Phosphorus application enhances alkane hydroxylase gene abundance in the rhizosphere of wild plants grown in petroleum-hydrocarbon-contaminated soil

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Highlights

- Phosphorus addition resulted in reduced total petroleum hydrocarbon (TPH) toxicity to wild plants
- Proteaceae species were sensitive to high phosphorus levels in soils
- Chlorophyll fluorescence can be used as an indicator of TPH toxicity in plants
- *AlkB* gene abundance was influenced by plant species and P input
- Removal of aliphatic hydrocarbons was associated with *AlkB* gene abundance

Abstract

This study assessed the ability of phosphorus (P) fertilizer to remediate the rhizosphere of three wild plant species (*Banksia seminuda*, a tree; *Chloris truncata*, a grass; and *Hakea prostrata*, a shrub) growing in a soil contaminated with total (aliphatic) petroleum hydrocarbon (TPH). Plant growth, photosynthesis (via chlorophyll fluorescence), soil microbial activity, alkane hydroxylase *AlkB* (aliphatic hydrocarbon-degrading) gene

abundance, and TPH removal were evaluated 120 days after planting. Overall, although TPH served as an additional carbon source for soil microorganisms, the presence of TPH in soil resulted in decreased plant growth and photosynthesis. However, growth, photosynthesis, microbial activities, and *AlkB* gene abundance were enhanced by the application of P fertilizer, thereby increasing TPH removal rates, although the extent and optimum P dosage varied among the plant species. The highest TPH removal (64.66%) was observed in soil planted with the Poaceae species, *C. truncata*, and amended with 100 mg P kg^{-1} soil, while *H. prostrata* showed higher TPH removal compared to the plant belonging to the same Proteaceae family, *B. seminuda*. The presence of plants resulted in higher *AlkB* gene abundance and TPH removal relative to the unplanted control. The removal of TPH was associated directly with *AlkB* gene abundance ($R^2 > 0.9$, $p < 0.001$), which was affected by plant identity and P levels. The results indicated that an integrated approach involving wild plant species and optimum P amendment, which was determined through experimentation using different plant species, was an efficient way to remediate soil contaminated with TPH.

Key words: wild plants, phosphorus application, total petroleum hydrocarbon, *AlkB* genes, rhizoremediation

1. Introduction

Spills, leaks, and other releases of petroleum hydrocarbon compounds are among the most frequently reported incidents of soil pollution and have drawn concerns from the public worldwide in recent decades. Such deleterious events have long-lasting, adverse impacts on human and ecological health, because of the toxicity and persistence of petroleum-related compounds in the environment (Tang et al., 2011). Total petroleum hydrocarbon (TPH) is a useful term for the measure of a complex mixture of different hydrocarbon compounds that

originate from crude oil, including both aliphatic and aromatic hydrocarbons (Coulon and Wu, 2014, Hoang et al., 2020). Although the aliphatic group of compounds is the main constituent of TPH in the soil environment (higher than 95% by volume), it has received less research attention relative to the aromatic counterparts (Stroud et al., 2007). Due to its toxic and persistent nature, the presence of aliphatic petroleum hydrocarbons in soil poses threats to human health and ecosystems (Abbasian et al., 2015). Therefore, it is necessary to develop environmentally friendly and cost-effective approaches to remediate soil contaminated with petroleum-derived aliphatic hydrocarbons.

Degradation by plant and soil microorganisms is a promising strategy for the remediation of TPH-polluted soils (Hoang et al., 2020, Hussain et al., 2017). Particularly, rhizosphere microorganisms ameliorate phytotoxicity of TPH via their pollutant-degrading abilities and metabolic activities as influenced by carbon and nutrients released from plant roots (Hussain et al., 2018a). Poaceae (grass) species form densely fibrous roots to support rhizosphere microorganisms, and these species have been widely used in the rhizoremediation of TPH-contaminated soils. However, if the contaminants are present at a depth, the use of such plant species is ineffective owing to their shallow root systems (Cook and Hesterberg, 2013). Therefore, the provision of plants with deeper root systems (such as trees and shrubs) is needed. In our previous screening study, the Proteaceae species (*Banksia seminuda*, a tree and *Hakea prostrata*, a shrub) showed their potential in rhizoremediation of TPH-contaminated soil (only lower than that of *Chloris truncata*, a grass species) (Hoang et al., 2021). To the best of the authors knowledge, this is the first time the Proteaceae species are deployed in the remediation of TPH-contaminated soils. Due to the hydrophobic nature of TPH, the availability of water-soluble nutrients to plants is reduced in TPH-contaminated soils (Tara et al., 2014). Additionally, competition for nutrients between the plants and rhizosphere microorganisms becomes prominent in such TPH-contaminated, nutrient-

deficient soils (Lugtenberg, 2015). Because nutrient levels affect the mineralization of TPH in soil, nutrients such as nitrogen (N) and phosphorus (P) act as critical and limiting factors in the rhizoremediation of TPH (Arslan et al., 2014).

Phosphorus is essential for many plant physiological functions (e.g., photosynthesis and carbohydrate transport) and enhances activities of the antioxidant defence system under stressful environments (Saleem et al., 2020). However, high levels of P in the soil can cause toxicity to plants due to dehydration, burning, and, in severe cases, plant death (Albornoz et al., 2020). Therefore, an optimum concentration of P is necessary to enhance plant growth and biomass production, which is essential for rhizoremediation (Huang et al., 2005). The Australian landscape is highly weathered and low in nutrient content due to the lack of recent geologic inputs from glaciation and volcanic activity (Lambers et al., 2013). As a result of these continental scale factors, the Australian native vegetation has evolved with unique properties, such as plants with a poor ability to down-regulate P uptake at high soil P availability (Denton et al., 2007, Lambers et al., 2013). However, external P application can enhance plant growth in TPH-polluted soils, especially under low P availability (Arslan et al., 2014). Although there are few studies on the effects of fertilizers on rhizoremediation of TPH-contaminated soils, the specific focus on phosphorus fertilization is still lacking. Therefore, since these plant species are sensitive to high levels of P in soil, it is essential to understand the effect of phosphorus fertilizers on rhizoremediation of TPH-contaminated soils by these plant species. It could be hypothesized that plants grown in TPH-impacted soils would respond to external P addition differently compared to those grown in uncontaminated soils. In addition, excessive P fertilizer might impede wild plant growth and rhizosphere microbial functions, which subsequently alter the TPH rhizoremediation potential of the plants. To test these hypotheses, three wild Australian plant species (*Banksia seminuda*, *Chloris truncata*, and *Hakea prostrata*) were grown in uncontaminated and TPH-

contaminated soils supplied with various levels of P fertilizer. Plant growth and photosynthesis, and rhizosphere microbial activity were determined to assess the influence of P fertilization on subsequent TPH rhizoremediation.

2. Materials and methods

2.1. Plant species and experimental setup

This study was conducted at the glasshouse facility of The University of Newcastle, Australia, under natural photoperiod conditions during January to April 2020. Three-week-old seedlings of *C. truncata* (Poaceae family), *B. seminuda* (Proteaceae family), and *H. prostrata* (Proteaceae family) were used. The seedlings were grown in plastic pots of 133 mm (diameter) x 140 mm (length) on 1 Jan 2020, each containing 1 kg of experimental soil, with one seedling per pot. The soil was a clay loam soil in texture based on USDA classification and characterized following standard methods to determine selected physical and chemical parameters (Supplementary Information, SI Table 1). The soil samples were amended with 75 mL of KH_2PO_4 solutions prepared at different concentrations to add different levels of P into the soil (0, 5, 10, 25, 50, and 100 mg P kg^{-1} soil) before transplanting the seedlings, and watered with 75 mL deionized water on alternate days. A preliminary study showed that cluster root formation by *B. seminuda* and *H. prostrata* was inhibited at P concentrations above 3 µM in soil solution, which was equivalent to around 10 mg P kg^{-1} soil based on the results of P sorption isotherms involving the experimental soil (SI Figure 1). The formation of cluster roots in the Proteaceae species is induced by P deficiency (Lambers et al., 2018, Shane et al., 2004a), and, thus, the result indicated that P level of 10 mg kg^{-1} is adequate for the plants' requirement. Therefore, the P levels were chosen with the aim of obtaining a range of inorganic P (Pi) concentrations in the soil below and above 3 µM level. Values from P

sorption isotherms were used to characterize the soil P status and to establish the P requirement.

Details about soil collection and characteristics, the glasshouse conditions, and seedling emergence and transplanting seedlings have been previously described (Hoang et al., 2021), and are given in SI. The target TPH concentration was 1.5% (15,000 mg TPH kg^{-1} soil) which resulted in initial pollutant level named TPH1. To achieve homogeneity, soil-TPH mixture was first mixed thoroughly using a dual tumbler bin (20 kg soil at a time) for 30 min and then using a concrete pan mixer for 15 min. Soil samples from different depths were collected and analysed for the initial TPH concentration (i.e., TPH1, SI Table 1) to ensure the contamination homogeneity. Soil samples used for DNA extraction were stored at -20 ^{o}C until analysis.

The experiment was arranged in a randomized complete block design with three factors (TPH, plant species, and P) involving two soil treatments (without and with TPH, i.e., TPH0 and TPH1), three plant treatments (i.e., *B. seminuda, C. truncata*, and *H. prostrata*) and an unplanted control, and six P treatments as mentioned above, with three replications for each treatment. The pots were rotated in positions fortnightly during the experiment to reduce systematic errors possibly caused by changes in environmental conditions.

2.2. Plant dry biomass

The biomass of plants was measured after harvesting at the end of the experiment (on 30 April 2020). All the plants were harvested, separately divided into roots and shoots, and then oven-dried at 70 ^{o}C for 72 h for dry weight determination (Hoang et al., 2021).

2.3. Chlorophyll fluorescence measurement

For each plant species, the youngest fully expanded leaves at the same developmental stage (100 days after planting) were selected by visual observation to measure chlorophyll fluorescence using a portable photosynthesis system (LI-6800, LI-COR Inc., Lincoln, NE, USA). Chlorophyll fluorescence is light re-emitted by chlorophyll molecules during return from excited to non-exited states. It is used to assess changes in photosynthesis. A dark-adapted leaf has minimal fluorescence (F_0). On the application of a saturating light, the value for fluorescence is raised from the ground state to maximal fluorescence (F_m). In the process, the first electron acceptor of photosystem II (PSII) is fully reduced (Kadir et al., 2007). Photosystem II is a specialized protein complex that uses light energy to oxidize water, resulting in the release of molecular oxygen into the atmosphere, and to reduce plastoquinone, which is a molecule involved in the electron transport chain in the light-dependent reactions of photosynthesis (Whitmarsh, 2001). Variable fluorescence, F_v, is a calculated term using F_0 and F_m. It is calculated as $F_v = F_m - F_0$. The term F_m' is another maximal fluorescence, but it is the maximal fluorescence of the light-adapted sample when a high intensity pulse has been applied. The ratio F_v/F_m [or $(F_m - F_0)/F_m$] represents an estimation of the maximal photochemical efficiency of PSII and is used to detect the loss of function of PSII reaction centre (Kadir et al., 2007). The efficiency of PSII can be calculated using F_m' and another fluorescence parameter, F_s, which is the steady-state terminal fluorescence. In this study, key fluorescence parameters were measured according to the method of Redondo-Gómez et al. (2010) under dark- and light-adapted conditions to investigate whether TPH pollution and external P addition affected the sensitivity of plants to photoinhibition, which is an indication of photosynthesis, as will be explained. After being dark-adapted for 20 min, the maximum fluorescence yield (F_m) was obtained during a 0.7 s saturation actinic light pulse of 15,000 μmol m^{-2} s^{-1} (Maxwell and Johnson, 2000). Steady-

state (F_s) and maximal (F_m') fluorescence were measured under light-adapted leaves (Van and Snel, 1990). While F_s was recorded after adapting plant leaves to ambient light conditions for 30 min, F_m' was measured under a saturating actinic light pulse of 15,000 µmol m^{-2} s^{-1} for 0.7 s, which caused a temporary inhibition of PSII, and, thus, this pulse produced the maximum fluorescence yield (Redondo-Gómez et al., 2010). Effective quantum efficiency of PSII [ΦPSII, $(F_m' - F_s)/F_m'$] and non-photochemical quenching [NPQ, $(F_m - F_m')/F_m'$] were calculated (Liu et al., 2017). All measurements were conducted during the late morning period (9:00 – 11:00 am), considered to be the most active time for photosynthesis.

2. 4. Soil dehydrogenase activity

Dehydrogenase activity (DHA) of the rhizosphere soil samples was determined according to the method proposed by Casida Jr et al. (1964), and is described in detail in SI.

2.5. Quantification of the abundance of AlkB gene copies in the TPH-contaminated soils

AlkB genes that encode for hydroxylase enzymes are critical for the microbial degradation of aliphatic petroleum hydrocarbons (Hoang et al., 2021, Khan et al., 2013). Quantification of *AlkB* gene copies (normalized using *16S rDNA*) in the TPH-contaminated soil with different plant and P treatments was performed according to the Synergy Brands, Inc. (SYBR) green-based real-time qPCR method developed by Liu et al. (2015) and Shahsavari et al. (2016) with some modification. The sequences, amplicon sizes, and annealing temperatures of the employed PCR primers are given in SI Table 2.

Soil DNA was extracted from a 0.25 g moist soil sample using a DNeasy® PowerSoil® Kit obtained from QIAGEN Pty Ltd., Australia (PO Box 169, Chadstone Centre, VIC 3148)

following the manufacturer's protocol. Conventional PCR was used to verify and recover the target or reference genes DNA. Each PCR reaction mixture contained 10 µL of 10X reaction buffer, 1 µL deoxynucleoside triphosphates (dNTPs) mixture, 1 µL Advantage 2 Polymerase Mix (Clontech), 1µL of both forward and reverse primers (10 µM each), and 2 µL of DNA template. Conditions for the conventional PCR were set according to Liu et al. (2015): initial denaturation at 94 °C for 5 min; 35 cycles of denaturing at 94 °C for 20 s; annealing temperature of the target or reference genes (50 and 58 °C for *AlkB and 16S rDNA*, respectively) for 2 min; and final extension at 72 °C for 7 min. PCR products were examined by 1.5% agarose gel electrophoresis, and the bands for the target and reference genes were extracted and purified using an agarose gel extraction kit (Cat#42600, Merck, Bayswater, VIC 3153, Australia). The purified PCR products were then used to construct standard curves (Shahsavari et al., 2016). The purified PCR products were sent for DNA sequencing (Australian Genome Research Facility, Melbourne, VIC 3000, Australia) to verify their authenticity. Gene copy numbers were calculated from the measured concentration of the purified PCR product concentration following the formula proposed by Shahsavari et al. (2016) (Eq. 1):

$$\mathrm{Y\ (molecules\ \mu L^{-1})} = \frac{\mathrm{X\ g\ \mu L^{-1}\ DNA}}{\mathrm{(PCR\ product\ length\ in\ base\ pairs \times 660)}} \times 6.022 \times 10^{23} \qquad \text{(Eq. 1)}$$

For each primer set, a standard curve was obtained from ten-fold serial dilutions of the template DNA (purified PCR product). qPCR reactions were carried out on a Bio-Rad CFX96 with iTaq^{TM} Universal SYBR Green Supermix (Cat# 1725121) (Gladesville, NSW 2111, Australia). Reaction mixtures contained 10 µL SYBR green supermix, 0.4 µL of forward primer (10 µM), 0.4 µL reverse primer (10 µM), 8.2 µL of PCR-grade water, and 1 µL of PCR product DNA and soil DNA as templates for the construction of standard curves and quantification of the target or reference genes, respectively. The temperature profile for

real-time qPCR was as follows: 94 °C for 30 s followed by 40 cycles of denaturing at 94 °C for 5 s, annealing temperature of primers (50 and 58 °C for *AlkB* and *16S rDNA*, respectively) for 15 s, followed by a melt curve from 55 °C to 95 °C (increment = 0.5 °C/10 s).

2.6. TPH analysis of the soil

After harvesting the plants from pots, soil from each pot was collected and homogenized for TPH analysis. Methods for extraction, analysis of residual TPH concentrations, and calculation for TPH removal rates have been described previsously (Hoang et al., 2021), and are given in SI.

2.7. Statistical analysis

Normal distribution of each data set was checked using SPSS Version 25 package for Windows (IBM Corp., NY, USA). One-way analysis of variance (ANOVA) was used at $\alpha = 0.05$ to determine the significant difference between treatments for plant dry biomass, chlorophyll fluorescence parameters, soil DHA, *AlkB* gene abundance, and TPH removal rates. Tukey's test at the 5% level of confidence was used for comparisons between treatments. Two-way ANOVA was used to examine the influence of two different independent variables (among TPH levels, plant species, and P levels) on the abovementioned dependent variables.

3. Results

3.1. Effects of TPH and P application on plant dry biomass

In the absence of TPH, shoot and root dry biomass continued to increase as P application level increased from 0 to 10 mg kg^{-1} for *B. seminuda* and *H. prostrata* whereas *C. truncata* increased steadily and obtained maximum shoot and root biomass at P application of 50 mg kg^{-1}, and the values tended to decrease at higher P levels (Figure 1). There were little changes in the root: shoot ratio among different P treatments for the three plant species (Table 1). Additionally, there was no obvious visual toxicity symptom at high P supply in all plant species in this study.

In TPH-contaminated soil, the plants showed significantly less growth and shoot biomass production than those cultivated in the uncontaminated soil ($p < 0.05$). However, P amendment up to a certain level enhanced plant growth and shoot biomass production in the contaminated soil (Figure 1), and the optimum P level varied among plant species. Similarly, root biomass production of *B. seminuda* and *C. truncata* were reduced in the presence of TPH in soil. Surprisingly, root biomass production of *H. prostrata* was not affected significantly by TPH (Figure 1F). Overall, the optimum P level for plant growth in TPH-contaminated soil was higher than that in the uncontaminated control for all the plant species. The two Proteaceae plants showed a slight fluctuation in root biomass production from P0- to P100-treated soils. Dry shoot and root biomass of *C. truncata* continuously increased as applied-P level increased from P0 to P100. Under TPH-contaminated conditions, the root: shoot ratios showed little changes among different P treatments for the three plant species. However, the ratios were significantly higher in all the plant species compared to those in the uncontaminated soil (Table 1).

3.2. Chlorophyll fluorescence

There were an initial increase in ΦPSII and decrease in NPQ for all the plant species in the TPH0-treated (uncontaminated) soil with increasing P application. High P levels caused the reverse pattern for ΦPSII and NPQ values, and the maximal P values varied among plant species (Figure 2). For *B. seminuda*, when soil P application was increased above 25 mg kg^{-1}, ΦPSII decreased and NPQ increased (Figure 2A, B). Similarly, P thresholds of 25 and 50 mg kg^{-1} were for *H. prostrata* and *C. truncata*, respectively (Figure 2C, D, E, F).

The presence of TPH in soil had an adverse effect on chlorophyll fluorescence parameters, which was exhibited by significantly reduced ΦPSII and increased NPQ ($p < 0.05$) (Figure 2). However, the declines in ΦPSII and increases in NPQ were alleviated significantly by the presence of external P, and changes observed varied with P levels for the different plant species. Similar to those in uncontaminated soil, high P levels caused adverse impacts on plant photosynthesis through decreased ΦPSII and increased NPQ in TPH-contaminated soil, and the threshold P application values varied among the plant species. Overall, the threshold P levels for the three plant species in the TPH-contaminated soil tended to be higher than those in the uncontaminated soil (Figure 2).

In this study, the selected chlorophyll fluorescence parameters (ΦPSII and NPQ) and total plant dry biomass were significantly correlated ($r = 0.48$, $p < 0.01$, and $r = -0.78$, $p < 0.01$ ($n = 36$), for ΦPSII and NPQ, respectively).

3.3. Soil dehydrogenase activity

Dehydrogenase activity varied significantly with increasing P levels in both TPH0- and TPH1-treated soil for the three plant species as well as the unplanted control. The DHA values tended to increase with increasing levels of P application up to 100 mg kg^{-1}. Among the plant species, DHA values in *C. truncata*-treated soil were significantly higher than those

in unplanted-, *B. seminuda*-, and *H. prostrata*-grown soil regardless of TPH level ($p < 0.05$) (Figure 3). Surprisingly, the presence of the two Proteaceae species did not show any significant difference in DHA values compared to unplanted control in both TPH0 and TPH1 treatments. The presence of TPH increased dehydrogenase activity in all planted treatments as well as unplanted control, and the increases were observed more obviously with elevated P levels (Figure 3).

3.4. AlkB gene abundance in TPH-contaminated soil

In the TPH-contaminated soil, numbers of *AlkB* gene copies, coding for *n*-alkane-degrading enzymes, differed significantly among unplanted- and planted treatments ($p < 0.05$) (Figure 4). The numbers tended to increase with increasing P levels with the exception of the two members belonging to Proteaceae family. For these two plant species, the abundance of *AlkB* genes declined at the similar P levels causing inhibited plant growth and photosynthesis in the contaminated soil (i.e., 25 and 50 mg P kg^{-1} for *B. seminuda* and *H. prostrata*, respectively). Highest numbers of *AlkB* genes were detected in the contaminated soil cultivated with *C. truncata* at all P levels compared to those in *B. seminuda*-, *H. prostrata*-, and unplanted treatments ($p < 0.05$).

3.5. Hydrocarbon removal rate

Higher TPH removal rates were found in the planted treatments than the unplanted control regardless of P levels (Figure 5). The TPH removal rate increased with increasing P application levels in the unplanted soil (although not significant, $p > 0.05$). Similarly, increasing P level from P0 to P100 resulted in steadily increased TPH removal in the *C.*

truncata-treatment. Additionally, the TPH removal at all P levels from P0 to P100 in this plant species treatment was higher than that in unplanted-, *B. seminuda*-, and *H. prostrata*-treatments. Conversely, for *B. seminuda* and *H. prostrata*, there was an initial increase in TPH removal rate with increasing P from P0 to a threshold P level (25 and 50 mg kg^{-1}, respectively), and then it tended to decrease at higher P levels (Figure 5).

There was a strong positive linear relationship between *AlkB* gene abundance and TPH removal rates (Figure 6), with Pearson's correlation coefficient of 0.957 ($p < 0.001$) (Eq. 2).

$$\text{TPH removal rate } (\%) = 2E-06x + 9.9808 \ (n = 24, R^2 = 0.91) \qquad \text{(E.q. 2)}$$

4. Discussion

4.1. Plant growth

In uncontaminated soil, an increase in P level resulted in an increased root and shoot dry biomass for the plant species only when the P supply was lower than the threshold levels (10 mg kg^{-1} for *B. seminuda* and *H. prostrata*, and 50 mg kg^{-1} for *C. truncata*) (Figure 1). Similar results have been reported previously with respect to the effects of P addition on the growth of a range of Australian plant species, which grow naturally in severely P-impoverished landscapes, under glasshouse conditions (Pang et al., 2010, Williams et al., 2019). The differences in the critical external P levels for plant species (from the same or different family) might be attributed to several factors such as intrinsic growth rates, internal P requirements, root morphology, and P acquisition efficiency (Hill et al., 2006). *C. truncata* favoured a relatively high external P supply compared to the other species; indeed, it was the only species growing well at a P level of 50 mg kg^{-1}, which is the P level barely found in natural environments in Australia (Kooyman et al., 2017).

Optimal plant growth as a result of colonization of degrading microorganisms is considered as a crucial factor for determining the effectiveness of the rhizoremediation process. In this study, the presence of TPH in soil reduced biomass production for all the plant species (Figure 1). It is well established that the presence of TPH causes negative effects on plant growth due to toxicity and impairment of nutrient and water availability under hydrophobicity of the contaminants (Hoang et al., 2021, Hussain et al., 2018b). However, the addition of P accelerated plant growth and biomass production, and the optimum P level for all the plant species was found to increase relative to that in the uncontaminated soil. The results indicated that the presence of TPH reduced plant P availability in soil, thereby reducing plant growth, and consequently, external P supply resulted in a better ability to grow in the contaminated soil. Earlier studies also reported that the application of P improved plant growth and development during remediation of polluted soil (Saleem et al., 2020, Zhou et al., 2020). Nevertheless, high P levels (> 25 mg kg^{-1} for *B. seminuda* and *H. prostrata*) led to a growth decrease for the two Proteaceae species, which are characterized by having a poor ability to down-regulate soil P. Down-regulation is the plant's ability to reduce the P-uptake capacity when supplied-P is in excess of what is required for growth. Plant species with a low capacity to down-regulate their P uptake, therefore, may develop symptoms of P toxicity when the plants are exposed to elevated soil P levels (de Campos et al., 2013, Shane et al., 2004b). Higher root: shoot ratios under the TPH-contaminated soil compared to those in the uncontaminated soil suggested that the plant species increased biomass allocation to roots in the contaminated soil, which was beneficial for plant survival in stressful conditions (Nie et al., 2010, Xu et al., 2015).

4.2. Chlorophyll fluorescence

Photosynthesis is among the most sensitive plant physiological processes likely to change under environmental stresses. Under stressful conditions, functionality of PSII, which is a membrane protein supercomplex that executes the initial reaction of photosynthesis in higher plants, is altered (Baker, 1991), and the extent can be assessed by measuring chlorophyll fluorescence parameters (Huang et al., 2014). Reduction of ΦPSII, which reflects a damage in PSII reaction centres, indicates plant stress (Hichem et al., 2009). Similarly, increased NPQ, which reflects increased dissipation of excess light energy as heat, occurs when plants are under stressful conditions (Gilmore, 1997). In this study, a decrease in ΦPSII and increase in NPQ were detected for the three plant species exposed to the TPH-contaminated soil (Figure 2). However, the application of P decreased NPQ and enhanced ΦPSII, indicating that the supplied-P enhanced resistance to TPH-induced stress. Similarly, after P was supplied, ΦPSII in TPH-stressed plants was improved, perhaps due to PSII repair and chlorophyll synthesis (Liu et al., 2017). In this study, severe P deficiency or toxicity altered the photosynthetic apparatus and photosystems. The results were consistent with previous findings that P deficiency or toxicity increased NPQ and decreased ΦPSII due to activating the regulated photoprotective NPQ-mechanism and photo-oxidative damage (Carstensen et al., 2019, Hernández and Munné-Bosch, 2015).

The correlation between the chlorophyll fluorescence parameters and total dry biomass observed in this study suggested that the quick fluorescence measurements could be used as a selection criterion for plant growth and development. Several studies have reported a similar correlation between plant biomass and leaf photosynthesis parameters (El-Sharkawy et al., 1990, Smethurst and Shabala, 2003). Studying chlorophyll fluorescence alone in this study, however, may not give a full picture of plant photosynthesis (Ikawa et al., 2019). As photosynthetic photon flux density and carbon dioxide are the primary inputs for the photosynthesis process, a combination of gas exchange and chlorophyll fluorescence

measurements is necessary to understand accurately the effects of stresses including soil contamination on leaf photosynthesis in plants.

4.3. Soil dehydrogenase activity

The DHA data indicate that overall microbial activity was enhanced in the presence of *C. truncata* compared to the unplanted control regardless of TPH level (Figure 3). This phenomenon is well-described in the literature, and it is attributed to the rhizosphere effect. However, no significant difference was found among unplanted- and *B. seminuda*- and *H. prostrata*-treatments and it could be attributed to root exudate characteristics and associated rhizosphere microorganisms of these plant species (Hoang et al., 2021). Phosphorus application stimulated microbial activity in both uncontaminated and TPH-contaminated soil and the effect was observed more clearly in the latter soil. The possible reason is that, in the contaminated soil, P becomes a limiting-factor for microbial growth and activity (Wang et al., 2013), especially when there was only a very small fraction of available P in the uncontaminated soil (SI Table 1). Increased DHA in the presence of TPH in soil compared to uncontaminated soil in both planted and unplanted treatments indicated that the aliphatic hydrocarbons can serve as a source of carbon and energy for soil microorganisms (Ramadass et al., 2017, Serrano et al., 2008).

Nonetheless, the overall microbial activity, as measured by DHA, may not be a good indicator for the presence and activity of hydrocarbon-degrading microorganisms in the soil because not all the microorganisms have the ability to metabolize petroleum hydrocarbon compounds (Hoang et al., 2021, Wu et al., 2017). Alkanes, which are saturated hydrocarbons, are the major constituents of diesel and engine oil used in this study. Microbial activity and catabolic-gene expression responsible for aerobic alkane degradation (e.g., *AlkB* genes) are,

therefore, a better indicator (Afzal et al., 2011, Shahsavari et al., 2013). Indeed, *AlkB* genes have been used in bioremediation of aliphatic hydrocarbons as a biomarker to monitor the remediation efficiency (Shahsavari et al., 2013).

4.4. AlkB gene abundance in TPH-contaminated soil

The overall number of gene copies was significantly higher in planted treatments in comparison to those in unplanted treatments, regardless of TPH level (Figure 4). Increasing evidence indicates that plant roots supply their associated rhizosphere micoorganisms with a constant source of alkanes via root exudates and rhizodeposits (Dutta et al., 2013). In this study, higher *AlkB* gene abundance was found in the rhizosphere of the three plant species compared to that in the unplanted control was reported, and this is attributed to a selective pressure induced by the plants on microbial populations in the rhizosphere. In the planted treatments, differences in the *AlkB* gene abundance among *B. seminuda*, *C. truncata*, and *H. prostrata* at the same P level (Figure 4) perhaps resulted from varying quantity and quality of root exudates and rhizodeposits of the plant species (Bulgarelli et al., 2013, Phillips et al., 2012).

The P application showed positive effects on the *AlkB* gene abundance in the TPH-contaminated planted treatments (Figure 4). Similar observations were reported in the literature (Chénier et al., 2003, Schloter, 2014, Tahseen et al., 2016), indicating that the degrading genes were amplified from nutrient addition. It appeared that growth of plants and their associated rhizosphere microorganisms were limited by the availability of nutrients including P in TPH-contaminated soil. Nevertheless, the pattern of varying *AlkB* gene abundance following P addition was not similar for the three plant species. For *C. truncata*, *AlkB* gene abundance increased with increasing P levels while the maximum values were

obtained at 25 and 50 mg kg^{-1} for *B. seminuda* and *H. prostrata*, respectively, then the values tended to decrease (Figure 4). It is worth noting that 25 and 50 mg kg^{-1} P were the P levels required for maximum shoot biomass production for *B. seminuda* and *H. prostrata*, respectively, in the TPH-contaminated soils (Figure 1). These results indicated that plant growth influenced their rhizosphere microbiota, which is in agreement with other studies (Dagher et al., 2019, Li et al., 2019). The *AlkB* gene abundance alone, however, does not allow determining whether the *AlkB*-containing community members are physiologically active or not. Hence, the confirmation that the presence of genes is related to their activity by determining the numbers of transcripts from such genes per cell is necessary. Therefore, it is important to explore the combined assessment of gene abundance and expression in the rhizoremediation of TPH in future studies.

4.5. Hydrocarbon removal rate

Very low TPH removal rates in the unplanted treatment receiving P from 0 to 100 mg kg^{-1}, as well as no significant differences between different P levels, were attributed to a reduction of bioavailable TPH for microbial degradation at the end of the experiment (Figure 5). The TPH used in this study was a mixture of diesel and engine oil, which are characterized with a high sorption potential to soil particles due to high K_{ow} values (Gaskin et al., 2008). In soil cultivated with the three plant species, higher removal rates were observed compared to the control soil with no plants, indicating the effect of plants in the remediation process. The Poaceae species, *C. truncata*, was found to be most efficient in the rhizoremediation of TPH in this study, perhaps due to its ability to develop fibrous roots, which provide sufficient surface for microbial growth and plant nutrient absorption (Gaskin and Bentham, 2010, Zuzolo et al., 2021) (SI Figure 2). Among the two Proteaceae species, *H. prostrata* showed

better growth, as well as higher *AlkB* gene abundance and greater TPH removal rate, in comparison to *B. seminuda*, indicating higher rhizoremediation potential of the former plant species.

Several previous studies have reported a correlation between hydrocarbon-degrading genes and TPH removal rates (Khan et al., 2013, Liu et al., 2015). The positive correlation between the TPH removal rates and *AlkB* gene abundance in the present study (Eq. 2 & Figure 6) confirmed that the quantification of the hydrocarbon-degrading genes could be used as one of the biomarkers to predict the rhizoremediation efficiency of indigenous rhizosphere microorganisms in TPH-contaminated soils. In this study, the gene abundance in the rhizosphere was probably driven by plant identity, plant biomass production, and photosynthesis. The results suggested that the selection of suitable plant species and enhancement of plant growth by addition of P are of utmost importance in the rhizoremediation of TPH-contaminated soil.

5. Conclusions

This study showed that addition of P can improve the growth of wild native plants in soils contaminated with aliphatic hydrocarbons in the rhizosphere. However, the sensitivity of Proteaceae species to P application should be taken into consideration. These results indicate that chlorophyll fluorescence measurement can be used as an assessment of plant performance in stressful conditions. Similarly, *AlkB* gene abundance could be useful as a biomarker to monitor the efficiency of the remediation process. The abundance of *AlkB* genes was strongly influenced by plant identity and P inputs. Future studies should be focused on determining the role of root exudates and rhizodeposits in the stimulation of hydrocarbon degraders to enhance rhizoremediation.

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Figures

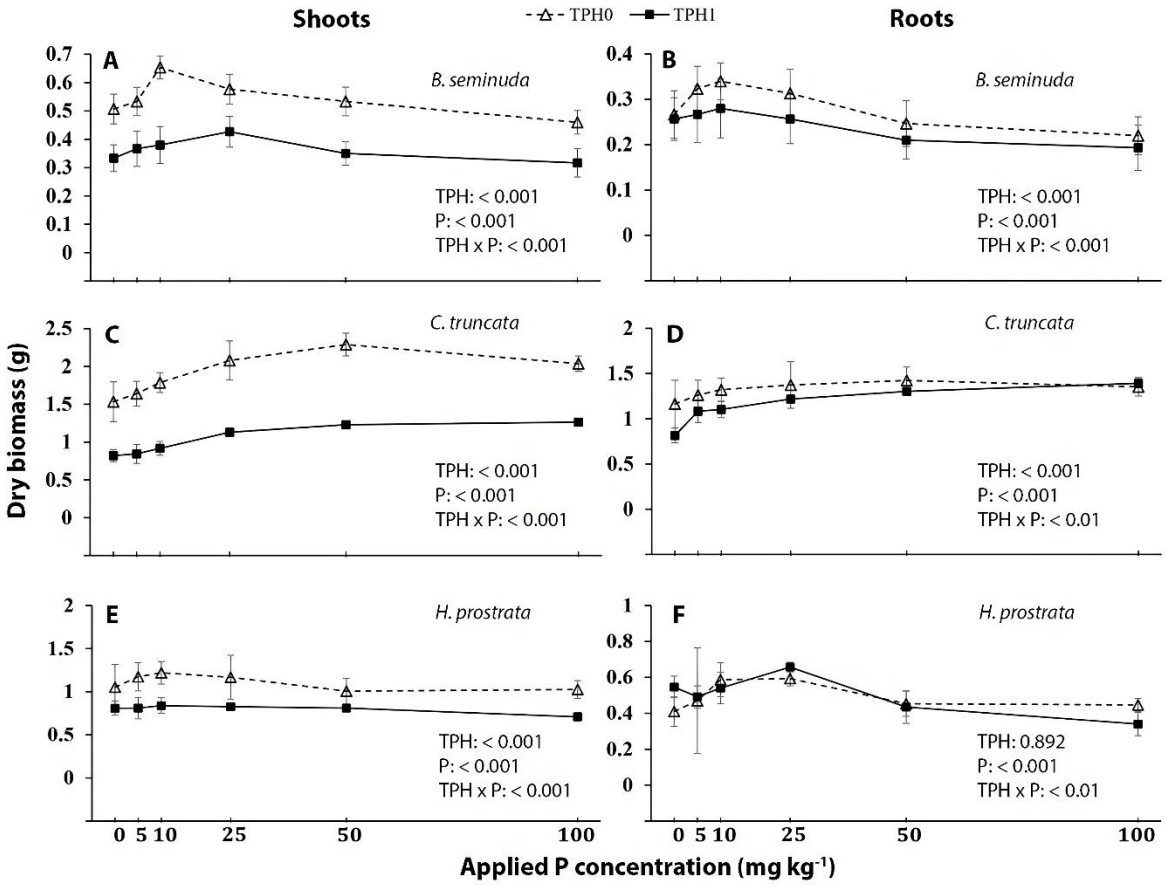

Figure 1. Shoot and root dry biomass of *B. seminuda*, *C. truncata*, and *H. prostrata* grown in uncontaminated (TPH0) and contaminated (TPH1) soils amended with different P levels. TPH: petroleum hydrocarbon effect; P: phosphorus effect; TPH x P: interactive effect of petroleum hydrocarbons and phosphorus. Values are given as mean ± standard deviation, SD (n = 3).

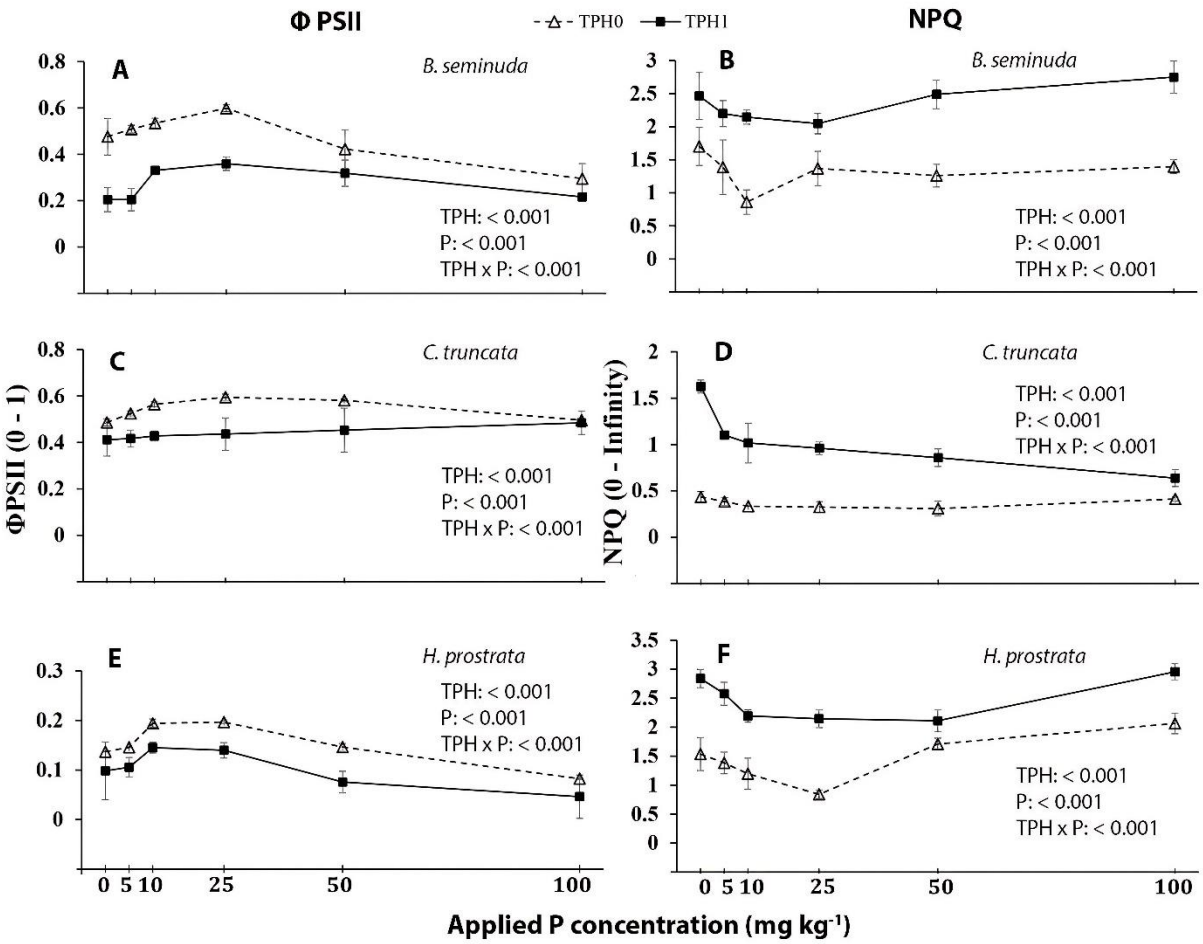

Figure 2. Quantum yield of PSII (ΦPSII) and non-photochemical quenching (NPQ) of *B. seminuda*, *C. truncata*, and *H. prostrata* grown in uncontaminated (TPH0) and contaminated (TPH1) soils amended with different P levels. TPH: petroleum hydrocarbon effect; P: phosphorus effect; TPH x P: interactive effect of petroleum hydrocarbons and phosphorus. Values are given as mean ± SD (n = 3).

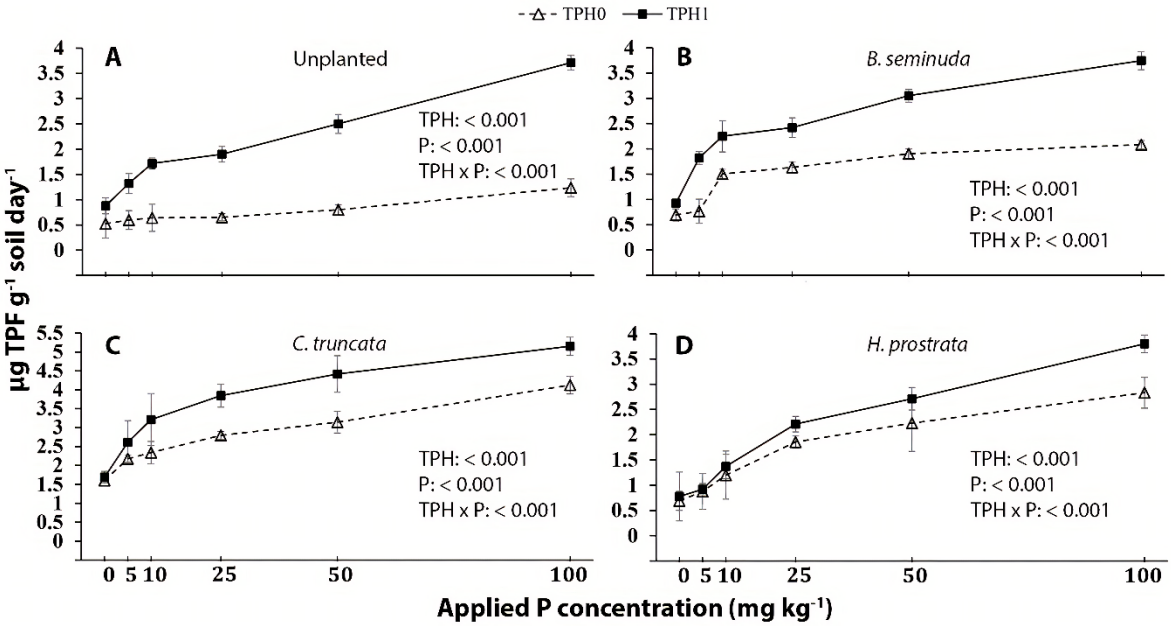

Figure 3. Dehydrogenase activity of unplanted-, *B. seminuda*-, *C. truncata*-, and *H. prostrata*- grown uncontaminated (TPH0) and contaminated (TPH1) soils amended with different P levels. TPH: petroleum hydrocarbon effect; P: phosphorus effect; TPH x P: interactive effect of petroleum hydrocarbons and phosphorus. Values are given as mean ± SD (n = 3).

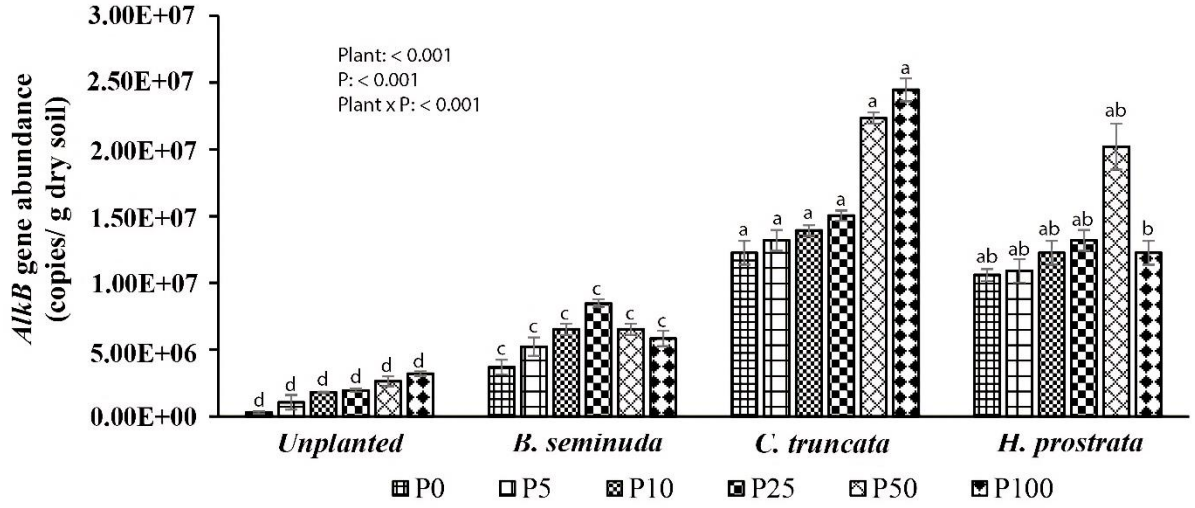

Figure 4. Abundance of *AlkB* genes in the unplanted- and planted-treatments in the TPH-contaminated soil amended with different P levels. Plant: plant species effect; P: phosphorus effect; Plant x P: interactive effect of plant species and phosphorus. Values are given as mean ± SD (n = 3). Values with different letters are significantly different at $p < 0.05$ among the unplanted, B. seminuda, C. truncata, and H. prostrata at the same P level.

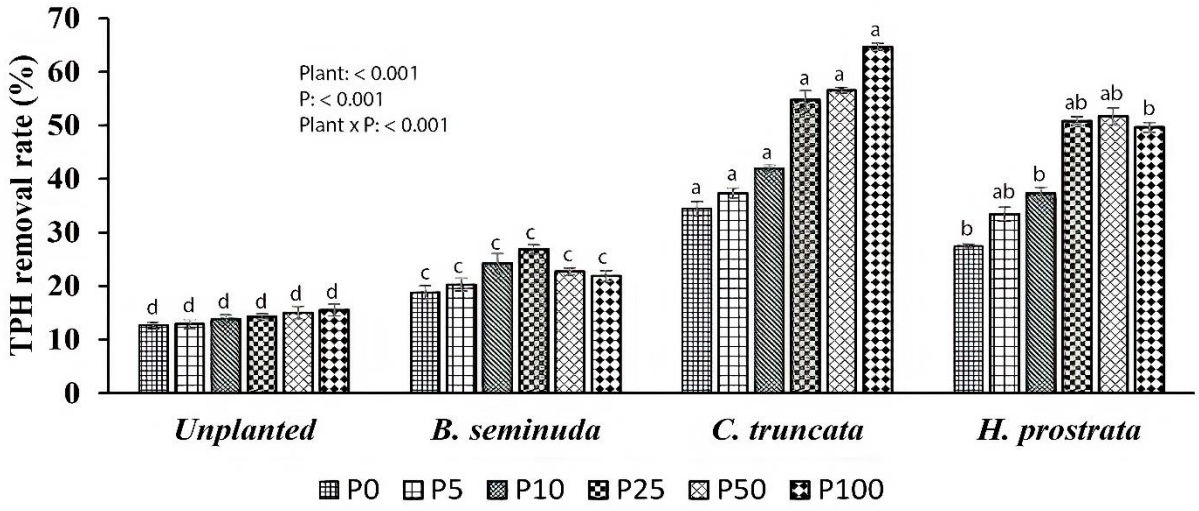

Figure 5. TPH removal rates under the unplanted- and planted-treatments in the TPH-contaminated soils amended with different P levels. Plant: plant species effect; P: phosphorus effect; Plant x P: interactive effect of plant species and phosphorus. Values are given as mean ± SD (n = 3). Values with different letters are significantly different at $p < 0.05$ among the unplanted, B. seminuda, C. truncata, and H. prostrata at the same P level.

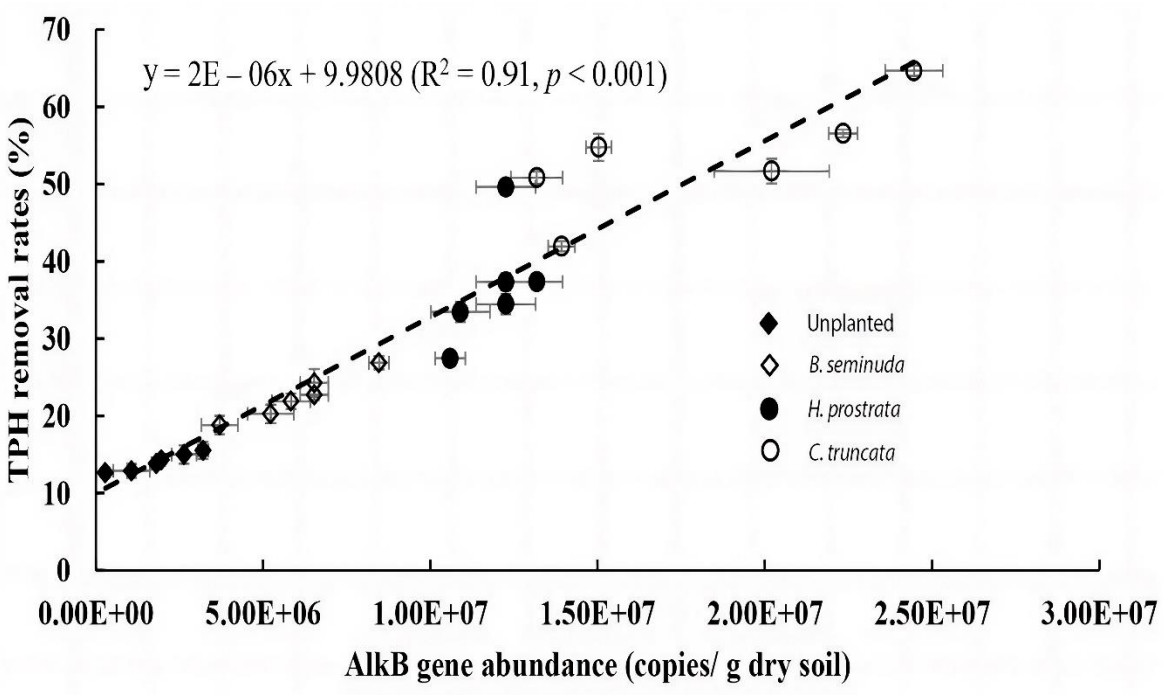

Figure 6. Correlation between TPH removal rate and *AlkB* gene abundance across the unplanted and planted treatments at all P levels.

Table

Table 1. Root: shoot ratios among different P and plant treatments in uncontaminated (TPH0) and TPH-contaminated (TPH1) soils

P levels	**Plant species**					
(mg kg^{-1})	***B. seminuda***		***C. truncata***		***H. prostrata***	
	TPH0	TPH1	TPH0	TPH1	TPH0	TPH1
P0	0.53 ± 0.04	1.01 ± 0.08	0.75 ± 0.01	0.99 ± 0.05	0.38 ± 0.01	0.59 ± 0.02
P5	0.61 ± 0.07	0.97 ± 0.05	0.77 ± 0.02	0.99 ± 0.01	0.4 ± 0.01	0.58 ± 0.04
P10	0.52 ± 0.02	0.99 ± 0.03	0.73 ± 0.01	0.98 ± 0.06	0.48 ± 0.01	0.64 ± 0.02
P25	0.54 ± 0.07	1.13 ± 0.04	0.66 ± 0.02	1.07 ± 0.01	0.5 ± 0.01	0.79 ± 0.03
P50	0.46 ± 0.04	1.09 ± 0.07	0.62 ± 0.01	1.05 ± 0.01	0.45 ± 0.01	0.53 ± 0.01
P100	0.48 ± 0.04	1.01 ± 0.07	0.66 ± 0.01	1.1. ± 0.03	0.43 ± 0.02	0.51 ± 0.01

Supplementary Information

1. Details about soil collection, characteristics, and initial TPH concentration

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).

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 mixture of diesel and engine oil (1:1, w/w) was used as source of total (aliphatic) petroleum hydrocarbon (TPH) in this study.

Table 1. Selected physicochemical characteristics of the experimental soil and initial TPH concentrations used in the rhizoremediation study

pH (H_2O)	**pH** ($CaCl_2$)	**EC** ($\mu S\ cm^{-1}$)	**Organic C** (%)	**Total N** (%)	**Available P** ($mg\ kg^{-1}$)	**Total S** (%)	**TPH1** ($mg\ kg^{-1}$)
6.04	5.41	280	0.78	0.08	3.0	0.002	11,370± 52 (n=3)

2. Phosphorus sorption isotherms

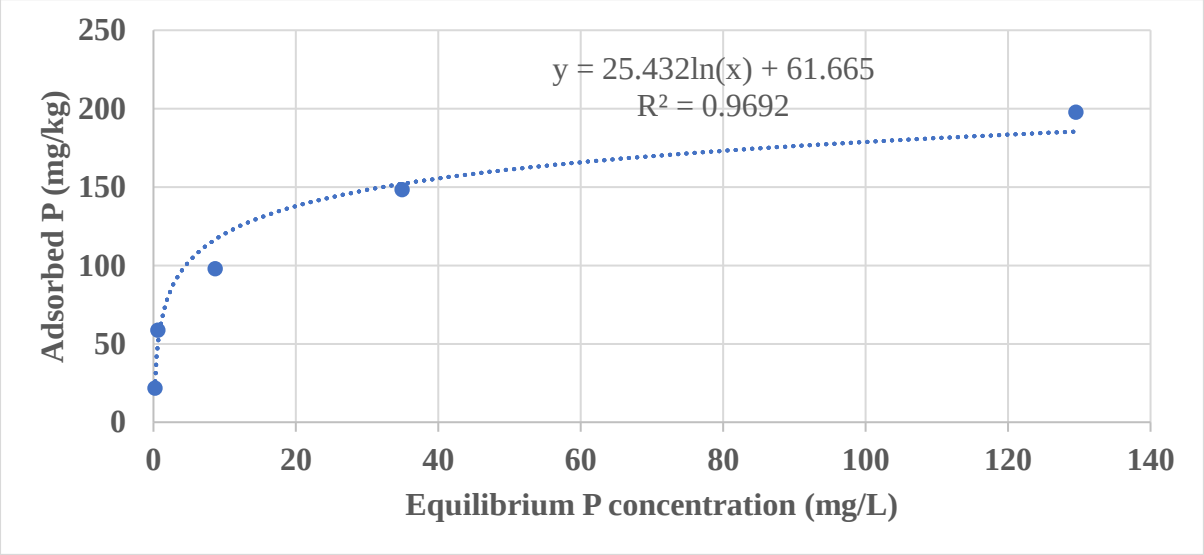

Figure 1. Phosphorus sorption isotherms for the experimental soil

3. Glasshouse conditions

The plant growth experiment was conducted under controlled glasshouse conditions (25ºC for 16 h (daytime), and 18ºC for 8 h (night), typical of the New South Wales State of Australia). The glasshouse received natural lights only.

4. Seedling emergence in Petri dishes and plant growth in micro-pots

Seeds of the wild plant species were commercially obtained from Nindethana Seed Service Pty, Ltd, (Western Australia, Australia). Seeds were soaked in 1% sodium hypochlorite for 5 min and rinsed thoroughly to eliminate any fungal pathogen.

To 120 x 120 x 17 mm (square) polystyrene Petri dishes, two layers of filter papers were placed, and evenly soaked with 3 mL of ultrapure water (Milli-Q®, 18.2 MΩ.cm). To each dish, 25 seeds of *B. seminuda* and *H. prostrata* were added in triplicate maintaining 5 x 5 grid spaces and sealed with Parafilm. All the plant species were germinated at 25ºC and monitored regularly for seven days. Once radicles emerged to a certain length (approx. 5 to 10 mm), the germinated seeds were placed in micro-plant pots (one seedling per pot) filled with autoclaved propagation sand (obtained from Bunnings, Australia).

Seeds of *C. truncata*, ten seeds were directly sown in each micro-plant pot (twenty replicated pots, 200 seeds in total) to obtain good "seed to soil" contact, which is considered as the key to grass seed germination. After 2 weeks, all pots were uniformly thinned out to one individual plant per pot to ensure homogeneity amongst all plant species.

Nutrient solution (20 mL) was added to every pot daily. The nutrient solution was prepared according to Asher and Loneragan (1967). Particularly, the following concentrations of nutrients were achieved (in µM) in the solution: calcium 250, magnesium 100, potassium 250, sulfur 100, nitrogen (as NO_3^-) 750, nitrogen (as NH_4^+) 100, phosphorus 1, chlorine 100, iron 2 (as Iron Ethylenediaminetetraacetic acid, FeEDTA), copper 0.1, manganese 1, zinc 0.5, cobalt 0.04, boron 3, and molybdenum 0.02. This nutrient solution was successfully tested for growing native plant species previously by Lamb et al. (2010).

5. Soil dehydrogenase activity determination

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.

6. The sequences, amplicon sizes, and annealing temperatures of the employed PCR primers

Table 2. The fragment length of PCR primers and annealing temperature conditions (Liu et al., 2015)

Primer	Proteins targeted	Sequence (5'-3')	Amplicon size (bp)	PCR annealing temperature (°C)
AlkB	Alkane monooxygenases	AACTACMTCGARCAYTACGG TGAMGATGTGGTYRCTGTTCC	100	50
16S rDNA	-	CGGTGAATACGTTCYCGG	126	58

7. TPH analysis of the soil

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™ 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 as follows:

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

8. An example of mass spectrum of initial and residual TPH after rhizoremediation

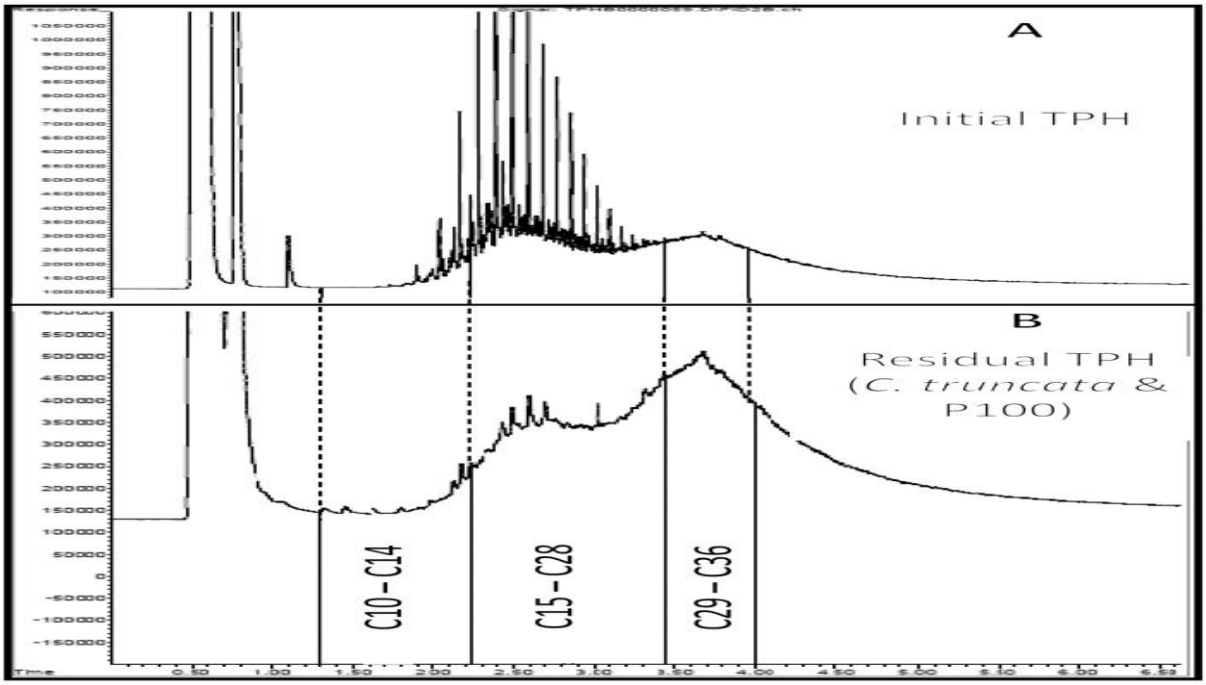

Figure 2. Mass spectrum of the initial (A) and residual TPH after rhizoremediation (B)