Root-induced changes of Zn and Pb dynamics in the rhizosphere of sunflower with different plant growth promoting treatments in a heavily contaminated soil

Seyed Majid Mousavi⁎, Babak Motesharezadeh⁎⁎, Hossein Mirseyed Hosseini⁎, Hoseiniali Alikhania, Ali Asghar Zolfaghari

⁎⁎⁎ Corresponding authors.
E-mail addresses: majidmousavi@ut.ac.ir (S.M. Mousavi), moteshare@ut.ac.ir (B. Motesharezadeh).

Abstract

Root induced changes are deemed to have an important role in the success of remediation techniques in contaminated soils. Here, the effects of two nano-particles [SiO₂ and zeolite] with an application rate of 200 mg kg⁻¹, and two bacteria [Bacillus safensis FO-036b(T) and Pseudomonas fluorescens p.f.169] in the rhizosphere of sunflower on Zn and Pb dynamics were studied in greenhouse conditions. The treatments reduced the exchangeable Zn (from 13.68% to 30.82%) and Pb (from 10.34% to 25.92%) in the rhizosphere compared to the control. The EC and microbial respiration/population of the rhizosphere and bulk soil had an opposite trend with the exchangeable fraction of Zn and Pb, but dissolved organic carbon followed a similar trend with the more bioavailable fractions. As a result, the accumulation of Pb and Zn in the plant tissues was significantly (p < 0.05) reduced by the application of amendments, which might be due to the shift of the metals to immobile forms induced by the nature of the treatments and changes in the rhizosphere process. The empirical conditions of this research produced the intensification of the rhizosphere process because the findings highlight those changes in the rhizosphere EC, pH and dissolved organic carbon can affect the efficiency of zeolite/SiO₂ NPs and bacteria to immobilize Pb and Zn in the soil, depending on the chemical character of the metals and the treatments. Generally, the affinity of the biotic treatment for Pb was more than the abiotic and conversely, the abiotic treatment showed a higher ability to immobilize Zn than the biotic treatment.

Keywords: Rhizosphere, Nano-particles, Bacteria, Zinc, Lead

1. Introduction

There are many anthropogenic activities, such as agricultural and industrial activities, transportation, coal combustion residues, spillage of petrochemicals and waste disposal which have increased the accumulation of heavy metals such as Zn and Pb in agricultural soils (Jamali et al., 2009; Zhang et al., 2010; Mousavi et al., 2013, 2017). Lead has no metabolic role in living organisms (Kabata-Pendias and Pendias, et al., 2009; Zhang et al., 2010; Mousavi et al., 2013, 2017). Thus, lead is readily absorbed and transported within plant tissue (Mousavi et al., 2010b, 2013). Therefore, it can readily enter the food chain, resulting in phytotoxicity (Chen and Kao, 2000) and consequently causing serious threats to public health. The maximum acceptable concentration range of Pb in soil and plants respectively is 2–300 mg kg⁻¹ and 0.2–20 mg kg⁻¹ (Alloway, 1990). By contrast, Zinc (Zn) is an essential micronutrient and has special physiological functions in all living systems, such as the maintenance of structural and functional unity of biological membranes and the promotion of protein synthesis and gene expression (Andreini et al., 2006). Tolerance to environmental stress conditions has a high requirement for Zn to regulate and maintain the expression of genes needed to protect cells from the detrimental effects of the stress (Cakmak, 2000). The concentration range of Zn in soil and plants is normally 1–900 mg kg⁻¹ and 1–400 mg kg⁻¹, respectively (Alloway, 1990). After entering the soil and sediment, Zn and Pb may be distributed among soil constituents and connected to them in different forms, which have often been related to as fractionation (Osakwe and Okolie, 2015; Li et al., 2016). To describe the activities of Zn and Pb in soil, knowledge about both total concentration and chemical fractionation is unavoidable. However, for measuring Zn and Pb activity in soil and to find how readily Zn and Pb uptake by plants happen, it is necessary to study the chemical fractionation rather than the total Zn and Pb content, as the former determines the mobility and bioavailability of Zn and Pb (Wu et al., 2006). Sequential extraction methods offer an efficient tool for studying the
et al. (1979) presented an extraction method that is the most widely applied technique. It enables the partitioning of the total metal content into five forms: exchangeable, carbonate bound, Fe/Mn oxide bound, organic bound and residual fraction. The procedure was suggested for sediments, but it can also be used for soils.

Nanoparticle materials are important supporting materials in remediation of soil contamination due to their large surface area and their physical/chemical reactivity. According to Dutta et al. (2000) a nanoparticle is defined as particle with an average characteristic dimension less than 100 nm. These materials show completely new or improved properties based on size, distribution, morphology, and phase compared with larger particles of the bulk material they are made of (Perez et al., 2004). Most of the atoms of a nanoparticle on the surface are unsaturated and can easily cohere with other ions, thus possessing considerable chemical reactivity (Liang et al., 2000).

The rhizosphere is a microenvironment where physical, chemical and biological properties may drastically differ in many respects from those in the bulk soil (Marschner and Rmheld, 1996) due to root-induced processes in the vicinity of the root, such as the release of exudates, adsorption and desorption of elements, removal of water and the physical forces of the plant roots. Therefore, the dynamics, transformations, phytoavailability and toxicity of heavy metals are expected to differ obviously from those in bulk soil (Bravin et al., 2012; Houben and Sonnet, 2012). Furthermore, soil application of different amendments for phytostabilization may also change the rhizosphere reactions, which may, in turn, impact the transformation, mobility and bioavailability of trace metals remarkably in response to change in soil pH, rhizodeposition and microbial community (Park et al., 2011).

Silicon from different sources has been demonstrated to reduce the harmful effects of trace metals on plants growing in contaminated soils (Rizwan et al., 2012; Shen et al., 2014; Keller et al., 2015). Zeolites are a group of alumino-silicates with a negative charge (Mohamed, 2001) which are commonly applied as toxic metal-immobilizing agents (Bolan et al., 2014; Navel and Martins, 2014). The fractionation of metals in the soil solution can be changed by Si through the formation of silicate complexes (Putwattana et al., 2010). Some researchers have reported that Fe-Mn oxides and organic matter fractions are the dominantgeochemical fractions in soils treated with Si (Chen et al., 2000; Liang et al., 2005), which consequently decreases the exchangeable fraction, thereby reducing trace metal bioavailability in the soil. However, little attention has been paid to the effects of nano-sized SiO₂ and zeolite on the chemical behavior of trace metals in the rhizosphere.

Bacteria that colonize plant roots and promote plant growth are referred to as plant growth-promoting rhizobacteria (PGPR) (Beneduzi et al., 2012). The ability of these bacteria in the alleviation of plant stress in metal-contaminated soils is well demonstrated (Mani et al., 2015; Kamran et al., 2016). Release of chelating agents and siderophores, acidification, phosphate solubilization, and redox changes are known as the main strategies of PGPRs to affect trace metal mobility and bioavailability (Ma et al., 2011). However, studies on PGPRs effects on the fate and behavior of trace metals in rhizosphere soil and in the presence of mineral amendments are still limited. Therefore, the aim of this work was to gain better insight about how the soil application of nanoparticles (SiO₂ and zeolite) and bacteria (Bacillus safensis FO-036b (T) and Pseudomonas fluorescens p.f.169) in the contaminated soil affect the rhizospheric reaction and, in turn, the fractionation and the bioavailability of Zn and Pb in the rhizosphere of sunflower (Helianthus annuus L.). Sunflower is commonly known as a heavy metal-tolerant plant and effective in phytoremediation (January et al., 2008; Huicheng et al., 2012), which is one of the most important oilseed crops in the world, and especially in Iran.

2. Materials and methods

2.1. Materials

The studied soil, from Calixerepts on the USDA system, was sampled (surface layer, 0–30 cm) from the vicinity of the National Iranian Lead and Zinc Company (NILZ) in Zanjan, Iran (36° 36' 40" and 36° 38' 40" N; 48° 37' 33" and 48° 38' 48" E) on Feb, 9, 2016, and after air-drying, it was sieved (2 mm) to determine some biological (microbial population (Alexander, 1982), microbial respiration (Anderson and John, 1982), physical (soil texture (Gee and Bauder, 1986)) and chemical properties (pH (Klute, 1986), EC (Brenner, 1982), CEC (Sumner and Miller, 1996), OC% (Walkley and Black, 1934), Calcite % (Loeppert and Suarez, 1996), Si (Hurney, 1973), N (Brenner, 1996), P (Olsen et al., 1954), K (Helme and Sparks, 1996), Zn and Pb concentration (Page, 1982)). The results of analysis are as follows; microbial population 150,000 MPN g⁻¹; microbial respiration 0.25 mg g⁻¹; soil texture, loamy; pH 7.19; EC 4.64 dS m⁻¹; CEC 17.94 meq. 100 g⁻¹; OC 0.39%; Calcite 15.25%; Si 158.98 mg kg⁻¹; N 0.07%; P 8.6 mg kg⁻¹; K 344 mg kg⁻¹; total Zn 7027.54 mg kg⁻¹; extracted Zn by DTPA 311.3 mg kg⁻¹; total Pb 4752.48 mg kg⁻¹ and extracted Pb by DTPA 174.23 mg kg⁻¹. Because of many adverse effects which are induced by sterilization of soil, especially in elemental composition, carbonate and organic compounds (Perkins et al., 2013), we decided to use non-sterilized soil in this work.

A natural clinoptilolite-zeolite powder (< 60 μm with purity > 95%) supplied by Afrand Tusco Co., Tehran, Iran, was used for this work. It was then changed into nano-sized particles (< 100 nm) by using a Planetary Ball Mill (PM 600) at the Material and Energy Research Center (https://en.merc.ac.ir/) in Karaj, Iran. Its initial characteristics were found as follows (unit %): SiO₂ 66.5, Al₂O₃ 11.8, CaO 3.1, K₂O 2.1, Na₂O 2, Fe₂O₃ 1.5, MgO 0.8, TiO 0.3, MnO 0.04, P₂O₅ 0.01 and its CEC was 160–180 meq. 100 g⁻¹. The studied SiO₂ NPs was obtained from Sigma-Aldrich (Purity > 95%). The morphology and characteristics of the zeolite/SiO₂-NPs were evaluated by field emission scanning electron microscopy (FE-SEM; Hitachi S-4700, Tokyo, Japan), and energy-dispersive X-ray spectra (EDS) were prepared using FE-SEM.

Two native strains of bacteria, namely Bacillus safensis FO-036b(T) and Pseudomonas fluorescens p.f.169, were separated and purified from the soils in the vicinity of a zinc and lead mine in Arak city, Markazi province, Iran, at longitude 35° 48' 35"E and latitude 50° 58' 18"N (Motesharezadeh and Savaghebi-Firoozabadi, 2010; Mohammadzadeh et al., 2014), and considered as biotic treatment, and their resistance to high levels of Pb and Zn in respect to colonization, were tested on plate (Saikia et al., 2015). For this test, the metals Pb and Zn were applied as PbNO₃ and ZnO. The salt solutions were prepared in high concentrations (Pb 9.652 mM and Zn 38.238 mM) and then added to a sterilized nutrient agar medium. Plates were then spot inoculated with the Bacillus safensis and Pseudomonas fluorescens and incubated at 37 °C. Colony forming units were determined after 2 days (Saikia et al., 2015).

To examine the other characteristics of the selected strains, the standard and routine protocols were used. After inoculating the plates and 2 days of incubation at 37 °C, the shape and colors of the colonies were examined under the microscope after Gram staining (Saikia et al., 2015). The isolates were also biochemically tested for siderophore in supernatants of culture fluids, and their characterization was performed directly on the agar plates. Orange halos around the colonies on blue agar were indicative of siderophore excretion (Schwyn and Neilands, 1987). Activity of ACC-Deaminase was determined, according to Penrose and Glick (2003), using 9 cm petri dishes, containing Rhizobial Mineral Medium (RMM) in three replicates. In order to study the potential of the strains in respect to indole-3-acetic acid production based on the Patten and Glick (2002) method, the selected strains were propagated overnight in 5 ml of DF salts minimal medium (Dworkin and Foster, 1958). After an incubation period of 42 h, the density of each...
culture was spectrophotometrically determined at 600 nm. After removing the bacterial cells from the culture medium by centrifugation, a 1-ml aliquot of the supernatant was mixed vigorously with 4 ml of Salkowski's reagent (Gordon and Weber, 1951) and allowed to stand at room temperature for 20 min before the absorbance at 535 nm was measured.

2.2. Experiment design

Plastic pots 14.5 cm tall with an 8.5 cm mouth opening diameter were used in the experiment. The pots had drainage and were filled with 3 kg of air-dried soil passed through a 4 mm sieve. A rhizobag technique was used for separating rhizosphere soil from bulk soil. In this method, cylindrical rhizobag (13.5 × 6.5 cm) were made of nylon with a mesh size of 60 μm. Then, these rhizobags were placed in the center of the pots and enclosed by the soil so that soil surfaces inside and outside the bags were at equal levels (McGrath, 1997; Silva Gonzaga et al., 2006). The content of soil inside of each rhizobag was about 500 g.

Abiotic treatments were uniformly applied at a rate of 200 mg kg⁻¹ on Feb, 16, 2016 in three levels: Zeolite- NPs (Z), SiO₂- NPs (S) and control (or without NPs application). Because in recent studies on silicon application in contaminated soils, the applied rate most was around 200 mg kg⁻¹ (da Cunha and do Nascimento, 2009; Li et al., 2009; Syu et al., 2016), therefore it was preferred level for both SiO₂ and zeolite. The pots were then incubated for 60 days (Wu et al., 2015) at room temperature (23–27 °C) and soil moisture was held near field capacity (FC) by watering every day and replacing the lost water (Huang et al., 2009). The soil of each pot was mixed well once every week to maintain intimate contact with the nano-particles. After the incubation step on Apr, 15, 2016, all the incubated soil samples were air dried and passed through a 2-mm sieve. The soil of each pot was sampled to determine the geochemical forms of Pb and Zn by Tessier et al. (1979) method. After that, sunflower seeds (Helianthus annuus L. ‘Azargol cultivar’ from Seed and Plant Improvement Institute in Karaj, Iran) were surface sterilized by soaking for 12 min in 1.5% sodium hypochlorite, thoroughly rinsed with sterile deionized water and then germinated. Prior to this step, the seeds response to inoculation was tested. Based on the obtained results, the strains did not restrict the germination (data not shown). In each pot, six germinated seeds were sown in the rhizobag, and for inoculation, the bacteria were inoculated at the rate of 1 ml inoculum in three levels (control treatment (or without bacterial inoculation), Bacillus safensis and Pseudomonas fluorescens with equal bacterial populations of 5 × 10⁸ cfu ml⁻¹ for each germinated seed which they were injected under the germinated seeds in the pot on Apr, 15, 2016. Irrigation was performed to field capacity (FC) with deionized water, and their moisture content was held constant by watering every day and restoring the lost water. During the study, the environmental condition was: temperature 28 °C/20 °C (day/night), average temperature 26 °C, relative humidity 48%, light intensity 14,000 lx and photoperiod 14 h/10 h (Huang et al., 2009).

2.3. Soil and plant analysis

After 2 months from the transplanting date, on Jun, 13, 2016, the plants were harvested and the roots were carefully separated. Soil material inside and outside the rhizobag was taken as rhizosphere and bulk soil, respectively, and were prepared for analysis. Then, microbial population, microbial respiration, dissolved organic carbon (Brandstetter et al., 1996), soil pH and electrical conductivity were measured in the soil samples. To study the Zn and Pb geochemical fractions in rhizosphere and bulk soil, the sequential extraction method was adopted (Tessier et al., 1979; Elliott et al., 1990). The zinc and lead were partitioned into five fractions: (1) exchangeable (EXCH: 1 M MgCl₂, pH 7); (2) bound to carbonates (CARB: 1 M NaOAc adjusted to pH 5 with acetic acid HOAc); (3) bound to Fe-Mn oxides (OXI: 0.04 M NH₄OH–HCl in 25% [v/v] HOAc); (4) bound to organic matter (ORG: 0.02 M HNO₃ and 5 ml of 30% H₂O₂ adjusted to pH 2 with HNO₃, 5 ml of 3.2 M NH₄OAc in 20% [v/v] HNO₃); and (5) residual (RES: HF-HClO₄ mixture). The steps were separated through decantation of the sample supernatant after centrifugation at 20,000 rpm for 30 min. Finally, Zn and Pb concentration was measured by Atomic Absorption (Shimadzu, Japan A-670). At each extraction step and in each measurement, a blank sample was analyzed in 3 replications to evaluate the quality of the reagents and to detect possible trace metal contaminations.

2.4. Statistical analysis

Statistical analysis of the data was done by MSTATC (MSTATC, East Lansing, Mich.) and SPSS 16.0 (SPSS Inc., Chicago, Ill.) software, and the means were compared according to Duncan’s multiple-range test (DMRT) (P < 0.05).

3. Results and discussion

3.1. Studying the properties of biotic and abiotic amendments

The results of laboratory studies showed that the studied strains have PGPR properties (Table 1) and potential to tolerate high levels of Pb and Zn (data not shown).

Fig. 1 presents the scanning electron microscopy (SEM) images of SiO₂ NPs and zeolite NPs at different magnifications (Fig. 1a–d), energy-dispersive X-ray spectra (EDS) of SiO₂ NPs (Fig. 1A) and zeolite NPs (Fig. 1B). Fig. 1c and d show that zeolite with an average particle size of 50 nm can be certainly recognized as the coarser powders than the SiO₂ which has an average particle size of 15 nm, so SiO₂ is considered as the NPs with the higher specific surface area compared to zeolite NPs that might be used as an effective stabilizer for toxic metals. Moreover, a reduction in the particle size leads to an increase in magnetic and electrical properties (Perez al, 2004). The energy-dispersive X-ray spectra (EDS) of the studied NPs further confirms the high purity of the applied SiO₂-NPs (> 95%) and the presence of Si, O, Fe, Al, Na and K in the zeolite- NPs.

As it was mentioned in the “Materials” section, prior to the research, the microbial population and respiration of the initial soil were measured (150,000 MPN g⁻¹ and 0.25 mg g⁻¹, respectively) and 2 months after transplanting, (harvesting stage), these traits were measured in the inoculated pots as well. The results showed that the microbial population and respiration in the inoculated pots was considerably higher than in the control treatment which in all of the traits was observed negative response.

### Table 1

The PGPR characteristics of the studied strains*.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Positive or negative gram</th>
<th>Siderophore</th>
<th>ACC-Deaminase</th>
<th>IAA*</th>
<th>Resistance to Pb (9.652 mM)</th>
<th>Resistance to Zn (38.238 mM)</th>
</tr>
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<tbody>
<tr>
<td>B2: Bacillus safensis</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B3: Pseudomonas fluorescens</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
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</table>

*B in order to verify the results of these experiments, the studied factors were measured in distilled water as the control treatment which in all of the traits was observed negative response.

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than in the initial soil and control treatment (i.e. non inoculated soil), which indicates a successful inoculation. For example, in the soil inoculated with the *Bacillus safensis*, microbial population and respiration were about 140% and 103.2% respectively, more than the initial soil. These contents for the *Pseudomonas fluorescens* were respectively about 177.33% and 54.8% (data not shown). Another confirmation of the successful inoculation was that the inoculated plants with the strains had more growth than the control treatment (data not shown).

### 3.2. Bulk soil and rhizosphere soil pH, EC and DOC

In the bulk soil, bacterial inoculation and NPs application affected the soil’s pH (Fig. 2). Zeolite NPs increased the pH from 7.21 in the control up to 7.31 in the BZ treatment, very likely due to the decomposition of metal oxides (e.g. CaO, K₂O and MgO) blended with zeolite, as well as the presence of –O⁻ groups at the surface of zeolites (Fig. 1), which can contribute to alkalinity through association with H⁺. The soils treated with the SiO₂ NPs had more pH compared to the zeolite treatments (variable from 7.33 to 7.38), however, due to the high buffering capacity of the calcareous soil, this increase is poor. The notable and significant result was the decreasing effect of BC and PC treatments on the pH. The reduction in the soil pH might be due to the production of protons, amino and organic acids through metabolic activities of the bacteria (Huang et al., 2002; Wu et al., 2006). The application of SiO₂-NPs increased the pH up to 7.38 separately and combined with the bacteria (Fig. 2). However, this increasing effect did not happen in the rhizosphere as its rhizospheric pH in the presence and in the absence of zeolite/SiO₂ NPs was lower than the bulk soil (Fig. 2). In the rhizosphere as well as the bulk soil, the lowest pH content was measured in the soil treated with the biotic treatment without any NPs, i.e. BC and PC treatments which had a significant difference with the other treatments. The decrease of soil pH was up to 1.1% and 3.2% at the *Bacillus safensis* and *Pseudomonas fluorescens* treatments respectively, when compared to the control treatment (Fig. 2). The decreasing effects of bacterial treatments on soil pH were confirmed in previous studies (Huang et al., 2002; Wu et al., 2006). Among soil properties, pH is one of the most important factors that controls metal bioavailability, and it has been observed that Si reduced metal availability by decreasing soil acidity (Treder and Gieslinski, 2005; Gu et al., 2011). Treating the soil with the biotic and abiotic amendments, not only in the bulk soil but also in the rhizosphere, significantly (p < 0.05) promoted the soil electro conductivity (EC), which in the rhizosphere was higher than the bulk soil (Fig. 2). As the application of zeolite-NPs in the bulk soil increased the EC from 3.03 dS m⁻¹ in the control up to 4.48 dS m⁻¹ and the application of SiO₂-NPs increased the EC up to 5.36 dS m⁻¹, separately and combined with the bacteria. In the rhizosphere soil, EC increased from 5.72 dS m⁻¹ in the control up to 8.77 ds m⁻¹ in the CS treatment. Generally, the inoculation of bacteria resulted in an increase of soil EC which may be due to the secretion of different ionic compounds through metabolic activities in the root zone (Wu et al., 2006). Also, a higher reduction in soil pH in the rhizosphere increases the dissolution and releasing of different ions from the organic and inorganic compounds into the soil solution, resulting in an increasing salinity and EC (Wu et al., 2006) as the difference in EC between rhizosphere and bulk soil was insignificant (Fig. 2). Based on this figure, the highest EC in the rhizosphere was measured in the soil treated with the SiO₂-NPs, which was about 53.3% more than the control. This finding emphasizes the highly alleviative effect of SiO₂ in the presence of toxic metals which increase the microbial population and consequently induces the promotion of microbial activities. In

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Fig. 1. Scanning electron microscopy (SEM) of (a) SiO₂-NPs at a high magnification (200 nm); (b) SiO₂-NPs (2 µm); (c) zeolite-NPs at a high magnification (200 nm); (d) zeolite-NPs (2 µm); (A) EDS of the SiO₂-NPs; and (B) EDS of the zeolite-NPs.
general, pH values between the rhizosphere and bulk soil differed slightly in the presence of different biotic and abiotic amendments. However, EC values of rhizosphere soil were significantly (p < 0.05) higher than those of bulk soil. For example, in the *Pseudomonas fluorescens* + SiO2 NPs treatment the rhizosphere EC was up to 63.3% more than the bulk soil EC (Fig. 2).

Dissolved organic carbon (DOC) is included in low molecular weight organic compounds such as polyphenols, simple aliphatic acids, amino acids and sugar acids, and it is one of the most important agents affecting metal mobility and bioavailability in soils (Kaiser et al., 2002).

The application of zeolite/SiO2 NPs and inoculation of bacteria increased DOC content of the soil compared to the control treatments (Fig. 3). All heterotrophic bacteria, such as the bacteria studied in this work, which required organic supplements for growth and energy supply, may participate in the promotion of DOC because they decompose these supplements and then release soluble low-molecular-weight compounds, such as organic acids, into the soil (Bosecker, 1997). However, this increasing effect is greatly weakened due to the presence of higher levels of soil contamination by heavy metals. In the rhizosphere and in the absence of any amendments, DOC content was about 65.5% more than that which was measured in the bulk soil. Application of different amendments significantly increased DOC content in the rhizosphere and bulk soil (p < 0.05), and this change in the rhizosphere was more than the bulk soil (Fig. 3). The *Pseudomonas fluorescens* + SiO2-NPs Treatment had the highest effect on DOC both in the rhizosphere and in the bulk soil, which was about 11.49% and 32.29% more than the control, respectively. The direct effect of the rhizosphere on microbial activity and population and also the considerable alleviative effect of the applied treatments as soil conditioners were the main reasons for the promotion of DOC content (Wu et al., 2006). In this work, the increasing effect of the rhizosphere on DOC content is greatly weakened due to the presence of higher concentrations of toxic metals and their adverse effect on microorganisms and microbial mediated processes in the soil (Lemire et al., 2013). The lowest and the maximum microbial respiration/population were measured in the control and *Pseudomonas fluorescens* + SiO2-NPs treatments respectively (data not shown).
3.3. Effects of the biotic and abiotic treatments on Pb and Zn speciation in bulk soil

The speciation of Pb and Zn in the soil was done using a sequential extraction procedure based on Tessier’s scheme (Tessier et al., 1979). Although sequential extractions have some drawbacks such as lack of selectivity and element redistribution during extraction, these methods have been used comprehensively by researchers to explain the speciation of heavy metal in soils, including the rhizosphere (Bacon and Davidson, 2008). In agreement with Hammer and Keller (2002), their use is especially vindicated when the purpose is to study differences created in the same soil by different treatments, such as the influence of treatment or plant growth on the chemical behavior of metals.

The fractionation experiment was conducted two times after incubation and after harvest. The results of the first experiment shows that a considerable portion of the total Pb and Zn content was in the F2, F3, and F5 fractions. Due to the calcareous nature of the soil, exchangeable and water soluble fraction was the lowest (variable from 0.028% to 0.18% of total Pb and variable from 0.12% to 0.24% of total Zn), and carbonated fraction was considerable. In general, the percentages of different fractions of Pb in the soil after incubation period were as follows: RES > OXI > CARB > ORG > EXCH Pb. The percentages for Zn were as follows: OXI > RES > CARB > ORG > EXCH Zn (data not shown).

In the second experiment (after harvest), results of Pb/Zn geochemical fractionation in the bulk soil revealed that the biotic and abiotic treatment applications reduced the amount of Pb and Zn in the exchangeable fraction (EXCH), with the exception of the biotic treatment, which had no significant effect on EXCH-Zn (Fig. 4). The highest reduction in EXCH-Zn was measured in SiO2-NPs with an approximate decrease of 12.6% decrease compared to the control, and had no significant difference with the zeolite-NPs.

The zeolite-NPs treatment was superior to the SiO2-NPs in the decrease of EXCH-Pb with a 25.38% decrease compared to the control, and followed by SiO2-NPs (22.11% decrease compared to the control). This difference in the decrease of EXCH pool of Pb/Zn is likely due to their different chemical nature. The ability of zeolite and SiO2 to reduce the mobility of heavy metals in soil was reported by Zorbas et al. (2000); Garau et al. (2007) and Adrees et al. (2015), and the formation of silicate complexes was assumed to be one of the main mechanisms by Putwattana et al. (2010). Studying the biotic treatment effect on EXCH pool of Pb shows that the bacteria had no significant difference with the control, but in combination with the abiotic treatment (i.e. P + Z, B + S, B + Z, P + S), it significantly decreased EXCH-Pb, and, in general, the biotic treatment was weaker than the abiotic treatment in reducing EXCH-Pb (Fig. 4). Wu et al. (2006) in a study on the influence of the two strains of Bacillus on chemical behavior of Pb and Zn reported that the bacteria could immobilize Pb due to the adsorption by bacterial cell walls and other possible sedimentation reactions that is inconsistent with the results of present work and might be the result of high levels of soil contamination, decreasing the ability of bacteria in the reduction of EXCH-Pb/Zn which was promoted in presence of zeolite/SiO2-NPs.

Even though it was not possible to conclude which fraction received the Pb/Zn from the EXCH fraction, based on the nature of treatments, different chemical behaviors were observed from Pb/Zn. Although the bacterial treatment had no significant effect on EXCH-Zn, the abiotic treatment significantly reduced this pool, and based on the results, Zn had significantly shifted from EXCH to OXI pool, which is similar to the obtained results for Pb. In nanoparticles, most of the atoms on the surface are unsaturated and can easily cohere with other ions, thus possessing considerable chemical reactivity. Therefore, such materials can selectively adsorb metal ions and have a significant adsorption capacity (Anjum et al., 2016). The zeolite-NPs were superior to the SiO2 with regard to an increase in OXI-Zn/Pb which most likely is due to its chemical composition (results of zeolite analysis are presented in the materials and methods). Therefore, a decrease in the EXCH pool of Zn/Pb in CS and CZ treatments induced the Zn/Pb shifts to OXI pool (Fig. 4).

![Fig. 4. Influence of different treatments on geochemical fractions of Pb and Zn at the bulk soil. PZ: Pseudomonas + zeolite; BS: Bacillus + SiO2; BZ: Bacillus + zeolite; PS: Pseudomonas + SiO2; CS: SiO2; CZ: zeolite; BC: Bacillus; PC: Pseudomonas. Means above the same column and each fraction followed by the same letter not significantly different according to DMRT (P < 0.05).](image-url)
In soil pH and EC were considered as two important factors in the shift of Zn/Pb from the EXCH to the OXI fraction, which consequently increased the precipitation of the metals (Lindsay, 1979). However, the soil pH, due to the high buffering capacity of the calcareous soil, slightly changed, but the EC in CS, P+S and CZ treatments was significantly (p < 0.05) more than in the control (Fig. 3). Based on Paalman et al. (1994), complexation of salt derived anions with heavy metals and competition of salt derived cations with positively charged heavy metal species for sorption sites on the solid phase are two mechanisms which play an important role in the fate of heavy metals. The highest EC was measured in the CS treatment, with an increase of about 76.9% compared to the control, and followed by the P+S and CZ treatments (Fig. 3), whose effect on the decrease of EXCH Pb in the bulk soil was observed because these treatments had the lowest concentration of EXCH-Pb compared to other treatments (Fig. 4). Also, the abiotic treatments significantly decreased EXCH-Zn compared to the control, which might be due to the high EC of the soil under these treatments. Influence of zeolite/SiO$_2$ NPs on the soil EC is related to their chemical composition and their nature.

Moreover, a reduction in the particle size to the nano-range leads to increased magnetic and electrical properties (Perez et al., 2004). The other effect of the studied NPs in this work with regards to changes of soil EC is related to their alleviative effects. In other words, because of the high levels of heavy metal contamination in the initial soil, microbial activity (e.g. microbial population/respiration) was low, but after the harvesting period, it was observed that these factors increased compared to the non-treated soil. For example, microbial respiration in the CS and CZ treatments was about 28.65% and 20.27% more than the control respectively. Based on Wu et al. (2006), microbial activity in the in-oculated soil by bacteria increases the soil EC.

In the combined abiotic and abiotic treatment applications, i.e. P+Z, P + S, B + Z and B + S treatments, the Pb shifted from the EXCH to ORG fraction, most likely due to the increase in microbial activity affected by zeolite/SiO$_2$-NPs. Based on Huang et al. (2002), soil microorganisms are the most active organic colloids in the soil, which due to their charged surface and secretion of different organic compounds have a significant role in determining the fate of the metals. Also, with the exception of B + Z, BC and PC treatments, the residual fractions (RES) dropped compared to the control, which indicates the influence of the studied treatments on the depletion of Pb from non-available form (RES fraction) to other forms with more availability (EXCH, OXI, ORG fractions) (Fig. 4). Wu et al. (2006) in a similar study but on the influence of mine tailings addition in presence of bacteria showed that ORG-Pb rose dramatically while RES fraction dropped sharply. They reported an increase of DOC concentration and other organic compounds mainly due to the bacterial activities. In separate biotic treatment applications, i.e. BC and PC treatments, EXCH Pb slightly changed compared to other treatments, which was most likely due to the adverse effect of high contamination in the studied soils (total Pb 4752 mg kg$^{-1}$ and total Zn 7027.5 mg kg$^{-1}$) on bacterial activity. However, the potential of the bacteria promoted in the presence of zeolite/SiO$_2$ NPs indicates the superiority of abiotic treatment in Pb/Zn speciation.

3.4. Effects of root induced changes on Pb/Zn speciation

The concentrations of Pb/Zn fractions in the rhizosphere are shown in Fig. 5. The data presented in this figure shows that the EXCH and ORG pools of Pb/Zn were decreased compared to the control, but the OXI pool was promoted. The averages of EXCH and ORG fractions of Zn in the rhizosphere soils were up to 0.48% of total and 3.09% of total respectively, while the averages of these fractions for Pb were up to 0.68% of total and 0.79% of total respectively. Also, the averages of OXI and RES of Zn were about 55.43% of total and 14.73% of total respectively, which for Pb were measured as 1749.95 mg kg$^{-1}$ (36.44% of total) and 2289.81 mg kg$^{-1}$ (47.69% of total) respectively. The highest decrease in EXCH-Pb happened in the B + S treatment (25.89% compared to the control), which was followed by P + S treatment (24.73% compared to the control) and is consistent with the behavior of B + S and P + S treatments in the OXI pool of Pb because the highest increase in OXI-Pb happened in these treatments compared to the control (Fig. 5). The other noticeable result about these treatments was their superiority in decreasing ORG-Pb compared to the other treatments with an average of 17.04% decrease compared to the control. The behavior of treatments on Zn was to some extent different from Pb. The maximum decrease in EXCH-Zn was recorded in the CS treatment (about 30.83% decrease compared to the control), which was followed by the P + S and CZ treatments (Fig. 5), and the minimum decrease of EXCH-Zn belonged to separate biotic treatment applications, i.e. BC and PC treatments. The decreasing effect of studied treatments on different fractions of Zn in the rhizosphere was observed in CARB (with the exception of CZ and B + C treatments), ORG and RES pools, as well (Fig. 5). Both separate and combined biotic and abiotic treatments clearly increased OXI pool. Therefore, it might imply that Zn is shifted from EXCH, CARB, ORG and RES pools to OXI pool in the rhizosphere. In most cases, the combined treatments, i.e. P + Z, B + S, B + Z and P + S treatments, were superior to other treatments. Therefore, it might be assumed that single applications of biotic and abiotic treatments do not have a high ability to shift the metals between different fractions, but their ability in the presence of an abiotic treatment significantly increased, which was most likely due to the high levels of soil contamination that plays the role of an inhibitor agent. The decrease of EXCH pool in the rhizosphere was reported by different researchers (Hu et al., 2011; Motaghian and Hosseinipour, 2015; Houben and Sonnet, 2012).

The exchangeable pools may be recomplexed by organic exudates of roots (Yang et al., 2012), thus these pools were decreased in the rhizosphere soil of the sunflowers. Hence, a reasonable interpretation for the change in EXCH pools of Pb/Zn is a shift from the EXCH pool to other pools. The increase in the Pb/Zn OXI fraction can be due to the oxidation of organic materials by microorganisms. Thus, Pb/Zn associated with organic matter would be released in the rhizosphere. Furthermore, hydrous iron oxides had a high affinity for Pb/Zn sorption, but this pool did not fix Pb/Zn in an unavailable fraction (Motaghian and Hosseinipour, 2014).

Microbial population in the rhizosphere soil, both in the control and in the treated soils, was significantly promoted compared to the control (data not shown). The P + S and B + S treatments had the highest population both in the rhizosphere and in the bulk soil. Also, the results of microbial respiration in the studied soil showed that the mentioned treatments, i.e. P + S and B + S treatments have the highest influence (38.23% and 32.6% increase compared to the control). Therefore, this outcome will be expectable that with increase of microbial activity in the rhizosphere, oxidation of organic materials increases, as well. Thus, Pb/Zn-ORG will be released with the oxidation of different organic compounds, and consequently, Pb/Zn-ORI will be increased in the sunflower rhizosphere. The results of Martinez-Alcala et al. (2010) showed that the reaction of organic anions with metals in soils depends on their complexation ability, their sorption/desorption reactions and their microbial degradability. They stated that the higher microbial biomass found in the rhizosphere soils compared to the bulk soil, may promote the degradation of the organic compounds produced by roots. Therefore, fixation mechanisms in the soil are more relevant for the metal.

Another important factor that was measured in the rhizosphere was dissolved organic carbon (DOC). As it was presented in Section 3.2, both biotic and abiotic treatments promoted the DOC content in the rhizosphere which, is consistent with the decrease of pH (Fig. 2). Although it is not reasonable to ignore the role of rhizosphere induced reactions on promotion of DOC level in the soil (Li et al., 2011). The data for the rhizosphere showed that P + S and B + S treatments induced the highest accumulation of DOC compared to the other treatments (Fig. 3). Based on Sherene (2009), DOC plays an important role in the
chemistry of soil, through its influence on acidity, nutrient availability, metal toxicity and transport. The organic acids present in the DOC can act as a chelating agent and promote the mobility of toxic heavy metals. Based on the results of Pb/Zn speciation, the EXCH Pb/Zn concentration significantly decreased, affected by different treatments, and in the P+S and B+S treatments was approximately recorded the minimum concentration of EXCH Pb/Zn which it was inconsistent with the results of Li et al. (2011). They reported that dissolved organic matter (DOM) derived from the rhizosphere could significantly reduce metal sorption by the soil components and promote its mobility through the formation of soluble DOM-metal complexes. Rashad et al. (2013) also stated that DOC facilitates metal mobility. But there are several studies which show that organic ligands do not necessarily promote the solubility and mobility of metals (Seuntjens et al., 2004; Wenzel, 2009; Hernandez-Soriano and Jimenez-Lopez, 2015).

Hernandez-Soriano and Jimenez-Lopez (2015) concluded that the mobilization of DOM and metals into the soluble phase induced a low concentration of free ion activities and promoted the formation of metal-organic complexes. The other reason for the decrease in solubility and mobility of metals by organic ligands might be due to the direct absorption of ligand or ligand-metal complexes by surface functional groups of the soil (Wenzel, 2009). In the superior treatments in term of maximum DOC concentration, i.e. B+S and P+S treatments, although the minimum EXCH-Pb and ORG-Pb/Zn was obtained, but the highest concentration of OXI-Pb/Zn were measured (Fig. 5).

3.5. Effects of zeolite/SiO2 NPs, bacteria and root-induced changes on Pb and Zn concentration in plant

The application of zeolite/SiO2 NPs and inoculation of bacteria significantly reduced the concentration of Pb and Zn in both the roots and the aboveground parts of the sunflower (P < 0.05) (Fig. 6), which confirms the reductive effect of zeolite/SiO2 and PGPRs on trace metal mobility and availability (Wu et al., 2006; Ma et al., 2011; Wen et al., 2013), and their accumulation in the roots was more than the aboveground (Mousavi et al., 2010a, 2010b, 2013, 2017). The abiotic treatment reduced the Zn concentration in plant tissues more than the biotic treatment with the exception of BC treatment which was the most effective treatment with respect to decrease in Zn concentration of roots (Fig. 6).

Findings of Janos et al. (2010) showed that low levels of zeolite can considerably redistribute trace metals in the soil between available (exchangeable and solution) and non-available (oxides, carbonates, bonded to organic matters, and residual) pools. According to Shi et al. (2005) and Adrees et al. (2015), the significant effect of SiO2 to reduce heavy metal mobility and uptake by plants may be due to the formation of insoluble compounds because of the low acidity of SiO2 and co-precipitation of metals in metabolically less active tissues, especially in the endodermal cell wall, pericycle, xylem, and phloem.

The separate biotic treatment application slightly decreased the Pb concentration in plant tissues more than the abiotic treatment (with the exception of CS treatment, which was superior to the biotic treatment in Pb root) (Fig. 6). The release of chelating agents and siderophores, acidification, phosphate solubilization, and redox changes are known as the main strategies of PGPRs to affect trace metal mobility and bioavailability (Ma et al., 2011), which directly and indirectly induces changes in the chemical speciation of these toxic metals in the soil, and depending on the chemical nature of the metals, bacteria may have both positive and negative effects on their mobility and bioavailability. Wu et al. (2006) reported that immobilization of Pb due to the adsorption by bacterial cell walls is the main mechanism while, sedimentation reactions with different compounds produced through bacterial metabolism are the most effective mechanism to immobilize Zn. Considering the lower ability of the bacteria than the NPs in terms of reducing EXCH fraction in the rhizosphere and also it can be deduced from the findings of Wu et al. (2006), that the zeolite/SiO2 NPs treatment makes more powerful complexes with the metal than the bacteria because sedimentation is their main mechanism as OXI pool, while in the bacteria treatment, adsorption is the predominant mechanism. Therefore, the biotic treatments, might be applied as complementary
agents which promote the potential of abiotic treatment, to reduce the availability of heavy metals.

In most cases, combined biotic and abiotic treatments, i.e. P + Z, B + S, B + Z and P + S treatments, were more effective than the separate biotic and abiotic treatment applications in terms of reducing Pb and Zn in aboveground parts. The efficiency of zeolite/SiO$_2$NPs application and the PGPRs inoculation to reduce metal uptake by plants was probably related to their nature and root induced changes on metal distribution in the rhizosphere. Results of geochemical speciation made it clear that the major mechanism of Pb and Zn immobilization after introducing zeolite/SiO$_2$ NPs and the bacteria was a shift of Pb and Zn from EXCH-pool to OXI-pool, which is consistent with the findings of Janos et al. (2010).

The presented data in Figs. 5 and 6 shows that the studied treatments affected the rhizosphere pH, EC and DOC which EC and DOC followed a similar trend, i.e. the studied treatments induced their promotion more compared to the control. The treatments, because of the high buffering capacity of the calcisol, slightly and insignificantly affected the soil pH. Therefore, the role of EC and DOC on mobility and availability of metals under this condition might be considerable.

The CS treatment caused the highest rhizosphere EC, followed by P + S and CZ treatments (Fig. 2), whose effect on the decrease of EXCH-Zn in the rhizosphere soil was clearly observed because these treatments had the lowest concentration of EXCH-Zn compared to other treatments, which consequently induced the minimum Zn concentration in the aboveground parts (Fig. 6). According to Hatje et al. (2003), under soil salinity conditions complexation of salt derived anions with metals decreased the activity of free metal ions in the soil solution and the metal speciation shifts towards complex forms. In this work, a similar response was observed for Pb in the P + S treatment, as well. Acosta et al. (2011) concluded that an increase in salinity did not produce a significant increase in the concentration of Zn in the soil solution, which indicates that Zn was not easily complexed by the existing ions in the soil solution, even when salinity was increased 50-times. Generally, it can be concluded that the chemical composition of the soil solution has a determinant role for trace metal mobility and availability in soil salinity condition.

The Pb concentration of aboveground in CS and CZ treatments was more than in the other treated pots, which was inconsistent with the Zn concentration under these treatments as the superior treatments in respect to the rhizosphere EC. Salinity effects are not limited to the complexation capacity of salt anions. For example, when Ca$^{2+}$ and Mg$^{2+}$ are abundant ions in the soil solution, heavy metals will also be mobilized from particles due to the competition between these divalent cations and the metal ions (Paalman et al., 1994), and therewith, their potential for absorption by plants is increased.

As far as was reported in the Section 3.2, the maximum DOC content in the rhizosphere was measured in the P + S and B + S treatments, while the lowest concentration was recorded in the control treatment. The fractionation experiment showed that these treatments, i.e. P + S and B + S, were superior than most treatments with respect to reduction of EXCH Pb and Zn in the rhizosphere (Fig. 5), and consequently, it is expectable that reduce their accumulation in the plant tissues which aboveground parts Pb and Zn were consistent with these results while, root Pb and Zn were variable in different treatments. Based on Hernandez-Soriano and Jimenez-Lopez (2015) organic ligands do not necessarily promote the solubility and mobility of metals. They showed that mobilization of DOC and metals into the soluble phase induced a low concentration of free ions and an increase of metal-organocomplexation. Also, the direct absorption of ligand or ligand-metal complexes by surface functional groups of the soil is considered as another reason for a decrease of solubility and mobility of metals by organic ligands (Wenzel, 2009).

4. Conclusion

From the findings of the present work, zeolite/SiO$_2$ applications and bacterial inoculation may stimulate the rhizosphere process to change the chemical behavior of metals depending on both the special character of the metal and the amendment. The single application of biotic treatments had no significant potential to shift the Zn between available fraction and fractions with low availability, but its ability in the presence of an abiotic treatment significantly increased. Conversely, the biotic treatment had a higher ability to reduce the mobility of Pb than the abiotic treatment, which might be due to the high affinity of bacterial cell walls for Pb adsorption and possible sedimentation reactions induced by bacterial metabolism. Furthermore, the bacteria indirectly affected the chemical behavior of Zn, e.g. by increasing the DOC concentration and decreasing the pH value, which promoted the Zn bioavailability. In general, results of geochemical speciation made it clear that the major mechanism of Pb and Zn immobilization after introducing zeolite/SiO$_2$ NPs and bacteria was a shift of Pb and Zn from EXCH pool to OXI pool. Therefore, the special structural/chemical nature of the treatments and metals plays an important role in the mobility/
immodity of metals. Furthermore, studies to determine the mechanisms involved that help to manage contaminated agricultural soils in the field condition.

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