Uptake of Co, Cs, Mn, Ni and Zn by Lemna minor and their effects on physiological and biochemical functions

Peer-reviewed author version

VAN DYCK, Isabelle; VANHOUDT, Nathalie; Vives i Batlle, Jordi; HOREMANS, Nele; Van Gompel, Axel; Nauts, Robin; Wannijn, Jean; WIJGAERTS, Ann; Vassilev, Andon & VANGRONSVELD, Jaco (2023) Uptake of Co, Cs, Mn, Ni and Zn by Lemna minor and their effects on physiological and biochemical functions. In: ENVIRONMENTAL AND EXPERIMENTAL BOTANY, 213 (Art N° 105440).

DOI: 10.1016/j.envexpbot.2023.105440
Handle: http://hdl.handle.net/1942/42411

Uptake of Co, Cs, Mn, Ni and Zn by *Lemna* minor and their effects on physiological and biochemical functions

Isabelle Van Dyck[1,2], Nathalie Vanhoudt[1], Jordi Vives i Batlle[1], Nele Horemans[1,2], Axel Van Gompel[1], Robin Nauts[1], Jean Wannijn[1], Ann Wijgaerts[2], Andon Vassilev[4] and Jaco Vangronsveld[2,3]

1 Belgian Nuclear Research Centre (SCK CEN), Biosphere Impact Studies, Boeretang 200, 2400 Mol, Belgium

2 UHasselt - Hasselt University, Centre for Environmental Sciences, Agoralaan Building D, 3590 Diepenbeek, Belgium

3 Maria Curie-Skłodowska University, Department of Plant Physiology and Biophysics, Institute of Biological Sciences, Akademicka 19, 20-033 Lublin, Poland

4 Agricultural University Plovdiv, Department of Plant Physiology and Biochemistry, Mendeleev blvd 12, 4000 Plovdiv, Bulgaria

1 Introduction

Pollutants into the environment origin from natural or anthropogenic sources (*e.g.* mining and milling, modern agricultural or industrial practices). Due to their persistence in the environment, non-biodegradability, potential for bioaccumulation and potential toxicity for biota, heavy metal pollution of aquatic environments is a considerable problem directly affecting plants and animals (Jamers et al., 2006; Martinez et al., 2019). A particular problem arises when pollutants do not exist in isolation but act in combination with each other, forming pollutant mixtures. It is even possible to find sites where additionally radionuclides are present (*e.g.* sites with naturally occurring radioactive materials) (Vanhoudt et al., 2021).

Lemna minor, commonly known as duckweed, is a small vascular plant from the *Lemnaceae* family, ubiquitous on the surface of still or slow-moving freshwater such as lakes and ponds where it is able to form dense mats (Driever et al., 2005; Lahive et al., 2011). It is characterized by a simple morphology, rapid growth and development, ease of cultivation and is sensitive to pollutants, making it of high interest in ecotoxicity tests (Monette et al., 2006; OECD, 2002; Oros et al., 2011). Moreover, *L. minor* can absorb and accumulate pollutants (*e.g.* heavy metals and radionuclides), making it interesting for phytoremediation applications (Oros & Toma, 2012; Ozyigit et al., 2021; Pratas et al., 2012).

L. minor and plants in general require essential elements for their growth, metabolism and reproduction. Essential elements consist of macronutrients (*e.g.* Ca, K, Mg, N, P and S) and micronutrients (*e.g.* Co, Cu, Fe, Cr, Mn, Ni and Zn). These are essential for organisms, but can also become toxic at higher concentrations and can accumulate. Other elements have no beneficial role (*e.g.* Cd, Cs, Hg and Pb). Despite being non-essential, they can be taken up, accumulate and cause toxic effects to the living organisms already at low concentrations (DalCorso, 2012).

Pollutants can be taken up and accumulated by *L. minor* by the same pathways as other elements and hence, pollutant uptake is influenced by the pH, temperature, pollutant concentrations in the water and other environmental factors (Arán et al., 2017; Nys et al., 2016; Ozyigit et al., 2021; Üçüncü et al., 2013). The uptake of pollutants by *L. minor* can be described as a two-step transfer process. Firstly, there is biosorption, which is a metabolically passive process of extracellular accumulation/precipitation and cell surface sorption/precipitation that occurs by complexation, co-ordination, chelation of metals, ion exchange, adsorption and micro precipitation (Chojnacka, 2010; Keskinkan et al., 2003). Biosorption is an initial, fast and reversible step lasting a few hours. Secondly, there is the bioaccumulation, namely the metabolically active intracellular accumulation of pollutants. Bioaccumulation involves a slow and irreversible step over a few days and includes transport of pollutants inside cells (Chojnacka, 2010; Keskinkan et al., 2003; Khellaf & Zerdaoui, 2009). When metals are taken up, they can interfere directly with the plant biological processes, resulting in physiological and biochemical changes (*e.g.* reduction of growth, inhibition of photosynthesis and respiration as well as functional modification of cell organelles) (Arán et al., 2017; Jayasri & Suthindhiran, 2017; Martinez et al., 2019; Vidaković-Cifrek et al., 2015). On the other hand, plants also have various mechanisms for metal detoxification and tolerance, such as control of metal influx, acceleration of metal efflux, metal chelation and sequestration, metal remobilization, scavenging of the metal-induced overproduction of reactive oxygen species by enzymatic antioxidants and production of phytohormones (which strengthen the antioxidative defense mechanism by activating antioxidative biosynthetic genes to mitigate metal stress) (Moustakas, 2023; Mustafa et al., 2023; Rahman et al., 2023).

Photosynthesis is the process in which light energy is absorbed by chlorophyll molecules in the thylakoid membrane and converted into ATP and NADPH. Both ATP and NADPH are, subsequently, used in the Calvin cycle to produce three carbon sugars in the stroma, like 3-phosphoglyceric acid,

which can be used for the formation of glucose, sucrose, starch and other carbohydrates. Plants use sugars for their general metabolism, growth and development. Excess of sugars can be stored in soluble form in vacuoles or in polymeric form as starch in plastids (Patrick et al., 2013). Starch and sugar content can be influenced by environmental parameters (salinity, nutrient deprivation, low or high temperatures, light intensity and photoperiod) (de Morais et al., 2019; Khavari-Nejad et al., 2009; Li et al., 2016; Pagliuso et al., 2018; Van Dyck et al., 2023; Yin et al., 2015; Zhao et al., 2014) or pollutants (*e.g.* heavy metals, radionuclides) (John et al., 2008; Mishra & Tripathi, 2008; Sree & Appenroth, 2014).

During photosynthesis, light energy is absorbed by chlorophyll molecules (present in pigment-protein complexes in photosystem II (PSII), PSI and light-harvesting complexes) and can be used in three competitive processes: (1) to drive photosynthesis, (2) to be re-emitted as heat or (3) to be re-emitted as light (chlorophyll fluorescence) (Maxwell & Johnson, 2000; Murchie & Lawson, 2013). Both photosynthetic pigments (Chl *a*, Chl *b* and carotenoids) and photosynthesis in general can be negatively affected by pollutant exposure (Jayasri & Suthindhiran, 2017). Photosynthesis can be followed by measuring *in vivo* chlorophyll fluorescence. In this technique, the quantification of the fluorescence yield is performed by illuminating a dark-adapted plant and measuring the re-emitted light. Thereafter the fluorescence level declines over a period of minutes, which is called fluorescence quenching (Maxwell & Johnson, 2000; Murchie & Lawson, 2013). The maximum quantum efficiency of PSII (F_v/F_m) is a simple and rapid measurement for monitoring stress (Baker, 2008). Additional stress indicators useful for the determination of plant toxicity are the photochemical and non-photochemical quenching (qP and NPQ, respectively), the photosynthetic efficiency or quantum yield of PSII (φPSII or Y(II)), the photosynthetic electron transport rate (ETR(II)), the fraction of open reaction centres (qL), the yield of non-photochemical quenching (Y(NPQ)), and the yield of the non-regulated energy dissipation (Y(NO)). Hence, measuring *in vivo* chlorophyll fluorescence can be an effective tool for sensing and assessing impact of metals and other toxins on *Lemnaceae*.

In this study, we selected five relevant trace elements (Co, Cs, Mn, Ni and Zn) based on the isotopic composition of waste waters typical for a nuclear installation. Due to the need to consider phytoremediation for mixtures of the (radio)elements ^{57}Co, ^{60}Co, ^{134}Cs, ^{137}Cs, generated and released to the environment by nuclear installations and medical facilities, in addition to the obvious interest in remediation of these elements as chemical pollutants, which can cause important toxic effects.

Cobalt is a component of vitamin B12 and an essential constituent of many enzymes (Sasmaz et al., 2016; Sree et al., 2015). It is involved in plant growth and development, but not proven to have an essential role for plants (Sasmaz et al., 2016). High concentrations can inhibit growth and photosynthesis, and can lead to chlorosis, decrease of chlorophyll content and starch accumulation (Begović et al., 2016; Hu et al., 2019).

Cesium has no known nutritional value for plants, however, it can rapidly incorporate into biological systems since it has large chemical similarities to K. Hence, Cs has been shown to be taken up through the K uptake channels and K presence can inhibit Cs uptake (Pinder III et al., 2006; Zhu & Smolders, 2000). In addition, Cs is phytotoxic at high concentrations, and will have negative effects on plant growth, disrupting photosynthetic pigments, changing ultrastructure of chloroplasts and causing necrosis. Critical Cs concentrations depend on species and concentration of other ions in solution (White & Broadley, 2000; Zhang & Liu, 2018).

Manganese is ubiquitous in soil, water, air and food (ATSDR, 2012; Zhou et al., 2019). It is an essential micronutrient for plant and animal growth and development. An excess of Mn is toxic and causes physiological and biochemical changes (Doganlar et al., 2012; Zhou et al., 2019), including photosynthesis inhibition by a decrease in pigment contents (Liu et al., 2017b).

Nickel has a low mobility in soil when pH > 6.5, but with environmental changes it can become more soluble and enter the groundwater (Ozyigit et al., 2021). It is an essential microelement for animals and plants, which improves plant growth and root development (Doganlar et al., 2012; Ozyigit et al., 2021). It can also induce toxic effects in plants when present in excess, such as changes in physiological and biochemical processes, *e.g.* inhibition of growth, oxidative stress, inhibition of enzymes, decrease of photosynthetic pigments and therefore inhibition of photosynthesis (Doganlar et al., 2012; Leblebici et al., 2017).

Lastly, Zinc is an essential element for all living organisms, which is present in the environment in a generally insoluble form (ATSDR, 2005). Zn is a cofactor for several enzymes related to protein synthesis and carbohydrate, nucleic acid and lipid metabolism (Martinez et al., 2019; Radić et al., 2010). Plants require it in trace amounts of Zn, and elevated concentrations cause phytotoxic effects (*e.g.* reduced growth, biomass reduction, chlorosis, reduced photosynthetic pigments) (Lahive et al., 2011; Lahive et al., 2012).

It is clear from the foregoing that the metals described above can have negative effects, but information is lacking for effects on *L. minor*'s physiological and biochemical functions. While effects on different levels in *L. minor* have been studied for some of these metals (*e.g.* Zn), information is scarce for others (*e.g.* Cs), and especially a comparison between these pollutants for their uptake and effects is not available (Ozyigit et al., 2021; Sree et al., 2015; Vidaković-Cifrek et al., 2015). Moreover, information related to the uptake mechanisms by *L. minor* (biosorption versus bioaccumulation) is missing for most of these metals. However, this knowledge is needed to better understand uptake and effects on *L. minor* when exposed to multiple pollutants, to improve impact assessments and to assess the possibility of using *L. minor* in phytoremediation applications. Therefore, the objective of this study was to have a more detailed and in depth comparison of the uptake mechanisms (biosorption and bioaccumulation) and effects (growth, photosynthetic pigments, photosynthesis, starch and soluble sugars content) of single pollutants (Co, Cs, Mn, Ni and Zn) on *L. minor*. This knowledge can contribute to the exploration of using *L. minor* in phytoremediation applications, since adverse effects of pollutants can interfere with *L. minor*'s removal capacity.

2 Materials and methods

2.1 *Plant material and pre-culture*

L. minor plants (Blarney plants, Serial number 1007, ID number 5500) originated from a pond in Blarney, Co. Cork, Ireland (University College Cork, Ireland), and were provided by Prof. Dr. M. Jansen and used in all experiments. Plants were cultivated in a non-sterile environment, in 1.5 L black containers (20 x 10 x 9 cm) filled with 0.65-0.70 L modified Hoagland solution (1:10) (1 mM KNO_3; 0.3 mM $Ca(NO_3)_2.4H_2O$; 0.2 mM $MgSO_4.7H_2O$; 0.1 mM $NH_4H_2PO_4$; 1.6 µM $FeSO_4.7H_2O$; 0.8 µM EDTA diNa-salt $2H_2O$; 4.6 µM H_3BO_3; 0.9 µM $MnCl_2.4H_2O$; 0.03 µM $CuSO_4.5H_2O$; 0.06 µM H_2MoO_4; 0.08 µM $ZnSO_4.7H_2O$; pH 5.6) and semi-closed with a square Petri dish to limit evaporation of the medium and prevent the potential drop of undesirable particles in the container.

The containers were placed into a plant growth chamber (Micro Clima MC1000, Snijders Scientific B.V., Tilburg, The Netherlands) under a 14h photoperiod with photosynthetic photon flux density (PPFD) of 98 µmol m^{-2} s^{-1} at frond level (supplied by three Valoya C-series C65 (spectrum NS12) LED lights producing a sun-like, wide spectrum), with day/night temperatures of 25°C and 65% relative humidity. To avoid growth of algae, the 1.5 L black containers were thoroughly washed with sodium hypochlorite (1-2%) and rinsed with demineralised water. The modified Hoagland solution was also sterilised before

use. To avoid crowding, every week a small amount (±650 mg) of *L. minor* plants with three or four fronds were transferred to fresh modified Hoagland solution.

2.2 *Experimental setup*

To study the effects of individual pollutants (Co, Cs, Mn, Ni and Zn) on *L. minor* growth, photosynthesis, photosynthetic pigments, starch and soluble sugars contents, five experiments were set up. For each experiment, a control condition was used together with four pollutant concentrations. The selection of the pollutant concentrations was made based on experimental half maximal effective concentrations (EC_{50} values) calculated from the fresh mass (FM) endpoint in a separate experiment within our lab (2.24 mg Co L^{-1}, 89.6 mg Cs L^{-1}, 84.7 mg Mn L^{-1}, 1.49 mg Ni L^{-1} and 9.0 mg Zn L^{-1}). For each metal, the lowest two concentrations were chosen below the EC_{50}, the third around the EC_{50} and the last one above the EC_{50}. The following concentrations were used: 0.5, 1, 1.75 and 2.5 mg Co L^{-1} (added as $Co(NO_3)_2.6H_2O$ (Merck Life Science B.V.)); 10, 25, 70 and 100 mg Mn L^{-1} (added as $MnSO_4.H_2O$ (Honeywell International Inc.)); 0.5, 0.75, 1.25 and 1.75 mg Ni L^{-1} (added as $NiSO_4.6H_2O$ (Merck Life Science B.V.)) and 1, 5, 10 and 20 mg Zn L^{-1} (added as $ZnSO_4.7H_2O$ (Honeywell International Inc.)). The concentration selection for Cs was based on a previous uptake experiment, where there was no Cs uptake at concentrations higher than the EC_{50} value. For this reason, concentrations below the EC_{50} were selected: 1, 10, 35 and 75 mg Cs L^{-1} (added as $CsNO_3$ (Alfa Aesar)). For the measurability of Cs, a radioactive ^{137}Cs tracer (Amersham International plc) was added to the solutions (50 kBq L^{-1}). Addition of this radioactive tracer resulted in a negligible increase of the total Cs concentration (1.5×10^{-5} mg L^{-1}).

At the start of each experiment, pollutant solutions were made in modified Hoagland solution, the pH was measured and 250 ml Nalgene polycarbonate pots (Thermo Scientific) were filled with 100 ml of the pollutant solutions. For the Cs experiment, the potassium concentration in the modified Hoagland solution was lowered to 10 mg L^{-1} to limit the competition between Cs and K for uptake by *L. minor*. No significant differences in specific growth rates were obtained between the modified Hoagland solution with 40 and 10 mg K L^{-1}. Also 10 ml water samples were taken of each Co, Ni, Mn and Zn conditions, filtered with an Acrodisc One 0.45 µm PTFE filter (Pall Corporation) and acidified with three droplets 1 M HCl from a Pasteur pipette for element analysis with ICP-OES. For the Cs conditions, 20 ml non-filtered water samples were taken for ^{137}Cs measurements with a gamma counter.

Non-sterile *L. minor* plants (50 mg) were transferred to the pots and a 1 cm surface-sterilised floating ruler was added to each pot as calibration for the images. Pots without plants were used for the exclusion of pollutant precipitation or adsorption on the pot. The pots were closed with a 9 cm Petri dish to avoid evaporation, pictures of the plants were taken and the total frond area at the beginning of the experiment was calculated using the pictures and the open source processing program ImageJ (version 1.52a) (Abràmoff et al., 2004). Pots were thereafter placed in a growth chamber (Micro Clima MC1000, Snijders Scientific B.V., Tilburg, The Netherlands) containing four Valoya C-serie C65 (spectrum NS12) LED lights producing a sun-like, wide spectrum. Reference growth conditions (25°C, 14h photoperiod, 148 µmol m^{-2} s^{-1} light intensity) were used as standard for all experiments. Plants were grown for 7 days, with at least three biological replicates (pots) per pollutant concentration and per endpoint analysis.

After an exposure time of 7 days, non-stirred water samples (10 ml) were taken of each pot in the climate chamber (pots with plants), filtered with a Acrodisc One 0.45 µm PTFE filter (Pall Corporation) and acidified with three droplets 1 M HCl from a Pasteur pipette for element analysis. For the Cs conditions, 20 ml non-stirred water samples were taken for ^{137}Cs measurements with a gamma

counter. Before taken water samples (10 or 20 ml) of the pots without plants, the pH of these solutions was adjusted to the pH of the pots with plants. Again, pictures of the plants were taken and the total frond area was calculated with the open source processing program ImageJ (version 1.52a). Subsequently, *L. minor* plants were harvested and used for different endpoint analyses: biosorption and bioaccumulation (2.3), *in vivo* chlorophyll fluorescence measurements (2.7), photosynthetic pigments (2.6), starch and soluble sugars (2.8). Specific growth rates based on FM of the plants after 7 days of growth were calculated according to OECD (2002), using the following equation:

$$\mu_{i-j} = \frac{\ln(N_j) - \ln(N_i)}{t_j - t_i} \quad (1)$$

where μ is the specific growth rate [day^{-1}] calculated from time point (*i*) and time point (*j*), *N* the amount of FM [mg] at the harvest of the plants, *i* the starting time point of the experiment [day] and *j* the end point of the experiment [day].

2.3 *Determination of biosorption and bioaccumulation fractions*

For *L. minor* plants that were exposed to Co, Mn, Ni and Zn, a distinction was made between the biosorption and bioaccumulation fractions. For plants exposed to Cs, no differentiation was made between both fractions due to safety restrictions related to working with open sources (radioactive ^{137}Cs tracer) in our facility. A washing protocol (adapted from Cuypers et al. (2011)) was executed for the determination of the biosorption fraction. All plants were harvested, patted dry and the FM in mg was determined. Afterwards, the plants exposed to Co, Mn, Ni and Zn were washed for 10 min with 15 ml 1mM $Pb(NO_3)_2$ at 4°C and twice for 10 min with 15 ml demineralised water at room temperature. All washing solutions were collected, filtered with a Acrodisc One 0.45 µm PTFE filter (Pall Corporation) and acidified with five droplets 1 M HCl from a Pasteur pipette for element analysis (see 2.5). These samples were considered the biosorption fractions.

After washing, the Co, Mn, Ni and Zn exposed plants were dried together with the Cs exposed plants at 55°C for at least 2 days to determine the dry mass (DM) in mg. The dried plant samples were transferred into 20 ml glass vials and put into the muffle furnace (Carbolite Gero CWF 1100) for the mineralisation (heating to 180°C, thereafter to 300°C and holding for 7 h, followed by heating to 550°C and an overnight temperature of 550°C) of the plant materials. Thereafter, samples were dissolved in 5 ml 1 M HCl and put on a sand bath to dry. A second run in the muffle furnace was performed (except for Mn samples). All Co, Mn, Ni and Zn samples were again dissolved in 10 ml 0.1 M HCl and filtered with a Acrodisc One 0.45 µm PTFE filter (Pall Corporation) for element analysis. These samples were considered the bioaccumulation fractions. The Cs samples were dissolved in 20 ml 0.1 M HCl for ^{137}Cs measurements using the gamma counter (see 2.5). These samples represent the Cs uptake after 7 days of growth.

2.4 *Calculation of pollutant removal parameters*

The amount of metal taken-up in the 7 day experiment by *L. minor* plants can be calculated by the difference in measured pollutant concentration in the pots without and with plants. Using this pollutant uptake and the DM of the *L. minor* plants after 7 days of exposure, the total removal per *L. minor* DM can be calculated. When expressing the pollutant removal per *L. minor* DM (*y*) [mg g^{-1}] as function of the different initial concentrations (*x*) [mg L^{-1}], a plateau phase is reached at a certain concentration. This plateau concentration is pollutant dependent and is modelled using the following Eq. (2), where *a* and *b* are two constants.

$$y = a\,(1 - e^{-bx}) \tag{2}$$

The percentages of pollutant removal, bioaccumulation, biosorption and residual faction are also calculated removal parameters using the amount of pollutants that is taken-up by *L. minor* in the 7 day experiment. The residual fraction is presented to define the theoretical loss of pollutants by harvesting the plants on absorbent paper or by performing the washing steps at which some pollutant can be lost. Presumably, the residual fraction is part of the reversible biosorption process.

$$Pollutant\ removal\ [\%] = \frac{pollutant\ uptake\ [mg\ L^{-1}]}{initial\ pollutant\ concentration\ [mg\ L^{-1}]} \times 100 \tag{3}$$

$$Bioaccumulation\ [\%] = \frac{bioaccumulation\ [mg\ L^{-1}]}{pollutant\ uptake\ [mg\ L^{-1}]} \times 100 \tag{4}$$

$$Biosorption\ [\%] = \frac{biosorption\ [mg\ L^{-1}]}{pollutant\ uptake\ [mg\ L^{-1}]} \times 100 \tag{5}$$

$$Residual\ fraction\ [\%] = \frac{pollutant\ uptake - \ bioaccumulation - biosorption\ [mg\ L^{-1}]}{pollutant\ uptake\ [mg\ L^{-1}]} \times 100 \tag{6}$$

The bioconcentration factor (BCF) was calculated (Eq. (7)) to determine the bioaccumulation capacity of *L. minor* for the initial pollutant concentration in the solution (Zayed et al., 1998). BCF is defined by the concentration of the pollutant in the plant tissue (C_{plant}) in [mg kg^{-1} DM] divided by the concentration of pollutant in the initial solution ($C_{initial\ solution}$) in [mg L^{-1}]. For Co, Mn, Ni and Zn, the bioaccumulated fraction is used for the calculation of the BCF. For Cs no differentiation was made between bioaccumulation and biosorption, so both fractions were taken into account for the calculation of the BCF for Cs.

$$BCF = \frac{C_{plant}}{C_{initial\ solution}} \tag{7}$$

2.5 *Element analysis*

The concentrations of the Co, Mn, Ni and Zn samples were determined by inductively coupled plasma - optical emission spectrometry (ICP-OES) (Agilent 710-ES ICP-OES, Agilent Technologies, Inc.). Multi-element standard solutions of 1000 mg L^{-1} for ICP (CPAchem) were diluted to concentrations of 0.1, 0.25, 0.5, 1, 2.5, 5, 10 and 25 mg L^{-1} and used in each analysis run. An internal control of 5 mg L^{-1} Co, Mn, Ni and Zn was also measured at least after every 20 measurements. Co, Mn, Ni and Zn concentrations were measured at the following wavelengths: 228.615; 257.610; 231.604 and 213.857 nm respectively.

The use of a radioactive ^{137}Cs tracer in combination with the stable Cs has the possibility to determine the ^{137}Cs concentration with a gamma counter and the stable Cs concentration after a conversion. The ^{137}Cs activities were determined using the Perkin Elmer Wallac 1480 Wizard 3 Gamma Counter. The detector (thallium activated, sodium iodide crystal) had the near 4π geometry of a well type detector and was surrounded by 50 mm of lead shielding above and below. The shielding against the conveyor was 75 mm of lead. All 20 ml Samples were measured directly in the gamma counter. Reagent blanks (demineralised water) and ^{137}Cs standards were included in each measurement to take background radiation into account and to determine measurement efficiency and energy calibration.

2.6 *Photosynthetic pigment analysis*

For determination of the photosynthetic pigments (chlorophyll *a*, chlorophyll *b* and carotenoids) FM samples between 10 and 20 mg were collected in 1.5 ml Eppendorf tubes and 0.5 ml dimethylformamide (DMF) was added. After vortexing the samples and a 1.5 min centrifugation at 21130 *g* (Eppendorf centrifuge 5424R), the extraction was performed in dark conditions for 24 h at 4°C. After vortexing all samples again and a 1.5 min centrifugation at 21130 *g* (Eppendorf centrifuge 5424R), the absorbance of the supernatant was measured at 664, 647 and 480 nm in a 96-well DMF resistant polypropylene plate (UV-Star microplate, Greiner Bio-One). Chl *a*, Chl *b* and carotenoid concentrations were calculated according to Wellburn (1994).

2.7 *In vivo measurement of chlorophyll fluorescence*

Measurements of chlorophyll fluorescence were performed using the Dual-PAM-100 Measuring System (Heinz Walz, Germany). After an exposure time of 7 days, 5-6 *L. minor* plants per condition were transferred to a sterile 6-well plate filled with 7.5 ml demineralised water in each well. *L. minor* plants were adapted to dark conditions for at least one hour before measuring the induction and light curve. For the measurements of the induction curve the dark-adapted *L. minor* plants were transferred to the demineralised water surface in a 1 by 1 cm cuvette and used for measuring. The minimal level of fluorescence (F_0) was measured with a very low light pulse (53 µmol m^{-2} s^{-1}), followed by the determination of the maximal level of fluorescence in the dark-adapted state (F_m) by using a saturation light pulse (15000 µmol m^{-2} s^{-1}, 0.8 s). After a delay time of 40 s, the actinic light is switched on to induce photosynthesis. Fluorescence yield will quickly rise to a second maximum peak (F_m'), followed by a biphasic decline (Kautsky effect) (Maxwell & Johnson, 2000). Thereafter, a second saturation pulse is given (1 s after the actinic light), followed by saturation pulses every 15 s during a total run time of 300 s. From these results, some other photosynthetic parameters can be calculated: the maximum quantum yield of PSII (F_v/F_m with $F_v = F_m - F_0$), the photochemical quenching (qP = (F_m' - F) / (F_m' - F_0)), non-photochemical quenching (NPQ), the photosynthetic efficiency of PSII (φPSII = (F_m' - F)/F_m'), the fraction of open reaction centres (qL), the regulated energy dissipation via the xanthophyll cycle (Y(NPQ)) and the non-regulated energy dissipation (Y(NO)).

Next, by measuring the rapid light curve from light adapted plants, the photosynthetic electron transport rate at PSII reaction centres (ETR(II) [µmol m^{-2} s^{-1}]) is determined by emitting increasing saturated light pulses at certain time intervals (30 s). ETR(II) values were plotted as function of different PPFD values and a curve was fit using the Levenberg-Marquardt nonlinear least-squares model (Moré, 1978). Three photosynthetic parameters (ETR_{max}, α, Ek) were calculated from the modelled curves (Ralph & Gademann, 2005). ETR_{max} is the maximal capacity of the electron transport chain to transfer electrons, α the photosynthetic rate in limited light conditions and Ek the minimal saturating irradiance, calculated as ETR_{max}/α.

2.8 *Starch and soluble sugars analysis*

Starch and soluble sugars contents in *L. minor* were determined using an anthrone reagent, following a protocol adapted from Hansen and Møller (1975). Freshly harvested plants were dried at 55°C for at least 2 days and DM samples between 10 and 20 mg were collected in a 2 ml Eppendorf tube. Three stainless steel beads (2.3 mm) were added to each sample and the samples were shred using the Mixer Mill MM400 (Retsch) for 4 min at 30 Hz. Interfering pigments were extracted with 4 times 1 ml 100% (v/v) acetone, vortexing, centrifugation for 1.5 minutes at 21130 *g* (Eppendorf centrifuge 5424R) and

removal of the supernatant. The stainless steel beads were also removed in the last extraction step. Subsequently, sugars were extracted with 2 times 1 ml 80% (v/v) ethanol and all transferred into a 15 ml tube. The total volume was set to 5 ml. All samples were centrifuged for 5 min at 953 *g* (Rotina 420R, Hettich Zentrifugen). The supernatant was transferred to another 15 ml tube for soluble sugars analysis. 5 ml 1.1% (v/v) HCl was added to the residue for hydrolyzing the remaining starch and homogenized by vortexing or shaking, followed by a 30 min heating in a 100°C water bath. The total volume was thereafter set to 10 ml by adding demineralised water.

For the starch and soluble sugars analysis, standard solutions of starch and glucose with following concentrations 0; 0.1; 0.2; 0.4; 0.6; 0.8 and 1.0 mg starch or glucose ml^{-1} were made in respectively 1.1% (v/v) HCl and 80% (v/v) ethanol. 100 µl of the samples or standards was pipetted into a 2 ml Eppendorf tube and put on ice. To each tube, 500 µl ice-cold anthrone reagent (0.5 g anthrone in 250 ml 72% (v/v) sulphuric acid) was added, all samples were vortexed and heated for 11 min in a 100°C water bath. Thereafter, all samples were rapidly cooled on ice and vortexed again. The absorbance was measured at 630 nm in a 96-well polypropylene plate (UV-Star microplate, Greiner Bio-One) and the concentration starch or soluble sugars was calculated using the standards.

2.9 *Statistical analysis*

Statistical analysis was performed with the open-source software package RStudio (2022.02.3 Build 492). Normal distribution of the data was tested using a Shapiro-Wilk test, the homogeneity of the variances was tested using a Breusch-Pagan test. Transformations (log or square root) were used where needed to have a normal distribution and homoscedasticity. To identify any statistical differences between conditions, a one-way ANOVA and Tukey HSD test was performed ($p < 0.05$). When data were not normally distributed and there is no homogeneity of variances, a Kruskal-Wallis test followed by a Conover's test (Multiple Comparisons of Mean Rank Sums) was performed to identify any statistical differences between the conditions.

3 Results and discussion

3.1 *Effects on growth*

Specific *L. minor* growth rates were calculated based on the *L. minor* FM accumulated over 7 days (Fig. 1). The control plants had a specific growth rate of 0.254 ± 0.005 day^{-1}. Almost all pollutant concentrations had a negative effect on the growth rate from the lowest concentration on. With further increase of the pollutant concentration, the specific growth rate decreased significantly.

Cobalt concentrations of 0.5 and 1 mg L^{-1} resulted in a significant decrease of the specific growth rate of respectively 12 and 11%. With increasing the Co concentrations, the specific growth rate further significantly decreased with 22% for both 1.75 and 2.5 mg L^{-1} Co. Similar growth rates were observed by Ince et al. (1999), who exposed *L. minor* to different concentrations of Co (using $CoCl_2$) for 7 days showing that higher concentrations of Co resulted in more growth inhibition. Begović et al. (2016), exposed *L. minor* to two concentrations of $CoCl_2$ (0.01 and 1 mM) for 24, 48 and 72h. The lowest concentration tested (0.01 mM $CoCl_2$ equals approximately 0.6 mg Co L^{-1}) had no significant effect on the growth rate compared to the control condition, whereas after 7 days in our experiment the growth rate already significantly decreased with 12% at 0.5 mg Co L^{-1}. Higher percentages of growth rate inhibition were also observed by Sree et al. (2015). They exposed *L. minor* plants for 7 days to different concentrations of $CoCl_2.6H_2O$ which makes it possible that the higher decreases in growth rates were

due to the different chemical forms of Co that were used ($CoCl_2.6H_2O$ instead of $Co(NO_3)_2.6H_2O$). Indeed, chloride ions create an environment which is more saline compared to the nitrate ions, which can result in a decrease of growth (Liu et al., 2017a).

The lowest Cs concentration (1 mg L^{-1}), had no significant effect on the specific growth rate of *L. minor* (Fig. 1). Higher Cs concentrations (10, 35 and 75 mg L^{-1}) resulted in a significant decrease of the specific growth rate compared to the control condition (16, 39 and 47% decrease, respectively). Little information is known about the effects of Cs on growth rates of *Lemnaceae*, but Zhang and Liu (2018) reported that excessive amounts of stable Cs induced slow and abnormal growth in *Brassica. juncea* seedlings. In the bacteria *Rhodococcus*, Cs inhibited bacterial growth and uptake at concentrations higher than 5 mM (approximately 665 mg Cs L^{-1}) (Ivshina et al., 2002).

Manganese concentrations of 10 and 25 mg L^{-1} had no effect on the specific growth rate of *L. minor* (Fig. 1). The growth rate significantly decreased from concentrations around and above the EC_{50} value (70 and 100 mg L^{-1}), with respectively 33 and 57%. Very similar results were reported by Liu et al. (2017b), who exposed *Spirodela polyrhiza* to 5, 30, 50 and 70 mg Mn L^{-1} for 1 to 9 days. After 7 days, no significant effects on specific growth rates were observed between the enhanced Mn concentrations and the control. In our study, however, 70 mg Mn L^{-1} resulted in a significant decrease. The difference in results is most likely due to the different plant species used by Liu et al. (2017b).

Elevated Ni concentrations in the Hoagland growth medium (0.5, 0.75, 1.25 and 1.75 mg Ni L^{-1}) all resulted in significant decreases of the specific growth rate (22, 26, 34 and 54% decrease) (Fig. 1). Between the Ni concentrations, also significant differences were observed. Our results are in line with those of Martinez et al. (2019), where specific growth rates of *L. gibba* after 7 days decreased with increasing Ni concentrations (0.18, 0.37, 0.92, 1.46, 3.61, 6 and 11.82 mg Ni L^{-1}). In that case, the decrease was only significant at the highest tested Ni concentration. Leblebici et al. (2017) studied growth rates of *L. minor* after 7 days exposure to 1, 5, 10 and 20 mg Ni L^{-1}. Here all growth rates significantly decreased with increasing Ni concentrations.

When Zn was added (1, 5, 10 and 20 mg L^{-1}), the specific growth rates significantly decreased with 16, 42, 58 and 69% respectively. Other studies also reported decreases of the growth rate when *L. minor* was exposed to different concentrations of Zn. Vidaković-Cifrek et al. (2015) reported significant decreases of the growth of *L. minor* up to 50% at Zn concentrations of 25 and 50 µmol L^{-1} (approximately 1.6 and 3.2 mg Zn L^{-1}) after 7 days. These concentrations are lower than in our experiment. Also in this case, salinity might explain the lower growth rates, because $ZnCl_2$ was used. Lahive et al. (2011) used one Zn concentration (10 mg L^{-1}) similar that what we used to expose *L. minor* for 7 days. They also applied the same as sulphate salt ($ZnSO_4.6H_2O$) as in our study and found a growth inhibition of about 45% compared to the control, which is in line with our results (42% of the control growth rate). All higher Zn concentrations also lead to a further decrease of the growth rate.

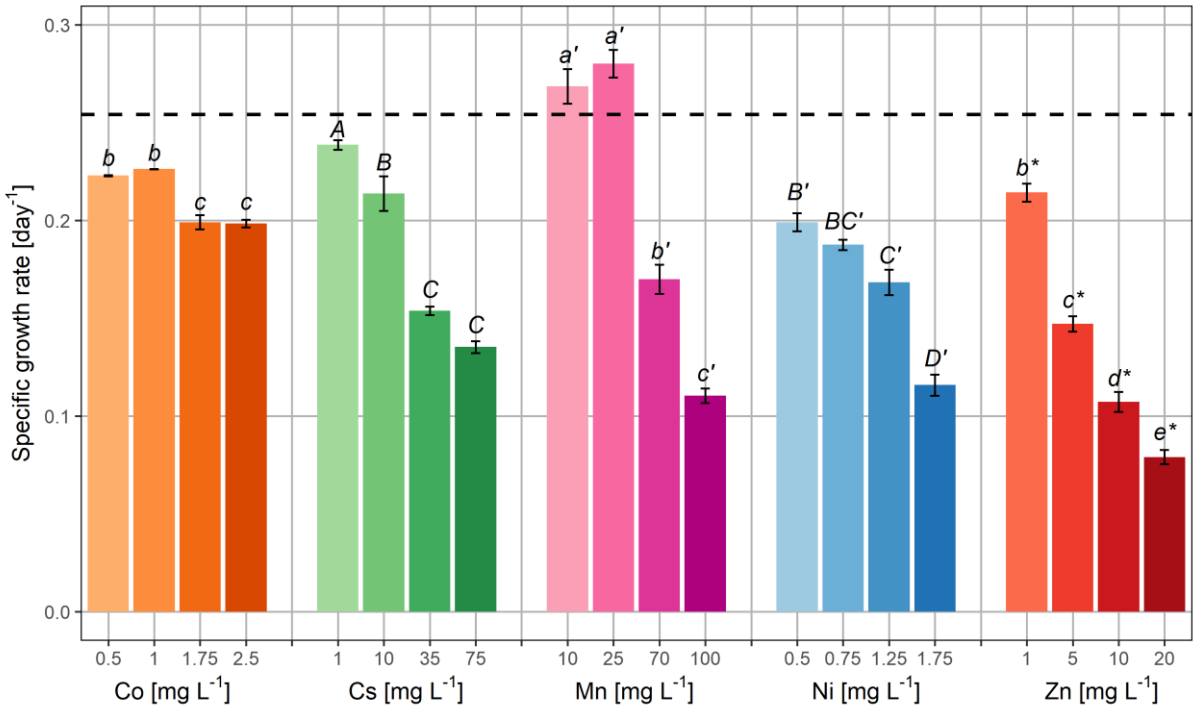


Fig. 1 Specific growth rate [days^{-1}] calculated for L. minor fresh mass (FM). Plants were exposed for 7 days to different metals and concentrations. Data points represent the mean value of at least three biological replicates together with the standard error of the means. Statistical analyses were performed on each data set. The control condition is represented by the black dashed line, with letter a, A, a', A' and a (not given for simplicity reasons). Different letters indicate significant differences between concentrations of a pollutant and the control condition ($p < 0.05$).*

3.2 *Effects on element uptake, biosorption and bioaccumulation*

After 7 days of exposure of *L. minor* plants to different pollutants and concentrations, uptake of the various elements was determined using ICP-OES (Co, Mn, Ni and Zn) or a gamma counter (Cs with radioactive ^{137}Cs tracer) (Fig. 2). In Table 1, distinction is made between bioaccumulation, biosorption and a residual fraction.

For Co and Zn, the amounts taken up during the experimental period (Fig. 2) significantly increased with increasing concentrations. The uptake of Mn significantly increased at 10 and 25 mg L^{-1}. At higher concentrations (70 and 100 mg Mn L^{-1}), however, the uptake did not change compared to that at 25 mg Mn L^{-1}. For Cs and Ni, significant increases were observed up to 35 mg Cs L^{-1} and 1.25 mg Ni L^{-1}, and no further increase occurred at 75 mg Cs L^{-1} and 1.75 mg Ni L^{-1}. In general, the higher the initial concentration of the pollutant, the higher the uptake. It is important to note that the K concentration was lowered in our Cs exposure experiments, since the presence of K can inhibit Cs uptake. It was reported that Cs is taken-up through the same transport channels as K, which can explain lower Cs removal in case higher K concentrations as a result of ion competition. The more K is present, the more competition between Cs and K, resulting in lower Cs uptake through the transport channels and conceivably less effects on *e.g.* growth (Smolders et al., 1996).

This is also the case when considering the pollutant removal per *L. minor* DM as function of the different initial concentrations. The following maximum removal values per *L. minor* DM were obtained from the modelled curves (Fig. S 1): 7.9 mg Co g^{-1} DM, 14.1 mg Cs g^{-1} DM, 29.6 mg Mn g^{-1} DM, 3.0 mg Ni g^{-1} DM and 13.1 mg Zn g^{-1} DM. Mn had the highest total removal value per *L. minor* DM and Ni the lowest.

When considering the removal percentage (Table 1), in which also the initial pollutant concentration is taken into account, there is an overall decrease with increasing pollutant concentrations. When comparing all tested metals and concentrations, we see that the highest removal percentage occurred for the lowest tested Co concentration (0.5 mg Co L^{-1}, 70.7% ± 0.9%), while the lowest removal percentage was observed for the highest Cs concentration (75 mg Cs L^{-1}, 2.8% ± 0.2%). The concentration range of Zn has the widest spread removal going from 67.3% ± 0.8% at 1 mg Zn L^{-1} to 8.1% ± 0.4% at 20 mg Zn L^{-1}. For the Ni concentrations, the removal percentage showed the narrowest range, meaning that even a concentration above the theoretical EC_{50} still results in some removal. Both Axtell et al. (2003) and Goswami and Majumder (2015) found overall Ni removal percentages of 87% and 82% respectively. These percentages are not in line with our observations, but another experimental setup, growth medium and plant species undoubtedly are reason for the observed differences. Saleh et al. (2020) studied Co and Cs uptake in the submerged species *Myriophyllum spicatum*, where more Co uptake was found compared to Cs as a result of the more unfavourable features of Cs for plants. Looking at the 1 mg L^{-1} concentration condition for Co and Cs in our experiments, more Co (57.5% ± 0.9%) than Cs (38.4% ± 2.1%) was removed by *L. minor*. Cs appears to be more toxic for *L. minor* than Co.

As said before, the removal of pollutants by *L. minor* can be described with a two-phase process, that is, biosorption (the initial, fast and reversible phase during the first hours of the uptake) followed by bioaccumulation, the slow and irreversible phase that continue up to a few days (Keskinkan et al., 2003; Khellaf & Zerdaoui, 2009). It is clear from our results in Table 1 that, for all metals tested, the bioaccumulation is more important in the total amount of metal uptake than biosorption. Both bioaccumulation (Fig. S 2) and biosorption (Fig. S 3) show the tendency to increase with increasing pollutant concentrations. In most cases, the bioaccumulation and biosorption fraction is not significantly different for the two highest pollutant concentrations comparted to the others. Other authors also observed a concentration-dependent bioaccumulation for Co and Zn (Begović et al., 2016; Hu et al., 2019; Vidaković-Cifrek et al., 2015). Sasmaz et al. (2016) performed an uptake experiment of Ni and Co over 8 days, where both Ni and Co were bioaccumulated by *L. minor*. The longer the exposure time to the pollutant, the more pollutant could be bioaccumulated.

Besides the bioaccumulated fraction, the BCF was calculated as well (Table 1), this being an important parameter for gaining more information on the metal uptake. In general the BCF decreased for each pollutant with increasing concentrations. With 0.5 mg Co L^{-1} the highest BCF (3260 ± 80) was observed, meaning that *L. minor* is able to accumulate a lot of Co in its biomass under the used experimental conditions. The 75 mg Cs L^{-1} had the lowest BCF (190.6 ± 0.8), resulting in less bioaccumulation, but still a sufficient amount is accumulated in *L. minor*'s biomass. Vidaković-Cifrek et al. (2015) presented two BCFs for exposure of *L. minor* to two different Zn concentrations (25 and 50 µmol L^{-1}, approximately 1.6 and 3.2 mg Zn L^{-1}). By increasing the Zn concentration, bioaccumulation significantly increased and the BCF significantly decreased (from BCF 2217.3 for 25 µmol Zn L^{-1} to BCF 1365.1 for 50 µmol Zn L^{-1}), which was the same in our experiments. Leblebici et al. (2017) studied the accumulation of Ni in *L. minor*. The amount of bioaccumulation increased with increasing Ni concentrations and the BCF decreased (from a BCF of approximately 3000 at 1 mg Ni L^{-1} to 1500 at 25 mg Ni L^{-1}), as in our experiments. A decrease in BCF with increasing Co concentrations was also observed for *L. gibba* (Al-Zurfi et al., 2018). We note that uptake experiments of Co and Cs with *M. spicatum* were performed by Saleh et al. (2020). Their BCF calculations were only performed for the experiments with ^{60}Co and ^{137}Cs and not with the stable elements. The BCF for ^{137}Cs (27.13) was higher than for ^{60}Co (10.80), but different initial activities were used, where ^{137}Cs almost had 50 times more activity compared to ^{60}Co. Looking at the BCF in our data for 1 mg L^{-1} Co (2690 ± 40) and Cs (2670 ± 170), almost no difference

was observed, meaning that using Co and Cs at the same concentrations, results in a similar bioaccumulation by *L. minor*.

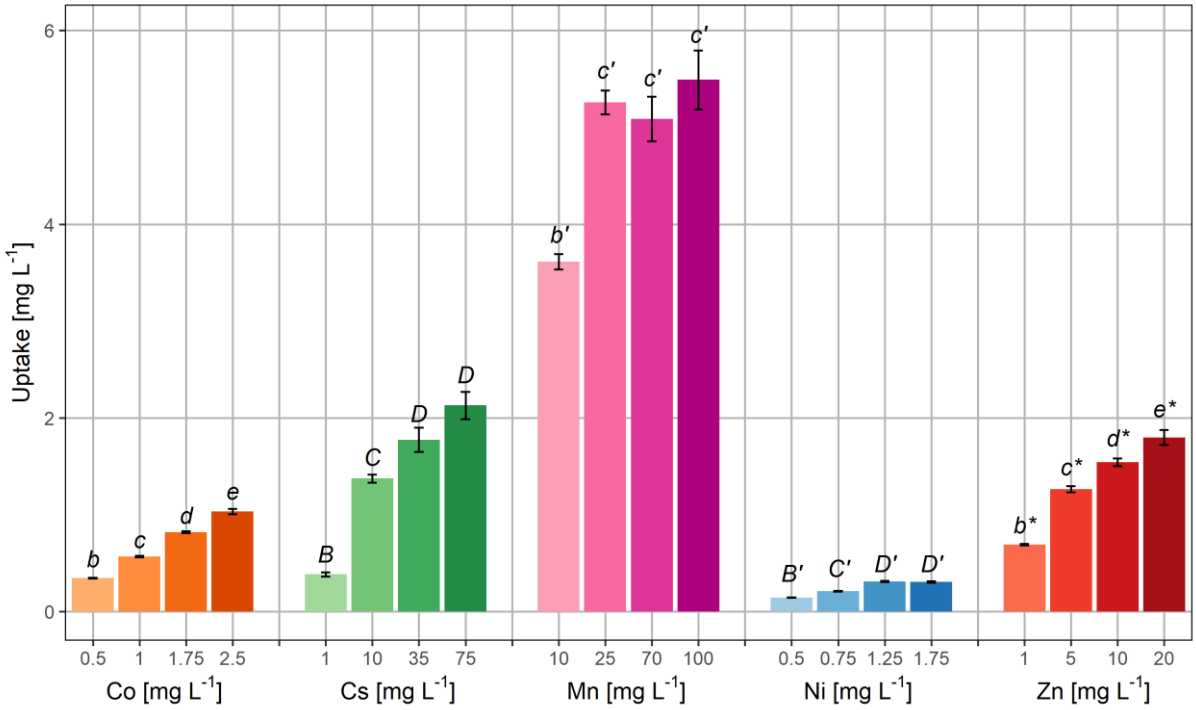


Fig. 2 Total amount of pollutant uptake after 7 days by L. minor exposed to different pollutants and concentrations. Data points represent the mean value of at least six biological replicates together with the standard error of the means. The control condition is not given on the graph, but represented with letter a, A, a', A' and a (not given for simplicity reasons). Different letters indicate significant differences between concentrations of a pollutant, including the control condition ($p < 0.05$).*

Table 1 Pollutant removal per L. minor DM [mg g^{-1}], pollutant removal [%] and BCF of L. minor after 7 days exposure to different pollutants and concentrations. For Co, Mn, Ni and Zn a division of the pollutant uptake in bioaccumulation, biosorption and a residual fraction in percentage is also given. Data represent the mean value of at least three biological replicates together with the standard error of the means. The control condition is not presented in the table and not included into the statistical analysis. Different letters indicate significant differences between concentrations of a pollutant ($p < 0.05$).

Condition		Pollutant removal per *L. minor* DM [mg g^{-1}]		Pollutant removal [%]		Bioaccumulation [%]		Biosorption [%]		Residual Fraction [%]		BCF	
Co [mg L^{-1}]	0.5	1.75 ± 0.06	a	70.7 ± 0.9	a	92 ± 5		1.4 ± 0.5		7 ± 5		3260 ± 80	a
	1	2.99 ± 0.08	b	57.5 ± 0.9	b	89 ± 3		2.1 ± 0.1		9 ± 3		2690 ± 40	b
	1.75	4.19 ± 0.09	c	46.5 ± 0.6	c	97 ± 3		2.3 ± 0.5		1 ± 3		2300 ± 40	c
	2.5	5.54 ± 0.18	d	41.7 ± 1.2	c	83 ± 3		1.8 ± 0.4		15 ± 3		1850 ± 20	d
Cs [mg L^{-1}]	1	1.98 ± 0.11	A	38 ± 2	A							2670 ± 170	A
	10	7.9 ± 0.3	B	13.7 ± 0.4	B							900 ± 16	B
	35	12.3 ± 0.9	C	5.1 ± 0.4	C							335 ± 13	C
	75	14.7 ± 1.0	C	2.8 ± 0.2	D							190.6 ± 0.8	D
Mn [mg L^{-1}]	10	15 ± 2	a'	34.6 ± 0.8	a'	94 ± 6		3.7 ± 0.5		2 ± 6		1420 ± 160	a'
	25	24.5 ± 1.4	ab'	18.9 ± 0.4	b'	92 ± 7		4.9 ± 0.3		4 ± 7		800 ± 18	b'
	70	27 ± 2	b'	6.9 ± 0.3	c'	93 ± 9		5.3 ± 0.3		1 ± 10		347 ± 9	c'
	100	31.6 ± 1.8	b'	5.0 ± 0.3	d'	96 ± 7		6.4 ± 0.8		-2 ± 8		274 ± 11	c'
Ni [mg L^{-1}]	0.5	0.80 ± 0.04	A'	30.1 ± 0.5	A'	80 ± 4		7.2 ± 0.9		13 ± 4		1340 ± 8	A'
	0.75	1.19 ± 0.05	B'	29.1 ± 0.6	AB'	70.3 ± 1.7		8.2 ± 1.0		22 ± 2		1170 ± 30	A'
	1.25	1.86 ± 0.06	C'	26.1 ± 0.5	B'	58 ± 3		7.5 ± 0.5		34 ± 3		920 ± 60	B'
	1.75	2.03 ± 0.06	C'	18.5 ± 0.6	C'	44 ± 5		9.4 ± 1.0		46 ± 6		550 ± 60	C'
Zn [mg L^{-1}]	1	3.53 ± 0.14	a*	67.3 ± 0.8	a*	43 ± 13		1.4 ± 0.4		55 ± 13		1500 ± 400	a*
	5	7.4 ± 0.5	b*	23.7 ± 0.6	b*	59 ± 3		7.2 ± 0.4		34 ± 4		820 ± 40	ab*
	10	10.8 ± 0.5	c*	14.1 ± 0.4	c*	56 ± 4		8.6 ± 0.3		36 ± 4		552 ± 18	b*
	20	13.1 ± 0.6	d*	8.1 ± 0.4	d*	48 ± 7		11.0 ± 0.6		41 ± 8		290 ± 40	c*

3.3 *Effects on photosynthetic pigments*

Photosynthetic pigments (Chl *a*, Chl *b* and carotenoids) were determined in *L. minor* plants after 7 days of exposure to different pollutants and concentrations. Results are presented relative to the control condition (Table 2). For each pollutant, the same trend is observed for Chl *a*, Chl *b* and carotenoid contents. In general, increasing the pollutant concentration resulted in a significant decrease of all photosynthetic pigments. The highest pollutant concentrations all resulted in the lowest photosynthetic pigment concentrations in *L. minor*. Some exposure conditions (1 and 10 mg Cs L^{-1}; 10, 25 and 70 mg Mn L^{-1} and 0.5 mg Ni L^{-1}) had little or no effect on the photosynthetic pigment levels compared to the control condition. Similar results have been reported before for *L. minor* or other duckweed/plants species where pigments contents also decreased with increasing concentrations of Co (Sree et al., 2015), Cs (Zhang & Liu, 2018), Mn (Liu et al., 2017b), Ni (Appenroth et al., 2010) and Zn (Radić et al., 2010).

The decreased concentrations of photosynthetic pigments as function of metal concentration, together with the lowered specific growth rates, can be attributed to metal toxicity (Radić et al., 2010). The rate of decrease is the same for all elements. The effect of Co, Cs, Mn, Ni and Zn is the highest on Chl *a* content, followed by Chl *b* and carotenoids. Chl *a* is an important photoreceptor for the absorption of light and the main pigment in photosynthesis, resulting in the inhibition of photosynthesis. Chl *b* and carotenoids are both accessory pigments and play an important role in protecting Chl *a*, but they also suppress damaging photochemical reactions (Jayasri & Suthindhiran, 2017). The lowered photosynthetic pigment contents could be due to (a) impairment of the electron transport chain; (b) replacement of Mg^{2+} ions present in the tetrapyrrole ring of Chl; (c) inhibition of enzymes involved in the biosynthesis of Chl; (d) over-accumulation of metals in chloroplasts leading to oxidative stress; (d) altered expression of genes shifting towards defence (Jayasri & Suthindhiran, 2017; Leblebici et al., 2017; Radić et al., 2010; Van Hoeck et al., 2017).

Table 2 Relative concentration of chlorophyll a, chlorophyll b and carotenoid [µg mg^{-1} FM] in L. minor plants exposed to different pollutants and concentrations for 7 days. Results are expressed relative to the control condition. Data represent the mean value of nine biological replicates together with the standard error of the means. Different letters indicate significant differences between concentrations of a pollutant (including the control condition) (p < 0.05).

Condition		Chlorophyll *a* [µg mg^{-1} FM]				Chlorophyll *b* [µg mg^{-1} FM]				Carotenoid [µg mg^{-1} FM]			
Control		1.000	±	0.007	a/A/a' A'/a*	1.000	±	0.006	a/A/a' A'/a*	1.000	±	0.006	a/A/ab' A'/a*
Co [mg L^{-1}]	0.5	0.930	±	0.008	a	0.937	±	0.010	a	0.971	±	0.008	ab
	1	0.835	±	0.006	b	0.832	±	0.006	b	0.891	±	0.006	bc
	1.75	0.753	±	0.008	c	0.798	±	0.010	bc	0.820	±	0.008	cd
	2.5	0.672	±	0.006	d	0.724	±	0.010	c	0.770	±	0.008	d
Cs [mg L^{-1}]	1	1.012	±	0.013	A	0.997	±	0.013	A	1.014	±	0.013	A
	10	0.991	±	0.014	A	1.024	±	0.015	A	0.965	±	0.014	A
	35	0.710	±	0.009	B	0.768	±	0.010	B	0.750	±	0.009	B
	75	0.642	±	0.008	B	0.674	±	0.008	B	0.728	±	0.008	B
Mn [mg L^{-1}]	10	1.055	±	0.014	a'	1.073	±	0.012	a'	1.050	±	0.013	a'
	25	0.952	±	0.006	a'	1.004	±	0.006	a'	0.991	±	0.006	a'
	70	1.018	±	0.008	a'	1.032	±	0.008	a'	1.046	±	0.008	a'
	100	0.641	±	0.017	b'	0.721	±	0.015	b'	0.776	±	0.015	b'
Ni [mg L^{-1}]	0.5	0.979	±	0.010	A'	1.001	±	0.009	A'	1.011	±	0.009	A'
	0.75	0.857	±	0.006	B'	0.864	±	0.006	B'	0.933	±	0.006	AB'
	1.25	0.841	±	0.009	B'	0.846	±	0.010	B'	0.895	±	0.008	B'
	1.75	0.658	±	0.010	C'	0.650	±	0.008	C'	0.786	±	0.010	C'
Zn [mg L^{-1}]	1	0.795	±	0.006	b*	0.828	±	0.006	b*	0.888	±	0.007	b*
	5	0.570	±	0.003	c*	0.690	±	0.005	c*	0.745	±	0.006	c*
	10	0.480	±	0.006	d*	0.612	±	0.006	c*	0.687	±	0.007	c*
	20	0.383	±	0.007	e*	0.527	±	0.008	d*	0.551	±	0.009	d*

3.4 *Effects on in vivo chlorophyll fluorescence*

Based on the chlorophyll fluorescence measurements, 10 photosynthetic parameters were calculated for each concentration of the five pollutants. It is stated that photosynthesis is very sensitive to heavy metals and that chlorophyll fluorescence is an effective tool for sensing and assessing the impact of metals and other toxins on *Lemnaceae* (Lahive et al., 2012). The maximum quantum efficiency of PSII (F_v/F_m) can provide information about photosynthetic efficiency and heat dissipation (Maxwell & Johnson, 2000). Plants that are not exposed to stress have F_v/F_m values of approximately 0.83. Decreases of the F_v/F_m values are observed when plants are exposed to abiotic and biotic stress (Baker, 2008). The control condition in our experiments has an average F_v/F_m value of 0.767 ± 0.003, which is lower than the 0.83 mentioned by Baker (2008). Several F_v/F_m values under control conditions have been published (Vanhoudt et al., 2014b; Vercampt et al., 2016; Vidaković-Cifrek et al., 2015), and it is likely that F_v/F_m is species specific and depends on the growth conditions. For Cs, Ni and Zn, a significant decrease of the F_v/F_m values is observed. These results are in line with Lahive et al. (2011) and Lahive et al. (2012) who reported that the maximum quantum efficiency of PSII of *L. minor* decreased with increasing Zn concentrations after 7 days of exposure. Also when *B. juncea* seedlings were exposed to different Cs concentrations (Zhang & Liu, 2018), decreased F_v/F_m values were obtained. For Co exposure no differences were observed for F_v/F_m while exposure to Mn resulted in a slight but significant increase in F_v/F_m values. This suggests that in case of both, Co and Mn exposure, the light harvesting capacity of PSII remains intact within the tested concentration range. Similar results as those seen for Mn were obtained when *A. thaliana* seedlings were exposed to different gamma doses (Vanhoudt et al., 2014b). F_v/F_m is by far the most reported fluorescence parameter for photosynthesis evaluations, but also proven to be the least sensitive endpoint (Oláh et al., 2021). For this reason, other photosynthetic parameters were also taken into account.

Steady-state levels of qP, NPQ, φPSII, qL, Y(NPQ) and Y(NO) are also used to evaluate photosynthesis. The tested concentration range of Zn on *L. minor* affected the photosynthetic parameters the most. The photochemical quenching (qP) and the fraction of the open reaction centres (qL) both showed a significant decrease up to 5 and 10 mg Zn L^{-1}. Further increasing Zn up to 20 mg L^{-1} resulted in a significant increase to values approximately the same as the control condition. The Zn concentrations of 5 and 10 mg L^{-1} had a negative effect on *L. minor*'s possibility to perform photosynthesis due to the more closed PSII reaction centres. Values of qP and qL were not affected at any concentrations of Co, Cs, Mn and Ni, except for the increase of qP at the highest tested Mn concentration (100 mg L^{-1}). This concentration could potentially have a stimulatory effect on *L. minor*'s photosynthesis.

The photosynthetic efficiency of PSII (φPSII) was reported to be a very sensitive endpoint (Oláh et al., 2021). Looking at the φPSII results, a general significant decrease is observed with the increasing Zn concentrations, starting from 5 mg Zn L^{-1}. This means that a smaller fraction of the incoming energy is used for photosynthesis through PSII (Biermans et al., 2014). In contrast, exposure to Mn resulted in a slight increase of φPSII, resulting in an enhanced capability to perform photosynthesis. Exposure to Co, Cs and Ni had no effect of the φPSII.

Regarding the NPQ for Zn, a similar pattern was observed as for qP and qL: a significant decrease up to 5 mg Zn L^{-1} followed by a significant increase. For both Co and Mn a general decrease of NPQ was observed while no effects on NPQ were detected for Cs and Ni. Mateos-Naranjo et al. (2013) studied the effect of Cu exposure on *Atriplex halimus* by measuring F_v/F_m, φPSII and NPQ values. They observed a general decrease of F_v/F_m and φPSII and an increase of NPQ. In our experiments only exposure to Zn caused decreases of F_v/F_m and φPSII, but the NPQ value of the highest tested Zn concentration remained the same as for the control condition, indicating that the excess of the absorbed light is not

quenched as heat. Cobalt exposure only resulted in a decrease of NPQ, whereas Cs and Ni exposure only resulted in a lowered F_v/F_m. Mn exposure also caused a decrease of NPQ together with an increase of F_v/F_m and φPSII. From these results it can be concluded that each pollutant leads to a different response of *L. minor*'s capability to perform photosynthesis. The effects of Mn and Zn on *L. minor* are the most contrasting from each other.

A differentiation can be made between the portion of the light that is used in photochemistry (φPSII) and the portion of the light that is lost (Y(NPQ) and Y(NO)). For Zn exposure we observed that besides the general decrease in φPSII, there is an increase in both Y(NPQ) and Y(NO), significant at 20 and 5 mg Zn L^{-1} respectively. For both Co and Mn, significant decreases of Y(NPQ) were observed and a significantly higher Y(NO) value at 1.75 mg Co L^{-1} compared to the control condition. For Cs and Ni, no significant differences were observed for both Y(NPQ) and Y(NO). A high Y(NPQ) indicates that both photochemical energy conversion and protective regulatory mechanisms are inefficient (Vanhoudt et al., 2014a), which in our experiments is only the case for Zn.

Finally, the photosynthetic parameters (ETR_{max}, α, Ek) were calculated from the ETR(II) values as function of the PPFD values using the Levenberg-Marquardt nonlinear least-squares model (Moré, 1978). According to Oláh et al. (2021), ETR_{max} is a very sensitive photosynthesis related endpoint for stress evaluation. This is not supported by our data, since no effects on ETR_{max} were observed. The photosynthetic rate (α) only decreased when Cs, Ni or Zn was applied, where the reduction of α was higher for Zn exposure. The calculated minimal saturation irradiance (Ek) was also only affected by Zn. An increase was observed at the lower Zn concentrations, followed by a significant increase at 20 mg Zn L^{-1}.

559 *Table 3 Chlorophyll fluorescence parameters (F_v/F_m, qP, NPQ, φPSII, qL, Y(NPQ), Y(NO), ETR_{max}, α and Ek), measured on L. minor plants. Plants were exposed for 7 days to different metals and*
560 *concentrations. Data represent the mean value of four biological replicates together with the standard error of the means. Different letters indicate significant differences between concentrations*
561 *of a pollutant ($p < 0.05$).*

Condition		F_v/F_m	qP	NPQ	φPSII	qL
Control		0.767 ± 0.002 a	0.787 ± 0.010 a	0.530 ± 0.013 a	0.538 ± 0.008 a	0.541 ± 0.014 a
Co [mg L^{-1}]	0.5	0.760 ± 0.003 a	0.806 ± 0.012 a	0.307 ± 0.019 b	0.571 ± 0.011 a	0.554 ± 0.019 a
	1	0.762 ± 0.001 a	0.816 ± 0.003 a	0.362 ± 0.014 b	0.573 ± 0.005 a	0.569 ± 0.004 a
	1.75	0.771 ± 0.003 a	0.779 ± 0.003 a	0.257 ± 0.009 b	0.569 ± 0.002 a	0.487 ± 0.007 a
	2.5	0.767 ± 0.004 a	0.812 ± 0.003 a	0.298 ± 0.015 b	0.589 ± 0.007 a	0.542 ± 0.009 a
Control		0.763 ± 0.001 A	0.814 ± 0.009 A	0.27 ± 0.02 A	0.585 ± 0.008 A	0.555 ± 0.016 A
Cs mg L^{-1}]	1	0.758 ± 0.002 AB	0.818 ± 0.003 A	0.351 ± 0.008 A	0.574 ± 0.002 A	0.572 ± 0.006 A
	10	0.741 ± 0.003 B	0.792 ± 0.012 A	0.297 ± 0.019 A	0.547 ± 0.009 A	0.545 ± 0.018 A
	35	0.722 ± 0.001 C	0.816 ± 0.007 A	0.27 ± 0.02 A	0.550 ± 0.007 A	0.592 ± 0.009 A
	75	0.726 ± 0.002 C	0.815 ± 0.004 A	0.28 ± 0.02 A	0.550 ± 0.001 A	0.589 ± 0.008 A
Control		0.746 ± 0.007 a'	0.749 ± 0.019 ab'	0.61 ± 0.03 a'	0.486 ± 0.018 a'	0.519 ± 0.018 a'
Mn [mg L^{-1}]	10	0.787 ± 0.001 b'	0.778 ± 0.006 ab'	0.424 ± 0.012 ab'	0.561 ± 0.006 b'	0.494 ± 0.007 a'
	25	0.765 ± 0.003 ab'	0.756 ± 0.006 a'	0.54 ± 0.04 ab'	0.515 ± 0.009 a'	0.497 ± 0.003 a'
	70	0.787 ± 0.001 b'	0.781 ± 0.002 ab'	0.389 ± 0.016 b'	0.568 ± 0.002 b'	0.492 ± 0.004 a'
	100	0.780 ± 0.006 ab'	0.814 ± 0.005 b'	0.42 ± 0.02 ab'	0.583 ± 0.007 b'	0.555 ± 0.007 a'
Control		0.780 ± 0.002 A'	0.785 ± 0.007 A'	0.437 ± 0.010 A'	0.558 ± 0.005 A'	0.514 ± 0.011 A'
Ni [mg L^{-1}]	0.5	0.772 ± 0.002 AB'	0.762 ± 0.008 A'	0.365 ± 0.010 A'	0.542 ± 0.004 A'	0.480 ± 0.013 A'
	0.75	0.753 ± 0.003 B'	0.785 ± 0.006 A'	0.437 ± 0.016 A'	0.533 ± 0.003 A'	0.540 ± 0.010 A'
	1.25	0.765 ± 0.002 AB'	0.799 ± 0.008 A'	0.379 ± 0.017 A'	0.562 ± 0.001 A'	0.541 ± 0.017 A'
	1.75	0.753 ± 0.005 B'	0.796 ± 0.001 A'	0.402 ± 0.015 A'	0.545 ± 0.005 A'	0.551 ± 0.003 A'
Control		0.778 ± 0.002 a*	0.845 ± 0.009 a*	0.480 ± 0.016 ab*	0.595 ± 0.008 a*	0.620 ± 0.016 a*
Zn [mg L^{-1}]	1	0.771 ± 0.001 a*	0.807 ± 0.006 ab*	0.412 ± 0.015 a*	0.570 ± 0.005 a*	0.554 ± 0.009 ab*
	5	0.713 ± 0.002 b*	0.734 ± 0.004 b*	0.325 ± 0.011 c*	0.480 ± 0.004 b*	0.488 ± 0.005 b*
	10	0.663 ± 0.005 bc*	0.738 ± 0.011 b*	0.392 ± 0.017 bc*	0.432 ± 0.006 c*	0.540 ± 0.017 ab*
	20	0.624 ± 0.006 c*	0.83 ± 0.02 ab*	0.59 ± 0.04 a*	0.418 ± 0.004 c*	0.70 ± 0.04 a*

562 *Table 3 (continued).*

Condition		Y(NPQ)			Y(NO)			ETR_{max}			α			Ek		
Control		0.160	± 0.004	a	0.302	± 0.006	a	55	± 4	a	0.241	± 0.006	a	230	± 20	a
Co [mg L^{-1}]	0.5	0.102	± 0.008	b	0.328	± 0.004	ab	61	± 3	a	0.257	± 0.007	a	239	± 12	a
	1	0.114	± 0.005	b	0.314	± 0.002	ab	57	± 3	a	0.261	± 0.001	a	217	± 12	a
	1.75	0.088	± 0.002	b	0.343	± 0.003	b	46.7	± 1.3	a	0.232	± 0.009	a	203	± 4	a
	2.5	0.097	± 0.005	b	0.335	± 0.004	ab	59	± 7	a	0.273	± 0.004	a	220	± 30	a
Control		0.087	± 0.007	A	0.329	± 0.006	A	73	± 7	A	0.266	± 0.002	A	280	± 30	A
Cs [mg L^{-1}]	1	0.111	± 0.002	A	0.316	± 0.001	A	63	± 2	A	0.264	± 0.001	A	238	± 8	A
	10	0.104	± 0.007	A	0.350	± 0.005	A	68	± 6	A	0.265	± 0.002	A	260	± 20	A
	35	0.094	± 0.008	A	0.356	± 0.001	A	62	± 4	A	0.239	± 0.003	B	258	± 18	A
	75	0.097	± 0.006	A	0.354	± 0.007	A	58.3	± 1.2	A	0.233	± 0.001	B	250	± 6	A
Control		0.195	± 0.010	a'	0.320	± 0.011	a'	49.7	± 0.9	a'	0.243	± 0.002	a'	209	± 6	a'
Mn [mg L^{-1}]	10	0.131	± 0.004	ab'	0.308	± 0.003	a'	69	± 4	a'	0.263	± 0.003	a'	262	± 17	a'
	25	0.168	± 0.010	b'	0.317	± 0.004	a'	52.1	± 1.8	a'	0.246	± 0.006	a'	212	± 5	a'
	70	0.121	± 0.004	b'	0.312	± 0.003	a'	49.4	± 1.1	a'	0.277	± 0.002	a'	179	± 4	a'
	100	0.122	± 0.005	b'	0.296	± 0.007	a'	51	± 4	a'	0.258	± 0.006	a'	198	± 13	a'
Control		0.134	± 0.003	A'	0.308	± 0.002	A'	50	± 3	A'	0.281	± 0.001	A'	179	± 10	A'
Ni [mg L^{-1}]	0.5	0.123	± 0.003	A'	0.335	± 0.003	A'	43.9	± 0.9	A'	0.278	± 0.002	A'	158	± 3	A'
	0.75	0.142	± 0.004	A'	0.326	± 0.003	A'	43.2	± 1.5	A'	0.268	± 0.002	AB'	162	± 6	A'
	1.25	0.120	± 0.004	A'	0.319	± 0.004	A'	48.8	± 0.0	A'	0.278	± 0.002	A'	177.1	± 1.5	A'
	1.75	0.130	± 0.004	A'	0.325	± 0.004	A'	45.6	± 0.6	A'	0.255	± 0.001	B'	177.7	± 1.7	A'
Control		0.131	± 0.004	a*	0.274	± 0.005	a*	68	± 3	a*	0.292	± 0.003	a*	232	± 8	a*
Zn [mg L^{-1}]	1	0.126	± 0.004	a*	0.305	± 0.002	a*	70	± 4	a*	0.265	± 0.001	b*	265	± 15	ab*
	5	0.128	± 0.004	a*	0.393	± 0.002	b*	51.7	± 1.7	a*	0.214	± 0.003	c*	242	± 9	a*
	10	0.159	± 0.005	a*	0.409	± 0.007	b*	59	± 5	a*	0.177	± 0.003	d*	330	± 20	ab*
	20	0.212	± 0.008	b*	0.370	± 0.009	b*	76	± 7	a*	0.166	± 0.003	d*	460	± 40	b*

563

3.5 *Effects on starch and soluble sugars content*

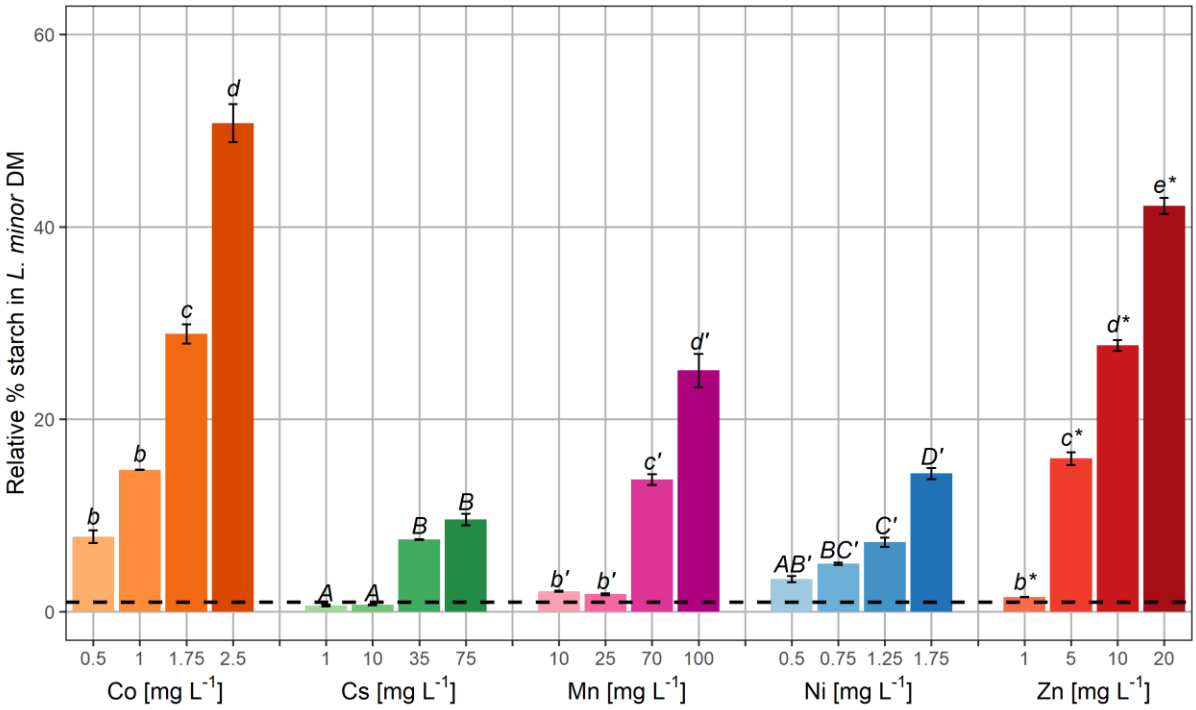
The starch content of *L. minor* is influenced by several environmental factors (salinity, nutrient deprivation, low or high temperatures, light intensity, photoperiod, *etc*.) and pollutants (*e.g.* heavy metals) (de Morais et al., 2019; Li et al., 2016; Sree & Appenroth, 2014; Yin et al., 2015; Zhao et al., 2014). Changing environmental conditions or adding pollutants results, in most cases, in stress conditions leading to growth inhibition and accumulation of starch. Reduced growth was already illustrated in Fig. 1: almost all pollutant concentrations had a negative effect of the specific growth rate of *L. minor.* Accumulation of starch is in this case definitely stress related.

Adding pollutants to the growth medium resulted in increases of the starch content (Fig. 3). Exposure to the lowest concentration of 0.5 mg Co L^{-1} resulted in 7 times more starch compared to the control condition. Further increasing Co to 1, 1.75 and 2.5 mg L^{-1} caused respectively 14, 28 and 50 times more starch compared to the control condition. Sree et al. (2015) also found increasing starch contents after exposure of *L. minor* to increasing Co concentrations (1, 3, 10 and 100 µM). Cs on the other hand, had no effect on starch at the two lowest concentrations (1 and 10 mg L^{-1}). There was a significant increase in starch from 35 and 75 mg Cs L^{-1} (7 and 9 times higher). Mn followed the same pattern as Cs, with the difference that 10 and 25 mg Mn L^{-1} resulted in a slight increase of starch and 70 and 100 mg Mn L^{-1} resulted in significant concentration dependent increases of the starch content (13 and 25 times higher). For Ni and Zn the significant increases of the starch content were concentration dependent and went up to 14 and 42 times more starch at the highest Ni and Zn concentrations (respectively 1.75 and 20 mg L^{-1}). Appenroth et al. (2010) found higher starch contents in *L. minor* after 7 days of growth exposed to increasing Ni concentrations up to 40 µM (2.35 mg L^{-1}). Even higher Ni concentrations led to a decrease of the starch content. This could not be confirmed in our experiments, since concentrations above 40 µM were not tested.

In general, when pollutant concentrations increased, significant increases of starch content were obtained. The extra formation of starch seems negatively correlated to the relative growth rates. Growth rates that are not significantly or slightly significantly lower than the control (Fig. 1) did not result in significant increases of the starch content. Starch accumulation in plants is clearly stress and pollutant related, and can be ascribed to inhibition of export of photosynthates or a greater inhibition of plant growth than photosynthesis, which in both cases results in more carbohydrates for starch biosynthesis or starch storage (Sree et al., 2015).

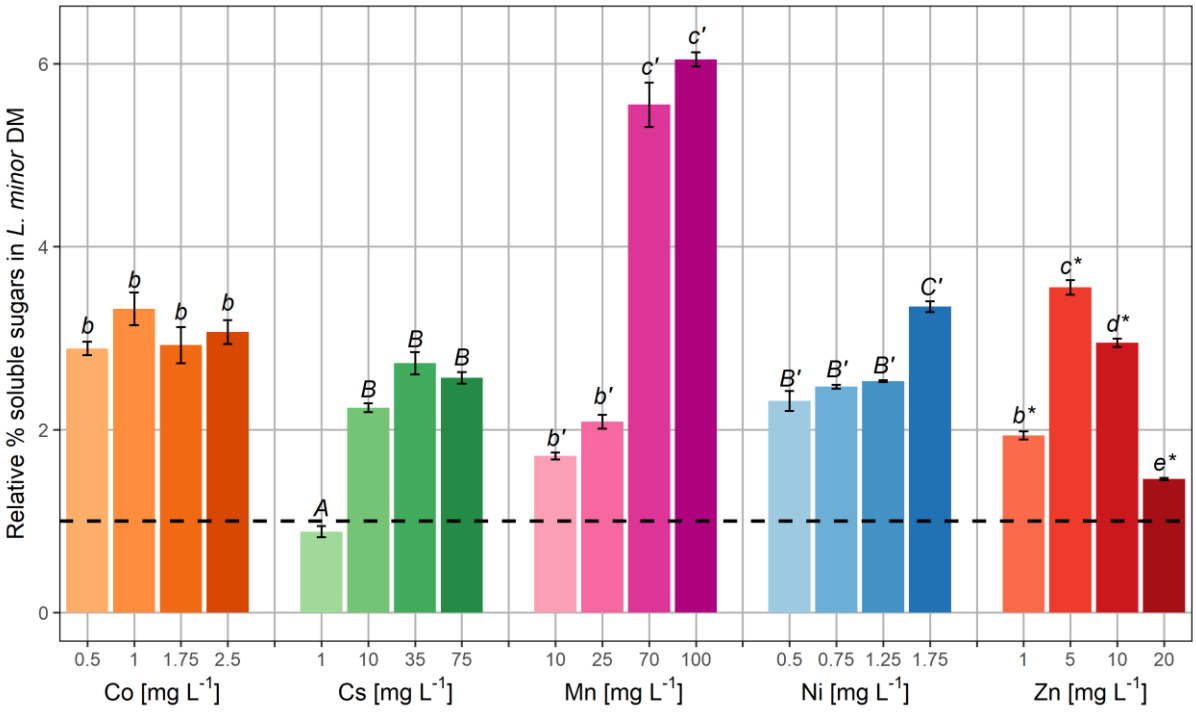
Little is known about effects on soluble sugars content of *L. minor.* Sugars are essential compounds for plants inherent to basic biochemical (*e.g.* photosynthesis, respiration) and physiological processes (*e.g.* transpiration). Sugar content can be influenced by several environmental parameters (like nutrient deprivation, temperature, light intensity, photoperiod) and pollutants (John et al., 2008; Khavari-Nejad et al., 2009; Mishra & Tripathi, 2008; Pagliuso et al., 2018).

In general, adding pollutants to the growth medium (except 1 mg Cs L^{-1}) resulted in higher soluble sugars contents (Fig. 4). In most cases, the soluble sugars content increased up to two or three times compared to the control. At the two highest Mn concentrations (70 and 100 mg L^{-1}) the highest concentrations of soluble sugars were observed (5.5 and 6 times more). These results are in contrast with the results of Mishra and Tripathi (2008), who reported that sugar concentrations decreased after addition of pollutants to the growth medium. The relation between lowering of sugar content and reduced photochemical activities and chlorophyll content is also not applicable in our results.



606

607 *Fig. 3 Relative percentage starch in L. minor DM exposed to different pollutants and concentrations for 7 days and expressed*
608 *relative to the control condition. Data represent the mean value of four biological replicates together with the standard error*
609 *of the means. The control condition is represented by the black dashed line, with letter a, A, a', A' and a* (not given for*
610 *simplicity reasons). Different letters indicate significant differences between concentrations of a pollutant, including the*
611 *control condition ($p < 0.05$).*



612

613 *Fig. 4 Relative percentage soluble sugars in L. minor DM exposed to different pollutants and concentrations for 7 days and*
614 *expressed relative to the control condition. Data represent the mean value of four biological replicates together with the*
615 *standard error of the means. The control condition is represented by the black dashed line, with letter a, A, a', A' and a* (not*
616 *given for simplicity reasons). Different letters indicate significant differences between concentrations of a pollutant and*
617 *towards the control condition ($p < 0.05$).*

4 Conclusion and perspectives for future work

We studied the uptake of Co, Cs, Mn, Ni and Zn by *L. minor* and their effects on growth, photosynthetic pigments, photosynthesis, starch and soluble sugars content. *L. minor* can remove these elements from polluted water. The percentage of pollutant removal and the BCF depend on the initial pollutant concentration in the water. In general, the higher the initial concentration, the lower the removal percentage and BCF. When pollutant concentrations keep increasing, no more pollutant will be taken up by plants when the maximum uptake is achieved. Therefore the percentage of pollutant removal and BCF will keep decreasing. For this reason both results should be evaluated critically. Looking at the chosen pollutant concentrations around their EC_{50} values (1.75 mg Co L^{-1}, 75 mg Cs L^{-1}, 70 mg Mn L^{-1}, 1.25 mg Ni L^{-1} and 10 mg Zn L^{-1}), the following order can be made based on the capacity of *L. minor* to remove and bioaccumulate pollutants from water: Co > Ni > Zn > Mn > Cs, where Co has the highest BCF and percentage of removal (2300 ± 40, 46.5%) and Cs the lowest (190.6 ± 0.8, 2.8%).

When expressing at the total pollutant removal on dry matter, a different ordering is obtained: Mn > Cs > Zn > Co > Ni. As Mn is an essential micronutrient for plant growth and development, it is logical that it will be highly accumulated (maximal removal of 29.6 mg Mn g^{-1} DM). On the other hand, only 7.9 mg Co g^{-1} DM and 3.0 mg Ni g^{-1} DM are removed. They both have lower EC_{50} values compared to the other metals, so the lower removal per *L. minor* DM can be a consequence of the higher toxicity of both metals. Lastly, due to the similarity between Cs and the essential element K (both monovalent cations in close proximity in the periodic table), Cs is taken up through the same transport channels as K, resulting also in a high removal per *L. minor* DM (14.1 mg Cs g^{-1} DM). However, it is important to note that the K concentration was lowered in our Cs experiments, because K presence can inhibit Cs uptake. A comprehensive study of the influence of K concentrations on Cs uptake can verify this, indicating the direction for future investigations.

We also showed that increasing pollutant concentrations significantly inhibit *L. minor* growth. Toxic effects are most visible as a decreases in photosynthetic pigments (Chl *a*, Chl *b* and carotenoids) and an increase in starch content in a concentration dependent manner. Both parameters are good pollutant stress indicators or biomarkers for *L. minor* since significant differences were obtained. The soluble sugars concentrations were also higher for all pollutant exposures, but no concentration dependency was observed. Zn and Mn are the two pollutants having the most pronounced effects on the photosynthetic capacity of *L. minor*. Zinc causes damage to the light harvesting capacity of PSII and by consequence a smaller fraction of the incoming energy can be used for photosynthesis trough PSII. In contrast, the light harvesting capacity of PSII remained intact during exposure to Mn and even an enhanced capability to perform photosynthesis was observed.

Our results contribute to the exploration of using *L. minor* in phytoremediation applications, highlighting the practical feasibility, possible adverse effects and the factual uptake of pollutants. The studied effects of the applied pollutants on growth, photosynthetic pigments, photosynthesis, starch and soluble sugars contents provide information on the toxicity of the pollutants. Moreover, stress caused by pollutants has a visible effect on *L. minor* growth which will result in less pollutant removal per gram dry matter and will eventually negatively affect the phytoremediation potential. This emphasizes again the importance of this in depth study on effects caused by different pollutants. Other factors, such as environmental conditions, are also proven to affect *L. minor* growth and possibly pollutant removal and should therefore also be considered when designing phytoremediation applications. Future research can focus on the influence of environmental conditions in combination with pollutant exposure. Additionally, exposure of plants to pollutant mixtures can have different effects on the above studied effects. Therefore, studying these various exposure conditions can help to select *L. minor* for phytoremediation. In general, our results are directly applicable in

664 phytoremediation technologies to mitigate aquatic pollution and its impacts on people and the
665 environment.

666 ## Acknowledgements

667 This work was supported by a PhD grant for Isabelle Van Dyck from the Belgian Nuclear Research
668 Centre (SCK CEN). The authors are grateful to Carine Put from the Centre for Environmental Sciences
669 (Hasselt University) for the ICP-OES measurements.

670 ## References

671 Abràmoff, M. D., Magalhães, P. J., & Ram, S. J. (2004). Image processing with ImageJ. *Biophotonics
672 international, 11*(7), 36-42.
673 Al-Zurfi, S. K. L., Alisaw, A. Y., & Al-Shafai, G. A. A. (2018). Bioaccumulation of some heavy metals by
674 the aquatic plant lemnagibba in vitro. *research Journal of Pharmacy and Technology, 11*(10),
675 4229-4236.
676 Appenroth, K. J., Krech, K., Keresztes, Á., Fischer, W., & Koloczek, H. (2010). Effects of nickel on the
677 chloroplasts of the duckweeds Spirodela polyrhiza and Lemna minor and their possible use in
678 biomonitoring and phytoremediation. *Chemosphere, 78*(3), 216-223.
679 Arán, D. S., Harguinteguy, C. A., Fernandez-Cirelli, A., & Pignata, M. L. (2017). Phytoextraction of Pb,
680 Cr, Ni, and Zn using the aquatic plant Limnobium laevigatum and its potential use in the
681 treatment of wastewater. *Environmental science and pollution research international, 24*(22),
682 18295-18308.
683 ATSDR. (2005). Toxicological profile for zinc. In P. H. S. U.S. Department of Health and Human
684 Services (Ed.). Atlanta.
685 ATSDR. (2012). Toxicological profile for manganese. In P. H. S. U.S. Department of Health and Human
686 Services (Ed.). Atlanta.
687 Axtell, N. R., Sternberg, S. P., & Claussen, K. (2003). Lead and nickel removal using Microspora and
688 Lemna minor. *Bioresource Technology, 89*(1), 41-48.
689 Baker, N. R. (2008). Chlorophyll fluorescence: a probe of photosynthesis in vivo. *Annu. Rev. Plant
690 Biol., 59*, 89-113.
691 Begović, L., Mlinarić, S., Antunović Dunić, J., Katanić, Z., Lončarić, Z., Lepeduš, H., et al. (2016).
692 Response of Lemna minor L. to short-term cobalt exposure: The effect on photosynthetic
693 electron transport chain and induction of oxidative damage. *Aquatic Toxicology, 175*, 117-
694 126.
695 Biermans, G., Horemans, N., Vanhoudt, N., Vandenhove, H., Saenen, E., Van Hees, M., et al. (2014).
696 An organ-based approach to dose calculation in the assessment of dose-dependent biological
697 effects of ionising radiation in Arabidopsis thaliana. *Journal of Environmental Radioactivity,
698 133*, 24-30.
699 Chojnacka, K. (2010). Biosorption and bioaccumulation–the prospects for practical applications.
700 *Environment international, 36*(3), 299-307.
701 Cuypers, A., Karen, S., Jos, R., Kelly, O., Els, K., Tony, R., et al. (2011). The cellular redox state as a
702 modulator in cadmium and copper responses in Arabidopsis thaliana seedlings. *Journal of
703 Plant Physiology, 168*(4), 309-316.
704 DalCorso, G. (2012). Heavy metal toxicity in plants. In A. Furini (Ed.), *Plants and heavy metals* (pp. 1-
705 25): Dordrecht: Springer Netherlands : Imprint Springer.
706 de Morais, M. B., Barbosa-Neto, A. G., Willadino, L., Ulisses, C., & Calsa Junior, T. (2019). Salt stress
707 induces increase in starch accumulation in duckweed (Lemna aequinoctialis, Lemnaceae):
708 Biochemical and physiological aspects. *Journal of Plant Growth Regulation, 38*(2), 683-700.
709 Doganlar, Z. B., Cakmak, S., & Yanik, T. (2012). Metal uptake and physiological changes in Lemna
710 gibba exposed to manganese and nickel. *International Journal of Biology, 4*(3), 148-157.
711 Driever, S. M., Nes, E. H. v., & Roijackers, R. M. M. (2005). Growth limitation of Lemna minor due to
712 high plant density. *Aquatic Botany, 81*(3), 245-251.

713 Goswami, C., & Majumder, A. (2015). Potential of Lemna minor in Ni and Cr removal from aqueous
714 solution. *Pollution (Tehran, Iran), 1*(4), 373-385.
715 Hansen, J., & Møller, I. (1975). Percolation of starch and soluble carbohydrates from plant tissue for
716 quantitative determination with anthrone. *Analytical biochemistry, 68*(1), 87-94.
717 Hu, D., Cheng, M., Hu, K., Zhang, W., Yang, Y., & Xu, Q. (2019). Evaluation of cobalt
718 hyperaccumulation and tolerance potential of the duckweed (Lemna minor L.). *Ecotoxicology*
719 *and Environmental Safety, 179*, 79-87.
720 Ince, N., Dirilgen, N., Apikyan, I., Tezcanli, G., & Üstün, B. (1999). Assessment of toxic interactions of
721 heavy metals in binary mixtures: a statistical approach. *Archives of Environmental*
722 *Contamination and Toxicology, 36*(4), 365-372.
723 Ivshina, I., Peshkur, T., & Korobov, V. (2002). Efficient uptake of cesium ions by Rhodococcus cells.
724 *Microbiology, 71*(3), 357-361.
725 Jamers, A., Van der Ven, K., Moens, L., Robbens, J., Potters, G., Guisez, Y., et al. (2006). Effect of
726 copper exposure on gene expression profiles in Chlamydomonas reinhardtii based on
727 microarray analysis. *Aquatic Toxicology, 80*, 249-260.
728 Jayasri, M. A., & Suthindhiran, K. (2017). Effect of zinc and lead on the physiological and biochemical
729 properties of aquatic plant Lemna minor: its potential role in phytoremediation. *Applied*
730 *Water Science, 7*(3), 1247-1253.
731 John, R., Ahmad, P., Gadgil, K., & Sharma, S. (2008). Effect of cadmium and lead on growth,
732 biochemical parameters and uptake in Lemna polyrrhiza L. *Plant Soil and Environment, 54*(6),
733 262.
734 Keskinkan, O., Goksu, M. Z. L., Yuceer, A., Basibuyuk, M., & Forster, C. F. (2003). Heavy metal
735 adsorption characteristics of a submerged aquatic plant (Myriophyllum spicatum). *Process*
736 *Biochemistry, 39*, 179-183.
737 Khavari-Nejad, R. A., Najafi, F., & Tofighi, C. (2009). Diverse responses of tomato to N and P
738 deficiency. *International Journal of Agriculture and biology, 11*(2), 209-213.
739 Khellaf, N., & Zerdaoui, M. (2009). Phytoaccumulation of zinc by the aquatic plant, Lemna gibba L.
740 *Bioresource Technology, 100*(23), 6137-6140.
741 Lahive, E., O' Halloran, J., & Jansen, M. A. K. (2011). Differential sensitivity of four Lemnaceae species
742 to zinc sulphate. *Environmental and Experimental Botany, 71*(1), 25-33.
743 Lahive, E., O'Halloran, J., & Jansen, M. A. K. (2012). Frond development gradients are a determinant
744 of the impact of zinc on photosynthesis in three species of Lemnaceae. *Aquatic Botany, 101*,
745 55-63.
746 Leblebici, Z., Kar, M., & Yalcin, V. (2017). Comparative Study of Cd, Pb, and Ni removal potential by
747 Salvinia natans (L.) All. and Lemna minor L.: Interactions with Growth Parameters. *Romanian*
748 *Biotechnological Letters, 23*(1), 1-14.
749 Li, Y., Zhang, F., Daroch, M., & Tang, J. (2016). Positive effects of duckweed polycultures on starch
750 and protein accumulation. *Bioscience Reports, 36*(5), 1-8.
751 Liu, C., Dai, Z., & Sun, H. (2017a). Potential of duckweed (Lemna minor) for removal of nitrogen and
752 phosphorus from water under salt stress. *Journal of Environmental Management, 187*, 497-
753 503.
754 Liu, Y., Sanguanphun, T., Yuan, W., Cheng, J. J., & Meetam, M. (2017b). The biological responses and
755 metal phytoaccumulation of duckweed Spirodela polyrhiza to manganese and chromium.
756 *Environmental science and pollution research international, 24*(23), 19104-19113.
757 Martinez, R. S., Sáenz, M. E., Alberdi, J. L., & Di Marzio, W. D. (2019). Comparative ecotoxicity of
758 single and binary mixtures exposures of nickel and zinc on growth and biomarkers of Lemna
759 gibba. *Ecotoxicology (London), 28*(6), 686-697.
760 Mateos-Naranjo, E., Andrades-Moreno, L., Cambrollé, J., & Perez-Martin, A. (2013). Assessing the
761 effect of copper on growth, copper accumulation and physiological responses of grazing
762 species Atriplex halimus: ecotoxicological implications. *Ecotoxicology and Environmental*
763 *Safety, 90*, 136-142.

764 Maxwell, K., & Johnson, G. N. (2000). Chlorophyll fluorescence—a practical guide. *Journal of*
765 *experimental botany, 51*(345), 659-668.
766 Mishra, V. K., & Tripathi, B. (2008). Concurrent removal and accumulation of heavy metals by the
767 three aquatic macrophytes. *Bioresource Technology, 99*(15), 7091-7097.
768 Monette, F., Lasfar, S., Millette, L., & Azzouz, A. (2006). Comprehensive modeling of mat density
769 effect on duckweed (Lemna minor) growth under controlled eutrophication. *Water Research,*
770 *40*(15), 2901-2910.
771 Moré, J. J. (1978). The Levenberg-Marquardt algorithm: implementation and theory. In *Numerical*
772 *analysis* (pp. 105-116): Springer.
773 Moustakas, M. (2023). Molecular mechanisms of metal toxicity and plant tolerance. In (Vol. 24, pp.
774 7810): MDPI.
775 Murchie, E. H., & Lawson, T. (2013). Chlorophyll fluorescence analysis: a guide to good practice and
776 understanding some new applications. *Journal of experimental botany, 64*(13), 3983-3998.
777 Mustafa, A., Zulfiqar, U., Mumtaz, M. Z., Radziemska, M., Haider, F. U., Holatko, J., et al. (2023).
778 Nickel (Ni) phytotoxicity and detoxification mechanisms: A review. *Chemosphere*, 138574.
779 Nys, C., Janssen, C. R., Van Sprang, P., & De Schamphelaere, K. A. C. (2016). The effect of pH on
780 chronic aquatic nickel toxicity is dependent on the pH itself: Extending the chronic nickel
781 bioavailability models. *Environmental Toxicology and Chemistry, 35*(5), 1097-1106.
782 OECD. (2002). OECD guidelines for the testing of chemicals: Lemna sp. growth inhibition test,
783 Guideline 221. In: Organisation for Economic Corporation and Development Berlin.
784 Oláh, V., Hepp, A., Irfan, M., & Mészáros, I. (2021). Chlorophyll fluorescence imaging-based
785 duckweed phenotyping to assess acute phytotoxic effects. *Plants, 10*(12), 2763.
786 Oros, V., & Toma, A. (2012). Ecotoxicological effects of heavy metals on duckweed plants (Lemna
787 minor). II. Tests for growth rate reducing by the Zinc. *Scientific Bulletin Series D : Mining,*
788 *Mineral Processing, Non-Ferrous Metallurgy, Geology and Environmental Engineering, 26*(1),
789 15-21.
790 Oros, V., Toma, A., & Tudoran, A. (2011). Preliminary tests on phytotoxicity of heavy metals Cu, Zn,
791 Cd and Fe on aquatic plants of duckweed (Lemna minor). *Scientific Bulletin Series D : Mining,*
792 *Mineral Processing, Non-Ferrous Metallurgy, Geology and Environmental Engineering, 25-*
793 *32*(2), 25-32.
794 Ozyigit, I. I., Arda, L., Yalcin, B., Yalcin, I. E., Ucar, B., & Hocaoglu-Ozyigit, A. (2021). Lemna minor, a
795 hyperaccumulator shows elevated levels of Cd accumulation and genomic template stability
796 in binary application of Cd and Ni: a physiological and genetic approach. *International Journal*
797 *of Phytoremediation*, 1-15.
798 Pagliuso, D., Grandis, A., Igarashi, E. S., Lam, E., & Buckeridge, M. S. (2018). Correlation of apiose
799 levels and growth rates in duckweeds. *Frontiers in chemistry, 6*, 291-291.
800 Patrick, J. W., Botha, F. C., & Birch, R. G. (2013). Metabolic engineering of sugars and simple sugar
801 derivatives in plants. *Plant biotechnology journal, 11*(2), 142-156.
802 Pinder III, J. E., Hinton, T. G., & Whicker, F. W. (2006). Foliar uptake of cesium from the water column
803 by aquatic macrophytes. *Journal of Environmental Radioactivity, 85*(1), 23-47.
804 Pratas, J., Favas, P. J., Paulo, C., Rodrigues, N., & Prasad, M. (2012). Uranium accumulation by aquatic
805 plants from uranium-contaminated water in Central Portugal. *International Journal of*
806 *Phytoremediation, 14*(3), 221-234.
807 Radić, S., Babić, M., Škobić, D., Roje, V., & Pevalek-Kozlina, B. (2010). Ecotoxicological effects of
808 aluminum and zinc on growth and antioxidants in Lemna minor L. *Ecotoxicology and*
809 *Environmental Safety, 73*(3), 336-342.
810 Rahman, S. U., Li, Y., Hussain, S., Hussain, B., Riaz, L., Ashraf, M. N., et al. (2023). Role of
811 phytohormones in heavy metal tolerance in plants: A review. *Ecological Indicators, 146*,
812 109844.
813 Ralph, P. J., & Gademann, R. (2005). Rapid light curves: A powerful tool to assess photosynthetic
814 activity. *Aquatic Botany, 82*(3), 222-237.

Saleh, H. M., Moussa, H. R., Mahmoud, H. H., El-Saied, F. A., Dawoud, M., & Wahed, R. S. A. (2020). Potential of the submerged plant Myriophyllum spicatum for treatment of aquatic environments contaminated with stable or radioactive cobalt and cesium. *Progress in Nuclear Energy, 118*, 103147.

Sasmaz, A., Dogan, I. M., & Sasmaz, M. (2016). Removal of Cr, Ni and Co in the water of chromium mining areas by using Lemna gibba L. and Lemna minor L. *Water and Environment Journal, 30*(3-4), 235-242.

Smolders, E., Kiebooms, L., Buysse, J., & Merckx, R. (1996). ^{137}Cs uptake in spring wheat (Triticum aestivum L. cv. Tonic) at varying K supply: II. A potted soil experiment. *Plant and Soil*, 211-220.

Sree, K. S., & Appenroth, K.-J. (2014). Increase of starch accumulation in the duckweed Lemna minor under abiotic stress. *Albanian Journal of Agricultural Sciences*, 11-14.

Sree, K. S., Keresztes, Á., Mueller-Roeber, B., Brandt, R., Eberius, M., Fischer, W., et al. (2015). Phytotoxicity of cobalt ions on the duckweed Lemna minor – Morphology, ion uptake, and starch accumulation. *Chemosphere, 131*, 149-156.

Üçüncü, E., Tunca, E., Fikirdeşici, Ş., Özkan, A. D., & Altındağ, A. (2013). Phytoremediation of Cu, Cr and Pb Mixtures by Lemna minor. *Bulletin of Environmental Contamination and Toxicology, 91*(5), 600-604.

Van Dyck, I., Vanhoudt, N., Vives i Batlle, J., Horemans, N., Van Gompel, A., Nauts, R., et al. (2023). Effects of environmental parameters on starch and soluble sugars in Lemna minor. *Plant Physiology and Biochemistry*, 107755.

Van Hoeck, A., Horemans, N., Nauts, R., Van Hees, M., Vandenhove, H., & Blust, R. (2017). Lemna minor plants chronically exposed to ionising radiation: RNA-seq analysis indicates a dose rate dependent shift from acclimation to survival strategies. *Plant Science, 257*, 84-95.

Vanhoudt, N., Horemans, N., Biermans, G., Saenen, E., Wannijn, J., Nauts, R., et al. (2014a). Uranium affects photosynthetic parameters in Arabidopsis thaliana. *Environmental and Experimental Botany, 97*, 22-29.

Vanhoudt, N., Horemans, N., Wannijn, J., Nauts, R., Van Hees, M., & Vandenhove, H. (2014b). Primary stress responses in Arabidopsis thaliana exposed to gamma radiation. *Journal of Environmental Radioactivity, 129*, 1-6.

Vanhoudt, N., Van Gompel, A., & i Batlle, J. V. (2021). Distribution and behaviour of naturally occurring radionuclides within a Scots pine forest grown on a CaF2 waste deposit related to the Belgian phosphate industry. *Journal of Environmental Radioactivity, 233*, 106591.

Vercampt, H., Koleva, L., Vassilev, A., Horemans, N., Biermans, G., Vangronsveld, J., et al. (2016). The functional role of the photosynthetic apparatus in the recovery of Brassica napus plants from pre-emergent metazachlor exposure. *Journal of Plant Physiology, 196*, 99-105.

Vidaković-Cifrek, Ž., Tkalec, M., Šikić, S., Tolić, S., Lepeduš, H., & Pevalek-Kozlina, B. (2015). Growth and photosynthetic responses of Lemna minor L. exposed to cadmium in combination with zinc or copper. *Archives of Industrial Hygiene and Toxicology, 66*(2), 141-152.

Wellburn, A. R. (1994). The spectral determination of chlorophylls a and b, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. *Journal of Plant Physiology, 144*(3), 307-313.

White, P. J., & Broadley, M. R. (2000). Mechanisms of caesium uptake by plants. *The New Phytologist, 147*(2), 241-256.

Yin, Y., Yu, L., Yu, C., Zhao, J., Sun, C., Ma, Y., et al. (2015). The influence of light intensity and photoperiod on duckweed biomass and starch accumulation for bioethanol production. *Bioresource Technology, 187*, 84-90.

Zayed, A., Gowthaman, S., & Terry, N. (1998). Phytoaccumulation of Trace Elements by Wetland Plants: I. Duckweed. *Journal of environmental quality, 27*(3), 715-721.

Zhang, Y., & Liu, G.-j. (2018). Effects of cesium accumulation on chlorophyll content and fluorescence of Brassica juncea L. *Journal of Environmental Radioactivity, 195*, 26-32.

Zhao, Z., Shi, H., Liu, Y., Zhao, H., Su, H., Wang, M., et al. (2014). The influence of duckweed species diversity on biomass productivity and nutrient removal efficiency in swine wastewater. *Bioresource Technology, 167*, 383-389.

Zhou, Y., Bai, T., & Kishchenko, O. (2019). Potential of Lemnoideae Species for Phytoremediation of Fresh Water with Elevated Manganese Concentration. *Innovative Biosystems and Bioengineering, 3*(4), 232-238.

Zhu, Y. G., & Smolders, E. (2000). Plant uptake of radiocaesium: a review of mechanisms, regulation and application. *Journal of experimental botany, 51*(351), 1635-1645.

Supplementary material

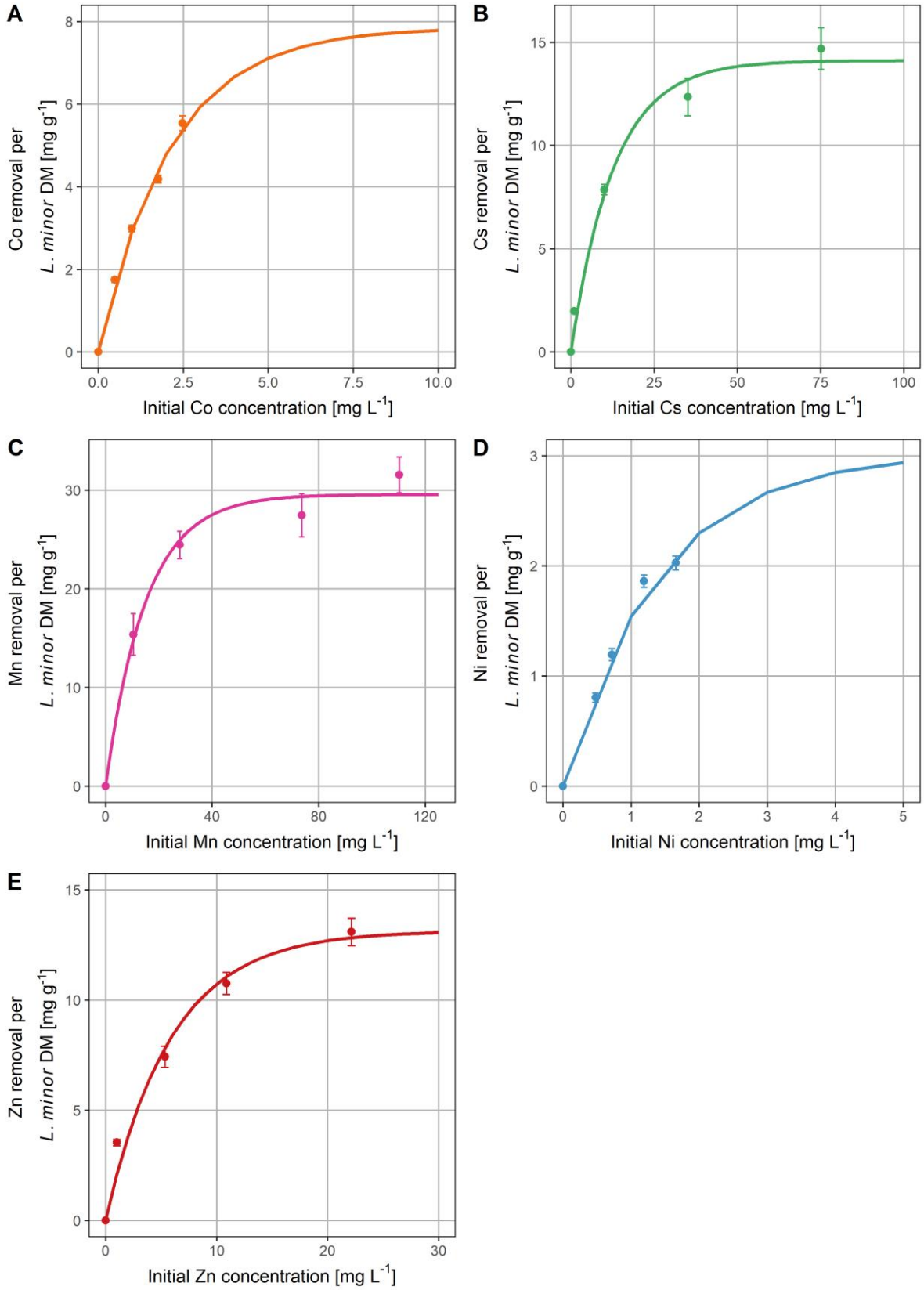


Fig. S 1 Pollutant removal per L. minor DM [mg g^{-1}] as function of the initial pollutant concentration in the solution [mg L^{-1}] for L. minor plants exposed to different pollutants and concentrations for 7 days: (A) Co, (B) Cs, (C) Mn, (D) Ni and (E) Zn. Data represent the mean value of at least three biological replicates, together with the standard error of the means and associated predicted curves.

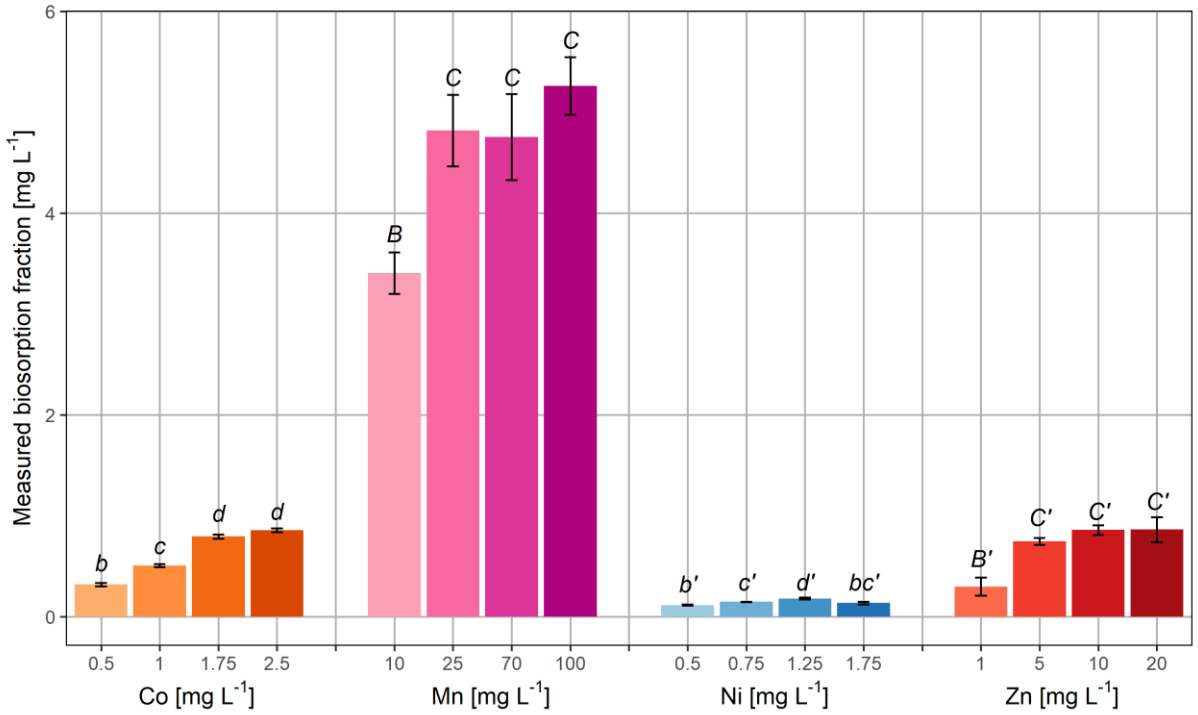


Fig. S 2 The measured bioaccumulation fraction of L. minor plants exposed for 7 days to different pollutants and concentrations. Data points represent the mean value of at least three biological replicates together with the standard error of the means. The control condition is not given on the graph, but represented with letter a, A, a' and A' (not given for simplicity reasons). Different letters indicate significant differences between concentrations of a pollutant, including the control condition ($p < 0.05$).

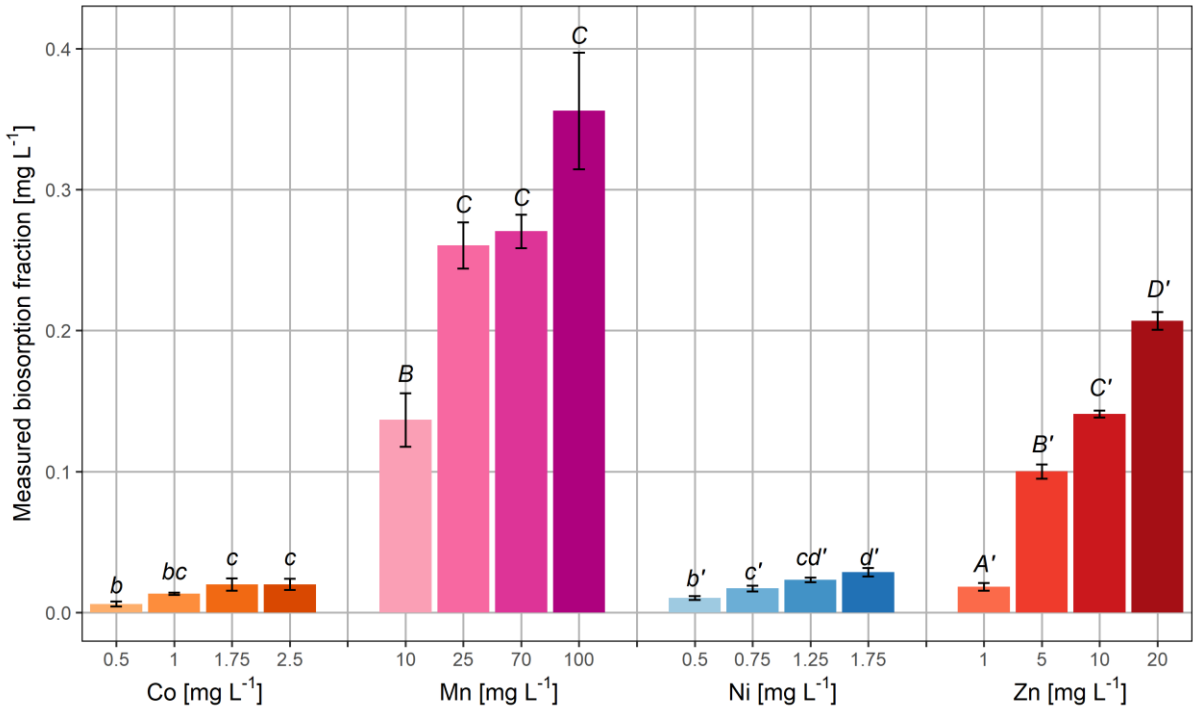


Fig. S 3 The measured biosorption of L. minor plants exposed for 7 days to different pollutants and concentrations. Data points represent the mean value of at least three biological replicates together with the standard error of the means. The control condition is not given on the graph, but represented with letter a, A, a' and A' (not given for simplicity reasons). Different letters indicate significant differences between concentrations of a pollutant, including the control condition ($p < 0.05$).