Implications of co-contamination with aged heavy metals and total petroleum hydrocarbons on natural attenuation and ecotoxicity in Australian soils.

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Abstract

The bioremediation of historic industrial contaminated sites is a complex process. Co-contamination, often with lead which was commonly added to gasoline until 16 years ago is one of the biggest challenges affecting the clean-up of these sites. In this study, the effect of heavy metals, as co-contaminant, together with total petroleum hydrocarbons (TPH) is reported, in terms of remaining soil toxicity and the

structure of the microbial communities. Contaminated soil samples from relatively a hot and dry climate in Western Australia were collected (n=27). Analysis of soils showed the presence of both contaminants, TPHs and heavy metals. The Microtox test confirmed that their co-presence elevated the remaining ecotoxicity. Toxicity was correlated with the presence of lead, zinc and TPH (0.893, 0.599 and 0.488), respectively, assessed using Pearson Correlation coefficient factor. Next Generation Sequencing of soil bacterial 16S rRNA, revealed a lack of dominate genera; however, despite the variation in soil type, a few genera including *Azospirillum* spp. and *Conexibacter* were present in most soil samples (85% and 82% of all soils, respectively). Likewise, many genera of hydrocarbon-degrading bacteria were identified in all soil samples. *Streptomyces* spp. was presented in 93% of the samples with abundance between 7% - 40%. In contrast, *Acinetobacter* spp. was found in only one sample but was a dominant member of (45%) of the microbial community. In addition, some bacterial genera were correlated to the presence of the heavy metals, such as *Geodermatophilus* spp., *Rhodovibrio* spp. and *Rubrobacter* spp. which were correlated with copper, lead and zinc, respectively. This study concludes that TPH and heavy metals co-contamination significantly elevated the associated toxicity. This is an important consideration when carrying out risk assessment associated with natural attenuation. This study also improves knowledge about the dynamics of microbial communities in mixed contamination scenarios.

Main Finding

Elevated ecotoxicity was associated with the presence of heavy metals along with TPH in soil samples. Many hydrocarbon degrading bacteria were observed with no major dominance.

1. Introduction

Globally, historic industrial sites contaminated with petroleum hydrocarbons represent a significant challenge as they pose serious risks to natural ecosystems and human health if left untreated. The 68th United Nations General Assembly declared the year 2015 as the International Year of Soil (Kostarelos et al. 2015), recognising the need and importance of soil remediation. The demand for re-using former industrial sites is driven globally by increased urbanisation, and as a result, many environmental agencies have prioritised the remediation of such sites. In Australia, around 160,000 sites are estimated to be contaminated (Naidu 2013); petroleum hydrocarbons represent the most common contaminant with metals, solvents, herbicides and pesticides making up the remainder of the contaminants (CSRIO 2016). Many of these sites have been exposed to extreme weather conditions including high temperature and low rainfall. Remediation of aged, weathered hydrocarbons-contaminated industrial sites can be challenging due to many environmental and technical issues as well as the complexity of the remaining petroleum hydrocarbons (Haleyur et al. 2018).

Total Petroleum Hydrocarbons (TPH) are a large group of chemicals which consist largely of carbon and hydrogen and comprise a mixture of short and long-chain aliphatic hydrocarbons (e.g. *n*-hexane and mineral oil, respectively) and a minor group of aromatic compounds (e.g. benzene). Crude oil is the main source of TPH and the concentration of each compound varies depending on the source (ATSDR 1999). TPH enter the environment due to transportation, oil spills and leaks, industrial activities and private use. They move through the soil and while some

compounds reach the groundwater, others attach to the soil particles (Maletić et al. 2011). It is known that exposure to TPH may cause permanent damage to the central nervous system; in addition, many compounds associated with petrogenic contamination are carcinogenic (ATSDR 1999).

The first step of removing TPH from the environment is performed naturally by the indigenous microorganisms, a process termed natural attenuation. The ability of soil's indigenous microbial population to degrade TPHs offer a natural bioremediation approach with many advantages over traditional techniques. Natural attenuation is proven to be an effective, economical and environmentally safe strategy to breakdown TPHs in field-contaminated soil (Guarino, Spada, and Sciarrillo 2017). However, many factors affect the biodegradation rate of the contaminant; for example, pH, temperature and moisture content (Wu et al. 2017). In addition, the dynamics of the microbial communities and the changes occurring following contamination are among the most important factors since they underpin any natural degradation process (Das and Chandran 2011). Understanding these changes is a crucial step to improving the modelling of the natural attenuation strategy. In this regard, the application of next-generation sequencing (NGS) or metagenomics provides comprehensive data regarding the structure and diversity of microbial communities, the potential roles of organisms within the community and also the interaction between the individuals in their communities (Mukherjee and Chattopadhyay 2017). The introduction of NGS to study hydrocarbon-contaminated environments has dramatically improved our knowledge in relation to microbial communities, especially the taxonomic classification of different organisms involved in the degradation of the contaminant, including bacteria, fungi, algae, archaea and protozoa (Hivrale et al. 2015).

Many enzymes have been associated with the degradation of petrogenic hydrocarbons including alkane hydroxylase, *AlkB*, dioxygenases and methane monooxygenases (Maeng et al. 1996; van Beilen and Funhoff 2007). Among all the known alkane-degrading enzymes, *alkB*-type enzymes represent the most widely involved in the microbial oil-degradation process (Guibert et al. 2016). As alkanes are major components of crude oil , the *alkB* gene is often used as an indicator to assess the hydrocarbons-degradation potential of the microbial communities in petroleum contaminated sites (Paisse et al. 2011).

One serious challenge impacting the remediation of TPH-contaminated environments is mixed contamination (Thavamani, Megharaj, and Naidu 2012). The presence of heavy metals, especially lead (Pb), as co-contaminants alongside TPH causes severe complications during the microbial degradation process of TPH breakdown due to their direct interaction with the enzymes (Olaniran, Balgobind, and Pillay 2013). It is assumed that Pb would be present as co-contaminant in most of the historic TPHs-contaminated sites in Australia since it was commonly added to the fuel prior to 2002. Although essential metals (e.g. Zn) have a crucial role in microbial life, non-essential metals (e.g. Pb) are toxic to microorganisms as they displace the essential metals from their binding site due to their higher affinities for oxygen sites and thiol-containing groups (Bruins, Kapil, and Oehme 2000).

Toxicity analysis of TPH-contaminated soils should be a crucial part of any ecological risk assessment of contaminated sites (Tang et al. 2011). The concentration of TPH does not necessarily indicate the associated toxicity of a contaminated soil (Khudur et al. 2015) as it is not just the concentration but also the chemical composition of the remaining TPH that has a major effect on the associated

toxicity. (Brils et al. 2002) reported that the fraction of TPH with a lower boiling point (C10-C19) was strongly correlated with the toxicity of the TPHs.

To examine the toxicity of the contamination on biota, inhibition of natural bacterial bioluminescence (Microtox test) can be used. A reduction in the emitted light produced by the marine bacterial species *Vibrio fischeri* (formerly known as *Photobacterium phosphoreum*) is measured after exposure to the toxicant for a certain time, usually 5 and 10 min. (Kamlet et al. 1986). The endpoint of this test is represented as effective concentration 50 (EC50) which refers to the concentration of a contaminant that causes a reduction in the emitted light of 50 % during the test time. The Microtox test has been used to assess the toxicity associated with contaminated environments in many studies over the last 30 years (Khudur et al. 2015; Plaza et al. 2010).

To the best of the authors' knowledge, the effect of mixed contamination on the structure of the soil microbial communities, as well as the combined ecotoxicity has not been previously reported in Australian soils. The co-contamination scenario has been reported in studies from Europe, Africa and Canada (Cook, Chu, and Goodman 2002; Plaza et al. 2010; Oriomah, Adelowo, and Adekanmbi 2015).

In this study, soil samples from various locations in Western Australia have been assessed for TPH and heavy metal concentrations and the ecotoxicity associated with these naturally attenuated soils assessed. In addition, the structure of the bacterial communities has been investigated using NGS to improve knowledge of the dynamics of bacterial communities in co-contaminated soils. Thus, a better understanding will be obtained for designing appropriate bioremediation strategies for co-contaminated soils.

2. Methods and Materials

2.1. Sample collection

Contaminated soil samples were collected from former diesel power stations in remote areas of Western Australia in May 2016. All samples were taken from known areas of aged (over 20 - 50 years ago) petroleum contamination. For each sample 12 subsamples were taken and combined in a sterile glass jar. All samples were then subdivided and shipped to RMIT and ALS for microbial and TPH/metal analysis, respectively. The samples were kept at an ambient temperature until analysed within 7 days. The contaminated sites are located in Marble Bar – Western Australia (21.18^0 S, 119.75^0 E). Due to the hot climate in the region, the soil has been exposed to relatively high temperatures. According to the Australian Bureau of Meteorology, the mean temperature in 2016 was 35.5 ^{0} C with a highest monthly mean of 44.9 ^{0} C and a lowest monthly mean of 24.1^0 C. In addition, an average rainfall rate of 392.3 mm was recorded in Marble Bar in 2016 (BOM 2018).

2.2. Soil texture, TPH and Heavy Metals Analysis

Soil samples were analysed for soil texture using methods previously described (Rayment and Higginson 1992) and divided into four different groups based on their texture (G1, G2, G3, and G4). Soil samples were analysed for TPH (C10–C40) and heavy metals by an accredited analytical reference laboratory (Australian Laboratory Services Pty. Ltd.) in Western Australia.

2.3. Bioluminescence inhibition testing (Microtox test)

Test samples were prepared in replicate as previously described (Khudur et al. 2015). One gram of air-dried soil was added to 9 mL of water in a 10 mL tube. Tubes were placed on a shaker overnight, moved to a sonic water bath for 30 min. and then centrifuged twice at 4500 rpm for 5 min. (Hubálek et al. 2007). The supernatant was subject to filtration through 0.45 µm syringe filters and tested for bioluminescence using the Microtox® Model 500 Analyser following the manufacturer's instructions. The acute Microtox reagent (MODERN WATER Microtox®) and the reconstitution medium were supplied by JW Industrial Instruments Pty. Ltd. The reconstituted freeze-dried marine bacteria *V. fischeri* was allowed to equilibrate to 4 °C in a Microtox® Analyser before starting the test. A solution containing 2% sodium chloride (NaCl) was used as a diluent and a solution containing 22% NaCl was used to adjust the osmotic pressure during the test. The inhibition of bioluminescence after 5, 10 and 15 min. was calculated for each sample and expressed as EC50 using the provided software (ASTM 2004). For the purpose of samples' toxicity comparison, the toxicity unit (TU) was calculated as TU= (1/EC50) x 100. Based on this scale, the toxicity of soil samples can be classified as No Toxicity (TU=0), Moderate Toxicity (TU<1), High Toxicity (TU=1-10) (Günesf, Günes, and Talınıe 2008).

2.4. DNA isolation

Genomic DNA was extracted from soil samples using a PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc. USA) according to the manufacturer's protocol. A NanoDrop Lite spectrophotometer (Thermo Scientific – USA) was used to check the quantity and quality of the extracted DNA. The DNA concentrations range was (14 – 30 ng μL^{-1}) and the purity ratio (Absorbance 260/280 nm ratio) ranged between 1.8

and 2.0 which is the recommended ratio by the manufacturer. After measuring the extracted DNA, samples were stored at -20°C until further analysis.

2.5. Quantification of total bacteria and the hydrocarbon-degrader bacteria

The abundance of genes encoding the 16S rRNA gene for total bacteria as well as alkane-degrading bacteria were determined by quantitative polymerase chain reaction (qPCR) using a QIAGEN Rotor-Gene machine; all samples were run in triplicate. The primer set 341-F (5′CCTACGGGAGGCAGCAG3′) and 518-R (5′ ATTACCGCGGCTGCTGG3′) (Schäfer and Muyzer 2001) were used to determine the total bacterial abundance and the primer set *alkB*-F (5′AAYACIGCICAYGARCTIGGICAYAA3′) and *alkB*-R (5′ GCRTGRTGRTCIGARTGICGYTG 3′) was used to determine the abundance of the *alkB* gene (Pérez-de-Mora, Engel, and Schloter 2011). For both amplifications, a total volume of 20 µL was used for each qPCR reaction which contained (8.2 µL) molecular-biology-grade water, (10 µL, x2, Kapa Biosystems) Kapa SYBR Fast qPCR Master Mix, (0.4 µL, 10 pmol/µL) forward primer, (0.4 µL, 10 pmol/µL) reverse primer and (1 µL) DNA sample. Amplification conditions for total bacteria were an initial denaturation step at 95°C (5 min.) followed by 40 cycles of 95°C denaturation (10 s), annealing at 55°C (30 s), 72°C extension (30 s), 80°C primer dimer removal and signal acquisition (10 s) (Shahsavari et al. 2013).

The amplification of alkB gene was performed as described by (Pérez-de-Mora, Engel, and Schloter 2011). The cycling conditions consisted of initial denaturation at 95°C for 10 min., 45 s at 95°C (5 cycles), 1 min. at 62°C and 45 s at 72°C followed by of 45 s at 95°C (40 cycles), 1 min. at 57°C and 45 s at 72°C. After completion of

each cycle, data were collected at 78°C. A final cycle at 95°C for 15 s, 60°C for 30 s, and 95°C for 15 s was performed for melting curve analysis.

For the generation of both standard curves, serial dilutions (up to 10^{-6}) of cleaned PCR products of the 16S rRNA gene from *Rhodococcus erythropolis* (isolated from control soil, data not shown) and the alkB fragment from *Pseudomonas putida* were used (Velasco et al. 2017).

The CT values from standard dilutions were plotted against the log of their initial copy number followed by the generation of a standard curve using linear regression. All samples and standards were amplified in duplicates and the gene copy numbers were expressed as $\log_{10}$ of gene copy numbers per g of dry soil.

2.6. Bacterial community analysis via Next Generation Sequencing (NGS)

In regards to NGS analysis, the V4-V5 region of the bacterial 16s rRNA gene was amplified using primer sets, V3-forward (5′CCTACGGGNGGCWGCAG3′) and V4-reverse (5′GACTACHVGGGTATCTAATCC3′) (Dehingia et al. 2015); the thermo-cycling conditions consisted of 1 cycle at 95 °C (5 min.); 25 cycles of 95 °C (30 s), 55 °C (30 s), 72 °C (30 s) and a final extension at 72 °C (5 min.) (Hou et al. 2015). Each PCR tube contained a total volume of 25 µL which consisted of (9.5 µL) molecular-biology-grade water, (12.5 µL) GoTaq Mix, (1 µL, 5 pmol/µL) forward primer, (1 µL, 5 pmol/µL) reverse primer and DNA sample (1 µL). Library preparation was performed according to the guidelines in 16S Metagenomic Sequencing Library Preparation guide (Illumina) using Nextera® XT Index Kit (Illumina, San Diego, CA). Quantification of the library DNA was performed using Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, CA) and 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). The sequencing of the samples was run on a MiSeq platform (Illumina,

San Diego, CA) at the School of Science, RMIT University (Koshlaf et al. 2016). Sequences were analysed using the 16S Metagenomics workflow available in Illumina BaseSpace (https://basespace.illumina.com/home/index) using a high-performance version of the RDP Naïve Bayes taxonomic classification algorithm (Wang et al. 2007). The operational taxonomic units (OTUs) table was visualized using MEGAN6 (Huson et al., 2016). Shannon diversity and Chao-1 (richness) indices were calculated with PAST software (Andreoni and Gianfreda 2007).

Data analysis including statistics, correlation tests and Principle Component Analysis (PCA) were performed using XLSTAT 2014 software.

3. Results and discussion

3.1. Soil and contaminants characterisation

Soil samples were classified into four diverse groups, based on the soil structure including silt loam, clay, sandy clay loam and sandy loam (Table 1). The concentrations of TPH, heavy metals and toxicity unit values of the contaminated soil samples are presented in Table 1. The TPH concentration was relatively low, ranging between 25 and 3340 mg kg^{-1}. Similarly, most heavy metals in the test soil samples showed low concentrations except for a few samples which have relatively high concentrations in comparison to other samples. The concentrations of heavy metals ranged between 6 – 338 mg kg^{-1} for Cr, 7 - 153 mg kg^{-1} for Cu, 2.5 – 151 mg kg^{-1} for Pb, 8 – 162 mg kg^{-1} for Ni and 19 – 130 mg kg^{-1} for Zn. The values of As and Cd were below detection limits.

Table 1 Soil characterisation and contaminants concentration in the test soil samples. TPH: total petroleum hydrocarbons, TU: toxicity unit, Cr: chromium, Cu: copper, Pb: lead, Ni: nickel, Zn: zinc.

Soil group and structure	Sample ID	TPH concentration (mg/kg)	Moisture content (%)	Heavy metal concentration (mg/kg)					TU
				Cr	Cu	Pb	Ni	Zn	
G1 (Silt loam)	S1	25	3.6	6	7	3	9	30	0
	S2	25	3.9	15	29	8	12	56	0
	S3	150	2.7	14	18	10	16	76	0
	S4	200	24	6	9	9	10	34	0
G2 (Clay)	S5	3340	5.6	12	51	2.5	15	59	0.346
	S6	3030	7.9	22	24	27	8	61	0.072
G3 (Sandy clay loam)	S7	900	2.2	19	60	9	9	19	0
	S8	1000	9.6	38	18	37	17	56	0
	S9	850	4.8	33	16	22	9	58	0
	S10	2170	7.3	37	86	151	14	105	1.01
	S11	430	9.9	32	32	27	17	121	0.09
	S12	1830	9.3	27	19	17	13	96	0.25
	S13	560	4.1	32	23	67	14	113	0.79
	S14	130	3.1	19	36	16	15	88	0
	S15	190	4.8	82	37	20	48	58	0
	S16	430	4	19	17	10	16	50	0
	S17	25	7.2	338	54	3	162	36	0
	S18	25	3.2	26	22	9	21	36	0
G4	S19	500	2.1	15	24	2.5	9	30	0

(Sandy loam)	**S20**	25	5.2	16	10	2.5	11	30	**0**
	S21	25	3.6	6	7	2.5	9	30	**0**
	S22	1000	3.5	26	11	15	11	31	**0**
	S23	850	5.1	49	70	42	17	130	**0.233**
	S24	770	2.4	22	16	13	12	65	**0**
	S25	25	4.4	14	12	7	13	40	**0**
	S26	25	2.9	39	153	13	38	52	**0**
	S27	260	3.2	11	18	2.5	10	26	**0**

3.2. Soil toxicity

Given the relatively low concentration of TPH and heavy metals, it was not surprising that most of the test samples showed little or no enhanced toxicity using the Microtox test (TU column in Table 1). However, several soil samples showed significant toxicity levels ($p \leq 0.05$) (Table 1). A comparison of the TPH concentration, metal concentrations and relative toxicity for each soil is presented in Figure 1. The most dominant factor regarding the toxicity was the presence of Pb; elevated toxicity was observed in samples with a relatively high concentration of Pb. A previous study (Zeb et al. 2016) showed that the toxicity of Pb, as a single contaminant, was 0.046 TU for *V. fischeri*. However, in this study Pb showed a much higher level of toxicity as it is combined with other contaminants. Two other contaminants additionally seemed to be responsible for the toxicity, Zn and TPH. The presence of each of these two contaminants, together with Pb correlated with the increased level of toxicity. The highest toxicity was observed in soil samples with an elevated concentration of all the contaminants at the same time (Figure 1). One possible

explanation of the elevated combined-toxicity is the increase in the bioavailability of the toxic heavy metal in the presence of an organic pollutant as has been previously observed (Olaniran, Balgobind, and Pillay 2013). In addition, co-contamination may affect the transportation activity of the cellular membrane of the microorganism and therefore inhibit the metal-ATPase activity (Gauthier et al. 2015). Although many studies have reported the toxicity of TPH and heavy metals individually, little is known about their combined ecotoxicity (Gauthier et al. 2014).

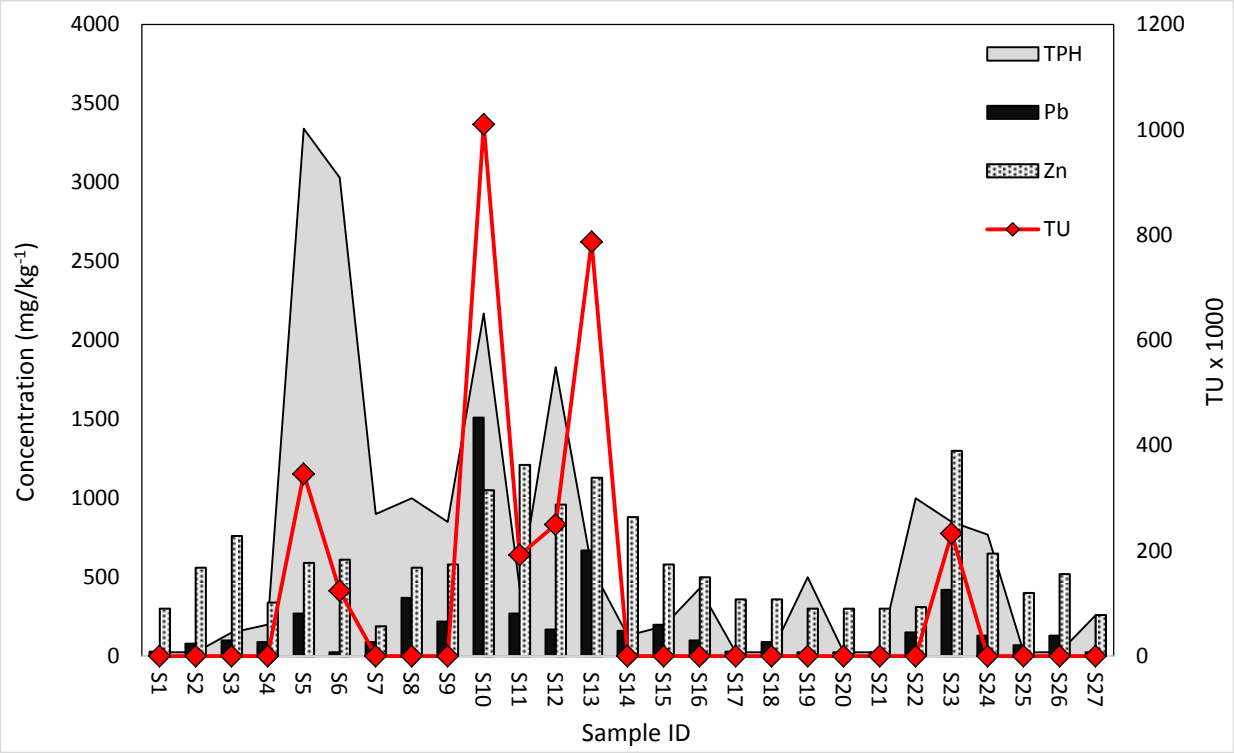

Figure 1. Toxicity of the test soil samples based on the presence of the contaminants in the soil. The concentration of Pb and Zn is presented as concentration mg kg^{-1} x 10 and toxicity is presented as Toxicity Unit x 1000.

The presence of Pb and Zn in high concentrations along with petroleum hydrocarbons showed a strong correlation with toxicity using Pearson correlation coefficient (0.893) and (0.599) respectively (Table 2). Similarly, TPH was correlated with the toxicity (0.488).

Table 2. Correlation matrix using Pearson Correlation coefficient factor between the contaminants and some of the soil variables.

Variables	TPH	Cr	Cu	Pb	Ni	Zn
Toxicity	**0.488** (0.010)	-0.034 (0.865)	0.264 (0.264)	**0.893** (0.0001)	-0.096 (0.633)	**0.599** (0.001)
***alkB* gene**	**0.544** (0.003)	-0.200 (0.345)	0.185 (0.355)	**0.416** (0.031)	-0.286 (0.148)	**0.517** (0.006)
Total Bac	0.180 (0.069)	-0.191 (0.339)	0.128 (0.525)	0.056 (0.781)	-0.214 (0.284)	0.338 (0.085)

Values in bold are different from 0 with a significance level alpha=0.05

Numbers in brackets represent P values

Further, Principal Component Analysis (PCA) showed that toxicity was closely associated with Pb, Zn and TPH respectively (Figure 3). In contrast, despite the relatively high concentration of Cr, Ni and Cu in some samples, (338, 162 and 153 mg kg^{-1}, respectively), none of these metals exhibited any relation to the toxicity. One possible explanation is that these metals concentrations are less than their EC50 (Zeb et al. 2016); in addition, the combined TPH concentration in these soil samples was very low (25 mg kg^{-1}).

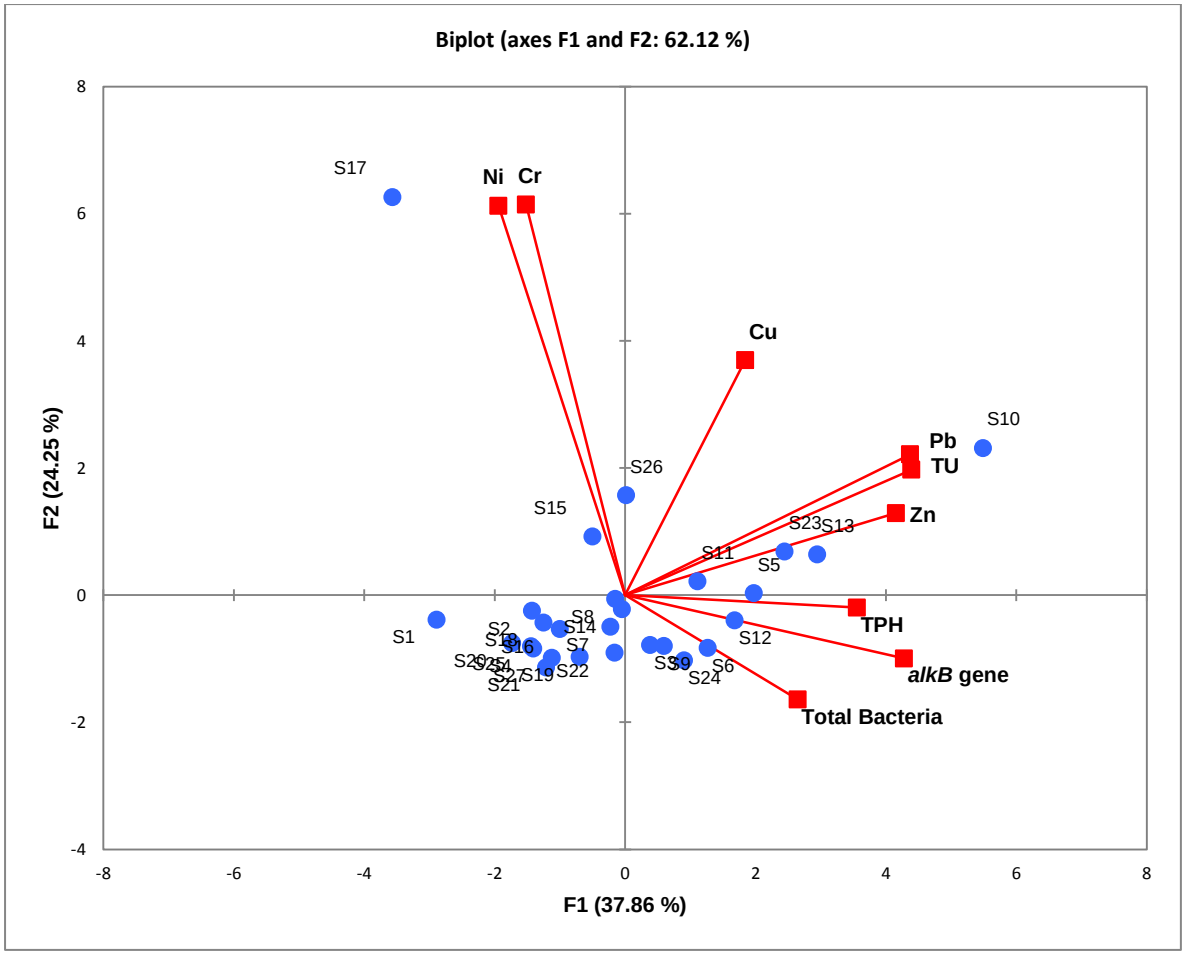

Figure 2. Principle Component Analysis (PCA) biplot depicting the relationship between the contaminants with the toxicity and number of gene copies in the test soil samples. Blue circles represent soil samples.

3.3. Analysis of soil bacterial community

3.3.1. The diversity of total bacteria and *alkB* gene copies

Assessment of the potential of the soil microbial community to degrade the contaminant TPH fraction, based on the number of copies of the *alkB* gene revealed a relationship between copy number and TPH concentration (Correlation factor, 0.544), (Table 2). Soil samples with higher TPH concentration showed an elevated

number of copies of the *alkB* gene, the gene of interest in this study, in comparison to soil samples with lower TPH concentration (Table 3). This is an indication of the extensive presence of hydrocarbon degrading bacteria in TPH contaminated sites and confirmation of their contribution to the degradation of TPH (van Beilen and Funhoff 2007; Paisse et al. 2011). Previous research had suggested a reduction in microbial diversity in contaminated soils. The diversity indices based on 16S rRNA sequencing in these soils showed that the diversity and richness of bacterial communities varied despite the presence of contaminants or observed toxicity. The Shannon index varied between 3.0 and 4.7 and chao-1 was between 212 and 467. Previous studies reported more diverse bacterial communities in highly TPH-contaminated soils in comparison to less contaminated soils (Liu et al. 2009; Jung et al. 2010). However the opposite trend have been reported by other studies, with reduced diversity in contaminated environments being reported (Pérez-de-Mora, Engel, and Schloter 2011; Qin et al. 2012; Yang et al. 2012). Other researchers have reported no change in overall bacterial diversity and richness despite exposure to different concentrations of contaminants (Hernandez-Raquet et al. 2006).

Table 3. Gene copies numbers of alkB gene and bacterial 16S rRNA and diversity indices. Gene copies numbers are presented as Log10 of the copies number ± standard deviation.

Sample ID	**Gene copies (log gene copies / g dry soil)**		**Shannon_H**	**Chao-1**
	***alkB* gene**	**Total bacteria**		
S1	3.9 ± 0.1	7.2 ± 0.0	3.8	261

S2	5.5 ± 0.1	8.6 ± 0.5	4.0	292
S3	6.5 ± 0.2	10.2 ± 0.2	4.5	412
S4	5.0 ± 0.0	9.0 ± 0.2	4.7	417
S5	6.0 ± 0.1	9.5 ± 0.0	3.8	212
S6	6.1 ± 0.2	10.0 ± 0.1	4.4	406
S7	6.0 ± 0.0	9.8 ± 0.0	4.6	374
S8	5.5 ± 0.1	9.2 ± 0.6	3.8	364
S9	6.4 ± 0.0	10.4 ± 0.0	4.5	365
S10	6.5 ± 0.0	9.4 ± 0.1	4.6	370
S11	5.6 ± 0.0	10.0 ± 0.4	4.5	377
S12	6.1 ± 0.0	10.3 ± 0.2	4.4	348
S13	5.7 ± 0.2	10.4 ± 0.0	3.2	275
S14	5.3 ± 0.1	9.8 ± 0.0	4.1	355
S15	5.6 ± 0.2	10.0 ± 0.3	3.9	336
S16	4.8 ± 0.1	9.7 ± 0.0	4.2	394
S17	4.3 ± 0.0	8.7 ± 0.0	4.6	392
S18	4.8 ± 0.1	10.0 ± 0.1	4.5	414
S19	5.5 ± 0.2	10.2 ± 0.0	4.5	382
S20	4.8 ± 0.2	9.1 ± 0.1	4.7	420
S21	5.3 ± 0.3	9.8 ± 0.1	4.3	430
S22	6.1 ± 0.0	9.8 ± 0.2	3.6	360
S23	6.6 ± 0.1	10.3 ± 0.3	4.4	350
S24	6.8 ± 0.1	10.8 ± 0.3	4.6	341
S25	4.3 ± 0.1	10.2 ± 0.5	3.0	252
S26	5.3 ± 0.1	10.3 ± 0.1	4.2	414

S27	5.1 ± 0.0	10.0 ± 0.0	4.6	467

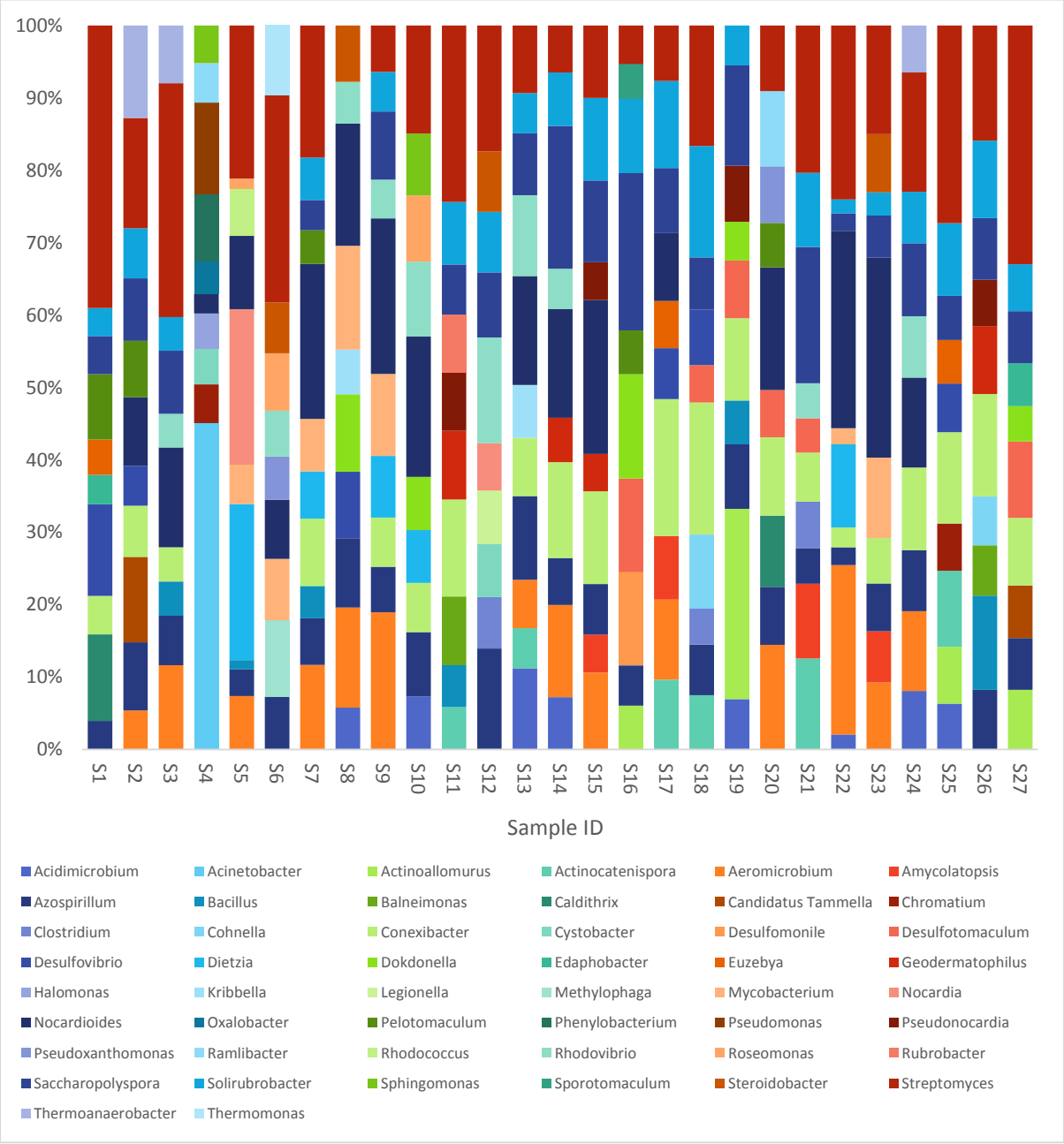

Figure 3. Comparison of the relative abundance (% of total sequences) of top 10 bacterial genera in the test soil samples.

3.3.2 NGS analysis

NGS data showed no major dominance among the top 10 dominant genera. Despite the different soil types, several bacterial genera were present in most soil samples; for example, *Azospirillum* spp. and *Conexibacter* spp. were present in 85% and 82% of all soils, respectively. Likewise, *Saccharopolyspora* spp. and *Solirubrobacter* spp. were present in 78% of all the samples (Figure 3). To the best of the authors' knowledge, none of these genera has been previously reported in a contaminated site. This might be due to the uniqueness of the studied sites and the weather conditions because many of these organisms have been found in (uncontaminated) soils from hot and extreme arid regions (Zhang et al. 2012; Bashan 1999).

Hydrocarbon-degrading bacteria were also present in all soil samples. Many of these bacterial genera have been previously found in TPH contaminated soils; for example, *Acinetobacter* spp. was reported to be present in a mixed contamination scenario (Markowicz, Cycoń, and Piotrowska-Seget 2016). *Pseudonocardia* spp., *Halomonas* spp., *Mycobacterium* spp. and *Streptomyces* spp. have also been reported in hydrocarbon-contaminated sites (Haleyur et al. 2018; Prince, Gramain, and McGenity 2010). In addition, many other genera were detected in the present study which have been previously reported in contaminated sites including, *Desulfotomaculum* spp., *Nocardia* spp*., Nocardioides* spp., *Dietzia* spp. and *R*hodococcus spp. (Wang, Wang, and Shao 2010; Prince, Gramain, and McGenity 2010), *Aeromicrobium* spp., *Pseudomonas* spp., *Pseudoxanthomonas* spp. (Abbasian et al. 2016; Guibert et al. 2016). Some hydrocarbon degraders were widely distributed in the soil samples, such as *Streptomyces* spp. which was found in 93% of the samples, although its relative abundance varied between 7% - 40%; *Nocardioides* spp. and *Aeromicrobium* spp. were present in 62% and 52% of soil

samples, respectively with abundance varying between 5% - 30% and 5% - 25%, respectively. In contrast, *Acinetobacter* spp. was present in only one sample but dominated the microbial community (45%). Also, *Halomonas* spp., *Nocardia* spp., *Rhodococcus* spp. and *Pseudoxanthomonas* spp. were found in a few samples at low concentration (Table 3).

The presence of *Dietzia* spp., *Methylophaga* spp., *Mycobacterium* spp., *Nocardia* spp., *Rhodococcus* spp. and *Roseomonas* spp. correlated with the presence of TPH in the soil (Table 3) suggesting a role for these bacteria in the TPH degradation process. These genera are well-known hydrocarbon degrading organisms (Haleyur et al. 2018) (Prince, Gramain, and McGenity 2010). Furthermore, many of these genera have been reported as metal-tolerant genera explaining their survivor in a mixed contamination environment, for example, *Bacillus* spp., *Pseudoxanthomonas* spp., *Acinetobacter* spp. and *Pseudomonas* spp. (Liu et al. 2017; Oriomah, Adelowo, and Adekanmbi 2015; Thavamani, Megharaj, and Naidu 2012).

The presence of other bacterial genera correlated with metal concentrations in soil samples. These bacteria included *Bacillus* spp., *Balneimonas* spp. and *Geodermatophilus* spp. all of which were correlated to the presence of Cu; *Rhodovibrio* spp. and *Sphingomonas* spp. were correlated to Pb and *Rubrobacter* spp. was the only bacterium correlated to Zn. No correlation was recorded between any bacterial genera in the soil samples to Cr or Ni.

Table 4. Correlation matrix using Pearson Correlation coefficient factor between the most common bacterial genera and the contaminants in the soil samples.

Bacterial genera	TPH	Cu	Pb	Zn
***Bacillus* spp.**	-0.077 (0.704)	**0.617** (0.001)	-0.132 (0.513)	-0.173 (0.389)
***Balneimonas* spp.**	-0.148 (0.462)	**0.539** (0.004)	-0.001 (0.995)	0.262 (0.187)
***Dietzia* spp.**	**0.486** (0.010)	0.110 (0.583)	0.012 (0.954)	-0.198 (0.323)
***Geodermatophilus* spp.**	-0.207 (0.301)	**0.605** (0.001)	-0.016 (0.937)	0.233 (0.243)
***Methylophaga* spp.**	**0.516** (0.006)	-0.055 (0.787)	0.045 (0.824)	0.014 (0.943)
***Mycobacterium* spp.**	**0.386** (0.047)	0.110 (0.585)	0.066 (0.743)	-0.025 (0.902)
***Nocardia* spp.**	**0.635** (0.000)	0.092 (0.649)	-0.121 (0.458)	0.065 (0.749)
***Rhodococcus* spp.**	**0.584** (0.001)	0.118 (0.559)	-0.118 (0.556)	0.002 (0.993)
***Rhodovibrio* spp.**	0.362 (0.064)	-0.099 (0.624)	**0.454** (0.017)	0.374 (0.055)
***Roseomonas* spp.**	**0.599** (0.001)	-0.037 (0.855)	0.027 (0.893)	0.015 (0.942)
***Rubrobacter* spp.**	-0.059 (0.771)	-0.004 (0.986)	0.045 (0.824)	**0.397** (0.040)
***Sphingomonas* spp.**	0.266 (0.181)	0.264 (0.183)	**0.792** (0.0001)	0.219 (0.273)
***Steroidobacter* spp.**	**0.444** (0.020)	0.000 (0.999)	0.157 (0.434)	0.343 (0.080)
***Thermomonas* spp.**	**0.516** (0.006)	-0.055 (0.787)	0.045 (0.824)	0.014 (0.943)

Values in bold are different from 0 with a significance level alpha=0.05

Numbers in brackets are P values

4. Conclusion

This study was conducted using soil samples from Australian contaminated sites which have been exposed to relatively high temperature and low rainfall. The outcome has shown increased toxicity in soil samples contaminated with both contaminants, TPHs and heavy metals. In addition, the most dominant factor regarding the toxicity was the presence of lead (Pb) followed by zinc (Zn) and then TPHs. Many hydrocarbon-degrading bacteria were identified in the microbial community structure. Some bacterial genera were found in the majority of the samples, e.g. *Streptomyces* spp. with an abundance level varied from 7% to 40%. In contrast, other genera were found in only one sample but at high concentration, representing 40% of the total bacterial community, e.g. *Acinetobacter* spp. Moreover, the presence of bacterial genera like *Geodermatophilus* spp., *Rhodovibrio* spp. and *Rubrobacter* spp. were correlated to heavy metals (Cu, Pb, and Zn, respectively) and have previously been reported to be present in mixed-contaminated soils. The overall conclusion of this study is that the toxicity increases in TPHs and heavy metals mixed contamination scenario in comparison to a single contaminant. This is an important consideration when carrying out risk assessment associated with natural attenuation. This study also improves knowledge about the dynamics of microbial communities in mixed contamination scenarios. NGS data provide valuable knowledge about the dynamics of microbial communities in mixed contamination scenarios since no major domination was reported and many genera were common among the test soil samples.

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