

## Article

# Phytoremediation of PAH- and Cu-Contaminated Soil by *Cannabis sativa* L.: Preliminary Experiments on a Laboratory Scale

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**Abstract:** This study proposes the phytoremediation of phenanthrene (PHE)-, pyrene (PYR)-, and copper (Cu)-contaminated soil by *Cannabis sativa* L. The experimental campaign was conducted in 300 mL volume pots over a 50 d period using different initial polycyclic aromatic hydrocarbon (PAH) concentrations, i.e., 100 (PC1), 200 (PC2), and 300 (PC3) mg  $\Sigma$ PAHs kg<sup>-1</sup> dry weight of soil, while maintaining a constant Cu concentration of 350 mg·kg<sup>-1</sup>. PHE and PYR removal was 93 and 61%, 98 and 48%, and 97 and 36% in PC1, PC2, and PC3, respectively, in the greenhouse condition. The highest Cu extraction amounted to 58 mg·kg<sup>-1</sup>. In general, the growth of *C. sativa* L. under the PC1, PC2, and PC3 conditions decreased by approximately 25, 65, and 71% (dry biomass), respectively, compared to the uncontaminated control. The present study is aimed at highlighting the phytoremediation potential of *C. sativa* L. and providing the preliminary results necessary for future field-scale investigations.

**Keywords:** hemp; phytoremediation; soil; polycyclic aromatic hydrocarbons; copper



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## 1. Introduction

Soil co-contamination by polycyclic aromatic hydrocarbons (PAHs) and heavy metals (HMs) due to productive activities, intensive farming practices, and waste incineration has caused growing concern in the last decade [1]. PAHs are widespread organic pollutants in the soil compartment and consist of two or more aromatic rings fused in different structures, thus resulting in high toxicity, mutability, and possible carcinogenic effects on humans [2,3]. HMs naturally occur throughout the rigid upper part of the lithosphere, whereas several HMs, such as copper (Cu), can be used by factories (e.g., the petrochemical industry) and agriculture practices and subsequently released into the environment [4,5]. Therefore, HMs can be present in the soil in different forms such as free metal ions and soluble metal complexes or incorporated into silicate structures and/or soil organic matter, oxides, hydroxides, and carbonates [6,7].

As a result of the adsorption of PAHs and HMs onto soil particles, urban soils generally reflect centuries of practices or the legacy of industrial activities. Indeed, soil is the main sink for PAHs due to their affinity to naturally occurring organic matter [8]. Adsorption of contaminants onto soil particles represents a key mechanism determining the fate of PAHs and HMs in the environment due to various interactions (e.g., hydrophobic, electrostatic) [9,10]. In addition, particle dimension and pore volume can improve PAH adsorption in the soil matrix [10] and affect the migration patterns of HMs at different scales [11]. For instance, Zhao et al. [12] showed PAH adsorption of up to 160 mg·kg<sup>-1</sup> onto organic substances in soil after 24 h. Additionally, Lim et al. [13] obtained adsorption (i.e., up to about 4 mg Pb·kg<sup>-1</sup>) of HMs ions onto soil particles after only 90 min.

An increase in the urban population has also led to new sources of the mentioned pollutants accumulating in soils [14]. Indeed, various types of combined pollution (e.g., multiple HMs, multiple PAHs, and HMs + PAHs) have been recently found in some global survey areas (e.g., China, Poland, Australia) and even in far-off Antarctic continents [15]. It has been estimated that approximately half a billion hectares of soil were contaminated worldwide by HMs and/or PAHs, which exceeded the regulatory levels [16]. To define a soil as harmless, EU national legislations impose maximum acceptable PAH and HM concentrations in soil (e.g., 50 mg each PAH kg<sup>-1</sup> for industrial use and 50 mg·Cu kg<sup>-1</sup>) [17,18].

The co-existence of both PAHs and HMs in the environment is a more complex condition compared to the presence of a single class of those pollutants [19]. Thus, the remediation of such polluted soils represents a systemic challenge for environmental science and management [20], and the use of sustainable, effective, and eco-friendly remediation practices is increasingly crucial to tackle the costs and preserve the environment [21]. In this context, several conventional strategies are currently available for the remediation of PAH- and HM-contaminated soils and can only deal with a single type of pollutant rather than a complex mixture, thus requiring a sequence of remediation processes to be performed [22]. Overlooking the literature, remediation techniques that can contemporarily remove both PAHs and HMs in contaminated soils mainly include physical–chemical processes (i.e., soil washing, electrokinetic technology, and supercritical fluid extraction), which are generally invasive and can cause deterioration and damage to natural resources [23]. Therefore, bioremediation can be alternatively employed [24].

Among the biological processes, phytoremediation has been effectively applied for the remediation of co-contaminated soils by PAHs and HMs [20]. Phytoremediation refers to the employment of plants and living species associated with them to remove contaminants from the soil through several mechanisms, such as phytoextraction and rhizodegradation [25]. Phytoremediation has largely been used in recent years due to its improved costs and benefits compared to other conventional techniques, in addition to the less impacting interaction with the ecosystem [26]. On the contrary, the thickness of the contaminated soil layer to be treated can be a problem as it can limit the application of the technique at the field scale [27]. However, only a small amount of information is available in the literature on the phytoremediation of co-contaminated soils by PAHs and HMs, indicating the need for further research [28]. For instance, PAHs could alter the HM uptake mechanism by plants, whereas HMs could influence the PAH biodegradation process by rhizobacteria [29–31].

*Cannabis sativa* L. has recently been reported as an affordable option for phytoremediation and bioenergy production purposes [32,33]. For instance, *C. sativa* L. was shown to grow under potential stress conditions induced by the presence of PAHs such as pyrene (PYR) in contaminated soil, being suitable for the phytoremediation of soils contaminated by up to 150 mg PYR kg<sup>-1</sup> [34]. *C. sativa* L. has also been observed to be capable of phytoextracting HMs, the distribution of which was found throughout the roots and aerial parts of the plant at different HM concentrations [33]. To the best of our knowledge, however, the phytoremediation potential of *C. sativa* L. under PAH and HM co-contamination conditions has never been tested.

In the present study, we investigate for the first time the phytoremediation of PAH- and HM-contaminated soil (i.e., 50–150 and 350 mg·kg<sup>-1</sup>, respectively) by *C. sativa* L. The type and concentration of pollutants were selected to represent the contamination of several real polluted sites in which the co-existence of PAHs and HMs is a common environmental issue [35,36]. Phytoremediation is a complex phenomenon involving several influencing factors to the used soil, the contaminant, the plant species, and external parameters (e.g., precipitation, temperature distribution, terrain slope) [27,37]. Since most of the above-mentioned variables are not directly manageable in the field condition due to the spatial and temporal variability of soil, which affect the processes occurring in the root layer, the experiments were conducted at a laboratory scale. The ultimate goal of this lab-scale

experiment is to provide the preliminary results necessary for the future implementation of a field-scale study.

Therefore, the initial and final concentrations of extractable PYR, PHE, and Cu in soil were evaluated. The height of *C. sativa* L. was monitored every week, while the dry hemp biomass of roots and aerial parts was determined after 50 d when harvested. Finally, a cost evaluation of phytoremediation to assess its upscaling perspective was conducted.

## 2. Materials and Methods

### 2.1. Chemicals

PYR (purity  $\geq 98\%$ ) and PHE (purity  $\geq 98\%$ ) were purchased from Sigma–Aldrich (Steinheim, Germany). Cu as copper (II) chloride anhydrous (purity  $\geq 99\%$ ) was provided by Acros Organics–Thermo Fisher Scientific (New Jersey, USA). Acetone (purity 100%) and acetonitrile (HPLC purity) were purchased by VWR (Fontenay-sous-Bois, France). MilliQ water with an electrolytic conductivity equal to  $0.05 \mu\text{S cm}^{-1}$  was used as the background for preparing all solutions.

### 2.2. Soil

A portion of uncontaminated soil was collected at a depth of 0–20 cm from topsoil in Sora, Lazio region, Frosinone province, Italy ( $41^{\circ}43'05''$  N,  $13^{\circ}36'48''$  E). The soil was placed in a hermetically sealed glass vessel, then transferred to the laboratory, dried in a TCF-50 oven (Argolab, Carpi, Italy) at  $45^{\circ}\text{C}$  until reaching a constant weight, and finally sieved through a 2 mm mesh. The soil was classified as silt loam, by containing sand (0.05–2.00 mm) at 32.4%, silt (0.002–0.050 mm) at 57.3%, and clay ( $<0.002$  mm) at 10.3%. The soil was characterized by total solid (TS) and volatile solid (VS) contents of 97.92 and 1.62%, respectively, a pH value of 7.80, an electric conductivity (EC) of  $1.79 \text{ mS}\cdot\text{cm}^{-1}$ , total organic carbon (TOC) of  $3.20 \text{ g kg}^{-1}$ , and total nitrogen (TN) of  $0.22 \text{ g kg}^{-1}$ .

### 2.3. Spiking Procedure for Soil

The same amount of PHE and PYR was firstly dissolved in acetone, which was subsequently added to the uncontaminated soil in order to obtain initial theoretical concentrations of 100 (PC1), 200 (PC2), and 300 (PC3)  $\text{mg } \Sigma\text{PAHs kg}^{-1}$  in the contaminated soil. An identical amount of acetone ( $1 \text{ L}\cdot\text{kg}^{-1}$ ) was also added to the uncontaminated control soil (PC0) to guarantee the same initial conditions [34]. After the complete evaporation of the solvent in the fume hood, copper (II) chloride anhydrous was dissolved in a water solution, which was subsequently added to the PAH-polluted soil by achieving a constant concentration of  $350 \text{ mg Cu}\cdot\text{kg}^{-1}$  under all conditions tested, as reported by Bonilla-Bird et al. [38].

### 2.4. Experimental Setup

The artificially contaminated soil was placed in 300 mL pots covered at the bottom with 5–13  $\mu\text{m}$  filter paper (VWR, Germany) to avoid soil and chemical losses. Three different concentrations of phenanthrene (PHE) and PYR, here used as model compounds for low (LMW) and high molecular weight (HMW) PAHs, respectively, were tested (i.e., 50, 100, and 150  $\text{mg each PAH}\cdot\text{kg}^{-1}$  dry weight of soil). Additionally, a constant copper concentration of  $350 \text{ mg}\cdot\text{kg}^{-1}$  was considered. Plants were placed in a grow box under controlled conditions for 50 d in 300 mL volume pots. Three replicates were prepared for each operating condition (i.e., PC1, PC2, and PC3 planted) in the presence of the plant by adding one seed of *C. sativa* L. (Carmagnola variety) in a single pot, and three replicates were prepared for the controls without the plant (i.e., PC1, PC2, and PC3 unplanted) [39]. Prior to sowing, the seeds were soaked in a water bath for 12 h. Afterwards, the pots with the planted PAH- and Cu-contaminated soil were placed in a growth chamber in the dark until the first leaves appeared [40]. The growth chamber was utilized to provide an 18/6 h light/dark cycle required for plant vegetative growth [41]. Additionally, unplanted pots (i.e., without the plant) were operated as control groups under the same operating conditions (i.e., duration of the test, water content, temperature, light/dark cycles) of the

planted tests. The temperature varied from 15 to 22 °C, while the humidity was between 45 and 50% during the 50-day growth period. Each pot was watered with distilled water at 50% of the soil water holding capacity to maintain a constant soil moisture, which was monitored by weighing the pots and avoiding unwanted drainage [42,43]. The position of the pots was sporadically and randomly changed to avoid edge effects [44]. Fresh and dry biomass of the plant was measured at the end of the 50 d treatment when harvested. The plant was divided into roots and aerial parts, which were weighed separately.

### 2.5. Analytical Methods and Sampling

TS and VS contents were determined according to standard methods [45]. TOC was extracted from soil by a 0.1 mol·L<sup>-1</sup> pyrophosphate and sodium hydroxide solution and subsequently evaluated by using a TOC-L Series (Shimadzu, Kyoto, Japan) apparatus [46]. pH was quantified with a Sentix/940 pH electrode (WTW, Weilheim, Germany) in a 1:2 suspension (soil:water). A TetraCon/925 (WTW, USA) conductivity cell was employed to determine the electrical conductivity (EC) in a 1:5 soil-to-water suspension. TN was spectrophotometrically measured using a Spectroquant<sup>®</sup> nitrogen cell test (Merk, Darmstadt, Germany). In order to constitute the representative samples for analysis, after the 50 d treatment, 3–4 sub-samples were taken from the selected pots and finally mixed. Possible anomalous areas such as topsoil and the edges of the pot were avoided, mainly sampling from the middle–central part of the pot. Afterwards, 0.5 g of the soil sample was dried under ventilated and dark conditions and finally extracted for PYR and PHE quantification according to Sun et al. [47]. The sample was filtered on a 0.45 µm glass microfiber filter (Whatman, Little Chalfont, UK) prior to the analysis. A LC-20AD HPLC (Shimadzu, Kyoto, Japan) equipped with a Kinetex<sup>®</sup> 3.5 µm PAH (150 × 4.6 mm) column (Phenomenex, Torrance, CA, USA) heated at 35 °C and an SPD-20A UV detector (Shimadzu, Japan) set at 254 nm was employed to analyze the amount of each PAH [34]. The total concentration of Cu in the soil samples, after 50 d phytoremediation, was obtained by an acidic mineralization process. Atomic adsorption spectrometry (AAS) using a Varian Model 55B SpectrAA (Agilent Technologies, Palo Alto, CA, USA) F-AAS equipped with a flame (acetylene/air) and a deuterium lamp for background correction was employed to evaluate the concentrations of Cu ions in the solution [48]. The plant height was measured every week to monitor the evolution of the biomass growth. At the end of the experiments (i.e., 50 d), the *C. sativa* L. plants were harvested in soil by cutting the stem at the bottom of the topsoil. The plants were divided into roots and above parts, washed with tap water, and then rinsed with deionized water. The fresh biomass weight was evaluated when harvested. The biomass was then oven-dried at 50 °C until a constant weight [49] and then weighed.

### 2.6. Statistical Analysis

Statistically significant differences between the obtained parameters were processed by a one-way analysis of variance (ANOVA) followed by a Tukey's honestly significant difference (HSD) test. According to the standard of the study, the results were assumed to be statistically significant when  $p < 0.05$ .

### 2.7. Economic Evaluation

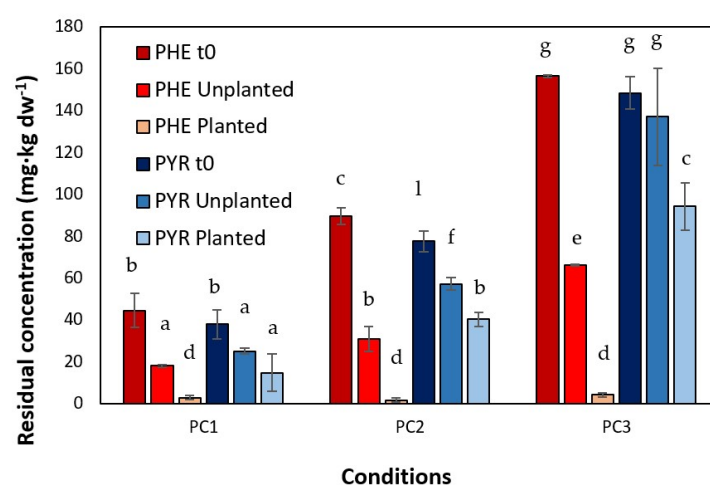
A rough cost assessment in dollar currency was conducted on the entire phytoremediation process. The cost considered for the purchase of the *C. sativa* L. seeds was 0.22 \$·seed<sup>-1</sup>. The input costs for planting, management, and monitoring procedures were estimated according to da Silva Benevenuto et al. [50]. Taking into account a biomass production of 1.23 kg·plant<sup>-1</sup>, the maximum hemp yield was 3600 plants·ha<sup>-1</sup>. The required working hours for *C. sativa* L. pre-plant cultivation, planting, harvesting and post-harvesting operations, and the general remediation management was 0.033, 0.033, 0.19, and 0.017 h plant<sup>-1</sup>, respectively. The used cost for hourly field and laboratory work and general expenses were USD 12 and 15, respectively. Among PC1, PC2 and PC3, the condition with the highest

biomass yield was selected for the economic evaluation [51]. A soil density of  $1.2 \text{ ton m}^{-3}$  was used for the calculation.

### 3. Results and Discussion

#### 3.1. Residual PAH Concentrations in Soil after the Growth of *C. sativa* L.

In the present study, plants of *C. sativa* L. were used for the phytoremediation of PAH- and HM-contaminated soil. The residual PYR and PHE concentrations in both planted and unplanted soil after 50 d of treatment in greenhouse conditions are illustrated in Figure 1. Final PHE concentrations of 2.93, 1.77, and  $4.28 \text{ mg PHE} \cdot \text{kg}^{-1}$  (Figure 1) were obtained in the planted conditions (i.e., PC1, PC2, and PC3, respectively). Under the same conditions, a significantly lower ( $p < 0.05$ ) removal was obtained for PYR than that achieved for PHE. Indeed, the residual concentrations of PYR in PC1, PC2, and PC3 were  $18.19, 31.14,$  and  $66.33 \text{ mg} \cdot \text{kg}^{-1}$  (Figure 1), respectively, which approximately halved under all planted conditions compared to the initial concentration (i.e., 100, 200, and  $300 \text{ mg } \Sigma\text{PAHs kg}^{-1}$ ).

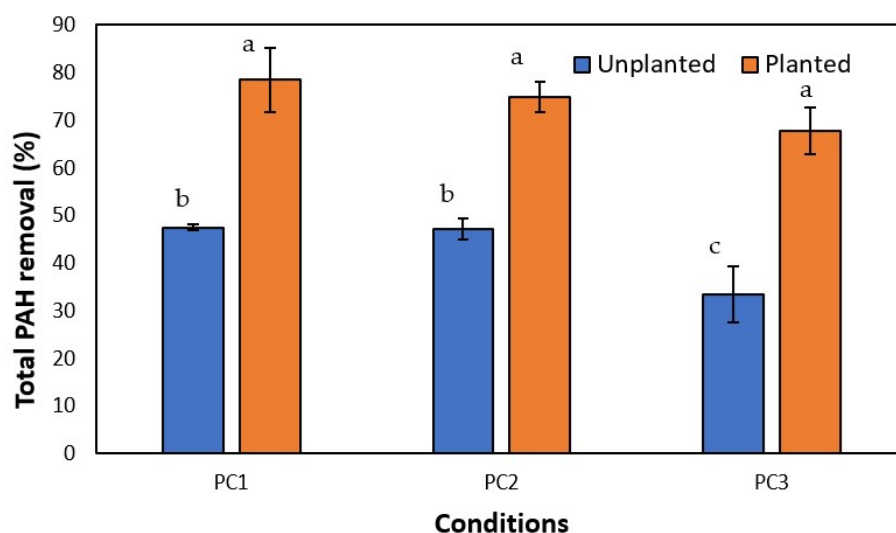


**Figure 1.** Residual pyrene (PYR) and phenanthrene (PHE) concentrations ( $\text{mg each PAH kg}^{-1}$ ) in the unplanted and planted soil, the latter being treated with 50 d phytoremediation by *Cannabis sativa* L. PYR t0 and PHE t0 represent the initial concentrations. The reported values are the means of three replicate analyses. The error bars display the RSD (residual standard deviations). The same letter represents no significant differences ( $p > 0.05$ ) among total PAH removal after 50 days of phytoremediation.

Being characterized by 3 and 4 rings, respectively, PHE and PYR have different structural features, which induce distinct ecological effects and consequently different responses of the bacterial communities towards their degradation [52]. Additionally, compared to HMW PAHs, an LMW compound can be more volatile, show higher solubility in water, and be less prone to be adsorbed onto suspended particles and biota, thus being more susceptible to biodegradation [53]. The results shown in Figure 1 are supported by previous studies reporting bioremediation techniques in which the biodegradation of LMW PAHs was faster and simpler than that of HMW compounds [54]. Similarly, Sun et al. [55] showed that PHE and PYR concentrations were reduced from  $100$  to  $1.6 \text{ mg kg}^{-1}$  and  $25 \text{ mg kg}^{-1}$ , respectively, after planting soil with ryegrass (*Lolium perenne*) for 60 d. Additionally, Campbell et al. [51] showed a lower removal of benzo[a]pyrene (i.e., 14–21%) compared to that obtained for chrysene (i.e., 20–30%) in soil remediated by *C. sativa* L. after 45 d of growth. The removal of PHE and PYR can also be deeply influenced by the initial PAH concentration in soil [56]. The PYR removal efficiency decreased as the initial  $\text{mg } \Sigma\text{PAHs kg}^{-1}$  in soil increased, while similar values of PHE removal efficiency were observed under the PC1, PC2, and PC3 conditions.

The total PAH removals in the planted PC1, PC2, and PC3 (i.e., 100, 200, and  $300 \text{ } \Sigma\text{PAHs kg}^{-1}$ , respectively) were 78, 75, and 68%, respectively, which were significantly

higher ( $p < 0.05$ ) than those obtained in the corresponding unplanted controls (Figure 2) after 50 d. These results suggest that an important fraction of PHE and PYR was removed through rhizodegradation mechanisms in the presence of *C. sativa* L., as recently reported by Gabriele et al. [34]. In the unplanted controls, both abiotic (i.e., volatilization, percolation, photo-degradation) and biotic (i.e., biodegradation by autochthonous microorganisms) contributions could occur (Figure 2) [55]. Plant-promoted PAH biodegradation was previously reported by Lu et al. [57] as the major mechanism involved in soil during PHE and PYR removal. The growth of *Kandelia candel* supported the removal of 48 and 38% of PHE and PYR, respectively, after 60 days, with an initial concentration of 20  $\Sigma$ PAHs  $\text{kg}^{-1}$  (i.e., 10 mg each PAH  $\text{kg}^{-1}$ ). Approximately 98 and 85% of PHE and PYR removal, respectively, was observed by Cheema et al. [58] in the planted soils with tall fescue (*Festuca arundinacea*) contaminated by about 400  $\Sigma$ PAHs  $\text{kg}^{-1}$ , due to increased biological activity in the rhizosphere. The lower PHE and PYR removals obtained here can be attributed to the presence of copper in the soil affecting rhizobacterial activity.



**Figure 2.** Total PAH removal (%) in the unplanted and planted conditions after 50 d of *Cannabis sativa* L.-induced phytoremediation. The reported values are the means of three replicate analyses. The error bars display the RSD (residual standard deviations). The same letter represents no significant differences ( $p > 0.05$ ) among total PAH removal after 50 days of phytoremediation.

The presence of nutrients and organic acids can stimulate the metabolic activity of bacteria, which in turn can produce enzymes (e.g., dehydrogenase) and other substances, improving plant growth [59,60]. Microorganisms in the rhizosphere could degrade PHE and PYR by releasing extracellular enzymes in the soil that mediate metabolic pathways, such as redox reactions, using these compounds as growth substrates and carbon sources [61]. For instance, Ghosal et al. [62] observed the assimilation of PHE by the *Ochrobactrum* sp. strain PWTJD, which was isolated from contaminated soil using PHE as a sole source of carbon and energy supply. Among different bacterial genera and species, the presence of bacterial varieties identified as being capable of biodegrading organic compounds such as *Bacteroidetes*, *Sphingomonas*, and *Rhodococcus* can be positively correlated with the degradation of PYR and PHE in soil [63–65]. In the co-presence of PHE and PYR, biodegradation likely occurred by co-metabolism, which consists of the microorganism's ability to degrade an HMW compound while using an LMW compound as a growth substrate [66].

### 3.2. Copper Removal from Soil after 50 d of Phytoremediation with *C. sativa* L.

The amount of Cu per gram of dry *C. sativa* L. biomass and the Cu removal efficiency after 50 d of phytoremediation under the PC1, PC2, and PC3 conditions are presented in Table 1.

**Table 1.** Cu removed per g of dry biomass ( $\text{mg g}^{-1}$ ) and Cu removal efficiency after 50 days of *Cannabis sativa* L. growth under the PC1, PC2, and PC3 operating conditions. The same letter represents no significant differences ( $p > 0.05$ ) among total Cu removal efficiency after 50 days of phytoremediation.

Conditions	Cu Removed per Gram of Dry Biomass ( $\text{mg}\cdot\text{g}^{-1}$ )	Cu Removal Efficiency	
		Values (%)	Tukey's HSD Test
PC1	46.04	$11.06 \pm 6.01$	a
PC2	57.66	$6.91 \pm 12.95$	a
PC3	n.d.	n.d.	n.a.

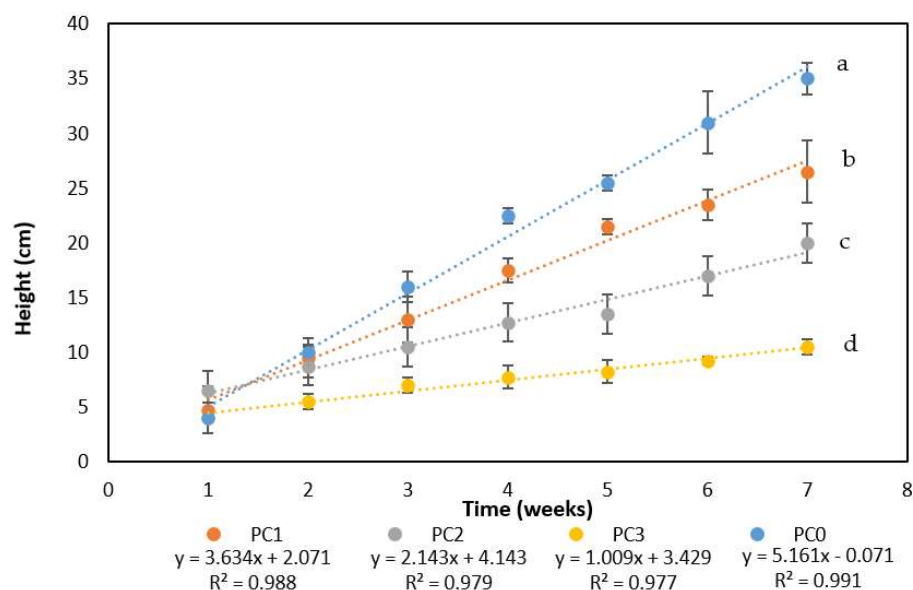
n.d. = not detectable; n.a. = not applicable.

In the present study, a removal of about 57 and 46  $\text{mg Cu g}^{-1}$  of dry biomass (Table 1) was achieved in PC1 and PC2, respectively. The obtained result was higher compared to the Cu removal of about  $1.5 \text{ mg}\cdot\text{g}^{-1}$  recently reported in the literature by Placido and Lee (2022) [67]. In this context, the presence of PAHs can alter the mechanisms of metal uptake, thus affecting the phytoextraction kinetics and extent [68]. The improvement of bacterial activity in the presence of hemp at a specific PAH concentration in the soil (i.e., up to  $200 \Sigma\text{PAHs kg}^{-1}$ ) can alleviate the stress on the plant induced by toxic metals and enhance metal extraction [69]. However, this effect vanished as the amount of PAHs increased above a certain threshold (i.e.,  $300 \Sigma\text{PAHs kg}^{-1}$ ), at which point the Cu concentration did not vary in the soil after the 50 d phytoremediation (Table 1). Therefore, the phytoextraction potential of *C. sativa* L. was also dependent on the biomass development and growth [70], which was significantly lower ( $p < 0.05$ ) in PC3 than the other examined conditions (Figure 3). Most of the existing literature reported the inhibition of biomass growth as a function of the initial PAH concentration in the presence of toxic metals above a certain threshold. For instance, Lin et al. [71] showed that the copper phytoextraction potential of corn (*Zea mays* L.) was reduced by 50% above  $100 \text{ mg}\cdot\text{kg}^{-1}$  PYR, thus leading to a decrease in the biomass growth rate [72].

With regard to the removal mechanisms, it has been reported that *C. sativa* L. could accumulate Cu in the plant tissue [73], likely due to glutathione-sulfide reductase and phospholipase D- $\alpha$  as the antioxidant enzymes, which can protect the plant from oxidative stress induced by reactive species in the presence of Cu [74]. Additionally, other studies reported an increase in aldo-keto reductase, an NAD(P)H-dependent enzyme in *C. sativa* L. grown under Cu stress. Consequently, the relative abundance of these enzymes could influence the accumulation of Cu in plant tissues [75]. Therefore, Cu can be highly accumulated in the root system architecture compared to the upper parts of the plant. This is further supported by the achievements of other studies that reported a maximum copper accumulation of 0.01, 1.5, and  $0.03 \text{ mg}\cdot\text{g}^{-1}$  of *C. sativa* L. in flowers, roots, and shoots, respectively [76–78].

### 3.3. *C. sativa* L. Biomass Response to PYR-PHE and Cu-Contaminated Soil over 50 d Phytoremediation

The occurrence of biomass growth in roots and aerial components is one of the key factors for PYR and PHE removal and Cu extraction from contaminated soil through phytoremediation [79,80]. The presence of a large root biomass promotes degradation through the action of rhizosphere microorganisms while providing a larger surface area for metal accumulation [81,82].



**Figure 3.** Measurements of the height (cm) of *Cannabis sativa* L. during the phytoremediation of PAH- and Cu-contaminated soil (PC1, PC2, and PC3 conditions) and the unspiked control. Values are the means of three replicate analyses. The error bars display the RSD (residual standard deviations). The means are interpolated with linear regressions, which are described by equations and determination coefficients ( $R^2$ ) on the graph. The same letter represents no significant differences ( $p > 0.05$ ) between the reached height at the end of the experiment.

In this study, the development of *C. sativa* L. biomass was monitored during the 50 d of phytoremediation, measuring the plant height under the PC1, PC2, and PC3 conditions tested (Figure 3). The results are summarized in Table 2. The height of the plants decreased with the increase of the initial concentration of PYR and PHE (Figure 3). The height of the plants grown under the PC3 condition was found to be the lowest, reaching a final value of 10.5 cm (Figure 3), about three times lower than the plants grown in the uncontaminated condition. The final heights achieved under the PC1 and PC2 conditions were 26.5 and 20 cm, respectively, which were 35 and 43% lower than those obtained in the uncontaminated controls (Figure 3). The most elevated plant (i.e., 35 cm) was detected in the PC0, which was the condition without PHE, PYR, and Cu contamination (Figure 3). These results are supported by Cameselle and Gouveia (2019) [83], who showed that a high PAH concentration of  $300 \text{ mg kg}^{-1}$  of soil led to a reduction of turnip (*Brassica rapa*) height from 11 to 9.9 cm after 26 days. Likewise, Reddy et al. [84] obtained a decreased height of tall fescue by 55% in PAH- and HM-contaminated soil (i.e., 150 and  $750 \text{ mg kg}^{-1}$ , respectively) compared to the uncontaminated control after 61 days of phytoremediation.

In general, by considering the dry biomass of *C. sativa* L., the root growth in the contaminated soil decreased by approximately 25, 65, and 71% (Table 2) in PC1, PC2, and PC3, respectively, compared to the uncontaminated soil. The condition PC2 (i.e.,  $200 \text{ mg } \Sigma\text{PAHs kg}^{-1}$ ) was found to be the threshold above which a severe biomass reduction was observed (i.e., 65% less, Table 2). This result is in agreement with those obtained by Lu et al. [19], who observed a drastic inhibition (i.e., approximately 50%) of tall fescue growth in PYR-contaminated soil (i.e.,  $200\text{--}500 \text{ mg}\cdot\text{kg}^{-1}$ ). This is likely due to contamination with both PAHs and Cu, which hindered plant development [85].

### 3.4. Preliminary Economic Evaluation

This study shows that PAH- and Cu-contaminated soil can be remediated through phytoremediation by *C. sativa* L. (Figure 2 and Table 1). Therefore, a rough economic assessment considering the seed purchase, the required working hours for *C. sativa* L. pre-planting, planting, harvesting, and post-harvesting operations, and the general remediation management was performed.



**Table 2.** Fresh and dry biomass (g) of aerial parts and roots of *Cannabis sativa* L. after 50 d of phytoremediation in PC1, PC2, and PC3 (i.e., 100, 200, and 300 mg  $\Sigma$ PAHs  $\text{kg}^{-1}$ ). The obtained values are the means of three replicate analyses and the RSD (residual standard deviations).

Conditions	Aerial Parts	Roots	Total
	Fresh weight (g)		
PC0	3.59 ± 0.13	0.59 ± 0.02	2.10 ± 0.01
PC1	3.33 ± 0.14	0.29 ± 0.04	1.81 ± 0.01
PC2	1.20 ± 0.03	0.22 ± 0.01	0.71 ± 0.01
PC3	1.05 ± 0.07	0.12 ± 0.02	0.59 ± 0.05
	Dry weight (g)		
PC0	0.37 ± 0.01	0.063 ± 0.01	0.43 ± 0.01
PC1	0.29 ± 0.01	0.031 ± 0.01	0.32 ± 0.01
PC2	0.12 ± 0.02	0.028 ± 0.01	0.15 ± 0.02
PC3	0.11 ± 0.02	0.016 ± 0.02	0.12 ± 0.02

A total cost of USD 47 per ton was obtained for the PC1 condition (i.e., 100 mg  $\Sigma$ PAHs  $\text{kg}^{-1}$ , Table 3), which achieved a higher PAH and Cu removal, and biomass production (i.e., 78 and 11%, and 0.32 g, respectively) compared to the PC2 and PC3 conditions (Figure 2, Tables 1 and 2) after 50 d. In a realistic approach, additional long-term monitoring and in-field studies are needed to verify time and cost data of in situ phytoremediation since several factors (e.g., autochthonous microorganisms, genetic assortment, and vegetation competition) could severely affect the remediation technique [86,87]. Additionally, the treatment cost of the obtained biomass after each phytoremediation cycle should be considered. In this context, the employment of plant biomass as an energy source is attractive due to further profits [88,89] deriving from bioenergy production (e.g., biogas). For instance, Hunce et al. [90] demonstrated that the presence of trace contaminants in plant biomass (e.g., HMs) did not limit their biomethane potential during anaerobic digestion. Moreover, an integrated approach combining soil remediation with post-process valuable elements recovery has been proven to enhance the economic feasibility of the phytoremediation treatment [91].

**Table 3.** Obtained costs for working hours (i.e., pre-plant cultivation, planting, harvesting and post-harvesting operations), management (i.e., field and laboratory work, and general expenses) and seed purchase.

Operations	Cost (USD per Ton)
Purchase of the <i>C. sativa</i> L. seeds	1.35
Pre-plant cultivation	
Planting	
Harvesting	25.30
Post-harvesting	
Management	20.24

The mentioned cost value (i.e., USD 47 per ton, Table 3) was slightly higher than that indicated by Cristaldi et al. [92] (i.e., USD 5–40 per ton), who did not take the monitoring costs into account. Indeed, the achieved cost here evaluated can be halved to approximately USD 27 per ton by neglecting the cost of monitoring. In each case, the phytoremediation cost was lower than that necessary for performing other conventional remediation techniques [93,94]. For instance, the employment of physical–chemical techniques (i.e., USD 100–500 per ton), such as soil washing, requires a high cost for extracting agents (e.g., surfactants, chelating agents), which should be further increased by the treatment of spent soil washing solution (e.g., about USD 50 per ton) [24]. Additionally, thermal remediation (e.g., vitrification) can show a cost of up to USD 425 per ton due to the involvement of a high energy demand to reach the desired temperatures and the need for long-term monitoring [16]. Other solutions such as landfilling are costly (i.e., up to USD 500 per ton) and

should be integrated with soil excavation and transportation procedures, as well as biogas production monitoring costs [92].

Considering the removal efficiencies, the application of the conventional Fenton technique resulted in a PAH removal comprised between 43 and 69% after 24 h [95]. Sequential application of chelating agents and innovative surfactants for enhanced electro-remediation exhibited a  $\Sigma$ PAH removal of 43 and 48%, respectively [96]. Up to 40 and 90% of PAHs (i.e., fluorene acenaphthene and fluoranthene) and HMs (i.e., Pb, Ni, and Zn), respectively, were removed after the application of soil flushing with Triton X-100 and EDTA at a flow rate of  $0.534 \text{ mL min}^{-1}$  through column experiments [22]. Although the higher HM removal efficiencies and shorter application times compared to the phytoremediation, the abovementioned techniques could lead to severe issues towards the ecosystem.

Moreover, it is largely accepted that a conventional remediation process of a site contaminated by hazardous compounds (e.g., PAHs, toxic metals) can also lead to greenhouse gas emissions (e.g., 58,000 ton of  $\text{CO}_2$  per year for thermal desorption) [97]. Thus, it is increasingly important to adopt soil remediation approaches entailing the fulfilment of sustainable development principles, promoting energy-saving and low carbon-emitting or even carbon-neutral technologies as much as possible [98].

#### 4. Conclusions

This study demonstrates that *C. sativa* L. can grow under stress conditions induced by the co-occurrence of PAHs and Cu in the soil. PHE and PYR removal up to 152 and  $54 \text{ mg}\cdot\text{kg}^{-1}$ , respectively, was obtained after a 50 d treatment. Additionally, approximately 57 and  $46 \text{ mg Cu}\cdot\text{g}^{-1}$  of dry *C. sativa* L. biomass were removed under the PC2 and PC1 conditions, respectively. Under the PC2 and PC3 conditions, a severe biomass reduction was achieved (i.e., >65%). Considering the plant height, the biomass decreased with the increase of the initial PYR and PHE concentrations. The economic assessment revealed a total cost of USD 47 per ton of soil, which was lower than that of conventional remediation techniques.

Although this study provides encouraging preliminary results, the influencing factors to calibrate a future field-scale experiment need further investigation. In order to understand the phytoremediation mechanisms primarily involved in this study, further analyses (e.g., microbiological analyses, plant tissue extractions) are also required. Finally, other studies should be aimed, for instance, at improving phytoremediation with soil conditioners and non-toxic chelating agents (e.g., ethylenediamine- $\text{N,N}'$ -disuccinic acid) for enhancing PAH rhizodegradation and metal mobilization, respectively.

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