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RESEARCH ARTICLE

Microbial community composition reflects water usage and storage conditions in a city-wide study of non-sewered wastewater (fecal sludge)

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Abstract

Nearly half (46%) the world's population is now served by non-sewered sanitation. In urban areas of low- and middle-income countries, this translates to onsite storage of wastewater in tanks and pits until it can be collected and transported by road to treatment, which is commonly referred to as fecal sludge management. The microbial communities that develop during storage of this wastewater remain understudied, leaving practitioners and scientists to speculate on best management practices such as downstream treatment and climate mitigation measures. In this study, we collected samples from 135 randomly selected containments across the city of Lusaka, Zambia, and evaluated statistical relations of 16S rRNA gene sequence data to types and volume of wastewater going into containments, disturbances (i.e., emptying events), characteristics of accumulated wastewater during storage, and metrics of downstream treatment processes. At the phyla level, 80% of the identified microorganisms belonged to *Firmicutes*, *Proteobacteria* and *Bacteroidota*. Focusing in at the genera level, microbial diversity and composition were statistically related to volumes of water usage, properties of wastewater in containments (total organic carbon, total kjeldahl nitrogen, ammonium nitrogen, pH), and metrics of stabilization and dewatering performance. In Lusaka, a core community was identified with 104 of the 1,247 identified genera being present in >90% of the containments. In contrast, 936 genera were present in <60% of the containments, indicating that niche or transient organisms may also be important in unravelling metabolic processes such as sulfur reduction, methanogenesis, and ammonia tolerance. Community similarity was independent of time since last emptied, indicating stability of microbial communities over time. Identified metabolic differences between pit latrines (i.e., less water usage) and septic tanks

Data availability statement: The data package for all analysis completed in this study is published open access with the Eawag Research Data Institutional Collection (ERIC), and can be accessed at https://doi.org/10.25678/0008ZW

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(i.e., more water usage) indicate that methanogens more actively convert organic matter to methane in the more dilute wastewater in septic tanks, which could be globally relevant for greenhouse gas mitigation from non-sewered sanitation.

1. Introduction

Almost half (46%) of the world's population is served by non-sewered sanitation, and this population is growing faster than the population with access to sewer-based sanitation [1]. In urban areas of low-income countries, non-sewered sanitation is commonly referred to as fecal sludge management [2]. Fecal sludge management relies on the onsite storage of wastewater (fecal sludge) in containments (pits and tanks) with periodic emptying of wastewater and transport to semi-centralized facilities for treatment [3]. Although containments are commonly referred to as "pit latrine" and "septic tank", they are not analogous to those designed for rural areas, which have adequate land available for soil-based treatment [3]. In dense urban areas, they are rather most often a mix of informal, self-constructed pits and tanks for storage purposes, with no level of standardization [4]. For example, In Lusaka, Zambia, only 2% of so called septic tanks are thought to be correctly constructed with discharge to a soak pit [5].

The wastewater going into containments in urban areas relying on fecal sludge management is typically less than 5% total solids (TS), with water originating from toilet flushing, bathing, cooking and cleaning. Non-household sources also represent around 50% of municipal wastewater, including offices, restaurants, markets, malls, small-scale manufacturing, and hotels [6]. Concentrations of solids, organic matter and nutrients in stored wastewater (fecal sludge) are highly variable, ranging across orders of magnitude and not following a normal distribution [7]. However, statistical patterns between characteristics and demographic (e.g., income), environmental (e.g., water table) and technical (e.g., drinking water source) forms of data are observed [8]. Although this management chain could provide a sustainable sanitation solution, the majority of wastewater ends up directly in the urban environment [9]. Prohibitive collection costs and transport challenges in urban areas, often result in bypassing the management chain, causing significant harm to public and environmental health [9].

For the development of improved management solutions for storage, emptying frequency, greenhouse gas mitigation and downstream treatment processes, more knowledge is urgently needed on the mechanistic role that microorganisms are playing during storage. To date, only a few studies have evaluated microbial communities in septic tanks [10,11], pit latrines [12–16], or both [17,18]. These studies focused on differences in community composition in relation to geographic location [16], pit latrine filling rates [14], mode of septic tank operation [11], efficiency of solar treatment [10], depth within pit latrines [15], and dewatering performance [17,18]. However, a systematic understanding of microbial community composition and properties in relation to storage conditions has not yet emerged, and the representativeness of results is not known due to the limited number of samples in these studies.

Usage patterns such as total volumes of water, amount of urine (i.e., ammonia), and inclusion of cooking wastewater (i.e., fats, oils and grease) [19], affect wastewater streams and could be expected to have an influence on microbial communities inside containments. For example, public toilets often have higher concentrations of urine [20], whereas ablution blocks with increased water usage generate more dilute wastewater [21]. In general, emptying frequencies range from very frequent (<1–2 weeks) to very infrequent (years or decades), and it is not known whether the frequency of emptying influences microbial communities. The role of microbial communities in the mineralization and stabilization processes during storage is not known, but the amount of organic matter that is degraded during storage is much less than previously thought [2]. An understanding of the roles could improve predictions of greenhouse gas emissions [2], and performance of downstream treatment processes such as dewatering [22].

In this study, samples were randomly collected from 135 containments across Lusaka, Zambia, with meta-data that included user-level questionnaire data, laboratory analysis of characteristics and metrics of treatment performance [22,23]. Based on the collected data, the objectives of this study were to i) characterize microbial communities during storage of non-sewered wastewater in containments in a statistically representative fashion across an entire city, ii) compare relative abundance and diversity metrics to other studies, and iii) to explore observed statistical patterns within this study in relation to water usage and wastewater type, disturbances (i.e., emptying events) and characteristics of accumulated wastewater, and downstream treatment.

2. Materials and methods

2.1. Sample collection

Zambia is located in Southern Africa on the Central African Plateau. Lusaka is the political and economic center of Zambia, and is one of the fastest growing cities in Southern Africa [5]. In 2018, the population was estimated to be 2,526,102, with 70% living in informal settlements, referred to as peri-urban areas [5]. 86% of the population relies on non-sewered sanitation, and of that 78% is considered not-safely managed [5].

The 135 samples collected in this study were from a range of onsite, non-sewered sanitation containments (pits and tanks). We collected the 135 samples for the extraction of DNA and microbial community analysis in this study, as a subset of the 465 stored wastewater (fecal sludge) samples from 421 onsite containments, as described in detail in Andriessen et al. and Ward et al. [23,24]. The open data package for all analysis completed in this study is available at https://doi.org/10.25678/0008ZW, and additional metadata [22] for the original 465 samples is available at https://doi.org/10.25678/00037X. In brief, as shown in Fig A in S1 Text, we overlaid a one-kilometer grid layer on a map of Lusaka in ArcMap, and randomly selected one sampling location per grid tile, with two sampling locations per tile in high-density areas, while excluding areas with access to sewer-based sanitation provision. We made composite samples from sub-samples taken at the bottom, middle and top layers of fecal sludge stored in containments (pits and tanks). Questionnaire data at sample collection included demographic (e.g., building use, income levels, occupation), environmental (e.g., seasonal changes), and technical (e.g., containment type, emptying frequency) categories. Complete characterization and metrics of dewatering performance are described in Ward et al. [23]. The characteristics of the 135 samples are provided in Table A in S1 Text. As shown by boxplot comparisons in Fig B in S1 Text, the subset of data in this study, follows the same distribution as the 465 samples in Ward et al.

Ethical clearances were approved in 2019 by Eawag and by ERES Converge. ERES is recognized by the National Health Research Authority in the Zambia's Ministry of Health, who is responsible for all Research Ethics Committees, or IRB's, on health-related research in the Country of Zambia. The study was also approved by the Lusaka City Council (LCC) Public Health Directorate, and the LCC Public Health Department participated in data collection with a member of LCC always being present with the data collection team. Participants were informed about the study by LCC, a consent form was signed by each participant, and data was collected anonymously. Samples were collected from stored wastewater (fecal sludge). Toilets are used by multiple people and/or multiple households, hence, no results could be directly

tracked back to individuals. No samples were taken directly from human subjects. GPS data was collected anonymously and kept confidential.

During collection, we thoroughly homogenized composite samples by shaking/stirring and a 2 mL subsample was centrifuged at 6,000 g for 10 min. The pellets were retained and 1 mL of RNAlater® (Sigma Aldrich, Steinheim, Germany) was added so that the samples could be used for either RNA or DNA analyses (note that the presence of RNAlater has no effect on DNA quality or integrity). Samples were stored at -20° C and then airfreighted to Eawag (Dübendorf, Switzerland) on dry ice.

2.2. DNA extraction

Prior to DNA extraction, we rinsed samples three times with 1X phosphate buffer to remove the RNAlater. We extracted DNA from all samples following the modified method of Griffiths et al. [25]. Briefly, 0.5 mL of hexadecyltrimethylammonium bromide buffer was added to each sample, mixed and transferred to a 2 mL lysing matrix tube. Lysing was done using a FastPrep®-24 (M.P. Biomedicals, Irvine, CA, USA) after adding 0.5 mL of phenol:chloroform:isoamylalcohol (PCI) (25:24:1, pH 6.8) (Sigma Aldrich) to the samples and bead-beating for 45 sec (4.5 m/s). We performed a second bead-beating step after cooling samples on ice for 5 minutes. Cell debris was removed by centrifugation at 14,000 g for 10 minutes. Supernatants were extracted again with 0.5 mL of chloroform:isoamylalcohol (CI) 24:1 and centrifuged. Nucleic acids were precipitated from the supernatant with polyethylene glycol 6000 (Sigma Aldrich) for two hours on ice followed by centrifugation for 60 min at 13,000 g and 4°C. Pellets were washed with ethanol, dried, and then dissolved in 100 µL of molecular grade water. DNA quantity (1.2-1200 ng/uL) was detected on a NanoDrop™ One/One (Thermo Fisher Scientific, USA) and purity was assessed by the 260/280 nm > 1.8 ng/uL and 260/230 nm > 2.0 ng/uL absorption ratios. Method (extraction) blanks were confirmed to be lower than 2.0 ng/ uL.

2.3. Sequencing and microbial community analysis

We shipped isolated and purified DNA samples to Novogene UK where the 16S rRNA gene was amplified and sequenced on an Illumina MiSeq platform using 2x300 paired-end sequencing with primers F515 (5'-GTGCCAGCMGCCGCGGTAA-3') and R806 (5'-GGACTACVSGGGTATCTAAT-3') [26] and Phusion® High-Fidelity PCR Master Mix (New England Biolabs), following standard Novogene quality control protocols. This generated raw reads of the V4 hypervariable region. Raw sequences were rarefied to an even sequencing depth (37,521 reads/sample) using the QIIME2 framework [27] and amplicon sequence variants (ASVs) were generated using the DADA2 pipeline [28]. The taxonomic assignment of each amplicon sequence variant (ASV) was performed by querying the sequences against the microbial database for activated sludge processes including anaerobic digestion (MIDAS4 database, accessed October 2022 [29]). From the sequence table generated, we carried out biostatistics using QIIME2 and the vegan, phyloseq [30] and ggplot2 [31] packages in R.

2.4. Statistical analysis

Alpha diversity indices (Shannon, Inverse Simpson and evenness) and Bray-Curtis beta diversity were quantified from the rarefied datasets. Next, Shapiro-Wilk tests were used to assess whether the data were normally distributed. After confirmation of non-normal distribution, Kruskal-Wallis and permutational multivariate analysis of variance (PERMANOVA) were applied to identify potential variables that could explain the variation in community composition with respect to alpha and beta diversities, respectfully. Then, relationships between fecal sludge characteristics and relative abundances of microbial communities were quantified using Spearman rank correlation test and p-values were corrected for multiple comparisons with the Bonferroni correction. The functional potentials of microbial communities were predicted using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) based on the least common ancestor approach [32].

3. Results and discussion

3.1. Overview of dataset

16S rRNA gene amplicon sequencing yielded 24,918–122,515 reads after quality filtering, and we obtained 7,742 high-quality 16S rRNA gene ASVs [33] after filtering and removal of chimeric sequences and singletons. On average, there were 1,217 ± 229 ASVs identified in each of the containments. The phylum *Firmicutes* represented over half of the total microbial community and the top five most abundant genera (Fig 1). The Phyla *Bacteroidota, Proteobacteria, Actinobacteria* and *Desulfobacteria* followed in abundance, and the top five phyla together represented ~87% of sequences *(*plots of the most abundant phyla, family, and genera are presented in Fig C in S1 Text). In general, the most abundant organisms at the phyla and family level were a mix of anaerobic, obligate or facultative aerobes and sulfate-reducing bacteria, that can be found in a broad range of environments, from the human gut, skin of animals, soils, sediments and marine environments. A high abundance of spore-forming *Clostridia* (anaerobic) and *Bacili* (aerobic) were identified, and being an amplicon-based study, it is not known whether or not populations are dormant. Since species-level taxonomic assignment is not yet reliable for stored wastewater (fecal sludge) microbial communities, we used genera-level taxonomy when possible, to gain the most information about the microbial communities.

We grouped the 1,247 identified genera as "AP: always present" (defined here as present in >90% of containments), "SP: sometimes present" (range of <90% to >60%), and "RP: rarely present" (<60%) [34]. Presented in Fig 2 are the top 20 most abundant genera by each of the groupings, and all genera listed by groupings and percent relative abundance are presented in Table B in S1 Text. 104 genera were identified in the AP grouping, representing 48.4 ± 8.8% of the total abundance. Of the top 20 most abundant genera in the AP grouping, 16 were in the phylum *Firmicutes* and were mainly anaerobic chemoorganotrophs (heterotrophs) that have been associated with the human gut microbiome. Although they are present in the gut microbiome, it is difficult to evaluate whether microbial communities in stored wastewater (fecal sludge) come from a core gut microbiome (or feces) population, as there is not agreement on what that core microbiome is [35]. In addition, the vast majority of gut microbiome studies have been conducted in Europe or North America, and the relation to Sub-Saharan Africa is not known [35]. In this study, a relatively small group of microorganisms (8% of those identified) made up half the total population. Due to their high prevalence, organisms in the AP grouping could be interpreted as a baseline or core microbial community in Lusaka, that is in general adapted to the ecological conditions of stored wastewater, and/or are consistent communities stemming from the continual input of human gut microorganisms

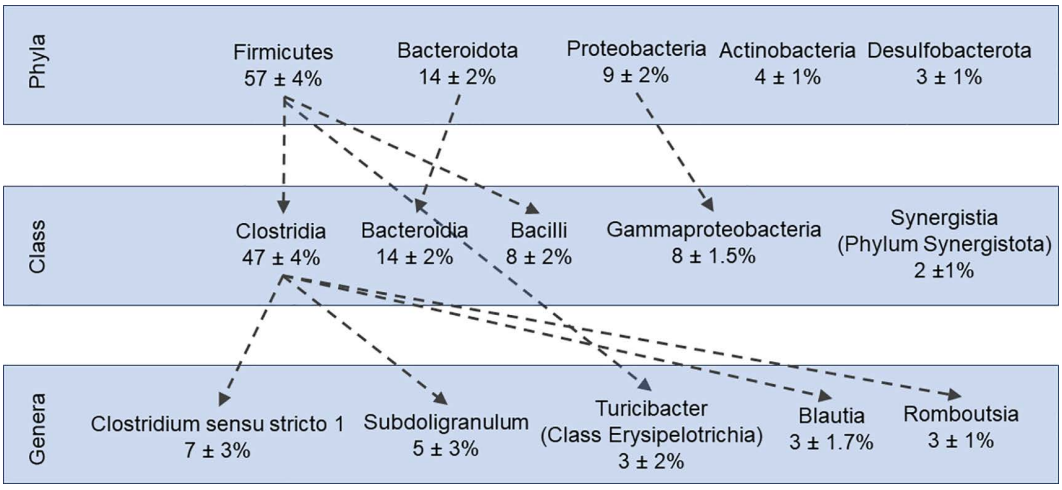

Fig 1. Top five most abundant phyla, class, and genera, and relation to higher taxonomic level (if relevant).

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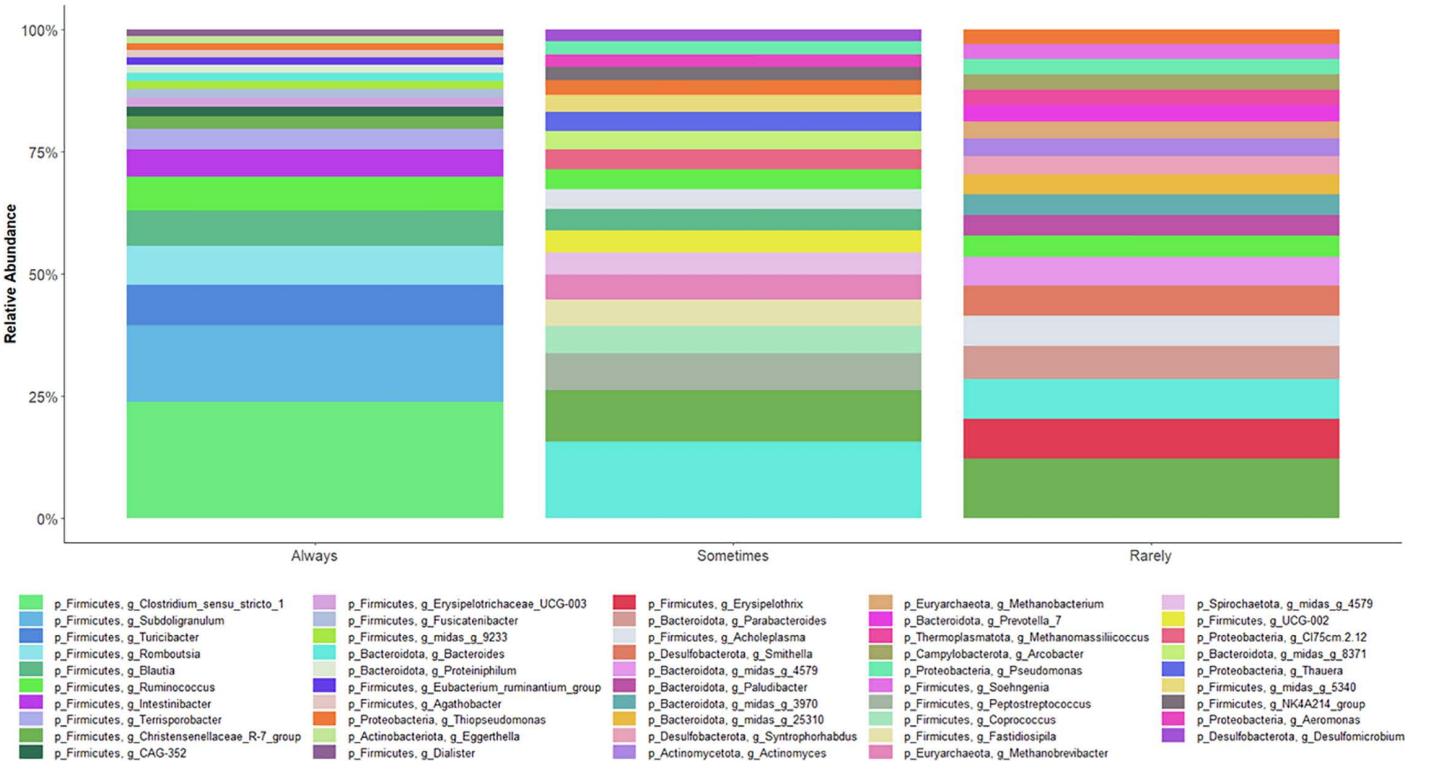

Fig 2. Relative abundances of the 20 most abundant genera in the categories as always present (defined as >90%), sometimes present (range of <90% to >60%), and rarely present (<60%) across the 135 onsite containments of stored wastewater in this study. All genera listed by groupings and percent relative abundance are presented in Table B in S1 Text.

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in feces [36,37]. Other studies reporting AP groupings in related environments include 12.4-84.8% of total abundance in activated sludge [38,39], 36.4-70.3% in anaerobic digesters [40], 21.7-82.8% in soil [41], 0–15% in human feces [42], and 8–93% in the human gut microbiome [43–47].

The SP grouping had 207 genera, which represented 27.3 ± 4.8% of the total abundance, and *Firmicutes* comprised half of the top 20 most abundant genera. SP genera could be expected to have more fluctuations in microbial community structure for a number of reasons, such as specific environmental conditions during storage that are beneficial for the growth of these organisms. The RP grouping had 936 genera, which cumulatively represented 24.3 ± 8.2% of the total abundance, indicating the much lower abundance of each of the RP genera ranging between 0.05 to 0.0002% abundance. It also reflects that very low abundance can occur at or near the method limit of detection, meaning the randomness of detection or non-detection can play a role. Only 5 of the 20 most abundant genera in this grouping were *Firmicutes* and diverse functional groups included methanogens (*Methanobacterium, Methanomassiliicoccus*), sulfate-reducers (*Smithella, Arcobacter*), and possible aerobes (*Pseudomonas*). RP taxa can be assumed to have a more specific niche environment with the greatest fluctuations over time. However, the samples in this study were taken at one point in time, and so we cannot infer from this specific analysis how populations of organisms change with time.

3.2. Comparison to previous studies

For comparison specifically to stored wastewater (fecal sludge), we found ten studies reporting on the microbial community composition in pit latrines and/or septic tanks, most of which report relative abundance at the phyla level (Fig 3).

Study	PL, ST	Country	Reported top 5 most abundant phyla												
			Actinobacteria	Bacteroidota	Chloroflexi	Cloacimonetes	Desulfobacterota	Euryarchaeota	Firmicutes	Halobacteria	Proteobacteria	Spirochaetota	Synergistota	Unknown	Verrucomicrobia
This study (n=135)	PL, ST	Zambia													
Beukes et al., 2019 (n=1, pooled)	PL	South Africa													
Bryne et al., 2017 (n=12)	PL	South Africa													
Connely et al., 2019) (n-NA)	ST	Thailand													
Ijaz et al., 2022 (n= 35)	PL	Tanzania													
Naphtali et al., 2020 (n=12)	ST	Canada													
Sam et al., 2023 (n=9)	PL, ST	8 countries*													
Smith et al. 2023 (n=55)	PL	Malawi													
Torondel et al., 2016 (n=55)	PL	Tanzania, Vietnam													
Ward et al. 2019 (n=25)	PL, ST	Tanzania, Senegal													
Ward et al. 2023 (n=20)	PL, ST	Uganda													
		*Canada, Ghana, Guatemala, India, Kenya, Lebanon, Senegal, Uganda													

Fig 3. Five most abundant phyla reported in the literature for pit latrines and septic tanks, with most abundant phyla represented by dark green shading, and decreasing in shading with decreasing abundance.

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In this study, organisms in the phyla *Firmicutes, Proteobacteria* and *Bacteroidota* represented 80% of the total microbial community, and a high abundance of these three phyla have also been consistently reported across studies (Fig 3). *Bacteroidota* and *Proteobacteria* are also abundant in activated sludge, anaerobic digesters, soil, human feces, gut microbiome, and compost [48–50]. When looking at the taxonomic level of family, in pit latrines in rural Tanzania Ijaz et al. [14] found a shift in microbial community structure from gut-associated families in the top layer to environmental- and sewer-based wastewater-associated taxa at greater depths [14]. However, when zooming in at the genera level Smith et al. [15] found that 124 of the 230 identified genera were unique to pit latrines when comparing communities with municipal activated sludge, sewage sludge anaerobic digesters, and the human gut.

For further comparison, we directly compared the genera level results in the cities of Blantyre, and Mzuzu, in Malawi, reported in Smith et al. [15], to all the containments from Lusaka, Zambia in this study. Overall, of the 230 genera found in Blantyre and Mzuzu pit latrines, 55% were also found in Lusaka. The specific abundance of the top ten most abundant genera from Blantyre and Mzuzu and Lusaka had an overlap of three genera, with a Jaccard similarity coefficient of 0.22 (Table 1). Of the genera that were reported in Smith et al. [15] as unique to pit latrines (i.e., not reported in the human gut, activated sludge, or anaerobic digestion), ten were also found in Lusaka (i.e., *Aerosphaera, CAG-352, Christensenellaceae R-7 group, Dechlorobacter, Fastidiosipila, Herbinix, Ignatzschineria, Lactivibrio, Methanobacterium, Prevotella 9*). It is not clear if this could indicate a core group of microorganisms in all stored wastewater (e.g., AP), together with regional diversity of less abundant organisms (e.g., SP, RP), as has been observed in communities in sewer-based wastewater [39].

3.3. Relation of microbial community composition to wastewater, storage, and downstream treatment

In Fig 4, we explored statistical patterns within the containments in Lusaka by: 1. relation of microbial community to wastewater coming into containment including types and volumes of wastewater; 2. relation of microbial community to conditions within containment including emptying frequency and wastewater characteristics; and 3. relation of microbial community to downstream treatment processes including level of stabilization and dewatering. For category 1, we used how people reported containment type (i.e., pit latrine or septic tank) as a proxy for relative volumes of water usage going

Table 1. Top ten most abundant genera during storage of wastewater in Blantyre and Mzuzu, Malawi [15] and pit latrines and septic tanks in Lusaka, Zambia, in order of most abundant to least abundant, overlap of genera underlined.

Blantyre and Mzuzu, Malawi		Lusaka, Zambia	
Genus	**Relative abundance (%)**	**Genus**	**Relative abundance (%)**
Clostridium sensu stricto 1	18.24	Clostridium sensu stricto 1	7.38
Methanothrix	9.91	Subdoligranulum	4.83
Romboutsia	5.90	Turicibacter	2.61
Succinivibrio	5.77	Blautia	2.58
Proteiniphilum	5.31	Ruminococcus	2.55
Faecalibacterium	4.68	Romboutsia	2.45
Fastidiosipila	3.34	Christensenellaceae R-7 group	2.15
midas g 1227	3.08	Bacteroides	2.14
Blautia	2.32	Intestinibacter	1.67
Prevotella_9	2.06	Terrisporobacter	1.26

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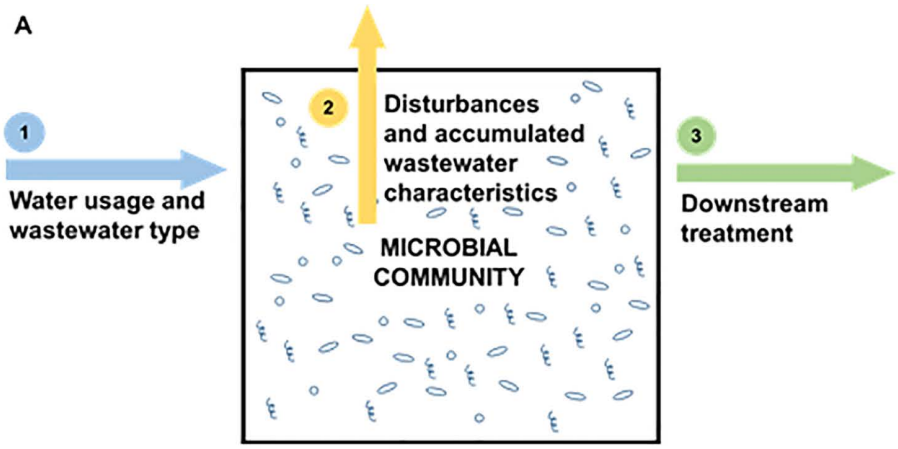
into containments. In this study, when participants reported having a pit latrine, 97% of them were reported as dry toilets and 64% as only blackwater going into the containment, and for septic tanks 93% were reported as flush toilets, with 75% as also including types of greywater going into containment. Moisture differences were also analytically confirmed, with higher moisture in septic tanks (98% median) than in pit latrine (85% median). It was therefore confirmed, that the reported labels were aligned with different water usage patterns.

As reported in Fig 4b, Shannon alpha diversity at the genera level did not differ significantly between pit latrine and septic tanks. As shown in Table 2, both Shannon alpha diversity, which is focused more on richness, and Simpson alpha diversity, which is focused more on evenness, were very similar for pit latrines and septic tanks, and also by whether pit latrines were lined, partially lined or unlined. How evenly the relative abundances were distributed among the organisms (Pielou's J), were also not significantly different among all types of containments (Table 2). In contrast, when comparing beta diversity, there was a significant difference between pit latrines and septic tanks (Fig 4), indicating different relative abundances of each of the genera by containments. When comparing the 20 most abundant at the phyla level between pit latrines and septic tanks, the top 19 most abundant were the same (Table C in S1 Text), whereas when focusing in at the genera level then only 12 of the top 20 were the same (Table D in S1 Text). It is logical that increased diversity will be seen when focusing in and making comparisons at lower taxonomic levels, and with the lower percent abundance at genera level, small differences in distribution can quickly affect beta diversity.

Building usage was evaluated as a proxy for production of different types of wastewater based on expected differences with bathing and kitchen water, including households (n = 116), schools (n = 5), restaurant (n = 1), and houses of worship (n = 2). Alpha diversity differed by building use (Fig 4), where the restaurant and house of worship wastewater had higher alpha diversity than the households and schools. However, beta diversity did not differ by building use (Fig 4), indicating a dissimilarity between wastewater microbial community evenness by building use but a similar overall microbial community composition. The results suggest the need for a larger sample size to detect statistically significant differences, for example Smith et al. postulate that with n = 8 sample size all taxa can be identified [15]. Or alternatively that the label "building usage" itself is not necessarily a useful grouping, unless usage patterns result in very clear differences in produced wastewater, such as public market toilets with higher total solids and NH_4^+-N versus ablution blocks for bathing and washing [17,51].

For category 2 in Fig 4, we analyzed time since last emptied and time since installation of the containment as proxies for disturbance of microbial communities. There were no statistically significant differences based on alpha and beta diversity (Fig 4). This is consistent with Smith et al. [15], who also reported no significant differences in microbial communities by

A



B

Factor	Kruskal-Wallis on Shannon alpha diversity p-value	PERMANOVA on Bray-Curtis beta diversity p-value
1. Water usage and wastewater type		
Water usage		
Pit latrine or septic tank	0.234	0.000215***
Flush or dry toilet	0.113	0.000215***
Building has water connection	0.595	0.000215***
Wastewater type		
Building usage	0.0358***	0.0509
2. Disturbances and accumulated wastewater characteristics		
Disturbance		
Time since last emptied	0.595	0.455
Time since installation	0.595	0.685
Wastewater characteristics		
Total organic carbon (TOC)	0.615	0.000215***
Ammonium Nitrogen (NH_4-N)	0.615	0.000215***
Total Kjeldahl nitrogen (TKN)	0.615	0.0140***
pH	0.615	0.000215***
3. Downstream treatment		
Stabilization		
Volatile solids/total solids (VS/TS)	0.615	0.0411***
Color (Bulk sludge saturation level)	0.639	0.000215***
Odor	0.217	0.000215***
Dewatering		
Capillary suction time (CST)	0.615	0.000215***
Turbidity following centrifugation	0.615	0.000400***
Cake solids following centrifugation	0.615	0.477

Fig 4. A. Stored wastewater (fecal sludge) containment factors are ordered by classification where blue is related to patterns of toilet and water usage, yellow is related to emptying frequency and laboratory characterization of wastewater characteristics, and green is related to laboratory metrics of stabilization and dewatering. B. Statistical comparisons between alpha diversity (Kruskal-Wallis), beta diversity (PERMANOVA), and factors associated with containments for storage of wastewater. P-values were adjusted for multiple comparisons using the Benjamini-Hochberg method and statistically significant values (≤5%) are indicated by ***.

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Table 2. Bacterial diversity, richness and evenness at the genera level of the 16S rRNA gene libraries for the different types of containments in Lusaka, Zambia (pit latrines and septic tanks), and the degree of lining reported for pit latrines (lined, partially lined, unlined).

Diversity index	Septic tank (n = 61)	Pit latrine (n = 64)	Lined pit latrine (n = 25)	Partially lined pit latrine (n = 31)	Unlined pit latrine (n = 8)
Alpha Diversity *(Shannon)*	5.51 ± 0.51	5.74 ± 0.25	5.71 ± 0.22	5.68 ± 0.27	5.76 ± 0.16
Alpha Diversity *(Simpson)*	0.98 ± 0.02	0.99 ± 0.01	0.98 ± 0.01	0.99 ± 0.01	0.99 ± 0.004
Evenness *(Pielou's J)*	0.23 ± 0.07	0.26 ± 0.05	0.27 ± 0.04	0.25 ± 0.06	0.27 ± 0.04

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emptying frequency. This could be due to relatively consistent environmental conditions within individual containments even with emptying. Based on associated infrastructure, types of wastewater and volumes of water going into individual containments could be expected to be relatively consistent. Emptying events also frequently do not remove 100% of the stored wastewater, which could leave behind a core community. However, ANOVA analysis identified differences of genera in the RP grouping with time since emptied, such as thermoacidophile bacteria in the class *Caldatribacteriia* [52], and heat and desiccant resistant bacteria in the genera *Deinococci* [53]. This could indicate that pockets of different environmental conditions are present within individual containments, as we evaluated composite samples, or that when looking across an entire city, anomalies will be identified. Although samples were collected at only one point in time, and real time series analyses are thus not possible, the lack of significant differences in alpha or beta diversity based on the variables time period since last emptied and since installed, suggest that core communities are relatively stable with time, and/or that there are large but possibly dormant populations.

Further for category 2, we evaluated the impact of in situ concentrations of total organic carbon (TOC), ammonium nitrogen (NH_4^+-N), total kjeldahl nitrogen (TKN) and pH in wastewater during storage in containment. Microbial communities did not differ by alpha diversity but did by beta diversity (Fig 4). The top 5 most correlated genera for NH_4^+-N and TKN were in the RP grouping of genera, with very low abundance of 0.03 to 0.0065%, whereas genera correlated to TOC were more abundant (Table E in S1 Text).

For category 3, we analyzed the relationship of laboratory metrics of stabilization (volatile to total solids ratio (VS/TS), color, odor) and dewatering (capillary suction time, turbidity, cake solids) and microbial diversity. These measures have potential relevance to downstream treatment following emptying and transport to treatment facilities. Differences in alpha diversity were not observed for any of these metrics, whereas differences in beta diversity were statistically significant for all of them other than cake solids (Fig 4). Similar to relations with NH_4^+-N and TKN, most correlated organisms with turbidity had very low abundance, indicating the importance of not only focusing on the role of most abundant organisms (Table F in S1 Text).

3.4. Potential metabolic activity

To further explore the statistical differences that we observed in this study (Fig 4b), we evaluated the functional potential of denitrification, methanogenesis, and acetoclastic methanogenesis with PICRUSt2 annotated marker gene sequence profiles by the categories pit latrine and septic tank. As illustrated in Fig 5, there was considerable overlap but also statistically significant differences ($p < 0.05$) with more counts of potential denitrification, methanogenesis, and acetoclastic methanogenesis pathways in septic tanks than in pit latrines. While associating the functional potential of microorganisms with 16S data only is limited and not fully validated for environmental samples, it provides an indication that the observed community differences could have functional consequences as well. Next generation microbiological tools can be useful for progressing knowledge of under-studied fecal sludge management, but will require further purposeful applications to build up sufficient databases.

These results suggest interesting metabolic differences between pit latrines and septic tanks; however, we can only speculate on possible reasons or implications. The greatest differences between reported pit latrines and septic tanks in this study were higher volumes of water usage associated with septic tanks, resulting in higher moisture content, and less

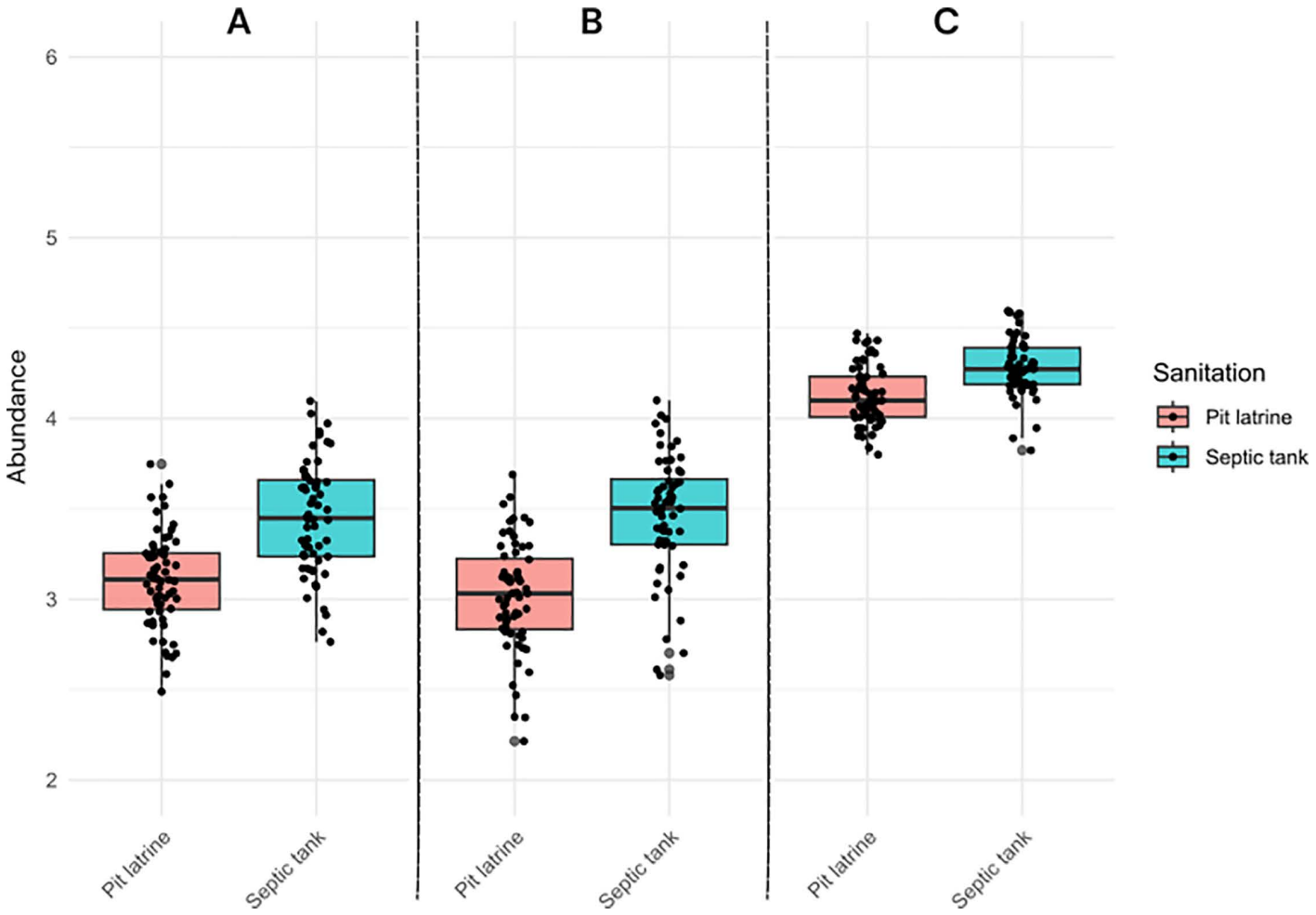

Fig 5. Log-transformed abundance (Y-axis) counts of biosynthesis/assimilation Kyoto Encyclopedia of Genes and Genomes (KEGG) ortholog (KO) pathways of A) denitrification, B) methanogenesis, and C) acetoclastic methanogenesis for pit latrines and septic tanks in this study.

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concentrated NH_4^+-Nand TOC in septic tanks (median: 0.4 g NH_4^+-N/L, 1.7 gTOC/L) than pit latrines (median: 2.8 g NH_4^+-N/L, 14.4 gTOC/L) (Fig D in S1 Text). It is generally thought that only the very thin surface layer of wastewater stored in containments is aerobic, with anaerobic pathways of degradation otherwise occurring [2]. It has also been reported that more dilute wastewaters in containments also have lower oxidation reduction potential (ORP), which are more conducive to methane production, including from -310 [54] to -489 mV [55] for septic tanks and -199–59 mV for pit latrines [56]. This is also consistent with literature reporting higher greenhouse gas emissions from septic tanks (211 Mt CO_2e) than pit latrines (166 Mt CO_2e) [57,58]. The higher NH_4^+-N in pit latrines could also result in more free ammonia nitrogen which can inhibit methanogenesis. Although using the median pH of 7.8 in this study, the calculated free ammonia nitrogen concentration would be less than 100 mg/L (at 25°C). As is the case with high solids and high free ammonia in the anaerobic digestion of swinery manure, it is not clear if this concentration would be inhibitory [59]. It remains to be seen how the inhibitory and buffering capacity of moisture and solids content, pH, volatile fatty acids, carbon dioxide and free ammonia nitrogen impact ammonia inhibition and methanogenesis, and the dynamics of how different microorganisms adapt to these conditions [59]. In containments at the higher end of observed NH_4^+-N, such as public toilets at markets that receive exceedingly higher proportions of urine, inhibition will be more likely. Although most of the methane coming from natural

systems originates from acetate [3], these results suggest the value of investigating methanogenesis from H_2 and CO_2 during storage of wastewater. These results could indicate that even though there is higher TOC in pit latrines, storage of this less dilute wastewater results in less methane emissions.

3.5. Study limitations

Although half the world's population is served by non-sewered sanitation, we currently have a very limited understanding of microbial processes that take place during storage of non-sewered wastewater during storage in containments. Although this study was not designed to evaluate causation, with the publication of this open-source data set, it can pave the way for further controlled field- and laboratory-based studies, to test hypotheses that increase our mechanistic understanding. Examples of limitations of the 16s amplicon results in this study include not being able to distinguish between active versus dormant bacteria and archaea populations, and ability to interpret low abundance values that occur at or near the method limit of detection. In addition to the enzymatic reactions carried out by bacteria and archaea, it also does not shed light on invertebrates, worms and fungi that could also be playing a role in the cycling of nutrients and organic matter in stored wastewater (fecal sludge). Time since emptied and time since constructed were used as proxies for time, whereas a time series performed over a period of years, together with sampling at depth within containments, could improve these interpretations.

A further understanding of these processes could lead to better management practices, such as selecting optimal downstream treatment technologies based on levels of stabilization. For example, anaerobic digestion technologies will obviously benefit from less stabilized wastewater, whereas dewatering technologies such as settling-thickening and drying beds perform better with more stabilized wastewater (fecal sludge) [18]. Additionally, this knowledge could lead to more well-informed decisions regarding potential benefits of greenhouse gas mitigation strategies, such as management practices that reduce dilution of wastewater (e.g., low-flush toilets, separate management of greywater, container-based sanitation), or retrofitting septic tanks with biofilters to reduce methane emissions.

4. Conclusions

Our conclusions from this representative study of microbial communities in non-sewered wastewater (fecal sludge) stored in containments (pits, tanks) across an entire city include:

- Communities are quite similar when looking at higher taxonomic levels and most abundant genera. In Lusaka, 80% of the identified microorganisms were in the phyla *Firmicutes, Proteobacteria* and *Bacteroidota*. There was a core community of 104 genera that were present in 90% of the containments, across containment type (pit latrine, septic tank). Based on alpha diversity, richness and evenness, there is a similar level of diversity in each individual containment across pit latrine and septic tank. Based on alpha and beta diversity, communities are similar with varying time since last emptied and time since installation, indicating they are stable with time and disturbances. Based on a comparison with the literature similarities can also be expected, including a 55% similarity of genera with a study in Malawi, together with regional differences,
- However, when focusing in at the lower taxonomic level of genera more diversity is observed, as is reflected in differences in beta diversity with water usage (i.e., toilet flush, bathing, kitchen), properties of sludge in containments, stabilization metrics and metrics of treatment performance.
- Metabolic differences between pit latrines and septic tanks are aligned with higher volumes of wastewater going into septic tanks resulting in lower ORP, TS and NH_4^+-N concentrations. Pit latrines have greater concentrations of TOC, but storage of this wastewater with lower moisture concentrations appears to be associated with lower methane production.

- The frequency of detection of niche organisms, with 936 genera being detected in less than 60% of containments, indicate it is important to move beyond most abundant organisms, to unravel specific ecological roles and metabolic processes, such as sulfur reduction, methanogenesis, and ammonia tolerance. Additionally, we need to move beyond amplicon analysis to understand whether organisms are dormant or in spore form, to understand the active component of the population.

Supporting information

S1 Text. All supplementary figures and tables.
(DOCX)

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