Tree Seedlings Suffer Oxidative Stress but Stimulate Soil Enzyme Activity in Oil Sludge-Contaminated Soil in A Species-Specific Manner

Naser Norouzi Haroni¹, Mehrdad Zarafshar^{*2}, Ziaedin Badehian^{*3}, Anket Sharma⁴, Martin Karl-Friedrich Bader^{5,6}

1 Ph.D. Student of Forest Science and Ecology, Faculty of Agriculture and Natural Resources, Lorestan University, I. R. Iran.

2 Natural Resources Department, Fars Agricultural and Natural Resources Research and Education Center, AREEO, Shiraz, I.R. Iran.

3 Assistant Prof. Department of Forestry, Faculty of Agriculture and Natural Resources, Lorestan University, I. R. Iran.

4 State Key Laboratory of Subtropical Silviculture, Zhejiang A&F University, Hangzhou, China

5 Institute for Applied Ecology, School of Science, Auckland University of Technology, 34 St. Paul Street, Auckland 1010, New Zealand.

6 Current address: Swiss Federal Institute of Forest, Snow and Landscape Research (WSL), Zürcherstrasse 111, Birmensdorf 8903, Switzerland

**Corresponding authors: e-mail address; M.zarafshar@areeo.ac.ir and badehian.z@lu.ac.ir, Telephone numbers; 0098- 9126055424 and 0098- 9177303977*

Abstract

The impact of **plants** on soil enzymatic activity and their potential for remediation of oil-contaminated soil has been widely studied but information about tree species is scarce. Here, we used seedlings of four tree species (*Ailanthus altissima* Mill, *Fraxinus rotundifolia* Mill, *Melia azedarach* L. and *Robinia pseudoacacia* L.) to investigate rhizosphere effects on key soil enzymes (dehydrogenase, urease, alkaline and acid phosphatase) and to evaluate oxidative stress in response to oil sludge-contaminated soil (0, 100, 200, 400 g kg⁻¹).

We observed a large species-specific stimulation of soil enzyme activity often far exceeding the figures in unplanted soil with peaks mostly occurring under low oil contamination (100 g kg⁻¹). The strongest stimulatory effect on dehydrogenase and urease activity produced by *R. pseudoacacia* at the low and intermediate and *F. rotundifolia* at the highest contamination level. No clear pattern emerged for acid phosphatase activity, which was equally stimulated by all species at the highest contamination level. Alkaline phosphatase stimulation was dominated by *F. rotundifolia* at the low and by *R. pseudoacacia* at the higher oil contamination levels. Foliar H₂O₂ rose significantly in a species-specific manner in response to oil contamination, triggering an upregulation of the antioxidant defense, which began to show signs of exhaustion at the highest pollution level and revealed that oxidative stress was highest in *A. altissima*. Our results imply that phytoremediation efforts can be optimized through carefully designed plant species assemblages aligning stimulatory effects on soil enzyme activity and oxidative stress tolerance with the severity of the oil pollution.

Keywords: Antioxidant enzymes, oil sludge, oil-soil contamination, phytoremediation, soil enzymes

1. Introduction

Soil contamination by petroleum sources is one of the big challenges for ecosystem and human health. Exploitation, drilling, and accidental leakage of oil during transportation are common causes for soil pollution (Brakorenko & Korotchenko 2016, Sajna et al. 2015, Varjani & Upasani 2017). Oil contamination has immensely negative impacts on the physicochemical and biological properties of soil (Kriksunov 2011, Osse et al. 2018). Petroleum compounds contribute to the contamination of groundwater and adversely affect microbial population density and soil organic matter, which ultimately causes reductions in crop yield (Xie et al. 2018).

Oil sludge is a complex mixture of compounds of NSO (nitrogen-sulfur-oxygen), aromatic hydrocarbons, alkanes, and asphaltenes (Beškoski et al. 2011). The presence of oil sludge in the soil affects various edaphic characteristics, including soil biological properties and may degrade the soil microbiome. Soil enzymes are mainly secreted by microorganisms and plants and play a vital role in soil metabolism (Li et al., 2018). As a consequence, their inhibition by heavy metal-contamination has received considerable attention and has been well documented over the last few decades (Namlı et al., 2010) but there is much less information available with regard to phytoremediation of contaminated sites by oil sludge.

Soil enzymes play a fundamental role in organic matter turnover and mineral nutrient supply for plant growth, decontamination of materials resistant to biodegradation, N₂-fixation, nitrification and denitrification (Das & Varma 2010). The activity of soil enzymes is widely used as an indicator for soil quality and health because soil enzymatic processes respond very sensitively to changes in the environment and are closely linked to the function and structure of the soil microbial community (Nanekar et al. 2015, Zarafshar et al. 2020). Therefore, investigating the link between environmental pollution and the activity of soil enzymes is crucial for understanding their effect on soil quality and fertility (Varjani & Upasani 2017). These enzymes may comprise amylase, arylsulfatase, beta-glucosidase, cellulase dehydrogenase, phosphatase, protease and released urease from plants, animals, organic compounds and soil microorganisms (Nannipieri et al. 1990). When talking about soil enzyme activity, we need to distinguish between intracellular activity associated with living cells of microorganisms, plants and wildlife, and extracellular activity, without direct linking to living cells (Burns 1982). Urease is an extracellular enzyme produced by microorganisms and involved in the decomposition of urea into ammonium carbonate, which makes it a key enzyme in the nitrogen cycle. The activity of urease has been shown to be more sensitive to pollutants compared to other enzymes (Banks et al. 2003). Phosphatase enzymes play a crucial part in the phosphorus cycle and organic phosphorus decomposition in various terrestrial and (semi-)aquatic ecosystems including lake and flood lands (Wyszkowska & Wyszkowski 2010, Lemanowicz, 2018). Alkaline phosphatase (intracellular) and acid phosphatase (extracellular) are the major enzymes responsible for the mineralization of organic phosphorus. Activities of both acid and alkaline phosphatase enzymes are influenced by different soil properties, soil organisms, vegetation cover and existence of inhibitor and

1 activator substances (Martinez-Salgado et al. 2010). Dehydrogenase enzyme activity is an indicator
2 of the oxidation and health status in biological systems and a marker of microbial activity and the
3 intensity of microbial metabolism in the soil since it only occurs in living microorganisms (Nannipieri
4 et al. 1990). Dindar et al. (2015) argued that soil enzymes are excellent soil health indicators, because:
5 (i) they are closely connected with organic matter, physical traits, microbial activity and biomass in
6 the soil; (ii) they give fundamental information on soil quality since soil enzymes such as urease and
7 phosphatase catalyze numerous reactions in nutrient cycling processes; and (iii) they are quantifiable.
8 In total, more than 100 enzymes operate in soil ecosystems (Dick and Kandeler, 2005) and many of
9 them may provide reliable information about soil quality (Zarafshar et al., 2020).

10 The close association of soil-borne microorganisms with plants in the rhizosphere provides a powerful
11 system for detoxification and decomposition of soil pollutants such as heavy metals and oil-derived
12 hydrocarbons. Recent studies have shown that the interactions of plants and microorganisms play a
13 critical role in detoxifying soil and removing PAHs from soils (Khan et al. 2013; Oliveira et al. 2014).
14 The positive effect of plants on the enzymatic activity in oil-contaminated soil has been shown
15 previously (Jeelani et al. 2018, Roy et al. 2014, Xun et al. 2015). For instance, Wang et al. (2008)
16 studied the effects of three grass species on enzyme activity in a soil contaminated by petroleum
17 compounds and found that the presence of plants stimulated dehydrogenase activity compared to
18 unplanted soil. Wyszowska et al. (2010) demonstrated that soil enzyme activity decreased with
19 increasing levels of soil contamination in the presence of herbaceous phytoremediator plants like
20 *Brassica napus* and *Avena sativa*. However, they also found species-specific differences in enzyme
21 stimulation, urease, dehydrogenase and alkaline phosphatase activities were higher in *B. napus* planted
22 soil, while *Avena sativa* planted soils displayed a higher rate of alkaline phosphatase activity.

23 A range of plant species shows potential for phytoremediation of petroleum compounds in soils, but
24 at the same time the exposure to oil can lead to severe oxidative stress and the deterioration of plant
25 health (Panchenko et al. 2017). An increase in reactive oxygen species (ROS), such as hydrogen
26 peroxide, is a clear sign of oxidative stress in plants (Mithöfer et al. 2004) and screening
27 phytoremediation candidates for oxidative stress markers can provide valuable information about their
28 abiotic stress tolerance or resistance and their suitability for remediation projects. At the same time,
29 investigating antioxidant enzyme activity in plants exposed to oil-contaminated soils may help
30 understand their defence mechanisms against oil-induced increases in oxidative stress (Panchenko et
31 al. 2017).

32 Information on the use of soil enzymes as bioindicators for petroleum hydrocarbon contamination is
33 scarce, which prompted us to investigate the relationship between soil enzyme activity and the level
34 of oil sludge contamination for soil health restoration. To quantify the rhizosphere effect and to
35 determine whether species-specific soil enzyme stimulation occurs, we compared the activities of four
36 key enzymes (dehydrogenase, urease, acid and alkaline phosphatase) in unplanted soil to soil planted
37 with seedlings of four forest tree species: *Ailanthus altissima* Mill., *Fraxinus rotundifolia* Mill., *Melia*

azedarach L. and *Robinia pseudoacacia* L. We also examined oil sludge-related oxidative stress and the response of the antioxidant defense system in the tree seedlings. We hypothesized a tree species-specific stimulation of soil enzymatic activity and expected *R. pseudoacacia* to have the greatest impact due to its symbiotic N₂-fixation and therefore increased availability of N in the soil. Furthermore, we anticipated an increase in oxidative stress (using hydrogen peroxide as a proxy) in response to increasing oil contamination with subsequent upregulation of the antioxidant system.

2. Materials and methods

2.1. Study objects and soil samples

For this experiment, seeds of *A. altissima*, *F. rotundifolia*, *M. azedarach* and *R. pseudoacacia* were soaked in water for 48 hours. The oil sludge was provided from the Isfahan Oil Refining Company and the uncontaminated soil was taken from a farm land in Lorestan Province, College of Agriculture and Natural Resources (33°26'14.4" N, 48°15'38.7" E), in the south west Khorramabad city. The soil was collected from the upper soil profile (0–30 cm, A horizon).

2.2. Soil and oil sludge characteristics

Single, mixed composite samples, consisting of three randomly collected subsamples, were formed from soil or oil sludge material and transferred to the soil laboratory for physical and chemical analyses. The samples were air-dried and passed through a 2 mm mesh. Soil texture and bulk density (BD) and actual density were determined using the hydrometer (Bouyoucos 1962) and clod method (Grossman & Reinsch 2002), respectively. Then, the pH value and electrical conductivity (EC) were recorded using pH meter and EC meter, respectively (Burt 2004). Finally, the concentration of some heavy metals and elements in the soil and oil sludge were determined by Inductively Coupled Plasma Emission Spectroscopy (ICP-ES-710 Varian, Australia) (Tables 1 and 2).

Table 1 General physical and chemical traits of the soil used in the pots.

pH	EC (ds/m)	Clay (%)	Silt (%)	Sand (%)	Texture	Bulk weight (g/cm ³)	Actual density (g/cm ³)
7.4	0.6	41	30	29	Clay loamy	1.3	2.63

Table 2 Chemical characteristics of soil and oil sludge used in the experiment on seedlings exposed to oil sludge contamination

chemical characteristics	Ag (g/kg)	Al (g/kg)	Be (mg/kg)	Ca (g/kg)	Cd (g/kg)	Cu (g/kg)	Fe (g/kg)	K (g/kg)
Soil	0.00029	45.722	0.0011	>10%	0.00034	0.033	28.709	10.744
Oil sludge	0.0006	12.615	<0.0001	36.473	0.0011	0.73	33.972	2.190
chemical characteristics	Mg (g/kg)	Mo (g/kg)	Ni (g/kg)	Pb (g/kg)	P (g/kg)	Sr (g/kg)	V (g/kg)	Zn (g/kg)
Soil	16.287	0.00067	0.103	0.042	0.544	0.196	0.078	0.501
Oil sludge	4.527	0.0157	0.152	0.093	10.933	0.495	0.227	1.332

2.3. Experimental design

The experiment was carried out in a greenhouse under controlled conditions. The average temperature during the experiment was 25 ± 10 °C with an average relative humidity of 44 % (85 % maximum, 21 % minimum). The greenhouse is located in Khorramabad city, Iran (33°26'22.4" N, 48°15'44.3" W) at 1148 m a.s.l. The climate is moderate and semi-humid Mediterranean. The mean annual precipitation and temperature are 512.9 mm and 17.6 °C respectively (IRIMO 2014). The study lasted for 240 days from 13 February to 11 October 2017.

The study was laid out as a factorial experiment following a fully randomized design with two fixed factors: species (four tree species including *A. altissima*, *F. rotundifolia*, *M. azedarach* and *R. pseudoacacia*) and oil sludge concentration (four levels: 0, 100, 200 and 400 g kg⁻¹). The range of oil sludge addition was guided by the results of a pre-treatment study showing extremely poor germination at contamination levels exceeding 400 g kg⁻¹. Seeds of the four tree species were planted in artificially oil sludge-contaminated soils. Seed-free oil sludge-contaminated soils served as controls. Before sowing the seeds, the dried oil sludge was manually mixed with the soil to form a homogenous combination. Contaminated soils were kept in the shade at ambient temperature for 5 days before they were allocated to experimental units (pots) with 5 kg capacity and dimensions of 20 × 25 cm. Next, fifteen seeds of the same species were sown in three pots (five seeds in each pot as subreplicates). The pots were transferred to the greenhouse and irrigated to field capacity every 2 days. At the end of the germination period, seedling emergence and survival under the various oil pollution levels were assessed (Table 3) and the number of seedlings in each pot was reduced to one healthy seedling. Those remaining seedlings were irrigated as described above until the end of the experiment.

After 240 days, 4-5 leaf samples were collected from the upper crown of the seedlings to determine antioxidative enzyme activities and H₂O₂ content. At the end of the study, soil samples were taken from all pots (three pots for each oil treatment by tree species combination) and immediately stored in a refrigerator at 4 °C for the soil enzyme assay.

2.4. Soil enzyme assays

Dehydrogenase activity

Soil dehydrogenase activity was determined by the reduction of triphenyltetrazolium chloride to triphenyl formazan (TPF) and reported as μg of TPF released per gram soil per hour (Casida Jr et al. 1964).

Urease activity

The assay for urease activity was based on the determination of NH_4^+ released by urease when the soil was incubated with the THAM (Tris (hydroxymethyl) aminomethane) buffer (pH= 9), a 0.02 M urea solution and toluene at 37 °C for 2 h (Tabatabai et al. 1972).

Soil phosphatase activity

Alkaline and acid phosphatase were determined by using samples of 0.5 g of dry soil according to the method of (Tabatabai et al. 1969). Each soil sample was mixed with 400 μL of modified universal sterile buffer (at pH 5.8 for acid phosphatase and pH 11 for alkaline phosphatase) and 100 μL of p-nitrophenyl phosphate solution (PNPP) prior to incubation for 1 h at 37 °C on a rotary shaker. The reaction was stopped by adding 100 μL of CaCl_2 and 400 μL of NaOH, and the soil suspensions were centrifuged at 12,000 rpm for 10 min. Then, absorbance readings were taken at 400 nm using a UV-VIS spectrophotometer (PhotonixAr 2015, Teifsanje Co. Ltd., Tehran, Iran).

2.5. Plant biochemical parameters

Hydrogen peroxide (H_2O_2)

Foliar hydrogen peroxide (H_2O_2) content in seedlings, was determined by extracting leaf samples (0.5 g) with 5 ml trichloroacetic acid (TCA) (0.1%, w/v) ice bath homogenate, followed by 12,000 $\times$ g centrifugation for 15 min (Velikova et al. 2000).

Peroxidase (POD)

POD extraction buffer contained 50 μM phosphate (pH 7.8), 25 μM guaiacol, 200 μM H_2O_2 , and the change in absorbance of the leaf samples was measured at 470 nm (Zhang & Qu 1990).

Ascorbate peroxidase (APX)

APX activity in leaves was assayed according to Nakano et al. (1981). The reaction mixture (1.5 cm^3) contained 50 mM phosphate buffer (pH= 6.0), 0.1 mM EDTA, 0.5 mM ascorbate, 1.0 mM H_2O_2 , and 0.05 cm^3 enzyme extract. The reaction was started by the addition of H_2O_2 , and ascorbate oxidation was measured at 290 nm for 1 min.

Catalase (CAT)

For measurements of the foliar CAT (EC 1.11.1.6) activity, extraction was done in a buffer containing 50 mM Tris-HCl (pH 7.0), 0.1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride (PMSF) and 0.3 g g^{-1} fw PVP. The enzyme extract was prepared as mentioned above. Activity was measured by the method of Aebi (1974). The 3 mL reaction mixture comprised 50 mM sodium phosphate buffer (pH

7.0), 20 mM H₂O₂ and enzyme extract. Decrease in the absorbance due to consumption of H₂O₂ was measured at 240 nm (extinction coefficient 39.4 mM⁻¹ cm⁻¹).

2.6. Statistical analysis

All data were analyzed using the SPSS statistical package version 16 (SPSS Inc., Chicago, IL, USA). A series of two-way analysis of variance (two-way ANOVA) models were used to test the main and interaction effects of tree species and oil-contamination on soil enzyme activity and four plant biochemical variables. Significant terms were followed up with a *post-hoc* analysis using Tukey's Honest Significant Difference (HSD) test.

The assumptions of normality and homogeneity of variance were checked on the raw data using the Kolmogorov-Smirnov test and Levene's test, respectively. After model fitting, we also inspected the residual plots (normalized residuals vs. fitted values and quantile-quantile plots) to evaluate the assumptions of the models graphically. No gross violations from normality or variance homogeneity were detected. All experiments were performed using three replicates.

3. Results

3.1. Impact of oil sludge contamination on soil enzyme activities of the tree species

The main effects of 'oil sludge contamination' and 'species' as well as their interaction were statistically significant for all studied soil enzyme activities (Table 3). The oil sludge × species interaction indicated that the effect of oil sludge contamination on soil enzymatic activity varied significantly with tree species which was further investigated with a *post-hoc* analysis (Fig. 1).

Table 3. Results of a series of two-way ANOVAs used to determine the effects of oil sludge contamination, tree species identity and their interaction on the activity of four soil enzyme. df_{num} = numerator degrees of freedom, df_{den} = denominator degrees of freedom

Effect	Soil enzymes	df_{num}	df_{den}	<i>F</i>	<i>P</i>
Oil Sludge	Dehydrogenase	3	40	143.167	< 0.001***
	Urease	3	40	182.911	< 0.001***
	Acid phosphatase	3	40	68.566	< 0.001***
	Alkaline phosphatase	3	40	71.885	< 0.001***
Species	Dehydrogenase	4	40	89.682	< 0.001***
	Urease	4	40	225.402	< 0.001***
	Acid phosphatase	4	40	54.461	< 0.001***
	Alkaline phosphatase	4	40	108.153	< 0.001***
Oil Sludge × Species	Dehydrogenase	12	40	33.948	< 0.001***
	Urease	12	40	46.489	< 0.001***
	Acid phosphatase	12	40	6.122	< 0.001***
	Alkaline phosphatase	12	40	24.462	< 0.001***

In the uncontaminated control treatment, no statistically significant differences in dehydrogenase activity occurred, apart from soil planted with *A. altissima* seedlings, which showed significantly higher activity than the unplanted soil (+44 %, Fig. 1 a). When oil sludge contamination was present, the activity of dehydrogenase in planted soil was always significantly higher than that of bare soil, except for *A. altissima* pots at 200 g kg⁻¹ contamination, which showed no significant difference in the activity of soil dehydrogenase compared to bare contaminated soil. However, even in the unplanted soil, dehydrogenase activity increased significantly by more than 50 % in response to low and medium

oil sludge contamination but dropped to the overall lowest activity when 400 g kg⁻¹ oil sludge were added (not significantly different from the uncontaminated control). The overall peak activity of dehydrogenase was observed in soil planted with *R. pseudoacacia* in the 100 g kg⁻¹ oil sludge treatment where it was significantly higher than all the other activities, exceeding the dehydrogenase activity in the unplanted control soil by a factor of 2.6 (Fig. 1 a). A similar but less pronounced pattern was seen at the 200 g kg⁻¹ oil contamination level, where dehydrogenase activity in *R. pseudoacacia*-planted soil was 44 – 94 % higher compared to soil planted with the remaining tree species and about twice as high as the activity measured in unplanted control soil. At the 400 g kg⁻¹ contamination treatment, all tree species stimulated dehydrogenase activity more strongly compared to the lower contamination levels, with the exception of pots planted with *R. pseudoacacia*, which showed significantly lower activity relative to the 100 and 200 g kg⁻¹ oil sludge treatments. At the highest oil contamination level, all dehydrogenase activities differed significantly: soil planted with *F. rotundifolia* showed the highest activity of dehydrogenase (+ 360 % compared to the seedling-free control), while soil planted with *A. altissima* and *M. azedarach* showed somewhat lower activity of dehydrogenase (270 – 290 % higher than the control) and the lowest level of enzymatic stimulation occurred in soil under *R. pseudoacacia* (Fig. 1 a). The peak magnitude of *R. pseudoacacia*'s stimulatory effect on dehydrogenase activity seen at 100 g kg⁻¹ oil sludge addition dropped by roughly 30 % with each doubling of the contamination level. The trend for urease activity strongly resembled the dehydrogenase pattern but urease activity in the unplanted pots remained unchanged with increasing oil sludge contamination (Fig. 1 b). Irrespective of oil contamination level, seedling presence significantly stimulated urease activity relative to the unplanted control soil by 60 – 380 % (Fig. 1 b). Overall, the highest activity occurred in soil planted with *R. pseudoacacia* at the 100 g kg⁻¹ oil sludge contamination level (Fig. 1 b). All tree species produced a significant increase in urease activity at 100 g kg⁻¹ compared to the activity levels seen in uncontaminated soil. The magnitude of this stimulatory effect on urease activity decreased significantly from the 100 to the 200 g kg⁻¹ oil contamination level in all tree species followed by a significant increase in the most severely contaminated soil, except for *R. pseudoacacia* whose effect showed a further significant decline at the highest contamination treatment (-46 % compared to the peak urease activity caused at 100 g kg⁻¹). Both, *F. rotundifolia* and *A. altissima* exerted their strongest effect on urease activity at the 400 g kg⁻¹ oil contamination treatment which was significantly higher than the increases in activity caused by those species at the lower contamination levels. The highest magnitude of urease stimulation linked to *M. azedarach* also occurred in the 400 g kg⁻¹ oil sludge treatment (although not statistically different from the effect observed at 100 g kg⁻¹) and was very similar to that of *A. altissima*.

The activity of acid phosphatase in unplanted soil increased significantly by 18 % in the 100 and 200 g kg⁻¹ contamination treatment compared to the uncontaminated control but returned to control levels in the most heavily contaminated soil (Fig. 1c). At each level of contamination, we observed consistently higher activity in soil planted with tree seedlings compared to unplanted soil (Fig. 1 c).

However, *R. pseudoacacia* did not show the same stimulatory effect it had on dehydrogenase and urease activity. The highest activities of acid phosphatase exceeded $1400 \mu\text{g pNP g}^{-1} \text{ dm h}^{-1}$ and were found in soil planted with *A. altissima* and *R. pseudoacacia* in the 200 g kg^{-1} oil sludge treatment and *F. rotundifolia* at the 100 g kg^{-1} level of contamination. At the highest oil contamination level of 400 g kg^{-1} , acid phosphatase activity was similarly stimulated across tree species but to a significantly lesser extent compared with the remaining contamination levels, apart from *F. rotundifolia* whose effect did not differ significantly from the one seen at 200 g kg^{-1} (Fig. 1 c).

The activity of alkaline phosphatase in unplanted control soil did not change significantly with increasing oil contamination level (Fig. 1 d). Tree seedling in the uncontaminated soil presence significantly stimulated alkaline phosphatase activity relative to the unplanted control, except for *A. altissima* which produced activity levels similar to the control. Overall, soil planted with *F. rotundifolia* in the 100 g kg^{-1} oil contamination treatment showed the strongest activity of the enzyme which was about 280 % higher than the control activity. However, the stimulatory effect of *F. rotundifolia* declined significantly with increasing soil contamination. At the 200 g kg^{-1} oil sludge treatment, *F. rotundifolia*-related enzyme activity dropped down to values seen in uncontaminated soil and even lower than that at the strongest contamination and in both instances the other tree species produced significantly larger effects. In the 200 and 400 g kg^{-1} oil contamination treatments, pots containing *R. pseudoacacia* showed peak activities of alkaline phosphatase which were significantly higher than all other enzyme activities in planted soil and translated into a 194 % and 234 % increase over the control in the 200 and 400 g kg^{-1} treatments, respectively. *A. altissima* and *M. azedarach* displayed the next larger effects on alkaline phosphatase activity in the 200 and 400 g kg^{-1} contamination treatments without significant differences in magnitude (Fig. 1 d).

Fig. 1 Activities of four key soil enzymes in unplanted soil (control) and soil planted with one of four species of forest tree seedlings in response to a 240-day oil sludge contamination treatment (0, 100, 200, 400 g kg^{-1} soil). Different lower-case letters indicate statistically significant differences based on Tukey's Honest Significant Difference (HSD) test ($\alpha = 0.05$). Means $\pm$ SE, $n = 3$.

3.2. Impact of oil sludge contamination on foliar H₂O₂ content and antioxidant enzymes

For all four leaf biochemical parameters, we detected statistically significant main effects of ‘oil sludge contamination’ and ‘species’ as well as a significant interaction between these two factors implying that the impact of oil sludge contamination on foliar plant biochemistry was species-dependent (Table 4).

Table 4. Results of a series of two-way ANOVAs used to determine the effects of oil sludge contamination, tree species identity and their interaction on four foliar biochemical parameters of the studied tree species. df_{num} = numerator degrees of freedom, df_{den} = denominator degrees of freedom

Effect	Plant biochemical parameters	df_{num}	df_{den}	<i>F</i>	<i>P</i>
Oil Sludge	Hydrogen peroxide	3	32	149.409	< 0.001***
	Peroxidase	3	32	98.721	< 0.001***
	Ascorbate peroxidase	3	32	95.981	< 0.001***
	Catalase	3	32	38.308	< 0.001***
Species	Hydrogen peroxide	3	32	4.488	0.010*
	Peroxidase	3	32	20.400	< 0.001***
	Ascorbate peroxidase	3	32	91.938	< 0.001***
	Catalase	3	32	54.509	< 0.001***
Oil Sludge × Species	Hydrogen peroxide	9	32	10.586	< 0.001***
	Peroxidase	9	32	4.615	< 0.001***
	Ascorbate peroxidase	9	32	5.038	< 0.001***
	Catalase	9	32	3.313	0.006**

The foliar H₂O₂ concentration was lowest in the uncontaminated control treatment and tended to increase in all species with increasing oil sludge contamination but this rise was not always statistically significant (Fig. 2 a). In uncontaminated soil, *R. pseudoacacia* showed significantly lower foliar H₂O₂ content (-14 %) than *F. rotundifolia* and *A. altissima*, while the intermediate values of *M. azedarach* were not significantly different from any of the other species (Fig. 2a). At the 100 g kg⁻¹ contamination level, H₂O₂ content in *R. pseudoacacia* foliage was significantly lower compared to the remaining species (ca. -17 %) and was still similar to the values seen in the uncontaminated control. When 200 g kg⁻¹ oil sludge were added, *F. rotundifolia* leaves showed the highest H₂O₂ content but differences to the other species were only statistically significant for *R. pseudoacacia* and *A. altissima*. Surprisingly, *A. altissima* was the only species whose foliar H₂O₂ content did not respond to a doubling of the oil sludge concentration from 100 to 200 g kg⁻¹. Under the most severe oil contamination (400 g kg⁻¹), all tree species showed significantly higher foliar H₂O₂ content compared with seedlings grown in uncontaminated soil and in the 100 g kg⁻¹ oil pollution but only *R. pseudoacacia* and *A. altissima* exhibited significant increases compared to the 200 g kg⁻¹ treatment (+32.5 % and +12.6 %, respectively). Overall, the maximum H₂O₂ content was observed in *R. pseudoacacia* seedlings at the highest oil sludge contamination level, which was on average 17 % higher than the H₂O₂ content seen in the three remaining species at this contamination level and 76 % higher compared to *R. pseudoacacia* seedlings growing in uncontaminated soil.

All tree species showed their lowest peroxidase (POD) activity under control conditions and a significant rise to maximal values in the 100 g kg⁻¹ oil sludge treatment followed by a return to control

1 levels with increasing contamination, except for *R. pseudoacacia* whose POD activity remained
2 significantly elevated at the strongest contamination (relative to the control) (Fig. 2 b). Overall, the
3 highest level of POD activity was found in seedlings of *A. altissima* exposed to 100 g kg⁻¹ oil sludge
4 concentration, which translated into a 158 % increase over the level of activity measured in conspecific
5 control seedlings.

6 Under control conditions, *A. altissima* and *M. azedarach* displayed similar but significantly higher
7 ascorbate peroxidase (APX) activity than the two other tree species. A steep increase in APX activity
8 from the uncontaminated control to the 100 g kg⁻¹ oil sludge concentration occurred in all species and
9 was followed by a significant decline at the two highest oil sludge additions back to the activity level
10 seen in the control, except for *A. altissima* (Fig. 1 c). Seedlings of *A. altissima* showed the highest
11 APX activity of all trees at 100 and 200 g kg⁻¹ oil sludge concentration (*ca.* 13 ΔA min⁻¹ μg⁻¹ protein)
12 and also displayed a significant activity reduction at the highest contamination treatment. However,
13 despite this decline the APX activity associated with the 400 g kg⁻¹ oil contamination was still
14 significantly higher than the activity in leaves of the control seedlings. The maximum foliar APX
15 activity of *F. rotundifolia* occurred at the 100 g kg⁻¹ contamination level followed by a significant
16 decline of 29 % in the 200 g kg⁻¹ contamination treatment and a further significant drop to values
17 similar to the control under the most severe oil-contamination. *F. rotundifolia* seedlings growing in
18 the uncontaminated and the most severely oil-contaminated soil had the lowest overall APX activity.
19 The APX activity levels associated with the 100 and 200 g kg⁻¹ contamination treatments were similar
20 to those of *R. pseudoacacia* (Fig. 1 c).

21 In uncontaminated soil, *A. altissima* showed significantly higher catalase (CAT) activity than *M.*
22 *azedarach* and both activities were significantly higher compared to the similar values observed in *R.*
23 *pseudoacacia* and *F. rotundifolia* (Fig. 2 d). Similar to APX, the highest activity of the catalase
24 enzyme occurred in seedlings of *A. altissima* under 100 and 200 g kg⁻¹ oil sludge concentration. CAT
25 activity in the remaining species also showed a significant increase over the control at 100 or 200 g
26 kg⁻¹ but returned to control levels at the highest oil contamination treatment, apart from *F. rotundifolia*
27 whose CAT activity also decreased significantly but yet remained significantly higher than the control.
28 Overall, the lowest recordings of CAT activity were observed in leaves of *F. rotundifolia* and *R.*
29 *pseudoacacia* growing in uncontaminated soil and in case of the latter species, also in the most
30 severely oil-contaminated soil (Fig.1 d).

Fig. 2 Oxidative stress (H_2O_2) and activity of antioxidant defense enzymes in leaves of four species of forest tree seedlings in response to a 240-day oil sludge contamination treatment (0, 100, 200, 400 g kg⁻¹ soil). Different lower-case letters indicate statistically significant differences based on Tukey's Honest Significant Difference (HSD) *post-hoc* test ($\alpha = 0.05$). Means + SE, $n = 3$. POD = peroxidase, APX = ascorbate peroxidase, CAT = catalase

4. Discussion

In the present study, we investigated the effects of soil contamination by oil sludge on the activity of key soil enzymes in the presence and absence of seedlings of four tree species frequently used in plantation projects in the Middle Eastern region in order to guide future afforestation and reforestation activities in oil-contaminated areas. In addition, we also explored changes in H_2O_2 content (as a proxy for oxidative stress) and the antioxidant defense system in the leaves of the four tree species in response to oil-induced oxidative stress. The same individuals used here were also used in a complementary study on the effects of oil-sludge contamination on seedling morphology and physiology, where seed germination, seedling survival and growth data were recorded as well, indicating that oil contamination dramatically affects tree growth (see Norouzi Haroni et al., 2019).

4.1. Soil enzyme activity in response to oil sludge contamination in planted and unplanted soil

In nearly all instances, we observed a tree-related stimulation of enzymatic activity in oil-contaminated soil relative to the plant-free control, regardless of enzyme type. As anticipated, the magnitude of this stimulatory effect varied significantly in a species-specific manner and also across contamination levels, which was supported by a significant two-way interaction between tree species and oil contamination treatment in the statistical models (Table 3).

Soil dehydrogenase is exclusively produced by living microbial cells and therefore serves as a reliable marker for microbial activity and changes in overall soil metabolism (Jeelani et al. 2018). The rather consistent seedling-driven stimulation of dehydrogenase in oil-contaminated soil suggests greater rhizodeposition or changes in exudate composition promoting microbial activity in comparison with uncontaminated control soil. This result is in line with the findings of Xie et al. (2018) indicating that

oil contamination may cause compositional changes in root exudates and thereby alter the soil microbial community and activity.

In the unplanted soil, the lack of labile soil carbon inputs and other stimulants from rhizodeposition must have limited microbial growth, which, together with the toxic effects of the oil sludge, was probably the main cause for the lower levels of dehydrogenase activity in seedling-free pots in our study (see also Movafagh et al. 2015). Nonetheless, dehydrogenase activity also rose significantly in unplanted soil in response to low and medium oil sludge contamination (Fig. 1 a) implying increased activity of heterotrophic bacteria decomposing oil compounds and using the intermediates or breakdown products as a source of energy and/or carbon. In line with this notion, Hernández-Ortega et al. (2012) demonstrated that oil sludge may add energy sources such as carbon, nitrogen and sulfur to soils which might promote both microbial enzyme production and enzyme activity.

Urease is one of the most important amidohydrolases and plays a crucial role in the nitrogen metabolism of soils (Acosta-Martinez & Tabatabai 2000). The trend for urease activity strongly resembled the dehydrogenase pattern but here we observed no increase in activity in unplanted soil with increasing oil contamination indicating that urease-producing soil microorganisms were not able to use oil compounds as a source of energy or carbon (Fig. 1 b). Unplanted soils always showed the lowest activities of urease and dehydrogenase compared to soil planted with tree seedlings highlighting the stimulatory effect of roots on microbial enzyme production and secretion (Yang et al., 2012). The large enzymatic stimulation of dehydrogenase and urease caused by *R. pseudoacacia* at the low and intermediate and by *F. rotundifolia* at the highest oil contamination level suggests that these species either release more root exudates or that their exudates undergo more beneficial changes in response to oil contamination at these levels compared to *A. altissima* and *M. azedarach*. The great magnitude of the stimulatory effect of *R. pseudoacacia* may be largely due to increases in dissolved organic nitrogen originating from the symbiotic nitrogen fixation. Between 1 and 2 % of the nitrogen fixed by *R. pseudoacacia* has been reported to find its way into the soil with root exudates (Please find and add the following citation: Uselman et al. 1999).

Oil sludge contains numerous heavy metals (Ca, Cd, Cu, Pb) which may cause denaturation of enzymes (Vig et al. 2003) and this was probably the reason for the decreased activity of dehydrogenase and urease in the rhizosphere of *R. pseudoacacia* seedlings growing in heavy metal-contaminated soil in a study by Huang et al. (2016). The heavy metal concentration in our oil-contaminated soils was lower compared to the experiment of Huang et al. (2016), but we have also recorded a decrease in dehydrogenase and urease activity with increasing oil-contamination in soil planted with *R. pseudoacacia*. However, if this decline in enzyme activity was mainly driven by heavy metal toxicity, then we should have seen a similar pattern of decline in soil under the remaining species too, unless we presume a protective rhizosphere effect that varies with tree species depending on the level of contamination. In the rhizosphere, the heavy metal-induced denaturation of soil enzymes is mitigated due to the strong chelating properties of organic acids in root exudates and therefore the extent of

denaturation processes may be controlled to a degree by the amount of root exudates or their composition (Mehrdad, please find and a couple of references in support of this and insert them here. I think the following book would work (mentioned in Rosselli, Keller & Boschi (2003)): Blaylock M J and Huang J W 2000 Phytoextraction of metals. In Phytoremediation of Toxic Metals: Using Plants to Clean up the Environment. Eds. Raskin I and Ensley B D. pp. 314. John Wiley and Sons Inc., New York.). This would imply a decrease in rhizodeposition or unfavourable compositional changes in root exudates of *R. pseudoacacia* with increasing oil-contamination, whereas the opposite should then be true for the three remaining tree species as they all showed a significant increase in their stimulatory effect on enzyme activity from the 200 to the 400 g kg⁻¹ contamination level. However, our foliar H₂O₂ data suggest oxidative damage as the most plausible explanation for the pattern associated with *R. pseudoacacia*. The significant decrease in the magnitude of the stimulation of dehydrogenase and urease activity with increasing oil contamination in *R. pseudoacacia*-planted pots was mirrored by a corresponding increase in foliar H₂O₂ content. Thus, we speculate that oxidative damage in leaves could have disrupted leaf carbohydrate metabolism resulting in curtailed resource allocation below ground which translates into reduced rhizodeposition and microbial stimulation.

The activity of phosphatase enzymes in soil is the major driver of organic phosphorus supply under mineral phosphorus deficiency (Helal & Dressler 1989). While acid phosphatase is formed by plant roots and microorganisms, alkaline phosphatase is predominantly produced by microorganisms (Marschner 2012). The significant increase in acid phosphatase activity in unpolluted soil planted with tree seedlings relative to the unplanted control highlights the plant contribution to the soil acid phosphatase pool through excretion and the rhizosphere effect. Acid phosphatase activity showed only minor changes in unplanted soil in response to rising oil pollution levels. However, in planted soil acid phosphatase activity generally increased with increasing oil sludge concentration but at the highest concentration enzymatic activity returned to levels similar to or slightly lower than those observed in the control (Fig. 1c). These findings suggest that exposure to oil sludge contamination can boost acid phosphatase production and secretion in tree roots but seems to have little or no effect on microbial acid phosphatase. Our data further indicate that this oil-driven stimulatory effect is vanishing at medium (see *F. rotundifolia*) to high oil contamination levels.

Alkaline phosphatase activity is also controlled by organic phosphorus availability and the pH in the soil and therefore changes in those variables associated with the presence of oil sludge or its decomposition processes may certainly have an effect on the activity of this enzyme (Lai et al. 2011). In our study, the activity of alkaline phosphatase in unplanted control soil did not change significantly with increasing oil contamination level suggesting no oil-related effects on the functioning of this enzyme or its microbial synthesis in the range of the oil sludge concentrations tested (Fig. 1 d). On the other hand, tree seedling presence significantly stimulated alkaline phosphatase activity relative to the unplanted control at each contamination level and this was most likely due to root secretions which have been shown to directly affect phosphatase activity in the rhizosphere (Thomson et al. 1986). Our

findings imply that seedling rhizodeposition in uncontaminated soil increased the activity of alkaline phosphatase, except for soil planted with *A. altissima* where alkaline phosphatase activity remained unchanged relative to the unplanted control suggesting that a key stimulating compound (or several) was lacking in its root exudates. As judged by the strong alkaline phosphatase stimulation in the uncontaminated control soil and in the 100 g kg⁻¹ contamination treatment, the composition of root exudates of *F. angustifolia* must have been very beneficial to soil microbes. However, the marked decrease by more than 50 % in the stronger contamination treatments implies changes in exudate composition rather than in volume since reductions in the amount of root exudates would have been reflected in a declining stimulatory effect on dehydrogenase and urease. *R. pseudoacacia* consistently stimulated alkaline phosphatase activity and this effect was significantly higher compared to the remaining species at the two highest contamination levels. A strong stimulatory effect of *R. pseudoacacia* on alkaline phosphatase was also found in a study from the Loess Plateau in western China by Qiu et al. (2010) who linked the stimulation to the increase in labile organic carbon and total nitrogen in the rhizosphere of *R. pseudoacacia*. (And here is the reference: Qui L et al. (2010) Effects of black locust (*Robinia pseudoacacia*) on soil properties in the loessial gully region of the Loess Plateau, China.).

4.2. Impact of oil sludge contamination on foliar H₂O₂ content and antioxidant enzymes

When oil sludge is introduced to a vegetated site, it commonly causes oxidative stress in plants due to the deficiency of oxygen molecules and generation of reactive oxygen species (ROS) (Liu et al. 2009). Oil products and hydrocarbons can induce the generation of ROS such as the superoxide anion, hydrogen peroxide and hydroxyl ions in plant cells (Habiba et al. 2015, Tauqeer et al. 2016, Zhang et al. 2007). This is corroborated by our results, which showed that H₂O₂ levels in tree seedlings increased with increasing oil sludge contamination. This is most likely due to the production of free radicals through Weiss-Haber reactions, which can ultimately raise the level of toxicity (Bouazizi et al. 2007). In our study, we have used H₂O₂ as a proxy for oxidative stress and observed increasing levels thereof in tree seedlings with increasing oil sludge contamination in a species-specific manner. Hydrogen peroxide can be eliminated by antioxidant enzymes such as POD, APX, and CAT (Sharma et al. 2017a, Sharma et al. 2017b, Sharma et al. 2012), therefore we also studied the activity of these enzymes in seedling foliage in response to oil contamination. The foliar H₂O₂ content as well as the activity of three major antioxidant enzymes varied significantly among species depending on oil contamination level, which was statistically supported by a significant two-way interaction. Interestingly, *R. pseudoacacia* seems to be able to ward off oxidative damage at low oil contamination as evidenced by the lack of increase in foliar H₂O₂ at the 100 g kg⁻¹ oil sludge treatment. However, the relatively strong accumulation of H₂O₂ in the most severe oil contamination treatment implies that the antioxidant pool responsible for the initial protection appears to become rapidly exhausted. The lack of a corresponding increase in activity of any of the major antioxidant enzyme classes in *R. pseudoacacia* foliage at 100 g kg⁻¹ oil sludge addition suggests that the antioxidant protection may

1 have been conferred by non-enzymatic antioxidants such as flavonoids, non-flavonoid phenolics,
2 tocopherols, carotenoids, ascorbic acid and other compounds, which have been reported to be present
3 in large numbers in *R. pseudoacacia* (Please add the following reference here: Tyśkiewicz et al.
4 (2019): Characterization of bioactive compounds in the biomass of black locust, poplar and willow.
5 *TREES*, 33, 1235-1263).

6 In line with our results, Yang et al. (2015) reported that the H₂O₂ level in the leaves of *R. pseudoacacia*
7 increased linearly with increasing lead (Pb) concentration in the soil. Our findings further indicate that
8 the antioxidant defense of *A. altissima* relies heavily on APX and to a somewhat lesser extent on CAT,
9 whereas POD only plays a major role at low oil contamination levels. APX has been shown to be more
10 efficient in the elimination of ROS in comparison with other peroxidase and catalase enzymes
11 (Mohsenzadeh 2018) and thus the strong reliance on such a strong ROS quencher indicates higher
12 oxidative stress in *A. altissima* than in the other species. The antioxidant defense system of *F.*
13 *rotundifolia* depends on all three enzymes at low and intermediate levels of oil contamination but
14 under more severe contamination CAT and possibly other compounds not investigated here play a
15 more dominant role in combating oxidative stress. Remarkably, all enzyme activities in *M. azedarach*
16 foliage dropped back down to control levels at the highest oil sludge exposure but its foliar H₂O₂
17 content did not significantly increase from the 200 g kg⁻¹ to the most severe oil contamination implying
18 a shift towards other enzymatic or non-enzymatic antioxidants. The reduced activity of POD in *F.*
19 *rotundifolia*, *A. altissima* and *M. azedarach* at the 200 and 400 g kg⁻¹ oil sludge additions might have
20 resulted from the accumulation of toxic compounds in the plant cell inhibiting enzyme functioning
21 and aside from this, increasing levels of H₂O₂ and other ROS may have caused molecular damage that
22 lessened the activity of POD (Patidar et al. 2017). Previous research has shown that peak POD activity
23 occurs at low levels of abiotic stress (Das & Roychoudhury 2014b, Kord et al. 2010, Sharma et al.
24 2016), which is in line with our findings. Moreover, POD can be regarded as an indicator of heavy
25 metal stress in the cytosol, cell wall and vacuoles, and its enzymatic activity depends on the production
26 of H₂O₂ and phenolic compounds (Ahmadvand et al. 2014). Because *R. pseudoacacia* was the only
27 species whose POD activity remained significantly elevated at the highest oil contamination level in
28 comparison with the control, it is plausible to assume that this species experienced more heavy metal-
29 related stress than the others. The declining enzyme activity observed in most cases at 400 g kg⁻¹
30 contamination level can probably be attributed to the accumulation of ROS and the progressing
31 exhaustion of the antioxidant defense system, which may even be accelerated through the activation
32 of peroxisomal proteases under extreme stress conditions (Tang et al. 2010, Das & Roychoudhury
33 2014a, Sharma et al. 2018).

34 Antioxidant enzymes are commonly regarded as oxidative stress markers and therefore the strong
35 activity of these enzymes (APX and CAT in particular) in *A. altissima* seedlings exposed to oil sludge
36 exposure suggests higher levels of oxidative stress compared to the remaining species. The antioxidant
37 defense system of *A. altissima* allowed H₂O₂ quenching to the same extent as *F. rotundifolia* and *M.*

azedarach but involved a higher metabolic cost and we cannot preclude that other, unmeasured ROS species have increased.

5. Conclusion

Our findings underline the importance of the rhizosphere effect for enzyme-driven reactions central to soil restoration. The observed species-specific stimulation of soil enzyme activity implies potential for enhancing phytoremediation efficiency through carefully engineered plant species assemblages. Using nitrogen-fixing woody phytoremediator species such as *R. pseudoacacia* may provide an extra boost for soil microbial activity, especially in nitrogen-poor soils. Our study indicates that *R. pseudoacacia* is particularly well-suited for phytoremediation projects dealing with low to intermediate oil pollution levels and also at highly oil-polluted sites with phosphorous-poor soil where a strong stimulatory effect on alkaline phosphatase is desirable to increase phosphorous availability. Greater seedling survival and growth under severe oil-contamination together with reasonably strong enzymatic stimulation make *M. azedarach* and *F. rotundifolia* (strong stimulation of dehydrogenase and urease) ideal phytoremediator species at highly oil-polluted sites. Moreover, our study identified oxidative stress tolerance as a functional trait to be considered in candidate tree species for phytoremediation of oil-contaminated soils. Among the species tested here, *A. altissima* seems to experience the highest level of oil-induced oxidative stress, which may translate into increased oxidative damage and decreased seedling growth and we therefore rank its phytoremediation potential lowest.

Conflict of Interest: The authors declare that they have no conflict of interest.

References

- Acosta-Martinez V, Tabatabai M (2000): Enzyme activities in a limed agricultural soil. *Biology and Fertility of Soils* 31, 85-91
- Aebi H (1974): Catalase, *Methods of enzymatic analysis*. Elsevier, pp. 673-684
- Agnello AC, Bagard M, Van Hullebusch ED, Esposito G, Huguenot D (2016): Comparative bioremediation of heavy metals and petroleum hydrocarbons co-contaminated soil by natural attenuation, phytoremediation, bioaugmentation and bioaugmentation-assisted phytoremediation. *Science of the total environment* 563, 693-703
- Ahmadvand H, Amiri H, Dehghani Elmi Z, Bagheri S (2014): Chemical composition and antioxidant properties of *Ferula-assa-foetida* leaves essential oil. *Iranian Journal of Pharmacology and Therapeutics* 12, 52-0
- Anandham R, Indiragandhi P, Madhaiyan M, Saravanan V, Sa T (2007): Isolation and characterization of thiosulfate oxidizing bacteria from rhizosphere soils of crop plants of Korea. *Environment Science* 5, 114
- Azevedo H, Glória Pinto CG, Santos C (2005): Cadmium effects in sunflower: membrane permeability and changes in catalase and peroxidase activity in leaves and calluses. *Journal of plant nutrition* 28, 2233-2241
- Banks M, Schwab P, Liu B, Kulakow P, Smith J, Kim R (2003): The effect of plants on the degradation and toxicity of petroleum contaminants in soil: a field assessment, *Phytoremediation*. Springer, pp. 75-96
- Beškoski VP, Gojgić-Cvijović G, Milić J, Ilić M, Miletić S, Šolević T, Vrvic MM (2011): Ex situ bioremediation of a soil contaminated by mazut (heavy residual fuel oil)—A field experiment. *Chemosphere* 83, 34-40

- 1 Bouazizi H, Jouili H, El Ferjani E (2007): Effects of Copper Excess on Growth, H₂O₂ Production and Peroxidase
- 2 Activities in Maize Seedlings (*Zea mays* L.). *Pakistan Journal of Biological Sciences* 10, 751-756
- 3 Bouyoucos GJ (1962): Hydrometer method improved for making particle size analyses of soils 1. 54, 464-
- 4 465
- 5 Brakorenko NN, Korotchenko TV (2016): Impact of petroleum products on soil composition and physical-
- 6 chemical properties, IOP Conference Series: Earth and Environmental Science. IOP Publishing, pp.
- 7 012028
- 8 Brandt R, Merkl N, Schultze-Kraft R, Infante C, Broll G (2006): Potential of vetiver (*Vetiveria zizanioides* (L.)
- 9 Nash) for phytoremediation of petroleum hydrocarbon-contaminated soils in Venezuela. *International*
- 10 *journal of phytoremediation* 8, 273-284
- 11 Burns RG (1982): Enzyme activity in soil: location and a possible role in microbial ecology. *Soil Biology*
- 12 *Biochemistry* 14, 423-427
- 13 Burt R (2004): Soil survey laboratory methods manual. Casida Jr L, Klein D, Santoro TJSs (1964): Soil
- 14 dehydrogenase activity. 98, 371-376
- 15 Das K, Roychoudhury A (2014a): Reactive oxygen species (ROS) and response of antioxidants as ROS-
- 16 scavengers during environmental stress in plants. *Frontiers in Environmental Science* 2, 53
- 17 Das K, Roychoudhury A (2014b): Reactive oxygen species (ROS) and response of antioxidants as ROS-
- 18 scavengers during environmental stress in plants. 2, 53
- 19 Das SK, Varma A (2010): Role of enzymes in maintaining soil health, *Soil enzymology*. Springer, pp. 25-42
- 20 Dick RP (1992): A review: long-term effects of agricultural systems on soil biochemical and microbial
- 21 parameters. *Agriculture, ecosystems & environment* 40, 25-36
- 22 Dick, R P, Kandeler, E (2005). Enzymes in soils. *Encyclopedia of Soils in the Environment*. 448-456.
- 23 Dindar E, Sağban FOT, Başkaya HS (2015): Variations of soil enzyme activities in petroleum-hydrocarbon
- 24 contaminated soil. *International Biodeterioration & Biodegradation* 105, 268-275
- 25 Gianfreda L, Rao MAJCRiES, Technology (2008): Interactions between xenobiotics and microbial and
- 26 enzymatic soil activity. 38, 269-310
- 27 Grossman R, Reinsch TJMosaPpm (2002): 2.1 Bulk density and linear extensibility. 201-228
- 28 Habiba U, Ali S, Farid M, Shakoor MB, Rizwan M, Ibrahim M, Abbasi GH, Hayat T, Ali B (2015): EDTA
- 29 enhanced plant growth, antioxidant defense system, and phytoextraction of copper by *Brassica napus*
- 30 L. *Environmental Science and Pollution Research* 22, 1534-1544
- 31 Helal HM, Dressler A (1989): Mobilization and turnover of soil phosphorus in the rhizosphere. *Zeitschrift für*
- 32 *Pflanzenernährung und Bodenkunde* 152, 175-180
- 33 Hernández-Ortega HA, Alarcón A, Ferrera-Cerrato R, Zavaleta-Mancera HA, López-Delgado HA, Mendoza-
- 34 López MR (2012): Arbuscular mycorrhizal fungi on growth, nutrient status, and total antioxidant
- 35 activity of *Melilotus albus* during phytoremediation of a diesel-contaminated substrate. *Journal of*
- 36 *environmental management* 95, S319-S324
- 37 Hsu S-Y, Kao CH (2003): Differential effect of sorbitol and polyethylene glycol on antioxidant enzymes in rice
- 38 leaves. *Plant Growth Regulation* 39, 83-90
- 39 Huang S, Jia X, Zhao Y, Chang Y, Bai BJAse (2016): Response of *Robinia pseudoacacia* L. rhizosphere
- 40 microenvironment to Cd and Pb contamination and elevated temperature. 108, 269-277
- 41 Institute S (1999): SAS Procedures Guide: Version 8, 1. Sas Inst
- 42 IRIMO (2014): IR of Iran Meteorological Org (IRIMO), 2014. The meteorology of the khoram abad
- 43 Township <http://www.irimo.ir/english/>. Accessed April 2014.
- 44 Jeelani N, Yang W, Qiao Y, Li J, An S, Leng X (2018): Individual and combined effects of cadmium and
- 45 polycyclic aromatic hydrocarbons on the phytoremediation potential of *Xanthium sibiricum* in co-
- 46 contaminated soil. *International journal of phytoremediation* 20, 773-779
- 47 Jia X, Zhang C, Zhao Y, Liu T, He YJJohn (2018): Three years of exposure to lead and elevated CO₂ affects
- 48 lead accumulation and leaf defenses in *Robinia pseudoacacia* L. seedlings. 349, 215-223
- 49 Kord B, Mataji A, Babaie SJIJoES, Technology (2010): Pine (*Pinus Eldarica* Medw.) needles as indicator for
- 50 heavy metals pollution. 7, 79-84
- 51 Kourtev PS, Ehrenfeld JG, Häggblom M (2002): Exotic plant species alter the microbial community structure
- 52 and function in the soil. *Ecology* 83, 3152-3166
- 53 Kriksunov E (2011): Marine oil spills: the causes, environmental impact, prevention methods, response
- 54 operations. *Water Resources* 38, 684-685

- Lemanowicz J (2018): Dynamics of phosphorus content and the activity of phosphatase in forest soil in the sustained nitrogen compounds emissions zone. *Environmental Science and Pollution Research* volume. 25, 33773–33782
- Lai J, Yu Z, Song X, Cao X, Han X (2011): Responses of the growth and biochemical composition of *Prorocentrum donghaiense* to different nitrogen and phosphorus concentrations. *Journal of Experimental Marine Biology and Ecology* 405, 6-17
- Liao C, Xu W, Lu G, Liang X, Guo C, Yang C, Dang Z (2015): Accumulation of hydrocarbons by maize (*Zea mays* L.) in remediation of soils contaminated with crude oil. *International journal of phytoremediation* 17, 693-700
- Liu F, Ying G-G, Tao R, Zhao J-L, Yang J-F, Zhao L-F (2009): Effects of six selected antibiotics on plant growth and soil microbial and enzymatic activities. *Environmental pollution* 157, 1636-1642
- Ma S-C, Zhang H-B, Ma S-T, Wang R, Wang G-X, Shao Y, Li C-XJE, safety e (2015): Effects of mine wastewater irrigation on activities of soil enzymes and physiological properties, heavy metal uptake and grain yield in winter wheat. 113, 483-490
- Maila MP, Cloete TE (2005): The use of biological activities to monitor the removal of fuel contaminants—perspective for monitoring hydrocarbon contamination: a review. *International Biodeterioration & Biodegradation* 55, 1-8
- Margalef O, Sardans J, Fernández-Martínez M, Molowny-Horas R, Janssens I, Ciais P, Goll D, Richter A, Obersteiner M, Asensio D (2017): Global patterns of phosphatase activity in natural soils. *Scientific reports* 7, 1337
- Marschner P (2012): Rhizosphere biology, Marschner's Mineral Nutrition of Higher Plants (Third Edition). Elsevier, pp. 369-388
- Martinez-Salgado M, Gutiérrez-Romero V, Janssens M, Ortega-Blu R (2010): Biological soil quality indicators: a review. *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology* 1, 319-328
- Mithöfer A, Schulze B, Boland W (2004): Biotic and heavy metal stress response in plants: evidence for common signals. *FEBS letters* 566, 1-5
- Mohsenzadeh F (2018): Removing of Benzo [a] pyrene using the Isolated Fungi from Petroleum-polluted Soils. *Toxicology and Environmental Health Sciences* 10, 123-131
- Movafagh S, Crook S, Vo K (2015): Regulation of hypoxia-inducible factor-1a by reactive oxygen species: new developments in an old debate. *Journal of cellular biochemistry* 116, 696-703
- Mueller JG, Middaugh DP, Lantz SE, Chapman P (1991): Biodegradation of creosote and pentachlorophenol in contaminated groundwater: chemical and biological assessment. *Applied and Environmental Microbiology* 57, 1277-1285
- Nakano Y, Asada KJP, physiology c (1981): Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. 22, 867-880
- Nanekar S, Dhote M, Kashyap S, Singh S, Juwarkar AA (2015): Microbe assisted phytoremediation of oil sludge and role of amendments: a mesocosm study. *International Journal of Environmental Science and Technology* 12, 193-202
- Nannipieri P, Grego S, Ceccanti B, Bollag J, Stotzky GJSb (1990): Ecological significance of the biological activity in soil, pp. 1990. 293-355.
- Osse M, Hamel J-F, Mercier A (2018): Markers of oil exposure in cold-water benthic environments: Insights and challenges from a study with echinoderms. *Ecotoxicology and environmental safety* 156, 56-66
- Panchenko L, Muratova A, Turkovskaya O (2017): Comparison of the phytoremediation potentials of *Medicago falcata* L. And *Medicago sativa* L. in aged oil-sludge-contaminated soil. *Environmental Science and Pollution Research* 24, 3117-3130
- Patidar K, Chouhan A, Thakur LS (2017): Removal of Heavy Metals from Water and Waste Water by Electrocoagulation Process—A Review. *Int. Res. J. Eng. Technol.(IRJET)* 4, 16-25
- Raiesi F, Beheshti A (2015): Microbiological indicators of soil quality and degradation following conversion of native forests to continuous croplands. *Ecological indicators* 50, 173-185
- Roy AS, Baruah R, Borah M, Singh AK, Boruah HPD, Saikia N, Deka M, Dutta N, Bora TC (2014): Bioremediation potential of native hydrocarbon degrading bacterial strains in crude oil contaminated soil under microcosm study. *International Biodeterioration & Biodegradation* 94, 79-89

- 1 Sajna KV, Sukumaran RK, Gottumukkala LD, Pandey A (2015): Crude oil biodegradation aided by
2 biosurfactants from *Pseudozyma* sp. NII 08165 or its culture broth. *Bioresource technology* 191, 133-
3 139
- 4 Sharma A, Kumar V, Thukral AK, Bhardwaj RJJoPP (2016): Epibrassinolide-imidacloprid interaction
5 enhances non-enzymatic antioxidants in *Brassica juncea* L. *Environmental Science and Pollution*
6 *Research* 21, 70-75
- 7 Sharma A, Kumar V, Kanwar M, Thukral A, Bhardwaj RJJoPP (2017a): Ameliorating imidacloprid induced
8 oxidative stress by 24-epibrassinolide in *Brassica juncea* L. *Environmental Science and Pollution*
9 *Research* 64, 509-517
- 10 Sharma A, Thakur S, Kumar V, Kesavan AK, Thukral AK, Bhardwaj RJBpb (2017b): 24-epibrassinolide
11 stimulates imidacloprid detoxification by modulating the gene expression of *Brassica juncea* L. *BMC*
12 *Plant Biology* 17, 56
- 13 Sharma A, Kumar V, Yuan H, Kanwar MK, Bhardwaj R, Thukral AK, Zheng BJFiPS (2018): Jasmonic acid
14 seed treatment stimulates insecticide detoxification in *Brassica juncea* L. *Frontiers in Plant Science* 9,
15 1-17
- 16 Sharma P, Jha AB, Dubey RS, Pessarakli M (2012): Reactive oxygen species, oxidative damage, and
17 antioxidative defense mechanism in plants under stressful conditions. *Journal of botany* 2012
- 18 Tabatabai M, Bremner JJSb, biochemistry (1969): Use of p-nitrophenyl phosphate for assay of soil phosphatase
19 activity. 1, 301-307
- 20 Tabatabai M, Bremner JJSB, biochemistry (1972): Assay of urease activity in soils. 4, 479-487
- 21 Tang J, Wang R, Niu X, Zhou Q (2010): Enhancement of soil petroleum remediation by using a combination
22 of ryegrass (*Lolium perenne*) and different microorganisms. *Soil and Tillage Research* 110, 87-93
- 23 Tauqeer HM, Ali S, Rizwan M, Ali Q, Saeed R, Iftikhar U, Ahmad R, Farid M, Abbasi GH (2016):
24 Phytoremediation of heavy metals by *Alternanthera bettzickiana*: growth and physiological response.
25 *Ecotoxicology and environmental safety* 126, 138-146
- 26 Thomson B, Robson A, Abbott L (1986): Effects of phosphorus on the formation of mycorrhizas by *Gigaspora*
27 *calospora* and *Glomus fasciculatum* in relation to root carbohydrates. *New phytologist* 103, 751-765
- 28 Tripathy S, Bhattacharyya P, Mohapatra R, Som A, Chowdhury DJEe (2014): Influence of different fractions
29 of heavy metals on microbial ecophysiological indicators and enzyme activities in century old municipal
30 solid waste amended soil. 70, 25-34
- 31 Varjani SJ, Upasani VN (2017): A new look on factors affecting microbial degradation of petroleum
32 hydrocarbon pollutants. *International Biodeterioration & Biodegradation* 120, 71-83
- 33 Velikova V, Yordanov I, Edreva AJP (2000): Oxidative stress and some antioxidant systems in acid rain-treated
34 bean plants: protective role of exogenous polyamines. 151, 59-66
- 35 Vig K, Megharaj M, Sethunathan N, Naidu RJAiER (2003): Bioavailability and toxicity of cadmium to
36 microorganisms and their activities in soil: a review. 8, 121-135
- 37 Wang J, Zhang Z, Su Y, He W, He F, Song H (2008): Phytoremediation of petroleum polluted soil. *Petroleum*
38 *Science* 5, 167-171
- 39 Wang W, Freemark K (1995): The use of plants for environmental monitoring and assessment. *Ecotoxicology*
40 *and environmental safety* 30, 289-301
- 41 Wyszowska J, Wyszowski M (2010): Activity of soil dehydrogenases, urease, and acid and alkaline
42 phosphatases in soil polluted with petroleum. *Journal of Toxicology and Environmental Health, Part A*
43 73, 1202-1210
- 44 Wyszowska J, Wyszowski MJJoT, Environmental Health PA (2010): Activity of soil dehydrogenases, urease,
45 and acid and alkaline phosphatases in soil polluted with petroleum. 73, 1202-1210
- 46 Xiao N, Liu R, Jin C, Dai Y (2015): Efficiency of five ornamental plant species in the phytoremediation of
47 polycyclic aromatic hydrocarbon (PAH)-contaminated soil. *Ecological Engineering* 75, 384-391
- 48 Xie W, Li R, Li X, Liu P, Yang H, Wu T, Zhang Y (2018): Different responses to soil petroleum contamination
49 in monocultured and mixed plant systems. *Ecotoxicology and environmental safety* 161, 763-768
- 50 Xun W, Huang T, Zhao J, Ran W, Wang B, Shen Q, Zhang R (2015): Environmental conditions rather than
51 microbial inoculum composition determine the bacterial composition, microbial biomass and enzymatic
52 activity of reconstructed soil microbial communities. *Soil Biology and Biochemistry* 90, 10-18
- 53 Yang Y, Han X, Liang Y, Ghosh A, Chen J, Tang MJPO (2015): The combined effects of arbuscular mycorrhizal
54 fungi (AMF) and lead (Pb) stress on Pb accumulation, plant growth parameters, photosynthesis, and
55 antioxidant enzymes in *Robinia pseudoacacia* L. 10, e0145726

- 1 Zamani J, Hajabbasi MA, Alaie E, Sepehri M, Leuchtmann A, Schulin R (2016): The effect of Piriformospora
2 indica on the root development of maize (*Zea mays* L.) and remediation of petroleum contaminated soil.
3 International journal of phytoremediation 18, 278-287.
- 4 Zarafshar Me, Bazot S, Matinizadeh M, Bordbar S K, Rousta M J, Kooch Y, Enayati K, Abbasi A,
5 Negahdarsaber M (2020): Do tree plantations or cultivated fields have the same ability to maintain soil
6 quality as natural forests?. Applied soil ecology. 151, 103536.
- 7 Zhang F-Q, Wang Y-S, Lou Z-P, Dong J-D (2007): Effect of heavy metal stress on antioxidative enzymes and
8 lipid peroxidation in leaves and roots of two mangrove plant seedlings (*Kandelia candel* and *Bruguiera*
9 *gymnorhiza*). Chemosphere 67, 44-50
- 10 Zhang Z, Qu W (1990): Experiment guide of plant physiology. High Education Press Beijin, China china.
11 Chinese
12