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Nanoscale zero-valent iron assisted phytoremediation of Pb in sediment: Impacts on metal accumulation and antioxidative system of *Lolium perenne*

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ABSTRACT

Lead (Pb) is a highly toxic environmental pollutant, and could result in toxic effects on living organisms. The effects of 0, 100, 200, 500, 1000 and 2000 mg/kg of nZVI on plant growth, Pb accumulation and antioxidative responses of *Lolium perenne* were investigated. Results showed that the total Pb contents in *L. perenne* with the treatment of low concentrations of nZVI (100, 200 and 500 mg/kg) were higher than those in the non-nZVI treatments, and the highest Pb accumulation capacity of 1175.40 μg per pot was observed in *L. perenne* with the treatment of 100 mg/kg nZVI. However, the total Pb contents in *L. perenne* decreased at high concentrations of nZVI (1000 and 2000 mg/kg). This might be resulted from the decrease of photosynthetic chlorophyll content and the aggravated oxidative stress induced by the high concentration of nZVI, which caused the decrease of plant biomass and metal accumulation capacity in plant. Moreover, the sequential extraction experiments results showed that the lowest acid soluble fraction of Pb in the sediments was found in the treatment with 100 mg/kg of nZVI, indicating that 100 mg/kg was the optimum concentration for nZVI to assist the phytoremediation of Pb-polluted sediment. To conclude, these findings provide a promising method to remediate Pb-polluted sediment by nZVI assisted phytoremediation.

1. Introduction

The rapid urbanization, industrial production, mining, as well as the progress of human civilization, have contributed to an increasing pollution of sediment with various heavy metals over the last few decades (Clemens, 2006; Huang et al., 2015). Heavy metals have persistence and toxicity, such as lead, nickel, chromium, cadmium, copper and zinc, and it can cause huge impacts on human activity and living organisms. Lead (Pb), as one of the most hazardous heavy metals pollution in sediment, has attracted particular attention (Huang et al., 2008; Rascio and Navari-Izzo, 2011). In fact, the remediation of Pb-contaminated sediment brings a technological challenge for researchers. Among the various remediation techniques, phytoremediation is the most promising, economical, and environment friendly remediation approach, which can eliminate the contaminants from sediment by the uptake and translocation of heavy metals in plants (Ehsan et al., 2014). Phytoremediation is a cost-effective way of environmental remediation using plants to remove, detoxify or immobilize contaminants in soil, water or sediments. Phytoextraction is a subprocess of phytoremediation by using plants to uptake and transfer contaminants from soils, sediments or water into harvestable plants. Phytostabilization is characterized by immobilizing the contaminants from soil or sediment on plant root surfaces, or precipitation the contaminants within the plant rhizosphere (Huang et al., 2017; Pulford and Watson, 2003). *Lolium perenne* produces high dry matter yields and has great tolerance to heavy metals, thus it could be suitable for the phytoremediation of Pb-polluted sediment and soils (Bidar et al., 2007). Karami et al. (2011) studied the Pb mobility and uptake by *L. perenne* in soil with the Pb concentration of up to 21,000 mg/kg, and they found that the accumulation of Pb in *L. perenne* could exceed 1500 mg/kg at day 120. In the study of Salazar et al. (2016), low (24.72 ± 3.73 mg/kg), medium (295.5 ± 35.5 mg/kg), and high (1222.63 ± 255.59 mg/kg) concentrations of Pb in soils were employed, and it was found that plants grown in high Pb-content soil tended to have a higher Pb extraction power than those grown in the soils with medium or low Pb contents.

Due to the strong reducibility and great specific surface area, nanoscale zero-valent iron (nZVI) has been applied in the remediation of environment polluted by heavy metals and organic pollutants. Previous studies indicated that using nZVI to remediate Pb-polluted soils is a practical and prospective strategy (Mar Gil-Díaz et al., 2014). Plants

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play an important part in providing power for life on earth, participating in soil formation, promoting the nature of material circulation and creating a habitat for creatures. Recent reporters have shown that the application of nZVI can bring about serious environmental concerns, due to their possible toxic effect on living organisms, particularly plants. Nanoscale zero-valent iron showed a strong nanotoxicity on *Typha* at higher nZVI concentrations (> 200 mg/L), while the enhanced plant growth was observed at lower concentrations of nZVI treatment (Ma et al., 2013). Wang et al. (2016) demonstrated that nZVI showed no adverse impacts on plant germination, but the inhibited effect of rice seedlings growth was observed in higher concentrations of nZVI (> 500 mg/kg). In contrast, the enhanced root length of *Arabidopsis thaliana* was observed in 500 mg/L nZVI-treatment (Kim et al., 2014). Gil-Díaz et al. (2016) studied a nanoremediation strategy for the As-polluted soil at concentrations of 1% and 10% of nZVI respectively, and demonstrated that the best effect of metal immobilization in soils was observed at the concentration of 10%, and the highest growing rate of plants was also observed in this treatment. Kumpiene et al. (2006) evaluated the effect of 1% nZVI on the mobility and bioavailability of Cr, Cu, As and Zn in soil, and found that nZVI restored the activities of most soil enzyme and reduced microbial toxicity in the soil. These previous studies focused on the nZVI toxicity to plant growth, but few studies investigated the effects of nZVI on metal accumulation in plants. Therefore, it is significant to investigate the effects of nZVI on metal accumulation in plants and metal stress to plants. Yet information on the toxicity of nZVI to *L. perenne* is still scant, it is necessary to choose a large nZVI concentration range to investigate the impacts on metal accumulation and antioxidative system of *L. perenne* by nZVI assisted phytoremediation clearly.

Indeed, heavy metals accumulation in plants may induce oxidative stress caused by overproduction of reactive oxygen species (ROS), including superoxide radicals ($O_2^{\cdot -}$), hydroxyl radicals ($HO^{\cdot}$) and hydrogen peroxide (H_2O_2). In present studies, the ROS cause adverse effects on the function of DNA and protein by lipid peroxidation, which further causes cell severe damages on living organisms (Ruley et al., 2004; Verma and Dubey, 2003). To prevent and eliminate the damages caused by ROS, the plants develop a defense mechanism based on antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT), as well as some low molecular weight antioxidants such as glutathione (GSH) (Gong et al., 2017).

The primary goal of this study was to investigate the remediation of Pb-polluted sediment by nZVI-assisted phytoremediation. Specifically, we studied: (i) the biomass and length of plants with nZVI treatment; (ii) the accumulation and translocation of Pb in plants through the nZVI assisted phytoremediation; (iii) the effects of nZVI and Pb on physiological structure and oxidative stress of plants after the remediation, including antioxidative system, soluble protein content, and chlorophyll content; (iv) the speciation transformation of heavy metals in sediment after the remediation; (v) the enzymes activities in sediment through the combination of plants and nZVI. This study could benefit the application of nanotechnology and phytoremediation for heavy metals, as well as the understanding of potential risks of nanoparticles to the organisms under environmental stress.

2. Materials and methods

2.1. Sediment and nZVI

Heavy metal contaminated sediment samples were collected from Xiangjiang River in the Changsha section in Hunan province, China, (28°11′N, 112°58′E). The sediment samples were sieved to a particle size of < 2 mm to exclude the coarse materials and large debris, and were air-dried (22 °C) for 1 week. To produce the Pb-contaminated sediment samples, we dissolved a pre-weighed amount of $Pb(NO_3)_2$ in deionized water (DI water) and used to spike the tested sediment with 45 days of cultivation in a dark room. A mixture of 2 g dried sediment (dried at 105 °C for 24 h) and 5 mL distilled water was shaken for 5 min and set for 1 h, then was used to measure sediment pH with a pH electrode. The organic carbon content was determined according to the Walkley-Black method, in which organic carbon is oxidized by potassium dichromate (Walkley and Black, 1934). The organic matter content was estimated based on loss on ignition (LOI) and determined as sample's percent weight loss after combustion at 550 °C for 6 h. Briefly, CEC (Cation Exchange Capacity) (cmol/kg) was determined by extracting base cations (Ca^{2+}, Mg^{2+}, K^+, and Na^+) and aluminum (Al^{3+}) with ammonium chloride, and the calculated CEC expressed in centimoles of charge per kg of sediment (cmol/kg). The percentage of sand, silt and clay contents of sediment were based on the particle size of the sediment, sand (50–2000 μm), silt (2–50 μm) and clay (0–2 μm). The sand, silt, and clay contents were determined using the proposed methods and standard hydrometer and pipette techniques (Feng et al., 2016). The sediment samples were digested in digestion tube with HNO_3–HF–$HClO_4$ to extract the total Pb content, and the products were diluted with DI water for analysis. Selected properties of the cultivated sediment are given in Table 1.

Table 1
Selected properties of the investigated sediment.

Feature	Sediment
pH	7.79 ± 0.12
Organic carbon (g/kg)	7.88 ± 0.15
Organic matter (g/kg)	13.59 ± 0.24
Cation Exchange Capacity (cmol/kg)	21.60 ± 1.83
Urease activity (NH_3-N mg/g)	7.24 ± 0.82
Catalase activity(mL^{-1}g 20 min)	1.57 ± 0.05
Sand (%)	65.17 ± 3.21
Silt (%)	32.01 ± 2.19
Clay (%)	3.76 ± 0.52
Total Pb content (mg/kg)	733.68 ± 12.72

In order to improve the stability of nZVI in the sediment, we use starch solution as a minor modification. Nanoscale zero-valent iron was synthesized through reductive precipitation of $FeSO_4{\cdot}7H_2O$ with $NaBH_4$ as described in the literature. In a typical preparation, an aliquot of the Fe^{2+} stock solution was mixed with a fraction of a starch solution as a stabilizer to yield a mixture of 50 mL containing 0.1 M Fe and a 0.2% (w/w) starch. Briefly, 50 mL of $NaBH_4$ in DI water (0.2 M) was added dropwise into 50 mL of $FeSO_4{\cdot}7H_2O$ solution in starch solution (0.1 M) while shaking constantly in a mechanical agitator at 500 rpm. DI water and starch solution were sparged with N_2 respectively before reaction, and the reaction process was conducted under nitrogen protection. Finally, the synthetic nZVI were collected using magnetic separating and dried in vacuum drying oven (DZF-6020, Shanghai) for 24 h (Alidokht et al., 2011; Sun et al., 2006). The TEM image of laboratory synthesized nZVI and the image of size distribution of nZVI by DLS (dynamic light scattering) were showed in Fig. S1. The selected nZVI concentration range was based on the results from our pre-experiments, which found that nZVI concentration of lower than 100 mg/kg had little influence on plant growth and metal accumulation, while that higher than 2000 mg/kg exhibited the completely inhibitory effects to plant survival. Considering the published literatures shown in 'Introduction', the nZVI concentration range was selected as 100–2000 mg/kg to evaluate the impacts on metal accumulation and antioxidative system of *L. perenne* by nZVI assisted phytoremediation in sediment.

2.2. Pot experiment

Air-dried sediment samples (1000 g) were placed in plastic pots and nZVI was added at 0, 100, 200, 500, 1000 and 2000 mg/kg. The sediment and nZVI were mixed thoroughly and wetted with DI water to 70% of the field water holding capacity of the sediment. Seeds were germinated on a constant temperature and humidity incubator with

25 °C and 70% humidity, and 1 g pregerminated seeds were cultivated to sediment which contained different concentrations of nZVI. Three replicates were done for this pot experiment and plants were kept in simulated natural conditions, which have temperature 26–27 °C and light intensity of 300 μmol m^{-2} s^{-1} in the daytime (6:00–18:00), 23–24 °C at night. *Lolium perenne* was harvested after 45 days grown, and the biomass and length of roots and shoots were measured at 45 days.

2.3. Determination of metals in plant organs

On the day of the plant harvest, plant roots and shoots were washed with DI water three times, dried at room temperature (22–24 °C) at low air humidity, and then was put in culture dish and oven-dried at 60 °C for 12 h. Plant samples (approximately 0.5 g) were cut into piece and digested in a digestion tube at a temperature of 100–230 °C with 1.0 mL of $HClO_4$ and 4.0 mL of HNO_3. After filtered through a 0.45 mm filter membrane, a clear solution was stored at 4 °C until analysis. The total Pb and Fe concentrations were analyzed by flame atomic absorption spectroscopy (AAS700, PerkinElmer, USA) (Wannaz et al., 2011). The BF and TF were defined as the ratio of Pb amount in plant shoots to that in sediment and Pb amount in shoots to that in roots, respectively (Wang et al., 2014).

2.4. Determination of plant antioxidative ability and plant physiology

2.4.1. MDA and H_2O_2

The determination of malondialdehyde (MDA) and H_2O_2 contents in leaves were made following the previous method described by Velikova et al. (2000). A fresh plant sample of 1.0 g was homogenized with 4 mL of 0.1% (w/v) trichloroacetic acid (TCA) in pre-chilled mortars. The milled mixture was centrifuged at 2572*g* at 4 °C for 15 min and supernatant was used for both MDA and H_2O_2 analysis. The thiobarbituric acid (TBA) reacted with MDA in samples generating red brown solution under the high temperature and acidity, which was used for the estimation of lipid peroxidation in the leaves. 1 mL of 20% (w/v) TCA composed of 0.5% (w/v) TBA was added to 1 mL of the supernatant. The mixture was reacted in thermostat water bath at 95 °C for 30 min and transferred to an ice bath for analysis. The red brown supernatant was measured by UV spectrophotometry at 450, 532, and 600 nm. As for the determination of H_2O_2 contents, 1 mL of 10 mM potassium phosphate buffer (pH 7.0) and 2 mL of 1 M KI was added to 1 mL of the supernatant, and the H_2O_2 contents were determined by UV spectrophotometry at 390 nm.

2.4.2. Antioxidant ability

Lolium perenne leaves (0.2 g) were homogenized with 1.5 mL 50 mM Tris–HCl (pH 7.8) buffer which contained 1.5% (w/w) polyvinylpyrrolidone and 1 mM EDTA in ice bath. The milled mixture was centrifuged at 2572*g* at 4 °C for 20 min and supernatant was used for enzyme assays. The activities of antioxidant enzyme (CAT, SOD and POD) were measured with an assay kit purchased from Nanjing JianCheng Bioengineering Institute, Nanjing, China. The activities of CAT, SOD and POD in plants were measured according to the method by Sun et al. (2014).

2.4.3. Soluble protein

The soluble protein content in leaves was measured following the method described by Bradford (1976). Fresh leaves (0.2 g) were homogenized with 8 mL extracting solution containing 50 mM Tris–HCl (pH 7.8), 1 mM EDTA, 1 mM dithiothreitol and 0.5 mM $MgCl_2$. The homogenate was centrifuged at 6583*g* at 4 °C for 25 min after grind, and 0.9 mL tris buffer solution and 5 mL coomassie brilliant blue G-250 staining solution were added into the 0.1 mL supernatant. Six bovine serum albumin (BSA) standards were prepared (0, 10, 30, 50, 70, and 90 μg/mL) as calibration. The ultramarine-colored complex were measured within 1 h using spectrophotometer at λ = 595 nm.

2.4.4. Chlorophyll content

The chlorophyll content of fresh leaves material was analyzed in a dark condition. 0.2 g leaves tissue avoiding painfully yellow or wormhole blade were grounded enough, and added a 5 mL solution of 90% ethyl alcohol. Milled mixture was then filtered into a 25 mL measuring flask and the process repeated twice until analysis. Chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and total chlorophyll content were measured by UV spectrophotometry at 663 nm and 645 nm, respectively (Brennan et al., 2014; Wellburn, 1994). And pay attention to avoiding light operation in the whole process, and the result calculated by as follows:

$$\text{Chl}\quad a = 12.72 \times \text{A663} - 2.59 \times \text{A645} \tag{1}$$

$$\text{Chl}\quad b = 22.88 \times \text{A645} - 4.67 \times \text{A663} \tag{2}$$

$$\text{Chl}\quad (\text{total}) = 20.29 \times \text{A645} + 8.05 \times \text{A663} \tag{3}$$

2.5. Fractions of Pb (II) in sediment

The BCR sequential extraction was conducted by Quevauviller et al. (1993) with some minor modifications. Briefly, sediment samples (0.5 g) were added into 50 mL centrifuge tube to measure the fraction of Pb. Firstly, 40 mL 0.11 M acetic acid was added into centrifuge tube, and which was shaken at 25 °C at 250 rpm for 16 h to extract acid soluble fraction. The complex was centrifuged at 1646*g* for 15 min, and the supernatant used to the determination of acid soluble fraction, and residual sediment samples used for the next step of experiment. Next, 40 mL 0.5 M hydroxylamine hydrochloride (pH 2.0) was added into 50 mL centrifuge tube with residual sediment samples, and the steps were the same for extraction of the acid soluble fraction; after which centrifuge tube was amended with 10 mL H_2O_2 at 85 °C in a bath for 1 h until the mixture less than 3 mL, and another 10 mL of 30% (m/v) H_2O_2 was then added until the mixture less than 1 mL. Subsequently, 50 mL 1 M NH_4OAc (pH 2.0) was added to extract the oxidizable fraction. Finally, the residual sediment samples were digested in digestion tube with HNO_3–HF–$HClO_4$ to extract the residual fraction, and the products were diluted with DI water for analysis.

2.6. Sediment enzyme activities

The activities of the urease and catalase in sediment were determined following the methods described by Kandeler and Gerber (1988) and Yan (1988), respectively. The determination of urease method involves the incubation of the sediment with urea and buffer for 24 h at 37 °C. Then, the incubated mixture was filtered into a 25 mL centrifuge tubes, 4 mL sodium phenate and 3 mL sodium hypochlorite were added into 1 mL filtrate. For calibration, nine standards were prepared (0, 1, 3, 5, 7, 9, 11, and 13 mg NH_4–N mL^{-1}). After a 20 min color reaction period, the blue-colored complexes were determined using spectrophotometer at λ = 578 nm within 1 h. The determination of catalase is potassium permanganate titration with a known concentration of potassium permanganate solution to the quantitative determination of hydrogen peroxide in system, through controlled trials can know the amount of hydrogen peroxide enzyme consumption, so as to determine the amount of enzymes. 5 g of sediment samples, 40 mL DI water and 5 mL 0.3% H_2O_2 were added to 50 mL conical flask, respectively. The conical flask was transferred into thermostatic oscillator at 37 °C for 20 min, and 5 mL 1.5 M sulfuric acid was added to stop reaction after incubation. The complex was filtered and titrated using standard 0.1 M $KMnO_4$ by alkali burette. Consumed amount of 0.1 M $KMnO_4$ per gram of dried sediment in 20 min was used to express the activity of CAT in sediment. The urease and catalase sediment measurements were used as early indicators of metal pollution.

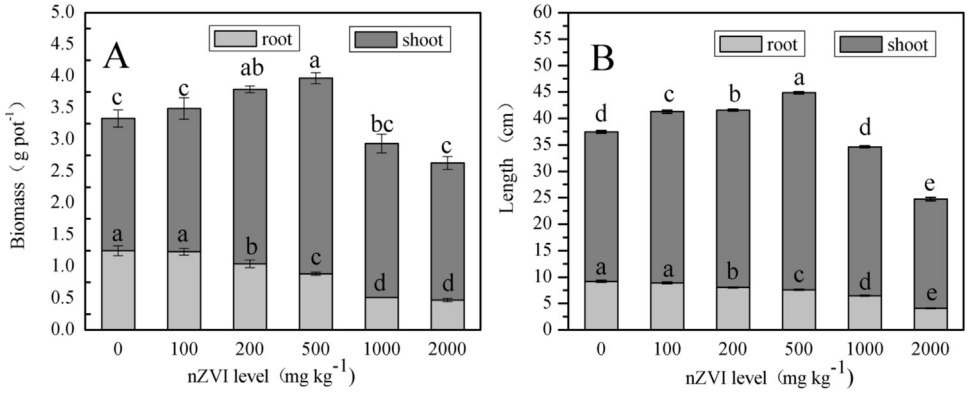

Fig. 1. The biomass (A) and length (B) of *L. perenne* grown in different nZVI-containing sediment samples at 45 days. Error bars represent standard deviation of triplicate samples. The different letters represent significant differences in roots and shoots, respectively, between the treatments using one-way ANOVA followed by Duncan test ($p < 0.05$).

2.7. Statistical analysis

All data were analyzed using Microsoft Excel 2003, Origin 8.0 and SPSS 19.0 (Germany). All results in this paper were presented as the mean with standard errors (n = 3). Data were analyzed using one-way Analysis of Variance (ANOVA), followed by the Duncan's analysis using SPSS 19.0 to test the differences in roots and shoots, respectively, between the treatments ($p < 0.05$).

3. Results and discussion

3.1. Plant growth and metal accumulation and translocation

To assess the effects of nZVI treatment on plant growth and metal accumulation, plant biomass and length of roots and shoots were measured after harvest. Fig. 1A shows that nZVI exposure significantly decreased roots biomass but increased shoots biomass of plants in all treatments, and the roots biomass decreased with the rising of nZVI concentrations. Compared with the control, the most significant increase (by 55.32%) of shoots biomass was achieved in 500 mg/kg nZVI treatment (Fig. 1A). The treatments with 100–500 mg/kg got a pronounced elongated growth compared with the 1000 and 2000 mg/kg nZVI treatments. The root length decreased with the rising of nZVI concentration, which coincided with the tendency of roots biomass (Fig. 1B). The growth of *L. perenne* in the treatment with 2000 mg/kg of nZVI (4.1 ± 0.06 cm for the roots and 20.67 ± 0.32 cm for the shoots) was distinctly inhibited compared to the growth observed in the control treatment. In addition, completely inhibitory effect appeared in the 5000 mg/kg treatment, *L. perenne* cannot grow normally from a supplementary test as shown in Fig. 2A. Consequently, significant inhibitory effect was observed in roots length and biomass of plant, which were reduced due to the sensibility of *L. Perenne* to high concentrations of nZVI. In summary, low concentrations of nZVI-sediment were favorable for the plant growth, while high concentrations of nZVI (1000 and 2000 mg/kg) sediment caused inhibition effect on *L. perenne* growth.

Based on the plants' absorption ability to heavy metals, phytoextraction means the accumulation in plants and the translocation of heavy metals to aerial part. The remediation of nZVI assisted phytoremediation of Pb-contaminated sediment was aimed at promoting Pb uptake and translocation in plants. Table 2 shows the Pb accumulation in plant roots and shoots in different treatments. The Pb concentrations in roots of *L. perenne* were 617.73 mg/kg, 583.49 mg/kg, 568.82 mg/kg, 610.06 mg/kg, and 650.17 mg/kg in the 100, 200, 500, 1000 and 2000 mg/kg nZVI treatments respectively, compared with 496.17 mg/kg of the control. The concentrations of Pb in the roots and shoots grown in the 2000 mg/kg treatment were 650.17 mg/kg and 244.59 mg/kg, with an increase of 31.04% and 58.76% respectively compared with the control. The contents of Pb in the roots were higher than those in the shoots, indicating that roots were the preferential heavy metal storage organ. The total Pb accumulation capacity was not only related to the Pb concentration in plants, but also related to the plant biomass, which was calculated in Fig. 2B. The highest total Pb accumulation capacity in *L. perenne* was in the 100 mg/kg nZVI treatment, which reached 1175.40 μg per pot as shown in Fig. 2B. The Pb accumulation capacities in *L. perenne* were 1175.40, 1141.58, 1133.53, 874.83, and 835.21 μg per pot in 100, 200, 500, 1000, and 2000 mg/kg nZVI treatments respectively compared with 937.30 μg per pot in the control treatment. These results indicated that addition of nZVI could effectively enhance the accumulation capacity of Pb in the plant-

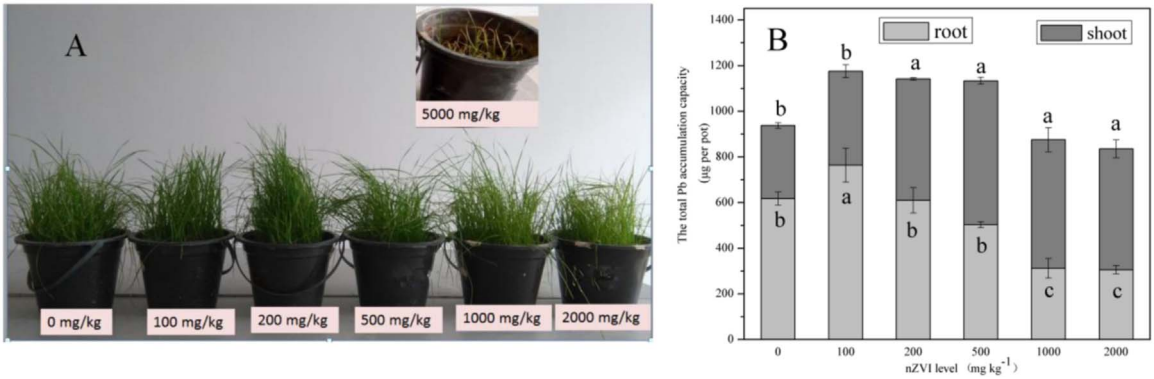

Fig. 2. Photos of *L. perenne* incubated in different nZVI-containing sediment samples at 45 days. The *L. perenne* incubated in 5000 mg/kg nZVI treatment (A). The Pb contents in roots and shoots of *L. perenne* grown in different sediment samples (B). Error bars represent standard deviation of triplicate samples. The different letters represent significant differences in roots and shoots, respectively, between the treatments using one-way ANOVA followed by Duncan test ($p < 0.05$).

Table 2
Pb and Fe distribution in roots and shoots of *L. perenne*, and bioconcentration factors (BF) and translocation factor (TF) of Pb grown in different sediment samples at 45 days. Error bars represent standard deviation of triplicate samples.

treatment (mg/kg)	Pb concentration (mg/kg)		Factors		Fe concentration (mg/kg)	
	roots	shoots	BF	TF	roots	shoots
0	496.17 ± 8.33^{b}	154.06 ± 5.30^{d}	0.207 ± 0.021^{d}	0.312 ± 0.032^{d}	682.42 ± 48.13^{c}	250.45 ± 14.44^{b}
100	617.73 ± 34.03^{ab}	182.37 ± 1.15^{c}	0.245 ± 0.019^{d}	0.295 ± 0.037^{cd}	966.38 ± 60.24^{b}	383.69 ± 20.24^{a}
200	583.49 ± 21.93^{ab}	193.42 ± 2.66^{bc}	0.269 ± 0.023^{c}	0.334 ± 0.037^{cd}	1005.67 ± 33.78^{b}	387.06 ± 8.92^{a}
500	568.82 ± 4.07^{ab}	204.44 ± 0.96^{b}	0.271 ± 0.027^{b}	0.361 ± 0.032^{b}	1121.34 ± 81.08^{b}	416.50 ± 25.64^{a}
1000	610.06 ± 79.54^{ab}	230.80 ± 7.83^{a}	0.299 ± 0.031^{b}	0.374 ± 0.029^{a}	1430.91 ± 50.31^{a}	387.35 ± 27.50^{a}
2000	650.17 ± 2.37^{a}	244.59 ± 7.12^{a}	0.347 ± 0.029^{a}	0.377 ± 0.030^{a}	1591.28 ± 46.08^{a}	398.69 ± 38.33^{a}

The different letters (a, b, c and d) represent significant differences in roots and shoots, respectively, between the treatments by Duncan's test ($p < 0.05$).

sediment, which was consistent with a previous result (Hu et al., 2014). Conversely, pot experiments showed that biochar-supported nZVI materials effectively reduced the upward translocation ability of Cr in the plant-soil system, and promoted the growth of plants (Su et al., 2016). It was suggested that the extent of promotion or inhibition is dependent on the types of plant and nanoparticles (Sahi et al., 2002).

To better estimate the effects of nZVI assisted phytoremediation of Pb on the metal accumulation and translocation in *L. perenne*, the bioconcentration factors (BF) and translocation factor (TF) were calculated, respectively, and the results are shown in Table 2. For each element, statistically significant increases of BF and TF values were observed in *L. perenne* grown in 100–2000 mg/kg treatment sediment, than those grown in the control sediment. Therefore, the nZVI-treated sediment increased the accumulation and translocation of Pb for *L. perenne* than the non-nZVI treatment. Previous studies showed that Pb extractable concentrations in sediment were strongly controlled by complexation with sediment organic matter, absorption on clays and oxides and precipitation processes (Lebourg et al., 1998; Huang et al., 2017b). We speculated that the nZVI increased sediment organic matter by means of plants root exudate, further enhanced the metal accumulation and translocation in *L. perenne* (Pueyo et al., 2004). According to the result of plants growth and accumulation capacity of Pb in plants, it was suggested that the using low concentration of nZVI to assist phytoremediation of Pb in sediment is practicable.

3.2. Fe analysis

To test the effect of nZVI assisted phytoremediation of Pb on Fe absorption, the Fe concentrations in all harvested plants in this study was measured, and the results are shown in Table 2. The total Fe concentrations in roots (682.42–1591.28 mg/kg) were higher than those in the shoots (250.45–416.50 mg/kg), which could be explained by the roots ' direct exposure to the nZVI and sediment. Fe contents in roots increased with the rising of nZVI concentration, and increased by 1.33 times in 2000 mg/kg nZVI treatment compared with the control. In 100–500 mg/kg nZVI treatment, the Fe contents in shoots increased with the rising of nZVI concentration, which indicated low concentrations of nZVI significantly enhanced Fe absorption and translocation by *L. perenne*. Interestingly, in high concentrations of nZVI (1000–2000 mg/kg), the total Fe contents in the shoots decreased slightly compared to that the low concentrations of nZVI and even the total Fe contents in the roots increased significantly at higher concentrations. The result demonstrated that high concentrations of nZVI were likely to result in the suppression of Fe absorption, which might be because the pathway of iron uptake and translocation from the root to the shoot was blocked by nZVI (Wang et al., 2016). Further, this reason consequently caused the decreased Pb contents in plant, which was in agreement with the result of Pb accumulation in plants. Overall, the low concentrations of nZVI significantly accelerated Fe translocation by shoot, while the high concentrations of nZVI caused the suppression of Fe absorption.

3.3. Plant leaf physiological stress

3.3.1. Antioxidant defense system in plant leaves

Accumulation of heavy metals in plants induces the generation and accumulation of ROS (including H_2O_2), which can cause oxidative damage to plants. MDA is a major end-product of membrane lipid peroxidation (Huang et al., 2017a), and the increase of MDA content indicates the aggravation of oxidative damage in plant tissues. The results about H_2O_2 and MDA are shown in Fig. 3A and B, respectively. The lower and similar content of H_2O_2 and MDA were observed at 100–500 mg/kg nZVI concentrations compared with the control, indicating a positive effect of low concentrations of nZVI. In contrast, 1000 and 2000 mg/kg nZVI significantly increased the content of H_2O_2 (46.15% and 108%, respectively) and MDA (34.93% and 48.66%, respectively). The experiment results indicated that excessive oxidative stress and lipid peroxidation occurred at the concentrations of 1000 and 2000 mg/kg nZVI due to the accumulation of metals and ROS (Demiral and Türkan, 2005). The high level of H_2O_2 was resulted from the action of SOD on superoxide radicals or by direct formation in biochemical pathways viz., photorespiration. Lipid peroxides are formed by direct action of redox-active metals or indirectly by lipoxygenase-mediated lipid peroxidation by non-redox active metals (Huang et al., 2017a; Mishra et al., 2006; Zhang et al., 2001).

The antioxidant enzymes including SOD, POD and CAT play a critical role in coping up with oxidative stress caused by ROS. SOD is the first line of defense against oxidation, catalyzing the disputation of O_2^- to H_2O_2 and O_2. In the present study, the treatment with 100 mg/kg of nZVI enhanced SOD activity (10.37 U/mg protein) compared with the control (9.41 U/mg protein), which was probably due to the increase of superoxide production as the reaction substrate. At the low concentrations of nZVI, the increase of CAT activity could be explained by the low lipid peroxidation, which was induced by the dismutation of O_2^- to H_2O_2 by SOD and the catabolism of H_2O_2 to H_2O and O_2 by CAT. Thus, the result indicated that these two enzymes synergistically quench ROS as an adaptive mechanism of the plants. Insignificant alteration of POD activity was observed at low concentrations of nZVI, but *L. perenne* exposed to 1000 mg/kg concentration of nZVI (18.85 U/mg protein) and 2000 mg/kg concentration of nZVI (15.27 U/mg protein) decreased POD activity than the control treatment (28.13 U/mg protein), which could be attributed to the severe stress of oxidative damage to antioxidant enzymes (Lin et al., 2009; Lopareva-Pohu et al., 2011; Schützendübel and Polle, 2002). Taken together, it was concluded that the low concentrations of nZVI slightly relieved the degree of oxidative stress in plants, which was in agreement with the conclusions of previous study conducted by Iannone et al. (2016). In contrast, the high accumulation of nZVI and Pb induced the severe oxidative stress and oxidative damage to antioxidant enzymes.

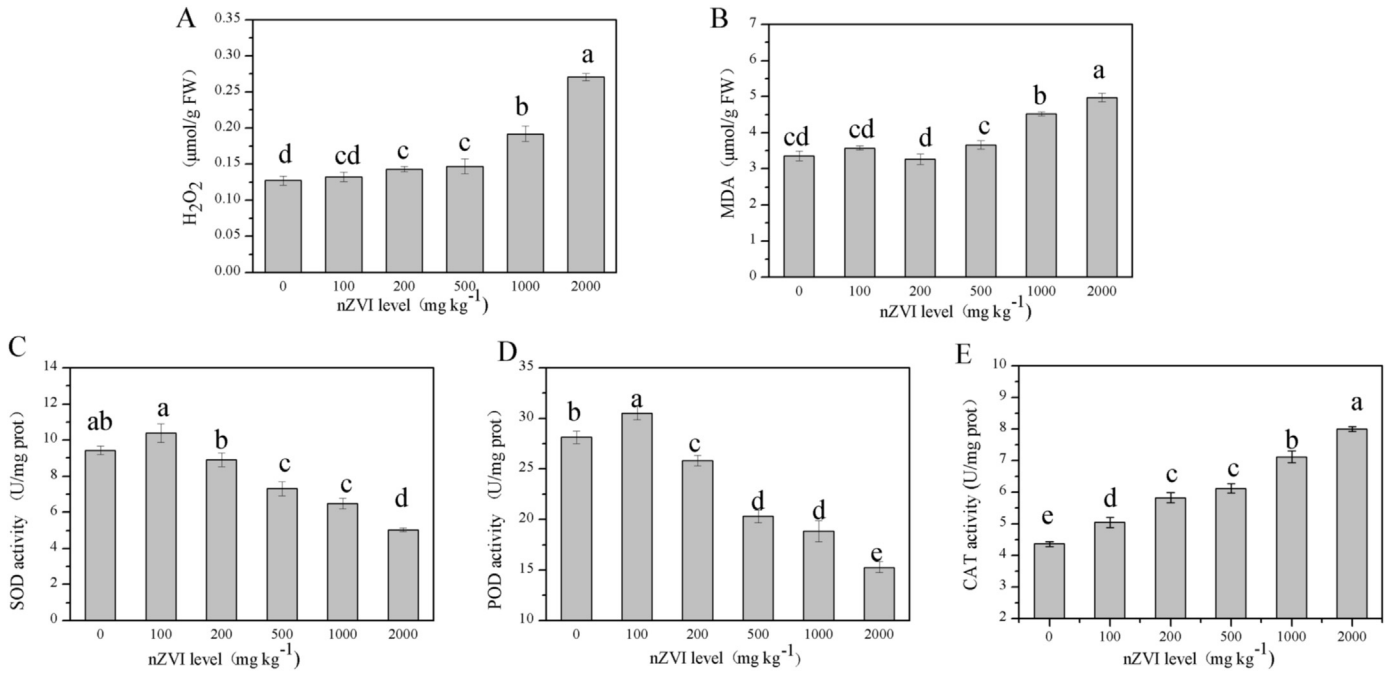

Fig. 3. H_2O_2 content (A), malondialdehyde (MDA) content (B), SOD activity (C), POD activity (D) and CAT activity (E) of *L. perenne* grown in different sediment samples at 45 days. Error bars represent standard deviation of triplicate samples. The different letters represent significant differences in roots and shoots, respectively, between the treatments using one-way ANOVA followed by Duncan test ($p < 0.05$).

3.3.2. Soluble proteins and chlorophyll content

The accumulation of nZVI and Pb in plants induced physiological changes. Fig. 4A shows that total soluble proteins increased in plants after 100–500 mg/kg nZVI exposure and reached at maximum values with 100 mg/kg nZVI. But the values decreased in 1000 mg/kg and 2000 mg/kg nZVI treatments compared with the control. When plants were exposed to low level of Pb and nZVI (100–500 mg/kg), soluble protein content maintained similar or slight increased, this may be explained by the reason that some soluble proteins functioned with anti-oxidation and detoxification were synthesized to cope with Pb stress (Yang et al., 2007). Conversely, the soluble protein content decreased by 58.91% in the treatment with 2000 mg/kg of nZVI compared with the control. The decrease of soluble protein content might be resulted from the damages in photosynthetic apparatus by nZVI-Pb, and the damages further triggered the decrease of protein content in plants (Grill et al., 1989).

Iron is an essential micronutrient for plants involving cellular mechanisms and functions, such as nitrogen fixation, DNA synthesis and photosynthesis (Hell and Stephan, 2003). Chl *a* and Chl *b* were used as the biomarker of photosynthesis ability. As shown in Fig. 4B, a slight decline was observed in Chl a compared with the control, and the total chlorophyll content significantly decreased with the increase of nZVI. Chl *a*/Chl *b* ratio was proposed as a useful indicator of environmental stress. In our studies, no pronounced difference of chlorophyll *a/b* (3.35–3.49) was observed in Fig. 4B, except for the increase (3.53) in the 2000 mg/kg nZVI treatment. The 2000 mg/kg nZVI treatment triggered the highest suppression rates for chlorophyll content at 30.85% compared with the control, which was attributed to the damaged chloroplast internal organization including the lower stomatal conductivity and transpiration rate (Islam et al., 2007). It was concluded that high concentrations of nZVI and Pb induced nontoxicity based on the negative effect of photosynthesis.

3.4. Percentage of four portions of Pb determined by BCR sequential extraction

The BCR sequential extraction was used to explore the speciation transformation of Pb (acid soluble fraction-F_1, reducible fraction-F_2, oxidizable fraction-F_3 and residual fraction-F_4) in sediment samples with different nZVI treatments. As shown in Fig. 5, the Pb species in the

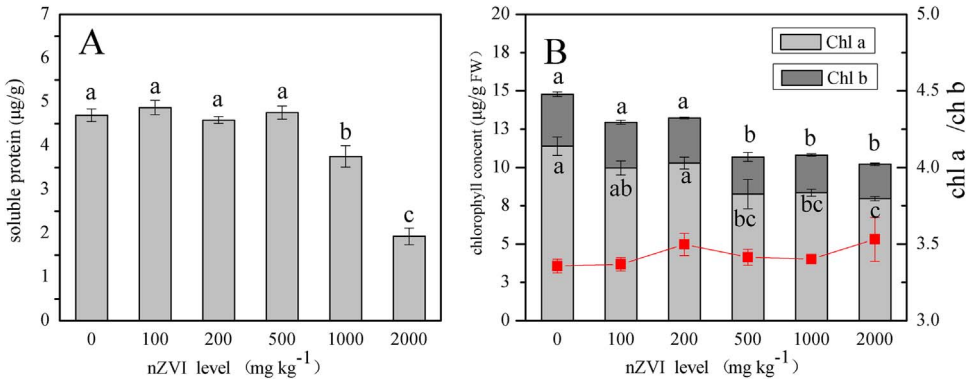

Fig. 4. Changes of the soluble proteins (A) and chlorophyll (B) content of *L. perenne* grown in different sediment samples at 45 days. Error bars represent standard deviation of triplicate samples. The different letters represent significant differences in roots and shoots, respectively, between the treatments using one-way ANOVA followed by Duncan test ($p < 0.05$).

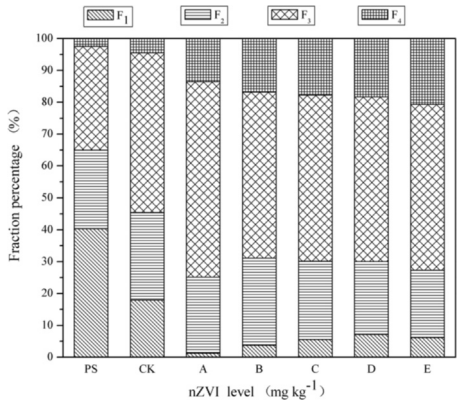

Fig. 5. Percentage of Pb speciation (F_1, F_2, F_3 and F_4) in primary sediment and changes of Pb speciation in sediment treated with different nZVI at 45 days. The PS, CK, A, B, C, D, and E represent the primary sediment, 0, 100, 200, 500, 1000 and 2000 mg/kg nZVI level respectively.

primary sediment were split into F_1 (40.32%), F_2 (24.59%), F_3 (32.57%) and F_4 (2.52%) respectively. After 45 days of remediation, the control sediment was split into F_1 (17.93%), F_2 (27.43%), F_3 (50.13%) and F_4 (4.51%). The acid soluble fraction of Pb includes the exchangeable fraction and bound to carbonates fraction, which was the most toxic fraction in sediment and easy to be absorbed by plants according to the previous study (Liu and Zhao, 2007). Lower acid soluble fraction was observed in all nZVI treatments, and the lowest acid soluble fraction (1.23%) was observed in the treatment with 100 mg/kg nZVI, which sharply decreased by 39.09% compared with that in the primary sediment. Phytostabilization means that heavy metals are immobilized in plant roots surface, and nZVI can also promote the immobilization of Pb in sediment. Therefore, the lowest acid soluble fraction could be attributed to the highest Pb accumulation capacity in 100 mg/kg nZVI treatment. The other reason might be the better precipitation within the plants rhizosphere, and nZVI promoted the conversion of acid soluble fraction into oxidizable and residual fraction (Gil-Díaz et al., 2014; Ruttens et al., 2006). According to the results, the percentage of residual fraction of Pb in different concentrations of nZVI treatment increased to 13.62%, 16.83%, 17.82%, 18.37% and 20.7% respectively. The decreased acid soluble fraction of Pb and increased residual fraction of Pb might be resulted from the combination effect of nZVI and phytoremediation, which indicated the decrease of heavy metals toxicity in sediment. It was concluded that using nZVI to assist the phytoremediation of Pb in sediment could be a promising method to remediate Pb-polluted sediment.

3.5. Urease and catalase enzymes in sediment

Previous studies have shown that enzyme activity in sediment would increase with the decrease of available heavy metals, which indicated that enzyme activity could reflect the degree of heavy metals pollution in sediment (Huang et al., 2016b). Therefore, enzyme activities in sediment can be sensitive indicators of sediment quality during the remediation of nZVI assisted phytoremediation, and analyzed enzyme activities (urease and catalase enzyme) in the sediment are shown in Fig. 6. The urease and catalase enzymes showed significantly greater activities in nZVI-treated sediment than in untreated sediment. In our study, urease activities of 0–200 mg/kg nZVI systems and 500–2000 mg/kg nZVI systems reached the peak on day 28 and day 14, respectively, then maintained at a steady decline. The highest value of urease (34.64 NH_3-N mg/g) was found in 2000 mg/kg nZVI treatment compared with the control (5.70 NH_3-N mg/g) at 45 days. The urease participates the biological activities via the cycle of nitrogen in sediment and its substrate, urea, which is incorporated into sediment as animal excreta and fertilizer (Wang et al., 2009). The results of urease activities indicated the decrease of heavy metals toxicity and the positive impacts on ecology and biology of sediment in remediation, which was in agreement with the result of the BCR sequential extraction in sediment. Catalase activities of all the six systems reached the peak on day 28, then maintained at a steady decline. In the present study, the activity of catalase increased slightly from day 7 to day 28 as shown in Fig. 6B. There were no significant differences of catalase activities among the different concentrations of nZVI treatments from day 7 to day 21, but a slightly lower catalase activity was observed in 2000 mg/kg nZVI treatment after 21 days. This might be because of the complexation of the excessive nZVI in sediment with the catalase substrate, combination of nZVI with the protein-active groups of the enzymes, or the reaction of nZVI with the enzyme-substrate complex (Hu et al., 2014). Catalase activity was supposed to reflect the intensity of metabolic activity for microorganism (Huang et al., 2016a), and the results supported that no adverse effects were observed on catalase activity in sediment with the addition of low concentration nZVI. Overall, it was concluded that nZVI assisted phytoremediation of Pb was beneficial to sediment quality with similar or increasing urease and catalase enzymes activities in sediment, and positive effects in sediment was observed in sediment in the treatment.

4. Conclusions

The aim of this research is to investigate the remediation of Pb-polluted sediment by nZVI assisted phytoremediation. The low concentrations of nZVI could effectively provide high accumulation capacity for Pb with the increase of biomass, and also promoted the plant growth indicated by the lower oxidative stress in plants. Moreover, the sequential extraction experiments demonstrated that the toxicity of

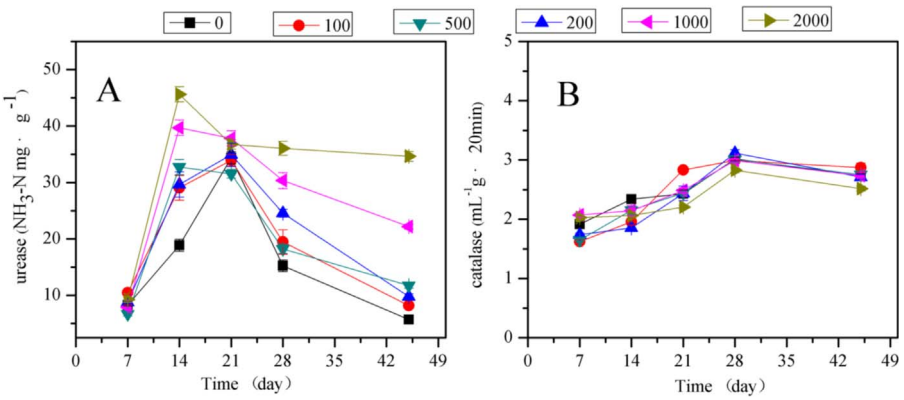

Fig. 6. Activities of urease (A) and catalase enzymes (B) with different nZVI treatments at various treatment times. Error bars represent standard deviation of triplicate samples.

heavy metal decreased in nZVI-treated sediment due to the lower acid soluble fraction and higher residual fraction of Pb. Hence, results supported that using low concentrations of nZVI (100–500 mg/kg) to assist phytoremediation of Pb by *L. perenne* is practicable. On the other hand, the high concentrations of nZVI showed inhibitory effects on plant growth and leaf physiological structure, which was attributed to the high oxidative stress in *L. perenne*. More cautions should be taken into account for the engineering use of nanomaterials. In addition, higher activities of urease and catalase enzymes were observed in nZVI assisted phytoremediation of sediment than those in the control in remediation. In conclusion, the addition of low concentrations of nZVI (100–500 mg/kg) could effectively promote plant growth and enhance the uptake and translocation of Pb in *L. perenne*, also promote the stabilization of Pb in sediment, which indicated that nZVI assisted phytoremediation could be a promising method to remediate Pb-polluted sediment.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2018.01.060.

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