The impact of lead co-contamination on ecotoxicity and the bacterial community during the bioremediation of total petroleum hydrocarbon-contaminated soils

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The continued increase in the global demand for oil, which reached 4,488 Mtoe in 2018, leads to large quantities of petroleum products entering the environment posing serious risks to natural ecosystems if left untreated. In this study, we evaluated the impact of co-contamination with lead on the efficacy of two bioremediation processes, natural attenuation and biostimulation of Total Petroleum Hydrocarbons (TPH) as well as the associated toxicity and the changes in the microbial community in contaminated soils. The biostimulated treatment resulted in 96% and 84% reduction in TPH concentration in a single and a co-contamination scenario, respectively, over 28 weeks of a mesocosm study. This reduction was significantly more in comparison to natural attenuation in a single and a co-contamination scenario, which was 56% and 59% respectively. In contrast, a significantly greater reduction in the associated toxicity of in soils undergoing natural attenuation was evident compared with soils undergoing bio-stimulation despite the lower TPH degradation when bioassays were applied. The earthworm toxicity test showed a decrease of 72% in the naturally attenuated toxicity versus only 62% in the biostimulated treatment of a single contamination scenario. In a co-contamination scenario, toxicity decreased only 30% and 8% after natural attenuation and biostimulation treatments, respectively. 16s rDNA sequence analysis was used to assess the impact of both the co-contamination and the bioremediation treatment. NGS data revealed major bacterial domination by Nocardioides spp., which reached 40% in week 20 of the natural attenuation treatment. In the biostimulated soil samples, more than 50% of the bacterial community was dominated by Alcanivorax spp. in week 12. The presence of Pb in the natural attenuation treatment resulted in an increased abundance of a few Pb-resistant genera such as Sphingopyxis spp. and Thermomonas spp in addition to Nocardioides spp. In contrast, Pb co-contamination completely shifted the bacterial pattern in the stimulated treatment with Pseudomonas spp. comprising approximately 45% of the bacterial profile in week 12. This study confirms the effectiveness of biostimulation over natural attenuation in remediating TPH and TPH-Pb contaminated soils. In addition, the presence of co-contaminants (e.g. Pb) results in serious impacts on the efficacy of bioremediation of TPH in contaminated soils, which must be considered prior to designing any bioremediation strategy.

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1. Introduction

The demand for petroleum products continues to increase; in 2018, world oil demand increased by 1.5 million barrels a day, 1.6% higher than the average in the last decade (IEA, 2018). Inevitably, during the exploration, recovery, storage and transport of such large quantities of petroleum products, vast amounts of petrogenic hydrocarbons enter the environment causing serious land contamination (Varjani, 2017).

Total Petroleum Hydrocarbons (TPHs), the main component of crude oil comprise a broad family of short (C8–C16) and long-chain (C17–C40) aliphatic hydrocarbons and a minor group of aromatic compounds (1–5 rings), which largely comprise carbon and hydrogen (Abbasian et al., 2015). TPHs are classified as priority...
Various chemical and physical techniques can be used to treat TPH contaminated soil, such as soil washing, soil vapour extraction, incineration and solidification (Jasmine and Mukherji, 2019; Chen et al., 2019). These techniques, are however relatively expensive due to operational costs and damage the natural properties of the soil (Xu and Lu, 2010). In contrast, bioremediation, which recruits indigenous biological agents to breakdown the contaminants, represents a simple, environmentally safe and cost-effective technique of contaminated soil remediation (Ron and Rosenberg, 2014). Indeed, the first response to any soil contamination takes place naturally through the action of the indigenous microflora of the soil. This process, natural attenuation occurs when biodegradation of the contaminant occurs without any enhancement or human intervention (Yu et al., 2005). The biodegradation rate can be accelerated by biostimulation where nutrients such as carbon, phosphorus, nitrogen and oxygen are added to the contaminated soil in order to stimulate the microflora and thus accelerate the biodegradation process (Andreolli et al., 2015). Biostimulation has been successfully applied to many contamination cases resulting in a significant decrease in TPH concentration. For example, higher degradation rates (78–90%) of TPH have been reported using bio-stimulation in comparison with natural attenuation (61–77%) (Khudur et al., 2015; Qin et al., 2013).

However, one major challenge impacting the efficiency of the bioremediation of TPH contaminated soil is co-contamination with heavy metals (Thavamani et al., 2012; Olaniran et al., 2013a; Liu et al., 2017; Khudur et al., 2018b). Lead (Pb) is likely to be present alongside TPH as a co-contaminant in many aged oil spills, as Pb was widely used as a fuel additive (Khudur et al., 2018a). A recent study confirmed that Pb concentrations (50–1750mg kg⁻¹) were detected in 58 surface soil samples collected from the Melbourne metropolitan area, Australia (Laidlaw et al., 2018). According to Australian guidelines established by the National Environment Protection Council, the Health Investigation Levels (HILs) of soil Pb is 300mg kg⁻¹ for residential areas with gardens or accessible soil and 1500mg kg⁻¹ for industrial or commercial areas (NEPC, 2011).

Lead is highly toxic to soil biota since it has a higher affinity for oxygen and thiol groups, enabling it to displace essential metals from their binding site (Bruins et al., 2000; Nouha et al., 2016). We recently reported an elevation in ecotoxicity in aged TPH-heavy metals co-contaminated soils (Khudur et al., 2018a). The presence of Pb in TPH-contaminated soil affects the structure of the microbial community which underpins any biodegradation process. Although numerous recent studies have addressed the biodegradation of TPH in co-contaminated environments, very little is known about the changes in dynamics of natural soil microbial communities during the bioremediation of co-contamination scenarios (Varjani, 2017; Liu et al., 2017). Understanding changes in the microbial communities’ structure following exposure to contaminants represents a crucial step in designing an effective bioremediation strategy (Chen et al., 2015; Klimek et al., 2016).

The aim of this study was to evaluate the efficacy of bioremediating TPH in TPH-Pb co-contaminated soils and to assess the subsequent impact of the remediation process on soil ecotoxicity. In addition, 16S amplicon sequencing was employed to assess the changes in the microbial community during the remediation process. In our previous studies, we evaluated the efficacy of natural attenuation and biostimulation in remediation TPH-contaminated soil by creating an optimal C:N:P molar ratio in the biostimulated soils (Khudur et al., 2015). We have also reported that TPH and heavy metal co-contamination significantly elevated the remaining ecotoxicity of the weathered, naturally attenuated soils (Khudur et al., 2018a). Here, we are reporting, for the first time, the efficacy of natural attenuation and biostimulation in remediating TPH-Pb co-contaminated soils, using RemActiv a commercially available biostimulator. In addition, to the best of the authors’ knowledge, the impact of Pb co-contamination together with bio-stimulation on the associated ecotoxicity and the soil bacterial community during the bioremediation process has not been previously reported. Thus, here we aim to achieve a solid basis upon which to design appropriate bioremediation strategies for co-contaminated soils.

2. Materials and methods

2.1. Experiment design

Clean pastureland soil from Victoria, Australia, was collected using a sterilized shovel and placed in clean plastic buckets and then transported to RMIT University, Bundoora campus (Schinner et al., 2012). The collected soil was stored overnight at ambient temperature and then sieved using a 6 mm sieve. The soil was divided into six sub-samples (12 kg each) to set up the experimental treatments. The treatments included (i) Natural attenuation (NA) and (ii) Biostimulation (BS) of TPH only contaminated soil; (iii) Natural attenuation (NA-H) and (iv) Biostimulation (BS-H) of TPH-Pb co-contaminated soil; (v) a Pb only contaminated soil (HM) and (vi) a control soil (CON). RemActiv (RA), a commercially available biostimulator, was used for the biostimulation treatments (Ziltek, 2015). RA was marketed by Ziltek Pty Ltd. The spiking and bio-stimulating protocols are shown in Table 1. Characterisation of the test soil and RA is shown in Table 2. The moisture content of all the treatments was maintained at 20% (W/W) throughout the experiment by adding the required amount of water twice a week. After spiking the soils, each treatment was divided into 3 replicates (4 kg of soil each) and placed in plastic pots (Khudur et al., 2015). A mesocosm experiment was set up for 28 weeks in a greenhouse. The inside temperature of the greenhouse was recorded every hour using an EL-GFX-1 temperature data logger. Soil samples were taken from each treatment every 4 weeks for further analysis, following the protocol previously applied (Koshfal et al., 2016).

2.2. Quantitative analysis of the contaminants

2.2.1. TPH concentration measurement

The TPH (C₁₀⁻C₄₀) concentration was determined using RemScan technology following the protocol previously described (Khudur and Ball, 2018). Soil samples of approximately 50 g were taken and air-dried overnight. The TPH concentration of the dried soil samples were measured using RemScan device, which uses a diffuse reflectance (mid)-infrared Fourier transform (DRIFT) spectrometer.

2.2.2. Lead (Pb) concentration measurement

Extraction of Pb from soil samples was performed using an acid digestion protocol. One gram of air-dried soil was weighed into glass test tubes which contained 3 ml concentrated nitric acid (HNO₃) and 1 ml concentrated hydrochloric acid (HCl). Tubes were heated at 85 °C for 3 h using a heating block and then cooled to room temperature. A volume of 5 ml of Milli-Q (MQ) water was
2.3. Ecotoxicity analysis

2.3.1. Earthworms’ acute toxicity test

An acute toxicity test was performed on all the treatments using earthworms, Eisenia andrei, which were obtained commercially from Bunnings Warehouse. To perform this test, five different concentrations for each replicate from selected time points were prepared and placed in glass jars by mixing the contaminated soil with clean soil in a total of 200 g of soil in each jar. For each concentration, ten adult earthworms were randomly selected, washed and placed into the soil (OECD, 1984). After 14 days of incubation at room temperature, the number of survivors was counted and the lethal concentration, which can be defined as the concentration that kills 50% of the test animal population; values were calculated for each treatment using ToxRat Professional software (Khudur et al., 2015). For the purpose of comparing the samples’ toxicity, the toxicity unit (TU) was calculated as TU= \((1/LC_{50}) \times 100\) (Khudur et al., 2018a).

2.3.2. Bioluminescence inhibition testing: the Microtox test

The Microtox test was performed following the standard method (ASTM, 2004). The acute Microtox reagent (MODERN WATER Microtox®) and the reconstitution medium were supplied by Streamline Hydro Pty Ltd. The test samples were prepared as previously described (Khudur et al., 2018a). For each replicate, 1 g of air-dried, sieved soil was added to 9 ml of water, placed on a shaker for 24 h and then centrifuged for 5 min at 5,000 rpm. The supernatant was taken and measured using the Microtox® Model 500 Analyzer. Effective Concentration 50 (EC_{50}) in which the light emission decreases by 50% at a given time, of each replicate sample, was calculated at 5, 10 and 15 min using the software provided.

2.4. Bacterial community’s analysis

2.4.1. Bacterial DNA extraction

Extraction of the genomic DNA from soil samples was performed using a PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc. USA) following the manufacturer’s protocol. The quality and quantity of the extracted DNA were determined using a NanoDrop Standard V2.1 spectrophotometer (Thermo Scientific – USA). The extracted DNA samples were stored at −20 °C until further analysis.

2.4.2. Quantification of bacterial 16s rRNA and alkB genes

Quantitative analysis of the 16s rRNA gene as an indication for total bacteria as well as the alkB gene, which is the most widely used indicator of TPH-degrading bacteria, was performed by real-time polymerase chain reaction (qPCR) using a QIAGEN RotorGene machine as previously described (Shahsavari et al., 2016).

2.4.3. Next generation sequencing (NGS) of the bacterial communities

In order to analyse the structure of the bacterial communities, primer set V3-forward (5’GCTACGGGNGGCWGCAG3’) and V4-reverse (5’GACTACHVGGGTATCTAATCC3’) were used to amplify the V4–V5 region of the bacterial 16s rRNA gene (Dehengia et al., 2015). The guidelines in the 16s Metagenomic Sequencing Library Preparation guide (Illumina) using Nextera XT Index Kit (Illumina, San Diego, CA) were used for the library preparation process. Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, CA) and 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) were used for the quantification of the library DNA. A MiSeq platform (Illumina, San Diego, CA) at the School of Science, RMIT University was used for sequencing (Khudur et al., 2018a). The 16s Metagenomics workflow available in Illumina BaseSpace (https://basespace.illumina.com/home/index) which uses a high-performance version of the RDP Naïve Bayes taxonomic classification algorithm was used to analyse the sequences (Wang et al., 2007). The visualisation of the operational taxonomic units (OTUs) table was performed using MEGAN6. The number of bacterial taxa was calculated using PAST software (Andreonii and Gianfreda, 2007).

2.5. Data analysis

The experimental data was subject to Analysis of Variance (ANOVA) using XLSTAT 2018 software. The separation of mean values was performed using the Least Significant Difference (LSD) test. The differences were considered significant at \((P = 0.05)\), where the F-value was significant. Data are presented as mean and standard deviation of three replicates.
3. Results and discussion

3.1. Concentration of contaminants

3.1.1. Assessing TPH concentration

The efficacy of bioremediation in the reduction of TPH in the TPH contaminated and TPH-Pb co-contaminated soils is shown in Fig. 1A. A significant reduction in TPH concentration was observed in both bioremediation strategies implemented, natural attenuation and biostimulation, for single and co-contamination scenarios.

The biostimulation strategy resulted in significantly higher efficiency in terms of bioremediation of TPH contaminated soil in comparison with natural attenuation. In a single contamination scenario (TPH only), a reduction of 95.9% in the starting concentration was observed in the biostimulated soil samples (BS) within 28 weeks in comparison with only 55.6% TPH reduction in natural attenuated soil samples (NA). Similarly, in a co-contaminated scenario (TPH and Pb), the biostimulated soil samples (BS-H) showed a higher reduction of 83.7% in TPH concentration compared to the natural attenuated soil samples (NA-H) which resulted in only 59.7% TPH reduction. The effectiveness of biostimulation over natural attenuation in remediating TPH contaminated soil in different contamination scenarios has also been demonstrated in previous studies (Khudur et al., 2015; Safdari et al., 2018; Xu and Lu, 2010). No TPH was detected in the HM and CON treatments.

In contrast to the findings of other studies that showed a rapid decrease (about 50%) of the TPH concentration in the first 2–4 weeks of the bioremediation processes followed by a slower degradation rate (Khudur et al., 2015; Bento et al., 2005; Suja et al., 2014, Koshlaf et al., 2016, Rodriguez-campos et al., 2019, Chen et al., 2019), the opposite degradation trend was observed in this study. All the treatments showed a relatively slow degradation of TPH in the first 4–8 weeks followed by a faster reduction of the TPH, especially in the biostimulated treatments after 8 weeks. One possible explanation could be the effect of temperature on the biodegradation rate. Temperature is the most crucial environmental factor influencing the degradation rate of TPH since elevated temperature increases the bioavailability of TPH during the biodegradation by changing their viscosity and diffusion (Coulon et al., 2007; Chaudhary and Kim, 2019). In addition, temperature enhances the metabolic activities, as well as the activity of bacterial enzymes involved in TPH degradation (Chaudhary et al., 2019; Abed et al., 2015). This experiment was set up as a mesocosm in the greenhouse without temperature control to simulate natural environmental conditions. The mean temperature at the start of the experiment was 14.8°C, rising to 27.4°C after 28 weeks (Fig. 1B). The optimum temperature for TPH biodegradation has been reported to lie within 20–40°C (Das and Chandran, 2011; Chaudhary et al., 2019; Sun et al., 2019).

3.1.2. Assessing Pb concentration

The Pb concentration in soils was determined using AAS. The Pb concentration in the NA-H, BS-H and HM treatments ranged between (1910–1980 mg kg\(^{-1}\)), showing no significant change in concentration during the 28 weeks of bioremediation. No Pb was detected in the NA, BS and CON treatments.

The presence of Pb affected the degradation of TPH in both bioremediation strategies. Despite the presence of the co-contaminant, the TPH in the NA-H treatment showed slightly higher (though not significant) degradation compared with the NA treatment (59.7% and 55.6%, respectively). The addition of nitrate, from Pb(NO\(_3\))\(_2\) which was added as a source of Pb, might have increased the nutrient level in the soil and therefore enhanced the degradation rate by marginally increasing bacterial growth and activity. In contrast, in the nutrient-rich (biostimulation) treatment the degradation date of TPH was negatively influenced by the presence of Pb. A significant reduction in the degradation rate was observed in BS-H over the BS treatment, 59.6% and 95.9%, respectively, indicating inhibition of bacterial activity by the co-contaminant. In addition to its toxicity, the co-presence of Pb inhibits many metabolic pathways, such as the enzymatic and respiratory processes of many hydrocarbon-degrading bacteria (Alisi et al., 2009; Al-saleh and Obuekwe, 2005; Dong et al., 2013; Deary et al., 2018).

![Fig. 1.](image-url) 

Fig. 1. (A) Reduction in TPH concentration over 28 weeks of bioremediation of TPH and TPH-Pb contaminated soils using natural attenuation and biostimulation strategies. Percentage of reduction data is presented as Mean ± SD, n = 3. (B) Temperature profile throughout the 28 weeks of bioremediation of TPH contaminated-soil. Data are presented as mean ± SD, n = 672.
3.2. Soil ecotoxicity

Since both contaminants involved in this study are known to pose high toxicity to the soil biota, the earthworm acute toxicity and Microtox tests were conducted to assess the toxicity of the bioremediated soils. The earthworm acute toxicity test (Fig. 2 A) showed that the NA treatment resulted in the highest decrease in toxicity, about 72%, where TU values dropped significantly from 9.2 to 2.6. The BS treatment also showed a significant drop in TU, from 9.2 to 3.5, which represents a 62% decrease. Similarly, the Microtox test (Fig. 2 B) showed that TU values dropped from 8.5 to 1.2 and 7.8 to 1.7 for NA and BS, respectively, which represents an 86% and 78% reduction in toxicity of NA and BS, respectively. Despite the higher reduction in the TPH concentration in the BS treatment, the associated ecotoxicity was higher in the BS rather than in the NA treatment. Many researchers have shown that in general, the decrease in ecotoxicity shows a positive correlation with a reduction in TPH concentration (Khudur et al., 2015; Shahsavari et al., 2017; Tang et al., 2011; Dorn and Salanitro, 2000). However, as seen in this study, previous studies have shown that a reduction in TPH concentration does not necessarily reflect the level of ecotoxicity and the remediated soil could still pose a potential toxicity risk to the biota (Khudur et al., 2015; Makadia et al., 2011; Phillips et al., 2000). While the results confirm the efficacy of RemActiv as a biostimulating agent, the ecotoxicity results suggest that care must be taken in the application of any biostimulating agent to ensure that the addition does not impact the toxicity of the soil through an imbalance in elemental soil composition.

The presence of Pb together with TPH significantly influenced the ecotoxicity associated with the co-contaminated soil. The toxicity of the NA-H treatment to the earthworms and the marine bioluminescent bacteria was significantly higher than the NA treatment. Likewise, the BS-H treatment showed significantly higher toxicity in comparison to the BS treatment. Throughout the 28-week incubation, the TU values for the earthworm toxicity test dropped from 10 to 7 and 13 to 12 representing 30% and 8% decrease in the associated toxicity for NA-H and BS-H treatments, respectively. In addition, the Microtox test showed a 50% and 38%
decrease in the associated toxicity of the NA-H and BS-H treatments, respectively. No toxicity was observed in the HM and CON treatments.

Since the ecotoxicity of the co-contamination treatments was significantly higher than the single contamination, the results of this study suggest that the addition of extra chemicals, even nutrients, to TPH contaminated soils negatively affects the associated toxicity. Besides the toxicity posed by an individual contaminant, a significantly elevated toxicity has been observed in aged co-contaminated samples in previous studies (Khudur et al., 2018a). The presence of Pb has been reported to increase the bioavailability of the organic contaminants as well as affect the transportation activity of the microbial cell membrane (Olaniran et al., 2013b; Gauthier et al., 2015).

3.3. Soil bacterial community

In the current study, changes in the community structure of the soil bacteria during the bioremediation process was investigated using qPCR and NGS. Since soil microorganisms represent the backbone of the contaminant biodegradation process, it is imperative to gather information on the dynamic structure of the microbial communities to design appropriate bioremediation strategies.

3.3.1. Quantification analysis of bacterial 16S rRNA and alkB genes

qPCR was performed to evaluate the copy number of bacterial 16S rRNA and alkB genes. The results revealed that the number of copies of the 16S rRNA gene significantly increased starting from week 4 after exposure of the microbiota to the contaminants (Fig. 3A). The highest number of copies observed in the BS treatment reached a peak of $3.0 \times 10^{10}$ in week 12, significantly higher than all other sampling points. This finding suggests that bio-stimulation has a positive effect on bacterial growth in contaminated soil. The NA treatment also showed a significant increase in copy number starting from week 4 until week 20, although the increase was significantly lower than the BS treatment.

Although BS-H showed a significant rise in the number of copies in week 8, reaching a peak of $2.1 \times 10^{10}$, the results suggest that the presence of Pb negatively affected the biostimulation effect on...
bacterial growth. In contrast, the Pb co-contamination has no significant effect on the total bacterial growth in natural attenuated treatments. The numbers of 16S rRNA gene copies averaged $4.8 \times 10^3$ and $4.7 \times 10^3$ for HN and CON treatments, respectively, showing no significant changes within 28 weeks. The results of the alkB gene, which is an indicator of the TPH degradation potential of the soil microflora (Guibert et al., 2016), also showed a significant increase in the number of copies starting from week 4 (Fig. 3B). A significant rise in the number of copies was observed in the BS treatment starting from week 8. The number of alkB genes reached the highest peak of $1.5 \times 10^6$ in week 12 which represents around 50% of the total soil bacteria at this sampling point. A similar trend was observed in the NA treatment, reaching the highest number of, $6.3 \times 10^5$ gene copies in week 12, significantly lower than in the BS treatment, perhaps explaining the positive effect of biostimulation, elevating the alkB gene copy number. The growth of hydrocarbon-degrading bacteria is enhanced by biostimulation (Shahi et al., 2016).

The presence of Pb significantly reduced the number of copies of the alkB gene in both natural attenuation and biostimulation treatments. Although the NA-H and BS-H treatment showed a significant increase in alkB gene number in week 4, a significantly lower number of copies was observed in comparison to the number detected in soils with only TPH contamination, NA and BS. These findings demonstrate the inhibitory effect of the co-contaminants on the growth of hydrocarbon-degrading bacteria (Ramadass et al., 2016).

3.4. Bacterial communities’ structure and composition

16S rRNA sequencing was carried out to evaluate the changes in the structure of the bacterial communities during the bioremediation process, as well as the effect of contaminants on the hydrocarbon-degrading bacteria. NGS data revealed that many changes in the structure of the bacterial communities occurred during 28 weeks of the bioremediation process depending on the presence of the contaminants and nutrients in each treatment. Bacterial diversity, using the number of taxa index, was significantly reduced in both natural attenuation treatments (NA and NA-H) in week 20 and 28; in contrast, bacterial diversity in both biostimulated soils (BS and BS-H) reduced early, in week 4 (Table 3). The number of taxa varied from 441-397 and 437-375 for HM and CON treatments, respectively, showing no significant changes in bacterial diversity. This finding suggests that the addition of nutrients to TPH contaminated soil significantly affected bacterial diversity despite the absence/presence of the co-contaminant. In addition, the earlier reduction in TPH concentration in biostimulated soils may explain the earlier changes in bacterial diversity. Several researchers have reported more diverse bacterial communities in high TPH-contaminated soils in comparison to less contaminated soils (Liu et al., 2009; Jung et al., 2010). The presence of Pb, however, significantly decreased the number of taxa, especially in week 20 and 28 in all treatments. It has previously been reported that although many indigenous bacteria are capable of degrading TPH, their activity is adversely affected by the presence of toxic co-contaminants (Xu and Lu, 2010).

Taxonomy profile analysis of the top 50 genera, which represent about 99.9% of the entire community, based on the total OTUs revealed differences in relative dominance in soils between different treatments during 28 weeks of bioremediation (Fig. 4). As expected, all treatments showed a similar taxonomy profile at their starting point. Many genera were common among all treatments showing no major dominance, including Nocardioides spp., Dokdonella spp., Pseudomonas spp., Alcanivorax spp., Sphingopyxis spp. Nocardioides spp. and Thermomonas spp. Except for CON (Fig. 4C) and HM (Fig. 4F) treatments, which showed no major changes, all other treatments showed changes in their taxonomy profile starting from week 4. A gradual increase in domination by Nocardioides spp., a well-known hydrocarbon-degrading bacterium (Schippers et al., 2005) was observed in NA treatment, attaining 40% and 35% of the total population in week 20 and 28, respectively (Fig 4A).

In contrast, biostimulation treatments showed a different trend of bacterial domination (Fig 4B). In BS treated soils, the abundance of a few hydrocarbon degrading bacteria increased in week 4, including Nocardioides spp., Pseudomonas spp., Sphingopyxis spp. and Nocardioides spp. Most notably, no one organism was dominant. However, Alcanivorax spp. represented 30% of the total population in week 8 and more than 50% in week 12. Alcanivorax spp. has been reported as a predominant bacterium in nutrient-rich TPH contaminated environments (Cappello et al., 2007). This major domination in BS treatment explains the significant rise in the copy number of the alkB gene in week 8 and 12 (Fig 3B).

In the presence of Pb, Nocardioides spp. was the dominant organism in NA-H treated soils, reaching around 25% and 35% of the total bacterial population in week 20 and 28, respectively (Fig 4D). However, an increase in the abundance of other hydrocarbon-degrading bacteria, such as Sphingopyxis spp., Thermomonas spp. and Nocardia spp. was also observed (Junfeng et al., 2010, Rodriguez-nava et al., 2007) in weeks 4, 8 and 12. This increase in hydrocarbonlastic bacteria may be responsible for the increased reduction of TPH concentration in NA-H soils compared with that observed in NA soils. The increased proliferation of Nocardioides spp. in weeks 20 and 28 in both natural attenuation soils, NA and NA-H may explain the significant changes in bacterial diversity observed at these time points.

The presence of Pb also affected the biostimulated treated soils. BS-H treated soil showed an elevated abundance of the same bacterial genera compared with BS in week 4 (Fig 4E). However, Pseudomonas spp. dominated, representing 45% and 40% of the BS-H bacterial profile in week 8 and 12, respectively. The difference in the major dominance between BS and BS-H could be due to the presence of Pb, as Pseudomonas spp. has been shown to be resistant to Pb (Oriomah et al., 2015; Liu et al., 2017). The major dominance in both BS and BS-H disappeared in weeks 20 and 28, possibly due to the reduced concentrations of TPH, as well as the nutrients provided. These changes in both biostimulation treatments (BS and BS-

<table>
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<tr>
<th>Treatment</th>
<th>Number of bacterial taxa</th>
<th>Week 0</th>
<th>Week 4</th>
<th>Week 8</th>
<th>Week 12</th>
<th>Week 20</th>
<th>Week 28</th>
</tr>
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<tr>
<td>NA</td>
<td>516</td>
<td>0.12</td>
<td>0.210</td>
<td>485</td>
<td>0.469</td>
<td>466</td>
<td>0.338</td>
</tr>
<tr>
<td>NA-H</td>
<td>540</td>
<td>0.225</td>
<td>0.380</td>
<td>414</td>
<td>0.291</td>
<td>428</td>
<td>0.220</td>
</tr>
<tr>
<td>BS</td>
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<td>0.400</td>
<td>0.900</td>
<td>452</td>
<td>0.001</td>
<td>445</td>
<td>0.0001</td>
</tr>
<tr>
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<td>534</td>
<td>0.233</td>
<td>0.816</td>
<td>403</td>
<td>0.010</td>
<td>391</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Values in bold are different from 0 with a significance level alpha – 0.05. Superscripted Numbers are P values.
H) may explain the significant reduction in the bacterial diversity observed in week 4.

Overall, the results of this mesocosm study suggested that bioremediation is an effective approach to clean up TPH and TPH-Pb contaminated soils. Biostimulation was found to be more effective than natural attenuation in terms of the reduction in TPH concentrations in both contamination scenarios. The addition of nutrients to contaminated soil samples enhanced the growth of various hydrocarbon-degrading bacteria resulting in increased degradation of TPH. However, the biostimulated soils samples showed relatively greater ecotoxicity despite the lower TPH concentration. The presence of Pb alongside TPH increased the associated ecotoxicity and inhibited the growth of soil biota, including hydrocarbon-degrading bacteria. These results confirm the complications caused by co-contamination which lead to a reduction in the bioremediation efficiency.

4. Conclusion

This mesocosm study concluded that the presence of Pb as a co-contaminant has negatively impacted the efficacy of TPH bioremediation, especially after biostimulation, in co-contaminated soils. The biostimulation treatment showed 84% reduction in TPH concentration representing a 24% greater reduction than natural attenuation in co-contaminated soils. However, this represents 11% less reduction in comparison to TPH-only contaminated soils. Also, in co-contaminated soils, the biostimulation treatment showed only 8% reduction in toxicity in comparison with 62% in single contamination scenarios.
contaminated soils. In terms of alkb gene numbers, although bio-
stimulation resulted in increasing the gene copies number to about 1.5 × 10^{10} in comparison with only 6.3 × 10^{9} in natural attenuated soil, the presence of Pb has significantly decreased these numbers. In addition, the presence of Pb caused distinct shifts in the structure of the soil microbial community. In natural attenuation, beside Nocardioides spp. which showed a major dominance in both contamination scenarios, Pb resulted in an increase in the abundance of Sphingopyxis spp., and Thermomonas spp. In contrast, in biostimulation treatments, major changes in bacterial domination were observed since Alcanivorax spp. showed dominance in TPH-
contaminated soil whereas, Pseudomonas spp. mainly dominated the co-contaminated soils. The overall conclusion of this study is that although biostimulation was more effective in remediating TPH in co-contaminated soils, the presence of Pb alongside TPH had deleterious effects on the bioremediation process. Therefore, the complications caused by the presence of co-contaminants (e.g. Pb) should be a priority consideration when designing any bioreme-
diation strategy for specific contamination scenarios.

Declaration of interests

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

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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