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Community diversity metrics, interactions, and metabolic functions of bacteria associated with municipal solid waste landfills at different maturation stages

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Abstract

Municipal landfills are hot spots of dynamic bioprocesses facilitated by complex interactions of a multifaceted microbiome, whose functioning in municipal landfills at different maturing stages is poorly understood. This study determined bacterial community composition, interaction conetworks, metabolic functions, and controlling physicochemical properties in two landfills aged 14 and 36 years. High throughput sequencing revealed a similar distribution of bacterial diversity, evenness, and richness in the 14- and 36-year-old landfills in the 0–90 cm depth. At deeper layers (120–150 cm), the 14-year-old landfill had significantly greater bacterial diversity and richness indicating that it is a more active microcosm than the 36-year-old landfill, where phylum *Epsilonbacteraeota* was overwhelmingly dominant. The taxonomic and functional diversity in the 14-year-old landfill was further reflected by the abundant presence of indicator genera *Pseudomonas*, *Lutispora*, *Hydrogenspora*, and *Sulfurimonas* coupled with the presence of biomarker enzymes associated with carbon (C), nitrogen (N), and sulfur (S) metabolism. Furthermore, canonical correspondence analysis revealed that bacteria in the 14-year-old landfill were positively correlated with high C, N, S, and phosphorus resulting in positive cooccurrence interactions. In the 36-year-old landfill, negative coexclusion interactions populated by members of N fixing *Rhizobiales* were dominant, with metabolic functions and biomarker enzymes predicting significant N fixation that, as indicated by interaction network, potentially inhibited ammonia-intolerant bacteria. Overall, our findings show that diverse bacterial community in the 14-year-old landfill was dominated by copiotrophs associated with positive conetworks, whereas the 36-year-old landfill was dominated by lithotrophs linked to coexclusion interactions that greatly reduced bacterial diversity and richness.

KEYWORDS
bacterial interaction networks, high throughput sequencing, indicator taxa, metabolic functions, municipal landfills

1 | INTRODUCTION

Municipal solid waste (MSW) landfills are characterized by interactions of highly diverse populations of syntrophic microorganisms that coexist and metabolize various forms of waste material (Barlaz, Schaefer, & Ham, 1989; He et al., 2014; Liu et al., 2019; Meyer-Dombard, Bogner, & Malas, 2020; Sawamura et al., 2010; Song, Wang, Tang, & Lei, 2015; Stanley, de los Reyes, & Barlaz, 2012; Wang, Cao, Zhao, Zhou, & Xu, 2017). A recent review by Sekhohola-Dlamini and Tekere (2019) revealed an increasing interest in understanding landfill microbiome and its ecological dynamics relating to bacterial, archaeal, and fungal populations' diversity and distribution as modulated by heterogeneous physical and chemical properties of landfills. However, only a few studies have investigated variations in bacterial composition and distribution between landfills at differing maturation stages (Xu et al., 2017; Zainun & Simarani, 2018) and at varying depths (Chen, Ueda, Sekiguchi, Ohashi, & Harada, 2003; Gomez, Yannarell, Sims, Cadavid-Restrepo, & Herrera, 2011; Liu et al., 2019; Wang et al., 2017). Even within these studies, the indicator taxa and dynamic *in situ* interactions among the coexisting bacterial groups, which are differentially influenced by landfill properties and directly impact waste bioprocessing and functioning of municipal landfills, remain ambiguous.

Due to their heterogeneous nature, MSW landfills present an intricate and challenging environment for the investigation of *in situ* microbial dynamics and interactions. For the most part, studies in MSW landfills have been limited to bacterial identification, especially at the phylum level, with the majority of studies reporting *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria* as most dominant phyla (He et al., 2014; Mwaikono et al., 2016; Song et al., 2015; Stanley, de los Reyes, & Barlaz, 2011; Thakur et al., 2020; Xu et al., 2017; Zainun & Simarani, 2018). However, identification at the genera level has revealed a greater diversity of the bacterial populations from one landfill to another as highlighted in a review by Sekhohola-Dlamini and Tekere (2019). Nonetheless, recently published literature indicates that there still exists a significant population of uncharacterized microorganisms in MSW landfills (Thakur et al., 2020). It is therefore pertinent for further research to focus on unraveling microbial populations of landfills, particularly at lower levels of classification. Furthermore, the relevance of bacterial indicator taxon under different environmental conditions is hardly ever explored, thus narrowing down the significance of taxonomic classification and characterization of landfill biodiversity.

Studies have shown that efficient waste degradation in MSW landfills emanates from microbial interactions and evolution of bacterial taxon dominance as some bacterial groups rely on others for the provision of metabolites and precursors (Bareither, Wolfe, McMahon, & Benson, 2013; Barlaz et al., 1989; Meyer-Dombard et al., 2020; Yan et al., 2019). The influence of physicochemical factors such as carbon (Barlaz, 1998), nitrogen (Yang & Song, 2019), moisture, and pH (Meyer-Dombard et al., 2020; Stanley et al., 2011) on bacterial composition and bioprocesses during decomposition of MSW has also been demonstrated, though mostly in bioreactor landfills. Thus, understanding the *in situ* complex microbial interactions, the metabolic functions involved in biodegradation of waste, and the influence of abiotic factors as the landfill matures needs continuous appraisal. Such investigations can advance the application of fundamental ecological indicators of municipal landfills that directly impact underlying mechanisms in bioprocess technology development to achieve more efficient MSW biodegradation.

The present work investigated bacterial community metrics, indicator taxa, their interactions, and associated perturbations due to abiotic properties in two MSW landfills aged 14 and 36 years at 0 to 150 cm depth using next-generation sequencing analysis. This comparative metagenomic approach allowed us to (1) decipher useful information on the indicators, interactions, and metabolic functions of the studied bacterial communities, and (2) determine the influences of physicochemical properties on bacterial communities in two landfills at different maturation stages.

2 | MATERIALS AND METHODS

2.1 | Description of the study area and sample collection

Sampling was done in two landfills that are 14- and 36-years-old, previously described and defined as traditional municipal landfills (Sekhohola-Dlamini & Tekere, 2019), located at a municipal landfill site in Johannesburg, South Africa (26.1201°S, 27.7870°E) as indicated in Figure 1. The sampled landfills are 0.5 km apart with an approximated size of 50,000 m^2 each. The landfill site receives varying quantities of typical mixed MSW including household and garden waste as well as industrial, construction, and demolition waste that amounts to approximately 144,000 tonnes annually. At the time of sampling, observed waste material at the 14-year-old landfill mostly included plastics, papers, clothing material, garden waste, rocks, and construction rubble while at the 36-year-old landfill most of the buried waste had transformed beyond recognition. For sample collection on each landfill, a Tractor loader back (TLB) was used to dig three pits within an area of 150 m by 150 m to a depth of 150 cm. From each pit, three replicate samples were taken at each depth interval and bulked into approximately 1 kg composite sample. The sampling depth was limited to 150 cm due to compaction of un-degraded waste, the majority of which was plastic material, at the 14-year-old landfill. The 150 cm depth was maintained at the 36-year-old landfill for comparison of the two landfills. Soil and buried waste samples were collected at 0–30, 30–60, 60–90, 90–120, and 120–150 cm intervals down each landfill profile. The sampling method aimed at investigating the vertical variability of the landfill profiles instead of spatial heterogeneity, which would require vast sampling across the landfills that was not permissible by the municipality. The samples were collected into sterile labeled zip-lock

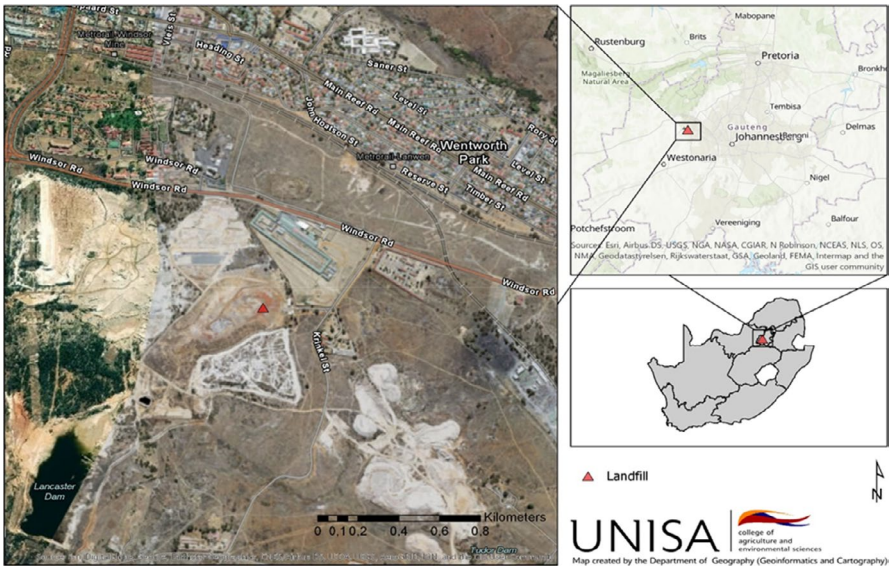


FIGURE 1 Location of municipal landfill site where the sampled landfills are situated in Gauteng province, South Africa

polyethylene sampling bags and delivered to the laboratory within 2 h of collection. At the laboratory, they were stored at −20°C for microbiological analyses.

2.2 | Physicochemical analyses

Samples were prepared for analyses by removing large components of waste material such as stones, glass, plastics, and pieces of wood and rubber, followed by grinding using pestle and mortar and thereafter sieving through a 2 mm sieve. Moisture content was determined by first drying samples in an oven at 105°C to constant weight and thereafter determining the percentage weight difference. The pH was quantified in a 1:5 (w/v) solution using Adwa AD11 pH meter (Adwa Instruments, Szeged, Hungary) as explained by Sekhohola and Cowan (2017). Total carbon (TC) and total nitrogen (TN) were determined by direct combustion of 0.2 g of the sample in a furnace at 1350°C of a LECO Trumac® Carbon, Nitrogen, and Protein analyzer fitted with boat sampler (Series 828 LECO, Michigan, US) as instructed by the manufacturer. Concentrations of heavy metals were measured with Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) (PerkinElmer Optima 5300 DV), following acid microwave digestion of samples (Sibanda, Selvarajan, Msagati, Venkatachalam, & Meddows-Taylor, 2019).

2.3 | Statistical analysis

Two-way analysis of variance (ANOVA) was used, followed by Fisher's protected least significant difference test to compare differences among means of selected properties (TC, TN, moisture content, and pH) in the 14- and 36-year-old landfills using Genstat (18th edition; Lawes Trust).

2.4 | DNA extraction and amplification

DNA extraction from the samples was done according to a protocol described by Sibanda et al. (2019), with slight modifications. Briefly, 2 g of the sample (with 3 sample replicates from each depth interval for each landfill) was mixed with 5 ml phosphate-buffered saline (PBS, pH 7.4) by vortexing. The mixture was allowed to stand for a few minutes at room temperature to dislodge bacterial cells adhering to solid waste. A supernatant aliquot of 400 µl was then used for extraction of total DNA using Faecal/Soil Total DNA™ extraction kit (Zymo Research Corporation, CA, USA) as instructed by the manufacturer. The extracted DNA was amplified following a two-step PCR method: firstly using 16S rDNA 27F and 1492R primers to cover the whole variable region and secondly to cover the V1-V3 region using 27F and 518R primer pairs with adapter sequences that are compatible with Illumina index.

2.5 | MiSeq library preparation and sequencing

Resultant PCR amplicons were purified through the AMPure XP purification system (Beckman Coulter, Massachusetts, USA) as per the manufacturer's instructions. After purification, the library preparation was done following a method described by Selvarajan et al. (2019). Briefly, the amplicon targets were mixed with the Illumina sequencing adapters and barcodes using Nextera XT indices (Illumina, Inc. San Diego, CA, USA). This was achieved through an 8 cycle PCR under the following conditions: 95°C for 3 min, 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 5 min, and cooling at 4°C. Following the second purification, the sizes of fragments were validated (~630 bp) using Bioanalyzer DNA 1000 chip (Agilent, Santa Clara, CA, USA) and quantified by fluorometric quantification method (Qubit, USA). This was followed by diluting

with Tris Buffer (10 mM, pH 8.5), based on the determined DNA concentrations. Five µl aliquots of diluted DNA were pooled from each library into 4 nM final DNA library for each depth interval for each landfill, which was denatured and loaded on the Illumina MiSeq System for sequencing of the V1-V3 gene region.

2.6 | Data analyses

Processing of the raw sequences (fastq files) was done according to the method described by Sibanda et al. (2019) with slight adjustments. Briefly, the trimming of adapters and primers was followed by the removal of PCR artifacts and poor-quality reads using *ngsShoRT* (next-generation sequencing short reads). Processing of the sequence data sets was done using Mothur pipeline v.1.40.0 (Schloss et al., 2009)and involved the initial joining of forward and reverse reads, followed by removal of reads containing low nucleotides (<50 nts), ambiguities (>2%), and homopolymers (7%) as well as sequences of mitochondrial and chloroplast origins. Thereafter, chimeric sequences were filtered using the UCHIME algorithm (Edgar, Haas, Clemente, Quince, & Knight, 2011), and resultant quality sequences were preclustered and classified using the Naïve Bayesian classifier algorithm (Wang, Garrity, Tiedje, & Cole, 2007). The taxonomic identity of bacteria was checked against the SILVA database version 132 at a confidence threshold of 80%.

A pairwise distance matrix (Euclidean distance matrix) was used to pick Operational Taxonomic Units (OTUs) at a sequence similarity of 97%, and the OTUs summarized at phylum, class, order, family, and genus levels. The alpha diversity indices (Shannon–Weaver index and Simpson index) and microbial community richness index (Chao 1) were calculated using Mothur at the genetic distance of 0.03 and presented in graphs computed using Sigma plot (Version 14, Systat Software Inc., CA, USA). The identified dominant OTUs at the phylum and genus level were used to generate a stacked bar chart and heat map using ggplot2 (Wickham, 2016) and heatmap.2 packages (Warnes et al., 2019) in R (v 3.6.1), respectively.

The presence and abundance of indicator genera were determined using the *indicspecies* package in R (v 3.6.1) based on 10^5 permutations (De Caceres, Legendre, & Moretti, 2010). Indicator values (IndVal) were determined from relative frequency and relative average abundance of genera OTUs in both landfills.

The metabolic functions of the landfill bacterial communities were predicted using the software package PICRUSt2 (phylogenetic investigation of communities by reconstruction of unobserved states) following the method outlined by Langille et al. (2013) and their reliability was validated using the Nearest Sequenced Taxon Index (NSTI) value. The predicted relative abundances of genes related to different functions were presented in heat maps generated using heatmap.2 package in R (v3.6.1). The presence of biomarker enzymes in the 14- and 36-year-old landfills was determined by Linear Discriminant Analysis Effect Size (LEfSe) according to Segata et al. (2011). This was achieved by applying the linear discriminant analysis (LDA) score of 2.0 and Kruskal–Wallis alpha significance threshold of ≤0.01. The detected functional biomarkers were visualized using GraPhlan (Asnicar, Weingart, Tickle, Huttenhower, & Segata, 2015).

Significant correlations among taxa were determined by Bray–Curtis dissimilarity, Kullback–Leibler dissimilarity, and Spearman correlation. Adjustment of *p*-values was achieved through multiple test correction described by Benjamini and Hochberg (1995), and the adjusted *p*-values were merged according to Brown's method. The bacterial conetworks were generated using CoNet (Faust & Raes, 2016) and thereafter visualized and customized using Cytoscape v3.7.2 (Shannon et al., 2003).

Canonical correspondence analysis (CCA) was used to determine the relationship between bacterial groups and the physicochemical properties of the landfills. CCA ordination triplot was generated using the PAST3 (v3.2) software package (Hammer, Harper, & Ryan, 2001) to visually display the patterns.

3 | RESULTS

3.1 | Basic physicochemical characteristics and bacterial community metrics in the landfills

Table 1 shows the basic physicochemical properties of the 14- and 36-year-old landfills investigated in this study. Total carbon, TN, and moisture content were significantly higher ($p < 0.05$) in the 14-year-old landfill compared to the 36-year-old landfill (Table A1). These parameters were also highly variable with landfill depth. In contrast, pH was higher in the 36-year-old landfill, with values ranging from 8.2 to 8.6 compared to 7.8–8.4 in the 14-year-old landfill. However, the pH did not vary significantly with depth.

A total of 6229 OTUs were detected among all samples, representing a broad taxonomic diversity at 97% sequence similarity. Overall, there were more OTUs found in samples from the 14-year-old landfill, ranging between 727 and 1064, compared to 170–771 OTUs found in samples from the 36-year-old landfill (Table A2). In total, 116,275 high-quality reads were retained after processing for quality and removal of chimeric sequences and the quality reads were within ranges of 5027–13,609 and 7883–17,417 for 14- and 36-year-old landfills respectively (Table A2). The Good's coverage for all samples was >99% and the rarefaction curve plots (Figure A1) were asymptotic, indicating that sequencing depth was sufficient to capture the whole representative bacterial community. The non-parametric diversity indices revealed that neither bacterial richness (Chao1 estimator) nor diversity (Shannon index) showed remarkable differences between the 14- and 36-year-old landfills from 0 to 90 cm depth ($p > 0.05$), as highlighted in Figure 2. However, beyond the 90 cm depth, bacterial diversity and richness were notably higher in the 14-year-old landfill compared to the 36-year-old landfill (Figure 2). Furthermore, bacterial evenness (E) appeared to be lower in the 36-year-old landfill than in the 14-year-old landfill (Figure 2). Overall, there was a consistent decrease in the bacterial community metrics with depth in the 36-year-old landfill, while in

the 14-year-old landfill a decrease from 0 to 90 cm was followed by an increase from 90 to 150 cm.

3.2 | Bacterial distribution and taxa dominance in the 14- and 36-year-old landfills

Sequence data analysis revealed a broad phylogenetic spectrum of known bacteria in both 14- and 36-year-old landfills grouped into 32 phyla, 63 classes, 139 orders, 252 families, and 490 genera. These phylogenetic groups accounted for more than 95% of all sequences analyzed in this study. Out of the 32 phyla detected, the most dominant (>2% cumulative abundance) were *Firmicutes*, *Epsilonbacteraeota*, *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Dependentiae*, and an unclassified phylum as illustrated in Figure 3a. Among the major phyla, *Firmicutes* was the most abundant in the 14-year-old landfill, with relative abundance ranging from 42% to 68%, but was very low in 36-year-old landfill with abundance ranging from 2% to 14%. In the 36-year-old landfill, phylum *Epsilonbacteraeota* was the most abundant, showing the highest relative abundance of 89% and 93% at 120 and 150 cm depths, respectively, but was very low at 60 cm with 0.4%. *Proteobacteria* was the next dominant phylum in the 14-year-old landfill, though present with relatively low abundance ranging from 6% to 17%. There was an even lower relative abundance of phyla *Actinobacteria* and *Dependentiae* in the 14-year-old landfill, whereby the relative abundance of *Dependentiae* ranged from 0.2% to 8.9% at 30 to 120 cm depth and completely absent at 150 cm. *Actinobacteria* was present with 0.1% abundance at 30 and 60 cm depths and completely absent at deeper levels of the 36-year-old landfill. Several minor bacterial phyla (abundance <2% of all assigned bacterial sequences) were also observed in both the 14- and 36-year-old landfills (Table A3).

A further classification at a lower level resulted in 67 bacterial classes shared in both 14- and 36-year-old landfills. Out of all detected classes, the most dominant (>2% cumulative abundance) were classes *Campylobacteria*, *Bacilli*, *Bacteroidia*, *γ-Proteobacteria*, *Clostridia*, *δ-Proteobacteria*, and *α-Proteobacteria* as well as an unclassified class, as illustrated in Figure 3b. Of the 10 dominant classes, *Clostridia* and *Bacilli* were generally the most abundant in the 14-year-old landfill, with highest cumulative abundances of 12% and 44%, respectively, but the least abundant in 36-year-old landfill with 0.6% and 5.9%, respectively. In the 36-year-old landfill, *Campylobacteria* was the most dominant with 37% abundance, followed by *Bacteroidia* at 16%. Bacterial members of class *Babeliae* were present with a cumulative abundance of 3.9% in 36-year-old landfill but not detected in the 14-year-old landfill, while class *Actinobacteria* was only present in 14-year-old landfill with a cumulative abundance of 4.6%. The abundance of classes *γ-* and *α-Proteobacteria* was comparatively similar in both landfills at 8% and 2%, respectively.

At the genus level, bacterial diversity decreased from the top (30 cm) to bottom (150 cm) layers of the 36-year-old landfill; however, this trend was not observed in the 14-year-old landfill (Figure 4a). The following were dominant genera present in both landfills though most abundant in the 14-year-old landfill; *Sulfuricurvum* (35.7% in the 14-year-old landfill and 0.1% in the 36-year-old landfill), *Bacillus* (14.7% in the 14-year-old landfill and 0.7% in the 36-year-old landfill), *Lysinibacillus* (5.3% in the 14-year-old landfill and 0.7% in the 36-year-old landfill), *Meniscus* (0.1% in the 14-year-old landfill and 9.0% in the 36-year-old landfill), and *Smithella* (0.3% in the 14-year-old landfill and 4.3% in the 36-year-old landfill). *Sulfurimonas* (5.7%) and *Thermobifida* (1.1%) were only present in the 14-year-old landfill while *Ureibacillus* (4.3%) and an unclassified genus of class *Babeliales* (1.4%) were only detected in the 36-year-old landfill.

The indicator species analysis showed 26 indicator genera in 14-year-old landfill with generally higher relative abundances, and 10 genera in 36-year-old landfill at comparatively lower relative abundances (Figure 4b). Mirroring the heat map results in Figure 4a, the key indicator genera associated with 14-year-old landfill samples included *Pseudomonas*, *Lutispora*, *Hydrogenspora*, *Sulfuricurvum*, *Sulfurimonas*, *Clostridium* sensu stricto_*18*, *Streptomyces*, *Pseudomonodaceae*_unclassified, *Arcobacter*, and

TABLE 1 Physical and chemical characteristics of the 14- and 36-year-old landfills at different depths

Landfill	Depth (cm)	TC (g/kg)	TN (g/kg)	Moisture (%)	pH
14-year-old landfill	0–30	67.02 ± 6.10^{d}	4.69 ± 0.05^{c}	56.42 ± 7.65^{f}	7.97 ± 0.15
	30–60	124.70 ± 5.01^{g}	9.44 ± 0.64^{f}	43.64 ± 6.05^{e}	8.03 ± 0.38
	60–90	90.0 ± 1.61^{f}	5.64 ± 0.83^{d}	33.35 ± 1.91^{d}	8.13 ± 0.06
	90–120	82.26 ± 3.73^{e}	6.38 ± 0.22^{e}	60.54 ± 2.35^{f}	8.07 ± 0.15
	120–150	87.79 ± 2.32^{f}	5.04 ± 0.08^{c}	43.41 ± 5.65^{e}	8.20 ± 0.17
36-year-old landfill	0–30	14.86 ± 0.20^{a}	1.80 ± 0.07^{ab}	12.02 ± 0.38^{a}	8.23 ± 0.06
	60–90	20.55 ± 0.36^{b}	1.53 ± 0.04^{ab}	14.21 ± 1.52^{ab}	8.47 ± 0.06
	60–90	25.75 ± 0.07^{c}	2.01 ± 0.04^{b}	21.21 ± 0.64^{c}	8.60 ± 0.00
	90–120	23.66 ± 0.32^{bc}	1.40 ± 0.08^{a}	18.82 ± 1.00^{bc}	8.53 ± 0.06
	120–150	23.66 ± 1.55^{bc}	2.02 ± 0.15^{b}	18.16 ± 0.21^{abc}	8.43 ± 0.06

Note: Data are presented as mean ±standard deviation of three replicates. For each parameter, values that are assigned different letters are significantly different ($p < 0.05$) while those with the same letters are not significantly different.

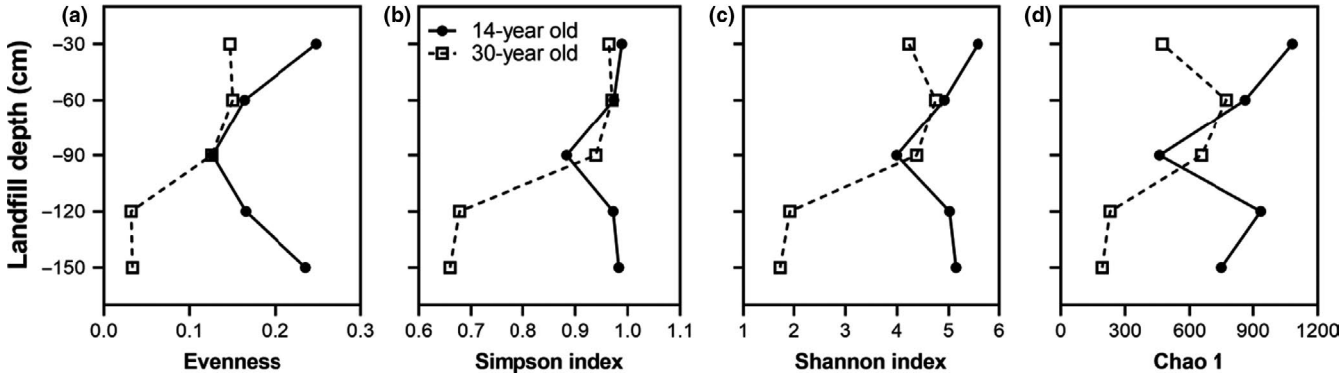


FIGURE 2 Alpha-beta indices showing species evenness (a), Simpson's diversity (b), Shannon's diversity (c), and Chao 1 species richness (d) down the profiles of the 14- and 36-year-old landfills

*Rhodobacteriaceae*_unclassified. In contrast, *Phyllobacterium* and *Sulfuricurvum* were the major indicator taxa (>50% relative abundance) associated with the 36-year-old landfill, with *Delftia*, *Pseudomonas*, and *Hydrogenspora* present at lower abundance.

3.3 | Metabolic functions and enzyme biomarkers in the 14- and 36-year-old landfills

Predicted functional profiles of the bacteria present in both 14- and 36-year-old landfills indicated the presence of putative metabolic and degradative genes across all samples. The obtained NSTI (nearest sequenced taxon index) value, which is an indicator of phylogenetic distance between OTUs in analyzed samples and the reference genomes, was low (0.06 - 0.17; Table A4) thus indicating accurate predictions. The analysis also revealed that metabolic genes were significantly higher ($p < 0.05$) than degradative genes in both landfills, particularly genes associated with the metabolism of compounds C, N, and S as well as their source substrates (Figure 5a). All metabolic functions predicted in the study were most abundant in the 36-year-old landfill at 150 cm depth followed by 60 cm depth, while at 90 and 120 cm only biotin and N metabolism genes were predicted. In the 14-year-old landfill, more metabolic functions were predicted in top layers (30 and 60 cm depths). Furthermore, 4433 enzymes were predicted from the community metagenome based on KO's abundance corresponding to OTUs, and out of 128 enzymes relating to energy metabolism, 24 were detected to be biomarkers relating to C, N, and S metabolism (Figure 5b). Sulfite reductase (k00380, k00381) and adenylylsulfate reductases (k00394, k00395) were predicted for S metabolism and nitrate reductases (k00372, k00373, and k00374) predicted for N metabolism in the 14-year-old landfill. Proteins associated with N fixation (*nif*A, *nif*X, *nif*W, *nif*Z, *nif*T, and *nif*V corresponding to KEGG orthologs k02584, k02595, k02597, k02593, and k02594, respectively) were predicted in the 36-year-old landfill. For C metabolism, fumarate hydratase (k01679) was predicted in the 14-year-old landfill and fumarate reductase (k18558) in the 36-year-old landfill.

3.4 | Bacterial interaction networks in the 14- and 36-year-old landfills

Microbial interaction network analysis revealed positive cooccurrence bacterial associations in 14-year-old landfill among members of family *Limnochordaceae*, family *Microbacteriaceae*, class *Clostridia*, and genus *Aminobacterium* (Figure 6a). The 6 nodes within the module in Figure 6a reflect the degree of connectivity among the bacterial groups, whereby the bigger the node the more connected the bacteria interacting. The network further reveals that the bacteria were interacting with each other frequently in the 14-year-old landfill and were more interconnected as indicated by the 10 positive links. Members of class *Clostridia* were most densely connected in the 14-year-old landfill, followed by family *Microbacteriaceae* and genus *Aminobacterium*. The bacterial network for the 36-year-old landfill consists of 3 modules mostly showing negative associations (Figure 6b). Positive associations were only found between *Aminobacterium* (genus of phylum *Synergistetes*) and *Phyllobacterium* (genus of phylum *Proteobacteria*) reflected in the major module (consisting of >2 nodes) and among members of phylum *Synergistetes* shown in a separate module. Strong and significant interactions were mostly linear in the 36-year-old landfill, occurring between different bacterial taxa as indicated by the connections of 12 nodes by 9 links, and *Sulfuricurvum* was the only node densely connected.

3.5 | Relationship between environmental parameters of the landfills and bacterial communities

Canonical correspondence analysis (CCA) resulted in CCA ordination triplot (Figure 7) showing the relationship between physicochemical properties of the two landfills and dominant bacterial communities at different depths. From the plot, 46.4% of the information corresponded to the first sequencing axis while 35.2% corresponded to the second sequencing axis. Figure 7 indicates that moisture, TN, TC, and P were positively correlated with bacterial classes *Actinobacteria*, *Bacilli*, and *Clostridia* in the 14-year-old landfill, showing that moisture content and concentrations of macronutrients in

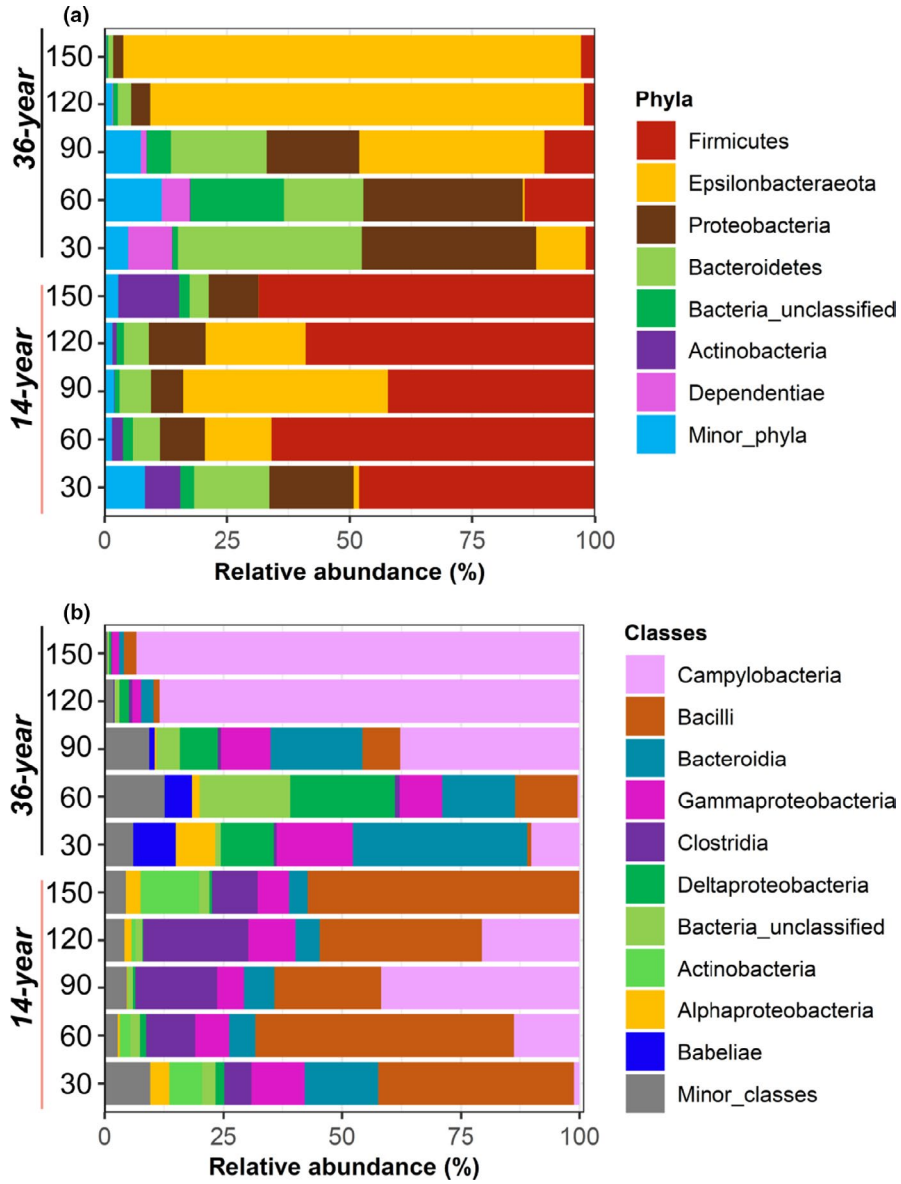


FIGURE 3 Relative abundance of bacterial phyla (a) and classes (b) in the 14- and 36-year-old landfills at different depths

this landfill were driving the proliferation of bacteria. In the 36-year-old landfill, heavy metals Fe and Pb were negatively correlated with *Spirochaetia*, *δ-Proteobacteria*, and *Babeliae*, indicating possible metal bio-toxicity, while the nutrients K, Mn and Ca showed a positive impact on members of class *Campylobacteria*, as indicated by strong positive correlations.

4 | DISCUSSION

In this study, we found that bacterial distribution, diversity, and richness were similar in the 14- and 36-year-old landfills in the upper layers (0–90 cm depth). This landfill depth was dominated by phyla *Firmicutes*, *Epsilonbacteraeota*, *Proteobacteria*, and *Bacteroidetes* in both the 14- and 36-year-old landfills. Several studies have also indicated the dominant presence of *Firmicutes*, *Proteobacteria*, and *Bacteroidetes* in different maturing landfills (Gomez et al., 2011; He et al., 2014; Song et al., 2015; Thakur et al., 2020; Wang et al., 2017; Xu et al., 2017). These mixotrophic phyla are among the most metabolically diverse bacterial groups that metabolize a variety of organic and inorganic substrates (Aislabie & Deslippe, 2013; Vetrovsky, Steffen, & Baldrian, 2014; Ximenes, Cowie, & Barlaz, 2018), as such are functionally key in the degradation of the heterogeneous MSW in landfills. Beyond 90 cm depth, there was notably higher bacterial evenness, diversity, and richness in the 14-year-old landfill than in the 36-year-old landfill, which indicated a highly active microcosm. In the 36-year-old landfill, bacterial diversity decreased sharply beyond the 90 cm depth, due to the overwhelming dominance of phylum *Epsilonbacteraeota*. The low bacterial evenness, diversity, and richness at 120–150 cm in 36-year-old landfill supports the notion

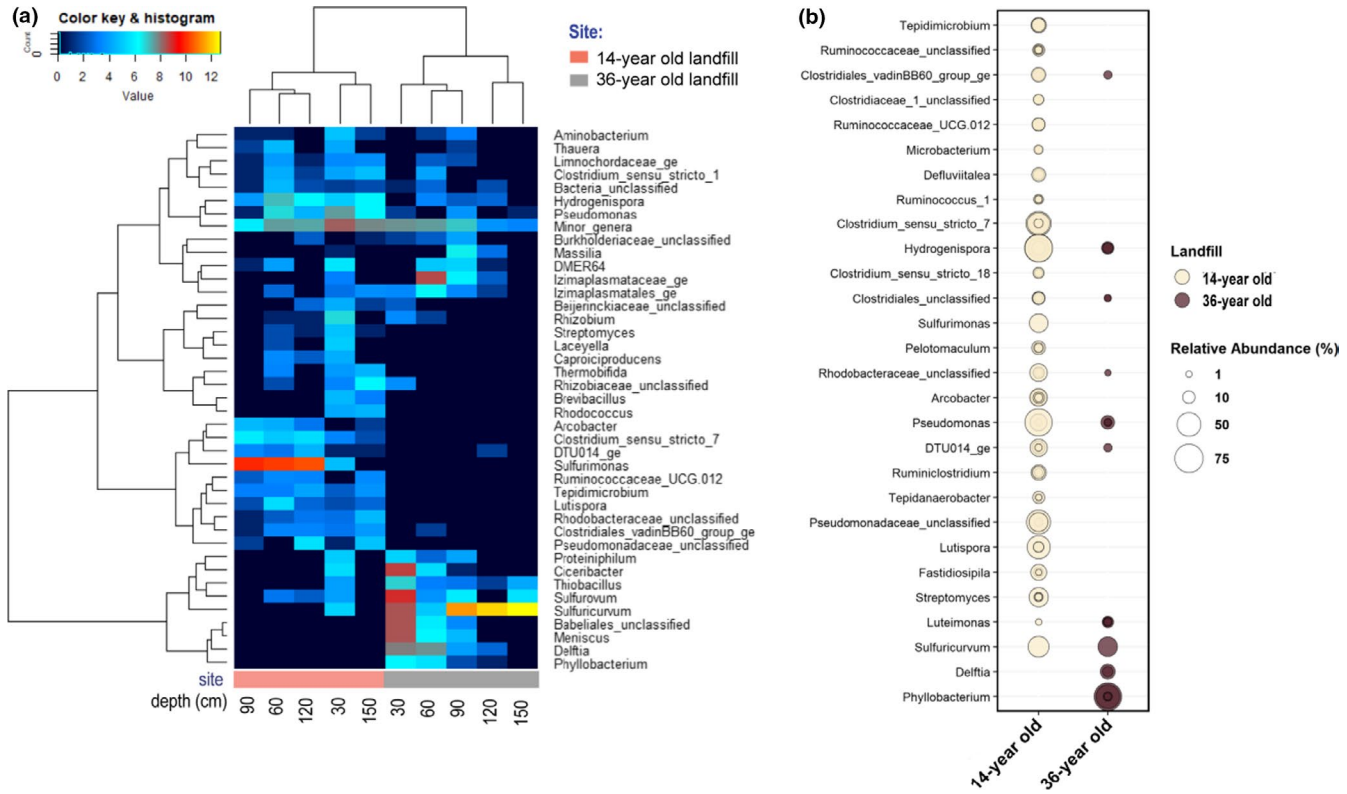
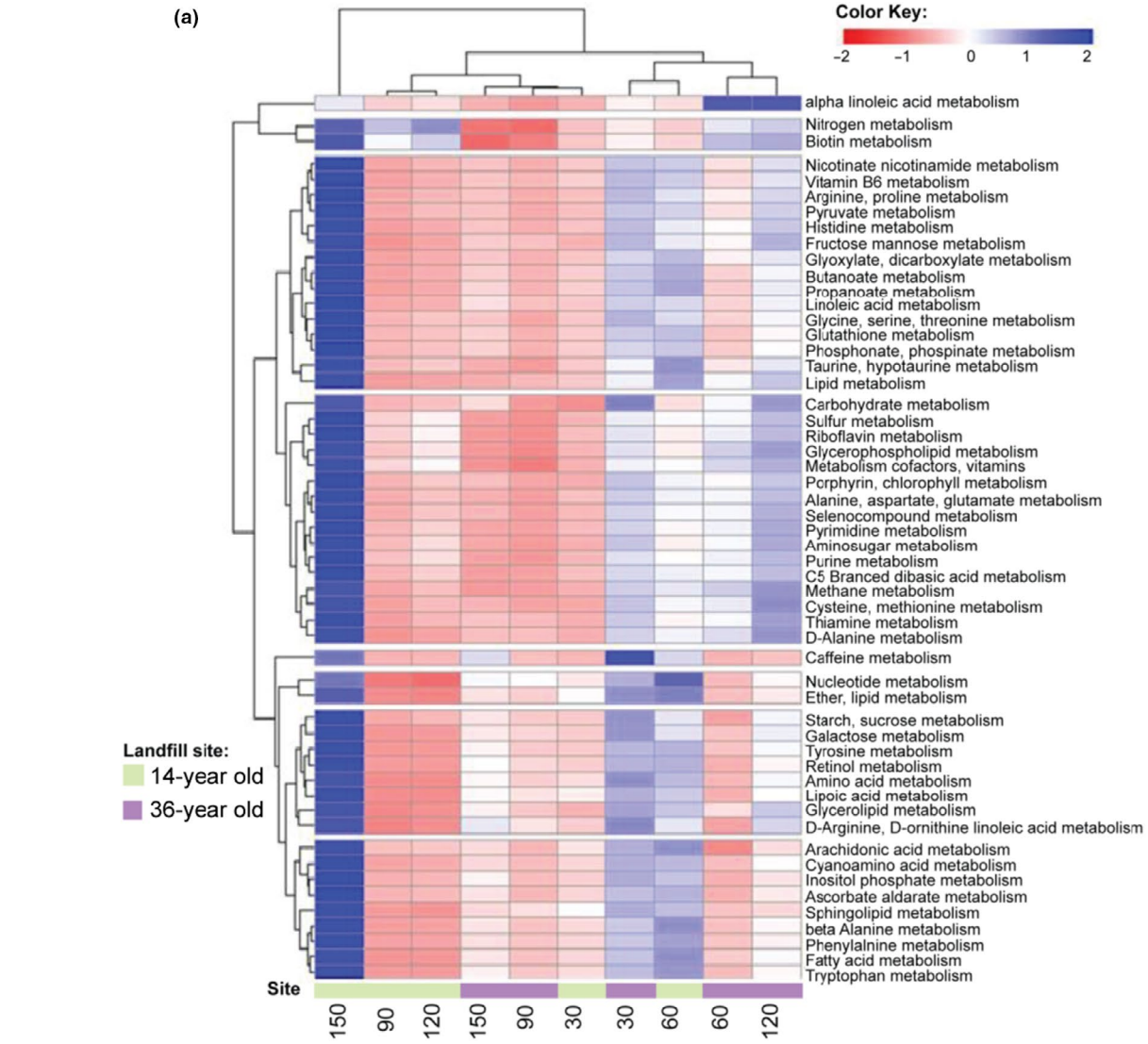


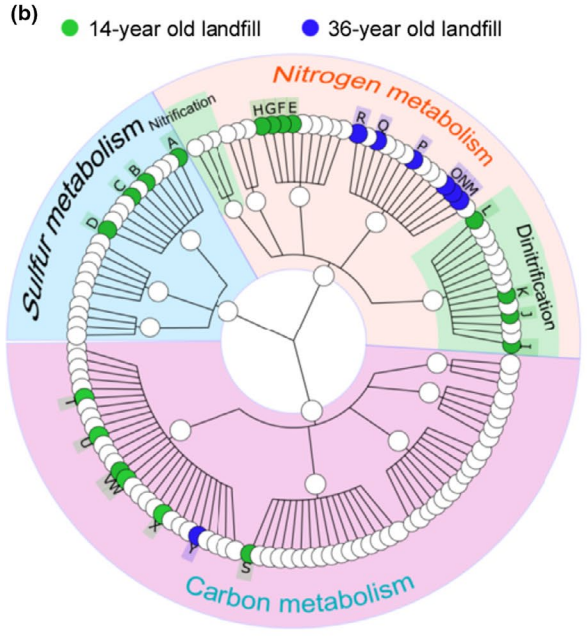
FIGURE 4 Heat map showing 20 most abundant bacterial genera whose relative abundance was >1% of all samples in the 14- and 36-year-old landfills at different depths (a) and indicator genera (b)

that as a landfill matures, changes in its environmental conditions at deeper levels reduce diversity and richness due to natural selection of certain bacterial groups and dominance by few (Barlaz et al., 1989; Sekhohola-Dlamini & Tekere, 2019). Furthermore, the negative bacterial correlations with heavy metals Fe and Pb may be an indication of metal concentrations inducing bio-toxicity often associated with maturing landfills (Abdu, Abdullahi, & Abdulkadir, 2017; Adelapo, Haris, Alo, Huddersman, & Jenkins, 2018; Xu et al., 2017; Zainun & Simarani, 2018), which would hinder the proliferation of metal-sensitive bacteria.

The predominant bacterial classes in the 14-year-old landfill were *Clostridia* and *Bacilli*, which are the most proliferate classes of phylum *Firmicutes* found in MSW landfills (Huang, Zhou, Zhu, & Qu, 2004; He et al., 2014; Mwaikono et al., 2016; Song et al., 2015; Xu et al., 2017). There was a positive correlation of these bacterial classes with moisture, TN, TC, S, and P in the 14-year-old landfill as revealed by canonical correspondence analysis, suggesting that they thrive under high moisture content and nutrient concentrations evident in the 14-year-old landfill. Also, the positive bacterial correlations with moisture, TN, TC, S, and P in the 14-year-old landfill suggest bacterial proliferation supported by predominant hydrolysis of organic waste substrates and bioavailability of the essential nutrients (Barlaz et al., 1989; Stanley et al., 2012). Previous studies have shown that TC, TN, P, and moisture are intrinsic to solubilization and mineralization of organic substrates by microorganisms (Bareither et al., 2013; Nwaokorie, Bareither, Mantell, & Leclaire, 2018; Smith et al., 2018). Other studies have demonstrated the critical role of moisture in enhancing the degradation of solid waste and bacterial proliferation through leachate re-circulation in landfill bioreactors (Barlaz et al., 1989; Stanley et al., 2012; Stanley, Xu, Cowie, Barlaz, & Hater, 2006). Our results also affirm the reported ability of *Clostridia* and *Bacilli* to metabolize N as well as complex and recalcitrant C sources (Aislabie & Deslippe, 2013; van Dyke & McCarthy, 2002; He et al., 2014; Stanley et al., 2011), which are typically found in aging MSW landfills. *Campylobacteria* was the most abundant class in the 36-year-old landfill and significantly dominant within 120 and 150 cm depths, showing strong positive correlations with K, Mn, and Ca, suggesting that their abundance is controlled by the availability of minerals. In the context of a landfill environment, this may indicate depletion of organic substrates, often reported in mature landfills, resulting in a shift in bacterial population from copiotrophs to lithotrophs as the landfill bioprocesses transition from substrate hydrolysis to subsequent mineralization processes such as acidogenesis, acetogenesis, and methanogenesis (Barlaz et al., 1989; Stanley et al., 2011).

At the genera level, our results revealed higher bacterial abundance and diversity of indicator taxa in the 14-year-old landfill than in the 36-year-old landfill. Specifically, *Pseudomonas*, *Lutispora*, *Hydrogenispora*, *Sulfurimonas*, *Streptomyces*, and *Arcobacter* were significantly abundant indicator taxa in the 14-year-old landfill. This group of complex C-catabolizing, S-oxidizing, and N-producing bacteria (Dai et al., 2016; Frey, Hietanen, Jürgens, Labrenz, & Voss,





A: *cys*I; sulfite reductase NADPH hemoprotein beta component
B: *cys*J; sulfite reductase NADPH flavoprotein alpha component
C: *apr*B; adenylylsulfate reductase subunit B
D: *apr*A; adenylylsulfate reductase subunit A
E: *nar*H; *nar*Y; *nxr*B; nitratereductase & nitriteoxidoreductase beta subunit
F: *nar*I; *nar*V; nitratereductase gamma subunit
G: *nap*A; periplasmic nitrate reductase
H: *nar*G; *nar*Z; *nxr*A; nitrate reductase & nitrite oxidoreductase alpha subunit
I: *nar*H; *nar*Y; *nxr*B; nitrate reductase & nitrite oxidoreductase beta subunit
J: *nap*A; periplasmic nitrate reductase
K: *nar*J; *nar*W; nitrate reductase molybdenum cofactor assembly chaperone
L: *nar*I; *nar*V; nitrate reductase gamma subunit
M: *nif*V; homocitrate synthase
N: *nif*T; nitrogen-fixation protein
O: *nif*A; Nif; specific regulatory protein
P: *nif*W; nitrogenase stabilizing and protective protein
Q: *nif*X; nitrogen-fixation protein
R: *nif*Z; nitrogen-fixation protein
S: *fum*A; *fum*B; fumarate hydratase class I
T: *kor*D; *oor*D; 2-oxoglutarate ferredoxin oxidoreductase subunit delta
U: *kor*C *oor*C 2-oxoglutarate ferredoxin oxidoreductase subunit gamma
V: *fum*A; *fum*B; fumarate hydratase class I
W: *kor*A; *oor*A; *ofor*A; 2-oxoglutarate & 2-oxoacid ferredoxin oxidoreductase subunit alpha
X: *kor*B; *oor*B; *ofor*B; 2-oxoglutarate & 2-oxoacid ferredoxin oxidoreductase subunit beta
Y: *frd*C; fumarate reductase subunit C

FIGURE 5 Relative abundance of predicted bacterial metabolic functions (a) and biomarker enzymes for energy metabolism (b) in the 14- and 36-year-old landfills

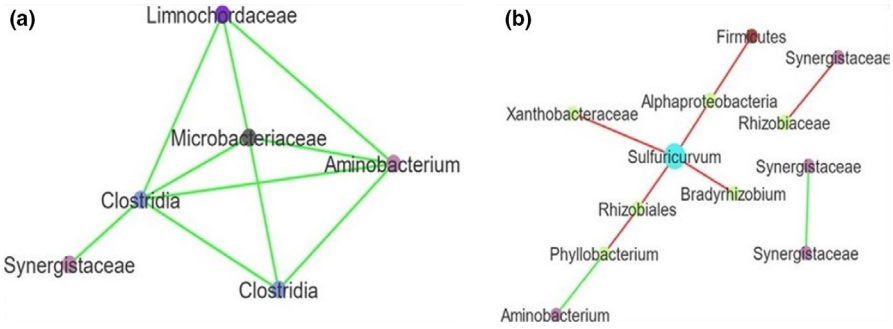


FIGURE 6 Bacterial interaction networks showing positive associations (green lines) among bacterial taxa in 14-year-old landfill (a) and negative associations (red lines) in 36-year-old landfill (b)

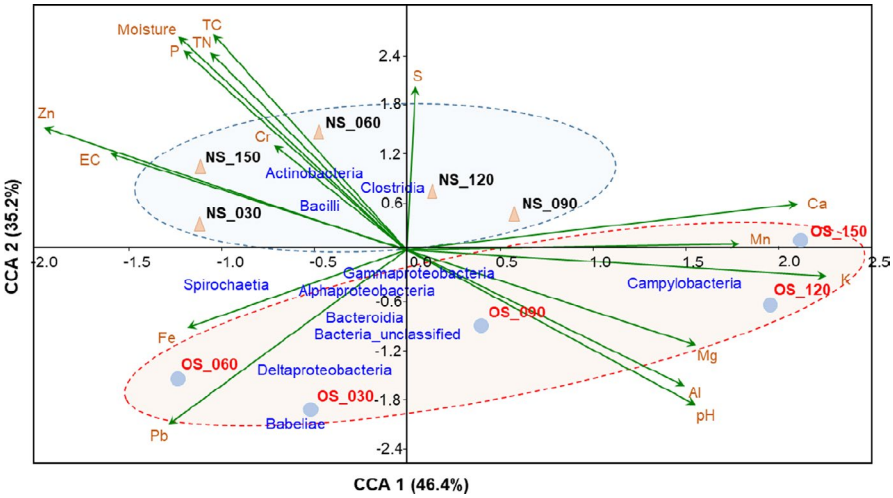


FIGURE 7 Canonical correlation analysis (CCA) ordination triplot showing correlations of physicochemical properties with dominant bacterial classes at different landfill depths. The codes NS and OS refer to Newer landfill Site and Older landfill Site, which represent the 14-year-old landfill and 36-year-old landfill respectively

2014; Wang et al., 2019; Yang et al., 2016) indicates the presence of the taxonomically and functionally diverse bacterial community in the 14-year-old landfill, as further predicted by the presence of biomarker enzymes associated with C, N, and S metabolism. Additionally, predicted genes associated with the metabolism of C, N, and S compounds as well as their source substrates were relatively higher at different depths of the 14-year-old landfill. The predicted biomarker enzymes and metabolic functions together strongly suggest the predominant hydrolysis of organic substrates consisting of C, N, and S compounds by bacteria as alluded to earlier. So far, there is no evidence in the literature indicating the presence of *Lutispora*, *Hydrogenispora*, *Sulfuricurvum*, and *Sulfurimonas* in municipal landfills. However, these genera have been found in anaerobic terrestrial and aquatic ecosystems where they have been shown to ferment complex organic compounds (Dai et al., 2016; Frey et al., 2014; Wang et al., 2019; Yang et al., 2016). Therefore, their occurrence at our study site indicates that fermentation of complex organic compounds, demonstrated in the degradation of MSW by Barlaz et al. as early as in 1989, is greater in the 14-year-old landfill compared to the 36-year-old landfill. In contrast, *Pseudomonas*, *Streptomyces*, and *Arcobacter* have been widely reported to be present in MSW landfills (Gupta, Rathour, Kumar, & Thakur, 2017; Huang, Zhu, Zhou, & Qu, 2005; McDonald, Allison, & McCarthy, 2010; Stamps et al., 2016). This bacterial group thrives under aerobic conditions where there is an abundant supply of organic and inorganic substrates. Furthermore, the interaction network analysis revealed that bacterial members of class *Clostridia*, family *Microbacteriaceae*, and genus *Aminobacterium* frequently interacted with each other and were more interconnected in the 14-year-old landfill. Members of class *Clostridia*, which were the most densely connected in the 14-year-old landfill, depolymerize recalcitrant C compounds and complex plant-derived C sources (Aislabie & Deslippe, 2013; van Dyke & McCarthy, 2002; Stanley et al., 2011). The degradation of polymers, in turn, facilitates the availability of substrates that would otherwise be inaccessible and at times toxic to some bacterial groups. Such beneficial cooccurrence of bacteria in the 14-year-old landfill is essential for efficient mineralization of the landfill's heterogeneous waste.

In the 36-year-old landfill, bacterial network analysis revealed negative coexclusion interactions mostly involving members of order *Rhizobiales* (family *Xanthobacteraceae* and genera *Phyllobacterium* and *Bradyrhizobium*), which are N fixing bacteria.

Predicted biomarker enzymes including *nif*A, *nif*X, *nif*W, *nif*Z, *nif*T, and *nif*V further reinforced the dominant occurrence of N fixation in the 36-year-old landfill. Conversely, in the 14-year-old landfill, predicted biomarker enzymes suggested that N metabolism was dominantly associated with nitrate reduction, which results in the production of the relatively nonreactive N gas. Indeed, profiling of bacterial metabolic functions during degradation of solid waste in a bioreactor showed that TN is one of the important factors that determine bacterial community structure, and the key functional genes detected at different phases of waste degradation revealed different N metabolic processes (Yang & Song, 2019). The high N fixation suggested by predicted biomarker enzymes could have resulted in increased production of ammonia, which has been reported to inhibit the growth of several bacterial groups (Bonk et al., 2018; Burton & Watson-Craik, 1998), thus explaining the coexclusion interactions in the 36-year-old landfill. On the other hand, a positive cooccurrence interaction was detected between *Phyllobacterium* and ammonia tolerant genus *Aminobacterium* in the 36-year-old landfill. In addition to the dynamic interactions of *Phyllobacterium* with other bacterial groups, this genus was the most abundant indicator taxa exclusively found in the 36-year-old landfill and is herein reported for the first time in an MSW landfill.

5 | CONCLUSIONS

Bacterial community structure and indicator taxa in municipal landfills aged 14 and 36 years were comparatively characterized through high throughput sequencing and the influences of selected landfill properties on bacterial distribution and interactions determined. Bacterial diversity, abundance, and species richness were higher in the 14-year-old landfill, with more diverse and abundant indicator genera, compared to the 36-year-old landfill. Furthermore, the bacterial community in the 14-year-old landfill was driven by positive cooccurrence interactions among different taxa, while negative coexclusion relations, highly influenced by N fixing *Rhizobiales*, dominated in the 36-year-old landfill. Dominating bacterial classes in the 14-year-old landfill were positively correlated with high concentrations of organic macronutrients C, N, S, and moisture, but in the 36-year-old landfill, there were positive bacterial correlations with minerals K, Mn, and Ca. Significant coexclusion interactions in the 36-year-old landfill prevailed possibly due to high N fixation that may have increased ammonia-induced inhibition of some bacterial groups and competition for lower and unevenly distributed organic substrate resources. It is therefore concluded that macronutrient availability invigorated bacterial proliferation, richness, and diversity in the 14-year-old landfill and promoted even distribution and beneficial symbiotic interactions among bacterial taxa. In the more mature 36-year-old landfill, species diversity and richness declined and the bacterial community structure shifted toward the dominance of lithotrophic population. Future research work should incorporate an in-depth biochemical investigation of the predicted bacterial functions and biomarker enzymes activity during waste decomposition. More quantification of the spatiotemporal variability of maturing landfills to better understand the biotic and abiotic parameters is also necessary during operation and maintenance.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Lerato Sekhohola-Dlamini: Conceptualization (lead); data curation (equal); formal analysis (equal); funding acquisition (supporting); investigation (equal); methodology (equal); project administration (supporting); validation (equal); visualization (equal); writing – original draft (lead); writing – review & editing (lead). **Ramganesh Selvarajan:** Data curation (equal); formal analysis (equal); investigation (supporting); methodology (equal); validation (equal); visualization (equal); writing – review & editing (supporting). **Henry Joseph Odour Ogola:** Data curation (equal); formal analysis (equal); investigation (supporting); methodology (equal); validation (equal); visualization (equal); writing – review & editing (supporting). **Memory Tekere:** Conceptualization (supporting); funding acquisition (lead); project administration (supporting); writing – review & editing (supporting).

ETHICS STATEMENT

None required.

DATA AVAILABILITY STATEMENT

The raw sequencing data have been deposited into the NCBI Sequence Read Archive database (accession number PRJNA563044): https://www.ncbi.nlm.nih.gov/bioproject/PRJNA563044.

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APPENDIX A

TABLE A1 Analysis of variance table for Total carbon, Total nitrogen, moisture and pH data from 14- and 36-year-old landfills

Total carbon					
Source of variation	***df***	**s.s.**	**m.s.**	**v.r.**	***F* pr.**
Age	1	35,350.91	35,350.91	4069.85	<0.001
Depth	4	3096.43	774.11	89.12	<0.001
Age.Depth	4	2509.60	627.4	72.23	<0.001
Residual	20	173.72	8.69		
Total	29	41,130.66			
Total nitrogen					
Source of variation	***df***	**s.s.**	**m.s.**	**v.r.**	***F* pr.**
Age	1	151.02	151.02	1266.83	<0.001
Depth	4	18.25	4.56	38.27	<0.001
Age.Depth	4	26.16	6.54	54.85	<0.001
Residual	20	2.38	0.12		
Total	29	197.81			
Moisture					
Source of variation	***df***	**s.s.**	**m.s.**	**v.r.**	***F* pr.**
Age	1	7016.11	7016.11	500.65	<0.001
Depth	4	581.35	145.34	10.37	<0.001
Age.Depth	4	1026.99	256.75	18.32	<0.001
Residual	20	280.28	14.01		
Total	29	8904.74			
pH					
Source of variation	***df***	**s.s.**	**m.s.**	**v.r.**	***F* pr.**
Age	1	1.05	1.05	44.17	<0.001
Depth	4	0.25	0.06	2.64	0.064
Age.Depth	4	0.08	0.02	0.82	0.525
Residual	20	0.47	0.02		
Total	29	1.85			

TABLE A2 Summary of sequencing outputs and diversity indices for bacterial communities in the 14- and 36-year-old landfills at different landfill depths

Landfill	**Depth (cm)**	**Quality reads**	**OTUs**
14-year-old landfill	0–30	12,286	1064
	30–60	13,609	835
	60–90	5027	429
	90–120	13,074	915
	120–150	10,147	727
36-year-old landfill	0–30	14,291	469
	30–60	17,417	771
	60–90	11,086	635
	90–120	7883	214
	120–150	11,455	170

TABLE A3 Minor bacterial phyla detected in 14- and 36-year-old landfills at different depths (The codes NS and OS refer to Newer landfill Site and Older landfill Site, which represent the 14-year-old landfill and 36-year-old landfill, respectively)

Minor Phyla	NS3	NS6	NS9	NS12	NS15	OS3	OS6	OS9	OS12	OS15
Spirochaetes	180	16	2	7	35	254	190	97	4	6
Tenericutes	61	38	37	27	124	33	223	48	7	0
Thermotogae	75	8	12	39	23	0	309	73	31	0
Marinimicrobia_ (SAR406_clade)	3	0	0	0	0	1	372	155	5	5
Synergistetes	288	20	7	18	25	4	58	54	14	0
Planctomycetes	42	22	11	8	5	112	169	62	7	2
Omnitrophicaeota	0	0	0	0	0	2	266	13	9	11
Acidobacteria	19	6	2	10	2	51	34	122	20	9
Atribacteria	17	23	8	10	1	0	109	41	6	0
Chloroflexi	48	11	2	1	21	0	112	7	1	0
Verrucomicrobia	21	0	0	0	0	69	56	0	0	0
Fibrobacteres	47	6	0	6	7	0	0	77	1	0
Cloacimonetes	61	0	6	7	9	0	10	28	0	0
Lentisphaerae	41	3	3	6	0	52	4	6	0	0
Kiritimatiellaeota	9	2	0	2	2	73	4	5	0	0
Hydrogenedentes	30	24	3	12	9	0	2	5	0	0
Halanaerobiaeota	9	0	2	34	3	0	2	0	14	2
unknown_unclassified	13	9	2	13	4	0	12	13	0	0
Armatimonadetes	6	11	0	2	3	9	5	2	0	0
Gemmatimonadetes	16	0	0	1	6	3	7	0	0	1
Rokubacteria	0	0	0	0	0	1	29	0	0	2
Patescibacteria	0	2	0	0	0	7	20	0	0	0
Cyanobacteria	8	0	0	2	0	5	4	4	0	0
Latescibacteria	1	0	0	0	0	5	11	3	0	0
BRC1	11	0	0	0	0	4	0	0	0	0
WS1	0	2	0	2	1	0	4	1	2	0
Caldiserica	0	1	0	0	0	0	8	0	0	2
WS4	0	0	0	0	0	2	3	0	0	0
Acetothermia	0	0	0	0	0	0	0	2	0	0
Elusimicrobia	2	0	0	0	0	0	0	0	0	0

TABLE A4 The obtained NSTI (nearest sequenced taxon index) values for samples collected from the 14- and 36-year-old landfills at different depths

Landfill	Depth (cm)	Metric	Value
14-year-old landfill	0–30	Weighted NSTI	0.130639
	30–60	Weighted NSTI	0.087618
	60–90	Weighted NSTI	0.075705
	90–120	Weighted NSTI	0.162
	120–150	Weighted NSTI	0.111167
36-year-old landfill	0–30	Weighted NSTI	0.131934
	30–60	Weighted NSTI	0.169019
	60–90	Weighted NSTI	0.162931
	90–120	Weighted NSTI	0.067717
	120–150	Weighted NSTI	0.057119

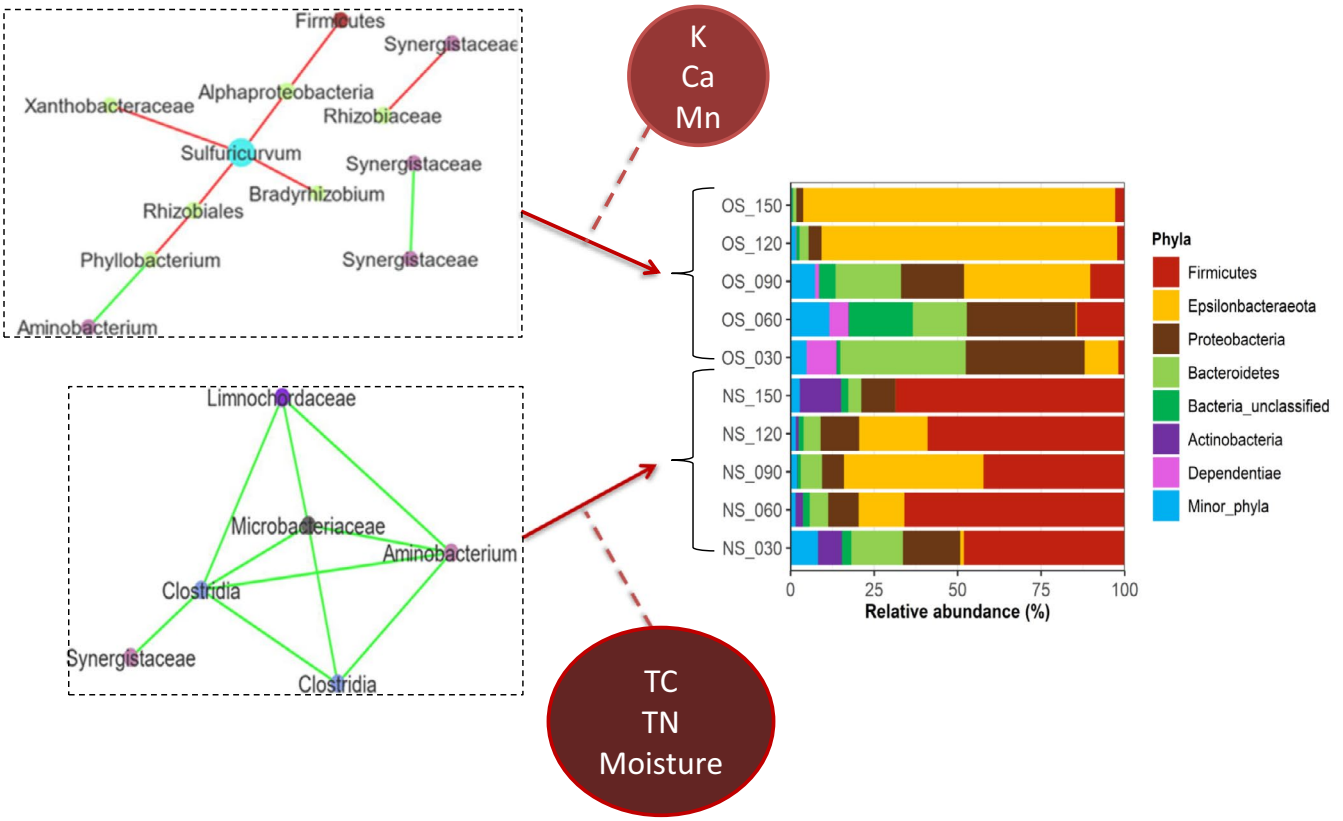

FIGURE A1 The rarefaction curve plots of sequences in the 14- and 36-year-old landfills at different depths. The codes NS and OS refer to Newer landfill Site and Older landfill Site, which represent the 14-year-old landfill and 36-year-old landfill, respectively