (Osram, Germany) with ~60 μmol photons m^{-2} s^{-1} at 22 ± 2 °C (Babić et al., 2009). For experimental purposes, colonies were harvested 24, 48 and 72 h after the start of the treatment.

2.2. Determination of mineral concentrations by ICP-OES

All samples were washed thoroughly with deionized water and then dried to constant weight at 60 °C. The dried samples were ground into a fine powder using a heavy metal-free ultra-centrifugal mill. All plant samples for measuring mineral concentrations were digested with 10 mL of a 5:1 mixture of HNO_3 and H_2O_2 at 180 °C for 60 min in microwave oven (CEM Mars 6). The concentrations of minerals in solutions of digested plant samples were determined by inductively-coupled plasma optical emission spectroscopy, ICP-OES (PerkinElmer Optima 2100 DV). The whole series of plant samples (consisted of four replicates) were analyzed with an internal pooled plasma control and with the reference material prepared in the same way as the other samples. All samples were prepared and analyzed in duplicate.

2.3. Growth rate

Plant growth, expressed as growth rate, was monitored 24, 48 and 72 h after inoculation by counting all visible fronds in control and treated plants. Growth rate (GR) was calculated by using formula $GR = lnN_{ti}$- $lnN_{t0}/t_i - t_0$ where N_{t0} represents frond number at the beginning of the experiment (t_0) and N_{ti} represents frond number 24, 48 and 72 h after the start of the exposure (t_i). Doubling time of frond number (T_d) was calculated for 72 h using the equation $T_d = \ln 2/GR$ (Vidaković-Cifrek et al., 2013). To evaluate the effect of 0.01 mM and 1 mM Co^{2+} on growth, percent inhibition of growth rate ($\%I_r$) was calculated using equation $\%I_r = (GR_C - GR_T)/GR_C$ x 100 where GR_C represents average values of growth rate in control and GR_T represents average values of growth rate in treated plants.

2.4. Determination of photosynthetic pigment content

Fronds and roots were collected and homogenized in absolute acetone followed by extraction for 15 min at +4 °C and centrifugation for 10 min at 3000 rpm. Procedure was repeated until the plant material was completely uncolored. The chlorophyll and carotenoid content was determined spectrophotometrically. Concentrations of total chlorophylls (Chl *a* + *b*), chlorophyll *a* to chlorophyll *b* ratio (Chl *a*/*b*) and carotenoids (Car) were calculated according to Lichtenthaler (1987).

2.5. Fast chlorophyll a fluorescence measurement

Fast chlorophyll *a* fluorescence transients of randomly selected control and treated *L. minor* fronds were recorded with Plant Efficiency Analyzer (Handy PEA Hansatech Instruments Ltd. Norfolk, UK). All measurements were performed on fully dark-adapted fronds at room temperature. After 30 min of dark adaptation, the fronds were exposed to a pulse of saturating red light (3200 μmol$m^{-2}s^{-1}$, peak at 650 nm) and recorded fluorescence transients were analyzed using JIP test. The JIP test represents translation of the original recorded data to biophysical parameters that quantify the stepwise energy flow through PSII (Strasser et al., 2000, 2004). In normal physiological conditions, fluorescence transient goes from initial O (F_0) to maximal P (F_m) fluorescence intensity and it includes two intermediate steps, J step at approximately 2 ms and I step at approximately 30 ms.

In order to compare recorded OJIP transients two types of normalization were used. Double normalization between O and P steps was used for calculation of relative variable fluorescence $W_{OP} = (F_t\text{-}F_0)/(F_P\text{-}F_0)$ and it is a measure of reduced Q_A fraction. Normalization between the steps O (50 μs) and J (2 ms) revealed variable fluorescence $W_{OJ} = (F_t\text{-}F_0)/(F_J\text{-}F_0)$ named K-band (300 μs). It was given as variable fluorescence and plotted with difference

kinetics $\Delta W_{OJ} = W_{OJ}-(W_{OJ})_{ref}$ in the 50 μs – 2 ms time range. The values measured for *L. minor* plants without Co^{2+} treatment 24 h, 48 h and 72 h after the start of the exposure (Control) were used as referent value $(W_{OJ})_{ref}$ (Strasser et al., 2004; Yusuf et al., 2010). Recorded data were: F_0 (fluorescence intensity at 50 μs), F_m (maximal fluorescence intensity), F_{300} (fluorescence intensity at 300 μs), F_I (fluorescence intensity at 2 ms), F_J (fluorescence intensity at 30 ms) and t_{max} (time needed to reach F_m). JIP test parameters calculated from the recorded values were: the maximum quantum yield of PSII (TR_0/ABS), the probability that trapped exciton moves an electron further than Q_A (ET_0/TR_0), the probability that absorbed photon moves an electron further than Q_A^- (ET_0/ABS), the absorption flux per active reaction center (ABS/RC), the trapping flux per active RC (TR_0/RC), the electron transport flux per active RC (ET_0/RC), the dissipation flux per active RC (DI_0/RC). The performance index (PI_{ABS}) was calculated using the density of RC on chlorophyll *a* basis (RC/ABS), the flux ratio of trapping per dissipation (TR_0/DI_0) and the electron transport beyond Q_A^- ($ET_0/(TR_0-ET_0)$). Detailed formulae and definitions of the used JIP-test parameters, according to Strasser et al. (2004) are shown in Table S1, Supplementary information.

2.6. Oxygen evolution measurement

Oxygen evolution was measured using the liquid-phase Clark-type oxygen electrode (Hansatech, UK) at 60 μmol photons m^{-2} s^{-1}. All measurements were performed at constant temperature (25 °C). *L. minor* plants were placed in a chamber filled with bicarbonate buffer described by Prasad et al. (2001).

2.7. Measurement of superoxide dismutase activity

Measurement of superoxide dismutase activity (SOD, EC 1.15.1.1) was carried out according to Giannopolitis and Ries (1977) with modifications described by Lepeduš et al. (2011). Fronds and roots were ground using mortar and pestle in liquid nitrogen and approximately 200 mg of powdered tissue was used. One unit of SOD activity was defined as the amount of enzyme that inhibits the NBT photoreduction by 50%. Protein content in extracts was determined according to Bradford (1976) using bovine serum albumin as a standard.

2.8. Measurement of lipid peroxidation level

Plant material, fronds and roots, was ground using mortar and pestle in liquid nitrogen and about two hundred milligrams of fine powder was used for analyses. The level of lipid peroxidation was determined as described by Verma and Dubey (2003) with modifications according to Lepeduš et al. (2011). The concentration of lipid peroxidation products were expressed as total thiobarbituric acid reactive substances level (TBARS) in nmol per g of fresh weight using an extinction coefficient of 155 mM^{-1} cm^{-1}.

2.9. Statistical analysis

Statistical analyses were performed between control and treated plants using one-way analysis of variance (ANOVA) followed by post hoc LSD test for each exposure time separately, if not stated otherwise. Results were expressed as arithmetic means of 9 replicates from three experiments ± standard deviation (SD) except for measurement of chlorophyll *a* fluorescence when 50 replicates were used and ICP-OES analysis when eight replicates were used. Different letters (a, b, c) represent statistical difference at $P \leq 0.05$. All statistical analyses were conducted with Statistica 12.5 Software (StatSoft Inc., USA).

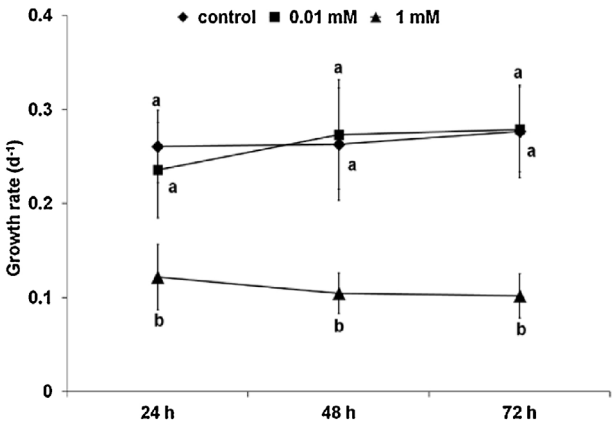

Fig. 1. Growth rate of *L. minor* 24, 48 and 72 h after the start of the exposure to 0.01 mM and 1 mM $CoCl_2$. Analyses by ANOVA showed significant differences between control and treated plants within each exposure time. Values are represented as means ± SD (n = 9).

3. Results and discussion

3.1. Cobalt content and influence on growth rate in L. minor

Results of cobalt content determination (Table 1) showed significant difference between control and treated plants and between two cobalt concentrations (0.01 and 1 mM). In vascular plants, growing in unpolluted freshwater, mean cobalt concentrations are 0.32 – 0.37 μg g^{-1} dry weight (0.005–0.006 μmol g^{-1} dry weight) (Outridge and Noller, 1991). These results are in concordance with measured Co^{2+} concentrations in control plants (Table 1). In plants treated with $CoCl_2$ significant increase in accumulation, up to 10^4-fold higher than in the control, approximately 3500 μg g^{-1} dry weight (58.7 μmol g^{-1} dry weight), can be observed 72 h after the start of the exposure to the higher concentration (1 mM) while at the lower concentration (0.01 mM) cobalt content was about 200-fold higher than in the control, 45.45 μg g^{-1} dry weight (0.771 μmol g^{-1} dry weight). In Sree et al. (2015) accumulation rate and consequent cobalt content was much higher. The authors reported approximately 900 μg g^{-1} fresh weight of cobalt content after 72 h and 21 mg g^{-1} dry weight was measured after 7 days in *L. minor* treated with the same Co^{2+} concentration as we used (0.01 mM).

Further, the authors reported on significant decrease in relative growth rate after seven days, up to 56% in plants treated with 0.01 mM and 93% in plants treated with 0.1 mM Co^{2+} (Sree et al., 2015). In our study the earliest influence of Co^{2+} on growth was observed 24 h after the start of the exposure, but only at higher concentration (1 mM) which caused inhibition of relative growth rate ($\%I_r$) by 53% compared to control. This effect was also seen 72 h after the start of the exposure when significant inhibition (63%) of relative growth rate (0.102 d^{-1}) with doubling time of 7.14 was observed (Fig. 1). Lower cobalt concentration (0.01 mM) did not influence relative growth rate (0.279 d^{-1}) at any exposure time as compared to control (Fig. 1). Doubling time at the lower concentration for 72 h was 2.55. In control plants relative growth rate for 72 h was 0.277 d^{-1} with a doubling time of 2.59.

Ince et al. (1999) showed that higher concentrations of Co^{2+} inhibited growth in *L. minor* while lower Co^{2+} concentration stimulated growth. Stimulating effect of cobalt on growth rate was also observed in algae (El-Sheekh et al., 2003; Horvatić and Peršić, 2007; Osman et al., 2004) and sweet pepper (Gad and Hassan, 2013). However, cobalt-induced stimulation of growth was not observed in our study.

Table 1
Cobalt and manganese content (μg g^{-1} dry weight ± SD) in *L. minor* plants 24, 48 and 72 h after the start of the exposure to 0.01 and 1 mM concentration of $CoCl_2$. Student' s *t*-test was used to analyze statistical difference between control and treated plants (n = 8).

	Co^{2+}			Mn^{2+}		
	24 h	48 h	72 h	24 h	48 h	72 h
Control	0.15 ± 0.03 (0.003 ± 0.001)	0.25 ± 0.08 (0.004 ± 0.001)	0.38 ± 0.10 (0.007 ± 0.002)	87.33 ± 8.59 (1.592 ± 0.137)	91.90 ± 12.09 (1.676 ± 0.220)	94.23 ± 1.49 (1.718 ± 0.082)
0.01 mM	40.11 ± 3.41* (0.68 ± 0.058)	52.03 ± 1.97* (0.883 ± 0.033)	45.45 ± 7.63* (0.771 ± 0.129)	80.25 ± 3.25* (1.463 ± 0.059)	86.87 ± 11.97* (1.584 ± 0.218)	82.19 ± 7.75* (1.498 ± 0.141)
1 mM	2037.00 ± 11.31* (34.566 ± 3.908)	2646.30 ± 467.74* (44.905 ± 7.937)	3459.00 ± 202.78* (58.697 ± 3.441)	63.10 ± 6.69* (1.150 ± 0.122)	58.79 ± 11.70* (1.072 ± 0.213)	41.15 ± 3.16* (0.750 ± 0.112)

Asterisks indicate values that are significantly different from control plants ($P \leq 0.05$). Values in parenthesis are expressed as (μmol g^{-1} dry weight ± SD).

Differences, such as cobalt content and effect on growth could be due to different mechanisms that algae and different plant species use to absorb ions. In addition, different *L. minor* clones, culture medium and growth conditions used in previous studies (Ince et al., 1999; Sree et al., 2015) might have affected the accumulation rate of cobalt and caused difference in the effect of cobalt on growth. Also, prolonged exposure time must not be overlooked.

3.2. Influence of cobalt on photosynthetic pigments and mineral content

Morphological changes, like chlorosis, are usually associated with metal toxicity (Assche and Clijsters, 1990; Chatterjee and Chatterjee, 2003; Khan and Khan, 2010; Tkalec et al., 2008). As described in the paper by Sree et al. (2015) younger fronds of *L. minor*, as opposed to mother fronds, showed chlorotic or necrotic features after exposure to 0.01 mM of Co^{2+}, while at higher concentration (0.1 mM) mother fronds were more prone to the damage than younger ones since their growth was evidently inhibited. In our study, after exposure to 0.01 mM and 1 mM of Co^{2+}, plants did not show visible morphological changes indicating that longer exposure time is needed to induce such changes or, as mentioned before, it could be because of the different cobalt content measured in plants upon exposure to 0.01 mM of Co^{2+}. Previous research has shown that cobalt can inhibit or induce synthesis of photosynthetic pigments depending on the concentration. Significant increase in total chlorophyll and carotenoid content was observed 24, 48 and 72 h after the start of treatment with 0.01 mM concentration compared to control plants (Fig. 2A and B). Increase in the ratio of chlorophyll *a* and *b* was also observed but only 24 h after treatment with 0.01 mM concentration of Co^{2+} (Fig. 2C). In the study by Sree et al. (2015) chlorophyll and carotenoid content did not change after four days of exposure to 0.01 mM Co^{2+}. However, our results showed that lower (0.01 mM) concentration of Co^{2+} stimulated production of chlorophylls and carotenoids, which could be associated with the absence of chlorosis in treated plants.

Stimulation of chlorophyll production was also reported by Csartoday et al. (1984) in algae *Anacystis nidulans*, Zeid (2001) in bean and Tewari et al. (2002) in mung bean. Authors suggested that increase of chlorophyll content is a result of enhanced accumulation of protoporphyrin XI, one of the precursors in chlorophyll biosynthesis.

On the other hand, some earlier studies have reported decreased chlorophyll content, when higher concentrations of cobalt were applied, as a result of the inhibitory effect of cobalt on chlorophyll synthesis (Chatterjee and Chatterjee, 2000; Csatorday et al., 1984; Tewari et al., 2002). In our study, higher concentration of Co^{2+} did not decrease total chlorophyll and carotenoid content when compared to control plants (Fig. 2A and B). Therefore, to achieve inhibition of chlorophyll and carotenoid synthesis a longer period of exposure is required as demonstrated by Sree et al. (2015) whose treatment with 0.01 mM and 0.1 mM Co^{2+} induced a decrease after seven days.

Zn content was not affected by cobalt treatment (Table S2). Analyses of Fe content revealed differences between control and treated plants. Results of Sree et al. (2015) showed that Fe content did not change in *L. minor* plants upon exposure especially to 0.01 mM Co^{2+} after 1 and 7 days. Although in our study Fe content was not significantly different between control and 0.01 Co^{2+} after 24 h, significant reduction of Fe content was observed 72 h after the start of the exposure to both concentrations of cobalt (Table S2). The main difference between our results and those of Sree et al. (2015) is due to the fact that in our control plants Fe content continuously increased for 72 h, while in the study by Sree et al. (2015) this trend was absent. As discussed previously, composition of nutrient medium,

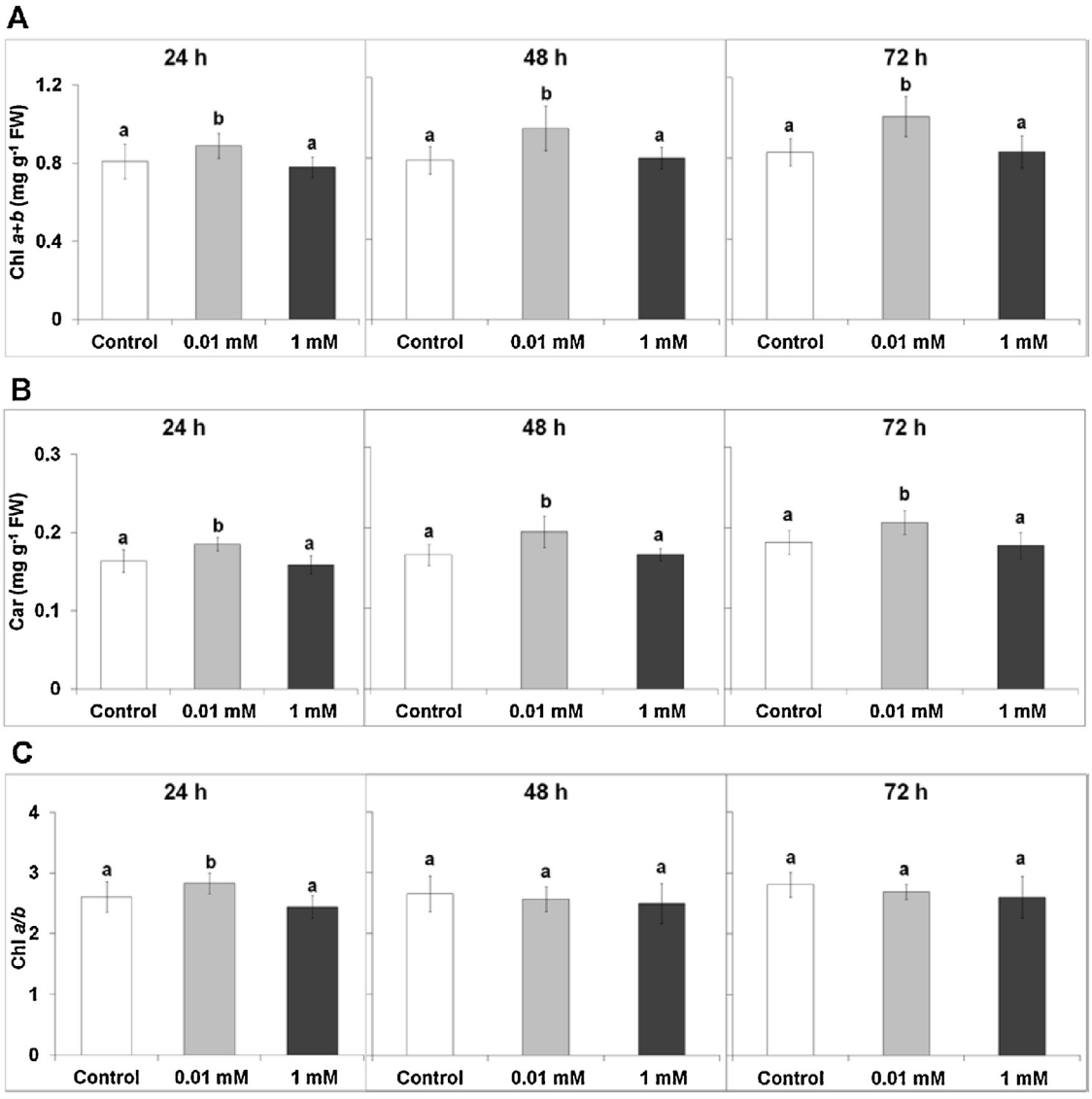

Fig. 2. Total chlorophyll content, Chl *a* + *b*, (A), carotenoid content, Car (B), and chlorophyll *a* to *b* ratio, Chl *a*/*b* (C) in *L. minor* 24, 48 and 72 h after the start of the exposure to 0.01 mM and 1 mM concentrations of Co^{2+}. Significant differences between control and treated plants were designated by different letters (ANOVA, $P \leq 0.05$). Values are represented as means ± SD (n = 9).

light intensity as well as different clones could have influenced Fe content.

Mg content in *L. minor* was not affected by treatment with 0.01 mM Co^{2+} whereas the higher concentration caused a reduction already 24 h after the start of the exposure and the decrease was observed up to 72 h (Table S2). We can assume that over the time Co^{2+} would inhibit the chlorophyll formation by disabling incorporation of Mg, confirming the suggestion by Sree et al. (2015) and Shalygo et al. (1999)

3.3. Fast chlorophyll a fluorescence transients after short exposure to Co^{2+}

Detail analysis of fast chlorophyll *a* fluorescence transients showed changes in functioning of photosynthetic apparatus of *L. minor* plants exposed to cobalt. Difference could be observed between treated and untreated plants, as well as between plants treated with different concentrations of cobalt starting from 24 h onwards. Both control and treated plants had typical chlorophyll *a* fluorescence rise kinetics consisting of clearly visible O, J, I and P steps indicating the ability for photosynthesis upon short exposure to cobalt.

In order to compare the effect of two concentrations of Co^{2+} we plotted the curves as a relative variable fluorescence derived from double O-P normalized transients ($W_{OP} = (F_t\text{-}F_0)/(F_P\text{-}F_0)$). The results showed that 1 mM concentration increased J and I step in treated plants whereas 0.01 mM concentration decreased them when compared to control. The observed changes were statistically significant (Fig. 3A-C). Rise of V_J (Table S3, Supplementary information) suggests an increase of the proportion of closed PSII RCs and of the proportion of reduced Q_A at J step. The increase of V_I (Table S3, Supplementary information) indicates accumulation of the reduced Q_A and plastoquinone which cannot transfer electrons further than Q_A (Strasser et al., 2004). Similar results were obtained by Pan et al. (2010) in maize treated with antimony (Sb). Lower fluorescence level on J and I step was the result of lower accumulation rate of the reduced Q_A electron acceptor.

Both tested concentrations of cobalt had negative effect on PSII primary photochemistry measured as a decrease of TR_0/ABS (Fig. 4). However, while treatment with the higher cobalt concentration led

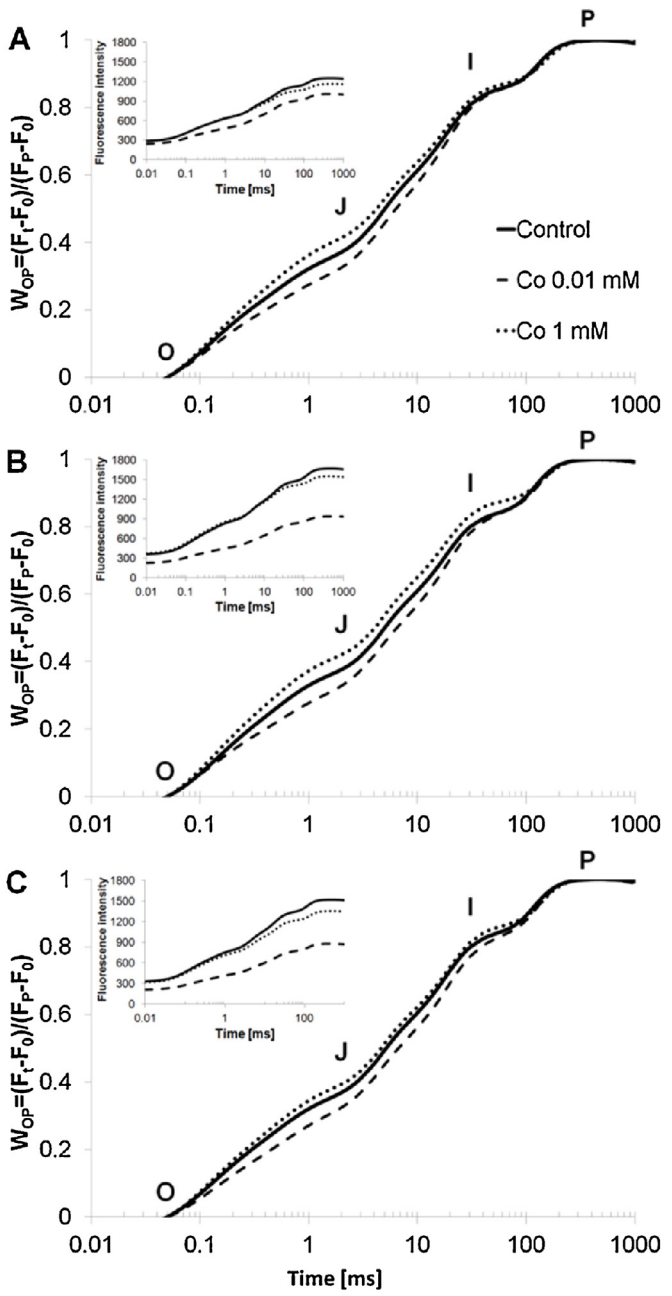

Fig. 3. Chlorophyll *a* fluorescence transients (OJIP) of dark adapted *L. minor* fronds in control plants and plants exposed to 0.01 mM and 1 mM Co^{2+} for 24 (A), 48 (B) and 72 h (C). Results are represented as double, O-P normalized transients ($W_{OP}=(F_t-F_0)/(F_P-F_0)$). Native curves are shown as inserts in each panel. Each curve represents average kinetics of 50 replicates (n = 50). The O, J, I and P steps are marked in the plots.

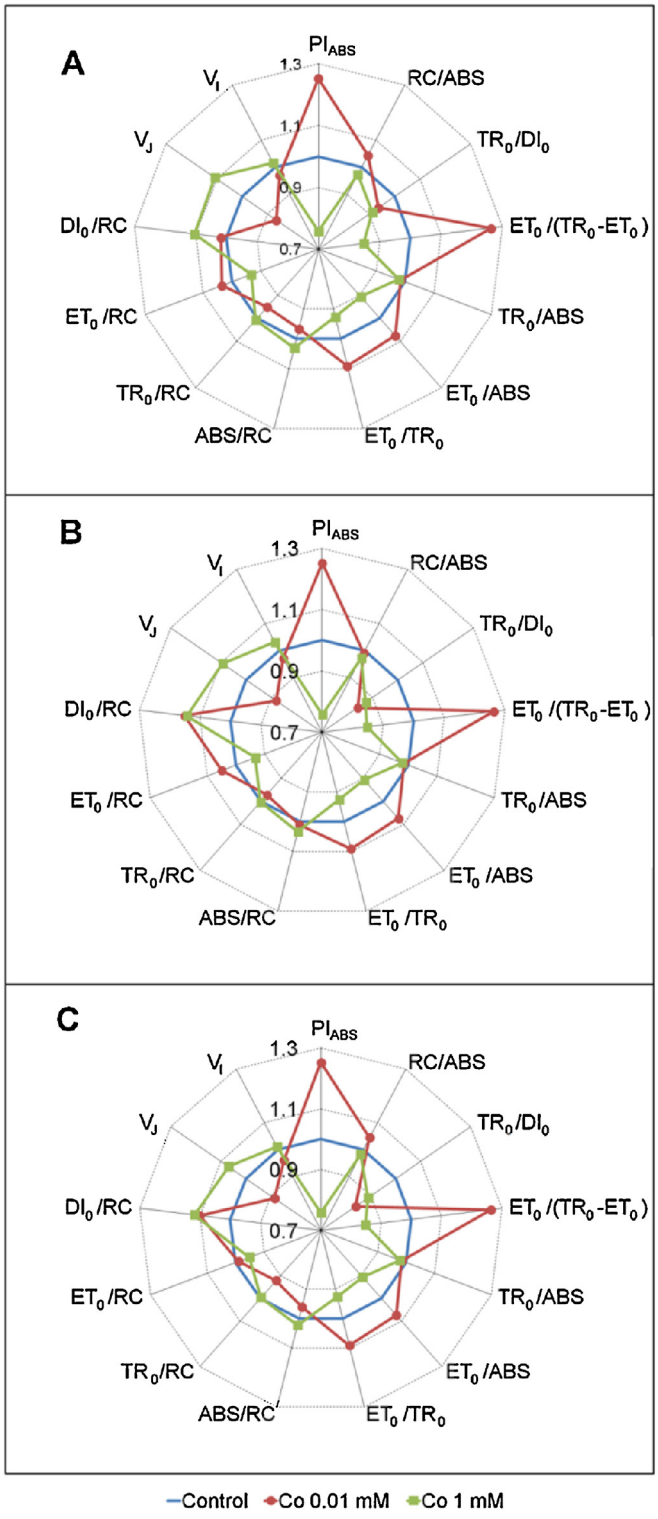

Fig. 4. Photosynthetic parameters determined by the JIP-test analysis of fluorescence transients. Every plot consists of 13 structural and functional photosynthetic parameters (average value of 50 replicates) derived by the JIP-test from the fluorescence transients. Values were normalized using the control treatment (without symbol) as reference. Normalization was obtained for each parameter, 24 (A), 48 (B) and 72 h (C) after the start of Co^{2+} treatment. Statistical analysis of presented parameters is shown in Table S3.

to decreased overall photosynthetic efficiency expressed as PI_{ABS}, treatment with the lower cobalt concentration had the opposite effect inducing an increase of PI_{ABS} value (Fig. 4).

PI_{ABS} is calculated as the product of three parameters: RC/ABS, TR_0/DI_0 and $ET_0/(TR_0-ET_0)$. Changes of $ET_0/(TR_0-ET_0)$ were shown to be the main reason for the observed differences in PI_{ABS}. Comparison of several other parameters describing the efficiency of electron transport such as ET_0/TR_0 or ET_0/ABS were decreased upon 1 mM cobalt treatment and increased upon 0.01 mM cobalt treatment. This indicated that differential effect of tested cobalt concentration on photosynthesis of *L. minor* is associated with effect on electron transport from reduced Q_A^- further downstream

(Fig. 4). Similar results were obtained by Perreault et al. (2014) who observed a biphasic response in electron transport in plants exposed to cooper. This conclusion is further supported by analysis of specific energy fluxes describing the distribution of energy through PSII at the reaction center level. The lower concentration of Co^{2+} decreased the value which expresses the rate by which an exciton is trapped by the RC resulting in the reduction of Q_A to Q_A^- ratio (TR_0/RC) but increased reoxidation of reduced Q_A^- via electron transport in an active RC (ET_0/RC) at 24 and 48 h (Table S3, Supplementary information). Also, lower concentration of Co^{2+} negatively affected antenna size of active reaction centers 24 and 72 h after the start of the exposure causing a decrease in ABS/RC ratio (Table S3, Supplementary information). The ABS/RC value expresses the total absorption of PSII antenna chlorophylls divided by the number of active (in the sense of Q_A reducing) reaction centers. Thus, it is influenced by the ratio of active/inactive RCs (Falqueto et al., 2013). An increase in ABS/RC induced by high Co^{2+} concentration could be due to inactivation of RCs that are being transferred to non-Q_A-reducing centers and/or due to increase in functional antenna size (Yusuf et al., 2010). Since the increase in ABS/RC in *L. minor* at 1 mM of Co^{2+} 24 h after the start of the exposure was accompanied by a decrease in TR_0/ABS (Fig. 4), but TR_0/RC (Table S3, Supplementary information) was not affected, it can be assumed that some part of RCs was inactivated. Possible causes of RC inactivation are inactivation of OEC and/or structural alteration of heat sinks (or silent centers) (Yusuf et al., 2010). In our case, both events occurred. Heat sinks are able to dissipate excess of absorbed energy safely as heat instead of utilizing it to reduce Q_A and presence of those is indicated by the increase in DI_0/RC (Table S3, Supplementary information).

These results indicated that exposure of *L. minor* to 0.01 mM concentration could be limited to PSII, at least for the first 72 h of exposure while 1 mM concentration could have more extensive effect on the whole photosynthetic electron transport chain, from donor side of PSII up to the reduction of end acceptor of PSI. Results observed after treatment with higher concentration of cobalt are in agreement with those obtained by Mohanty et al. (1989).

3.4. Influence of cobalt on OEC

Appearance of positive K-band, which is considered to reflect inactivation of OEC (especially of the Mn complex) and/or increase the functional PSII antenna size (Yusuf et al., 2010), has been mainly used as an indicator of drought stress (Oukarroum et al., 2007) and heat stress in plants before visible symptoms appear (Lazár et al., 1997; Srivastava et al., 1997). Results of our investigation showed that K-band could be used as an indicator in the assessment of early stress caused by cobalt, particularly intactness of OEC. Although hidden in OJIP transients, further analyses using data normalized between O and J steps (Fig. 5) revealed visible K-band and confirmed the influence of cobalt on electron transport between OEC and the reaction centres of PSII (Srivastava et al., 1997). Positive K-band in our study appeared at higher concentration (Fig. 5A-C) thus indicating inactivation of OEC by dissociation, thereby enabling electron donation from non-water electron donors to the reaction centres (De Ronde et al., 2004; Tóth et al., 2007).

Reports of cobalt influence on plants shows that cobalt has the ability to displace metal ions from protein complexes as well as reducing their translocation from root to shoot (Chatterjee and Chatterjee, 2000). In studies on green microalgae *Scenedesmus* (Mallick and Mohn, 2003) and poplar (Velikova et al., 2011) exposed to metal stress authors suggested that the metals used replace Mn ion from OEC. Dissociation of a manganese-stabilizing protein, bound to the donor side of the reaction center of PS II and the release of Mn atoms is associated with the loss of manganese cluster (Brestic and Zivcak, 2013) and therefore OEC inactivation. Indeed, analyses of manganese content in treated plants revealed

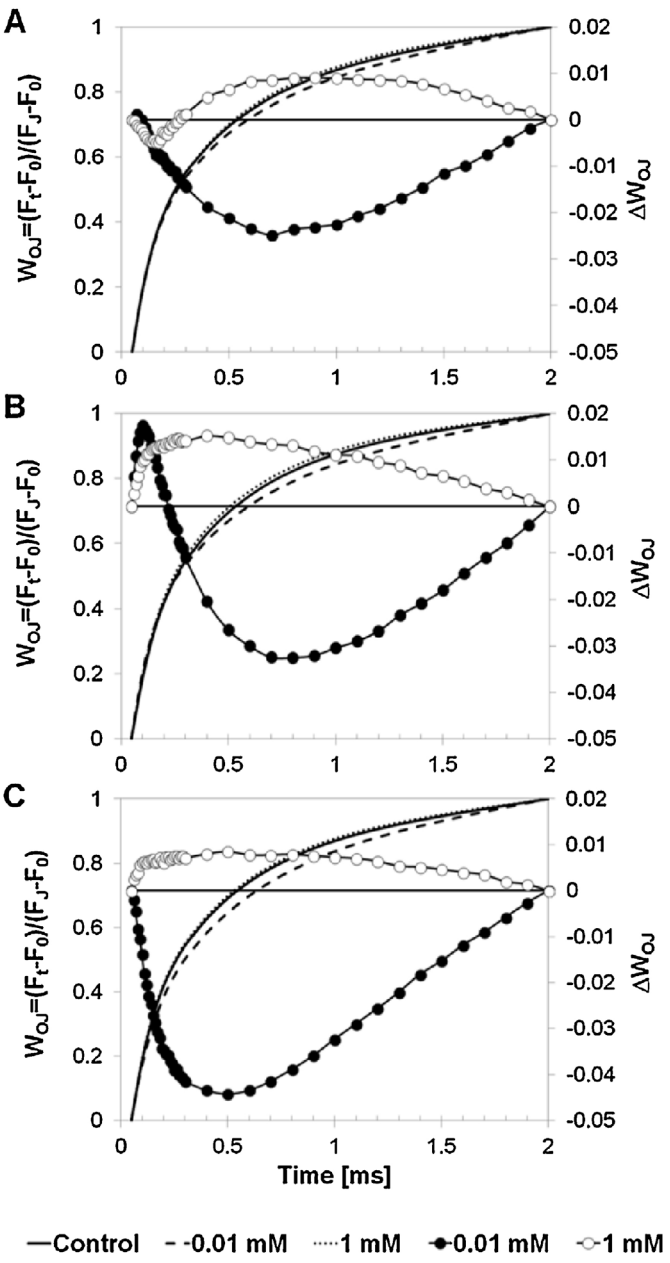

Fig. 5. Changes in the shape of the chlorophyll *a* fluorescence transient curves of *L. minor* exposed to 0.01 mM and 1 mM Co^{2+} 24 (A), 48 (B) and 72 h (C) after the start of exposure. Fluorescence data (average value of 50 replicates) are normalized between O and J steps (K-band) expressed as variable fluorescence $W_{OJ}=(F_t-F_0)/(F_J-F_0)$ and then plotted with difference kinetics $\Delta W_{OJ}=W_{OJ}-(W_{OJ})_{ref}$ in the 50 μs–2 ms time range. Arithmetic mean from values measured in control plants was used as referent value $(W_{OJ})_{ref}$. Lines (solid, dashed and dotted) refer to difference kinetics (ΔW_{OJ}) curves, while solid lines with marks (open and closed) refer to variable kinetics (W_{OJ}) curves.

significant decrease in comparison with control plants confirming the hypothesis of cobalt action on Mn ions (Table 1).

Described limitation on donor side of PSII under the influence of cobalt was not observed when lower cobalt concentration was applied in nutrient solution. On the contrary, occurrence of negative K-band was proved (Fig. 5A-C). Negative K-band has previously been demonstrated as an indicator of better performance of plants under stress conditions (De Ronde et al., 2004; Tewari et al., 2002; Yusuf et al., 2010). In this study it could indicate a mechanism by which the plant is trying to diminish the negative effect of small excess of cobalt caused by 0.01 mM Co^{2+} treatment.

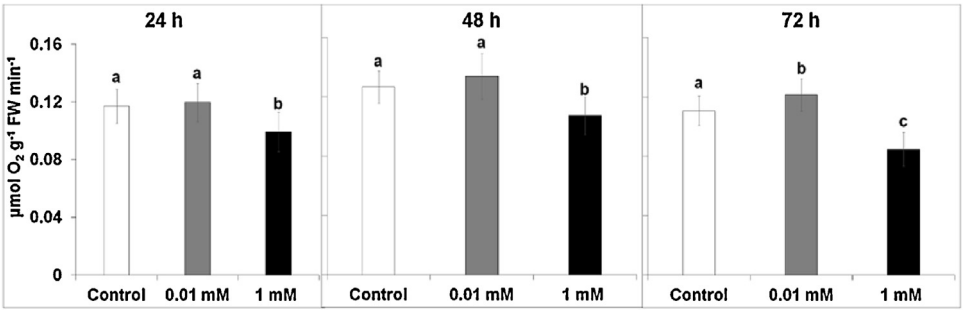

Fig. 6. Influence of two cobalt concentrations (0.01 and 1 mM) on oxygen production in *L. minor* 24, 48 and 72 h after the start of exposure. Significant differences between control and treated plants were designated by different letters (ANOVA, $P \leq 0.05$). Values are given as means ± SD (n = 9).

The conclusion about the above described influence of cobalt on OEC was further supported by oxygen evolution measurement (Fig. 6). The exposure to higher Co^{2+} concentration caused a decrease in oxygen production confirming inactivation of OEC indicated by the positive K-band (Fig. 5A–C), while lower Co^{2+} concentration increased oxygen production after 72 h (Fig. 6C).

3.5. SOD activity and TBARS levels in L. minor after exposure to Co^{2+}

Although SOD is the first line of antioxidative defense in plants and is therefore an important biomarker of oxidative stress, in our study SOD was not activated. On a contrary, SOD activity decreased in plants treated with 0.01 mM cobalt concentration 24, 48 and 72 h after the start of the exposure and 24 and 72 h after the start of the exposure to 1 mM concentration of cobalt (Fig. 7A). Increased activity of SOD was observed 48 h after the start of the exposure to the higher concentration of Co^{2+} (Fig. 7A). The higher value of SOD activity measured in control plants 24 h after the start of the exposure (Fig. 7A) could be caused by activation of antioxidative enzymes as a part of the unspecific reaction/response to changes in the environment. Namely, according to Lichtenthaler (1996) in order to acclimate to the new conditions plants can activate their stress-coping mechanism which include different metabolic processes. This is so called general acclimatization syndrome (GAS) that includes initial oxidative burst, i.e. biologically unspecific reaction. On the other hand, prolonged and continuous stress will lead to damage or activation of defense mechanisms. Accordingly, in our study, the higher values of SOD activity, measured in control plants (24 h), could be a result of unspecific signaling as a response to inoculation to the nutrient medium prior to the beginning of the experiment. This statement is corroborated by the obtained results at 48 and 72 h which show lower SOD activities in control plants. It would be expected that treated plants also exhibit higher SOD activity after 24 h because of unspecific signaling caused by mechanical stress, but this was not the case in our study. Such result could be explained by the fact that increased *in vivo* cobalt content in *L. minor* was detectable as early as ten minutes after the start of the exposure demonstrating high accumulation rate (Sree et al., 2015). Under these conditions the amount of Co^{2+} ions present in the nutrient medium was most likely sufficient enough to reduce the SOD activity in treated plants.

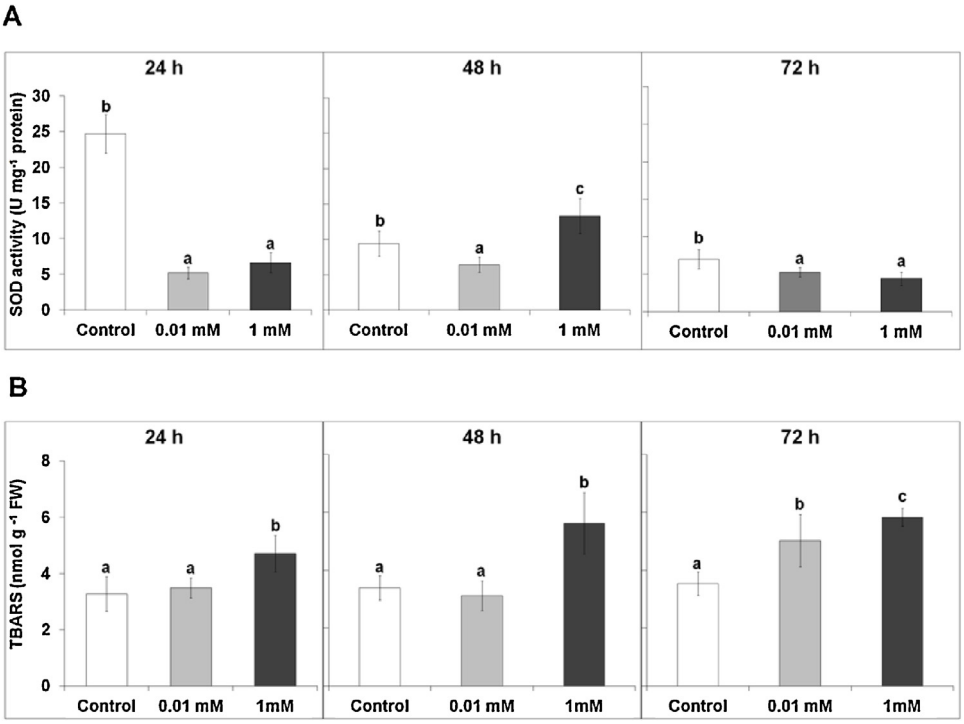

Fig. 7. Specific activity of SOD (A) and TBARS content (B) in *L. minor* 24, 48 and 72 h after the start of the exposure to 0.01 mM and 1 mM $CoCl_2$. Significant differences between control and treated plants were designated by different letters (ANOVA, $P \leq 0.05$). Values are represented as means ± SD (n = 9).

Decreased activity of SOD, in plants exposed to cadmium stress, was observed in pea (Sandalio et al., 2001) and wheat (Milone et al., 2003). In pigeon pea plants treated with cobalt Gopal (2014) reported a reduced SOD activity with increased concentration. The author suggested that decreased SOD activity is due to inhibition of SOD synthesis or inactivation of particular SOD isoforms as well as inactivation of H_2O_2. Because Co^{2+} has affinity for complexes that possess Fe, Mn, Zn and Cu, it can switch place with these metals, whereby the activity of enzymes possessing them is reduced (Chatterjee and Chatterjee, 2000). In addition, inhibition of electron transport further than Q_B could diminish production of superoxide anion ($O_2^{\cdot -}$) production at PSI site (Chen et al., 2012; Pospíšil, 2012) which could be a consequence of decreased SOD activity in our study.

Action of ROS on the cell membrane results in the process of peroxidation of polyunsaturated fatty acids of lipids leading to lipid peroxidation (Wojtaszek, 1997). Previous studies indicated differential impact of Co^{2+} on level of lipid peroxidation in treated plants. While Gopal (2014) reported on reduced lipid peroxidation level in pigeon pea, Zeid et al. (2013) reported increased TBARS content in alfalfa treated with increasing concentrations of cobalt. The latter is in agreement with our results. Exposure of *L. minor* to 1 mM of Co^{2+} significantly increased lipid peroxidation levels (TBARS) already 24 h after the start of the exposure, compared to control (Fig. 7B). This increment was observed at 48 and 72 h also. Lipid peroxidation levels in plants treated with 0.01 mM concentration of Co^{2+} significantly increased only 72 h after the start of the exposure, compared to control, but this increase was lower when compared to 1 mM concentration. Increased TBARS levels 24 h after the start of the exposure showed that higher concentration of cobalt induced early oxidative damage in *L. minor* while at lower concentration plant is able to prevent oxidative damage for some time, but the negative effect of cobalt is evident 72 h after the start of the treatment.

4. Conclusion

It is important to identify how metals impact biochemical pathways in plants before visible toxic effects take place. Induction of defense mechanisms like antioxidative response and adjustment of photosynthetic performance enables the plant to protect itself when exposed to metals. In this study, toxic effects of two cobalt concentrations on *L. minor* photosynthetic activity and oxidative damage after short exposure were demonstrated. Both concentrations of cobalt mainly prevented the activation of SOD. On the other hand, TBARS content increased 24 h after the start of the exposure to 1 mM Co^{2+} and 72 h after the start of the exposure to 0.01 mM indicating oxidative damage and showing that the integrity of the membranes in the cell was compromised. Therefore, the lipid peroxidation level could be a good marker for an early assessment of Co^{2+} toxicity. Our results confirmed that Co^{2+} influences photosynthetic performance altering the electron transport trough PSII and induces OEC impairment, particularly at the higher concentration. At the lower concentration plants were increasing photosynthetic electron transport trying to adapt and to reduce the toxic effect of cobalt.

In toxicological studies it is important to detect physiological parameters of early stress in order to reduce or prevent the harmful consequences of metal toxicity. In the present study, chlorophyll *a* fluorescence parameters, especially K-band, were shown to be useful tools for assessment and screening of early physiological stress in *L. minor* induced by cobalt. Further investigations should be undertaken to validate the application of K-band for assessment of early toxic effect of other metals.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2016.03.009.

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