The use of sulfidated nano zero-valent iron for stabilization of soil contaminants – implication on plants

PhD Thesis

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This thesis is submitted in fulfilment of the requirements for the PhD degree at the Czech University of Life Sciences Prague, Faculty of Environmental Sciences.

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Abstract

Nanoparticles have shown promise in metal(loid)s stabilization; however, risks associated with using engineered nanoparticles cannot be neglected. The main aim of the thesis was to evaluate the influence of sulfidated nano zero-valent iron (S-nZVI) with or without the co-application of sewage sludge in stabilizing metal(loid)s in soil and simultaneously investigating its influence on plants grown on such soil. Sewage sludge has been commonly used as a fertilizer and a source of organic matter in soils, and its use could be beneficial during the stabilization process; nevertheless, it may also include several contaminants, including leachable metals. The study's first phase investigated how the co-application of S-nZVI and sewage sludge can reduce the leaching of Cd, Pb, Fe, and Zn. Two leaching procedures (SPLP and TCLP) were used with five treatments (control, 1% Fe grit, 1% composted sludge, 1% S-nZVI, 1% composted sludge, 1% S-nZVI). Samples were incubated for 1 week, 1 month, 3 months, and 6 months. The combination of composted sludge and S-nZVI was the most effective in reducing the leachability of metals in the column systems. S-nZVI alone was the most efficient in reducing approximately 80% of the Zn concentration in soil solution. Iron grit was the most effective in reducing the concentration of extractable metals in the batch experiment. Therefore, the most practical combination of two amendments for decreasing metal leaching and potentially minimizing the related dangers was S-nZVI combined with composted sewage sludge and Fe grit. The second phase of the experiment investigated the role of the co-application of S-nZVI and sewage sludge on plant performance in terms of growth, plant pigment, antioxidant enzymes, and metal uptake, using two plant species (*Arrhenatherum elatius* L., *Festuca rubra* L.). Six different amendments (control, 1% S-nZVI, 2% S-nZVI, 3% composted sludge, 1% S-nZVI + 3% sewage sludge, and 2% S-nZVI+ 3% sewage sludge) were used, and plants were grown for a total of 75 days after which plants were harvested and analyzed. Applying S-nZVI with or without sewage sludge effectively improved plant height and above-ground biomass, and increased plant pigment was also observed. 1% S-nZVI alone promotes the reduction of about 45% Pb in *Festuca rubra* roots. The coapplication of S-nZVI with sewage sludge did not significantly influence the growth and biomass of both plants, while the co-application of 2% S-nZVI with 3% sewage sludge was the best in stabilizing Pb and Cd in the soil, thus reducing its uptake by plants. Variations were observed in pigments in both plants, with most amendments resulting in increased plant pigments. There was little to no influence of antioxidant enzymes in most variants across treatments for both plants; *Festuca rubra* is the most tolerant plant. Thus, the co-application of S-nZVI and sewage sludge is a promising amendment. However, additional studies are still needed to evaluate its full potential and eliminate the risks associated with its usage.

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1. Introduction

1.1. Background & scope

Contamination damages soil's ecological services, making it an international issue (Khalid et al., 2012). Transportation, industrialization, smelting, application of pesticides, mining, and sewage sludge to land are among the artificial activities that account for most pollution sources. Unlike traditional physicochemical technologies, nanotechnology has recently enabled the construction of novel, affordable, and ecologically friendly remediation options (Hidangmayum et al., 2022). Nanoparticles react with contaminants more quickly because of their huge surface area (Roy et al., 2021). Metal(loid)s in soils can be stabilized by using nano zero-valent iron (nZVI), which has been employed as a soil stabilizing agent (Galdames et al., 2020). Because of its low oxidation-reduction potential, nano zero-valent iron has long been an affordable, effective, and ecologically acceptable reductant (Galdames et al., 2020).

According to Li et al. (2007), nZVI has also been studied for its capacity to precipitate and reduce metals such as Ni in biosolids, reducing the metal's mobility and bioavailability. It has been shown that sulfidation modification further boosts the nZVI's reactivity and selectivity toward target contaminants (Fan et al., 2017; Brumovsky et al., 2020). In the bulk material or on the surface of the nZVI particle, this process modifies nZVI by adding lower valent sulfur molecules, forming weakly crystalline iron sulfide (Dong et al., 2018). It has been shown that sulfidated nZVI particles (S-nZVI) may rapidly stabilize a range of metals, metalloids, and organic pollutants (Rajajayavel & Ghoshal, 2015; Xu et al., 2019).

Several studies have demonstrated the potential of S-nZVI in soil remediation (Galdames et al., 2020). However, the potential toxicological risks after nZVI application cannot be neglected. In order to minimize these unwanted effects, it is essential to study the risks and benefits of utilizing S-nZVI in terms of their interaction with soil biota and to use other co-amendments promoting the stabilization and lowering the associated risks.

Sewage sludge is a by-product of wastewater treatment (Li et al., 2007). It blends organic and inorganic components from different wastewater sources and water. Globally, a substantial volume of these sludges is produced in the European Union alone; the yearly production of these sludges amounts to over 11 million tons (Ñurdevic et al., 2022). These sludges need to be utilized in some way. Sludge cannot be utilized again unless it has undergone some treatment. The disposal method determines the kind of treatment needed. The environmental implications of these disposal methods are significant since improper disposal can contaminate soil, water, and atmospheric resources and endanger human health. Therefore, adequate and proper management is essential (Abubakar et al., 2022). The qualities of sludge are altered by treatment techniques, which also affect the end product's quality. Because of this, choosing the optimal sludge treatment is essential; failing to do so could adversely affect the environment, society, and economy (Rorat et al., 2019). Treatment techniques include pyrolysis, thermal treatments, composting, anaerobic and aerobic digestion, and stabilization using chemical or alkaline materials (Wu et al., 2017).

After treatment, sewage sludge can be added to the soil, changing its physical, biological, and chemical characteristics (Lonova et al., 2022). Its use may enhance the nutritional quality of the soil. However, because of its propensity for fermentation and dangerous chemicals, sewage sludge deposited on the soil's surface can also cause harm to soil biota (Boudjabi & Chenchouni, 2021). It has many pathogens, metals, metalloids, and other contaminants (Fijalkowski et al., 2017). Since metals and metalloids do not biodegrade, they can collect and biomagnify in soil and sediment after their introduction (Melake et al., 2023).

Overall, since sewage sludge has the potential to improve plant growth due to the presence of nutrients but with the concern of increasing metal(loid)s in plants and, nZVI has proven to be a good stabilization agent for contaminants in soils. Hence, the principal goal of this research is to investigate how the application of S-nZVI and sewage sludge can lead to the stabilization of soil contaminants while simultaneously improving plant growth and overall health.

The thesis is divided into the following two primary research aims with the given objectives:

Aim 1: To evaluate the efficiency of S-nZVI in stabilizing metal(loid)s in contaminated soils using column and batch experiments. This was achieved under the following objectives:

i) Determine the beneficial effect of the co-application of sewage sludge with S-nZVI to stabilize metal(loid)s in contaminated soils.

ii) Evaluate the leaching of Zn, Pb, Cd, and Fe from amended soils.

iii) Discuss the findings in the context of environmental risks versus benefits of utilizing S-nZVI.

Aim 2: To investigate the effect of the application of S-nZVI with or without sewage sludge as a stabilization agent for soil contaminants and its effect on plant performance. This will be achieved under the following objectives:

i) To investigate the influence of co-applying S-nZVI with sewage sludge on plant performance.

ii) To understand how S-nZVI with or without sewage sludge modulates plant stress.

iii) To evaluate the toxic potential of S-nZVI with or without sewage sludge on plants.

2. Literature review

2.1. Soil pollutants

Soil is an essential part of the terrestrial ecosystem since it is a living matrix and a significant repository of chemical components. The primary causes of soil contamination are various human activities like mining/smelting and agricultural and industrial activities (Palansooriyaa et al., 2020). Metal contamination of soil poses a risk to the ecosystem. There are 5 million soil contamination locations worldwide where metal(loid)s have current concentrations exceeding permissible limits (Li et al., 2019). Metals are primarily categorized and may become potentially accessible through their ionic state in soil solutions, binding to solids in soil solutions, or adsorption on the surfaces of microbes, organic matter, and the complexes they are associated with (Yang et al., 2014; Chen et al., 2021). Moreover, speciation and bioavailability require precipitation and chelation with different minerals and organic matter components. Different mineral organic complexes, or chelates, are produced due to several physical, chemical, and biological reactions between these biotic and abiotic components (Chen et al., 2014; Du et al., 2018a). Mineral-organic interactions alter the surface charges, specific surface areas, and functional groups of soils, which affect the fixation of metals at multiphase soil interfaces (Chen et al., 2020; Engel et al., 2021). To predict metal cation interfacial reactions in natural systems and comprehend the cycle processes of these reactions, precise characterization of component interactions and reactivity with pertinent elements is necessary.

An estimated 11 million tons of sewage sludge are produced yearly in the European Union as a by-product of wastewater treatment. Land application, industrial reuse, landfill disposal, incineration, and ocean discharge are ways sewage sludge can be disposed of (Twardowska, 2004). Typically, sludge is treated before being applied to land; this helps eliminate pathogens, minimize odors, and lessen the material's appeal to disease-carrying vectors, including rats, flies, and mosquitoes (EPA, 2000). Most organisms become poisoned when exposed to metal(loid)s concentrations in sludge beyond the limit threshold (Rastetter and Gerhardt, 2017). Additionally, many organic pollutants, such as microplastics, are in sewage sludge (Nizzetto et al., 2016). Other emerging pollutants, such as pharmaceuticals, can also be in sludge (Aydun et al., 2022).

Metal(loid) pollutants are difficult to remove from soil because they do not break down like some organic pollutants. They may impact soil microorganism morphology and metabolism (Ojuederie and Babalola, 2017). Compared to plants, microorganisms are more vulnerable to stress. According to Abdul Dayem et al. (2017), pollutants in sludge also affect plants, producing reactive oxygen species (ROS) that damage proteins, lipids, and nucleic acids.

Several remediation techniques have been employed to return the soil to its original, pollution-free form. According to Yeung (2016), a few soil remediation methods include thermal desorption, bioremediation, and soil washing. Other remedial processes include chemical stabilization, etc. Utilizing water and surfactants, soil washing is a method of eliminating contaminants from the soil. Thermal desorption is the process of using heat to increase the volatility of contaminants so that they may be removed from the solid substance. Bioremediation uses live microorganisms, such as bacteria and fungi, to break down organic pollutants in the soil (Cooper, 2013). Chemical stabilization reduces the mobility, bioavailability, and bioaccessibility of heavy metals and metalloids in soil. It consists of adding specific immobilizing agents. These agents promote precipitation, complexation, or adsorption to immobilize the pollutants (Raffa et al., 2021)

Due to its potential for broader applicability, better reactivity, and cost-effectiveness compared to other iron products and other *in situ* approaches, nanoscale zero-valent iron (nZVI) has recently been used to remediate polluted sites (Mueller et al., 2011). The nZVI treats about 60% of the remediated locations using nanoparticles (Karn, 2011). In order to stabilize metal(loid)s in soils without impairing plant functions and growth, nZVI has been applied as a stabilizing agent for polluted soils (Martinez-Fernandez and Komarek, 2016; Martinez-Fernandez et al., 2016).

2.1.2. Impacts of soil pollution

Diverse microbes live in soil, and these organisms are crucial to the mineralization of soil organic matter and the cycling of nutrients. A valuable tool for monitoring the health and state of the soil environment is the presence of soil microbes. By using microbiological indices of the soil environment, one may assess the ecological state of the soil. According to Smalla and Van Elsas (2010), the application of sewage sludge has a long-term impact on the variety of soil microbial populations. Nevertheless, it is still unclear how long-term pollutants will affect the microbial communities in sludge-amended soils (Giller et al., 2009).

The degree to which soil microbial communities are experiencing environmental stress has been measured using biological markers, including microbial biomass carbon, soil basal respiration, enzymatic activity, and soil DNA analysis

The study of soil microbial communities has also benefited from applying molecular approaches. The investigation of changes in soil microbial communities has been aided by the analysis of multiplex terminal restriction length fragment polymorphism and phospholipid fatty acid indicators (Singh et al., 2006).

Many studies show that plants develop faster under favorable soil and climatic conditions and produce more biomass (Togay et al., 2008; Angın and Yaganoğlu, 2011). Plant toxicity brought on by metal buildup in the soil is the main issue with using sewage sludge (McGrath et al., 2002). Plants are direct targets of potential pollutants, so genotoxic assays using higher plants have been employed to monitor soils supplemented with sewage sludge. Apart from identifying harmful compounds even in minute quantities, several genetic indicators, ranging from point mutations to chromosomal abnormalities, can be assessed from various organs such as leaves, endosperm, pollen grains, and roots (Geras'kin et al., 2011).

Metals can bind to specific amino acids, DNA, or locations to interact with the plant cellular machinery in distinct ways (Mateuca et al., 2006). Emamverdian et al. (2015) state that Cr, Cu, Mn, and Fe can induce oxidative injury that generates oxygen-free radical species. This disturbs cell homeostasis, breaks DNA strands, defragments proteins, damages photosynthetic pigments, and ultimately results in cell death. It attaches to protein sulfhydryl groups and blocks antioxidative enzymes Al, Cd, Ni, Hg, and Zn, causing oxidative stress by depleting glutathione (Emamverdian et al., 2015). According to McBride et al. (2022), sewage sludge added to the soil considerably raised the amounts of Cd, Ni, Cu, and Zn in the plant. Moreover, Li et al. (2005) found that adding sludge to soil on which the alfalfa plant was grown raised the amounts of Zn and Cd in the plant tissue. As a result, adding contaminated sewage sludge raises the possibility of environmental pollution.

2.2. Soil remediation techniques

Techniques for remediating contaminated areas can be divided into two main types: *in-situ* and *ex-situ* techniques. *In-situ* remediation involves treating contaminants at their source. This strategy aims to treat the contaminants in soil and sediment without displacing the soil or sediment itself. *Ex-situ* techniques include excavating or treating soil or sediment outside the contaminated site (Thomé et al., 2018).

2.2.1. Physical processes

Physical processes to immobilize or remove contaminants from soil or sediment include surface coatings, vapor extraction, and electrokinetic methods. Surface capping involves covering contaminated sites with low-permeability materials that prevent the passage of water, reduce the movement of contaminants in the soil, and reduce the risk of human exposure in contaminated areas. Steam extraction involves installing underground vertical or horizontal extraction wells to draw steam into underground cavities. The vapor is treated before being released into the atmosphere. In electrokinetic remediation, a low-density electrical current is introduced into the soil, and the accumulated electric field transports cations from the soil to the cathode and anions to the anode (Vasilachi and Gavrilescu, 2021).

2.2.2. Biological processes

Biological processes involve decontaminating the environment by living organisms such as plants, animals, and microorganisms. Phytoremediation is a type of bioremediation that uses plants to decontaminate soil and sediments (Truu et al., 2015). The scientific community widely accepts this environmentally friendly technology. This technology uses several methods, e.g., phytoextraction, which uses plant uptake to remove pollutants. The pollutants then accumulate in the plant tissue. It is one of the most successful methods for removing metal(oid) from contaminated soil. Plants take pollutants from the soil, water, or sediments through their roots and transfer them to the aboveground biomass, like shoots (Singh and Santal, 2015; Sarwar et al., 2017). Many factors, such as metal bioavailability, soil properties, metal speciation, and the plant's ability to absorb metals and accumulate aboveground components, determine how effective phytoextraction may be as a potential environmental cleaning solution (Yan et al., 2020). It has been determined that between 450 and 500 distinct plants are hyperaccumulators (Chaudhary et al., 2018). For a plant species to be appropriate for phytoextraction, it needs to possess the following qualities: (i) high biomass output, (ii) resistance to hazardous metals, and (iii) active accumulation of metals in sections that are readily harvested (Vangronsveld et al., 2009; Suman et al., 2018). The fundamental principle of phytoextraction for contaminated sites is to grow appropriate plant species locally, gather biomass that contains metals, and treat it to reduce its mass and size. This can be done by compressing, dehydrating, composting, or thermally breaking down the biomass (McGrath et al., 2002; Sheoran et al., 2009; Suman et al., 2018).

Phytodegradation involves the uptake of pollutants by the roots and their subsequent degradation through plant metabolism or the release of enzymes that accelerate decomposition (Ojuederie and Babalola, 2017). Plant volatilization also occurs through the uptake of pollutants by the roots, subsequent metabolism and transport within the plant, and volatilization through the plant surface. It is only suitable for soluble and volatile contaminants (Limmer and Burken, 2016). Phytostabilization helps to limit pollutants through deposition by roots or precipitation inside the rhizosphere. This prevents the movement of contaminants through soil dispersion, leaching, and erosion from wind and water. Adding a layer of vegetation to the surface of polluted soils to lessen their exposure to wind and direct human or animal contact is another definition of phytostabilization. Utilizing plant species that can withstand high pollution levels and using efficient soil additives to immobilize metal(loid)s can improve phytostabilization (Kumpiene et al., 2007; Park et al., 2011a). This approach does not produce polluted secondary waste and requires additional processing. Additionally, it improves soil fertility, which leads to ecosystem restoration. The site must be regularly monitored to maintain the ideal stabilizing conditions because the contaminants are left in place. It could be necessary to reapply soil amendments regularly to preserve their efficacy if they are utilized to improve immobilization (Bolan et al., 2003a; Keller et al., 2005).

Plant stabilization mechanisms fix pollutants in the plant roots. Immobilization can occur through adsorption, precipitation due to pH changes, formation of metal complexes, or changes in the redox state of the pollutant (Radziemska et al., 2017). The root nodule decomposition mechanism promotes root aeration and creates more favorable conditions for bioremediation. In addition, roots release exudates in the form of sugars, amino acids, and other compounds that stimulate microbial growth and degradation of pollutants (Rohrbacher and St-Arnaud, 2016).

2.2.3. Nanotechnology for soil remediation

Zero-valent nanometals (e.g., iron, nickel, palladium) are commonly used for *in-situ* and *ex-situ* remediation of contaminated sites (O'Carroll et al., 2013). Nanoparticles are essential pollutant scavengers mainly due to their high reactivity and large surface area (Michalkova et al., 2014). Nanosized metal oxides have a particular affinity for adsorbing metals and metalloids, and their applications in environmental problems are rapidly increasing (Martinez-Fernandez et al. 2016). The physicochemical properties of oxides enable the adsorption of various chemical species, leading to specific (chemical) and nonspecific (physical) adsorption. Specific adsorption involves surface complexation processes and reactions between metal ions and surface functional groups (Komarek et al., 2013).

Compared to other nanomaterials, nZVI is widely used because it is non-toxic, cheap, abundant, and easy to fabricate (Karn et al., 2011). Due to its relatively low redox potential, the nZVI has long been used as a cost-effective, efficient, and environmentally friendly reducing agent. In addition, it is effective in transforming and immobilizing many contaminants present in biosolids, offering potential advantages over traditional methods for stabilizing biosolids (Wang et al., 2008).

2.2.4. Uptake and distribution of nZVI in plants

Plants make up the majority of the ecosphere. Comprehending the effects of nZVI exposure on plants requires a detailed characterization of nZVI and its absorption and distribution in particular tissues. Plant uptake and bioaccumulation can also affect the fate and transport of nZVI in the environment (Ma and Chuanxin et al. 2015; Tripathi et al. 2017).

The distribution of nZVI in plant cells and their adherence on plant surfaces are frequently seen using transmission electron microscopy (TEM) and scanning electron microscopy (SEM). Aggregates of nZVI were found inside *Sinapis alba* and *Sorghum saccharatum* (Libralato et al., 2016). Black coats and dots were found on the roots of both species under a microscope. In the meanwhile, Wang et al. (2016) observed that the Casparian strip and suberin deposition between radial cell walls prevented nZVI from crossing the endodermis in rice roots.

These findings suggested that nZVI might not enter the transmembrane channel for Fe absorption and transportation but rather travel across the cortex and epidermis by apoplastic pathways. Even though most nZVI stays attached to the surface of roots, some can go up the stem from roots to leaves and eventually disperse throughout the entire plant after being absorbed by the roots.

Ma et al. (2013) found that there was restricted upward transport of nZVI to shoots or stems in poplar and cattail plants. Conversely, Navarro et al. (2008) proposed that nZVI may enter the plant through trichomes or stomata after amassing on the leaf surface and then move to other tissues in vivo.

Iron particles have been shown to accumulate more readily on smooth, waxy leaf surfaces than on nonwaxy, wrinkled surfaces (Da Silva et al., 2006). nZVI can enter epidermal cells, pass through the cortex, and breach plant root cell walls and membranes when present in medium or soil.

2.2.5.nZVI phytotoxicity

The toxicogenic mechanism of nZVI could not be singular but a synthesis of several pathways. The mechanism of toxicity may also involve the strong adsorption capabilities of nZVI, which allows it to adsorb hazardous and toxic substances from the environment actively. nZVI modifications improved nZVI's stability, reactivity, and mobility while decreasing nZVI's aggregation or passivation. On the other hand, changes to nZVI could result in materials that become more efficient in eliminating pollutants and build-up within living things. Studies have shown that, compared to other nanoparticles, the toxic effects of nZVI are minimal (Reijnders, 2006). Several mechanisms can contribute to nanoparticles' toxicity, most of which converge with those that lead them to be highly reactive. It has been shown that nZVI toxicity affects seed germination, seedling elongation, germination index, and biomass in connection to plant growth and development. In addition, the level mentioned above of nZVI allows the antioxidant enzyme activities to be maintained longer with the non-damaged seedling membrane. At higher doses concentration, nZVI has been demonstrated to significantly inhibit growth, while the opposite effect was seen at lower concentrations, where it promoted plant development (Li et al. 2015). Kim et al. (2019) found that while there were no notable variations in cotyledon growth or toxicity in alfalfa, there was an increase in root length compared to the control group.

Overall, low Fe concentrations have been shown to promote the production of high-affinity iron transporters, whereas high iron concentrations have been shown to hinder the expression of phytosiderophores (Shao et al., 2007). Numerous studies' findings demonstrate that nZVI has no detrimental effects on plant growth. nZVI, for instance, can pierce the seed coverings of tomatoes and peanuts, enhancing water absorption and promoting seed germination (Li et al. 2015; Lin et al. 2009). nZVI may increase root elongation; additional mechanistic research revealed this through a non-enzymatic process called nZVI-mediated hydroxyl radical-induced cell wall relaxation. Root elongation resulted from the breakdown of pectin polysaccharide, which alleviated pressure on the longitudinal axis of the cell wall (Müller et al. 2009; Schopfer 2001). On the other hand, plant development and endocytosis benefited from cell wall loosening, which may also lead to thinner roots and biased cellulose microfibrils. Producing extremely reactive oxygen species, which build up in the cell environment and denature macromolecules like lipids, proteins, and nucleic acids, damaging intracellular structures and ultimately causing cell death, is another frequently proposed mechanism contributing to nZVI toxicity. They interact with molecular oxygen (O_2) to create reactive oxygen species (ROS). This is because of their small size, large specific surface area, and numerous electron donor and acceptor activity sites on the particle surface. Unbalanced intracellular oxidation and antioxidant status can arise from this excessive reactive oxygen species, which the intracellular antioxidant defense system cannot eliminate in time. Oxidative stress is the outcome, and as a result, macromolecules, including lipids, proteins, and nucleic acids, denature, harming cell structure and ultimately leading to cell death (Keller et al., 2012).

2.2.6. nZVI toxicity on plant metabolism.

Iron is a necessary nutrient for plant growth; if Fe levels are excessively high, they can lead to nutritional disorders and poor nutrition, particularly in absorbing nutrients like K, P, Mg, and Ca. nZVI can easily agglomerate and cling to the root surface, blocking root membrane pores and hindering plants' ability to absorb water and nutrients (Ma et al., 2015b). Furthermore, by producing ROS, nZVI can indirectly cause membrane damage, which lowers plants' ability to absorb nutrients. On the other hand, nZVI can also serve as a nutrient storage station accessible in low-nutrient situations because of its excellent adsorption capability and large specific surface area (Lütz-Meindl and Lütz, 2006). Through several processes, including blocking the synthesis of chlorophyll and photosynthesis, modifying the rates of transpiration and photosynthetic reactions, and regulating heat, nZVI can impact plant metabolism (Grantz et al., 2003). Chlorosis can result from a Fe deficiency since Fe is a necessary microelement for photophosphorylation and chlorophyll production (Varotto et al., 2002). High Fe concentrations, however, may have a comparable impact. Wang et al. (2016) showed that exposure to high concentrations greater than 750 mg/kg nZVI can drastically lower the levels of chlorophyll and carotenoid, which has an immediate impact on seedling photosynthesis. In the meantime, plant stomata may clog once nZVI is applied to the photosynthetic apparatus on the leaf surface, disrupting gas exchange and raising the foliar temperature. As a result, photosynthetic activity may be directly suppressed, and cellular metabolism may decline (Da Silva et al., 2006).

2.2.7. Plant defense mechanism against nZVI toxicity

Oxidative damage and antioxidant enzyme defense mechanisms are typical cellular metabolic byproducts of redox reactions experienced by all living things

(Ma et al., 2015b). In plants, certain antioxidant enzymes can neutralize or eliminate the ROS generated in non-stressful environmental circumstances. On the other hand, excessive ROS production in microcells causes oxidative damage in plants, which includes chlorophyll content loss, DNA damage, pollen membrane disintegration, and cell death. nZVI can produce H_2O_2 and ·OH radicals through the Fenton reaction. Highly reactive ROS called hydroxyl radicals can permanently damage biomolecules such as proteins, DNA, and lipids (de la Rosa et al., 2017; Kim et al., 2019). According to studies (de la Rosa et al., 2017; Gill & Tuteja, 2010), the damage that nanoparticles induced to the cell membrane increased as the number of lipid peroxides (LPO) rose. This suggests that the creation of ROS could lead to the death of the cells. Plants have evolved various antioxidant defense mechanisms, such as low molecular weight oxide scavengers and several enzymes that can detoxify superoxide and hydroperoxides to combat excessive ROS formation. Superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and glutathione peroxidase (GPx) in animals (Chen et al. 2012), and ascorbic acid peroxidase (APx) in plants (Ma et al. 2015) are examples of crucial intracellular antioxidant enzymes that can be activated to detoxify ROS and subsequently protect against oxidative damage.O_2^- can be quickly converted to H_2O_2 by SOD, and H_2O_2 can be quickly converted to H_2O and O_2 by CAT (Frei et al., 2016; Ghosh et al., 2017; Ma et al., 2015b). According to Wang et al. (2016), a Fe deficit can impact the antioxidant enzyme defense system's effectiveness since iron is a fundamental component of POD and CAT.

2.3. Plant bioassays

Biological tests, especially short-term plant bioassays, are the best method to detect and estimate contaminant loads in any matrix approach. Many plant species are widely used to indicate the cytogenetic and mutagenic effects of environmental physical and chemical contaminants (Juchimiuk and Maluszynska, 2005). Several enzymatic and non-enzymatic strategies remove free reactive oxidative species (ROS) in plant cells (Das and Roychoudhury, 2014). ROS are produced in chloroplasts and mitochondria as stress markers in plants. Both antioxidant enzymes and enzyme-dependent mechanisms also control ROS levels in plants. The most crucial stress markers include catalases, peroxidases, and superoxide dismutases. They function by inhibiting or preventing the formation of free radicals or reactive species in cells and breaking down hydrogen peroxide and hydroperoxides into harmless molecules (Grene., 2002). Stress triggers are usually quantified by homogenizing plant tissues in a buffer solution, followed by centrifugation and analyzing the supernatant for enzyme assays (Shalini and Dubey, 2003).

3. Materials and Methods

3.1. Experiment one

3.1.1. Characterization of tested soil and amendments

The soil sample utilized in this investigation came from the Příbram smelting district, Czech Republic. The Příbram soil has been contaminated by metallurgical activities, which have caused pollutants to be deposited in soil due to atmospheric deposition and past floods that have added hazardous metal(loid) to the soil. Metals Cd and Zn are primarily present in the most mobile fractions, based on past research done in the study area (Nováková et al., 2015). The mining stopped in 1978, but the smelting process continues. The top 20 cm of the soil was collected, allowed to dry naturally, homogenized, and then sieved using a 2 mm sieve. Michálková et al. (2016) provided a previous description of the soil (Table 1).

The S-nZVI slurry used in the experiment was purchased from Nano Iron Ltd. (product NANOFER 25DS, Czech Republic), and the Fe grit originating from the cast iron cutting was provided by the company Dekonta. The wastewater treatment plant in the Czech Republic (the facility wanted to stay anonymous) provided the composted sludge. The biomass for composting was created by combining raw sewage sludge with grass clippings in various composting piles. The total duration of composting was eight months. The microwave acid digestion was carried out by US EPA method 3050A using a representative 0.5 g sample in a mixture of HNO_3, HCl, and HF (9:3:1); the total elemental concentrations in the composted sludge and Fe chips were determined using ICP OES (iCAP 7000 series, ICP spectrometer, Thermo Scientific, USA). Additionally, blanks for analysis that contained just the reagent were made.

After digestion, the samples were dried at 100°C and dissolved in 10 mL of 2% HNO_3.

Quality control of sample digestion was done by analyzing certified reference materials BCR-483-70G and NIST2710A and comparing the results obtained with the certified values. Results of the physicochemical characterization of the study soil and selected elemental composition of the soil, composted sludge, and Fe grit are shown in Table 1 below.

Table 1

Physicochemical characteristic of the study soil represented as mean ± standard deviation (n=3)

pH_{H_2O}	5.95 ± 0.02
pH_{KCl}	5.14 ± 0.04
CEC (nmol/kg)	9.08 ± 0.52
TOC (%)	2.15 ± 0.05

Particle size distribution (%)

Clay	Silt	Sand
7	31	62

Soil classification

Sandy loam

Total metal concentrations mg kg^{-1} (n=3)

Soil		**Composted sludge**	**Fe grit**
As	296 ± 60	<dl	<dl
Pb	3539 ± 37	28.3 ± 3.4	42.2 ± 8.8
Cd	39 ± 10	<dl	<dl
Zn	4002 ± 68	358 ± 10	1226 ± 86
Cu	68 ± 30	109 ± 2	1392 ± 59
Fe	37408 ± 19	17592 ± 414	817888 ± 65610
Mn	4276 ± 34	386 ± 3	7369 ± 477

<dl result lower than the detection limits of the ICP OES

3.2. Batch experiment

Incubation batch tests were carried out to determine the impact of the tested amendments on the mobility of metal(loid)s in the polluted soils. Five different treatments were used for the experiment: control (C), 1% S-nZVI (CS), 1% Fe grit (CF) (w/w), 1% composted sludge (CC) (w/w), and 1% composted sludge (w/w) + 1% S-nZVI (CCS) (w/w). A mass of 200 grams of soil - either control or amended soil was incubated at different time intervals of 1 week, 1 month, 3 months, and 6 months. Following each incubation cycle, soil samples were dried for 48 hours at 60 °C. After drying the soils, 2 g were put into an extraction vessel. A 1:20 (w/v) ratio was used to apply the appropriate volumes of either the TCLP solution (pH 4.93 ± 0.05) (US EPA method 1311) or the SPLP solution (pH 5.00) (US EPA method 1312) to the soil after the vessel was allowed to agitate for 18 hours. Following agitation, samples were centrifuged at 5,000 rpm for 10 minutes. The centrifuged samples were then filtered using a 0.45 µm cellulose acetate syringe filter to separate the liquid and solid phases. The collected leachates were examined for metals using ICP OES and anions using ion chromatography (IC, Dionex 5000+, Thermo Fisher Scientific, USA). They dissolved organic carbon (DOC) using a TOC/TC analyzer (TOC-LCPH, Shimadzu, Japan. The pH and Eh values were also recorded.

3.3. Column experiment

A column experiment was performed to examine the impact of different amendments on the transport behavior of pollutants in the examined soils. The same amendments described in the batch experiment was used. To understand how the leachability of pollutants changes over time, soil samples were preincubated for varying lengths of time (1 week, 1 month, 3 months, and 6 months) before soil samples were leached in the columns. A mass of 30 grams of soil - either controlled or amended – was added to the pot for this purpose, and the mass was recalculated based on the proportion of each amendment. To sustain the soil moisture at field capacity, the pots were routinely watered with deionized water samples from each time interval, subsequently used for the column experiment.

Leaching solutions included two distinct solutions: (i) Toxicity Precipitation Leaching Procedure (TCLP) at pH 4.93 ± 0.05 and (ii) Synthetic Precipitation Leaching Procedure (SPLP) at pH 5.00. The goal of the TCLP is to mimic the leaching of contaminants in municipal landfills. Conversely, the SPLP attempts to simulate the leaching of pollutants under acidic precipitation. Samples were moved into a glass column tube (2.5 x 30 cm BIO-RAD Econo-column) and run down-flow at a flow rate of 0.5 mL min^{-1}. A peristaltic pump controlled the flow rate. The leachates collected were analyzed for metals, anions, and dissolved organic carbon (DOC); the pH and Eh values were recorded.

3.4. Soil pore water analyses

The impact of the amendments and incubation period on the chemistry of soil pore water was investigated. The experimental design was similar to the batch experiment; 200 g of dry soil was used per pot for subsequent soil pore water sampling. Rhizon sampler (Rhizosphere Research Products, Netherlands) was attached to the base of each container. The pots were watered twice weekly to maintain the moisture level at the field capacity. The soil was incubated at different time intervals of 1 week, 1 month, 3 months, and 6 months. After each incubation period, the soil pore water was analyzed using the Rhizon sampler. The contents of metal(loid)s, DOC, and anions were determined, and pH and Eh values were recorded.

3.5. Statistical analysis

Statistical analyses were performed using SigmaPlot 14.0 (Statsoft Inc., USA). Experimental data were evaluated using analysis of variance (ANOVA) at $P < 0.05$ using the Tukey post hoc test.

3.6. Experiment two

3.6.1 Characterization of tested soil and amendments

The experiment investigated the effect of applying S-nZVI with or without sewage sludge as a stabilization agent for soil contaminants and its effect on plant performance. The soil used was obtained from the area of a smelter in the Czech (the company wanted to stay anonymous) and classified as Technosol. The soil was collected from a depth of 0 to 20 cm. The collected soil samples were air-dried and sieved through a 2-mm mesh sieve. The S-nZVI slurry used was produced by Nano Iron Ltd. (NANOFER 25DS, Czech Republic). The thermally treated sludge (dried at 90°C) was obtained from a wastewater treatment plant in the Czech Republic. Total elemental concentrations in the soil and sludge were determined using ICP OES (iCAP 7000 series, ICP spectrometer, Thermo Scientific, USA) after microwave acid digestion, which was performed following US EPA method 3051A using a representative 0.5 g of sample in a mixture of HNO_3, HCl, and HF (9:3:1). Analytical blanks containing only the reagent were also prepared. After the microwave digestion, samples were evaporated to dryness at 100°C and dissolved in 10 mL 2% HNO_3. Quality control of sample digestion was done by analyzing certified reference materials BCR-483-70G and NIST2710A and comparing the results obtained with the certified values. The result obtained from the chemical characterization of soil is presented in Table 2, and the initial elemental composition of all amendments and the pseudototal elemental composition of the sludge are presented in Appendix Table S1 and S6, respectively.

Table 2

The physicochemical characteristic of the study soil represented as mean ± standard deviation (n=3)

pH_{H2O}	7.50 ± 0.02
pH_{KCl}	6.70 ± 0.04
CEC (nmol/kg)	9.08 ± 0.52
TOC (%)	12.7 ± 2.7

Particle size distribution (%)

Clay	Silt	Sand
8	11	81

Total elemental concentrations mg kg^{-1} (n=3)

As	348 ± 41
Pb	9073 ± 778
Cd	47.2 ± 3.3
Zn	5607 ± 60
Cu	703 ± 21
Fe	41441 ± 1048
Mn	1877 ± 113

3.7. Pot experiment – experimental setup

A 75-day pot experiment was conducted to assess the influence of plants and amendments in the changes in metal concentration (As, Cd, Pb, Zn). in both plants and soil. The soil was pretreated with six different amendments: Control (soil without any amendment), 1% S-nZVI (w/w), 2% S-nZVI (w/w), 3% sludge (w/w), 1% S-nZVI + 3% sludge (w/w), 2% S-nZVI + 3% sludge (w/w). One kg of amended or control soil was placed in a plastic pot with the dimensions (18.2

cm height x 24.5 cm width x 24.5 cm depth), and the mass was recalculated depending on the percentage of each amendment. The soil was watered to maintain the moisture at field capacity and kept in the greenhouse to equilibrate for 8 weeks before seed planting. Two plant species were selected for the experiment, *Arrhenatherum elatius* L. (AE) and *Festuca rubra* L. (FR), following a pilot study in which four plant species (*Lolium perenne, Agrostis capillaris, Arrhenatherum elatius*, *Festuca rubra* were used, and the best two in terms of phytostabilization potential were selected. Six amendments and five replicates of each amendment were used to give 60 pots. The seeds were sown in the top 1.5 to 2.0 cm of soil and received weekly irrigations. The following parameters were maintained during the plant growth: 20 - 25°C, 12 hours of sunlight and 12 hours of darkness, 225 $\mu E\ m^{-2}\ s^{-1}$ of photosynthetically active radiation, and 60 - 80% humidity. Following 75 days of growth, all the plants were collected and divided into stems and roots, and measurements were made of the length of the shoots and the above-ground biomass. Following cleaning, the plant tissues were dried for 48 hours at 60°C in an oven to determine the dry weight of the biomass and get them ready for digestion. Some fresh samples were immersed in liquid nitrogen and stored at -80 °C before plant pigment and antioxidant enzyme analysis.

3.7.1. Determination of elemental concentration in plant tissue

Following harvesting, the plant's roots and shoots were carefully cleaned with distilled water and dried for 48 hours at 60°C. The collected samples underwent a modified methodology of Zinzala et al. (2023) for digestion. Five mL of HNO_3 and 1 mL of H_2O_2 were used to digest 0.25 g of plant tissue. After microwave digestion, samples were evaporated on the hot plate at 75°C, and the dried samples were then dissolved in 10 mL of 2% HNO_3. The dissolved plant material was filtered through an acetate filter with a pore size of 0.45 µm; the total elemental concentration was determined using ICP OES. Quality control of the digestion process was done by analyzing the certified reference material ERM-CD281 RYE GRASS.

3.7.2. Quantification of pigments (chlorophylls and carotenoids)

Pigments were extracted from 5 mg of freeze-dried samples with 1 mL of acetone and centrifuged (microcentrifuge Sigma 1-14, Germany) for 5 min at 9000×g. The supernatant was then evaporated under nitrogen flow. Dry pigment mixtures were stored at -80 °C and dissolved in 200 □L of acetone before analyses. The presence of pigments, such as chlorophyll a and chlorophyll b, was detected using the HPLC-UV/VIS system, consisting of Gradient Pump Beta, Autosampler HTA 300, Watrex Nucleosil 120-5-C18 column, UV detector Sapphire, and Vacuum Degasser DG 3014 (ECOM, Czech Republic). The gradient: acetonitrile/methanol/water – 80:12:10 and methanol/ethylacetate – 95:5, both solvent mixtures contained 0.01% of antioxidant butylated hydroxytoluene (BHT), flow rate 1 mL·min^{-1}, total analysis time was 25 min and gradient ran during 2-6 min, detection at 445 nm. Quantification of detected pigments was performed with Clarity software (DataApex). The carotenoid content was expressed in micrograms of particular carotenoid per gram of dry leaves (µg·g^{-1} DW). Pigment standards were isolated from lyophilized tobacco leaves, and HPLC separated and purified carotenoids using the same device and conditions described above. Components were identified and quantified spectrophotometrically (Spectrophotometer Hitachi 2000) using spectral parameters and absorption coefficients of individual pigments. The HPLC system was calibrated with individual carotenoids using three different concentrations.

3.7.3. Antioxidant enzyme quantification.

Plant leaves were homogenized in liquid nitrogen; the homogenized samples were extracted in an extraction buffer (50 mM KH_2PO_4; pH 7, 0.1 mM EDTA, 1% PVP K 30, 0.5% Triton-X 100) and centrifuged (14,000 g, 10 min, 4 °C), the supernatant was used for enzymes assay. Protein content was measured according to Bradford (1976). The activities of catalase (CAT), glutathione S-transferase (GST), ascorbate peroxidase (APX), and guaiacol-specific peroxidase (POX) were measured spectrophotometrically following the methods of standard methods (Stuchlíková, 2018). Glutathione reductase (GR) activity was measured according to Carlberg and Mannervik (1985). The specific activities of the enzyme were calculated per protein unit.

3.7.4. Soil digestion

Total digestion of the soil samples before and after plant harvest was performed according to the US EPA method 3051A. An amount of 0.5 g of soil sample reacted with a mixture of 9 mL of HNO_3, 3 mL of HCl, and 1 mL of HF concentrated acids overnight before being placed in a microwave unit (Multiwave PRO microwave reaction system SOLV, Anton Paar, Germany). After microwave digestion, the samples were cooled for 30 minutes and poured into digestion vessels. The vessels were placed on a hot plate for approximately 3 h at 100 °C for complete evaporation. The residues were then dissolved in 10 mL of 2% HNO_3 and filtered using a 0.45 µm acetate filter. All the samples were analyzed in triplicate with procedural blanks. The filtrates collected were analyzed for metals using ICP OES and anions using ion chromatography (IC). The values of pH and Eh were also measured using standardized procedures.

3.8. Statistical analysis

Statistical analyses were performed using SigmaPlot 14.0 (Statsoft Inc., USA). Experimental data were evaluated using analysis of variance (ANOVA) at P < ß0.05 using the Tukey post hoc test.

4. Results and discussion

4.1. Experiment one

4.1.1 Batch experiments

The pH values for all tested samples ranged from 4.86 to 4.96 due to the buffering nature of the TCLP solution (Pinto & Al-Abed, 2017). Fig. 1a shows the variations in TCLP-extractable Pb concentrations throughout 6 months. Within the control group, Pb concentrations varied throughout the several incubation periods, from 30.9 ± 1.1 to 35.6 ± 1.3 mg kg^{-1}. Pb concentrations were increased in the Fe grit-treated samples (47.9 ± 5.6 mg kg^{-1}) and S-nZVI-treated samples (45.7 ± 2.3 mg kg^{-1}) after 1 week of incubation. This might be attributed to the dissolution of Fe oxides, as acetic acid could increase the number of H^+ ions that react with the –OH functional group. An increased number of positively charged Fe-OH groups could increase the desorption of metal cations, which were then extracted (Daniela & Janusevicius, 2022). There was no difference between the samples treated with composted sludge alone and the mixture of composted sludge and S-nZVI compared to the control samples at the end of 1 week incubation. After 1 month of incubation, the concentration of Pb extracted was lower in Fe grit-treated samples (33.3 ± 0.9 mg kg^{-1}) compared to control samples. After 3 months of incubation, a further decrease was observed (28.1 ± 1.6 mg kg^{-1}), suggesting that Pb changed from an exchangeable to an oxide-bound fraction. Thus, its availability decreased over time (Parvin et al., 2022). When compared to the control, no difference was observed in the concentration of Pb extracted from the Fe grit-treated sample after 6 months of incubation. The S-nZVI treatment resulted in an increase in Pb extracted over the incubation period. This is in contrast to the results of Gil-Dias et al. (2018), who found that the concentration of Pb extracted using TCLP significantly decreased over 45 days. However, a previous study by Mitzia et al.

(2019) using the same soil reported an increase in the exchangeable Pb fraction compared to the control after adding nZVI. This could explain the increase in Pb concentration after extraction in S-nZVI treated samples. After 1 week of incubation, there was no difference in the amount of Pb extracted from samples amended with a mixture of composted sludge and S-nZVI. However, compared to the control, there was a significant increase of 22%, 33%, and 25% in Pb extracted after 1, 3, and 6 months of incubation. Because the addition of soluble organic matter contained in composted sludge can elevate metal concentration in soil solution due to the formation of soluble organo-metal complexes, a slight increase in Pb extracted was observed for samples treated with composted sludge alone as incubation time increased (Zhou & Haynes, 2010). According to several studies (Bolan et al., 2014; Van Herwinjnen et al., 2007; Schwab et al., 2007), the addition of organic material, such as composted green waste and sewage sludge, increased the concentration of Pb in the soil. Overall, samples treated with S-nZVI alone or a combination of composted sludge and S-nZVI showed increased Pb concentration at most incubation times (1, 3, and 6 months). In contrast, Fe grit caused a reduction in the concentration of extractable Pb for the first 3 months.

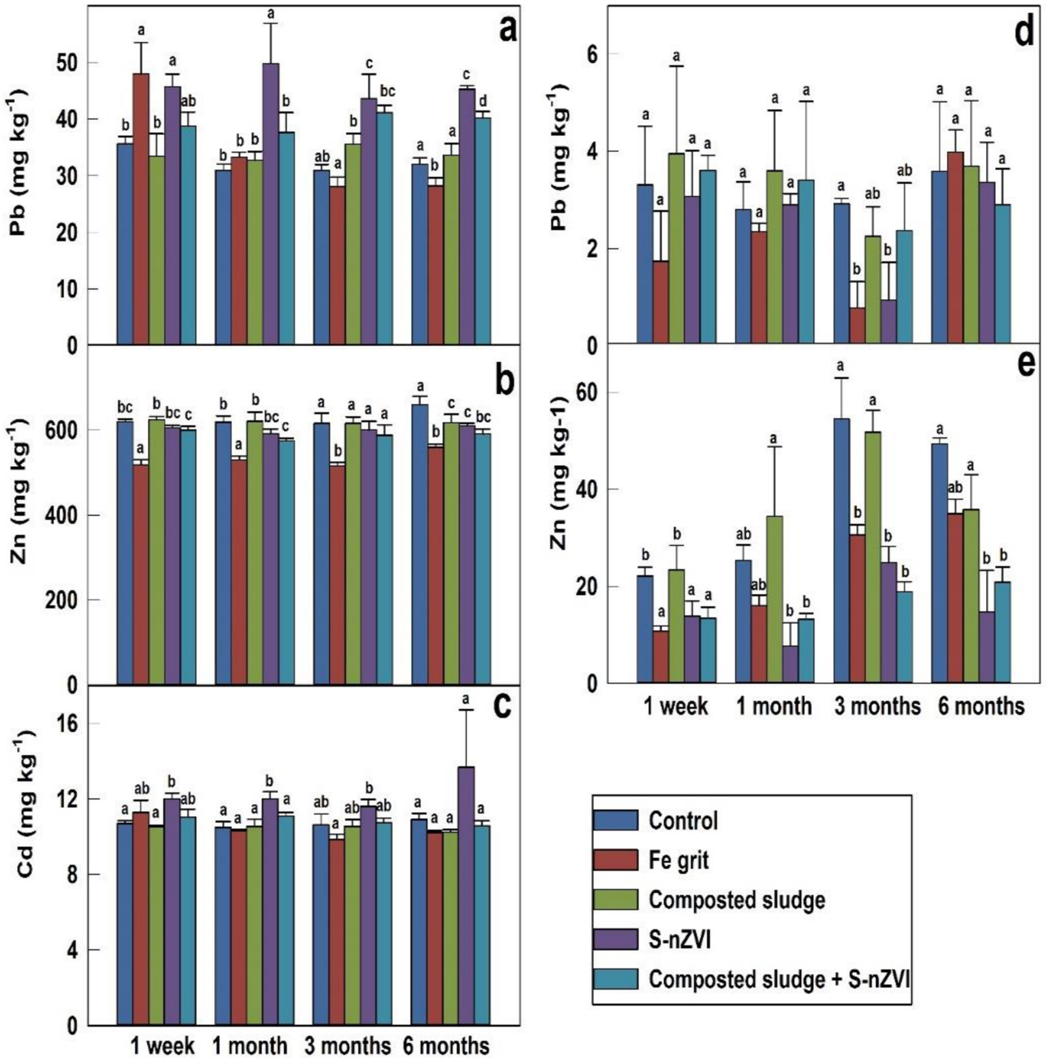

Fig. 1. Concentration of TCLP-extractable Pb (**a**), Zn (**b**), Cd (**c**), and SPLP-extractable Pb (**d**) and Zn (**e**) after application of different amendments at different incubation times. Data with the same letter represent statistically identical values ($P < 0.05$) ($n = 3$).

Fig. 1b shows the concentration of TCLP-extractable Zn, one of the major contaminants in the soil. Compared to the control, the Fe grit amendment decreases the amount of Zn extracted by 16%, 14%, and 16% after 1 week, 1 month, and 3 months of incubation, respectively. During the first 3 months, there was little to no difference in the amount of Zn extracted with other treatments compared to the control. After 6 months, samples treated with Fe grit, composted sludge, S-nZVI, and a combination of composted sludge and S-nZVI had the least amount of Zn extracted – the extractable amount decreased by 15%, 6%, 8%, and 10%, respectively.

The control group had the lowest quantity of Cd extracted after 3 months (10.9 ± 0.3 mg kg^{-1}) (Fig. 1c), representing about 28% of the total Cd leached from the soil sample. After 1 week of incubation, the amount of Cd extracted was higher with the S-nZVI treatment (12.0 ± 0.3 mg kg^{-1}) than with the control. The concentration of Cd extracted in samples treated with S-nZVI showed no change after 1 or 3 months, but after 6 months, there was an increase (13.7 ± 3.0 mg kg^{-1}). This contradicts the results of Chen et al. (2016) and Xue et al. (2023), who found that the concentration of Cd recovered by TCLP decreased following stabilization with nZVI/Activated carbon composite (nZVI/AC) and S-nZVI, respectively. Given that the addition of nZVI/AC and S-nZVI reduced the exchangeable and carbonated fraction and transformed it into the residual fraction in both investigations, an understanding of the metal speciation before and after the addition of these amendments may aid in the understanding of the process of Cd stabilization. After 6 months of incubation, other amendments did not influence the concentration of Cd extracted compared to the control.

Compared to TCLP, all elements are released in comparatively small amounts in SPLP, as shown in Figs. 1d, 1e. The chemistry of the extraction fluid explains this discrepancy. Acetic acid in the TCLP fluid can create acetate chelates, which increase Pb solubility (Pinto & Al-Abed, 2017). Samples extracted with SPLP have a pH ranging from 5.95 to 6.75. The SPLP fluid permits the sample's pH to fluctuate, in contrast to the buffering properties of TCLP (Dungan & Dees, 2009). After 1 month, there was no difference in the Pb concentrations extracted in any of the variants (Fig. 1d). When compared to the control, the samples treated with Fe grit and S-nZVI showed a reduction by 73% and 68% in the amount of Pb extracted at the end of 3 months incubation period, respectively. The adsorption of Pb with the soil organic acids may cause variations in Pb extracted from SPLP-treated samples as opposed to TCLP-treated samples (Al-Abed et al., 2006). Pb and acetate ions may combine to produce complexes. Since hydrolyzed metal species and metal complexes are preferentially adsorbed over free metal ions, these complexes would compete with Pb ions' adsorption reaction on the surface. As a result, the concentration of dissolved Pb may decrease as a more thermodynamically favorable precipitate form (Al-Abed et al., 2006). Chen et al. (2016) and Pinto & Al-Abed (2017) also showed a similar decrease in Pb concentration in the SPLP-leached sample compared to the TCLP-leached samples. When compared to the control, there was a significant decrease in the concentration of Zn extracted from variants with Fe grit (52%, 44%, 29%), S-nZVI (38%, 55%, 70%), and a mixture of composted sludge and S-nZVI treated samples (39%, 65%, 59%) after 1 week, 3 months, and 6 months of incubation, respectively (Fig. 1e). Samples treated with composted sludge alone had no influence in the concentration of Zn extracted at all incubation times.

4.2. Column leaching

4.2.1 TCLP

Column leaching was performed to understand how different amendments influence the mobility of metal(loid)s under dynamic flow conditions.

Figure 2 below shows the variations in Zn, Pb, and Cd concentrations leached by TCLP. The most difference observed among different amendments occurs in the first 120 minutes, after which equilibrium is reached, and no changes occur afterward, except samples treated with a mixture of S-nZVI and composted sludge, which continuously decreased the concentrations of most elements overall sampling times when compared to the control. Fe grit caused an increase in the concentration of Pb (Fig. 2e) and Cd (Fig. 3a) leached in the first 75 minutes after 1 week incubation period. However, this effect was short-lived, as no difference occurred in the concentration of Zn (Fig. 2b), Pb (Fig. 2f), and Cd (Fig. 3b) leached after a 1 month incubation period when compared to the control. However, at the end of 3 and 6 months of incubation, Fe grit reduced the Cd, Zn, and Pb concentration up to the first 120 minutes, with Fe grit being the best amendment for reduction of Pb leaching at the end of the 6-month incubation period. Leachates from columns containing soils treated with S-nZVI showed substantially decreased Pb (up to 1 month) and Zn (up to 6 months) concentrations than the control samples. Zn was less successfully immobilized than Pb, and this discrepancy is explained by the two metals' different chemical characteristics. According to Li and Zhang (2007), Zn ions are attached to the Fe surface by sorption or surface complex formation, while the removal mechanism for Pb by nZVI is sorption and partial chemical reduction. Gil-Diaz et al. (2014) reported a similar reduction in the concentration of Pb and Zn leached from columns containing soils treated with nZVI.

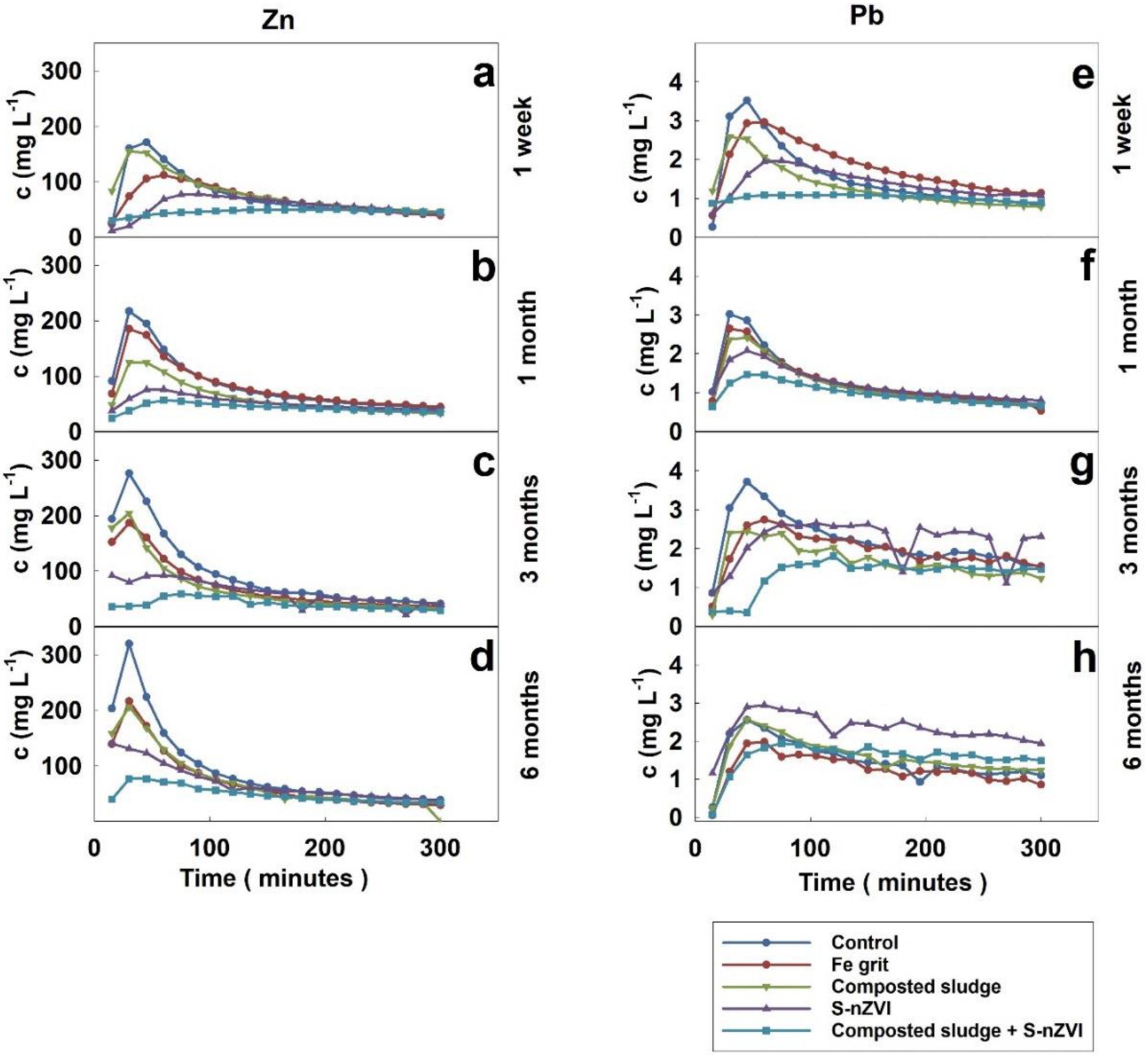

Fig. 2. The amount of Zn and Pb leached by the TCLP in the column experiment after applying various soil amendments at different incubation times.

When compared to the control, the S-nZVI amendment increased the concentration of Cd towards the end of the sampling time (105 minutes) after 1 week (Fig. 3a) and 3 months (Fig. 3c) incubation period. Samples treated with composted sludge alone have little to no influence on the concentration of Zn and Pb leached after 1 week incubation period compared to the control.

However, Pb and Zn concentrations were significantly reduced after 1 month and 3 months, after which the effect phased out and at 6 months, as no difference was observed compared to the control. Several studies have demonstrated that changes in Zn mobility driven by sewage sludge application are a result of the adsorption of Zn to the soil solid phase, thus causing a reduction in the amount of Zn leached from the column (Ashworth and Alloway 2004, Jalali and Arfania 2010). This suggests that the soil's solid surface removed soluble Zn from its organic ligand. Moreover, Zn can bind to carbonates or organic materials found in sewage sludge. Throughout the incubation period, combining composted sludge and S-nZVI treatment was the most effective way to reduce the leaching of all elements (Zn, Cd, and Pb) at all sampling intervals. However, after 6 months, compared to the control, this amendment increased the concentration of Pb (Fig. 2h) and Cd (Fig. 3d).

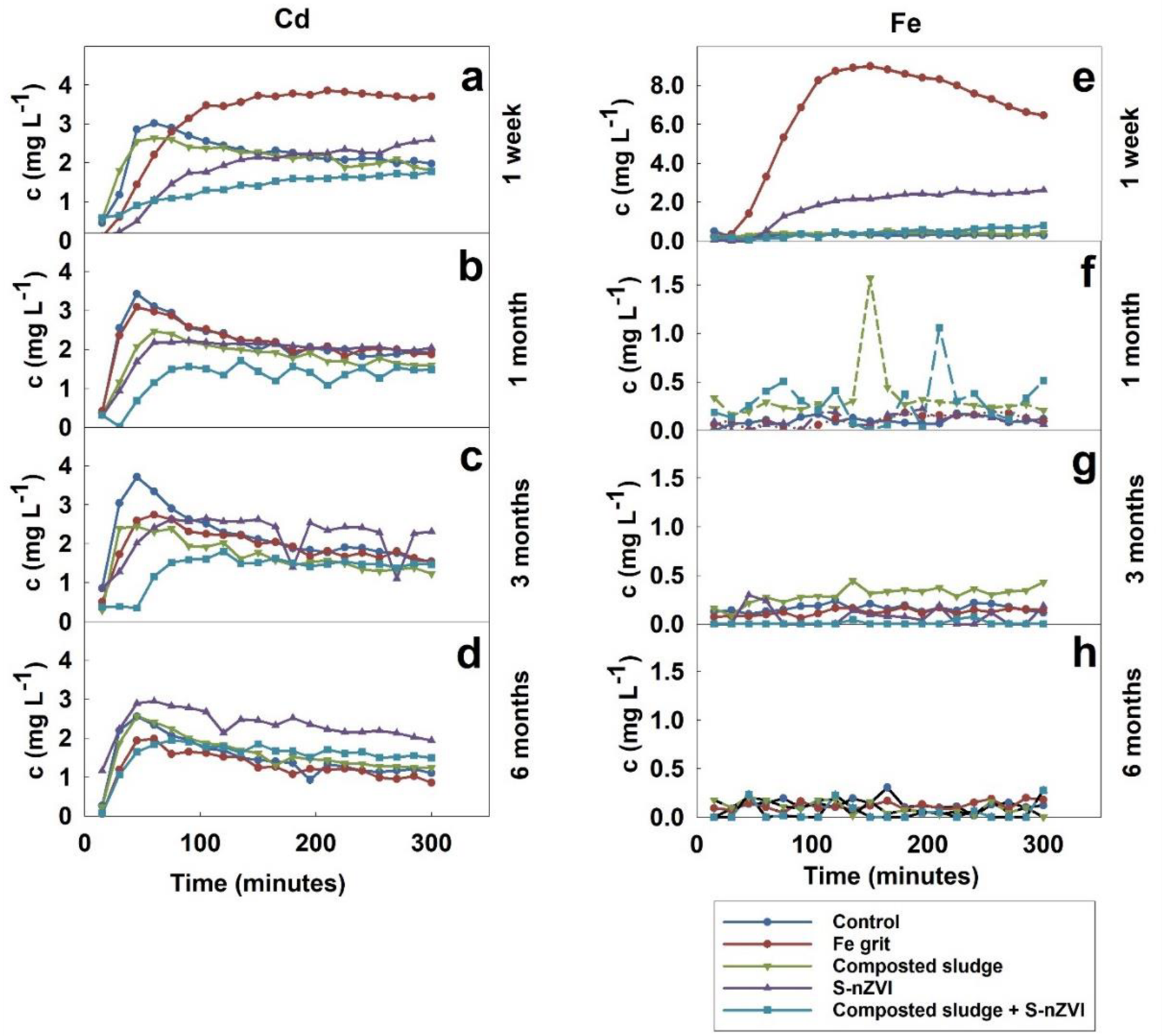

Fig. 3. The concentration of Cd and Fe leached by the TCLP in the column experiment after applying various soil amendments at different incubation times.

4.2.2 SPLP

Figure 4 below shows the concentration of Zn and Fe leached from different soil variants using SPLP. At all incubation periods, the impact of amendments on Zn leaching is most noticeable within the first 45 minutes. It implies that samples leached with SPLP attain equilibrium more quickly than samples leached with TCLP. The concentrations of Cd demonstrated the impact of fluid chemistry on metal leaching, and Pb leached using SPLP, which were below the ICP OES detection limit. In contrast, acetic acid in TCLP can facilitate Pb leaching by forming soluble acetate chelates (Pinto and Al-Abed, 2017).

There was an increase in the concentration of Zn in the first 15 minutes in samples treated with composted sludge, after which the concentration of Zn remained unchanged compared with the control. After 1 week and 1 month of incubation, S-nZVI decreases Zn leaching after the first 45 minutes but increases Zn leaching during the first sample time (15 minutes), after which there is no difference between the treated and control groups. Only during the first 15 minutes of incubation does the Fe grit reduce Zn leaching; the concentrations remain unchanged relative to the control after that. Compared to the control, samples treated with a combination of S-nZVI and composted sludge only reduced Zn leaching during the first 60 minutes at all incubation times.

Figures 4e, f, g, and h show how applied amendments affect Fe leaching; for all samples except those treated with a combination of composted sludge and S-nZVI, there was an increase in the concentration of Fe leached at 1 week. At 1 month, the Fe concentration was lower in the S-nZVI and Fe grit amendment. However, samples treated with a combination of S-nZVI and composted sludge showed increased Fe concentration throughout the incubation period. After 3 and 6 months, there is no variation in Fe concentrations across all amendments. However, for 3 and 6 months, most Fe concentrations leached from samples containing composted sludge and S-nZVI mix were below the ICP OES detection limits.

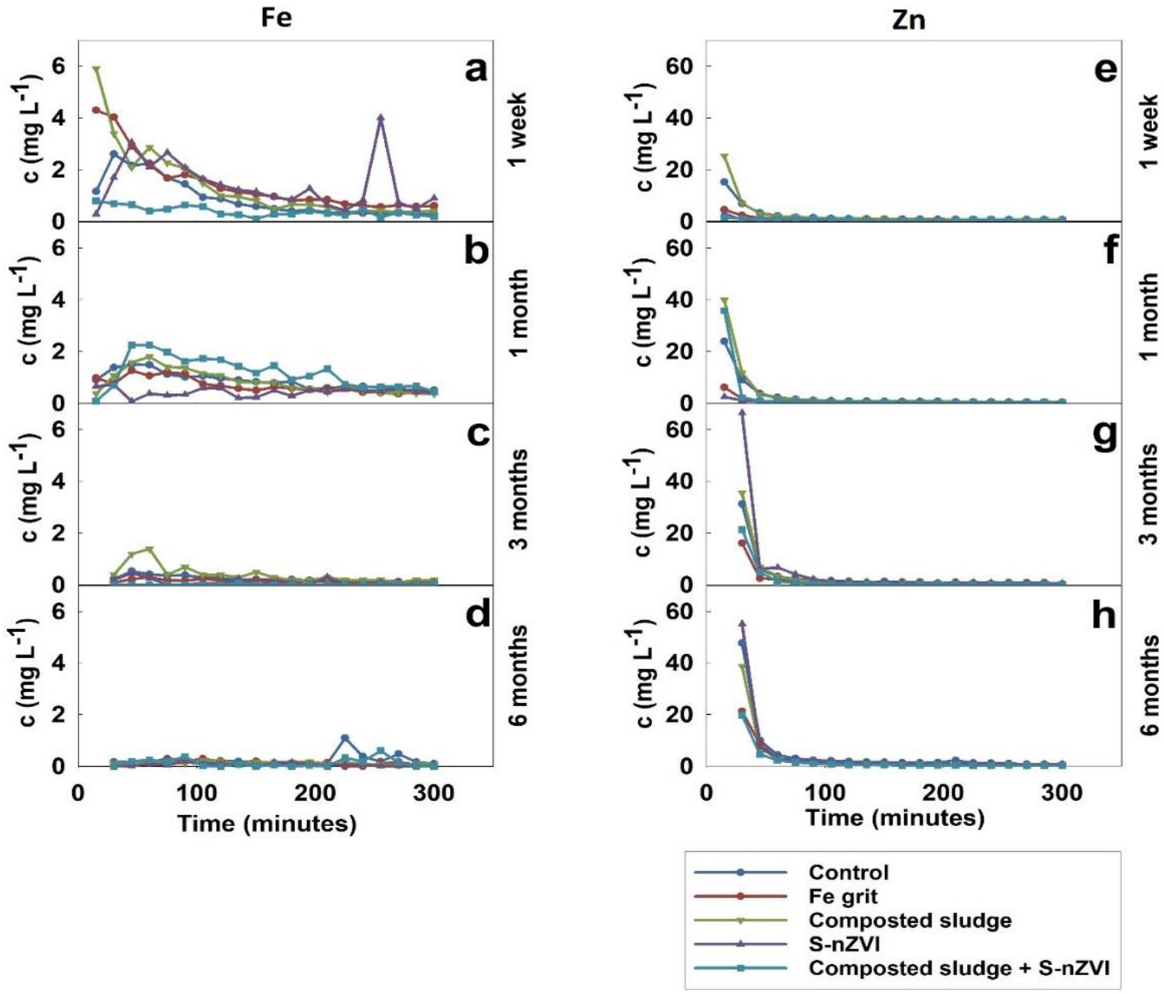

Fig. 4. Fe and Zn leached from the columns with amended soils using SPLP at different incubation times.

4.2.3 Pore water

This section will focus solely on the results for Zn because the amounts of the other elements (Pb, Cd, and Fe) in the pore water were all below the ICP OES detection limit. The impact of different amendments on the concentration of Zn in soil solution is seen in Fig. 5a. Composted sludge caused an initial increase in pH (6.45 ± 0.29). Then, it reduced the pH until the end of the incubation period (5.69 ± 0.28). The presence of NH_4^+ in the sludge may have contributed to the initial rise in soil pH, and an increase in nitrate content was likely the cause of the following pH decrease (Fig. 5b). This is in line with the findings of Kokina et al. (2022). An initial rise in pH was noted, followed by a pH drop due to nitrification from adding N-rich residue and the production of breakdown products (Jalali and Arfania 2010).

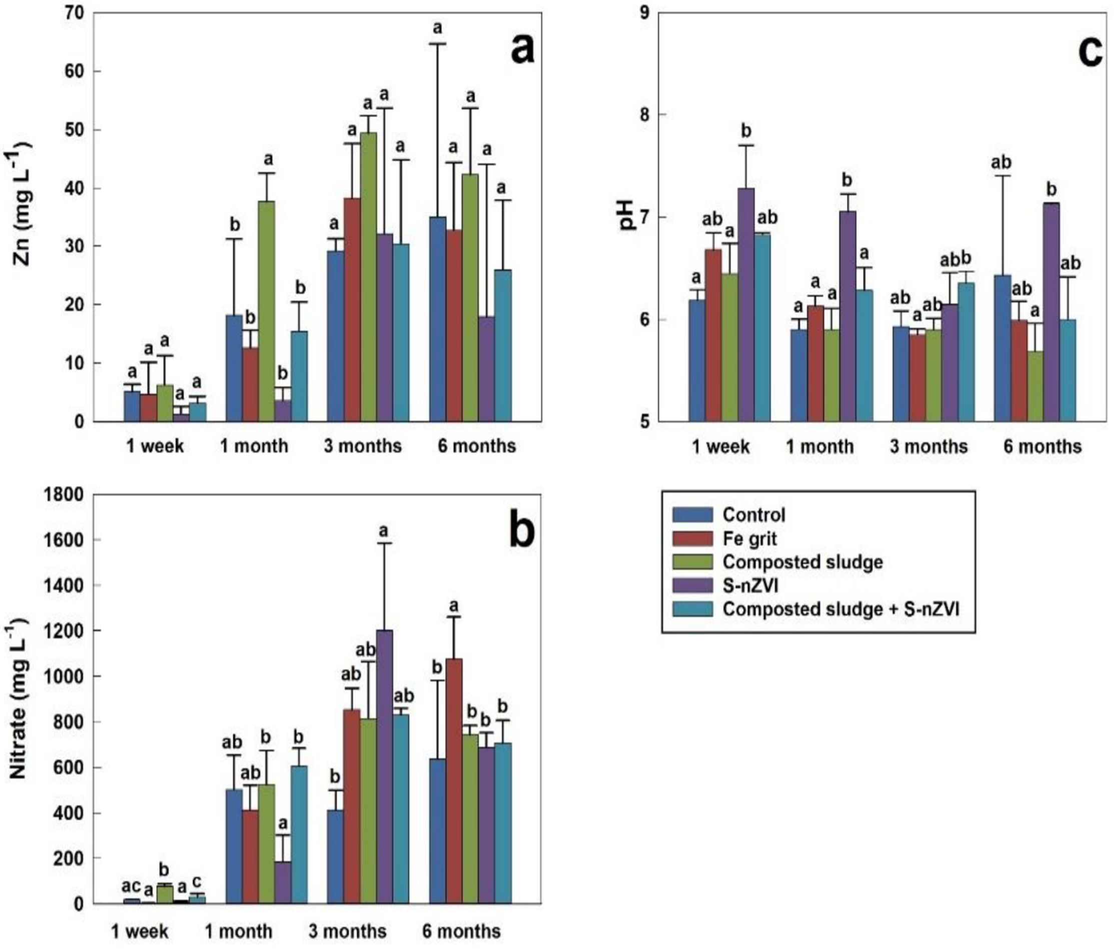

Fig. 5. Changes in the concentration of Zn (**a**), pH value (**b**), and nitrates (**c**) in soil solution in amended soils at different incubation times. Data with the same letter represent statistically identical values ($P < 0.05$) ($n = 3$).

Variations in Zn concentrations after 1 week, 1 month, 3 months, and 6 months are shown in Fig. 5a. The concentration of Zn in solution increased in all samples amended with composted sludge after 1 week, 1 month, and 3 months of incubation; however, at these incubation times, the effects were not statistically significant. After 1 month, S-nZVI increased the pH, and a similar Zn removal efficacy of nZVI was demonstrated by Yan et al. (2010); adsorption, complexation, and (co)precipitation were the primary mechanisms for removing Zn (Liang et al., 2014). The removal of Zn by nZVI is significantly influenced by pH; the observation that Zn uptake increases with increasing pH is supported by Kishimoto et al. (2011), who found that Zn uptake increases gradually with pH. The highest Zn bound to nZVI concentration occurs at the pH of 7. Krzisnik et al. (2014) observed a similar result at pH 7 since strong magnetic force increases particle aggregation at neutral pH, enhancing the potential of contaminant removal (Redzauddin et al., 2015).

Composted sludge increased the concentration of Zn in soil solutions by 107%. This denotes that composted sludge significantly increases the available Zn phases and mobility (Bowszyz et al., 2015). As observed in a previous study by Zaragüeta et al. (2021), Zn may be more mobile and biologically accessible in sludge-treated soils. Additionally, McBride (2022) clarified that incorporating biosolids into soil can increase the percentage of Zn that is bioavailable. Due to its ability to form soluble chelates with both humified and non-humidified types of organic matter, this mobility has increased over time following the addition of sewage sludge to the soil (Zaragüeta et al., 2021). When compared to the control, the percentage reduction in Zn concentration in soil solution collected after 1 month in samples treated with Fe grit, S-nZVI, and a combination of composted sludge and S-nZVI were 30.6%, 80.2%, and 15.5%, respectively. With S-nZVI being the most effective method for reducing the content of Zn in soil solution, it is S-nZVI.

4.3. Experiment two

4.3.1. Plant growth parameters

Plant height and biomass were measured to determine the impact of amendments on plant growth and potential yield. Plant height indicates dwarfism caused by environmental factors or genetics (Kroupin et al., 2019). Higher biomass values, however, may indicate a robust and healthy plant (Marin et al., 2020). Fig. 6b illustrates how the amendments affected plant height following a 75-day pot experiment. Both *Arrhenatherum elatius* (AE) and *Festuca rubra* (FR) plant species showed an increase in plant height in samples treated with 1%, 2% S-nZVI, and 3% sewage sludge compared to the control. This indicates a direct correlation between plant height and biomass production, as increasing plant heights resulted in increased above-ground biomass for these amendments (Uslu et al., 2020; Su et al., 2022) compared to the control group. The highest increase in plant height was observed in samples treated with 2% S-nZVI alone for both *Arrhenatherum elatius* and *Festuca rubra* plants.

Radziemska et al. (2021) noticed that applying nZVI to pots resulted in a 38% boost in the production of above-ground biomass of *Festuca rubra* and *Lolium perenne* plants. Xie et al. (2016) and Libralato et al. (2016) also showed that plants subjected to the impacts of nZVI display diverse effects, including the promotion of sprouting or increased biomass. Additionally, Yoon et al. (2019) showed that adding nZVI resulted in a 38% increase in *Arabidopsis thaliana* biomass. This improved biomass may occur because nZVI provides bioavailable Fe as a nutrient or increases phytohormone content and antioxidant enzyme activity (Yoon et al., 2019).

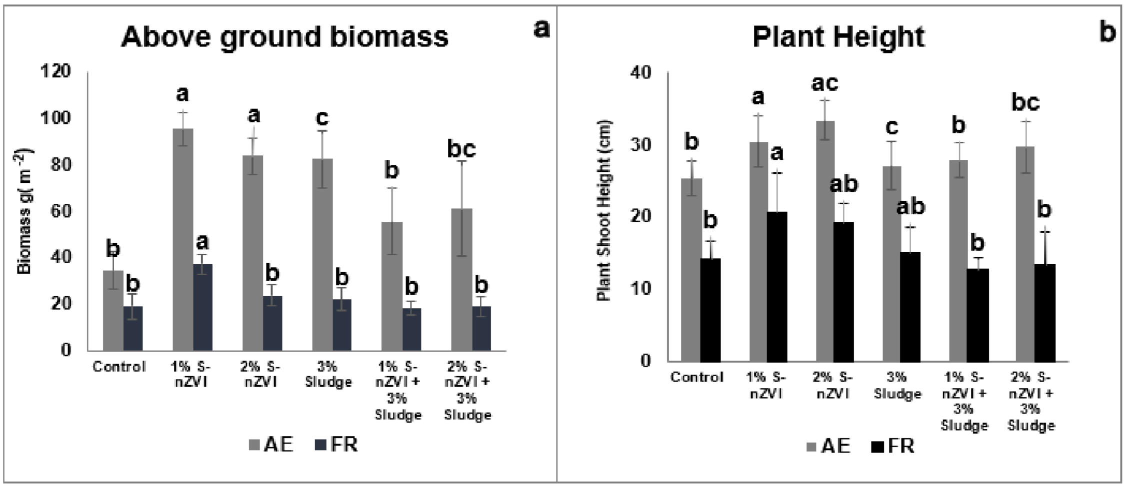

Fig. 6. Above-ground biomass of (**a**) *Festuca rubra* (FR) and *Arrhenatherum elatius,* (**b**) Plant height of *Festuca rubra* (FR) and *Arrhenatherum elatius* (AE) at the end of pot experiment for different amendments. Statistical evaluation was performed separately for each plant species. Data with the same letter represent statistically identical values ($P < 0.05$) ($n = 5$).

Large amounts of nutrients found in sewage sludge (Appendix Table 1), which are crucial for plant growth, may cause increases in plant height and biomass in samples treated with 3% sludge alone (Alaoui-Sossé et al., 2018). The results support Bai et al. (2022), who found that sewage sludge increased biomass and extensive vegetation cover in Mongolian pine trees. Additionally, Gubišová et al. (2020) note that the addition of sewage sludge results in a 1.2-2.7 increase in yield of *Arundo donax*. For *Festuca rubra*, the co-application of 3% sewage sludge with 1% and 2% S-nZVI did not affect plant height or above-ground biomass. A pictorial representation of the plants during growth at the greenhouse is also presented in Appendix Fig. 7 below.

4.4. Plant pigment analysis

Cellular and molecular barriers abound for photosynthetic systems in dynamic environmental circumstances. Under stressful conditions, the main reasons for photosynthetic inhibition are poor membrane function and membrane disruption (Ashraf & Harris, 2013). The total carotenoid value, chlorophyll a/b ratio, and chlorophyll a and b levels were assessed to assess amendments' impact on plant health and indirectly determine stress response. In the complex process of photosynthesis, chlorophyll is an essential pigment used in photosynthetic processes. Metal accumulation patterns elicited by different plants have been shown to impact photosynthetic pigment production adversely (Jalmi et al., 2018).

Metal poisoning either directly inhibits chlorophyll biosynthesis by causing the enzymes involved to degrade or indirectly by replacing necessary components of chlorophyll structure. In addition to being potential antioxidants under stress, carotenoids are necessary for a variety of plant functions, such as membrane stabilizers, dissipators of surplus detrimental energy under stress, light harvesters, quenchers, and scavengers of singlet oxygen species and triple-state chlorophylls (Uarrota et al., 2018). An additional indicator of plant stress is the chlorophyll a/b ratio. The plant's ability to withstand stress decreased with increasing chlorophyll a/b ratio (Soudek et al., 2024)

In both plant species, all amendments increase in chlorophyll a (Fig. 7a). Chlorophyll b (Fig. 7b) levels in samples treated with 1% and 2% S-nZVI as well as a combination of 2% S-nZVI and 3% composted sludge show an increase in *Arrhenatherum elatius* plants as compared to the control. Adding 3% sewage sludge alone and combining 1% S-nZVI and 3% sewage sludge do not affect the amount of chlorophyll b in the *Arrhenatherum elatius* plant compared to control samples. Chlorophyll b levels increased in the *Festuca rubra* plant after the application of all the amendments when compared to the control.

According to Kim et al. (2019), the rise in chlorophyll content in S-nZVI treated samples suggested that the Fe ions released due to S-nZVI treatments were tolerable to both plants and appropriate for plant growth. Since Fe is essential for producing chlorophyll, plant-available Fe ions, and S-nZVI inclusion may, therefore, be contributing factors to the elevated chlorophyll concentration (Li et al., 2024). According to several studies, adding Fe nanoparticles has been linked to a beneficial rise in chlorophyll a. Kim et al. (2019) found that nZVI treatments increased the amount of chlorophyll a in alfalfa. Additionally, after adding different amounts of nZVI, Mielcarz-Skalska et al. (2021) found an increase in chlorophyll a content by 10% and chlorophyll b between 45% and 60%. The presence of critical metal ions and the high concentration of readily available nitrogen (N) and phosphorus (P) in sewage sludge may be the cause of the increased chlorophyll content in sewage sludge amended samples, as both N and P are necessary elements for chlorophyll biosynthesis (Pengcheng et al., 2008).

For many species, there is a positive relationship between nitrogen levels and leaf chlorophyll concentrations (Zhang et al., 2013). This is because nitrogen is a part of the structure of chlorophyll molecules (Martinez et al., 2015). In addition, Burducea et al. (2019) found that basil leaves grown on sewage sludge and substrates modified by sewage sludge had higher levels of chlorophyll b.

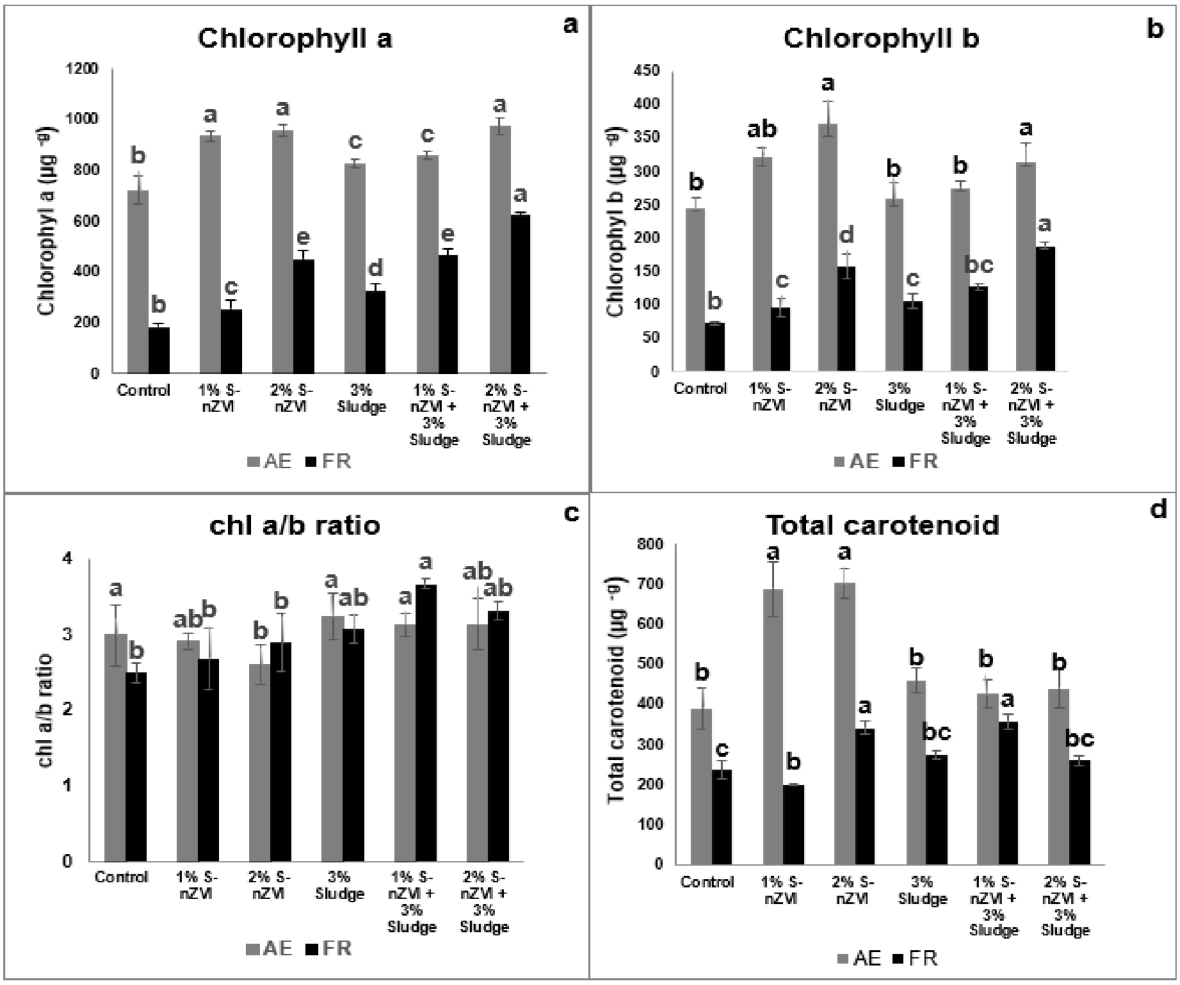

Fig. 7. chlorophyll a content of **(a)** *Festuca rubra* (FR) and *Arrhenatherum elatius*, **(b)** chlorophyll b content of *Festuca rubra* (FR) and *Arrhenatherum elatius,* **(c)** chlorophyll a/b ratio of *Festuca rubra* (FR) and *Arrhenatherum elatius,* **(d)** total carotenoid of *Festuca rubra* (FR) and *Arrhenatherum elatius* at the end of pot experiment for different amendments. Statistical evaluation was performed separately for each plant species. Data with the same letter represent statistically identical values ($P < 0.05$) ($n = 5$).

The ratio of chlorophyll a/b is depicted in Fig. 7c. For *Arrhenatherum elatius* plants, the chlorophyll a/b ratio did not differ among treatments, however, for *Festuca rubra* samples treated with 3% sludge, 1% S-nZVI, and the co-application of 3% sludge and 2% nZVI, the level of chlorophyll a/b ratio rose, which may indicate stress on the plant.

Fig. 7d shows the total carotenoid contents of *Festuca rubra* and *Arrhenatherum elatius*. Other amendments had no effect on the total carotenoid for Arrhenatherum elatius plants except for samples treated with 1% S-nZVI, which led to an increase in the total carotenoid value of *Arrhenatherum elatius* plants relative to the control. All amendments for *Festuca rubra* plants caused an increase in total carotenoid for *Festuca rubra* species, except for samples treated with 2% S-nZVI and 3% composted sludge, which showed no effect compared to the control. Prior research on rice seedlings treated with nZVI had comparable outcomes (Guha et al., 2018). Following nZVI treatment, Brasili et al. (2020) also observed a rise in the amount of leaf carotenoid in tomatoes. The increase in carotenoid levels observed in *Festuca rubra* plants grown on sewage sludge most likely be as a result of elevated concentrations of copper (Cu) and zinc (Zn) concentrations (Burducea et al., 2019).

4.5 Antioxidant enzyme assay

The exposure of plants to metal(oid)s can lead to oxidative stress (Killian et al., 2020). Plants possess various defense mechanisms to cope with oxidative stress, one of which is an increase in the activities of antioxidant enzymes (Hasanuzzaman et al., 2020). Several enzymes, e.g., glutathione S-transferase (GST), catalase (CAT), ascorbate peroxidase (APX), peroxidase (POX), superoxide dismutase (SOD), and glutathione reductase (GR), play an essential role in plants along with the changes in the expression and activities of these enzymes represent a vital defense system against stress conditions (Rajput et al., 2021).

The result obtained from the activity of peroxidase, catalase, and glutathione reductase quantified in plant leaves is presented in Fig. 8 abc. All amendments did not influence peroxidase activity compared to the control samples for *Festuca rubra* and *Arrhenatherum elatius* plants. Catalase activity was significantly increased in *Arrhenatherum elatius* plant leaves grown at 1% S-nZVI compared to the control. This corroborates the findings of Mohammadi et al. (2020), where catalase activity increased in the shoot of the *Helianthus annuus* plant treated with Fe nanoparticles under Cr stress. Haydar et al. (2022) also reported a similar increase in catalase activity in mulberry after treatment with nano iron chelate; other amendments did not influence the change in the catalase enzyme for *Arrhenatherum elatius* plants compared to the control. There was also no difference in the activity of glutathione reductase in all amendments for both *Festuca rubra* and *Arrhenatherum elatius* plants when compared to the control, with the exception found in plants grown on 1% S-nZVI in combination with 3% sewage sludge for *Festuca rubra* plants, which lead to an increase in the activity of glutathione reductase when compared with the control.

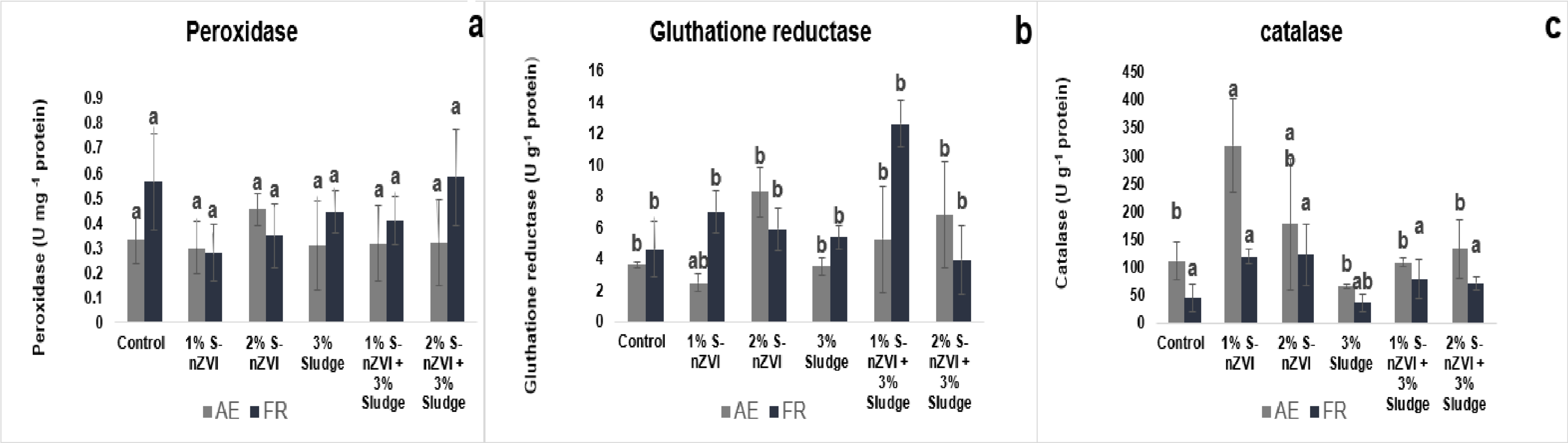

Fig. 8 **(a)** Peroxidase **(b)** Gluthatione reductase **(c)** Catalase values in *Festuca Rubra* (FR) and *Arrhenatherum elatius* (AE) at the end of the pot experiment for different amendments. Statistical evaluation was performed separately for each plant species. Data with the same letter represent statistically identical values ($P < 0.05$) ($n = 5$)

4.6. Plant digestion

The concentration of As and Pb in plant tissues is depicted in Fig. 9 and 10 below. In all amended and controlled soils, As and Pb concentrations in the roots and shoots of both plants demonstrated that roots were the preferred storage organ for these metal(loid)s. For both *Arrhenatherum elatius* and *Festuca rubra* plants, there was no difference in the concentration of As in the shoots when compared to the control, except 2% S-nZVI, which caused a decrease in the concentration of As in the shoots of *Arrhenatherum elatius* plants. For *Festuca rubra* plants, the concentration of As was decreased in samples treated with 1% S-nZVI, 2% S-nZVI, and combination of 2% S-nZVI and 3% sewage sludge, while samples amended with 3% sewage sludge alone and samples amended with 1% S-nZVI and 3% sewage sludge tended to show the increase in the concentration of As in the roots of *Arrhenatherum elatius* plants but the difference was statistically insignificant.

The *Arrhenatherum elatius* plants treated with 1% S-nZVI, 2% S-nZVI, a mixture of 1% S-nZVI with 3% sewage sludge, and 2% S-nZVI with 3% sewage sludge showed a decrease in the As root concentrations with the minor uptake of As in the samples amended with a combination of 2% S-nZVI with 3% sewage sludge.

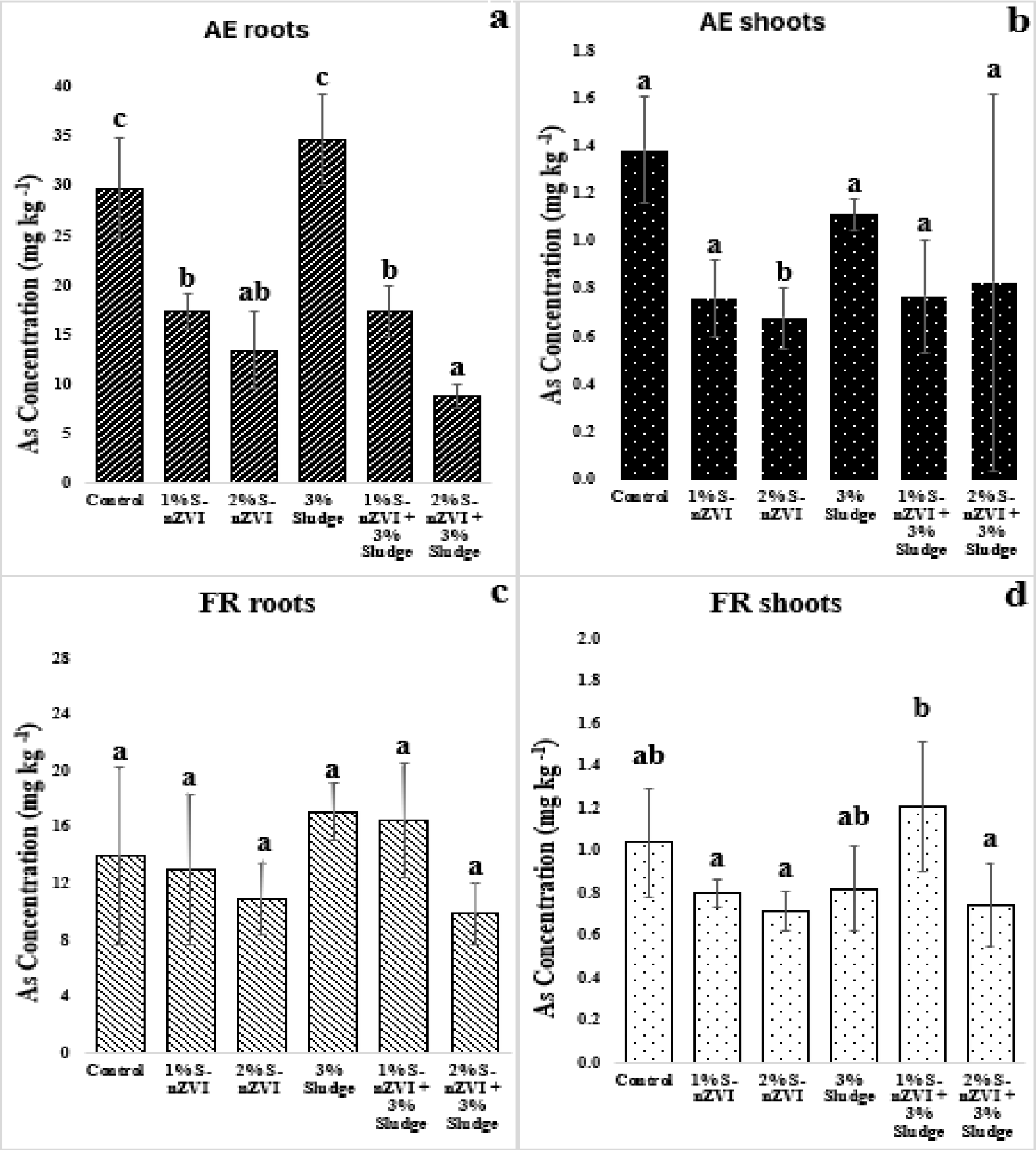

Fig. 9. Concentration of **(a)** As in *Arrhenatherum elatius* roots, **(b)** As in *Arrhenatherum elatius* shoots, **(c)** As in *Festuca rubra* roots, **(d)** As in *Festuca rubra* shoot at the end of pot experiment for different amendments. Statistical evaluation was performed separately for each plant species Data with the same letter represent statistically identical values ($P < 0.05$) ($n = 5$).

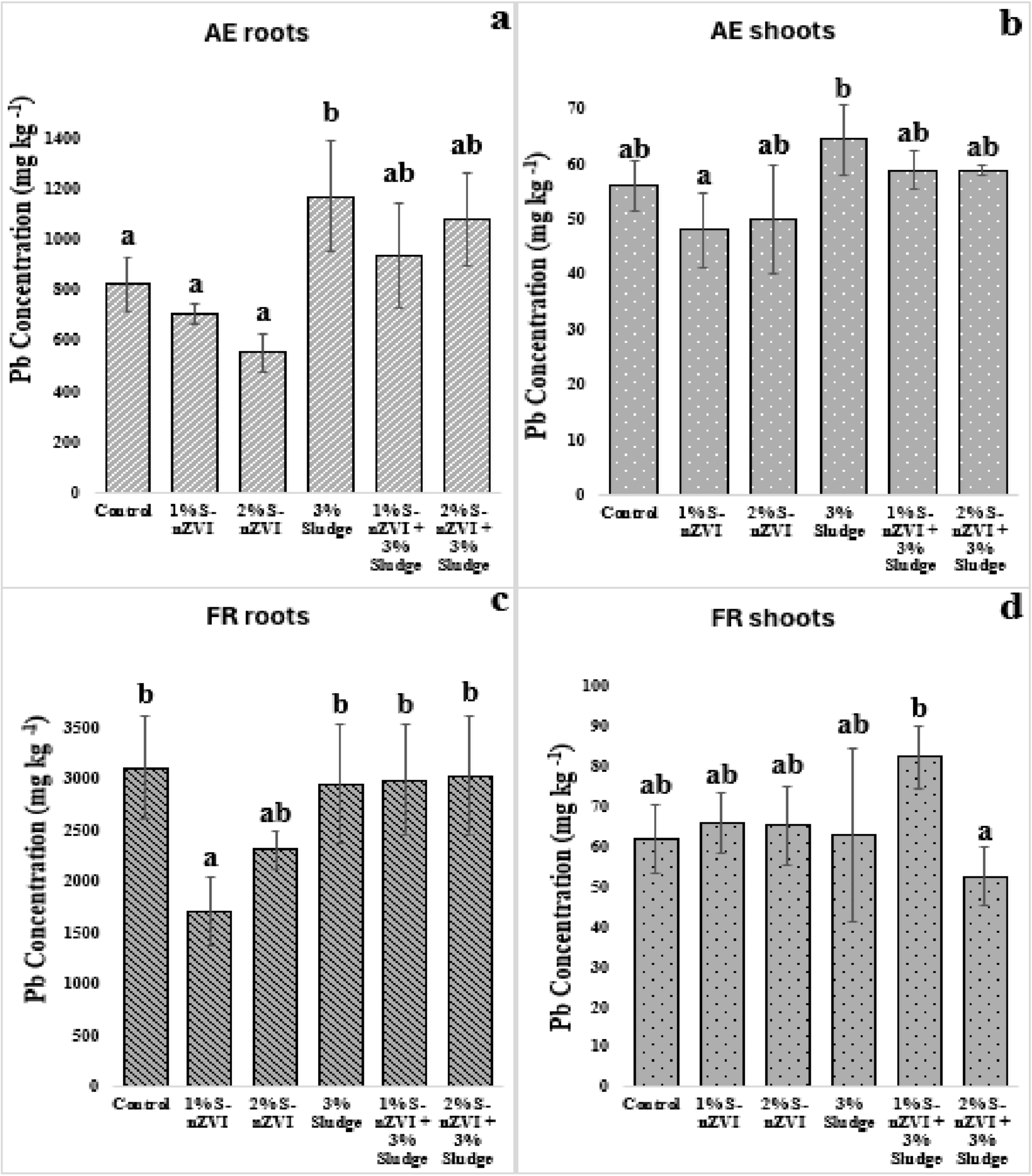

Fig. 10. Concentration of **(a)** Pb in *Arrhenatherum elatius* roots, **(b)**Pb in *Arrhenatherum elatius* shoots, **(c)** Pb in *Festuca rubra* roots, **(d)** Pb in *Festuca rubra* shoot at the end of pot experiment for different amendments. Statistical evaluation was performed separately for each plant species. Data with the same letter represent statistically identical values ($P < 0.05$) ($n = 5$).

No amendment influenced the concentration of Pb in shoots of both *Festuca rubra* and *Arrhenatherum elatius* plants. At the same time, 3% sewage sludge increased the concentration of Pb in the root of the *Arrhenatherum elatius* plant. Dar et al. (2015) also recorded an increase in the concentration of Pb in the roots of mustard after treatment with sewage sludge. For *Festuca rubra* plants, 1% S-nZVI led to a 45% decrease in the concentration of Pb in the root of *Festuca rubra* plants compared to the control.

The concentration of Cd and Zn in both *Arrhenatherum elatius* and *Festuca rubra* plants is depicted in Fig. 11 and 12 below. The concentrations of Zn and Cd in the shoots were much lower than in the roots, indicating that both plants prevent the translocation of Zn and Cd to the shoot, thus protecting the shoot from severe contamination. Namdari et al. (2024) reported a similar finding in *Zea mays* L., where the roots prevent the translocation of Zn and Cd to the edible part of the plant. Other researchers made similar observations while studying the metal accumulation by different plant species (Rezapour et al., 2019). For *Arrhenatherum elatius* plants, no amendment had an influence on the uptake of Cd in both shoots and roots. For *Festuca rubra* shoots, 2% S- nZVI caused an increase in the concentration of Cd. In comparison, samples amended with 3% sewage sludge and the mixture of 1% S-nZVI and 2% S-nZVI with 3% sewage sludge showed a decrease in the concentration of Cd, with the least Cd found in the 3% sewage sludge variant. Cd concentration in *Festuca rubra* roots was increased in variants treated with 1% and 2% S-nZVI compared to the control. The concentration of Zn in the shoots of *Arrhenatherum elatius* was reduced in samples treated with 2% S-nZVI when compared to the control, while 3% sewage sludge and the co-application of 2% S-nZVI and a mixture of 3%

sewage sludge caused an increase in the concentration of Zn in shoots of *Arrhenatherum elatius* plants in comparison to the control, a similar trend was also observed in the roots of *Arrhenatherum elatius* plants.

This increase in the concentration of Zn in all variants with sewage sludge (3% sewage sludge, 1% S-nZVI + 3% sewage sludge, and 2% S-nZVI + 3% sewage sludge) may be attributed to the bioavailability of Zn in sewage sludge-treated samples as Zn has higher mobility than other studied metals (Rezapour et al., 2019). Results for other elemental concentrations in *Arrhenatherum elatius* plant shoots, *Arrhenatherum elatius* roots, *Festuca rubra* shoots, and *Festuca rubra* roots are presented in Appendix 1, 2, 3, and 4, respectively.

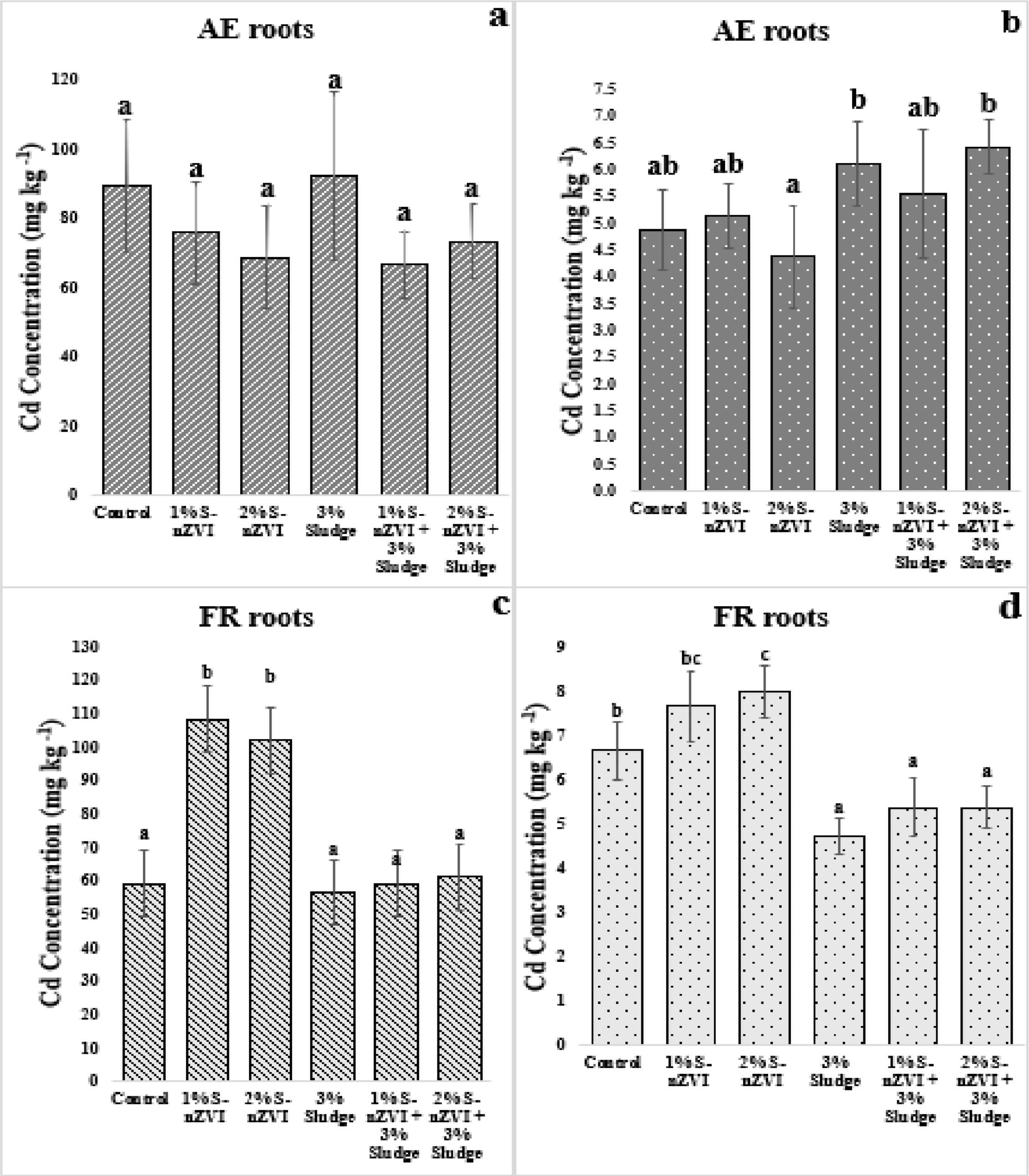

Fig. 11. Concentration of **(a)** Cd in *Arrhenatherum elatius* roots, **(b)** Cd in *Arrhenatherum elatius* shoots, **(c)** Cd in *Festuca rubra* roots, **(d)** Cd in *Festuca rubra* shoot at the end of pot experiment for different amendments. Statistical evaluation was performed separately for each plant species. Data with the same letter represent statistically identical values ($P < 0.05$) ($n = 5$)

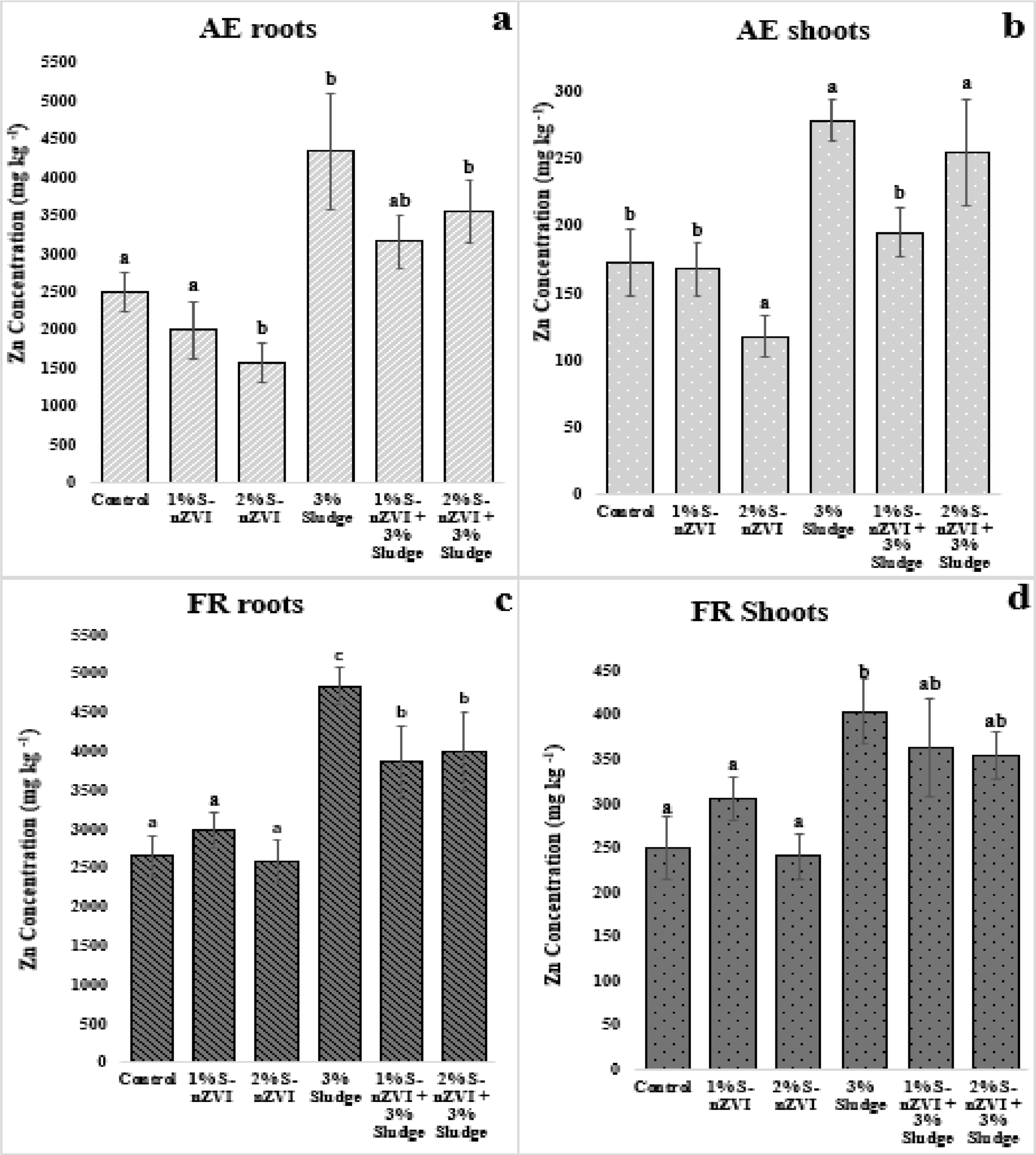

Fig. 12. Concentration of **(a)** Cd in *Arrhenatherum elatius* roots, **(b)** Cd in *Arrhenatherum elatius* shoots, **(c)** Cd in *Festuca rubra* roots, **(d)** Cd in *Festuca rubra* shoot at the end of pot experiment for different amendments. Statistical evaluation was performed separately for each plant species. Data with the same letter represent statistically identical values ($P < 0.05$) ($n = 5$).

5. Conclusions

5.1. Experiment one

Composted sewage sludge was combined with sulfidated nano zero-valent iron (S-nZVI) to test their potential to lower Pb, Cd, and Zn leachability. The release of Fe from the applied amendments was also examined. Interestingly, the most effective method for lowering the release of certain pollutants in batch trials was the addition of Fe grit. In contrast, column studies showed that the combined application of S-nZVI and composted sludge was the most effective. For TCLP, the leachability decreased in the following order: Zn > Pb > Cd. The pH is the most crucial driving parameter for Zn removal by nZVI in soil solution, as is the case with many, if not most, of the research examining metal(loid) leaching. Depending on the testing conditions as observed by our findings, both amendments (Fe grit and a combination of composted sludge and S-nZVI) have shown to be successful; Fe grit is the more economical of the two and may be taking advantage of using inexpensive waste material.

The short- and long-term toxicological impacts of Fe grit and S-nZVI should be assessed. It is advised that future research employ larger doses beyond 1% to examine the stabilization potential of samples amended with composted sludge, as they had little to no effect on the stability of pollutants in the batch and column experiments conducted in this work. The results demonstrate the effectiveness of S-nZVI in stabilizing metal(loid)s.

However, since most of the toxicological data available now come from short-term tests, more research is required to explore the long-term assessment of S-nZVI, particularly in the field, to understand any possible environmental risk better. Finally, we recommend utilizing both short- and long-term environmental monitoring, emphasizing S-nZVI's ecotoxicological effects and its persistence and transport.

5.2. Experiment two

The effect of S-nZVI with and without the co-application of sewage sludge was observed on two plant species (*Arrhenatherum elatius* and *Festuca rubra*), including plant growth (plant height, and biomass), plant pigments, oxidative stress, and nutrient uptake. In general, the coapplication of S-nZVI with sewage sludge did not significantly influence the growth and biomass of both plants, with 2% S-nZVI having the highest impact in terms of growth and biomass increase in plants. Variations were observed in the plant pigment in both plants, with most amendments resulting in increased plant pigments. Individual and co-application of S-nZVI with sewage sludge (1% S-nZVI; 2% S-nZVI; 1% S-nZVI + 3 % sewage sludge and 2% S-nZVI + 3% sewage sludge) was able to stabilize Pb and Cd in soils, thus reducing its uptake in plants, with the combination of 2% S-nZVI + 3% sewage sludge being the most effective of the four variants.

There was little to no influence of antioxidant enzymes in most variants across treatments for both plants. Although both plant responses regarding growth and biomass were the same regarding treatments, their tolerance and uptake of metal(loid)s differ from our findings, showing *Festuca rubra* as the most tolerant plant of the two species. Future work should try different concentrations of sewage sludge in the coapplication with S-nZVi in plant research to better understand this amendment's dose dependency, synergy, or antagonistic behavior. Also, investigation should dive deeper into plant genetic material to better understand the signaling pathway controlling the co-application of these two amendments.

6. Limitations and recommendations

Several limitations of the work presented here remain for future exploration:

1) All studies presented were conducted under controlled situations, e.g., laboratory or pot experiments. Therefore, field-scale testing is required to understand amendments' influence under natural environmental conditions.

2) Detailed or elaborate photosynthesis parameters—like the rate at which carbon dioxide (CO_2) is assimilated, the amount of CO_2 inside cells, the rate at which plants transpire, and how the stomatal conductance of leaves exposed to nZVI—have changed. It should be studied to assess how the application of nZVI affects plant photosynthesis more thoroughly.

3) Several doses of sewage sludge should be used below and above in these studies to understand better the ideal concentration suitable to enhance overall plant health and performance.

4) Further research should probe the synergy or antagonist influence of the co-application of sewage sludge and S-nZVI and the molecular mechanism that drives the pathways of their interactions with plants.