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Petroleum hydrocarbon rhizoremediation and soil microbial activity improvement via cluster root formation by wild Proteaceae plant species

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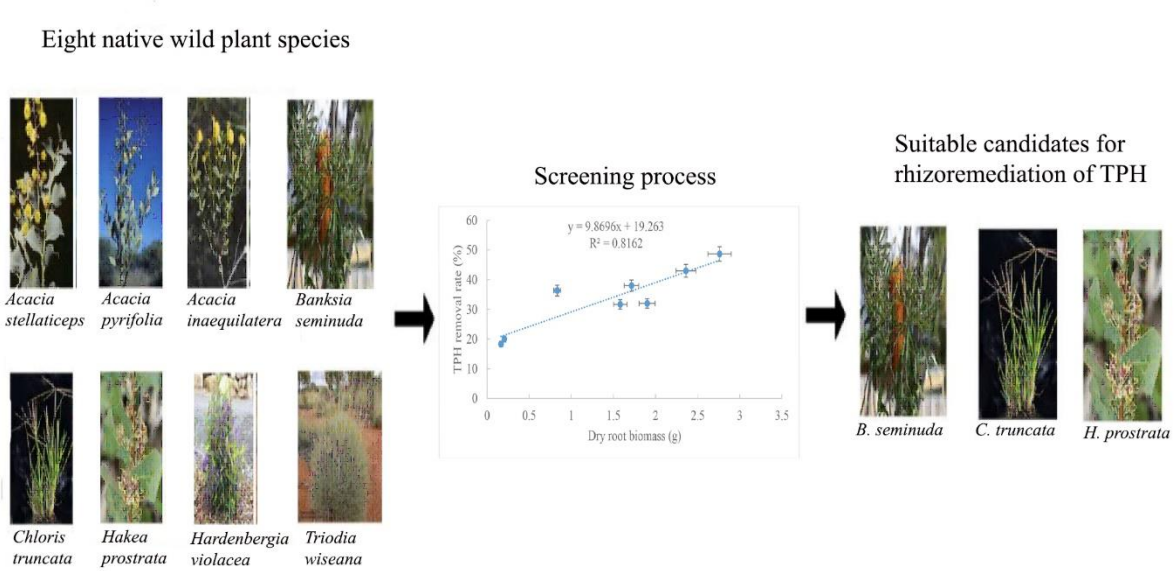
Highlights

- Wild plant species varied in tolerance to total (aliphatic) petroleum hydrocarbon (TPH)
- Rhizosphere microbial activity was strongly associated with plant growth status
- Poaceae and Proteaceae plants exhibited high TPH rhizoremediation potential
- The TPH removal rates were associated with root biomass production
- Overall microbial abundance was not significantly correlated with TPH removal rates

GRAPHICAL ABSTRACT



Abstract

Rhizoremediation potential of different wild plant species for total (aliphatic) petroleum hydrocarbon (TPH)-contaminated soils was investigated. Three-week-old seedlings of *Acacia inaequilatera*, *Acacia pyrifolia*, *Acacia stellaticeps*, *Banksia seminuda*, *Chloris truncata*, *Hakea prostrata*, *Hardenbergia violacea*, and *Triodia wiseana* were transplanted in a soil contaminated with diesel and engine oil as TPH at pollution levels of 4,370 (TPH1) and 7,500 (TPH2) mg kg^{-1}, and an uncontaminated control (TPH0). After 150 days, the presence of TPH negatively affected the plant growth, but the growth inhibition effect varied between the plant species. Plant growth and associated root biomass influenced the activity of rhizo-microbiome. The presence of *B. seminuda*, *C. truncata*, and *H. prostrata* significantly increased the TPH removal rate (up to 30% compared to the unplanted treatment) due to the stimulation of rhizosphere microorganisms. No significant difference was observed between TPH1 and TPH2 regarding the plant tolerance and rhizoremediation potentials of the three plant species. The presence of TPH stimulated cluster root formation in *B. seminuda* and *H. prostrata* which was associated with enhanced TPH remediation of these two members of Proteaceae family. These results indicated that *B. seminuda*, *C. truncata*, and *H. prostrata* wild plant species could be suitable candidates for the rhizoremediation of TPH-contaminated soil.

***Key words**:* Petroleum hydrocarbon remediation; Soil dehydrogenase activity; Proteaceae plants; Rhizosphere; Soil respiration; Wild plant species

Abbreviations:

NEPC	National Environment Protection Council
TPH	Total Petroleum Hydrocarbon
FeEDTA	Iron-ethylenediaminetetraacetic acid
DHA	Dehydrogenase Activity
TTC	2,3,5-Triphenyltetrazolium Chloride
TPF	Triphenyl Formazan
ASE	Accelerated Solvent Extractor
GC-FID	Gas Chromatography fitted with Flame Ionization Detector
ANOVA	Analysis of Variance

1. Introduction

Soils contaminated with hydrocarbons have been of great concern worldwide due to the ever-growing usage of petroleum fossil fuels. In Australia alone, nearly 80,000 sites were reported to be seriously contaminated by petroleum hydrocarbons, according to the National Environment Protection Council (NEPC 1999). Total petroleum hydrocarbon (TPH) is the measurable amount of any mixture of hydrocarbons that have originated from crude oil. There are several hundred of these compounds which are made up of two types: volatile petroleum hydrocarbons and extractable petroleum hydrocarbons. With respect to chemical structure, TPH can be classified as either aliphatic or aromatic hydrocarbon compounds (Kuppusamy et al. 2020). Although aliphatic petroleum hydrocarbons are significant pollutants in the soil environment, this class of chemicals have been receiving much less research attention compared to the aromatic counterparts, which only equates to less than 5% of TPH by volume (Stroud et al. 2007). Similar to aromatic hydrocarbons, the toxicity and persistence of aliphatic compounds in the environment adversely affect not only the human health but also ecosystems (Abbasian et al. 2015). Consequently, it is necessary to develop cost-effective and eco-friendly remediation technologies for the sustainable management of aliphatic hydrocarbon-contaminated soils.

Among various bioremediation approaches, rhizoremediation (i.e., the use of plants and their associated microorganisms in the root zone) is an emerging method, and especially effective for degrading organic contaminants, offering both ecological and engineering advantages (Ahkami et al. 2017; Gupta et al. 2020; Hoang et al. 2020). The rhizoremediation method examined in this research work could be applicable to remove degradable organic contaminants such as aliphatic hydrocarbons and aromatic hydrocarbons from contaminated soils (Gaskin and Bentham 2010; Hoang et al. 2020; Sivaram et al. 2018). The proposed method is suitable for the remediation of especially derelict field sites contaminated with

aliphatic hydrocarbons within plant tolerance levels. In contrast to conventional methods (e.g., excavation, incineration), rhizoremediation is non-intrusive and protects soils from any functional and structural damage while reducing the mass flux of the contaminants to receptors (Cundy et al. 2016). Plants produce varying quantity and quality of root exudates which may fuel an initial substrate-driven microbial community establishment followed by a shift in rhizosphere dynamics (chemistry and biology) (Bulgarelli et al. 2013). As a result, even closely related plant genotypes may harbour notably diverse microbial populations in the rhizosphere, which are able to degrade contaminants with different rates (Dagher et al. 2019). Owing to the cooperative evolution and adaptation of microorganisms in the soil-plant-microbe systems, the use of native plants is generally preferred over introduced plant species for hydrocarbon biodegradation (Hatami et al. 2019). Native plants offer better interactions with local beneficial soil microbiota than invasive plant species, enabling efficient rhizoremediation of contaminants (Bolan et al. 2013; Chowdhury et al. 2017). Studies on the use of wild plant species for rhizoremediation of TPH is limited, and mostly focused on grass species belonging to the Poaceae family (Gaskin and Bentham 2010; Kiamarsi et al. 2020; Steliga and Kluk 2020). However, shallow root systems of grasses cannot reach deep soil layers, and hence become ineffective to remediate contaminants residing at a depth (Cook and Hesterberg 2013). Plants outside grasses (Poaceae family) also showed rhizoremediation potential, such as shrubs and trees (e.g., *Chromolaena odorata*, *Haematosylum campechianum*) (Anyasi and Atagana 2018; Pérez-Hernández et al. 2017), legumes (e.g., *Cajanus cajan*, *Lotus corniculatus*) (Allamin et al. 2020; Hussain et al. 2019), ornamental plants (e.g., *Hylotelephium spectabile*) (Cheng et al. 2019), and even agronomic crops (e.g., *Zea mays*, *Vicia faba*) (Baoune et al. 2019; Ghalamboran et al. 2020). Hence, it is important to test the suitability of non-Poaceae plant species for TPH biodegradation under different contamination scenarios and biomes. In this context, testing the tolerance of wild

plant species to various levels of hydrocarbon contaminations, and the plants' degree of stimulation of the rhizosphere microbial activity are a need of the hour for achieving successful rhizoremediation of TPH contaminants. Because wild plants have developed synergistic relationships with other plants, soil microorganisms and the local environment, the proposed rhizoremediation process can be considered as an *in-situ* 'rhizo-engineering' technique which do not require intensive and intrusive engineering techniques involving excavation and *off-site* treatments such as incineration and soil washing. These special adaptations of wild plants have evolved over long periods of time and they allow wild plants to thrive under extreme conditions (e.g., low nutrient concentration, low soil moisture) (Bolan et al. 2011). Consequently, wild plants are easier to cultivate and manage, and therefore they have been utilized effectively for rhizoremediation and phytostabilization of contaminants including TPH in different studies (Abbaspour et al. 2020; Bolan et al. 2011; Cheng et al. 2017).

This study aims to evaluate eight wild plant species for the purpose of rhizoremediation of TPH, with a particular focus on aliphatic hydrocarbon contaminants. For the first time, members belong to Proteaceae family (*B.seminuda* and *H.* prostrata), which can develop cluster roots as an underground adaption for tolerance in extreme environments, were investigated for their potential for the rhizoremediation of TPH. The specific objectives are to determine the plant height and dry biomass parameters, soil respiration, soil dehydrogenase activity, and TPH removal rate under different plant and TPH concentration treatments. The provision of suitable wild plant species would offer an economically feasible and environmentally sustainable option for the remediation of TPH-contaminated sites.

2. Materials and methods

2.1. Experimental design

A factorial randomized block design with five replications was adopted to investigate the effect of TPH-contaminated soils (two pollution levels of 4,370 and 7,500 mg kg^{-1} denoted as TPH1 and TPH2, respectively, and an uncontaminated control) on the growth of eight wild plant species from three families, namely Fabaceae, Poaceae and Proteaceae (Supplementary Information; SI Table 1). These plants were selected due to their habitation of different climates and soil conditions, diverse taxa, and varied root morphologies. Furthermore, some of these plant species have been found to be effective in the phytoremediation of organic contaminants (Khan et al. 2018). The experiment also investigated the plants' potential for enhanced removal of TPH in contaminated soils. The plant growth experiment was conducted under controlled glasshouse conditions (25^{o}C for 16 h (daytime), and 18^{o}C for 8 h (night), typical of the New South Wales State of Australia). The glasshouse received natural lights, and plants were irrigated with 50 mL of deionized water on alternate days over 150 days of the experiment. No external nutrients were applied to the soil during the plant growth experiment to mimic uncropped Australian soils and considering the nature of the native wild plants tested.

2.2. Preparation of TPH-contaminated soils

An uncontaminated soil (Red Kandosol; Australian Soil Classification) without any hydrocarbon contamination history was collected from an area in Cobar, New South Wales (-31.502085, 145.781435). The soil was transported to the glasshouse, air-dried, sieved through a 4-mm screen (mesh No. 5), and identified as a clay loam soil in texture (with 35, 37, and 28% of clay, silt, and sand, respectively). Selected physical and chemical properties of the soil determined following standard methods (Burt 2004) are shown in SI Table 2. Pro Quip Platinum Diesel and Castrol GTX Engine Oil 20W-50 were obtained commercially at a local service station in Newcastle, New South Wales. The sieved soil was artificially contaminated

166 with the mixture of diesel and engine oil (1:1, w/w) at target concentrations of 0.5% (5,000
167 mg kg^{-1}) and 1% (10,000 mg kg^{-1}), which resulted in initial pollutant levels named TPH1 and
168 TPH2, respectively, as mentioned above. The mixture was chosen to represent aliphatic
169 hydrocarbons covering a wide range of carbon chains, likely to show different degrees of
170 biodegradation. In addition, by artificially contaminating a 'clean' site soil with known TPH
171 concentrations, a greater experimental control was ensured in terms of the ability to evaluate
172 growth and TPH remediation at a range of TPH levels for individual plant species. Therefore,
173 data could be compared with growth performance in the uncontaminated controls and
174 literature data.
175 The 0.5% contamination level was chosen representing the highest level in the majority of
176 hydrocarbon-contaminated mine sites worldwide (Gaskin et al. 2008; Sun et al. 2004). The
177 1% pollution level was chosen to increase the hydrocarbon-derived stress on plants but
178 without causing any serious damage (Sun et al. 2004). To achieve homogeneity in the TPH-
179 soil mixtures, the soil with each TPH concentration was mixed thoroughly using a dual
180 tumbler bin (15 kg soil at a time) for 30 min, then using a concrete pan mixer for 15 min, and
181 left for 14 days before starting the plant growth experiment. After mixing, three sub-samples
182 from the top (0 – 10 cm), middle (10 – 20 cm), and bottom (> 20 cm) layers of the soil at
183 each pollution level (totally nine samples) were analysed for initial TPH concentrations to
184 ensure the contamination homogeneity (SI Table 2).
185
186

187

188 *2.3. Seedling emergence in Petri dishes and plant growth in micro-pots*

189 Seeds of the selected wild plant species were commercially obtained from Nindethana Seed
190 Service Pty, Ltd, (Western Australia, Australia). Seeds were soaked in 1% sodium
191 hypochlorite for 5 min and rinsed thoroughly to eliminate any fungal pathogen. Preliminary
192 tests (data not shown) showed that germination of all seeds was compromised at the TPH1
193 pollution level. Therefore, a standard germination test was applied for all plant seeds (Groves
194 et al. 1982), with the exception of the species belonging to Poaceae (i.e., *C. truncata* and *T.*
195 *wiseana*). To 120 x 120 x 17 mm (square) polystyrene Petri dishes, two layers of filter papers
196 were placed, and evenly soaked with 3 mL of ultrapure water (Milli-Q®, 18.2 MΩ.cm). To
197 each dish, 25 seeds of each selected plants (*A. inaequilatera*, *A. pyrifolia*, *A. stellaticeps*, *B.*
198 *seminuda*, *H. prostrata*, and *H. violacea*) were added in triplicate maintaining 5 x 5 grid
199 spaces, and sealed with Parafilm (Lamb et al. 2012). Seeds of *A. pyrifolia* were boiled at
200 100^{o}C for 1 min to break dormancy and allowed to cool for 15 min at room temperature
201 before adding to the Petri dishes.

202 All the plant species were germinated at 25^{o}C and monitored regularly for seven days. Once
203 radicles emerged to a certain length (approx. 5 to 10 mm), the germinated seeds were placed
204 in micro-plant pots (one seedling per pot) filled with autoclaved propagation sand (obtained
205 from Bunnings, Australia). For *C. truncata* and *T. wiseana*, ten seeds were directly sown in
206 each micro-plant pot (twenty replicated pots, 200 seeds in total) to obtain good "seed to soil"
207 contact, which is considered as the key to grass seed germination (Chauhan et al. 2018b).
208 Nutrient solution (20 mL) was added to every pot daily. The nutrient solution was prepared
209 according to Asher and Loneragan (1967), with a modified phosphorus concentration.
210 Particularly, the following concentrations of nutrients were achieved (in µM) in the solution:
211 calcium 250, magnesium 100, potassium 250, sulfur 100, nitrogen (as NO_3^-) 750, nitrogen (as

212 NH_4^+) 100, phosphorus 1, chlorine 100, iron 2 (as Iron Ethylenediaminetetraacetic acid,
213 FeEDTA), copper 0.1, manganese 1, zinc 0.5, cobalt 0.04, boron 3, and molybdenum 0.02.
214 This nutrient solution was successfully tested for growing native plant species previously
215 (Lamb et al. 2010).
216
217 *2.4. Plant growth test in TPH-contaminated soils*
218 For each pollution level, 1 kg of soil was filled in each plastic grow pot (13.3 cm in diameter
219 and 14 cm tall) which was previously lined with 0.1 mm textile fabric to promote drainage
220 and to avoid soil loss. A saucer was placed under each pot to collect the leachate, which was
221 reused for irrigation. For each plant species, when the seedlings grew approx. 5 to 10 cm
222 height in the micro-pots, plants with similar height were transplanted to the TPH
223 contaminated (TPH1 and TPH2) and control (TPH0) soils. Unplanted control pots received
224 identical treatments as the planted pots. After 2 weeks, all pots with *C. truncata* and *T.*
225 *wiseana* were uniformly thinned out to one individual plant per pot to ensure homogeneity
226 amongst all plant species.
227 One day prior to harvesting the plants (day 150), shoot height (length between shoot base and
228 apical tip) was measured. After harvesting, shoot and root biomasses were gently washed
229 with deionized water to remove soil particles. Root and shoot biomasses (g dry weight) were
230 determined for each pot after drying at 70^{o}C for 48 h. In addition to plant height and dry
231 biomass, relative growth performance (R) of TPH-contaminated soil (TPH1 and TPH2) as a
232 percentage of growth of uncontaminated control soil (TPH0) within each plant species were
233 calculated. The R values were used as an indicator of plant tolerance to TPH contamination
234 (Kulakow et al. 2000). Plants whose R values were less than 25%, between 25 and 50%, and
235 higher than 50% were respectively considered as strongly susceptible, moderately tolerant,
236 and considerably tolerant plants (Sun et al. 2004). Furthermore, the relative growth of root to

whole plant of all pots were calculated by root: shoot ratios (i.e., the dry mass of roots divided by the dry mass of shoots) for the estimation of the extent of root/soil contact, which might play an important role in the degradation of TPH in the rhizosphere (Shahsavari et al. 2013).

Soils adhered to plant roots were collected by gentle hand tapping (Rovira 1974). For TPH measurement, all soil samples were stored in a freezer (- 20^{o}C) until extraction. For soil dehydrogenase activity determination, subsamples were stored at 4^{o}C until analysis. Unplanted control soils were processed and stored the same way as the treatment samples.

2.5. Soil respiration

Soil respiration (CO_2 flux) was measured in the glasshouse by an automated soil respirometer (Li-8100A, LICOR, Lincoln, NE, USA) within 30 min after harvesting the plant shoots from pots (with the roots still remaining in the soil) (Zainul et al. 2017). Measurements of CO_2 fluxes (μmol CO_2 m^{-2} s^{-1}) were made with a cylindrical metal column (20 cm in diameter and 20 cm tall) which was sealed at the bottom. The collar was perfectly fitted with a 20 cm survey chamber (8100-103, LICOR) used in combination with the respirometer. Pots were placed inside the metal column during soil respiration measurement. The offset (height between the soil surface and column top) and soil area of each pot was measured, and entered into the Li-8100A software for calculation of the system volume, which was used for calculating the carbon flux (Zainul et al. 2017). In addition, a soil moisture probe (8150-204, LICOR) fitted with the respirometer was used for soil moisture measurement to ensure no significant difference of the soil moisture content between different treatments.

The SoilFluxPro^{TM} software from LICOR was used to compute data files for chamber measurements. This software used the ideal gas law (Eq. 1) and linear regression ($R^2 > 0.99$) to calculate soil CO_2 fluxes.

$$PV = nRT \quad (Eq.\ 1)$$

where, p is the pressure of the gas (N m^{-2}), V is the volume of the gas (m^3), n is the number of moles of gas, R is the ideal gas constant (8.3145 J mol^{-1} K^{-1}), and T is the absolute temperature (K).

2.6. Soil dehydrogenase activity (DHA)

Soil DHA for rhizosphere samples was determined according to the method described by Casida Jr et al. (1964), where 2,3,5-triphenyltetrazolium chloride (TTC) was reduced to tri-phenyl formazan (TPF). Field moist soil (3 g) was added to a 50 mL centrifuge tube, along with 0.5 mL of 3% aqueous TTC solution, 30 mg $CaCO_3$, and 1.25 mL of ultrapure water (Milli-Q®, 18.2 MΩ.cm), successively. The samples were incubated for 24 h at 37ºC. After incubation, 10 mL methanol was added to the sample, and thoroughly mixed using a vortex mixer. The content was then centrifuged at 4000 rpm for 15 min. After filtering through a syringe filter (0.2 µm), the absorbance of the clear supernatant was measured at 485 nm wavelength by a spectrophotometer (Microplate Reader; Ensight ™, Multimode, Perkin Elmer, USA). The result was presented as µg TPF per g^{-1} dry soil day^{-1}. Assays without any soil but $CaCO_3$ and TTC served as the controls. Additionally, a set of the contaminated soil was sterilized using an autoclave, which received the same procedure to determine DHA value to evaluate the interference of TPH itself to the DHA measurement.

2.7. Sample preparation and extraction of TPH

Concentration of TPH in soil was analysed according to Richter (2000). For sample extraction, 3 g of moist soil was taken into a 50 mL glass beaker. Diatomaceous earth (1.0 g) was added to the beaker and mixed thoroughly. The mixture was placed into extraction cells through a screw-on funnel to ensure that the entire soil was removed from the beaker. Glass fibre filters were fitted in the outlet before loading cells to the extraction system.

All extractions were carried out using a Dionex ASE 350 Accelerated Solvent Extractor (Thermofisher, USA) in Dionex ASE 150/350 Stainless Steel cells. Extractions were performed at 175^{o}C and 1500 psi, with hexane: acetone (1:1, v/v). Heating and static time were 8 and 5 min, respectively. The flush volume was 75%, and purge time was 60 s, with 2 cycles. After extraction, the extracts were collected in clean 40 mL glass vials. The vials were placed into a Turbo Vap II evaporator until the solvent fully disappeared. Subsequently, 5 mL hexane was added to every vial, and 1 mL aliquot of sample was transferred into 2 mL auto-sampler vial for analysis by gas chromatography. In preliminary tests, the recoveries achieved were > 84% for the accelerated solvent extraction method on a model soil containing high clay content (50%, representing a hard case scenario for extraction) spiked with 5,000 and 10,000 mg TPH kg^{-1} soil.

Analytical determination of TPH in soil samples was performed by gas chromatography fitted with flame ionization detector, GC-FID (Model No. 7697A, Headspace Sampler 7697A, Agilent Technology, USA). The following conditions were applied for all analyses: capillary column (30 m x 0.25 mm, ID = 0.25 mm); H_2 carrier gas; FID at 330^{o}C; injector temperature 300^{o}C; oven temperature programmed from 40 to 300^{o}C at 12^{o}C min^{-1} after 2-min hold with a 15-min hold at the final temperature; column flow rate 1.5 mL min^{-1}. External calibration standards were prepared from Hydrocarbon Window Defining Standard stocks in chloroform (Novachem, Australia). The linear standard curves were prepared with six concentrations (1, 5, 10, 20, 60 and 100 µg mL^{-1}) for three common TPH fractions: C_{10} – C_{14}, C_{15} – C_{28}, and C_{29} – C_{36} in the aliphatic hydrocarbons (diesel and engine oil) used in this study. Varian Star^{TM} Version 4.5 software was used to integrate the total chromatogram areas of the three TPH fractions. Sample concentrations were measured using the standard curves, and TPH values were calculated as the sum of C_{10} – C_{36} and presented as mg TPH kg^{-1} dry soil. The GC-FID system was purged by injecting *n*-hexane (solvent blank) to ensure the system was free of

contamination. In addition, a soil-free sample was subjected to the same extraction procedure mentioned above to detect any potential interferences. Limits of detection for $C_{10} – C_{14}$, $C_{15} – C_{28}$, and $C_{29} – C_{36}$ were 35, 79, and 69 ng mL^{-1}, respectively, according to the calibration standard curve (Guideline 2005). In addition, accuracy was evaluated using the recovery for real soil samples spiked with 16.67 µg mL^{-1}, which was diluted from the stock standard solution of 500 µg mL^{-1}. The recovery rate was determined using the measured concentrations in the enriched samples and the added concentration. The average recovery was 89% (n = 3), which conformed satisfactory performance of the method, and the value was in accordance with ISO 16703 standard (Standardization 2004).

The TPH removal rates under different plant and soil treatments were calculated using Eq. 2 (Qi et al. 2015):

$$\text{TPH removal rate (\%)} = \frac{(\text{Initial soil TPH} - \text{Final soil TPH})}{\text{Initial soil TPH}} \times 100 \quad \text{(Eq. 2)}$$

2.8. Statistical analysis

All data were analysed for normality and homogeneity using SPSS Version 25 package for Windows. Analysis of variance (ANOVA) test was conducted at $\alpha = 0.05$ to determine significant difference between treatments for plant height, dry biomass, soil respiration, DHA, and TPH removal rate. Treatment means showing significance were separated with Tukey's test at 5% level of confidence.

3. Results

3.1. Effect of TPH on plant height and biomass

After 150 days, plant growth was evaluated by comparing plant heights and dry biomasses between TPH-untreated and TPH-treated plants within each plant species. Overall, the results from one-way ANOVA showed that there were significant differences ($p < 0.05$) in plant

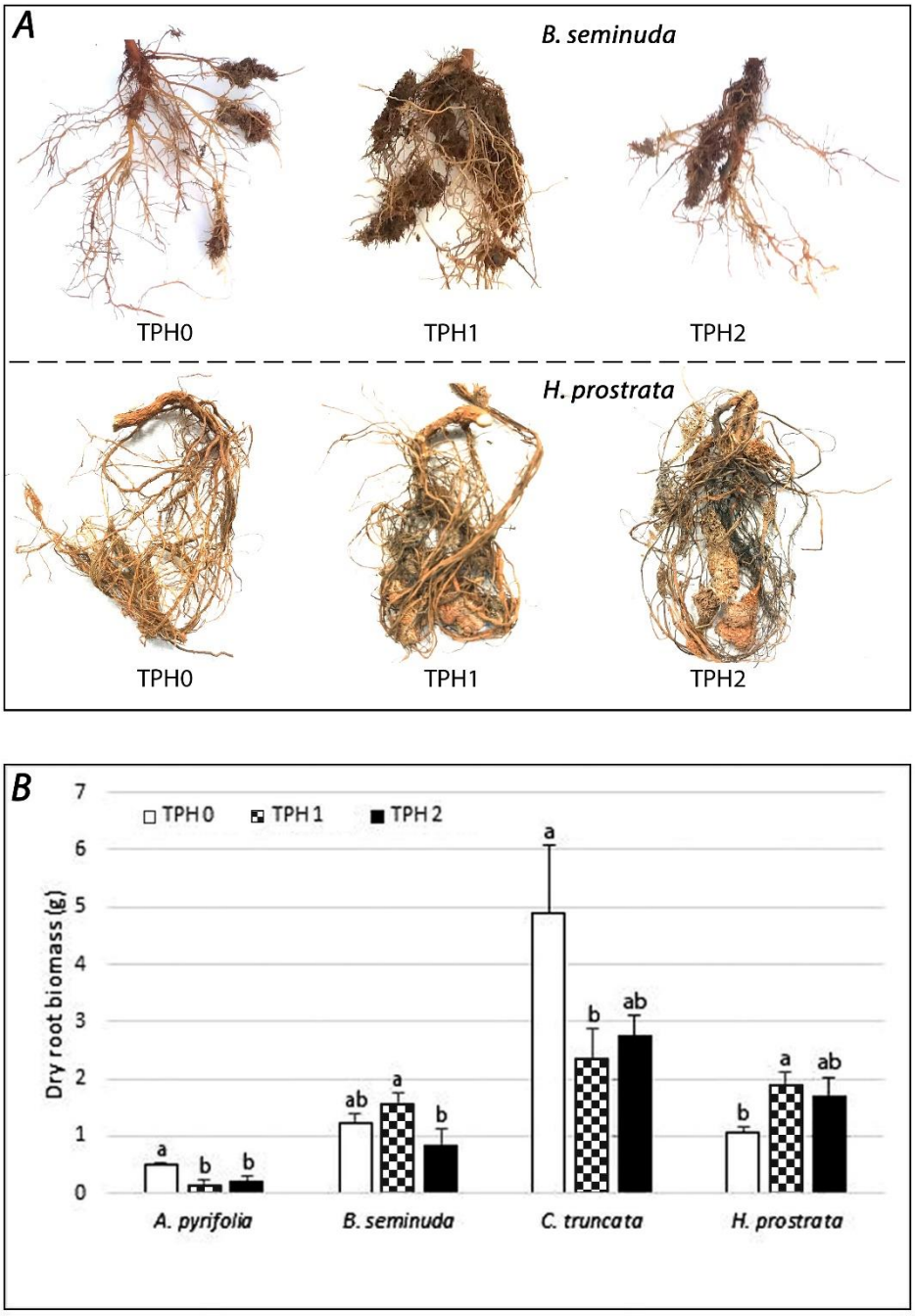
height and dry biomass between TPH0 and TPH1- or TPH2-treated plants within each plant species (Tables 1 & 2).

Table 1. Plant height (in cm) of eight Australian wild plant species in different pollution levels. Mean values ± standard deviation (n = 3). * Significantly different at $p = 0.05$ for each plant species. Numbers within parenthesis represent R values.

Plant species	**TPH concentration**		
	TPH0	TPH1	TPH2
A. inaequilatera	22.13 ± 1.90*	10.83 ± 1.31 (0.49)	10.2 ± 1.25 (0.46)
A. pyrifolia	22.87 ± 1.51*	8.63 ± 0.84 (0.38)	12.47 ± 0.97 (0.55)
A. stellaticeps	10.83 ± 0.17*	4.70 ± 0.14 (0.43)	4.57 ± 0.66 (0.42)
B. seminuda	14.90 ± 0.22	13.00 ± 0.57 (0.87)	12.36 ± 0.52 (0.83)
C. truncata	30.97 ± 3.20*	24.20 ± 1.98 (0.78)	21.87 ± 0.26 (0.71)
H. prostrata	29.13 ± 5.31*	14.90 ± 0.54 (0.51)	14.83 ± 0.47 (0.51)
H. violacea	20.37 ± 2.05*	3.83 ± 0.85 (0.19)	4.60 ± 0.37 (0.23)
T. wiseana	19.17 ± 2.05*	11.00 ± 0.71 (0.57)	10.77 ± 1.11 (0.56)

Seven of the tested plant species, i.e., *A. inaequilatera*, *A. pyrifolia*, *A. stellaticeps*, *C. truncata*, *H. prostrata*, *H. violacea*, and *T. wiseana* (except *B. seminuda*) were susceptible to TPH1 and TPH2 levels, although the extent varied among the plant species. For example, *A. inaequilatera*, *A. stellaticeps*, *H. violacea*, *T. wiseana* were strongly susceptible to TPH1 and TPH2 levels, according to their relative growth (Tables 1 & 2). In contrast, *B. seminuda* and *C. truncata* showed considerable tolerance to either of the contamination levels. Interestingly, despite significant reductions in plant height and biomass in the TPH-contaminated soils, all of the eight plant species were able to survive until the end of the experiment, and some

353 species produced considerably increased root biomass compared to those grown in the
354 uncontaminated soil (Figure 1).
355 Shoot length (plant height) values showed that the response varied among plant species and
356 TPH concentrations (Table 1). For almost all plant species, TPH-contaminated soils led to
357 considerable reductions in shoot lengths compared to the control soil ($p < 0.05$), although no
358 significant difference was observed between TPH1 and TPH2 pollution levels ($p > 0.05$). The
359 most significant growth inhibition was observed for *H. violacea* at TPH1 and TPH2
360 contamination levels (R values were 19 and 23% that of the control, respectively).
361 Conversely, shoot length of *B. seminuda* showed no significant difference between different
362 pollution levels ($p > 0.05$). This indicated that *B. seminuda* was the least affected plant by
363 TPH contamination with respect to shoot length development, regardless of TPH
364 concentrations.



365

366 **Figure 1.** Development of cluster roots in the members belong to Proteaceae family (A), and
367 dry root biomass of the four tolerant plant species grown in different TPH levels after 150
368 days (B). The error bars indicate the standard deviation of the means (n = 3). Different
369 lowercase letters indicate significant difference within the same plant species.

370

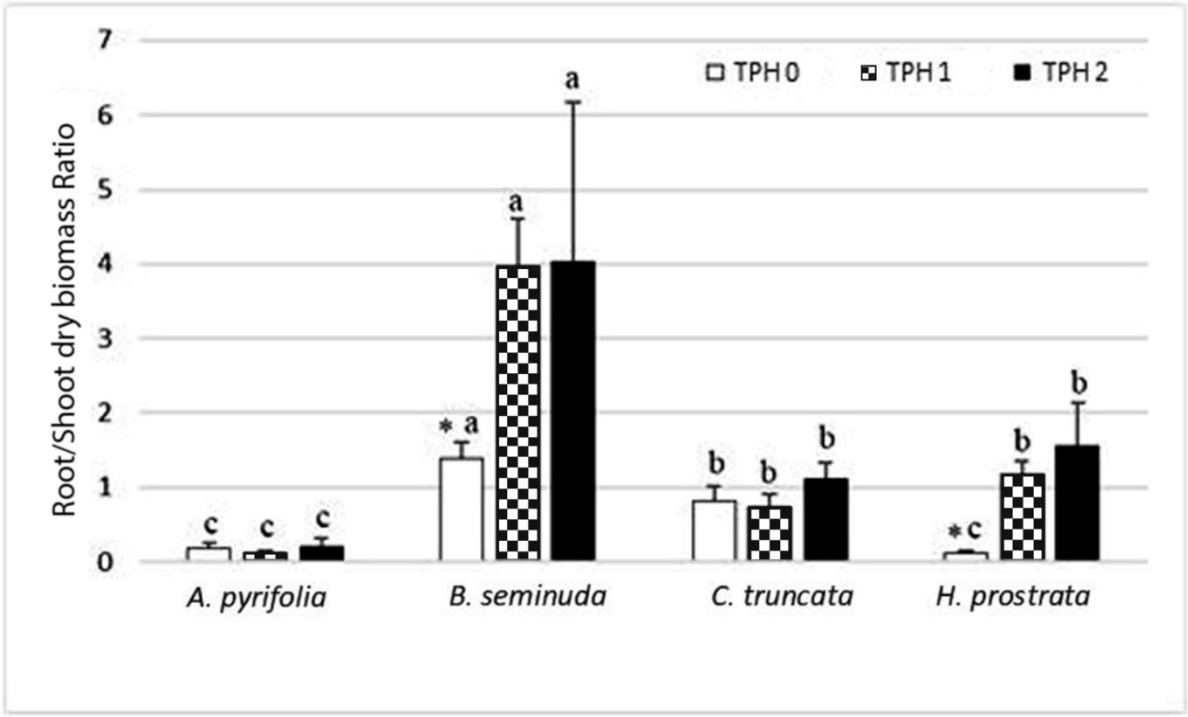
Similarly, within individual species, plant dry biomass from the contaminated soils was significantly different to that of the control in almost all plants tested. No significant difference was detected between TPH1- and TPH2-contaminated soils ($p > 0.05$) (Table 2). After 150 days of growth, dry biomass of the eight plant species decreased in the contaminated soils (both TPH1 and TPH2). Compared with the control, > 75% reduction in dry biomass was found in *A. inaequilatera*, *A. stellaticeps*, *H. violacea*, and *T. wiseana* (Table 2). Interestingly, adding diesel/oil to the soil at TPH1 level caused no significant decrease in the biomass of *B. seminuda* plant. Nevertheless, plant biomass at TPH2-contaminated soil was significantly lower than in the control soil for this plant species ($p < 0.01$).

Table 2. Plant dry biomass (in g) of eight Australian wild plant species in different pollution levels. Mean values ± standard deviation (n = 3). * Significantly different at $p = 0.05$ for each plant species. Numbers within parenthesis represent R values.

Plant species	**TPH concentration**		
	TPH0	TPH1	TPH2
A. inaequilatera	2.99 ± 0.38*	0.61 ± 0.30 (0.20)	0.64 ± 0.06 (0.21)
A. pyrifolia	2.80 ± 0.05*	1.36 ± 0.43 (0.49)	1.24 ± 0.38 (0.44)
A. stellaticeps	1.44 ± 0.04*	0.26 ± 0.04 (0.18)	0.20 ± 0.03 (0.14)
B. seminuda	2.17 ± 0.13	2.00 ± 0.28 (0.92)	1.09 ± 0.22 (0.50)*
C. truncata	10.87 ± 1.27*	5.56 ± 0.50 (0.51)	5.27 ± 0.20 (0.49)
H. prostrata	9.40 ± 1.52*	3.53 ± 0.29 (0.38)	2.90 ± 0.10 (0.31)
H. violacea	8.97 ± 1.28*	0.22 ± 0.01 (0.02)	0.22 ± 0.01 (0.02)
T. wiseana	5.39 ± 0.56*	1.04 ± 0.11 (0.19)	0.24 ± 0.06 (0.04)

Plant height and dry biomass were not compared among the different plant species because their initial heights and biomass as well as growth rates were different. With respect to plants' tolerance to TPH via R values in this study, the growth rates were significantly lower in plants grown in the contaminated soils than in the control soil for almost all species tested and varied among the plant species. These results indicated phytotoxicity of TPH at varying extents to the plant species (Tables 1 & 2). *A. inaequilatera*, *A. stellaticeps*, *H. violacea*, and *T. wiseana* were strongly susceptible to both TPH1 and TPH2 pollution levels (R values below 25%). Since these four plant species were strongly sensitive to both TPH contamination levels, they were not used for further experimentation.

For further assessment of plant growth in the TPH-contaminated soils, the root: shoot dry biomass ratio was determined. In this study, the root: shoot ratio varied significantly between the four tolerant plant species in the contaminated and control soils. Of these, *A. pyrifolia* and *C. truncata* showed no significant difference in root: shoot ratios under both levels of TPH and control soils. In contrast, considerable differences were detected among soil treatments in the case of *B. seminuda* and *H. prostrata*. The two Proteaceae species showed significantly increased root: shoot ratio ($p < 0.05$) in response to TPH contamination (Figure 2).



404

405 **Figure 2.** Root: Shoot ratio of the four tolerant plant species grown in different TPH levels
406 after 150 days. The error bars indicate the standard deviation of the means (n = 3).
407 *Significantly different within the same plant species at $p = 0.05$. Different lowercase letters
408 indicate significant difference between plant species at the same TPH level.

409

410 *3.2. Soil respiration*

411 Overall, soil respiration was higher in the planted compared to unplanted pots at all pollution
412 levels (Figure 3). For each plant species and unplanted control, no significant difference was
413 detected between TPH1 and TPH2-contaminated soils ($p > 0.05$). At the end of the
414 experiment, *A. pyrifolia* and *C. truncata* showed higher (nearly double) soil respiration
415 relative to those in *B. seminuda*, *H. prostrata*, and unplanted control treatments. Interestingly,
416 there was no significant difference among plant treatments and unplanted control at TPH1
417 and TPH2 pollution levels for *B. seminuda* and *H. prostrata* ($p > 0.05$). While the presence
418 of TPH tended to decrease soil repiration in all plant species, an opposite pattern was

observed in unplanted treatment, although no significant difference was observed in the latter case (Figure 3).

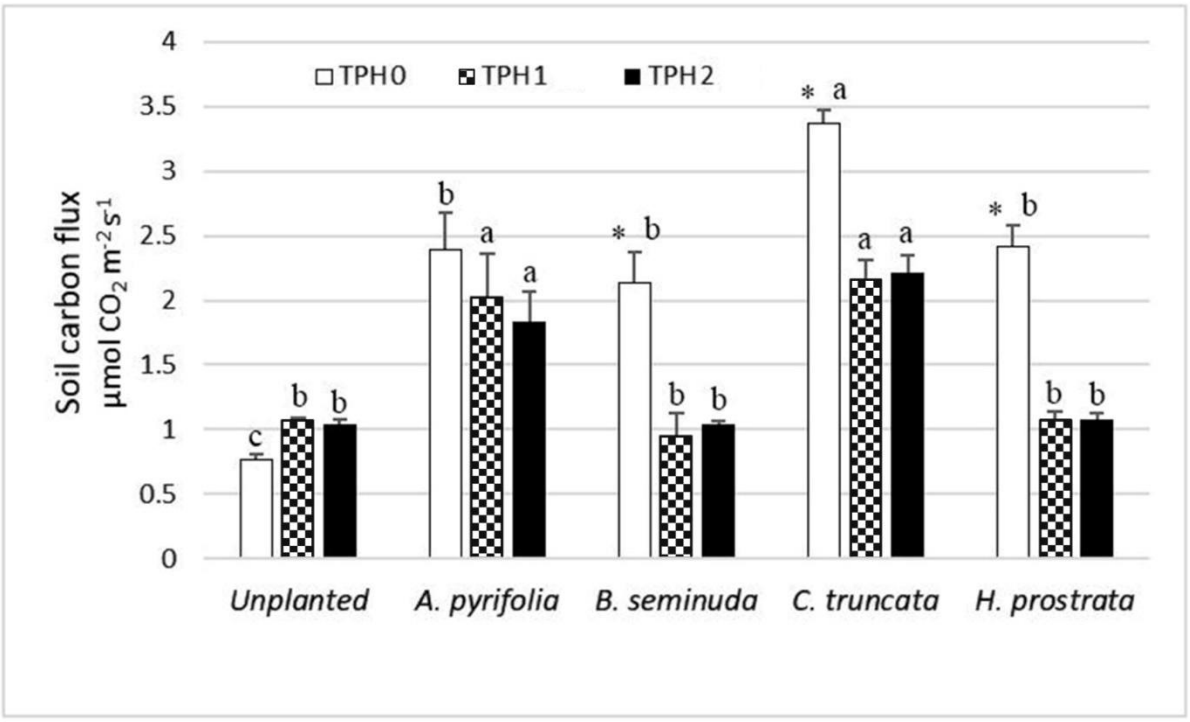


Figure 3. Soil carbon flux from unplanted pots and the four plants grown under different TPH concentrations. The error bars indicate the standard deviation of the means (n = 3). *Significantly different within the same plant species at $p = 0.05$. Different lowercase letters indicate significant difference between plant species at the same TPH level.

3.3. Soil DHA

Tests confirmed that TPH did not interfere with the colour development step of the DHA assay. Although the presence of plants stimulated DHA in all the soil treatments, both *B. seminuda* and *H. prostrata* showed no significant difference in DHA values at TPH1 and TPH2 pollution levels compared to those in the unplanted control ($p > 0.05$) after 150 days (Figure 4). This result suggests that although these species had a rhizosphere effect on the

microbial activity in the uncontaminated soil, the effect was not evident in the presence of TPH.

At both TPH pollution levels, DHA values in the rhizosphere of the four plant species and unplanted control after 150 days tended to be higher than those in the uncontaminated soil. However, at TPH2 pollution level, soil DHA in *C. truncata* decreased significantly compared to that in the uncontaminated soil ($p < 0.05$). This decline indicated that any TPH pollution level higher than the TPH1 level used in this study may be the critical level for the change in the activity of dehydrogenase enzyme for this plant species. The TPH1 pollution level enhanced soil DHA of *C. truncata*, while the TPH2 pollution level inhibited the DHA (Figure 4).

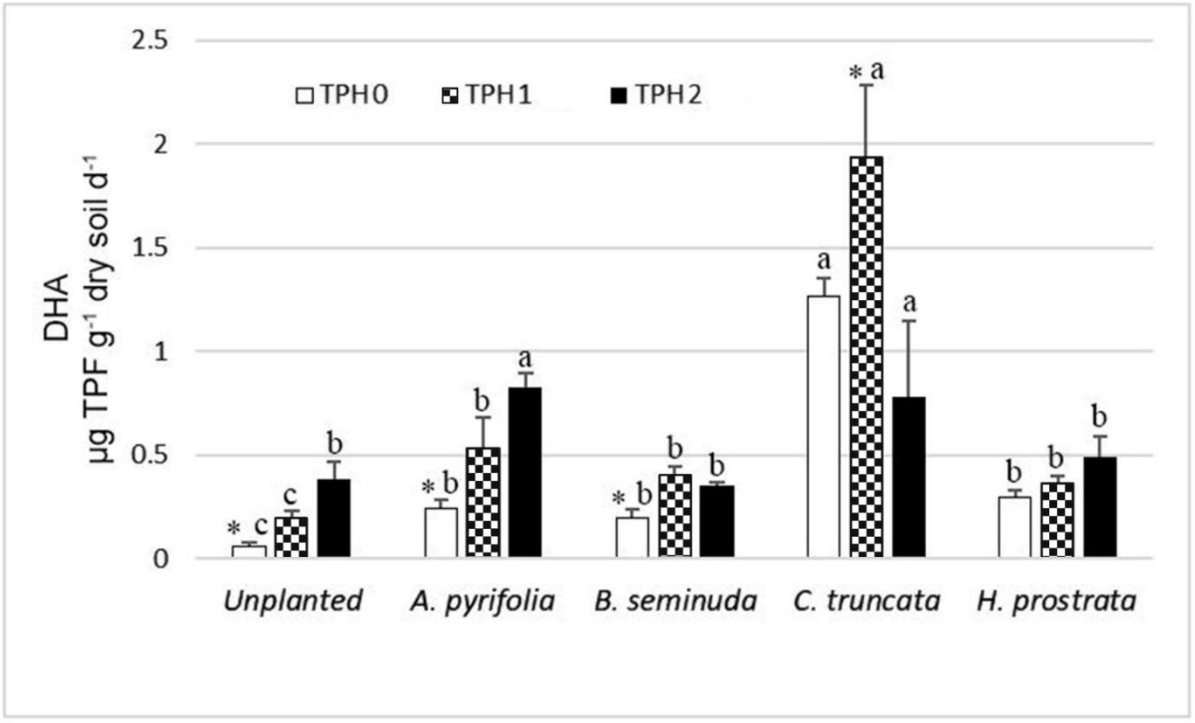


Figure 4. Effect of TPH contamination on soil DHA in the rhizosphere of tested plants and unplanted control. The error bars indicate the standard deviation of the means (n = 3). *Significantly different within the same plant species at $p = 0.05$. Different lowercase letters indicate significant difference between plant species at the same TPH level.

3.4. Hydrocarbon removal rate

Figure 5 shows that with the exception of *A. pyrifolia*, the removal rates (%) of the four tested tolerant plants at TPH1 and TPH2 contamination levels in the rhizosphere soils had higher values than those in the unplanted control treatment ($p < 0.05$), at the end of the experiment. A 10 to 30% enhancement in TPH removal rate in the planted soils was observed relative to the unplanted control. In addition, as the concentration level increased from TPH1 to TPH2, the removal rate did not change significantly for any plant species and unplanted treatment. At TPH1 and TPH2 pollution levels, only three plants (*B. seminuda*, *C. truncata*, and *H. prostrata*) showed significant removal rates relative to the unplanted control ($p < 0.05$). The highest removal rate was observed for *C. truncata*, around two times than that in the unplanted control. In contrast, no significant difference was detected in the presence of *A. pyrofolia* compared to unplanted control ($p > 0.05$). In the rhizosphere soil, *B. seminuda* and *H. prostrata* had the second highest removal rates (about 1.5 times that of the unplanted control), with no significant difference between the two plant species ($p > 0.05$).

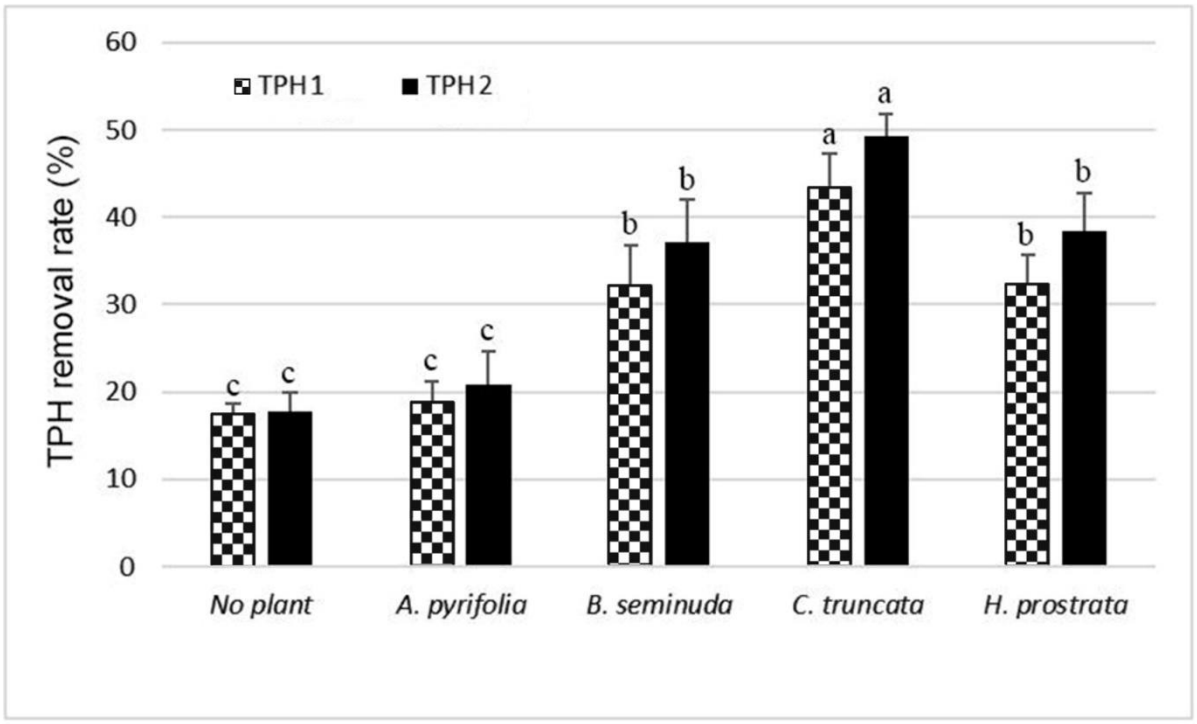


Figure 5. The removal rate of two TPH levels by the four native tolerant plant species and unplanted control. The error bars indicate the standard deviation of the means (n = 3). Different lowercase letters indicate significant difference between plant species at the same TPH level.

4. Discussion

4.1. Plant growth performance

It was reported the addition of 0.5% and 1% (w/w) of TPH to soil caused significant decrease in plant growth (Robson et al. 2003; Wei et al. 2019). These results were in agreement with our experimental data that the growth of all the plant species were inhibited in TPH1- and TPH2-treated soils. The inhibition of plant growth caused by TPH contamination could be explained by the fact that plant roots were in direct contact with the contaminants. On the one hand, low-molecular-weight (lighter, C_{10} - C_{19}) fractions of TPH could enter the plant body through roots causing direct toxic effects on the plant cells (Basumatary et al. 2012; Bell et al. 2014). On the other hand, high-molecular-weight (heavier, > C_{19}) fraction could hinder the

absorption of nutrients and water from the soil into the roots by forming a water-repellent film of oil covering the root surface, which in turn also would affect the respiration of roots (Jonker et al. 2006; Lin and Mendelssohn 2009). The TPH might also negatively affect soil properties such as water holding capacity and soil aeration, further hindering the plant growth (Chen et al. 2015; Mena et al. 2016). The freshly contaminated clay loam soil with low organic content coupled with low-molecular-weight aliphatic hydrocarbons in diesel used in the current study could be expected to allow considerable bioavailability and contribute to the observed outcomes of plant growth. In addition, high-molecular-weight aliphatic hydrocarbon compounds, mainly found in the engine oil, could have negative effects on soil properties, thereby impeding plant performance in the contaminated soil.

Increased root biomass could be an adaptive strategy to reduce phytotoxicity in plants (Qi et al. 2019; Shahsavari et al. 2013). In this study, the members belonging to Proteaceae family (i.e., *B. seminuda* and *H. prostrata*) increased root biomass in the TPH-treated soils by forming cluster roots. Exudates of cluster roots are characterized with relatively high proportion of readily available substrates for enhancing rhizosphere microorganism abundance and activities, which is advantageous in rhizoremediation of the TPH-contaminated soil (Martin et al. 2014; Shane et al. 2004).

Root: shoot ratio is an indicator of plant stress and it could allow comparison of different soil treatments within each plant species and also amongst various plants (Agathokleous et al. 2019; Husáková et al. 2018). Higher root: shoot ratio in *B. seminuda* and *H. prostrata* under TPH1 and TPH2 levels suggested that the plant species increased biomass allocation to roots in the TPH-contaminated soil. Plants with a higher proportion of roots were considered to perform better in rhizoremediation approach (Huang et al. 2005; Nie et al. 2010). Regarding root biomass as the lone determinant of rhizoremediation, *B. seminuda* and *H. prostrata* showed the potential to remediate soil up to TPH2 contamination level in this study.

507

508 *4.2. Soil respiration*

509 Soil respiration, which is related to plant root and microbial respiration, has been
510 recommended to be a quick and accurate assessment of metabolically active microbial
511 communities in contaminated soils, and it gives an idea of the quantity of easily mineralizable
512 substrates present in the soil (Gielnik et al. 2019; Kim et al. 2018; Wang et al. 2014).
513 Therefore, any change in soil respiration observed in the TPH-contaminated soils compared
514 to the corresponding uncontaminated soil could reflect the metabolic state of soil microbial
515 communities, and the abundance of metabolically active microbes in the soil (Salazar et al.
516 2019).

517 Increased soil respiration in the planted compared to the unplanted treatments in this study
518 indicated that all the four plant species enhanced their rhizosphere microbial activity, a
519 phenomenon termed as *the rhizosphere effect* (Barati et al. 2018). The positive effect of
520 plants on soil respiration with and without TPH addition was similar to that found by
521 Muratova et al. (2012). The authors reported an increased CO_2 flux in 1% TPH-contaminated
522 soil cultivated by *Lolium perenne* L. (ryegrass) compared to soil without plants after three
523 weeks of plant growth. Similarly, the soil respiration rate was significantly higher in barley-
524 cultivated soils than in uncultivated treatment at 0, 4, 6, and 8% TPH contamination rates,
525 indicating that plant roots stimulated microbial activity in the rhizosphere in all the pollution
526 levels (Barati et al. 2018). However, in the present study, the CO_2 fluxes were not observed to
527 increase at the end of the experiment for two plant species (i.e., *B. seminuda* and *H.*
528 *prostrata*) compared to unplanted controls at both TPH pollution levels (Figure 3). Since this
529 is the first time the two plant species have been assessed for rhizoremediation of TPH-
530 contaminated soils, the reason for this disparity is not fully understood. It could be explained
531 in part by the true extent of rhizosphere effect to the associated microrganisms in these plant

532 species, and also in terms of their exudation patterns (Gaskin and Bentham 2010). Further
533 investigations are therefore warranted to elucidate the role of root exudates of these two plant
534 species in influencing the rhizosphere microbiota.
535 Within each plant species, the presence of TPH at both the pollution levels showed a negative
536 effect on the CO_2 flux (Figure 3). Similar observation was reported in the literature. For
537 example, Khan et al. (2018) found that hydrocarbons significantly decreased the diversity and
538 abundance of rhizosphere microorganisms. The microbial activity decreased with the addition
539 of diesel in the rhizosphere of *C. truncata* and *Triticum aestivum* (wheat) because of
540 hydrocarbon toxicity (Khan et al. 2018). Additionally, any adverse effect on plant growth due
541 to TPH contamination might have an additional inhibitory effect on soil microorganisms in
542 the rhizosphere and plant root productivity (Dagher et al. 2019; Merkl et al. 2006; Saraeian et
543 al. 2018). Increased competition between roots and rhizosphere microorganisms for nutrients
544 such as nitrogen, phosphorus and potassium in such nutrient-poor soils, resulting from TPH
545 contamination, could also be a possibility (Arslan et al. 2014; Kuzyakov and Xu 2013). These
546 reasons altogether led to the decreased soil respiration in the contaminated than
547 uncontaminated soils in this study. Nevertheless, an increased CO_2 flux (although not
548 significant) in TPH1 and TPH2 pollution levels compared to uncontaminated soil in the
549 unplanted treatment indicated that soil microorganisms might not suffer from TPH toxicity
550 (Figure 3). Therefore, the reasons for the reduction of soil respiration in the TPH-
551 contaminated planted soils compared to the control soil could be linked to plant root-driven
552 decreases in the abundance of metabolically active rhizosphere microbes.
553
554 *4.3. Dehydrogenase activity*
555 Dehydrogenase activity (DHA) is a measure of total microbial activity in soil, and can
556 indicate the onset of biodegradation process (Kaimi et al. 2007; Maila and Cloete 2005). In

this study, increased DHA was observed in the presence of plants in comparison to the unplanted treatment. Similar observations were reported by Zamani et al. (2018) that DHA was higher in planted than in unplanted soils during TPH treatment. The exact role of plants in stimulating microbial activity might result from the release of root exudates which supply nutrient and carbon sources for soil microorganisms (Herz et al. 2018). Furthermore, the physical effect of roots in improving soil aeration and harbouring microorganisms through the soil, is also a possibility (Jacoby et al. 2017). However, that trend was not observed in the presence of *B. seminuda* and *H. prostrata* compared to the unplanted treatment at both TPH pollution levels (Figure 4). The reason for this disparity might be related to root exudate characteristics of these plant species as the rhizosphere microbial community is greatly affected by root exudates that depend on plant species (Dagher et al. 2019; Gaskin and Bentham 2010). In this study, the two Proteaceae species (i.e., *B. seminuda* and *H. prostrata*) formed cluster roots in TPH-contaminated soils regardless of TPH concentrations, but not in the control soil (Figure 1). The rhizosphere of cluster roots is often characterised by high concentrations of readily available carbon sources (mainly low molecular weight organic anions and phenolics) that mobilize soil nutrients (de Britto Costa et al. 2016). Additionally, acid phosphatase enzyme is exuded at high rates in the rhizosphere of cluster roots (Lambers et al. 2018; Shane et al. 2004). The enzyme is hypothesised to not only enhance phosphorus supply to the plants but also provide an easily degradable energy source for rhizosphere microorganisms, and increase the bioavailability of TPH (Martin et al. 2014). Consequently, the development of strong nutrition depletion zones around the roots of these species caused the reduced soil DHA at the end of the experiment (Kaimi et al. 2006; Luo et al. 2015). Dehydrogenase is an enzyme that occurs in all viable microbial cells (Järvan et al. 2014). In the current study, DHA was not affected by the presence of TPH in a similar way to soil respiration. This is possibly because, a part of the microflora (i.e., plant root–associated

582 microorganisms) was in a dormant state in the rhizosphere due to the plant root-derived
583 growth-substrate deficiency in the TPH-contaminated soil (Blagodatskaya et al. 2014). As a
584 result, the presence of TPH in planted treatment decreased soil respiration via plant root
585 (Jiang et al. 2017; Yu et al. 2015). On the other hand, the increased DHA in contaminated
586 soils might be due to the adaptation of microorganims by secreting enzymes in the stressful
587 environment. In addition, TPH compounds such as saturated and aliphatic (n-alkanes)
588 compounds in the mixture could serve as available carbon source, which would enhance soil
589 microbial activity (Ikeura et al. 2016; Stroud et al. 2007). The stimulatory effect of TPH on
590 soil microbial activity in planted treatments was reported earlier too (Dhote et al. 2017; Ebadi
591 et al. 2018). In the present study, the relatively low DHA values were resulted from the
592 absence of glucose in the measurement method as a growth substrate for microorganisms.
593 Indeed, Petrisor et al. (2004) reported that the value with glucose addition was able to
594 evaluate the activity of whole DHA in the soil, and can be 10 times higher than that without
595 glucose.
596
597 *4.4. Hydrocarbon removal rates*
598 The level of soil microbial activity (measured from soil respiration and/or DHA) could reflect
599 the soil microorganisms' ability to degrade petroleum pollutants, consequently TPH removal
600 from polluted soils (Rodríguez-Rodríguez et al. 2016; Zamani et al. 2018). However, that
601 trend was not clear in this study. For example, high values of soil respiration and DHA in
602 *A.pyrifolia* did not lead to high TPH removal rates (Figures 3, 4 & 5). Therefore, an
603 enhancement of soil respiration and/or DHA did not necessarily relate to the increase of TPH
604 removal rate in the rhizosphere, compared to the unplanted treatment. The true extent of TPH
605 rhizodegradation, in fact, might not depend on the whole microbial community as indicated
606 by soil DHA, but the activity of specific hydrocarbon-degrading microorganisms (Bastida et

al. 2016; Yergeau et al. 2014). These microorganisms, which are selectively enriched under the combined selective pressure of TPH and rhizosphere, could utilize TPH compounds as carbon and energy sources in order to protect their roots from the toxic effects of petroleum contaminants (Afzal et al. 2011; Khan et al. 2013). The exploration of shifts in the rhizosphere microbial community composition and hydrocarbon-degrading gene expression would shed more light on the efficiency of TPH rhizoremediation, could be a future study. Different plant species, and even closely related plant genotypes, vary in their potential for rhizoremediation of TPH (Dagher et al. 2019; Ikeura et al. 2016). Grasses have been shown as suitable candidates for the elimination of TPH in soils in numerous studies (Gaskin and Bentham 2010; Hussain et al. 2019; Kiamarsi et al. 2020; Steliga and Kluk 2020). *C. truncata* is a perennial grass with fibrous roots, which provide sufficient surface for microbial growth and plant nutrient absorption (Chauhan et al. 2018a). Similarly, *B. seminuda* and *H. prostrata*, from Proteaceae family, developed cluster roots in the nutrient-poor soils resulting from the TPH contamination (Abbasian et al. 2016a; Andreolli et al. 2015; Ávila-Valdés et al. 2019), thereby enhancing root biomass relative to that in the control soil (Figure 1). This pattern might be the main reason for the relatively high removal rates of TPH in the soil by the three plants compared to the unplanted control and *A.pyrifolia*, which developed much less root biomass than *B. seminuda*, *C. truncata*, and *H. prostrata* (Figures 1 & 5). It is worth noting that *A. pyrifolia* is a member belonging to Fabaceae family, which is able to fix nitrogen directly from the atmosphere through nodules on the plant roots. The use of legumes could generally increase soil enzymatic activity more than non-legumes (Maseko and Dakora 2013; Zhou et al. 2017); therefore, it could explain the high soil respiration and DHA observed in the *A. pyrifolia* plant treatment, which did not result in high TPH removal in the contaminated soil.

Aliphatic hydrocarbons (e.g., *n*-alkane) are readily degraded by aerobic microorganisms that use molecular oxygen for the initial activation (Abbasian et al. 2015). Three possible peripheral pathways for aerobic aliphatic hydrocarbon biodegradation are proposed (Varjani 2017) (SI Figure 1). However, the most frequently encountered mechanism pathway is terminal oxidation (Zhang et al. 2011). The process starts with oxidation of the substrate molecules which introduces an oxygen atom inside the terminal methyl group to form an alcohol group (Meng et al. 2017). Alkane hydroxylases, which mainly consists of the integral-membrane alkane monooxygenase (AlkB) and the cytochrome P450 CY153 family, are the key enzymes for the first and foremost oxidation step in aerobic degradation of *n*-alkanes (Smits et al. 2002). Generally, these enzymes could hydroxylate *n*-alkanes with short- and medium-chain length (up to C_{16}). However, *n*-alkanes with the chain length up to C_{32} could be hydroxylated by some Actinomycetes whose AlkB-type alkane hydroxylases were fused with rubredoxin protein (Nie et al. 2011; Piccolo et al. 2011). The alcohol can be further oxidized to the corresponding aldehyde and then to fatty acid prior to entering the β-oxidation and tricarboxylic acid cycles (Abbasian et al. 2016b). The terminal oxidation of aliphatic TPH contaminants can be described as follows:

$$R\text{-}CH_3 \xrightarrow[hydroxylases]{Alkane} R\text{-}CH_2OH \xrightarrow[dehydrogenase]{Alcohol} R\text{-}CHO \xrightarrow[dehydrogenase]{Aldehyde} R\text{-}COOH \longrightarrow \beta\text{-oxidation pathways} \longrightarrow \text{Tricarboxylic acid cycles} \longrightarrow \text{Microbial biomass} + CO_2 + H_2O$$

Microorganisms present at the rhizosphere of plants growing in a TPH-contaminated soil are equipped with metabilic machinery to use the contaminants as a carbon and energy source (Van Hamme et al. 2003; Yergeau et al. 2014). The extent of aliphatic hydrocarbon degradation, indeed, is connected with the abundance and activity of hydrocarbon-degrading microorganisms with functional enzymes encoded in functional genes (Gielnik et al. 2020).

Therefore, studies concerning the genes, such as *alkB*, involved in the degradation of aliphatic hydrocarbons, could give a better indication of the "soil bioremediation potential", which warrants future investigation.

Numerous studies report that microbially-facilitated rhizoremediation is a principal pathway for TPH removal from soil (Correa-García et al. 2018; dos Santos and Maranho 2018; Thijs et al. 2016). Plants able to promote the degradation of TPH though the rhizosphere effect have been reported (Allamin et al. 2020; Cheng et al. 2019). Indeed, plant roots could directly absorb hydrocarbon compounds (Wei et al. 2019). Moreover, root exudates, whose amount are proportionally linked with root biomass, could enhance the degradation of TPH in the rhizosphere by specifically promoting efficient bioremediation communities (Bell et al. 2014; Eisenhauer et al. 2017). As a result, increased root biomass has been considered as the key to success in rhizoremediation technology (Huang et al. 2005; Saraeian et al. 2018; Wang et al. 2011; Wei et al. 2019).

A simple linear regression was carried out to investigate the relationship between plant dry biomass, soil respiration and DHA and TPH removal rate. However, there was only a strong positive linear relationship between dry root biomass (g) and TPH removal rate (%) (Figure 6), with Pearson's correlation coefficient of 0.9034 ($p < 0.001$). The relationship can be presented as follows (Eq. 3):

$$\text{TPH removal rate (\%)} = 9.8696 \times \text{dry biomass (g)} + 19.263 \ (R^2 = 0.8162, \quad N = 8) \quad \text{(Eq. 3)}$$

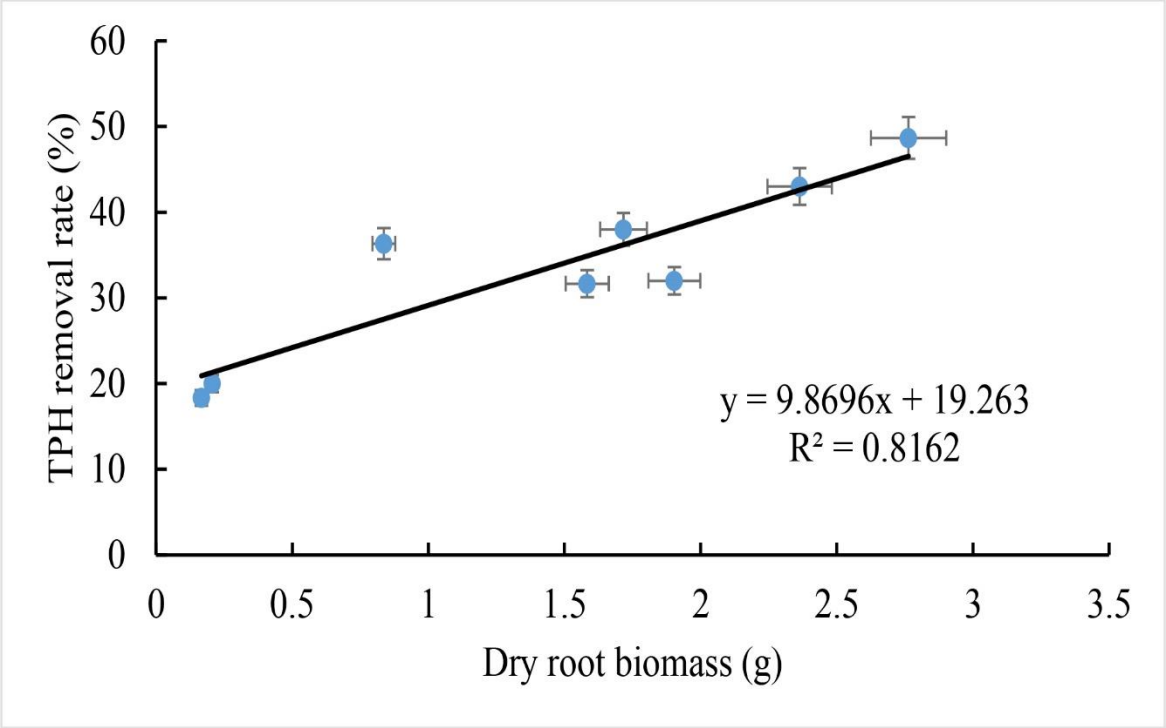


Figure 6. Correlation between TPH removal rate and dry root biomass across the four tolerant plant species at both TPH pollution levels

The finding that the production of root matter in TPH-contaminated soil across plant species was related to TPH removal rate confirms the importance of screening plant species that are able to tolerate TPH. In addition, to promote root biomass production, plant species adapted to local conditions is a requisiste for successful phytoremediation of TPH-degraded sites. In this study, the Proteacea species and *C. truncata* showed the greatest root production, and the increased root biomass was associated with the enhanced TPH removal at the end of the study. The stimulation of cluster roots in the presence of TPH contamination concurrently with enhanced TPH removal indicates the promise of the Proteaceae family plants for TPH rhizoremediation.

5. Conclusion

The tolerance of the eight wild plant species to two levels of TPH contamination were investigated in this study. The presence of aliphatic hydrocarbon compounds had negative effects on the plant growth, and inhibited soil respiration and dehydrogenase activity for almost all plant species tested, although to varying extents. *A. pyrifolia*, *B. seminuda*, *C. truncata*, and *H. prostrata* showed greater potential of TPH rhizoremediation than the other four species due to the higher tolerance of the former species to TPH contamination than the latter ones. The grass species (*C. truncata*) was the most efficient for the rhizoremediation of TPH-contaminated soil. *B. seminuda* and *H. prostrata* (tree and shrub species, respectively) also showed remarkable rhizoremediation potential due to their ability to form cluster roots in the contaminated soil. While selecting plants for rhizoremediation their ability to develop high root biomass reaching to contaminants in impacted soils should be considered, to maximize the removal rates. Further studies should consider the ways to enhance (e.g., rhizosphere engineering) the remediation efficiency of suitable/selected plant species, particularly species from Proteaceae family, which has largely been neglected in rhizoremediation investigation. There is also scope for future work exploring rhizosphere microbiota alteration in composition and hydrocarbon-degrading gene expression, and plant-specific exudates being deposited in the rhizosphere during the remediation process.

References

Abbasian F, Lockington R, Mallavarapu M, Naidu R (2015) A comprehensive review of aliphatic hydrocarbon biodegradation by bacteria. Appl. Biochem. Biotechnol. 176: 670-699.

Abbasian F, Lockington R, Megharaj M, Naidu R (2016a) The biodiversity changes in the microbial population of soils contaminated with crude oil. Curr. Microbiol. 72: 663-670.

Abbasian F, Lockington R, Megharaj M, Naidu R (2016b) A review on the genetics of aliphatic and aromatic hydrocarbon degradation. Appl. Biochem. Biotechnol. 178: 224-250.

Abbaspour A, Zohrabi F, Dorostkar V, Faz A, Acosta JA (2020) Remediation of an oil-contaminated soil by two native plants treated with biochar and mycorrhizae. J. Environ. Manage. 254: 109755.

721 Afzal M, Yousaf S, Reichenauer TG, Kuffner M, Sessitsch A (2011) Soil type affects plant
722 colonization, activity and catabolic gene expression of inoculated bacterial strains
723 during phytoremediation of diesel. J. Hazard. Mater. 186: 1568-1575.
724 Agathokleous E, Belz RG, Kitao M, Koike T, Calabrese EJ (2019) Does the root to shoot ratio
725 show a hormetic response to stress? An ecological and environmental perspective. J.
726 For. Res 30: 1569-1580.
727 Ahkami AH, White III RA, Handakumbura PP, Jansson C (2017) Rhizosphere engineering:
728 Enhancing sustainable plant ecosystem productivity. Rhizosphere 3: 233-243.
729 Allamin IA, Halmi MIE, Yasid NA, Ahmad SA, Abdullah SRS, Shukor Y (2020)
730 Rhizodegradation of Petroleum Oily Sludge-contaminated Soil Using Cajanus cajan
731 Increases the Diversity of Soil Microbial Community. Sci. Rep. 10: 1-11.
732 Andreolli M, Lampis S, Brignoli P, Vallini G (2015) Bioaugmentation and biostimulation as
733 strategies for the bioremediation of a burned woodland soil contaminated by toxic
734 hydrocarbons: a comparative study. J. Environ. Manage. 153: 121-131.
735 Anyasi RO, Atagana HI (2018) Profiling of plants at petroleum contaminated site for
736 phytoremediation. Int. J. Phytoremediation 20: 352-361.
737 Arslan M, Afzal M, Amin I, Iqbal S, Khan QM (2014) Nutrients can enhance the abundance
738 and expression of alkane hydroxylase CYP153 gene in the rhizosphere of ryegrass
739 planted in hydrocarbon-polluted soil. PLoS One 9: e111208.
740 Asher C, Loneragan J (1967) Response of plants to phosphate concentration in solution culture:
741 I. Growth and phosphorus content. Soil Sci. 103: 225-233.
742 Ávila-Valdés A, Piper FI, Zúñiga-Feest A (2019) Cluster root formation and function vary in
743 two species with contrasting geographic ranges. Plant Soil 440: 25-38.
744 Baoune H, Aparicio JD, Acuña A, El Hadj-khelil AO, Sanchez L, Polti MA, Alvarez A (2019)
745 Effectiveness of the Zea mays-Streptomyces association for the phytoremediation of
746 petroleum hydrocarbons impacted soils. Ecotoxicol. Environ. Saf. 184: 109591.
747 Barati M, Bakhtiari F, Mowla D, Safarzadeh S (2018) Comparison of the effects of poultry
748 manure and its biochar on barley growth in petroleum-contaminated soils. Int. J.
749 Phytoremediation 20: 98-103.
750 Bastida F, Jehmlich N, Lima K, Morris B, Richnow H, Hernández T, Von Bergen M, García
751 C (2016) The ecological and physiological responses of the microbial community from
752 a semiarid soil to hydrocarbon contamination and its bioremediation using compost
753 amendment. Journal of Proteomics 135: 162-169.
754 Basumatary B, Bordoloi S, Sarma HP (2012) Crude oil-contaminated soil phytoremediation by
755 using Cyperus brevifolius (Rottb.) Hassk. Water, Air, Soil Pollut. 223: 3373-3383.
756 Bell TH, Hassan SE-D, Lauron-Moreau A, Al-Otaibi F, Hijri M, Yergeau E, St-Arnaud M
757 (2014) Linkage between bacterial and fungal rhizosphere communities in hydrocarbon-
758 contaminated soils is related to plant phylogeny. ISME J 8: 331-343.
759 Blagodatskaya E, Blagodatsky S, Anderson T-H, Kuzyakov Y (2014) Microbial growth and
760 carbon use efficiency in the rhizosphere and root-free soil. PLoS One 9.
761 Bolan N, Kunhikrishnan A, Gibbs J (2013) Rhizoreduction of arsenate and chromate in
762 Australian native grass, shrub and tree vegetation. Plant Soil 367: 615-625.
763 Bolan NS, Park JH, Robinson B, Naidu R, Huh KY (2011) Phytostabilization: a green approach
764 to contaminant containment. Adv. Agron. Elsevier.
765 Bulgarelli D, Schlaeppi K, Spaepen S, Van Themaat EVL, Schulze-Lefert P (2013) Structure
766 and functions of the bacterial microbiota of plants. Annu. Rev. Plant Biol. 64: 807-838.
767 Burt R (2004) Soil survey laboratory methods manual.
768 Casida Jr L, Klein D, Santoro T (1964) Soil dehydrogenase activity. Soil Sci. 98: 371-376.
769 Chauhan BS, Manalil S, Florentine S, Jha P (2018a) Germination ecology of Chloris truncata
770 and its implication for weed management. PLoS One 13.

771 Chauhan BS, Manalil S, Florentine S, Jha P (2018b) Germination ecology of Chloris truncata
772 and its implication for weed management. PLoS One 13: e0199949.
773 Chen M, Xu P, Zeng G, Yang C, Huang D, Zhang J (2015) Bioremediation of soils
774 contaminated with polycyclic aromatic hydrocarbons, petroleum, pesticides,
775 chlorophenols and heavy metals by composting: applications, microbes and future
776 research needs. Biotechnol. Adv. 33: 745-755.
777 Cheng L, Wang Y, Cai Z, Liu J, Yu B, Zhou Q (2017) Phytoremediation of petroleum
778 hydrocarbon-contaminated saline-alkali soil by wild ornamental Iridaceae species. Int.
779 J. Phytoremediation 19: 300-308.
780 Cheng L, Zhou Q, Yu B (2019) Responses and roles of roots, microbes, and degrading genes
781 in rhizosphere during phytoremediation of petroleum hydrocarbons contaminated soil.
782 Int. J. Phytoremediation 21: 1161-1169.
783 Chowdhury S, Thangarajan R, Bolan N, O'Reilly-Wapstra J, Kunhikrishnan A, Naidu R (2017)
784 Nitrification potential in the rhizosphere of Australian native vegetation. Soil Res. 55:
785 58-69.
786 Cook RL, Hesterberg D (2013) Comparison of trees and grasses for rhizoremediation of
787 petroleum hydrocarbons. Int. J. Phytoremediation 15: 844-860.
788 Correa-García S, Pande P, Séguin A, St-Arnaud M, Yergeau E (2018) Rhizoremediation of
789 petroleum hydrocarbons: a model system for plant microbiome manipulation. Microb.
790 Biotechnol. 11: 819-832.
791 Cundy A, Bardos R, Puschenreiter M, Mench M, Bert V, Friesl-Hanl W, Müller I, Li X,
792 Weyens N, Witters N (2016) Brownfields to green fields: realising wider benefits from
793 practical contaminant phytomanagement strategies. J. Environ. Manage. 184: 67-77.
794 Dagher D, de la Providencia I, Pitre F, St-Arnaud M, Hijri M (2019) Plant identity shaped
795 rhizospheric microbial communities more strongly than bacterial bioaugmentation in
796 petroleum hydrocarbon-polluted sediments. Front. Microbiol. 10: 2144.
797 de Britto Costa P, Abrahão A, Viani RAG, Brancalion PHS, Lambers H, Sawaya ACHF,
798 Oliveira RS (2016) Cluster-root formation and carboxylate release in Euplassa
799 cantareirae (Proteaceae) from a neotropical biodiversity hotspot. Plant Soil 403: 267-
800 275.
801 Dhote M, Kumar A, Jajoo A, Juwarkar A (2017) Assessment of hydrocarbon degradation
802 potentials in a plant–microbe interaction system with oil sludge contamination: A
803 sustainable solution. Int. J. Phytoremediation 19: 1085-1092.
804 dos Santos JJ, Maranho LT (2018) Rhizospheric microorganisms as a solution for the recovery
805 of soils contaminated by petroleum: A review. J. Environ. Manage. 210: 104-113.
806 Ebadi A, Sima NAK, Olamaee M, Hashemi M, Nasrabadi RG (2018) Remediation of saline
807 soils contaminated with crude oil using the halophyte Salicornia persica in conjunction
808 with hydrocarbon-degrading bacteria. J. Environ. Manage. 219: 260-268.
809 Eisenhauer N, Lanoue A, Strecker T, Scheu S, Steinauer K, Thakur MP, Mommer L (2017)
810 Root biomass and exudates link plant diversity with soil bacterial and fungal biomass.
811 Sci. Rep. 7: 1-8.
812 Gaskin S, Soole K, Bentham R (2008) Screening of Australian native grasses for
813 rhizoremediation of aliphatic hydrocarbon-contaminated soil. Int. J. Phytoremediation
814 10: 378-389.
815 Gaskin SE, Bentham RH (2010) Rhizoremediation of hydrocarbon contaminated soil using
816 Australian native grasses. Sci. Total Environ. 408: 3683-3688.
817 Ghalamboran M, Kordkheli S, Bernard F (2020) Enzymatic response and metal ion content in
818 roots of corn and broad beans planted in soil contaminated with gasoline. Int. J.
819 Environ. Sci. Technol. 17: 973-982.

Gielnik A, Pechaud Y, Huguenot D, Cébron A, Esposito G, van Hullebusch ED (2020) Functional potential of sewage sludge digestate microbes to degrade aliphatic hydrocarbons during bioremediation of a petroleum hydrocarbons contaminated soil. J. Environ. Manage.: 111648.

Gielnik A, Pechaud Y, Huguenot D, Cébron A, Riom J-M, Guibaud G, Esposito G, van Hullebusch ED (2019) Effect of digestate application on microbial respiration and bacterial communities' diversity during bioremediation of weathered petroleum hydrocarbons contaminated soils. Sci. Total Environ. 670: 271-281.

Groves R, Hagon M, Ramakrishnan P (1982) Dormancy and germination of seed of eight populations of Themeda australis. Aust. J. Bot. 30: 373-386.

Guideline IHT (2005) Validation of analytical procedures: text and methodology Q2 (R1). International conference on harmonization, Geneva, Switzerland.

Gupta A, Patel AK, Gupta D, Singh G, Mishra VK (2020) Rhizospheric remediation of organic pollutants from the soil; a green and sustainable technology for soil clean up. Abatement of Environmental Pollutants. Elsevier.

Hatami E, Abbaspour A, Dorostkar V (2019) Phytoremediation of a petroleum-polluted soil by native plant species in Lorestan Province, Iran. Environ. Sci. Pollut. Res. 26: 24323-24330.

Herz K, Dietz S, Gorzolka K, Haider S, Jandt U, Scheel D, Bruelheide H (2018) Linking root exudates to functional plant traits. PLoS One 13.

Hoang SA, Lamb D, Seshadri B, Sarkar B, Choppala G, Kirkham M, Bolan NS (2020) Rhizoremediation as a green technology for the remediation of petroleum hydrocarbon-contaminated soils. J. Hazard. Mater.: 123282.

Huang X-D, El-Alawi Y, Gurska J, Glick BR, Greenberg BM (2005) A multi-process phytoremediation system for decontamination of persistent total petroleum hydrocarbons (TPHs) from soils. Microchem. J. 81: 139-147.

Husáková I, Weiner J, Münzbergová Z (2018) Species traits and shoot–root biomass allocation in 20 dry-grassland species. J Plant Ecol 11: 273-285.

Hussain I, Puschenreiter M, Gerhard S, Sani SGAS, Reichenauer TG (2019) Differentiation between physical and chemical effects of oil presence in freshly spiked soil during rhizoremediation trial. Environ. Sci. Pollut. Res. 26: 18451-18464.

Ikeura H, Kawasaki Y, Kaimi E, Nishiwaki J, Noborio K, Tamaki M (2016) Screening of plants for phytoremediation of oil-contaminated soil. Int. J. Phytoremediation 18: 460-466.

Jacoby R, Peukert M, Succurro A, Koprivova A, Kopriva S (2017) The role of soil microorganisms in plant mineral nutrition—current knowledge and future directions. Front. Plant Sci. 8: 1617.

Järvan M, Edesi L, Adamson A, Võsa T (2014) Soil microbial communities and dehydrogenase activity depending on farming systems. Plant Soil Environ. 60: 459-463.

Jiang L, Ma S, Zhou Z, Zheng T, Jiang X, Cai Q, Li P, Zhu J, Li Y, Fang J (2017) Soil respiration and its partitioning in different components in tropical primary and secondary mountain rain forests in Hainan Island, China. J Plant Ecol 10: 791-799.

Jonker MT, Brils JM, Sinke AJ, Murk AJ, Koelmans AA (2006) Weathering and toxicity of marine sediments contaminated with oils and polycyclic aromatic hydrocarbons. Environ. Toxicol. Chem. 25: 1345-1353.

Kaimi E, Mukaidani T, Miyoshi S, Tamaki M (2006) Ryegrass enhancement of biodegradation in diesel-contaminated soil. Environ. Exp. Bot. 55: 110-119.

Kaimi E, Mukaidani T, Tamaki M (2007) Screening of twelve plant species for phytoremediation of petroleum hydrocarbon-contaminated soil. Plant Prod. Sci. 10: 211-218.

Khan MAI, Biswas B, Smith E, Mahmud SA, Hasan NA, Khan MAW, Naidu R, Megharaj M (2018) Microbial diversity changes with rhizosphere and hydrocarbons in contrasting soils. Ecotoxicol. Environ. Saf. 156: 434-442.
Khan S, Afzal M, Iqbal S, Mirza MS, Khan QM (2013) Inoculum pretreatment affects bacterial survival, activity and catabolic gene expression during phytoremediation of diesel contaminated soil. Chemosphere 91: 663-668.
Kiamarsi Z, Kafi M, Soleimani M, Nezami A, Lutts S (2020) Conjunction of Vetiveria zizanioides L. and oil-degrading bacteria as a promising technique for remediation of crude oil-contaminated soils. J. Clean. Prod. 253: 119719.
Kim J, Lee AH, Chang W (2018) Enhanced bioremediation of nutrient-amended, petroleum hydrocarbon-contaminated soils over a cold-climate winter: the rate and extent of hydrocarbon biodegradation and microbial response in a pilot-scale biopile subjected to natural seasonal freeze-thaw temperatures. Sci. Total Environ. 612: 903-913.
Kulakow PA, Schwab A, Banks M (2000) Screening plant species for growth on weathered, petroleum hydrocarbon-contaminated sediments. Int. J. Phytoremediation 2: 297-317.
Kuppusamy S, Maddela NR, Megharaj M, Venkateswarlu K (2020) An overview of total petroleum hydrocarbons. Total petroleum hydrocarbons: 1-27.
Kuzyakov Y, Xu X (2013) Competition between roots and microorganisms for nitrogen: mechanisms and ecological relevance. New Phytol. 198: 656-669.
Lamb DT, Ming H, Megharaj M, Naidu R (2010) Phytotoxicity and accumulation of lead in Australian native vegetation. Arch. Environ. Contam. Toxicol. 58: 613-621.
Lamb DT, Naidu R, Ming H, Megharaj M (2012) Copper phytotoxicity in native and agronomical plant species. Ecotoxicol. Environ. Saf. 85: 23-29.
Lambers H, Albornoz F, Kotula L, Laliberté E, Ranathunge K, Teste FP, Zemunik G (2018) How belowground interactions contribute to the coexistence of mycorrhizal and non-mycorrhizal species in severely phosphorus-impoverished hyperdiverse ecosystems. Plant Soil 424: 11-33.
Lin Q, Mendelssohn IA (2009) Potential of restoration and phytoremediation with Juncus roemerianus for diesel-contaminated coastal wetlands. Ecol. Eng. 35: 85-91.
Luo P, Han X, Wang Y, Han M, Shi H, Liu N, Bai H (2015) Influence of long-term fertilization on soil microbial biomass, dehydrogenase activity, and bacterial and fungal community structure in a brown soil of northeast China. Ann. Microbiol. 65: 533-542.
Maila MP, Cloete TE (2005) The use of biological activities to monitor the removal of fuel contaminants—perspective for monitoring hydrocarbon contamination: a review. Int Biodeterior Biodegrad. 55: 1-8.
Martin BC, George SJ, Price CA, Ryan MH, Tibbett M (2014) The role of root exuded low molecular weight organic anions in facilitating petroleum hydrocarbon degradation: current knowledge and future directions. Sci. Total Environ. 472: 642-653.
Maseko S, Dakora F (2013) Rhizosphere acid and alkaline phosphatase activity as a marker of P nutrition in nodulated Cyclopia and Aspalathus species in the Cape fynbos of South Africa. S. Afr. J. Bot. 89: 289-295.
Mena E, Villaseñor J, Rodrigo MA, Cañizares P (2016) Electrokinetic remediation of soil polluted with insoluble organics using biological permeable reactive barriers: effect of periodic polarity reversal and voltage gradient. Chem. Eng. J. 299: 30-36.
Meng L, Li H, Bao M, Sun P (2017) Metabolic pathway for a new strain Pseudomonas synxantha LSH-7′: from chemotaxis to uptake of n-hexadecane. Sci. Rep. 7: 1-13.
Merkl N, Schultze-Kraft R, Arias M (2006) Effect of the tropical grass Brachiaria brizantha (Hochst. ex A. Rich.) Stapf on microbial population and activity in petroleum-contaminated soil. Microbiol. Res. 161: 80-91.

Muratova AY, Golubev SN, Dubrovskaya EV, Pozdnyakova NN, Panchenko LV, Pleshakova EV, Chernyshova MP, Turkovskaya OV (2012) Remediating abilities of different plant species grown in diesel-fuel-contaminated leached chernozem. Appl. Soil Ecol. 56: 51-57.

NEPC (1999) National environment protection measure for the assessment of site contamination: impact statement.

Nie M, Yang Q, Jiang L-F, Fang C-M, Chen J-K, Li B (2010) Do plants modulate biomass allocation in response to petroleum pollution? Biol. Lett. 6: 811-814.

Nie Y, Liang J, Fang H, Tang Y-Q, Wu X-L (2011) Two novel alkane hydroxylase-rubredoxin fusion genes isolated from a Dietzia bacterium and the functions of fused rubredoxin domains in long-chain n-alkane degradation. Appl. Environ. Microbiol. 77: 7279-7288.

Pérez-Hernández I, Ochoa-Gaona S, Adams R, Rivera-Cruz M, Pérez-Hernández V, Jarquín-Sánchez A, Geissen V, Martínez-Zurimendi P (2017) Growth of four tropical tree species in petroleum-contaminated soil and effects of crude oil contamination. Environ. Sci. Pollut. Res. 24: 1769-1783.

Petrisor IG, Dobrota S, Komnitsas K, Lazar I, Kuperberg JM, Serban M (2004) Artificial inoculation—perspectives in tailings phytostabilization. Int. J. Phytoremediation 6: 1-15.

Piccolo LL, De Pasquale C, Fodale R, Puglia AM, Quatrini P (2011) Involvement of an alkane hydroxylase system of Gordonia sp. strain SoCg in degradation of solid n-alkanes. Appl. Environ. Microbiol. 77: 1204-1213.

Qi Y, Wang J, Tong Y, Hu X, Liu G, Li Y (2015) Screening of weed plants for phytoremediation of petroleum-contaminated soils [J]. Ecol. Sci. 1: 148-153.

Qi Y, Wei W, Chen C, Chen L (2019) Plant root-shoot biomass allocation over diverse biomes: A global synthesis. Glob. Ecol. Conserv. 18: e00606.

Richter BE (2000) Extraction of hydrocarbon contamination from soils using accelerated solvent extraction. J. Chromatogr. 874: 217-224.

Robson DB, Knight JD, Farrell RE, Germida JJ (2003) Ability of cold-tolerant plants to grow in hydrocarbon-contaminated soil. Int. J. Phytoremediation 5: 105-123.

Rodríguez-Rodríguez N, Rivera-Cruz M, Trujillo-Narcía A, Almaráz-Suárez J, Salgado-García S (2016) Spatial distribution of oil and biostimulation through the rhizosphere of Leersia hexandra in degraded soil. Water, Air, Soil Pollut. 227: 319.

Rovira A (1974) Biology of the rhizosphere. The plant root and its environment: 153-204.

Salazar A, Lennon JT, Dukes JS (2019) Microbial dormancy improves predictability of soil respiration at the seasonal time scale. Biogeochemistry 144: 103-116.

Saraeian Z, Haghighi M, Etemadi N, HajAbbasi MA, Afyuni M (2018) Phytoremediation effect and growth responses of Cynodon spp. and Agropyron desertorum in a petroleum-contaminated soil. Soil Sediment Contam 27: 393-407.

Shahsavari E, Adetutu EM, Anderson PA, Ball AS (2013) Tolerance of selected plant species to petrogenic hydrocarbons and effect of plant rhizosphere on the microbial removal of hydrocarbons in contaminated soil. Water, Air, Soil Pollut. 224: 1495.

Shane MW, Cramer MD, Funayama-Noguchi S, Cawthray GR, Millar AH, Day DA, Lambers H (2004) Developmental physiology of cluster-root carboxylate synthesis and exudation in harsh hakea. Expression of phosphoenolpyruvate carboxylase and the alternative oxidase. Plant Physiol. 135: 549-560.

Sivaram AK, Logeshwaran P, Subashchandrabose SR, Lockington R, Naidu R, Megharaj M (2018) Comparison of plants with C3 and C4 carbon fixation pathways for remediation of polycyclic aromatic hydrocarbon contaminated soils. Sci. Rep. 8: 2100.

966 Smits TH, Balada SB, Witholt B, Van Beilen JB (2002) Functional analysis of alkane
967 hydroxylases from gram-negative and gram-positive bacteria. J. Bacteriol. 184: 1733-
968 1742.
969 Standardization IOf (2004) ISO 16703: Soil Quality: Determination of Content of Hydrocarbon
970 in the Range C10 to C40 by Gas Chromatography. ISO.
971 Steliga T, Kluk D (2020) Application of Festuca arundinacea in phytoremediation of soils
972 contaminated with Pb, Ni, Cd and petroleum hydrocarbons. Ecotoxicol. Environ. Saf.
973 194: 110409.
974 Stroud J, Paton G, Semple KT (2007) Microbe-aliphatic hydrocarbon interactions in soil:
975 implications for biodegradation and bioremediation. J. Appl. Microbiol. 102: 1239-
976 1253.
977 Sun WH, Lo JB, Robert FM, Ray C, Tang C-S (2004) Phytoremediation of petroleum
978 hydrocarbons in tropical coastal soils I. Selection of promising woody plants. Environ.
979 Sci. Pollut. Res. 11: 260-266.
980 Thijs S, Sillen W, Rineau F, Weyens N, Vangronsveld J (2016) Towards an enhanced
981 understanding of plant–microbiome interactions to improve phytoremediation:
982 engineering the metaorganism. Front. Microbiol. 7: 341.
983 Van Hamme JD, Singh A, Ward OP (2003) Recent advances in petroleum microbiology.
984 Microbiol. Mol. Biol. Rev. 67: 503-549.
985 Varjani SJ (2017) Microbial degradation of petroleum hydrocarbons. Bioresour. Technol. 223:
986 277-286.
987 Wang G, Mayes MA, Gu L, Schadt CW (2014) Representation of dormant and active microbial
988 dynamics for ecosystem modeling. PLoS One 9.
989 Wang Z, Xu Y, Zhao J, Li F, Gao D, Xing B (2011) Remediation of petroleum contaminated
990 soils through composting and rhizosphere degradation. J. Hazard. Mater. 190: 677-685.
991 Wei Y, Wang Y, Duan M, Han J, Li G (2019) Growth tolerance and remediation potential of
992 six plants in oil-polluted soil. J. Soils Sed. 19: 3773-3785.
993 Yergeau E, Sanschagrin S, Maynard C, St-Arnaud M, Greer CW (2014) Microbial expression
994 profiles in the rhizosphere of willows depend on soil contamination. ISME J 8: 344.
995 Yu L, Wang Y, Wang Y, Sun S, Liu L (2015) Quantifying components of soil respiration and
996 their response to abiotic factors in two typical subtropical forest stands, southwest
997 China. PLoS One 10.
998 Zainul A, Koyro H-W, Huchzermeyer B, Bilquees G, Khan MA (2017) Impact of a Biochar or
999 a Compost-Biochar Mixture on Water relation, Nutrient uptake and Photosynthesis of
1000 Phragmites karka. Pedosphere.
1001 Zamani J, Hajabbasi MA, Mosaddeghi MR, Soleimani M, Shirvani M, Schulin R (2018)
1002 Experimentation on degradation of petroleum in contaminated soils in the root zone of
1003 maize (Zea Mays L.) inoculated with Piriformospora indica. Soil Sediment Contam 27:
1004 13-30.
1005 Zhang Z, Hou Z, Yang C, Ma C, Tao F, Xu P (2011) Degradation of n-alkanes and polycyclic
1006 aromatic hydrocarbons in petroleum by a newly isolated Pseudomonas aeruginosa
1007 DQ8. Bioresour. Technol. 102: 4111-4116.
1008 Zhou Y, Zhu H, Fu S, Yao Q (2017) Variation in soil microbial community structure associated
1009 with different legume species is greater than that associated with different grass species.
1010 Front. Microbiol. 8: 1007.

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Supplementary Information for:

Petroleum hydrocarbon rhizoremediation and soil microbial activity improvement via cluster root formation by wild Proteaceae plant species

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Supplementary Tables

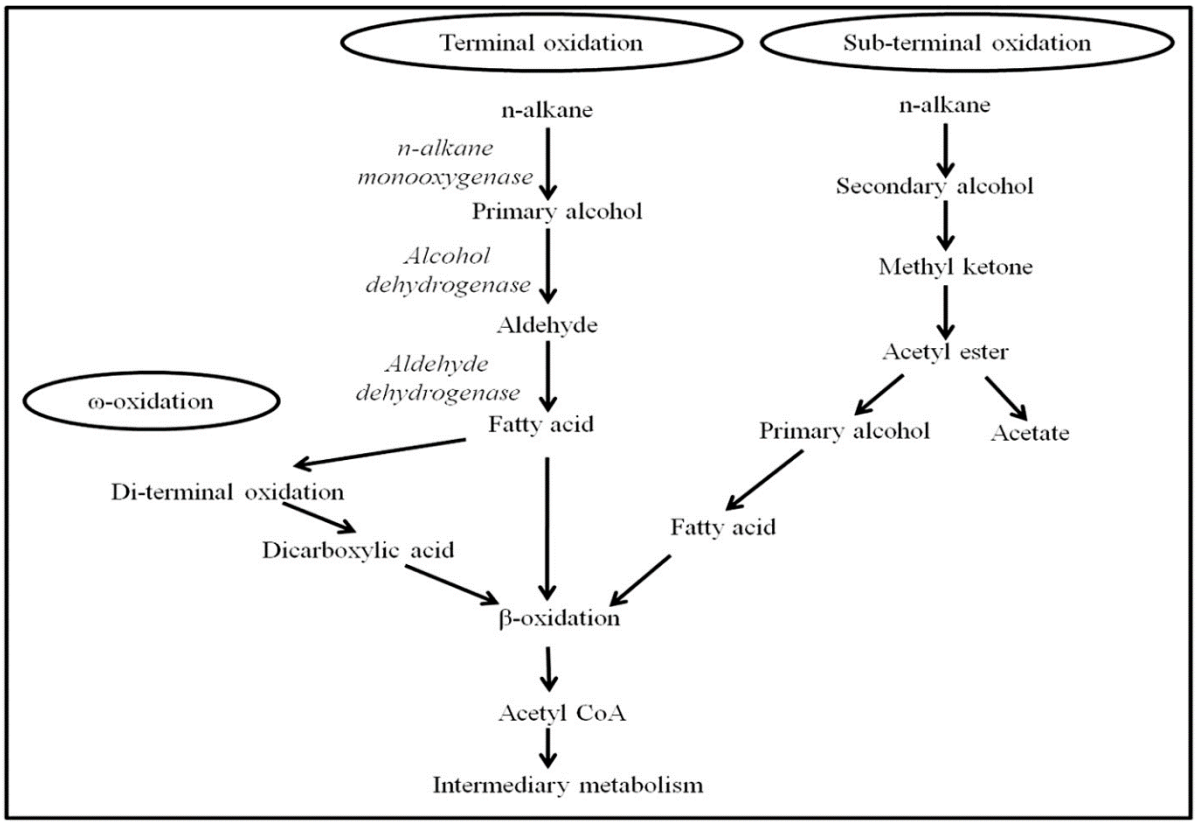
SI Table 1. Characteristics of Australian wild plant species used for the rhizoremediation of TPH

Plant type	Plant species	Common name	Family	Life cycle	Soil grown
Tree	*Acacia inaequilatera*	Kanji Bush	Fabaceae	Perennial	Sandy loam
	Banksia seminuda	River Banksia	Proteaceae	Perennial	Sandy
Shrub	*Acacia pyrifolia*	Ranji Bush	Fabaceae	Perennial	Sandy
	Acacia stellaticeps	Poverty Bush	Fabaceae	Perennial	Clayey sand
	Hakea prostrata	Harsh Hakea	Proteaceae	Perennial	Sandy loam
	Hardenbergia violacea	Purple Coral Pea	Fabaceae	Perennial	Sandy loam
Grass	*Chloris truncata*	Windmill Grass	Poaceae	Perennial	Sandy
	Triodia wiseana	Hard Spinifex	Poaceae	Perennial	Sandy

SI Table 2. Selected physicochemical characteristics of the experimental soil and initial TPH concentrations used in the rhizoremediation study

pH (H_2O)	**pH** ($CaCl_2$)	**EC** (µS cm^{-1})	**Organic C** (%)	**Total N** (%)	**Available P** mg kg^{-1}	**Total S** (%)	**TPH1** (mg kg^{-1})	**TPH2** (mg kg^{-1})
6.04	5.41	280	0.78	0.08	3.0	0.002	4,370± 52	7,500± 166

Supplementary Figure



SI Figure 1. Three possible pathways for aerobic aliphatic hydrocarbon biodegradation