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Study of plasma activated water effect on heavy metal bioaccumulation by *Cannabis sativa* Using Laser-Induced Breakdown Spectroscopy

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ABSTRACT

Contamination of the environment with toxic metals such as cadmium or lead is a worldwide issue. The accumulator of metals *Cannabis sativa* L. has potential to be utilized in phytoremediation, which is an environmentally friendly way of soil decontamination. Novel non-thermal plasma-based technologies may be a helpful tool in this process. Plasma activated water (PAW), prepared by contact of gaseous plasma with water, contains reactive oxygen and nitrogen species, which enhance the growth of plants. In this study, *C. sativa* was grown in a short-term toxicity test in a medium which consisted of plasma activated water prepared by dielectric barrier discharge with liquid electrode and different concentrations of cadmium or lead. Application of PAW on heavy metal contaminated *C. sativa* resulted in increased growth under Pb contamination as was determined by ecotoxicology tests. Furthermore, the PAW influence on the bioaccumulation of these metals as well as the influence on the nutrient composition of plants was studied primarily by applying Laser-induced breakdown spectroscopy (LIBS). The LIBS elemental maps show that *C. sativa* accumulates heavy metals mainly in the roots. The results present a new proof-of-concept in which PAW could be used to improve the growth of plants in heavy metal contaminated environment, while LIBS can be implemented to study the phytoremediation efficiency.

1. Introduction

Pollution of the environment is a worldwide burning issue. One of the biggest problems is the heavy metal pollution. Heavy metals are present in all layers of biosphere including soil. The accumulation of heavy metals in soil negatively affects agriculture production. Heavy metals affect plants by their phytotoxicity (e.g. growth reduction) as well as the health of soil organisms and health of final consumers - animals and humans. Crops grown on heavy metal contaminated soil show a reduced growth, changed plant metabolism, biomass production and heavy metal bioaccumulation. In the worst cases, heavy metal bioaccumulation may cause death of organisms (Khan et al., 2015; Nagajyoti et al., 2010).

Rock formations are natural sources of metals in nature. However, industrialization has greatly increased the content of heavy metals in the biosphere, especially in soil and water ecosystems. Through bioabsorption of nutrients from soil, heavy metals are also absorbed by plants. They are bioaccumulated in roots and may be translocated to above-ground parts of the plants (Nagajyoti et al., 2010; Shahid et al., 2017). The translocation of heavy metals into above-ground parts depends on the type of heavy metal, as well as the plant. Elements such as Cd, Fe or Cu usually accumulate in the roots with partial translocation to the above-ground part. Elements such as Pb, Ag or Cr accumulate in the root with very little translocation to the above ground part, while elements such as Zn, Mn or Ni are distributed uniformly between roots and the above-ground parts (Siedlecka and Siedlecka, 1995). Lead contamination covers approximately 10 % of total heavy metal pollution. The plant uptake of lead affects plant metabolic functions, growth of the root and even photosynthetic activity (Collin et al., 2022). Cadmium is a trace element which is unessential to plants. Due to the anthropogenic activities such as mining, metal manufacturing or urban refuse, the concentrations of Cd in the environment have increased. Cd is carcinogenic and its uptake from the soil by plants causes disruption of plant metabolism, inhibition of plant growth and causes oxidative stress

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(Haider et al., 2021). Cadmium in soil also originates from applications of mineral fertilizers (Marini et al., 2020) However, heavy metals can be extracted from the soil by the means of phytoremediation. Phytoremediation is an affordable and environmentally friendly technique of soil decontamination and remediation. It is based on utilizing the plants' ability to extract toxic metals from the polluted soil and bioaccumulating them in parts of the plant (roots, as well as leaves) (Linger et al., 2002; Golia et al., 2023).

Naturally occurring plants classified as hyperaccumulators are slowly growing and producing low biomass (Sarwar et al., 2017). Thus, the extraction of heavy metals can be enhanced utilizing chelating agents such as EDTA, which may enhance bioavailability of heavy metals and thus, its uptake by plants (Saifullah et al., 2009). Some studies propose the biochar-assisted phytoremediation, because of its specific physical-chemical properties, such as a large surface area for sorption of heavy metal cations (Lu et al., 2012). Alternatively, biochar is also reported to enhance plant growth and biomass production, which may be utilized in combination with hyperaccumulating plants (Fellet et al., 2014; Paz-Ferreiro et al., 2014). Microorganisms associated with plants may also have an influence on heavy metal availability and uptake by plants, such as plant growth promoting bacteria (Sheng and Xia, 2006; Chen et al., 2003). Minuț et al. report on the plant growth-promoting bacteria from the genus of *Azotobacter, Bacillus* and *Pseudomonas*. Their results show that all three bacteria had different effects on plants (Minuț et al., 2022). Lastly, genetically engineered plants may also play an important role in enhancing the phytoremediation (Van Aken, 2008).

The potential of *Cannabis sativa* L. for phytoremediation was previously described by (Golia et al., 2023). The advantage of *C. sativa* for phytoremediation is its short life cycle. *C. sativa* bioaccumulates heavy metals in the underground parts, leaving the stems and leaves free of such pollutants. It was found that Cd and Pb concentration in the fibres of *C. sativa* was below the heavy metal threshold for textile product and therefore, safe to use (De Vos et al., 2022).

One of the promising ways for increased growth of plants and potentially higher efficiency of phytoremediation lies in the application of low-temperature plasma treatment of seeds and water used for the plant watering. The positive effects of non-thermal plasma lie in the presence of charged particles (electrons, ions, radicals), electric field and ultraviolet radiation produced by the plasma discharge at low temperature. The reactive oxygen and nitrogen species (RONS) present in plasma may enhance or contribute to the seed germination, seed disinfection, and subsequently, to the plant growth and increased food production (Ohta, 2016). Zhao et al. (2021) report on treating seeds of three Cd-tolerant plants, *Bidens pilosa* L., *Solanum nigrum* L. and *Trifolium repens* L. Plasma seed treatment increased both plant dry biomass and Cd accumulation (Zhao et al., 2021). Cold plasma treatment was also utilized for treatment of *Medicago sativa* L. seeds, which resulted in enhanced the removal of heavy fuel oil by the *M. sativa* plant grown from the plasma-treated seeds (Žaltauskaitė et al., 2024).

Apart from the direct plasma treatment of seeds, plasma treatment of water and its subsequent use as a liquid fertilizer is a very discussed topic. As mentioned above, the gas plasma consists of both positively and negatively charged particles. In atmospheric air plasma, it is mostly reactive oxygen and nitrogen species (RONS). When the air plasma is in direct (and sometimes indirect) contact with liquid, the RONS present in the gas plasma phase are transported through the interface into the bulk liquid. When entered the liquid phase, the short-lived RONS such as $\bullet OH$, $NO\bullet$, O_2^-, OH^- or $ONOO^-$ form stable long-lived molecules such as NO_2^-, NO_3^-, O_3 or H_2O_2 (Bruggeman et al., 2016; Judée et al., 2018). Due to these RONS, the water gains its unique properties such as antibacterial effect or promoting plant growth. In literature, the most common term associated with this water is Plasma Activated Water (PAW). Depending on the pH of PAW and the storage conditions, the concentration of reactive species remains stable for days. When the pH is relatively neutral, the concentrations of NO_2^- and H_2O_2 are initially lower, due to their mutual reaction and formation of NO_3^- (Ranieri et al., 2021). Thanks to the presence of RONS, PAW may trigger the release of dormancy and multiple other signalling pathways including gibberellin and abscisic acid (ABA) metabolism. Chemical composition of PAW can directly affect the chemical action on cell walls which results in the premature endosperm weakening. Triggering multiple signalling pathways removes dormancy blocks and results in faster germination of seeds (Grainge et al., 2022).

Laser-induced Breakdown Spectroscopy (LIBS) is a multi-elemental analysis technique based on optical emission collection from laser-induced plasma (LIP). The observed spectral radiation yields qualitative information about the elemental composition of the sample (Buday et al., 2021). During the last years, LIBS was established as an important tool in bioimaging of biological tissues, including soft tissues such as murine kidney (Šindelářová et al., 2021) or skin tumours (Kopřivová et al., 2024), hard tissues such as teeth (Pořízka et al., 2023), and most importantly plant tissue (Brennecke et al., 2023).

Thanks to the fast analysis, its ability to analyse broad range of elements including organogenic elements (C, O, H, N, P), macro nutrient elements (Ca, Mg, K, Na) and even minor and trace elements (Zn, Cu, Cd, Pb) and the possibility to implement bioimaging makes it a complementary technique to other established bioimaging techniques such as Laser Ablation Inductively Coupled Plasma Mass Spectrometry (LA-ICP-MS) (Pořízka et al., 2022). Insights into the spatial distribution of elements help to unveil the correlation between the precise location of an element and its toxic impact (Modlitbová et al., 2020a).

The main objective of this study is to determine the PAW effects on growth and accumulation of heavy metals (Cd, Pb) in the selected model plant – *Cannabis sativa* L. Due to its metal accumulating properties, *C. sativa* was chosen to study the effect of different heavy metal concentrations on its growth and heavy metal bioaccumulation. Cadmium and lead were chosen as representatives of toxic heavy metals. The effect of different concentrations of both contaminants (Cd, Pb) in PAW on growth of the plant was studied in a short-term toxicity test, simulating different concentrations of heavy metals in the polluted environment. These results are confirmed by bulk ICP-OES analysis. The advantage of LIBS is the possibility of spatial analysis of pollutants in plant tissues. Therefore, it explains the migration of pollutants such as toxic metals and its relationship to other elements within the plant tissue. The results of this study represent a proof-of-concept where non-thermal plasma and the PAW can assist in phytoremediation strategies and improve the plant growth.

2. Materials and methods

2.1. Plasma activated water

Plasma activated water was prepared by dielectric barrier discharge generating plasma above the water surface. The experimental device was constructed at the Faculty of Chemistry, BUT (Czech Republic) specifically for plasma interaction with water. It consisted of a Petri dish with a volume of 75 mL and a bottom graphite electrode which was covered by a ceramic plate with the outer silver electrode. The distance of 2–3 mm between the ceramic plate and the water surface ensures a sufficient gap for the plasma ignition by the AC high voltage applications (Šimečková et al., 2020). The pulsed power supply (Lifetech s.r.o., Czech Republic) was operating at a frequency of 11 kHz and giving a rectangular peak voltage of 16 kV. Total energy supplied from the electrical network was (36±2) W. Water was treated for 2, 5 and 10 minutes. For better assessment of the plasma treatment effect on the water composition, distilled water was used.

The characterization of PAW was done for its following physical-chemical properties: pH, conductivity, concentrations of H_2O_2, NO_2^- and NO_3^-. The concentrations of NO_2^- and NO_3^- were measured colorimetrically using a commercial kit by Sigma-Aldrich based on the Griess reaction and UV–VIS spectrometer Helios (ThermoFisher Scientific, USA). Concentration of H_2O_2 was determined colorimetrically using a titanium reagent (Sigma-Aldrich, Germany) and UV–VIS spectrometer.

2.2. Plant toxicity test

The root and the stem measurements of contaminated plants is a standard methodology in ecotoxicology, previously described in other publications, and it was modified for the purpose to test PAW effects (Brennecke et al., 2023; Modlitbová et al., 2020b). Firstly, seeds of *C. sativa* were germinated for 72 h in a Petri dish with filtration paper wetted with distilled water. After 72 h, seeds with the similar-size roots of approximately 1.0–1.5 cm were chosen for the toxicity test. To assess the PAW effect on the plant growth and heavy metal uptake, two types of heavy metal solutions were prepared:

- Control solutions of cadmium chloride ($CdCl_2$) or lead chloride ($PbCl_2$) in distilled water in concentration of 2 mg/L, 5 mg/L, 10 mg/L and 25 mg/L.
- Solution of $CdCl_2$ or $PbCl_2$ in plasma activated distilled water, with the concentration of 2 mg/L, 5 mg/L, 10 mg/L and 25 mg/L. Based on the treatment time, the properties of PAW can be altered. The times of treatments were 2 minutes, 5 minutes and 10 minutes.
- Distilled water without heavy metals as negative control.

Distilled water was used in this study to make sure that only Cd(II) or Pb(II) ions and RONS from PAW interacted with the plant and there were no other influences on the results. The grown seedlings were placed into Eppendorf tubes (P-lab, Czech Republic) with 2 mL of either control heavy metal solution or PAW solution. They were cultivated for 72 h in growth boxes with the light/dark cycle of 16/8. After 72 h, the toxicological endpoints were measured to determine the PAW and heavy metal effect on the plant growth (the root, the stem, and the whole plant length).

2.3. Statistical analysis

The statistical analysis was performed by the Mann Whitney U test of statistical significance in the OriginPro software (OriginLab corporation, USA). The levels of significance were accepted at $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$. The statistical analysis of growth reduction in tested plant groups was compared with the growth obtained under distilled water (negative control).

2.4. Element content analysis

The inductively coupled plasma optical emission spectroscopy (ICP-OES) was chosen as a reference method to determine the exact concentration of Cd(II) and Pb(II) in *C. sativa* as well as the content of selected nutrients (Ca, Mg, P, K). Nutrients were not supplied in the solution, thus the nutrients determined by ICP-OES were stored within the seeds. Each concentration of Cd(II) and Pb(II) was tested in three replicates. Plants were split into two parts – the root and the above-ground part (stem and leaves). Then, they were dried at 60 °C for 24 h and weighed on analytical scales. Separated parts were digested in the mixture of 2.2 mL of 65 % HNO_3 and 1 mL of 30 % H_2O_2 in Savillex PFA vials for at least 1.5 h at 200 °C. Digested solutions were diluted to 25 mL with distilled water and analysed using the spectrometer iCAP PRO series ICP-OES (Thermo Fisher Scientific, USA). Thus, the concentrations were obtained for the root part and for the above-ground part.

Apart from plants, the concentrations of heavy metals in mediums before and after the exposure were measured by ICP-OES. To quantify the uptake and translocation of Cd(II) and Pb(II) in PAW and non-PAW treated plants, the bioaccumulation factor (BAF) and the translocation factor (TF) were determined. The BAF was calculated according to Eq. (1), as the ratio of heavy metal content in the plant versus the concentration in the medium after the exposure.

$$BAF_{Cd,Pb} = \frac{m_{Cd,Pb}}{c_{Cd,Pb}} \tag{1}$$

The TF was calculated according to Eq. (2), as the ratio of heavy metals concentration in the above-ground part (stem and leaves) versus their concentration in the underground part (Kummerová et al., 2012).

$$TF_{Cd,Pb} = \frac{m_{above-ground,Cd,Pb}}{m_{undergrownd,Cd,Pb}} \tag{2}$$

2.5. Plant bioimaging

The plant samples for the laser-induced breakdown spectroscopy were prepared according to the methodology previously presented by Modlitbová et al. (2020) (Modlitbová et al., 2020b). Dried and moulded plants were fixed on the microscopy slides by epoxy resin. Plants were photographed with an optical microscope (Pomeas, China, magnification x25) prior to the measurement.

The 2D LIBS elemental maps were recorded using the LIBS FireFly system (Lightigo, Czech Republic). The setup, previously described in other publications (Brennecke et al., 2023; Modlitbová et al., 2020b), consists of a Nd:YAG laser (20 mJ, 1064 nm, 20 Hz, 10 ns, Litron). The mapping was done with a step size of 100 μm with the map size varying according to the sample area. Spectra were collected by two Czerny-Turner type spectrometers. The high-resolution spectrometer (Spectrometer1, resolution of 0.01 nm) with modifiable wavelength range was focused on the heavy metal line of interest (Cd I 228.80 nm or Pb I 405.78 nm) and was equipped with the ICCD camera. The lower resolution spectrometer (Spectrometer2, resolution of 0.1 nm) with a broad wavelength range covering the range of 245–407 nm was equipped with the integrated SCMOS camera. Spectra from both spectrometers were collected simultaneously with the gate delay of 0.25 μs and the gate width of 50 μs. Then, both data sets were processed in Epina software and a custom python script.

Firstly, the background was corrected for both data sets by the moving minimum method. Then, the background corrected spectra were used in the rest of the data processing pipeline. The high-resolution spectrometer was focused on the line of the contaminant – either Cd I 228.80 nm or Pb I 405.78 nm so the line was highlighted in the data and its signal-to-noise ratio (SNR) was determined for all spectra by dividing the integral of the line area by an integral of the noise area near the line with the same width. This was done also for lines Mg II 279.55 nm and Ca II 393.37 nm at the data from the lower resolution spectrometer. These elements were chosen to acquire an elemental map of the whole plant and as a reference for the analysis of different sample sets.

Only data coming from the plant tissue had to be selected for the rest of the analysis. To do this, the SNR maps were used to create masks. These masks were in the form of polygons containing only the plant tissue spectra and all other pixels from each sample were omitted from the data set. The data evaluation was done by extracting the mean and standard deviation values of the SNR for all specified lines (Cd/Pb, Mg, Ca) and for ratios of the contaminant and nutrients. The Cd(II) or Pb(II) line area integral was divided by the Mg line area integral to gain a new value – contaminant/Mg ratio. Additionally, this was repeated for the root area, only, as the root accumulates most of the contamination.

3. Results and discussion

3.1. Plasma activated water characterization

Results shown in Table 1 demonstrate that depending on the time of the plasma treatment, the concentration of RONS in PAW increases significantly, especially in the case of NO_2^- and NO_3^-. Subsequently, the conductivity increases with the increased treatment time and concentration of RONS. With this, pH is radically reduced from pH 7.5 (after

Table 1
Characterization of plasma activated water prepared by dielectric barrier discharge with the liquid electrode.

Treatment time (min)	pH	Conductivity (μS/cm)	H_2O_2 (mg/L)	NO_2^- (mg/L)	NO_3^- (mg/L)
2	4.9	40	0.715 ±0.002	0.05 ±0.02	15±6
5	4.2	106	0.912 ±0.002	0.13 ±0.01	20±2
10	3.8	270	0.937 ±0.003	0.22 ±0.03	42±2

2 minutes of the plasma treatment) to 3.9 (10 minutes of the plasma treatment). The PAW gains its properties thanks to contact of the water with plasma discharge. The short-live RONS generated in plasma discharge in the air such as nitric oxide radical ($NO\bullet$), hydroxyl radical ($\bullet OH$), superoxide anion radical ($\bullet O_2^-$), atomic oxygen (O), nitrogen ions (N_2^+) are transported through the plasma-liquid interface and form long-lived species such as hydrogen peroxide (H_2O_2), nitrites (NO_2^-), nitrates (NO_3^-) (Bruggeman et al., 2016). The concentrations of these long-lived RONS are dependent not only on the type of plasma reactor, but also on the treatment time. Therefore, the longer the treatment time is, the higher the concentrations of RONS is produced (Xiang et al., 2022). In the presence of hydrogen peroxide (H_2O_2), nitrites (NO_2^-), nitrates (NO_3^-) in the aqueous solution the pH of water is decreased while the conductivity increases.

3.2. Toxicology tests

After the 72 h exposure of *C. sativa* seedlings to various concentrations of heavy metals in distilled water and plasma activated water, the biometric toxicity endpoints were measured.

In the case of cadmium, both growth of the root and the stem were affected (Fig. 1). Although the PAW reduced the negative effect of Cd(II) on the growth of the root more than with the stem (as seen in the Figs. 1–3), the overall plant growth was still significantly affected by cadmium compared to the control (DW without addition of heavy metals). The Fig. 1A (PAW with the 2-minute treatment time) shows that the growth of the root decreased at the $p<0.01$ level of significance for all concentrations of Cd(II) in DW compared to the negative control (DW), with the exception of 10 mg/L, where the growth of the root decreased at the $p<0.001$ level of significance. In the case of Cd(II)

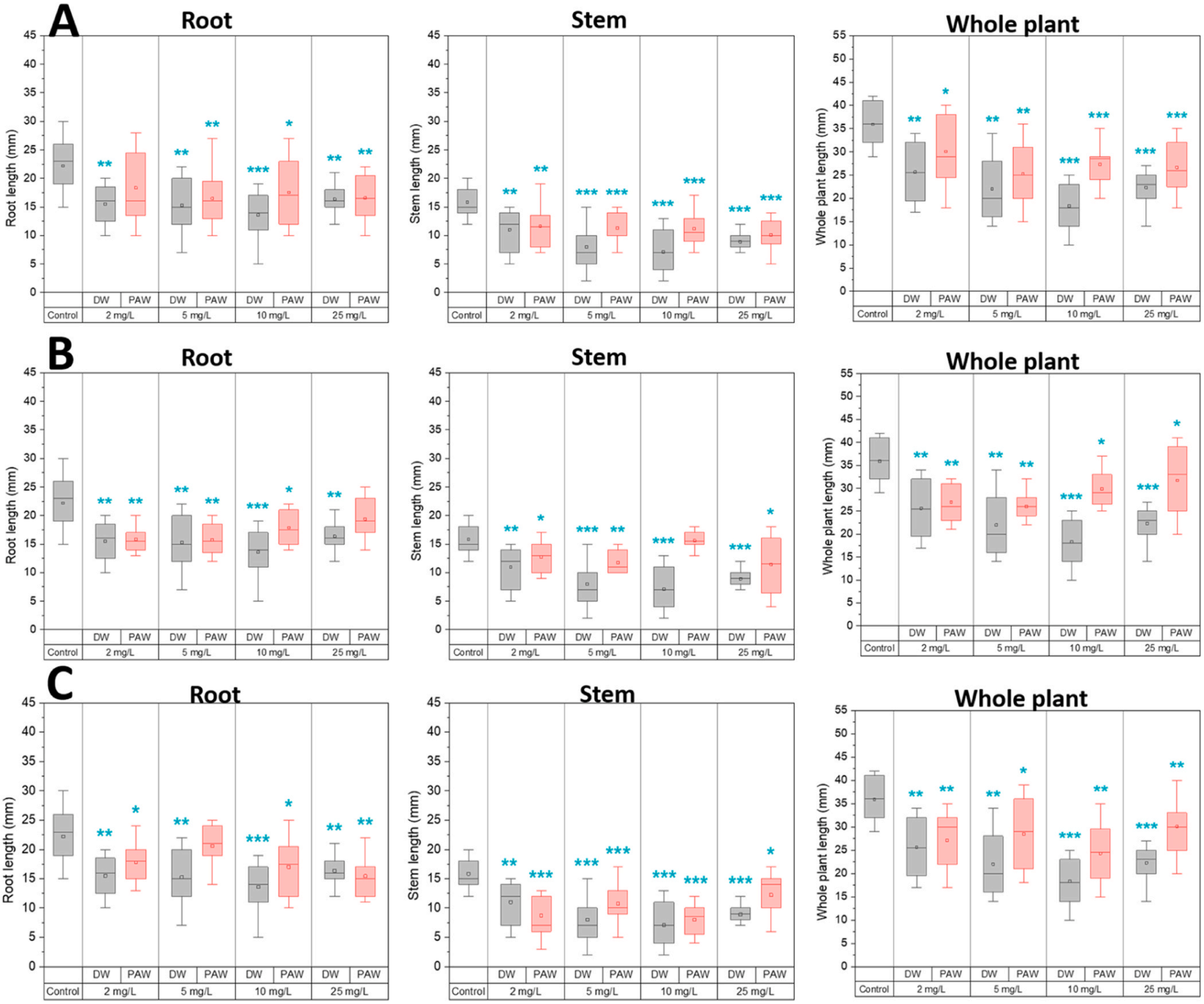

Fig. 1. Results of the toxicology test after 72 h C. sativa exposure to $CdCl_2$ in distilled water or $CdCl_2$ in plasma activated water (PAW). The control group was grown in distilled water, only. The level of significance is expressed by blue stars above each box (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Time of plasma treatment of water was: A – 2 minutes; B – 5 minutes; C – 10 minutes.

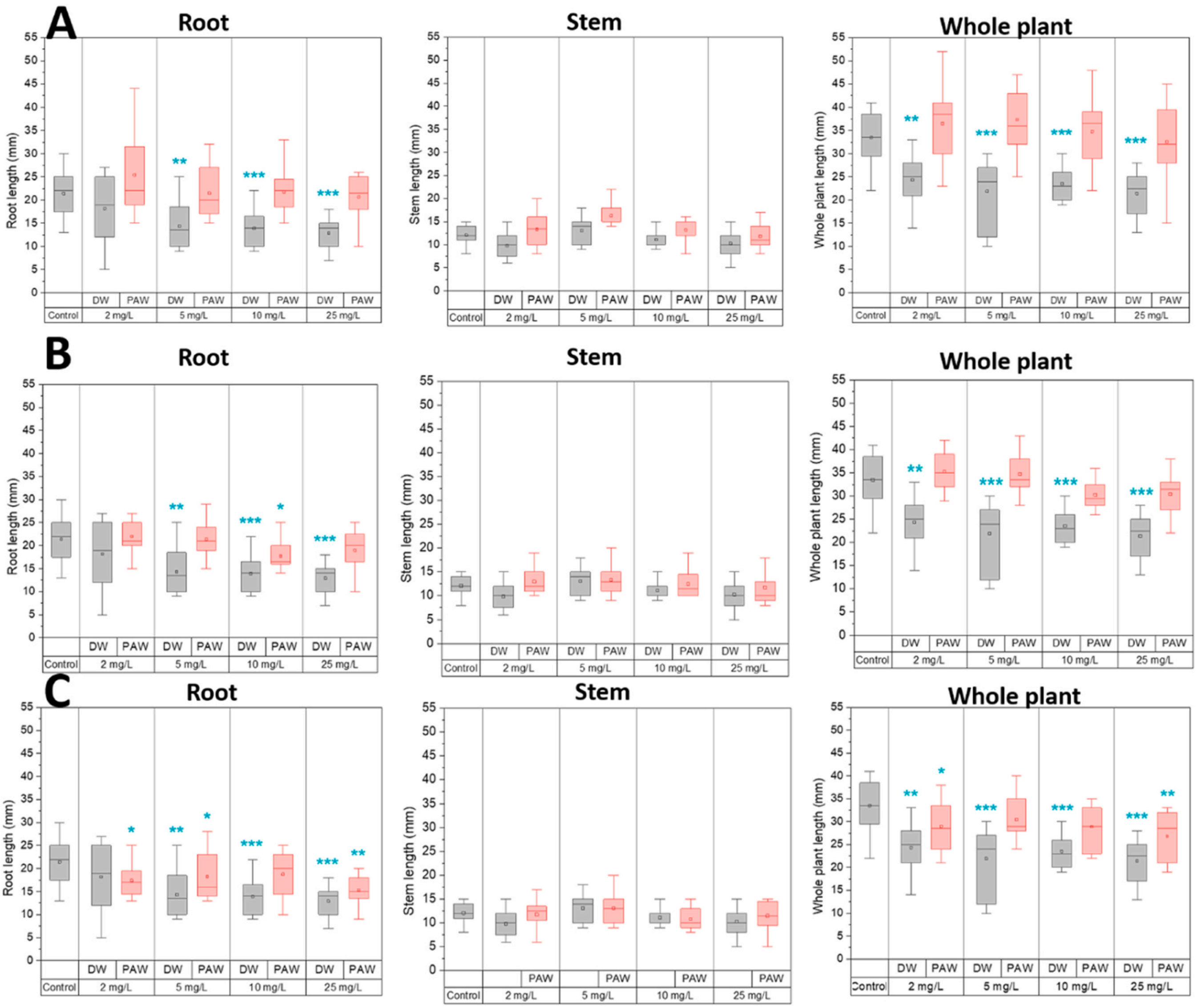

Fig. 2. Results of the toxicology test after 72 h C. sativa exposure to $PbCl_2$ in distilled water or $PbCl_2$ in plasma activated water (PAW). The control group was plants grown in distilled water, only. The level of significance is expressed by blue stars above each box (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Time of plasma treatment of water was A – 2 minutes; B – 5 minutes; C – 10 minutes.

2 mg/L concentration in PAW, the growth was not significantly affected compared to the negative control. However, there is a significant ($p<0.01$) reduction of growth in concentration of 5 and 25 mg/L of Cd (II) in PAW, as well as 10 mg/L, where the growth was reduced at the $p<0.05$ level of significance. The growth of the stem was negatively affected at the $p<0.01$ level of significance for the 2 mg/L concentration of Cd(II) in both DW and PAW, and at the $p<0.001$ level of significance for higher concentrations in both DW and PAW. This was reflected at the reduction of the whole plant growth. In the case of PAW with 5-min treatment time (Fig. 1B), the root reduction growth is significant at the $p<0.01$ level of significance for 2 and 5 mg/L of Cd(II) in both DW and PAW and 25 mg/L of Cd(II) in DW. The concentration of 10 mg/L in DW caused growth reduction at the $p<0.001$ level of significance, while in PAW it was at the $p<0.05$ level of significance. Interestingly, 25 mg/L of Cd(II) in PAW was not significantly different compared to the negative control. The stem growth reduction of 2 and 25 mg/L of Cd(II) in PAW was at the $p<0.05$ level of significance and at the $p<0.01$ level of significance for 5 mg/L of Cd(II) in PAW. The stem growth was not significantly effected for 10 mg/L of Cd(II) in PAW. The growth of the whole plant was affected at the $p<0.01$ level of significance for 2 and 5 mg/L of Cd(II) in PAW and at the $p<0.05$ level of significance for 10 and 25 mg/L of Cd(II) in PAW. For PAW with 10-minute treatment time (Fig. 1C), the root growth was decreased at the $p<0.05$ level of significance for 2 and 10 mg/L of Cd(II) in PAW, $p<0.01$ level of significance for 25 mg/L of Cd in PAW and not significantly affected for 5 mg/L of Cd (II) in PAW. The stem growth was reduced at the $p<0.001$ level of significance for 2, 5 and 10 mg/L of Cd(II) in PAW and $p<0.05$ level of significance for 25 mg/L of Cd(II) in PAW. The whole plant length was significantly reduced at the $p<0.01$ level of significance in the case of 2, 10 and 25 mg/L of Cd(II) in PAW and $p<0.05$ level of significance of Cd (II) in PAW. These results suggest that even with the application of PAW, Cd(II) still negatively affects growth of plants.

The nitrogen species in PAW cause improved growth when applied to plants. However, the growth of *C. sativa* was not significantly improved by application of PAW. This was probably caused by Cd(II) toxicity. Chang et al. (2013) also report that application of nitrogen fertilizer did not improve the growth of Pentas lanceolata under Cd(II) presence in growth media (Chang et al., 2013).

In the case of lead, only inhibition of the root growth was impacted. This was also observed in previous studies (Brennecke et al., 2023). The

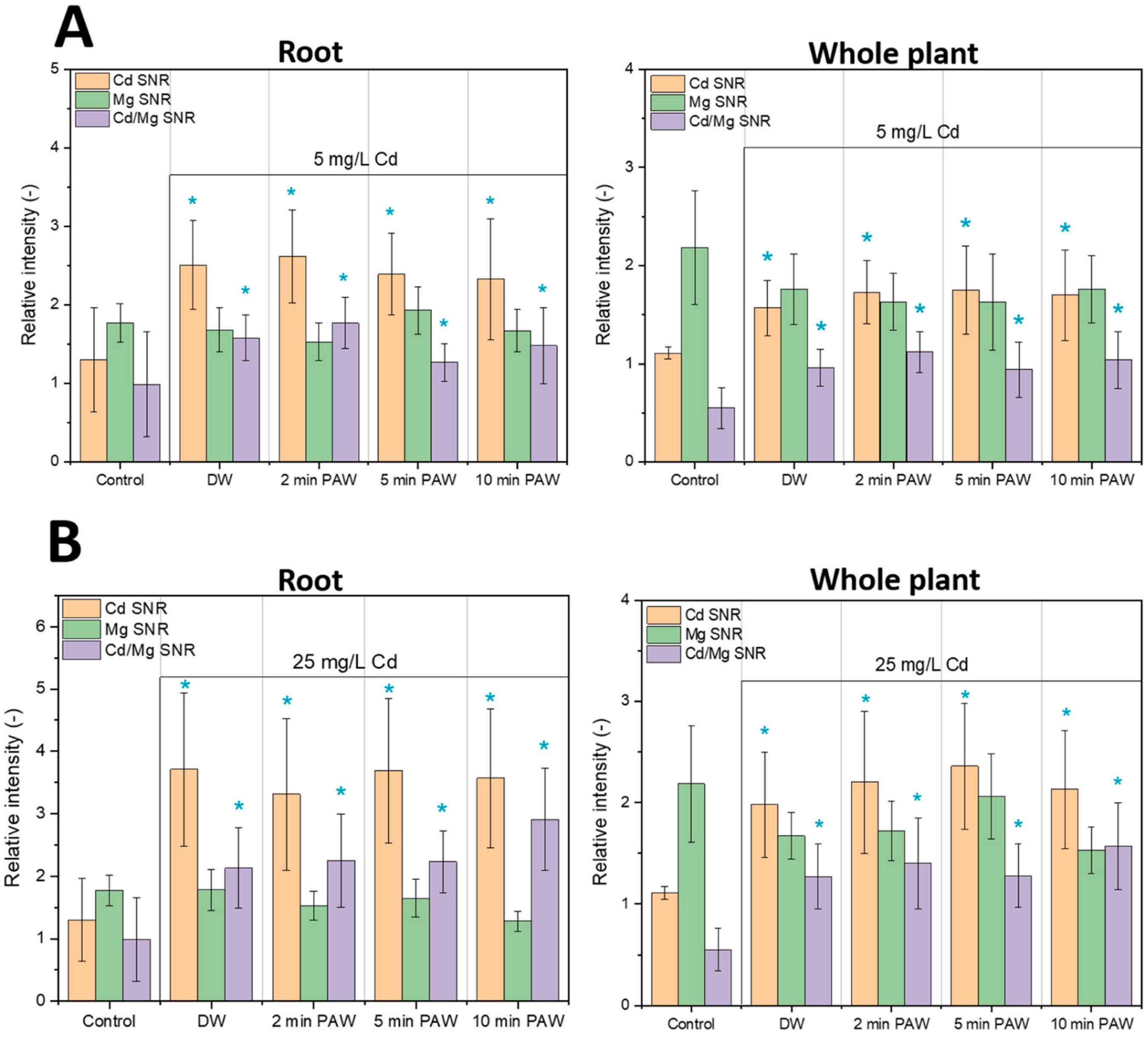

Fig. 3. Comparison of SNR of Cd(II), Mg and Cd(II)/Mg in the root and the whole plant. Concentration of $CdCl_2$ was: A – 5 mg/L; B – 25 mg/L. The control group was plants grown in distilled water only. DW stands for for solution of Cd(II) in distilled water. Each group was statistically compared to the control (distilled water without Cd(II)). The level of significance is expressed by blue stars above each box (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). For single elements SNR, values around 1 mean the level of noise – no detected signal. This was preserved for calculation of Cd/Mg ratio.

PAW had a reduced the negative effect of lead. The growth of root was affected in the case of 5 mg/L of Pb(II) at the $p<0.01$ level of significance, 10 and 25 mg/L of Pb(II) in the DW at the $p<0.001$ level of significance. For all Pb(II) concentrations in PAW, growth of the root was not significantly affected. The stem growth was not affected by Pb(II) in both DW and PAW. The whole plant length was reduced by Pb(II) in DW, at the $p<0.01$ level of significance for 2 mg/L of Pb(II) and at the $p<0.001$ level of significance for 5, 10 and 25 mg/L of Pb(II). The whole plant length for Pb(II) concentrations in PAW was not significantly decreased. Similar results were found for the 5–minute treatment time PAW (Fig. 2B), where only the root growth in 10 mg/L of Pb(II) in PAW was significantly decreased at the $p<0.05$ level of significance. This did not have an effect on the whole plant growth, which was not significantly affected in the case of all Pb(II) concentrations in PAW. After the 10–minute plasma treatment (Fig. 2C), PAW seemed to be less effective, with decreased root growth at the $p<0.05$ levels of significance for 2 and 5 mg/L of Pb(II) in PAW and at the $p<0.01$ level of significance for 25 mg/L of Pb(II). This was probably caused by the low pH of PAW after 10 minutes of treatment.

The growth of *C. sativa* under PAW and Pb application may be primarily caused by the nitrogen species in PAW. It was previously proven that nitrogen fertilizer causes a significant increase in plant growth and dry biomass production of *C. sativa* grown in Pb spiked soil (Deng et al., 2021).

The RONS present in PAW may play the role of a stress factor, which in return makes the plants more resilient against other stress factors, such as heavy metal contamination. However, the oxidative stress analysis would need to be performed to test this hypothesis. For example, Kostoláni et al. (2021) report that PAW causes moderate oxidative stress and elevate the antioxidant enzymes. RONS present in PAW do not cause any DNA damage in pea seedlings (Kostoláni et al., 2021). Lukacova et al. (2021) report that PAW pre-treatment increased the growth of corn seedlings exposed to arsenic in a hydroponic test. PAW also increased the guaiacol peroxidase (POX) activity and chlorophyll content in PAW treated plants combined with As. However, the As accumulation was not affected by the PAW treatment (Lukacova et al., 2021). Modlitbová et al. (2018b) report on toxicity of Cd-based quantum dots (QDs) for the model plant *Allium cepa* L. while CdTe QDs showed the highest bioaccumulation among all exposure groups, the core/shell CdTe/ZnS QDs showed no toxicity and low bioaccumulation in *A. cepa (*Modlitbová et al., 2018a*)*.

The results from toxicology tests serve as a proof-of-concept for further investigation of PAW on heavy metal contaminated plants and accumulators of metals. For example, if accumulators such as *C. sativa* are grown on the heavily contaminated land, application of PAW may help with the resilience of the plants to the contamination, higher growth, and potentially more effective remediation. However, the results from the short-term toxicology study presented in this article must be supported by further investigation on plants grown in contaminated soil in longer time periods. The potential of non-thermal plasma technologies in enhancement of phytoremediation is strongly dependent on the scalability of the plasma discharges. The advantage of the dielectric barrier discharge (DBD), used in this study, is its scalability and a relatively homogeneous operation at atmospheric pressure. The most common applications of DBD in the industry is ozone production or treatment of surfaces (Kogelschatz, 2003; Xu, 2001), which points to the possibility of using this type of discharge in agriculture as well.

Alternatively, PAW can also be used in conjunction with other phytoremediation strategies, such as utilizing chelating agents such as EDTA (Saifullah et al., 2009), the biochar-assisted phytoremediation (Lu et al., 2012) or microorganisms assisted phytoremediation (Sheng and Xia, 2006; Chen et al., 2003).

3.3. Element content analysis by ICP-OES

The concentration of Cd(II) and Pb(II) in the dry mass of *C. sativa* was determined by the ICP-OES analysis. The plants were separated to the underground part (roots) and the above-ground parts (roots and leaves). Additionally, for the purpose of the BAF calculation, the growth solutions were analysed as well. Based on the ICP-OES, the concentration of Cd(II) in roots and stems of *C. sativa* increases with the increasing concentration of the initial growth solutions. In the case of 10 mg/L and 25 mg/L solutions of Cd(II), there appears to be a lower concentration in the PAW treated plants compared to the non-PAW treated plants (Figure S1 in Supplementary). Nevertheless, according to the Mann-Whitney statistical test, this difference is not statistically significant. Similarly, the BAF is lower in the cases of PAW treated plants, but not statistically significant (Figure S1 in Supplementary). According to the translocation factor (TF) calculated for the stem in Table S1 (in Supplementary), after 3 days of the growth there was no significant translocation from the root to the stem of the plant. TF was not affected by PAW. The TF value is low due to accumulation of Cd(II) in roots with low concentrations of Cd(II) found in the stem.

The concentration of lead in both the root and the stem of *C. sativa* was not significantly affected by the PAW treatment, as seen in Figure S2 (in Supplementary). The TF again showed a low translocation of lead with PAW not playing a significant role in the translocation (Table S2 Supplementary). The TF value is low due to accumulation of Pb(II) in roots with low concentrations of Pb(II) found in the stem.

The PAW did not significantly affect the bioaccumulation of both Cd (II) and Pb(II). Both Cd(II) and Pb(II) were accumulated mostly in the roots of plants. This was mainly caused because both Cd(II) and Pb(II) are reported to be heavy metals which accumulate mostly in the root (Siedlecka and Siedlecka, 1995). Heavy metals accumulation is also plant-specific, and *C. sativa* was reported in many studies that it accumulated heavy metals mainly in its roots (De Vos et al., 2022). Depending on the chosen plant, PAW can have different effects on bioaccumulation of heavy metals. Study by Hou et al. (2021) suggests that the cold plasma treatment of seeds together with the PAW treatment suppressed the Cd(II) absorption by water spinach but did not positively affect the growth of the plants. Bioaccumulation of heavy metals in water spinach was affected by heavy metal concentration in soil (Hou et al., 2021). Another study shows that PAW helped with a faster germination of soybeans and produced higher biomass, but also lowered the uptake of lead by plants when combined with zinc-oxide nanoparticles (ZnONPs) (Mahanta et al., 2022).

Different strategies to either decrease or improve the uptake of heavy metals by *C. sativa* have been tested. Effect of silicon (Si) application on *C. sativa* plants exposed to Cd in a short-term 1 week hydroponic was studied by Luyckx et al., (2021). It was found that Si application decreased Cd accumulation in all organs and improved water use efficiency. However, the plat growth did not improve under additional Si application (Luyckx et al., 2021). Application of mineral fertilizer also resulted in elevated concentration of Cd in seeds of *C. sativa* variety Henola (Wielgusz et al., 2022). Nitrogen fertilizer was reported to cause a significant increase in plant growth and dry biomass production of *C. sativa* grown in Pb spiked soil. Pb was accumulated mainly in the roots and its uptake and accumulation was increased with the N fertilizer application (Deng et al., 2021). The pH of the environment may also play an important role in bioaccumulation of heavy metals. Higher concentrations of Cd in seeds in an alkaline soil was observed in study by Wielgusz et al. (Wielgusz et al., 2022). Because of PAWs low pH, the bioaccumulation of Cd(II) and Pb(II) was not affected. However, due to the increased growth of C. sativa under Pb contamination, PAW could be applied together with other strategies to improve the uptake of heavy metals.

Yan et al. suggest that nanomaterials are a promising tool to mitigate and decrease the bioaccumulation of Cd(II) by plants (Yan et al., 2023). A study done by Jing et al. (2024) with *Apocynum venetum*, another plant suitable for phytoremediation, reports that *A. venetum* bioaccumulated Cd(II) primarily in the root of the plant and Cd(II) stress promoted *A. venetum* root development (Jing et al., 2024). The application of Abscisic acid (ABA), growth and stress hormone, in different concentrations in a hydroponic experiment increased the Cd(II) bioaccumulation of *Comos bipinnatus* (Yu et al., 2023). Wierzbicka et al. report that application of "InCa" (Plant Impact), a Ca transport activator on leaves of various plants *Cucumis sativus*, *Linum usitatissimum*, *Medicago sativa* and *Solanum lycopersicum* reduced the concentration of Pb(II) in both plant roots and shoots (Wierzbicka et al., 2023).

Additionally, concentrations of selected nutrients (P, Mg, Ca, and K) were measured for deeper understanding of the PAW and heavy metal's effect on plants (Table 2). The concentrations of nutrient elements seems to be lower in the roots of plants contaminated with Cd(II) compared to control (distilled water), especially in the case of Mg(II) and K(I). The most noticeable difference was with samples contaminated with 25 mg/L of $CdCl_2$. It is important to note that the plants were grown in solution of heavy metals in either DW or PAW. The nutrient elements were not supplied from the solution. Moreover, more date would need to be collected to support this claim. It was previously reported that heavy metals interfere with uptake of the above-mentioned nutrients. For example, both Cd(II) and Pb(II) decreased level of nutrients such as Fe, Mg, K or Ca in roots and shoots of *Raphanus sativa* or *Cucumis sativus* (Siedlecka and Siedlecka, 1995). The increased concentrations of Cd(II) and Pb(II) resulted in decreased levels of Ca, K, Na and Fe in spinach *Spinacia olerace*a (Alia et al., 2015). Cd(II) may also reduce the uptake of water and absorption of nitrate by inhibiting nitrate reductase, while Pb (II) is reported to cause chlorosis (iron deficiency) in plants (Ghori et al., 2019). The deficiencies of macro and micronutrients in *C. sativa* were previously described by Cockson et al. (2019). The symptoms of Mg(II) deficiency in *C. sativa* is reported to develop after 7 weeks of growth by slight yellowing of the interveinal regions on the leaves, while K(I) deficiency develops after 9 weeks of growth as a yellowing of leaf margins. Ca(II) deficiency develops 5 weeks after the treatment and is displayed as a slight stunting and irregular growth. However, these

Table 2
Concentrations of P(V), Mg(II), Ca(II), and K(I) in C. sativa contaminated with Cd(II) and Pb(II) obtained by the ICP-OES.

Cadmium Cd(II)									
Element	Root (mg/g)			Stem			Whole plant		
	Control	25 mg/L	PAW treated 25 mg/L	Control	25 mg/L	PAW treated 25 mg/L	Control	25 mg/L	PAW treated 25 mg/L
P	13.0±2.0	11.2±0.9	12.0±2.0	13.0±3.0	9.3±1.2	12.3±4.0	26.0±4.0	20.5±1.9	24.0±6.0
Mg	3.8±0.3	2.4±0.2	2.4±0.6	6.3±0.9	4.3±0.7	5.6±0.8	9.1±0.9	6.8±0.7	9.0±3.0
Ca	2.1±0.7	4.0±2.0	4.4±0.9	0.9±0.3	0.4±0.1	0.5±0.2	2.9±0.7	5.0±3.0	4.9±0.9
K	25.9±4.0	9.7±1.7	9.7±2.5	8.4±0.6	6.2±0.3	7.5±1.5	34.0±4.0	15.0±2.0	17.0±6.0
Lead Pb(II)									
Element	Root (mg/g)			Stem			Whole plant		
	Control	25 mg/L	PAW treated 25 mg/L	Control	25 mg/L	PAW treated 25 mg/L	Control	25 mg/L	PAW treated 25 mg/L
P	13.0±2.0	12.0±2.0	14.0±2.0	13.0±3.0	15.7±2.9	13.0±3.0	26.0±4.0	28.0±3.0	27.0±4.0
Mg	3.8±0.3	2.7±0.4	2.9±0.4	6.3±0.9	6.9±0.9	5.7±0.6	9.1±0.9	9.6±1.2	8.7±0.8
Ca	2.1±0.7	1.4±0.3	1.8±0.5	0.9±0.3	1.5±0.2	0.5±0.2	2.9±0.7	2.9±0.2	2.4±0.7
K	25.9±4.0	19.8±5.6	19.0±2.0	8.4±0.6	8.0±3.0	8.0±2.0	34.0±4.0	27.0±5.0	27.0±4.0

effects could not be observed in this study after only 3-day exposition to Cd(II) and Pb(II).

3.4. *Plant bioimaging by LIBS*

For a better insight into the PAW effect on heavy metal bioaccumulation in *C. sativa*, LIBS was used to determine the exact distribution of metals in plants. A statistical evaluation was done by extracting the mean and standard deviation value of the SNR for all specified lines (Cd/Pb, Mg, Ca) and for ratios of the contaminant and nutrients. The SNR values of Cd (Fig. 3) and Pb (Figure S3) were compared with the control group (distilled water without heavy metals) and contaminated plant without PAW (DW) and with PAW (2, 5, 10 min). The SNR value of Mg and Cd/Mg SNR ration (Fig. 3) and Pb/Mg SNR ration (Figure S3) was also compared with the control group. The SNR values of Cd were significantly different at the $p<0.05$ level of significance compared to the control group in all cased of Cd application (Cd in DW, 2 min PAW, 5 min PAW, 10 min PAW). The SNR values of Mg in plants contaminated with Cd(II) (Fig. 3) show no statistical difference compared to the control without Cd(II). The ration of Cd/Mg SNR, which indicates the relationship between Cd(II) bioaccumulation and content of Mg(II) in plants, shows significant difference at the $p<0.05$ level of significance compared to the control group in all cased of (Cd(II) in DW, 2 min PAW, 5 min PAW, 10 min PAW).

Fig. 4 shows the maximum emission spectra from the plants contaminated with Cd(II) and Pb(II) to show clear visibility of all discussed emission lines. Spectra were recorded with the Spectrometer1 (Figs. 4A, 4B). The spectra of Cd(II) show lower maximum intensities of Cd(II) lines in plants treated with PAW. Furthermore, nutrient elements spectra (Fig. 4C) were recorded using the Spectrometer2.

The elemental maps in Fig. 5 demonstrate the relationship between Cd(II) or Pb(II) bioaccumulation and biodistribution of magnesium. For the 2D maps, the emission lines of Cd I 228.82 nm, Pb I 405.81 nm and Mg II 279.47 nm were chosen. The maps show a decreased intensity of Mg(II) in parts or the root where Cd(II) or Pb(II) were accumulated, compared to the control map without contaminants. This confirms the findings of ICP-OES analysis and further increases the precision of the contaminant localization in the plant tissue. It is not possible to achieve such a precision with ICP-OES as the whole sample or the area of the sample is milled to get the results. Based on the LIBS elemental maps, it can be deduced that contaminants are distributed equally along the root area.

Both the LIBS data analysis and the elemental maps show that most of the contaminant concentration being either cadmium or lead is mainly localized in the root part of the plant. Thus, confirming that *C. sativa* is a metal accumulator. Additionally, in all concentrations of the contaminant except the lowest one, the level of nutrients slightly decreases with the prolonged PAW treatment as it is visible from the magnesium SNR levels. However, according to Mann-Whitney test, this decrease is not statistically different. This may be connected to the contaminant negatively influencing the ability to provide sufficient nutrition to the whole mass of the plant. With almost the same concentrations of the two contaminants in each group, the LIBS analysis has difficulties detecting Cd(II) in the lowest concentration. This is because of the Cd(II) line belonging to the UV range, where the losses of the optical system carrying the plasma signal are higher than in the visible range.

Overall, the LIBS results were confirmed by the results gained by ICP-OES. Based on the spatially resolved analysis, they also confirm the toxicology results of this work. Both contaminants were detected in a sufficient amount and the localization of the contaminants could be evaluated on the elemental maps.

During the years, LIBS was utilized in several studies for bioimaging of contaminants as well as essential elements in plants. The earlies studies focused mainly on the Pb(II) content in *Helianthus annuus* L (Kaiser et al., 2009; Galiová et al., 2007). or *Capsicum annuum* L (Galiová et al., 2011). with a spatial resolution of only 500 μm. The sample was a few square mm in size and the method did not capture the whole plant sample. Cd(II) was detected in an aquatic plant *Lemna minor* L. which was exposed to $CdCl_2$ and Cd-based quantum dots solution at various concentrations with a resolution of 200 μm (Modlitbová et al., 2018b). Essential elements such as P, K, Mg, Ca, etc. were analysed in sugar cane leaves in the study from Guerra et al. (2015) with a spot size of 750 μm and the area of 9 mm × 9 mm (Bueno Guerra et al., 2015). Bioimaging of essential elements such as Mg or K in *Zea mays* L. leaves was done with a resolution of 200 μm (Zhao et al., 2016). Zhao et al. detected Cd(II) in leaves of *Lactuca sativa* L. grown in a solution of $Cd(NO_3)_2$ using the nanoparticle-enhanced LIBS with a resolution of 300 μm (Zhao et al., 2019). The application of silver NPs on the plants surface was done to enhance the Cd(II) signal. Furthermore, Modlitbová et al. (2020) were able to map the whole plant sample of *Sinapis alba* L. after a 72 h short-term toxicity test with the resolution of 100 μm and parts of the plants with a 25 μm resolution (Modlitbová et al., 2020b). A new study from Brennecke et al. focuses on bioimaging of *C. sativa* contaminated with Cd(II) and Pb(II) with a resolution of 100 μm (Brennecke et al., 2023).

It is clear, that the capabilities of the LIBS method in spatial resolution, possibility to map larger samples and detection limits improved over time to better suit the needs of plant biologists and toxicologists. We are now presenting results from imaging samples with a size of approx. 3–5 square cm with a resolution of 100 μm which is a great improvement from imaging of samples of only a few square mm with spatial resolutions of 200–500 μm. The better the resolution is the more precise the method becomes, with better abilities to pinpoint the exact distribution of contaminants. Moreover, LIBS data analysis was able to compare different PAW treatments of plants and bioaccumulation of Cd (II) and Pb(II) on the roots of plants.

The advantage of LIBS in the phytoremediation lies in the possibility

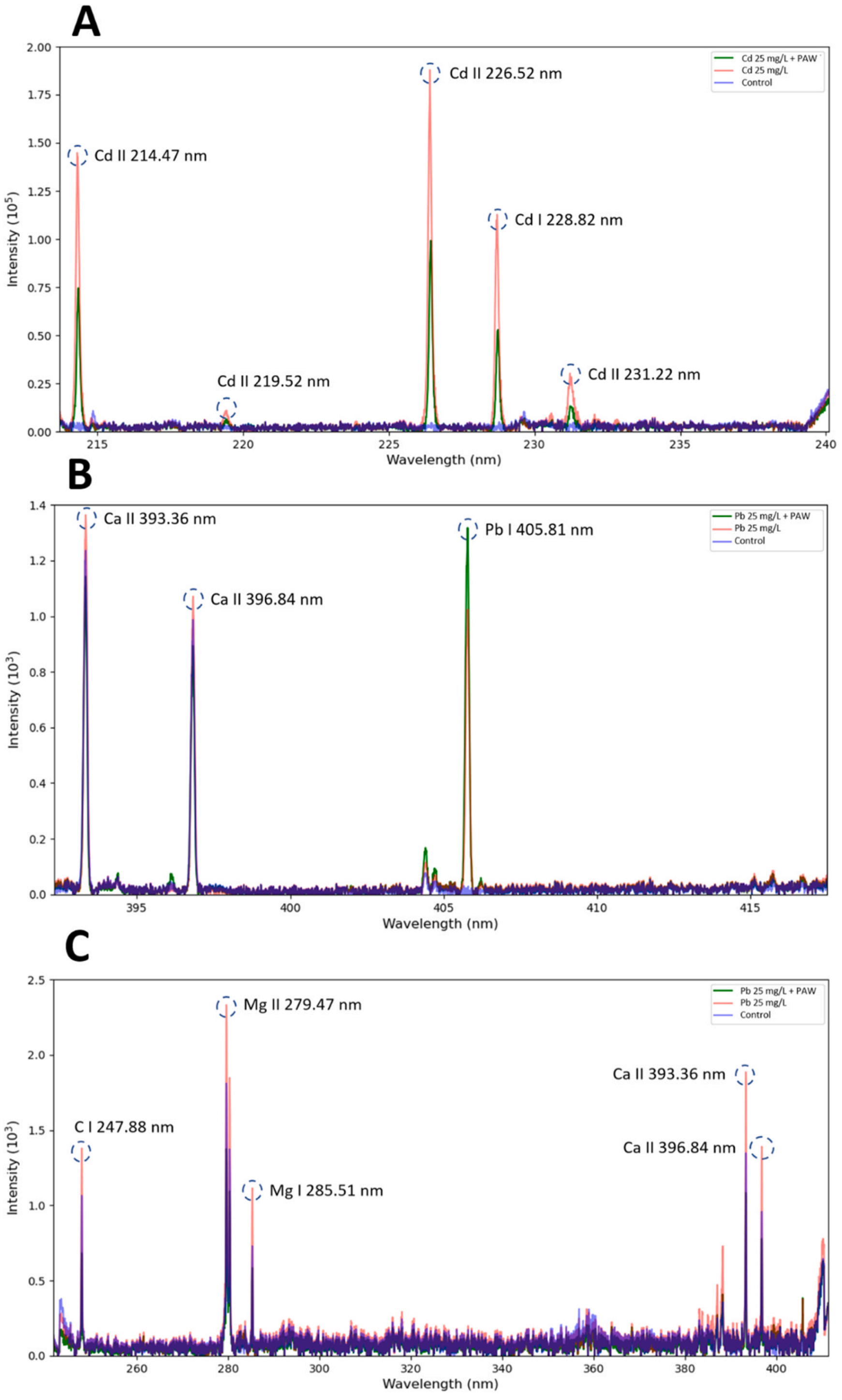

Fig. 4. Example of maximum emission spectra from the root of C. sativa plant. A: The samples were exposed to 25 mg/L Cd(II) (red), 25 mg/L Cd(II) together with PAW (green) and control plant in distilled water without Cd(II). Spectra were recorded with the Spectrometer1. B: Example of total spectra from the root of C. sativa plant. The samples were exposed to 25 mg/L Pb(II) (red), 25 mg/L Pb(II) together with PAW (green) and control plant in distilled water without Pb(IISpectra were recorded with the Spectrometer1. C: Example of total spectra of nutrients from the whole C. sativa plant recorded with the Spectrometer2. The samples were exposed to 25 mg/L Pb(II) (red), 25 mg/L Pb(II) together with PAW (green) and control plant in distilled water without Pb(II).

of fast determination of heavy metals and nutrient elements in different plant parts. We can assess the accumulating properties of different plant species by analysis of upper parts of the plants (stem, leaves). Furthermore, nutrient deficiencies can be better understood by detection of the exact distribution of elements.

4. Conclusions

In this study, we have explored the synergic effect of Plasma Activated Water and toxic metals on the growth and bioaccumulation of the hyperaccumulator *C. sativa*. Due to their co-application in hydroponic tests, we were able to observe the positive effect on the better growth of *C. sativa* with the PAW treatment in the case of the lead contamination.

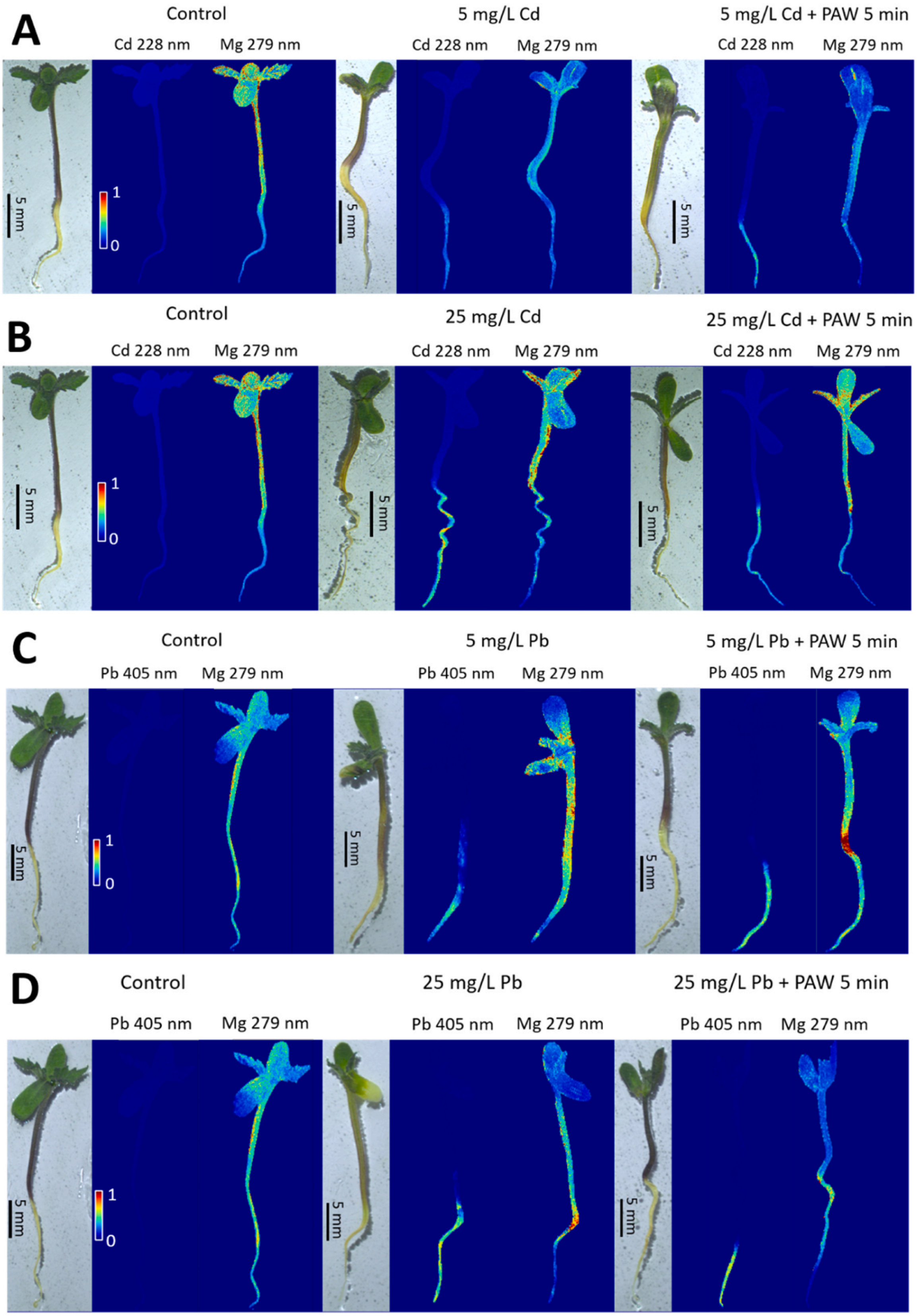

Fig. 5. Results of LIBS analysis of C. sativa seedlings after 3-day exposure to: A – 5 mg/L of Cd(II) and Cd(II) with PAW; B – 25 mg/L of Cd(II) and Cd(II) with PAW; C – 5 mg/L of Pb(II) and Pb(II) with PAW; D – 25 mg/L of Pb(II) and Pb(II) with PAW. All maps were recorded using the LIBS FireFly system. The colour scale is the same within the same element.

The impact of PAW on the plant bioaccumulation properties is still not fully understood. Hence, a robust methodology to investigate this phenomenon was proposed to bring more insights into this problem. The elemental maps recorded by LIBS showed that cadmium and lead accumulated primarily in the root of the plant. Although the PAW does not have a significant effect on the bioaccumulation of toxic metals, the accelerated growth may be of high benefits in phytoremediation, especially in the case of lead contamination, where there was no significant decrease of the growth with the exposition to lead together with PAW. These results serve as a proof-of-concept for further investigation of PAW on heavy metal contaminated plants and accumulators of metals. Application of PAW, either alone or in co-application with other phytoremediation strategies may be beneficial in better growth and higher yield of the plants in the contaminated areas, and potentially more effective remediation. However, further investigation must be carried out on plants grown in contaminated soil.

CRediT authorship contribution statement

Zdenka Kozáková: Writing – review & editing, Methodology, Conceptualization. **Pavel Pořízka:** Supervision, Funding acquisition. **Karel Novotný:** Supervision. **Pavlína Modlitbová:** Writing – review & editing, Methodology, Conceptualization. **Lucie Šimoníková:** Investigation, Data curation. **Daniel Holub:** Investigation, Data curation. **Ludmila Čechová:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Jozef Kaiser:** Supervision, Funding acquisition. **František Krčma:** Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Author contribution statement

Ludmila Čechová, Zdenka Kozáková and Pavlína Modlitbová conceived and designed the experiment; Ludmila Čechová prepared and analysed plasma activated water; Ludmila Čechová, Daniel Holub and Lucie Šimoníková prepared the plant samples, performed the experiments and processed all data; Karel Novotný and Pavel Pořízka supervised the ICP-OES and LIBS experiments; Ludmila Čechová and Daniel Holub wrote the original draft; Ludmila Čechová, Zdenka Kozáková and Pavlína Modlitbová edited the draft; František Krčma and Jozef Kaiser supervised all steps. All authors contributed to the article and approved the submitted version.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2024.116807.

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