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Bacterial community dynamics and associated genes in hydrocarbon contaminated soil during bioremediation using brewery spent grain

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Original Research

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Abstract

Brewery spent grain (BSG) has previously been exploited in bioremediation. However, detailed knowledge of the associated bacterial community dynamics and changes in relevant metabolites and genes over time, is limited. This study investigated the bioremediation of diesel contaminated soil amended with BSG. We observed the complete degradation of three Total Petroleum Hydrocarbon (TPH C10 – C28) fractions in amended treatments as compared to one fraction in the unamended, natural attenuation treatments. The biodegradation rate constant was higher in amended treatments (0.1021 k) than in unamended (0.059 k) and bacterial CFUs increased significantly in amended treatments. The degradation compounds observed fitted into the elucidated diesel degradation pathways and quantitative PCR results showed that the gene copy numbers of all three associated degradation genes, *alkB*, *catA* and *xylE,* were significantly higher in amended treatments. High-throughput sequencing of 16S rRNA gene amplicons showed that amendment with BSG enriched autochthonous hydrocarbon degraders. Also, community shifts of the genera *Acinetobacter* and *Pseudomonas* correlated with the abundance of catabolic genes and degradation compounds observed. This study showed that these two genera are present in BSG and thus, may be associated with the enhanced biodegradation observed in amended treatments. The results suggest that the combined evaluation of TPH, microbiological, metabolite and genetic analysis provides a useful wholistic approach to assessing bioremediation.

Data summary and availability

The DNA sequencing data generated and analysed during the current study is publicly available and has been deposited with NCBI Sequence Read Archive (SRA) with BioProject accession number PRJNA861128. https://www.ncbi.nlm.nih.gov/sra/PRJNA861128

Impact Statement

This report summarises findings relating to enhancing the bioremediation of diesel fuel using brewery spent grain as an amendment, and the associated bacterial populations, during incubation in an organic soil. The data provides information on the biodegradation of hydrocarbons, relates the presence of signature metabolites in the degradation pathway to specific hydrocarbon degradation genes and changes in the microbial population. The use of various amendments to enhance bioremediation has received increased interest in recent years and is recognised as having value in industrial practice. This is enhanced when routine amendment materials such as spent grain is used, rather than expensive waste treatments. Our data is novel in using the same treatment samples for a combination of biochemical analysis of hydrocarbons and their degradation metabolites with the genetic identification of genes and microbial communities to illuminate correlations at each time point during the degradation. Intrinsic BSG bacterial populations have also been identified and profiled.

Declaration – This work is based on the corresponding author's publicly available doctoral thesis, which is not considered a prior publication.

Introduction

Environmental contamination by petrogenic hydrocarbons is ubiquitous and diesel fuel is one of the most common pollutants of this type (Tellechea et al. 2017; Aziz et al. 2020). It is introduced to the environment mainly by spillage during transportation and storage, thereby contaminating water and soil (Yergeau et al. 2012; Tellechea et al. 2017). The impacts of diesel pollution go beyond environmental degradation to include health risks for humans and other organisms due to the toxic, mutagenic and carcinogenic nature of the constituent hydrocarbons (Souza et al. 2014; Varjani, 2017). Another concern is that hydrocarbon pollutants can accumulate in the high trophic levels of the food chain (Semple et al. 2001, Truskewycz et al. 2019).

Soil is a key natural resource that is essential to sustain life on Earth. Many key functions of soil are carried out by microorganisms, which also play important roles in various biogeochemical cycles (Gao et al. 2014; Jansson and Hofmockel, 2018). However, soil also acts as an ultimate repository for contaminants, accumulating contamination through precipitation and sedimentation from air and water. This contamination affects the soil microbiota, leading to a disruption of its functions (Gao et al. 2014; Wyszkowski et al. 2020). While physiochemical remediation methods such as landfilling, and incineration are available, more environmentally safe, efficient and cost-effective remediation methods are sought to mitigate the impacts of soil diesel contamination (Shahsavari et al. 2013).

Biodegradation is the natural attenuation of toxic contaminants by autochthonous microorganisms. However, the process is slow, and enhancement is needed to deal with high contaminant concentrations, the associated nutritional imbalances and toxicity to intrinsic microbial populations (Sarkar et al. 2016). Thus, bioremediation techniques are employed under controlled conditions to enhance biodegradation. Bioremediation is an exploitation of the metabolic capabilities of microorganisms to transform contaminants into innocuous, mineralised products (Guarino et al. 2017). The efficiency of the biodegradation process is associated with the enzymatic potential of the resident microorganisms, enabling them to digest the various substrates in the relevant degradation pathways (Das and Chandran, 2011; Eze et al. 2022; Bekele et al. 2022).

Hence, microbial populations are the agents of biodegradation and play a vital role in the decontamination of petrogenic hydrocarbons in soil (Sheppard et al. 2011, Shahsavari et al. 2013). As such, analysis of the microbial community dynamics during bioremediation is essential to understand the response and adaptation of microbes to pollution and monitor the bioremediation process (Fuentes et al. 2014). More specifically, analysis targeting the 16S rRNA gene as a molecular marker enables profiling of microbial communities and has been usefully applied to characterise microbial communities involved in bioremediation of various environments (Yergeau et al. 2012; Sarkar et al. 2016; Wu et al. 2020). Monitoring the presence and abundance of hydrocarbon degradation genes in the degrading microbiome is also required to fully appreciate its catabolic potential. This can also provide useful biomarkers for estimating the bioremediation potential of contaminated sites (Yang et al. 2015; Lima-Morales et al. 2016). For example, the *alkB* gene is very important in the aerobic transformation of aliphatic hydrocarbons as it encodes an alkane monooxygenase enzyme that hydrolyses alkanes to their corresponding primary or secondary alcohols and has been evaluated in a number of studies (Gielnik et al. 2019; Garrido-Sanz et al. 2019; Kawagoe et al. 2019).

Bacteria are instrumental in degrading hydrocarbon pollutants in soil and utilising the resulting metabolites for energy and growth, via the tricarboxylic acid (TCA) cycle (Kulshreshtha, 2012). Metabolites associated with hydrocarbon degradation have also been successfully identified in studies (Yanto and Tachibana 2013; Umar et al. 2017). Following soil contamination, biodegradative bacterial strains that are resistant to the toxicity of soil pollutants, can detoxify soil and make nutrients available for other quiescent populations to grow, leading to changes in microbial community structure and succession (Zucchi et al. 2003; Wu et al. 2016). As a result, community composition varies over time, with those species best able to exploit the metabolic breakdown products dominating as bioremediation progresses (Reddy et al. 2011). Bacterial genera associated with the biodegradation of petroleum hydrocarbons have been shown to include *Acinetobacter, Arthrobacter, Bacillus, Flavobacterium, Nocardia, Pseudomonas* and *Vibrio* (Chandra et al. 2013; Varjani, 2017). Among the different species associated with bioremediation, *Pseudomonas* spp. in particular are known to be versatile in the biodegradation of hydrocarbons, especially those found in diesel fuel (Dussán and Numpaque, 2012). However, knowledge of the microbial species involved in diesel remediation using food wastes in temperate countries is limited.

Shahsavari et al. (2013) observed high degradation rates in diesel and gasoline contaminated soil amended with crop residues. As soil is the most expensive medium to decontaminate, the use of crop residues and food waste is cost efficient and advantageous (Agamuthu et al. 2013). Brewery spent grain is a readily available food by-product with high nutritional content (Thomas and Rahman, 2006; Lynch et al. 2016). It has been successfully used to stimulate bioremediation and its effluents have been used for diesel bioremediation, indicating strong

potential (Agarry and Latinwo, 2015). However, these uses have been mostly under tropical conditions (Agamuthu et al. 2010; Abioye et al. 2012). BSG was chosen as a biostimulant in this study because, as well as releasing nutrients for autochthonous bacteria during biodegradation, it is known to have its own resident microflora and so may provide both biostimulation and bioaugmentation benefits (Robertson et al. 2010). It would also allow reuse of an industrial by-product that might otherwise be put to landfill as waste (Bianco et al. 2020).

Previous work on diesel biodegradation using BSG has tended to focus on the reduction of TPH and culturable bacterial count. Changes in the associated catabolic genes, metabolites, and community succession over time during diesel bioremediation using BSG in a temperate soil have not been investigated. This time-course study was designed to illuminate any correlations between breakdown metabolites, gene abundance and community shifts in response to biodegradation, with the aims of evaluating the impact of BSG supplementation on diesel bioremediation in soil and answering the following questions. How does BSG influence biodegradation? What are the metabolites associated with this biodegradation process and how do they fit into known hydrocarbon breakdown pathways? Which bacterial species are associated with biodegradation, how does their abundance change over time and does BSG supplementation favour known hydrocarbon-degrading bacteria and catabolic genes?

2. Methods

2.1 Soil preparation and experimental design

Soil was collected (10–30 cm depth) from an uncontaminated pristine agricultural farmland using sterile implements, air dried and sieved using a 4 mm mesh (Shahsavari et al. 2013). The pH of the soil was determined to be 6.8 after a 1:2.5 soil: distilled water dilution (Emmett et al. 2010). Soil characteristics were determined (by Anglian Soil Analysis Ltd, UK) as listed in Table 1. The three treatments used in this study were soil + diesel (S+D) unamended natural attenuation, soil + diesel + BSG (S+D+G) amendment, and soil alone (S) control. In triplicate, 20 ml of diesel was spiked into 2000 g soil to achieve a 1% (v/w) contamination. To ensure homogeneity the diesel was added to 25 % of the soil and mixed thoroughly with a stainless-steel implement before adding the remaining soil (Fernández et al. 2011). BSG (10 %) was then added to treatments as required and mixed in thoroughly. BSG had a moisture content of 75%, a pH of 5.3 and was stored at 4 °C for four days prior to use.

Table 1 Physical and chemical characteristic of the soil used in this study

Soil property	Value
Soil texture	Sandy/Loam
pH	6.8
Moisture (%)	26.04
Organic matter (%)	12.0
Sand (%)	50.44
Silt (%)	41.84
Clay (%)	7.72
Total organic carbon (%)	6.96
Potassium (mg kg^{-1})	15.4
Magnesium (mg kg^{-1})	21.1
Phosphate (mg kg^{-1})	17.5
Nitrate (mg kg^{-1})	3.5

Sterile counterparts of treatments (sterile soil + diesel, and sterile soil + diesel + sterile BSG) were also analysed to confirm biotic loss. Sterilisation was achieved prior to diesel spiking by autoclaving for one hour at 121 °C on three alternate days (Molina-Barahona et al. 2004; Emam et al. 2014). For each treatment, 2000 g soil was incubated in triplicate 5 litre pots. Treatment pots were covered with Gore-Tex cloth and incubated at 15 °C ± 3 °C. Treatments were oxygenated by mixing twice a week using a sterile spatula and the moisture content was maintained by the weekly addition of 5 % (v/w) sterile distilled water. Composite samples for analysis were obtained from each pot on days 0, 2, 5, 7, 12, 14, 21, and 28 by collecting 5 g samples from the four corners and the centre and mixing together (Chagas-Spinelli et al. 2012).

2.2 Determination of TPH removal and detection of hydrocarbon metabolites

2.2.1 GC Analysis

TPH was determined using a modified US EPA 8015 technique (Bento et al. 2005; Suja et al. 2014). Hexane was used for diesel extraction with mechanical shaking (Geilnik et al. 2019). Aliquots of the extract (1 ml) were transferred in triplicate to 1.5 ml gas chromatography vials and GC analysis was carried out using an Agilent Technologies 7890A system equipped with a flame ionization detector (FID) and autosampler (7693). A 30 m x 0.32 mm x 0.25 µm capillary column (19091J-413E HP-5, Agilent Technologies, UK) was used with helium as the carrier gas at a flow rate of 2 ml min^{-1}, hydrogen gas at a flow rate of 30 ml min^{-1} and air at a flow rate of 300 ml min^{-1}. The temperature program used was a modified version of that given by Bento et al. (2005). The initial temperature was 50 °C with isothermal operation for 5 min, followed by heating to 270 °C at a constant rate of 10 °C min^{-1} and a final 5 min isothermal operation. Samples from each time point and triplicate standards were analysed on the same run.

2.2.2 Percentage TPH reduction, extent of aliphatic TPH (C10-C28) fractions removal and biodegradation rate

Percentage TPH reduction was calculated using the formula:
% TPH reduction $=$ [(TPH of control – TPH treatment) / TPH control] x 100, with day 0 TPH being used as a control for each treatment (Bento et al. (2005). The TPH Standard Mix 1 (Sigma Aldrich, UK), with known concentration for each of the (C10 – C28) analytes, was used to obtain the calibration curve for each fraction. Concentrations of each fraction in the soil hydrocarbon mixture were then determined based on the calibration curve of each corresponding standard fraction. Retention times of each fraction (analyte) in the standard were compared to the sample chromatogram to determine target compounds and the total peak areas of both standard and analyte fractions were determined. The biodegradation rate was determined using the formula $\frac{C}{C_0} = e^{-kt}$ (Baek, et al. 2004), which is same as: $C = C_0 e^{-kt}$, (Abioye et al. 2012); where C is the concentration of the TPH fractions (mg kg^{-1}) at time t, C_0 is the initial concentration of the TPH fractions (mg kg^{-1}), t is time (day $^{-1}$) and k is the biodegradation rate constant (day^{-1}).

2.2.3 GS-MS Analysis

GC-MS analysis was carried out using an Agilent GC-MS 7890A/5975C series instrument with a 30 m x 0.32 mm x 0.25 µm capillary column (19091S-433E HP-5MS, Agilent Technologies, UK). Helium was the carrier gas with a flow velocity of 1 ml min^{-1} and I µl of sample was injected into the column in a splitless mode. The analytical conditions were an initial temperature of 50 °C, with isothermal operation for 1 min followed by heating to 120 °C at a constant rate of 20 °C min^{-1} and a final heating to 310 °C at a constant rate of 4 °C min^{-1} (Xu and Lu, 2010) with a 5 min isothermal operation. The column was directly connected to an electron ionisation mass spectrometer with an electron energy of 70 eV, producing ions that are characterised according to mass-to-charge ratio and relative abundance.

2.2.4 Detection of hydrocarbon degradation metabolites and BSG metabolic potential

Following the GC-MS analysis to identify compounds present in the treatments at each time point, the G3835AA Mass Hunter Mass Profiler Professional Software (Agilent Technologies, UK) was used to analyse the MS data (identified compounds) and the identified metabolites were used to determine the oxidative pathways utilised in the breakdown of diesel with and without BSG. The abundance of each compound in the treatments, and compounds differing significantly between the treatments overall, were determined.

2.3 Determination of colony forming units (CFUs) for the enumeration of heterotrophic and hydrocarbon degrading bacteria

Composite samples of 10 g from each treatment pot were transferred to sterile bottles containing 100 ml of 0.2 % (v/v) sterilised sodium pyrophosphate and mixed on a shaker at 150 rpm for 30 minutes at 20 °C. Thereafter, 1 ml of the soil suspension from each bottle was 10-fold serially diluted in sterile saline to give dilutions 10^{-1} to 10^{-6}. Following dilution, 0.1 ml of each suspension was plated onto R2A agar for enumeration of heterotrophic bacteria, and onto oil agar for enumeration of hydrocarbon-degrading bacteria. These media were incubated at 30 °C for 24 hours and 25 °C for 7 days, respectively.

2.4 Quantification of catabolic genes and bacterial community composition profiling

2.4.1 DNA extraction from treatments

Microbial community DNA was extracted from bioremediation treatment samples (1 g), with and without BSG at each time point (days 0, 5, 12 and 21), using the EZNZA soil DNA kit (Omega Bio-Tek, Inc., USA) (Dineen et al. 2010). Purity of extracted DNA was estimated by measuring absorbance at 260nm and 280nm, using a micro volume spectrophotometer (Nanodrop technologies, USA) and calculating the 260/280 ratio, which was required to be between 1.8 to 2.0.

2.4.2 qPCR quantification of diesel catabolic genes

Three hydrocarbon catabolic genes, *alkB* (Powel et al. 2006), *catA* and *xylE* (Shahsavari et al. 2016), were assayed quantitatively using PCR primers as listed in Table 2. Quantification was performed by real time PCR (qPCR) using 1:10 dilutions of the extracted community DNA in sterile nuclease-free water (Shahsavari et al. 2016). The assays were performed in a Rotor Gene Q thermocycler (Qiagen, UK) using the 2x Kapa Sybr® Fast qPCR Master Mix Universal kit (Sigma Aldrich, UK). Each reaction (20 µl) contained 2x Kapa Sybr Fast qPCR Master Mix (10 µl), forward primer (0.2 µM), reverse primer (0.2 µM), PCR-grade water (8.2 µl) and DNA template (1 µl). The amplification programme for the *alkB* gene included initial denaturation at 95^{o}C (5 mins), followed by 40 cycles of denaturation at 95^{o}C (10 s), annealing at 50^{o}C (30 s), extension at 72^{o}C for 30 seconds and primer-dimer removal and signal acquisition at 80^{o}C for 10 seconds. Thermal cycling programmes for both *catA* and *xylE* genes comprised an initial denaturation step at 95^{o}C (5 mins), followed by 40 cycles of denaturation at 95^{o}C (10 s), annealing at 58^{o}C (30 s), extension at 72^{o}C (30 s), with primer-dimer removal and signal acquisition at 80^{o}C for 10 seconds. Reactions were run in triplicate and negative controls (PCR-grade water) were included in all amplifications.

In an initial experiment to evaluate the methodology (Nnadi, 2019), PCR amplicons from genes of interest in the amended treatment were verified to be of correct band size by electrophoresis on a 2% (w/v) agarose gel. Bands were visualised using a Chemi Doc™ MP Imaging System (Bio-Rad Laboratories Ltd. Hertfordshire, UK), excised under UV radiation and extracted using the QIAquick Gel Extraction Kit (QIAGEN, USA). Following confirmation of their DNA sequences, the cleaned PCR products were used as positive controls for the standard curve. CT values of the treatments were related to the standard curve. Gene copy numbers were calculated using the formula: number of copies = (ng/ul DNA x 6.022 x10^{23}) / (PCR product length in base pairs x 1 x 10^{9} x 650 (Staroscik, 2004) where Avogadro's number of 6.022x10^{23} is the number of molecules/mole DNA and 650 Da is the average weight of a base pair. Results were expressed as $\log_{10}$ of gene copy numbers per g dry soil (log 10 $^{g-1}$).

Table 2 Details of the primers used for the detection and quantification of target hydrocarbon catabolic genes

Target gene	Primer name	Annealing temperature ^{o}C	Sequence (5' – 3')	Amplicon size	References
Alkane monoxygenase	*alkB*	50	F: AAC TAC ATC GAG CAC TAC GG R: TGA AGA TGT GGT TGC TGT TCC	100 bp	Powell et al. 2006
Catechol-1,2-dioxygenase	*catA* (C12O)	58	F: ACVCCVCGHACCATYGAAGG R: CGSGTNGCAWANGCAAAGT	470 bp	Sei et al. 1999 Shahsavari et al. 2016
Catechol-2,3-dioxygenase	*xylE* (C23O)	58	F: AAGAGGCATGGGGGCGCACCGGTTCGATCA R: CCAGCAAACACCTCGTTGCGGTTGCC	380 bp	Azhari et al. 2010 Shahsavari et al. 2016

2.4.3 High-throughput sequencing of 16SrRNA gene amplicons for bacterial community profiling

Samples of microbial community DNA extracted from bioremediation treatments with and without BSG at each time point (days 0, 5, 12 and 21), as used for catabolic gene quantification, were also used for community profiling via analysis of the 16S rRNA gene. V4 variable region PCR primers 515 – 806 (Caporaso et al. 2011) were used in a single-step 30 cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94°C (3 mins), followed by 28 cycles (5 cycle used on PCR products) of 94°C (30s), 53°C for (40 s) and 72°C (1 min), after which a final elongation step at 72°C (5 mins) was performed.

Sequencing was performed at MR DNA, Shallowater, TX, USA using an Ion Torrent PGM system. Sequence data were processed using a proprietary analysis pipeline (MR DNA, Shallowater, TX, USA). In summary,

sequences were depleted of barcodes and primers, then sequences < 150bp, with ambiguous base calls and with homopolymer runs exceeding 6bp were removed. Sequences were denoised, OTUs generated, and chimeras removed. Operational taxonomic units (OTUs) were defined by clustering at 3% divergence (97% similarity). Final OTUs were taxonomically classified using BLAST against a curated database derived from Green Genes, RDPII and NCBI (www.ncbi.nlm.nih.gov, DeSantis et al. (2006), http://rdp.cme.msu.edu). Data was submitted to the Sequence Read Archive (SRA) at NCBI with the BioProject accession number PRJNA861128. https://www.ncbi.nlm.nih.gov/sra/PRJNA861128

2.5 Statistical Analysis

IBM SPSS 24 statistics software was used to determine significant differences among treatments in respect to TPH reduction, microbial count, and catabolic gene copy numbers. All data were tested for normality using the Shapiro-Wilk test. Analysis of variance (ANOVA) and the least significant difference (LSD) *post hoc* test were used to determine significant differences among treatment means of more than two independent variables, while the independent T test was used to determine significant differences between two independent variables. Significance was determined as $p < 0.05$.

3 Results

3.1. Determination of TPH removal and detection of hydrocarbon metabolites

3.1.1 Percentage TPH reduction

The natural attenuation treatment (S+D) and the treatment amended with BSG (S+D+G) produced a rapid reduction in TPH of 78% and 84% respectively by day two (Table 3). Further reductions of 92% in the natural attenuation, and 96% in the amended treatments were observed after one week of incubation. After 28 days of incubation, a final 99% reduction was observed in the amended treatment and a 93% reduction in the natural attenuation treatment. Statistical analysis revealed a significant difference in TPH total peak heights between the two treatments throughout the experiment except at day 0. The treatment amended with BSG showed a significantly higher percentage TPH reduction compared to the natural attenuation, unamended treatment.

Table 3 Percentage reduction of TPH during bioremediation of diesel contaminated soil treatments with (S+D+G) and without (S+D) BSG

	% TPH Reduction in treatments	
Days	S+D	S+D+G
2	78	84
5	89	92
7	92	96
12	92	97
14	92	97
21	93	98
28	93	99

3.1.2 Extent of aliphatic TPH (C10-C28) fractions removal and biodegradation rate

The extent of aliphatic TPH fractions (C10-C28) removal in both natural attenuation (S+D) and BSG amended treatments (S+D+G) was evidenced by the reduced concentrations of the TPH fractions over time, during the bioremediation (Fig 1). The results show a 100% decline of the C10 fraction by day 12 in both treatments. This was the only hydrocarbon fraction to be fully removed in the natural attenuation treatment. However, a complete decline of the C10, C12 and C28 fractions was observed in the amended treatment by day 28. These three fractions had initial concentrations of 700 mg kg^{-1}, 2400 mg kg^{-1} and 500 mg kg^{-1} respectively at the start of the incubation. The concentrations of the C14 to C26 fractions reduced over time but persisted until day 21 in both treatments. However, the reduction of these fractions was significantly greater in the amended treatment than in the natural attenuation treatment. The biodegradation reaction rate constant (k) was significantly higher in the amended treatment (0.1021 day^{-1}) compared to the natural attenuation treatment (0.0590 day^{-1}). The first order linear model r values were 0.8699 and 0.9585 respectively for these two treatments.

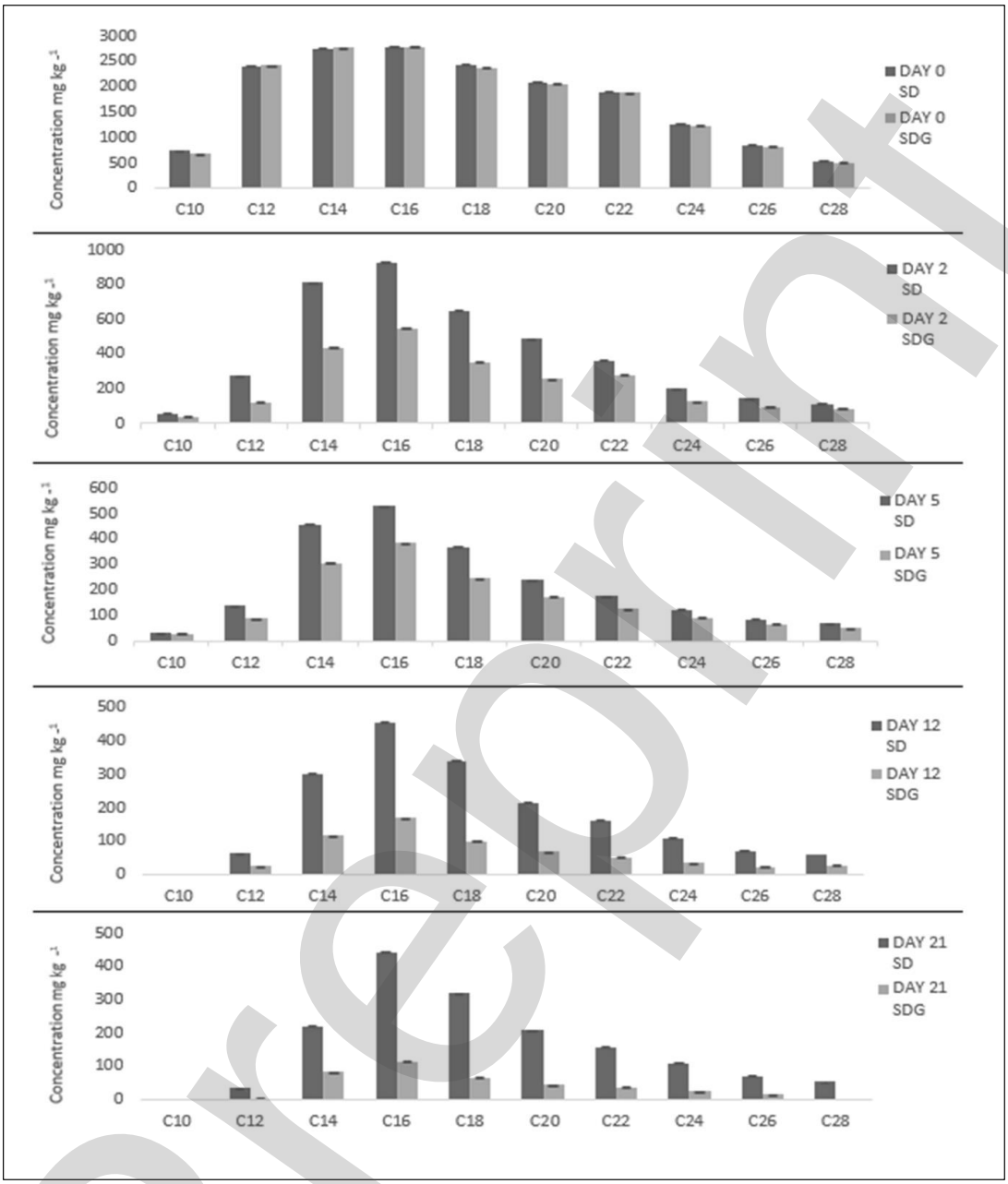

Fig 1 Extent of aliphatic TPH (C10 - C28) fractions removal, with and without BSG, over time, during diesel biodegradation. Results represent the means of three replicates. Error bars show standard error. SD = soil + diesel, SDG = soil + diesel + BSG amendment.

3.1.3 Detection of hydrocarbon degradation metabolites and BSG metabolic potential

Compounds detected by GC-MS from the treatments (S+D and S+D+G) at different stages during the bioremediation, were assessed to determine the presence of known metabolites in the degradation pathways of both aliphatic and aromatic hydrocarbons, based on their functional groups such as alcohols, ketones, esters, aldehydes, carboxylic acids, and esters.

Results for aliphatic hydrocarbon degradation (Table 4) revealed that ketones, being metabolites of the subterminal oxidation pathway, were present in both unamended (S+D) and amended (S+D+G) treatments, throughout the bioremediation process, while aldehydes, being metabolites of the terminal oxidation pathway, were absent. However, carboxylic acids, which are further metabolites of the terminal oxidation pathway, resulting from aldehyde oxidation (van Elsas 2007; Chikere et al. 2011; Varjani, 2017), were present at the start and up until day 5 in the unamended treatment but were only present at the start in the amended treatment. For aromatic hydrocarbons however, aromatic ketones, which are oxidation products of the *ortho*-cleavage pathway, were present in both treatments throughout the bioremediation. Aromatic aldehydes, which are oxidation products of the *meta*-cleavage pathway, were only observed from the start of the experiment on days 0 and 5 in unamended treatments but were present on days 5, 12 and 21 in the amended treatments.

338 *Table 4 Hydrocarbon degradation metabolic compounds present in treatments, over time during bioremediation, including diesel control*

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				TREATMENTS							
				Day 0		Day 5		Day 12		Day 21	
HYDROCARBON TYPE	FUNCTIONAL GROUP	TYPE OF COMPOUND	DIESEL CONTROL	SOIL AND DIESEL	SOIL DIESEL AND BSG	SOIL AND DIESEL	SOIL DIESEL AND BSG	SOIL AND DIESEL	SOIL DIESEL AND BSG	SOIL AND DIESEL	SOIL DIESEL AND BSG
ALIPHATIC	C-H	STRAIGHT CHAIN	+	+	+	+	+	+	+	+	+
HYDROCARBONS	–OH	ALCOHOLS		+	+	+	+	+	+	+	+
	–H–C=O	ALDEHYDES		-	-	-	-	-	-	-	-
	–R–C=O	KETONES		+	+	+	+	+	+	+	+
	–OH–C=O	CARBOXYLIC ACIDS		+	+	+	-	-	-	-	-
	–O–C=O	FATTY ACID ESTER		+	+	+	+	+	+	+	+
AROMATIC		BENZENE RINGED	+	+	+	+	+	+	+	+	+
HYDROCARBONS	–OH	CATECHOL (Alcohol)	+	+	+	+	+	+	+	+	+
	–H–C=O	ALDEHYDES		+	-	+	+	-	+	-	+
	–R–C=O	KETONES		+	+	+	+	+	+	+	+
	–OH–C=O	CARBOXYLIC ACIDS		+	+	+	+	+	+	-	-
	–O–C=O	FATTY ACID ESTER		+	+	+	+	+	+	+	+

3.2 Determination of colony forming units (CFUs) for the enumeration of heterotrophic and hydrocarbon degrading bacteria

Changes in heterotrophic bacterial CFUs are shown in Fig 2a. CFUs in the unamended diesel contaminated soil samples (S+D) increased at the start of the experiment and peaked at 5.0 log 10 g^{-1} on day 7 after which they decreased continually. The CFUs in the diesel contaminated samples amended with BSG (S+D+G), increased continually from day 0 and peaked at 5.6 log 10 g^{-1} on days 12 and 14 before decreasing gradually. However, the heterotrophic bacterial CFUs remained significantly higher in the amended treatment (S+D+G). The soil control treatment (S) also showed increased CFUs after day 0 but peaked at 4.2 log 10 g^{-1} on day 2. This treatment had the lowest CFUs.

The soil control treatment (S) had no hydrocarbon utilising bacterial growth until day 7 after which the population slowly declined. Hydrocarbonoclastic CFUs in the unamended treatment (S+D) remained similar throughout the experiment (Fig 2b), with a 6% increase on day 12 when it peaked at 3.6 log 10 g^{-1}. The CFUs in the amended treatment (S+D+G) had the highest CFUs and showed a rapid CFU increase of 34% on day 2 as compared to the level at day 0. It then peaked at 4.9 log 10 g^{-1} on day 7 with a 43% increase compared to the unamended treatment (S+D). After this time, a gradual decrease in CFU's occurred until day 28 at which point a 12% increase in CFUs was seen as compared to the unamended treatment. A negative correlation was observed between TPH concentration and hydrocarbon degrading CFUs in both unamended (r = -0.858) and amended (r = -0.926) treatments.

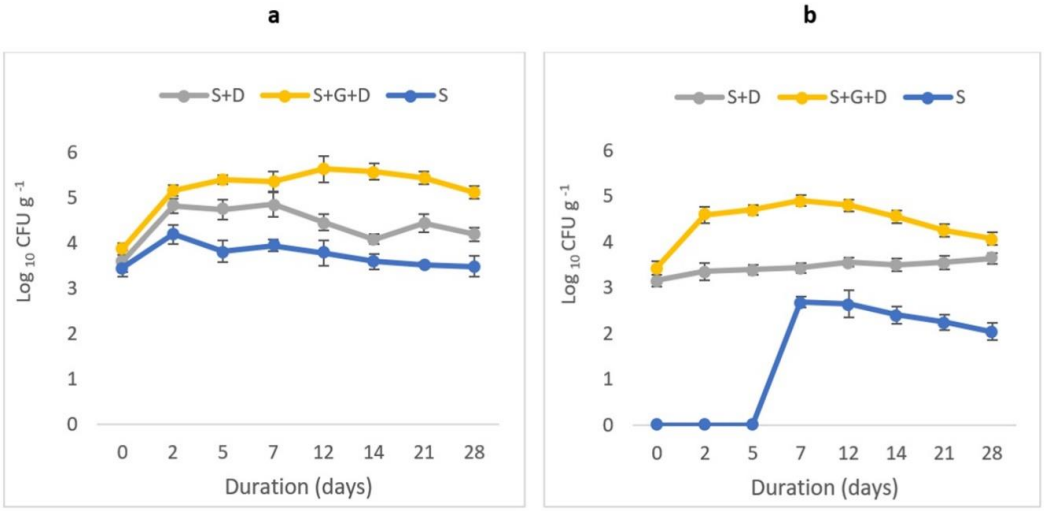

Fig 2 Mean colony forming units (CFU's) for the enumeration of heterotrophic bacteria (**a**) and hydrocarbon degrading bacteria (**b**) in treatments with and without BSG. Results represent the means of three replicates. Error bars show standard error. S+D = soil + diesel, S+D+G = soil + diesel + gain amendment, S = control soil.

3.3 Quantification of diesel catabolic genes and bacterial community composition.

3.3.1 Quantification of diesel catabolic genes by qPCR

Gene copy number quantification in this study was limited to the contaminated soils, with and without BSG, to determine the bioremediation potential of BSG. The gene copy numbers of all three catabolic genes in this study, increased with the addition of BSG. From day 5 until the end of the experiment, the *alkB* gene copy numbers (Fig 3a) in the amended treatment (S+D+G) were significantly higher than that of the unamended treatment (S+D). Also, a very distinct difference in gene copy numbers was observed in the amended treatment on day 12 while that in the unamended treatment remained constant. Nevertheless, there was an increase in gene copy numbers in both treatments after this time. From the start to the end of the experiment, gene copy numbers in the unamended treatment (S+D) increased by 22.8% from 4.57 $\log_{10}$ g^{-1} to 5.61 $\log_{10}$ g^{-1} while that in the amended treatment (S+D+G) increased by 40.4% from 5.65 $\log_{10}$ g^{-1} to 7.93 $\log_{10}$ g^{-1}.

A significant difference in the *catA* gene copy numbers was observed (Fig 3b), between the amended (S+D+G) and unamended (S+D) treatments from the start and throughout the experiment. The rapid reduction in percentage TPH and concentration of aliphatic hydrocarbon (C10 – C28) fractions also occurred at the start of the experiment and was associated with a negative correlation in hydrocarbon degrading bacterial CFUs. However, like the *alkB* gene, a distinct difference in gene copy numbers was evident between the amended and unamended treatments from day 12, after which it plateaued until the end of the experiment. Nevertheless, a 13.2% decrease in the *catA* gene copy numbers, from 6.59 $\log_{10}$ g^{-1} to 5.72 $\log_{10}$ g^{-1}, was observed in the natural attenuation treatment S+D, from day 0 to the end of the experiment. In the amended treatment, S+D+G, *catA* gene copy numbers increased by 4.7% from 7.10 $\log_{10}$ g^{-1} to 7.43 $\log_{10}$ g^{-1}.

Gene copy numbers for the *xylE* gene (Fig 3c), like the *alkB* gene, were significantly higher in the amended treatment from day 5 and throughout the experiment. However, as with both the *alkB* and *catA* genes, a distint difference in gene copy numbers was evident from day 12. A 1.9% increase in copy number of the *xylE* genes was observed in the naturally amended treatment, S+D, from day 0 to the end of the experiment increasing from 4.84 $\log_{10}$ g^{-1} to 4.93 $\log_{10}$ g^{-1} while a 15.4% increase was observed in the amended treatment, S+D+G, from 4.89 $\log_{10}$ g^{-1} to 5.64% $\log_{10}$ g^{-1}.

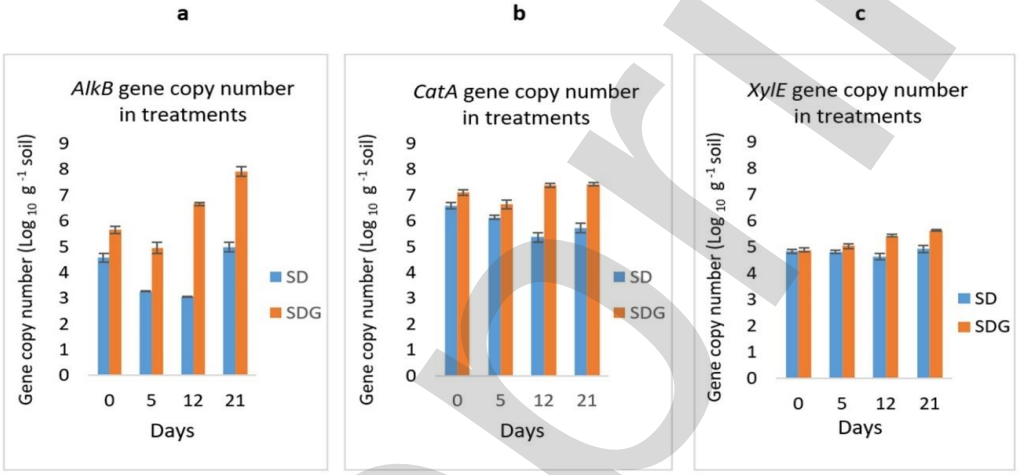

Fig 3 Gene copy numbers of the *alkB* gene (**a**), *catA* gene (**b**) and *xylE* gene (**c**) in treatments with and without BSG over time, during the bioremediation of diesel contaminated soil. Results represent the means of three replicates. Error bars show standard error. S+D = soil + diesel, S+D+G = soil + diesel + gain amendment.

3.3.2 High-throughput Sequencing of 16S rRNA gene amplicons for community profiling

Results monitoring the bacterial community changes and dynamics during the transformation of aliphatic and aromatic hydrocarbons in the diesel contaminated soil during bioremediation, were profiled in terms of the relative abundance (Fig 4) and percentage abundance (Fig 5) of the twelve most dominant bacterial populations in the treatments. Aliquots from the same community genomic DNA samples, with and without BSG, used in the qPCR assay for the three catabolic genes in this study, were used for this analysis for comparability with each other and with the control soil alone and BSG alone samples. DNA yield from contaminated samples (ug) were all high and the purity based on absorbance at 260nm and 280nm using a Nanodrop (ND-2000, Thermo Fisher Scientific, UK) were between 1.8 to 2.0 as expected. Diesel contamination did not appear to impact genomic DNA recovery from the soil.

The results in Fig 4, show that the treatment amended with BSG had the highest relative abundance of bacterial populations. These results are consistent with those from culture-based methods in which treatments amended with BSG supported the highest heterotrophic and hydrocarbonoclastic bacterial populations. However, the bacterial population on day 0 were similar for all treatments except that of the grain control. The grain control, however, did not continually sustain microbial growth on its own.

As shown in Fig 5, we observed in the soil alone control treatment (S), that the percentage abundance of *Flavobacterium succinicans* increased from around 7% on day 0 to 20% on day 12. Thereafter, it reduced to 11% on day 21. Culturable hydrocarbon degrading bacteria were first observed on day 7 in this treatment. Apart from these changes, no significant shifts in the percentage composition of other bacterial genera in this treatment were observed.

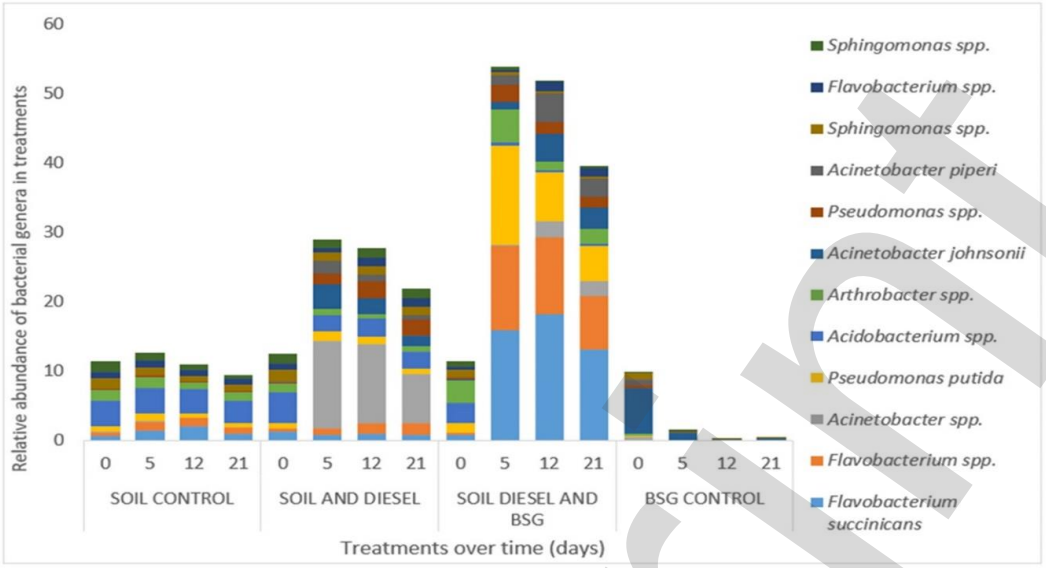

Fig 4 Relative abundance of bacterial genera present in samples showing the bacterial population profile of treatments during diesel bioremediation over time.

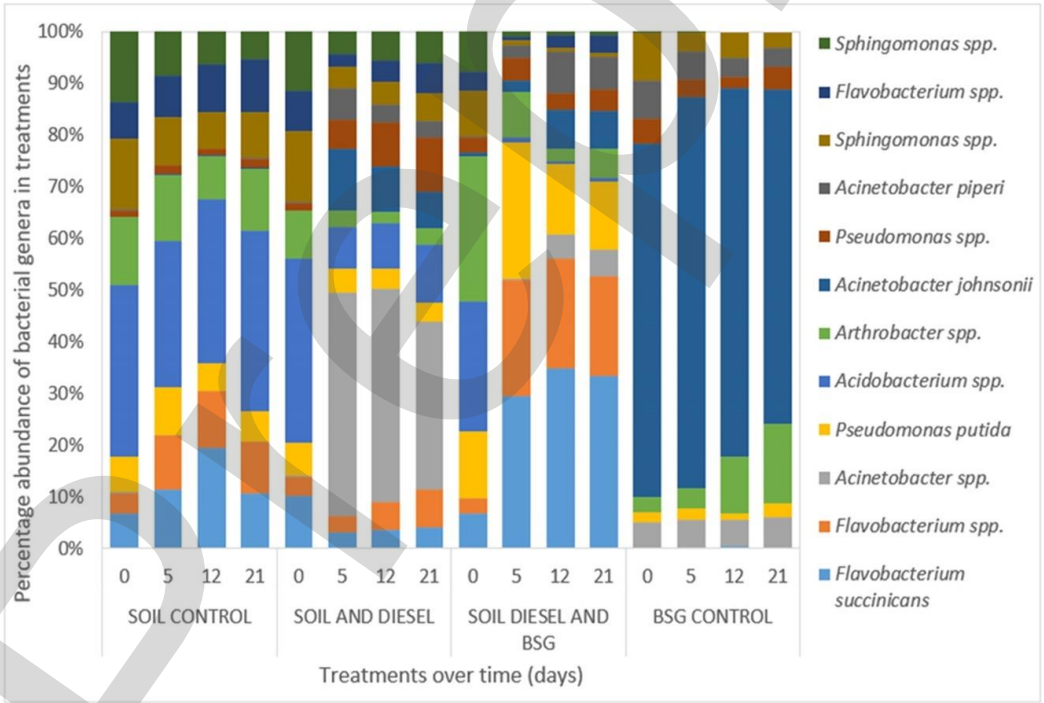

Fig 5 Bacterial community profile showing percentage abundance of bacterial populations during diesel bioremediation.

In the BSG alone control treatment, the percentage abundance of *Arthrobacter* spp. increased from 2% to 15% from the start of the experiment up till day 21 (Fig 5). Slight decreases in the abundance of *Pseudomonas* spp., *Acinetobacter piperi* and *Sphingomonas* spp. were also observed. As with the control soil alone treatment, there were no significant changes in the percentage composition of other bacterial populations in this treatment with *Acinetobacter johnsoni* being the most abundant species in this treatment throughout the experiment.

In contrast, the percentage composition of bacterial populations in the diesel contaminated soil treatments changed extensively. A rapid shift in bacterial community composition was observed in the unamended soil and diesel treatment after day 0. The population profile revealed three *Acinetobacter* species in the treatment by day 5 namely: *Acinetobacter* species 45%, *Acinetobacter johnsonii* 12%, and *Acinetobacter piperi* 5% while *Acidobacterium* spp. reduced. Changes in species abundance were observed hereafter though no more shifts in community composition were seen. After day 5 the three *Acinetobacter* species gradually reduced while two *Flavobacterium* species increased. *Acinetobacter* spp. were the most abundant species in this treatment though it reduced from 45% to 41% and 32% on days 5, 12 and 21, respectively. *Acinetobacter johnsonii*, reduced from 12% to 8% and 5% on days 5, 12 and 21 respectively and *Acinetobacter piperi* reduced from 5% to 3% and 2.5% on days 5, 12 and 21. *Flavobacterium* spp., however, increased from 3% to 5% and finally to 9% on days 5, 12 and 21 while *Flavobacterium succinicans* increased from 2% to 3% and 5% on the same days. *Pseudomonas* spp. also increased from 5% to 8% and finally to 10%.

Addition of BSG to the contaminated soil on day 0 may have contributed to the increased percentage abundance of the *Arthrobacter* spp. and *Pseudomonas putida* observed in the amended treatment (S+D+G) compared to the unamended soil and diesel (S+D), and soil control (S) treatments (Fig 5). A rapid shift in community composition was observed in this treatment by day 5, resulting in the increased abundance of *Flavobacterium succinicans*, *Flavobacterium* spp. and *Pseudomonas putida.* Thereafter, this treatment sustained these three species as the most abundant throughout the experiment. At the same time, *Arthrobacter* spp. declined and *Acidobacterium* spp. were almost eliminated. As with the unamended treatment, a reduction in the abundance of *Acidobacterium* spp. following contamination was also observed in the amended treatment. *Acinetobacter piperi* was also observed in this treatment after day 0. After day 5, a second shift in bacterial community composition was observed in the amended treatment during which *Acinetobacter* spp. was seen and the abundance of two *Acinetobacter* species increased. *Acinetobacter johnsonii* increased from 2% to 8% and 9% and *Acinetobacter piperi* increased from 4% to 9% and 8% on days 5, 12 and 21 respectively. At the same time, however, the abundance of *Pseudomonas putida*, reduced from 28% to 14%.

Discussion

The enhancement of the bioremediation process by stimulating with BSG was evident by the significant reduction in percentage TPH and high biodegradation rates observed. Similarly, Agarry and Latinwo (2015) reported a high rate of 79% TPH reduction after 28 days when using brewery spent effluent for the bioremediation of a 10% (w/w) diesel contaminated soil. The potential of BSG to enhance the bioremediation process was also made obvious by the significant increase in both heterotrophic and hydrocarbonoclastic bacterial populations, along with a negative correlation between petroleum hydrocarbon degradation and bacterial CFUs (Margesin et al. 2003; Shahsavari et al. 2013). The CFUs observed in the soil only control reveal that the soil has got indigenous hydrocarbon degraders which began utilising diesel as their only carbon source after a lag phase of about one week. Similarly in their study, Alisi et al (2009) reported that in soils without prior hydrocarbon contamination, a 2-day lag phase was observed before CO_2 evolution and 6 days were required for the soil microbial community to become active.

The initial rapid TPH reduction observed in this study was contributed to by all the aliphatic TPH (C10 – C28) hydrocarbon fractions. However, although the C10, C12 and C28 aliphatic fractions in the amended treatment were completely degraded, the C12 fraction, having a lower molecular weight despite its higher initial concentration, degraded faster than the C28 fraction. Thus, it is likely that the most labile and low molecular weight fractions were metabolised in the initial rapid phase while the more resistant and higher molecular weight fractions were degraded in a later, second phase (Ros et al. 2010; Karamalidis et al. 2010; Varjani and Upasani, 2017). The initial rapid degradation in both the natural attenuation and amended treatments may also have resulted from autochthonous hydrocarbon degraders being present in the soil (Shahsavari et al. 2013). Although the soil was pristine, hydrocarbonoclastic microbes are known to be ubiquitous and following contamination, it has been observed that microbial communities in pristine soils adapt well to contaminants resulting in rapid degradation (Kingston, 2002; Margesin et al. 2013; Schwarz et al. 2018).

The degradation pathways of aliphatic hydrocarbons show that, depending on the position of the methyl group initially attacked by the oxygenase enzyme, alkanes may be oxidised to either primary or secondary alcohols (van Elsas et al. 2007). Further oxidation of primary alcohols produce aldehydes and fatty acids while secondary alcohols produce ketones and esters (Varjani, 2017). The degradation pathways of aromatic hydrocarbons, however, indicate that they are initially oxidised to catechol (Benzene-1, 2-diol) (Das and Chandran 2011; Eze et al. 2021). Thereafter, cleavage of the benzene ring occurs in either of two routes: The *ortho*-cleavage pathway, which involves cleavage between carbons 1 and 2 catalysed by catechol-1, 2-dioxygenase to produce

ketones and esters, and the *meta*-cleavage pathway, which involves cleavage between carbons 2 and 3 by catechol-2, 3-dioxygenase to produce aldehydes and carboxylic acids (Chikere et al. 2011; Olajire and Essien, 2014). From the elucidated pathways, and as observed during this study, ketones and aldehydes are key distinguishing metabolites in determining the catabolic pathways utilised by the bacterial community. Aldehydes being the distinguishing metabolite for the terminal oxidation pathway of aliphatics and *meta*-oxidation pathway of aromatics (Okoh 2006; van Elsas 2007; Olajire and Essien 2014). Ketones on the other hand, distinguish the activity of the subterminal oxidation pathway of aliphatics and the *ortho*-oxidation pathway of aromatics (van Elsas 2007; Varjani et al. 2017).

According to Tsugawa et al. (2011), to deduce the metabolic activity of microbes associated with hydrocarbon biodegradation, an analysis of metabolites in the biological samples is required. Thus, to deduce the metabolic pathways in this study, we identified ketones and aldehydes from the literature (Varjani 2017) as the signature metabolites that differentiate between the terminal and subterminal pathways in aliphatic hydrocarbon degradation catalysed by alkane monoxygenase and the *ortho* and *meta* degradation pathways of aromatic hydrocarbon degradation catalysed by catechol-1, 2-dioxygenase and catechol-2, 3-dioxygenase respectively (Varjani and Upasani, 2017; Fuchs et al. 2011). Aliphatic hydrocarbons may have been degraded mainly via the subterminal oxidative pathway as suggested by the presence of aliphatic ketones and esters, in both the natural attenuation and amended treatments, throughout the biodegradation process (Table 4). However, the presence of aliphatic fatty acids (carboxylic acids), though aliphatic aldehydes were not detected, suggests that a rapid oxidation of aldehydes to carboxylic acids may have occurred, particularly in the BSG amended treatments. The carboxylic acids may then have been oxidised via the β-oxidation pathway to the tricarboxylic acid (TCA) cycle.

For aromatic hydrocarbon degradation however, aromatic ketones were present throughout the degradation indicating that degradation was mostly via the *ortho*-cleavage pathway catalysed by catechol 1, 2-dioxygenase (Fuchs et al. 2011). Aromatic aldehydes, which are the key differentiating metabolites of the *meta*-cleavage pathway catalysed by catechol-2, 3-dioxygenase (Varjani and Upasani, 2017), were only present at the start of the degradation in the natural attenuation treatment. However, the amended treatment with BSG supported its presence as from day 5 of the degradation process and throughout the experiment (Table 4). Thus, the metabolites present at different times during the bioremediation process in this study, have shown that the breakdown of diesel was mostly via the subterminal oxidation pathway for the aliphatic hydrocarbon content of diesel and via the *ortho*-oxidation pathway for the aromatic hydrocarbon content of diesel.

The amendment of diesel contaminated soil with BSG appeared to enhance metabolism via these two pathways, while also sustaining metabolism via the *meta*-cleavage pathway for aromatic hydrocarbon degradation and speeding up the metabolism of carboxylic acids via the β-oxidation pathway for aliphatic hydrocarbons in terms of synthesis or appearance of metabolic substrates and catabolism of metabolic products. Thus, diesel degradation in this study fits the elucidated pathways, is consistent with the literature (Yanto and Tachibana, 2013; Ghosal et al. 2016; Varjani and Upasani, 2017) and provides evidence that the amendment of contaminated soil with BSG enhances the oxidative breakdown of hydrocarbons.

Knowledge of the degradation pathway, as determined by the metabolites present, provided information on the degradation genes to be assayed and related the degradation pathways to their associated degradation genes. DNA sequencing targeting the 16S rRNA gene amplicon, on the other hand, provided information regarding the community bacterial populations associated with the biodegradation; thus, correlating these degradation genes with the bacterial population harbouring them and further confirming the bioremediation potential of BSG. Among the three associated genes of the degradation pathways in this study, the *alkB* gene, which is key in the aerobic biodegradation of aliphatics (Powell et al. 2006; Rojo, 2009; Wang et al. 2010; Garrido-Sanz et al. 2019), had the highest percentage increase from the start up until the end of the bioremediation especially in the amended treatment. This, interestingly, tallies with the presence of the aliphatic ketone metabolites all through the biodegradation experiment. Similar increase in *alkB* gene copy numbers have been recorded during the bioremediation of a 1% diesel and engine oil contaminated soil using plant residues (Shahsavari et al. 2013).

The combination of microbiome analysis with GC-MS evaluation of metabolites adopted in this study, is useful in elucidating the bacterial community dynamics during diesel bioremediation. The results tally with that of the elucidated degradation pathways such that the abundance of aromatic ketone metabolites from the start of the experiment (Table 4), was reflected in the abundance of the *catA* gene, which was the only gene having a significantly higher copy number from the start of the degradation. This coincided with the rapid hydrocarbon biodegradation observed at the onset of the degradation process. Since the *catA* gene encodes the catechol-1, 2-dioxygenase enzyme, responsible for metabolising aromatic hydrocarbons through the *ortho*-cleavage pathway and having ketone metabolites, its activity may have contributed to the statistically significant reduction of hydrocarbons in the treatments amended with BSG. A similar rapid initial degradation phase was also observed in the study of Ros et al. (2010).

Pseudomonas putida, which was present in abundance in the treatment amended with BSG from the start of the experiment and sustained all through the experiment in this treatment (Fig 5), declined after day 5. Similarly, the *catA* gene copy numbers (Fig 3b) also declined by day 5. *Pseudomonas putida* is known to metabolise both aliphatic and aromatic hydrocarbons (Marcus, 2003) and have the *catA* gene that metabolises aromatic hydrocarbons via the *ortho*-cleavage pathway catalysed by catechol-1, 2-dioxygenase (Harwood and Parales, 1996; Nelson et al. 2002). *Pseudomonas putida* was also present in the treatment without BSG, in which hydrocarbons were also reduced during the initial rapid biodegradation. Nevertheless, the abundance of *P. putida* was greater in the amended treatment than in the unamended treatment. This suggests that *P. putida* may have been actively involved in the metabolism of aromatics catalysed by catechol-1, 2-dioxygenase to produce ketones at the start of the experiment and contributed to the rapid initial degradation observed. Further experiments showing the breakdown pattern of aromatic hydrocarbons, revealed that aromatic hydrocarbons were mostly degraded at the initial degradation phase (Nnadi, 2019).

A major shift in microbial community in favour of *Acinetobacter* species was observed following the introduction of diesel to the natural attenuation treatment. Acinetobacter is a known hydrocarbon degrading genus, identified as having the *xylE* gene encoding catechol-2, 3- dioxygenase that catalyses the degradation of aromatic hydrocarbons via aldehyde metabolites (Méndez et al. 2010; Fuentes et al, 2014). This gene has also been detected in *Pseudomonas* spp. (Méndez et al. 2010; Fuentes et al. 2014). *Acinetobacter* was identified in the unamended soil and diesel treatment immediately after the start of the degradation process. It is not surprising, then, that aromatic aldehydes, were identified in this treatment at the start of the degradation. However, as the abundance of *Acinetobacter johsonii* and *Acinetobacter piperi* decreased in the unamended treatment after day 5, both species increased in abundance until the end of the experiment in the amended treatment. This was also reflected by the presence of aromatic aldehydes in the amended treatment as from day 5 up till the end of the experiment and although *Acinetobacter* spp. persisted in the unamended treatment, aldehydes were no longer seen in the unamended treatment.

Community profiling has shown that the genera *Acinetobacter* and *Pseudomonas,* among others, may be responsible for the metabolism of hydrocarbons in this study. These genera were also observed to be present in BSG. *Flavobacterium*, however, was seen to be the most abundant genera in the amended treatment. Nevertheless, it is likely that a synergy of microbes is necessary for the complete degradation of hydrocarbon contaminants rather than a single species. This tallies with previous studies showing that species of *Acinetobacter, Arthrobacter, Flavobacterium, Nocardia, Pseudomonas,* and *Vibrio* are associated with petroleum hydrocarbon degradation *(*Varjani, 2017; Chandra et al. 2013). Since *Flavobacterium* have been identified as degraders of hydrocarbons (Zhang et al. 2006; Shahsavari et al. 2013; Varjani, 2017), their increase in the soil alone control treatment may be the reason behind the presence of hydrocarbon utilising bacterial CFUs in this treatment as from day 7. *Acidobacterium* spp. however, reduced in abundance following hydrocarbon contamination. This is interesting to note as they are ubiquitous and mostly found in soils but not known hydrocarbon degraders (Naether et al. 2012).

The adoption of molecular techniques in this study provided a culture independent approach in the elucidation the bacterial population dynamics during the biodegradation of diesel contaminated soil amended with BSG. Changes in the abundance and an enrichment of autochthonous aerobic hydrocarbon degraders resulting in shifts in the bacterial population in their favour, was made evident. Also, the community profiling of the BSG alone control treatment showed that the grain supports its own microflora; most of which are known hydrocarbon degraders. This supports the potential of supplementation with BSG as not just a biostimulation treatment but also bioaugmentation.

5. Conclusion

The findings of this study, including microbiological, metabolite and genetic analysis to assess bioremediation provides a more informed understanding of the process. It also elucidates the correlation between these parameters in monitoring bioremediation to allow for the design of more effective interventions. It has demonstrated that the amendment of diesel contaminated soil with BSG enhanced biodegradation under controlled conditions reflecting temperate environments. BSG contains intrinsic hydrocarbon degrading species including *Pseudomonas putida* and *Acinetobacter piperi* and promoted increased gene copy numbers of the *alkB*, *catA* and *xylE* genes. Organic by-products such as BSG can thus provide a valuable contribution to bioremediation as well as reducing potential landfill disposal making it an environmentally viable option.

Conflicts of interest

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.

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Ethical approval and consent to participate

Not applicable

Consent for publication

This work has not been published before and is not under consideration for publication elsewhere. All authors have agreed with the content of the manuscript and have consented to its submission for publication.

Author contributions

Conceptualization: [Mabel Owupele Nnadi, Keith Thomas]; Methodology: [Mabel Owupele Nnadi, Keith Thomas, Lewis Bingle]; Formal analysis and investigation: [Mabel Owupele Nnadi]; Writing - original draft preparation: [Mabel Owupele Nnadi]; Writing - review and editing: [Mabel Owupele Nnadi, Keith Thomas, Lewis Bingle]; Funding acquisition: [N/A]; Resources: [N/A]; Supervision: [Keith Thomas]

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References

Abioye OP, Agamuthu P, Abdul-Aziz RA (2012) Biodegradation of used motor oil using organic waste amendment. Biotech Res Intl 2012. https://doi.org/10.1155/2012/587041

Agamuthu P, Tan YS, Fauziah SH (2013) Bioremediation of hydrocarbon contaminated soil using selected organic wastes. Procedia Environ Sci. https://doi.org/10.1016/j.proenv.2013.04.094

Agamuthu P, Abioye OP, Abdul Aziz A (2010) Phytoremediation of soil contaminated with used lubricating oil using *Jatropha curcas*. Journal of Hazardous Materials. https://doi.org/10.1016/j.jhazmat.2010.03.088

Agarry S, Latinwo KG (2015) Biodegradation of diesel oil in soil and its enhancement by application of bioventing and amendment with brewery waste effluents as biostimulation - bioaugmentation agents. J. Ecol. Eng. https://doi.org/10.12911/22998993/1861

Alisi C, Musella R, Tasso F, Ubaldi C, Manzo S, Cremisini C, Sprocati AR (2009) Bioremediation of diesel oil in a co-contaminated soil by bioaugmentation with a microbial formula tailored with native strains selected for heavy metals resistance Science of the Total Environment http://dx.doi.org/10.1016/j.scitotenv.2009.01.011

Azhari NE, Devers-Lamrani M, Chatagnier G, Rouard N, Martin-Laurent F (2010) Molecular analysis of the catechol-degrading bacterial community in a coal wasteland heavily contaminated with PAHs. J. Hazard. Mater. https://doi.org/10.1016/j.jhazmat.2009.12.074

Aziz S, Ali MI, Farooq U, Jamal A, Liu F, He H, Guo H, Urynowicz M, Huang Z (2020) Enhanced bioremediation of diesel range hydrocarbons in soil using biochar made from organic wastes. Environ. Monitoring. Assess. https://doi.org/10.1007/s10661-020-08540-7

Baek KH, Kim H, Moon S, Lee I, Oh H, Yoon B (2004) Effects of soil types on the biodegradation of crude oil by Nocardia sp. H17. J. Microbiol. Biotechnol. 15,901 – 905.

Bento FM, Flavio AOC, Benedict CO, William TF (2005) Comparative bioremediation of soils contaminated with diesel oil by natural attenuation, biostimulation and bioaugmentation. Bioresource. Technol. https://doi.org/10.1016/j.biortech.2004.09.008

Bekele GK, Gebrie SA, Mekonen E, Fida TL, Woldesemayat AA, Abda EM, Tafesse M, Assefa F (2022) Isolation and characterization of diesel degrading bacteria from hydrocarbon contaminated sites, flower farms, and soda lakes. Int J Microbiol, 2022:5655767. https://doi.org/10.1155/2022/5655767

Bianco A, Budroni M, Zara S, Mannazzu I, Fancello F, Zara G (2020) The role of microorganisms on biotransformation of brewers' spent grain. App. Microbiol. Biotechnol. https://doi.org/10.1007/s00253-020-10843-1

Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Turnbaugh PJ (2011) Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. PNAS https://doi.org/10.1073/pnas.1000080107

Chagas-Spinelli ACO, Kato MT, de Lima ES, Gavazza S (2012) Bioremediation of a tropical clay soil contaminated with diesel oil. J. Environ. Manag. https://doi.org/10.1016/j.jenvman.2012.05.027

Chandra S, Sharma R, Singh K, Sharma A (2013) Application of bioremediation technology in the environment contaminated with petroleum hydrocarbon. Ann Microbiol. https://doi.org/10.1007/s13213-012-0543-3

Chikere, C. B., Surridge, K. J., Cloete, T. E., Okpokwasili, G. C. (2011) Phylogenetic diversity of dominant bacterial communities during bioremediation of crude oil-polluted soil. Revista Ambiente & Água - An Interdisciplinary Journal of App Science: 6, 61 -76.

Das N, Chandran P (2011) Microbial degradation of petroleum hydrocarbon contaminants: An overview. Biotechnol. Res. Int. https://doi.org/10.4061/2011/941810

DeSantis TZ, Hugenholtz P, Larsen N, et al (2006) Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Applied and Environmental Microbiology https://doi.org/10.1128/AEM.03006-05

Dineen SM, Aranda IVR, Anders DL, Robertson JM (2010) An evaluation of commercial DNA extraction kits for the isolation of bacterial spore DNA from soil. J. App. Microbiol. https://doi.org/10.1111/j.1365-2672.2010.04816.x

Dussán J, Numpaque M (2012) Degradation of diesel, a component of the explosive ANFO, by bacteria selected from an open cast coal mine in La Guajira, Colombia. J Bioproces Biotechniq. https://doi.org/10.4172/2155-9821.1000126

Emam TM, Erin KE, Mathew JR (2014) Soil sterilization alters interactions between the native grass *Bouteloua gracillis* and invasive *Bromus tectorum*. J Arid Environ. 111, 91–97. https://doi.org/10.1016/j.jaridenv.2014.08.006

Emmett B, Reynolds B, Chamberlain P, Rowe E, Spurgeon D, Brittain S, Frogbrook Z, Hughes S, Lawlor A, Poskitt J, Potter E, Robinson D, Scott A, Wood C (2010) Countryside survey: Soils report from 2007. Centre for Ecology and Hydrology, Wallingford, Oxon UK, 192 pp.

Eze M O (2021) Metagenome analysis of a hydrocarbon-degrading bacterial consortium reveals the specific roles of BTEX biodegraders. Genes 12*, 98.* https://doi.org/10.3390/genes12010098

Eze MO, Thiel V, Hose GC, George SC, Daniel, R (2022) Bacteria-plant interactions synergistically enhance biodegradation of diesel fuel hydrocarbons. Commun Earth Environ 3, 192. https://doi.org/10.1038/s43247-022-00526-2

Fernández MD, Javier P, Alonso C, Aragonese P, Tarazona JV (2011) Terrestrial microcosms in a feasibility study on the remediation of diesel-contaminated soils. Ecotoxicol. Environ. Safety https://doi.org/10.1016/j.ecoenv.2011.08.009

Fuchs G, Boll M, Heider J (2011) Microbial degradation of aromatic compounds – from one strategy to four. Nat. Rev. Microbiol. https://doi.org/10.1038/nrmicro2652

Fuentes S, Méndez V, Aguila P, Seeger, M (2014) Bioremediation of petroleum hydrocarbons: catabolic genes, microbial communities, and applications. Appl. Microbiol. Biotechnol. https://doi.org/10.1007/s00253-014-5684-9

Gao Y, Shu-hai G, Jia-ning W, Dan L, Hui Wang, De-Hui Z (2014) Effects of different remediation treatments on crude oil contaminated saline soil. Chemosphere. https://doi.org/10.1016/j.chemosphere.2014.08.070

Garrido-Sanz D, Redondo-Nieto M, Guirado M, Jimenez OP, Millan R, Martin M, Ricilla R (2019) Metagenomic Insights into the bacterial functions of a diesel-degrading consortium for the rhizoremediation of diesel-polluted soil. Genes https://doi.org/10.3390/genes10060456

Ghosal D**,** Ghosh S, Dutta TK, Ahn Y (2016) Current state of knowledge in microbial degradation of polycyclic aromatic hydrocarbons (PAHs): A review. *Front Microbiol. https://doi.org/10.3389/fmicb.2016.01369*

Gielnik A, Pechaud Y, Huguenot D, Cebron A, Esposito G, van Hullebusch ED (2019) Bacterial seeding potential of digestate in bioremediation of diesel contaminated soil. Intl. biodeterioration and biodegradation. https://doi.org/10.1016/j.ibiod.2019.06.003

Guarino C, Spada V, Sciarrillo R (2017) Assessment of three approaches of bioremediation (natural attenuation, landfarming and bioagumentation - assisted landfarming) for a petroleum hydrocarbons contaminated soil, Chemosphere. https://doi.org/10.1016/j.chemosphere.2016.11.165

Harwood CS, Parales RE (1996) The beta-ketoadipate pathway and the biology of self-identity. Ann Rev Microbiol https://doi.org/10.1146/annurev.micro.50.1.553

Jansson JK and Hofmockel K (2018) The soil microbiome – from metagenomics to metaphenomics. Curr. Opin. in Microbiol. https://doi.org/10.1016/j.mib.2018.01.013

Karamalidis AK, Evangelou AC, Karabika E, Koukkou AI, Drainas C, Voudrias, EA (2010) Laboratory scale bioremediation of petroleum-contaminated soil by indigenous microorganisms and added *Pseudomonas aeruginosa* strain Spet. Biores. Technol. https://doi.org/10.1016/j.biortech.2010.03.055

Kawagoe T, Kubota K, Kiwako SA, Kubo M (2019) Analysis of the alkane hydroxylase gene and long-chain cyclic alkane degradation in *Rhodococcus*. Advances in Microbiology, https://doi.org/10.4236/aim.2019.93012

Kingston P F (2002) Long-term environmental impact of oil spills. Spill Science & Technology Bulletin, https://doi.org/10.1016/S1353-2561(02)00051-8

Kulshreshtha (2012) Current trends in bioremediation and biodegradation. Bioremediation and Biodegradation https://doi.org/10.4172/2155-6199.1000e114

Lima-Morales D, Jáuregui R, Camarinha-Silva A, Geffers R, Pieper DH, Vilchez-Vargasa R (2016) Linking microbial community and catabolic gene structures during the adaptation of three contaminated soils under continuous long term pollutant stress. appl. environ. microbiol. https://doi.org/10.1128/AEM.03482-15

Lynch K M, Steffen EJ, Arendt EK (2016) Brewers' spent grain: a review with an emphasis on food and health. J. Inst. Brew. https://doi.org/10.1002/jib.363

Marcus Adam (2003) Versatile soil-dwelling microbe is mapped http://www.genomenewsnetwork.org/articles/01_03/soil_microbe.shtml. Accessed 27 June 2022

Margesin R, Labbe D, Schinner F, Greer CW, Whyte LG (2003) Characterization of hydrocarbon-degrading microbial populations in contaminated and pristine alpine soils. App. Env. Microbiol. https://doi.org/10.1128/AEM.69.6.3085-3092.2003

Margesin R, Moertelmaier C, Mair J (2013) Low-temperature biodegradation of petroleum hydrocarbons (n-alkanes, phenol, anthracene, pyrene) by four *Actinobacterial* strains Int. Biodeter. Biodegr. https://doi.org/10.1016/j.ibiod.2012.05.004

Méndez V, Fuentes S, Hernández M, Morgante V, González M, Moore E, Seeger M (2010) Isolation of hydrocarbon-degrading heavy-metal resistant bacteria from crude oil-contaminated soil in central Chile. J Biotechnol. https://doi.org/10.1016/j.jbiotec.2010.09.225

Molina-Barahona L, Rodrıguez-Vázquez R, Hernández-Velasco M, Vega-Jarquın C, Zapata-Pérez O, Mendoza-Cantú A, Albores A (2004) Diesel removal from contaminated soils by biostimulation and supplementation with crop residues. App Soil Ecol. 27,165–175.

Naether A**,** Foesel BU, Naegele V, Wüst PK, Weinert J, Bonkowski M, Alt F, Oelmann Y, et al (2012) Environmental factors affect *Acidobacterial* communities below the subgroup level in grassland and forest soils. App Environ Microbiol. 78, 7398–7406.

Nelson KE, Weinel C, Paulsen IT, Dodson RJ, Hilbert H, Martins dos Santos VA, Fouts DE, Gill SR et al (2002) Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. Environ. Microbiolol. https://doi.org/10.1046/j.1462-2920.2002.00366.x

Nnadi MO (2019) Bacterial community dynamics during the bioremediation of diesel contaminated soil using brewery spent grain. Dissertation, University of Sunderland.

Okoh IA (2006) Biodegradation alternative in the cleanup of petroleum hydrocarbon pollutants. Biotechnol Mol Biol Rev 1, 38-50.

Olajire AA, Essien JP (2014) Aerobic degradation of petroleum components by microbial consortia. J. Petroleum. Environ. Biotechnol. http://dx.doi.org/10.4172/2157-7463.1000195

Powell SM, Ferguson SH, Bowman JP, Snape IS (2006) Using Real-Time PCR to assess changes in the hydrocarbon-degrading microbial community in Antarctic soil during bioremediation. Microbial. Ecology. https://doi.org/10.1007/s00248-006-9131-z

Reddy MV, Devi MP, Chandrasekhar K, Goud RKS, Mohan V (2011) Aerobic remediation of petroleum sludge through soil supplementation: Microbial community analysis. J Hazard Mat. https://doi.org/10.1016/j.jhazmat.2011.09.061

Robertson JA, I'Anson KJA, Treimo J, Faulds CB, Brocklehurst TF, Eijsink Vincent GH, Waldron KW (2010) Profiling brewers' spent grain for composition and microbial ecology at the site of production. LWT – Food. Sc. Technol. https://doi.org/10.1016/j.lwt.2010.01.019

Rojo F (2009) Degradation of alkanes by bacteria. Environ. Microbiol. https://doi.org/10.1111/j.1462-2920.2009.01948.x

Ros M, Rodriguez I, Garcia C, Hernandez T (2010) Microbial communities involved in the bioremediation of an aged recalcitrant hydrocarbon polluted soil by using organic amendments. Bioresource. Technol. https://doi.org/10.1016/j.biortech.2010.03.126

Sarkar J, Sufia K, Kazy S K, Gupta A, Dutta A, Mohapatra B, Roy A, Bera P, Adinpunya Sar M, Sar P (2016) Biostimulation of indigenous microbial community for bioremediation of petroleum refinery sludge. Front. Microbiol. https://doi.org/10.3389/fmicb.2016.01407

Schwarz A, Adetutu EM, Juhasz AL et al (2018) Microbial degradation of phenanthrene in pristine and contaminated sandy soils. Microb Ecol. https://doi.org/10.1007/s00248-017-1094-8

Sei K, Asano K, Tateishi N, Mori K, Ike M, Fujita M (1999) Design of PCR primers and gene probes for the general detection of bacterial populations capable of degrading aromatic compounds via catechol cleavage pathways. J Biosci Bioeng. https://doi.org/10.1016/S1389-1723(00)87673-2

Semple KT**,** Reid BJ, Fermor TR (2001) Impact of composting strategies on the treatment of soils contaminated with organic pollutants. Environ Pollut. https://doi.org/10.1016/S0269-7491(00)00099-3

Shahsavari E, Aburto-Medina A, Taha M, Ball A S (2016) A quantitative PCR approach for quantification of functional genes involved in the degradation of polycyclic aromatic hydrocarbons in contaminated soils. MethodsX. https://doi.org/10.1016/j.mex.2016.02.005

Shahsavari E, Adetutu EA, Anderson PA, Ball AS (2013) Plant residues – A low cost, effective bioremediation treatment for petrogenic hydrocarbon-contaminated soil Sci Total Environ. https://doi.org/10.1016/j.scitotenv.2012.11.029

Sheppard, PJ**,** Adetutu EM, Makadia TH, Ball AS (2011) Microbial community and ecotoxicity analysis of bioremediated weathered hydrocarbon-contaminated soil. Soil Research. https://doi.org/10.1071/SR10159

Souza EC, Vessoni-Penna TC, Oliveira RPDS (2014) Biosurfactant-enhanced hydrocarbon bioremediation: an overview. Int. Biodeterior. Biodegrad. https://doi.org/10.1016/j.ibiod.2014.01.007

Staroscik A (2004) ds DNA copy number calculator. http://cels.uri.edu/gsc/cndna.html Accessed 27 June 2022

Suja F, Rahim F, Taha MR, Hambali N, Razali MR, Khalid A, Hamzah A (2014) Effects of local microbial bioaugmentation and biostimulation on the bioremediation of total petroleum hydrocarbons (TPH) in crude oil contaminated soil based on laboratory and field observations. Int. Biodeter. Biodegr. 90, 115–122. http://doi.org10.1016/j.ibiod.2014.03.006

Tellechea FR, Martins MA, Silva AO, Netto AF, Martins ML (2017) Ex situ Bioremediation of a Tropical Soil Contaminated with Diesel. JSM Biol 2: 1, 1007.

Thomas KR, Rahman PKSM (2006) Brewery wastes. Strategies for sustainability. A review. Aspects. Appl. Biol. 80,147-153.

Truskewycz A, Gundry TD, Khudur LS, Kolobaric A, Taha M, Aburto-Medina A, Ball AS, Shahsavari E (2019) Petroleum Hydrocarbon Contamination in Terrestrial Ecosystems—Fate and Microbial Responses. Molecules. https://doi.org/10.3390/molecules24183400

807 Tsugawa H, Tsujimoto Y, Arita M, Bamba T, Fukusaki E (2011) GC/MS based metabolomics: development of
808 a data mining system for metabolite identification by using soft independent modelling of class analogy
809 (SIMCA). BMC Bioinformatics https://doi.org/10.1186/1471-2105-12-131

810 Umar ZD, Aziz NA, Zulkiflia SZ, Mustafa M (2017) Rapid biodegradation of polycyclic aromatic hydrocarbons
811 (PAHs) using effective Cronobacter sakazakii MM045 (KT933253). MethodsX
812 https://doi.org/10.1016/j.mex.2017.02.003

813 van Elsas JD, Jansson JK, Trevors JK (2007) Modern soil microbiology, 2nd edition. CRC Press, New York, pp
814 387–429. https://doi.org/10.1201/9781420015201

815 Varjani SJ, Upasani VN (2017) A new look on factors affecting microbial degradation of petroleum
816 hydrocarbon pollutants. Int. Biodeter. Biodegr. https://doi.org/10.1016/j.ibiod.2017.02.006

817 Varjani SJ, 2017 Microbial degradation of petroleum hydrocarbons. Bioresource. Technol.
818 https://doi.org/10.1016/j.biortech.2016.10.037

819 Wang W, Wang L, Shao Z (2010) Diversity and abundance of oil degrading bacteria and alkane hydroxylase
820 (alkB) genes in the subtropical sea water of Xiamen Island. Microbial. Ecol.
821 https://doi.org/10.1007/s00248-010-9724-4

822 Wu M, Dick WA, Li W, Wang X, Yang Q, Wang T, Limei Xu, Zhang M, Chen L (2016) Bioaugmentation and
823 biostimulation of hydrocarbon degradation and the microbial community in a petroleum-contaminated
824 soil. Inter. Biodeter. Biodegr. https://doi.org/10.1016/j.chemosphere.2017.01.012

825 Wu M, Guo X, Wu J, Chen K (2020) Effect of compost amendment and bioaugmentation on PAH degradation
826 and microbial community shifting in petroleum contaminated soil. Chemosphere
827 https://doi.org/10.1016/j.chemosphere.2020.126998

828 Wyszkowski M, Wyszkowska J, Borowik A, Kordala N (2020) Contamination of Soil with diesel oil,
829 application of sewage sludge and content of macroelements in oats. Water. Air. Soil. Pollut.
830 https://doi.org/10.1007/s11270-020-04914-2

831 Xu Y, Lu M (2010) Bioremediation of crude oil-contaminated soil: Comparison of different biostimulation and
832 bioaugmentation treatments. J Hazard Mater https://doi.org/10.1016/j.jhazmat.2010.07.038

833 Yang Y, Wang J, Liao J et al (2015) Abundance and diversity of soil petroleum hydrocarbon-degrading
834 microbial communities in oil exploring areas. Appl Microbiol Biotechn https://doi.org/10.1007/s00253-
835 014-6074-z

836 Yanto DHY, Tachibana S (2013) Biodegradation of petroleum hydrocarbons by a newly isolated Pestalotiopsis
837 sp. NG007. Int. Biodeterior. Biodegrad 10.1016/j.ibiod.2013.09.008

838 Yergeau E, Sanschagrin S, Beaumier D, Greer CW (2012) Metagenomic Analysis of the Bioremediation of
839 Diesel Contaminated Canadian Arctic Soils. Plos. One 10.1371/journal.pone.0030058

840 Zhang, X., Cheng, S., Zhu, C., Sun, S. (2006) Microbial PAH-degradation in soil: Degradation pathways and
841 contributing factors. Pedoshere. 16, 555–565. https://citationsy.com/archives/q?doi=10.1016/S1002-
842 0160(06)60088-X

843 Zucchi M, Angiolini L, Borin S, Brusetti L, Dietrich N, Gigliotti C, Barbieri P, Sorlini C, Daffonchio D (2003)
844 Response of bacterial community during bioremediation of an oil polluted soil. J App 94, 2, 248 – 257.
845 https://doi.org/10.1046/j.1365-2672.2003.01826.x

Dear Editor and Reviewers,

Thank you for your suggestions and feedback. We have studied the comments carefully and made corrections to the manuscript which we hope will meet your approval.

Manuscript number: ACMI-D-22-00154
Title: Bacterial community dynamics and associated genes in hydrocarbon contaminated soil during bioremediation using brewery spent grain
Authors: Mabel Owupele Nnadi; Lewis Bingle; Keith Thomas

Reviewer 1 Comments to Author: General comments

This paper presents an interesting study dealing with the enhanced degradation of total petroleum hydrocarbons through the addition of brewery spent grain. This is of interest to researchers in the field of environmental bioremediation. The results are clear and convincing. However, in some cases the results were also discussed in the results section, which is not appropriate. In addition, a number of claims lack supporting references. I recommend moving all discussions to the "Discussion" section unless the authors want to combine both sections. Specific comments that address these and other changes are provided below.

Response: Thank you for the informative comments and helpful suggestions. The discussion previously in Section 3.1.3 of the Results section, have been moved to the Discussion section in lines 480 - 494 of the manuscript and more references have been included as suggested.

Introduction

Line 82-84: "The efficiency…degradation pathways."
Add one or two more refences after this sentence. One relevant reference is the recent study: https://www.nature.com/articles/s43247-022-00526-2

Response: Thank you for this suggestion for improvement. The two references below have been cited in line 84 of the manuscript and added to the reference list in lines 691 and 656.

Eze MO, Thiel V, Hose GC, George SC, Daniel, R (2022) Bacteria-plant interactions synergistically enhance biodegradation of diesel fuel hydrocarbons. Commun Earth Environ 3, 192. https://doi.org/10.1038/s43247-022-00526-2

Bekele GK, Gebrie SA, Mekonen E, Fida TL, Woldesemayat AA, Abda EM, Tafesse M, Assefa F (2022) Isolation and characterization of diesel degrading bacteria from hydrocarbon contaminated sites, flower farms, and soda lakes. Int J Microbiol, 2022:5655767. https://doi.org/10.1155/2022/5655767

Materials and Methods

Line 140: Add the word "in" between the words "used" and "this".

Response: The word ‘in’ has been added to the manuscript on line 140. Thank you for identifying this clarification.

Line 171-175: How many times was extraction performed per sample? Granted, the effectiveness of n-hexane over other solvents such as DCM for the extraction of diesel fuel hydrocarbons was clearly demonstrated in the study http://dx.doi.org/10.1039/C9RA10919F. However, using non-standardized instruments like shakers (without sonication) once may not lead to a complete extraction of residual diesel fuel. The standard methods of extraction include Soxhlet extraction (USEPA Method 3540C), ultrasonic extraction (USEPA Method 3550C), accelerated solvent extraction (ASE) and microwave-based extraction method.

Response: Thank you for identifying this limitation and suggestions for improvement. The extractions were performed for triplicate samples of each treatment per time point. Thereafter, three aliquots were analysed from each triplicate with a total of nine samples per treatment at each time point. The method is comparable to reported literature (Gielink et al. 2019) in terms of mechanically shaking in hexane and was also evaluated for comparability during initial experiments. And although all the diesel fuel may not have been completely extracted, the same method was used all through the experiment to give comparable results of percentage degradation. We have edited the manuscript in lines 171 – 173, to reflect the wording in the literature referred to, during the experiment.

Line 186-187: Use the formula function in MS Word to express %TPH reduction in order to avoid any ambiguity associated with subtraction, division and multiplication here.

Response: Thank you for this clarification. The formular has been updated in line 185 of the manuscript.

Line 188-190: "The concentration…in the samples." How?
Did you make use of an internal standard that have the same chemical characteristics as your target analyte? Or did you prepare calibration curves for all target compounds and then determined the concentration of each analyte using the parameters of its calibration curve? Please provide more details of the approach you used.

Response: Thank you for suggesting this improvement. The TPH Standard Mix 1 (Sigma Aldrich, UK), with known concentration for each of the (C10 – C28) analytes, was used to obtain the calibration curve for each fraction. Concentrations of each fraction in the soil hydrocarbon mixture were then determined based on the calibration curve of each corresponding standard fraction. Retention times of the analytes were compared to the sample chromatogram to determine target compounds and total peak areas of both standard and analyte fractions were determined. The manuscript has been updated in lines 186 - 191 to include this information.

Results

The results are clearly presented. In some cases, some of the results were also discussed in this section, which is not appropriate, unless the authors decide to combine "results and discussion". I recommend moving discussions to the "Discussion" section. See specific comments below.

Line 325-333: Move this part to "Discussion" section and provide sufficient references as mentioned below.

Response: Thank you for this suggestion. Lines 325 – 337 have been moved to lines 480 – 494 in the Discussion section of the manuscript. The references have also been included in the reference section of the manuscript.

Line 328-329: "The degradation pathways…to catechol".
Provide supporting references from published articles. For example, a detailed analysis of these pathways including the mechanisms leading to catechol as well as meta/ortho-cleavages of catechol can be found in doi.org/10.3390/genes12010098 and doi.org/10.1007/BF00186968

Response: Thank you for suggesting this improvement. The sentence has been updated in line 483 – 485 and the references added to the reference section of the manuscript.

Line 335-337: "Aldehydes being…pathways of aromatics." Provide supporting references.

Response: Thank you for identifying and suggesting this improvement. References have been added to this statement in lines 491 – 492.

Line 341-342: "However, carboxylic acids…resulting from aldehyde oxidation,"
Provide supporting references just after the word "oxidation" and before the continuation of the sentence.

Response: Thank you for identifying and suggesting this improvement. References have been added to this statement in line 332 of the Results section.

Reviewer 2 Comments to Author: In this study, the authors examined the bioremediation of diesel-contaminated soil amended with BSG. They monitored for degradation of petroleum hydrocarbons and for recovery of CFUs. They then determined the bacterial pathways involved in degradation of petroleum hydrocarbons and determined that the bacterial community shifts towards Acinetobacter and Pseudomonas. Overall, the authors found that amendment with BSG resulted in an improved reduction in TPH, and that the bacterial community shifts to enrich towards bacteria that are able to break down the hydrocarbons.

The introduction was well-written and made the topic accessible to microbiologists outside of the bioremediation field.

Some minor points to address/areas to improve:

Figure 2: The scale of the y-axes should be the same in Fig 2A and 2B

Response: Thank you for the informative comments and suggestions improvement. Figures 2A and 2B have been updated in line 359 of the manuscript.

What is the cause of the sudden increase in CFUs of hydrocarbon degrading bacteria at Day 5 in the control soil?

Response: Thank you for noting this. The limited growth of hydrocarbon degrading bacteria CFUs up till day 5 is not very clear but may be the result of a long lag phase due to the very small initial numbers of culturable hydrocarbon degrading bacteria in the soil prior to exponential growth by day 5. Similarly, Alisi et al (2009) reported that in soils without prior hydrocarbon contamination, 6 days were required for the soil microbial community to become active. They also observed a 2-day lag phase before CO_2 evolution. This has been updated in the manuscript in lines 463 – 467.

Figure 3: The scale of the y-axes should be the same in Fig 3A- C.

Response: Thank you for identifying this clarification. Figures 3A – C have now been updated in line 392 of the manuscript.

In Fig. 3, are gene copy numbers in the soil+diesel and soil+diesel+BSG shown relative to gene copy numbers in control soil? If so, this should be made clear. If not, how do they compare to the gene copy numbers of the control soil?

Response: Thank you for identifying this clarification. The scope of this study was limited to the quantification of gene copy numbers in the contaminated soil, with and without BSG, to determine the biodegradation potential of BSG. Nevertheless, as this knowledge is useful, it would be considered in future evaluations. The manuscript has been updated to clarify this in lines 367 - 368.

Does diesel impact the recovery of genomic DNA from the soil?

Response: Thank you for identifying this clarification. From the results obtained during this study, the extracted DNA yield from contaminated samples in terms of concentration (ug), were all high and the purity based on absorbance at 260nm and 280nm using a Nanodrop (ND-2000, Thermo Fisher Scientific, UK) were between 1.8 to 2.0 as expected. Thus, it is unlikely that diesel impacted genomic DNA recovery. Lines 403 – 406 of the manuscript has been updated to include this.