Microbial life beyond the grave: *16S rRNA* gene-based metagenomic analysis of bacteria diversity and their functional profiles in cemetery environments

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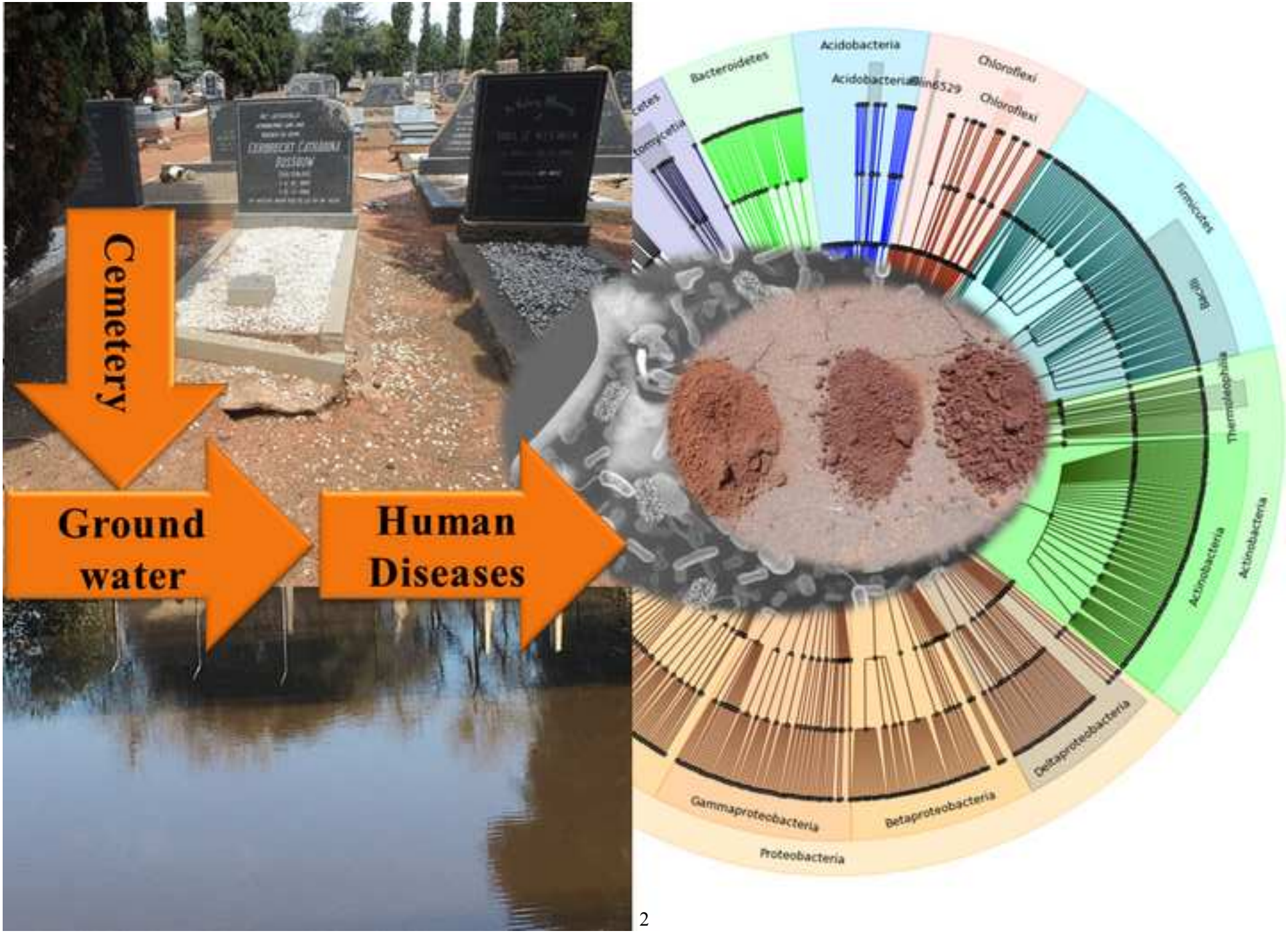
*Graphical Abstract



Highlights

- Cemetery soil bacterial diversity and function were analysed using metagenomics
- Samples were examined at the surface (0 m) and below burial depth (2 m)
- The 2 m-depth samples had more human disease functional profiles
- Infectious diseases signatures including cholera were the most commonly identified
- Cemeteries could pollute groundwater posing heath threats in shallow aquifer areas

Abstract

Recent studies have identified cemeteries as potential environmental reservoirs of multi-drug resistant pathogenic bacteria that could contaminate groundwater sources posing public health threats. However, these findings were based on the identification of culturable bacteria and at times not below burial grounds. Investigation on the bacterial diversity and functional profiles of bacterial communities above and below burial grounds in human cemeteries are few. The current study used high-throughput sequencing techniques to determine the bacterial composition and their associated functional profiles in cemetery soil samples collected at the surface and below burial ground in two South African cemeteries (Maitland Cemetery in Cape Town and Fontein Street Cemetery in Middelburg) to evaluate the potential health threat to surrounding populations through contamination of groundwater. Significant differences were observed between sample depths with the clustering of the surface (0 m) and the 2 m samples into separate groups. *Pseudomonas* and *Corynebacterium* were the most abundant genera across all samples. *Pseudomonas* and *Rhodococcus* were the dominant genera in the 2 m samples while *Prauserella* and *Staphylococcus* were dominant in the surface samples. The 2 m samples showed a lower alpha diversity but recorded higher proportions of human diseases functional classes compared to the surface samples. Human disease functional profiles revealed involvement, in infectious (cholera), neurodegenerative (Alzheimer's disease) cardiovascular (hypertrophic cardiomyopathy) immune system (Systemic lupus erythematosus) metabolic (Type I & II diabetes) diseases and cancer. Antibiotic resistance and antibiotics synthesis signatures were also identified. Thus, cemeteries could be potential sources of microbial and antibiotic pollution in groundwater, especially in areas with shallow water tables such as Maitland. Selection of sites for use as cemeteries should, therefore, require a proper understanding of the hydrogeological

characteristics of the selected site. However, further studies are required to trace the actual movement of these pollutants into groundwater resources.

Keywords: Cemeteries; human burial; microbial communities; functional metagenomics; groundwater contamination; public health

Abbreviations

KEGG; Kyoto Encyclopedia of Genes and Genomes

NGS; Next Generation Sequencing

OTUs; Operational Taxonomic Units

PICRUSt; Phylogenetic Investigation of Communities by Reconstruction of Unobserved States

SA; South Africa

SRA: Sequence Read Archive

1. Introduction

The ubiquitous nature of microorganisms has been demonstrated through numerous scientific studies. Microorganisms have been isolated from the air, water, soil, humans and animals. These organisms have even been isolated from extreme environments such as hot springs (Jardine et al., 2017), high salt environments (Shi et al., 2012), permafrost (Zhang et al., 2013), and

environments with extremely elevated metal concentrations (Kimiran Erdem et al., 2015). In these natural environments, these organisms live in a balanced state, providing various ecological functions that maintain a balanced ecosystem (Dobrovol'skaya et al., 2015).

Although most environmental microorganisms are naturally occurring, others (especially pathogenic ones) are introduced by different anthropogenic activities (Sood et al., 2014). For example, the application of animal waste (Tien et al., 2017) and sludge from wastewater treatment plants (Chen et al., 2016) in agricultural fields leads to the introduction of numerous human and animal pathogens into the soil. Also, the discharge of untreated or poorly treated sewage into the environment has been shown to cause devastating environmental pollution (Bradbury et al., 2013). Similarly, the lack of sanitary facilities in informal settlements has been recognised as a significant contributor to environmental pollution (Van Der Hoven et al., 2017). These human activities introduce not only the pathogens but also nutrients that allow the microorganisms to grow and survive in the environment (Zhao et al., 2017). At times, these pollutants may percolate into and negatively impact groundwater quality with possible adverse health effects on users of untreated water from such sources (Amin et al., 2013; Forslund et al., 2011; Urbaniak et al., 2016).

Although diverse microorganisms have been found in almost all environments known to man, most studies have relied on conventional culture methods for the isolation of specific organisms. However, culture-based methods are biased as they allow for the growth of specific organisms at the expense of others as there exist a significant number of organisms in the environment that are non-culturable with the existing culture media (Malik et al., 2008; Rastogi and Sani, 2011). As such, advanced molecular tools such as next-generation sequencing have been employed not only to study microbial diversity in different environmental sample types but also to determine

the functional profiles of the identified microorganisms (Marathe et al., 2018; Su et al., 2014). Unlike structural metagenomics that aims at studying the structure (abundance and diversity) of a given microbial population, functional metagenomics on the other hand goes further to determine the potential of a given metagenome (Alves et al., 2018). Specific genes are involved in specific activities in nature. Many of these genes and their related functions have been studied and are annotated in known databases such as the KEGG (Kyoto Encyclopedia of Genes and Genomes), COG (Counts of orthologs) and EggNOG (evolutionary genealogy of genes: Non-supervised Orthologous Groups) (Carr and Borenstein, 2014). Thus, the functional profile of a given community is achieved by mapping the obtained sequencing reads orthologous gene groups in these databases to identify corresponding genes or proteins with known and annotated functions.

Despite extensive research on the effects of anthropogenic activities on the modification of microbial communities in the environment, little attention has been put on the impact of cemeteries on the microbial communities in the environment. Although cemeteries or graveyards are believed to prevent any potential public health dangers arising from decomposing bodies, these dedicated areas could still negatively impact human and environmental health. For example, contaminated cemetery soils could be a health risk for grave diggers through injuries and subsequent contamination of wounds during digging (Całkosiński et al., 2015). Also, decomposing bodies may release toxic substances and microorganisms into the environment and contaminate groundwater (Całkosiński et al., 2015; Spongberg and Becks, 1999; Żychowski and Bryndal, 2015). Such waters could become a public health threat to communities living around cemeteries. Thus, the current study was conducted to investigate the diversity and functional profile of bacterial species above and below burial depths in two cemeteries in South Africa to

ascertain if this land use could pose a potential threat to surrounding populations through contamination of groundwater sources. Such information would be crucial to guide town planners and policymakers on the selection of sites for the creation of cemeteries.

2. Material and Methods

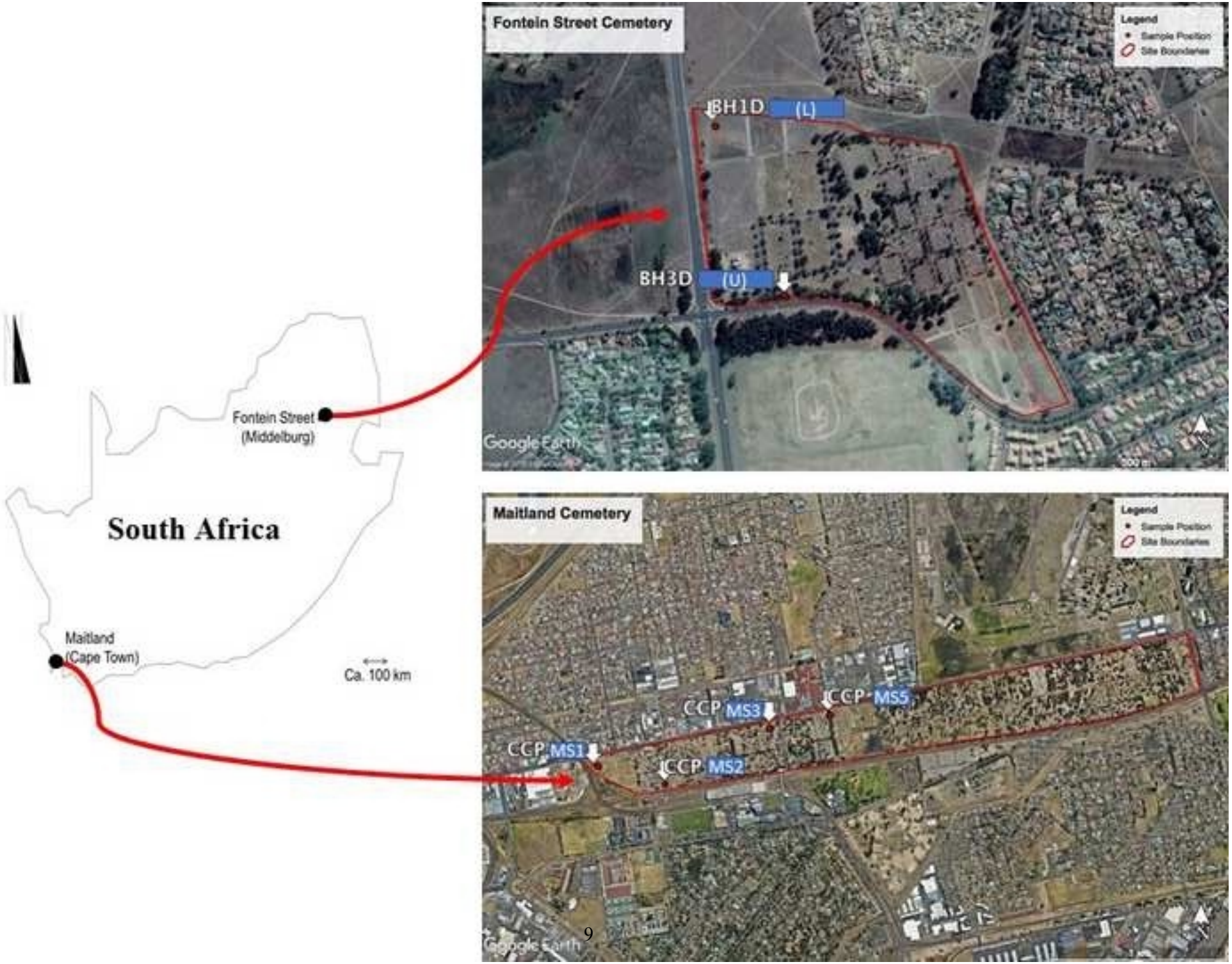
2.1 Study sites

The present study was conducted in two cemeteries located in two provinces of South Africa namely, the Maitland Cemetery in Cape Town (Western Cape Province) and Fontein Street Cemetery in Middelburg (Mpumalanga Province). The localities are shown in Fig. 1 and the geology and burial history are detailed in Table 1.

* maps published jointly by Geological Survey/ Council for Geoscience and Chamber of Mines/ Minerals Council

The Maitland Cemetery in Cape Town has previously been described (Abia et al., 2018b). A major characteristic of this cemetery is that it is located in the Cape Flats, where the aquifer is mainly intergranular and almost entirely saturated. The water table here intersecting the ground surface in most cases, forming wetlands or marsh areas (Seyler et al., 2016).

Fig. . ap of st d site within the o th African bo ndaries (o rce oo le arth)



. Physical and anthropogenic conditions at the relevant cemeteries

	Maitland Cemetery	**Fontein Street Cemetery**
Locality	Cape Town, Western Cape Province	Middelburg, Mpumalanga Province
Geology*	Tertiary to Quaternary unconsolidated sedimentary deposits comprising mostly sands (1:250 000-scale 3418 Cape Town geological sheet)	Karoo Supergroup mudrocks intruded by Jurassic dolerite (1:250 000-scale 2528 Pretoria geological sheet)
Age and size (source: relevant municipality)	Ca. 113 ha 2200 graves/ha 1856-present	Ca. 15 ha Density uncertain Late 1900s to present

2.2. Sample collection

Sample sites were selected based on areas that could be accessed according to the cemetery authorities. At the Maitland Cemetery, samples were collected using a hand auger at four sampling sites (MS1, MS2, MS3 and MS5) while in the Fontein Cemetery, samples were collected using a professional motor operated drill (U; uphill, and L; downhill). For each cemetery and each sampling point, approximately 500 g of soil samples were collected in triplicates from the surface (first 10 cm of soil) and at 2 m below the surface. The 2 m samples were collected as this depth was just below the burial depth of 1.8 m recommended by the South African Department of Health (National Department of Health SA, 2013). Samples were transferred into sterile zip lock bags and transported to the laboratory on ice for further processing and analysis.

2.2. DNA extraction and high throughput amplicon Sequencing

Triplicate samples were pooled together into one sterile ziplock bag and mixed by agitating the bag several times manually. Genomic DNA was directly extracted from 250 mg of each soil sample using the ZR Soil Microbe DNA MicroPrep™ (Zymo Research Corp., Irvine, California, USA), following the manufacturer's instructions. The NanoDropsND-2000 spectrometer (NanoDrop Technologies, Wilmington, DE, USA) was used to determine the concentration and purity of the extracted DNA, and the DNA was sequenced on an Illumina® MiSeq platform as previously described (Abia et al., 2018a).

2.3. Data analysis

All software and methods used were previously described by Abia et al. (2018b). Briefly, Microbial composition was determined using Mothur (Schloss et al., 2009) and CLC Genomics Workbench (CLC Bio Qiagen) after conducting quality (Q>30) and length (150 bp) trimming Operational taxonomic units (OTUs) were obtained based on an updated Greengene database (DeSantis et al., 2006) enriched with NCBI nr BLAST database. Venn plotter at http://bioinformatics.psb.ugent.be/webtools/Venn/ was used to identify the abundance and similarities of the OTUs. Stacked barplots and rarefaction curves plotted using CLC Genomics Workbench (CLC Bio Qiagen), R package Phyloseq (McMurdie and Holmes, 2013) and Microsoft Excel (2013). GraPhlAn (Asnicar et al., 2015) on galaxy was used to depict the core microbial composition across all samples. ClustVis (Metsalu and Vilo, 2015) and R (ComplexHeatmap package) were used to generate heatmap and to perform Principle component analysis (PCA) for sample bacterial diversity. PICRUSt (Langille et al., 2013) on Galaxy was used for functional prediction of bacterial population based on the KEGG database. Further profile analysis was performed using Piphillin with updated KEGG database of May 2017 with 99% identity cutoff (Iwai et al., 2016). Random Forest supervised learning models (mtry=200 and bootstrapping= 1000) was conducted to identify the most important OTUs by calculating the Gini coefficient using R package "RandomForest".

3. Results

3.1. General characteristics of cemetery soil samples

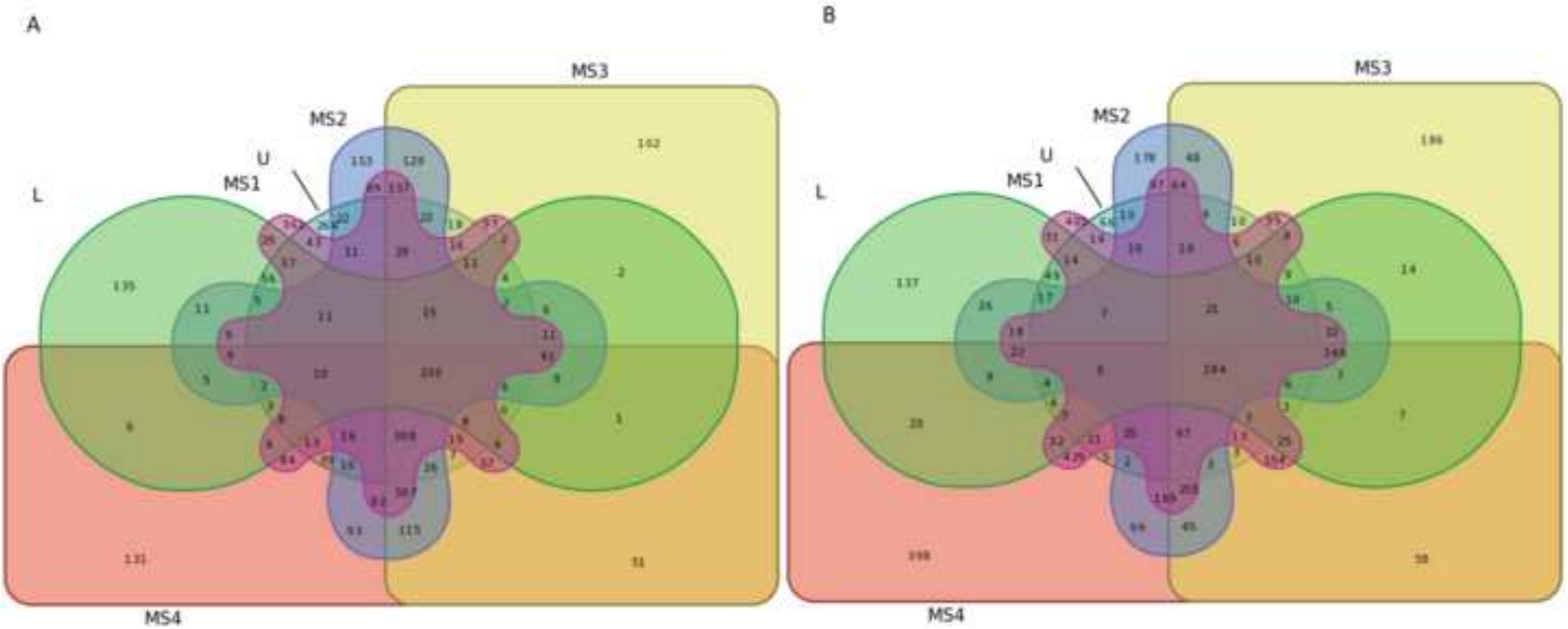
Soil samples were collected from the surface (0 m) and 2 m below the surface at two cemeteries in South Africa and analysed to compare the bacterial diversity and functional profiles of the identified bacteria with respect to depth. A total of 2,888,646 reads were obtained from sequencing of the 16S rRNA gene. Following sequence trimming 2,884,851 sequences passed quality trimming and were considered for further analysis. The average read length of sequences consisted of 296.75 nucleotides (Table 2).

Except for the U site of the Fontein Cemetery and the MS1 site of the Maitland Cemetery, all sampling sites yielded higher reads at 2 m compared to 0 m. A total number of 4670 OTUs were predicted across all samples based on Greengene database (Table S1). Results revealed 110 common OTUs across all samples (**Table S2**). Among the six surface samples, the MS1 samples harboured the highest number of unique OTUs (362 OTUs) while the highest number of unique OTUs in the samples obtained at a depth of 2 m was recorded at MS4 (398 OTUs) as (Fig. 2).

Table 2 . General characteristics of samples, including the total number of reads, average length of sequences, and number of OTUs.

Cemetery	Sampling point	Depth	Total number of reads	Avg. length of reads	Number of predicted OTUs
Fontein	**L**	**0m**	147,712	296.3	709
		2m	184,878	296.8	890
	U	**0m**	216,938	296.6	1266
		2m	183,654	295.1	629
Maitland	**MS1**	**0m**	255,794	297.3	2342
		2m	224,328	297.4	2396
	MS2	**0m**	248,096	297.3	2168
		2m	513,568	297.2	1599
	MS3	**0m**	183,034	297.3	2027
		2m	196,000	296.2	1504
	MS5	**0m**	215,192	297.0	1916
		2m	319,452	296.6	2237

Fig. 2 Comparison of OTUs between six locations at 0 m depth (A) and 2 m depth (B).



On the other hand, the lowest number of OTUs was recorded for the surface samples obtained from MS3 (102 OTUs) and the U-2m sample (Fig. 2). In general, MS4-2m, and U-0m showed the highest number of unique OTUs across all samples (Table S1).

3.2. Bacterial composition of soil samples obtained from the cemeteries

A total of 53 phyla, 162 classes, 323 orders, 516 families, and 795 genera were obtained for all soil samples analysed (Table 3).

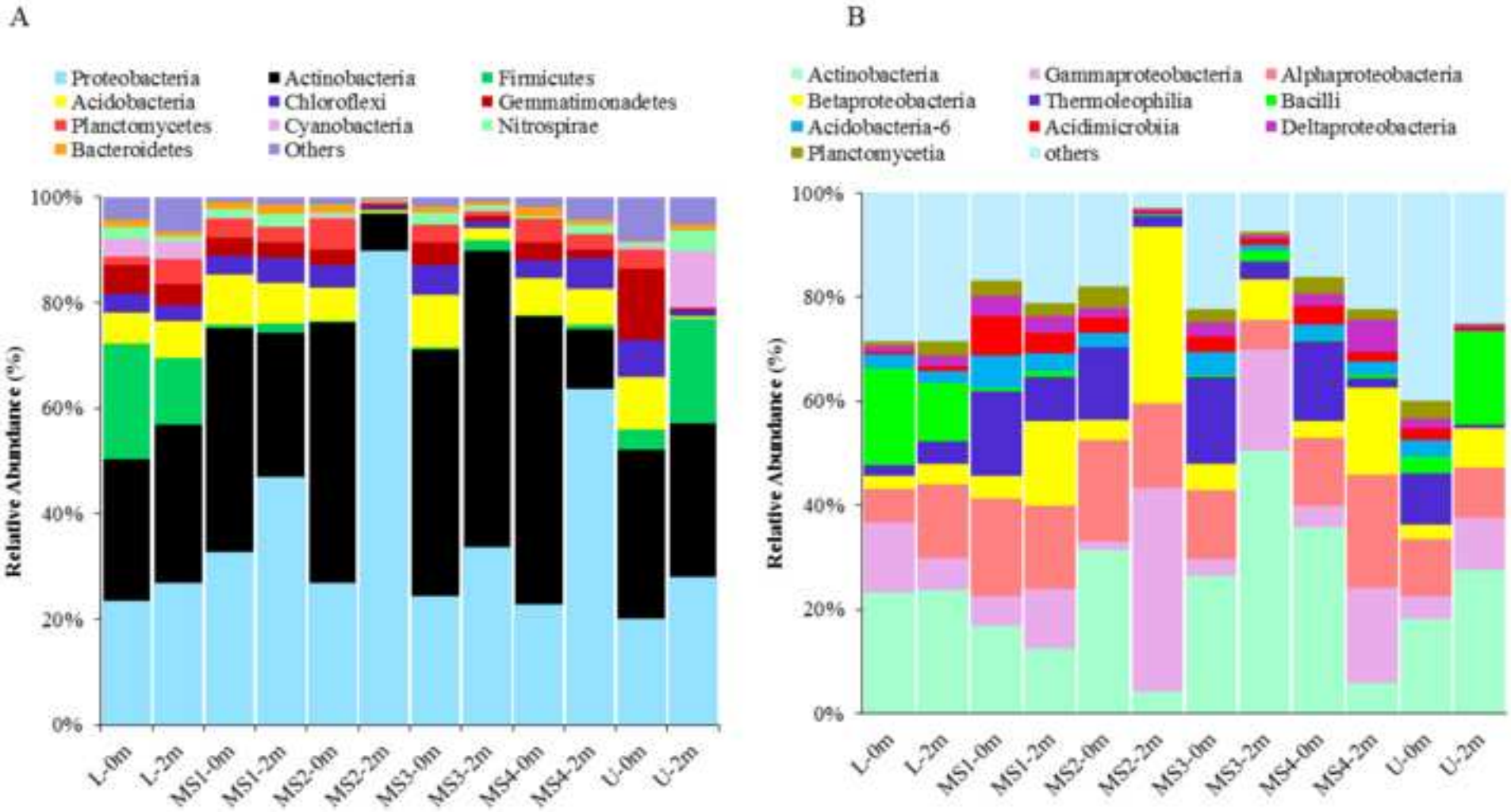
The Pearson's Chi-squared test showed statistically significant differences (df = 99, $p < 0.01$) in the bacterial composition of the cemetery samples. A pairwise analysis revealed that there were statistically significant differences (FDR adj. $p < 0.05$) between sampling locations and studied depths (Table S3).

Proteobacteria (36.6%) and *Actinobacteria* (34.4%) were the most abundant phyla across all samples, followed by *Acidobacteria* (6.1%), and *Firmicutes* (5.4%) (Fig. 3A, Table S1). Also, the bacterial composition of the studied samples differed at the class level (Fig. 3B). The three classes *Actinobacteria* (23%), *Alphaproteobacteria* (13.8%) and *Gammaproteobacteria* (11.5%) were recorded as the most dominant categories in all samples (Fig. 3B, Table S1).

Table 3. The number of predicted OTUs for each sample at phylum, class, order, family, genus and species le els.

Taxonomy	Maitland Cemetery (Western Cape)								Fontein Cemetery			
	MS1		MS2		MS3		MS4		L		U	
	0m	2m	0m	2m	0m	2m	0m	2m	0m	2m	0m	2m
Phylum	53	52	50	45	36	33	44	51	43	35	34	42
Class	162	160	152	161	152	137	155	159	134	131	156	129
Order	321	321	316	322	319	321	319	323	321	319	318	317
Family	513	514	507	515	510	514	510	516	513	511	509	508
Genus	794	792	787	793	749	774	783	795	763	767	785	790

Fig. 3. Comparison of the bacterial composition obtained from cemeter soil. A and B represents the ab ndance of OTUs at ph l m and class le els respecti el



Applying the Fisher Exact test, statistically significant differences (FDR adj. $p < 0.05$) were recorded between the studied samples at the genus level (Table S3). *Pseudomonas* and *Rhodococcus* were the dominant genera in samples obtained at a depth of 2 m, while *Prauserella* and *Staphylococcus* were identified as the most abundant genera in samples obtained from the surface (Fig. 4A). Generally, *Pseudomonas* and *Corynebacterium* were the most dominant genera across all cemetery samples (Fig. 4A).

The different samples displayed varying relative abundances (%) of the bacteria genera. *Pseudomonas*, *Corynebacterium*, and *Staphylococcus* were generally among the most abundant genera recorded (Table 4).

Fig 4. Comparison of the bacterial composition obtained from cemeter soil. A and B represents the ab ndance of OTUs at en s and species le els respecti el.

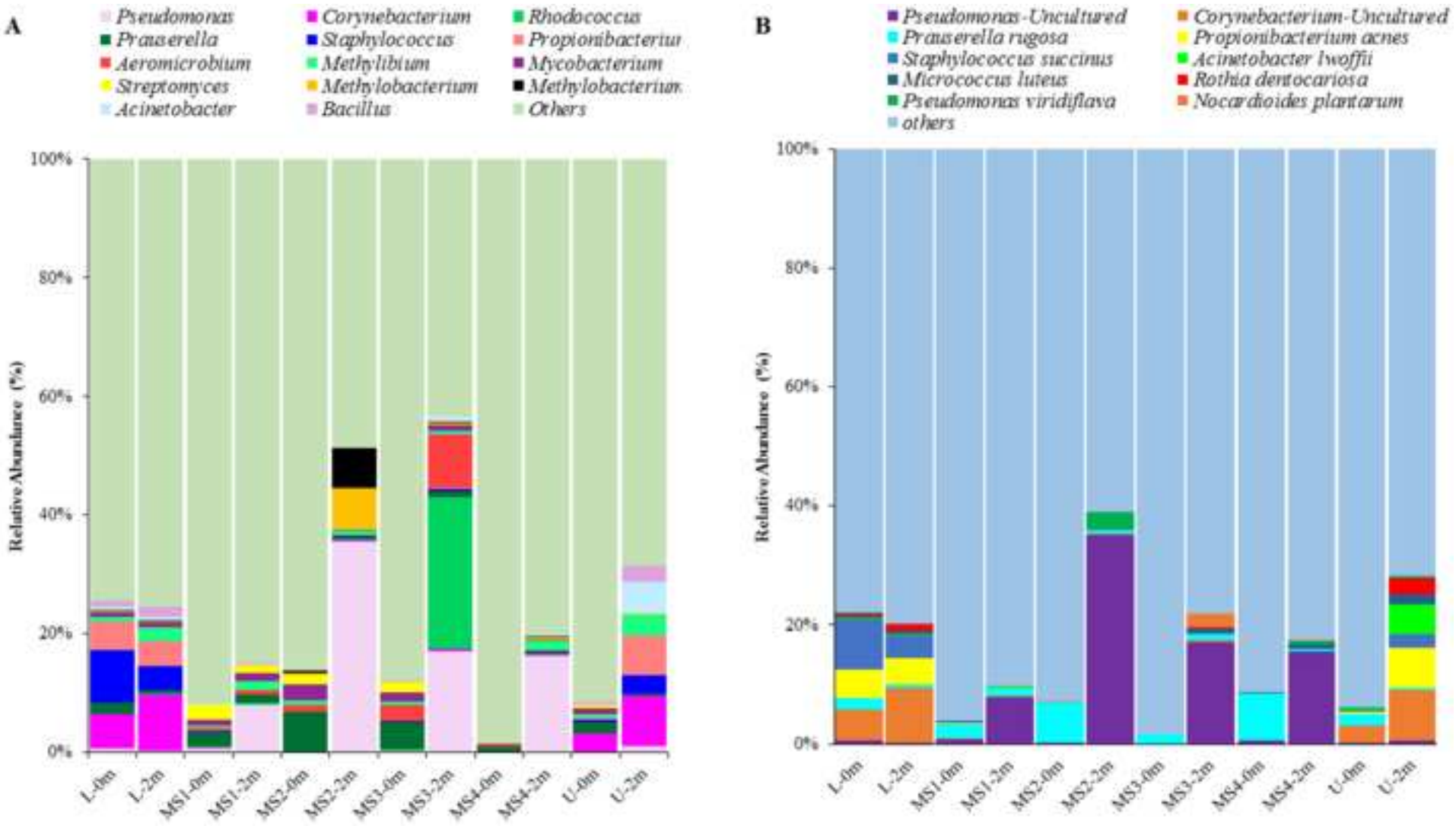


Table 4. Relative abundance (%) of common bacterial genera in soil samples obtained from the cemeteries.

	Maitland Cemetery (Cape Town)								**Fontein Cemetery (Mpumalanga)**			
Taxonomy	**MS1**		**MS2**		**MS3**		**MS4**		**L**		**U**	
	0m	**2m**	**0m**	**2m**	**0m**	**2m**	**0m**	**2m**	**0m**	**2m**	**0m**	**2m**
Bacillus	0.11	0.23	0.05	0.02	0.20	0.16	0.01	0.03	0.88	1.57	0.39	2.86
Bacteroides	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00
Campylobacter	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.20	0.00	0.15
Corynebacterium	0.25	0.11	0.05	0.27	0.05	0.44	0.01	0.03	5.63	9.62	3.01	8.47
Haemophilus	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.08	0.03	0.01	0.04
Legionella	0.01	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.19	0.00	0.00
Mycobacterium	1.03	1.25	2.83	0.27	1.36	0.82	0.32	0.27	0.78	0.66	0.90	0.02
Neisseria	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.30
Nocardia	0.01	0.08	0.04	0.02	0.03	0.01	0.00	0.00	0.16	0.00	0.05	0.00
Pseudomonas	0.74	8.00	0.13	38.08	0.31	17.11	0.06	16.39	0.76	0.22	0.19	1.02
Staphylococcus	0.10	0.06	0.01	0.08	0.01	0.41	0.00	0.02	8.87	4.01	0.37	3.15
Streptococcus	0.02	0.02	0.00	0.01	0.00	0.04	0.00	0.00	1.24	1.90	0.20	2.21
Treponema	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.00	0.00	0.00
Vibrio	0.00	0.01	0.00	0.01	0.00	0.01	0.00	0.00	0.06	0.00	0.04	0.00

Although most of the species were identified as being uncultured, *Prauserella rugosa*, *Propionibacterium acnes* and *Staphylococcus succinus* were found among the dominant known species (Fig. 4B).

3.3. Bacterial Diversity with respect to sampling depth and location

Variations within each sample were calculated based on the number of OTUs in each sample. Based on rarefaction, samples from MS1-2m had the highest variation (richness) when compared to the other samples. However, the samples obtained from 2m depth demonstrated a lower alpha diversity compared to those obtained from the surface (Fig. 5).

The diversity of the bacterial composition among the samples was obtained through the principal component analysis (PCA). A marked difference was observed between the samples obtained from different depth except for MS2-2m which was the most distinct sample having a lower amount of similarity compared to the other samples (Fig. 6).

Fig. 5. Bacterial diversity within samples (alpha diversity). Rarefaction curves were obtained based on the number of OTUs**.**

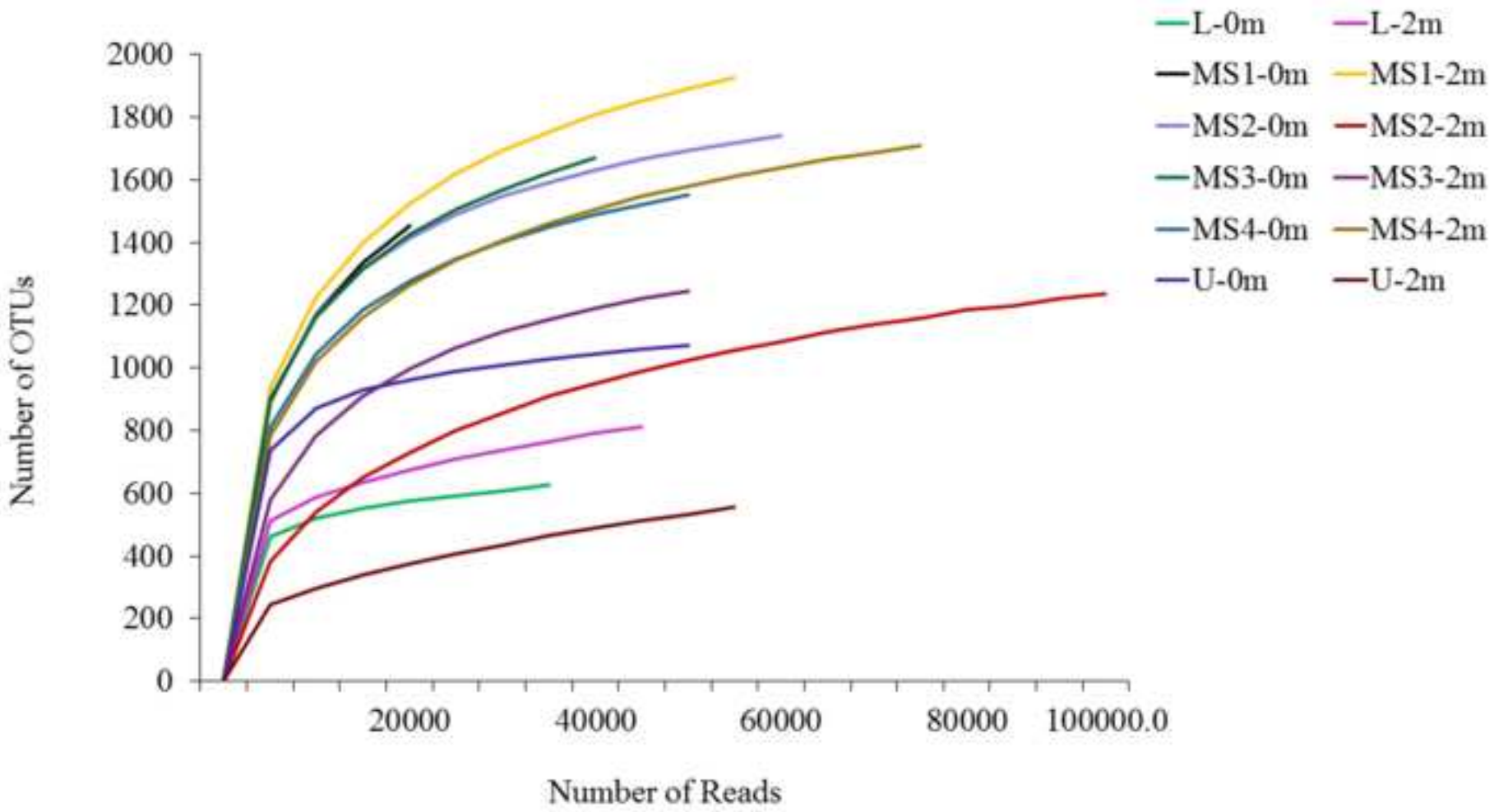
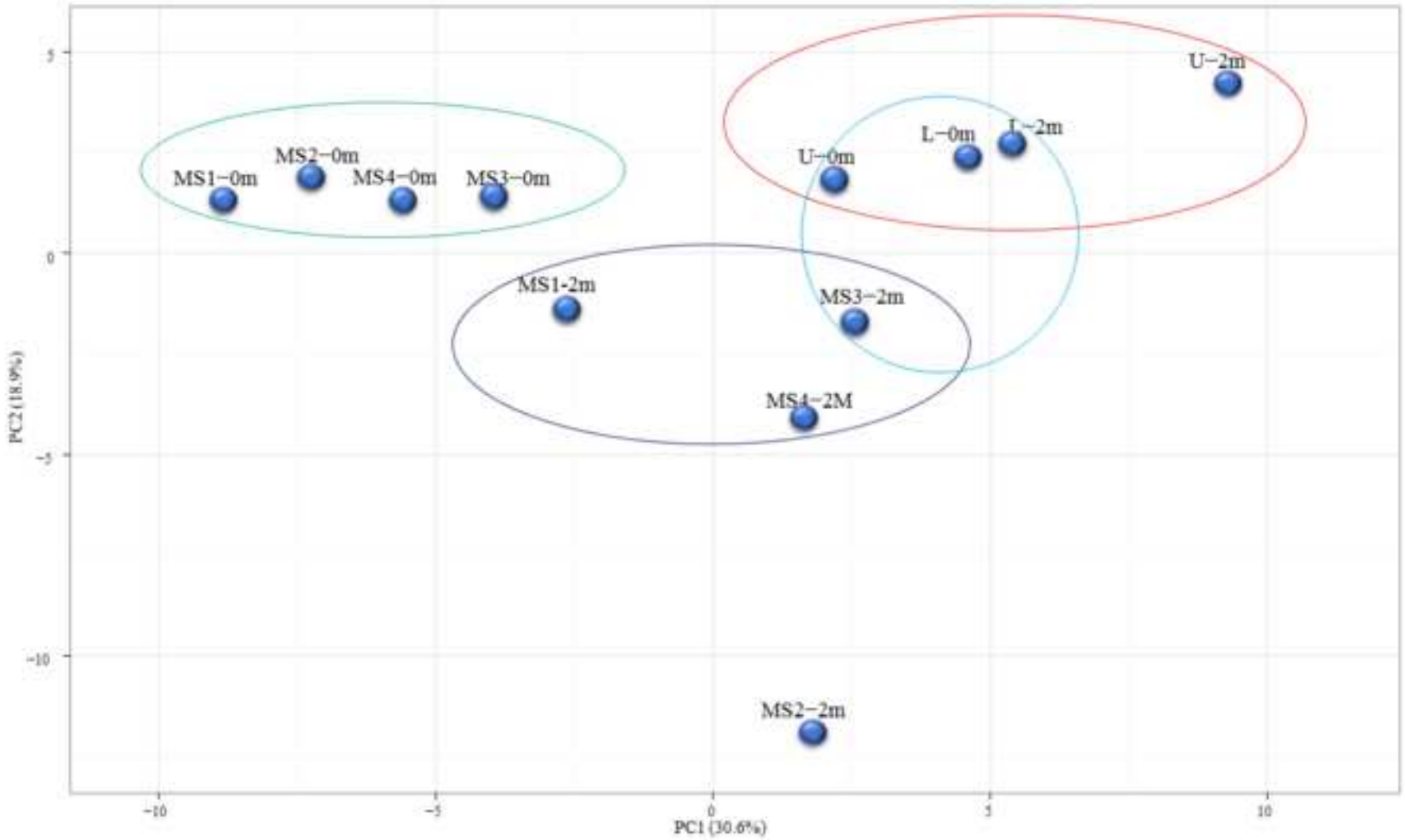


Fig. 6. Bacterial diversity among cemetery soil samples. Principal component analysis (PCA) obtained based on the correlation of the 150 most abundant OTUs across samples.



Despite the observed diversity, PCA analysis revealed a high similarity between U and L (Fontein Cemetery) samples regardless of sampling depth (Fig. 6).

A Heatmap of the most abundant OTUs also revealed differences between cemetery samples (Fig. 7). Like the PCA, the heatmap clustered the U and L samples together (Fig. 7). Interestingly, in all cases, samples obtained from different depths (0 and 2m) were distinguished in separate clusters based on correlation of the most abundant OTUs (Fig. 7).

3.4 Functional profiles of identified bacterial species in cemetery soil samples

Functional contributions of bacteria in cemetery soil samples were explored based on OTUs using the PICRUSt on Galaxy and Piphillin. Findings revealed a total of 306 groups at level 3 KEGG Orthology using PICRUSt (Table S4), whereas more than 1000 enzymes involved in 240 KEGG pathways have been identified based on Piphillin (Tables S5 and S6). Overall, the functional profiles were identified into seven categories including cellular processes, environmental information processing, genetic information processing, human diseases, metabolism and organismal systems; a considerable amount of the top OTUs was unclassified (Table S4). Pathways involved in metabolism, transporting, environmental and genetic information processing were top functional identified classes using PICRUSt and Piphillin (Fig. 8, Table S6).

Fig. 7. Clustering of the cemetery samples using Heatmap based on the 150 most abundant OTUs. The colour legend illustrates the correlation of samples.

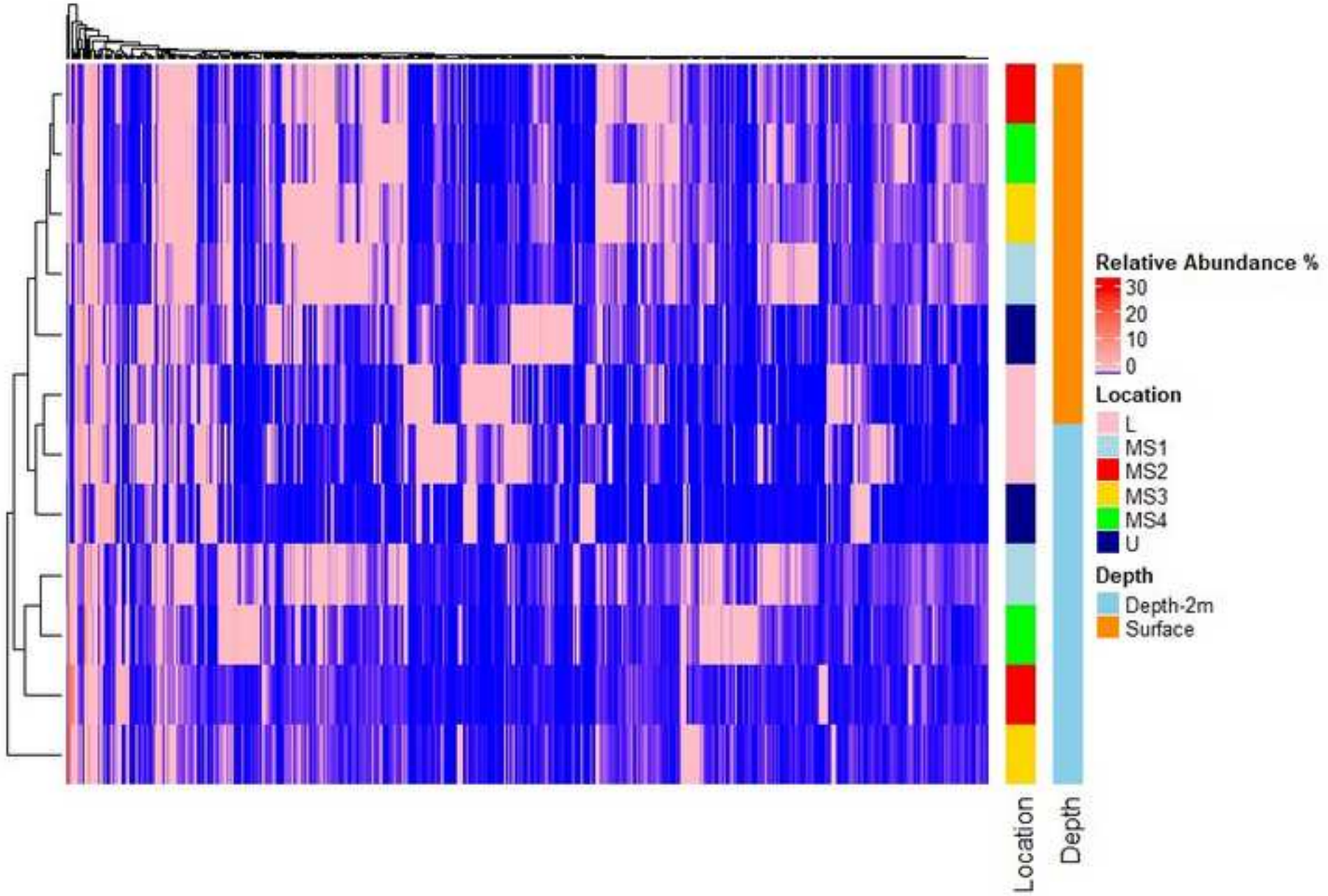
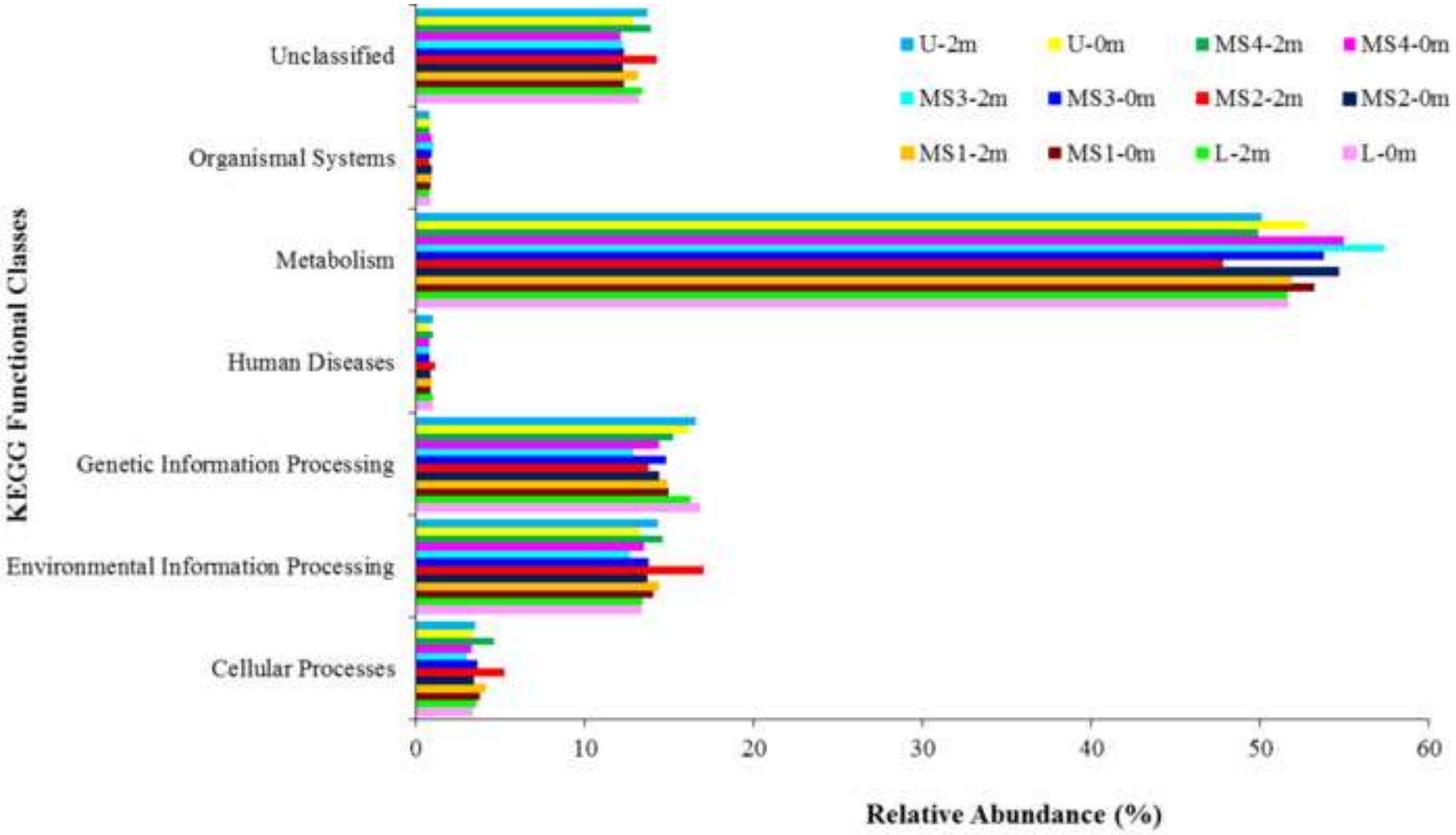


Fig. 8. General predicted functional categories of bacterial populations obtained from different cemeteries based on KEGG (Level 1).



Based on PICRUSt, the PPAR (Peroxisome proliferator-activated receptors) signalling pathway, phosphotransferase system (PTS), cytoskeleton proteins, toluene degradation, cyanoamino acid metabolism, primary bile acid biosynthesis, inositol phosphate metabolism, and bacterial invasion of epithelial cells were among the top identified functional classes (Table S4). Results of Piphillin predicted a higher frequency of some enzymes, including sedoheptulose-bisphosphatase, phosphatase and tensin homolog PTEN, 3',5'-cyclic-nucleotide phosphodiesterase, arylsulfatase, iron (III) transport system ATP-binding protein, mannosyl-oligosaccharide alpha-1,2-mannosidase, and pullulanase across all cemetery soil samples (Table S5). To find the impact of cemetery soil on human health, we targeted human diseases functional classes (Table 5).

Four main human disease classes were identified. These included cancers, cardiovascular diseases, immune system diseases, infectious diseases, metabolic diseases and neurodegenerative diseases, with infectious diseases being the most common (Table S4).

Generally, samples obtained from 2 m harboured higher amounts of human disease functional classes compared to samples collected from the surface. The findings also revealed that the bacterial population in the cemetery soil samples were mainly related to tuberculosis, Alzheimer,

Table 5. OTUs related to human diseases in cemetery soil (relative abundance; %).

	Maitland Cemetery (Cape Town)								Fontein Cemetery (Mpumalanga)			
Disease	**MS1**		**MS2**		**MS3**		**MS4**		**L**		**U**	
	0m	**2m**	**0m**	**2m**	**0m**	**2m**	**0m**	**2m**	**0m**	**2m**	**0m**	**2m**
Tuberculosis	0.23	0.22	0.22	0.19	0.22	0.18	0.21	0.25	0.26	0.26	0.24	0.28
Alzheimer's disease	0.12	0.13	0.10	0.17	0.10	0.10	0.10	0.16	0.12	0.13	0.10	0.15
Huntington's disease	0.11	0.14	0.11	0.17	0.10	0.09	0.10	0.16	0.12	0.14	0.10	0.15
Vibrio cholerae pathogenic cycle	0.10	0.11	0.09	0.18	0.10	0.10	0.09	0.13	0.15	0.11	0.09	0.14
Type I diabetes mellitus	0.08	0.07	0.08	0.06	0.08	0.08	0.08	0.07	0.09	0.09	0.08	0.09
Pathways in cancer	0.07	0.08	0.07	0.10	0.07	0.06	0.07	0.09	0.08	0.09	0.07	0.09
Primary immunodeficiency	0.08	0.07	0.08	0.08	0.08	0.08	0.09	0.06	0.08	0.08	0.08	0.07
Parkinson's disease	0.06	0.08	0.05	0.12	0.05	0.04	0.05	0.10	0.05	0.07	0.04	0.07
Amyotrophic lateral sclerosis (ALS)	0.05	0.06	0.05	0.08	0.04	0.08	0.05	0.07	0.06	0.06	0.04	0.06
Type II diabetes mellitus	0.05	0.05	0.05	0.06	0.05	0.05	0.04	0.06	0.06	0.06	0.05	0.07
Pertussis	0.03	0.05	0.02	0.14	0.02	0.04	0.02	0.08	0.04	0.04	0.03	0.05
Epithelial cell signaling in *Helicobacter pylori* infection	0.03	0.04	0.03	0.07	0.03	0.03	0.03	0.05	0.05	0.05	0.04	0.06
Renal cell carcinoma	0.03	0.04	0.03	0.04	0.03	0.03	0.03	0.04	0.04	0.04	0.03	0.04
African trypanosomiasis	0.02	0.03	0.02	0.04	0.02	0.04	0.02	0.03	0.03	0.03	0.02	0.03
Amoebiasis	0.03	0.03	0.03	0.02	0.03	0.01	0.03	0.03	0.03	0.03	0.03	0.03
Prostate cancer	0.02	0.02	0.02	0.03	0.02	0.02	0.02	0.03	0.03	0.03	0.03	0.03
Chagas disease (American trypanosomiasis)	0.02	0.02	0.02	0.03	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Influenza A	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.03	0.01	0.02	0.01	0.02
Small cell lung cancer	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.03	0.01	0.02	0.01	0.02
Viral myocarditis	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.03	0.01	0.02	0.01	0.02
Toxoplasmosis	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.03	0.01	0.02	0.01	0.02

Huntington, and *Vibrio cholerae* pathogenic cycle diseases as well as pathways in cancer (Table 5, Table S6).

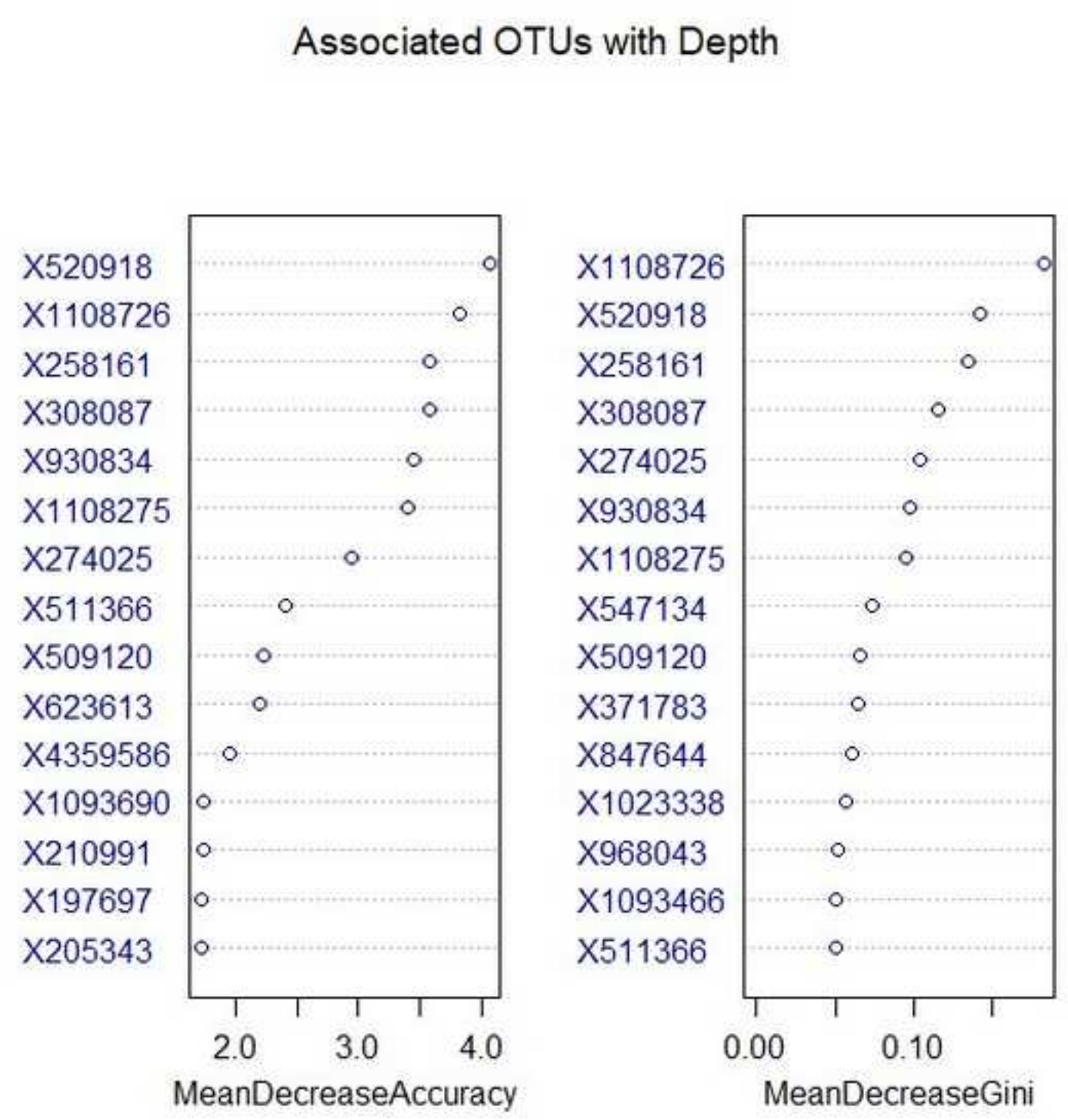
3.5 Key OTUs differentiate soil samples obtained from surface and 2 m depth

The Random Forest supervised learning models ((Out-of-bag (OOB) error rate: 16.67%)) to identify the most essential OTUs between the surface (0 m) and 2 m depth, using mean decrease of accuracy and mean decrease of Gini index (Figures 8). A higher value in both indices represents a higher importance of a particular OTU. Accordingly, *Pseudomonas fragi* (X930834), *Methylibium* (X1108726), TM7(X520918), *Pseudonocardiaceae* (X258161, X623613), *Myxococcus* (X274025), *Lactobacillales* (X308087), *Janibacter* (X1084157), *Limnohabitans* (X1108275), *Isosphaeraceae* (X847644) and Solirubrobacteraceae (X371783) were recorded as the most important OTUs separating samples obtained from surface and 2 m depth (Figure 9).

4. Discussion

To investigate if the burial of human remains could have negative impacts on the quality of groundwater, the present study investigated the bacterial diversity and functional profiles of identified species in soil samples collected above and below burial ground in two cemeteries in South Africa using high throughput sequencing of the *16S rRNA* gene. Marked bacterial diversity was observed in samples from both cemeteries and at both depths. The samples obtained from 2

Fig. 9. Mean decrease of accuracy and mean decrease of Gini index calculated using Random Forest supervised learning models.



m depth demonstrated a lower alpha diversity compared to those obtained from the surface. Despite their lower richness, 2 m samples harboured higher numbers of human disease functional classes compared to samples collected from the surface. Overall, species identified belonged to four main human disease classes with infectious diseases being the most detected class.

3.2. Bacterial composition above and below burial depths in the cemetery environment

Bacteria naturally occur in different environmental matrices such as water, air and soil, where they perform different ecological functions. However, human activities significantly alter the bacteria composition in the environment. For example, Yan et al. (2016) demonstrated that urban development such as the construction of urban ring roads considerably altered the soil bacterial composition in the affected area. Similarly, a recent study conducted in Beijing, China, showed that the bacterial communities under different vegetation types were similar to each other but differed significantly from the communities under impermeable surfaces and permeable pavement (Hu et al., 2018). In the current study, statistically significant differences were observed between the bacterial composition in the 0 and 2 m samples in both cemeteries (Table S3). *Pseudomonas* and *Corynebacterium* were the most abundant genera in all cemetery soil samples analysed. *Pseudomonas* spp. have been historically shown to be the most abundant species in soil communities based on *16S rRNA* gene analysis (Janssen, 2006). Thus, it was not surprising that this genus was the most abundant in the current study as their enormous metabolic versatility has permitted them to occupy various ecological niches including water, clouds, plants and animals (Özen and Ussery, 2012).

Regarding the sample depth, the 2 m samples were dominated by *Pseudomonas* and *Rhodococcus* while *Prauserella* and *Staphylococcus* were the most abundant genera in surface

samples. Sampling depth and other environmental factors such as oxygen availability and soil moisture content have been reported to have significant effects on bacterial composition in the soil (Du et al., 2015; Li et al., 2017). Although it is generally shown that available oxygen in the soil would decrease with increasing depth (Cook and Knight, 2015), other physical properties such as soil texture and structure, pore size distribution, and connectivity of soil particles could also affect the diffusion of oxygen into the soil (Neira et al., 2015). In the current study, aerobes (*Staphylococcus* and *Rhodococcus*) were found at both sampling depths meaning that oxygen availability had little influence on the bacterial composition observed. The constant digging and filling of graves could have affected soil structure and connectivity, thus, increasing pore sizes and favouring oxygen diffusion.

Nevertheless, some genera like *Campylobacter* were only detected at 2 m while *Bacteroides* were only detected at the surface (Table 4). Another factor that could have accounted for the difference in the microbial composition between the 0 and 2 m samples is soil moisture. The 2 m samples had a higher moisture content (visual observation) than the 0 m samples. The effect of soil moisture on bacterial composition and diversity has previously been reported (Li et al., 2017).

3.3. Bacterial Diversity above and below burial depths in the cemetery environment

In a study conducted in the Qinghai-Tibetan Plateau, Zhang et al. (2016) suggested that human activities (and climate change) had a significant effect on soil microbial diversity through the modification of soil moisture and nutrient content. Similarly, human activities such as the use of fertilisers in agriculture modify the nutrient content of soils and strongly affect the microbial

diversity belowground levels (Levine et al., 2011). Similarly, decomposing bodies within a cemetery release substantial amounts of nutrients into the soil that may affect soil microbial diversity and function (Singh et al., 2018). In the present study, the rarefaction curves obtained based on the number of OTUs revealed that the 2 m-depth samples had a lower alpha diversity compared to those obtained from the surface (Fig. 5). This was further supported by the results of the PCA (Fig. 6) and the heatmap (Fig. 7). Burial of human remains in a cemetery leads to the introduction of nutrients and chemicals in the environment, especially belowground (Guttman et al., 2009). Also, the corrosion of metals used for the making of coffins results in the release of toxic substances in the cemetery environment (Dippenaar, 2014; Jonker and Olivier, 2012). These heavy metals have been shown to have an impact on microbial diversity in soil. For example, Feng et al. (2018) found out that Cadmium contamination considerably reduced soil microbial diversity. Thus, this could have accounted for the lower species richness observed in the samples collected below burial ground compared to those obtained from the surface.

Several studies have reported on the change in microbial composition during the decay of buried bodies (Carter and Tibbett, 2008; Cobaugh et al., 2015; DeBruyn and Hauther, 2017; Marais-Werner et al., 2017; Singh et al., 2018). As bodies decompose, oxygen is depleted rapidly and anaerobic conditions set. This results in a change of the microbial communities from obligate aerobes such as the *Bacteroides* to anaerobes like the *Clostridium* (Carter and Tibbett, 2008; DeBruyn and Hauther, 2017). This could also explain the fact that *Bacteroides*, which are obligate aerobes, were only found in surface samples in the current study while the facultative anaerobes *Pseudomonas* were more present at 2 m than at the surface (Table 4). Such a change in soil microbial diversity due to the presence of human cadavers can last for up to two years without complete recovery to the original conditions (Singh et al., 2018).

3.4 Functional profiles of identified bacterial species in cemetery soil samples

The introduction of high-throughput sequencing techniques has revolutionised the study and understanding of microbial communities in diverse environmental matrices such as water (Abia et al., 2018a), soil (Su et al., 2014) and even air (Be et al., 2014). Furthermore, functional screening has led to the understanding of the role of these microbial communities and has aided in the discovery of novel genes that could be missed through conventional culture-dependent and sequence analysis only (Su et al., 2014).

In the current study, seven different functional profiles were identified with involvement in metabolic activities being the most recorded (Fig. 8; Tables S5 and S6). Although the burial of human remains in cemeteries is said to reduce public health risk, the current study revealed that a considerable proportion of the microbial community in the cemetery soil samples was linked to human diseases. Also, of significance was the fact that samples obtained below burial depth harboured higher amounts of human disease functional classes compared to samples collected from the surface (Table S4). These results suggest that in areas with shallow aquifers as in Cape Town, cemeteries could be a source of microbial contamination of groundwater resources. This is particularly important as infectious diseases were the most detected of the four human diseases classes identified with signatures related to waterborne infections such as cholera, amoebiasis and shigellosis, and *Helicobacter pylori* and *Staphylococcus aureus* infections also being detected (Table S4). It could be argued that culture-independent methods such as metagenomics do not provide information regarding the viability of identified bacterial species. However, it is well established that bacteria can be modified through horizontal gene transfer (Brown-Jaque et al., 2015; Jutkina et al., 2016; Rumbo et al., 2011; Von Wintersdorff et al., 2016). One of such

mechanisms is transformation which involves the uptake and incorporation of free or naked DNA from the surrounding (Dzidic and Bedekovic, 2003).

Results of Piphillin further revealed genes involved in antibiotic resistance to beta-lactams. As with genes involved in disease pathways, antibiotic resistance genes could be transferred to non-resistant bacteria in the environment (Djordjevic et al., 2013). There is a current global public health concerned due to the increasing reports on antibiotic resistance with an estimated 10 million death per year projected by 2050 as a result of antibiotic resistance (de Kraker et al., 2016). Thus, the presence of more antibiotic resistance signatures below burial depths compared to the surface as recorded in the current study could further support the fact that cemeteries could represent a health risk through the contamination of groundwater resources. Also, there was a considerable involvement in the biosynthesis of numerous antibiotics including vancomycin, tetracycline, butirosine and neomycin, novobiocin, penicillins and cephalosporins, clavulanic acid, streptomycin, ansamysin, and 12-, 14- and 16-membered macrolides (Table S4). These substances are produced by soil microorganisms as a protective mechanism allowing them to outcompete other organisms and survive longer in the environment (Fernandes et al., 2016). Although produced in small quantities, these active compounds can still induce resistance in soil organisms. Low concentrations of antibiotics have been associated with the development of resistance in previously susceptible organisms (Burow et al., 2014).

Nevertheless, it has been reported that some soil microorganisms produce antibiotics in appreciable amounts of antibiotics that are stable in the environment especially in subsurface soils (Berendsen et al., 2013). The transportation of these antibiotics through subsurface water paths could lead to the contamination of groundwater. It has recently been reported that water

flow paths could induce the development of antibiotic resistance as they transport antibiotics including drug-resistant bacteria and free resistance genes through the soil (Lüneberg et al., 2018). However, further studies would be needed to determine the concentration of antibiotics in the cemetery environments and to understand if the identified concentrations could induce resistance in bacteria in the environment. Also, while bacteria may not easily infiltrate into groundwater due to their sizes, it may be easy for the chemicals to migrate to groundwater. As such, investigating for the presence of antibiotics in groundwater sources around cemeteries could provide valuable information on the potential impact of cemeteries on these sources to ascertain the health consequences that may arise from such contamination.

5. Conclusion

The current study investigated the bacterial composition and their associated functional profiles in cemetery soil samples collected at the surface and below burial ground to ascertain if cemeteries could represent a potential health threat to surrounding populations through contamination of groundwater. There was a distinctive clustering of the surface (0 m) and the 2 m samples into separate groups. Although the bacterial communities at the 2 m depth were less diverse, they were more abundant and harboured higher numbers of human disease functional classes compared to samples collected from the surface. Human disease functional profiles were involved in pathogenic *Escherichia coli* infections, shigellosis, cholera and even cancer. Signatures related to antibiotic resistance and in the synthesis of potent antibiotics were also identified. Thus, this study suggests that cemeteries could be potential sources of microbial and antibiotic pollution into groundwater, especially in areas with shallow water tables such as the

Maitland Cemetery. The buried bodies could be providing nutrients for the survival of bacteria while digging of the graves and the burying of the coffins modify the soil ecosystem hence the bacterial community. Selection of a site for use as a cemetery would, therefore, require a careful and proper understanding of the hydrogeological characteristics of the selected site. However, further studies are required to trace the actual movement of these pollutants into groundwater resources.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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Supplementary materials

Table S1. Total OTUs across all cemetery soil samples based on Greengene database.

Table S2. Similarities and differences of OTUs between soil samples obtained from different cemeteries.

Table S3. Comparison of samples based on Fisher exact test (P value<0.05) at genus level using R companion package.

Table S4. Predicted functional classes of the bacterial populations extracted from soils of different cemeteries located in South Africa. The functional classes were explored using Pi-based on the KEGG database (Level 3).

Table S5. Predicted functional classes of the bacterial populations extracted from soils of different cemeteries located in South Africa. The functional classes were predicted using Piphillin based on the KEGG database.

Table S6. Predicted pathways based on Piphillin results using the KEGG pathway database.