CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

FACULTY OF ENVIRONMENTAL SCIENCES

DEPARTIMENT OF ENVIRONMENTAL GEOSCIENCES

DIPLOMA THESIS

Nanoparticles as sorbents of metals/metalloids: implications for plant physiology

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Prague 2015

CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

Department of Environmental Geosciences

Faculty of Environmental Sciences

DIPLOMA THESIS ASSIGNMENT

Didac Barroso

Exchange programs

Thesis title

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Objectives of thesis

Metal nanoparticles such as nano-maghemite can have many applications in environmental remediation. The high reactivity of these compounds can allow stabilizing the metal/metalloids contaminants from the soil reducing its availability for the plants or other living organisms. In order to introduce the nano-maghemite compounds in the soil for contaminant fixation is important to know its effect on the organisms living in these environment. High toxicity of these compounds by them or combined with other compounds can be a huge disadvantage for the implementation of nano-maghemite in remediation. In the other hand a good interaction with some organisms can be a good argument for the use of these nanoparticles in some metal contaminated terrains. The knowledge about the uptake of NPs, the mechanisms by which they penetrate plants, interactions with cells, and potential toxic effects, is scarce and is hindering their full application.

The aim of this experiment is study the effects of nano-sized maghemite (Fe2O3; NM) on plant physiology focusing on the root hydraulic conductivity since the effects on plants may not be derived directly from chemical phytotoxicity of NPs and roots are the first organ which can suffer from physical interferences with NPs in the soil.

This research will provide information of high scientific and practical importance concerning the uptake of NPs and their effects for plants.

Methodology

A hydroponic culture experiment was performed in order to investigate the direct effect of the NPs on the plant physiology of a model plant, sunflower, during NPs exposure.

The interactions of NPs with roots were investigated taking into account the effects of NPs on transport of water and dissolved elements through roots, and potential transport of NPs through roots and their incorporation in plant tissues.

The proposed extent of the thesis

40

Keywords

Nanoparticle, plant, physiology, root, uptake

Recommended information sources

Domingo Martínez-Fernández, Deniz Bingol, Michael Komárek. 2014. Trace elements and nutrients adsorption onto nano-maghemite in a contaminated-soil solution: A geochemical/statistical approach. Journal of Hazardous Material 276, 271–277.

Domingo Martínez-Fernández, Martina Vítková, M. Pilar Bernal, Michael Komárek. Effects of Nano-Maghemite on Trace Elements Accumulation and Drought Response of Helianthus annuus L. in a Contaminated Soil. In press Water Air and Soil Pullution. March 2015, 226:101. DOI: 10.1007/s11270-015-2365-y.

Lukáš Trakal, Domingo Martínez-Fernández, Martina Vítková and Michael Komárek. 2015: Phytoextraction of metals: Modeling root metal uptake and associated processes. In: Anasari A.A., Gill S.S., Gill R., Lanza G.R., Newman L. (eds.): Phytoremediation: Management of Environmental Contaminants Vol. 1, Springer International Publishing Switzerland, 348s. DOI 10.1007/978-3-319-10395-2.

Expected date of thesis defence

2015/06 (červen)

The Diploma Thesis Supervisor

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Electronic approval: 20. 4. 2015

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Electronic approval: 20. 4. 2015

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Prague on 20. 04. 2015

Declaration

I hereby certify that work presented in this thesis and entitled as "*Nanoparticles as sorbents of metals/metalloids: implications for plant physiology*", is, to the best of my knowledge and belief, original, and that the material has not been submitted, either in whole or in part, for a degree at this or any other university. The literature and other sources, which I used, are stated list of references, which are attached to this work.

Prague, 20th April 2015 Didac Barroso Bergadà

Acknowledgements

I would like to express my gratitude to my research supervisor Domingo Martínez-Fernández Ph.D., for his guidance during my work. I would like to extend my thanks to the laboratory of the Department of Environmental Geosciences at the Czech University of Life Sciences. During the course of my experiment my work was partly supported by the European project Postdok ČZU (ESF/MŠMT CZ.1.07/2.3.00/30.0040). I am grateful for the opportunity to participate in this works, get to knowledge while doing my practical work and for the chance to make my input into its development.

ABSTRACT

Nanoparticles (NPs) have become an important option for the soil remediation in recent years. Many investigations have been done to determine the potential applicability of NPs for fixing or modifying the availability of contaminants such as metal(loid)s. However, physical and chemical interactions between NPs and living organisms in soils have not been properly studied yet. The application of NPs for remediation could also become a secondary contamination problem if all factors are not taken in consideration. In the following work, the promising NP nanomaghemite (NM) has been evaluated during its interaction with a bioremediation model plant, sunflower (*Helianthus annuus*), in a hydroponic experiment describing the root water transport, nutrients uptake and metabolic effects for the plants. Significant water uptake reduction has been found by the roots, causing the subsequent reduction of macronutrients presence in the shoots, with important effects in the energetic plant metabolism. This study will provide useful information for the combined use of NM and crops during soil remediation tasks.

Key words: contamination, phytoremediation, metal, nanoparticles, iron, oxides, soil, hydroponic, stress.

1. Introduction

1.1. Contaminated soils

Environmental pollution, especially by chemicals, is one of the most effective factors in the destruction of biosphere components. Among all chemical contaminants, metal(loid)s are believed to be of a specific ecological, biological, and/or health significance. The input–output balance of metals in soils shows that trace metal concentrations in surface soil are likely to increase on a global scale, with growing industrial and agricultural activities. Specially mining activity has led to significant build up a wide range this elements in soils, such as arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), mercury (Hg), lead (Pb), selenium (Se), and zinc (Zn). Soil contamination has become an important environmental problem worldwide because of its detrimental effects on human and ecosystem health, soil productivity, and socioeconomic well-being. The German Advisory Council on Global Change estimated 22 million ha of contaminated soils worldwide (GACGC 1994) and the European Environment Agency estimated the total costs for their cleanup in Europe to be between EUR 59 and 109 billion (CEC 2004).

Entry of soil-borne metal(loid)s into the food chain depends on the amount and source of metal(loid)input, the properties of the soil, the rate and magnitude of uptake by plants, and the extent of absorption by animals. High concentrations of metal(loid)s in soils have often been reported in number of countries, but soil contaminated with trace metals can produce apparently normal crops that may be unsafe for human or animal consumption. For example, significant adverse impacts of As on human health have been recorded in Bangladesh, India, and China and it is claimed that millions of people are potentially at risk from As poisoning. Metal(loid)s are deposited in soil usually by waste water flows coming from mining exploitations as a result of cleaning process of mineral. Similarly, there have been concerns about urban development of horticultural sites which contained toxic levels of metal(loid)s in soils resulting from excessive use of fungicides and herbicides that are rich in these metal(loid)s. Unlike organic contaminants, metal(loid)s do not undergo microbial or chemical degradation, and the total concentration of these metal(loid)s persist for a long time after their introduction in soils. Meta(loid)s use to affect all living organisms metabolism, being able to produce the dead of those organisms with low doses.

1.2. Green technologies and phytoremediation

Solving metal(loid)s contamination in soils even could allow to recover the agricultural use of the previously contaminated soil, recovering its economic potential and landscape view. Phytoremediation is a technology that involves application of selected plants to degrade, assimilate, metabolize or detoxify undesirable substances like metal(loid)s, pesticides, hydrocarbons, organic solvents and crude oil from soil and water to improve its quality. It is defined as the combined use of plants, soil amendments and agronomic practices to remove, retain environmental pollutants, or to reduce their toxicity (Salt et at., 1995).

Soil remediation techniques comprise in situ (non excavated soil) and ex situ techniques (soil is excavated). Ex situ remediation requires that the soil be transported to a treatment facility (e.g., soil washing). The in situ method on the other hand is a technology which try to remove or immobilize the metal(loid)s from contaminated soil without performing excavation and transport of contaminants. Experts tend to favour in situ techniques, which imply soil disposal as close to the source of contamination as possible. Compared to conventional strategies (in situ vitrification, soil incineration, excavation and landfill, soil washing, soil flushing and solidification), phytoremediation is an aesthetically pleasing, efficient and eco-friendly process in removing contaminants from low to moderate levels. It is also a low cost method which requires less than half of the price of the conventional methods. It also provides an added advantage by not only cleaning polluted soil but by also preventing soil erosion and metal leaching. This method causes less ecological disturbance and its economically aspect makes it a better alternative than ex situ technology. This method is further divided into different categories to remove toxic metals from soil and water: phytoextraction, phytostabilization, phytovolatilization, phytodegradation, and rhizofiltration (Gaur et al., 2013).

Phytostabilization seems to be the most promising technique of phytoremediation, especially for multicontaminated soils. Most contaminants cannot be degraded or volatilized. Filtration and extraction normally are only possible in liquid media and became very difficult in soils. Therefore phytostabilization provides a more promising approach for major part of contamination solving. Plants can help to keep contaminants in soil avoiding its mobility to water sources or other living organisms avoiding the consequences of the presence of these contaminants.

1.3. Oxides for stabilization of contaminants

Soil oxides are ubiquitous natural soil components present virtually in all soil types. These weathering products occur in soils as discrete crystals, coatings on other particles and as mixed gels and although they are not usually present in large quantities, they play a major role in soil chemistry. Soil oxides are usually characterized by their small particle size (tens nm to thousands mm) and low solubility in the range of common pH values found in soils. Due to their important sorption properties, metal oxides (especially Fe) have been extensively studied as potential stabilization amendments in soils contaminated with metals and As. Their application, either direct or indirect through the application of their precursors (e.g., bulk iron or Fe sulfates) is supposed to decrease mobile, bioavailable and bioaccessible fractions of the metal(loid)s and minimize thus possible risks of environmental contamination, leaching and uptake by soil organisms, plants, crops and humans (Komárek et al., 2012).

1.4. Nanoparticles

1.4.1. Applications

Nanotechnology is an emerging applied science with a mission in designing tools and devices of submicroscopic size between 1 to 100 nm harbouring specific function at atomic, molecular and cellular levels. The nanomaterials produced by industries have been used widely in electronics, medicine, physics, chemistry, biology, but also in food and cosmetic industries. Nanotechnology produces for these sectors are steadily increasing in the last years, but the predictions are for a further and rapid increase. The global market for nanomaterials is estimated at 11 million tonnes at a market value of 26 billion USD in 2014, reaching 64 billion USD in 2019 (20 bn €). The current direct employment in the nanomaterial sector is estimated at 300000 to 400000 in Europe (European Comision of Enterprise and Industry).

Nanoparticles (NPs) are being incorporated into many products of daily use, e.g. fillers, opacifiers, catalysts, pharmaceuticals, lubricants, cosmetics, pharmaceuticals, electronic devices or other domestic appliances (Nel et al., 2006). Generally speaking, the following NPs are already used on the industrial scale: Ag, Al–Ox, Ce-Ox, Fe–Ox, SiO_2, TiO_2, ZnO.

Since environmental remediation has been one important focus of research in the last 40 years, NPs application also has been studied in this field. E.g. NPs have been found effective for solving As contamination problems acting as sorbents combined with some plant cultures

(Singh et al., 2014); Fe-Mn NP also have been found useful in immobilization in metal(loid)s such as Se with promising results (Xie et al., 2014); Fe NPs are also effective in solving plastic contamination problem such as PCB declorating this compounds due its great oxidative power (Gomes et al., 2015).

1.4.2. Risks for the environment

It is inevitable that this increasing application of nanotechnology will result in potentially significant release of engineered nanomaterial (ENMs) into the environment. Unfortunately, our understanding of NPs fate and effects in terrestrial and aquatic ecosystems has lagged far behind implementation of the technology. Given the lack of regulatory guidance and requirements for NPs use, critical knowledge gaps may remain unanswered in the short to medium term and, as such, judicious use of NPs is warranted (Gardea-Torresdey et al., 2014; Holdem et al., 2013). Nanoparticles can be released to the environment (Nowack and Bucheli, 2007) as bare NPs, functionalised NPs, aggregates, or embedded in a matrix. Nanoparticles released to the environment, deliberately or accidentally, disperse in the environment reaching water, soil and the air. There, they can persist for a long time or be taken up by biological organisms. They can act as ecotoxicological hazard, undergo biodegradation or bioaccumulate in the food chain (European Comision of Health and food Safety). For example, it has been shown that nanodispersed platinum group elements can be transferred to animal tissues (Ek et al., 2004); and Hawthorne et al., (2014) found that NPs CeO_2 accumulates in zucchini at greater levels than equivalent bulk materials and that this greater NPs intake results in trophic transfer and possible food chain contamination.

1.4.3. Nanotoxicity for plants

Plants play a critical role in maintaining ecosystem health and function and as a food source for humans. Plant uptake of ENMs represents an important pathway for human exposure to these NPs through food consumption. Consequently, investigation of the uptake and accumulation of ENMs by agricultural crops is not only warranted but also critical to food safety and human health. However, there are only a small number of studies in the literature that have addressed the interactions of NPs with terrestrial plants.

Kirchner et al., (2005) distinguish three main causes of NPs toxicity following contact with live cells:

(1) Due to chemical toxicity of materials from which they have been made: e.g. Cd_2^+ is released from NPs of cadmium selenide.

(2) Due to their small size: NPs may stick to cellular membranes and enter the cells. Attachment of NPs to the membranes and storage of NPs inside the cells can impair cellular functions even in the case of chemically inert NPs, which do not decompose and do not react with other matrix components.

(3) Due to their shape: e.g. carbon nanotubes can easily pierce cell membrane.

It is now evident that particle size is relevant for the particle toxicity (Nowack and Bucheli, 2007; Zhang et al., 2014). A classical example of toxicity due to the shape, in addition to chemical composition, is toxicity of asbestos (Maynard et al., 2006; Poland et al., 2008). In the past, a side effect of long-term utilisation of asbestos was emission to the environment of asbestos fibres, which can also be considered nanomaterials according to the present classification. They have been shown to induce cytogenotoxicity and therefore are regarded as carcinogens (Takeuchi et al., 1999). At present, we know that one of the reasons for its high toxicity was the shape of released NPs which could pierce cells.

The toxicity of carbon nanomaterials also depends on their geometric structure (Jia et al., 2005). Carbon nanotubes have been shown to cause necrosis, cell degeneration and apoptosis in macrophage cell lines (Jia et al., 2005). Recent studies point to the similarity of the toxicity of asbestos and carbon nanotubes and therefore suggest great caution before introducing nanoproducts to the market (Poland et al., 2008). Even more attention needs to be paid when introducing NPs containing metal(loid)s since, in this case, one may anticipate a magnified toxic interaction with cells – due to size/shape as well as chemical composition.

Surface modifications of NPs can also have an influence on the uptake and toxicity of NPs (Kostarelos et al., 2007; Verma et al., 2008). Until now, there have been few attempts of quantitative assessment of differences in the uptake and toxicity of chemically modified NPs. Surface modifications may also affect aggregation and agglomeration of NPs which will also influence their toxicity and behaviour in the environment (Buzea et al., 2007; Kostarelos et al., 2007).

1.4.4. Nano-Maghemite

Nano-maghemite is a nano sized (from 1 to 100 nm) iron oxide (Fe_2O_3). The reduced size of this oxides increase in a huge way the available reactive surface regarding the bulk iron oxide. The increased reactive surface possibilities a wider reaction capacity. In bioremediation NM forms a coordinated compound with metal(oids) present in soil reducing their availability for all the living forms (Vitkova et al., 2015). NM uses as contrast in magnetic resonance or in hyperthermia treatment thanks to their biocompatibility and biodegradability. NM has been already tested in bioremediation fixing metal(loid)s in contaminated soils (Grieger et al., 2010). However more information is needed in order to understand the complete affect of NM in living organisms such as plants. If NM is used in phytoremediation without knowing properly their effects, further contamination can be caused.

2. Objectives

Metal NPs such as nano-maghemite (NM) can have many applications in environmental remediation. The high reactivity of these compounds can allow stabilizing the metal/metalloids contaminants from the soil reducing its availability for the plants or other living organisms. In order to introduce the nano-maghemite compounds in the soil for contaminant fixation is important to know its effect on the organisms living in these environment. High toxicity of these compounds by them or combined with other compounds can be a huge disadvantage for the implementation of nano-maghemite in remediation. In the other hand a good interaction with some organisms can be a good argument for the use of these NPs in some metal contaminated terrains. The knowledge about the uptake of NPs, the mechanisms by which they penetrate plants, interactions with cells, and potential toxic effects, is scarce and is hindering their full application.

The aim of this experiment is study the effects of nano-sized maghemite (Fe_2O_3; NM) on plant physiology focusing on the root hydraulic conductivity since the effects on plants may not be derived directly from chemical phytotoxicity of NPs and roots are the first organ which can suffer from physical interferences with NPs in the soil.

This research will provide information of high scientific and practical importance concerning the uptake of NPs and their effects for plants.

3. Materials and Methods

3.1. Design of experiment

A hydroponic culture experiment was performed in order to investigate the direct effect of the NM on the plant physiology of a model plant, sunflower, during NPs exposure. The interactions of NM with roots were investigated taking into account the effects of this NPs on transport of water and dissolved elements through roots, and potential transport of NM through roots and their incorporation in plant tissues.

Sunflower (*Heliantus annuus*) was chosen as candidate because is a high studied plant which has a good resistant to metal(loid) contamination and has even shown the capacity of removing certain concentrations of metal(loids) from water (Nehnevajova et al., 2012). *H. annuus* also has fast germination and growth and large biomass production, important arguments for the implementation of sunflower in phytoremediation crops in metal(loid) contaminated soils. The ability of drought resistance (Ghobadi et al., 2013) also makes that sunflower can grow in not well irrigated soils decreasing the irrigation expenses and making the crops cheaper. Products obtained from sunflower crops have a huge range of application, going from human and animal alimentation to biomass usage for biocombustible production (Zhao et al., 2013) or industrial uses of oil obtained from seeds. All the analyzed factors seems to point to the good option of sunflower as a good candidate for uses in metal(loid) remediation. The axonomorph root also helps to measure the root hydraulic conductivity.

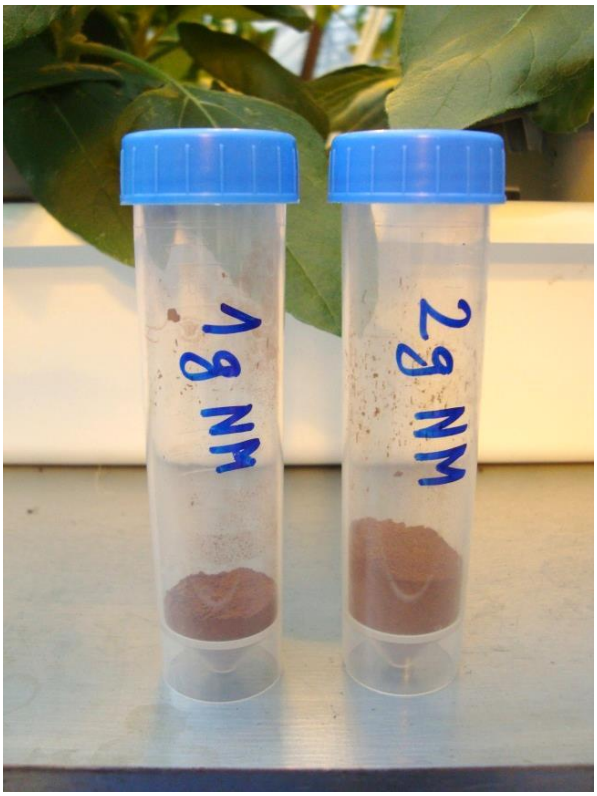
3.1.1. Growing the sunflower plants

Seeds of *Helianthus annuus* (L.) were planted in perlite:substrate (2:1 w/w) and after 20 days, representative specimens were subsequently transferred into hydroponic culture solution after proper root cleaning. The hydroponic culture was performed in in opaque containers, 40x20x20 sized, with 5 l of a modified Hoagland solution (Table 1). The containers (5 replicates per treatment) were randomly distributed on a bench in a glasshouse under controlled conditions (20-25°C, 13 h daylight/11 h darkness and humidity 60-80%). The solution was replaced every 7 days. Four plants were placed at the cover plate of each recipient with the roots introduced in the solution. Each container had an aquarium aerator to provide oxygen to the roots and later to maintain the NPs in suspension.

Table 1 *Modified Hoagland Solution used during the grow period of the hydroponic experiment*

Hydroponic Sol. Reactive	Concentration (µM)
Macronutrients	
$Ca(NO_3)_2.4H_2O$	2000
KNO_3	2000
NH_4NO_3	1000
KH_2PO_4	1000
$MgSO_4.7H_2O$	500
Micronutrients	
H_3BO_3	25
$MnSO_4.H_2O$	2
$ZnSO_4.7H_20$	2
$CuSO_4.5H_2O$	0,5
$(NH_4)_6Mo_7O_{24}.4H_2O$	0,1
NaCl	50
FeEDDHA	20
pH	6,5

Image 1 *NM particles in falcon tubes*



3.1.2. NM treatment

After 25 days of grow, three treatments with the NM were imposed: Control without NM; 50 and 100 mg l^{-1} of NM. Pure and previously-characterised NM, purchased from Sigma Aldrich (Germany) as Fe (III) (γ-Fe_2O_3; particle size 20-100 nm; pH = 3.0; pHzpc = 7.4; BET = 46.6 m^2 g^{-1}; Michalkova et al., 2014), was chosen in order to ensure the reproducibility of the studies with this material. The iron source of the Hoagland solution was changed by 20 µM $FeCl_3$ (replacing the FeEDDHA with $FeCl_3$ during the treatment with NM, to avoid that the chelant interferes with the absorption of NM).

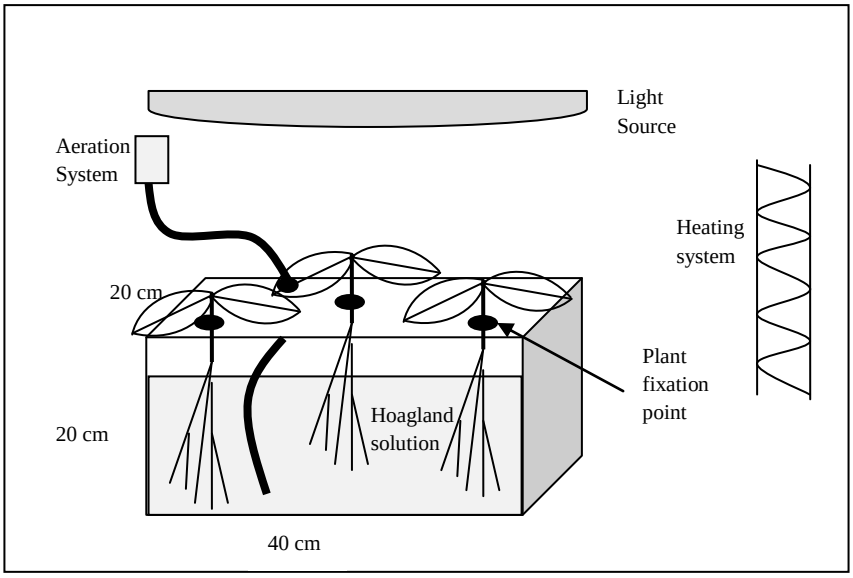


Image 2 *Diagram of one container of the hydroponic culture.*

3.1.3. Harvest and conservation of samples

After 7 days of NM treatment, plants were harvested. The plants were cut separating the shoot or aerial part and the root and weight separately. After weighting, plants were placed in paper envelopes and frozen. After lyophilization, samples were weight again and milled. The milled material was stored in tubes for further analysis.

Image 3 *Hydroponic culture of H. annuus during the experiment with NPs in the glasshouse.*

3.2. Analysis

3.2.1. During Harvest

- *Root hydraulic conductivity* (L_o): Once cut, roots were introduced in the pressure Scholander chamber (PMS instrument 06 Corvallis, Oregon USA MODEL 600). At more than five different pressures between 0.1 and 1.5 MPa the flux of sap was measured. For measuring the flux of sap, it was collected in different eppendorf tubes every 60 seconds using a syringe. The weight of the eppendorf tube with the sap was taken later. L_o was calculated as the slope of the resultant straight line of representing mg of gathered sap per g of dry root and per hour against the pressure imposed (mg g^{-1} DW h^{-1} MPa^{-1}): L_o = mg of sap / (g of root dry weight x time to fill every eppendorf (h) x MPa.

Image 4 *L_0 test using a Scholander chamber with each complete root system.*

- *Leaf water potential* (Ψ_w): the xylem pressure potential was measured for a leaf of each plant which had been covered with aluminum foil 2 h earlier, in a Scholander pressure chamber. Pressure was increased until the first water drop appears in the petiole. The pressure data was recorded. The water potential was measured in one leaf after two hours of darkness, therefore the hydric potential in that leaf was the same as all the aerial part of the plant.

- *Relative water content* (RWC): 8 mm diameter discs were cut from the leaves of plants from each container according to Walker et al. (2010). The discs were weight (FW) and introduced in Petri dishes with miliQ water. After 24 hours, the discs were weight again in order to obtain

the turgor weight (TW), and introduced in a stove at 60 ºC during 24 h for the dry weight (DW) determination. Relative water content was calculated using the Turner equation: RWC (%) = [(FW-DW) / (TW-DW)] * 100. From the total area and dry weight of the discs, the specific leaf area (SLA) was calculated. Both RWC and SLA were determined for plants to compare these parameters in each treatment.

- *Biomass*: Roots and shoot were weight just after harvesting. The plants (leaves, roots) were lyophilized until constant weight in order to conserve the organic components structure. The metals and nutrients were extracted from the freeze-dried plant material by microwave acid (HNO_3/H_2O_2) digestion at 210°C, and determined by ICP-OES (Varian, VistaPro, Australia). For each set of samples, a reference plant material (Bowen's Kale; IUPAC 1979) was also digested. After lyophilization they were weight again for the dry weight determination (DW).

- *Chlorophylls*: At the end of the experimental period, photosynthetic pigments were extracted from fully expanded leaves of plants grown under each treatment, using 200 mg of fresh plant material in a mortar with 80% aqueous acetone. The homogenized was filtered and completed to 25 ml with the same acetone. Chlorophyll a (Chl a) and chlorophyll b (Chl b) contents were determined with a spectrophotometer, using two wavelengths (663 and 645 nm). Pigment concentrations (μg g^{-1} FW) were calculated following the method of Lichtenthaler & Wellburn (1983), by the equations to determine Chl a and Chl b content in 80% acetone extracts: Chl a (mg l^{-1}) = 12,21 x A_{663} – 2,81 x A_{645} and Chl b (mg l^{-1}) = 20,13 x A_{645} – 5,03 x A_{663}.

3.2.2. Lyophilized and milled samples

- *Proline* (stress indicator) was extracted from 150 mg freeze-dried tissue mixed with 15 ml of d-water and incubated in a water bath at 100 °C for 1 h. After filtration, the determination was carried out by the methods of Bates et al., (1973).

- *Determination of proline*: 2 ml of the extraction were mixed with 2 ml of acid Ninhidrin and 2ml of glacial acetic acid. The mix was incubated 1 hour at 100ºC. Then, 4 ml of toluene were added and mixed, obtaining two phases. The toluene phase was transferred to a spectrophotometer crystal cuvette. The product dissolved in toluene was measured on a UV/VIS spectrophotometer at 520 nm with a calibration curve using L-proline (Sigma) from 0 to 200 μM proline standards.

- *Ascorbate*: the extraction was done with 100 mg of milled samples mixed with 200ml metaphosphoric acid 5%, at 4°C to prevent oxidation of ascorbate. The homogenates were centrifuged at 18,000 xg for 20 min and 4°C.

- *Ascorbate determination* (ASC, in reduced state): 250 µl phosphate buffer -Na 150 mM pH 7,4 (NaH_2PO_4+NaOH; 1M +Na), 250 µl EDTA 5 mM and 100 µl of the extract (supernatant) were mixed. The reaction was carried out in Eppendor tubes, where 150 µl of TCA 10% were added, and 200 µl of phosphoric acid 44 %, 200 µl of α-α´dipiridil 4% in ethanol at 70%, and 50 µl of $FeCl_3$ in TCA at 10%. The reaction mixture was allowed to incubate for 40 min at 40°C (in water) and subsequently read the ASC at 525 nm using a spectrophotometer. For ASC quantification, the results were extrapolated from standards prepared from commercial ascorbic acid (10-60 µM).

3.3. Statistical treatment

Each treatment was conducted with five replicates. The experimental results were compared using the ANOVA-one way statistical treatment with SPSS software for Windows (Version 19.0). Statistical significance was accepted when the probability of the result assuming the null hypothesis (p) was less than 0.05. The normality of the data was checked with the Kolmogorov–Smirnov test for all the parameters measured. In case of negative results from the K-S test, data were transformed in order to get the normality. Differences among means were determined using Tukey's test at the 0.05 probability level.

4. Results and Discussion

4.1. Effects in the water transport

Significant reduction of L_o was found. The treatment with 50 mg l^{-1} NM caused a L_o reduction of 46 % respect the control and 100 mg l^{-1} NM caused a reduction up to 74%. No significant differences were found in the RWC, SLA and Ψ_w.

Table 2 *Water relations of H. annuus cultivated hydroponically after 7 days of exposure to NM, when the plants were harvested: Root hydraulic conductivity (L_o), leaf relative water content (RWC), specific leaf area (SLA), leaf water potential (Ψw). Within each column, values not sharing a common letter are significantly different ($P < 0.05$) according to Tukey's test (ns=no significant).*

Treatment	L_o (mg g^{-1} h^{-1} Mpa^{-1})	RWC (%)	SLA ($m^2Kg^{-1}DW$)	Ψ_w (MPa)
Control	1303.9 a	82.173	64.520	6.960
+50 mg l^{-1} NM	740.9 b	81.660	67.483	7.940
+100 mg l^{-1} NM	344.3 c	81.152	68.716	7.980
ANOVA	**	ns	ns	ns

The reduction of L_o involves a reduced plant capacity to obtain water and the pass of this water to the shoot, although in this experiment any other change in the water relation was related to the treatment with NM (RWC, SLA or Ψ_w). Maybe the high reactive capacity of NM due its reduced size could produce a big binding capacity to the epithelial root cell wall. The accumulation of NM in epithelial cells could interfere in the water transport because of several physical and chemical interactions, with the consequent L_o reduction. Root cell water transport pore diameter is found to be 6.6 nm, and NM particles diameter oscillates between 20 and 100 nm, therefore the most of solved NPs cannot cross the pore and in consequence, stay blocking the pores stopping the water absorption capacity by the epithelial root cells. Aggregation of NM particles (observed in the roots of this experiment; Image 5) also formed a cake which could cover the epithelial cells stopping all the interactions between these cells and the media. The accumulation of NM in cell wall surface also changes the potential charge preventing the interactions of these cells with the media (Dietz & Herth 2012). Huge reactivity of NPs also produces the interactions with proteins or phospholipids in membrane

responsible of osmotic regulation modifying these compounds in an irreversible way (Tang & Lo 2013). Osmotic regulation problems can also cause an impediment for root cells to transport water from outside part of roots to inside the xylem.

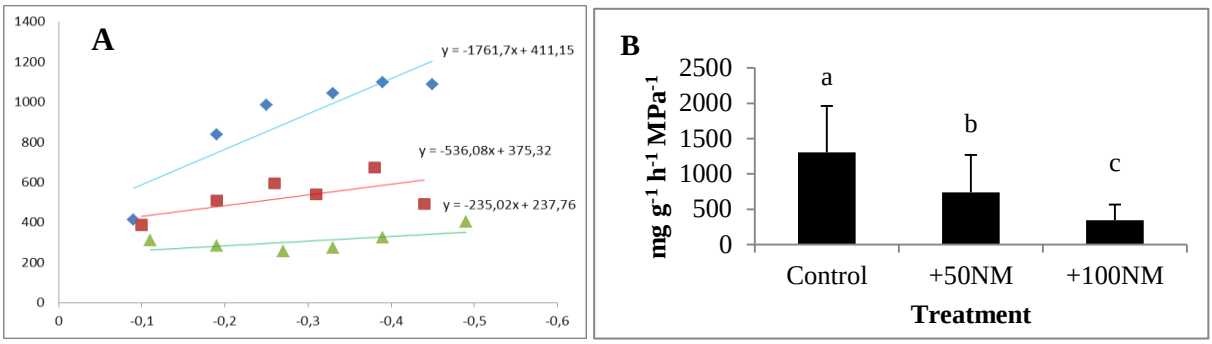


Figure 1 (A) *Example of the root hydraulic conductivity (L_o) of three plants of H. annuus from each treatment (blue=control; red= +50 mg l^{-1} NM; green= +100 mg l^{-1} NM). It was calculated as the slope of the resultant straight line of representing mg of gathered sap per g of dry root and per hour (x) against the pressure imposed in MPa (y).* **(B)** *Relation between the averages L_0 in the plants of each treatment with NM during 7 days.*

Several previous works have shown disruption of plant water relations by NPs in different experiments with different kinds of NPs. In agreement to our results, Asli & Neuman (2009) found the effect of titanium oxides NPs to water transport increasing due the exposition time to these NPs in hydroponic culture. Only 4h of exposition to TiO_2 was enough to find a reduction of 25% in water transport capacity. This data can explain the big difference between control and treatments in the experiment due the long exposition of sunflower plants to the NM, 7 days. This knowledge can let to deduce that plants grown in a NPs treated soil can find its water transport capacity reduced.

Root growth was also found to affected by the metallic NPs. Moon et al., (2014) found that the growth of roots is directly affected by the concentration of Cu nano-oxides and seed germination. The effects in plant growth have not been found in this experiment (Figure 4), being the root biomass no different between control and treatments by NM, but the effect in root growth found by Moon et al., (2014) can lead to think in an effect to the epithelial meristem cells in roots. Meristem cells need some space for growing and an important connection between the cells to coordinate growing. Physical impediment caused by NM cake and chemical interaction between NM and cell wall can explain the problems for root growing. It is also necessary to test a longer root exposition to NPs to investigate their effects.

On the other hand, Lü et al., (2010) found that treatment of silver NPs improve the water transport in rose cuts. This effect shows that NPs can affect the epithelial root cells but seems that they do not have an important effect in the xylem of the plant. The low effect in the remaining water transport system is a positive fact for the uses of NPs in bioremediation since it only seems to affect the root system. Improvement of water transport respect to the control can be caused because of the interaction of silver NPs with the damaged tissue keeping it from healing (Lü et al., 2010). The fact that there are no changes in the water potential shows that the plant has enough water and the water transport system in the shoots was not dramatically affected as is found in Lü et al., (2010).

Even though the L_o was seriously affected by the NM treatment, the RWC did not change by the treatment, maybe because of the hydroponic culture and the high availability of water in excess for plants. In hydroponic cultures plants have more than enough water, so even a damaged water transport system can collect all the water needed by the plant. Further research is needed to clarify whether root functionality is affected by NPs in different types of soils, where their effect on the surfaces of roots could be less dramatic. So, it is important to investigate this behavior in a soil culture with a more privative water presence. Martínez-Fernández et al. (2015) did not found changes in RWC of *H. annuus* in contaminated soil treated with NM. This fact may be due to the adherence of the NPs to soil particles and sand grains.

4.2. Effects in nutrient concentration

As expected, Fe concentration in roots was different among treatments. Increment of 18.29 mg l^{-1} from control to 100 mg l^{-1} NM treatment was found. No difference in sap and shoot were found between controls. Macronutrients concentration showed significant differences in roots. Decreasing of 48% in Mg concentration and 33% in S concentration in shoots between control and 100 mg l^{-1} NM treatments were found. Mo concentration in roots and sap also shows significant differences, being the only micronutrient with significant differences caused by the treatment with NM.

Table 3 *Concentration of nutrients in the sap (mg l^{-1}) and in the shoot and root (mg g^{-1}DW) of H. annuus after 7 days of treatment with NM in hydroponic (nd= no determined).*

Treatment	Material	Ca	Cu	Fe	K	Mg	Mn	Mo	Na	S	Zn
Control	Sap	32.681	nd	0.173	178.452	14.524	0.242	0.074 ab	4.636	nd	0.860
+50NM	Sap	41.595	nd	0.290	222.879	16.425	0.334	0.087 a	3.803	nd	1.532
+100NM	Sap	42.950	nd	0.315	200.378	15.161	0.290	0.049 b	3.309	nd	1.429
ANOVA		ns		ns	ns	ns	ns	*	ns		ns
Control	Shoot	25.01 a	0.002	0.320	25.24 a	4.684 a	0.071	0.014	0.437	3.897 a	0.347
+50NM	Shoot	19.85 ab	0.002	0.346	20.72 a	3.355 b	0.065	0.012	0.443	3.078 b	0.317
+100NM	Shoot	16.86 b	0.002	0.327	17.80 b	2.922 b	0.073	0.012	0.487	2.622 b	0.278
ANOVA		*	ns	ns	*	***	ns	ns	ns	***	ns
Control	Root	5.437	0.024	0.756 c	22.744	1.707	0.055	0.019 c	2.108	2.485	0.136
+50NM	Root	7.290	0.024	8.277 b	20.553	1.989	0.042	0.026 b	1.982	2.429	0.166
+100NM	Root	5.489	0.025	19.048 a	22.753	1.613	0.044	0.037 a	2.124	1.957	0.141
ANOVA		*	ns	***	ns	ns	ns	***	ns	ns	ns

Image 5 *NM aggregation onto the roots of H. annuus at the end of the hydroponic experiment with NM.*

The interaction between de NM particles and the epithelial root cells described previously causes a huge accumulation of iron Fe compounds in the epithelial part of roots (Image 5). As expected, the concentration of treatment has a direct influence in the Fe concentration because of the adherence (Figure 2). Mainly all the accumulation of Fe was caused by the adherence of NM particles to the roots, however little amount of Fe is absorbed from the Hoagland

solution. The uptake from the epithelial cells to the central part of the root was not deduced from the sap analysis, which was taken from the root, and it did not show significant variance on the Fe concentration between treatments. This fact looks to prove that Fe NPs suspended in the media cannot cross the membrane, maybe because of their size and charge but more research must be done. The absorption of Fe in sunflower takes place using the IRT-1 membrane transporters, these transporters are only expressed when plant is in an iron deficiency situation, then, Fe concentration in plant remains controlled in function of plant needs (Conte & Walker 2011). This fact is supported in the experiment data by the absence of differences between concentrations of Fe by the treatments in the aerial part. Therefore, Fe accumulation is only obtained from $FeCl_3$ dissolved in Hoagland solution and no from NM. Highly reactive NM particles can also react with the IRT-1 producing a blockage of these absorption channels by the adherence to the proteins or changing the spatial distribution of these proteins as described by Dietz & Herth (2012).

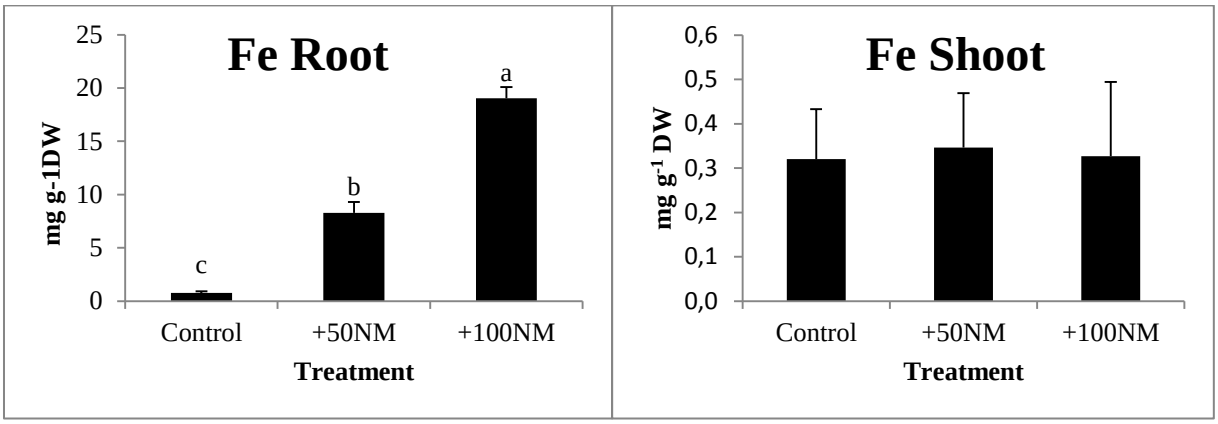


Figure 2 *Comparison of Fe concentration (mg of Fe g^{-1} of plant) in Root and Shoot with 50 and 100 mg l^{-1} NM.*

However, the majority of the NPs appeared to remain in the root tissues, raising concerns on the heightened accumulation of NPs by root vegetables (Boxal et al., 2007). Zhang et al. (2014) found that ZnO NPs were able to reach the shoots of *Schoenoplectus tabernaemontani*. Nanoparticles absorbed increase in function of the time of treatment in a linear behavior. The amount of NPs taken after 7 days of treatments can reach the 3% in the aerial part of the plant (Zhang et al., 2014). This only happens when plants are treated with low concentrations of NPs such as 10 mg l^{-1}, higher concentration reduce the uptake of the ZnO NPs in a huge way. These results are consistent with the results of this experiment because the concentrations of

the treatments with NM are 50 mg l^{-1} and 100 mg l^{-1}, this amount means and increasing of ten times of concentration compared with Zhang et al. (2014). Therefore, the uptake of NPs maybe was reduced to non-detectable differences because of the interferences of absorption described upper in the text. At the experiment short time treatment doesn't let. Then, the uptake of NPs is not only related to one kind of trace element absorption channels and the effect can be extrapolated to the major part of the metalloids. In order to understand the effects of NM in the plant physiology it is important to understand properly the changes in the macronutrient absorption and distribution. Significant differences were no detected for the concentrations of the macronutrients Mg, K, Ca and S between treatments in root and sap (Figure 3). Although the used Hoagland solution could provide to the plant all the necessary nutrients, the plants had some impediment to transport these nutrients to the shoots since significant differences were detected for these macronutrients among treatments in the shoot.

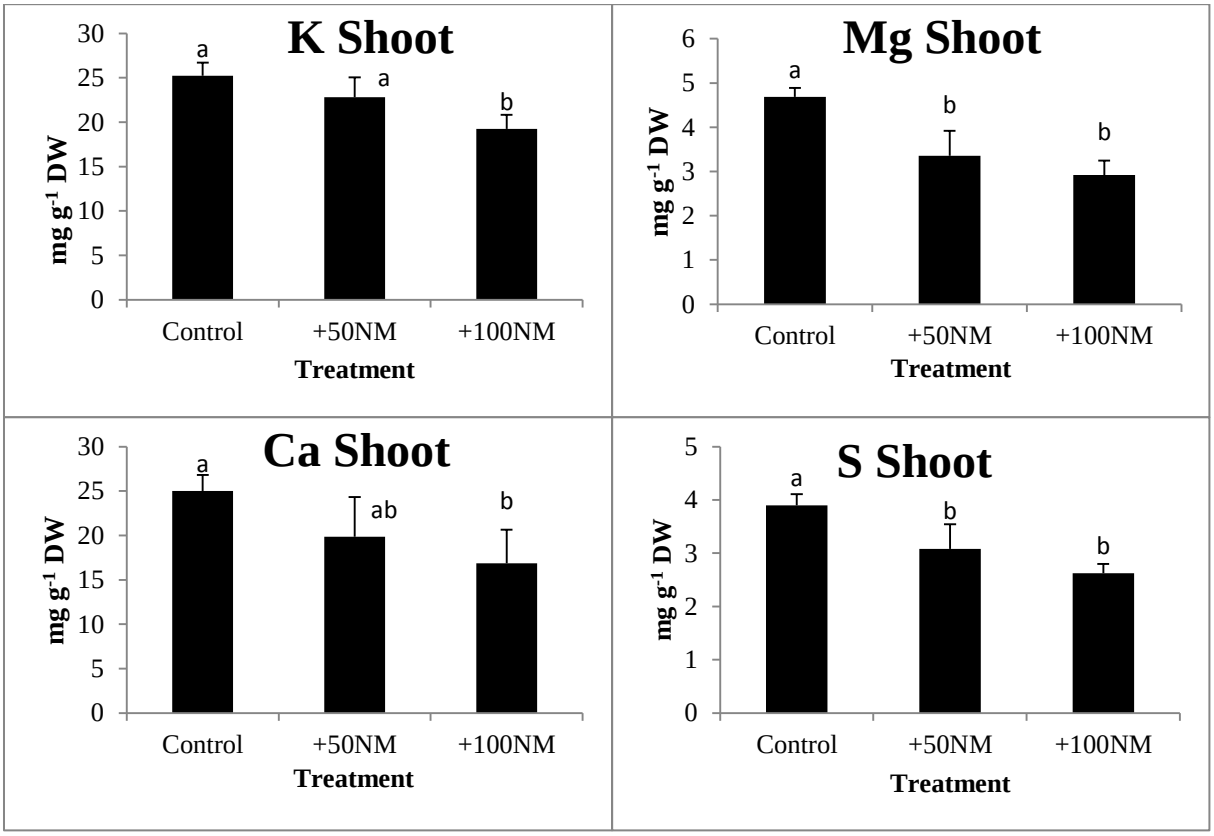


Figure 3 *Comparison of macronutrient concentration (mg of macronutrient per g of DW) on of H. annuus grown in hydroponic after 7 days of treatment with 50 and 100 mg l^{-1} NM.*

This fact could be explained because root cell vacuoles keep major part of the obtained K, Ca and Mg to balance the high osmotic potential caused for the high amount of NM particles adhered in the outside part of the roots. The absorption of macronutrients to the roots also can be limited because the NM damages or blocks the transporters involved in the uptake of Ca, K, Mg and S as the same way as the Fe transporter described previously. A damaged water transport system involves a lower capacity to pass the water to the shoot, making more difficult the transport of cations. Besides, a lower flow of water could cause a deficiency of these macronutrients in the shoot according to the plant requirements. These three combined effects (osmotic effect in roots, reduction of L_o and effects in nutrient uptake) of NM caused a reduction of the macronutrients concentration in the aerial part of the plants treated with the NM. According to Martínez-Fernández et al. (2015), a significant reduction of macronutrients concentration in roots and shoots can be caused by the effect of NM on *Helianthus annuus* grown in contaminated soil during 42 days. Maybe the effect found also in the roots was due by the longer period of exposition which affected the root cell transport channel in a more important way.

Molybdenum is a trace element important for many proteins for its correct function such as related with nitrogen fixation or purine degradation (Bittner 2014). The concentration of Mo increased related with the dose of NM. This fact could happen because of the close relation between the Fe and Mo adsorption systems. The increase of Fe concentration in the media facilitates the transport of Mo to the interior of the plant (Bittner 2014). The increase of Mo in roots does not cause a significant augment in shoots, even a little reduction was found. This fact can be caused for the difficult of transport from roots shoots derived from a lower L_o in treatments.

Concentration of any other micronutrient in shoot did not show any difference between treatments. In that case, in spite of the reduction of the water flow through the roots, the plants had no enough impediments to obtain the needed amount of micronutrients for their metabolism and no significant differences between the concentrations of micronutrients in the shoots were detected.

4.3. Effects in plant growth

The low macronutrients level did not produce a different growth observed between treatments (Figure 4). The absence of effect on the biomass could be explained by the time of the treatment and by the use of NM treatment in already grown plants or because macronutrients lower concentration does not reach deficiency levels. However irons NPs such as NM have been proved to have an important long term effect on plant growth.

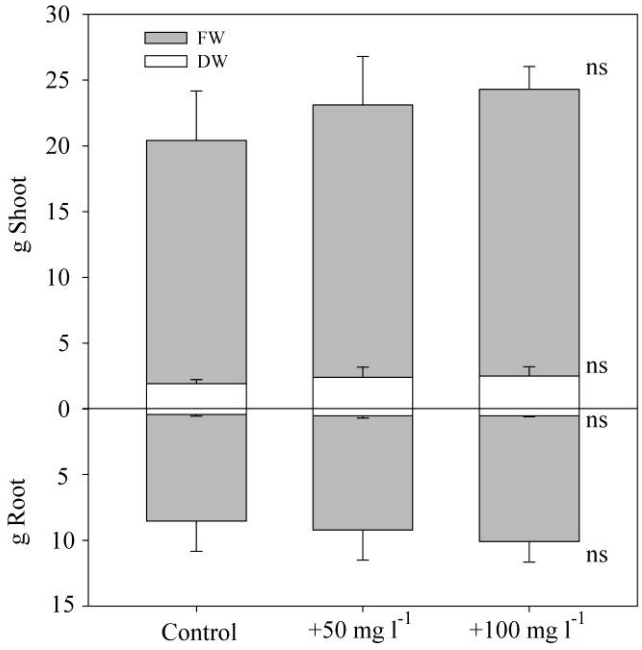


Figure 4 *Fresh (FW) and Dry Weight (DW) of the biomass per plant of H. annuus of the shoot and root matter at the end of the experiment with NM.*

Garcia et al. (2011) found that 74% of *Cucumis sativus* or 87% of *Solanum lycopersicum* did not germinate for the effect of iron NPs. Also Song et al. (2015) found some inhibition on the growth rate of *S. pholyrhiza* after 7 days of treatment with Cu NPs. Long term exposition to NPs also is shown to affect the growth. Bandyopadhyay et al. (2015) found that *Medicago sativa* has an 80% of root biomass less than the control after 30 days of treatment. However *H. annuus* treated with NM in soil by Martínez-Fernández et al. (2015) showed a 25% increase in shoot biomass with no differences in root biomass. This fact can be caused because soil keeps the NM and highly reduces its viability for roots and the little amount viable helps the macronutrient transport as found by Zhang et al. (2014).

4.4. Effects in metabolism

Significant differences were found in the *Chl a* and *Chl b* concentrations. The total chlorophyll concentration was reduced from 1771.46 µg g^{-1} at control to 1563.11 µg g^{-1} in 50 mg l^{-1} NM treatment and to 1154.76 µg g^{-1} in 100 mg l^{-1} NM treatment.

Table 4 *Chlorophylls a (Chl a) and b(Chl b) and ascorbate (ASC)contents in the fresh leaves of H. annuus and the proline content in the shoot and root dry matter (lyophilised). Within each column, values not sharing a common letter are significantly different (P < 0.05) according to Tukey's test (ns=no significant).*

Treatment	*Chl a* µg g^{-1} FW	*Chl b* µg g^{-1} FW	ASC nmol g^{-1}FW	Shoot Proline µmol g^{-1}DM	Root Proline µmol g^{-1}DM
Control	1382.44 a	389.02 a	132.08	5.424	1.785
+50 mg l^{-1} NM	1207.41 ab	355.70 ab	109.29	5.596	1.924
+100 mg l^{-1} NM	887.77 b	266.98 b	112.55	5.140	1.933
ANOVA	**	*	ns	ns	ns

The low concentration of macronutrients did not have an important effect in biomass after the treatment maybe by the sort duration of the treatment (7 days), but it was enough to cause an effect in the plant metabolism. So, the treatment with NM reduced the concentration of *Chl a* and *Chl b* in the plants related to the concentration of NM (Figure 4).

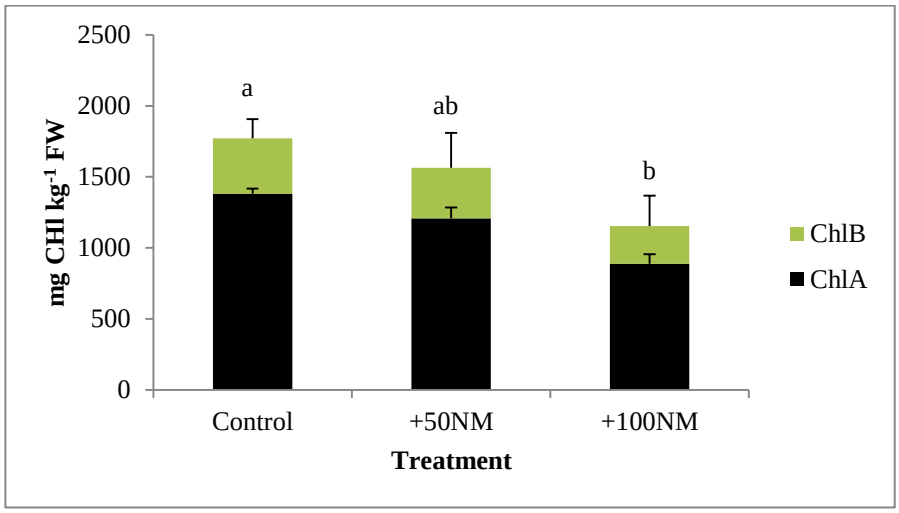


Figure 4 *Chlorophyll A and B (mg of Chlorophyll per kg of FW shoot) in the leaves of H. annuus after the assay.*

Regarding the bulk materials, NPs addition can impact on the membrane, by a physical adsorption on root surface and blockage of nutrient uptake by plant roots may also occur (Zhang et al., 2014). It is possible that such impacts on the roots may have affected the uptake of elements such as magnesium, nutrient associated with the synthesis of chlorophyll. A decrease in the concentration of these essential nutrients might have contributed to the decrease in relative chlorophyll content observed in the treatments. As it can be seen in the Image 6, the basic structure of chlorophyll is a porphyrin ring with a central atom of magnesium. Chlorophyll has a basic importance in the energetic metabolism being important in the sun energy obtaining and Chlorophyll loss is associated to environmental stress and may be a good indicator of stress in plants (Hendry and Price, 1993). Cambrollé et al. (2013) also found a reduction of chlorophyll *a* and *b* after 30 days of treatment with high Cu doses (up to 570 mg l^{-1}) dissolved in the growing solution in function of the concentration of the metalloid in plants of genus *Vitia*. The low levels of chlorophyll can lead to a deficient plant growing after several days of treatment. Water absence can also affect the chlorophyll content. Ghobadi et al. (2013) found that after five month of exposure to drought, *H. annuus* chlorophylls concentration can be reduced up to 30 %. The combination between the low macronutrient transport and the reduction of water transport capacity in roots can explain the significant reduction of chlorophylls. Low concentration of chlorophylls can be traduced in an important energetic problem that can affect the plant development in a long term exposition to NM.

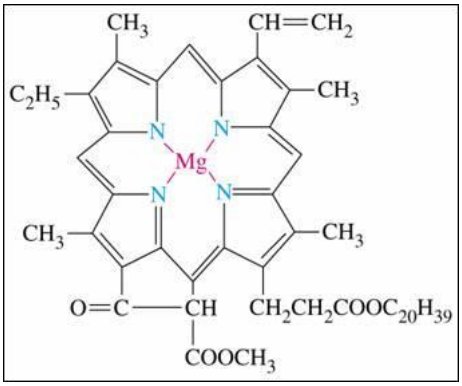


Image 6 *Representation of chlorophyll molecule.*

Other aspects of chlorophyll synthesis or degradation could have been affected as well, and a more detailed study will be required to understand the extent or severity of effects of NPs on chlorophyll metabolism.

The ascorbate and proline concentrations in roots and shoots did not show any differences among treatments. Ascorbate and proline metabolism is related with the plant response to stress. Changes in plant metabolism were expected after analyzing the data of L_o which show a deficient water transport to shoots. As found by Mannivannan et al. (2008), after 50 days of drought, *H. annuus* shows proline metabolism changes in response to drought condition increasing the concentration of proline. This drought situation can be compared with hydric stress found in the experiment. However, it is expected that after more days of exposure to treatment the shoot relative water content get lower. The lower relative water content affect the proline metabolism because proline accumulation occurred only when RWC fell below 70–75 % (Martínez-Fernández et al., 2012). Proline accumulation is a common metabolic response to water deficit in higher plants and has been advocated as a plant stress index (Verbruggen and Hermans 2008). Indeed, increased proline accumulation was reported in sunflower under drought stress (Manivannan et al., 2008). After more days of hydric stress it is expected that *H. annuus* show changes in Proline concentration as response to this stress.

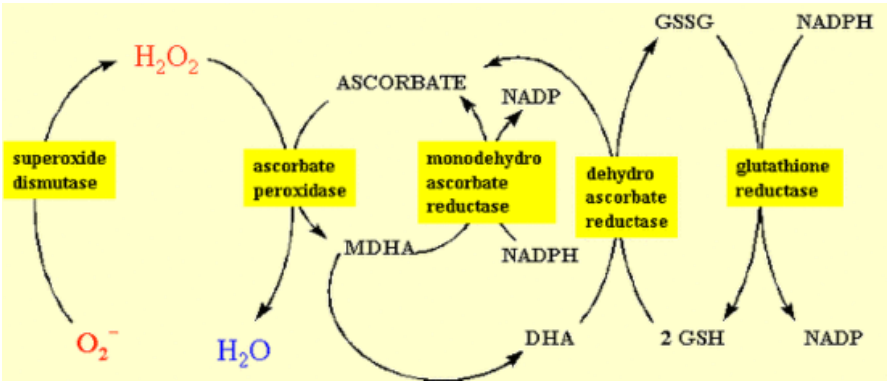


Image 7 *The ascorbate cycle is a metabolic pathway that detoxifies hydrogen peroxide (H_2O_2), which is a reactive oxygen species that is produced as a waste product in metabolism.*

NM could take part in multiple redox reactions. These reactions can provide an important effect in plant metabolism. To respond to this oxidative stress plant uses different strategies to compensate the redox reaction. One of this strategies involve ascorbate metabolism (Image 7). Ascorbate concentration increases in case of oxidative stress as found in Ranieri et al. (2000). In the experiment no changes of ascorbate concentrations were found. This fact can be caused by the low exposure time to treatment that prevents to find a metabolic response or because NM do not produces a significant oxidative stress to activate the metabolic response to this stress.

5. Proposals for further experiments

Further information is needed to describe properly the effects of NM before the full application of these NPs as an environmental amendment in contaminated soil. Long term effects in water transport by NM are interesting, so a deeper research with a longer experiment could be very useful for the understanding if they can completely block the L_o. Variation of concentration of NM treatments should be also interesting, being able to detect safe and dangerous doses for plants. NM effects have been already tested in soil conditions in *Helianthus annuus* by Matínez-Fernández et al., (2015), since is concluded by Bolan et al., (2014), studies with NPs are needed at the root–soil interface, including measurements of plant water relations.

When combined with soil, NPs do not seem the same adherence to root exterior and remain in soil pores (Whiteley et al., 2013). However interaction between NM and epithelial root cells needs further analysis. Studies at cellular levels should be interesting for understanding toxic effects and levels of NM. Epithelial cell cultures of plants treated with NPs could let to know the death concentration level and the specific transport problems caused by NPs.

In case of implementation of *H. annuus* crops combined with contaminated soils treated with NM, it is important to know the capacity of seed for germinating under the effects of NM. An experiment of germination with NM treatments should provide enough information to confirm *H. annuus* as a good candidate Since germination is a critical point in the plant crop yield. A low germinating rate under NM could provide a non beneficial yield for *H. annuus* production.

Oil from *H. annuus* seeds and ethanol produced from its biomass are good candidates as products obtained from contaminated soils since these products are no used for human or animal consumption. Analyzing the quality of the obtained oil and ethanol should be a interesting complementary information for further use.

Testing effects of NM in other plants species should provide information to understand if the L_0 problems found in *H. annuus* only happen in this specie. Other interesting plants for bioremediation such as *Salix* or *Populus* can have different responses to the water transport problems providing a better candidate for working together with NM.

Other kinds of NP are already available for remediation. Other NPs like Zn or Cd ones can also have a different plant interaction becoming better for NP treatment combined with crops.

6. Conclusions

Short time treatments with NM at concentrations of 50 and 100 mg l^{-1} had important effects on *H. annuus* plants. Hydraulic conductivity in roots was reduced up to 26 % of its full capacity. The inhibition of L_o combined with the block of the root nutrient transporters by NM caused reductions in the macronutrient concentrations in shoots. Roots did not seem to be affected by the problems of nutrient absorption. The lower concentration of macronutrients in shoot affected to the chlorophyll concentration and consequently the energetic metabolism was reduced in plants treated with NM. Biomass growth and RWC were not affected in this short term treatment. The water balance response was not affected after five days of treatment. Further experiments must be done to ensure the effects of NM in all the crop time. Increment of the Fe uptake was no found during the treatments. In case of application of NM combined with *H. annuus* should be important to take into account the important reduction of L_o because of the important effect it can have in the plant development.

In summary, this study reported the first study on the fate and phytotoxicity of NM to a commonly encountered plant species. The results suggested that NM at the concentrations used in hydroponic conditions could lead to phytotoxic effects on plants because of a water balance disruption in the root uptake. The result indicated that large scale introduction of NM to the environmental could lead to serious environmental consequences and the environmental impact of such application warrants further attention.

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