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Zero valent iron nanoparticles and organic fertilizer assisted phytoremediation in a mining soil: Arsenic and mercury accumulation and effects on the antioxidative system of *Medicago sativa* L.

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HIGHLIGHTS

- Organic fertilizer coupled to nZVI improved phytostabilization.
- Plants increased NPT content in order to avoid negative impact on metabolism.
- nZVI treatment immobilized As and Hg, while organic fertilizer mobilized them.
- nZVI treatment diminished oxidative stress in *Medicago sativa*.
- Organic fertilizer treatment improved the bioaccumulation of As and Hg.

GRAPHICAL ABSTRACT
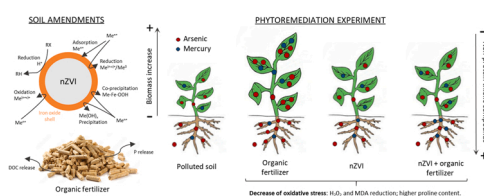
ARTICLE INFO

ABSTRACT

Zero valent iron nanoparticles (nZVI) attract interest given their effectiveness in soil remediation. However, little attention has been given to their impacts on plants. Likewise, although fertilizers are commonly used to enhance phytoremediation, their effects on As mobilization, resulting in potential toxic effects, require further study. In this context, we examined the impact of As and Hg accumulation on the antioxidative system of *Medicago sativa* grown in a soil amended with organic fertilizer and/or nZVI. The experiment consisted of 60 pots. Plants were pre-grown and transferred to pots, which were withdrawn along time for monitoring purposes. As and Hg were monitored in the soil-plant system, and parameters related to oxidative stress, photosynthetic pigments, and non-protein thiol compounds (NPTs) were measured. In general, the application of nZVI immobilized As in soil and increased Hg accumulation in the plant, although it surprisingly decreased oxidative stress. Plants in nZVI-treated soil also showed an increase in NPT content in roots. In contrast, the application of the fertilizer mobilized As, thereby improving bioaccumulation factors. However, when combining fertilizer with nZVI, the As accumulation is mitigated. This observation reveals that simultaneous amendments are a promising approach for soil stabilization and the phytomanagement of As/Hg-polluted soils.

1. Introduction

The use of organic amendments for the reclamation of degraded soils, such as those found in mining regions, has grown in recent decades (Conesa and Párraga-Aguado, 2021; Karami et al., 2011; Kumpiene et al., 2008). However, these amendments have several disadvantages that need to be addressed. Organic fertilizers made from animal waste commonly contain certain metal(loid)s, as well as considerable amounts of dissolved organic carbon (DOC) and P, which are factors that can mobilize metalloids such as As (Arabi et al., 2021; Souza et al., 2020).

Therefore, these types of amendments require modification before being applied to soils, for example, by presenting them in the form of slow-release fertilizers or by combining them with other types of treatments, such as nanoremediation (Baragaño et al., 2020a; Mitzia et al., 2020).

Nanomaterials comprise a new cross-disciplinary research field that has developed along with the implementation of sustainable development strategies (Baragaño et al., 2021; Luo and Deng, 2018). Given their results at laboratory and field scale, nanoparticles, in particular zero valent iron nanoparticles (nZVI), emerge as a promising approach for the treatment of polluted soils (Baragaño et al., 2020b; Gil-Díaz et al., 2019; Teodoro et al., 2020). The immobilization of metal(loid)s in soils by nZVI involves adsorption reactions between the metal(loid) and the nanoparticles, coprecipitation of the metal(loid) with newly formed oxides, and the formation of secondary minerals (Chapman et al., 2020; Hiller et al., 2021). However, nZVI do not provide the soil with nutrients, so their application cannot solve a deficit of nutrients or organic matter. In this regard, nZVI can be combined with organic amendments (Baragaño et al., 2021, 2020a; Liu and Lal, 2012). This combined strategy leads to successful yields (Hemati Matin et al., 2020; Xu et al., 2016), and synergistic effects (Mitzia et al., 2020).

Mining activities are a potential source of highly toxic pollutants, as for example As and Hg. These elements are attracting high attention in the last years due to their risk to public health (Boente et al., 2022). In this sense, there is a global need to mitigate the pollution produced by both, especially for Hg, as it was claimed in the Minamata Convention. To solve this, stable vegetation cover on such mining sites should be developed, and it can be achieved through the application of organic amendments, which improve soil quality (Forján et al., 2018). However, such amendments tend to mobilize anions, such as As, due to the increase in pH, and the interaction between the DOC and the P that they provide (Beesley et al., 2010). Given these considerations, the effect of combining these amendments with nZVI is of great interest. nZVI are effective at decreasing available As in soil (Baragaño et al., 2020c; Gil-Díaz et al., 2019); however, there is little information about the effects of this nanomaterial on plant physiology (Gul et al., 2020; Martínez-Fernández et al., 2015; Rizwan et al., 2019). For example, Huang et al. (2018) revealed that the application of low doses of nZVI promote Pb phytoremediation using *L. perenne*, although high doses of nZVI showed inhibitory effects on plant growth and high oxidative stress.

When metal(loid)s are taken up by roots and reach the cell cytoplasm, the most common detoxification mechanism is chelation and consequent compartmentalization in the vacuole (Vázquez et al., 2009). Low molecular weight compounds such as phytochelatins, homophytochelatins, and other thiolic compounds are synthesized by plants to bind to metal(loid)s through functional thiol groups, thereby forming less toxic complexes that are later compartmentalized in the vacuole (Fernández et al., 2013). In contrast, metal(loid)s that are not chelated through thiols or other mechanisms, and that remain free inside cells, enhance the generation of reactive oxygen species (ROS). These molecules then disrupt metabolic pathways and cause severe damage to proteins, membrane lipids, and DNA, thus leading to oxidative stress (Del Río and López-Huertas, 2016). To cope with these stress conditions, plants have antioxidant systems involving enzymes and non-enzymatic antioxidant compounds that maintain homeostasis in all compartments (Martí et al., 2009). The effectiveness of the chelation and antioxidant systems depends mainly on the tolerance of the plant to metal(loid) exposure.

La Soterraña site (Asturias, Spain) is greatly affected by Hg and As pollution. It was abandoned in the 1970s after the cessation of mining and metallurgy activities, and no environmental remediation efforts have been made (Fernández et al., 2020). The extractive and processing activities caused the mobilization of As and Hg, thus affecting the surrounding soils (Boente et al., 2022). Soil pollution on this site is also accompanied by a deficit in nutrients and organic matter, thereby hindering the development of stable vegetation cover (Matanzas et al.,

2017). The characteristics of La Soterraña make it an ideal site on which to apply a soil chemical stabilization strategy combined with a phytoremediation approach using *Medicago sativa L.*, a species that is highly resistant to high metal(loid) concentrations in soil, and it is a widely used fodder plant of great interest.

In this context, here we examined the efficacy of the chemical stabilization of soil coupled to phytoremediation using nZVI and organic fertilizer, alone and combined, in the soil of a paradigmatic As- and Hg-polluted site. In addition, to understand the impact of the amendments on plant physiology, we monitored oxidative stress, photosynthetic pigments, and thiolic compound content.

2. Materials and methods

2.1. Soil sampling and amendments

Located in northern Spain (Asturias), La Soterraña mine is one of the largest abandoned Hg mine sites in Europe. In this area, mining, ore processing and metallurgy activities were performed for decades (Matanzas et al., 2017). In addition to high Hg concentrations (0.6–860 mg/kg), this site also has high As concentrations (22–9920 mg/kg) because of the paragenesis of the ores from which the Hg was extracted (Boente et al., 2022). The development of these activities, in particular polluting steams and particles, and the dumping of several waste products has affected the environmental compartments (Fernández et al., 2020).

To conduct the remediation feasibility study, a composite soil sample of 20 kg was collected from the first 25 cm of soil in the surroundings of La Soterraña (N 43°11′38.15″, W 5°50′41.40″). Once in the laboratory, the soil was air-dried, mixed for 30 min in a concrete mixer to maximize homogeneity, and then passed through a 2-mm sieve. The organic fertilizer, Phenix, was supplied by the company Italpollina (Verona, Italia). It is made from animal manure and plant debris and has an N:P:K ratio of 6:8:15, respectively. The zero valent iron nanoparticles (nZVI), namely NANOFER 25S, were supplied by NANO IRON s.r.o. (Brno, Czech Republic). According to commercial specifications, this product contains 14–18% of iron and 2–6% of magnetite. Nanoparticles are covered with a polyacrylic acid (PAA) to prevent particles agglomeration. The particles have an average size of around 60 nm, the suspension is strongly alkaline (pH 11–12), and the active surface area is 20 m^2/g (additional details are available at www.nanoiron.cz). A more extensive characterization is provided in a previous study (Baragaño et al., 2020b), revealing that zeta potential was negative due to the polyacrylic acid (PAA) used for coating the nanoparticles and decreasing agglomeration in water suspension.

2.2. Physico-chemical analysis of soil samples

In brief, pH was measured in a 1:2.5 soil-to-water ratio (w/v) using a multimeter Mettler Toledo, organic matter was assessed by the loss-on-ignition method, and grain size was determined using a Laser Diffraction Particle Analyzer (Beckman Coulter Inc.). The concentration of pseudo-total metal(loid)s was determined after acid digestion with a mixture of 6 mL nitric acid (69% purity) and 2 mL of hydrochloric acid (37% purity), in a microwave reaction system (Milestone ETHOS 1, Italy). The concentration of As, Cd, Cu, Hg, Ni, Pb and Zn in the digestion extract was quantified by Inductively Coupled Plasma Mass Spectrometry (ICP-MS 7700; Agilent Technologies, California, USA) using Isotope Dilution Analysis (IDA) with a spike solution from ISC Science Spain. High-purity standards (Charleston, SC, USA) for calibration and a Certified Reference Material (CRM) (soil, ERM-CC018) were used. Detection limit for all elements is 0.1 µg/l, except for Cu, Ni and Zn, which is 0.25 µg/l. Available As and Hg was determined by means of EDTA 0.05 M, adjusting pH to 7 using NH_4OH (Quevauviller, 1998). The extractant was added to the soil in a 1:10 ratio (soil:extractant, w-v) and was shaken for 1 h at room temperature. Samples were centrifuged at 4000 *g*

for 15 min, and the supernatant was analyzed using ICP-MS. Triplicate samples were measured in order to diminished standard deviation for all analysis.

2.3. Plant material and growing conditions

Medicago sativa L. (alfalfa) was selected because it is a very fast-growing grass species and it has been widely used in metal(loid) studies (Carrasco-Gil et al., 2012; Zhou et al., 2008). Seeds of *M. sativa* L. were sterilized with 5% commercial bleach (50 g sodium hypochlorite L^{-1}) in a laminar flow chamber for 5 min followed by 3 × 5-min rinses with distilled water. They were then maintained for 3 days in a cold chamber to break dormancy. After this period, they were placed in Petri dishes with filter paper and sterile distilled water and were kept in darkness at 25 °C for 4 days in a growth chamber. They were then subjected to a 16-h photoperiod for 7 days. Seedlings were transferred to multipots filled with peat and then moved to a greenhouse for 35 days until they reached a height of between 9 and 11 cm. The shoot and root length, and fresh weight of each plant were measured before transplanting into pots with 150 g of treated or untreated soil. The pots with untreated soil (non-amended polluted soil from La Soterraña) were labeled as S. The following three amendment strategies were tested: for the nanoparticle treatment (SN), and based on previous studies (Baragaño et al., 2020b, 2020c, 2020d), a 2% in weight of a nZVI water suspension was added to the soil (which means a dose of 0.4% of nZVI); for the organic fertilizer treatment (SF), soil was treated with a 5% dose of organic fertilizer; and for the combined organic fertilizer plus nZVI treatment (SNF), soil was treated with nZVI and the organic fertilizer at the same doses, 2% and 5% respectively. These soil treatments were applied 10 days before transferring plants to the pots for achieving soil stabilization. A total of 30 plants per treatment were prepared. After 1, 3 and 10 days, 10 plants from each treatment were washed with water, rinsed 3 times with double deionized water for 5 min, and divided into shoots (leaves and stems) and roots. Shoot and root length, and fresh weight were again measured. Samples were then powdered using liquid nitrogen and stored at − 80 °C before analysis.

2.4. Metal(loid) concentrations and thiolic compounds in plants

To determine the concentration of metal(loid)s in the plants, 100 mg of dried and powdered shoots and roots was digested using 3 mL of concentrated HNO$_3$ and highly purified in a microwave oven for 6 min at 240 W. After cooling the digestion extracts, two consecutive dilutions were made and a solution of 10 μg Rh kg^{-1} concentrated HNO$_3$ was added as an internal standard (Fernández et al., 2008). Blanks without plant biomass were also performed following the same procedure, and the method was validated by measuring a certified reference sample (BCR-CRM 100 beech leaves). Relative standard deviation (RSD) values during the measurement for As and Hg were below 2%. The concentration of As and Hg in plant tissues was determined by ICP-MS.

The translocation factor (TF), defined by Baker and Brooks (1989), was determined using the following equation:

$$TF = \frac{C_s}{C_r} \qquad (1)$$

where C_s is the As or Hg concentration in the plant shoots and C_r is the As or Hg concentration in the plant roots, both in mg kg^{-1}. A plant translocates As or Hg effectively from the roots to shoots when TF > 1.

We also determined the bioaccumulation factor (BAF) in the plants, which measures their efficiency to take up metal(loid)s from the soil, assessed by taking the available forms instead of the soil pseudo-total concentrations as a reference (Rodríguez-Vila et al., 2014), as expressed by the following equation:

$$BAF = \frac{C_{ap}}{C_s} \qquad (2)$$

where C_{ap} is the metal(loid) concentration in the aerial part (mg kg^{-1}) and C_s is the metal(loid) concentration in the soil (mg kg^{-1}).

Non-protein thiol (NPTs) compounds were analyzed by high-performance liquid chromatography (HPLC) following the method described by Rauser (1991) and modified by Fernández et al. (2012). To this end, 500 mg of powdered frozen shoots or root was mixed with 1% of polyvinylpolypyrrolidone and 750 μl of 0.1 N HCl. The mixture was shaken for 30 s, sonicated for 5 min and, after shaking again for another 30 s, it was centrifuged at 15,000 *g* for 15 min at 4 °C. The supernatant was passed through a Millex-HV filter (45 μm diameter, Merck Millipore). Finally, the filtrate (100 μl) was injected directly into the HPLC apparatus (Waters 600, Water Corporation) and subjected to post-column derivatization with Ellman's reagent (Ellman, 1959). The derivatized thiols were detected at 412 nm using a Waters 996 photo-diode array detector, and the peaks obtained were identified by comparison with the standards of GSH and a mix of PCs (PC2, PC3, PC4, PC5 and PC6) and hPCs (hPC2, hPC3 and hPC4). The quantitative changes in the NPTs were calculated by the integration of their peak areas at 412 nm of absorbance converted into nmol and quantified in terms of GSH equivalents.

2.5. Parameters related to oxidative stress

Chlorophyll and carotenoids were extracted from 200 mg of frozen leaves using 2 mL of a solution of 80% acetone. The mixture was then centrifuged at 6000 *g* for 5 min before measuring absorbance. The pigment content was determined following Porra (2002) and Lichtenthaler and Wellburn (1983) using the equations:

$$Chlorophyll\ a\ (Chl\ a) = 12.21\ Abs_{663} - 2.81\ Abs_{646} \qquad (3)$$

$$Chlorophyll\ b\ (Chl\ b) = 20.13\ Abs_{646} - 5.03\ Abs_{663} \qquad (4)$$

$$Carotenoids = \frac{1,000\ Abs_{470} - 3.27\ C_a - 104\ C_b}{229} \qquad (5)$$

The hydrogen peroxide (H$_2$O$_2$) content was measured following Singh et al. (2006) with some modifications. First, 100 mg of leaves or roots were mixed with 2.5 mL of 0.1% trichloroacetic acid (TCA) and centrifuged at 10,000 *g* for 15 min at 4 °C. Next, 0.5 mL of supernatant was mixed with 0.5 mL of 100 mM phosphate buffer (pH 7.6) and 1 mL of 1 M KI. The absorbance of the samples was measured at 390 nm, and the H$_2$O$_2$ content was determined using a calibration curve and expressed as μmol g^{-1} fresh weight (FW).

Lipid peroxidation was monitored by measuring the formation of malondialdehyde (MDA) in leaves and roots (Demiral and Türkan, 2005; Fernández-Fuego et al., 2017). MDA was extracted by homogenizing 100 mg of frozen leaves or roots with 2.5 mL of 0.1% TCA. The mixture was then centrifuged at 10,000 *g* for 15 min at 4 °C, and 2 mL of 20% TCA with 0.5% thiobarbituric acid was then added to a 0.5 mL aliquot of the supernatant. The samples were heated at 95 °C for 30 min and quickly cooled in an ice bath. Finally, they were centrifuged again at 10,000 *g* for 15 min, and the absorbance of the supernatant was measured at 532 nm (ε = 155 mM^{-1} cm^{-1}). Absorbance at 600 nm was used to correct non-specific turbidity. MDA content was determined using the Lambert-Beer equation and was expressed as μmol g^{-1} FW.

$$MDA = \frac{Abs_{532} - Abs_{600}}{\varepsilon} \qquad (6)$$

Proline content was determined using the method described by Bates et al. (1973) and slightly modified by Demiral and Türkan (2005). To this end, 100 mg of sample—leaves or roots—was homogenized with 1.5 mL 3% sulfosalicylic acid and centrifuged at 10,000 *g* for 15 min at 4 °C. Next, 0.5 mL of supernatant was mixed with 0.5 mL of acid ninhydrin and glacial acetic acid (1:1 v/v) and heated in a water bath at 100 °C for 1 h. Samples were cooled using ice, and 2.5 mL of toluene was added to each tube. After two phases were generated, the upper one was

measured, determining the absorbance at 520 nm. Proline content was determined using a calibrating curve and expressed as µmol g^{-1} FW.

2.6. Statistical analysis

All analytical determinations were performed in triplicate. The data obtained were statistically treated using the SPSS program, version 27.0 for Windows. Analysis of variance (ANOVA) and test of homogeneity of variance were carried out. In the case of homogeneity, a post hoc least significant difference (LSD) test was done. If there was no homogeneity, Dunnett's T3 test was performed.

3. Results and discussion

3.1. Chemical characterization of soil

The untreated soil (S) markedly exceeded the concentrations of As and Hg established by the Soil Screening Levels (SSL) for metal(loid)s in Asturias (BOPA, 2014; the limits established for As and Hg are 40 and 1 mg/kg respectively) (Table S1). However, the available concentrations of these two elements (Table S1) were clearly lower as they were present in the residual fraction. Their presence in this fraction is attributable to the paragenesis of the mineralization, which is formed by cinnabar in the case of Hg and iron sulfides or oxides for As (Boente et al., 2022). Although the available concentration of As and Hg was low, there is a significant risk of these metal(loid)s entering the trophic chain because they can be directly absorbed by plant root systems and can accumulate at toxic levels in edible parts (Li et al., 2010).

Regarding the other metal(loid)s, namely Cd, Cu, Ni, Pb and Zn, they did not exceed the limits established by the SSL (Table S1). The soil had an acidic pH, which could hinder the development of stable vegetation, although the organic matter content (%) was within the normal values for a soil (Table S1).

3.2. Impact of amendments on soil pH and available As and Hg

The soil in the S, SF, SN and SNF treatments showed an acidic pH (Table S2). The addition of fertilizer did not cause a significant change in pH; however, we observed an increase when nZVI were used. In this regard, at the end of the experiment (10 days), the SN treatment showed the highest soil pH values (Table S2). The increase in pH caused by this treatment is related to the alkaline water suspension (pH = 11) in which the nanoparticles were previously prepared, as observed in previous studies (Baragaño et al., 2020b, 2020d).

The available As did not change in the untreated soil (S) during the experiment (Fig. 1a). In contrast, three well-differentiated behaviors were detected in the treated soils. The application of SF increased the available As with respect to the control soil after 1 day of treatment, although no differences between S and SF were observed after 10 days. However, opposite effects were detected in the SN treatment after 1 and 10 days compared to S and SF. In the SNF treatment, the available As did not change at any monitoring point with respect to the control (Fig. 1a).

After 1 day, the highest As availability was observed in the SF treatment (Fig. 1a). This increase could be due to the composition of the organic fertilizer added, namely animal manure and plant residues, which supply DOC and P to the soil and usually lead to an increase in available concentrations of metalloids such as As (Kumpiene et al., 2008; Pardo et al., 2014). Other authors also (Hou et al., 2014; Liu and Lin, 2013) concluded that one of the main factors involved in the increased availability of As by organic fertilizers is the increase in DOC content in soil. The ligands of DOC facilitate the transfer of As from the unavailable to the available fractions through the formation of soluble complexes. In addition, the increase in soil phosphate content caused by the addition of a fertilizer can also enhance the amount of available As (Gunes et al., 2009).

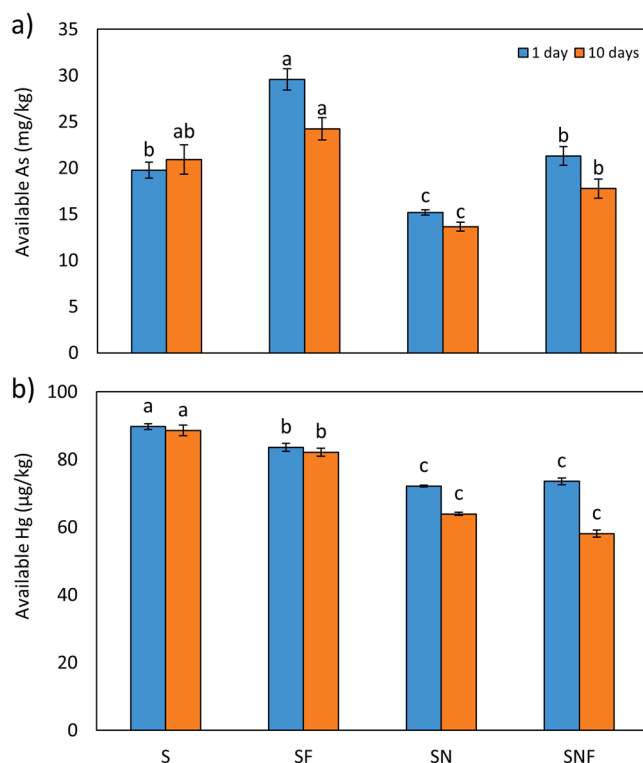The decrease in available As after the application of nZVI when

Fig. 1. Available concentrations of As (a) and Hg (b) in the untreated soil (S) and soil after treatment with organic fertilizer (SF), nZVI (SN), and both amendments (SNF) after 1 and 10 days. For each time, data with the same letter do not differ significantly (p < 0.05).

compared to the S treatment is consistent with the data obtained by Baragaño et al. (2020b, 2020d). Arsenic is immobilized by nZVI via inner-sphere surface complexation onto iron oxides in the shell circling the nanoparticle (Gil-Díaz et al., 2019). As mentioned above, the direct application of nZVI does not provide nutrients, but this can be compensated by adding organic amendments without causing an increase in As mobility (Baragaño et al., 2020a). This was corroborated in this study as the SNF treatment did not lead to a significant increase in available As with respect to the control soil at the end of the experiment (10 days).

On days 1 and 10, the amendments (SF, SN, SFN) caused a significant decrease in the available Hg in soil when compared to the control (Fig. 1b). The treatments that included nZVI (SN, SFN) showed the best Hg immobilization results at the end of the experiment (Fig. 1b). The organic matter provided by the fertilizer can form complexes with Hg since this metal binds to organic particles, thereby decreasing its availability (Mahbub et al., 2017). The addition of organic matter to the soil by the organic fertilizer could increase the concentrations of OH$^-$ and therefore augmented the negative charges, resulting in enhanced adsorption efficacy (Wang et al., 2020). Boosting soil nutrient content also increases microbial activity, and some microorganisms can reduce Hg^{2+} to less toxic and insoluble Hg^0 (Pepi et al., 2013; Wagner-Döbler, 2003). In turn, the increase in pH caused by nZVI in the SN and SFN treatments may be one of the reasons for the decrease in available Hg, in agreement with Wang et al. (2020). The main mechanism by which nZVI decreases available Hg is via reduction (Latif et al., 2020). Given that the SN treatment caused the highest increase in soil pH and also considering the strong reduction process caused by the nanoparticles, it is consistent that the SN treatment was the most effective at decreasing available Hg, even when used in combination with the organic fertilizer. The efficacy of nZVI in decreasing available Hg is in agreement with the results obtained by Gil-Díaz et al. (2019).

3.3. Plant growth

The results regarding growth parameters and biomass production revealed the great capacity of *M. sativa* to adapt and tolerate the presence of metal(loid)s in soil, although the addition of fertilizer caused a significant increase in the harvested biomass of the plant compared to the S, SN and SNF treatments (Fig. 2). The second most effective treatments were SN and SFN, probably due to the immobilization of As and the addition of nutrients, respectively. This was expected since it has been demonstrated that the nutritional supply provided by organic fertilizers enhances plant development (Forján et al., 2017; Pedrol et al., 2014). However, the application of a fertilizer in combination with nZVI can cause the immobilization of some nutrients, such as P, which may explain the differences between the SF and SFN treatments (Baragaño et al., 2020d). On the other hand, although nZVI can improve plant development by preventing the translocation of pollutants to the plant, the nZVI application in combination with organic amendments or other fertilizers provides better yields (Baragaño et al., 2020a). Furthermore, we also monitored the length of the aerial part of the plants and detected similar trends to those of harvested biomass (Fig. 2).

3.4. As-Hg accumulation in plants

The concentration of As was higher than that of Hg in both the shoots and roots of *M. sativa* (Fig. 3), and several differences were found between the treatments. Regarding As, its concentration was always higher in roots than shoots (Fig. 3a). Similar results were also obtained by authors working with other species (Chen et al., 2015; González et al., 2019). These observations are logical as the root is the first organ of the plant in contact with the soil and highly toxic metal(loid)s can be adsorbed to the walls of root cells (Fernández et al., 2013) or can be quickly chelated and stored in cell vacuoles to prevent undesirable toxic effects (Fernández-Fuego et al., 2017; Navazas et al., 2021). Comparison

a)

b)

Fig. 2. Harvested biomass (a) and shoot length (b) of *M. sativa* after 10 days of growth in the polluted soil (S) and soil after treatment with organic fertilizer (SF), nZVI (SN), and both amendments (SNF). Data with the same letter do not differ significantly (p < 0.05).

of the amount of As accumulated in the different treatments revealed that the SF treatment caused the highest concentration in both shoots and root (Fig. 3a). This greater increase in the concentration of As in the shoots of the SF treatment and, to a lesser extent in the SFN treatment, was corroborated by significantly positive correlations between available As in the soil and As accumulated in the plants (0.81, P < 0.05). This increase could be due to As acting as a P analog and being taken up by plants via a P transporter system (Navazas et al., 2021). Phosphorus is more strongly adsorbed to soil than As and hence it competes for sorption sites, thereby facilitating the desorption of As to soil solution and its subsequent uptake by plants (Bolan et al., 2014). In addition to this P effect, the As mobilization caused by the fertilizer through the DOC would also make this metalloid more available to the plant (Gunes et al., 2009). Treatments involving nZVI did not show significant differences in the As accumulation at the end of the experiment when compared to the untreated soil (Fig. 3a). This observation can be explained by the capacity of the nanomaterial to reduce As availability. However, after nZVI application, As accumulation in plants could be decreased in the long-term because of ZVI aging and transformation onto iron oxides, which may suppose new reactive phases for As immobilization in soils.

Regarding Hg, the concentration of this metal was also consistently higher in roots than in shoots, although it accumulated in lower concentrations than As (Fig. 3b). However, such accumulation is also relevant due to the greater toxicity of Hg. Once again, the application of fertilizer caused an increase in Hg concentration in both the shoots and roots compared to the other treatments (Fig. 3b).

It is striking that the plants grown in the SF treatment accumulated the greatest amount of As and Hg and they also showed the longest shoots. Given that both elements are highly toxic for the plant, these observations suggest that the plant has a detoxification mechanism to reduce the toxicity of these elements, which could involve down-regulation of aquaporins or other transporters, as previously proposed (Lafuente et al., 2010; Navazas et al., 2021). In our case, this down-regulation would not have occurred since the concentration of As and Hg was higher in the SF treatment than in the untreated soil. Alternatively, the plants may have a chelation system for these elements that involves phytochelatins or other compounds (González et al., 2019).

Regarding the TF for As and Hg, both values decreased over the days in all the treatments (Table 1). For As, this value was always below 1, indicating the low capacity of this plant to translocate this element (Baker and Brooks, 1989). Reduced metal(loid) translocation to aerial parts has been reported to be a mechanism that allows plants to survive in extremely polluted soils. In the context of *M. sativa,* this mechanism is advantageous as this plant is used as fodder in cattle feed and since cattle eat only the aerial part of the plant, this mechanism would prevent As from entering the trophic chain in high concentrations. For Hg, the TF was also higher on day 1 than on day 10. In the SF and SNF treatments, this factor was greater than 1 but it decreased notably by day 10. This reduction in the accumulation of As and Hg along the experiment could be attributed to a plant defense mechanism. In this regard, these elements are highly toxic, and elevated concentrations of them in leaves could affect the photosynthetic apparatus or other processes and could cause irreversible effects on the plant (Mourato et al., 2019).

On the whole, these TF values indicate that *Medicago* cannot be considered a phytoextractor for As and Hg. Nevertheless, the use of this species in phytostabilization would reduce the time needed to clean contaminated soil, with the advantage that it has a low TF of metals to aerial tissues (Nouri and Haddioui, 2016; Pinto et al., 2015) and thus is not harmful to cattle. With regard to the bioaccumulation factor (BAF), two differentiated behaviors were observed. In the treatments that included nZVI (SN, SNF), the efficiency of *M. sativa* to take up As from the soil increased over the days. However, in the untreated soil (S) and soil treated only with fertilizer (SF), this efficiency decreased (Table 1).

The BAF values for Hg were much higher than those obtained for As (Table 1). Our results indicate that in untreated soil and soil amended with fertilizers or nZVI, *M. sativa* was more efficient at taking up Hg than
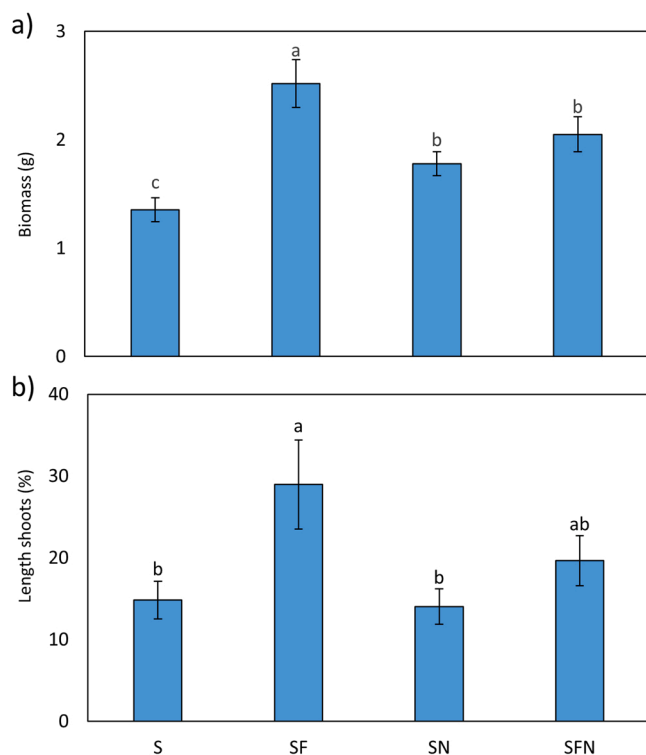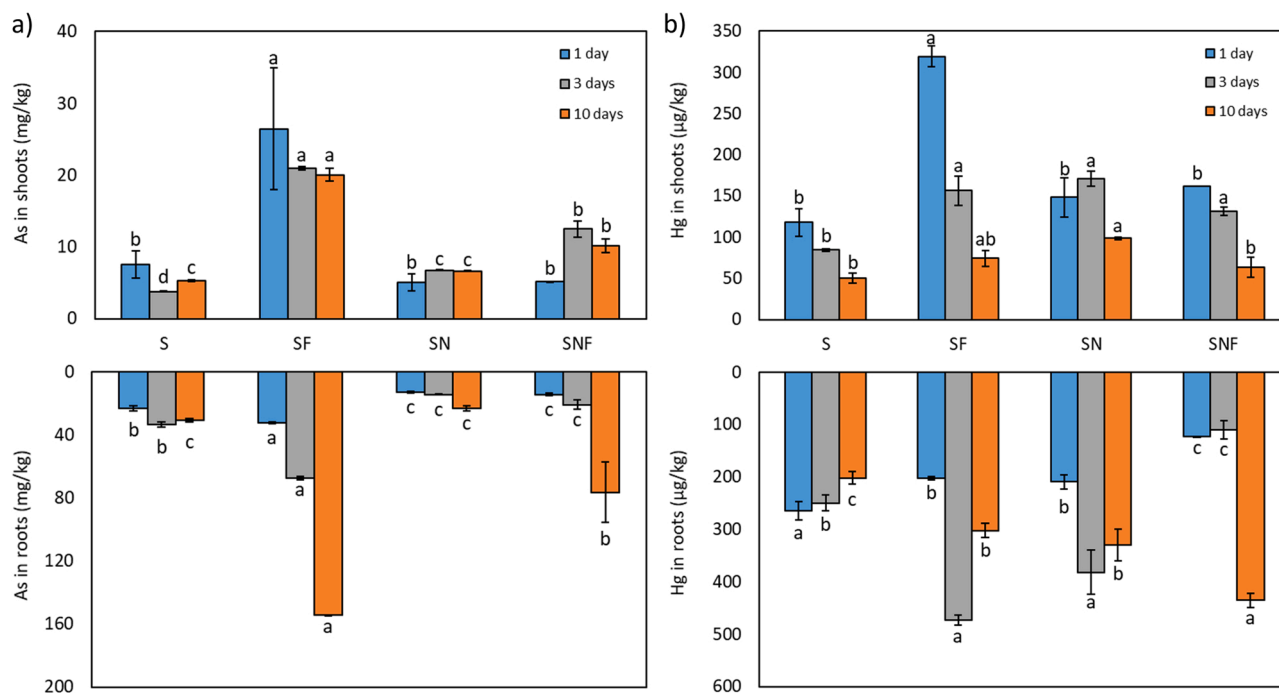
Fig. 3. As (a) and Hg (b) accumulation in the shoots and roots of *M. sativa* under the different soil treatments at days 1, 3 and 10 days. Different letters denote significant differences among treatments and in the same culture time. (p < 0.05).

Table 1

Transfer of As and Hg to *M. Sativa* according to the transfer factor (TF) and bioaccumulation factor (BAF) in polluted soil (S), and soil after treatment with organic fertilizer (SF), nZVI (SN), and both amendments (SNF) at days 1 and 10 of the experiment.

Factors	Pollutant	Time (days)	S	SF	SN	SNF
TF	As	1	0.33	0.82	0.39	0.36
		10	0.17	0.13	0.29	0.13
	Hg	1	0.44	1.58	0.71	1.32
		10	0.25	0.25	0.30	0.15
BAF	As	1	0.38	0.89	0.33	0.24
		10	0.25	0.83	0.49	0.57
	Hg	1	5.97	10.80	9.78	7.62
		10	2.40	3.06	7.21	3.57

As, although after 10 days this efficiency also decreased; however, it was always greater for Hg than for As (Table 1). This decrease in Hg adsorption by the alfalfa plant over time was also reported by Li et al. (2021), which can be due to the increase of phytochelatins along the experimental time.

3.5. Non-protein thiolic compounds

Table 2 shows the NPT content of the shoots and roots of *M. sativa* grown in the different experimental conditions. In all treatments except SNF, the total concentration of NPTs was higher in roots than in shoots, which is in agreement with a higher accumulation of As and Hg in these organs. Similar results were also obtained by various researchers working with other plant species (Chen et al., 2015; Fernández et al., 2013; González et al., 2019). The shoots of plants grown in the S treatment showed six NPTs, of which the most abundant were GSH and hGSH (Table 2). Furthermore, we also observed the presence of three compounds, which we were unable to identify and which we named CT2, CT5 and CT6 on the basis of their retention time. Both GSH and hGSH are low molecular weight thiol compounds that have a detoxification function against metal(loid)s but they are also precursors of the synthesis of phytochelatins and homophytochelatins, respectively

(Zagorchev et al., 2013). These thiols form complexes with toxic metal (loid)s and are eventually stored in the vacuoles to prevent interference with physiological and metabolic processes in the leaves (Lebrun et al., 2019; Balzano et al., 2020). Shoots grown in the SF treatment showed a higher concentration of NPTs (more than two-fold) than that obtained in the S treatment. This increase was due primarily to *de novo* synthesis of hPC2, cysPC2, PC3, CysPC3, hPC4 and PC5 and also higher concentrations mainly of hGSH (Table 2). This higher concentration of NPTs in the SF treatment with respect to S may be related to a greater concentration of metal(loid)s both in shoots and roots (Dazy et al., 2009; Leão et al., 2014). The shoots and roots of plants in the SN treatment showed the same NPTs and in similar concentrations to those observed in the S treatment; however, the SNF treatment showed the highest concentration of thiols (4 fold higher than SF). Although we did not detect any new NPTs, we observed a very large increase in the concentration of those thiols already present in the SF treatment (Table 2), mainly GSH and hGSH. Note that some authors hypothesize that these thiols, apart from being precursors of PCs and participating as chelating compounds, can also protect against oxidative stress (Shri et al., 2009). This could be the reason why the plants grown in this SNF treatment had a lower content of H_2O_2 and MDA. It is not easy to explain why the shoots of plants grown in the SNF treatment had the highest concentration of thiols as these plants did not show the highest accumulation of As and Hg. One hypothesis is that the TF in SNF was greater than 1 for Hg and, since this metal is highly toxic for the plant, a greater presence of NPTs would allow it to accumulate As and Hg in cells and thus they could be quickly chelated. Another possibility could be that the fertilizer reacted with the nZVI and the plant absorbed the nanomaterial together with the mineral elements from the soil and, since Fe is a metal, the plant synthesized phytochelatins and other thiolic compounds to chelate this excess Fe. Some authors also observed that nZVI can be absorbed and accumulated in the tissues of *Populus deltoides* and *Populus nigra* (Ma et al., 2013), which could give rise to toxicity and perhaps cause an increase in NPT synthesis. However, this behavior was not observed when only nZVI are added to the soil.

We found that the roots of the S treatment showed 11 NPTs (Table 2) compared to the 6 in shoots. In the SF treatment, the total concentration

Table 2
Non-protein thiolic compounds (NPTs) (nmol g^{-1} FW) in shoots of *M. sativa* grown for 10 days in polluted soil (S), and soil after treatment with organic fertilizer (SNF), nZVI (SN), and both amendments (SNF). Different letters within the same row and the same organ denote significant differences (p < 0.05). Values below detection limit are presented by -.

NPTs	Shoots				Roots			
	S	SF	SN	SNF	S	SF	SN	SNF
Cys	0.34 ± 0.04b	1.22 ± 0.09b	0.31 ± 0.06b	9.82 ± 1.26a	0.13 ± 0.00c	0.47 ± 0.01b	0.19 ± 0.02c	0.79 ± 0.09a
CT1	–	–	–	–	1.23 ± 0.16a	–	–	0.26 ± 0.12b
GSH	1.66 ± 0.14b	1.41 ± 0.13b	1.09 ± 0.04b	8.54 ± 1.08a	0.63 ± 0.02b	1.98 ± 0.06a	1.72 ± 0.08a	3.02 ± 0.01a
hGSH	1.01 ± 0.03c	3.24 ± 0.06b	1.99 ± 0.20c	12.91 ± 1.32a	1.17 ± 0.26b	2.25 ± 0.07a	3.13 ± 0.04a	5.03 ± 0.70a
CT2	0.53 ± 0.07c	1.78 ± 0.11a	0.56 ± 0.11c	–	0.06 ± 0.03a	–	–	–
PC2	–	–	–	–	–	1.53 ± 0.05a	0.12 ± 0.02b	1.35 ± 1.15a
CT3	–	–	–	–	–	–	–	0.10 ± 0.15a
desglyPC2	–	–	–	–	0.19 ± 0.06b	3.22 ± 0.09a	0.38 ± 0.08b	3.49 ± 0.24a
h-PC2	–	0.46 ± 0.03b	–	2.21 ± 0.23a	0.17 ± 0.01a	–	0.06 ± 0.01b	–
cysPC2	–	0.28 ± 0.03b	–	0.92 ± 0.23a	–	–	–	–
PC3	–	0.56 ± 0.11b	–	4.36 ± 0.55a	1.12 ± 0.00b	3.91 ± 0.07a	0.48 ± 0.04c	2.04 ± 0.02b
desglyPC3	–	–	–	–	0.29 ± 0.01b	2.46 ± 0.06a	–	0.60 ± 0.73b
cysPC3	–	0.14 ± 0.03a	–	0.39 ± 0.06a	–	–	0.35 ± 0.01a	0.62 ± 0.42a
h-PC4	–	0.30 ± 0.01a	–	0.42 ± 0.07a	–	–	–	–
PC5	–	0.31 ± 0.08a	–	0.64 ± 0.03a	–	–	–	–
CT4	–	–	–	–	0.89 ± 0.13a	1.04 ± 0.01a	0.83 ± 0.04a	1.23 ± 0.30a
CT5	0.04 ± 0.01b	0.13 ± 0.03a	0.12 ± 0.00a	–	–	–	–	–
CT6	0.70 ± 0.04a	0.41 ± 0.05b	0.40 ± 0.03b	–	0.03 ± 1.23a	–	–	–
Total NPTs	4.28 ± 0.73c	10.25 ± 0.54b	4.47 ± 1.59c	40.20 ± 0.28a	5.92 ± 0.14b	16.86 ± 0.24a	7.26 ± 0.47b	18.54 ± 0.34a

of thiols in roots was more than two-fold that obtained in the S treatment. Moreover, we also observed new NPTs that we had not detected in the shoots, such as PC2, desglyPC2, desglyPC3 and the unidentified compound CT4. These observations appear to indicate that, in the root, these thiols play a key role in chelating metal(loid)s (metal(loid) concentration is higher in SF than in S) to protect the plant from damage (van der Ent et al., 2013). In the SN and SNF treatments, new thiols were also observed in shoots, although the concentrations of NPTs were lower than those of shoots and similar to those obtained in the SF treatment. Given that the accumulation of As is 5 or 6 times greater in roots than in shoots, we expected that the concentration of thiols would also be higher, but this was not the case. It should be considered that plants may simultaneously use other detoxification mechanisms, such as binding to cell walls and the synthesizing of organic acids or phenolic compounds, as others have observed when working with various metal(loid)s in a variety of plant species (Fernández-Fuego et al., 2017; Fernández et al., 2013; Sun et al., 2011).

3.6. Parameters related to oxidative stress

3.6.1. Photosynthetic pigments

The SF treatment reduced chlorophyll (chl) content, significantly decreasing both chl a and chl b (Table 3). This reduction could be related to the increase in As and Hg concentrations in shoots since these metalloids can inhibit the synthesis of photosynthetic pigments and affect metabolic processes related to photosynthesis (Chen et al., 2015; Mishra et al., 2016; Navazas et al., 2021). In our case, a significantly negative correlation was obtained between shoot and root As content, BTF and chl a content, with values of − 0.73, − 0.78 and − 0.82 respectively (P < 0.05). In contrast, *M. sativa* grown in the treatments where nZVI

Table 3
Photosynthetic pigments (µg g^{-1} FW) in leaves of *M. sativa* at 10 days of growth in the polluted soil (S), and soil after treatment with organic fertilizer (SF), nZVI (SN), and both amendments (SNF).

| Treatments	Chlorophyll a	Chlorophyll b	Chlorophyll total	Carotenoids
S	178 ± 6a	58 ± 7b	236 ± 4a	54 ± 6b
SF	32 ± 12b	21 ± 4c	53 ± 16b	64 ± 10b
SN	171 ± 35a	78 ± 9b	249 ± 39a	94 ± 11a
SNF	144 ± 31a	110 ± 14a	254 ± 45a	69 ± 1b

For each column, different letters in distinct samples indicate significant differences (n = 3, ANOVA; P < 0.05). Typical deviation is represented by ±.

were applied alone (SN) or in combination with the organic fertilizer (SNF) generally showed higher values of chl b and carotenoids (Table 3). This observation could be attributed to the As and Hg concentrations in the leaves (Navazas et al., 2021). Regarding carotenoids, we observed an increase in the SN treatment compared to S. This finding could be due to the fact that, in addition to participating in photosynthetic activity, carotenoids have an antioxidant function to counteract the toxic effects caused by As and Hg (Mascher et al., 2002; Singh et al., 2006).

3.6.2. Measurements of hydrogen peroxide, lipid peroxidation and free proline

The leaves and roots of plants in the S treatment showed a higher H_2O_2 content than in the other treatments, the leaves of the plants in the SF and SNF treatments presenting the lowest content of H_2O_2 (Fig. 4a). These findings would suggest that the fertilizer contributes to reducing the oxidative stress caused by As and Hg or that the antioxidant system of *M. sativa* grown in these conditions was able to cope with metal(loid)-induced H_2O_2 production, as reported by Sytar et al. (2013). The accumulation of these metal(loid)s in plant cells increases the production of ROS, which can cause oxidative damage (Shahid et al., 2014). The treatments applied led to a decrease in H_2O_2 concentration, thereby suggesting that the decrease in the SN treatment is contrary to that observed by other authors (Ma et al., 2010), who concluded that the use of nZVI impairs the redox processes of *Typha*, thus causing a toxic effect. Other authors (Huang et al., 2018) propose that this decrease in H_2O_2 content could be due to an increase in the production of antioxidant enzymes.

Membrane lipids are highly sensitive to oxidation by ROS and are therefore considered primary targets for metal(loid) toxicity (Del Río and López-Huertas, 2016). Measurements of the MDA content in leaves and roots revealed that, like H_2O_2, this parameter was significantly higher in the untreated soil (S) than in the SF, SN and SNF treatments (Fig. 4b), thereby revealing a positive effect of both fertilizer and nZVI on the reduction of oxidative stress. In general, MDA and H_2O_2 content are correlated, because while MDA is an indicator of cell membrane lipid peroxidation, H_2O_2 is a ROS involved in the same reaction (Gunes et al., 2009; Huang et al., 2018). In this experiment, MDA and H_2O_2 content showed a significantly positive correlation of 0.99 (P < 0.05). Overall, the combination of organic fertilizer and nZVI resulted in a lower increase in MDA in harvested *M. sativa*. This finding could be because the organic fertilizer provided nutrients while nZVI fixed As and Hg in the soil matrix, thus allowing plants to grow under better conditions. On the
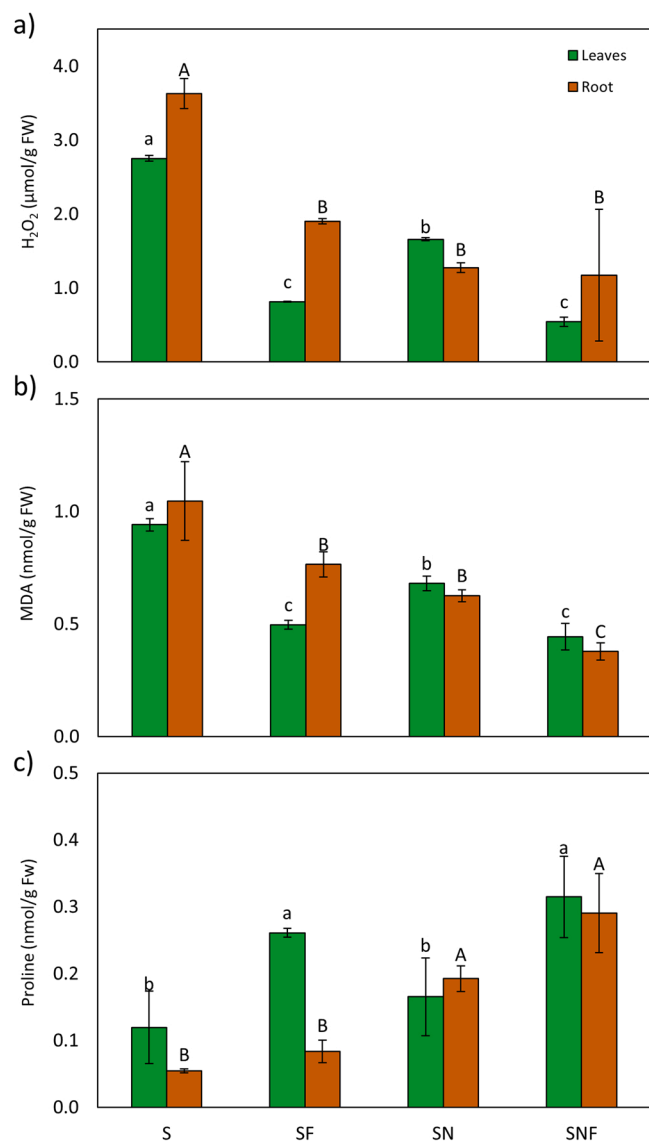
Fig. 4. Concentrations of H$_2$O$_2$ (a), MDA (b) and free proline (c) in leaves and roots of *M. sativa* growing in polluted soil (S), and soil after treatment with organic fertilizer (SNF), nZVI (SN), and both amendments (SNF) at day 10. Different lowercase letters indicate significant differences between leaves while different capital letters indicate significant differences between roots.

other hand, the capacity of nZVI (SN) to increase the MDA content in leaves was greater than when the fertilizer (SF and SNF) was added. These results are in agreement with those obtained by authors such as Rao and Shekhawat (2016), Shaw and Hossain (2013), Yusefi-Tanha et al. (2020).

Finally, the accumulation of proline (Fig. 4c) in cells is a plant strategy to protect itself against the oxidative stress caused by different agents, including metal(loid)s (Ashraf and Foolad, 2007). However, there is no clear consensus about the mechanisms by which proline alleviates metal stress (Huang and Wang, 2010). Apart from being an osmotic regulator, free proline can also play a role in the stabilization of membranes as a ROS scavenger, enzyme protector and metal(loid) chelator (Bonet et al., 2016). In this assay, the proline content increased in leaves when the soil was treated with the organic fertilizer alone or in combination with nZVI (SF, SNF). This increase could be related to the greater accumulation of As and Hg observed in both leaves and roots, thus the plant may be responding to the toxic effects of these metal(loid)s by increasing the synthesis of proline, as reported by other authors

working with different metal(loid)s (Fernández et al., 2013; Huang and Wang, 2010).

4. Conclusions

The application of nZVI and organic fertilizers for soil remediation has recently been documented. However, little attention has been given to the simultaneous use of these two amendments and their effect on the antioxidative system of plants such as *Medicago sativa*. In this regard, our results revealed that, in our case, the combined amendment was a promising strategy for the remediation of As- and Hg-polluted areas with low organic matter and nutrients content.

In fact, the application of nZVI promoted increased plant biomass, possibly due to a decrease in the availability of As and Hg in the soil. In the long term, this increase could result in a lower accumulation of As in the edible part of the plant. With respect to the antioxidative system of *Medicago sativa*, the oxidative stress in leaves and roots was also diminished in response to nZVI, as reflected by the low content of H$_2$O$_2$ and MDA, and the high content of proline, mainly in roots. Moreover, as a protective response, plants growing in the nZVI-treated soil increased NPT content in roots due to the higher concentration of pollutants in this organ with respect to the aerial part. On the other hand, the sole application of the organic fertilizer mobilized As in soil, improving As accumulation in shoots and roots. This treatment might be a suitable strategy for phytoremediation, as plants increased the NPT content of roots and aerial parts in order to avoid the negative impact of As accumulation on their metabolism. Nevertheless, to circumvent the harmful effects of As accumulation when an organic fertilizer is applied, the combination of this fertilizer with nZVI emerges as a useful approach to improve plant growth. In this sense, our findings fully endorse the advantages of a phytostabilization approach using both amendments simultaneously for soils affected by As and Hg pollution. However, new perspectives should be address for understanding interaction between nZVI and organic fertilizer in soils.

CRediT authorship contribution statement

Diego Baragaño: Conceptualization, Methodology, Formal analysis, Writing – original draft. **Rubén Forján:** Formal analysis, Writing – review & editing. **Nicolás Álvarez:** Investigation, Formal analysis. **José Luis R. Gallego:** Writing – review & editing. **Aida González:** Conceptualization, Methodology, Investigation, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2022.128748.

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