soil systems

Article

Enhanced Lead Phytoextraction by Endophytes from Indigenous Plants

Ilaria Pietrini [1], Martina Grifoni [2], Elisabetta Franchi [1], Anna Cardaci [1], Francesca Pedron [2], Meri Barbafieri [2], Gianniantonio Petruzzelli [2] and Marco Vocciante [3,*]

1 Eni S.p.A., Renewable Energy & Environmental Laboratories, Via F. Maritano 26, 20097 San Donato Milanese, Italy; ilaria.pietrini@eni.com (I.P.); elisabetta.franchi@eni.com (E.F.); anna.cardaci@eni.com (A.C.)
2 Institute of Research on Terrestrial Ecosystems, National Council of Research, Via Moruzzi 1, 56124 Pisa, Italy; martina.grifoni@iret.cnr.it (M.G.); francesca.pedron@cnr.it (F.P.); meri.barbafieri@cnr.it (M.B.); gianniantonio.petruzzelli@cnr.it (G.P.)
3 Department of Chemistry and Industrial Chemistry, University of Genova, Via Dodecaneso 31, 16146 Genova, Italy
* Correspondence: marco.vocciante@unige.it

Abstract: Lead (Pb) is one of the most common metal pollutants in soil, and phytoextraction is a sustainable and cost-effective way to remove it. The purpose of this work was to develop a phytoextraction strategy able to efficiently remove Pb from the soil of a decommissioned fuel depot located in Italy by the combined use of EDTA and endophytic bacteria isolated from indigenous plants. A total of 12 endophytic strains from three native species (*Lotus cornicolatus*, *Sonchus tenerrimus*, *Bromus sterilis*) were isolated and selected to prepare a microbial consortium used to inoculate microcosms of *Brassica juncea* and *Helianthus annuus*. As for *B. juncea*, experimental data showed that treatment with microbial inoculum alone was the most effective in improving Pb phytoextraction in shoots (up to 25 times more than the control). In *H. annuus*, on the other hand, the most effective treatment was the combined treatment (EDTA and inoculum) with up to three times more Pb uptake values. These results, also validated by the metagenomic analysis, confirm that plant-microbe interaction is a crucial key point in phytoremediation.

Keywords: soil remediation; phytoextraction; mobilizing agents; microbial endophytes; lead pollution

Citation: Pietrini, I.; Grifoni, M.; Franchi, E.; Cardaci, A.; Pedron, F.; Barbafieri, M.; Petruzzelli, G.; Vocciante, M. Enhanced Lead Phytoextraction by Endophytes from Indigenous Plants. *Soil Syst.* **2021**, *5*, 55. https://doi.org/10.3390/soilsystems5030055

Academic Editors: Matteo Spagnuolo, Paola Adamo and Giovanni Garau

Received: 9 July 2021
Accepted: 2 September 2021
Published: 3 September 2021

1. Introduction

The overture of remediation of polluted soils for sustainable innovation is one of the critical steps in addressing current global environmental issues.

Nowadays, several physicochemical and biological solutions have been developed for the recovery of contaminated water [1–3] and soils [4,5], making the optimal selection a problematic yet crucial step for the success of the reclamation [6,7].

However, remediation activities also have an environmental impact since they often use chemical products or processes, with consequent consumption of raw materials and energy that could compromise the sustainability of the approach or even invalidate its beneficial aspects [8].

In the light of this, environmentally unsound technologies for the remediation of heavy metal contaminated soils (chemical extraction, chemical oxidation, stabilization/solidification, solvent extraction, etc.) should be replaced by green and sustainable technologies. The aim of the latter is not only to eliminate or reduce contamination but also to minimize the environmental impact (reduction of air emissions, minimization of energy use, decrease of waste, etc.) and create synergies between different sectors and activities (ecosystems protection, circular economy, climate change and resilience). Concerning this, sustainable phytomanagement can make a significant contribution to supporting this transition towards sustainable remediation.

Remediation technologies based on the new natural-based solution (NBS) approach enable the achievement of the goals established by current environmental policies to protect natural resources [9]. Among the NBS remediation measures, a growing focus is on phytoremediation [10–12], which is the set of remediation technologies with plants as the main actors to remediate organic and inorganic contaminants in soil and other environmental matrices (sediments, water). The interest in these phytotechnologies has increased over time, given some significant advantages in terms of low cost, simplicity of operation and environmental benefits, as highlighted by some recent LCA-based studies [13,14]. In addition, the combined use with other solutions to further increase the overall sustainability is under investigation [15].

Among the different phytoremediation technologies, phytoextraction is considered a non-invasive technique to remove heavy metals from contaminated soil in an environmentally friendly and economical way [16,17]. In phytoextraction, metals are absorbed by roots from the soil solution and then transferred and accumulated in various tissues of the plant. Traditional phytoextraction requires hyperaccumulating species able to accumulate high amounts of metal without suffering physiological damage. However, these species exhibit low biomass production and slow growth rate, making phytoextraction a slow process with long implementation times [17,18].

Several studies have focused on improving phytoremediation efficiency using fast-growing, high biomass tolerant species, and the aid of chelating agents for increased metal uptake from the soil. The main chelating agents include ethylenediaminetetraacetic acid (EDTA), diethylenetriamine pentaacetate (DTPA), ethylene glycol tetraacetic acid (EGTA), hydroxyethyliminodiacetic acid (HEIDA), and ethylenediamine disuccinic acid (EDDS). These compounds can accelerate the release of heavy metals bound to soil particles into the soil solution, thus increasing the phytoavailable metal fraction [19]. Indeed, the only metals that plants can absorb are those in bioavailable form, i.e., present in soluble forms in the soil solution [20,21].

Among the various assisted-phytoextraction approaches to maximize the technique's effectiveness, an exciting alternative is the use of plant growth-promoting rhizobacteria (PGPR) [22]. This strategy involves rhizobacteria that can stimulate plant growth by both facilitating the bioavailability of soil nutrients and modulating the production of phytohormones (including auxins, cytokines, gibberellic acid, 1-aminocyclopropane-1-carboxylate deaminase—ACCD) and modulating plant hormone levels [22,23]. In addition, through microbial processes active in the rhizosphere, PGPR can also promote the mobility and bioavailability of metals in the soil, increasing their uptake by plants [23,24].

This work aimed to investigate the single and synergistic effect of PGPR and EDTA on the growth and Pb uptake in two tolerant species, *Brassica juncea* L. (Indian mustard) and *Helianthus annuus* L. (sunflower), to evaluate the phytoremediation potential of a Pb-contaminated site.

The selection of bacterial strains capable of improving the efficiency of phytoremediation is a fundamental step. In this study, the addition of a microbial consortium with indigenous endophytic bacteria allowed detectable levels of phytoextraction to be obtained even without the addition of mobilizing chemical agents.

2. Materials and Methods

2.1. Site Description and Soil Sampling

The contaminated site under investigation is located in an area of 40,000 m^2 near an urban area in Italy, previously affected for many years by a deposit for the storage of industrial wastes, active until the beginning of the 90s.

Being adjacent to a pond and at a distance of about 1 km from the sea, the site is characterized by sediments of marine and alluvial origin (silts, clayey silts, fluvio-lacustrine and marshy clays, arenaceous and conglomeratic, as well as alluvial deposits) and by a superficial layer (4–5 m depth) mainly composed of homogeneous artificial backfill soils of clayey, silty sand nature.

Previous chemical analyses performed on the soil of the area under examination revealed a relevant Pb contamination, which exceeded the contamination threshold concentrations (CSC) established by the Italian regulation (D.Lgs 152/2006) for sites intended for industrial use (1000 mg kg^{-1}) [25]. The highest Pb concentration found in the area was approximately 2200 mg kg^{-1}.

For the soil sampling to be addressed for the laboratory activities, 6 sampling points (SP1 to SP6) were chosen within the site. The selection was based on the geological and morphological characteristics of the soils and favoring the points where previous analytical campaigns had already detected the presence of Pb.

Approximately 50 kg of soil was collected from each sampling point with an excavator to a maximum depth of 2.5 m.

The soil samples were sieved at 2 cm on-site and then transported in special containers to the laboratories for the soil characterization analysis. Based on the results of characterization, which showed that the soil samples were homogeneous, a single soil sample was prepared for the phytoextraction tests, obtained by mixing the soil aliquots (~20 kg) from each soil sample. On this soil (Pb-soil), the determination of the total and bioavailable lead content was performed again.

2.2. Soil Characterization and Pb Analysis

The soil samples were air-dried, ground, and sieved (0–2 mm) before analysis. The physicochemical characteristics of the soil were determined following the procedures reported in Methods of soil analysis [26]. Soil pH and electrical conductivity (EC) were determined by glass electrodes with a soil/water ratio of 1:2.5 and 1:2, respectively. The cation exchange capacity (CEC) was measured by exchange with barium acetate (pH 8.1) and titration with EDTA (0.05 N). Particle size distribution (sand, silt, and clay) was evaluated by the pipette method [27].

For the determination of total Pb content, soil samples were digested in a mixture of HNO_3 (65%, *v*/*v*) and H_2O_2 (30%, *v*/*v*) using a microwave oven (FKV-ETHOS 900), according to EPA method 3051-A [28].

Potentially bioavailable concentrations of Pb in soil were determined according to an extraction procedure that sequentially involves the use of H_2O (to extract soluble Pb), KNO_3 1 M (to extract exchangeable Pb), and EDTA 1% (to extract Pb retained with bonds also of covalent nature) with a soil/extractant ratio of 1:5 and extraction time 5 h [29,30].

EDTA was selected because it is one of the most effective mobilizing agents for Pb and can be applied effectively in various soil types [31–33].

A single extraction with EDTA was also performed. The concentration selected was 2 mM, frequently used in phytoextraction tests [15,31,34]. Higher concentrations of EDTA would mobilize excessive quantities of metal, which could give rise to leaching phenomena towards the aquifer [18,19,35].

The extraction was carried out by shaking the soil and extractant (ratio of 1:5) for 2 h. All extracts were centrifuged at 15,000 rpm for 15 min and filtered.

All analyses were performed in triplicate, and the mean value was recorded.

2.3. Microcosm Experimental Design

To study the role of bacterial inocula on phytoremediation processes, alone or in combination with the EDTA mobilizing agent, a microcosm experimental campaign was performed.

Each microcosm was prepared by adding 300 g of Pb-soil and sowing *B. juncea* var. Scala or *H. annuus* var. Paola, at doses of 0.5 g and 5 seeds per pot, respectively.

The experimentation lasted about 30 days and was conducted in a growth chamber (CCL300BH-AS S.p.A., Perugia, Italy). The growth conditions were the following: photoperiod of 14 h light at 25 °C and 10 h dark at 19 °C, photosynthetic photon flux density (PPFD) of 200 µmol m^{-2} s^{-1}, and relative humidity 65%.

The experiment consisted of the following treatments: Pb-soil treated with 10^8 CFU (Colony-Forming Unit) per gram of soil of bacterial consortium SG_1 (PGPR) prepared as described in Section 2.6, Pb-soil treated with PGPR and EDTA 2 mM (PGPR + EDTA), and Pb-soil without any treatment (CT, control).

The study design consisted of a completely random design with three replicates per treatment. Bacterial inocula were added approximately 9 days after planting. EDTA addition was carried out after approximately 15 days, splitting the total dose over 5 days to avoid possible Pb toxic effects on plant growth resulting from rapid release and high metal mobilization [36].

Pots were thoroughly watered with tap water at field capacity, maintaining this condition throughout the experiment without fertilizer addition.

Plants were harvested 15 days after the addition of EDTA. Plant organs (roots and shoots) were washed with deionized water and then dried at 40 °C to constant weight to determine their Pb content. Roots were further treated in an ultrasonic bath (Branson Sonifier 250 ultrasonic processor, Branson Ultrasonic Corporation, Brookfield, CT, USA) to remove any residual soil.

Dried plant tissues were weighed, dry weight (DW) recorded, and powdered. Total Pb content was determined in the dried vegetal samples after overnight acid pre-digestion with HNO_3 (65%, v/v) and H_2O_2 (30%, v/v) according to US-EPA 3052 [37]. Quantification of Pb in soil, extract, and vegetal samples was performed by inductively coupled plasma optical emission spectroscopy (ICP-OES) Liberty AX, Varian.

2.4. Test of Phytotoxicity

Soil toxicity was assessed by a phytotoxicity screening test based on germination inhibition and root extension.

The assay was performed in a Petri dish (10 cm diameter) filled with 10 g of Pb-soil moistened with deionized water to saturation, over which was placed a Whatman #1 filter and 10 seeds of *Lepidium sativum* L. [38]. *L. sativum* is a biological indicator of soil toxicity screening highly sensitive to phytotoxic compounds.

Hydrated quartz sand was used as a negative control. Five replicates were performed for each soil (Pb-soil and negative control). The closed Petri dishes were placed in a germination chamber in the dark at 25 ± 1 °C. After 72 h, the number of germinated seeds was counted, and the root length was measured. The germination index (*GI*%) and the radical extension inhibition (*Inh*%) were estimated by combining the values of seed germination and root elongation:

$$GI\% = \frac{G_s * L_s}{G_c * L_c} * 100 \quad (1)$$

$$Inh\% = \frac{L_c - L_s}{L_c} * 100 \quad (2)$$

G_s and G_c are the average numbers of seeds germinated in the contaminated soil samples and in the negative control, respectively. L_s and L_c are the radical lengths (mm) for the contaminated soil sample and negative control, respectively.

2.5. Endophyte Isolation

The contaminated site is rich in spontaneous species that have developed on the reported soil, most of which were probably already present as seed banks in the fill soil. The prevalent herbaceous vegetation has the homogeneous characteristics of the surrounding area.

For this reason, endophytic bacteria were isolated and characterized from three of the most commonly represented spontaneous species: *Lotus cornicolatus*, *Sonchus tenerrimus*, and *Bromus sterilis*. The soil around the roots was removed by repeatedly rinsing with tap water. To sterilize the root surface, they were treated with 70% EtOH for 5 min, with NaClO for 2 min and again 70% EtOH for another 5 min. They were then thoroughly rinsed at

least three times with sterile H_2O. The roots, finely chopped with a sterile scalpel, were placed in sterile flasks containing TYEG (Trypticase Yeast Extract Glucose) medium and incubated for 16 h at 30 °C. Serial dilutions (10-4, 10-6, 10-8) of the obtained suspension were prepared, and 100 µL of each dilution (in triplicate) was spread over R2A Agar (Merck®) plates. Then 100 µL of the third rinsing water was plated to confirm the efficiency of sterilization. Endophyte colonies appeared after 4–5 days.

About twenty phenotypically different colonies for each root type were isolated and pure cultures were used for DNA extraction and taxonomic classification as already described in Franchi et al. [23].

2.6. PGPR Characterization

Endophytes isolated were subjected to a series of in vitro assays to assess their plant growth-promoting potential. The production of auxin indole-3-acetic acid (IAA) was estimated following the method proposed by Shahab et al. [39], siderophore molecules release was determined as described by Milagres et al. [40]. Their ability to solubilize mineral phosphorus (P) was determined by growing the strains in NBRIP (National Botanic Research Institute's Phosphate) according to the protocol developed by Nautiyal [41]. Production of exopolysaccharide (EPS) was estimated using a modified Weaver mineral medium enriched with sucrose [42]. Proteolytic activity was determined as described by Nielsen and Sørensen [43]. The production of ammonia was determined in peptone water (5 g L^{-1} peptone and 5% NaCl, pH 7.2), following the method proposed by Kifle and Laing [44].

The isolated strains were also tested for their capacity to form biofilm in vitro, inoculating them in glass tubes with 7 mL of LB (*Luria Broth*) medium. The tubes were incubated at 30 °C for 7 days without agitation. The formation of a visible layer (pellicle) at the interface between medium and air indicated a potential capability to produce biofilms. The potential capacity to fix atmospheric nitrogen was evaluated by growing the isolates with a specific nitrogen-free medium (NFb) [45]. The strains that showed at least three growth-promoting properties were selected. With the 12 potentially most promising (Figure 1), a microbial consortium (SG_1) was prepared in the form of lyophilizate and was then used as an inoculum in the phytoremediation tests.

Endophytes features

Bacterial isolates	Closest described relative [BLAST search]	Origin	Acc. N°	Family	IAA	Siderophores	N_2 fix	iP Solub.	NH_3	EPS	Pellide	Proteases	SCORE
SMV297	*Pseudomonas lactis*	*Lotus cornicolatus*	MN538912	Pseudomonadaceae	−	−	+	+	+	+	+	+	6
SMV298	*Pseudomonas baetica*		MN538913	Pseudomonadaceae	+	+	−	+	+	−	+	+	6
SMV303	*Pseudomonas putida*		MN538914	Pseudomonadaceae	+	−	−	+	+	−	+	−	4
SMV304	*Pseudomonas azotoformans*		MN538915	Pseudomonadaceae	−	−	+	+	+	+	+	+	6
SMV305	*Bacillus toyonensis*		MN538916	Bacillaceae	+	−	−	−	+	−	+	+	4
SMV311	*Lysinibacillus macroides*	*Sonchus tenerrimus*	MN538917	Bacillaceae	+	+	+	−	+	−	+	+	6
SMV313	*Pseudomonas plecoglossicida*		MN538918	Pseudomonadaceae	−	+	+	+	+	−	−	−	4
SMV316	*Micrococcus aloeverae*		MN538919	Bacillaceae	−	+	−	+	+	−	−	+	4
SMV321	*Pseudomonas koreensis*	*Bromus sterilis*	MN538920	Pseudomonadaceae	−	+	+	+	+	−	−	+	5
SMV326	*Pseudomonas stutzeri*		MN538921	Pseudomonadaceae	−	−	−	+	−	−	+	+	3
SMV328	*Pseudomonas taiwanensis*		MN538922	Pseudomonadaceae	−	−	−	+	−	+	+	+	4
SMV329	*Lysinibacillus macroides*		MN538923	Bacillaceae	+	+	+	−	+	−	+	−	5

Figure 1. The 12 selected endophytes are listed. In vitro, PGP properties and Genbank Accession number are shown. The + sign indicates the presence of the PGP property, the − sign its absence. Each strain was assigned a score calculated considering the number of PGP properties displayed.

2.7. Next-Generation Ion Torrent Sequencing (NGS)

An amount of 3 ng of the genomic DNA, obtained by extraction of 500 mg of soil samples and about 200 mg of roots samples through the Fast DNA® Spin Kit for Soil (MP Biomedicals, Irvine, CA, USA) and quantified by Qubit® 2.0 fluorometer (Invitrogen, Waltham, MA, USA), was amplified using the 16S Metagenomics Kit (Thermo Fischer Scientific, Waltham, MA, USA).

The amplification program was set up as follows: 95 °C for 10 min, followed by 25 cycles at 95 °C per 30 s, 58 °C for 30 s, and 72 °C for 20 s, a final hold time for 7 min at 72 °C and a cooling step at 4 °C.

The subsequent purification of the amplicons, the preparation, and the sequencing of the libraries followed the protocols for the Ion OneTouch™ 2 System, the Ion OneTouch™ ES, and the Ion PGM™, respectively, as previously described in Conte et al. [46].

The run was based on the workflow Metagenomics 16S w1.1 handling the Database Curated microSEQ®16 S and the reference Library 2013.1. The primers detected both ends to obtain 250 base pairs sequences. Alignment in Torrent Suite™ Software (version 5.8) was performed using the Torrent Mapping Alignment Program (TMAP). The sequences that occurred only once in the entire dataset were removed, and the representative sequences were defined with a 97% similarity cut-off.

After classifying the Operational Taxonomic Unit (OTU) representative sequences, the output was elaborated to obtain a relative abundance (%) of each OTU in the total amounts of the entire sample.

2.8. Quality Assurance and Quality Control

Quality assurance and quality control were performed by testing two standard solutions (0.5 and 2 mg L^{-1}) for every 10 samples. CRM ERM—CC141 for soil and CRM ERM—CD281 for plants were used as certified reference materials. The values obtained for Pb were 31.8 ± 1.2 mg kg^{-1} for CRM ERM—CC 141 and 1.69 ± 0.20 mg kg^{-1} for CRM ERM—CD281, in agreement with the certified values of 32.2 ± 1.4 mg kg^{-1} and 1.67 ± 0.11 mg kg^{-1}, respectively.

The detection limit for Pb was 5 µg L^{-1}, and the recovery of samples with spikes (5%) ranged from 93% to 101% with a relative standard deviation (RSD) of 1.92% of the mean.

2.9. Statistical Analysis

Data referred to the phytoextraction test are reported as the mean of three replicate microcosms, and analyses were performed in triplicate, recording the mean value ± standard deviation (±SD). Data statistical analyses were performed using Statistica version 6.0 (StatSoft, Inc., Tulsa, OK, USA). Treatment effects were analyzed using a one-way analysis of variance (ANOVA, San Francisco, CA, USA). Differences between means were compared, and a post hoc analysis of variance was performed using Tukey's honestly significant difference test ($p < 0.05$).

3. Results

3.1. Soil Analysis

Based on the data (Table 1), the soil samples under study (SP1-SP6) were homogeneous with each other, with a CEC in the range of 16–20 $Cmol_{(+)}$ kg^{-1} and an alkaline pH (~8.3 pH), which suggested a low Pb availability. Soil texture was also rather similar among the soil samples, with a predominance of the sandy fraction. The high conductivity values (on average 585 ± 54.5 µS cm^{-1}) raised a particular concern initially due to possible effects on plant growth, which may have a different sensitivity to soil salinity. However, during the phytoextraction test, no growth problems due to the high salt content in the soil were observed.

Table 1. Chemical properties in individual samples collected at the site under investigation (SP1-SP6). Values are reported as mean (n = 3) ± SD.

	SP1	SP2	SP3	SP4	SP5	SP6
pH	8.22 ± 0.1	8.44 ± 0.2	8.42 ± 0.1	8.51 ± 0.2	8.38 ± 0.1	8.12 ± 0.1
EC (µS cm^{-1})	596 ± 12	644 ± 10	548 ± 13	621 ± 8.5	495 ± 11	607 ± 6.1
Clay (%)	14.4 ± 0.2	8.74 ± 0.2	12.9 ± 0.4	9.78 ± 0.3	10.2 ± 1.0	13.1 ± 1.1
Silt (%)	18.5 ± 1.5	13.2 ± 0.9	17.4 ± 1.4	15.6 ± 1.1	16.2 ± 0.9	14.8 ± 1.3
Sand (%)	67.1 ± 1.1	78.1 ± 0.1	70.1 ± 0.3	67.8 ± 1.3	71.5 ± 0.4	72.3 ± 0.5
CEC ($Cmol_{(+)}$ kg^{-1})	18.7 ± 0.2	18.7 ± 1.2	20.5 ± 0.4	17.5 ± 1.2	16.2 ± 0.8	19.4 ± 0.5

Therefore, the homogeneity of the main characteristics of the sampled soils allowed the composition of a single sample of contaminated soil (Pb-soil) on which to perform the phytoextraction tests.

As reported in Table 2, the soils had significant amounts of Pb, with a mean value between samples SP1-SP6 of 108 ± 3.45 mg kg^{-1}. The total Pb content in the Pb-soil was in the range of values found in the original samples. These values are higher than the limit of the values for public, private, and residential green use, established by the Italian legislation [25], thus implying a potential environmental risk.

Table 2. Pb content (mg kg^{-1}) in individual samples collected at the site under investigation (SP1-SP6) and in the Pb-soil composite sample. Values are reported as mean (n = 3) ± SD.

	SP1	SP2	SP3	SP4	SP5	SP6	Pb-Soil
Total	106 ± 2.2	112 ± 8.1	106 ± 3.5	111 ± 6.9	107 ± 6.1	137 ± 3.5	112 ± 4.8
H_2O	bdl	bdl	bdl	bdl	bdl	bdl	bdl
KNO_3 1 M	1.30 ± 0.2	0.77 ± 0.1	1.70 ± 0.6	0.92 ± 0.3	1.34 ± 0.6	1.04 ± 0.1	1.15 ± 0.2
EDTA 1%	21.5 ± 2.3	21.4 ± 3.1	20.3 ± 2.0	20.5 ± 1.9	20.6 ± 2.5	20.9 ± 1.7	20.8 ± 2.8
EDTA 2 mM	6.89 ± 1.8	7.27 ± 0.9	7.66 ± 1.3	6.55 ± 1.3	7.47 ± 1.1	7.72 ± 2.4	7.68 ± 2.1

bdl: below detection limit.

The sequential extraction procedure (SEP), which allowed the evaluation of potentially phytoavailable Pb samples, showed that most of the Pb was in a form not available for uptake by plants (Table 2). The Pb amounts solubilized by H_2O and KNO_3 were negligible, amounting to about 1% of the total Pb concentration. The results are ascribable to the basic pH of the soil, whereas the amount extractable in EDTA 1% was about 20%. Also, the extraction with 2 mM EDTA significantly increased the Pb phytoavailability, extracting on average 6.7% of total Pb.

3.2. Phytotoxicity Test

The phytotoxicity test was considered as a preliminary investigation to assess plant growth to provide supporting information for further investigations performed in this study.

GI% and Inh% data for Pb-soil, 85 ± 10.8% and 14 ± 10.5%, respectively, suggest the lack of adverse conditions for plant growth. High GI% and low Inh% values indicate reduced phytotoxicity and good quality of the contaminated soil.

The results confirmed the low values of Pb bioavailability, which did not cause significant toxic effects in the species used. Moreover, thanks to the phytotoxicity test, it was possible to verify that the high values of EC did not affect the plant germination.

3.3. Effect of PGPR and EDTA on Plant Growth and Pb Phytoextraction Efficiency

Small-scale phytoremediation experiments, performed under controlled conditions (microcosm), are essential for evaluating both the species performance and the effectiveness of treatments to be used.

Therefore, the first parameter to be analyzed is biomass, which provides information about plant growth under stress conditions.

B. juncea and *H. annuus* species showed no visible toxic symptoms during growth in the Pb-contaminated soil, even after applying the different treatments.

The results presented in Figure 2 indicate some significant differences in dry biomass of roots and shoots between treatments in the two species.

PGPR utilization positively affected the growth of both species, both when applied alone and in combination with EDTA.

In particular, the aerial biomass of *H. annuus* increased by 13.4% and 11.5% compared to the control when PGPRs were applied individually and combined with EDTA, respectively. In contrast, a relevant effect (36.5%) on *B. juncea* can be observed only in the combined treatment (Figure 2B). As for the roots, PGPR did not significantly influence both species' biomass production, except for the combined treatment PGPR + EDTA on *B. juncea* (Figure 2A).

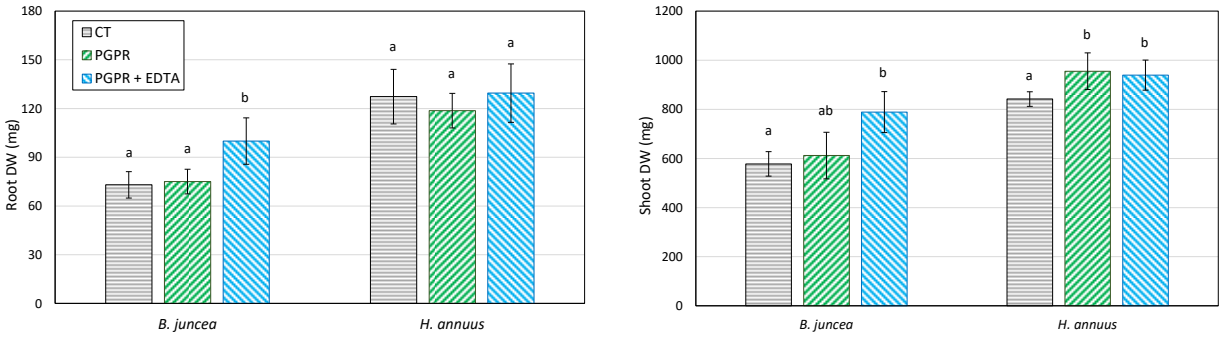


Figure 2. (**A**) Root and (**B**) shoot dry weight (mg pot^{-1}) of *B. juncea* and *H. annuus* grown on Pb-soil control (CT) and Pb-soil treated with, PGPR, and PGPR + EDTA. The values are the mean of three replicates, and the error bars show ± standard deviation. Values with different letters are significantly different at the 5% probability level (Tukey's test).

Under the experimental conditions investigated, and it is worth highlighting this, both *B. juncea* and *H. annuus* well tolerated the amounts of Pb released by EDTA.

The absence of adverse effects of EDTA on plant growth could be attributed to its ability to chelate Pb, which prevents binding between the metal and cellular components, neutralizing cytological impacts [47,48].

Pb concentrations in roots and shoots of *B. juncea* and *H. annuus* are presented in Table 3.

Table 3. Effect of PGPR and PGPR + EDTA treatment on Pb concentration in root and shoot of *B. juncea* and *H. annuus* grown on Pb-soil. The values are the mean of three replicates, and the error bars show ± standard deviation. The different letters within the same column represent different significance levels at $p < 0.05$ (Tukey's test).

Treatment	*B. juncea*		*H. annuus*	
	Root	**Shoot**	**Root**	**Shoot**
CT	1.33 ± 0.51 a	0.20 ± 0.05 a	2.14 ± 0.58 a	1.34 ± 0.25 a
PGPR	55.8 ± 4.89 c	4.82 ± 0.77 c	19.0 ± 3.46 b	1.14 ± 0.13 a
PGPR + EDTA	52.3 ± 4.74 c	2.04 ± 0.54 b	42.0 ± 5.10 c	3.46 ± 0.69 c

As suggested by SEP, which did not reveal significant amounts of metal in an immediately bioavailable form, the lowest amounts of Pb were found in the control plants of both species. These minimal concentrations could be due to the radical exudates promoting the Pb solubilization.

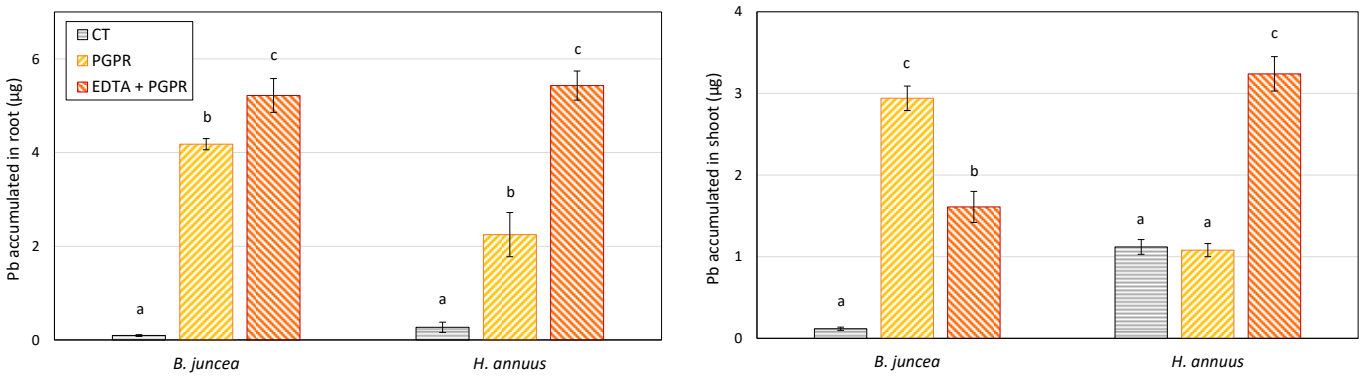
The application of EDTA improved Pb uptake in both species with respect to the control. Indeed, significant increases in Pb concentration in the soil solution due to the EDTA addition, as indicated by the soil bioavailability results, resulted in substantial increases in Pb concentrations in the roots and shoots of both plants.

However, concerning *B. juncea*, the highest Pb concentrations were found in the roots and shoots of plants treated with only PGPR. The shoots of *B. juncea* treated exclusively with PGPR had approximately 24 times more Pb than control shoots. The PGPR + EDTA combined treatment mainly affected *B. juncea* roots, which showed Pb values not significantly different from plants treated with PGPR alone treatment. In contrast, the PGPR + EDTA combined treatment did not increase Pb amounts in *B. juncea* shoots.

In *H. annuus*, the highest Pb concentration was found in the roots and shoots of the plants treated with PGPR + EDTA.

The phytoextraction efficiency is the relation between the metal concentration in the plants and the biomass produced by evaluating the total accumulation (total uptake), obtained by multiplying the Pb concentration in plant tissues by the corresponding biomass

produced. The effects of the treatments on Pb total uptake in *B. juncea* and *H. annuus* are illustrated in Figure 3.



(**A**) (**B**)

Figure 3. Effect of PGPR and PGPR + EDTA treatment on Pb uptake by (**A**) root and (**B**) shoot of *B. juncea* and *H. annuus* grown on Pb-soil. The values are the mean of three replicates, and the error bars show ± standard deviation. Values with different letters are significantly different at the 5% probability level (Tukey's test).

Our results indicated that treatment with PGPR, alone or in combination with EDTA, effectively improved Pb phytoextraction by *B. juncea* and *H. annuus*.

In *B. juncea* roots, the combined treatment PGPR + EDTA increased Pb accumulation by about 58 times compared to the control. Concerning *B. juncea* shoots, the highest Pb accumulation was observed in plants treated with only PGPR.

In contrast, in *H. annuus*, the highest Pb accumulation in shoots and roots was attributable exclusively to the PGPR + EDTA treatment. However, bacterial inocula alone increased the *H. annuus* root biomass to the levels of the combined treatment, without showing a significant difference.

3.4. NGS Analysis Results

The bacterial communities (Figure 4) were mainly constituted by the family Pseudomonadaceae (maximum value of 51% in the sample 1 R), followed by the families Caulobacteraceae (maximum value of 30% in the sample 6 R), Xanthomonadanceae (maxi-mum value of 18% in the sample 6 R) and Sphingomonadaceae (maximum value of 15% in the sample 3S). These bacteria are often correlated to hydrocarbon and metal contamination [49,50]. Considering the known PGPR strains [51] found in these communities and mainly related to Pb tolerance, the Pseudomonadaceae and Bacillaceae were isolated in the site and used for the inoculum. Pseudomonadaceae maintained a constant abundance in all the samples, while the abundance of Bacillaceae seemed to be affected by the inoculum, with values from 2% (both Controls) to 8% (sample 2S: PGPR *B. juncea*) and to 5–10% (respectively, in samples 5S: PGPR in *B. juncea* and 6S: EDTA + PGPR in *H. annuus*). Moreover, this family can be found only in the soil samples. Similarly, the family Rhodospirillaceae, mainly represented by the genus *Azospirillum sp.*, can be found, even if only with low relative abundance (maximum value 2%), in the samples belonging to *B. juncea*. Continuing to consider PGPR families, also known for their tolerance to Pb, there is a slight increase of the Alcaligenaceae in the sample's roots in the treatment added with the inoculum. In detail, these families increase their relative abundance from 0% to 2% in sample 3 R (i.e., PGPR in *B. juncea*) and from 0% to 1–2%, respectively in the samples 5 R (i.e., PGPR in *H. annuus*) and 6 R (i.e., EDTA + PGPR in *H. annuus*). The Enterobacteraceae represents another family of interest showing its increase in the root samples treated with the mobilizing agent EDTA in the presence of both *B. juncea* and *H. annuus* (i.e., increase from 0% to 4% in sample 2 R: EDTA with *B. juncea* and increase from 14% to 27% in sample 6 R: EDTA with *H. annuus*).

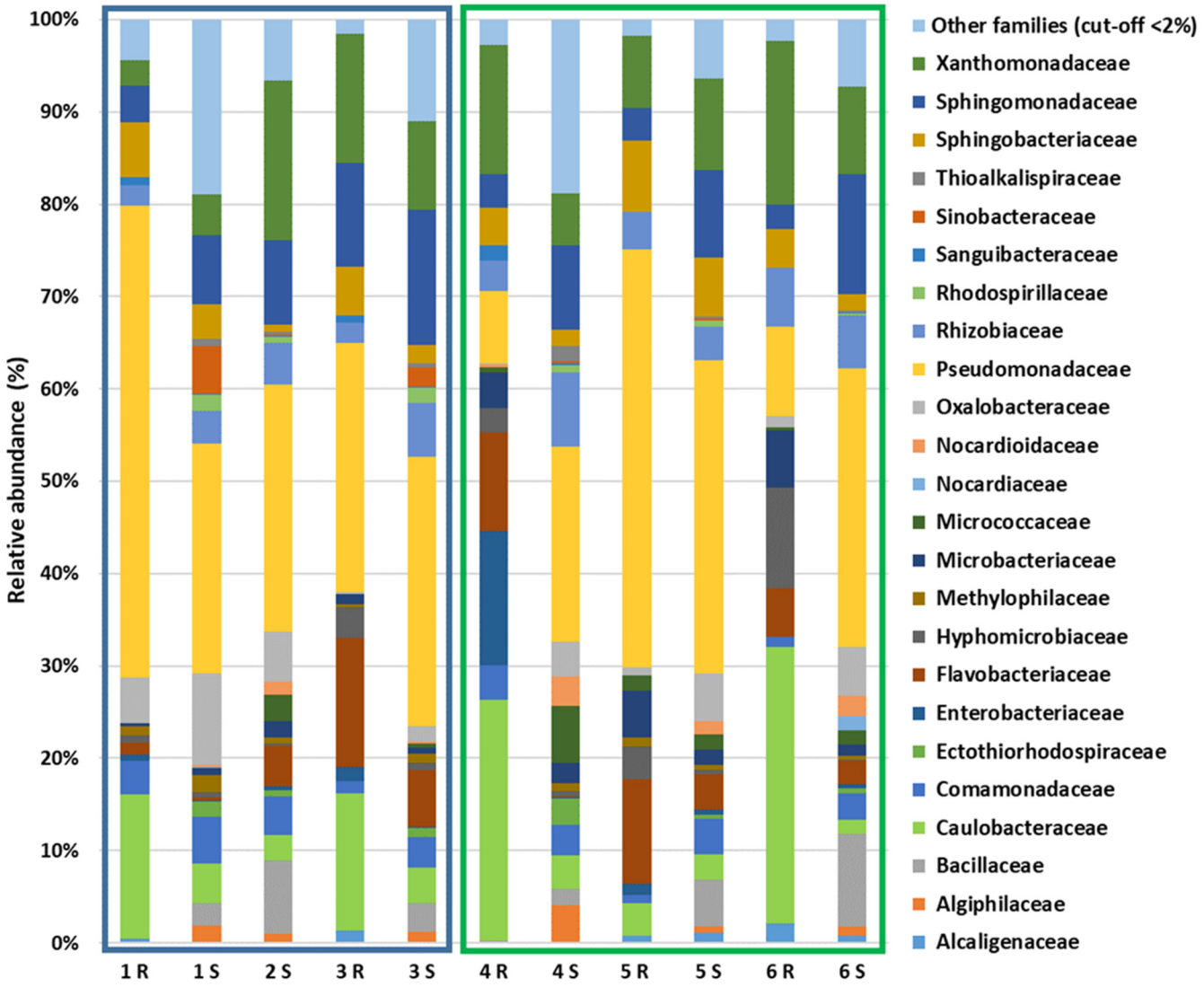


Figure 4. NGS Ion Torrent analysis of all the samples divided by the *B. juncea* (i.e., samples 1–3) and *H. annuus* (i.e., samples 5–8). The numbers represent the different treatments applied (i.e., Controls: 1 and 4; PGPR: 2 and 5; EDTA + PGPR: 3 and 6), and S stands for Soils and R for Roots. Sample 2 R was not included due to the low quality of the data required. Due to the high number of results obtained, the calculations, at the family level, were carried on by setting a cut-off < 2%.

Expanding the attention to other PGPR families, not known for their Pb tolerance, it can be observed that both the families Flavobacteriaceae and Nocardioacea were mainly represented in the microcosms with *H. annuus*; the first one with higher values in the root samples, while the Nocardioacea was present almost exclusively in soil samples.

4. Discussion

Among soil contaminants, Pb is of particular concern due to its ability to biomagnify through the food chain, threatening food safety [47]. Lead is highly toxic for most plants, causing severe physiological damage [52]. However, some plant species have proven to be particularly resistant to lead toxicity and are widely used in the phytoremediation of Pb-contaminated soils [31,47]. The plant species tested in this experiment showed a growth capacity not affected by lead contamination. The reason for the selection of these two species is twofold: in addition to their satisfactory performances, as shown in other studies on Pb phytoremediation [53–57], they have recently been recognized as energy crop species, capable of providing a high level of biomass that can be reused in the bioenergy field [58–61].

This last aspect is currently very relevant as it allows further support for phytoremediation both in economic and sustainability terms.

The phytoextraction of Pb-contaminated soils with EDTA has been extensively studied. The results obtained in our study are in agreement with previous works that have shown how the addition of this complexing agent was recognized as an effective technique to increase Pb absorption by plants [31,47,62,63].

Although several studies have reported that EDTA can promote plant growth in metal-polluted soils by stimulating auxin production and aiding nutrient translocation in plants [33,64,65], the rapid release of bioavailable metal in a short period could cause harmful transient plant phytotoxicity. In this regard, a good strategy for phytoremediation of Pb-contaminated sites could be the combined use of PGPRs and EDTA to simultaneously improve metal bioavailability in soil and plant growth under metal stress.

The stress caused in plants by the presence of heavy metals triggers complex physiological and molecular mechanisms. Among these, the production of radical exudates containing, e.g., low-molecular-weight organic acids (LMWOAs) that stimulate microbial growth of the rhizosphere and solubilize essential trace elements such as insoluble or poorly soluble (e.g., phosphorus, iron and zinc), can complex some metals such as arsenic, cadmium and lead [66].

Most metals are present in the soil in the form of insoluble and non-bioavailable salts. Chemical compounds such as EDTA, dipotassium phosphate or ammonium sulfate can separate metals from complexes bound to soil particles, favoring the absorption by the plants' roots.

PGPRs can increase the bioavailability of metals by producing microbial metabolites and siderophore molecules. Many studies show that adding chelating agents (such as EDTA) would further improve plant growth and metal uptake when combined with PGPR inoculation [67,68].

In this work, we have shown that the simple addition of the microbial inoculum (PGPR) led in *B. juncea* to a significant increase in the absorption of lead in the aerial part. A similar result was also shown in the work of He et al. [69], where the inoculation of two *Bacillus* strains, isolated from the soil, improved the rhizosphere soil environment promoting absorption of Pb by plants, enhancing the dry weight of shoots of plants growing in Pb-contaminated soil, and significantly increasing the total Pb content in aerial parts.

The possibility of avoiding the addition of chemical compounds to increase the bioavailability of the metals is undoubtedly a fascinating aspect that significantly increases the sustainability of phytoremediation.

New experiments with different plant species and endophytes isolated from metal contaminated soils are underway.

5. Conclusions

Heavy metal contamination is an important aspect of environmental pollution, and phytoextraction is one of the most effective technologies in removing many metal species, including radionuclides.

In these contamination cases, the selection of remediation technology should be directed towards the application of phytoremediation if it is to be sustainable.

The interaction between plants and microorganisms creates a fascinating ecosystem that can lead to encouraging results. As highlighted in this study, the microbial inoculation of endophytic bacteria isolated from the roots of spontaneous plants grown on the Pb-contaminated soil allowed a significant percentage of metal phytoextraction to be obtained without chemical mobilizers.

Case studies showing positive results of assisted phytoextraction only with the support of PGPRs are limited, and research in this field must undoubtedly be supported and encouraged.

Author Contributions: Conceptualization, M.G., E.F. and G.P.; methodology, M.G., F.P., M.B., E.F. and G.P.; validation, M.G., F.P., M.B., I.P., E.F. and G.P.; formal analysis, M.G., F.P., M.B. and I.P.; investigation, E.F., M.G., F.P., M.B., I.P. and G.P.; resources, I.P., M.G., F.P., M.B. and A.C.; data curation, E.F., I.P. and M.V.; writing—original draft preparation, E.F., G.P., M.G. and M.V.; writing—review and editing, M.V., G.P., I.P. and E.F.; visualization, M.V.; supervision, M.V.; project administration, G.P.; funding acquisition, E.F. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by Eni S.p.A, Research & Technological Innovation Department, San Donato Milanese (Italy) and fully funded by Syndial S.p.A. (now Eni Rewind S.p.A.), agreement number 3500038857.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data is contained within the present article.

Acknowledgments: The Authors thank Irene Rosellini, IRET-CNR, for technical assistance in Phytoremediation experiments.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Farhadian, M.; Vachelard, C.; Duchez, D.; Larroche, C. In situ bioremediation of monoaromatic pollutants in groundwater: A review. *Biores. Technol.* **2008**, *99*, 5296–5308.
2. Vocciante, M.; Finocchi, A.; De Folly D'Auris, A.; Conte, A.; Tonziello, J.; Pola, A.; Reverberi, A. Enhanced oil spill remediation by adsorption with interlinked multilayered graphene. *Materials* **2019**, *12*, 2231. [CrossRef]
3. Pietrelli, L.; Francolini, I.; Piozzi, A.; Sighicelli, M.; Vocciante, M. Chromium (III) Removal from Wastewater by Chitosan Flakes. *Appl. Sci.* **2020**, *10*, 1925. [CrossRef]
4. Khan, F.I.; Husain, T.; Hejazi, R. An overview and analysis of site remediation technologies. *J. Environ. Manag.* **2004**, *71*, 95–122.
5. Pavel, L.V.; Gavrilescu, M. Overview of ex situ decontamination techniques for soil cleanup. *Environ. Eng. Manag. J.* **2008**, *7*, 815–834.
6. Vocciante, M.; Reverberi, A.P.; Pietrelli, L.; Dovì, V.G. Improved Remediation Processes through Cost-effective Estimation of Soil Properties from Surface Measurements. *J. Clean. Prod.* **2017**, *167*, 680–686.
7. da Silva, B.M.; Maranho, L.T. Petroleum-contaminated sites: Decision framework for selecting remediation technologies. *J. Hazard. Mat.* **2019**, *378*, 120722.
8. Vocciante, M.; Dovì, V.G.; Ferro, S. Sustainability in ElectroKinetic Remediation Processes: A Critical Analysis. *Sustainability* **2021**, *13*, 770. [CrossRef]
9. Song, Y.; Kirkwood, N.; Maksimović, Č.; Zhen, X.; O'Connor, D.; Jin, Y.; Hou, D. Nature based solutions for contaminated land remediation and brownfield redevelopment in cities: A review. *Sci. Total Environ.* **2019**, *663*, 568–579. [CrossRef]
10. Grifoni, M.; Pedron, F.; Barbafieri, M.; Rosellini, I.; Petruzzelli, G.; Franchi, E. Sustainable Valorization of Biomass: From Assisted Phytoremediation to Green Energy Production. In *Handbook on Assisted and Amendments Enhanced Sustainable Remediation Technology*; Prasad, M.N.V., Ed.; John Wiley & Sons: Hoboken, NJ, USA, 2020.
11. Hou, D.; Bolan, N.S.; Tsang, D.C.W.; Kirkham, M.B.; O'Connor, D. Sustainable soil use and management: An interdisciplinary and systematic approach. *Sci. Total Environ.* **2020**, *729*, 138961. [CrossRef]
12. O'Connor, D.; Zheng, X.; Hou, D.; Shen, Z.; Li, G.; Miao, G.; O'Connell, S.; Guo, M. Phytoremediation: Climate change resilience and sustainability assessment at a coastal brownfield redevelopment. *Environ. Int.* **2019**, *130*, 104945. [CrossRef]
13. Vocciante, M.; Caretta, A.; Bua, L.; Bagatin, R.; Franchi, E.; Petruzzelli, G.; Ferro, S. Enhancements in phytoremediation technology: Environmental assessment including different options of biomass disposal and comparison with a consolidated approach. *J. Environ. Manag.* **2019**, *237*, 560–568. [CrossRef]
14. Vocciante, M.; De Follis D'Auris, A.; Franchi, E.; Petruzzelli, G.; Ferro, S. CO_2 footprint analysis of consolidated and innovative technologies in remediation activities. *J. Clean. Prod.* **2021**, 126723. [CrossRef]
15. Pedron, F.; Grifoni, M.; Barbafieri, M.; Petruzzelli, G.; Franchi, E.; Samà, C.; Gila, L.; Zanardi, S.; Palmery, S.; Proto, A.; et al. New Light on Phytoremediation: The Use of Luminescent Solar Concentrators. *Appl. Sci.* **2021**, *11*, 1923. [CrossRef]
16. Ashraf, S.S.; Ali, Q.; Zahir, Z.A.; Ashraf, S.S.; Asghar, H.N. Phytoremediation: Environmentally sustainable way for reclamation of heavy metal polluted soils. *Ecotoxicol. Environ. Saf.* **2019**, *174*, 714–727. [CrossRef]
17. Shah, V.; Daverey, A. Phytoremediation: A multidisciplinary approach to clean up heavy metal contaminated soil. *Environ. Technol. Innov.* **2020**, *18*, 100774. [CrossRef]
18. Sheoran, V.; Singh Sheoran, A.; Poonia, P. Factors affecting phytoextraction: A review. *Pedosphere* **2016**, *26*, 148–166. [CrossRef]
19. Evangelou, M.W.H.; Ebel, M.; Schaeffer, A. Chelate assisted phytoextraction of heavy metals from soil. Effect, mechanism, toxicity, and fate of chelating agents. *Chemosphere* **2007**, *68*, 989–1003. [CrossRef]

20. Pedron, F.; Grifoni, M.; Barbafieri, M.; Petruzzelli, G.; Rosellini, I.; Franchi, E.; Bagatin, R.; Vocciante, M. Applicability of a Freundlich-like model for plant uptake at an industrial contaminated site with a high variable arsenic concentration. *Environments* **2017**, *4*, 67. [CrossRef]
21. Petruzzelli, G.; Grifoni, M.; Barbafieri, M.; Rosellini, I.; Pedron, F. Sorption: Release processes in soil-the basis of phytoremediation efficiency. In *Phytoremediation*; Ansari, A.A., Gill, S.S., Gill, R., Lanza, G.R., Newman, L., Eds.; Springer: Cham, Switzerland, 2018; pp. 91–112. [CrossRef]
22. Manoj, S.R.; Karthik, C.; Kadirvelu, K.; Arulselvi, P.I.; Shanmugasundaram, T.; Bruno, B.; Rajkumar, M. Understanding the molecular mechanisms for the enhanced phytoremediation of heavy metals through plant growth promoting rhizobacteria: A review. *J. Environ. Manag.* **2020**, *254*, 109779. [CrossRef]
23. Franchi, E.; Cosmina, P.; Pedron, F.; Rosellini, I.; Barbafieri, M.; Petruzzelli, G.; Vocciante, M. Improved arsenic phytoextraction by combined use of mobilizing chemicals and autochthonous soil bacteria. *Sci. Total Environ.* **2019**, *655*, 328–336. [CrossRef]
24. Abbaszadeh-Dahaji, P.; Omidvari, M.; Ghorbanpour, M. Increasing phytoremediation efficiency of heavy metal-contaminated soil using PGPR for sustainable agriculture. In *Plant-Microbe Interaction: An Approach to Sustainable Agriculture*; Choudhary, D.K., Varma, A., Narendra, T., Eds.; Springer: Singapore, 2017; pp. 187–204. [CrossRef]
25. Legislative Decree 152/2006. Rules in environmental field. In *Italian Official Journal*; National Legislation: Italy, 2006.
26. Sparks, D.L.; Page, A.L.; Helmke, P.A.; Loeppert, R.H. *Methods of Soil Analysis Part 3—Chemical Methods*; Soil Science Society of America, American Society of Agronomy: Madison, WI, USA, 1996. [CrossRef]
27. Gee, G.W.; Bauder, J.W. Particle-size analysis. In *Methods of Soil Analysis: Part 1d—Physical and Mineralogical Methods*; Klute, A., Ed.; Soil Science Society of America, American Society of Agronomy: Madison, MI, USA, 1986; pp. 383–411.
28. EPA—United State Environmental Protection Agency. Method 3051A Microwave assisted acid digestion of sediments, sludges, soils, and oils. In *Test Methods for Evaluating Solid Waste: Physical/Chemical Methods (SW-846)*; U.S. Environmental Protection Agency: Washington, DC, USA, 1995.
29. Grifoni, M.; Petruzzelli, G.; Barbafieri, M.; Rosellini, I.; Pedron, F. Soil quality protection at heavy metal-contaminated manufactured gas plant sites: Role of biological remediation. In *Enhancing Cleanup of Environmental Pollutants*; Anjum, N., Gill, S., Tuteja, N., Eds.; Springer: Cham, Switzerland, 2017; pp. 231–260. [CrossRef]
30. Pedron, F.; Petruzzelli, G.; Barbafieri, M.; Tassi, E. Strategies to use phytoextraction in very acidic soil contaminated by heavy metals. *Chemosphere* **2009**, *75*, 808–814. [CrossRef]
31. Barbafieri, M.; Pedron, F.; Petruzzelli, G.; Rosellini, I.; Franchi, E.; Bagatin, R.; Vocciante, M. Assisted phytoremediation of a multi-contaminated soil: Investigation on arsenic and lead combined mobilization and removal. *J. Environ. Manag.* **2017**, *203*, 316–329. [CrossRef]
32. Jez, E.; Lestan, D. EDTA retention and emissions from remediated soil. *Chemosphere* **2016**, *151*, 202–209. [CrossRef]
33. Kanwal, U.; Ali, S.; Shakoor, M.B.; Farid, M.; Hussain, S.; Yasmeen, T.; Adrees, M.; Bharwana, S.A.; Abbas, F. EDTA ameliorates phytoextraction of lead and plant growth by reducing morphological and biochemical injuries in *Brassica napus* L. under lead stress. *Environ. Sci. Pollut. Res.* **2014**, *21*, 9899–9910. [CrossRef]
34. Li, F.L.; Qiu, Y.; Xu, X.; Yang, F.; Wang, Z.; Feng, J.; Wang, J. EDTA-enhanced phytoremediation of heavy metals from sludge soil by Italian ryegrass (*Lolium perenne* L.). *Ecotox. Environ. Safe.* **2020**, *191*, 110185. [CrossRef]
35. Grifoni, M.; Rosellini, I.; Petruzzelli, G.; Pedron, F.; Franchi, E.; Barbafieri, M. Application of sulphate and cytokinin in assisted arsenic phytoextraction by industrial *Cannabis sativa* L. *Environ. Sci. Pollut. Res.* **2021**, *28*, 47294–47305. [CrossRef]
36. Tassi, E.; Pouget, J.; Petruzzelli, G.; Barbafieri, M. The effects of exogenous plant growth regulators in the phytoextraction of heavy metals. *Chemosphere* **2008**, *71*, 66–73. [CrossRef]
37. EPA—United State Environmental Protection Agency. Method 3052 Microwave assisted acid digestion of siliceous and organically based matrices. In *Test Methods for Evaluating Soild Waste: Physical/Chemical Methods (SW-846)*; U.S. Environmental Protection Agency: Washington, DC, USA, 1995.
38. ISO—International Organization for Standardization. *ISO 18763:2016 Soil Quality–Determination of the Toxic Effects of Pollutants on Germination and Early Growth of Higher Plants*; International Organization for Standardization: Geneva, Switzerland, 2016.
39. Shahab, S.; Ahmed, N.; Khan, N.S. Indole acetic acid production and enhanced plant growth promotion by indigenous PSBs. *J. Plant Pathol.* **2009**, *91*, 61–63.
40. Milagres, A.M.F.; Machuca, A.; Napoleão, D. Detection of siderophore production from several fungi and bacteria by a modification of chrome azurol S (CAS) agar plate assay. *J. Microbiol. Methods* **1999**, *37*, 1–6.
41. Nautiyal, C.S. An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. *FEMS Microbiol. Lett.* **1999**, *170*, 265–270. [CrossRef]
42. Santaella, C.; Schue, M.; Berge, O.; Heulin, T.; Achouak, W. The exopolysaccharide of Rhizobium sp. YAS34 is not necessary for biofilm formation on Arabidopsis thaliana and Brassica napus roots but contributes to root colonization. *Environ. Microbiol.* **2008**, *10*, 2150–2163. [CrossRef]
43. Nielsen, P.; Sørensen, J. Multi-target and medium-independent fungal antagonism by hydrolytic enzymes in *Paenibacillus polymyxa* and *Bacillus pumilus* strains from barley rhizosphere. *FEMS Microbiol. Ecol.* **1997**, *22*, 183–192. [CrossRef]
44. Kifle, M.H.; Laing, M.D. Isolation and screening of bacteria for their diazotrophic potential and their influence on growth promotion of maize seedlings in greenhouses. *Front. Plant Sci.* **2015**, *6*, 1225. [CrossRef]

45. Liba, C.M.; Ferrara, F.I.S.; Manfio, G.P.; Fantinatti-Garboggini, F.; Albuquerque, R.C.; Pavan, C.; Ramos, P.L.; Moreira-Filho, C.A.; Barbosa, H.R. Nitrogen-fixing chemo-organotrophic bacteria isolated from Cyanobacteria-deprived lichens and their ability to solubilize phosphate and to release amino acids and phytohormones. *J. Appl. Microbiol.* **2006**, *101*, 1076–1086. [CrossRef]
46. Conte, A.; Chiaberge, S.; Pedron, F.; Barbafieri, M.; Petruzzelli, G.; Vocciante, M.; Franchi, E.; Pietrini, I. Dealing with complex contamination: A novel approach with a combined bio-phytoremediation strategy and effective analytical techniques. *J. Environ. Manag.* **2021**, *288*, 112381. [CrossRef]
47. Saifullah; Meers, E.; Qadir, M.; de Caritat, P.; Tack, F.M.; Du Laing, G.; Zia, M.H. EDTA-assisted Pb phytoextraction. *Chemosphere* **2009**, *74*, 1279–1291. [CrossRef]
48. Xu, Y.; Yamaji, N.; Shen, R.; Ma, J.F. Sorghum roots are inefficient in uptake of EDTA-chelated lead. *Ann. Bot.* **2007**, *99*, 869–875. [CrossRef]
49. Li, Q.; Huang, Y.; Wen, D.; Fu, R.; Feng, L. Application of alkyl polyglycosides for enhanced bioremediation of petroleum hydrocarbon-contaminated soil using *Sphingomonas changbaiensis* and *Pseudomonas stutzeri*. *Sci. Total Environ.* **2020**, *719*, 137456. [CrossRef]
50. Rathi, M.; Yogalakshmi, K.N. Brevundimonas diminuta MYS6 associated *Helianthus annuus* L. for enhanced copper phytoremediation. *Chemosphere* **2021**, *263*, 128195. [CrossRef] [PubMed]
51. Nazli, F.; Mustafa, A.; Ahmad, M.; Hussain, A.; Jamil, M.; Wang, X.; Shakeel, Q.; Imtiaz, M.; El-Esawi, M.A. A Review on Practical Application and Potentials of Phytohormone-Producing Plant Growth-Promoting Rhizobacteria for Inducing Heavy Metal Tolerance in Crops. *Sustainability* **2020**, *12*, 9056. [CrossRef]
52. Barbafieri, M.; Morelli, E.; Tassi, E.; Pedron, F.; Remorini, D.; Petruzzelli, G. Overcoming limitation of "recalcitrant areas" to phytoextraction process: The synergistic effects of exogenous cytokinins and nitrogen treatments. *Sci. Total Environ.* **2018**, *639*, 1520–1529. [CrossRef]
53. Alaboudi, K.A.; Ahmed, B.; Brodie, G. Phytoremediation of Pb and Cd contaminated soils by using sunflower (*Helianthus annuus*) plant. *Ann. Agric. Sci.* **2018**, *63*, 123–127. [CrossRef]
54. Bassegio, C.; Campagnolo, M.A.; Schwantes, D.; Gonçalves Junior, A.C.; Manfrin, J.; Schiller, A.D.P.; Bassegio, D. Growth and accumulation of Pb by roots and shoots of *Brassica juncea* L. *Int. J. Phytoremediat.* **2020**, *22*, 134–139. [CrossRef]
55. Chauhan, P.; Rajguru, A.B.; Dudhe, M.Y.; Mathur, J. Efficacy of lead (Pb) phytoextraction of five varieties of *Helianthus annuus* L. from contaminated soil. *Environ. Technol. Innov.* **2020**, *18*, 100718. [CrossRef]
56. Gurajala, H.K.; Cao, X.; Tang, L.; Ramesh, T.M.; Lu, M.; Yang, X. Comparative assessment of Indian mustard (*Brassica juncea* L.) genotypes for phytoremediation of Cd and Pb contaminated soils. *Environ. Pollut.* **2019**, *254*, 113085. [CrossRef]
57. Rathika, R.; Srinivasan, P.; Alkahtani, J.; Al-Humaid, L.A.; Alwahibi, M.S.; Mythili, R.; Selvankumar, T. Influence of biochar and EDTA on enhanced phytoremediation of lead contaminated soil by *Brassica juncea*. *Chemosphere* **2021**, *271*, 129513. [CrossRef] [PubMed]
58. Ahmed, S.; Hassan, M.H.; Kalam, M.A.; Ashrafur Rahman, S.M.; Abedin, M.J.; Shahir, A. An experimental investigation of biodiesel production, characterization, engine performance, emission and noise of *Brassica juncea* methyl ester and its blends. *J. Clean. Prod.* **2014**, *79*, 74–81. [CrossRef]
59. Hunce, S.Y.; Clemente, R.; Bernal, M.P. Energy production potential of phytoremediation plant biomass: Helianthus annuus and Silybum marianum. *Ind. Crops Prod.* **2019**, *135*, 206–216. [CrossRef]
60. Iram, S.; Tariq, I.; Ahmad, K.S.; Jaffri, S.B. *Helianthus annuus* based biodiesel production from seed oil garnered from a phytoremediated terrain. *Int. J. Ambient Energy* **2020**. [CrossRef]
61. Pant, S.; Ritika; Komesu, A.; Penteado, E.D.; Diniz, A.A.R.; Rahman, M.A.; Kuila, A. NaOH pretreatment and enzymatic hydrolysis of Brassica juncea using mixture of cellulases. *Environ. Technol. Innov.* **2021**, *21*, 101324. [CrossRef]
62. Luo, J.; Qi, S.; Gu, X.W.S.; Wang, J.; Xie, X. An evaluation of EDTA additions for improving the phytoremediation efficiency of different plants under various cultivation systems. *Ecotoxicology* **2016**, *25*, 646–654. [CrossRef] [PubMed]
63. Mahmood-ul-Hassan, M.; Suthar, V.; Ahmad, R.; Yousra, M. Heavy metal phytoextraction—natural and EDTA-assisted remediation of contaminated calcareous soils by sorghum and oat. *Environ. Monit. Assess.* **2017**, *189*, 1–10. [CrossRef]
64. Saleem, M.H.; Ali, S.; Kamran, M.; Iqbal, N.; Azeem, M.; Tariq Javed, M.; Ali, Q.; Zulqurnain Haider, M.; Irshad, S.; Rizwan, M.; et al. Ethylenediaminetetraacetic Acid (EDTA) Mitigates the Toxic Effect of Excessive Copper Concentrations on Growth, Gaseous Exchange and Chloroplast Ultrastructure of *Corchorus capsularis* L. and Improves Copper Accumulation Capabilities. *Plants* **2020**, *9*, 756. [CrossRef]
65. Sharma, P.; Pandey, A.K.; Udayan, A.; Kumar, S. Role of microbial community and metal-binding proteins in phytoremediation of heavy metals from industrial wastewater. *Bioresour. Technol.* **2021**, *326*, 124750. [CrossRef]
66. Ma, Y.; Rajkumar, M.; Zhang, C.; Freitas, H. Beneficial role of bacterial endophytes in heavy metal phytoremediation. *J. Environ. Manag.* **2016**, *174*, 14–25. [CrossRef]
67. Franchi, E.; Fusini, D. Plant Growth-Promoting Rhizobacteria (PGPR) Assisted Phytoremediation of Inorganic and Organic Contaminants Including Amelioration of Perturbed Marginal Soils. In *Handbook of Assisted and Amendment-Enhanced Sustainable Remediation Technology*; Prasad, M.N.V., Ed.; John Wiley & Sons Ltd.: Hoboken, NJ, USA, 2021; pp. 477–500.
68. Ullah, A.; Heng, S.; Munis, M.F.H.; Fahad, S.; Yang, X. Phytoremediation of heavy metals assisted by plant growth promoting (PGP) bacteria: A review. *Environ. Exp. Bot.* **2015**, *117*, 28–40. [CrossRef]
69. He, X.; Xu, M.; Wei, Q.; Tang, M.; Guan, L.; Lou, L.; Xu, X.; Hu, Z.; Chen, Y.; Shen, Z.; et al. Promotion of growth and phytoextraction of cadmium and lead in *Solanum nigrum* L. mediated by plant-growth-promoting rhizobacteria. *Ecotoxicol. Environ. Saf.* **2020**, *205*, 111333. [CrossRef] [PubMed]