

**CZECH UNIVERSITY OF LIFE
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Combined influence of nZVI and arbuscular
mycorrhizal fungi on metal(loid) uptake by
maize (*Zea mays* L.)

Diploma thesis

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Declaration

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ABSTRACT

Nanoscale zero-valent iron (nZVI) is an efficient amendment for immobilizing metal(loid)s (e. g. Zn, Cd, Cr, Pb, As...etc) in the soils, due to its high specific surface area, strong reducibility and high surface reactivity. However, nZVI functions may be influenced by plants and microbes, and nZVI can also pose toxicity to those living organisms. Therefore, before application, nZVI behavior and its functions in plant-microbial-soil system should be elucidated. Arbuscular mycorrhizal (AM) fungi are ubiquitous soil fungi that can make symbioses with most terrestrial plants, they can provide plants with mineral nutrients, and in return get carbohydrates from plant partners. AM symbiosis can also enhance plant tolerance to metal(loid)s and influence metal(loid) behavior in plant-soil system, but its influence on nZVI functions towards metal(loid) is still unclear. Thus the present study mainly focused on the influence of AM symbiosis on nZVI functions, as well as the effects of nZVI on AM symbioses formation.

Maize (*Zea mays* L.) plants were inoculated with/without AM strains *Rhizophagus irregularis*, and grown in a “real” metal(loid) contaminated soil amended with/without 0.5% nZVI. AM colonization status was assessed, and uptake of mineral nutrients (P, Fe, Ca, K, Mg), as well as various metal(loid)s (i.e. Zn, Pb, Cu, Mn, Cd, Cr, As, Ni) by plants were determined by inductively coupled plasma-optical emission spectrometry (ICP-OES). The results showed that although in a heavily contaminated situation, AM fungi can still make symbiosis with maize plants, but nZVI addition showed certain negative influence on AM symbioses formation. nZVI and AM generally had no influence on plant growth and macro nutrients (e.g. P, K, Ca, Mg) acquisition. nZVI amendment generally decreased some metal(loid)s (e.g. Mn, Pb, Cr, Ni, As) uptake by plant roots, showing stabilization functions of nZVI on metal(loid)s. However, when plants were inoculated with AM fungi, the stabilization functions of nZVI decreased, showing the negative influence of AM symbiosis on nZVI functions. Especially, it is interesting to find that AM symbiosis generally increased metal(loid)s uptake by plant roots when nZVI was amended into the soils. Besides, AM symbiosis generally enhance metal(loid) immobilization in plant roots and reduce metal(loid) transport from roots to shoots.

In summary, the study confirmed the counteractive effects of AM symbiosis and nZVI on plant metal(loid) uptake, and revealed the negative influence of nZVI on AM development,

as well as the significant influence of AM symbiosis on nZVI functions towards metal(loid) stabilization.

Keywords: Arbuscular mycorrhizal fungi, Nano zero-valent iron, Metal(loid)s uptake, *Zea mays*. L, Soil contamination

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1. Introduction

Currently, with the development of the society, numerous metal(loid)s (e.g Zn, Cd, Pb, As etc) were discharged during industrial production. Those metal(loid)s went into soils, threatening the soil ecosystem and human beings.

Recently a lot of efforts have been made to develop suitable and sustainable technologies towards remediation of metal(loid) contaminated soils. Nano zero-valent iron (nZVI) is a newly developed products that has a great potential in remediation of soils contaminated with metal(loid)s (such as Zn, Cd, Cr, Pb, As etc), because of its huge specific surface area, high surface reactivity and strong reducibility (Komarek et al., 2013; Ponder et al., 2000). However, nZVI may show toxicity to plants and microbes in the soil, and metabolism of plants and microbes may also influence nZVI functions. While few studies have addressed on this issue. Considering the great potential of nZVI in soil remediation it is essential to investigate interactions between nZVI and plants and their associated microbes in the soils.

As a widely distributed soil fungi that can make symbiosis with most terrestrial plants, arbuscular mycorrhizal (AM) fungi can usually enhance plant growth under metal(loid) (e.g. Zn, Cr, As, Cu etc) contamination and influence metal(loid) uptake by plants (Chen et al., 2007; Wu et al., 2014). Once the symbiosis established, numerous extraradical mycelium will develop in the rhizosphere. Those mycelium only has a diameter of several micro meters, and can interact with nZVI and metal(loid)s, and thus influence nZVI functions in the soil. However, little information are available towards the interactions between nZVI and AM symbiosis under metal(loid) contamination.

2. Aims of the study

The present study mainly aims to investigate the combined effects of nZVI and AM symbiosis on metal(loid) uptake by plants, which will indicate the influence of AM symbiosis on nZVI functions towards metal(loid) stabilization. Besides, the effects of

nZVI on AM formation and development under metal(loid) contamination will also been investigated.

A Fluvisol soil from the alluvium of Litavka River (Prábram District, Czech Republic) that heavily contaminated with Zn and Pb (and also with Cd and As to a lesser extent) was chosen. A widely used plant maize (*Zea mays* L.) was chosen for study, as this plant can be easily cultivated and shows a high mycorrhizal potential. Maize was inoculated with/without AM fungi, and the metal contaminated soil was amended with/without nZVI. AM development and nutrients and metal(loid) uptake by plants was detected. We predict that nZVI amendment can decrease mycorrhizal colonization, and AM fungi can enhance metal(loid) uptake by plant roots when nZVI was added.

3. Literature review

3.1 Soil metal(loid) contamination

Nowadays, a high amount of soils are exposed to metal(loid)s contamination. It has been estimated by the United States Environmental Protection Agency (USEPA) that over 50 million cubic meters at current National Priorities List (NPL) sites are currently contaminated with metals (USEPA, 2004). Those metal(loid)s mainly include Zn, Pb, Cu, Cr, As, Cd etc, posing great threats to our human beings and the environment. Metal(loid) contamination in soils could be caused by anthropogenic activities like mining, agriculture, industry and urban waste etc. Some of these elements (e.g. Zn, Cu etc) are essential for life, they may become toxic if taken up in higher quantities. Different from organic compounds these metal(loid)s are non-degradable by microbial and plant activities (Naidu et al., 1999), but their bioavailability and toxicity can be changed through alteration of chemical forms (speciation) (Maslin et al., 2000). Here characteristics of some key metal(loid)s such as Zn, Pb, Cd, As, Cr and their phytotoxicity were described separately.

(1) Zinc

Zinc(Zn) is a transition metal which production is augmenting progressively, thus its input into the soils is increasing nowadays. It is among the most extensive phytotoxic microelement in acidic soils (Chaney, 1993). Zn solubility, uptake and phytotoxicity increases with decreasing soil pH. At comparable soil pH and Zn concentrations, Zn

phytotoxicity is more severe in light-textured soils than in heavy textured-soils. This is mainly due to different adsorption capacities of the soil (Rattan and Skukla, 1984). Numerous studies have shown significant physiological toxicity of Zn to plants. A high amount of species become chlorotic in presence of excessive soil Zn, it is believed that this situation occurs due to its interference in Fe uptake, translocation (Ambler et al., 1971) or Fe utilization in leaves, perhaps in chlorophyll biosynthesis (Rosen et al., 1977; White et al., 1979a). It has also been observed that P uptake is lower, most likely because of Zn inhibiting the root length or the production of insoluble Zn-phosphates present in root cells.

(2) Lead

Lead (Pb) is one of the most abundant hazardous heavy metals in the environment (Sengar et al., 2008). Typical mean Pb concentration for surface soils worldwide averages 32mg kg⁻¹ and ranges from 10 to 67mg kg⁻¹ (Kabata-Pendias and Pendias, 2001). Pb ranks fifth behind Fe, Cu, Al, and Zn in industrial production of metals. About half of the Pb goes for the manufacture of Pb storage batteries. Being largely immobile in nature, Pb persists and pollutes the soil. The most stable forms of Pb are Pb (II) and Pb-hydroxy complexes. Pb (II) is the most common and reactive form of Pb, forming mononuclear and polynuclear oxides and hydroxides (GWRTAC, 1997). Once released into the environment, it gets easily adsorbed in the soil, therefore its mobility is not high (Sharma and Dubey, 2005). In general, most plants cannot absorb or accumulate Pb. However, in soils high in Pb contamination, it is possible that some Pb can be taken up (Wuana and Okieimen, 2011). Pb exposure usually causes oxidative stress and affects growth and physiology of plants; and disrupts various biochemical attributes (Singh et al., 2011). The oxidative stress in plants usually due to production of free radicals, which in turn act on the unsaturated lipids in the membranes, leading to lipid peroxidation and cell membrane damage (Kumar et al., 2013). Pb has been reported to restrain root growth (Eun et al., 2000).

(3) Cadmium

Just like Pb, Cadmium(Cd) is also not essential in living organisms. It is right underneath Zn in the periodic table so it presents many chemical similarities, so it might be introduced into living organisms through Zn transporters, causing dysfunctional effects. Cd is a very biopersistent element that can remain inside the organisms for prolonged periods of time and tends to biomagnify in the food chain.

As a free ionic metal, Cd (and other metallic elements) in the environment could affect the performance of plants at various aspects (Ernst et al., 1992). The direct interaction of free ionic metals with cellular components can initiate, in a time-scale of seconds, a variety of metabolic responses, sometimes with direct or indirect generation of reactive oxygen species (Sandmann and Böger, 1980 ; Babu et al., 2001). These metabolic disturbances could lead to decrease plant growth (Vangronsveld et al., 1997) and even genetic changes of plant population after decades (Ernst and Peterson, 1994).

(4) Arsenic

Arsenic (As) is a metalloid and is generally known because of its high toxicity. As usually has high mobility in nature and shows a great threat to human beings, animals and plants. As exists in several oxidation states with trivalent (arsenate, As(III)) and pentavalent (arsenite, As(V)) forms as the most common in the environment. In terms of toxicity, inorganic forms, As(V) and As(III), show higher toxicity than those organic forms, e.g. methylated forms of arsenic, and As(III) is far more toxic As(V) (Wu et al., 2011). The pH of the soil plays an important role in the mobility and bioavailability of As. Under acidic conditions, As(V), the predominant As form in agricultural soil, is primarily sorbed onto iron and aluminum oxides, whereas this species is sorbed by calcium oxides under alkaline conditions (Pongratz, 1998). As is taken up by plants through the roots easily and its toxicity depends on chemical species. As(V) has the ability to mimic phosphate because of their chemical structure similarity and As can enter into plants cells via phosphate transporters in the plasma membrane. Once go into the cell, As can interfere with phosphate-based energy-generating processes, thus inhibit oxidative phosphorylation (Markley and Herbert, 2009). However, As(III) enters cells via a different route (aquaglycerolporins) and targets a broader range of cellular processes, e.g. binding to the thiol groups of important cellular proteins, e.g. pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase (Meharg and Hartley-Whitaker, 2002).

(5) Chromium

Chromium (Cr) is the seventh most abundant metal in the earth's crust (Katz and Salem, 1994), it is also another transition metal that is raising more than a significant concern in the past few years due to its contamination to soil and water. Although being essential for glucose metabolism in animals (Katz and Salem, 1994;

Shrivastava et al., 2002) Cr is a non-essential element for plants. Cr can usually affect many plants physiological activities; e.g. photosynthesis, respiration processes etc. R can also influence enzymatic activities and in higher proportions can even cause plant death (Shanker et al., 2005; Singh et al., 2013a). Cr can be present in the soil media in various forms. Two most common Cr oxidation status are trivalent chromium (Cr(III)) and hexavalent chromium (Cr(VI)). Chromium toxicity, mobility, solubility and bioavailability is strongly dependent on its oxidation status. Cr(VI) form, Cr is more mobile and toxic than Cr(III). The toxicity of Cr(VI) strongly depends on its concentration in the soil and its uptake mechanism. Cr(III) is very stable in soil, but usually well immobilized on iron and manganese oxides and hydroxides or complexes to organic matter (Gardea et al., 2005).

When the concentration of these potentially hazardous elements in a certain location are high enough to represent risks to the environment or human health, this locality should be categorized as a contaminated site. This situation creates the prime need to elaborate a remediation strategy, and as soon as possible commence with the remediation tasks.

3.2 Techniques for remediation of metal(loid) contaminated soils

The basic idea of remediation would be the action that aims the removal of pollutants, or reduce their mobility and toxicity, and thus eliminate their environmental risks. The overall objective of any soil remediation approach is to create a solution that is protective of human health and the environment (Martin and Ruby, 2004). Till now, many technologies have been developed towards remediation of metal(loid) contaminated soils.

These techniques can be divided into two major categories: physical-chemical and biological technologies, the details are shown in Table 1. Both of them aim to remove metal(loid) from soils or reduce their bioavailability and transform them into a less toxic speciation. Soil remediation techniques can be used either ‘*in situ*’ or ‘*ex situ*’ that will depend on each specific situation.

The chosen technique will be dependent on many factors and all should be considered, such as; the short-term and/or long-term effectiveness to reach remediation goals, the effectiveness of contaminant reduction in site, reduction of contaminant toxicity and the cost effectiveness of remediation (Burlakovs and Vircavs, 2011)

The following table displays the main heavy metal remediation technologies, including physical separation technologies, chemical treatments and biological treatments as well as their main advantages and disadvantages.

Table 1. Soil remediation technologies for metal(loid)s.

Physical separation	Involves phase transfer of metal(loid)s from the contaminated media by exploiting differences in certain physical characteristics between metal bearing and soil particles considering their size, density, magnetism, and hydrophobic surface properties
Main technologies	Elektrokinetic technology, soil washing (hydrodynamic classification, gravity concentration, froth flotation, magnetic separation, electrostatic separation, attrition scrubbing), soil flushing, vitrification and thermal desorption.
Advantages	It can treat organic and metal contaminants simultaneously. Soil volumes that subsequently have to be treated (metal recovery) or disposed off-site are considerably reduced. Processed soils can be returned to the site at a lower cost. Unit systems are mobile and can be used for full-scale on-site remediation. Technologies are usually well established in the mineral processing industry.
Disadvantages	Physical separation normally cannot treat sorbed forms of metals. These systems require large equipments and large spaces for soil treatment and may involve huge excavations and cause secondary pollution. Sometimes, they are not sustainable and cost a lot.
Chemical treatments	Chemical processes include reduction of the bioavailability/mobility of metal(loid)s through chemical reactions with specific reagents. Technologies for heavy metal remediation are based on precipitation, oxidation-reduction, and organic-metal complexation reaction
Main processes	Chemical leaching, chemical stabilization or fixation using different materials e.g. biochar, nanoparticles etc.
Advantages	Sorbed metal forms can be treated. Certain metal compounds can be dissolved. Fine-grain soils may be treated in certain cases. The extracted metals may be easily recovered by a wide variety

	of methods.
Disadvantages	The use of chemical agents increase the processing costs. Physical-chemical and microbiological properties of soil can be affected. Chemical reagents may generate secondary toxicity.
Biological treatments	They take advantage of the pathways developed by microorganisms to protect themselves from metals or the usage of plants or animals to remove, or stabilize metal(loid)s in the soil.
Main technologies	Biological treatment with microorganisms, phytoremediation (phytoextraction, phytostabilization and phytofiltration)
Advantages	It is a natural solution. Cost-effective alternative to physical-chemical remediation systems. It is less disruptive to the environment and does not involve waiting for new plant communities to recolonize the site. It is more aesthetically pleasing than traditional methods. It avoids excavation and transport of polluted media thus reducing the risk of spreading the contamination.
Disadvantages	Long time is needed to remediate the site. It cannot decontaminate soils where pollutants are deep thus the remediation process is dependent on the root depth and it can be only applicable in shallow soils. It is limited by specific metal resist species. It is dependent on the growing conditions required by the plant (i.e., climate, geology, altitude, and temperature).

Among those technologies stated in Table 1, chemical stabilization and phytoremediation are more attracted for large metal(loid) contaminated areas and their combination may be more effective (Mench et al. 2003, 2006a, b, 2009; 2010 Adriano et al., 2004; Marques et al. 2009). In order to show more clearly on this emerging field, details on phytoremediation and chemical remediation were described in the following.

(1) Phytoremediation

Phytoremediation can be defined as an *in situ* remediation strategy that uses vegetation and associated microbiota, soil amendments, and agronomic techniques to remove, stabilize environmental contaminants (Cunningham et al., 1996; Helmisaari et al., 2007). Phytoremediation is a promising new method that uses green plants to assimilate or detoxify metals and organic chemicals. The phytoremediation of metal-contaminated soils offers a low cost method for soil remediation and some extracted metals may be recycled for value (Chaney et al. 1997). For remediation of heavy metal-contaminated soils, there are three main existing technologies available that include phytoextraction, phytostabilization, and phytofiltration (Garbisu and Alkorta, 2001).

For the purpose of phytoextraction, it is necessary to use plants that accumulate metals to high concentrations that are normally referred to as “hyperaccumulators” (Visoottiviseth et al. 2002). Metal(loid)s can be accumulated in their aerial parts, but the high amounts and variety of heavy metals present in a certain location can decrease plants tolerance to heavy metals, hence their extraction yields would be reduced. Therefore other strategies have been investigated, such as the one proposed by Luo (2000), which aims to grow high-biomass crop plants with lower capacity to accumulate metals, but metal removal of soil metals would be enhanced by chelate phytoextraction.

For phytostabilization, plants are used to immobilize metal(loid)s in the soil, where plant roots are the main plant component for this technique. Plant roots are able to absorb, adsorb or precipitate contaminants in the rhizosphere, hence reduce their mobility and protect the environment from their hazardous effects. This technique has proved to be useful for the cleanup of metal(oid)s such as, Pb, As, Cd, Cr, Cu, and Zn (Jadia and Fulekar, 2009).

(2) Chemical stabilization

Chemical stabilization includes the addition of reagents or materials into the contaminated soil and thus decreasing the migration of heavy metals to water, plant and other environmental media (Zhou et al., 2004). Remediation efficiency of these treatments, will be greatly dependent on the soil geochemistry, soil texture, cation exchange capacity, buffering capacity, and organic matter content as well as the

metal contamination characteristics; type, concentration, fractionation and speciation of metals.

Within chemical stabilization, the usage of oxides as stabilizing soil amendments have been extensively studied in the recent years. Due to their small size, their low solubility (in normal soil pH ranges) (Sposito, 2008) and high sorption capacities, they have been established as a relevant option for remediating soil contaminated with metal(loid)s. Among those oxides, iron oxides, manganese oxides and aluminum are the most common ones. The increase of iron oxides can be practically achieved by the application of their precursors such as, Fe sulfates or elemental Fe(0) (Komarek et al., 2012). The use of Fe(0) as a precursor of Fe oxides in soils has proved to be potentially effective and cheap for decreasing the mobility and potential bioavailability of various metal(oids) in contaminated soils (Kumpiene et al., 2007). Although the oxidation reactions for Fe(0) are slower than Fe applied as sulfate salts.

Chemical stabilization has been favored by the increased development of nanotechnology. Nanoscale materials are of interest for environmental applications, as they maximize some of the properties of the already existing natural sized materials. For example, the surface areas of the Fe(0) particles would be larger when compared with their volumes; therefore, their reactivity in chemical or biological surface mediated reactions can be greatly enhanced in comparison to the same material at much larger sizes. Several studies have shown, that these nanoparticles have greater decontaminating yields compared with larger iron particles. For instance, an increase of 25 times for the removal rate of hexavalent chromium happened with nano zero valent iron (nZVI) (Cao and Zhang, 2006). Nanoscale materials that contain iron are the most widely used nanoscale material in full-scale applications for site remediation (EPA, 2011). Regarding the remediation of heavy metals, the use of iron-based nanomaterials (e.g. nano-magnetite (Fe_3O_4), nano-maghemite ($\gamma\text{-Fe}_2\text{O}_3$), nano-hematite ($\alpha\text{-Fe}_2\text{O}_3$) or nano zero valent iron (nZVI)) are prevalent.

3.3 Nano zero valent iron (nZVI) and its role in metal(loid) stabilization in soil

Nanoscale zero valent iron (nZVI) is the most commonly used nanomaterial in the United States and has received increasing amounts of attention within the last

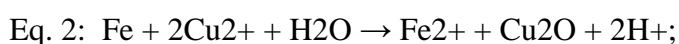
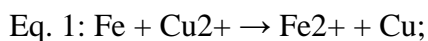
decade, primarily due to its potential for broader application, higher reactivity, and cost-effectiveness compared to conventional zero-valent iron applications and other *in situ* methods. The most basic form of nZVI consists of spherical iron (Fe^0) nanoparticles with individual particle dimensions less than 100nm (Grieger et al., 2010). nZVI particles behavior will vary from one to another depending on the synthesis method used to produce them, making them differ in their properties such as; different size, shape, specific surface area etc. Bare nZVI has low colloidal stability (Lowry and Casman, 2009; Tratnyek and Johnson, 2006) and tend to aggregate between each other, thus reduce their reactive area and mobility, hence their remediation potential (Theron et al., 2008). To solve the issues regarding nZVI changes in their properties and behavior when applied in soils and groundwater, they are typically coated with different surface modifiers to better control their reactivity and mobility (Sellers et al., 2009). Polymers, polyelectrolytes, and surfactants are in the main types of coatings used for nZVI, including starch (He and Zhao, 2005). Poly (acrylic acid) (PAA) (Schrick et al., 2005), poly(styrene sulfonate) (Phenrat et al., 2009a), carboxymethyl cellulose (CMC) (He et al., 2007) are examples of commonly used coatings.

To date, nZVI have been used and developed to degrade a wide range of organic and inorganic soil and water contaminants, including halogenated organic compounds, polycyclic aromatic hydrocarbons (PAHs), pesticides and heavy metals (Chang and Kang, 2009, Li et al. 2006, Liu and Lowry. 2006, Yoo et al., 2007).

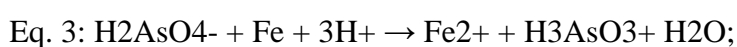
(1) Mechanism of nZVI functions

nZVI has a metallic iron core and an iron oxide shell. Its metallic iron core possesses the well-characterized reducing or electron-donating capacity, while the surface iron hydroxides offer the coordinative and electrostatic functions to attract and absorb charged ions (Li and Wang, 2008). nZVI thus has two nano-components with distinct and complementary functions in order to remove oxyanions (e.g., As(V), Cr(VI)), and cations (e.g. Cu(II), Zn(II), Cd(II), Pd(II), Ni(II)) (Tang and Lo, 2007; Tomasevic et al., 2014; Shi et al., 2011; Giasuddin et al., 2007; Yan et al., 2010; Zhang et al., 2011; Eglal and Ramamurthy, 2015; Wang et al., 2014). nZVI has a relatively low standard potential allowing it to efficiently donate electrons to pollutants, converting them into their reduced forms. In the process, Fe is transformed from Fe^0 to Fe^{2+} (Li and Zhang, 2006). Therefore, theoretically Fe^0

can reduce any pollutant that has a higher reduction potential than -0.440 V. This occurs for Pb(II) or Cr(VI) removal (Wang et al., 2016; Shi et al., 2011). When heavy metals have much more positive standard reduction potentials, such as Cu(II), they can be reduced rapidly (Eq. 1 and 2) (Li et al., 2014; Rangsviek and Jekel, 2005):



nZVI can also reduce oxyanions, such as arsenate (AsO_4^{3-}) (Eq. 3) and selenite (SeO_4^{2-}) (Yan et al., 2010; Tang et al., 2014)



Compared with chemical precipitation and sorption, the chemical reduction of Cu(II) and As(V) by nZVI is thermodynamically much more favorable, less affected by pH changes and chelates (Li et al., 2016).

For metal cations such as Zn(II) and Ni(II), whose standard potentials are negative than or close to that of iron, electrostatic attraction and chemical adsorption, precipitation (with hydroxide ions) and co-precipitation within iron corrosion products are likely the primary mechanisms (Zhang et al., 2011; Yan et al., 2013; Rangsviek and Jekel, 2005). In the Figure 1 it can be seen a structural model of nZVI and its reactions with several contaminants.

The end products are iron oxides and hydroxides (Liu and Lowry, 2006; Sohn et al., 2006; Kumar et al., 2014), which are environmentally harmless. In behalf of these properties, nZVI its positioned a as very promising technology for remediating soils and groundwater with heavy metals.

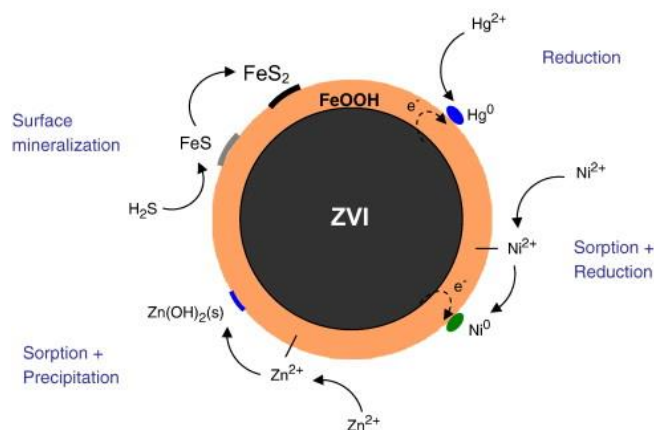


Figure 1. The core-shell model of nZVI and schematic representations of the reaction mechanisms for the removal of Hg(II), Ni(II), Zn(II) and H₂S (Yan et al., 2010)

(2) nZVI application in the field

Despite the importance of the laboratory tests, these cannot reflect reality in a complete way, as in natural conditions there are far more factors that can affect nZVI remediation performance. Good results obtained in pilot test increased the attempts of water and soils remediation with full-scale application of nZVI. The majority of projects involving the usage of nZVI are performed in the United States. 25 sites compiled by US EPA where nZVI was tested or applied on a large scale for soil remediation. The data shows that 56% of the cases the load of contaminants was reduced with distinct decreasing trend of concentration by an average of 70%. The suspension concentration that was most frequently applied was 8g/L (in a range from 0.2g/l to 30g/L) (City Chlor, 2013). For example, in Europe the first time that nZVI was applied was in 2007 for full scale of chloroorganic contaminants in Bornheim (Germany). Previous the introduction of nZVI in the site, the application of steam extraction was unsuccessfully being the remediation technique used. Therefore, the remediation method had to be changed, obtaining satisfactory results. Within a short time, around 90% of the contaminants were eliminated including a lower cost (N. C. Mueller et al., 2012). After the macro scale application in Germany, the next one was realized in Horice and Pisečna in Czech Republic. Reduction of contaminants varied from 60% to 90% (N. C. Mueller et al., 2012).

(3) Potential negative influence of nZVI on plants and microbes

Although their great potential in soil remediation, the application of nZVI is raising some concerns regarding the potential adverse effects on plants and soil microbial communities, as well as the ecosystem stability.

The potential ecotoxicological impact of large scale nZVI has undergone limited scrutiny in the past. Recently, due to its emerging application, more attention has been paid to assess the potential ecotoxicology of nZVI in the environment, after mounting evidences suggesting that nanoscale materials could carry significant ecotoxicological risks (Peralta-Video et al., 2011). Recent studies revealed that nZVI could exert some degree of toxicity towards many microbial species. Besides, the negative effects of nZVI on soil microbial community have also been elucidated (Němeček et al., 2014).

Many studies suggest that cell membrane disruption and oxidative stress through the generation of Fe^{2+} and reactive oxygen species by nZVI are the main mechanisms of nZVI toxicity (Lefevre et al., 2016), see Figure 2.

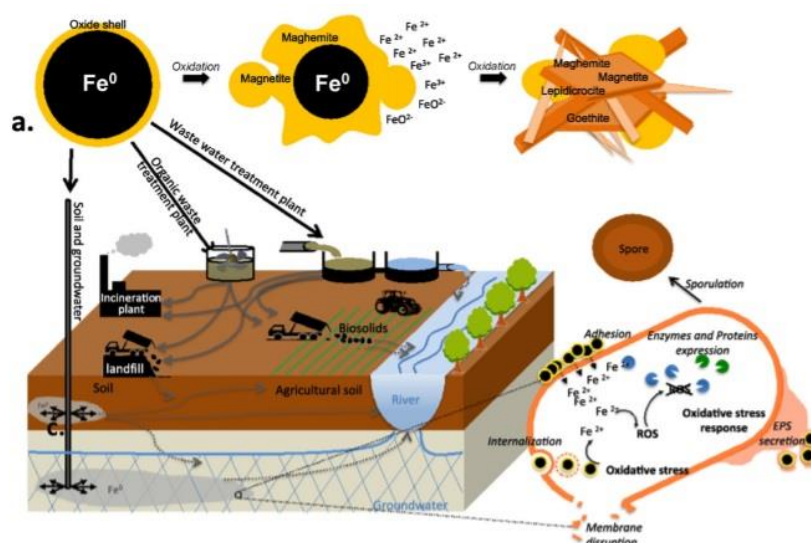


Figure 2. nZVI oxidation, cytotoxicity, cellular defense mechanisms mediated by nZVI, and potential routes of nZVI in the natural environment (Lefevre et al., 2016)

Note: Illustration of (a.) nZVI oxidation process,(b.) bacterial toxicity and defense mechanisms, and (c.) potential routes followed by nZVI in the environment. Plain gray arrows represent deliberate injection or amendment of nZVI, and deliberate transport of material potentially containing nZVI. Dashed gray arrows represent potential non-deliberate transport of nZVI in non-target environments. Abbreviations: EPS, Extracellular polymeric substances; ROS, Reactive oxygen species.

The toxic effects may be due to the particle surface oxidation, leading to the formation of a nanoparticle with Fe⁰ surrounded by an outer layer of iron oxide of about 3nm in thickness (Crane and Scott, 2012). Ferrous (Fe²⁺) and ferric (Fe³⁺) ions, known to have cytotoxic effects, are initially released near the nanoparticle surface and progressively oxidize to form Fe(II) and Fe(III) oxides. Besides, during oxidation of nZVI, reactive oxygen species (ROS) may be produced, and thus pose oxidative stress to organisms (Lefevre et al., 2016).

Several factors can influence nZVI toxicity, such as microbial species, exposure time and level, nZVI types, soil physio-chemical characteristics etc. Toxicity of nZVI depends on microbial species. For example, exposure to high nZVI concentrations did not lead to any toxic effects on *Klebsiella planticola* or *Klebsiella oxytoca* (Fajardo et al. , 2012, Sacca et al., 2013), but resulted in a severe toxic effect on *Bacillus nealsonii* (Fajardo et al., 2012). The toxicity is also highly dependent on the exposure dosage and time, as well as soil properties such as, organic matter content, soil texture etc. Pawlett et al. (2013) showed that soil microbial biomass of Gram - bacteria and arbuscular mycorrhizal fungi decreased when exposed to nZVI and the effects are more significant in sandy soils than in clay. Another important factor influencing nZVI toxicity is the presence of a coat surrounding the nZVI. As it is mentioned in the previous section, nZVI are usually coated by some organic compounds to increase their mobility, and these coats can also influence nZVI toxicity by reducing it (He and Zhao, 2005). nZVI coatings may limit adhesion to bacterial cells, likely by increasing electrostatic repulsion between them (Li et al., 2010). These coatings can be degradable and further studies are needed to investigate their behavior in the soils and how they affect nZVI properties and toxicity.

So far, only a few studies have investigated the toxic effects of nZVI on terrestrial plants. These studies have shown that the effects of nZVI on seed germination and seedlings growth of barley, flax and ryegrass are related to the concentration of nZVI and plant species (El-Temsah and Joner 2012a, b). The results obtained by Ma (2013) showed that nZVI is phytotoxic at concentrations normally used in the field. Several mechanisms could be attributed to nZVIs phytotoxicity. The formation of black coatings on the roots surface could effectively block the root membrane pores and interfere with the water and nutrient uptake process. The black coating could be formed from the oxidation of the outer layer of the nZVI creating a cover of

insoluble Fe^{3+} on the root surface or it could be also the result of the direct deposition of the nZVI on the surface. In a study carried out by Martínez-Fernández and Komárek (2016) the different effects of nZVI and nano-maghemite (nFe_2O_3) on plant physiology was studied, showing the adherence of nFe_2O_3 to the root surface, but the nZVI seemed to be less harmful than nFe_2O_3 regarding water uptake.

(4) Influence of plants and microbes on nZVI functions

Considering the great potential of nZVI in stabilization of metal(loid)s in contaminated areas, it is essential to test if key soil microbes like AM fungi can influence nZVI functions, which will provide important information for future usage of nZVI in phytoremediation. For instance, Adeleye et al. (2016) found that Fe0 could be oxidized into Fe(III) by *Chlamydomonas reinhardtii*, resulting in the formation of Fe_2O_3 , $\text{Fe}(\text{OH})_3$ and $\gamma\text{-FeOOH}$. The transformation of nZVI may further influence its adsorption capacity towards metal(loid)s. From other perspectives, even metal(loid)s are adsorbed by nZVI, they may be dissolved under the functions of plants and rhizosphere microbes (Smits et al., 2009; Martino and Perotto, 2010). For example, some low-molecular-weight organic acids (LMWOAs) excreted by organisms can increase mobility of metals even in nano-maghemite amended contaminated soils (Vitkova et al., 2015).

3.4 Arbuscular mycorrhizal fungi

In the natural ecosystem, plants usually establish association with soil microbes. Among them, arbuscular mycorrhizal (AM) fungi are one of the most common and can form symbiotic associations with more than 80% terrestrial plants (Smith and Read, 2008). AMF are a group of endomycorrhizal fungus, characterized by intraradical mycelium growth and intracellular fungal proliferation belonging to the Glomeromycota. Symbiosis development results from the formation of a tree-shaped subcellular structures inside plant cells. These structures, which are called arbuscules, are thought to be the main site of nutrient exchange between the fungal and plant symbiotic partners (Parniske, 2008), see Figure 3. AMF obtain carbohydrates from their host plants and in return they provide plants with mineral nutrients such as phosphorus (P) and nitrogen (N), etc. (Smith and Read, 2008). AMF can also enhance plant resistance to various biotic and abiotic stresses, such as drought (Li et al., 2014), salinity (Garg and Pandey, 2015), pathogen infection

(Campos-Soriano et al., 2012), and heavy metal contamination (Wu et al., 2013). Additionally, apart from enhancing and being an active contributor in maintaining plant biodiversity and consequently ecosystem stability (Van der Heijden, 1998), AMF can stabilize soil structure by excreting organic compounds such as glomalin (Rillig and Steinberg, 2002).

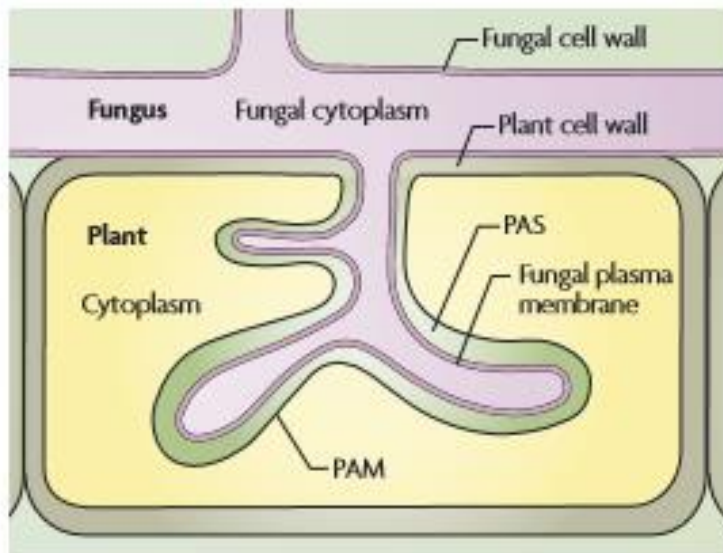


Figure 3. Pictures showing the arbuscule structures in arbuscular mycorrhizal symbioses (Parniske, 2008). Note: Schematic drawing of an arbuscule, the symbiotic structure and arbuscular mycorrhiza (AM). Each fungal branch within a plant cell is surrounded by a plant-derived periarbuscular membrane (PAM) that is continuous with the plant plasma membrane and excludes the fungus from the plant cytoplasm. The apoplastic interface between the fungal plasma membrane and the plant-derived PAM is called the periarbuscular space (PAS). Because of the cell-wall synthesizing potential of both the fungal membrane and the PAM, the PAS comprises fungal and plant cell-wall material.

(1) The influence of metal(loid)s AM symbiosis

Arbuscular mycorrhizal (AM) fungi, can survive in soils highly contaminated with heavy metals, although, the colonization is often reduced under these conditions. Various heavy metals are toxic to fungi, for this reason, spore germination is reduced, mycelium development is also reduced and consequently, the colonization rate (Jamal, 2002). For instance, in an experiment where AMF were exposed to different concentrations of Zn, the mycorrhizal colonization did not decrease

compared to the control receiving no Zn (Chen et al., 2003). On the other hand, Chen et al. (2004) indicated that in soils polluted with Zn, *Glomus caledonium*, colonized the roots at a rate above 70%, when soil was polluted with Zn at concentration levels of 300 and 600mg/Kg the colonization rate decreased to 50%. While Wu et al. (2014), observed that 20mg/kg Cr (VI) addition decreased AM colonization by dandelion. This indicates that AM symbiosis development depends on the plant species, metal species and concentration, etc.

(2) Role of AM fungi in plant resistance to heavy metal stress

As mentioned in the previous section, AMF can enhance plant resistance to heavy metal contamination. In this sense, AMF could be a key factor in phytoremediation for soils contaminated with heavy metals. It has been proved that this symbiosis has a beneficial effect, as it can immobilize heavy metals in the plants root, therefore reducing its translocation to the shoot, consequently, avoiding these metals to be introduced in the food chain (Pawlowska et al., 2000; Del Val et al., 1999; Pawlowska et al., 1996; Barea et al., 1995; Leyval et al., 1995). Furthermore, mycorrhizal dependency on plants increased with increasing metal stress levels, indicating the influence of metal contamination levels on AM functions (Yang et al., 2015).

In fact Chen et al. (2004) pointed out that AM function on plant metal uptake can depends on metal concentrations in the soils. They found that there may exist a critical Zn level below which Zn uptake by plants can be enhanced by AM symbiosis, while above the level, Zn uptake can be restrained by AM symbiosis.

3.5 Combined influence of AM fungi and nZVI on plant metal(loid) uptake and resistance needs to be elucidated

Considering the great potential of nZVI in soil remediation, and the fact that AM symbiosis is widely distributed in the nature, it is interesting to know the interactions between nZVI and AM fungi under metal contamination. For one thing, nZVI may pose toxic effects on AM functions and influence AM functions. For another, AM symbioses may influence nZVI functions on metal(loid) stabilization, as AM fungal exudates (e.g. glomalin-related soil protein (GRSP)) may possibly affect stability of nZVI-metal(loid) complexes, leading to desorption of metal(loid)s from nZVI. However, little information are available on this issue. Therefore it is essential to

investigate AM-nZVI-metal(loid) interactions, which will provide important information on assessment of nZVI application in the natural environment. Besides, given that AM symbiosis can usually immobilize metal(loid)s in the rhizosphere or roots, this study can also provide information for developing new methods combining AMF and nZVI towards metal phytostabilization.

4. Materials and Methods

4.1 Soil

The soil was from the alluvium of Litavka River (Prábram District, Czech Republic), which consists of 75% sand (50-2000 μ m), 20% silt (2-50 μ m), 5% clay (<2 μ m), with pH (2.5 in water) 5.95, CEC 9.08 cmol kg⁻¹, Soil organic matter 21.5 g kg⁻¹, extractable P (0.4% HCl and 0.07% H₂SO₄) 1.53 mg kg⁻¹, Eh 426 mV. Metal(loid) concentration are shown in Table 1. Soil was mixed with quartz sands (soil: sand=5:1) before use.

Table 2. Metal(loid) concentration and speciation in the soil.

Metal	Total	Acid			CaCl ₂	EDTA
		extractable	Reducible	Oxidizable	extractable	extractable
Fe (mg kg ⁻¹)	37400	12.4	1690	8.36	0.06	793
Mn (mg kg ⁻¹)	4280	111	240	545	9.30	1410
Zn (mg kg ⁻¹)	4000	1670	541	307	440	1660
Cu (mg kg ⁻¹)	68.3	8.08	<DL	28.5	<DL	32.9
Pb (mg kg ⁻¹)	3540	207	892	596	0.89	2040
Cd (mg kg ⁻¹)	39.1	21.31	8.44	2.13	7.49	24.6
Cr (mg kg ⁻¹)	61.3	<DL	<DL	0.92	<DL	<DL
Ni (mg kg ⁻¹)	24.4	1.79	<DL	<DL	0.31	1.32
As (mg kg ⁻¹)	296	<DL	<DL	2.02	<DL	5.18

4.2 Host plant

The plants used in this experiment were Maize (*Zea mays* L.) and the plant seeds were purchased from REIN SAAT (Reinsaat KG-3572 St. Leonhard am Hornerwald 69 – AUSTRIA). The seeds were surface sterilized in 10% H₂O₂ for 15min, after carefully washed with Milli-Q water, and then pregerminated on 3 moist filter paper layer until the emergence of radicles.

4.3 AM fungi

The AM fungi species utilized for this study was *Rhizophagus irregularis* (previously known as *Glomus intraradices*), pH5, isolated from Pb contaminated soils) purchased from the Department of Mycorrhizal Symbioses, Institute of Botany, Czech Academy of Sciences, Czech Republic, the AM fungal inoculum includes mycorrhizal roots, spores (80 spores/g), and sands.

4.4 nZVI

Nano zero valent iron was from NANO IRON, Ltd, Czech Republic, which was in the form of surface stabilised air-stable nano powder (NANOFER STAR). The nZVI particle was coated by a thin layer of Fe oxides that protects nZVI from immediate oxidation.

4.5 Experimental design

The soils were treated with/without 0.5 % (w/w) nZVI, and the plants were inoculated with/without AM fungi. Therefore there were 4 different treatments, where each treatment had 6 replicates, thus 24 pots in total were prepared with the fluvisol soil collected from the alluvium of Litavka river. Treatments amended with nZVI were mixed directly with the soil and the nZVI powder, and after, water was poured into the soil to activate the nZVI, as the NANOFER STAR product would have become very reactive in water environment. When treatments were inoculated with AM fungi, 700 g amended soils were previously put into the pot, afterwards 300 g soil containing 30g AM inoculum were added. For non-inoculated controls, 30 g gamma-radiation sterilized AM inoculum were added together with 10 mL AM inoculum filtrate in order to put back the soil microbes except for AM fungi. Germinated seeds were transplanted into pots, and 200 g soil was amended on the surface. Seedlings were thinned to 2 per pot one week after emergence. The experiment was realized in a controlled environment greenhouse that belongs Czech

University of Life Sciences Prague at 14/10 h and 25/20°C (light/dark). The light intensity was 500–1,100 $\mu\text{mol m}^{-2}\text{s}^{-1}$, provided by natural light and supplementary lights from high pressure sodium lamps. Each pot was daily watered by weighing to maintain water content of 18% on a dry weight basis. Basal nutrients including 60 mg kg^{-1} N, 60 mg kg^{-1} K, and 15 mg kg^{-1} P were carefully added to support the plant growth.

4.6 Plant harvest

After two 65 days, the plants were harvested. Plants shoots and roots were harvested separately. Plant samples were then lyophilized with a freeze-dryer at $-50\text{ }^{\circ}\text{C}$ for 48 h to get the dry weight. The dried samples were motor-homogenized for metal and nutrient analysis. Besides, a little fraction of fresh root samples were kept for mycorrhizal assessment.

4.7 AM colonization assessment

Roots sub-samples that were previously collected were washed and cut in 1cm long fragments. As Phillips and Hayman (1970) stipulated for root staining, the roots were cleared in 10% (w/v) KOH for 1h at 90°C in an oven, then rinsed with water for three times and covered the roots with 2% HCl for 5 minutes. The HCl was thrown away and the roots were covered with 0.05% (w/v) trypan blue at 90°C for 30 minutes and finally the roots were placed in a Petri dish with glycerol-lactate. 30 root fragments were selected for each pot and gently placed on a slide. In accordance with mycorrhizal colonization estimation method described by Trouvelot et al. (1986), the selected stained roots were observed with a light microscope and the AMF colonization was assessed by using MYOCALC software (www2.dijon.inra.fr/mychintec/Mycocalc-prg/download) (Trouvelot et al., 1986). The parameters that were measured included the frequency of mycorrhizal colonization in the root system (F%), that is the ratio between the number of root fragments colonized by AMF and the total number of root fragments that were analyzed, the mycorrhizal colonization intensity in the root system (M%), which is an estimate of the percentage of AM structures within the root system, and the arbuscule abundance in the root system (A%), that is an estimation of the percentage of the arbuscule occurrence in the entire root system.

4.8 Metal determination

The lyophilized and homogenized plant samples were digested in $\text{HNO}_3/\text{H}_2\text{O}_2$ at 210 °C, and diluted to 50 mL with Milli-Q water and filtered through a quantified filter paper (Grade 392, Ahlstrom Munktell, Finland). P and metal(loid)s (e.g. Zn, Fe, Cu, Pb, As, Cd, Mn, Ni, Cr etc) were determined by inductively coupled plasma-optical emission spectrometry (ICP-OES) (Varian, VistaPro, Australia). The digestion and elemental analysis processes have been proved to be completely suitable for plant samples by (Martinez-Fernandez et al., 2016).

4.9 Statistical analysis

SPSS 18.0 statistical package was utilized to carry out the entire statistical analysis. All data were analyzed by one-way analysis of variance (ANOVA), followed by Duncan's test ($P < 0.05$) to determine the significance of differences between treatments.

5. Results

5.1 AM colonization

Generally, AM fungi can make well symbiosis with maize plants under metal(loid) contamination, as there existed typical fungal structures such as arbuscules, mycelium etc inside plant roots (Figure 4a,b). However, for control treatment, there were no fungal structures (Figure 4c).

Plants without AMF inoculation remained practically uncolonized, as seen in Table 3. Mycorrhizal treatment presented a high colonization frequency (more than 80%), and the intensity of mycorrhizal colonization (M%) and arbuscule abundance (A%) in the whole root system were 29.43% and 18.78% respectively. The amendment of nano zero valent iron (nZVI) reduced M% and A% values to 13.63% and 4.87% respectively ($P < 0.05$, Table 3).

Table 3. Mycorrhizal development parameters in roots of maize (*Zea mays* L.) grown in Zn/Pb contaminated soils with different treatments. Note: "+/-M" represent with/without arbuscular mycorrhizal fungal inoculation; "+/- nZVI" represent with/without nano zero valent iron amendment. Different letters show significant difference between different treatments.

Treatment	F%	M%	A%
-M-nZVI	1.67±1.82b	0.5±0.55c	0.5±0.05c
-M+nZVI	1.11±2.72b	0.33±0.82c	0.33±0.82c
+M-nZVI	88.4±8.9a	29.4±10.0a	18.8±6.16a
+M+nZVI	80.5±11.0a	13.6±3.89b	4.87±2.05b

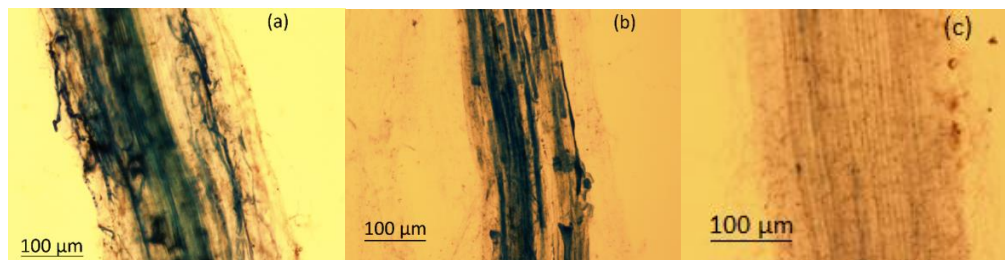


Figure 4. Pictures of arbuscular mycorrhizal roots (a,b) and nonmycorrhizal roots (c) in the present study.

5.2 Plant growth

Generally, AM symbiosis and nZVI amendment had no influence on plant shoot and root dry weight, although a combined AM and nZVI treatment slightly (not significantly) decreased shoot dry weights ($P < 0.05$, Figure 5.)

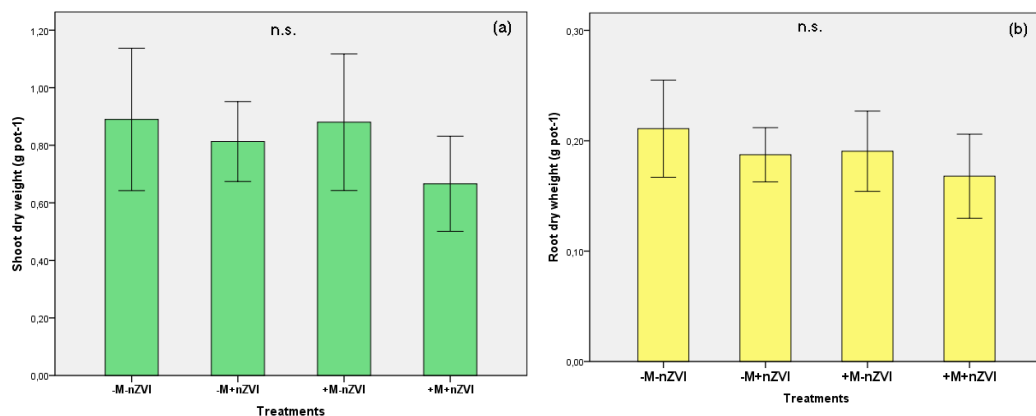


Figure 5. Plant shoot (a) and root (b) dry weights from different treatments. Note: “+/-M” represent with/without arbuscular mycorrhizal fungal inoculation; “+/-nZVI” represent with/without nano zero valent iron amendment.

5.3 Plant nutrition

Plant nutrients, such as phosphorus (P) ($P < 0.05$, Figure 6a, b), calcium (Ca), potassium (K), sodium (Na) and magnesium (Mg) were determined (Figure 7, a, b, c, d, e, f, g and h). Generally, there were no significant difference among different treatments, indicating that AM symbiosis and nZVI did not influence on those nutrients uptake. Root P concentration in the “+M+nZVI” treatment was significantly higher compared with that in “+M-nZVI” treatment ($P < 0.05$).

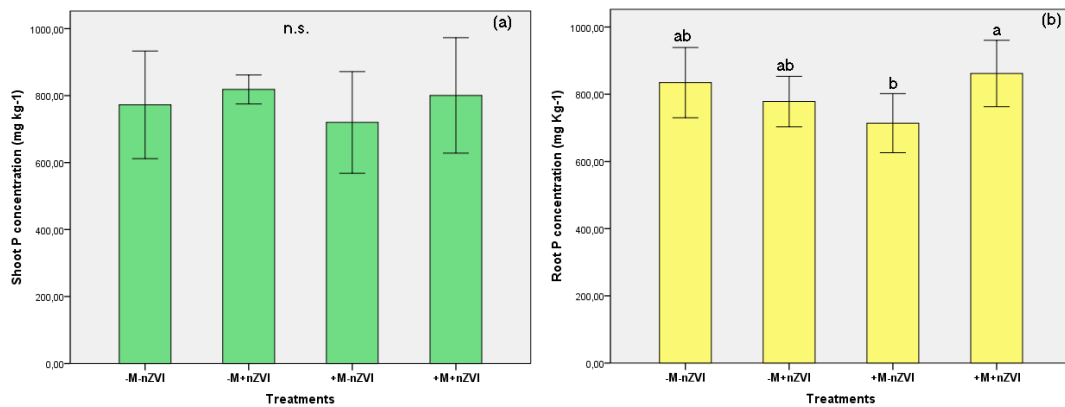


Figure 6. Plant shoot (a) and root (b) P concentrations from different treatments. Note: “+/-M” represent with/without arbuscular mycorrhizal fungal inoculation; “+/-nZVI” represent with/without nano zero valent iron amendment. Different letters above the columns show significant difference among different treatments.

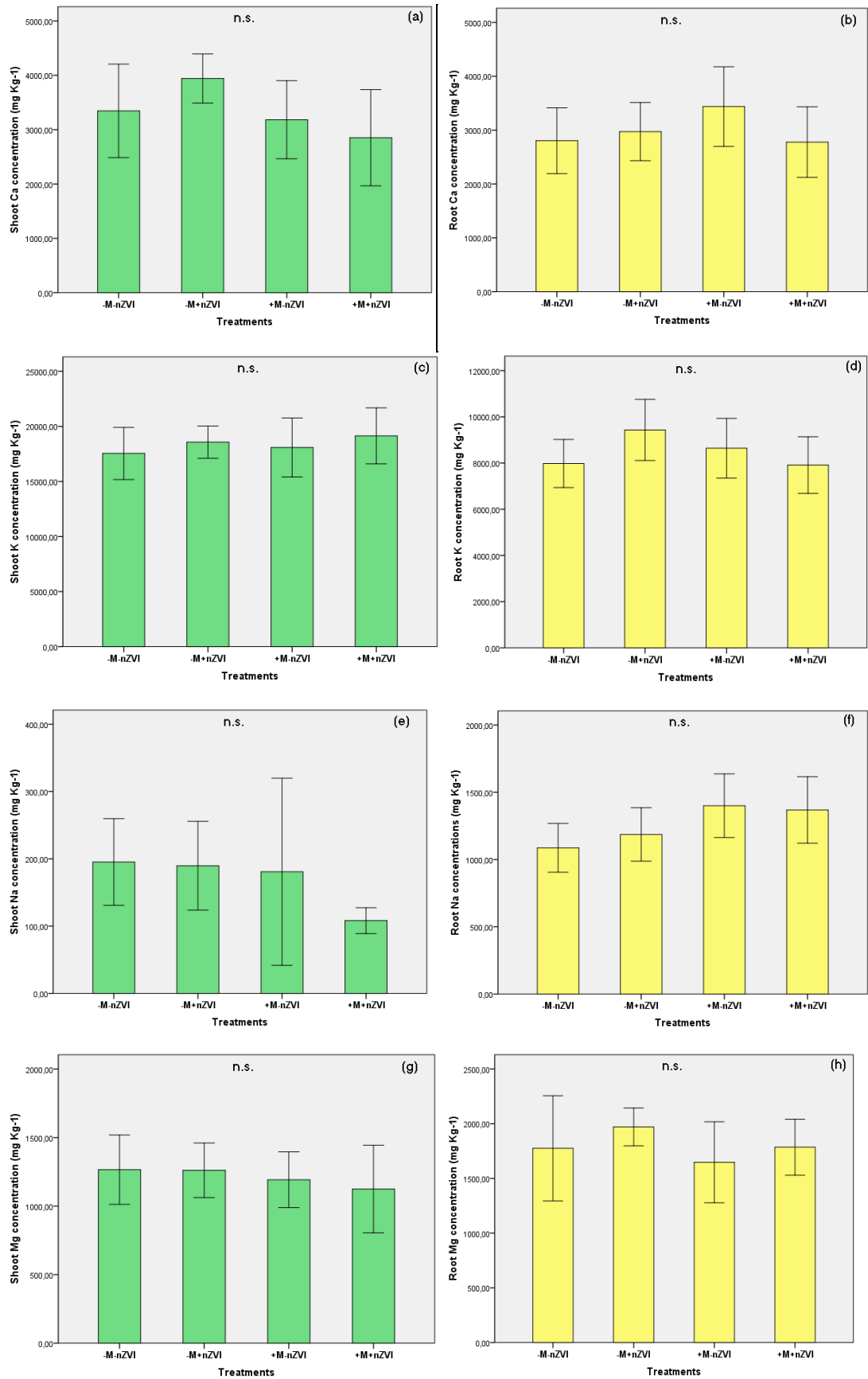


Figure 7. Plant shoot and root, Ca(a, b), K(c, d), Na(e, f) and Mg(g, h) concentrations. Note:“+/-M” represent with/without AM fungal inoculation, “+/-nZVI” represent with/without nano zero valent iron amendment.

5.4 plant metal(loid) uptake

As for heavy metals that were abundant in the present soils, such as Zn, Fe, Mn, Pb. Zn concentrations in plant shoots kept stable within all treatments, while root Zn concentrations were different. Plant roots from “-M-nZVI” and “+M+nZVI” treatments contained higher Zn compared with those roots from treatments of “-M+nZVI” and “+M-nZVI” ($P < 0.05$, Figure 8a), while there was no difference on plant shoot Zn concentrations among different treatments (Figure 8b). These indicated that AM symbiosis and nZVI had no influence on shoot Zn concentration, while mycorrhizal treatment decreased root Zn concentration when nZVI was not amended ($P < 0.05$), but tended to increase Zn concentration in roots when nZVI was amended (Figure 8a). Root Pb concentration values showed practically the same pattern as root Zn concentrations among all treatments, although in this case the lowest Pb content value was for the “+M-nZVI” treatment. For shoot Pb concentrations, it must be highlighted that for the “-M+nZVI” treatment the presence of Pb was approximately double with respect to the rest of the treatments ($P < 0.05$, Figure 8c). AM symbiosis increased root Pb concentrations under nZVI amendment ($P < 0.05$, Figure 8d). Fe concentration in the roots is not significantly different among treatments (Figure 8f). Shoot Fe concentration could be divided into two significantly different homogeneous subsets: (1) -M+nZVI and -M-nZVI treatments, with a higher content of Fe concentration in the shoot, and (2) +M+nZVI and +M-nZVI corresponding to a lower Fe concentration in the shoot ($P < 0.05$, Figure 8e). Respecting Mn, its presence in the shoot was significantly higher in the “-M+nZVI” treatment compared to other treatments ($P < 0.05$, Figure 8g). Mn concentration in the roots is not significantly different among treatments (Figure 8h).

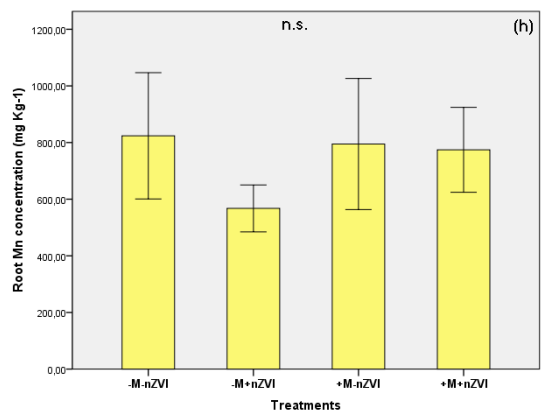
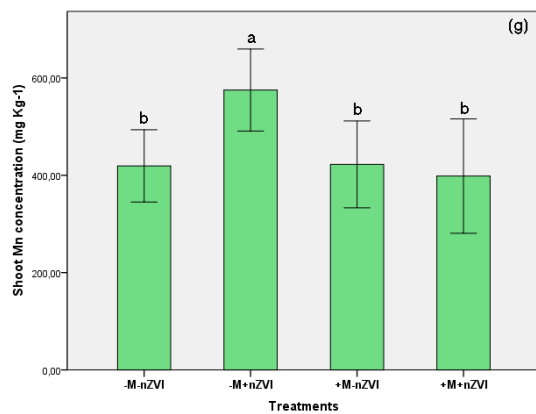
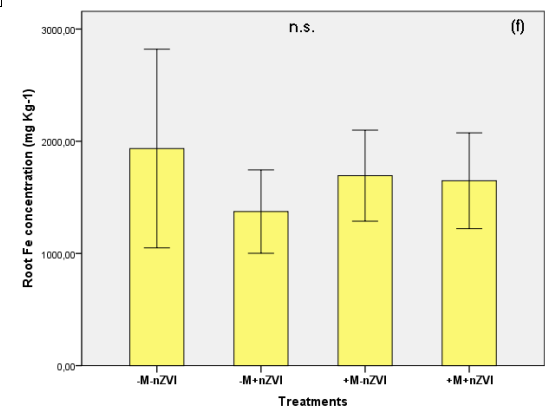
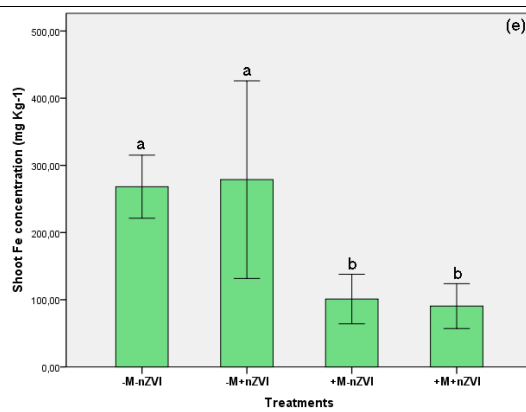
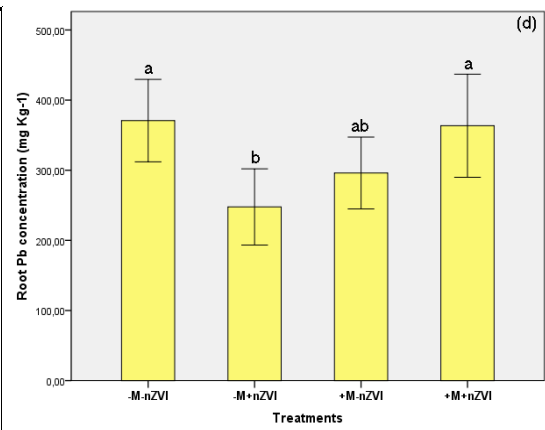
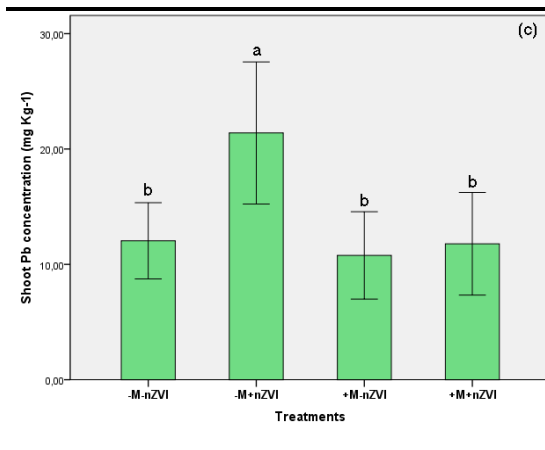
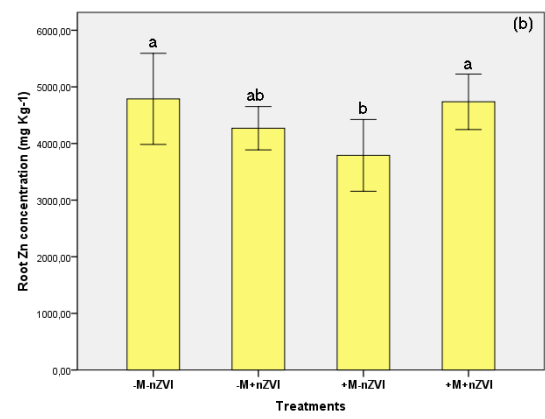
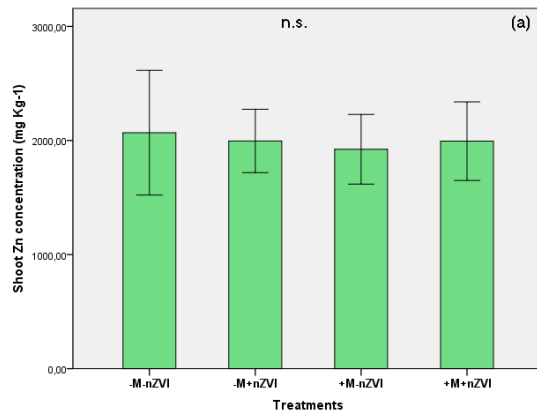
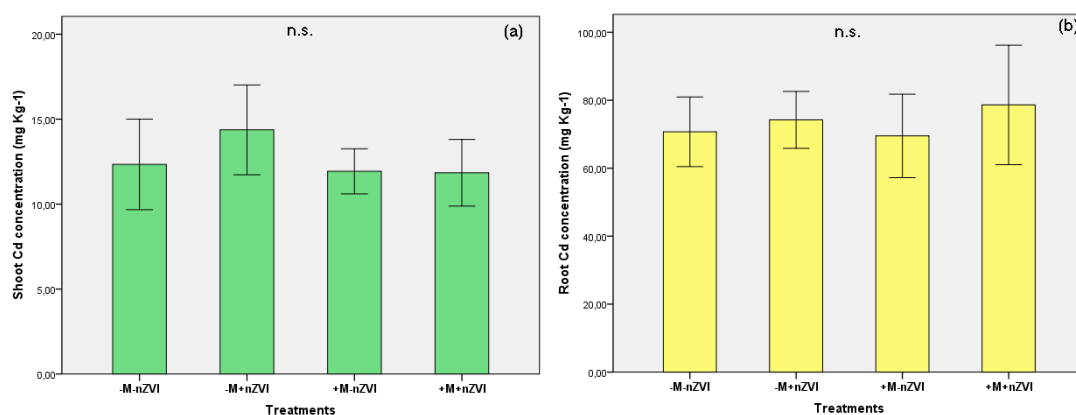
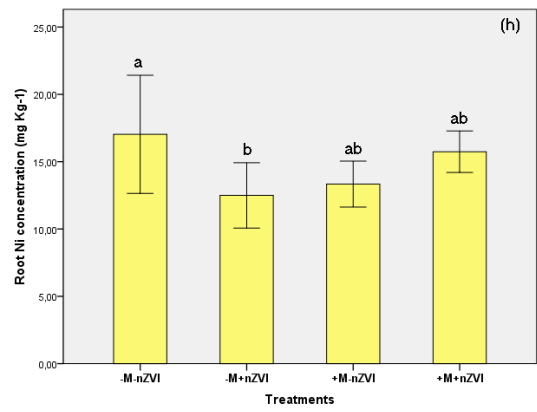
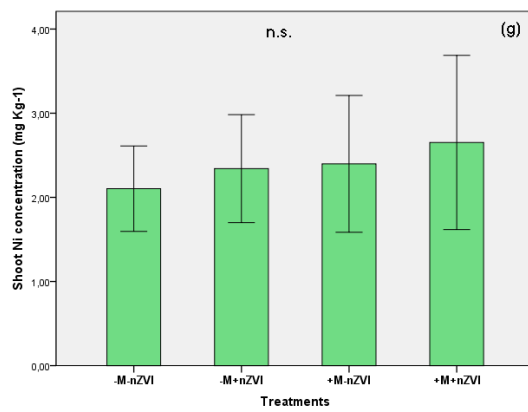
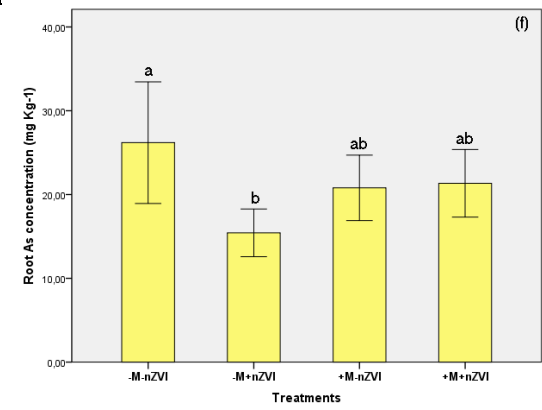
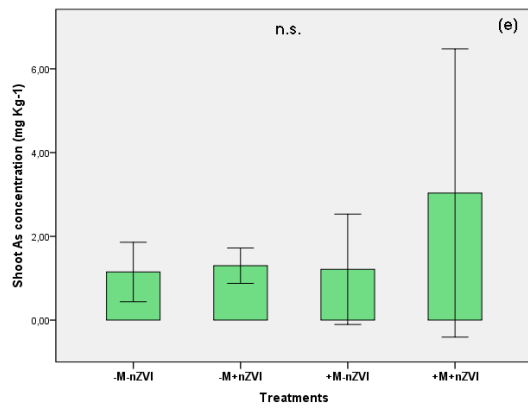
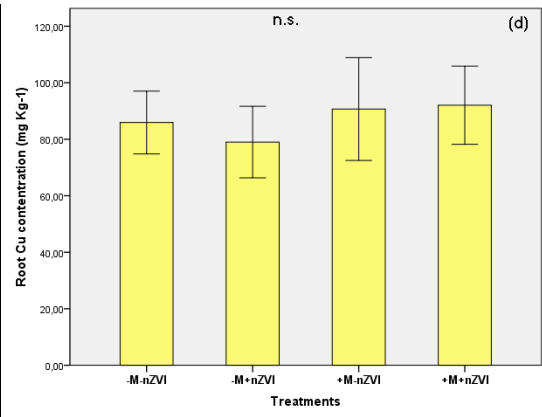
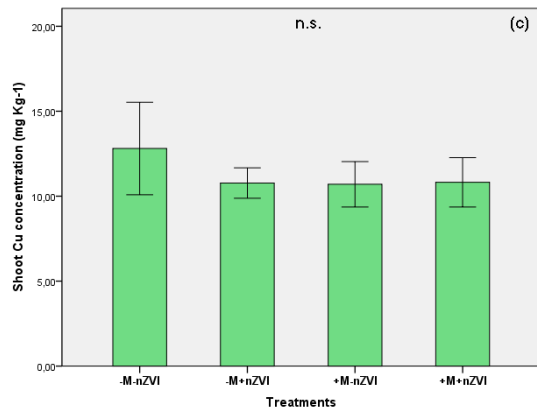


Figure 8. Plant shoot and root, Zn(a, b), Pb(c, d), Fe(e, f) and Mn(g, h) concentrations. Note:“+/-M” represent with/without AM fungal inoculation, “+/-nZVI” represent with/without nano zero valent iron amendment. Different letters above the columns show significant difference among different treatments

For Cd and Cu root and shoot concentrations did not differ significantly ($P < 0.05$, Figure 9(a, b) and 9(c, d)). Regarding As concentration there was no significant difference on shoot As concentrations among different treatments ($P < 0.05$, Figure 9e). On the other hand, As in the root differed significantly between treatments ($P < 0.05$). The lowest amount of As was found in “-M+nZVI” treatment, while the highest was in the “-M-nZVI” treatment. nZVI decreased As uptake by roots without AM colonization while had no influence on As uptake by mycorrhizal roots ($P < 0.05$, Figure 9f). AM symbiosis tended to increase root As concentration under nZVI amendment. Nickel concentrations in roots were significantly higher ($P < 0.05$) in “-M-nZVI” and “+M+nZVI” treatments compared to those in other treatments ($P < 0.05$, Figure 9h). AM had no influence on root Ni concentration, while significantly increased root Ni concentration when nZVI was added. nZVI addition decreased Cr uptake by roots for non-mycorrhizal treatment, but had no influence on root Cr concentration for mycorrhizal treatment. AM symbiosis decreased Cr concentrations in shoots, while increased Cr uptake by roots after nZVI amendment ($P < 0.05$, Figure 9).





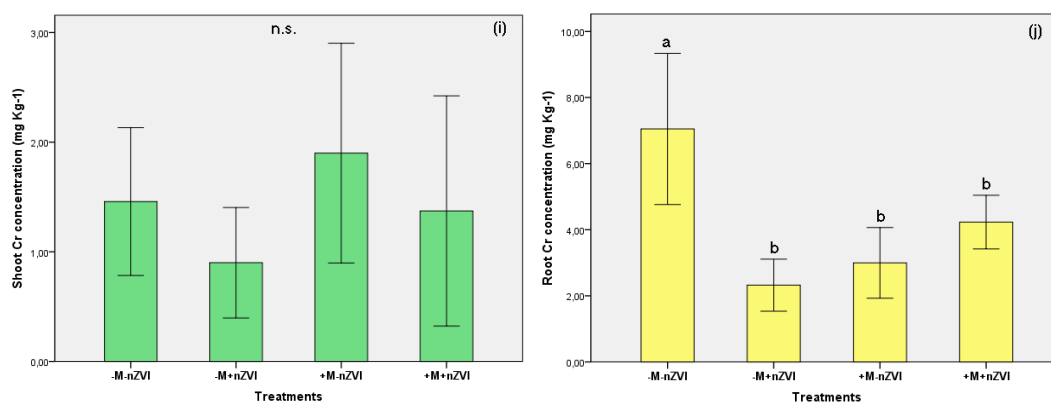


Figure 9. Plants shoot and root Cd (a, b), Cu (c, d), As (e, f), Ni (g, h), Cr (i, j) concentrations. Note:“+/-M” represent with/without AM fungal inoculation, “+/-nZVI” represent with/without nano zero valent iron amendment. Different letters above the columns show significant difference among treatments.

6. Discussion

Low colonization intensity (M%) and arbuscule abundance (A%) values were observed throughout the entire study. These can be attributed to the high amounts of heavy metals in the present soils. Studies which use high concentrations of certain heavy metals have shown similar trends, although in many cases not to such a low extent (Cheng et al., 2003, Wu et al., 2014, Duponnois et al. 2006), but these results can be explained if it is taken into account that our soil is greatly loaded with not only one, but by various heavy metal(loid)s. For instance, in a study realized by Yu et al. (2009) when As addition levels reached 100 mg kg⁻¹, the colonization rate dropped down to 69%, but in our soil, As content was 296 mg kg⁻¹. In the study of Chen et al. (2004), amendment of 600 mg kg⁻¹ Zn in the soil decreased colonization intensity (M%) to 50%, while the present soil was contaminated with 4000 mg kg⁻¹ Zn. Yan et al. (2016) conducted an experiment where the highest Pb stress level (1500 mg kg⁻¹) greatly decreased the mycorrhizal colonization of legumes, in our case Pb soil concentration was up to 3540 mg kg⁻¹. In this study all these elements and their respective concentrations were present together in the soil, plus the addition of Cd, Cr among others, can logically demonstrate the low M% and A%.

The present study proved negative effects of nZVI amendment on mycorrhizal infection. As nZVI significantly reduced M% and A% with respect to the other

treatment inoculated with AMF, yet without nZVI amendment. Application of nZVI can implicate toxicity to microbial soil organisms and communities, although it is context dependent. For instance, Pawlett et al. (2013) found the negative influence of nZVI on AM fungi in soils. The toxicity of nZVI may be due to oxidative stress, resulting from generation of Fe^{2+} and reactive oxygen species (ROS) by the nZVI particles (Auffan and Bottero 2009; Klaine and Lead 2008).

Plant growth did not differ among treatments. Neither the application of nZVI nor the AM symbiosis enhanced plant growth. High quantities of nZVI introduced into the soil may pose phytotoxic risks, although it is plant species dependent (Ma et al., 2013) and could counter the remediation effects of this technology. This assumption can partially explain why the nZVI amendment did not improve plant growth. Ma et al. (2013) observed that at low concentrations of bare nZVI, plant growth was enhanced, on the other hand at higher quantities ($>200\text{mg/Kg}$) demonstrated severe toxic effects, for instance, with the introduction of 100mg/kg of nZVI it could already be observed a decrease of 6.93% of the plant weight compared to the initial weight, due to the apparition of dry leaves. It was also seen that as nZVI concentration increased, the plant roots turned darker in behalf of the adherence of the nZVI particle onto the root surface, forming irregular iron aggregates. Although further studies should continue gathering knowledge about nZVI potential phytotoxic effects. As for AM fungi inoculation, it was mentioned during the literature review, that under heavy metal stress conditions, the AMF would alleviate their toxic effects, therefore strengthen plant growth. However, AMF in this study, could not significantly improve plant development, which may be linked to the massive amount of heavy metals present in the soil.

The main mechanism of AM symbiosis is to improve the plants mineral nutrient uptake, such as phosphorus and nitrogen, therefore directly influence plant growth. Numerous studies have proved this fact. When P is poorly available, AM symbiosis highly increases plant growth compared to non-mycorrhizal plants (Bolan, 1991). The extra-radical mycelium in the rhizosphere can provide a massive surface area to interact with these nutrients. The major advantage of the AM symbiosis for plants in acquiring P is that AM fungi provide a very effective pathway by which P is scavenged from large volumes of soil and rapidly delivered to cortical cells within the root, bypassing direct uptake (Smith et al., 2011). In the present study, treatments

inoculated with AM fungi did not improve P uptake, or translocation to the shoots. A reason could be that the available P concentration in the present soil was very low (i.e. 1.5 mg kg⁻¹). Such a low P concentration may also restrain P uptake by AM fungal mycelium. As growth differences between plants with AM symbiosis and plants without AM symbiosis tend to disappear as available soil P is increased, because of lower P depletion in rhizospheres.

Several studies have observed how nZVI as well as other nanomaterials deposit over the root surface forming aggregates and can potentially block water and nutrient uptake (El-Temsah and Joner, 2012; Ma et al., 2013; Stefaniuk et al., 2016). Another effect of nZVI on nutrient uptake by plants is that nZVI can possibly absorb nutrients and impede their uptake by the plant. In this study nZVI amendment had no significant effects on plant macro nutrient uptake (e.g. Ca, K, Mg). This may be because that there were high amounts of bioavailable K, Ca, Mg in the present soil which are enough for plants.

In general, nZVI reduced the metal(loid)s concentrations in the plants roots, most probably due to its high specific area and sorption capacities. The nZVI functions may result from direct adsorption of metal(loid)s by nZVI in the soils (Komarek et al., 2013; Vitkova et al., 2016), or alteration of metal(loid)s speciation, or aggregation of nZVI on root surface and thus reduce metal(loid) uptake by plants (Martínez-Fernández and Komárek, 2016). However, nZVI had no influence on metal(loid) uptake when plants were inoculated with AM fungi, suggesting that the functions of nZVI was influenced by AM symbiosis. Special attention should be paid to the influence of AMF symbiosis on plant root metal concentration. Generally, similar with our previous study (Wu et al., 2014), AM symbiosis decreased metal (e.g. Zn, Cr, Pb, Ni, etc) concentrations in roots, which may result from change of metal(loid) speciation in the rhizosphere soils (Wu et al., 2014). However, on the contrary, when nZVI was amended, AM symbiosis increased root metal(loid) e.g. Mn, Cu, Cr, Ni, As (also Zn, a little) concentration (Figure 10, 11). This was in accordance with our assumption that AM symbiosis can effect nZVI functions and increase metal(loid)s uptake when nZVI was added. The reasons may lie in (1) Direct interaction of AM fungi with nZVI, which can change nZVI speciation and influence its functions; (2) metal(loid) bioavailability in the soil was influenced by AM symbiosis through changing soil physio-chemical traits; (3) some

exudates of AM symbioses e.g. glomalin related soil protein (GRSP) can interact with nZVI and metal(loid)s and influence metal(loid) bioavailability (Nichols, 2003). For the shoot metal(loid) concentration, AM symbiosis decreased metal concentration, indicating enhanced stabilization of metal(loid)s by AM symbiosis, which may be due to the compartmentation of metal(loid)s in fungal structures within plant roots (Joner et al., 2000; Wu et al., 2015; 2016a; 2016b).

7. Conclusion

The present study revealed the negative effects of nZVI on AM symbiosis formation and development. Besides, the study also showed that nZVI could reduce metal(loid) uptake by plants, but its functions were restrained by AM symbiosis, which increased metal(loid) uptake by plant roots under nZVI amendment. The study provides important information on assessment of nZVI functions in the “real” metal(loid) contaminated soils with the existence of AM fungi. However, the mechanisms of the influence of AM symbiosis on nZVI functions are still unclear. Besides, future studies should also pay attention to the influence of other soil microbes on nZVI functions.

8. References

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