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RESEARCH ARTICLE

Phytoextraction of arsenic, nickel, selenium and zinc from sewage sludge: from laboratory to pilot scale

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Abstract

Aims The present study aimed at: *(i)* verifying the suitability of pure sewage sludge (SS) as growing medium for the hyperaccumulator species (*Pteris vittata*, *Odontarrhena chalcidica, Astragalus bisulcatus* and *Noccaea caerulescens*); *(ii)* evaluating the removal of As, Ni, Se and Zn operated by the chosen species; *(iii)* estimating the potential metal yields (bio-ore production) and connected monetary rewards in a small-scale field experiment.

Methods Hyperaccumulator plants were first tested under controlled conditions, on three different SS (P1, P2, P3) characterized by the presence of one or more contaminants among As, Ni, Se and Zn. P1 sludge was then chosen for a small-scale field experiment. Hyperaccumulator seedlings were transferred on SS and cultivated for 16 weeks before harvesting.

Results All hyperaccumulator species grew healthy on P1 SS, with *A. bisulcatus* and *O. chalcidica* reaching an average biomass of 40.2 and 21.5 g DW/plant. Trace metal concentrations in aerial parts were: As (*P. vittata)* 380 mg/kg DW, Ni (*O. chalcidica)* 683 mg/kg DW, Se (*A. bisulcatus)* 165 mg/kg DW, Zn (*N. caerulescens)* 461 mg/kg DW. The total removal of As, Ni, Se and Zn from SS due to phytoextraction was 5.8, 19, 18, 29% respectively.

Conclusions This study demonstrated that phytoextraction can be applied to SS for the removal contaminants while recovering valuable metals. Se and As were identified as the most promising target element, while Ni and Zn removal was poorly efficient under the present experimental conditions.

Keywords Hyperaccumulator · Trace metal · Biosolid · Phytomining · Bio-ore · Phytoremediation

Abbreviations

SS	Sewage sludge
TF	Translocation factor
BF	Bio-accumulation factor
WWTP	Wastewater treatment plant
EC	Electrical conductivity
OC	Organic carbon

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Introduction

Rising population and urbanization have led to a worldwide increase in the production of sewage sludges (SSs), the solid or semi-solid by-product of wastewater treatment. In Europe, the production of SS has reached a value of approximately 11 million tonnes per year and it is projected to increase in the future (Wójcik and Stachowicz 2019). As a consequence, SS management is of utmost importance.

Actually, the most common SS disposal strategies are landfill, incineration and agricultural reuse (Raheem et al. 2018). While landfill is usually discouraged, a resource recovery approach has been increasingly implemented in many countries, including European ones (Fijalkowski et al. 2017). Energy recovery through sludge incineration may reduce the SS volume by 90% with the disadvantage of generating air pollution and ashes to be disposed of (Elmi et al. 2020). Agricultural use, on the other hand, is considered a great opportunity to recover resources from SS, while decreasing the need for synthetic fertilizers (Elmi et al. 2020). Sewage sludge are in fact rich in organic carbon (OC), nitrogen (N), phosphorous (P) and micronutrients (Geng et al. 2020; Kołodziej et al. 2022), which are precious resources to maximize agricultural land productivity. The main issue associated with SSs agricultural use is the presence of hazardous contaminants. In particular, the presence of trace elements such as As, Cd, Cu, Ni, Pb, Se and Zn is of great concern, as they are commonly found in sludge and may enter the food chain, posing risks for human health and ecosystems (Yakamercan et al. 2021). Land application of SS is strictly regulated in Europe through the Sewage Sludge Directive (Directive 86/278/EEC), which sets all the requirements to prevent harmful effects on human health and the environment. Following this Directive, EU Members States have then produced specific national legislations. For example, in Italy two dedicated legislation decrees (D.Lgs. 99/1992 ; D.Lgs. 109/2018) have set the framework establishing the minimum nutrient requirements (OC $\geq$ 20%, N $\geq$ 1.5%, P $\geq$ 0.4% dry weight (DW) in SS) and the limits for harmful substances, including many trace elements (Cd 20 mg/kg DW, Ni: 300 mg/kg DW, Pb: 750 mg/kg DW, Cu: 1000 mg/kg DW, Zn: 2500 mg/kg DW; As 20 mg/kg DW, Se 10 mg/kg DW, Cr 200 mg/kg DW).

To decrease trace elements concentration in SSs and make them suitable for agricultural use, a wide variety of technologies, such as chemical washing/leaching or the use of adsorbent resins, were applied even though most of them are too costly to be scaled up to large volumes (Khalid et al. 2017).

Phytoextraction is one of the most promising green technologies for the removal of trace metals from SS (Antonkiewicz et al. 2022). This strategy makes use of hyperaccumulator plants which can actively remove contaminants from the substrate and concentrate them in their harvestable biomasses (Awa and Hadibarata 2020). Phytoextraction has the potential not only to remove trace elements, making SS suitable for agricultural use, but also to create profit from their recovery through further processing of the enriched biomass (bio-ores) (Ghori et al. 2016). However, availability and climatic limitations of hyperaccumulators may restrict the field of use of this technology on SS. Currently, 746 hyperaccumulator species were reported globally: 532 for Ni, 53 for Cu, 42 for Co, 42 for Mn, 41 for Se, 20 for Zn, 7 for Cd, 5 for As, 2 for Tl and 2 for rare earth elements (REEs) (Reeves et al. 2018).

In order to maximize environmental and economic benefits from phytoextraction, target trace elements to be removed from SS should be highly toxic (environmental benefit) and/or valuable (economic benefit) (Corzo Remigio et al. 2020). Taking into account all previous considerations, As, Se, Ni and Zn are those elements present in significant concentrations and possible targets for phytoextraction from SS. Arsenic is considered the most toxic element in the US Agency for Toxic Substances and Disease Register (ATSDR) (ATSDR 2019). Selenium, on the other hand, is one of the most valuable elements present in SS (44 USD/kg) according to the U.S. Geological Survey (USGS 2021). Nickel (14 USD/kg) and Zn (2 USD/kg) are often abundant in SS, they do not have a particularly high economic value and are both within the top 100 most toxic elements (ATSDR 2019).

When working in temperate climates, the selection of possible species suitable for phytoextraction is quite limited. *Pteris vittata* L. is one of the first and most studied As hyperaccumulators, and was reported to accumulate more than 20,000 mg/kg DW under greenhouse culture conditions (Danh et al. 2014). In Mediterranean areas, *Odontarrhena chalcidica* (Waldst. & Kit.) Endl. is considered one of the most

efficient Ni hyperaccumulator, being able to accumulate Ni between 10,000 and 20,000 mg/kg DW on ultramafic soils (Bani et al. 2015b). *Astragalus bisulcatus* (Hook.) A. Gray is a well-known and well-studied Se hyperaccumulator reported to accumulate up to 10,000 mg/kg DW of Se in its natural environment (Statwick et al. 2016). Lastly, *Noccaea caerulescens* J. Presl & C. Presl is considered a model hyperaccumulator plant, being able to accumulate not only Zn (up to 40,000 mg/kg DW) (Dinh et al. 2015), but also significant concentrations of Cd (up to 2000 mg/kg DW) and Ni (up to 10,000 mg/kg DW) depending on the accessions (Hazotte et al. 2017).

The phytoextraction potential of these hyperaccumulator species was already studied on polluted soils, natural ultramafic soils and mine tailings (Bani et al. 2015b; Barrutia et al. 2011; Chen et al. 2018). To date, very few studies focused on the application of phytoextraction to achieve trace elements removal from pure sewage sludge (Nissim et al. 2018; Salinitro et al. 2021), and even less used hyperaccumulator species (Qiu et al. 2014). In particular, Salinitro et al. (2021) demonstrated that phytoextraction is technically applicable to pure SS by growing commonly cultivated crop species such as *Brassica juncea* L., *B. napus* L., *Helianthus annuus* L. and *Zea mays* L. on 6 different SSs, obtaining in all cases plant biomasses comparable or superior to those of control soil. Nonetheless, the study highlighted that crop plants were only capable of low trace element removal (around 1% of the total for As, Cd, Cr, Cu, Ni, Se, Zn). Conversely, hyperaccumulators showed better performances, as demonstrated by *Sedum alfredii* Hance plants accumulating up to 14,065 mg/kg DW Zn when grown on pure SS (Qiu et al. 2014).

In this scenario, new studies on the use of hyperaccumulators to achieve trace element removal from SSs are of primary importance to understand the efficiency of the process (i.e. quantify metal removal), the plant performances (i.e. biomass yields) and to forecast possible scenarios for the process scale-up. Therefore, the aims of the present study were: (i) to verify the suitability of pure SS as growing medium for four selected hyperaccumulator species (*P. vittata*, *O. chalcidica, A. bisulcatus* and *N. caerulescens*); (ii) evaluate the amount of As, Ni, Se and Zn removed from three different SSs; (iii) to estimate the potential metal yields (bio-ores production) and connected monetary rewards in small scale field experiments.

Materials and methods

Seedling production

Accession of *N. caerulescens* and *O. chalcidica* seeds were obtained from University of Bologna Botanical Garden, and *A. bisulcatus* seeds were purchased from Prairie Moon Nursery (Winona, Minnesota, US). Seeds were placed in Ø 3,5 cm plastic pots filled with 70% turf and 30% perlite and subjected to cold stratification at 4 °C for one week. After that, seeds were placed in a growth chamber at a temperature of 20 °C with a photoperiod of 16/8 hours light/dark. *Pteris vittata* spores (obtained from the University of Bologna Botanical Garden) were germinated on pure turf and were not subjected to cold stratification. One-month old (three-months old for *P. vittata*) seedling were then transferred on SS.

Sewage sludge collection

For the laboratory experiment sewage sludges were collected from 3 different wastewater treatment plants (WWTPs) of the Emilia-Romagna region (northern Italy). The 3 WWTPs, will be indicated as P1 (N44.4121565, E11.5830100), P2 (N44.6743771, E10.9418852), P3 (N44.2306459, E12.0887208), to ensure anonymity as requested by the multiutility company management. WWTPs were heterogeneous in terms of size, type of wastewater collected and sludge characteristics. Each sludge was characterized by the marked presence of one or more contaminants among As, Ni, Se and Zn investigated in the present study. P1 is a small WWTP of 25,000 population equivalent (PE) and is characterized by the presence of Ni and As in SS (928 and 35 mg/kg DW), P2 is a medium-large WWTP of 450,000 PE and produces sludge enriched in Se (18 mg/kg DW), P3 is a large WWTP of 500,000 PE and produces Zn-enriched SS (1882 mg/kg DW) (Salinitro et al. 2021). Each WWTP was sampled twice, two months apart, to take into consideration sludge variability. The sludge was collected after the dehydration centrifuge and every sample consisted of 40 kg of solid SS, with a water content of around 70–75% (w/w). After collection samples were air dried and kept in plastic bags at room temperature until use. Before use, sludges from the two samplings of the same WWTP were carefully mixed in a single homogeneous sample.

Laboratory scale trial

For the lab trial 40×30×20 (LxWxH) cm undrilled plastic boxes were used. In each box a 5 cm layer of coarse gravel covered with a sheet of permeable non-woven fabric, was placed at the bottom to avoid eventual stagnant water to be in direct contact with the sludge (Fig. 1a). On the top of the fabric 4 kg DW of SS were spread to a form a 15 cm layer (cultivation substrate). Each SS was tested at 3 concentrations: (1) 80:20% SS:compost ratio, (2) 95:5%, (3) 100:0%. Compost (Terriccio Universale Geolia, Vigorplant Italia srl. Fombio, Italy) was used as control soil. Three replicates (boxes) for each treatment were produced. Seedlings were transferred on sludge one day after its spreading. In each box two plants per species were planted with their original clod of soil (total of 8 plant/box, Fig. 1c) and irrigated with deionised water 3 times a week using 200–250 mL each. This amount of water was adequate to sustain plants while avoiding water stagnation in the boxes. Plants were grown for 8 weeks before harvest.

Pilot scale trial

For the pilot scale trial, SS coming from P1 was chosen as growing substrate as it showed the simultaneous presence of more contaminants allowing the testing of all hyperaccumulator plants on the same substrate. In a 80 m^2 fully contained concrete growing bed, 30 cm of coarse gravel covered with a sheet of non-woven fabric were placed to allow drainage. A 15 cm layer of P1 SS was spread on the fabric, the sludge was taken directly after the dehydration centrifuge and manually spread to assure constant thickness. In total 3.4 tons of sludge with a water content around 75% were used to fill the whole growing bed (equal to 24.5 kg DW of SS/m^2). Growing beds were covered with a plastic tunnel and endowed with an irrigation system (Fig. 1b). In the pilot scale experiment 400 plants per species were

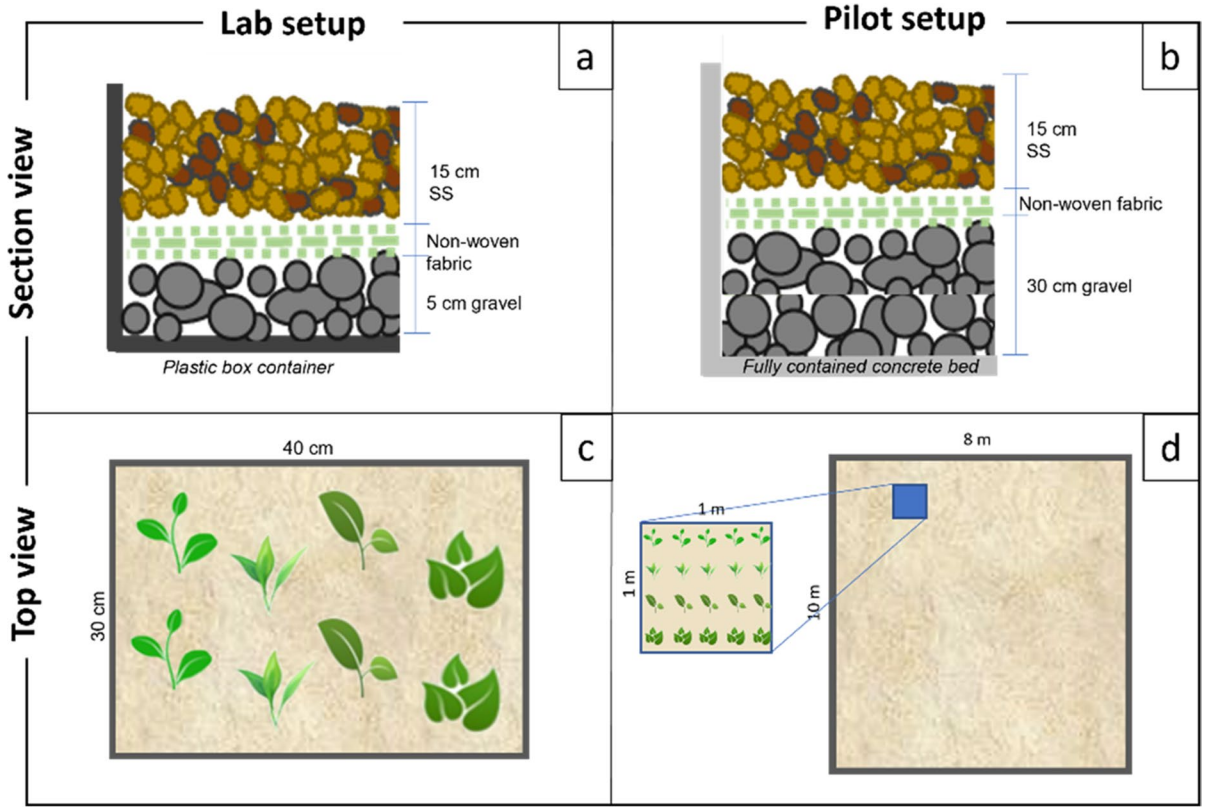

Fig. 1 Laboratory and pilot trials setup. **a** Section view of box stratification. **b** Section view of the growing bed stratification. **c** Plant arrangement in laboratory scale boxes. **d** Plant arrangement in the pilot scale growing bed

planted in alternated rows (1 row per species, row distance of 25 cm, plant distance 20 cm) on all the surface (Fig. 1d). Pilot experiment was automatically irrigated with tap water with the following volumes: 35 L/m^2/day during first week, 15 L/m^2/day during second week, 7.5 L/m^2/day during third week, 1.5 L/m^2/day from 4th to 16th week. Excess water (leachate) was collected in a specific tank then sampled for analysis. Plants were cultivated for 16 weeks from March to June before harvest. A control plot was not included in the field trial.

Sludge and plant sampling

Sewage sludge samples were collected before and after plant cultivation to evaluate the impact of the phytoextraction process. Three samples of SS were randomly taken on the whole cultivated surface (either the box or the growing bed) and mixed together in a single sample (replicate); for each treatment 5 replicates were collected for analysis.

After 8 weeks of growth, plants from laboratory boxes were harvested collecting shoots and roots separately. In the pilot experiment plant samples were collected weekly from the 7th to 15th weeks to monitor the uptake of trace metal. For this purpose, the top leaves of 10 plants for each species were collected and mixed in a single replicate; 5 replicates per species were collected each week. At the end of the 16th week all plants were harvested collecting shoots and roots separately.

Roots and shoots were washed under tap water to remove sludge particles then, rinsed with deionised water. To calculate dry weight, SS and plant samples were dried in a ventilated oven at 105 °C for 24 h until constant weight. Root biomass was not calculated since the removal of all roots from SS was not feasible. Plant parts were powdered in an A11 basic analytical mill (IKA, Staufen, Germany) to obtain homogeneous samples which were stored at room temperature for further analysis. Sewage sludge samples were placed in a MM 400 ball-mill (Retsch GmbH, Haan, Germany) and grinded at 20 shake/sec for one min to obtain a fine powder.

Sludge and plant analysis

The bioavailable trace metals were quantified in sludge samples by means of diethylenetriaminepentaacetic acid (DTPA) extraction (Lindsay and Norvell 1978). The extracting solution was composed of 14.92 g/L triethanolamine (TEA), 1.47 g/L CaCl2_2H2O and 1.97 g/L DTPA, adjusted to pH 7.3 ± 0.5 with 2 M HCl. Amounts of 2 g DW of sludge powder were extracted with 20 mL of DTPA solution and placed in a shaker for 2 h. The samples were filtered over Whatman 42 (Maidstone, UK) ashless filter paper before ICP-OES analysis. To quantify total metal content an *aqua regia* digestion was performed following a modified method from EPA-ROC (1994). 0.25 g DW of SS powder were placed in TFM (tetrafluoroethylene) digestion tubes for close vessel digestion and 6 mL of 37% (v/v) HCl, 2 mL of 69% (v/v) HNO_3 and 0.5 mL of 35% (v/v) H_2O_2 were added. All reagents were suprapure and purchased from Merck (Darmstadt, Germany). Sewage sludge samples were subjected to a digestion cycle of 2 min at 250 W, 2 min at 400 W, 1 min at 0 W, 3 min at 750 W and 32 min cooling, in a microwave digestion system (Milestone, Sorisole, BG, Italy).

To quantify total metal content in plant samples, the digestion was performed with 0.25 g DW of root or shoot powder, placed in TFM digestion tubes with 6 mL of 69% (v/v) HNO_3 and 0.5 mL 35% (v/v) H_2O_2 following the method proposed by Tüzen (2003). For plant material the microwave digestion cycle was slightly different: 2 min at 250 W, 2 min at 400 W, 1 min at 0 W, 2 min at 600 W and 33 min cooling. After digestion, samples were brought up to the volume of 20 mL with milliQ water and filtered with Whatman 42 (Maidstone, UK) ashless filter paper. The quantification of trace elements was carried out with a Spectro Arcos 2 ICP-OES (Ametek, Kleve, Germany). The limits of detection (LoD) of the analysed elements were for plant and sludge respectively 2.44 and 2.66 μg/kg DW (As), 0.49 and 2.41 μg/kg DW (Ni), 1.46 and 2.2 μg/kg DW (Se) and 0.01 and 0.15 μg/kg DW (Zn). For quality control, BCR®-143R sewage sludge amended soil and BCR®-482 lichen *Pseudovernia furfuracea* (L.) Zopf certified reference materials (CRM) (JRC-Joint Research Centre, Geel, Belgium) were digested together with SS and plant samples, respectively. Recovery rates of all elements were within ±5% of the CRM target concentrations. Quality control solutions were also included in the measurements to assure instrumental stability. All data were expressed as mg of element per kg of sample dry weight (mg/kg DW). For each plant sample bioaccumulation factor (shoot metal concentration/soil metal concentration, BF) and translocation factor (shoot metal concentration/root metal concentration, TF) were calculated. For both indexes a value > 1 means an efficient uptake and translocation of the target element.

Sewage sludge samples were also tested for pH and electrical conductivity (EC). To carry out these analyses

3 and 5 g DW of SS powder were dispersed in 50 mL of deionized water then shaken for 30 min and filtered with Extra Rapida-Perfecte 2 (Gruppo Cordenons, Milan, Italy) filter papers. Samples were tested for pH with a Coulter 360 pH-meter (Beckman, Irvine, CA, USA) and for EC with a CDM210 conductivity meter (MeterLab, Terni, Italy).

Finally, SS samples were tested for total organic carbon (TOC) and total N content. A 0.8 mg of sludge powder was put inside small tin capsules and weighted with a precision scale. The tin capsules were then pressed to remove all the air inside and placed in the instrument autosampler. The elemental C and N quantification was carried out with a CHNS-O Flash 2000 Elemental Analyzer (Thermo-Fisher Scientific, Waltham, MA, USA).

Data analysis

Five biological replicates for each SS treatment before and after phytoextraction, 5 biological replicates of roots and shoots per species at each sampling time were analysed. Resulting data were then organized in 5 datasets (Supplementary Tables S1–S5). All plant and SS variables were tested for homogeneity using the Levene's test for homogeneity of variance and for normality using the Shapiro-Wilk normality test from the package *car* (https://cran.r-project.org/web/packages/car/index.html (accessed on 6 July 2021)). For parametric data, ANOVA was performed followed by a Tukey HSD test, while for non-parametric data, a Kruskal-Wallis rank sum test was performed followed by the Dunn's test for multiple comparisons using rank sums (https://cran.r-project.org/web/packages/dunn.test/index.html (accessed on 6 July 2021)) to evaluate the differences among compared groups. To compare single parameters (i.e., pH, EC, etc.) before and after phytoremediation, the Student's *t*-test (parametric data) and the Wilcoxon rank-sum tests (non-parametric data) were used ($p < 0.05$). Statistical and graphical analyses were carried out using R 4.0.2 software and Microsoft Excel© for Microsoft 365.

Results

Plant trace elements uptake and efficiency under laboratory scale conditions

Four different hyperaccumulator species (*O. chalcidica, A. bisulcatus, P. vittata* and *N. caerulescens*) targeting respectively Ni, Se, As, Zn were grown for 8 weeks under controlled conditions on 3 different sewage sludge (SS) containing different concentrations of the above contaminants. At each SS, compost was added at decreasing concentrations leading to the following SS:compost ratios: 80:20%, 95:5% and 100:0%, whereas pure compost was used as control soil. All selected species successfully grew on the three SSs at different ratios.

Plant shoot dry weight (DW) showed several differences among treatments within the same species, though it was impossible to infer a specific influence of the substrate on this parameter. In general, the highest biomass values were reached by *O. chalcidica* growing on P1_80:20, P3_95:5 and P_100:0, with 2.7 g DW/plant compared to an average of 0.78 g DW/plant in the other treatments ($p < 0.05$) (Table 1).

In *O. chalcidica* BF was on average 1.5 in P1 and P2 sludges, while it was on average 0.5 on P3 sludge ($p < 0.05$). TF was instead > 1 in all treatments, indicating a marked efficiency of this species in transferring Ni from roots to shoots. BF and TF for *A. bisulcatus* were always < 1 and often not available, because Se content was below the limit of detection (LoD) in plant roots. *P. vittata* and *N. caerulescens'* BF and TF resulted always higher than 1, showing the efficiency of these plants in the uptake and transfer of the targeted contaminants (Table 1).

The uptake of As was maximum in the shoots of *P. vittata* growing on control substrate (187 mg/kg DW) and on P1_95:5 and P3_100:0 sludges (average 150 mg/kg DW, $p < 0.05$). Arsenic was not absorbed by any other species, which showed shoot concentrations below the limit of detection (LoD) (Table 1).

Nickel uptake was strictly correlated with its concentration in SS and all plants showed their maximum Ni uptake when growing on P1 sludge. The highest Ni concentrations were reached by *N. caerulescens* growing on P1_80:20 and P1_100:0, with an average concentration in plant shoots of 1872 mg/kg DW (Table 1). On the same substrates, also *O. chalcidica* efficiently accumulated Ni showing an average concentration of 908 mg/kg DW in shoots ($p < 0.05$) (Table 1). Selenium uptake was below the LoD in all species except *A. bisulcatus*. Nonetheless, despite being a Se hyperaccumulator, this species had a limited uptake also when grown on the Se-rich P2 sludge, reaching an average concentration of only 1.8 mg/kg DW in aerial parts.

The maximum Zn uptake was detected in *N. caerulescens* growing on P3_80:20, with a concentration of 4755 mg/kg DW in shoots. In general, this

Table 1 Trace element concentration in shoots of the four hyperaccumulator plant species

Species	Treatment	DW (g/plant)	BF	TF	As (mg/kg DW)	Ni (mg/kg DW)	Se (mg/kg DW)	Zn (mg/kg DW)
Odontarrhena chalcidica	Control	0.69 ± 0.03^{a}	28 ± 1^{a}	7.1 ± 0.2^{a}	< LoD	427 ± 8^{a}	< LoD	458 ± 16^{a}
	P1_80:20	3.30 ± 0.20^{b}	1.32 ± 0.07^{b}	3.7 ± 0.2^{b}	< LoD	976 ± 52^{b}	< LoD	652 ± 46^{b}
	P1_95:5	0.58 ± 0.02^{a}	0.34 ± 0.02^{c}	3.9 ± 0.1^{b}	< LoD	312 ± 20^{c}	< LoD	463 ± 29^{a}
	P1_100:0	0.83 ± 0.04^{c}	0.91 ± 0.09^{b}	4 ± 0.3^{b}	< LoD	841 ± 64^{b}	< LoD	616 ± 41^{b}
	P2_80:20	0.63 ± 0.02^{a}	2.3 ± 0.2^{d}	7 ± 1^{a}	< LoD	83 ± 8^{d}	< LoD	547 ± 38^{c}
	P2_95:5	0.85 ± 0.04^{c}	2.3 ± 0.3^{d}	6 ± 1^{a}	< LoD	89 ± 17^{d}	< LoD	458 ± 54^{a}
	P2_100:0	0.99 ± 0.04^{c}	1.8 ± 0.3^{b}	5 ± 1^{c}	< LoD	74 ± 18^{d}	< LoD	323 ± 72^{d}
	P3_80:20	0.92 ± 0.03^{c}	0.58 ± 0.06^{c}	1.85 ± 0.03^{d}	< LoD	41 ± 1^{e}	< LoD	508 ± 21^{c}
	P3_95:5	2.48 ± 0.08^{d}	0.45 ± 0.02^{c}	1.6 ± 0.03^{d}	< LoD	39 ± 1^{e}	< LoD	585 ± 31^{c}
	P3_100:0	2.39 ± 0.08^{d}	0.44 ± 0.04^{c}	1.8 ± 0.4^{d}	< LoD	38 ± 1^{e}	< LoD	411 ± 23^{a}
Astragalus bisulcatus	Control	0.10 ± 0.04^{a}	N.A.	N.A.	< LoD	0.84 ± 0.03^{a}	< LoD	102 ± 4^{a}
	P1_80:20	0.15 ± 0.01^{b}	N.A.	N.A.	< LoD	13.1 ± 0.3^{b}	< LoD	129 ± 7^{b}
	P1_95:5	0.19 ± 0.04^{c}	0.21 ± 0.03^{a}	N.A.	< LoD	25 ± 1^{c}	1.21 ± 0.06^{a}	104 ± 3^{a}
	P1_100:0	0.04 ± 0.01^{d}	0.36 ± 0.04^{b}	N.A.	< LoD	1.3 ± 0.6^{d}	2.1 ± 0.07^{b}	64 ± 3^{c}
	P2_80:20	0.27 ± 0.01^{e}	0.13 ± 0.01^{c}	N.A.	< LoD	3.4 ± 0.1^{e}	1.83 ± 0.07^{c}	115 ± 4^{a}
	P2_95:5	0.55 ± 0.02^{f}	N.A	N.A.	< LoD	0.4 ± 0.3^{a}	< LoD	35 ± 23^{d}
	P2_100:0	0.26 ± 0.01^{e}	0.02 ± 0.01^{d}	N.A.	< LoD	3.2 ± 0.4^{e}	0.4 ± 0.1^{d}	109 ± 6^{a}
	P3_80:20	0.51 ± 0.01^{g}	N.A	N.A.	< LoD	0.4 ± 0.3^{a}	< LoD	45 ± 6^{d}
	P3_95:5	0.36 ± 0.02^{h}	N.A	N.A.	< LoD	< LoD	< LoD	35 ± 2^{d}
	P3_100:0	0.22 ± 0.01^{i}	N.A	N.A.	< LoD	1.5 ± 0.3^{a}	< LoD	101 ± 2^{a}
Pteris vittata	Control	0.43 ± 0.02^{a}	66 ± 4^{a}	55 ± 4^{a}	187 ± 3^{a}	3.7 ± 0.6^{a}	< LoD	68 ± 4^{a}
	P1_80:20	0.32 ± 0.01^{b}	3.8 ± 0.2^{b}	66 ± 2^{b}	122 ± 6^{b}	24.7 ± 0.8^{b}	< LoD	75 ± 2^{a}
	P1_95:5	0.21 ± 0.01^{c}	4.1 ± 0.2^{b}	21 ± 0.4^{c}	154 ± 7^{c}	9.1 ± 0.4^{c}	< LoD	41 ± 1^{b}
	P1_100:0	0.29 ± 0.01^{b}	2.3 ± 0.2^{b}	24 ± 2^{c}	90 ± 4^{d}	36 ± 5^{d}	< LoD	78 ± 8^{a}
	P2_80:20	0.67 ± 0.03^{d}	25 ± 2^{c}	48 ± 3^{d}	88 ± 4^{d}	0.65 ± 0.02^{a}	< LoD	45 ± 5^{b}
	P2_95:5	0.43 ± 0.01^{a}	33 ± 2^{d}	49 ± 2^{d}	121 ± 5^{b}	0.2 ± 0.2^{a}	< LoD	40 ± 1^{b}
	P2_100:0	0.34 ± 0.01^{b}	19 ± 2^{e}	N.A.	70 ± 6^{d}	0.9 ± 0.1^{a}	< LoD	53 ± 6^{a}
	P3_80:20	0.73 ± 0.02^{e}	7 ± 4^{b}	N.A.	45 ± 30^{e}	2 ± 1^{a}	< LoD	65 ± 43^{a}
	P3_95:5	0.57 ± 0.01^{f}	12.1 ± 0.4^{f}	7.9 ± 0.3^{e}	91 ± 3^{d}	0.7 ± 0.1^{a}	< LoD	43 ± 8^{b}
	P3_100:0	0.28 ± 0.01^{b}	20 ± 2^{e}	53.6 ± 2.6^{a}	147 ± 7^{c}	3.3 ± 0.3^{a}	< LoD	85 ± 16^{a}
Noccaea caerulescens	Control	0.37 ± 0.01^{a}	13 ± 2^{a}	5 ± 0.1^{a}	2.6 ± 0.1	412 ± 17^{a}	< LoD	3387 ± 96^{a}
	P1_80:20	0.56 ± 0.02^{b}	3.5 ± 0.4^{b}	4.2 ± 0.3^{b}	< LoD	1816 ± 121^{b}	< LoD	3889 ± 383^{a}
	P1_95:5	0.5 ± 0.02^{c}	1.45 ± 0.09^{b}	4.6 ± 0.1^{a}	< LoD	687 ± 45^{c}	< LoD	1926 ± 124^{b}
	P1_100:0	0.41 ± 0.02^{a}	2.6 ± 0.3^{b}	3.1 ± 0.2^{c}	< LoD	1928 ± 127^{b}	< LoD	3521 ± 254^{a}
	P2_80:20	0.61 ± 0.01^{b}	3.8 ± 0.2^{c}	3 ± 0.1^{c}	< LoD	160 ± 6^{d}	< LoD	3695 ± 114^{a}
	P2_95:5	0.58 ± 0.03^{b}	2.6 ± 0.2^{b}	3.1 ± 0.2^{c}	< LoD	153 ± 9^{d}	< LoD	2842 ± 121^{c}
	P2_100:0	0.5 ± 0.01^{c}	2.18 ± 0.08^{b}	2.9 ± 0.1^{c}	< LoD	156 ± 3^{d}	< LoD	2538 ± 74^{c}
	P3_80:20	0.95 ± 0.04^{d}	3.1 ± 0.4^{b}	2.6 ± 0.1^{c}	< LoD	110 ± 3^{d}	< LoD	4755 ± 396^{d}
	P3_95:5	0.47 ± 0.01^{c}	0.6 ± 0.09^{d}	2.4 ± 0.3^{c}	< LoD	118 ± 7^{d}	< LoD	1119 ± 164^{e}
	P3_100:0	0.65 ± 0.04^{b}	1.2 ± 0.3^{d}	2.8 ± 0.5^{c}	< LoD	126 ± 5^{d}	< LoD	2118 ± 388^{b}

Data represent the average of 5 biological replicates ± SD. Different small letters indicate significant differences among treatments within the same species for the considered parameters according to ANOVA/Kruskal-Wallis followed by Tukey HSD/Dunn tests ($p < 0.05$). DW, dry weight, BF: bioaccumulation factor, TF: translocation factor, LoD: limit of detection, N.A: data not available: Complete dataset in Supplementary Table S1

species accumulated large quantitates of Zn, with an average of 2934 mg/kg DW in all treatments. *O. chalcidica* was the second most efficient species for Zn accumulation with values in shoots of 634 mg/kg DW reached on P1_80:20 and P_100:0 substrates (Table 1, Supplementary Table S1).

Effectiveness of SS phytoextraction under laboratory scale conditions

The initial chemical composition of the three analysed SSs was highly heterogeneous and it was influenced by the sewage network served by each wastewater treatment plant (WWTP), especially their dimension and the different final stabilization processes. Organic carbon (OC), N, P and K percentages ranged between 24 and 27%, 3.3-5%, 2.5-3%, 0.28–0.48%, respectively, and they did not show substantial differences among P1, P2, P3 sludges (Table 2). Similarly, pH and electrical conductivity (EC) were between 6.8 and 7.8 and 1.3 to 1.5 dS/m in all tested sludges. On the other hand, important differences were present when considering contaminants' content. P1 sludge was enriched in As and Ni, with concentrations in pure SS around 39 and 924 mg/kg DW, respectively (Table 2). The main contaminant of P2 was Se, present at a concentration of 18.2 mg/kg DW. Finally, P3 sludge was characterized by the presence of Zn at levels up to 1871 mg/kg DW. In control soil the content of contaminants was from 6 (Se and Zn), 10 (As) and 60-folds (Ni) lower than in sludges (Table 2).

The bioavailability of analysed trace metals is summarized in Table 3. Overall, bioavailability of As was the lowest ranging from 1 to 10% of the total. Selenium bioavailability was higher, between 9 and 28% finally Ni and Zn were similar ranging from 18 to 39% of the total metal concentration in sludge.

The effects of plant phytoextraction resulted in a general decrease of macronutrients, hyperaccumulator targeted contaminants and EC, while pH slightly increased during the 8 weeks of plant cultivation under laboratory scale trial. OC removal was similar in control, P1 and P2 sludges, with an average of around 5.7% ($p = 0.61$), while it was higher in P3 (8.7%) (Table 2). N removal was similar in all tested substrates, on average around 10%. P and K were largely removed by plants especially in control soils (45% and 25% respectively) in which removal values 4 to 7-folds higher than in SS were reached. All treatments consistently experienced an increase in pH, which varied on average from 7.3 to 7.7. The impact of phytoextraction was particularly prominent on EC, with an average decrease of 44%. Except for the case of Se, the removal (% removed compared to initial concentration) of contaminants was always higher in control soils compared to sludge (2 to 6-fold more) because their overall concentration was lower from the beginning. Removal rates were different also among the three tested sludges. Particularly, the highest removals were obtained for As, Ni and Zn in the P3 sludge (14, 18, 15%, respectively), and for Se in the sludge P1 (15%) (Table 2).

Plant trace element uptake and efficiency in pilot scale trial

In pilot scale the four hyperaccumulator species (*O. chalcidica, A. bisulcatus, P. vittata* and *N. caerulescens*) were cultivated on pure P1 sludge which was that most contaminated from Ni and As (see Tables 2 and 4). Plants were grown for 16 weeks and irrigated with tap water. All species grew successfully on this substrate reaching considerable dimensions and biomasses in a short period of time (Fig. 2). *A. bisulcatus* reached an average biomass of 40.2 g DW/plant, followed by *O. chalcidica* with 21.5 g DW/plant, *P. vittata* 5.9 g DW/plant and *N. caerulescens* with 1.2 g DW/plant. Both *A. bisulcatus* and *O. chalcidica* were able to flower before harvesting at week 16.

From the 7th to the 15th week of culture, the uptake of the target trace metals was monitored in plants through the harvesting and analysis of apical young leaves, and at the 16th week it was finally quantified on the whole biomass. As uptake in *P. vittata* reached its maximum at weeks 8–9, with an average concentration in leaves of 814 mg/kg DW (Fig. 3a). At harvesting, *P. vittata* had accumulated 380 mg/kg DW of As in the aerial and 58 mg/kg DW in the root biomass (Fig. 3a, Supplementary Table S3). Nickel uptake in *O. chalcidica* was maximum at weeks 13–14, with an average content in leaves of 870 mg/kg DW (Fig. 3b), to decrease during the two last weeks of cultivation to the values of 683 and 393 mg/kg DW in shoots and roots, respectively (Fig. 3b, Supplementary Table S3). Se uptake in *A. bisulcatus* followed a general decreasing trend from the start to the end of the monitoring period. In young leaves Se varied from a maximum of 256 mg/kg DW

Table 2 Sewage sludge physico-chemical parameters before and after phytoextraction in laboratory scale trial and average of each element removal

	Sample	OC (%)	N (%)	P (%)	K (%)	pH	EC (dS/m)	As (mg/kg DW)	Ni (mg/kg DW)	Se (mg/kg DW)	Zn (mg/kg DW)
SS before phytoextraction	Control	30 ± 1	1.3 ± 0.1	0.12 ± 0.02	0.50 ± 0.03	7.1 ± 0.3	0.7 ± 0.1	2.8 ± 0.2	15.4 ± 0.3	2.9 ± 0.6	263 ± 38
	P1_80:20	25.2 ± 0.6	4.2 ± 0.1	2.4 ± 0.1	0.43 ± 0.01	6.9 ± 0.3	1.24 ± 0.04	31.7 ± 0.5	740 ± 10	5.4 ± 0.5	1130 ± 27
	P1_95:5	25 ± 1	4.7 ± 0.2	2.8 ± 0.1	0.39 ± 0.02	6.8 ± 0.2	1.3 ± 0.1	37.2 ± 0.6	906 ± 31	5.8 ± 0.6	1331 ± 31
	P1_100:0	24.8 ± 0.7	5.0 ± 0.1	2.9 ± 0.1	0.39 ± 0.03	6.8 ± 0.2	1.4 ± 0.1	39 ± 0.7	924 ± 47	6.0 ± 0.6	1350 ± 62
	P2_80:20	27.6 ± 0.9	3.8 ± 0.2	2.48 ± 0.04	0.34 ± 0.01	7.5 ± 0.3	1.2 ± 0.1	3.6 ± 0.2	36 ± 3	14.5 ± 0.4	969 ± 35
	P2_95:5	28 ± 2	4.3 ± 0.2	2.92 ± 0.04	0.30 ± 0.02	7.7 ± 0.3	1.2 ± 0.1	3.7 ± 0.2	39 ± 4	17.2 ± 0.8	1093 ± 42
	P2_100:0	27.4 ± 0.9	4.6 ± 0.2	3.04 ± 0.01	0.28 ± 0.02	7.8 ± 0.3	1.31 ± 0.04	3.6 ± 0.2	42 ± 4	18.2 ± 0.4	1167 ± 34
	P3_80:20	25 ± 1	2.9 ± 0.2	2.0 ± 0.1	0.49 ± 0.03	7.4 ± 0.1	1.42 ± 0.05	6.5 ± 0.4	71 ± 6	4.0 ± 0.3	1517 ± 106
	P3_95:5	24.4 ± 0.9	3.2 ± 0.1	2.41 ± 0.01	0.47 ± 0.01	7.6 ± 0.4	1.4 ± 0.1	7.5 ± 0.2	87 ± 2	4.5 ± 0.4	1865 ± 26
	P3_100:0	24 ± 1	3.3 ± 0.1	2.5 ± 0.1	0.48 ± 0.03	7.5 ± 0.1	1.5 ± 0.1	7.5 ± 0.5	87 ± 7	5 ± 0.5	1871 ± 136
SS after phytoextraction	Control	28.4 ± 0.8*	1.1 ± 0.1*	0.07 ± 0.01*	0.38 ± 0.01*	7.6 ± 0.2*	0.39 ± 0.01	2.3 ± 0.1*	7 ± 1	2.7 ± 0.6*	121 ± 3
	P1_80:20	23.4 ± 0.6*	3.5 ± 0.1*	2.21 ± 0.03*	0.34 ± 0.01*	7.4 ± 0.2	0.7 ± 0.1*	28.6 ± 0.3*	732 ± 25	4.6 ± 0.4*	1069 ± 42*
	P1_95:5	23.2 ± 0.5	4.1 ± 0.1*	2.62 ± 0.01	0.36 ± 0.01*	7.4 ± 0.1*	0.84 ± 0.02	33.5 ± 0.3*	846 ± 36*	5.0 ± 0.6*	1252 ± 76*
	P1_100:0	24.2 ± 0.3	4.4 ± 0.2	2.7 ± 0.1*	0.37 ± 0.02	7.5 ± 0.1	1.1 ± 0.1*	35.1 ± 0.4*	883 ± 29	5.1 ± 0.6*	1278 ± 72
	P2_80:20	26.4 ± 0.8*	3.6 ± 0.1	2.4 ± 0.2	0.29 ± 0.01*	7.7 ± 0.1	0.61 ± 0.03*	3.4 ± 0.3	35 ± 2	14.4 ± 0.6	934 ± 44*
	P2_95:5	26.3 ± 0.8	4.0 ± 0.1*	2.8 ± 0.1	0.29 ± 0.02	7.9 ± 0.1	0.64 ± 0.01	3.5 ± 0.2	35.3 ± 0.9	16.1 ± 0.6	1073 ± 53
	P2_100:0	25.5 ± 1.4	4.1 ± 0.4	3.0 ± 0.1	0.27 ± 0.01	7.6 ± 0.2	0.8 ± 0.2	3.4 ± 0.3	37 ± 3	16 ± 0.8*	1133 ± 36
	P3_80:20	23.5 ± 0.5*	2.6 ± 0.1*	1.78 ± 0.05*	0.47 ± 0.01	7.6 ± 0.2*	0.56 ± 0.01	5.4 ± 0.2*	56 ± 2	3.6 ± 0.1	1247 ± 54*
	P3_95:5	21.7 ± 0.4*	3.0 ± 0.1*	2.28 ± 0.02*	0.46 ± 0.01	7.6 ± 0.2	0.56 ± 0.01	6.3 ± 0.1*	70 ± 2*	4.1 ± 0.2*	1576 ± 68*
	P3_100:0	22.2 ± 0.7*	3.1 ± 0.1*	2.31 ± 0.05	0.46 ± 0.02	7.7 ± 0.3*	0.8 ± 0.2	6.8 ± 0.3	73 ± 1	4.3 ± 0.4	1643 ± 71
Removal (%)	Control	6.1 ± 0.1	10.7 ± 0.1	45.2 ± 0.2	24 ± 1	-7.0 ± 0.2	45.2 ± 0.5	20.9 ± 0.1	53.8 ± 0.7	4.4 ± 0.2	53.9 ± 0.1
	P1	5.5 ± 2.8	13.4 ± 3	6.9 ± 0.7	11.2 ± 8.2	-9 ± 1	32 ± 13	9.9 ± 0.1	4 ± 3	14.7 ± 0.6	5.6 ± 0.3
	P2	5.5 ± 1.4	8 ± 2.3	2.8 ± 0.3	6.1 ± 5.6	-0.3 ± 0.4	44 ± 7	5 ± 1	7 ± 4	6 ± 5	2.8 ± 0.9
	P3	8.7 ± 2.2	7.9 ± 3.1	7.7 ± 3.2	3.3 ± 1	-1.8 ± 0.7	55 ± 6	14 ± 4	18 ± 2	10 ± 2	15 ± 3

Data represent the average of 5 biological replicates ± SD. The star symbol (*) indicates significant differences among samples before and after phytoextraction according to T/Wilcoxon tests ($p < 0.05$). OC: organic carbon, EC: electrical conductivity. Negative numbers in the removal section indicate an increase of the parameter compared to its initial values. Complete dataset in Supplementary Table S2

Table 3 Bioavailable fraction (DTPA) of trace elements in sewage sludge

Sample	As	Ni	Se	Zn
Control	9.0 ± 0.6%	29 ± 2%	16 ± 1%	26 ± 0.9%
P1_80:20	1.0 ± 0.2%	34 ± 2%	13 ± 1%	39 ± 3%
P1_95:5	1.4 ± 0.3%	32 ± 4%	14 ± 1%	37 ± 2%
P1_100:0	1.3 ± 0.5%	32 ± 3%	13 ± 2%	36 ± 2%
P2_80:20	9 ± 1%	18 ± 2%	10 ± 1%	34 ± 1%
P2_95:5	9 ± 1%	24 ± 2%	9 ± 2%	36 ± 2%
P2_100:0	10 ± 1%	19 ± 2%	9 ± 1%	31 ± 2%
P3_80:20	5.1 ± 0.8%	22 ± 1%	23 ± 3%	26 ± 3%
P3_95:5	4.3 ± 0.2%	24 ± 3%	28 ± 2%	30 ± 2%
P3_100:0	4.5 ± 0.4%	23 ± 2%	23 ± 2%	29 ± 3%
P1 (pilot)	2.0 ± 0.3%	23 ± 2%	9.7 ± 0.8%	21.0 ± 1%

Data represent the average of 5 biological replicates ± SD. Data are expressed as percentage of the total trace element concentration

at week 7 to a minimum of 132 mg/kg DW at week 15. The average Se content in the final harvested biomass was 165 and 44 mg/kg DW in shoots and roots respectively (Fig. 3c, Supplementary Table S3). Zinc uptake in *N. caerulescens* followed a similar trend to that of Se with the maximum concentration detected in leaves at weeks 7–8 (2039 mg/kg DW) to decrease to 1400 mg/kg DW at weeks 13–15 (Fig. 3d) and a final content in the harvested biomass of 1461 and 251 mg/kg DW in shoots and roots, respectively (Fig. 3d, Supplementary Table S3). *N. caerulescens* was also able to accumulate large quantities of Ni with a peak in leaves (748 mg/kg DW) between the 11th and 12th week, while in the final harvested biomass, Ni content was 591 and 133 mg/kg DW shoots and roots, respectively (Supplementary Table S3).

Overall, the most efficient species in taking up and transferring the respective targeted elements were *A. bisulcatus* and *P. vittata*, showing a BF of 19 and 10.6 and a TF of 3.8 and 5.7 respectively for Se and As. On the other hand, *O. chalcidica* and *N. caerulescens*, Ni and Zn hyperaccumulators, were not able to concentrate target metals in their aerial parts when growing on P1 sludge and showed in fact BF values of 0.6 and 0.9 for Ni and Zn, respectively. However, the translocation of these elements from roots to shoots was quite efficient (Ni TF = 1.7 in *O. chalcidica*, Zn TF = 5.8 in *N. caerulescens*).

Effectiveness of SS phytoextraction at pilot scale and metal yields

P1 sludge was characterized by the presence of As (35.7 mg/kg DW) and Ni (1103 mg/kg DW) at concentrations above the Italian law limits (respectively for Ni < 300 mg/kg DW and As < 20 mg/kg DW) (D.Lgs. 99/1992 ; D.Lgs. 109/2018) while all the other parameters were compliant with regulations for the SS use in agriculture. Table 4 reports trace metal and nutrient contents as g/m^2 or mg/m^2, taking into consideration the pilot scale trial set up in each m^2 of the growing bed 24.5 kg DW of P1 sludge were deposited and 5 plants per each species were planted. The trace metal yield was 8.7, 73.7, 33 and 11.3 mg/m^2 for As, Se, Ni and Zn, respectively, only considering

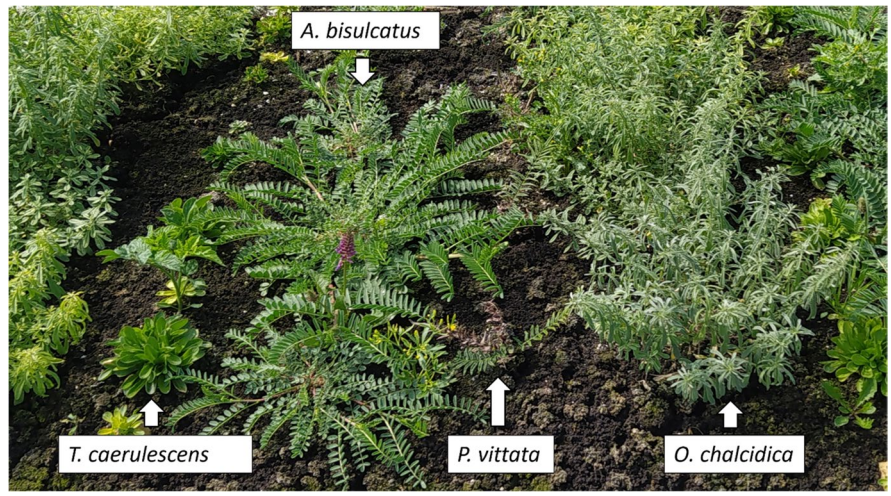

Fig. 2 Hyperaccumulator plants growing on P1 sewage sludge at the 12th week of culture

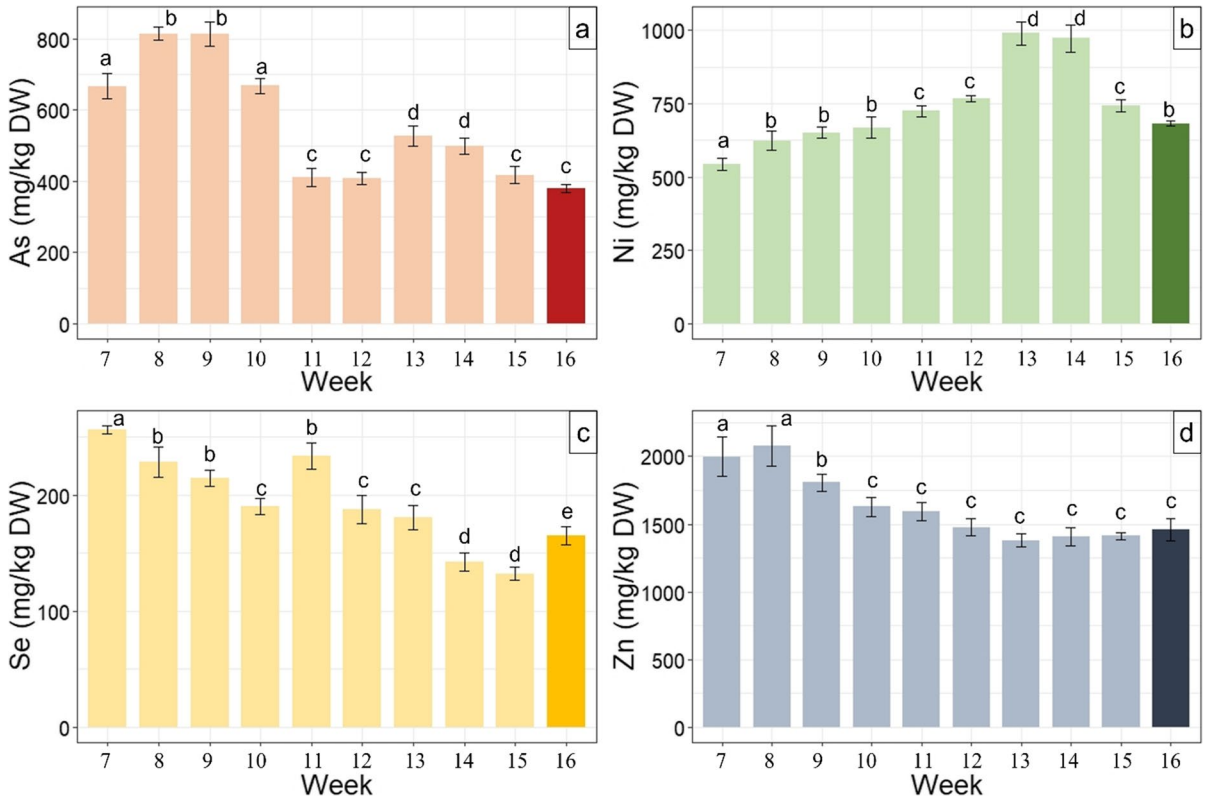

Fig. 3 Trace metal accumulation in the shoot (aerial parts) of the four selected species. **a** As uptake in *P. vittata.* **b** Ni uptake in *O. chalcidica*. **c** Se uptake in *A. bisulcatus*. **d** Zn uptake in *N. caerulescens.* Data represent the average of 5 biological replicates ± SD. Small letters indicate significant differences in metal uptake among samples during weeks 7 to 16 according to ANOVA/Kruskal-Wallis followed by Tukey HSD/Dunn tests ($p < 0.05$). Data from weeks 7–15 represent trace metal concentration in young leaves, while the darker bar represents the average trace metal content in whole plant aerial biomass collected at week 16. Complete dataset (including root data) is available in Supplementary Table S3

the element uptake of the specific hyperaccumulator plant (Table 4). During the 16 weeks of growth, plants contributed to the depletion of macronutrients and trace elements from SS. However, not all element losses from SS could be ascribed to plant activity, as for some elements it could be estimated that irrigation water contributed to the removal of 26, 16 and 24% of the initial content of K, Ni and Zn, respectively, while plants only removed 6.9, 0.3 and 0.2% of these elements. Conversely, As and Se were mainly removed by plants (1.3 and 13%) compared to the irrigation water (0.8 and 0.6%) (Table 4). Plant trace metal uptake, coupled with the losses caused by irrigation, were however not sufficient to bring the content of As and Ni below the law limits (34.2 and 894 mg/kg DW, respectively, after phytoextraction). After the phytoextraction, OC, N and P levels, despite being impacted by the process, were still above the minimum limits for agricultural reuse (OC 31% with law limit > 20% DW, N 5.9% with limit > 1.5% DW, P 3.1% with limit > 0.4% DW).

Irrigation was abundant during the first two weeks after the transplant to be then progressively reduced. Irrigation water removed the most concentrated elements such as N (34 g/m^2), K (19 g/m^2), Ni (4 g/m^2) and Zn (10 g/m^2) (Table 4). Leachate changed in volume and composition during the pilot trial (Fig. 4). Leaching rate was to 28.13, 8.25, 3, 0.34, 0.26, 0.17, 0.05 L/m^2/day during 1st, 2nd, 3rd, 4-7th, 8-10th, 11-12th, 13th week respectively. At the beginning of the test (weeks 2 and 3) N was equally present in the form of NH_4^+ and NO_3^- (112 and 93 mg/L, respectively). Then, NH_4^+ sharply decreased reaching concentrations of 0.7 mg/L during weeks 10–13, whereas NO_3^- levels stabilized around 113 mg/L from week 10 to 13 (Fig. 4a). Overall, N leaching was maximum during

Table 4 Effectiveness of phytoextraction and trace metal yields in pilot scale trial

	OC (g/m^2)	N (g/m^2)	P (g/m^2)	K (g/m^2)	As (mg/m^2)	Ni (mg/m^2)	Se (mg/m^2)	Zn (mg/m^2)
P1 before phytoextraction	8147 ± 81	1550 ± 42	817 ± 15	74 ± 3	875 ± 19	27,031 ± 622	259 ± 4	41,481 ± 837
Leaching losses	116 ± 2	34 ± 1	3.02 ± 0.03	18.9 ± 0.5	7.21 ± 0.06	4209 ± 45	1.5 ± 0.1	9771 ± 134
N. caerulescens shoot uptake	N.A.	N.A.	0.03 ± 0.04	0.13 ± 0.5	< LoD	3.5 ± 0.1	0.001 ± 0.001	8.7 ± 0.5
O. chalcidica shoot uptake	N.A.	N.A.	0.367 ± 0.003	1.703 ± 0.002	< LoD	73.7 ± 0.8	< LoD	54 ± 2
A. bisulcatus shoot uptake	N.A.	N.A.	0.655 ± 0.006	3.01 ± 0.03	< LoD	9.4 ± 0.5	33 ± 2	8.8 ± 0.4
P. vittata shoot uptake	N.A.	N.A.	0.04 ± 0.01	0.29 ± 0.08	11.3 ± 0.3	1.2 ± 0.03	< LoD	1.57 ± 0.06
P1 after phytoextraction	7522 ± 124*	1420 ± 35*	762 ± 21*	50 ± 1	824 ± 25	21,900 ± 605*	213 ± 2*	29,895 ± 614*
	OC (%)	N (%)	P (%)	K (%)	As (%)	Ni (%)	Se (%)	Zn (%)
Plant removal	N.A.	N.A.	0.13 ± 0.02	6.89 ± 0.04	1.29 ± 0.02	0.32 ± 0.02	12.64 ± 0.01	0.18 ± 0.01
Irrigation water removal	1.43 ± 0.02	2.17 ± 0.03	0.37 ± 0.01	25.52 ± 0.03	0.82 ± 0.01	15.57 ± 0.01	0.57 ± 0.01	23.6 ± 0.3
Other causes of removal	6.24 ± 0.07	6.19 ± 0.02	6.2 ± 0.3	0.01 ± 0.02	3.7 ± 0.1	3.08 ± 0.03	4.6 ± 0.2	4.20 ± 0.05
Total removal	7.7 ± 0.1	8.3 ± 0.1	6.7 ± 0.2	32.4 ± 0.6	5.8 ± 0.2	18.9 ± 0.4	17.8 ± 0.3	27.9 ± 0.5

All data were normalized by m^2 and are the average of minimum five biological replicates ± SD. In each m^2 of the growing bed 24.5 kg DW of P1 sludge were deposited and 5 plants per species were planted. Other causes of removal may include: growth of weeds, phytovolatilization, insect larvae, etc

The star symbol (*) indicates significant differences among samples before and after phytoextraction according to T/Wilcoxon tests ($p < 0.05$). OC: organic carbon: LOD: limit of detection; N.A.: data not available. Complete datasets available in Supplementary Tables S4 and S5

weeks 2 and 3, with a N total level ($NH_4^+ + NO_3^-$) of 205 mg/L to then stabilize around 113 mg/L in the weeks 7 to 13. P and K concentrations in leachate steadily decreased during the trial from 8.2 to 58.7 mg/L to 0.5 and 39 mg/L, respectively (Fig. 4a). Similarly, leaching was maximum at week 2 for Zn and Ni trace metals (36.1 and 20.8 mg/L respectively). As and Se concentrations were instead very low during the first 2 weeks (0.01 and 0.02 mg/L, respectively) and below the LoD from week 5 till the end of the trial (Fig. 4b).

Discussion

In the present research phytoextraction was applied on contaminated SS both at laboratory and pilot scale, using 4 hyperaccumulator species, targeting As, Ni, Se and Zn. The concentration of trace metal contaminants was characteristic of each selected SS with P1 enriched in As and Ni (39 and 924 mg/kg DW, respectively), P2 with high levels of Se (18.2 mg/kg DW), while P3 was characterized by the presence of Zn (1871 mg/kg DW) (Table 2). If compared to other SS both from European and south American WWTPs, the concentrations of As, Ni and Se in the studied SS were above the ranges found by Nascimento et al. (2020) and Cěrne et al. (2019) (As 1.9–11 mg/kg DW, Ni 6.5–523 mg/kg DW; Se 0.9–11 mg/kg DW), while Zn was in line with the previously reported values (245–4592 mg/kg DW). According to European and Italian legislations, the here detected levels of As, Ni and Se were above the limits set for SS re-use in agriculture (As < 20 mg/kg DW, Ni < 300 mg/kg DW, Se < 10 mg/kg DW) (D.Lgs. 99/1992; D.Lgs. 109/2018; Directive 86/278/EEC n.d.). Galvanic industries in the area served by the WWTP can be identified as the major sources of Ni and Zn in wastewater (Duan et al. 2017), whereas As could come from industrial detergents, wood preservative and fuel

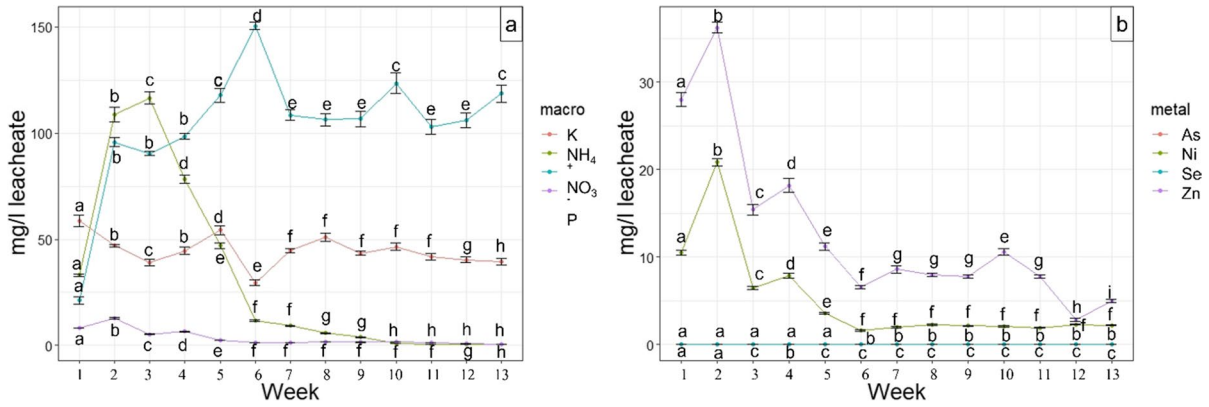

Fig. 4 Macronutrient and trace metal content in leachate. **a** Macronutrients (N (NH_4^+, NO_3^-), P, K, mg/l), **b** trace metals (As, Ni, Se, Zn, mg/l). Data represent the average of 5 biological replicates ± SD. Small letters indicate significant differences in the analysed parameters across weeks 1 to 13 according to ANOVA/Kruskal-Wallis followed by Tukey HSD/Dunn tests ($p < 0.05$). Data from weeks 14–16 were unavailable for lack of leachate. Complete dataset available in Supplementary Table S5

combustion residues from urban areas (Garelick et al. 2008). Se enrichment originates from industrial activities such as glass and ceramic pigments (Cappon 1991) and from Se-supplements used in intensive farming, agricultural fertilizers and pesticides (Tan et al. 2016).

Regarding hyperaccumulator plant metal uptake, *P. vittata* reached an As concentration of 350 mg/kg DW at the end of the growing period in the pilot experiment, compared to only 103 mg/kg DW in the laboratory scale trial (Fig. 3a). These concentrations were significantly lower than those usually reported in the literature (Danh et al. 2014; Ha et al. 2011; Visoottiviseth et al. 2002) where *P. vittata* plants growing on soil containing 150 − 35,900 mg/kg DW of As could accumulate it in fronds from 1750 to 9677 mg/kg DW. In the present research however, it has to be reported that, As concentration in P1 SS was 10 to 100 order of magnitude lower than that of natural soils studied in the previous papers and bioavailability of this element only the 2% of the total (Table 3). This species resulted the most efficient in the bioaccumulation and translocation of the targeted element, reaching an average bioaccumulation factor (BF) and translocation factor (TF) respectively of 10.6 and 5.7 at pilot scale (Supplementary Table S3). These values were comparable with those reported for *P. vittata* growing on As-contaminated soils in the Yunnan Province (China) (BF 3.58–5.51 and TF 9.95–13.78) (Ma et al. 2018). The efficiency of *P. vittata* when growing on P1 SS remained unvaried if compared to that measured on naturally contaminated soils (Ma et al. 2018).

During the pilot scale experiment Se reached a concentration of 165 mg/kg DW in *A. bisulcatus* aerial biomass at the end of the growing period, whereas at laboratory scale only 0.6 mg/kg DW of Se were recorded (Table 1; Fig. 3c). Nevertheless, despite the good results reached at pilot scale, the concentration of Se in shoots, was markedly lower than that present in the literature for the same species. Several authors (Galeas et al. 2007; Statwick et al. 2016; Sura-de Jong et al. 2015) reported an accumulation up to 5000-10,000 mg/kg DW in *A. bisulcatus* aerial parts when grown on soils containing 5–1000 mg/kg DW of Se. This species did not show significant bioaccumulation nor translocation of Se at laboratory scale, as BF remained < 1 and TF was impossible to calculated as Se in roots was below LoD. At pilot scale, despite the overall low content this species efficiently concentrated Se in its aerial parts (average of 165 mg/kg DW with a BF of 19 and a TF of 3.8 (Supplementary Table S3). The initial concentration of Se in P1 sludge (8.7 mg/kg DW, Supplementary Table S4) seemed to be close to the sensitivity threshold of the plant in agreements with previous data reporting that a concentration of Se in soil below of 2–5 mg/kg DW do not allow *A. bisulcatus* to accumulate this element in shoots (Statwick et al. 2016). Selenium bioavailability (9.7%) is strictly connected to the high presence of organic matter (Dinh et al. 2019) and the presence

of 33% OC in P1 SS (Supplementary Table S4) could in fact be responsible of its immobilization leading to a reduced uptake in plants. This is in agreement with the results from the laboratory trial where *A. bisulcatus* growing on P3 sludge, with a double amount of Se respect to P1, but similar OC% did not show significant accumulation either (Table 1).

O. chalcidica accumulated larger quantities of Ni at pilot scale (870 mg/kg DW) compared to laboratory scale (277 mg/kg DW) (Table 1; Fig. 3b). The concentration reached by this species on SS were significantly lower compared to those previously reported in soils (up to 8483-14,100 mg/kg DW when cultivated in soils with 2070–3500 mg/kg DW Ni) (Bani et al. 2007, 2015a). One of the reasons explaining the reduced uptake of this element could be the pH value of the substrate. In fact, despite the high Ni concentration in P1 SS (1103 mg/kg DW, Table 4), pH was 7.54. Under these conditions Ni results unavailable to plants, since the optimal range to assure its efficient accumulation is pH 5.0-6.5 (Nkrumah et al. 2016). The low bioavailability of Ni during the pilot experiment (23%) is also reflected in the unusually low BF (0.6) obtained for this species (Supplementary Table S3). When compared with values of BF of 2.7 reported by Li et al. (2003) that obtained a Ni accumulation in shoots of 4600 mg/kg DW when this species was grown in soils with analogous Ni concentrations (1720 mg/kg DW) as P1 SS but a pH of 6.04.

In contrast with the previous trends, *N. caerulescens* accumulated more Zn at laboratory scale (on average 2934 mg/kg DW in shoots, Table 1) than at pilot scale (1461 mg/kg DW, Fig. 3). Even in this case the maximum values were lower than most of those reported in literature, where Zn shoot concentration ranged from 5000 to 52,000 mg/kg for plants cultivated in soils with 50 − 40,000 mg/kg DW of Zn (Balafrej et al. 2020; Peer et al. 2006; Zhao et al. 2003). At the laboratory scale BF was higher (2) compared to the pilot scale (0.9), although the species showed a more efficient translocation of Zn in the field compared to glasshouse conditions (TF 5.8 and 3, respectively, Supplementary Tables S1 and S3).

At the end of the 16th week of cultivation in pilot scale, P1 SS main contaminants (As and Ni) decreased by 5.8% and 19% passing from 35.7 to 34.2 mg/kg DW and from 1103 to 894 mg/kg DW, respectively (Table 4). In the present study, trace element reduction resulted higher in a shorter cultivation period if compared with previous studies. Niazi et al. (2012) obtained a proportionally lower (16% in more than 2 years) As decrease after cultivating *P. vittata* on As-contaminated soils. Li et al. (2003) obtained a lower Ni removal (6.3%) when cultivating *O. chalcidica* on Ni-contaminated agricultural soils for approximately 17 weeks. Similarly, 18% of Se and 28% of Zn were removed during SS phytoextraction, and literature data on *N. caerulescens* grown on soils from southern China, showed and average Zn removal of the 25% per year in the superficial 5 cm (He et al. 2021). It has to be said that the higher removal rates obtained in the present study can be ascribed to the limited layer of sludge (15 cm) that was easily and fully explored by plant roots. Unlike plants cultivated in the fields, this confined environment forced plant roots to create a dense network which enhanced phytoextraction. Furthermore, the final removal cannot be ascribed uniquely to the plant uptake. Ni and Zn removal were mostly associated to irrigation water (16% and 24%, respectively), while plant removal accounted only for a small part of the total (0.32% and 0.18%, respectively) (Table 4). Under the present conditions for the previous two elements SS phytoextraction can be thus considered highly inefficient. On the other hand, As and Se were mostly removed by plants (1.3% and 13%, respectively), whereas irrigation water removal resulted less significant (0.82% and 0.57%). The fact that the latter two elements were less washed out by irrigation water can be explained by both their low concentration (2 order of magnitude lower than Zn and Ni) and their stronger adsorption onto organic matter particles (Karaca 2004).

Generally, the higher obtained biomasses at the end of pilot scale (Supplementary Table S3) were one order of magnitude higher than those reported in literature for similar cultivation periods. DeTar et al. (2015), for example, obtained a biomass of only 0.04 g/plant DW when cultivating *A. bisulcatus* for 7 weeks on soil. Similarly, Jiang et al. (2015), obtained *O. chalcidica* plants of 2 g/plant DW when cultivating this species for 8 weeks on soils fertilized with 26.2 mg/kg of P. In the present data the higher biomasses achieved in 16 weeks, could be explained by the use of SS enriched in macronutrient and organic carbon which can boost plant growth. OC, N, P and K concentrations in the studied sludge (Table 4) were in line with the average values reported for 25 WWTPs located in Europe and south America (OC% 20–49; N% 3.0-7.2; P% 0.8–2.4; K% 0.05–0.46)

(Černe et al. 2019; Nascimento et al. 2020). On the other hand, if from one side the high nutrient availability enhances plant growth, on the other side may reduce trace element uptake as previously mentioned for *Spinacia oleracea* and *Lactuca sativa* (Waheed et al. 2019).

Together with the trace metals reduction, N, P, K and OC also diminished at the end of the pilot trial (8.3%, 6.7%, 32% and 7.7%, respectively) (Table 4). Nonetheless, their concentrations remained sufficiently high for SS use in agriculture according to present regulations (D.Lgs. 99/1992). The use of hyperaccumulator species caused a lower N and OC depletion if compared to high biomass species like *Echinochloa crus-galli* and *Hibiscus cannabinus* (12% and 14%, respectively) used for the phytostabilization of municipal SS (Almasi et al. 2021).

Metal yields and potential rewards have been estimated based on data collected during the pilot experiment. Under the presented experimental conditions, it is possible to extract 1130 g/ha As, 8780 g/ha Ni, 330 g/ha Se and 7310 g/ha Zn. The market price of different elements was taken from USGS (2021): 1.7, 2.4, 14.1 and 44 USD/kg (USGS 2021). From this scenario the recovery of As and Zn from SS would result unprofitable (~2 USD/ha). Nonetheless, despite its poor economic value, As removal from SS could anyhow lead to an important environmental gain since As is considered the most toxic substance for humans (ATSDR 2019). Selenium is particularly valuable on the market being an important nutrient for both humans and animals. Selenium deficiency, is considered of great concern in many areas of the world and this element is often added to agricultural fertilizers and animal feeds (Stillings 2017). The economic gain deriving from Se bio-ore selling could be up to 322 USD/ha while that of Ni bio-ore can be instead up to 123 USD/ha, however the selling of bio-ores, would not cover production costs with these foreseen yields and market prices. Finally, if successfully applied this process can lead to the reduction of SS disposal costs for WWTPs holders. In fact, for each ton of remediated SS usable in agriculture, about 175 USD are saved for the avoided incineration. (https://ec.europa.eu/environment/archives/waste/sludge/pdf/sludge_disposal4.pdf).

Conclusions

Phytoextraction proved to be a feasible and environmental-friendly method for the removal of As, Ni, Se and Zn from SS especially at pilot scale, while maintaining OC and macronutrient concentrations high enough for SS re-use in agriculture. With this method would be possible to achieve the simultaneous clean-up of contaminated SS and their recovery from plant biomasses leading to potential economic gains. The present data showed that because of the leaching losses and poor accumulation efficiency of *N. caerulescens* and *O. chalcidica* when growing on SS, Zn and Ni phytoextraction is at present poorly efficient and not applicable to SS. Conversely *P. vittata, and A. bisulcatus* showed higher accumulation rates when grown on sludge, despite the relatively low concentration of targeted elements in SS (As, Se). Considering these results, As and Se are definitely the most promising targets for which phytoextraction could constitute a viable alternative in the near future.

However, to develop large scale application of such process further studies are necessary to maximize plant uptake efficiency while reducing metal losses from substrate after irrigation. Future research should be therefore focussed on the trace metal speciation and connected plant availability in SS (for example by testing these species at different pH). And in addition, these species have to be individually grown on SS to ascertain advantages/disadvantages of the polyculture method here presented.

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Author contributions Mirko Salinitro contributed to the study conception, design and material preparation. Data collection was performed by Mirko Salinitro and Sofia Montanari. Analyses were performed by Mirko salinitro, Sofia Montanari and Andrea Simoni. Annalisa Tassoni and Claudio Ciavatta provided the resources and supervised the study. The draft of the manuscript was written by Sofia Montanari and Mirko Salinitro and Annalisa Tassoni revised and edited the final version.

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Data availability The datasets generated during the current study are available as Supplementary Tables S1, S2, S3, S4, S5. Additional data and pictures are available from the corresponding author upon reasonable request.

Code availability Software and codes utilized for data processing are open source and available at the reported links in the subsection "data processing".

Declarations

Ethics approval Not applicable.

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