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Helichrysum microphyllum subsp. *tyrrhenicum*, Its Root-Associated Microorganisms, and Wood Chips Represent an Integrated Green Technology for the Remediation of Petroleum Hydrocarbon-Contaminated Soils

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Abstract: Phytoremediation and the use of suitable amendments are well-known technologies for the mitigation of petroleum hydrocarbon (PHC) contaminations in terrestrial ecosystems. Our study is aimed at combining these two approaches to maximize their favorable effects. To this purpose, *Helichrysum microphyllum* subsp. *tyrrhenicum*, a Mediterranean shrub growing on sandy and semiarid soils, was selected. The weathered PHC-polluted matrix (3.3 ± 0.8 g kg^{-1} dry weight) from a disused industrial site was employed as the cultivation substrate with (WCAM) or without (UNAM) the addition and mixing of wood chips. Under the greenhouse conditions, the species showed a survival rate higher than 90% in the UNAM while the amendment administration restored the totality of the plant survival. At the end of the greenhouse test (nine months), the treatment with the wood chips significantly increased the moisture, dehydrogenase activity and abundance of the microbial populations of the PHC degraders in the substrate. Cogently, the residual amount of PHCs was significantly lower in the UNAM (3–92% of the initial quantity) than in the WCAM (3–14% of the initial quantity). Moreover, the crown diameter was significantly higher in the WCAM plants. Overall, the results establish the combined technology as a novel approach for landscaping and the bioremediation of sites chronically injured by PHC-weathered contaminations.

Keywords: phytoremediation; organic amendments; rhizodegradation; weathered hydrocarbons; landscaping

1. Introduction

Phytoremediation is the use of plants and their associated microbial communities to stabilize or eliminate organic (i.e., petrogenic hydrocarbons and xenobiotics) and inorganic contaminants (i.e., metals and metalloids) in soils [1,2]. Moreover, the technology can offer economic, societal and aesthetic benefits, thus allowing for the requalification of the contaminated sites, urban design and landscape architecture [3].

In the last decades, phytoremediation has been recognized as an eco-friendly and cost-effective technology for the restoration of sites contaminated by petrogenic hydrocarbons (PHCs), a complex mixture of hydrocarbons primarily derived from crude oils [4]. In the process, the degradation occurs through different mechanisms [5]. A wide spectrum of root-associated microorganisms, including bacteria and fungi, accomplishes the pollutant degradation and detoxification (rhizodegradation) using PHCs as carbon and energy sources [6]. The plants contribute to the process of degrading PHCs by secreting enzymes in the root exudates [7,8] as well as employing contaminant uptakes and subsequent sequestration [9]. Therefore, phytoremediation can be considered an integrated approach where both plants and microorganisms mediate the PHC breakdown via the use of their enzymatic machineries [5]. At the same time, plants and their associated microbial communities interact synergically and symbiotically in the rhizosphere. There, the plants release the root exudates that modify the physical–chemical and nutritional properties of the contaminated soils (e.g., increase the aeration, contaminant desorption from the soil particles, induction of the microbial hydrocarbon-degrading genes), thus stimulating the microbial degradation [6,10,11]. On the other hand, microorganisms promote plant growth and increase the plant tolerance to environmental stresses [5,12].

A key aspect in the implementation of phytoremediation is the selection of the appropriate plant species [6]. The criteria that should be considered in the plant selection are not only its tolerance to contaminants but also its adaptability to other stress factors (e.g., drought and low nutrient contents), fast growth with the production of consistent biomass, dense root and shoot systems and suitability for various substrate types. Pioneer plant species that are able to spontaneously colonize contaminated sites should be preferred, but plants with a good adaptability to the local climate [13,14] are also desirable. Furthermore, perennial plants would guarantee the restoration of a stable vegetation cover for environmental landscaping, as observed for *Cistus salviifolius* L., *Euphorbia pithyusa* L. subsp. *cupanii* (Guss. ex Bertol.) Radcl.-Sm., *Helichrysum microphyllum* (Willd.) Cambess. subsp. *tyrrhenicum* Bacch., Brullo and Giusso, *Pistacia lentiscus* L., *Pinus halepensis* Mill. and *Scrophularia canina* L. subsp. *bicolor* (Sm.) Greuter on metal(loid) contaminations [15–31]. For the reasons mentioned above, *Helichrysum microphyllum* subsp. *tyrrhenicum* (hereafter *H. tyrrhenicum*) was used in this study. The genus *Helichrysum*, belonging the Asteraceae family, is widely distributed in Africa, Madagascar, the Mediterranean Basin, Macaronesia, central Asia and India and includes approx. 500 to 600 species according to different authors [32–34]. *Helichrysum tyrrhenicum* is a Cyrno-Sardinian endemic species [35] well adapted to the Mediterranean climatic conditions. It spontaneously grows on different substrates, especially sandy and muddy soils, as well as abandoned mine sites, where it tolerates highly harsh conditions, such as aridity, high metal(loid) levels (in particular Zn, Pb, Cd and As) and low nutrient contents [17–20,36–40]. Moreover, the anticandidal activity of its essential oils has been demonstrated [34].

In the scenario of the soil contamination by PHCs, the challenge is still open regarding the long-term contaminations that are frequently associated with crude oil extraction and refining, as well as industrial storage plants [41]. As the duration of the contact between PHCs and the soil increases, the readily available fraction decreases through degradation, loss, or transformation into a recalcitrant fraction, a process known as aging [42,43]. The aged recalcitrant fraction results from the contaminant sorption and entrapment within the soil micropores and a slow diffusion within the organic matter [43]. The biodegradation kinetics of aged PHCs are mainly limited by mass transport phenomena (desorption, diffusion, convection, etc.) [44]. Therefore, even if soil microorganisms have the genetic ability to degrade contaminants, the low microbial availability of the aged fraction causes its persistency in the soil [43,45]. Up to now, a limited number of herbaceous species have been tested for phytoremediation in the soils contaminated by aged PHCs [14,46–49]. A complementary approach to enhance the aged PHC degradation is biostimulation through the addition of carbon organic amendments, such as compost [50], sewage sludge [51], composted plant biomass [52] and rice straw [53]. Nevertheless, relevant studies on

the aged contaminations have been mainly performed from the perspective of a single technology while their integration has not been previously tested.

With these premises, the aims of the present study were to (i) test the applicability of *H. tyrrhenicum* for the revegetation of the substrate with weathered hydrocarbon contamination and to (ii) evaluate whether the addition of wood chips as substrate amendment enhances hydrocarbon removal by the studied plant–microbe system. In order to achieve the aforementioned objectives, a multidisciplinary approach was employed, including an evaluation of the plant growth, soil microbial activity and abundance, as well as the hydrocarbon degradation.

2. Materials and Methods

2.1. Substrates, Plants and Wood Chips

The polluted substrate was obtained from a coastal site located in Sardinia (the former SIPSA S.R.L. in Torregrande, Italy), where petroleum refining was first carried out followed by the storage of heavy oils and bitumen since the 1960s. The cessation of the industrial activities in 2001 was followed by dismantling the plant installations. The site remediation is currently in progress. In October 2020, a volume of 2 m^3 of the substrate not yet subjected to remediation treatments (39°53′56.02″ N, 8°32′17.80″ E) was collected (Figure S1). After a thorough mixing, the substrate was immediately used for the phytoremediation greenhouse test.

A commercial garden preparation was used as the optimal growth substrate in the test. The substrate was composed of a mixture of Irish peats, 10–20 mm of calibrated blond peat, 10–30 mm of calibrated Baltic peat, 3–8 mm of pumice, expanded silicate, soluble ternary fertilizer (1000 gr m^{-3}) and microelements. The chemical–physical parameters of the commercial substrate were a pH of 5–6, an electrical conductivity of 0.20–0.25 ds m^{-1}, a density of 0.27 g cm^{-3} and a porosity of 90%.

The wood chips were obtained by green pruning and dry wood from the city green and hardwoods (pH 7.2, C/N 22). The plants of *H. tyrrhenicum* were grown on the uncontaminated substrate for two years before the transplantation into the contaminated substrate. The commercial garden substrate, plants and wood chips were provided by Sgaravatti Land S.R.L, (Capoterra, Italy).

The samples (three replicates) of the contaminated substrate and wood chips were collected before the greenhouse test for the determination of the initial physico–chemical and microbiological parameters.

2.2. Greenhouse Test and Samplings

A phytoremediation test was conducted in a greenhouse at Sgaravatti Land (39° 7′44.31″ N, 9° 0′13.12″ E). Three experimental conditions were tested: (i) a contaminated substrate without amendments (UNAM), (ii) a contaminated substrate mixed with wood chips at a proportion of 1:1 *v/v* as the amendment (WCAM) and (iii) a commercial garden substrate (GARD) as the reference condition. The planting was performed by transferring one plant in every pot with a 14 L substrate. For each experimental condition, 30 pots were prepared. Each pot was named with a unique alphanumerical label as follows: the vegetal species (Hm), the substrate (i.e., UNAM, WCAM, GARD) and a progressive number (1–30). The pots setup was completed within 12 h of the substrate sampling.

Throughout the phytoremediation test, the plants were irrigated weekly with tap water. The following growth parameters were monitored fortnightly: the plant height, stem base diameter and crown diameter (Figure 1). The height of the plant was determined using a rigid meter, starting from the base and reaching the highest branch. A digital caliber (IP65, Alpa Metrology, Italy) was used to measure the base and crown diameters. In order to measure the height from the same point of the base, on the day of the first measurement, a point was marked on the stem and it was used as a reference for the subsequent determinations. The base diameter was assessed at the same point and was

used as a reference for the height measurement. The diameter of the crown was measured from the edges of the outermost branches of the plant.

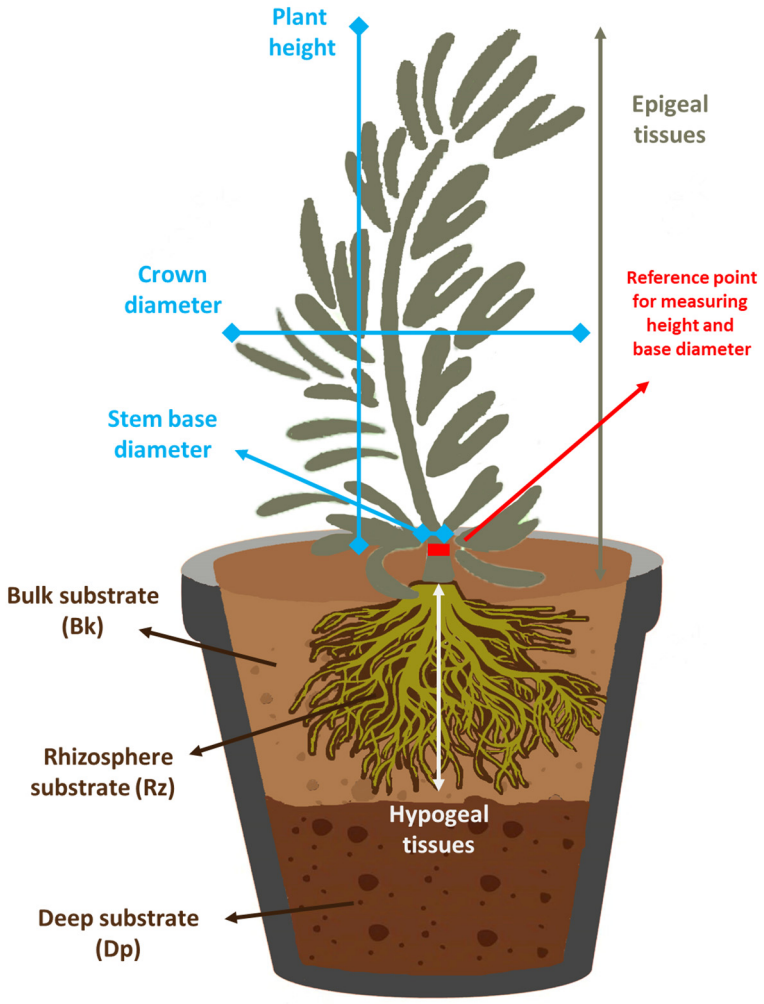

Figure 1. Schematic representation of the plant growth parameters and the different matrices used for determination of the substrate parameters.

The experimental test lasted for nine months. Then, five pots for each experimental condition were randomly chosen and used for the chemical, microbiological and enzymatic analyses (Figure S2). In the greenhouse, the plants were manually eradicated, and three different matrices were separated from each pot, as illustrated in Figure 1. More specifically, the substrates that did not tightly adhere to the plant roots were collected by shaking the roots, weighing them and considering the matrix as the bulk substrate. The substrate under the hypogeal tissues, not in direct contact with roots, were separately collected using a small disinfected shovel, weighed and considered as the deep substrate. The substrate firmly adhering to the hypogeal tissues was considered as the rhizosphere substrate. For every pot, a sample was obtained for each matrix. In order to limit the changes in the chemical and microbiological parameters, the samples (five replicates) were immediately transported to the laboratories under refrigeration at 4 °C.

To determine the plant dry biomass, specimens of the other five pots for each experimental condition were randomly chosen. The plants were manually harvested, gently hand cleaned and split with scissors into epigeal and hypogeal fractions (Figure 1), which were dried in an oven (Binder ED 155) at 40 °C until a constant weight was obtained.

2.3. Physico–Chemical Analyses

The texture was determined according to the Unified Soil Classification System. The substrate moisture was evaluated through oven drying at 105 °C for 24 h, as a percentage of the wet-weight loss. The pH was measured in a 1:2.5 substrate to water ratio after 1 h mixing and subsequent settling. For the wood chips, an aliquot (1 g) was crumbled into 10 mL of water in an Ultra-Turrax tube disperser with stainless steel balls (IKA, Staufen, Germany). The pH was measured after settling. The water used for the pH measurement was previously boiled to remove interfering carbon dioxide.

To determine the total petroleum hydrocarbons (TPHs), the subsamples were stored at −20 °C immediately after collection until the analysis. For each of the three experimental conditions, the bulk substrate (250 g), deep substrate (250 g) and rhizosphere substrate (except for the quantity used for the microbiological and enzymatic analyses) of the five replicate pots were separately analyzed (Figure 1). An analysis was carried out according to the ISPRA Method 75/2011 [54]. More specifically, each substrate aliquot was air dried and sieved. The <2 mm particle size fraction was placed in a glass container with an acetone/hexane solution (2:1, acetone for gas chromatography ECD and FID SupraSolv®, *n*-hexane Chromasolv for GC, Sigma Aldrich, Milan, Italy). The mixture was sonicated to facilitate the extraction of the organic compounds. The resulting mixture was filtered and shaken with water in a separating funnel. The organic phase was purified through a solid phase extraction with sodium sulfate and florisil (Supelclean™ LC-Florisil® SPE Tube, bed wt. 1 g, 6 mL). The filtrate was dried and resumed with a known volume of hexane. The extract was analyzed using gas chromatography coupled with mass spectrometry (GC-MS 7820A GC system, Agilent Technologies, Santa Clara, United States). A DB5 column was used for the chromatographic separation with the following ramp heating: 60 °C for 10 min, 15 °C/min to 280 °C and 280 °C for 30 min.

The total amount of hydrocarbons was determined by the total area of the peaks in the range of the *n*-dodecane and *n*-tetracontane retention times. A standard mixture of linear hydrocarbons (C12-C40 Alkanes Calibration Standard, Merck, Milan, Italy) and a standard mixture of mineral oils (BAM-K010, mineral oil standard mixture Type A and B for EN 14039 and ISO 16703, Merck) were used as the reference solutions. The identity of the organic compounds was checked by comparing the retention times of the standards and by a mass spectra analysis.

The residual content of the TPHs in the substrate was calculated for each pot, summing the amount of the TPHs in the bulk, deep and rhizosphere substrates, and expressed as percentage with respect to the initial amount of the TPHs in the substrate. The concentration of the TPHs in the hypogeal tissues were also quantified, and the amount of the TPHs accumulated in the hypogeal tissues was calculated based on the measured dry biomass.

2.4. Microbiological and Enzymatic Analyses

The microbiological and enzymatic analyses were performed on the refinery substrate and wood chips before the greenhouse test as well as on the bulk and rhizosphere substrates of the unamended (UNAM) and amended (WCAM) pots (five replicates) at the end of the greenhouse test.

For the wood chips, an aliquot (1 g) was aseptically crumbled into 10 mL of a sterile saline solution in an Ultra-Turrax tube disperser with stainless steel balls. For the analyses of the rhizosphere substrates, the root material with the tightly adhering substrate particles (25 g) were washed in a sterile saline solution (8.5 g L^{-1} NaCl) for two hours. Then, the root tissues were removed and the substrate suspension was recovered. The extracts of the wood chips, rhizosphere substrate suspensions and bulk substrates were used for the selective enumeration of the microorganisms and the dehydrogenase assay.

2.4.1. Selective Enumeration of Microorganisms by the Most Probable Number

The selective enumeration of the four different microbial groups was performed by most probable number (MPN) procedure in the five replicates (five-tube MPN) [55]. For

the rhizosphere substrates and wood chips, the substrate suspension and ground materials were used, respectively. For the bulk substrates, the subsamples (1.0 g wet w) were suspended in a sterile saline solution and the suspension was vigorously shaken for two min. The samples were left to settle for 15 min and the selective enumeration was carried out in 96-well microtiter plates in a total culture volume of 200 µL per well. The inoculation was performed by adding 20 µL of each 10-fold dilution (up to 10^{-10}) to five wells per plate. For each plate, five wells were used as the sterile control, omitting the sample inoculation. The selective enumeration of the microorganisms capable of degrading hydrocarbons was carried out in a Bushnell–Haas (BH, BD Difco, Milan, Italy) minimum culture medium, free of a carbon source, to which the different carbon and energy sources of interest were added [56,57]. The viable titers of the following different groups of microorganisms were selectively determined: (i) heterotrophic bacteria in Tryptic Soy broth (Microbiol, Cagliari, Italy) with cycloheximide (300 µg L^{-1}) to inhibit fungal growth, (ii) fungi in Sabouraud Broth (Microbiol, Cagliari, Italy), (iii) medium-chain hydrocarbons degraders, providing diesel as the sole carbon source and (iv) long-chain hydrocarbon degraders, providing paraffin as the sole carbon source. The commercial diesel was sterilized using filtration (cutoff 0.2 µm). The paraffin was sterilized using autoclaving for 15 min at 120 °C and 1 atm. The preliminary analysis of the paraffin before and after the sterilization confirmed the hydrocarbon mixture was not altered by the sterilization procedure. The microtiter plates were wrapped in plastic bags and incubated at 28 ± 1 °C for 10 days for fungi and 21 days for bacteria and the PHC degraders. The cultures setup was completed within six hours of the sampling. At the end of the incubation periods, the positive wells were visually inspected for heterotrophic bacteria and fungi. For the PHC degraders, 20 µL of an iodonitrotetrazolium violet (INT) sterile water solution (3 g L^{-1}) was added to each well and the plates were incubated for 24 h at 28 °C. The wells were scored as positive if a red precipitate was detected due to an INT reduction and an intracellular deposition of the red-colored formazan (INTF). The viable titer (MPN g^{-1} wet w) was calculated according to Alexander [55].

2.4.2. Dehydrogenase Assay

The dehydrogenase activity (DHase) was measured using INT as substrate [58]. For the bulk substrates, 1 g of wet w was suspended in 1.2 mL of a sterile saline solution and mixed. The determinations for the rhizosphere substrates and wood chips were performed on 1.2 mL of the substrate suspension and ground material, respectively. A volume of 0.3 mL of an INT solution (as above) was added. For each sample, a control was also prepared by substituting an equal volume of saline solution to the substrate solution. The setup of the assays was completed within six hours of the sampling. The assays were incubated overnight in an orbital shaker at 30 °C and 180 rpm, and then stopped by adding 3 mL of acetonitrile [59]. The insoluble INTF was extracted by shaking at 180 rpm at 30 °C for 30 min, and the absorbance was measured as 490 nm. The value measured from the non-INT substrate control was subtracted from the value obtained for each of the three replicate INT samples. The INTF concentration was determined from the linear least squares best-fit line from a standard curve of the INTF solutions (5–100 mg L^{-1}) in acetonitrile. All the determinations were performed in triplicate.

2.5. Statistical Analyses

A Shapiro–Wilk test was used as the normality test and the homoscedasticity was checked using Levene's test. Since not every variable met the criteria of the parametric procedure, the Kruskal–Wallis rank non-parametric ANOVA was performed for each measured variable to test whether the differences between the tested experimental conditions were significant ($p < 0.05$). For multiple comparisons, the post-hoc Mann–Whitney pairwise test with the Bonferroni correction was applied. The univariate analyses were carried out using the PAST software package v4.03 [60].

The relationships between the variables measured in the UNAM and the WCAM were explored by the principal component analysis (PCA), a statistical technique that linearly transforms a set of original multiple variables into a substantially smaller set of principal components (PC) for easier interpretation. As a pre-treatment transformation, the values for each variable had their mean subtracted and they were then divided by their standard deviation (i.e., *z*-score transformation). The two-way cross analysis of similarity (ANOSIM, 9999 permutations) was performed on the Euclidean distance matrix of the abiotic variables measured in the substrates and plants to test the significant difference between the groups (i.e., the UNAM, the WCAM). Multivariate analyses (i.e., PCA and ANOSIM) were carried out as implemented in the PRIMER v.7 software [61].

The labels for the variables were as follows: U%: moisture, h: plant height, DMc: crown diameter, DMs: stem base diameter, DWep: dry weight of the epigeal tissues, DWhy: dry weight of the hypogeal tissues, DHase: dehydrogenase activity, F: viable titer of the fungi, B: viable titer of the bacteria, D: viable titer of the diesel degraders, P: viable titer of the paraffin degraders, TPH: total petroleum hydrocarbons. The labels for the matrices were as follows: Bk: bulk substrate, Rz: rhizosphere substrate, Dp: deep substrate.

3. Results

3.1. Properties of the Substrate and Wood Chips

The chemical, enzymatic and microbiological properties of the refinery substrate and wood chips before the greenhouse test are shown in Supplementary Table S1. The refinery substrate was classified as silty sand (texture SM). It was slightly alkaline (8.4 $\pm$ 0.2), with a low moisture (12%) and a DHase of 6.0 $\pm$ 5.4 INT-F g^{-1} wet w. The concentration of the TPHs was 3.3 $\pm$ 0.8 g kg^{-1} dry w. The fungi were found to have the lowest abundance among the four enumerated microbial groups with a viable titer of 3.2 $\pm$ 0.2 log MPN g^{-1} wet w. The heterotrophic bacteria (5.1 $\pm$ 0.4 log MPN g^{-1} wet w) exhibited a viable titer two order of magnitude higher than the fungi. The viable titers comparable to the heterotrophic bacteria were found for the microorganisms able to be used as carbon source medium- (5.4 $\pm$ 0.3 log MPN g^{-1} wet w) and long-chain PHCs (4.9 $\pm$ 0.1 log MPN g^{-1} wet w).

The wood chips were characterized using a neutral pH (7.2 $\pm$ 0.1), a moisture of 49% and an abundant microbial community. More specifically, the viable titers were 7.6 $\pm$ 0.4 log MPN g^{-1} wet w for the fungi and 7.7 $\pm$ 0.2 log MPN g^{-1} wet w for the heterotrophic bacteria. The degraders of the medium- and long-chain PHCs were the most abundant populations with 8.5 $\pm$ 0.4 and 8.0 $\pm$ 0.5 log MPN g^{-1} wet w, respectively. The amendment exhibited a DHase of 15.8 $\pm$ 0.3 INTF g^{-1} wet w.

3.2. Plant Growth and Biomass Production

The plant growth parameters monitored during the greenhouse test are shown in Figure 2, and their statistical analysis is shown in Supplementary Tables S2 and S3. The plant height increased in all the tested substrates until 102 days after transplantation (Figure 2a—left). Then, the parameter gradually decreased in the UNAM, while plant growth continued for the entire duration of the test in the GARD and the WCAM, with a faster growth rate in the GARD than in the WCAM. Indeed, the values at the end of the greenhouse test (199 days) were significantly higher than the initial values in the GARD and the WCAM, however the difference was not significant in the UNAM (Table S2). Comparing the three tested substrates, the values of the parameters were significantly higher in the GARD than in the UNAM starting at 158 days (Figure 2a, Table S3). Intermediate values of the plant height were found in the WCAM, which were not significantly different compared to the UNAM and the GARD. The only exception was at 199 days when the parameter was significantly higher in the GARD than the WCAM (Figure 2a—right).

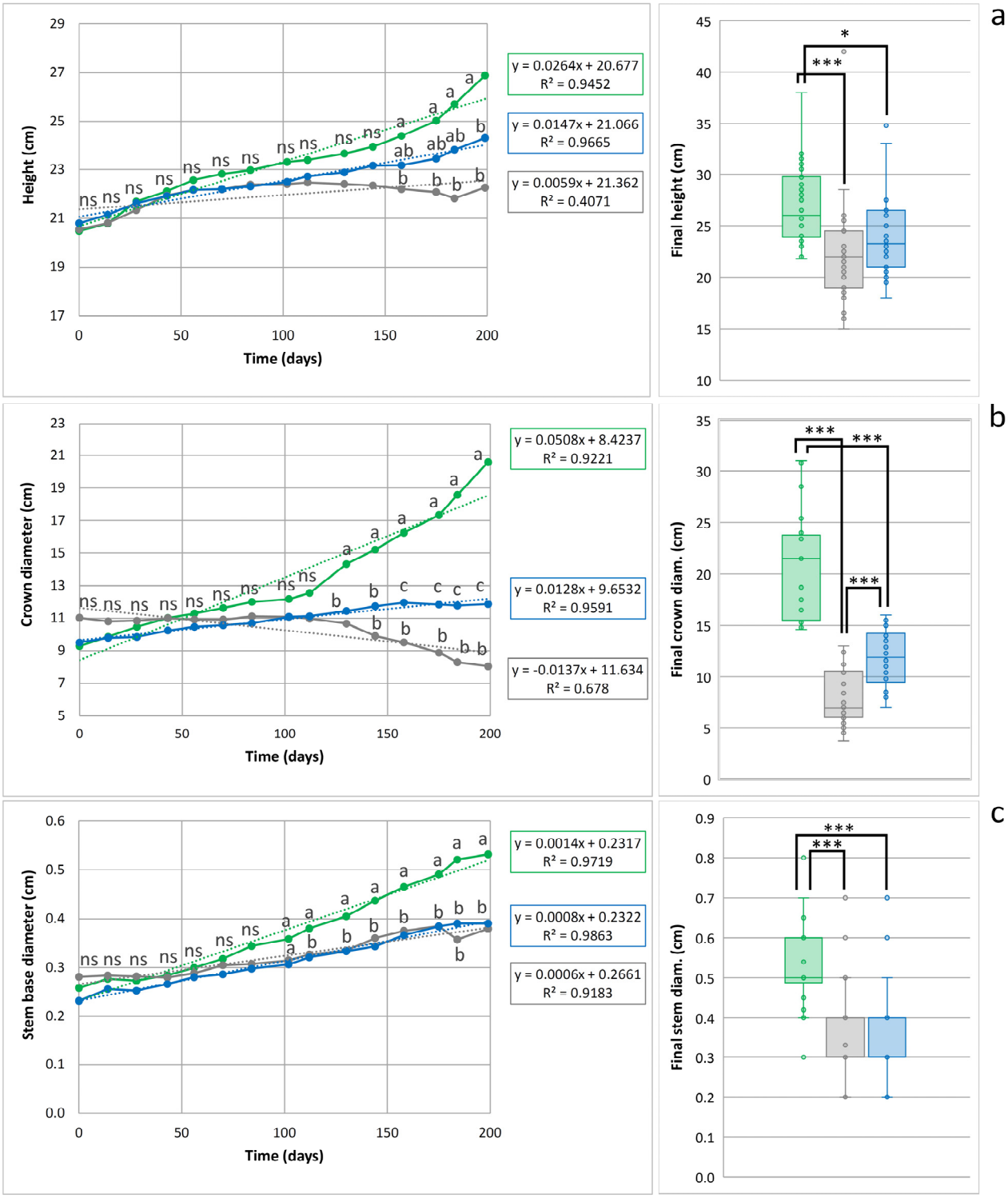

Figure 2. Growth of *Helichrysum microphyllum* subsp. *tyrrhenicum* in the greenhouse test. (**a**) Plant height, (**b**) crown diameter, (**c**) stem base diameter. Left panel. Kinetics and linear least squares best-fit line of the growth parameters. Each value is the mean of the measurements on the 30 pots for each experimental condition. Significant differences between the tested conditions in the pairwise comparisons ($p < 0.05$) are represented by different lowercase letter labels. ns: $p > 0.05$. Right panel. Box plot of the growth parameters measured at the end of the test. Bars are drawn from each box to the higher and lower data within 1.5 times the interquartile range. * $p < 0.05$, *** $p < 0.001$. Green: commercial garden substrate (GARD), Gray: contaminated substrate without amendment (UNAM), Blue: contaminated substrate amended with wood chips (WCAM).

The crown diameter remained almost constant in the UNAM until 112 days and then it decreased (Figure 2b), resulting in a final value significantly lower than the initial one (Table S2). On the contrary, the parameter increased throughout the test in the GARD and the WCAM. In both conditions, significantly higher values at the end of the greenhouse test were found compared to the initial ones (Table S2). The crown diameter was significantly lower in the UNAM than in the GARD starting at 130 days, and in the UNAM than in the WCAM at 158 days, even though the rate was substantially slower in the WCAM than in the GARD. Indeed, significantly higher values in the GARD than in the WCAM were found at 130 days (Figure 2b, Table S3).

The stem base diameter increased in all the tested conditions and the differences between the initial and final values were significant (Figure 2c, Table S2). Among the substrates, the fastest growth rate was found in the GARD. The values of the parameter were significantly higher in the GARD than in the UNAM at 130 days and in the GARD than in the WCAM at 84 days (Table S3). In contrast, the differences between the UNAM and the WCAM were not significant.

The plant survival and dry biomass of the epigeal and hypogeal tissues measured at the end of the greenhouse test (199 days) are shown in Table 1, and the statistical analysis is shown in Supplementary Table S4. At the end of the experimentation, the plant survival was 93% in the UNAM and 100% in both the GARD and the WCAM. The plants grown in the GARD exhibited an epigeal dry biomass significantly higher than the plants in the UNAM and the WCAM (Table 1). No significant differences were found between the UNAM and the WCAM in the epigeal dry biomass. The differences in the hypogeal dry biomass were not significant among the three tested conditions.

Table 1. Survival and dry biomasses of *Helichrysum microphyllum* subsp. *tyrrhenicum* at the end of the greenhouse test.

Condition	Plant Survival	Dry Biomass (g dry w)	
		Epigeal Tissues	Hypogeal Tissues
GARD	100.0%	40.9 ± 12.2^{a}	8.0 ± 4.2^{ns}
UNAM	93.3%	15.6 ± 4.3^{b}	5.6 ± 4.8^{ns}
WCAM	100.0%	14.4 ± 8.8^{b}	3.1 ± 2.7^{ns}

GARD: commercial garden substrate, UNAM: contaminated substrate without amendment, WCAM: contaminated substrate amended with wood chips. Each value is the mean ± SD of the measurements on the five pots for each experimental condition. Significant differences between the tested conditions in the pairwise comparisons ($p < 0.05$) are represented by different lowercase letter labels. ns: $p > 0.05$.

3.3. Substrate Properties, Microbial Activity and Abundance of the Different Microbial Populations after the Phytoremediation Greenhouse Test

The substrate parameters measured at the end of the phytoremediation test with *H. tyrrhenicum* are shown in Table 2. Compared to the initial properties (Table S1), the substrate at the end of the test without amendments (UNAM) showed a moderate decrease in the pH, reaching the value of 8.1 ± 0.2. The substrate moisture remained almost constant and less than 20%. A 1-log increase in the viable titers of the fungi, heterotrophic bacteria and degraders of medium- and long-chain PHCs was observed in the bulk substrate of the UNAM test compared to the initial refinery substrate. Moreover, the DHase increased from 6.0 ± 5.4 INTF g^{-1} wet w in the substrate before the greenhouse test to 42.0 ± 6.7 INT-F g^{-1} wet w in the bulk substrate of the UNAM.

Table 2. Effect of wood chips on the properties of the bulk substrate (Bk), rhizosphere substrate (Rz) and deep substrate (Dp) at the end of the phytoremediation test with *Helichrysum microphyllum* subsp. *tyrrhenicum* in the refinery substrate with aged contamination unamended (UNAM) and amended (WCAM) with wood chips.

Parameter	Matrix	Condition	Mean ± SD	
Substrate moisture (%)	Bk	UNAM WCAM	10% ± 6% 33% ± 8%	**
Substrate pH (H_2O)	Bk	UNAM WCAM	8.1 ± 0.2 7.84 ± 0.05	*
Dehydrogenase activity (INTF g^{-1} wet w)	Bk	UNAM WCAM	42.0 ± 6.7 50.5 ± 5.9	*
	Rz	UNAM WCAM	203.2 ± 35.4 244.6 ± 105.7	ns
Viable titer of fungi (log MPN g^{-1} wet w)	Bk	UNAM WCAM	4.0 ± 0.5 5.7 ± 0.3	**
	Rz	UNAM WCAM	4.8 ± 0.3 5.6 ± 0.1	**
Viable titer of bacteria (log MPN g^{-1} wet w)	Bk	UNAM WCAM	6.1 ± 0.1 6.5 ± 0.2	**
	Rz	UNAM WCAM	7.0 ± 0.3 7.2 ± 0.7	ns
Viable titer of diesel degraders (log MPN g^{-1} wet w)	Bk	UNAM WCAM	6.4 ± 0.2 7.2 ± 0.2	**
	Rz	UNAM WCAM	7.5 ± 0.3 7.4 ± 0.6	ns
Viable titer of paraffin degraders (log MPN g^{-1} wet w)	Bk	UNAM WCAM	6.7 ± 0.3 7.1 ± 0.2	*
	Rz	UNAM WCAM	7.6 ± 0.3 7.5 ± 0.3	ns
Total petroleum hydrocarbons (g kg^{-1} dry w)	Bk	UNAM WCAM	2 ± 1 0.6 ± 0.3	ns
	Rz	UNAM WCAM	0.03 ± 0.02 0.1 ± 0.1	*
	Dp	UNAM WCAM	3 ± 2 1.3 ± 0.7	*

Each value is the mean ± SD of the measurements on the five pots for each experimental condition. ns: $p > 0.05$, * $p < 0.05$, ** $p < 0.01$.

Comparing the rhizosphere and the bulk substrate at end of the UNAM test, the DHase was five-fold higher and the viable titers of the four analyzed microbial populations were one order of magnitude more abundant in the rhizosphere than in the bulk substrate (Table 2). After phytoremediation without amendment, the residual TPHs were in the range of 9–92% of the initial contamination (Figure 3). Moreover, marked differences were observed in the contaminant distribution among the three analyzed substrate matrices with the concentrations decreasing in the order of the deep substrate (3 ± 2 g kg^{-1} dry w), the bulk substrate (2 ± 1 g kg^{-1} dry w) and the rhizosphere substrate (0.03 ± 0.02 g kg^{-1} dry w). The GC-MS analysis showed that the contamination was essentially due to an unresolvable mixture of hydrocarbon compounds, among which some middle and long-chain alkenes, such as dodecene, tetradecene, hexadecene and octadecene, stood out due to their high content in the deep substrate.

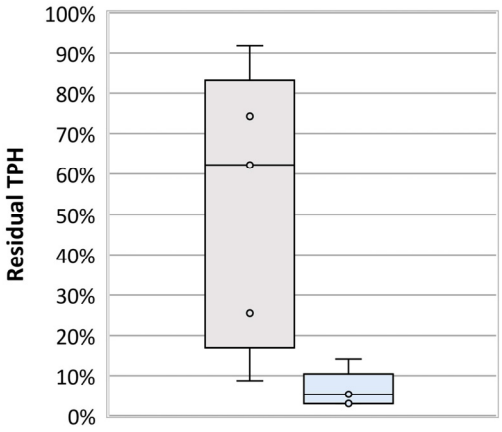

Figure 3. Residual TPHs at the end of the phytoremediation test with *Helichrysum microphyllum* subsp. *tyrrhenicum* in the refinery substrate with aged contamination unamended (UNAM, grey) and amended with wood chips (WCAM, blue). Residual TPHs were express as a percentage of the initial quantity for each experimental condition. Bars are drawn from each box to the higher and lower data within 1.5 times the interquartile range. Five pots for each experimental condition.

The comparison between the UNAM and the WCAM the of variables measured in the substrates at the end of the phytoremediation test and their statistical analysis are shown in Table 2 and Supplementary Table S5, respectively.

The treatment with the wood chips significantly increased the substrate moisture from 10% ± 6% in the UNAM to 33% ± 8% in the WCAM and decreased the substrate pH from 8.1 ± 0.2 in the UNAM to 7.84 ± 0.05 in the WCAM. Moreover, the DHase was significantly higher in the WCAM than in the UNAM. The treatment affected the populations of the four analyzed microbial groups, which showed significantly higher viable titers in the WCAM than in the UNAM. A significantly higher viable titer of the fungi in the WCAM than in the UNAM was also found in the rhizosphere substrate. In contrast, the DHase and the abundances of bacteria and PHC degraders in the rhizosphere substrates did not differ significantly between the UNAM and the WCAM. The TPHs were three-fold less concentrated in the bulk substrate of the WCAM (0.6 ± 0.3 g kg^{-1} dry w) than the UNAM (2 ± 1 g kg^{-1} dry w), even though the difference was not significant due to the high heterogenicity in the contaminant concentrations. In the deep substrate, the concentration of the TPHs was significantly higher in the UNAM (3 ± 2 g kg^{-1} dry w) than in the WCAM (1.3 ± 0.7 g kg^{-1} dry w). On the contrary, the amendment administration resulted in a significant increase in the pollutant level in the rhizosphere substrate in the WCAM as compared to the UNAM. Overall, the residual TPHs in the substrate (Figure 3) were significantly lower in the WCAM (in the range from 3% to 14%) than in the UNAM (in the range from 9% to 92%). On the other hand, the treatment did not significantly increase the total amount of TPHs accumulated by the root tissues in the WCAM (≤14 mg) compared to the UNAM (≤5 mg).

In order to explore the relationships between the physico–chemical and biological properties and how they were affected by the treatment with the wood chips, a PCA was carried out on the variables measured on both the plant and substrate matrices (Figure 4a). The first two components accounted for 70.8% of the total variance. The WCAM pots were separated from the UNAM pots on the first component based on their lower pH values, TPH concentrations in the bulk and deep substrates, as well as their higher values of substrate moisture, viable titers of the four microbial groups in the bulk substrate, viable titers of the fungi in the rhizosphere and the concentration of TPHs in the rhizosphere (Figure 4b).

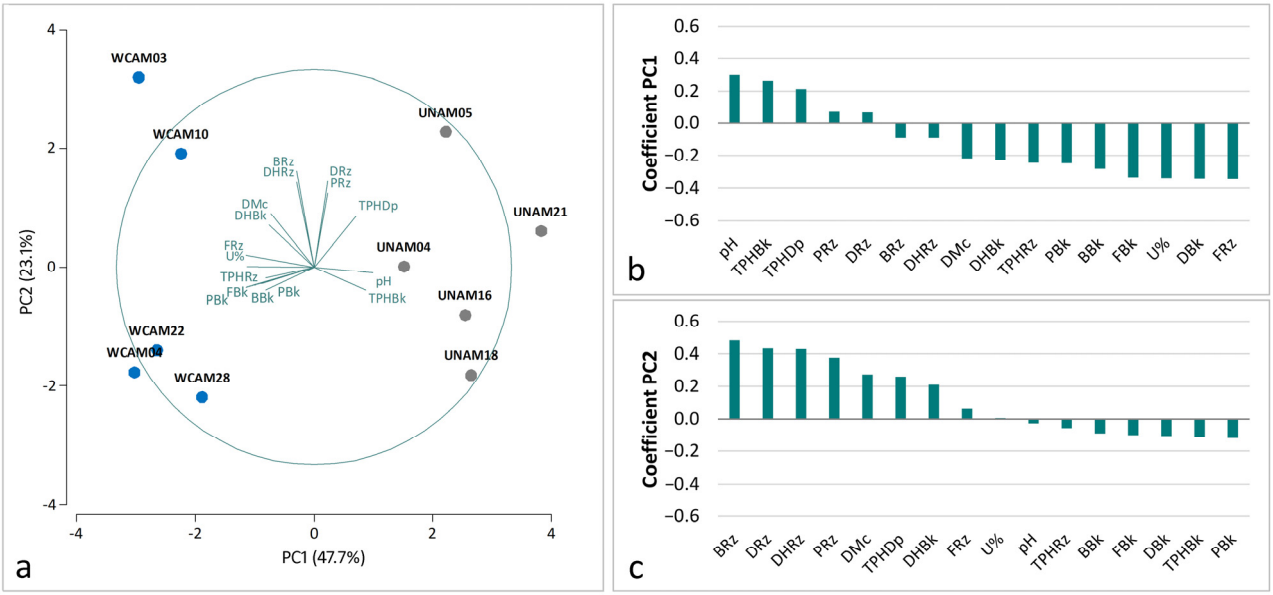

Figure 4. Principal component analysis of the physico–chemical and biological variables measured in the plants and substrates at the end of the phytoremediation test with *Helichrysum microphyllum* subsp. *tyrrhenicum* in the refinery substrate with aged contamination unamended (UNAM, grey) and amended with wood chips (WCAM, blue). (**a**) PC1 vs. PC2 plot. The percentage of the variation explained by each axis is indicated in parentheses after the axis label. Coefficients in the linear combinations of the variables that make up PC1 (**b**) and PC2 (**c**). Variable labels are defined in the text.

The second component separated the pots without clearly differentiating between the two tested groups based on their higher diameter of plant crown values, the viable titers of bacteria and PHC degraders in the rhizosphere and the DHase in the rhizosphere (Figure 4c). Overall, the patterns of the environmental variables were significantly different between the tested conditions, as demonstrated by the one-way ANOSIM (R = 0.896, p = 0.008).

4. Discussion

Helichrysum microphyllum subsp. *tyrrhenicum* displayed a high tolerance to the aged contamination in our experiments and roughly the totality of plants survived until the end of the greenhouse test in the unamended substrate. Nevertheless, the plant biomass production and growth were impaired under the stress conditions imposed by the contaminated substrate. In the literature, sub-optimal plant biomass accumulation in contaminated soils has been attributed to nutrient deficiency and oxidative stress [5,62]. In the present study, the refinery substrate was characterized by the presence of the TPHs above the threshold contamination level established by the Italian law D.Lgs. 152/2006 for industrial use (0.75 g kg^{-1}). Moreover, the contaminant hydrophobicity and the fine texture that limited water diffusion resulted in a low substrate moisture.

A slight reduction in the total amount of contaminants occurred in the employed substrate even without the addition of amendments (Figure 3), which could be ascribed to the degradation activity of the autochthonous microorganisms already present in it. Indeed, the substrate was colonized by a microbial community dominated by degraders of medium- and long-chain PHCs, which was consistent with the previous studies demonstrating a selective enrichment of degraders after a long-term permeance of soil contaminants [63,64]. The proliferation of the PHC degraders and the enhancement of the microbial activity occurred in the bulk substrate after the unamended phytoremediation test (Table 2, UNAM), which could be mainly ascribed to the higher temperature and aeration under the greenhouse conditions compared to the original site, even if the influence of the plant on the microbial community in the bulk substrate could not be excluded. In the rhizosphere, a further increase in the microbial abundance and a parallel improvement of the contaminant

removal were evident. In the literature, the stimulating effect of the plant roots on the associated microbial communities has been attributed to several mechanisms, such as the selective enrichment of the PHC degraders [65], the induction of the hydrocarbon-degrading genes [66], the provision of nutrients [67] and an increase in the hydrocarbon solubility [68]. Moreover, hypogeal tissues mechanically increased the soil aeration, thus improving the microbial degradation [5,69]. An opposite situation was found in the substrate portion under the plant roots (i.e., the deep substrate). There, the contamination level was almost the same as the initial substrate concentration, without evidence of degradation in the unamended condition. The higher contaminant concentration in the deep substrate compared to bulk substrate could be partially the result of the vertical movement of the contaminant into the pot. Nevertheless, many studies have shown that oxygen depletion in soils lead to a sharp reduction in the biodegradation activities due to the major degradative pathways for both the saturates and the aromatics involved the intervention of hydroxylation by mono- or di-oxygenases [70,71]. In the absence of oxygen, anaerobic bioremediation has been proven to be a very slow process compared to aerobic degradation [72,73]. With concern for the presence of alkenes in the deep soil fraction, the anaerobic way to metabolize alkanes by dehydrogenation to 1-alkenes was described [74]. The latter would be hydrated using a hydratase of the corresponding alcohols, or alternatively epoxidized when oxygen was present. However, this alkene-involving pathway has been questioned, although there is some convincing evidence in its favor [75].

After an initial phase of constancy in the plant morphometric parameters, which lasted for three months, the plant height and crown diameter decreased during the following three months in the UNAM plants (Figure 2). This finding could be the consequence of the nutrient depletion under the confined conditions [76]. On the other hand, the processes involved in the microbial degradation, such as the enhancement of the contaminant bioavailability and the synthesis of the low molecular weight intermediates, could justify the worsening of the plant conditions during the experiment. It is a well-known fact that insolubility substantially limits the possible interactions between the PHCs and the microbial cells, primarily as it regards medium- and high-chain alkanes [71]. The problem of the alkane uptake [77] by the degrading microorganisms is more or less effectively overcome with the production of extracellular surface-active compounds (SACs) as high molecular weight emulsifiers and low molecular weight biosurfactants [78–80]. These amphiphilic metabolites allow microbial cells to access the hydrophobic substrates, a prerequisite for their internalization and metabolism [57,81–83]. The microbial SACs stimulate the desorption and transport of the soil-sorbed contaminants, thus improving the contaminant bioavailability in the soil [79,80].

The treatment with the wood chips restored the totality of the plant survival, improved the plant growth in the short-term of the greenhouse experiment and stimulated the contaminant removal (Figures 2 and 3). The positive effects of the wood chips addition to the substrate can be traced back to the direct and indirect mechanisms. Wood chips host an abundant microbial community (Table S1). Therefore, their administration operates the bioaugmentation of the allochthonous hydrocarbon degraders in the substrate. The amendment also improves the substrate properties by increasing the substrate moisture and introducing nutrients (i.e., lignocellulosics, other organic compounds and nitrogen) (Table 2). Both the amendment itself and the metabolites produced by the biodegradation processes resulted in the acidification of the substrate. The lowering of the substrate pH resulted in an increase in the availability of important nutrients, such as iron and magnesium. Overall, the amendment determined an increase in the microbial abundances in the bulk substrate and stimulated their activity.

The obtained results are in good agreement with the previous studies demonstrating the effectiveness of wood chips as bulking agents for substrate bioremediation [62,84–86]. In our study, the stimulating effects of the amendment on hydrocarbon removal were more pronounced in the deep portion that in the bulk substrate (Figure 1, Table 2), supporting the hypothesis that the wood chips facilitated aeration, thereby ensuring sufficient aeration

for enhancing the growth of the aerobic microorganisms in the substrate below the plant roots where oxygen was a limiting factor for microbial degradation. On the contrary, the contaminant levels in the rhizosphere were improved by the wood chips. As previously mentioned, the improvement in the contaminant bioavailability was a prerequisite for the microbial degradation of the PHCs, especially in the substrate affected by the aged contaminations [44,80,81]. The increase in the contaminant concentration in the rhizosphere realized by the amendment administration denoted a higher contaminant mobility in the substrate.

5. Conclusions

Overall, our results demonstrated that *H. tyrrhenicum* has ecological characteristics that make it suitable for phytoremediation and the landscaping actions of polluted sites with weathered hydrocarbon contamination. To the best of our knowledge, we provided the first demonstration of the applicability of wood chips as an amendment for improving phytoremediation of aged contaminations by PHCs. The results obtained in this study at the greenhouse scale could set the foundation for further tests of the technology under field conditions. Based on our previous studies demonstrating that *H. tyrrhenicum* tolerates high metal(loid) concentrations, behaving as a species suitable for phytostabilization [17–20,39,40], future studies will be aimed at evaluating the integrated green technology based on *H. tyrrhenicum*, plant-associated microorganisms and wood chips for the phytomanagement of highly anthropized areas impacted by multiple contamination from metals and PHCs.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13030812/s1, Figure S1: Sampling of the contaminated substrate at the former SIPSA Srl plant (Brabau-Torregrande, OR, Italy); Figure S2: Samplings of the substrates and plants at the end of the phytoremediation test with *Helichrysum microphyllum* subsp. *tyrrhenicum* in the refinery substrate with an aged contamination (July 2021); Table S1: Main characteristics of the refinery substrate and organic amendment; Table S2: Statistical analysis of the growth parameters (plant height, crown diameter, stem base diameter) of *Helichrysum microphyllum* subsp. *tyrrhenicum* measured at the beginning and end of the greenhouse test in the three different substrates; Table S3: Statistical analysis of the growth parameters (plant height, crown diameter, stem base diameter) of *Helichrysum microphyllum* subsp. *tyrrhenicum* measured at each kinetic time during the greenhouse test in the three different substrates; Table S4: Statistical analysis of the dry biomasses of *Helichrysum microphyllum* subsp. *tyrrhenicum* at the end of the greenhouse test in the three different substrates; Table S5: Statistical analysis of the properties measured in the bulk, rhizosphere and deep substrates at the end of the phytoremediation test with *Helichrysum microphyllum* subsp. *tyrrhenicum* in the refinery substrate with an aged contamination unamended (UNAM) and amended (WCAM) with wood chips.

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Data Availability Statement: All datasets from this study are included in the article and supplementary materials.

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