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Phytoremediation of a pyrene-contaminated soil by *Cannabis sativa* L. at different initial pyrene concentrations

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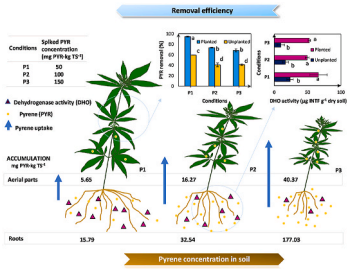
HIGHLIGHTS

- Phytoremediation of pyrene (PYR)-contaminated soil by *C. sativa* L. was investigated.
- A PYR removal of 95% was achieved at a PYR concentration of 50 mg kg TS^{-1} of soil.
- Growth of *C. sativa* L. biomass was negatively affected at higher PYR concentrations.
- Dehydrogenase activity was a good indicator of PYR degradation in planted soil.
- *Proteobacteria* and *Firmicutes* were the most abundant PYR-degrading phyla.

GRAPHICAL ABSTRACT



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ABSTRACT

This study proposes the phytoremediation of a pyrene (PYR)-contaminated soil by *Cannabis sativa* L. The experimental campaign was conducted along a 60 days period using three different initial PYR concentrations (i.e., 50, 100 and 150 mg kg TS^{-1} of soil) in 300 mL volume pots under greenhouse conditions (18–25 °C and 45–55% humidity). After 60 days of hemp growth and flourishing, the highest PYR removal reached approximately 95% in planted soil, 35% higher than in the unplanted control. PYR accumulation was observed in both roots and aerial parts of the plant, with a higher PYR uptake at increasing initial PYR concentrations in soil. The initial PYR concentration affected the growth and, thus, the phytoremediation potential of *C. sativa* L., which was the result of different removal mechanisms. Overall, the lowest initial PYR concentration was the one that resulted in the highest PYR removal. The interaction between the plant roots and microorganisms in rhizosphere was likely associated with PYR removal in this study. The highest DHO activity of 66.26 μg INTF g^{-1} TS^{-1} was observed in the soil spiked with 50 mg PYR·kg TS^{-1}.

1. Introduction

Soil contamination by hazardous organic compounds has increased over the years due to productive activities, fossil fuel combustion and vehicular traffic, posing relevant risks for natural ecosystems, biodiversity and human safety (Salehi et al., 2020). Polycyclic aromatic

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hydrocarbons (PAHs) are among the main organic compounds released by anthropogenic sources (Sánchez-Avila et al., 2009). PAHs contain two or more benzene rings fused in different structures (Lawal, 2017), and this results in high toxicity, mutability and carcinogenic effects induced by PAHs (Lee, 2010; Sørensen et al., 2020). In particular, pyrene (PYR) has four fused rings and is included in the 16 PAHs of priority concern listed by the United States Environmental Protection Agency (US EPA). PYR is ubiquitous in the environment and has been found in soil in a great amount from different regions worldwide (Gabriele et al., 2021; Jiang et al., 2009; Liu et al., 2016). Due to its hydrophobic and lipophilic characteristics, PYR tends to be sorbed onto solid particles and accumulate in soils and sediments by atmospheric transport, deposition and rains (Jiang et al., 2014). Consequently, a constant need for effective techniques capable to remediate PYR-contaminated sites exists, with the aim to ensure human and environmental protection (de Boer and Wagelmans, 2016).

Traditional remediation technologies, e.g. thermal treatment, soil washing and chemical oxidation, are frequently invasive and involve elevated energy supply entailing high operating costs (Bianco et al., 2020a,b; Li et al., 2019). On the contrary, green technologies offer cost benefits in addition to a more sustainable interaction with the environment (Nouri et al., 2017). In this view, phytoremediation has successfully been applied over the years to treat different contaminated soils and groundwater, with research still ongoing to find new approaches to improve the process efficiencies (AL Sbani et al., 2020; Rostami and Azhdarpoor, 2019). Phytoremediation consists in the use of plants and associated microorganisms in soil to remove different compounds from environmental compartments (Panneerselvam and Priya, 2021; Saier and Trevors, 2010). With regard to PAHs, phytoremediation is capable for PAH removal from contaminated soils by different mechanisms (Awa and Hadibarata, 2020; Batty and Dolan, 2013), which generally involve accumulation, degradation, stabilization and volatilization (Cristaldi et al., 2017; Muthusaravanan et al., 2018). Enzymes can actively participate during PAH degradation catalyzing many important biochemical reactions in soil, such as the synthesis and decomposition of humus, the hydrolysis of organic compounds and their conversion into more bioavailable forms (Abbas et al., 2021; Gangola et al., 2019; Pandey et al., 2019; Rao et al., 2010; Sanchez-Hernandez et al., 2018). Finally, plants interact with PAHs in the rhizosphere via the transpiration stream (Harvey et al., 2002).

Among the plant species used to remediate PAH-contaminated soils, *Cannabis sativa* L. has wide margins for investigation. Hemp, a fibrous plant with a high biomass productivity, has been reported to be suitable to survive under potential stress conditions associated with the presence of toxic elements (Ahmad, 2015). It was suggested that hemp could have the ability to avoid cell damage induced by heavy contamination by activating molecular protection mechanisms (Citterio et al., 2003). *C. sativa* L. showed a high tolerance to chrysene and benzo(a)pyrene at concentrations of 25, 50, and 75 μg g^{-1}, being suitable for the remediation of the contaminated fortified soil (Campbell et al., 2002). Moreover, *C. sativa* L. is capable of extracting high concentrations of potential toxic metals in its roots and aerial parts (Deng et al., 2021). Thus, the application of industrial hemp in phytoremediation of PYR-contaminated soils would represent a promising remediation strategy, also to generate positive feedbacks in terms of land use emissions and creation of circular schemes (Rheay et al., 2020). Indeed, besides the phytoremediation potential, industrial hemp is a precious resource to implement novel second-generation biorefineries aimed at bioenergy and biomaterials recovery (Kumar et al., 2017; Moscariello et al., 2021; Papirio et al., 2020; Rheay et al., 2020). The energy supplied from renewable resources will play a significant role in the global energy demand in the next decades to fulfill the transformations required by the ecological transition and mitigate the impact of climate change (Beringer et al., 2011; Daioglou et al., 2019; Sri Shalini et al., 2021).

In the present work, we propose for the first time the phytoremediation of a PYR-contaminated soil by *C. sativa* L. Three increasing PYR concentrations were tested (i.e., 50, 100 and 150 mg kg^{-1} dry soil). Plants were grown in greenhouse conditions for 60 days in 300 mL volume pots. The initial and final concentration of total and butanol extractable PYR in soil along with the total PYR concentration in plant tissues on day 60 were evaluated. The *C. sativa* L. height was monitored every week, while hemp dry biomass of roots and aerial parts was determined after 60 days when harvested. Soil dehydrogenase (DHO) activity was measured at the end of the experimental campaign. The specific objectives of this study were to: 1) investigate the PYR removal potential of hemp; 2) explore the mechanisms of phytoremediation focusing on a) PYR bioavailability and b) enzymatic activity in soil; 3) evaluate the effect of PYR on plant growth, biomass production and phytoextraction.

2. Materials and methods

2.1. Experimental soil

20 kg of soil, with no history of contamination, was manually collected at a depth of approximately 20 cm from the rural level in Sora, Lazio region, Italy (41°43′05″N, 13°36′48″ E). The soil was stored in a hermetic glass vessel and transported to the laboratory. The collected soil was air dried in a laboratory oven (Argolab TCF-50, China) at 45 °C until constant weight, passed through a 2 mm mesh to remove the coarse fraction and ensure homogeneity and kept at room temperature prior to being used (Dai et al., 2020). The soil characterization is reported in Table 1. Based on the Textural Classification System of the United States Department for Agriculture, the soil texture was typical of that of a silt loam.

2.2. Spiking procedure for soil

The selected PAH was PYR (analytical grade ≥98%), which was purchased from Sigma-Aldrich (Germany). PYR was dissolved in acetone (VWR, Italy grade 100%) and added to the uncontaminated soil to obtain three initial theoretical concentrations of 50, 100 and 150 mg PYR·kg^{-1} dry soil (P1, P2 and P3 conditions, respectively). The same amount of acetone but no PYR was added to another portion of uncontaminated soil to obtain the PYR-free control (P0 condition). The above mentioned PYR concentrations were selected to simulate the contamination values of representative real contaminated soils (Anastasi et al., 2009; Sousa et al., 2020; Wang et al., 2012). Also, the concentrations used reflect the concentration values tolerated by different plants. For instance, PYR concentrations of 50, 100, and 150 mg kg^{-1} were reported to be lower than the upper tolerable concentration of alfalfa and ryegrass (Wang et al., 2012). Afterwards, the contaminated soil was placed under a fume hood and manually mixed daily to completely evaporate the solvent (Lu et al., 2014). Finally, to reproduce

Table 1
Physical-chemical properties of the soil used in the experimental campaign.

Parameter	Soil
	Average
Sand (%) [a]	32.4 ± 0.9
Silt (%) [a]	57.3 ± 1.6
Clay (%) [a]	10.3 ± 1.5
pH	7.8 ± 0.02
Total solids (%)	97.92 ± 0.03
Volatile solids (%)	1.62 ± 0.01
Total organic carbon (g·kg TS^{-1})	4.07 ± 0.25
Total nitrogen (g N·kg TS^{-1})	1.71 ± 0.3
Electric conductivity (mS·cm^{-1})	1.79 ± 0.01

[a] Tested samples were sieved through different mesh diameters (i.e., sand 0.05–2.00 mm, silt 0.002–0.050 mm, clay <0.002 mm).

the effect of aging and better incorporate PYR in the soil matrix, the contaminated soil was stored in the darkness at room temperature based on the aging procedure described by Lukić et al. (2016). The aging period was 2 months according to Wang et al. (2012). After the 2 months aging, the measured initial PYR concentrations were slightly different from the theoretical values, as reported in Table 2.

2.3. Experimental design

The experimental soil was placed in plastic pots of 300 mL volume and shut with filter paper at the bottom to avoid soil and chemical loss when watering (Jeelani et al., 2017). Three replicates of each operating condition (i.e., 50, 100, 150 mg PYR·kg^{-1} dry soil with plant) were prepared. Three further pots per each PYR concentration were used as controls in the sole presence of soil without the plant. Three controls were run with the plant in the absence of PYR. The same operating conditions (i.e., humidity, temperature, light/dark cycles) and experimental duration of the main tests were used for the controls.

The feminized seeds of *C. sativa* L. (i.e., certified Carmagnola variety) were preliminarily soaked in warm water for 12 h before planting them in each single pot at a depth of about 1 cm (Wang et al., 2011). The pots with the PYR-contaminated soil and the controls were then placed in a growth chamber in the dark for one week or until the first leaves appeared (Cook et al., 2009). From that moment, the phytoremediation experiments were officially started. For the vegetative growth (i.e., 40 days) and flourishing (i.e., 20 days) periods, 18/6 h and 12/12 h light/dark cycles were used, respectively (Borille et al., 2017; Vogelmann et al., 1988). The temperature ranged between 18 and 25 °C, while the humidity varied from 45 to 55% for the whole duration of the experiment. Each pot was irrigated three times per week using deionized water at 50% of soil water holding capacity to maintain a constant soil moisture and avoid excessive drainage (Liao et al., 2015). The soil moisture content was monitored by weighing the pots (Kaya et al., 2006). The position of the pots was changed periodically at random (Kucey, 1987; Xu et al., 2009). The plants were observed and measured every week to take note of changes in appearance and biomass growth. Finally, the plants were harvested after 60 days of phytoremediation.

2.4. Analytical procedures

Total (TS) and volatile solids (VS) were measured in accordance with standard methods reported in APHA (1998) and U.S. EPA 2001 (2001). A Sentix/940 pH-electrode (WTW, USA) was used in a suspension 1:2 (soil:water) to determine the pH. The electrical conductivity (EC) was determined by measuring the electrical resistance of a 1:5 (soil:water) suspension with a TetraCon/925 (WTW, USA) conductivity cell. Total organic carbon (TOC) in soil was measured by using 1 g of soil and a 0.1 mol L^{-1} pyrophosphate and sodium hydroxide solution, prior to being analyzed with a TOC-L Series (Shimadzu, Japan) equipment. Total Kjeldahl nitrogen (TKN) analysis was performed to measure the concentration of ammonia-N and organic-N using a K-425 speed digester (Büchi, Switzerland). N–NH_4^+ was measured with an A6/25 ammonium cell test (WTW, Germany). Organic-N was measured as a difference between TKN and N–NH_4^+. Sulfate concentrations were analyzed by ion chromatography (IC) using an 883 Basic IC Plus (Metrohm, Switzerland).

PYR was extracted from contaminated soil samples using the procedure described by Sun et al. (1998). 3–4 sub-samples were taken from each pot and then mixed. The collection point of each single sub-sample was chosen avoiding any anomalous areas and the edges of the pot, mainly focusing on the middle-central part of the pot. Briefly, a contaminated soil sample of 0.5 g, dried under ventilated and dark conditions, was placed in a 25 mL flask with 10 mL acetone (VWR, Italy grade 100%) and 1 mL of 10 mg L^{-1} chrysene (VWR, GC/MS grade 100%) solution as internal standard (recovery efficiency of 99 ± 1%). Subsequently, the flask was ultrasonicated for 30 min keeping the temperature between 39 and 41 °C before filtering the sample on a 0.45 μm glass microfiber filter (Whatman, USA) prior to analysis. PYR was analyzed using a LC-20AD HPLC (Shimadzu, Japan) equipped with a 159 Kinetex® 3.5 μm PAH (150 × 4.6 mm) column (Phenomenex, USA) heated at 35 °C and an SPD-20A UV detector (Shimadzu, Japan) set at 254 nm. A water/acetonitrile (VWR, HPLC grade) solution (50:50) was used as eluent phase at a flowrate of 1.2 mL min^{-1} (Volk, 2011).

All harvested hemp plants were cut into roots and above parts, washed with tap water, and then rinsed with deionized water. The roots and aboveground parts were weighed and then subjected to oven-dried at 50 °C overnight and their oven-dried weights were measured until a constant value was reached (Wang et al., 2012). The PYR in the aboveground parts and roots was extracted according to the procedure reported by Zhang et al. (2012). According to Kelsey et al. (1997), the amount of PYR extracted with butanol is correlated with the bioavailable PYR fraction. For the determination of PYR bioavailability, a solution with 20 mL butanol (VWR, Italy grade ≥96%) and 1 mL of 50 mg L^{-1} chrysene was placed in a 25 mL flask. Then, 2 g of dried soil were added and the flask was shaken on rotary tables at 160 rpm for 16 h in the dark (Zhou et al., 2018). The extracted sample was filtered on a Whatman glass microfiber filter (0.45 μm) before analysis.

Soil DHO activity was determined as modified from Trevors et al. (1982), using iodonitrotetrazolium chloride (INT) (Alfa Aesar, Italy grade 95%) as substrate and iodonitrotetrazolium formazan (INTF) (Sigma-Aldrich, Germany grade ≥98%) as product of the enzymatic activity, according to the methods reported by Madrid et al. (2016). Briefly, 0.2 mL of INT (0.4%) solution and 0.4 mL of deionized water were added to 1 g of dried contaminated sample. As a control, the same procedure was performed for 1 g of soil with 0.6 mL of deionized water. The samples were incubated in the dark at room temperature and shaken on a rotary table for 20 h. Afterwards, 10 mL of methanol was added, and the solution was further shaken. The extracted sample was filtered on a Whatman glass microfiber filter (0.45 μm). The INTF formed was determined by using an 8453 UV–visible spectrophotometer (Agilent, USA). INTF was quantified spectrophotometrically at 490 nm.

The composition of the microbial community populating the 150 mg kg^{-1} PYR-contaminated soil (P3) after 60 days of treatment in both planted and unplanted conditions was analyzed through a preliminary DNA extraction with a ZR Fecal/Soil Microbe DNA MiniPrep™ Kit (Zymo Research, USA). The income of DNA extracted from the sample was assessed as described by Claassen et al. (2013). Polymerase chain reaction (PCR) amplification, Illumina sequencing, sequence filtering and taxonomic classification were performed as according to Haleyur et al. (2019). Library quantification was determined through a 2100 Bioanalyzer (Agilent Technologies, USA).

Table 2
Initial and final pyrene (PYR) concentration under the different experimental conditions.

Conditions	PYR (mg·kg TS^{-1})			
	t_0		t_{60}	
P1 + plant	38.52	±0.53	2.11	±1.01
P2 + plant	89.04	±2.72	23.60	±0.82
P3 + plant	155.23	±3.59	49.32	±3.01
P1 no plant	38.52	±0.53	15.65	±0.10
P2 no plant	89.04	±2.72	52.15	±3.72
P3 no plant	155.23	±3.59	91.01	±1.36

2.5. Statistical analysis

The obtained data were processed by one-way analysis of variance (ANOVA) followed by a Tukey test to evaluate statistically significant differences between the tested working parameters. The correlation between different conditions (i.e., PYR removal, DHO activities and

hemp biomass production) was estimated by the standard Pearson sample correlation coefficient ($r_{variable,1\text{-}variable,2}$) with both-sided alternative. All statistical analyses were performed with the XLStat statistical software for Microsoft Excel (2021.1. version, Addinsoft, USA). The result, by the standard of the study, was assumed statistically significant with $p < 0.05$.

2.6. Calculations

The shoot concentration factor (SCF) and the root concentration factor (RCF) were calculated as follows (Zhang et al., 2012):

SCF = PYR concentration in the aerial parts/PYR concentration in soils

RCF = PYR concentration in the aerial parts/PYR concentration in soils

The translocation factor (TF) was calculated as:

The components used for calculating each mass balance were the accumulation of PYR in the roots and the aerial parts, the PYR loss in the unplanted control, the accumulation of PYR in the aerial parts of *C. sativa* L. grown in the PYR-free control.

TF (%) = (PYR concentration in aerial parts/PYR concentration in root)·100

3. Results and discussion

3.1. PYR removal from soil after 60 days phytoremediation with C. sativa L

In the present study, *C. sativa* L. was used for PYR removal from an artificially spiked soil over a 60-days treatment. The PYR removal percentage after 60 days in both planted and unplanted soil is depicted in Fig. 1. The final PYR removal efficiency exceeded 68% under all operating conditions. The P1 condition (i.e., 50 mg PYR·kg^{-1} dry soil) resulted in the highest PYR removal efficiency (i.e., 94.52%), while P2 and P3 conditions allowed to achieve a PYR removal of 73.50 and 68.22%, respectively. All planted conditions showed a significant statistical difference with the corresponding unplanted control tests (Fig. 1), indicating that the presence of *C. sativa* L. was crucial in PYR depletion from soil. PYR removal was not significantly different ($p > 0.05$) in the unplanted controls of P2 and P3 conditions. The residual PYR concentrations after 60 days of treatment under each operating condition are reported in Table 2 (see Fig. 1).

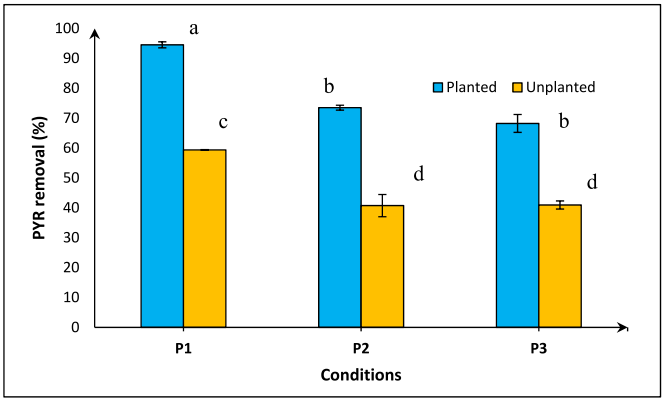

Fig. 1. Pyrene (PYR) removal (%) after 60 days of phytoremediation in an artificially spiked soil having an initial PYR concentration of 50 (P1), 100 (P2) and 150 (P3) mg·kg TS^{-1}. The same letters (Tukey test) do not represent statistical differences ($p > 0.05$) between the experimental conditions. Values are the averages of triplicate analyses and error bars indicate the standard deviation.

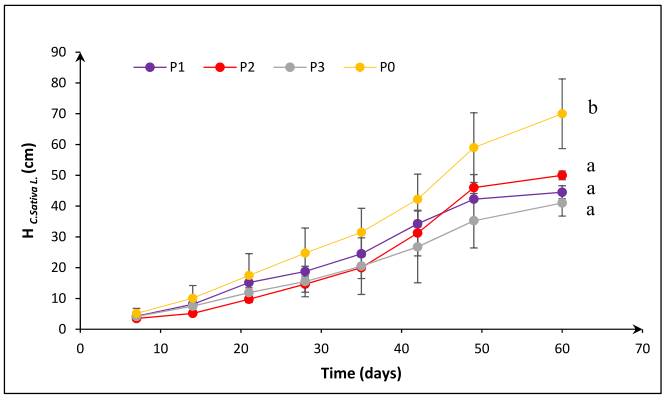

Fig. 2. Evolution of the height of the *C. sativa* L. plants used in the PYR-contaminated soil (P1, P2 and P3 conditions) and in the unspiked soil. The same letters (Tukey test) do not represent statistical differences ($p > 0.05$) between the experimental conditions. Values are the averages of triplicate analyses and error bars indicate the standard deviation.

Findings of previous studies confirm that PYR removal is higher in the presence of plants, with different plant species investigated for this purpose (D'Orazio et al., 2013; Rostami et al., 2016). Zhang et al. (2011) used the species *Scirpus triqueter* and reported that the degradation of 80 mg PYR·kg TS^{-1} reached a PYR removal percentage of approximately 65 and 54% in the rhizospheric and non-rhizospheric soil, respectively, compared to a 43% removal efficiency observed in an unplanted soil after 80 days of treatment. High PYR degradation efficiencies were achieved when using *Vetiveria zizanioides*, *Rohdea japonica* and *Typhafr orientalis* species (86, 84, and 77%, respectively) after 14 weeks, and were 66% higher than those observed in the unplanted control experiments (Wang et al., 2008). Our results are also consistent with Rostami et al. (2016), where the removal percentage of PYR in unplanted controls was 47% at the PYR concentration of 150 mg*kg^{-1} in a soil with 2% organic matter.

EU national legislations impose a maximum acceptable PYR concentration in soil in order to define that soil as safe. For instance, in Dutch legislation, the tolerable threshold is 40 mg PYR kg^{-1}. In Italian legislation, the Decree 152/2006 reports a PYR threshold contamination concentration of 50 mg kg^{-1} for industrial soil (Grifoni et al., 2020). The "Interim Canadian Environmental Quality Criteria for Contaminated Sites", issued in 1991, indicate a PYR concentration of 100 mg kg^{-1} for the industrial and commercial sites as the threshold to protect environmental and human health. However, a real environmental quality standars (EQSs) assessment to improve the understanding of how to implement the PAHs soil quality guidelines for PYR is lacking in the guidelines revised in 2010. In any case, these considerations imply that the process investigated in this study would allow to efficiently decrease PYR concentration below the above mentioned threshold values.

3.2. Bioavailable PYR fraction in soil

PYR bioavailability in soils is a fundamental parameter to determine the effectiveness of any biological remediation technique, including phytoremediation (Bianco et al., 2020a,b; Wijayawardena et al., 2015).

Table 3
Initial and residual bioavailable pyrene (PYR) concentrations after 60 days of phytoremediation with *C. sativa* L. under P1, P2 and P3 operating conditions. The concentrations of bioavailable PYR in the unplanted control tests are also reported.

Conditions	Bioavailable PYR (mg·kg TS^{-1})			
	t_0		t_{60}	
P1 + plant	33.49	±0.41	2.55	±0.65
P2 + plant	75.06	±2.67	23.93	±0.93
P3 + plant	136.86	±1.21	46.01	±1.22
P1 no plant	33.49	±0.41	13.88	±0.33
P2 no plant	75.06	±2.67	45.10	±0.04
P3 no plant	136.86	±1.21	82.66	±9.92

The bioavailable PYR fractions, for instance the water-soluble and acid-soluble fractions, can be desorbed by root exudates and be directly accessible to plants (Wu and Zhu, 2016). The concentration of the bioavailable PYR fraction measured at the beginning and after 60 days of phytoremediation is reported in Table 3.

The high bioavailability along with the high total PYR removal efficiency suggests that PYR removal was most likely the result of biotic processes, such as microbial degradation, opposed to sorption onto soil constituents. This is also corroborated by the low presence of soil organic matter (1.6%, expressed as VS, over the total dry matter of soil), which is normally associated with a low PYR bioavailability but rather with the abiotic sequestration of PYR in soil (Lin et al., 2018). Moreover, a higher reduction of the bioavailable PYR fraction after 60 days of treatment was observed in planted soils compared to unplanted controls. These results are consistent with what observed by Parrish et al. (2005), who found a reduction of PYR bioavailability in the presence of plant roots as long as phytoremediation was performed.

3.3. PYR content, accumulation and translocation in roots and shoots of *C. sativa L*

After 60 days of growth and flourishing of hemp in contaminated soils, the amount of PYR adsorbed onto the root surface was 15.79, 32.54 and 177.03 mg PYR·kg TS^{-1} under the P1, P2 and P3 conditions, respectively. In the aerial parts, the detected PYR amounts were 5.65, 16.27, 40.37 mg PYR·kg TS^{-1} of biomass.

It has been reported that PYR is not prone to be susceptible to translocation mechanisms in plants due to its lipophilic characteristics (Gao and Zhu, 2004). Hence, the SCF, RCF and TF were used to evaluate the translocation potential of PYR in *C. sativa* L (see Table 4).

As shown in Table 4, the calculated TFs values were not higher than 0.50 in this study. Nonetheless, the TFs calculated did not show a regular trend with the initial PYR concentration used in soil. Low TF values indicate that *C. sativa* L. took up PYR from soil and transferred it to the aerial parts with a low efficiency (Sun and Zhou, 2016).

PYR was not detected in the roots of the plant grown in the unspiked control, while a PYR amount of 1.87 ± 0.81 mg kg^{-1} of biomass was found in the aerial parts. The accumulation of PYR in the aerial parts of the plants was also reported to occur from ambient air, possibly due to the entrapment of fine contaminated soil particles (Schroll et al., 1994). Yet, the uptake of soil-bound PYR directly via the stomata or the PYR deposition on the leaves surface are pathways for PYR accumulation in plant shoots (Sun et al., 2010). In this study, the extraction of PYR by the plant grown in the unspiked control served as a control for aerial adsorption.

Table 4
Shoot/root concentration factors (SCFs/RCFs) and the root-to-shoot transfer factors (TFs) obtained under the P1, P2 and P3 conditions after 60 days of *C. Sativa* L. growth.

Conditions	SCF	RCF	TF (%)
P1	0.14	0.40	0.35
P2	0.18	0.36	0.50
P3	0.26	1.14	0.22

Table 5
Fresh and dry biomass (g) of roots and aboveground parts of *C. sativa* L. after 60 days of phytoremediation at three different initial pyrene (PYR) concentrations and in an unspiked soil. The same letters (a, b, c and d) obtained with the Tukey test do not represent statistical differences ($p > 0.05$) between the experimental conditions. Values are the averages of triplicate analyses and error bars indicate the standard deviation.

Spiked PYR concentration (mg·kg TS^{-1})				
Conditions	0	50	100	150
Fresh weight				
Roots (g)	0.52 ± 0.05 [a]	0.30 ± 0.04 [a]	0.21 ± 0.08 [b]	0.19 ± 0.08 [b]
Above parts (g)	3.49 ± 0.11 [c]	2.84 ± 0.07 [c]	1.11 ± 0.08 [c]	0.96 ± 0.08 [d]
Dry weight				
Roots (g)	0.12 ± 0.03 [a]	0.07 ± 0.02 [a]	0.06 ± 0.02 [b]	0.05 ± 0.02 [b]
Above parts (g)	0.70 ± 0.08 [c]	0.56 ± 0.03 [c]	0.23 ± 0.02 [d]	0.21 ± 0.05 [d]

In literature, PYR accumulation in plants has been attributed to the lipid content present in the roots (Arvanaghi et al., 2017; Chiou et al., 2001; Gao and Zhu, 2004). The PYR accumulation in roots and aerial parts here observed can, thus, be due to the high content of lipids and sugars normally observed in the tissues of *C. sativa* L. cultivars (Huang et al., 2017). A previous study showed a PYR concentration of 462 ± 18 mg PYR·kg^{-1} in the roots of *S. triqueter* grown on 100 mg PYR·kg TS^{-1} of contaminated soil (Chen et al., 2017). This indicates that different physiological characteristics of plants and especially the root system developed likely play a pivotal role in governing the PYR removal from soil (Wang et al., 2021). However, once accumulated in the roots, it is possible that PYR degradation is not complete, negatively affecting both plant growth and further PYR accumulation (Chen et al., 2017).

The amount of PYR taken up by roots and aerial parts is highly correlated with the bioavailable PYR fraction, as well as the total PYR concentration, soil water content and the microbial community in soil (Wu and Zhu, 2016). For instance, an increased PYR accumulation in plant tissues as a function of initial PYR concentration in soil was observed by Gao and Zhu (2004), where PYR in roots increased from 21.5 ± 3.20 to 112 ± 6.49 mg kg^{-1} of biomass with an initial PYR concentration in soil of 48.7 ± 3.76 and 172 ± 9.21 mg PYR kg^{-1} of contaminated soil, respectively.

Besides plant accumulation, the fate of PYR in spiked soils during phytoremediation can be ruled by percolating, photo-degradation, volatilization and biodegradation mechanisms as well as supplementary abiotic losses (Lu et al., 2010). Carrying out some simple mass balances, we observed that 40.62, 58.56 and 58.64% of PYR removal was attributed to abiotic mechanisms under the P1, P2 and P3 operating conditions, respectively. The amount of PYR found in roots made up only the 0.004, 0.005 and 0.010% of the PYR removed for P1, P2 and P3, respectively. Similarly, the contribution of the aerial parts of *C. sativa* L. in PYR removal was irrelevant. The remaining percentages of 59.38, 41.44 and 41,35% for P1, P2 and P3, respectively, can be ascribed to microbial degradation, which resulted in the highest PYR depletion contribution, confirming what reported in section 3.2. According to Zhang et al. (2009), plant root exudates might enhance PYR biodegradation promoting the activity of rhizosphere microorganisms and enzymes. In contrast, in the unplanted soil, PYR removal may be due to the metabolic activity of the microorganisms naturally present in the soil and/or partially to photochemical oxidation and leaching (Abdel-Shafy and Mansour, 2016).

3.4. Production of C. sativa L. biomass in PYR-contaminated and uncontaminated soil

According to previous studies, increasing PYR concentrations in soil are associated with the decrease of the plant biomass growth, as for

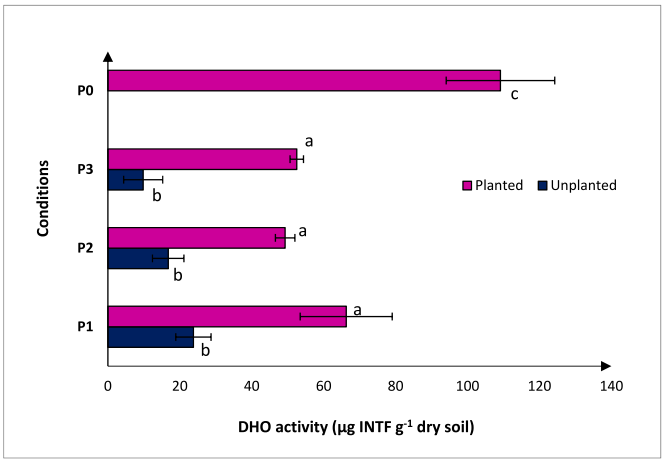

Fig. 3. DHO activity in 50 (P1), 100 (P2) and 150 (P3) mg PYR·kg^{-1} dry contaminated soil and uncontaminated control (P0) after 60-days phytoremediation with *C. sativa* L. The same letters (Tukey test) do not represent statistical differences ($p > 0.05$) between the experimental conditions. Values are the averages of triplicate analyses and error bars indicate the standard deviation.

instance observed for alfalfa by Alves et al. (2018). Therefore, the height of the plants here used was measured every week to investigate the ability of hemp to grow in the PYR-contaminated soil. The height of *C. sativa* L. during the 60-days experiment is shown in Fig. 2. Under P1, P2, and P3 conditions, the final height of the plant was averagely 36, 29 and 42% lower than that observed in the PYR-free control ($p < 0.05$), respectively. The height of the plant in the uncontaminated soil reached the highest value of about 70 cm after 60 days. No significant difference ($p > 0.05$) was observed among the plants grown at the three different initial PYR concentrations. Plants developed an average height of 45 cm after 60 days (Fig. 2) in the PYR-contaminated soil.

Yet, PYR concentration showed adverse effects on roots and shoot dry biomass, as reported in Table 5. Considering the dry biomass weight, the above parts decreased by 28, 36 and 42% in P1, P2 and P3 respectively, compared to the uncontaminated control (P0). A more severe biomass reduction was observed in roots. Increasing the PYR concentration, the root biomass decreased by 41, 50 and 56% compared to the roots grown in the unspiked soil (P0). Statistical analysis showed no influence on the biomass weight up to P1 concentration (Table 4). At higher PYR concentrations (i.e., P2 and P3 conditions), a significant difference ($p < 0.05$) was observed in comparison to P1 and P0. This can be attributed to a more severe effect of PYR concentrations higher than 50 mg kg^{-1} on the biomass growth (Houshani et al., 2019). The presence of such PYR concentrations could alter root morphology, hindering the nutrients and water uptake and resulting in a decreased biomass response (Chen et al., 2017). Negative effects of PYR on biomass growth were also reported by Wang et al. (2014), who observed that the total biomass of a mangrove species (i.e., *Kandelia obovata*) decreased by approximately 28% when grown in a contaminated soil with a PYR concentration of 10 mg kg^{-1}. Similarly, Zhang et al. (2018) reported that PYR contamination reduced the height and biomass of spruce. Despite reduced biomass, *C. sativa* L. did not exhibit visible sign of stress and toxicity (e.g., seeds germination, appearance of spots, yellowing tissues and damage in leaves) proving that phytoremediation in PYR-contaminated soils is feasible with this plant.

3.5. Assessment of DHO activity in planted and unplanted soil

The DHO activity in soil measured after 60 days of treatment is shown in Fig. 3. DHO was monitored as an indicator of microbial activity in soil and a possible indirect indicator related to PYR degradation (Liu et al., 2015), as DHO functions are associated with microbial oxidoreduction processes in soil (Wolinska and Stepniewsk, 2012).

The role of PYR, either stimulating or inhibitory, can increase or decrease the levels of DHO activity in soils (Hong et al., 2015; Jeelani et al., 2017). Different studies reported an increase in DHO activity possibly related to PYR and its intermediates, which could serve as carbon source for microbial metabolism and growth.

In the present study, after 60 days of hemp growth, the DHO activity ranged between 50 and 110 μg INTF g^{-1} dry soil in planted soils. In unplanted controls, DHO was between 10 and 24 μg INTF g^{-1} dry soil, indicating that the DHO activity was up to 10 times higher in planted soils. Hence, the presence of hemp roots significantly ($p < 0.05$) enhanced the activity of the native microbial community in soil. The highest DHO activity was found under the P1 operating condition, which was also associated with the highest PYR removal efficiency. This is also indicated by the Pearson coefficient (i.e., rDHO-PYR = 0.761) that demonstrates a good correlation between the DHO activity and PYR removal, being consistent with previous studies, which observed a direct correlation between PYR degradation and the dehydrogenation process (Cheema et al., 2010; Soleimani et al., 2010). Indeed, a higher DHO activity is frequently indicated as the main reason of PYR degradation in planted soils by rhizospheric microflora (Zhang et al., 2009).

Increasing initial PYR concentrations had a negative impact on DHO activity (Fig. 3), as also reported in previous studies. According to Sivaram et al. (2018), a significant reduction in DHO activity was observed in PYR-spiked soil compared to an uncontaminated control. In contrast, Baran et al. (2004) observed a positive correlation between the presence of PYR and DHO activity. This was ascribed to an adaptation of the soil microflora to PYR, which was used as a carbon and energy source.

3.6. Identification of PYR-degrading bacteria

The phylogenetic analysis showed that *Proteobacteria* and *Firmicutes* were the most abundant bacterial phyla in soil accounting for 16 and 25%, respectively, after the 60 days of growth and flourishing of *C. sativa* L. under the P3 condition (Fig. 4). Species belonging to archaea were completely absent. The genus *Pseudomonas*, 2.13% abundant in the analyzed samples, was associated with PYR degradation according to previous studies (Ma et al., 2013) as well as the *Bacteroidota* phylum (Charalampous et al., 2021), which showed an abundance of 5%. The *Actinobacteria* phylum was relatively abundant in the soil (i.e., 5%) and the bacteria belonging to it can be considered active PYR-degraders according to Ho et al. (2000), including the *Mycobacterium* genus that was the most abundant among *Actinobacteria* (DeBruyn et al., 2012; Kim et al., 2018).

In the unplanted controls, the microbial community considerably shifted and was mostly enriched in *Cyanobacteria* accounting for more than 42% of the total reads in the sample (Fig. 4). A genus of *Cyanobacteria* termed Leptolyngbya_EcFYyyy-00 was found in the unplanted control, while it was not detected in the planted soil. A genus of *Bacillus* was also found at a higher extent in the control rather than in the planted soil sample (i.e., 20 and 10%, respectively). Thus, the presence of *C. sativa* L. could promote the growth of different species associated with PYR degradation.

In our study, the bacterial diversity is limited due to the PYR concentrations used. We only report the bacterial abundance relative to the P3 condition because those referred to the other two conditions (i.e. P1 and P2) were not significantly different. Based on reported studies, loss of bacterial diversity was detected even in the low (i.e., 20 mg kg^{-1}) PYR-contaminated soil (Zhou et al., 2009). Since in the present study the PYR concentration is higher (i.e. from 50 to 150 mg kg^{-1}), we assume that a substantial change in the bacterial community in soil is more likely to occur at lower PYR concentrations than those used.

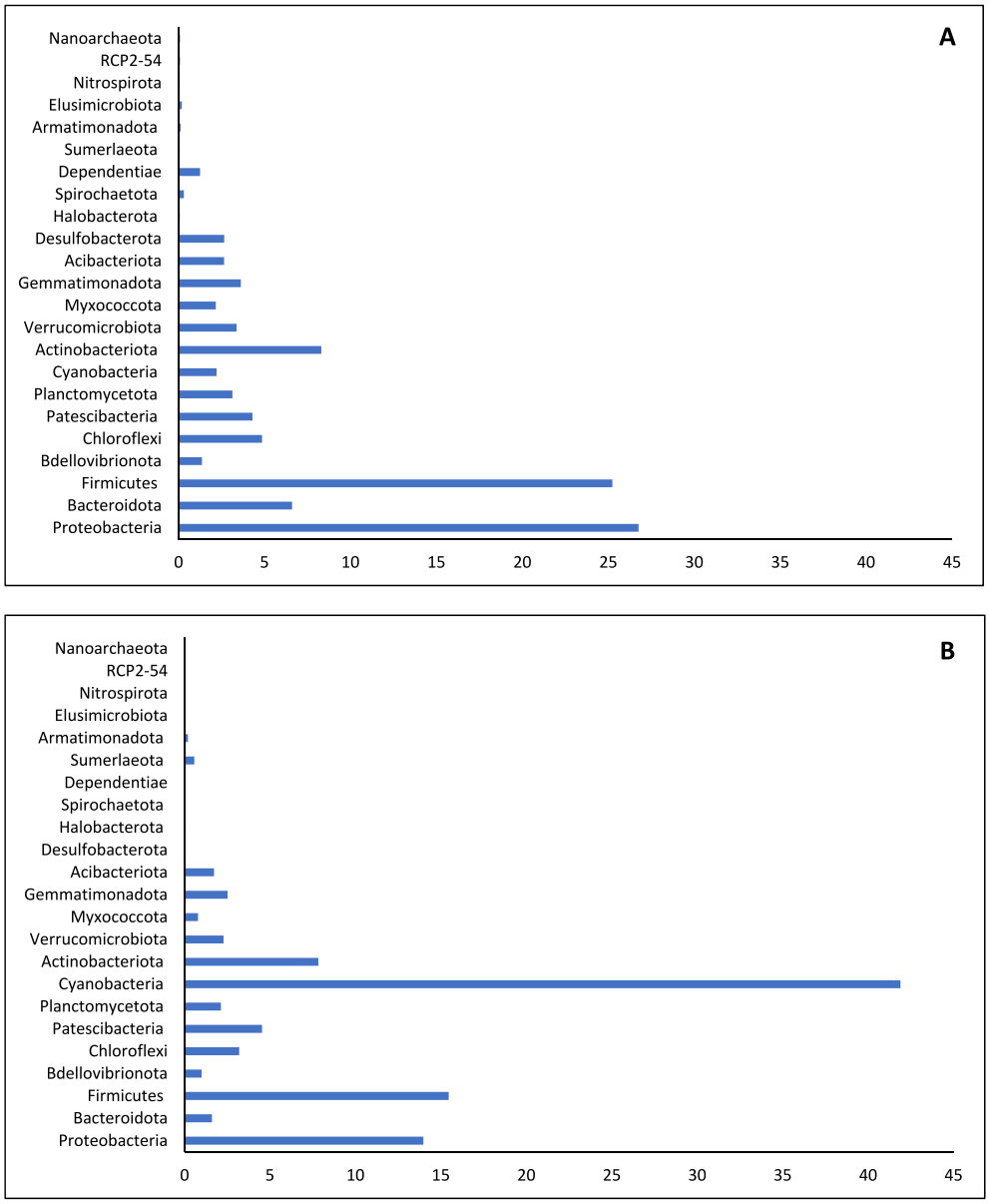

Fig. 4. Abundance (%) of bacterial diversity (at phylum level) living in the rhizosphere around *C. sativa* L. roots in P3 (150 mg PYR kg TS^{-1}) after 60 days of growth (A). Panel B is related to the unplanted control.

4. Conclusions

This study shows that phytoremediation with *C. sativa* L. for PYR-contaminated soil is capable for removing PYR up to approximately 95%, when an initial PYR concentration of 50 mg kg TS^{-1} is used. After 60 days of treatment, concentrations of 15.79, 32.54 and 177.03 mg PYR·kg TS^{-1} under the P1, P2 and P3 conditions, respectively, were found in the roots of *C. sativa* L. Hence, higher initial PYR concentrations in soil resulted in a higher PYR uptake in the plant, and this led to a decrease of the weight of roots and above parts by 41–58 and 28–52%, respectively, when compared to the PYR-free controls. Positive correlations were found between PYR degradation and the elevated DHO presence in soil, likely indicating that the presence of *C. sativa* L. enhanced the activity of the rhizospheric PYR-degrading flora. The high level of PYR bioavailability suggests that PYR removal was mostly the result of biotic processes rather than abiotic loss.

Credit author statement

Ilaria Gabriele: Conceptualization, Data curation, Investigation, Writing – original draft, Writing – review & editing. **Marco Race**: Conceptualization, Supervision, Writing – review & editing. **Patrizia Papetti**: Data curation and elaboration, Writing – review & editing. **Stefano Papirio**: Conceptualization, Supervision, Writing – review & editing. **Giovanni Esposito**: Supervision, Writing – review & editing, Administration, Funding acquisition.

Declaration of competing interest

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

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