MICROBIOME OF VIP LATRINES

Report to the

Water Research Commission

by

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EXECUTIVE SUMMARY

Ventilated Improved Pit (VIP) latrines are designed to accumulate human excreta and anal cleansing materials and serve as a digestion system for accumulating matter that undergoes stabilisation through natural biological processes. The faecal material added becomes layered over time resulting in discrete zones with different biological, chemical, and physical conditions found within. The organic materials rely heavily on microorganisms to break down the matter to allow biological settling and reduction of both the organic constituents and the physical height of the sludge. These processes are performed by microorganisms with an abundance that is estimated to reach up to about 10^{13} CFU/g in fresh faecal material.

The two major biodegradation processes that occur within a VIP latrine are aerobic biodegradation which occurs in the presence of oxygen, and anaerobic biodegradation which occurs in the absence of oxygen. Aerobic degradation is responsible for about 30% of the reduction of total solid mass within VIP's. Anaerobic degradation is slower than aerobic degradation but results in a 70% reduction of the total solid mass within VIP latrines. Anaerobic degradation is the dominant form of decomposition of faecal matter in pit latrines and mainly results in the by-product formation of gasses which are released into the surrounding environment. Complete degradation results in the conversion of faecal sludge into products that are either soluble, gaseous, or non-biodegradable solids. The soluble and gaseous products are released out of the pit through leaching and ventilation while the stabilised non-biodegradable matter accumulates at the bottom of the pit.

This biological activity allows to some degree for the extended life span of the pit before maximum capacity is reached. The microbial communities that predominate in faecal sludge and their biodegradation activities are determined and regulated by various physical and chemical conditions (temperature, pH, oxygen, moisture content, presence of antimicrobial chemicals, presence of metals, and organic and inorganic load) prevailing within the pit. This consequently determines the degradation capacity of the faecal sludge and the rate at which matter stabilises to the bottom of the pit, and in turn, determines the lifespan of the pit. Deleterious compounds and conditions directly influence the efficiency of the microorganisms within the pit sludge and is an aspect that should be constantly monitored.

Previous models of filling rate of VIP latrines considered only the macro-scale characteristics of the system and provided a relatively crude predictive method to determine the height of accumulated material after a specific time. In particular, all biodegradable material within the pit was lumped into a single layer, undergoing the same transformations. Various experimental studies, including this project, have shown that there are distinct layers within the pits, which are subject to different transformations, and are dependent on the microbial composition of each layer.

This report provides an overview of a study undertaken to determine the microbiome of VIP contents by assessing the microbial community composition using genetic sequencing and analysis to gain a deeper understanding of the activities within VIP latrines to enable the development of a microbiome-based model of the pit latrine, based on the concept of semi-batch reactors in series.

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LIST OF ABBREVIATIONS

Abbreviation	Description
AA	Amino acids
AOB	Ammonia oxidizing bacteria
BOD	Biological oxygen demand
BREC	Biomedical Research Ethics Committee
ca	Approximately
Cd	Cadmium
CFU/g	Colony forming units per gram
CH_4	Methane
Co	Cobalt
CO_2	Carbon dioxide
COD	Chemical oxygen demand
COD_P	Particulate chemical oxygen demand
COD_S	Soluble chemical oxygen demand
COD_T	Total chemical oxygen demand
Cr	Chromium
Cu	Copper
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
E. coli	Escherichia coli
EBPR	Enhanced biological phosphorus removal
EC	Electrical conductivity
FDA	Fluorescein diacetate
GAO	Glycogen accumulating organisms
H_2	Hydrogen (gas)
H_2O	Water
Hbu	Butyric acid
Hva	Valeric acid
K	Potassium
k	New non-biodegradable material
LC 50	Concentration lethal to 50% of the test objects/animals….
LFCA	Long chain fatty acids
MO	Microorganisms
MPN	Most probable number
MS	Monosaccharides
N	Nitrogen
NaOH	Sodium hydroxide
NGS	Next generation sequencing
NOB	Nitrite oxidizing bacteria
OUT	Operational taxonomic unit
P	Phosphorus
PAO	Polyphosphate accumulating organisms
Pb	Lead

Abbreviation	Description
pp	Per person
PHAs	Polyhydroxyalkanoates
r	First order rate constant for biodegradation
SA	South Africa
SCFA	Short chain fatty acids
SOP	Standard operating procedure
t	Time
T	Total age of the pit
TKN	Total Kjeldahl nitrogen
TS	Total solids
TSS	Total suspended solids
UKZN	University of KwaZulu-Natal
USA	United States of America
UV	Ultraviolet
V	Volume
vb0	Initial volume of biodegradable material
VFA	Volatile fatty acids
VIP	Ventilated improved pit
VS	Volatile solids
vuo	Initial volume of non-biodegradable material
WASH R&D	Water, Sanitation & Hygiene Research & Development
WRC	Water research commission
WWTP	Wastewater treatment plant

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1 CHAPTER 1: INTRODUCTION

Data from the 2022 Census (StatsSA, 2022) estimates that approximately 30% of South African households rely on on-site sanitation such as the Ventilated Improved Pit (VIP) latrine compared to 70% of households with sewered (flushing toilets) sanitation. The technical resources and knowledge devoted to serving the on-site sanitation section of the population is small compared to the sewer-served section of the population. The perceived wisdom by sanitation service providers is that on-site sanitation provision is as simple as providing a suitable underground and above ground structure and the selection of an appropriate pedestal. The complexity of the pit microbiome is seldom considered.

VIP latrine sludge is a mixed composite of organic and inorganic materials originating from human excreta, but is also known to contain a wide variety of added foreign materials such as newspapers, magazines, broken glass, stones, rubble, underwear, toys and metals to name a few. The faecal material added become layered over time resulting in discrete zones with different biological, chemical and physical conditions found within. The organic materials rely heavily on microorganisms to break down the matter to allow biological settling and reduction of both the organic constituents and the physical height of the sludge. This biological activity allows to some degree for the extended life span of the pit before maximum capacity is reached. Deleterious compounds and conditions directly influence the efficiency of the microorganisms within the pit sludge and is an aspect that should be constantly monitored.

The literature on the microbiome of on-site sanitation is sparse compared to the biomic literature of the process feeding the material in the sanitation system. A simple Google scholar search revealed that there is a wealth of pre-existing knowledge which can be applied to the better understanding of on-site sanitation systems. Data from a previous sampling campaign in South Africa (Byrne et al., 2017) has been published with a preliminary interpretation of the data, and this data, together with the data generated from this project will provide contextual in-depth interpretation. This study encompasses determining the microbiome of VIP contents by assessing the microbial community composition using genetic sequencing and analysis to gain a deeper understanding of the activities within VIP latrines

The hypothesis of this project is that an understanding of the microbial metabolic reactions taking place is necessary for the rational design of improved on-site sanitation systems, which will contribute to the better understanding of the transformational processes in VIPs.

1.1 Contextualisation

The microbiome within dry storage non-sewered sanitation systems (drop and store) such as dry VIP latrines is dominated by that of the input material which is generally that of the gut microbiome, as the material is deposited layer by layer and there is little to no mixing between layers. The material is removed infrequently (ca 5 to 10 years). In process engineering terms, such a system can be considered as a series of unmixed non-steady-state batch reactors of increasing residence time.

In wet drop and store systems the deposited material tends to breakup and disperse in the liquid in the tank and a degree of mixing occurs every time liquid is added to the tank. Emptying occurs more regularly (6 months to 3 years) and not all the liquid is removed. In this case the microbiome might be altered from that of the gut microbiome via selection of the organisms promoted by all previous fill and empty cycles (weighted by the fraction of material from the previous cycle that was left behind). In process engineering terms, such a system can be considered as a fill-and-draw poorly mixed compartmentalised reactor. The performance of such a system is very dependent on the previous emptying history of the system.

To date the common analyses and interpretation of on-site systems has been limited to the types of analyses used in conventional biological wastewater treatment systems such as moisture content, total solids, volatile solids, ash, chemical oxygen demand, speciated COD (e.g. soluble, particulate and biodegradable) nitrogen species and phosphate species. The dominant mechanisms in engineered wastewater treatment are due to separation stages, selector processes and recycle streams. These calculated engineered stages and flows are absent in on-site systems. This is primarily because the basic transformation processes have not been determined and quantified in reaction kinetic terms.

By starting to understand the microbiome and the matching metabolic activity of on-site sanitation systems, a process engineering approach can be initiated to better understand the mechanisms and opportunities of different designs and operating / emptying strategies. Additional benefits of a deeper understanding of the microbiome is that it prepares for future research into understanding the medical state of the population using the on-site sanitation system and on improving pit latrine performance.

1.2 Aims and Outcomes

The aims of this project are to:

- Undertake a literature review of microbiome characteristics and biochemical transformations in VIP latrines
- Examine, process and interpret the existing VIP latrine genomic and chemical data from on-site systems, supplemented if necessary by new data
- Develop a working hypothesis of the major transformation processes in VIP latrines
- Undertake a targeted VIP latrine sampling campaign and submit samples for analysis
- Interpretation of the South African VIP latrine and septic tank microbiome from a reactor perspective

The outcomes of this project would lead to:

- A deeper understanding of the microbiome in South African on-site sanitation systems, and in particular, VIP latrines
- The possibility of developing systems designed to enhance the natural microbiological processes that catalyse the turnover of solid materials present which could occur in VIP latrines and septic tanks.

1.3 Ethical clearance

Ethical clearance for this study was obtained from the Biomedical Research Ethics Committee (BREC) at the University of KwaZulu-Natal (BREC/00003778/2022)

1.4 Structure of the report

Chapter 2 of the report provides a comprehensive literature review on available information regarding the chemical, physical and biological aspects of VIP latrines. As an outcome of this review, a desktop analysis was undertaken to determine the available chemical, physical and biological data from VIP latrines from internal and external sources, and the available genomic data from VIP latrines from internal and external sources.

Chapter 3 provides an overview of the methodology for the experimental work. This covers the selection of the most suitable heterogeneous VIP's for sampling (undertaken together with eThekwini Water and Sanitation unit), the collection of sludge samples following standard operating procedures (SOPs) developed by the Water, Sanitation & Hygiene Research & Development (WASH R&D) Centre at the University of KwaZulu-Natal (UKZN), the preparation of sub-samples for genome sequencing and the small-scale laboratory tests conducted on the sludge in order to determine the chemical, physical and biological parameters that influence VIP sludge composition. The results of the analyses are provided in **Chapter 4**.

Chapter 5 discusses the results from the genetic sequencing, while **Chapter 6** describes the development of the model and provides an overview of the demonstration model (Appendix 1) which is attached as a separate Excel spread sheet model. The conclusions and recommendations from this project are presented in **Chapter 7.**

2 CHAPTER 2: LITERATURE REVIEW

A detailed literature review was undertaken in order to obtain an understanding of the current information and level of knowledge in this area. This involved data mining of information on the microbiome of the human gut and similar engineered systems such as biogas reactors, (which in many ways resembles the human gut community) including the microbiome characteristics and underlying biochemical transformations, as well as information on the characteristics of the contents of VIP latrines. The aim of this literature review was to draw some correlations between the two in order to inform the sampling and experimental campaign of the project.

2.1 Introduction

Ventilated improved pit latrines (VIP) are a widely used and common basic form of sanitation facility used in many low- and middle-income countries. They are a minimum acceptable level of on-site sanitation designed mainly for the accumulation and storage of human excreta prior to being emptied or disposed of (Bakare et al., 2012; Nakagiri et al., 2017). An estimated 1.77 billion people rely on pit latrines as a primary means of human excreta disposal (Graham and Polizzotto, 2013). In South Africa, 21.9% of the population makes use of VIP latrines (StatsSA, 2018).

A typical structure of a VIP latrine (Figure 2-1) consists of a hole dug in the ground, covered by a platform with a drop hole, a superstructure that provides privacy, and a vertical pipe for ventilation beside or within the latrine superstructure. Though they are similar to the standard pit latrines, the fundamental design of a VIP latrine is the airflow down into the pit through the drop hole and up the ventilation pipe, thus removing odours from the latrines. In addition, the ventilation pipe is fitted with a fly screen at the top to control flies. The pit is dug to a depth of 1.5 m, which may be lined or unlined with reinforcing materials to prevent the collapse of the surrounding soil into the pit (Mara, 1984; Buckley et al., 2008; Obeng et al., 2019). It must be noted that all VIP's are considered as pit latrines, but all pit latrines are not VIP's. These two terms will be used interchangeably herein, as waste material is still deposited, degraded, collected and studied from a hole below ground.

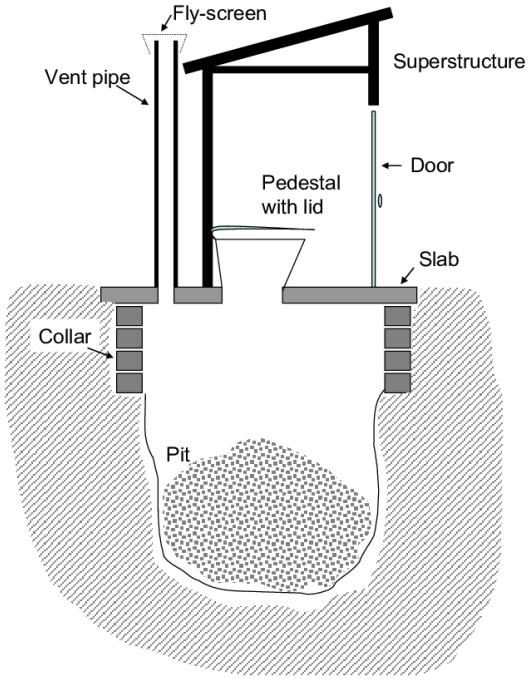

Figure 2-1: A typical structure of a VIP latrine (Buckley et al., 2008).

The main challenge with VIP latrines is that the pit eventually reaches its capacity, and without a long-term plan of maintenance, the latrine becomes unusable as a sanitation solution to users. This causes a major problem in densely populated areas, as there is a lack of space for the relocation of the toilets, while the accumulation of high amounts of faecal sludge can lead to the spread of waterborne diseases and pollution of the environment (Jenkins et al., 2015; Septien et al., 2018; Jung et al., 2023). Pits are allowed to fill within a certain proximity to the top of the pit. In the eThekwini Municipality in South Africa, pits are allowed to fill up to 300 mm within the top, and the pits are emptied on a five-year cycle. Pits fill at different rates depending on a number of factors with some pits reported filled after 18 months, while others have been reported to have an infinite lifespan (Zuma et al., 2015).

The pit volume is designed to accommodate excreta and cleansing materials for a specific number of users over a set period of time. The life of a pit is determined by in- and out-flows in the latrine and by decomposition of pit content (Grolle et al., 2018). If used appropriately, the faecal sludge in any general pit should be composed of faecal matter, urine, and cleansing materials. Faecal matter is a major feed into a pit, and it comprises of 80% biodegradable organic matter and 20% of inert material, which stabilises and accumulates from the bottom of the pit. Freshly added faecal matter undergoes decomposition, where microorganisms occurring naturally in faeces play a key role in the degradation of faecal sludge within the pit, thus gradually reducing the volume and/or mass of solid material pit. It is assumed that the greater the rate of biodegradation, the more rapid the total mass is reduced and the more rapid the faecal sludge will stabilise (Brouckaert et al., 2013).

However, VIP latrines were found to fill prematurely (Gudda et al., 2019). Consequently, some households and even municipalities have resorted to using additives that are marketed for their ability to reduce the accumulation of faecal sludge and thus extend the life of the VIP latrine (Basamykina et al., 2021). It is assumed that the degradation rates can be increased with the addition of additives into the pits. The efficacy of such additives is, however, based on anecdotal evidence. There is a lack of scientific verification on the effectiveness of additives to increase decomposition of faecal sludge, with some studies even reporting that there is no significant benefit to adding additives in VIP latrines (Foxon et al., 2006; Awere and Edu-Buandoh, 2016).

Microorganisms play a key role in the degradation of faecal sludge within the pit, making up to 30% of the total dry mass of faeces (MacNeal et al., 1909; Stephen and Cummings, 1980; Foxon, 2008) . However, studies of the microbes and their association with the degradation processes of faecal sludge in VIP latrines are limited (Torondel et al., 2016). The role of microorganisms is well documented for sludge treatment processes in wastewater treatment plants (WWTPs) and biogas reactors. The advancement of molecular techniques has enabled the analysis of microbial communities present, their abundance and possible function during sludge treatment, and their response and performance under different conditions. Such developments have enabled the development of strategies to enhance the activities of beneficial microbial communities, thus improving the efficiency of critical processes (LaMartina et al., 2021). Moreover, the insight into the microbial community enabled better control over operational parameters by predicting a possible change of behaviour of the microbial communities under different conditions (Hoshino et al., 1978; de Celis et al., 2020). Studies on the microbial communities in VIP latrines and their association with faecal sludge degradation is still limited. Understanding the structure of microbial communities is crucial as they determine the success or the failure of a process in any given environment.

2.2 Pit sludge composition

2.2.1 Typical faecal sludge composition

VIP latrine sludge is primarily composed of fresh, decaying and inactive / recalcitrant organic matter, depending on the time frame and depth within the sludge. Depending on the type of pit, VIP latrine contents can become layered over time resulting in layered data / zones (Nwaneri et al., 2008; Bakare, 2011; Zuma, 2016). Within these pits, the topmost layer is where the readily biodegradable material is found and where rapid aerobic degradation takes place. This layer is negligibly small and not realistically measurable. The second layer is still an aerobic section, the third layer is anaerobic and the last layer is anaerobic and contains old stable matter (Nwaneri et al., 2008).

Table 2-1: Data differences with depth in sludge contents (Bakare, 2011)

	Surface (%)	1,5 m depth (%)
Moisture	76.88	67.22
COD	60.30	24.40
Volatile solids	57.89	36.57
Biodegradability	52.46	16.55

A study by Bakare (2011) showed a difference in the characteristics of the sludge contents at 1.5 m depth compared to the surface layer (Table 2-1). The surface layer showed the highest percentage results, but also the highest variability in the data. This depth pattern and variability is also seen in the data presented by Zuma et al. (2015), and the moisture difference is also presented by Septien et al. (2018). All data variables decreased from the surface down to 1.5 m depth with reduced data variability with increasing depth (Bakare, 2011). A study performed on latrine contents in Kampala City, Uganda, also showed decreasing measurements in chemical oxygen demand (COD), temperature and dissolved oxygen (DO) with depth, but an increase in moisture content across the pit types (lined and unlined) and across the seasons (rainy and dry). This was due to the pits being below the groundwater table (Kimuli et al., 2016). Pit latrine COD data from Kumasi, Ghana, was 45.61 g/L (Fanyin-Martin et al., 2017).

Other studies present a range of data from pit sludge; for example, moisture content between 38 and 85%, COD values of between 76 and 178% and VS values between 10 and 84% (Zuma, 2016). Sludge composition can also vary horizontally with variations between the front and back sections. Zuma et al. (2015) showed a lower moisture content within the back section of the pit compared to the front, at the same depth.

Although the contents of any VIP latrine can potentially contain a wide range of materials (Bakare et al., 2012; Zuma et al., 2015) the majority of this is composed of faeces and urine, with some toilet paper. Toilet paper alone can constitute as much COD as 706 mg/sheet, TS of 578 mg/sheet and TSS of 546 mg/sheet (Almeida et al., 1999). These total organic excreta material accounts for, on average, 87% of pit contents, with the remainder consisting of such wastes as paper, plastics and textiles (Zuma et al., 2015). However, as pointed out by Magagna (2006), Buckley et al. (2008), Bakare et al. (2012), Niwagaba et al. (2014a) and Changara et al. (2018), pit sludge properties, characteristics and contents can differ widely from one pit, and location, to the next.

2.2.2 Organic vs. inorganic constituents

A dry mass percentage representation of the major constituents of faecal matter is presented in Figure 2-2.

A large percentage of faecal matter is comprised of liquids being mostly water, digestive juices, mucus, etc. (Rose et al., 2015; Hamilton, 2018). What remains are the solids, of which bacterial mass makes up roughly 25-54% of this by weight, including dead cells (Stephen and Cummings, 1980; Guyton and Hall, 2000; Achour et al., 2007) The values presented in the above section and in Figure 2-2 are approximations and are entirely dependent on diet, age, fitness, sex, geographic location and ethnicity of the individual depositing the material (for example, the data presented above comes primarily from Western origins). Fanyin-Martin et al. (2017) present faecal sludge data from Kumasi, Ghana, showing average lipid content to be only 8.82-9.66%, much lower than the above-mentioned values. This variance is solely due to difference in diet.

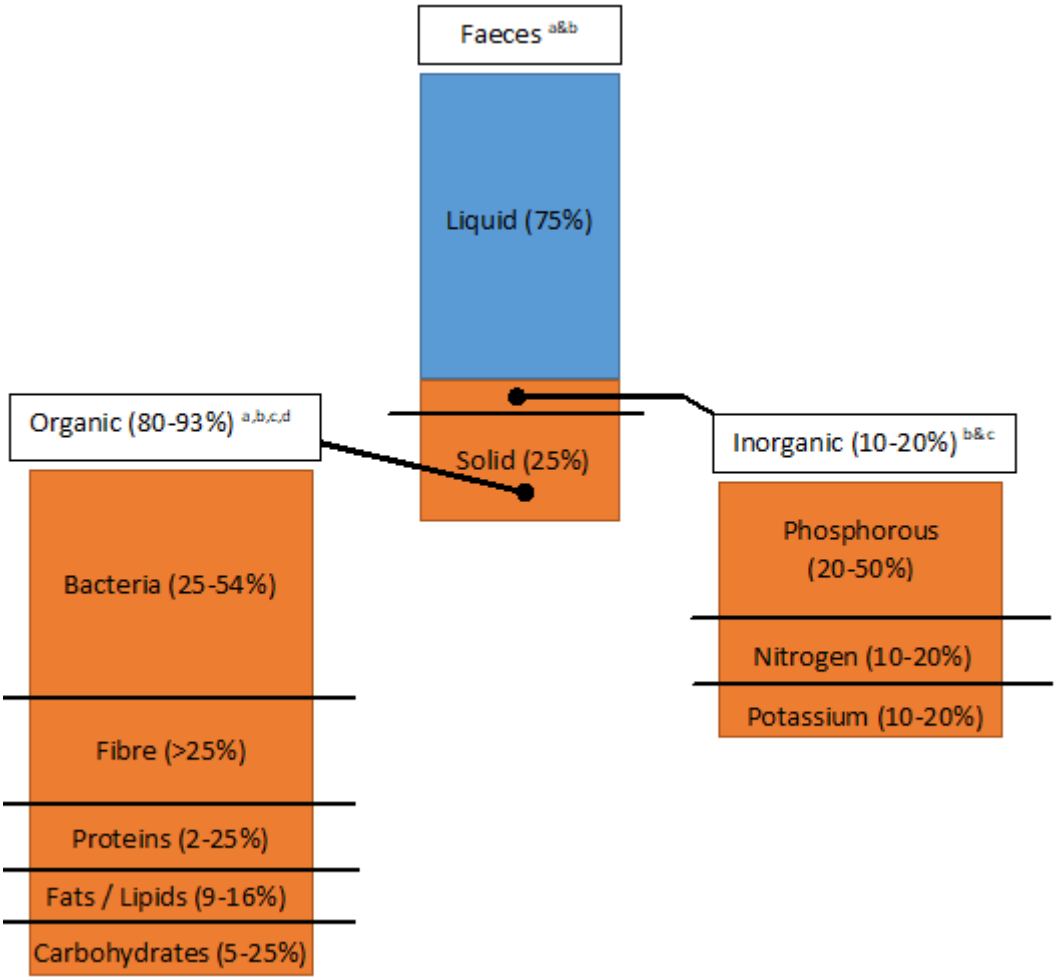

Figure 2-2: Generalised breakdown of faecal matter by its percentage components by dry mass: a, Nwaneri et al. (2008); b, Rose et al. (2015); c, Jónsson et al. (2005) and d, Still and Foxon (2012)

Urine generation from humans contains approximately 91-96% water and is usually the major fraction of liquids added into VIP latrines (Zuma, 2016). The remaining few percent from urine account for roughly 1.4% inorganic electrolytes, 1.3% urea, 0.4% organics and 0.4% organic ammonia (Still and Foxon, 2012; Zuma, 2016).

The inorganic components (Figure 2-2) from faecal origin make up the remaining ±10-20% and are made up of primarily of undigested dietary elements in the common form of inorganic salts such as calcium phosphate and iron phosphate (Nwaneri et al., 2008; Rose et al., 2015). These inorganics can be considered as the nutrient fraction that is excreted (Niwagaba, 2009; Zuma, 2016). Rose et al. (2015) breaks down the major elements found within total faeces into 74% oxygen, 10% hydrogen, 5% carbon and 0.7% nitrogen.

Both nitrogen and phosphorous concentrations are usually much higher in sludge compared to domestic wastewater (10-100 times and 2-50 times respectively) (Niwagaba et al., 2014a). Nitrogen compounds within pits are usually ammonium / ammonia, nitrate, nitrite and the nitrogen organic forms (amino acids and amines), while for phosphorous these are phosphate, orthophosphoric acid and the phosphorous organic forms (nucleic acids, phospholipids and phosphorylated proteins) (Niwagaba et al., 2014a). Some available data on these nutrient concentrations is presented in Table 2-2 and elemental concentrations in Table 2-3.

Table 2-2: Average concentrations of nutrients found in pit latrine excreta

	Kampala City, Uganda				Kumasi, Ghana[b]	Hanoi, Vietnam[c]
	Rainy Season		Dry Season			
	Unlined Pits	Lined Pits	Unlined Pits	Lined Pits		
Ammonia (mg/kg)	2,64 ± 22	2,02 ± 57				± 260.00
Ammonium (mg/kg)			227.00 ± 71	191.00 ± 43		
Nitrate (mg/kg)	705.00 ± 59	506.00 ± 27	194.00 ± 59	254.00 ± 27		
Total Nitrogen (mg/kg)	42,59 ± 270	45,23 ± 198	39,21 ± 39	40,03 ± 91		
Total Phosphates (mg/kg)	8,44 ± 738	7,59 ± 428	8,81 ± 544	12,39 ± 1390		
Potassium (mg/kg)	31,70 ± 5066	21,79 ± 869	30,75 ± 815	20,50 ± 1087		
Average Nitrogen (mg/L)					4479.03 ± 2323.77	
Total Phosphorous (mg/L)						± 300.00
Phosphate (mg/L)						± 18.00

a: Kimuli et al. (2016), b: Fanyin-Martin et al. (2017), c: Englund et al. (2020)

Table 2-3: Elemental concentrations found within faeces (Rose et al., 2015)

Elements	(g/kg)
Total Phosphorous	1.83-9.86
Total Potassium	1.78-7.16
Sodium	0.80-4.94
Calcium	2.68-4.27
Chlorine	0.60
Sulphur	0.60-0.87

Urine also contributes a large fraction of the available carbon (13%) N (14-18%), P (3.7%) and K (3.7%) into the pit contents from individuals (Rose et al., 2015). Some data on urine nutrient input per person are; total P: 1 g/pe/day and total K: 2.73 g/pe/day (Jónsson et al., 2005). The majority of the inorganic components within VIP's appears to come from foreign materials added into the pit, including covering materials such as sand and ash (Zuma, 2016).

Unlike other tested pit parameters (e.g. COD, moisture, etc.), the nutrient concentrations found within sludge did not vary significantly with depth, as found by Kimuli et al. (2016).

2.2.3 Foreign materials present in VIP latrines

Foreign materials that have been found in VIP latrines include; newspapers, magazines, broken glass, glass bottles, rags, stones, rubble, chip packets, vegetable waste, maize cobs, plastic bottles, jars, polystyrene, blankets, hats, underwear, toys, metals, wood, synthetic hair, feminine products, paper, diapers, soil, plant seeds, sponges, bone, wood and other household waste materials (Buckley et al., 2008; Nwaneri, 2009; Bakare et al., 2012; Still and Foxon, 2012; Niwagaba et al., 2014a; Zuma et al., 2015; Cunningham et al., 2016; Zuma, 2016; Septien et al., 2018). Maggots, flies and worms are also abundantly present in VIP's. However, VIP's that had missing back pipes, or slabs that were damaged, or had poor back slabs or missing pedestals, had a significantly higher population of maggots (Buckley et al., 2008; Nwaneri, 2009).

It is estimated that such "trash" makes up 5-10% of the pit volume, and since these materials generally do not decompose, after approximately 10 years, they will constitute 25% of the pit volume (Still and Foxon, 2012; Zuma, 2016).

2.3 Microorganisms in VIP latrines

VIP latrines are designed to accumulate human excreta and anal cleansing materials and serve as a digestion system for accumulating matter that undergoes stabilization through natural biological processes (Buckley et al., 2008; Nakagiri et al., 2017). Biodegradation of faecal sludge in pit latrines heavily depends on mutual and syntrophic interaction of consortium microorganisms to break down complex organic matter into soluble and gaseous monomers (Anukam et al., 2019). Microbial sewage communities represent a combination of inputs from human faecal microbes and enrichment of specific microbes from the environment to form a unique microbial structure (McLellan et al., 2010). Similarly, microbial communities of faecal sludge would likely be composed of microbes from human excreta as they are the major feed into the pit and microbes from the surrounding environment as the pit is dug into the ground.

2.3.1 Microbes of human excreta origin

The human gastrointestinal tract harbours a complex and dynamic population of microorganisms with abundance estimated to range between 10^{13} and 10^{14} CFU/g, of which 90% are assumed anaerobic, and only 10% are either aerobic or facultative anaerobic microorganisms (Thursby and Juge, 2017; Jalili-Firoozinezhad et al., 2019). Bacteria are the most important and the largest components of the microbial community in the gut and are often heterotrophic, hence, they rely on an organic carbon source for energy and biomass synthesis (Stephen and Cummings, 1980; Thursby and Juge, 2017). A number of microbiome studies revealed that the human gut is dominated by bacteria from four phyla, *Proteobacteria* (now Pseudomonadota but will be referred to as the *Proteobacteria* throughout the report), *Firmicutes (*now *Bacillota* but will be referred to as *Firmicutes* throughout the report*)*, *Actinobacteria (*now *Actinomycetota* but will be referred to as *Actinobacteria* throughout the report*),* and *Bacteroidetes (now Bacteroidota* but referred to *Bacteroidetes* as throughout the report), together with *Verrucomicrobi (*now *Verrucomicrobiota* but referred to as *Verrucomicrobi* throughout the report*), Cyanobacteria (*e.g. members of the non-photosynthetic class cand. Melainobacteria*),* and *Fusobacteria (*now *Fusobacteriota* but referred to as *Fusobacteria* throughout the report) making up almost all of the bacterial species found in the human gut (Browne et al., 2017; Tasnim et al., 2017; Rinninella et al., 2019). A study by Oduaran et al. (2020) reported that the gut of a South African representative of urban and rural populations was dominated by bacterial species from phyla of *Firmicutes* (43.2%), *Bacteroidetes* (40.2%), and *Proteobacteria* (12.4%).

When microbes are excreted from the gut, their survival and persistence depend on their ability to adapt and respond to changing environmental conditions such as atmospheric oxygen and temperature. Microorganisms can make up to 30% of the total dry mass of faeces, with some studies reporting even up to 55% of microorganisms in faeces (Stephen and Cummings, 1980; Achour et al., 2007; Rose et al., 2015). It is estimated that about 10^{11} bacteria per gram are present in wet human faeces, and their survival rate is estimated as 87% when exposed to oxygen for only 2 minutes, decreasing to >50% if exposed for 2 hours (Bellali et al., 2019). Once adapted to the new environment, microbial activity can be beneficial as they utilise organic and inorganic substances from the environment as nutrients for energy synthesis and growth, thus playing a major role in many biochemical processes. The presence and the role of microbes from the human gut have been demonstrated in WWTPs, which receive sewage waste from households and institutions (Cai et al., 2014). Studies of the microbiome in WWTPs have demonstrated that the microbial communities during the sludge treatment process are dominated by microbes of the human gut origin (Newton et al., 2015; Shchegolkova et al., 2016). *Proteobacteria* is the most abundant phylum in many WWTPs, accounting for 30-80% of the total microbial community, followed by *Bacteroidetes*, *Firmicutes*, and *Actinobacteria* (Nascimento et al., 2018; de Celis et al., 2020). These communities include taxa involved in different metabolic pathways, some of which include nitrogen fixation, nitrification, denitrification, desulphurication as well as sulphur oxidation (de Celis et al., 2020). Xu et al. (2018) reported prevalent occurrence of bacterial species from phyla *Proteobacteria* (26.7-48.9%), *Bacteroidetes* (19.3-37.3%), *Chloroflexi* (2.9-17.1%), and *Acidobacteria* (1.5-13.8%) in WWTPs in the north of Xinjiang Uygur Autonomous Region of China, while 19 WWTPs in São Paulo State, Brazil, were dominated by bacteria representing the phyla *Proteobacteria*, *Bacteroidetes*, and *Firmicutes* (Nascimento et al., 2018).

Faeces are the major feed in pit latrines, and thus, microorganisms present would play a leading role in the initial formation of microbial communities. Faecal sludge provides a good habitat for many microorganisms, and the prevailing physicochemical conditions within the pit would determine the predominant microbial communities (Appiah-Effah et al., 2015). The microbiota present in pit latrines and their association with faecal sludge decomposition is still not well understood. The diversity and physiology of microorganisms of the human gut were traditionally analysed with culture-dependent methods; such methods are, however, limiting as some microbes are not amenable to culturing and thus do not reflect the actual microbial communities present (Wright et al., 2019). However, recent approaches such as “culturomics” have provided access to microbes that were not detected previously (Chang et al., 2019; Matar and Bilen, 2022; Quaranta et al., 2022). The presence of typical gut bacteria belonging to the order *Enterobacterales* in the phylum *Proteobacteria* (i.e. *Escherichia and Citrobacter*) and phylum *Bacillota* (i.e. *Staphylococcus*) detected in human faeces have been isolated from pit latrine faecal sludge (Beukes et al., 2017, Kumwenda et al., 2017, Beukes and Schmidt, 2018, Capone et al., 2021). Culture-independent methods have revealed a greater diversity than previously recognised and identified. Only a few studies have been conducted to identify the microbiome in a pit latrine. Torondel et al. (2016) reported that the most abundant bacteria were species from the phylum *Firmicutes* with 66% prevalence from selected pit latrines in Tanzania and 37% prevalence from selected pit latrines in Vietnam, followed by phyla *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria* in the order of decreasing abundance, were also detected in pit latrines. This study also demonstrates that diet is a factor in determining the structure of microbial communities and their relative abundance in faecal sludge.

A recent study by Smith et al. (2023) on the microbial communities in pit latrines in peri-urban Malawi, indicated that of their sampled pits, the bacterial population was dominated by fermenters (such as *Clostridium sensu stricto*) at each of their sampled depths and locations. Sugar fermenters, such as *Faecalibacterium*, were found mostly within the surface layers of the pit latrine samples, while the acetogens (*Proteiniphilum*) were detected at every depth, with slightly higher relative abundance within the surface layers of the pits (Smith et al., 2023).

2.3.2 Microbes from environmental origin

Pit latrines consist of a hole dug 1.5 m (usually) into the ground. Therefore, microorganisms that inhabit the soil and surrounding environments would enter the pit through percolation or water runoff and form part of the microbial communities of faecal sludge. Furthermore, some pit latrines are used

for the disposal of household waste and domestic water, which may contain microbes. Soil is a complex environment and is inhabited by diverse microorganisms (Coller et al., 2019), with bacteria being the most abundant (10^8 to 10^9 bacteria per gram) and a diverse group of other microorganisms such as algae, fungi and protozoa present (Köberl et al., 2020). Microorganisms mediate the majority of processes occurring in the soil, including mineralisation of organic matter, transformation of phosphorus, sulphur, and nitrogen (Furtak and Gajda, 2018). Microbiome analysis revealed that agricultural soil of 10-30 cm depth is inhabited by diverse bacterial species from phyla such as *Proteobacteria*, *Acidobacteria*, *Verrucomicrobia*, *Actinobacteria*, *Bacteroidetes*, and *Chloroflexi* (Köberl et al., 2020). Smith et al. (2023), compared the microbial community data from their sampled pit latrines against the literature data of the microbial communities generally found within the human gut. They determined that about half (55%) of the human gut genera were found within the pit latrines, indicating that the remaining 45% is potentially from environmental origin.

2.4 Biological processes occurring in pit latrines

Faeces are the major feed in the pit, thus a major contributor of organic substances. Faecal matter is high in organic substances, composed of 80% biodegradable substances and 20% non-degradable substances (Stephen and Cummings, 1980; Rose et al., 2015). Two major biodegradation processes occur in a pit, i.e. aerobic degradation and anaerobic degradation. A complete degradation results in the conversion of faecal sludge into products that are either soluble, gaseous, or non-biodegradable solids. The soluble and gaseous products are released out of the pit through leaching and ventilation while the stabilised non-biodegradable matter accumulates at the bottom of the pit (Buckley et al., 2008; Nwaneri et al., 2008).

2.4.1 Aerobic biodegradation

Aerobic degradation occurs when faecal sludge comes into contact with oxygen and is limited to the top layer in the pit. As new contents enter the pit, it covers the older sludge, restricting diffusion of oxygen and reducing the rate of degradation (Nwaneri et al., 2008; Bakare et al., 2012). Though it is a rapid process, aerobic degradation is only responsible for about 30% of the reduction of total solid mass. Oxygen-dependent and facultative anaerobic microorganisms utilise nutrients in faecal sludge and oxygen as an electron acceptor to convert organic matter into carbon dioxide and water for energy used to generate new cells (Foxon, 2008; Kliem et al., 2020). The carbon dioxide and water released then exit the pit through leaching and ventilation while the non-biodegradable matter stabilises to the bottom of the pit (Nwaneri et al., 2008).

2.4.2 Anaerobic biodegradation

As the depth of the pit increases, oxygen diffusion is reduced and the COD decreases. Anaerobic degradation is slower than aerobic degradation, however, the former process results in a 70% reduction of the total solid mass and is the dominant decomposition of faecal matter in pit latrines (Foxon, 2008; van Eekert et al., 2019). In the absence of oxygen, organic matter is typically converted by anaerobic microorganisms representing bacteria and archaea into methane (55-70% of the total volume makeup), carbon dioxide (30-45% volume), and trace concentrations of hydrogen, hydrogen sulphide, and water through a series of reactions, i.e. hydrolysis, acidogenesis, acetogenesis and the final step methanogenesis (Forbis-Stokes et al., 2016). The polymers in faeces (proteins, polysaccharides, and lipids) are hydrolysed into soluble organic compounds. The organic compounds are transformed by the process of acidogenesis into propionic acid, butyric acid, carbon dioxide, and hydrogen. Organic acids formed are further catabolised by the process of acetogenesis, forming acetic acid, hydrogen, and water. The final step of the anaerobic process is the formation of methane by the process of methanogenesis, carried out by a specialised group of *Archaea*, the so-called methanogens, converting acetate (acetoclastic) or hydrogen and CO_2 (hydrogenotrophic) into methane (Anukam et al., 2019).

Biological processes occurring in a VIP latrine can be separated into four theoretical zones (Figure 2-3). The top zone (i) is composed of fresh faeces that undergo rapid biodegradation. This zone is in contact with oxygen, and aerobic processes convert readily biodegradable organic components into simple compounds. As new faeces enter the pit, they cover an old layer of faecal sludge, creating a second zone (ii) characterised by a decrease in oxygen diffusion. In this zone, aerobic processes

convert complex organic molecules to simpler molecules via aerobic hydrolysis. The build-up of faecal sludge in the pit creates an anaerobic zone (iii) that lacks oxygen, where anaerobic hydrolysis takes place and complex organic molecules are catabolised. Non-biodegradable components then settle in the fourth zone (iv) at the bottom of the pit; no further stabilisation of faecal sludge occurs within the remaining life of the pit (Buckley et al., 2008).

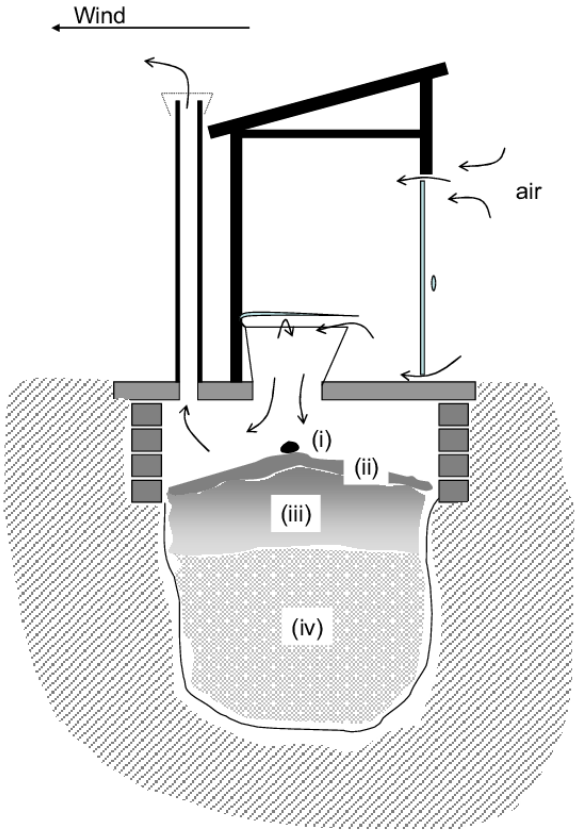

Figure 2-3: Theoretical zones of biological processes occurring in a VIP latrine (Buckley et al., 2008)

2.4.3 Factors affecting microbial degradation of faecal sludge

Microorganisms in faecal sludge are likely derived from human faeces, the environment (soil, groundwater), and other waste added to the pit. When microbes leave the gut, their survival and persistence depend on their ability to respond and adapt to changing environments. The human gut is composed of various microbial communities that may have a different response to changing conditions (Bellali et al., 2019). Environmental factors such as temperature, pH, oxygen availability, and moisture content are a major influence on the dynamics of microbial communities, which subsequently influence the degradative capacity of faecal sludge in pit latrines.

2.4.3.1 Temperature

Temperature is one of the major factors influencing microbial growth and biological reactions by affecting enzyme-catalysed reactions and substrate diffusion into cells. Most microorganisms, particularly those of faecal origin, exhibit a narrow temperature range over which they can be active (Wang et al., 2019). Little is known about the effect of temperature on the biodegradation of faecal sludge in pit latrines. The temperature within the pit is influenced by the air temperature outside the pit latrine structure. Nakagiri et al. (2017) reported temperatures in a pit ranging from 21 to 30.7°C in urban slums of Kampala, Uganda, while Nabateesa et al. (2017) reported temperatures ranging from

22.3 to 30°C in unlined pit latrines in Kampala slums. A temperature of up to 33°C has been reported for pit latrines in Ifakara, Tanzania, (Irish et al., 2013). Thus, mesophilic conditions are most likely to predominate in the pit. Mesophilic organisms operate at a temperature range between 10 and 45°C. At temperatures below 10°C, enzymatic reaction and growth rates are slow, while higher temperatures (> 45°C) may deactivate mesophilic organisms (Lopez Zavala et al., 2004). A dramatic change in temperature may cause a severe disturbance of microbial processes, even shifting microbial community dynamics, and the system may require a long period to adapt to a stable state.

2.4.3.2 pH

Generally, the pH of pit contents is influenced by users' diet and varies from pit to pit. pH in faecal sludge normally ranges from 6.5 to 8.0 (Zuma et al., 2015), while Irish et al. (2013) reported pH values ranging from 5.2 to 8.2 for pit latrines in the town of Ifakara, Tanzania. Nakagiri et al. (2017) reported a pH range of 5.0 to 11.8 in pit latrines of urban slums of Kampala, Uganda. The pH at the top surface was found to differ from the pH in the lower layer of faecal sludge in the pit, which can be attributed to different stages of biodegradation of faecal sludge (Couderc et al., 2008). Nabateesa et al. (2017) reported a neutral pH (7.1) of the sludge at the top surface, which became more alkaline with the decrease in depth (pH 9). pH is influenced by the hydraulic flow of water, increased organic loads, and the addition of toxic substances. Stability of pH in faecal sludge is crucial as pH outside the normal range indicates an upset in the biological process and can inhibit anaerobic digestion. For example, pH values below 6.0 are known to inhibit methanogenesis and can even inactivate methanogens (Ingallinella et al., 2002).

2.4.3.3 Dissolved oxygen

Oxygen is only important for the aerobic process, which is limited to the top layer of faecal sludge in the pit. Dissolved oxygen decreases with an increase in pit depth and is influenced by the runoff water entering the pit. During a rainy season, dissolved oxygen ranged between 0.96 and 1.72 mg/L in a lined pit and between 0.97 and 1.32 mg/L in an unlined pit from Kampala city slum in Uganda. During a dry season, dissolved oxygen was lower than in a rainy season, with an average oxygen concentration of 0.66 mg/ L for lined pit and 0.58 mg/L for unlined pit (Kimuli et al., 2016). Fresh faeces entering the pit create a layer covering the old faecal sludge, thus creating anaerobic conditions. Anaerobic biodegradation is the predominant process of faecal digestion, typically resulting in a 70% reduction in total solid mass compared to the aerobic process, which only reduces 30% of the total solid mass (Buckley et al., 2008). Increased oxygen levels in faecal sludge can inhibit the activity of strictly anaerobic microorganisms that are effectively reducing the solid mass within the pit.

2.4.3.4 Moisture content

Moisture is an absolute requirement for microbial growth and activity. The majority of microorganisms require moisture contents of $\geq$ 60%, though some have been reported to survive and multiply at about 20% moisture content. Bakare et al. (2012) stated that a moisture content above 50% provides a suitable environment for microbial activity. Nabateesa et al. (2017) reported moisture content ranging from 60 to 90% in pit latrines in Kampala slums in Uganda, while Irish et al. (2013) reported an average moisture content of 70% in pit latrines in the town of Ifakara, Tanzania. Finally, Zziwa et al. (2016) observed that moisture content increased with the increase in pit depth and reported a moisture content ranging from 64 to 99% in Kampala Slums, Uganda. Pit latrines located in areas near high water tables are characterised by high moisture content. Moreover, disposal of domestic water and greywater in the pit increases moisture. Accumulation rates were reported to be lower in wet pits than in dry pit due to high rate of anaerobic degradation. The optimal moisture content for methane production is 90%, anaerobic degradation does not occur at low moisture content and the rate of hydrolysis decreases with a decrease in moisture content (Couderc et al., 2008). Aerobic degradation under mesophilic conditions is optimal at 60% moisture content, after which it decreases with an increase in moisture content due to a low supply of oxygen. The availability of water is important for stabilising the intracellular water potential, enabling the activity of hydrolases that use water as co-substrate, as solvent for substrates, microbial mobility, and oxygen diffusion. Low moisture content can make an environment less hospitable for microbial growth, thus influencing microbial community structure and dynamics (Stark and Firestone, 1995; Stres et al., 2008).

2.4.4 Pit sludge turnover / catabolism

One of the earliest guides by Franceys et al. (1992) on the biological process in pits, indicated that excreta undergo immediate decomposition when deposited. Upon deposition, complex organic compounds (protein, urea, etc.) would be broken down into simpler forms and gasses produced (primarily CO_2, methane, ammonia and N) that would mix with the atmosphere.

SANDEC (1997) within the tested parameters (BOD, TS, TKN and volume), found that the composition of pit contents was lower than that of fresh faecal matter. Zuma (2016) also reported average COD values of fresh faeces to be higher than those in the pit, with Chaggu (2004) and Nwaneri et al. (2008) showing similar patterns. Nwaneri et al. (2008) reports COD values of the surface layer to be 539 mg COD/g dry sample, while for fresh faeces this was 1130 mg COD/g dry sample. This means that almost half of the biodegradable COD is already utilised within the surface layer (Nwaneri et al., 2008). This supports Franceys et al. (1992) earlier indications and the theory described by Buckley et al. (2008).

The biological decomposition of latrine sludge has been theorised to be anaerobic within the deeper layers of the pit and aerobic closer to the surface layers (Buckley et al., 2008; Nwaneri et al., 2008). Model results by Zavala et al. (2004) show that of the 80% biodegradable fraction of faeces, 15% of this is easily hydrolysed, while the remainder is slowly hydrolysed. This slowly hydrolysable portion must first be broken down by means of extra-cellular hydrolytic enzymes (Zavala et al., 2004), before being utilised by microorganisms (MO). However, the degree of biodegradation depends on the age of the faecal sludge (Nwaneri et al., 2008; Nwaneri, 2009) with fresher surface material being more easily biodegradable. This external breakdown process is performed by MO and termed hydrolysis, in which complex organic material is converted into soluble substrates by the action of extra-cellular secreted enzymes (Batstone et al., 2002). The products of hydrolysis are commonly amino acids, sugars, long chain fatty acids and glycerol These products are further broken down anaerobically by the process of acidogenesis followed by acetogenesis and finally methanogenesis, by different classes of MO (Anderson et al., 2003).

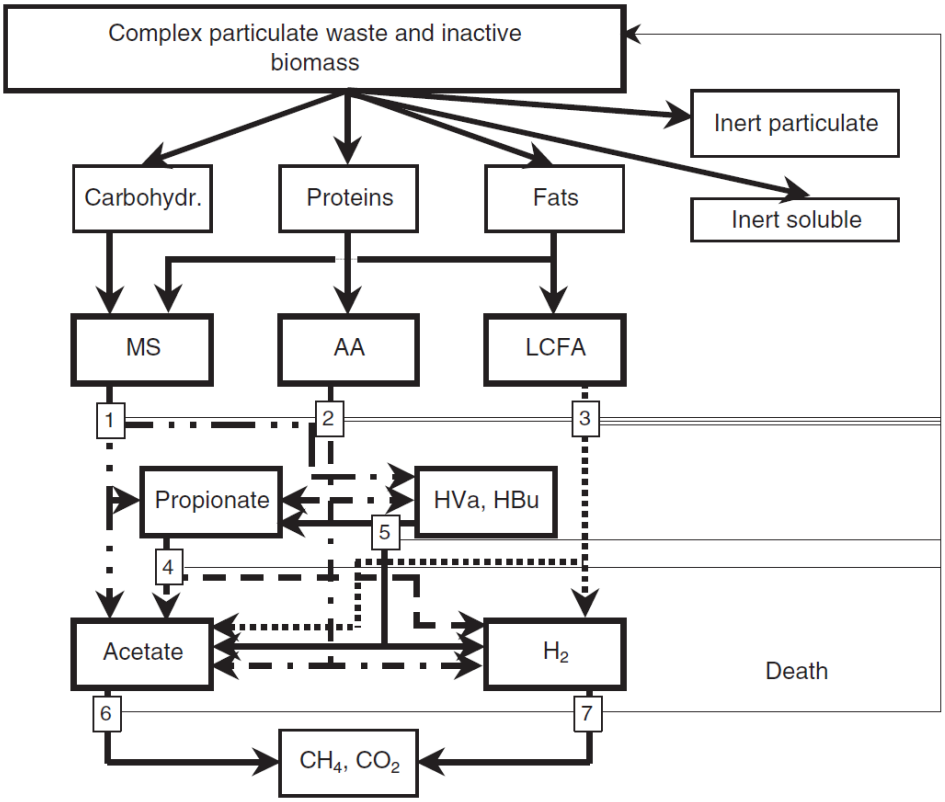

Figure 2-4 shows the general anaerobic model including the biochemical processes.

Over time, the biodegradable organic material will eventually become a stable material, composed almost entirely of inorganic products, suitable for agricultural use with no unpleasant odour (Franceys et al., 1992; Still and Foxon, 2012). In some instances, the nutrients that can be derived from sludge are considered economically feasible to recycle (Kimuli et al., 2016). Still and Foxon (2012) calculated that for a set mass under aerobic conditions, 27% of the original mass would remain as un-biodegradable with the remaining 73% converted into CO_2, but if the same mass underwent anaerobic digestion, only 21% of the initial mass would remain, with the remaining 79% of its decomposed mass converted into methane.

2.4.5 Abiotic and biotic processes occurring

The biotic processes in a pit are both aerobic and anaerobic digestion which occur simultaneously, at different locations and depths (Zuma, 2016; Byrne et al., 2017). Anaerobic digestion is however, the predominant process occurring. In aerobic digestion, MO consume biodegradable material in the presence of oxygen for their own energy requirements to grow, reproduce, expel CO_2 and water as a waste product. In anaerobic digestion, MO decompose (convert high molecular weight polymers into low molecular weight compounds) biodegradable material via hydrolysis, etc. in the absence of oxygen, also for their own energy requirements, but this process is much slower (Still and Foxon, 2012). The general waste products for anaerobic digestion are CO_2, water and methane gas (Batstone et al., 2002). A graphic representation of the conversion ratios is presented in Figure 2-5.

Insects and other invertebrates are known to inhabit, breed and feed on defecated material. While these are not considered beneficial to the degradation of pit sludge, they do digest pit material aiding in some reduction and stabilisation. Their movements in the surface layer aids in the supply of oxygen into a deeper layer of the sludge than would otherwise occur in their absence (Buckley et al., 2008).

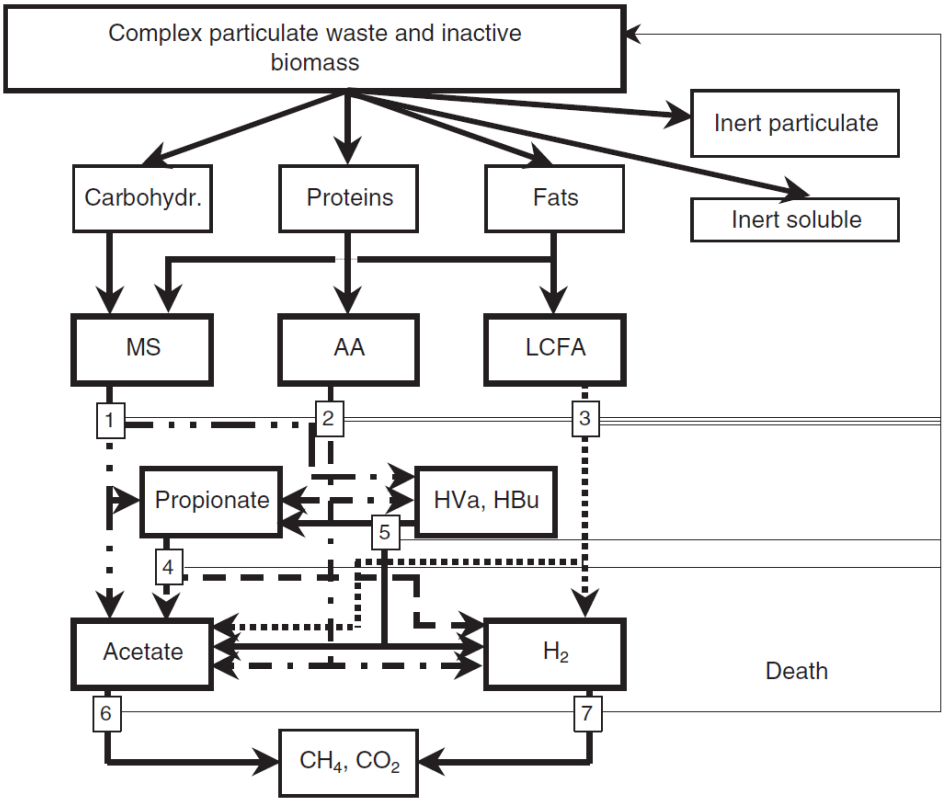

Figure 2-4: The anaerobic model as implemented including biochemical processes: (1) acidogenesis from sugars, (2) acidogenesis from amino acids, (3) acetogenesis from LCFA, (4) acetogenesis from propionate, (5) acetogenesis from butyrate and valerate, (6) aceticlastic methanogenesis, and (7) hydrogenotrophic methanogenesis (Batstone et al., 2002)

For many pits, the main abiotic process occurring is dehydration. Factors affecting dehydration in pits include temperature, moisture content, oxygen, humidity, surrounding soil characteristics, pit dimensions and storage time (Still and Foxon, 2012; Zuma, 2016). While some drainage in VIP latrines is preferred (Still and Foxon, 2012), too much drainage, or high temperatures or wind can result in a dehydrated chamber and limit biological activity. In low-income countries, fibre intake by the population is usually higher which results in greater (2 times) faecal wet mass value compared to higher income countries, allowing for better hydrated pits (Niwagaba et al., 2014a; Rose et al., 2015). This is however meaningless if the moisture subtraction rate is greater than the addition rate.

Compaction by new material being added on top, and the degradation and density increase of older sludge, can compact the pit material lower down. This may cause moisture loss from squeezing and therefore loss of moisture from the sludge (Buckley et al., 2008).

The pH range of many MO is very narrow and moving outside of this range either results in decreased functionality or cell death. The average pH range of biological activity seems to be neutral, however a range between pH 6.5-7.8 is believed to be optimal for anaerobic degradation (Anderson et al., 2003). In some cases where ash is added as a covering agent, the pH of the sludge was above 9, which makes for an unfavourable environment for biological degradation (Chaggu, 2004; Niwagaba et al., 2014a). Zuma et al. (2015) reported a pH of 8 for non-functional urine diversion pits and a pH range of 4.7-8.6 for faecal sludge with very little difference in pH within the space of the pit, however, the pH of individual pits varied. Fanyin-Martin et al. (2017) reported an average pH of 7.77 ± 0.13 for Ghana while Appiah-Effah et al. (2020) pH was 7.3 in similar rural areas. Englund et al. (2020) reported an average pH of ± 7.6 across 60 samples in Hanoi, Vietnam. Fresh faeces have an average pH of 6.64 and a range of 5.3-7.5, while urine has an average pH of 6.2 (Rose et al., 2015).

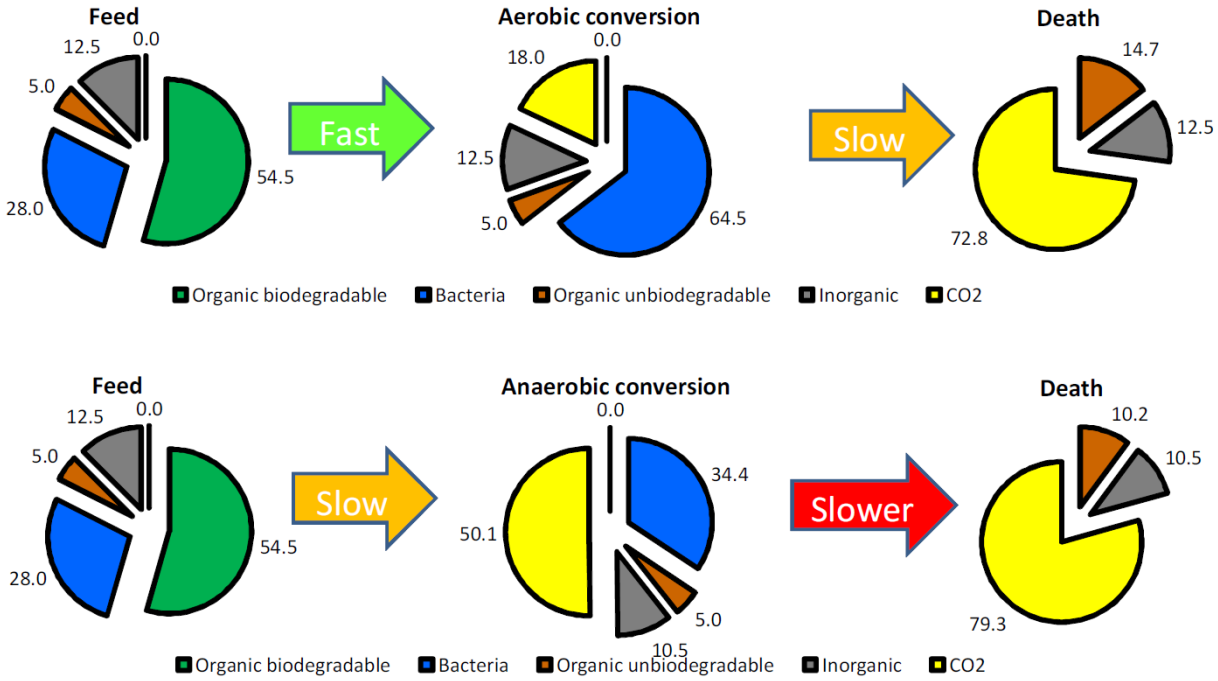

Figure 2-5: Aerobic vs. anaerobic conversion (Still and Foxon, 2012)

2.4.6 Possible factors limiting biodegradation

Dehydration from open pits / damaged pits / lack of added liquids / soil composition results in decreased moisture levels which is a primary factor in biological degradation limitation. Moisture availability is a limiting factor to the rate and extent of biological transformation within pit latrines (Buckley et al., 2008; Zuma, 2016). Contrary to this, over hydration from flooding or excessive water addition may seriously dilute the sludge and allow for the majority of the MO, and useable soluble

compounds, to be removed via the porous sediment (Buckley et al., 2008). The only study found on the effect of moisture content on anaerobic digestion in latrine pit sludge was by Couderc et al. (2008) where the addition of moisture showed some correlative data between moisture content and gas production rate. Singh et al. (2017) does relate, that a high moisture content (90%) in sludge is known to increase methane production. A similar study performed on sludge cake from a wastewater treatment plant, showed that methanogenic activity dropped from 100% to 53% after the moisture content was reduced from 96% to 90% (Lay et al., 1997). A similar relationship can be expected from pit sludge. The average faecal moisture content is 74.6% (Rose et al., 2015) with a sludge range of 70-98% (Septien et al., 2020), so any deviation from this is a good indication of pit over-hydration or dehydration.

Microorganisms themselves can alter the pH in their surroundings from their by-products (Anderson et al., 2003). Anaerobic respiration results in products that have the capability to acidify the surrounding media from the production of organic acids / volatile fatty acids or the accumulation of dissolved hydrogen gas (Chaggu, 2004; Buckley et al., 2008). Veeken et al. (2000) show that at a pH of 5-5.5, the production of fermentation products was slower, than the total soluble COD produced. This effect increased with lower pH values, suggesting that fermentation is inhibited under acidic conditions. Couderc et al. (2008) attempted to increase the alkalinity in collected latrine sludge, but data indicated no increase in gas production with increased alkalinity. A study presented in Buckley et al. (2008) showed that the addition of an alkaline solution to sludge resulted in stunted gas production for the initial 20 days (compared to the control). This was followed by similar gas production rates as the controls and was thought that the MO had overcome the inhibitory conditions during this duration. Other factors such as redox condition and initial pH have been shown to increase solids hydrolysis by aiding in substrate solubility making it easier for biological conversion (Grolle et al., 2018).

Heavy metals are toxic to MO at specific concentrations and have the potential to cause serious negative effects on biological activity and efficiency. The effects of heavy metals on wastewater treatment plant sludge was reported by Bhat et al. (2020), who tested metal concentrations of Cu between 1-100 mg/L, and Cd and Pb between 0.1-10 mg/L. Data showed that heavy metal toxicity was related to decreased bacterial activity, community composition and COD which decreased from 87% to 26% under different metal concentrations (Bhat et al., 2020). Chua et al. (1999) however reported that in their wastewater research the decreased COD removal efficiency at sub-lethal heavy metal concentrations was due to the metal ions acting as competition on the active sites on the organic compounds, rather than acting as a toxic microbial inhibitor. A similar finding was also reported by Sin et al. (2000). It must also be noted that anaerobic digestion is known to release heavy metals from the substrate matrix (Anjum et al., 2017).

Table 2-4 presents some heavy metal concentration found in urine and faeces from global rural communities. Heavy metals LC50 established by El Bestawy et al. (2013) on activated sludge showed that copper possessed the highest toxicity towards oxygen uptake rate and COD removal (Cu>Cd>Cr>Co) when testing the effects of industrial effluent in sewerage water.

Table 2-4: Estimated heavy metal concentrations found in urine and faeces

	Urine (mg/pe/day)[a,b]	Faeces (mg/pe/day)[a,b]	Faeces (mg/kg)[c]
Lead	0.0020	0,02-1,26	28-59
Cadmium	0.0068	0,07-1,23	1,8-2
Mercury	0.0082	0,007-0.009	0,8-0,9
Copper	0.10	1.00-2,1	114-216
Chromium	0.010	0,02-0,18	401-485

Nickel	0.0071	0,08-0.3	24-30
Zinc	0.045	5-13,31	646-918
Magnesium		180-1120	
Iron		30-1000	
Arsenic			0,6-2,8

a: Jónsson et al. (2005), b: Rose et al. (2015), c: Cunningham et al. (2016)

As for pH, the optimal temperature range of most MO is narrow and mainly affects anaerobic digestion (Speece, 1996). This occurs by (i) the pH of a system is affected by temperature fluctuations, and (ii) temperature effects the biological activity of all MO (Nwaneri, 2009). Depending on the temperature, different groups of MO will be active (Speece, 1996). Kimuli et al. (2016) reported internal temperature variations for lined pits of 24.2 ± 0.7°C and 26.2 ± 0.7°C, and unlined pits of 24.1 ± 0.7°C and 25.4 ± 0.7°C during the rainy and dry season respectively, with an average temperature decrease between the surface and bottom of the sludge (1.5 m) of approximately 2°C, irrespective of pit type or season. Henze et al. (1997) stated that for every 1°C drop in temperature below 30°C, the conversion rate of anaerobic digestion decreases by 11%. It has been said that pit latrines can operate at temperatures between 0°C and 30°C (Nwaneri, 2009), although the temperature ranges for pit latrines in South Africa have been reported between 15°C and 30°C ((Foxon et al., 2006).

Oils and grease have been mentioned as a possible factor in hampering microbial degradation (Niwagaba et al., 2014a). Although there are several natural oils (meats, seeds, nuts, etc.), it is the synthetic oils (kerosene, lubricating oils, etc.) which can pose a problem due gas diffusion limitation (Niwagaba et al., 2014a).

2.5 Increasing VIP latrine biodegradability

2.5.1 Status of knowledge on VIP latrine biodegradability

There have been limited studies reported on the decomposition rate within VIP's with circumstantial evidence into the possible factors that can speed up this process (Buckley et al., 2008; Torondel et al., 2016). Past studies include Couderc et al. (2008) who investigated the effectiveness of increasing the moisture and/or alkalinity factors on the rate of anaerobic digestion. The authors found that the additional alkalinity showed no effect, while there was some correlative data between moisture content and gas production and biological stability. A study by Veeken et al. (2000) showed that slightly acidic conditions are optimal for hydrolysis, but negatively impact fermentation, with findings that pH is the primary variable controlling the hydrolysis rate of anaerobic digestion in solid state waste. However, the authors do say that the relationship depends on the composition of the waste, which as reported above, changes.

Grolle et al. (2018) also investigated the effects of pH, temperature and moisture and determined that both temperature and moisture content had little impact on the hydrolysis of solids but concluded that extended aeration and increased pH (9) had the potential to enhance faecal solids breakdown. The authors discuss that the effect of increased pH:

> "…tends to solubilise substrate by increasing the solubility of proteins and ionising VFA's, making it easier to convert biologically as long as the biomass is active at the given pH." (Grolle et al., 2018)

Other researchers concluded that adequate moisture assists in the solubility of compounds and allows for the relative movement of compounds through stationary solid products (Martin et al., 2003). Faecal crust formation would certainly be slowed dramatically in a moist environment, a parameter that would aid liquid additives, and prevent them from running off the faecal sludge (Grolle et al.,

2018). Nwaneri et al. (2008) reported on the conceptual theory of biological degradation processes occurring, but not on speeds or rates.

While the aerobic degradation of faecal sludge material is fast, the conversion of organic matter into cellular biomass is much higher (1 g COD sludge = 0.50-0.70 g COD bacterial biomass). This results in a much faster reduction of sludge volume, but increased volumes of bacterial biomass. Contrary to this, anaerobic digestion is much slower and results in a much lower biomass conversion (1 g COD sludge = 0.05-0.10 g COD bacterial biomass) (Still and Foxon, 2012). In essence, to speed up the degradation process to stability, the pit would require an aerobic environment, but to reduce the volume of the pit, an anaerobic environment would be required (Still and Foxon, 2012). This is usually why for wet pit latrines, the contents accumulate much slower compared to dry pit latrines (Still and Foxon, 2012). In older compacted pits, moisture addition does very little to improve degradation (Couderc et al., 2008), however moisture addition to fresher VIP material has the potential to increase the degradation rate (Nwaneri, 2009), while increased leachate recirculation rates enhance aerobic degradation, as shown in bio-waste batch reactors (Veeken and Hamelers, 2000).

Bioavailability throughout most of the pit sludge does not seem to be a limiting factor. The majority of the easily biodegradable matter at the surface is consumed by aerobic processes which leaves the more recalcitrant material to be covered with fresh excreta, and become anaerobic (Buckley et al., 2008). Organic products are still constantly broken down and utilised by MO, their neighbours and microbial waste products are an energy source for other microbe species. Although organic materials are available, the process of degradation to make it biologically usable, is much slower once the easily degradable fraction is used up. Bioavailability only becomes an issue in the final phases of the pit life span where nearly all the organic fractions have been used and what remains is foreign artefacts, highly recalcitrant materials and inorganic matter (Franceys et al., 1992; Still and Foxon, 2012).

Faecal crust formation as reported by Grolle et al. (2018) could be a hindrance to additive addition, as additives require contact with the substrate to act. During their experiments, Grolle et al. (2018) reported faecal crust formation within 3-5 hours. Crust formation persisted even after being covered in water for 15 mins (47% crust reduction softness) and with fresh faecal matter for 4.75 hrs (8.5% crust reduction softness). The chances of urine or liquids remaining on faecal matter for these periods is slim, the higher probability is the liquids running off or seeping into faecal cracks (Grolle et al., 2018).

2.5.2 Pit Additives

Commercial (bio) additives which are often based on enzymes, bacteria or nutrients have in the past claimed to increase pit decomposition rates, reduce odour and flies and extend the pit life span (Buckley et al., 2008). In South Africa, there are dozens of marketed products that claim to either enhance degradation or prevent the filling of pits (Still and Foxon, 2012).

One of the earliest studies found was Jere et al. (1998) who used spore forming non-pathogenic bacteria on latrine sludge in Harare, Zimbabwe. The authors dosed 4 pit latrines weekly with 300 g each of the additive, for 4 weeks and found that the treatment reduced the height of the pit contents significantly. However, no control was performed to enable a comparison, no graphical data is available and no unit data (height / volume) was mentioned in text.

Studies carried out by Taljaard et al. (2003) and by (Foxon et al., 2009) assessed the ability of microbes or their derived products to treat pit latrine contents. However, the dosages of the additives used in the laboratory were in far excess for normal practise, and when field testing two of the products that proved effective in removing COD and TSS, some reduction was seen, but only as much as 22 cm over a three-month period (Taljaard et al., 2003). This was followed by larger field testing by Taljaard et al. (2005) who tested three pit additives. At the end of the three-month test period, the most effective additive resulted in a 160 mm drop of the solid waste, compared to 60 mm with another additive brand, while the third additive showed no drop. Some of the observations made included that the better performing additive showed signs of liquefaction in the pit and that the bulk of the solids were looser and softer, but some caveats of the study included; (i) faecal samples were collected weeks apart and (ii) five-day delay in testing collected samples (Taljaard et al., 2005) cited in Bindoff (2008).

In 2006 an experiment was conducted in Naivasha, Kenya, using five bio-additives (two chemical (Ikati and Soda), two biological (Ecotreat and Sannitree) and wood ash) on fresh pig faeces (Zingoda, 2016). The results concluded that there was no evidence that any bio-additives either enhanced or inhibited anaerobic degradation.

Bindoff (2008) attempted to test the efficacy of additive "M", which was presented as a representative group of additives composed of aerobic microbes and enzymes. Results concluded that there was insufficient precision and reliability in the data to make any conclusive decision as to whether the additive was effective or not.

In 2007, the South African Water Research Commission tested a single commercially available pit additive on nine VIP latrines and no reduction in sludge was observed (Buckley et al., 2008). In 2009, additional laboratory tests were conducted using nine more additive products. None of the anaerobic tests showed any statistically significant rate of mass loss from the sludge. There was some mass loss from aerobic tests, but no significant difference between any of the different treatments (Foxon et al., 2009). However, data analysis revealed that the aerobic mass loss was due to a combination of dehydration through evaporation and biological stabilisation processes and not from the addition of the pit additive product (Foxon et al., 2009).

Additional experimentation continued in 2009/2010 testing the efficacy of two different pit additives. Over the 6-month duration, the results concluded that while there was a significant variation in mass loss rate in the experiments, there was no significant difference in the mass loss rate within each of the treatments. Even the addition of only water gave similar results to using pit additives (Still and Foxon, 2012). In 2009, the results from an experiment to test a new pit additive at the request of the manufacturer not only showed no reduction, but rather an increased rate in sludge accumulation (Still and Foxon, 2012).

A study in 2016 performed in Ghana by Awere and Edu-Buandoh (2016) tested two commercial additives (acidic disinfectant and septonic) and household ash under laboratory conditions for 30 days. Results showed that all additives showed a sludge mass loss ranging from 2.6% to 7.7% of their original mass. The authors continue to report that although there was mass loss for each additive, only additive B (acidic disinfectant) had any significant effect on mass loss, but this was not conclusively tested (Awere and Edu-Buandoh, 2016). All tested additives did reduce flies and odour.

Experimentation continued in 2010 where Bakare et al. (2010) performed field and laboratory trials on two different bio-additives. Additive 1 was described as a concentrated bacterial powder (5 billion CFU/g, yeast like, pH range 5.5-10.5) with multiple uses, and Additive 2 was described as being able to eliminate odours, remove flies, stop the spread of disease, reduce solids level and aid in composting. In both cases, the additives proved ineffectual in reducing either the accumulation rate or the mass loss rate of VIP sludge compared against a water only control (Bakare et al., 2010).

The most numerous additive study found was performed by Grolle et al. (2018) who tested 47 different pit latrine additives in small scale batch tests on both black water and faecal sludge. The types of additives used for batch test screening for faecal solids hydrolysis were:

> "…two soils (including soil bacteria); three inorganic conditioners; fifteen pure strain bacteria species spores; three bacterial spore mixes; one fungus spore mix; four commercial bacterial spores products; two live bacteria consortia; six enzyme concentrates; plus one mix and ten faeces extracts of herbivores." (Grolle et al., 2018)

Grolle et al. (2018) name the additives used, except for the commercial ones, in table format (see Table 3, (pg. 217) cited in Grolle et al. (2018). However, of all the tested additives, the only positive COD solubilisation percentage was from *Rhodococcus pyridinivorans* (COD solubilisation +5%) after 21 days under aerobic conditions, and with llama (COD solubilisation +4%) and ring tail lemur (COD solubilisation +2%) faeces extracts under aerobic-anaerobic conditions. None of the remaining additives either enhanced or hindered COD hydrolysis. The authors also tested a simulated pit latrine over a period of 4 months using three bacterial mixes and a commercial bacterial product (Table 2, pg. 210 in (Grolle et al., 2018)). Results from this showed that the commercial product slightly enhanced COD removal but did not reduce pit height. The remaining three bacterial mixes had no effect on COD or pit height.

The most recently found study on pit additives, tested the effects of two “mostly improvised chemical additives”, calcium carbide and lambda super 2.5 EC (Appiah-Effah et al., 2020). After 30 days, data of the control vs. the calcium carbide and lambda super showed a reduction of BOD of 30%, to 47.4% and 40.6%, an increase in COD of 34.7%, to 47.3% and 47.9% and a slight increase in sludge mass from 55%, to 61% and 58% reduction respectively. This experiment was performed under fully aerobic conditions with surfaces exposed to the ambient atmosphere, which may account for the moisture loss range of 39-74%, and with faecal material that comprised mostly of slowly biodegradable material.

With some additives, the product claims to add aerobic bacteria or nutrients into the pit, resulting in increased aerobic metabolic conditions, however oxygen is the limiting factor that determines aerobic respiration and this cannot be manipulated by known/current pit additives (Still and Foxon, 2012).

Of important mention by Buckley et al. (2008), was that the high concentration of naturally occurring degrading MO in latrines was of a similar order of magnitude to that of added biological additives, but that the overall number of naturally occurring MO was far greater than those added via the additive. Thus, the impact of the additive was negligible given the already high degree of degradation occurring at the surface layer. In addition, pit latrine additive studies need to be carefully designed to separate the natural biological activity vs. the treatment being added.

2.5.3 Laboratory scale methodologies

2.5.3.1 Collection and storage

Appiah-Effah et al. (2020) collected samples of faecal sludge from a depth of 1 m beneath the pedestal of the pits after peak hour usage using a 5 point arbitrary sampling method to collect randomly distributed sludge samples. The samples were then homogenised and stored in pre-rinsed (distilled water), air tight sterile plastic containers then transported and stored at 4°C (Appiah-Effah et al., 2020). Other researchers collected sludge samples from the surface of the faecal sludge, directly underneath the pedestal (Foxon et al., 2009; Awere and Edu-Buandoh, 2016), while Bindoff (2008) collected from only the upper 150 mm of the pit sludge and stored theirs at 10°C. Awere and Edu-Buandoh (2016) also randomly collected samples from five different locations within the same pit and homogenised the samples to obtain a composite sample. They also wrapped their samples in black polyethylene bags before transport to imitate the dark pit environment (limit any light induced bio-activity) and transported and stored their samples at 4°C. All samples were collected in the morning after use of the facilities.

Buckley et al. (2008) mention that it has been clearly shown that pit latrine contents vary greatly in their composition, and thus different pit latrine samples can be expected to give different results. It is therefore not possible to homogenise the material to an extent that sub-samples would give identical results in any testing protocol. Data variation in experimental data is expected from different pits or collections, if not well homogenised, and should always be factored into the final analysis. Buckley et al. (2008) also say that since pit latrine additive products only make contact with the surface material of the VIP pit sludge, samples taken for laboratory testing should only be taken from this uppermost layer. That said, the location of sample collection should relate to the type of experiments being conducted or additives being tested.

2.5.3.2 Experimental protocols

Foxon et al. (2009) describe useful protocols for the laboratory testing of additives. Randomly collected samples of VIP sludge, from the same depth, are homogenised and divided equally by weight (300 g) into individual 300 ml screw top honey jars (Figure 2-6). The additive of choice is then added and a unified terminology defined (Foxon et al., 2009). Trials consist of multiple treatments, including a control and reference treatments, while treatments consist of three or five units, each being identical, with a unit being a single honey jar (Buckley et al., 2008; Foxon et al., 2009). The two reference treatments used are: i) no water and no additive (control) and ii) the addition of water only (water reference) (Foxon et al., 2009; Bakare et al., 2010; Still and Foxon, 2012; Awere and Edu-Buandoh, 2016; Zingoda, 2016). Buckley et al. (2008) did use a third reference treatment, that of an alkaline solution.

If the experiment is aerobic, the units must be left open and unhindered to any air movement (Figure 2-6), while for anaerobic experiments the units should be closed, limiting atmospheric interference (Buckley et al., 2008; Foxon et al., 2009) or held in an anaerobic chamber. For aerobic experiments the additives are added to the top layer of the sample (Awere and Edu-Buandoh, 2016). Grolle et al. (2018) however, mixed their additives into the substrate before adding this mixture into their testing bottles to assess the overall effect of solids hydrolysis of their additive.

Figure 2-6: Laboratory trials of pit additive using honey jars under aerobic conditions (Foxon et al., 2009)

This general honey jar experimental setup has been used by Bakare et al. (2010), Still and Foxon (2012) and Appiah-Effah et al. (2020). Awere and Edu-Buandoh (2016) followed the protocol devised by Buckley et al. (2008), which is similarly described by Foxon et al. (2009) as the same honey jar methodology described above.

The units or containers of choice are incubated for a set period of time at approximate constant temperature in a humidity controlled (usually) fume cupboard to limit the rate of evaporation. This is done so that any mass change would be mostly due to biological activity, and not evaporation (Buckley et al., 2008; Bakare et al., 2010). Some options for this process include covering the containers with open ended plastic boxes to reduce evaporation from the forced ventilation by the fume hood extractor fans, and/or to saturate the air supply to the fume hood by running the air supply through water (Buckley et al., 2008). An ambient incubation temperature of 22°C has been used (Awere and Edu-Buandoh, 2016), while others have used a much higher temperature of 30°C (Grolle et al., 2018). It is also important to remember to allow cold stored samples to reach room temperature before any tests are conducted (Awere and Edu-Buandoh, 2016).

Mass change is monitored the most frequently with the containers weighed while empty, immediately after filling and again every 3 d for between 27 d and 46 d (Foxon et al., 2009; Awere and Edu-Buandoh, 2016). Laboratory scale lab experiments are generally run for 30 d (WRC-Report1745 ; Buckley et al., 2008; Bakare et al., 2010; Still and Foxon, 2012; Awere and Edu-Buandoh, 2016; Appiah-Effah et al., 2020) while some do run longer, up to 6 weeks (Bindoff, 2008). Twelve weeks have been reported in Taljaard et al. (2005) cited in Bindoff (2008). Some larger scale experiments have run up to four months, where additives and faecal matter were added on a monthly basis (Grolle et al., 2018).

Other researchers have used more intricate design experiments to test the effectiveness of additives (Figure 2-7). Here Bindoff (2008) cleared as much non-biodegradable material as possible and then added 500-900 g of sludge to each vessel and flattened the sludge with a glass beaker. Experimentation continued with varied water reference treatments and additive use. Jere et al. (1998), while not a very intricate system, directly injected their tested additive into the pit sludge (in the field) by use of a pressurised perforated tube, while Couderc et al. (2008) used an adapted serum bottle test and also measured gas production via a glass syringe lubricated with distilled water. Grolle et al. (2018) devised a small-scale simulated pit latrine by cutting up a two-litre water bottle (Figure 2-8). Sugden (2006) in Bakare (2011) used a bucket system to test the effectiveness of additives on

pig faeces. Here, holes were drilled at the bottom and sides of 25 L buckets and 3 L of alpine grit added. Each bucket was then put into a 60 L bucket which was covered with a lid. Pig faecal matter was added on top of the alpine grit to which the different additives were added (Sugden, 2006) in (Bakare, 2011). Zingoda (2016) simply used large 10 L plastic buckets, instead of smaller honey jars for their experiments using faecal sludge, they however, stirred their samples manually for two minutes prior to taking samples for sludge characterisation.

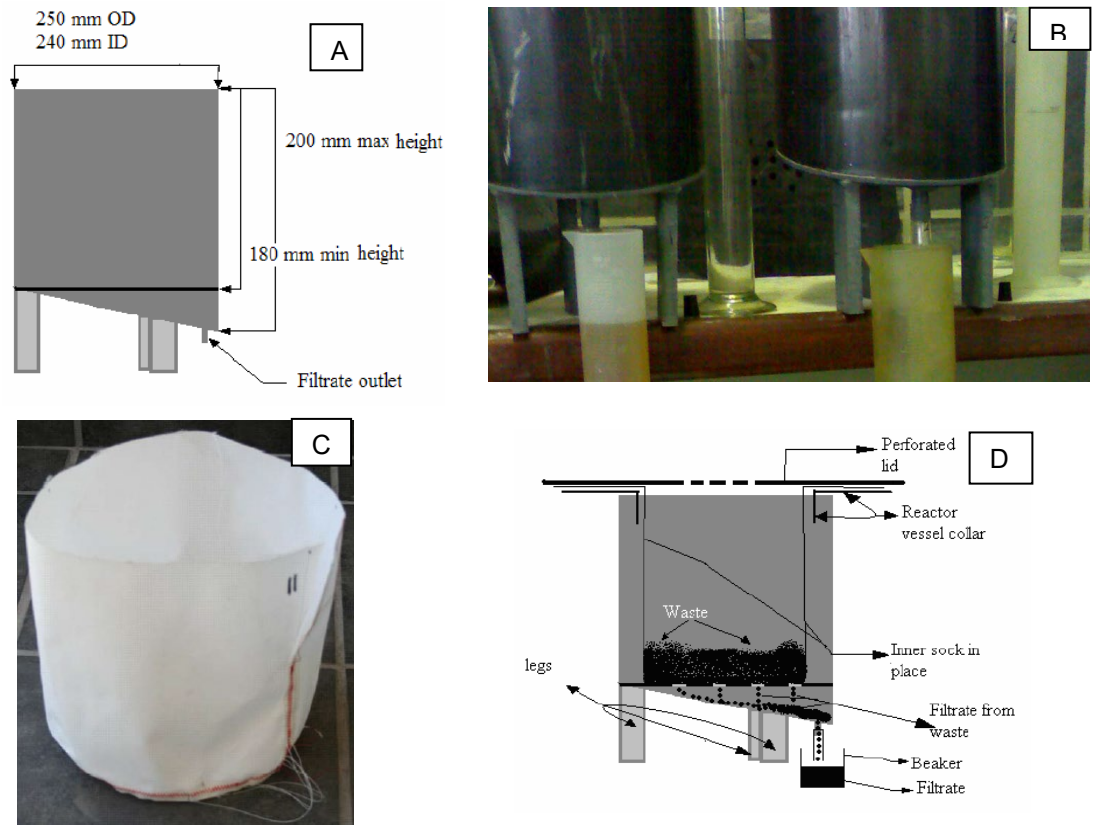

Figure 2-7: Reaction vessel as designed by Bindoff (2008). A: Schematic side view, B: Base of the reaction vessel, C: The inner bag, D: Schematic diagram of the basic set-up

Other anaerobic digester experimental designs, although not used for additive experiments are shown in Figure 2-9, where the authors used the experimental setup to collect biogas. Although their small-scale biogas collection efforts failed, the designs can be modified to test for gas production if desired.

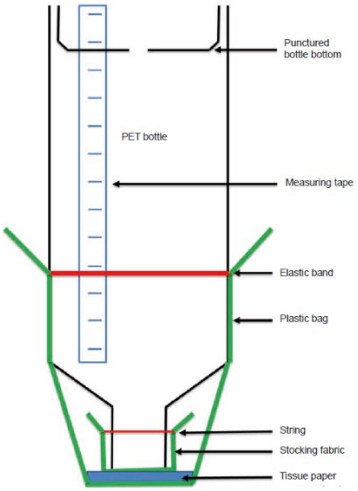

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Figure 2-8: Schematic drawing of a two litre PET bottle used as a miniature pit latrine simulation (Grolle et al., 2018)

Figure 2-9: Left image, a perplex digester with a tube for gas collection (white arrow). Right image, 2nd larger type of digester using 200 L containers, with attached car tyre tubes (a) and crank handle for mixing (b) (Madikizela et al., 2017)

Additive dosing rates (mass / volume) need to be scaled down from the pit area to the experimental container area (g/m^2 or ml/mm^2, etc.) (Buckley et al., 2008). Awere and Edu-Buandoh (2016) provide a useful equation for this calculation (Equation 1) and used a surface area of 5 mm^2 for their honey jar experiments.

$$\boldsymbol{Dose\ applied\ (ml)} = \frac{\boldsymbol{manufacturer's\ dosage\ (ml) \times surface\ area\ of\ honey\ jar\ (mm^2)}}{\boldsymbol{surface\ area\ of\ pit\ latrine\ (mm^2)}} \textbf{... (Equation 1)}$$

2.5.4 Standard tests

Especially for lab trials, the three main groups of testing are chemical, physical and biological tests. Chemical oxygen demand concentrations (g COD/g sample), moisture content (g H_2O/g sample), total solids (g TS/g sample) and mass change (g) are the most basic tests required for assessing additive effectiveness. These tests are run at the start, during (by some) and at the end of the experiment (Taljaard et al., 2005; Bindoff, 2008; Buckley et al., 2008; Couderc et al., 2008; Foxon et al., 2009; Awere and Edu-Buandoh, 2016; Grolle et al., 2018). Initial testing on collected faecal sludge is usually, and preferably performed, within 24 hr (Awere and Edu-Buandoh, 2016; Appiah-Effah et al., 2020) to reduce any data deviations due to ongoing chemical, physical and biological reactions. For mass change, the container is measured every few days (Foxon et al., 2009) or weekly (Appiah-Effah et al., 2020) and the rate of loss calculated over time (Bakare et al., 2010).

2.5.4.1 Chemical tests

The pH is measured to determine the degree of acidity or alkalinity of the sample. The pH measurement is usually initially conducted on site (if possible) using a portable hand-held meter (Appiah-Effah et al., 2020) to prevent any data change between the field and the laboratory. It is also measured during the experiment, if required, using a benchtop pH probe (Taljaard et al., 2005; Zingoda, 2016; Grolle et al., 2018). Appiah-Effah et al. (2020) measured pH every other day for 15 days.

COD is measured to quantify the oxidisable organic matter within samples (Jere et al., 1998; Taljaard et al., 2005; Bindoff, 2008; Awere and Edu-Buandoh, 2016; Grolle et al., 2018; Changara et al., 2019; Appiah-Effah et al., 2020). COD can be targeting particulate COD (COD_P) and soluble COD (COD_S). COD_P combined with COD_S make up the total COD (COD_T) of the sample. Appiah-Effah et al. (2020) measured COD weekly for 4 weeks. COD in the past was performed by the open reflux method according to Standard Analytical Methods (APHA 1998), but is now more currently done using a Spectrophotometric Method.

Biological / Biochemical oxygen demand (BOD) is performed to quantify the amount of oxygen consumed by microorganisms while they decompose organic matter (USGS 2021). So far, only three articles have been found where researches tested BOD while testing additives, Jere et al. (1998), Changara et al. (2019) and Appiah-Effah et al. (2020) who tested BOD weekly for 4 weeks. BOD is determined either chemically via spectroscopy, or biologically with a respirometer.

Some of the lesser performed chemical tests include: volatile fatty acids; total nitrogen; phosphorous; phosphates; potassium; calcium; magnesium; sodium; sulphates and ammonia (Jere et al., 1998; Grolle et al., 2018; Changara et al., 2019). Odour is less common in faecal sludge additive experiments. So far, odour has only found to be reported by Zingoda (2016) during additive trials, who used the threshold odour test 2150 B (NEMI 2021).

2.5.4.2 Physical tests

Temperature is an important parameter as pH changes with temperature so they are commonly taken in tandem, as well as being an important biologically. Temperature is easily measured with the use of a standard thermometer (Zingoda, 2016) or a laser thermometer for surface measurements. Appiah-Effah et al. (2020) measured temperature every other day for 15 d.

Sludge mass tracks the loss, or gain, of mass from the experimental containers over time (Appiah-Effah et al., 2020). This is measured by weighing the containers over time. Moisture content is an important parameter as this mass difference is an indication of either high evaporation, or mass loss / gain due to additive effectiveness (Bindoff, 2008; Awere and Edu-Buandoh, 2016; Appiah-Effah et al., 2020). Moisture content is measured by drying a known mass of sample in an oven for 24 hr at 105°C. Appiah-Effah et al. (2020) measured moisture content weekly for 4 weeks.

Total solids (TS) is a step to determine the moisture content (organic and inorganic) of a sample (Nwaneri et al., 2008). TS is comprised of total suspended solids (TSS), volatile solids (VS) and ash (Jere et al., 1998; Taljaard et al., 2005; Bindoff, 2008; Zingoda, 2016; Grolle et al., 2018; Changara et al., 2019). This test involves filtering a known homogenised mass sample through filter paper, followed by a series of oven drying and furnace ashing of the residual material remaining on the filter.

The rate of sludge accumulation (height) can be easily and accurately measured by use of a laser distance measure (Still and Foxon, 2012). For field trials, the distance is measured from the pedestal to the sludge surface, three measurements within an area of 0.06 m^2 (Bakare et al., 2010), while some authors suggest up to 10 random measurements within the observable portion and averaged (Still and Foxon, 2012). Although, the cheapest option is a tape measure or a ruler (Zingoda, 2016), automated methodologies using cameras and scanners have been used for assessing sludge height, colour and quantification (Still and Foxon, 2012; Ward et al., 2021). Bakare (2011) designed a supporting camera system that fits over a VIP pedestal to ensure accurate image capture for stereographic imaging (Figure 2-10 A) and Still and Foxon (2012) mention the use of automated laser scanner to measure the height of the sludge and to create 3D images of the sludge surface (Figure 2-10 B).

A lesser-known physical parameter tested is faecal crust formation (Grolle et al., 2018). Faecal crust formation, while not commonly tested during additive experiments, can influence the effectiveness of additive addition by the physical formation of a hard surface barrier limiting the surface interaction between the additive and the faecal sludge surface, which is the main point of interaction (Grolle et al., 2018).

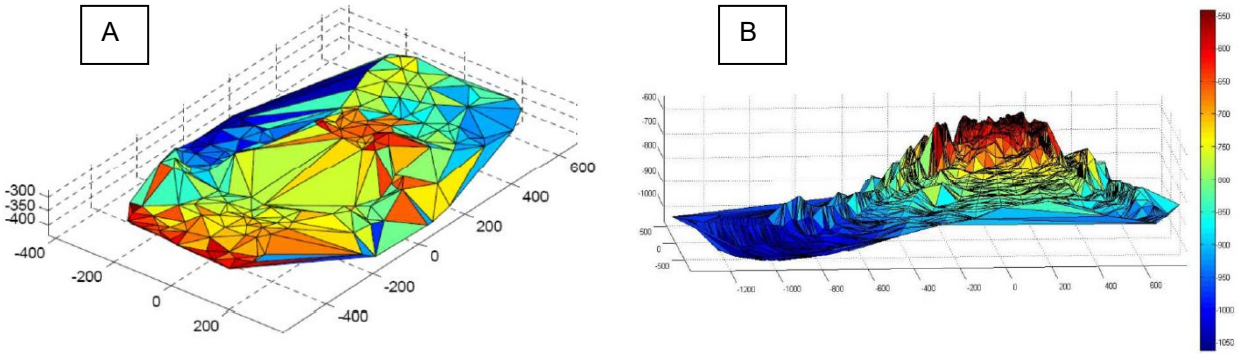


Figure 2-10: A) Stereographic imaging technique of a pit latrine surface (Bakare, 2011) and B) 3D modelling of the pit latrine surface using automated laser scanner (Dahmani, 2010) in Still and Foxon (2012).

2.5.4.3 Biological tests

Simple biological tests are not usually conducted during faecal additive trials because many additives either add a biological agent or alter the sludge chemistry. The biological tests that are performed test for the presence and abundance of faecal pathogens such as helminth eggs, total coliforms (Changara et al., 2019; Appiah-Effah et al., 2020) and *Escherichia coli* (Zingoda, 2016; Changara et al., 2019) and are usually performed to assess the health and safety impacts of faecal sludge (Nabateesa et al., 2017) or the effects of additive choice on VIP sludge microbiology also linked to health and safety. There are numerous studies into the bacterial community structure (sequence based) of the human gut microbiome and that of waste water, but data on the bacterial community structure within VIP sludge is scarce. The three studies found are i) Torondel et al. (2016), who used the FastDNA SPIN kit for soil for DNA extraction, ii) Beukes (2019) who used the uBiome gut sampling kit from VIP's in KwaMashu, South Africa and iii) Smith et al. (2023) who sampled pit latrines in peri-urban Malawi. The data generated from the three studies is presented in Figure 2-11, Figure 2-12 and Figure 2-13.

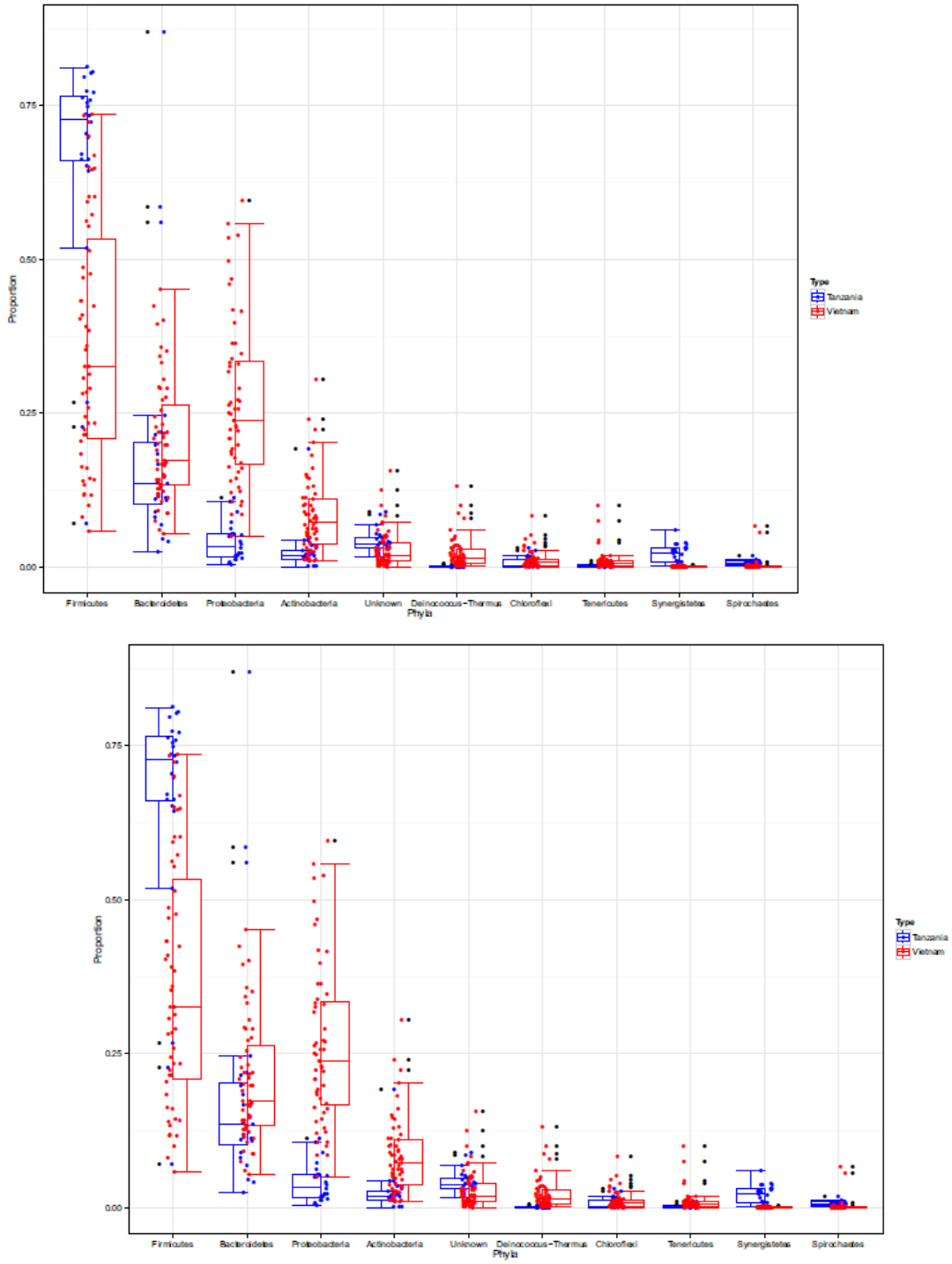


Figure 2-11: Mean phylum level abundance in the Vietnamese and Tanzanian pit latrines (Torondel et al., 2016)

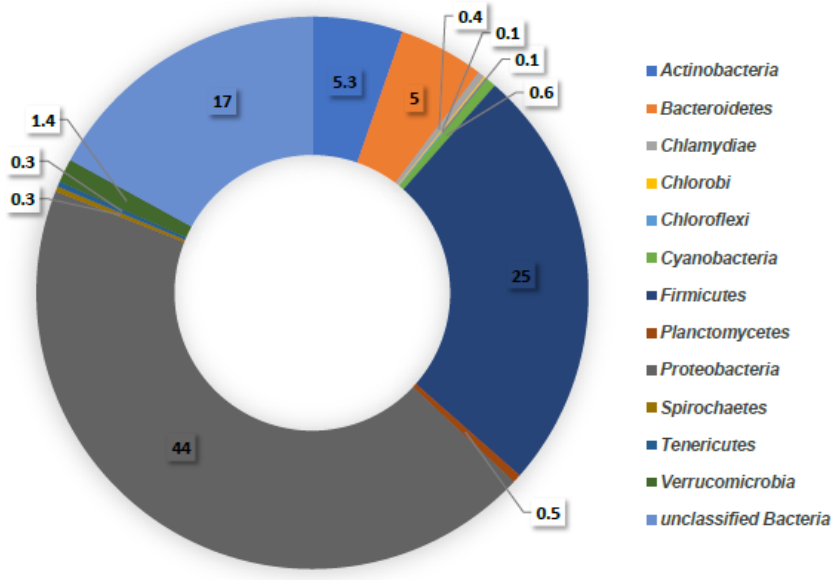


Figure 2-12: Bacterial phyla abundance (%) in pit latrine faecal sludge from KwaMashu, South Africa (Beukes, 2019)

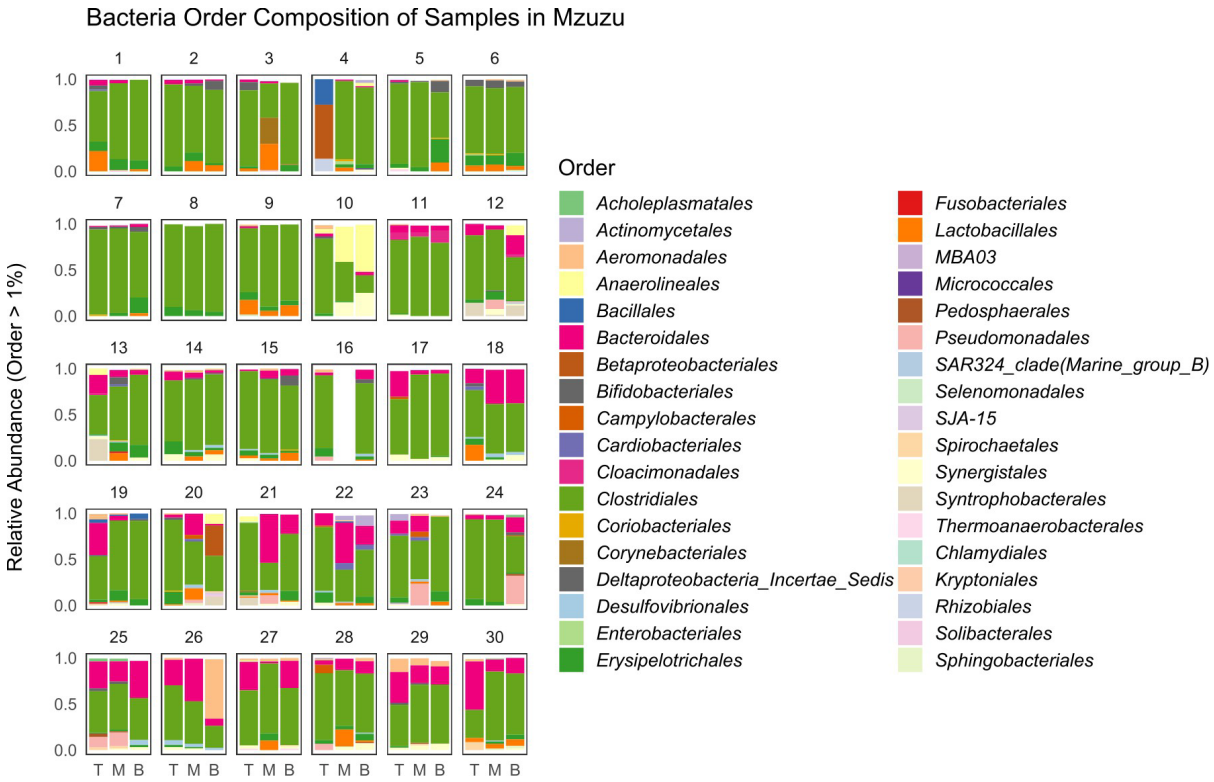


Figure 2-13: Relative abundance of bacteria at order level for each pit latrine sampled at the three depths for the Mzuzu site (Smith et al., 2023)

Gas production (Couderc et al., 2008), aerobic gas production (Changara et al., 2019) and pressure (Grolle et al., 2018) are useful tools to test the efficiency of digestion by measuring the volume of gas produced. For anaerobic gas production the use NaOH pellets, or a 5% solution, is recommended to absorb CO_2 ensuring that only methane and other gasses are collected (Grolle et al., 2018; Changara et al., 2019). Figure 2-14 shows the laboratory set-up for anaerobic digestion of pit latrine sludge, which can be altered for smaller scale experiments.

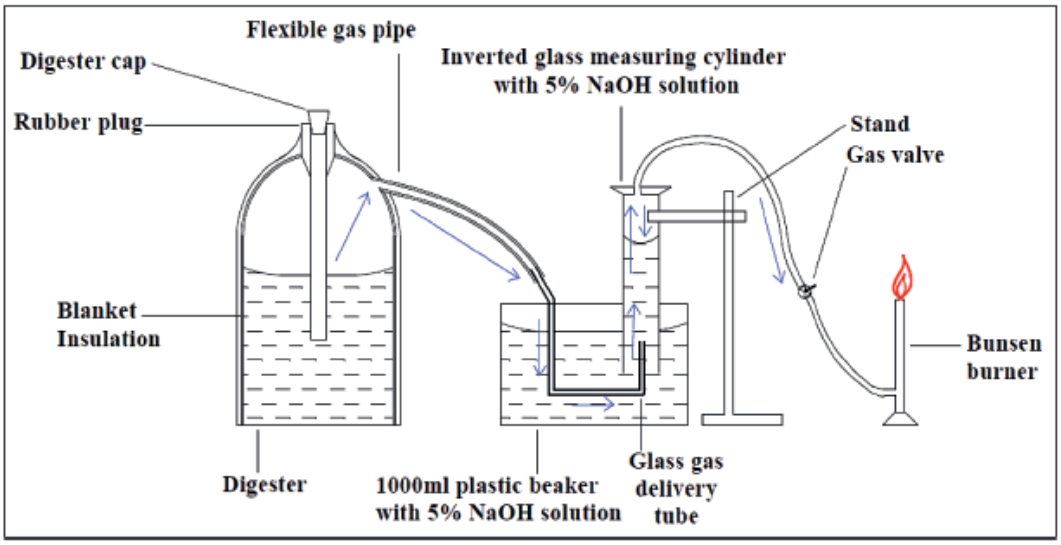


Figure 2-14: Laboratory set-up for anaerobic digestion of pit latrine sludge (Changara et al., 2019).

2.5.5 Inhibitory components in VIP latrines

Nwaneri (2009) reported that some households added disinfectants such as Jik® / Domestos® / Jeyes Fluid® into the VIP latrines in an attempt to reduce the unpleasant smell. These chemicals contain microbiocidal properties and may certainly limit the functionality of MO within the pit sludge influencing sludge biodegradation (Still and Foxon, 2012). Disinfectants have also been reportedly used by caretakers to control the flies and odour (Awere and Edu-Buandoh, 2016). Insecticides may also be added by households (no existing data), which will reduce the activity of fly larvae which may also inhibit sludge breakdown (Still and Foxon, 2012).

2.6 Data Gaps

Some evident data gaps, or studies that needs to be expanded on, or data that is important for further research into the characteristics of pit latrine sludge, include, but are not limited to the following:

- **Microbiome in faecal sludge**: Microorganisms are important in the decomposition of faecal sludge. However, studies on microbial communities and their association with faecal sludge decomposition are limited. Understanding microbes present in the sludge enables the engineering of interventional strategies to enhance microbial processes in pit latrines.
- **Biodegradation limiting step**: A large data gap is not knowing for certain, what the main limiting step hampering effective biodegradation is in the pit.
- **Decomposition rates**: There are limited studies on the rates of decomposition of VIP latrine sludge. The studies that are available, researchers focus and manipulate on one or two variables to determine any change. Additional research needs to be carried out on simple biodegradation rates throughout the entirety of the sludge contents.
- **VIP sludge composition**: Although some data do exist, it is usually as a partial note or paragraph relating to what was found within the sludge. Additional research into this, including the state of decay of certain degradable foreign materials would be useful.

- **Temperature gradients**: Data on VIP latrine pit temperature gradients was extremely limited. Since microorganisms rely on the temperature to dictate the degree of biological activity, knowing the temperature gradients within the sludge profile would aid in determining where the warmest and most active areas are.
- **pH Gradients**: This data would be useful for the same reasoning as for temperature. In addition, more research needs to be done on pH regulation and stability in pits, as it is evident that while acidic conditions might aid in hydrolysis, neutral to slightly basic conditions are preferred for methane production as they reduce the hydrogen sulphide toxicity.
- **Moisture gradients:** This data would be useful for the same reasoning as for temperature.
- **Soil composition**: Many studies supply data on the moisture levels within VIP latrine sludge, but extremely few give the soil characteristics of where the pit lies. Soil characteristics is one of the primary means of advanced water loss or gain, allowing for either a nice and "controlled" moist environment for optimal biological degradation, or very dry conditions, possibly from localised moisture absorption into the surrounding soil. Flooding, causing dilution of matter and nutrients followed by possible heavy drainage, removes MO and key soluble nutrients from the system.
- **Non-seepage study:** So far, all pits appear to be "lined" or unlined, but there is limited information on 100% sealed pits.
- **Heavy metals**: The effect of heavy metal concentrations on the efficiency of bacteria seems to be the dominant platform with wastewater and especially WWTPs (many authors relate the waste discharge coming from industrial complexes). For VIP latrines, some researchers report on heavy metal concentrations in fresh faeces, urine and in latrine sludge, but so far, no research has been found on the toxicity, rates, or effects of heavy metals on microorganisms in latrine sludge.
- **Volatile fatty acids**: Some reports indicate that at the end of the pit lifecycle, VFA's should be zero, but work on this is limited. This niche needs more attention as it has been shown that high levels of VFA (33 g/L) inhibit bio-waste hydrolysis and at very high levels (40-50 g/L) anaerobic hydrolysis stops (Veeken et al., 2000).
- **Other factors**. Pharmaceutical compounds such as antibiotics when excreted via human faeces can affect the performance of key members of the anaerobic food chain. However, no such data on the presence of antibiotic residues in pit latrines appear to be available for South Africa even though studies highlighted the presence of antibiotic resistant MOs (or similar).

2.7 Conclusions

VIP latrine sludge is a mixed composite of organic and inorganic materials originating mostly from human faeces, which become layered over time, resulting in discrete zones with different biological, chemical, and physical conditions found within. This biological activity allows, to some degree, for the extended life span of the pit contents before maximum capacity is reached but is influenced by the physical and chemical characteristics within each discrete layer within the pit. By determining the unique physical, chemical, and biological characteristics, combined with the next-generation sequencing analyses of pit latrine samples to assess the microbial composition and diversity within these layers, a more holistic kinetic rate equation can be determined to better calculate the filling rates of VIP latrines. A better understanding of the microbial communities' present will potentially aid developers in designing effective VIP additives.

3 CHAPTER 3: METHODOLOGY

3.1 Development of Hypothesis

There are many chemical, physical and biological process occurring simultaneously within VIP latrine sludge material, and it is very difficult to combine them all together within a single, all round, encompassing hypothesis.

One simplistic all-encompassing hypothesis would potentially be:

- Deposited organic waste matter / material is degraded over time by microbiological catabolism resulting in the; i) reduction of solid waste mass, ii) production and emission of gaseous by-products, and iii) chemical, physical and biological alteration of the waste material properties.

More specific hypotheses relating to individual or grouped processes may include the following potential hypotheses:

- Organic matter present within the VIP sludge is initially degraded aerobically, followed by anaerobic degradation with sludge depth or coverage by fresh material
- Shifts in the base bacterial population depends primarily on the available organic material and the oxygen availability within the VIP sludge
- Waste organic material is converted into soluble substrates by microbes, which are in turn converted into inorganic materials, gasses and biomass
- The microbial communities and microbial processes occurring within each layer of the pit is majorly influenced by the prevailing physical and chemical characteristics and would each exhibit a distinctive microbial community structure
- The hydrolytic activity of microbes in the faecal sludge predominates in the two upper layers within the pit where complex organic compounds and oxygen are prevalent
- The redox potential of faecal sludge decreases with the depth of the pit, potentially indicating different biodegradation processes occurring in faecal sludge
- Substrate distribution and variation are heterogenous throughout the VIP sludge material, but homogenous within unique layers
- Microbial population dynamics vary greatly within VIP's, amongst different VIP's, and across geographic location
- Specific / grouped substrate utilisation is unique to grouped microbial genera

The above hypotheses constitute some that are theoretically possible for the major transformation processes in VIP latrines based on the available literature. Not all of the mentioned hypotheses were tested during this study.

3.2 Objectives

As discussed in Chapter 2, the biodegradation of faecal sludge in pit latrines is facilitated by the microbial communities present, thus reducing the volume of faecal sludge and accumulation rates in the pit. The activity of the microbes are, however, influenced by physical and chemical properties prevailing in the faecal sludge in pit latrines. Therefore, understanding the microbial ecology and diversity in these systems is crucial as they determine the success or the failure of the process in any given environment. Moreover, it allows for the formulation of appropriate tools that may optimise their functional role within the systems.

Samples were collected from four VIP latrines from four different layers as described in Figure 2-3 within the VIP latrine pit with the following objectives:

- To undertake an investigation of the diversity and composition of bacterial communities found in the faecal sludge from each layer within the pit;
- To analyse the various physicochemical properties of faecal sludge from each layer within the pit;
- To enumerate the total coliforms and *E. coli* present in faecal sludge from each layer within the pit; and
- To understand the change in the biological, chemical, and physical characteristics of faecal sludge from the VIP latrines after 15 days and 30 days

To achieve these objectives and test the hypothesis stated in Section 3.1, various analytical methods were employed. This included:

- **Next Generation Sequencing** – to study the specific community of microorganisms within each zone in the faecal sludge. This allows determination of the microbial diversity and possible functional role of each microbial community present based on the identification and abundance of microbes via the analysis of reads generated.
- **Physicochemical properties** – affect the structure of microbial communities, as well as microbial growth and activities. Properties studied are:
  - **pH** – influences the occurrence and distribution of microorganisms and affects enzyme activity as well as the toxicity of metabolites such as H_2S.
  - **Electrical conductivity (EC) –** indicates the concentration of ions from water-soluble salts in faecal sludge, which is important for regulating microbial composition.
  - **Moisture content** – availability of water is important for stabilising the intracellular water potential, enabling the activity of hydrolases that use water as a co-substrate, and as a solvent for substrates, microbial mobility, and oxygen diffusion
  - **Total solids** – affects the performance of biological processes as it contains the organic matter that is broken down and used as carbon and energy source
  - **Volatile solids –** strongly influences the availability of nutrients or volatile fatty acids for microbes which consequently influence bacterial diversity and community composition
  - **Nitrogen and phosphate species** – both microelements are important for the synthesis of proteins, amino acids, and nucleic acids. They can affect microbial abundance, diversity, and community composition
- **Total coliforms and *E. coli*** – fresh human faeces contain high numbers of faecal coliforms and *E. coli*. Similar to other microbes, the numbers and distribution of faecal coliforms is determined by the prevailing conditions in faecal sludge within the pit.
- **Mass loss and change in faecal sludge characteristics** – the faecal sludge within the pit undergoes a series of biological reactions that reduce the mass within the pit. These reactions occurring in the faecal sludge within the pit determine the biological, chemical and physical characteristics of faecal sludge.

3.3 VIP Latrine sample collection

The faecal sludge was collected from four VIP latrines located in Edendale in Pietermaritzburg, KwaZulu-Natal, South Africa (Figure 3-1).

Samples were collected during the emptying of the VIP latrine by contractors. Samples were removed from the surface, and at each of the theoretical 3 underlying layers. The sampling was carried out in this way to allow for some limited segmented data in identifying the presence, absence or changes of any chemical, physical, biological or genetic data across each sampled layer and across all the sampled depths. The maximum depth of the surface sample was 15 cm, as below this is the theoretical transition towards the anaerobic zone.

3.3.1 Description of VIP latrines

This section provides a description of the VIP latrines sampled for the project. All images used are with the permission of the photographer (Mr T Kunnen, WASH R&D Centre). All images captured were with permission of a resident household member. All and any persons captured within any and all images were with permission, and permission was granted for images containing person/s to be used for the project and or scientific use.

(A) (B)

Figure 3-1: Sample collection (A) location of VIP latrines (Google Earth, 2022), and (B) Tools used to collect faecal sludge sample from the pit

*3.3.1.1 **VIP 1** (29°40'45.5''S 30°17'34.7''E **OR** -29.679316, 30.292968)*

VIP 1 contents were rather wet but did not contain any trash, and the VIP was in functional use. The resident understood the use of the VIP and that trash impedes the system. The resident stated that they do not add any water or other liquids to the VIP, so the sludge may be wet potentially due to a high-water table. The household and surroundings are on the side of a hill, and therefore the high amount of water could be from the down flow of water and the higher water table. While sampling the sludge it was odorous and pungent, with the unique faecal smell. However, the deeper the pit was sampled, especially at layer 3, the smell of the sludge gave off a "eggier" / hydrogen sulphide smell and not as much of a pungent faecal smell. Even the lower layers were very wet. Sampling was carried out through the pedestal access hole (Figure 3-2).

*3.3.1.2 **VIP 2** (29°40'45.2''S 30°17'50.6''E **OR** 29.679233, 30.297377)*

VIP 2 contents were very dry and contained a lot of trash / rubbish, mostly plastics and clothing. Where needed, the rubbish was scraped away or removed to get access to "cleaner" sludge for sampling. VIP 2 contents did not give off a strong Faecal smell, and looked very soil like with a crumbly texture with small ball like particles. VIP 2 was very full, and was reported to be used occasionally (the residents had constructed their own "VIP" which was mainly used). Sampling was done from rear access through the removable concrete slab (Figure 3-3)

Figure 3-2: Sampling of VIP 1

Figure 3-3: Sampling of VIP 2

*3.3.1.3 **VIP 3** (29°40'53.4''S 30°17'29.6''E **OR** -29.681502, 30.291566)*

VIP 3 (Figure 3-4) was approaching being full, had been in operation for about 10 years, and had never been emptied. This pit contained a high amount of trash, mostly chip packets, glass bottles and loose plastics. Contents were wet and there was what looked like a marsh or wetland next door. Contents were sloppy and had a dark appearance. Once the surface layer was broken, the smell was not as pungent and changed from a fecal smell to an anaerobic egg-like smell. Contents became darker the deeper the pit was emptied. There was a bottle of Jeyes Fluid® next to the toilet pedestal which implied that the users add the Jeyes Fluid® to the pit contents, but this was not confirmed at the time of the emptying.

Figure 3-4: Sampling of VIP 3

3.3.1.4 ***VIP 4*** *(29°40'26.7''S 30°44.5''E* ***OR*** *-29.674095, 30.295691)*

VIP 4 (Figure 3-5) was very full and had been in use for approximately 8 years. Due to an increase in the number of household residents, the residents built another unlined pit. VIP 4 was full of trash, including chip packets, plastic bottles, string / synthetic / weave hair, used condoms and loose plastics. The surface layers were very odorous, but became more egg-like (e.g. H_2S) in the deeper samples.

3.3.2 Collection and storage of Samples

Samples were collected following the SOPs developed by the WASH R&D Centre for the collection and storage of sludge. About 0.8-1.0 kg of faecal sludge (wet weight) was collected from each of the four different layers within the pit from the top surface to the bottom of the pit, from each of the four VIP latrines. Pit latrine samples were collected in sterile containers and transported to the WASH R&D Centre laboratory. All samples were stored in the cold room 4°C until analysis. For most of the analyses, the samples can be stored for 24 hrs before any detrimental changes start occurring which can impact on the test results.

Figure 3-5: Sampling of VIP 4

3.4 Experimental set up

From each sampled VIP, 300 g of material from each sampled layer was weighed out into clean plastic 375 ml honey jars. The surface layer jar was left open to the atmosphere while the remaining layers were closed with a lid. Samples were taken at day 0, day 15 and day 30 from each of the honey jars and tested for the following parameters: moisture content, total solids, volatile solids, ash, pH, EC, mass change, NGS (next generation sequencing), CODt, CODs, ammonium, nitrate, ortho-phosphate, *E. coli* and total coliforms. After day 30, the remaining materials from the honey jars was safely discarded.

3.5 Sample Analysis

Samples were taken at day 0, day 15 and day 30 to account for any change in bacterial population structure as the VIP material undergoes natural changes overtime.

3.5.1 Microbiome analysis

A single spoonful (± 1 g) of the faecal sludge samples from each layer (from each pit) were transferred into individually labelled DNA/RNA Shield Faecal Collection Tubes (Zymo Research, Cat #

R1101, with no beads) prefilled with preservatives and shaken vigorously to ensure proper stabilisation. The collection tubes were stored at 4°C and then shipped to the University of Columbia Genome Center (USA) for DNA extraction, library preparation and Next Generation Sequencing (NGS) of DNA amplicons following the in-house method from the University of Columbia Genome Centre.

The reads obtained were processed using a Bioinformatic pipeline for quality trimming using the 16S nextflow pipeline from Epi2Me (Bioinformatics resources from Oxford Nanopore), Operational Taxonomic Unit (OTU) clustering, and taxonomic assignment as reported in chapter 5.

3.5.2 Physicochemical characteristics of faecal sludge

Different analytical tests were performed on the faecal sludge samples using the SOPs from the WASH R&D Centre.

3.5.2.1 Physical analysis

- **pH and Electrical conductivity:** The pH and the Electrical conductivity of the faecal sludge samples collected were measured using a pH meter fitted with a pH and EC probe. For sample preparation, 10 g of faecal sludge was suspended in 20 mL distilled water and homogenised.

- **Moisture content, total solids, volatile solids, and ash content**: 20 g of faecal sludge was transferred into pre-weighed ashed crucibles (550°C for 1 hour) and dried in an oven at 105°C for 24 hours. After drying, the crucibles were allowed to cool down and weighed to determine the total solids and moisture content. The crucibles containing dried faecal sludge (105°C for 24 hrs) were further ashed in a pre-heated muffle furnace at 550°C for 2 hours. After ashing, the crucibles were allowed to cool and then weighed to determine the volatile solids and ash content.

3.5.2.2 Chemical analysis

The same procedure was followed for the analysis of:

- Total and soluble Chemical Oxygen Demand
- Ammonium (NH_4-N)
- Orthophosphate (PO_4-P)
- Nitrate (NO_3-N)

Initially, 1 g of sludge sample was mixed with 1000 mL of distilled water and the solution blended to homogeneity (30 sec or until visually mixed). This however resulted in low levels of CODs, below the detection range of the kit. To counteract this, the mass of sludge was increased to 6 g, and the volume of distilled water reduced to 100 mL. In instances where this now increased the chemical range of some kits to above the maximum, these samples were diluted proportionally to be within the working range of the kit.

These tests were conducted using Merck's Spectroquant photometric test kits by following the instructions provided with the test kits.

3.5.2.3 Enumeration of coliforms and Escherichia coli

The total coliforms and *E. coli* were quantified using the Idexx Quanti-Tray® 2000 with the Colilert-18 substrate. Faecal sludge sample (1-5 g) was suspended in 100 mL sterile 0.85% NaCl and appropriate serial dilutions were established. For the Colilert analysis, 100 mL of each sample was poured into a sterile plastic bottle. The Colilert substrate supplement was then added, and the sample vessel was shaken to mix and dissolve the substrate. The mixed solution was then poured into an Idexx Quanti-Tray® 2000, any bubbles removed by manually tapping, sealed using the Quanti-Tray sealer and then incubated for 19 h at 35°C. The Quanti-Tray® 2000 has 49 large wells and 48 small wells with a quantification range of 1-2419.6 MPN per gram of faecal sludge, with the final data calculated based on the dilution ranges used.

Following incubation, *E. coli* were enumerated. First, total coliform-positive wells (yellow/gold in colour) were identified and enumerated from the 49 large and 48 small Idexx Quanti-Tray® 2000 wells. Then, the Quanti-Tray was placed in a 365-nm Longwave UV viewing cabinet, and the wells were assessed. The yellow/gold wells that fluoresced or glowed blue under the UV lamp were counted as *E. coli*-positive wells. The number of large wells and small wells were then compared to the Colilert MPN table for Quanti-Tray® 2000.

3.5.2.4 Mass reduction and change in characteristics of faecal sludge

300 g of faecal sludge collected from each layer within each pit latrine was transferred into individual honey jars and placed in the fume hood at room temperature. The change in faecal sludge characteristics was monitored at day 0, day 15, and then day 30 by analysing the following parameters:

- Biological characteristics – microbiome, total coliforms, and *E. coli*
- Physical characteristics – mass reduction, moisture content, pH, EC, total solids, volatile solids, ash content
- Chemical characteristics – total and soluble COD, ammonium, nitrate, and orthophosphate

4 CHAPTER 4: RESULTS AND DISCUSSION

4.1 Physicochemical characteristics of faecal sludge from different layers within the VIP latrines

The physical and chemical characteristics prevailing within the pit determine the microbial communities of the faecal sludge and influence the activity of the microbes present therein. This can influence the rate at which the faecal sludge is degraded. Equally so, the biotic and abiotic processes occurring within the pit can change the properties of the faecal sludge over time. Therefore, various analytical tests were carried out to determine the physicochemical characteristics of faecal sludge collected and their change over 30 days.

4.1.1 Physical characteristics

This section provides the results obtained for the physical characteristics of the sludge, and represented by graphical trends for the VIP latrines. This includes pH (Figure 4-1); electrical conductivity (EC – Figure 4-2); moisture content (Figure 4-3); total solids (Figure 4-4); volatile solids (Figure 4-5); and ash content (Figure 4-6).

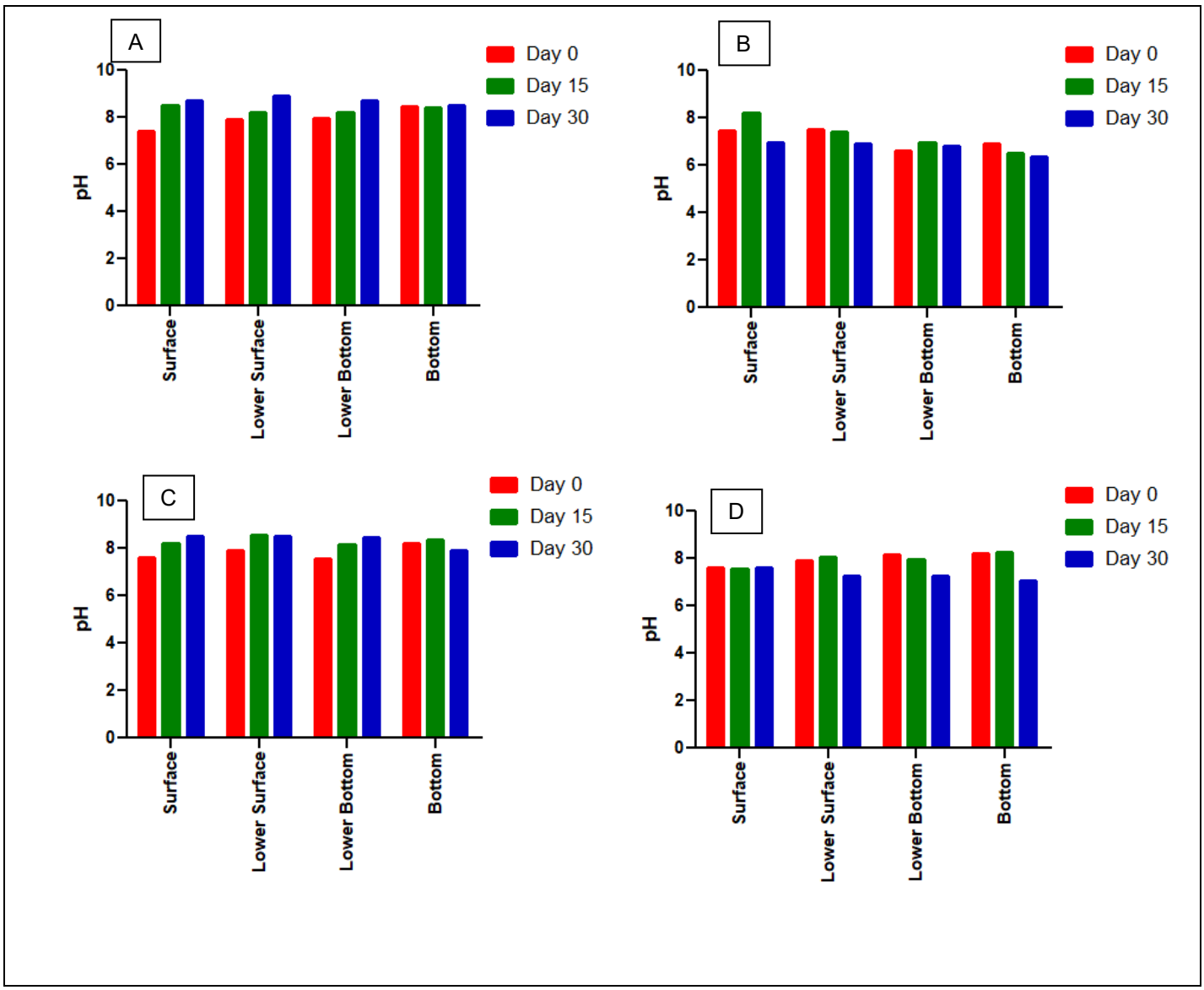


Figure 4-1: The pH of faecal sludge from different layers of VIP latrines for 30 days in honey jars at room temperature. A – VIP 1, B – VIP 2, C – VIP 3, C – VIP 4

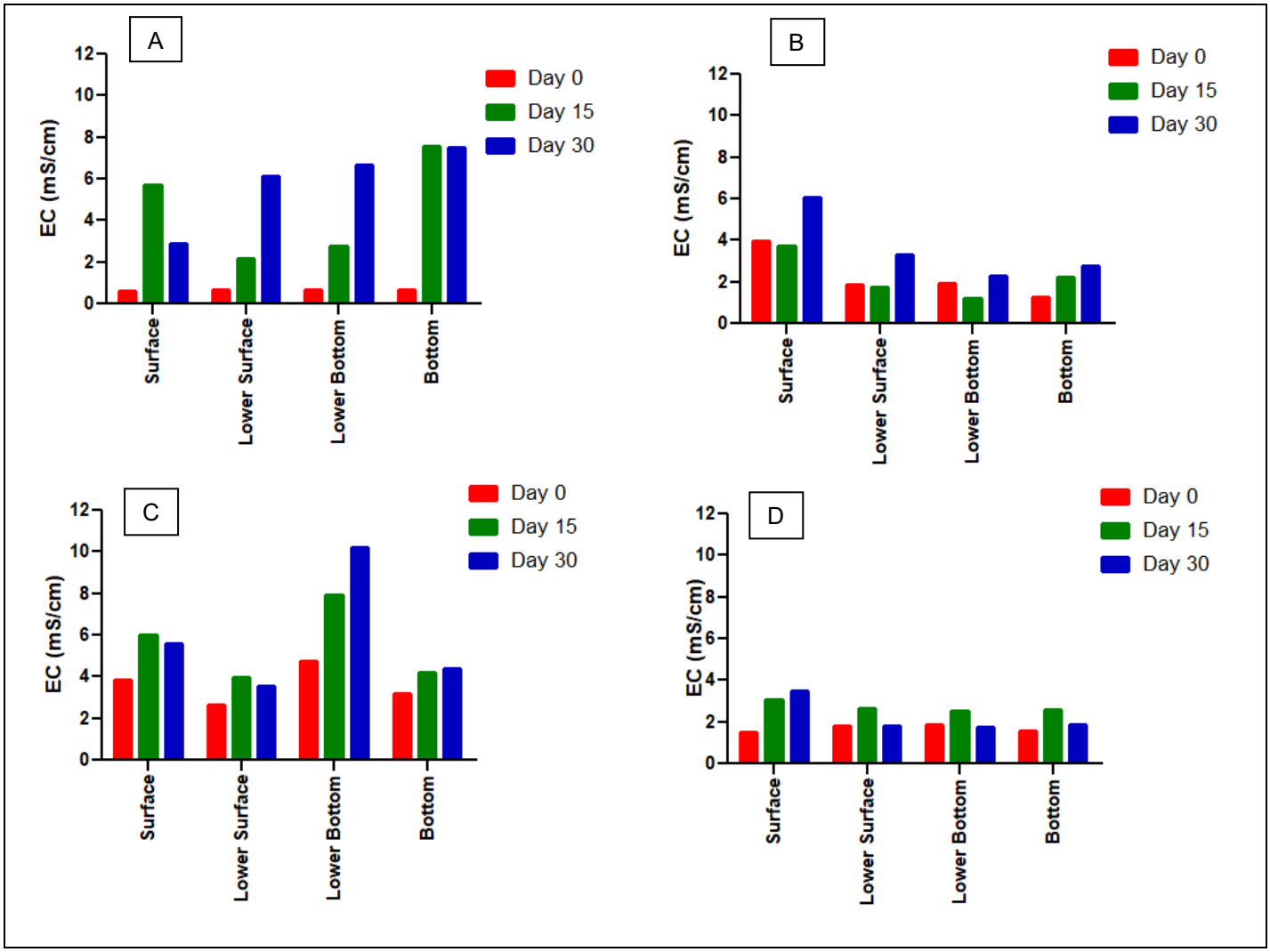


Figure 4-2: The Electrical conductivity (EC) of faecal sludge from different layers of VIP latrines for 30 days in honey jars at room temperature. A – VIP 1, B – VIP 2, C – VIP 3, C – VIP 4

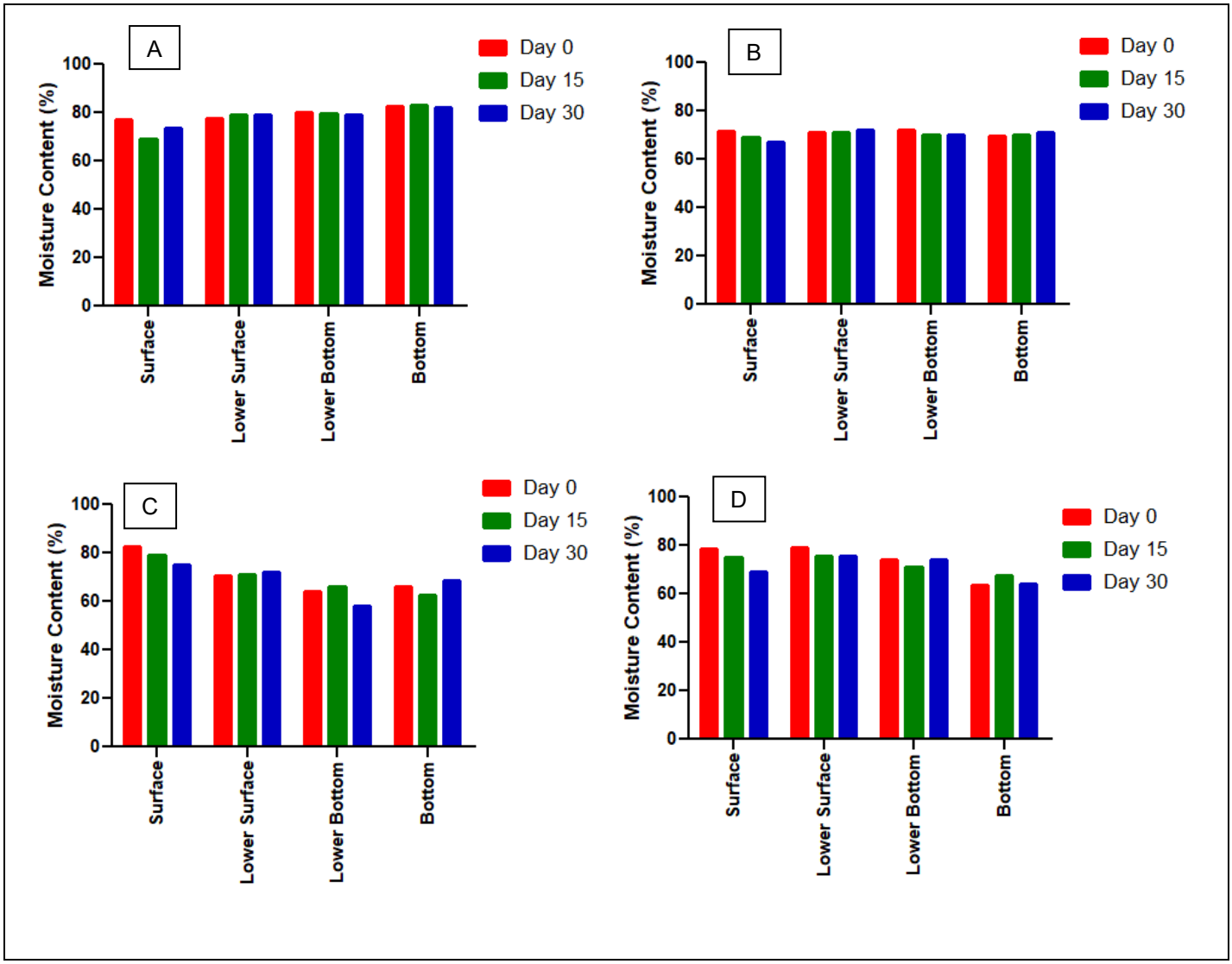


Figure 4-3: The moisture content of faecal sludge from different layers of VIP latrines for 30 days in honey jars at room temperature. A – VIP 1, B – VIP 2, C – VIP 3, C – VIP 4

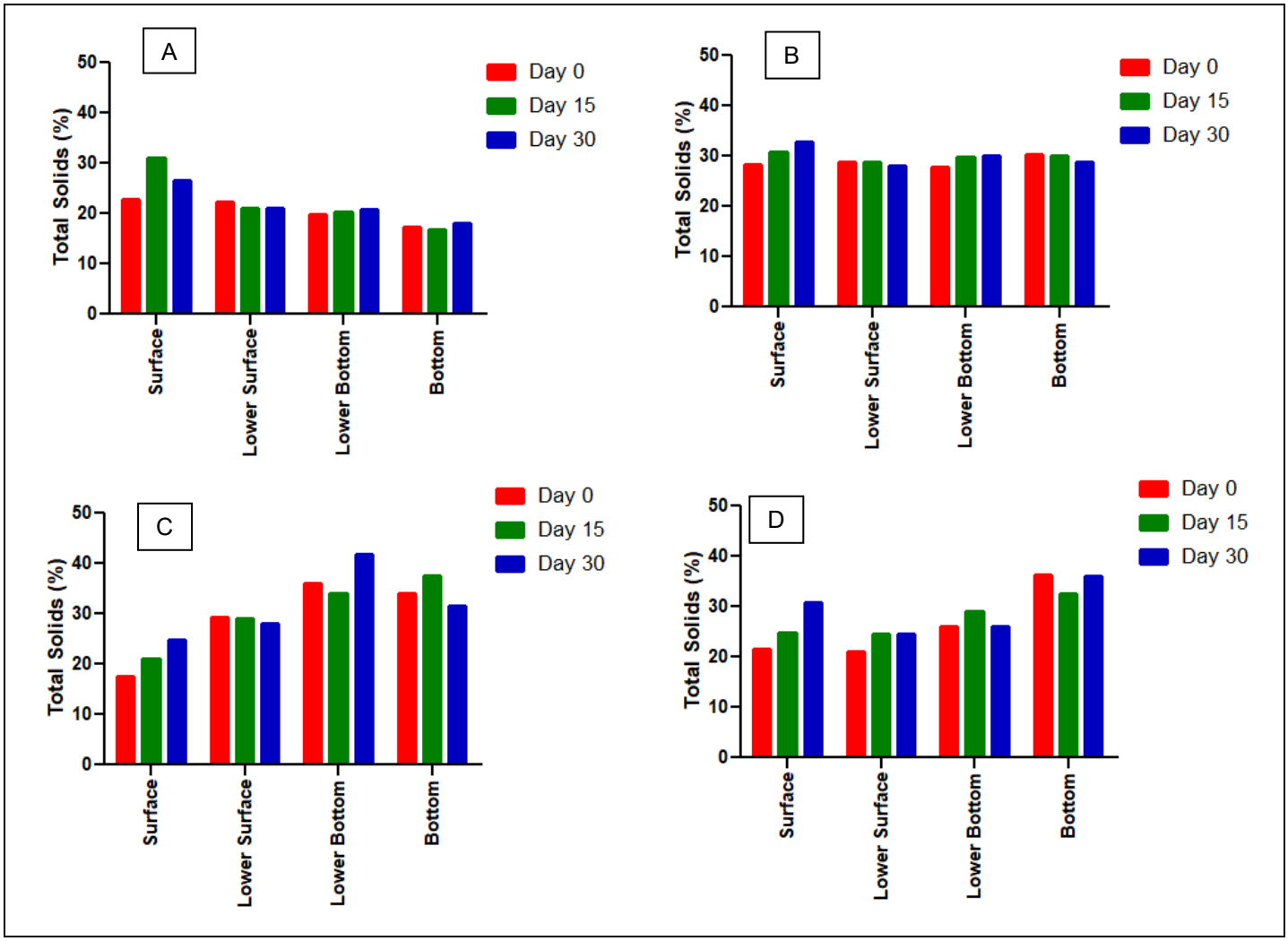


Figure 4-4: The total solids in faecal sludge from different layers of VIP latrines for 30 days in honey jars at room temperature. A – VIP 1, B – VIP 2, C – VIP 3, C – VIP 4

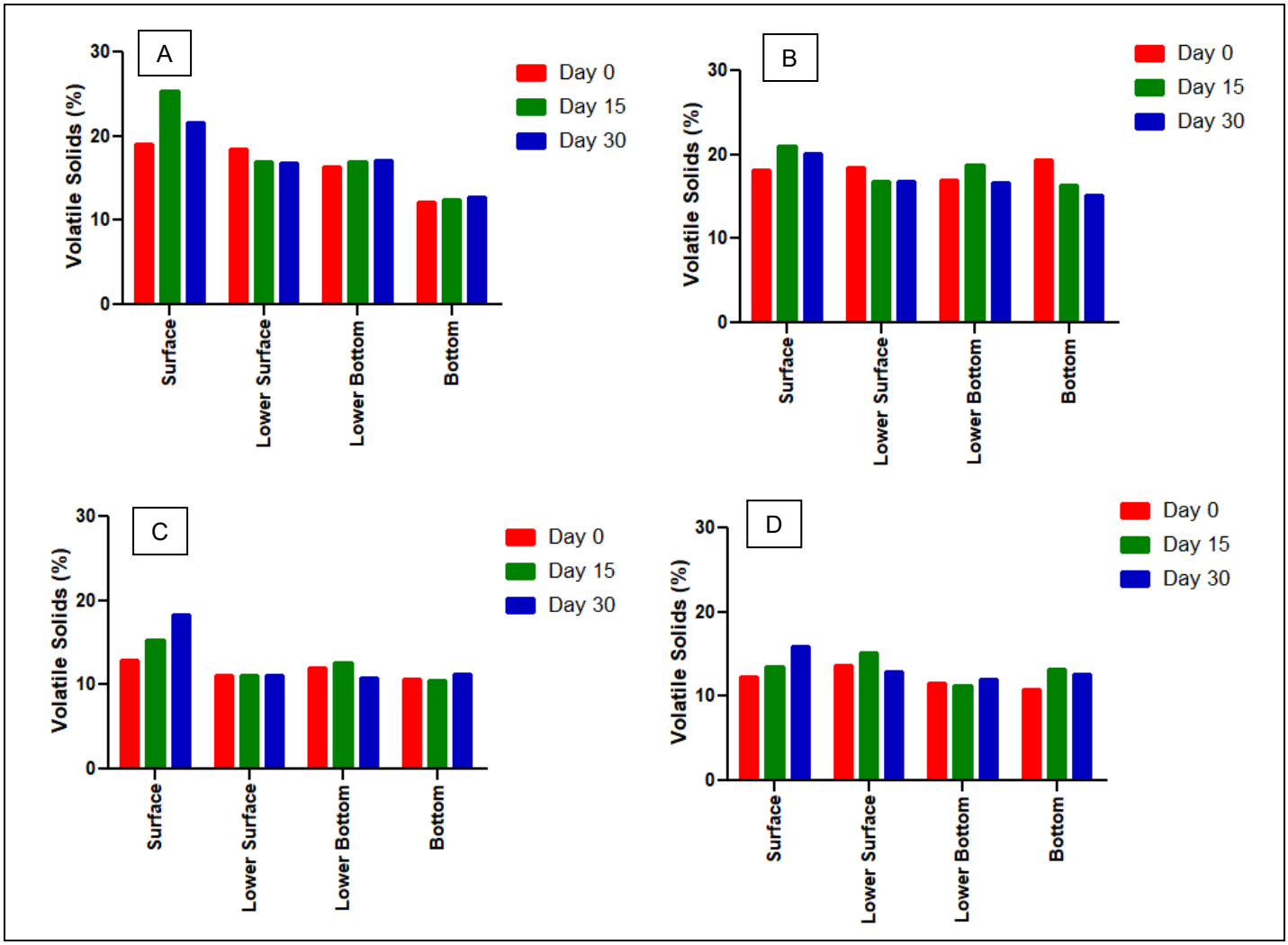


Figure 4-5: The volatile solids in faecal sludge from different layers of VIP latrines for 30 days in honey jars at room temperature. A – VIP 1, B – VIP 2, C – VIP 3, C – VIP 4

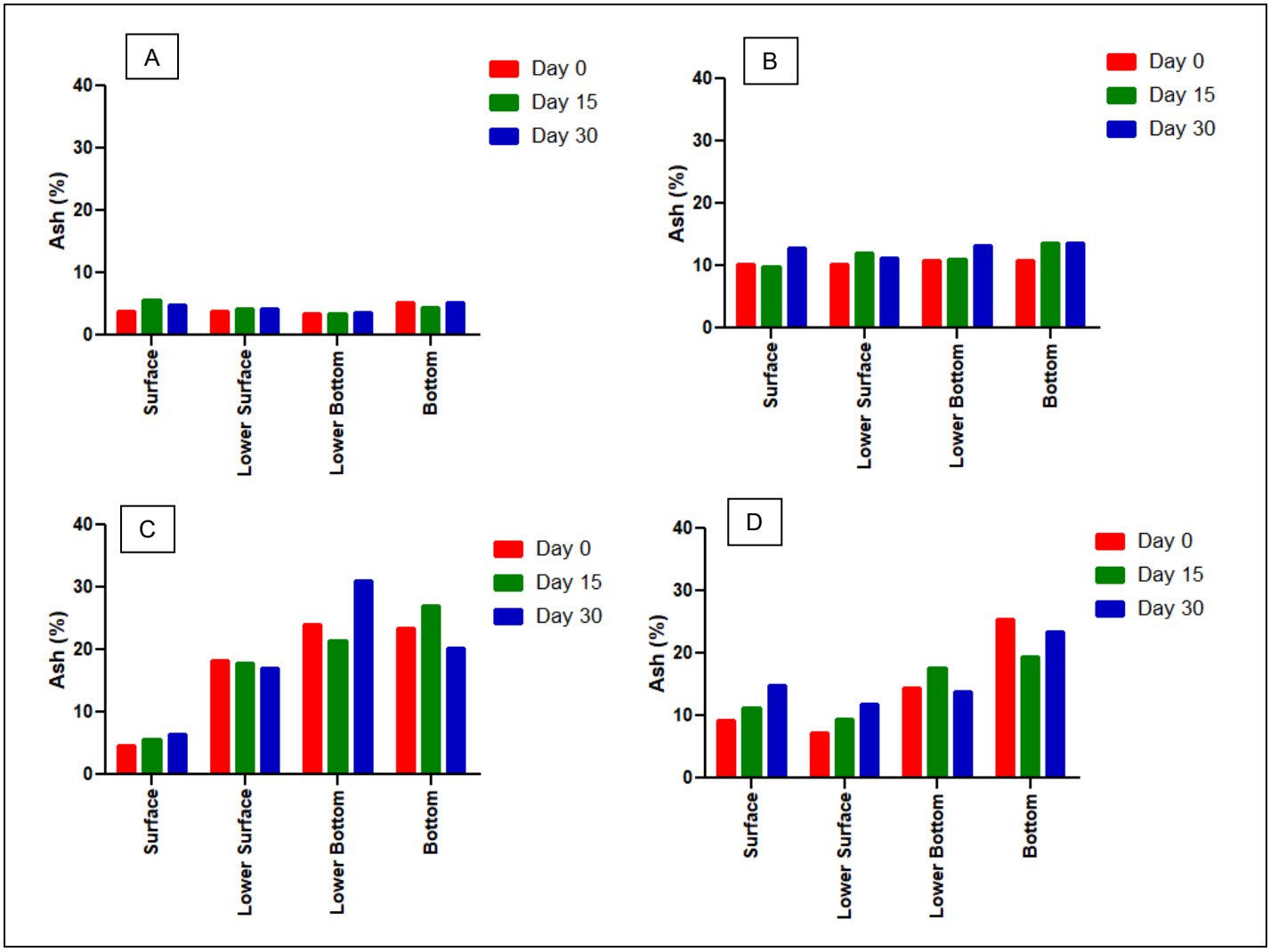


Figure 4-6: The ash content in faecal sludge from different layers of VIP latrines for 30 days in honey jars at room temperature. A – VIP 1, B – VIP 2, C – VIP 3, C – VIP 4

4.1.2 Chemical characteristics

This section provides the results obtained for the physical characteristics of the sludge, and represented by graphical trends for the VIP latrines. This includes total COD (Figure 4-7); soluble COD (Figure 4-8); percentage soluble COD_S/COD_T (Figure 4-9); ammonium (Figure 4-10); nitrate (Figure 4-11); and orthophosphate (Figure 4-12).

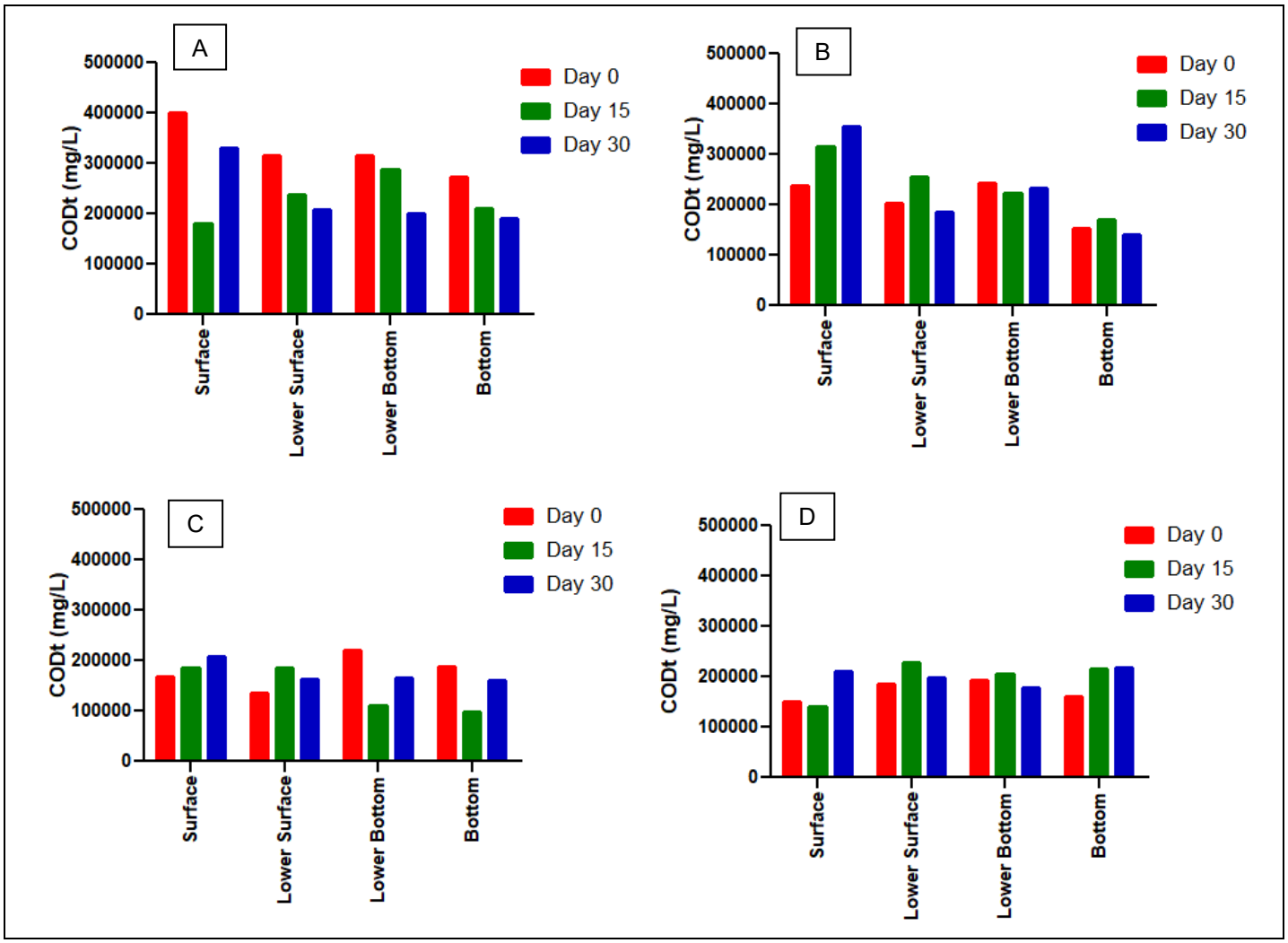


Figure 4-7: The total COD in faecal sludge from different layers in VIP latrines for 30 days in honey jars at room temperature. A – VIP 1, B – VIP 2, C – VIP 3, C – VIP 4

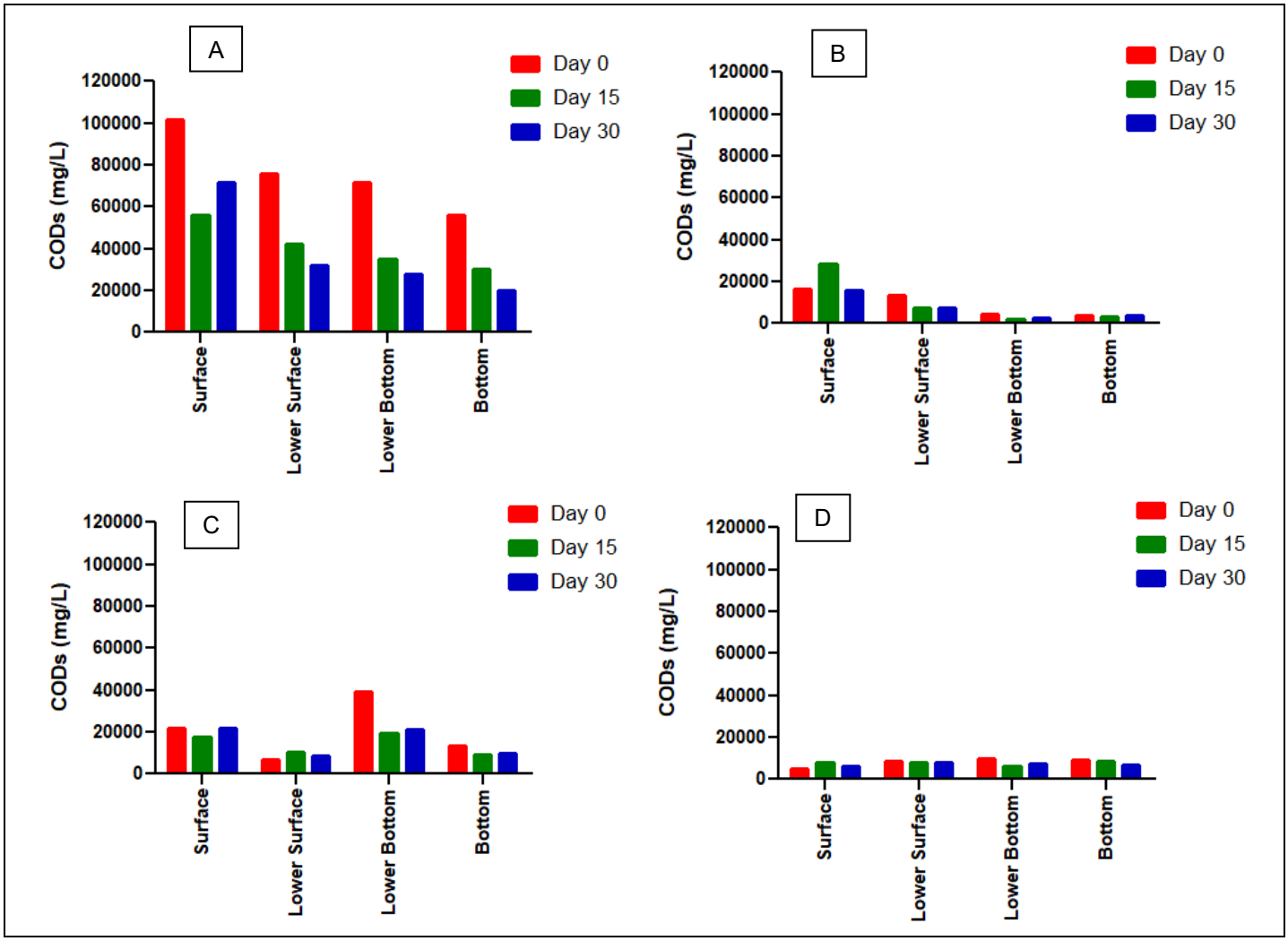


Figure 4-8: The soluble COD in faecal sludge from different layers in VIP latrines for 30 days honey jars at room temperature. A – VIP 1, B – VIP 2, C – VIP 3, C – VIP 4

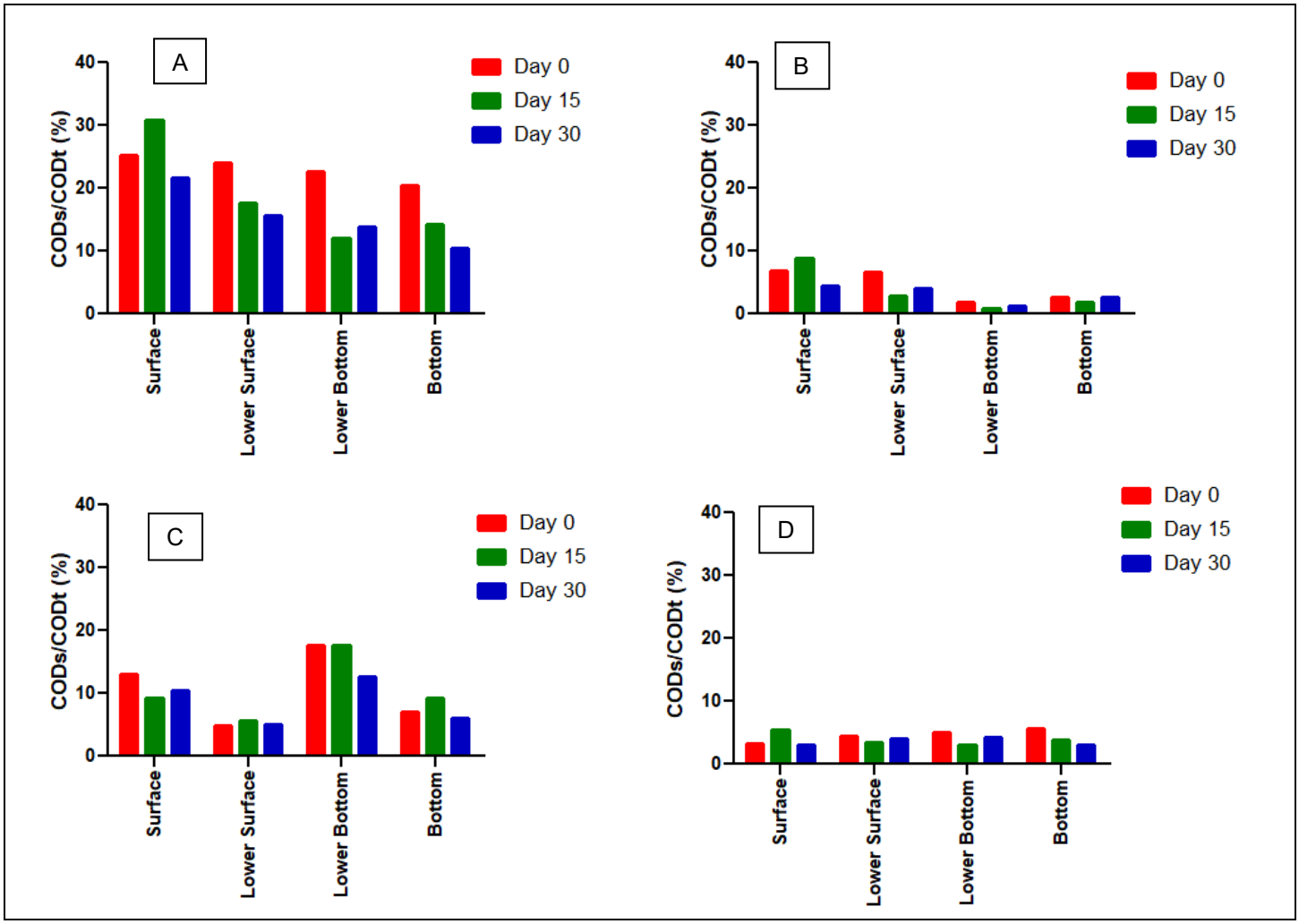


Figure 4-9: The percentage soluble COD_S/COD_T in faecal sludge from different layers in VIP latrines for 30 days in honey jars at room temperature. A – VIP 1, B – VIP 2, C – VIP 3, C – VIP 4

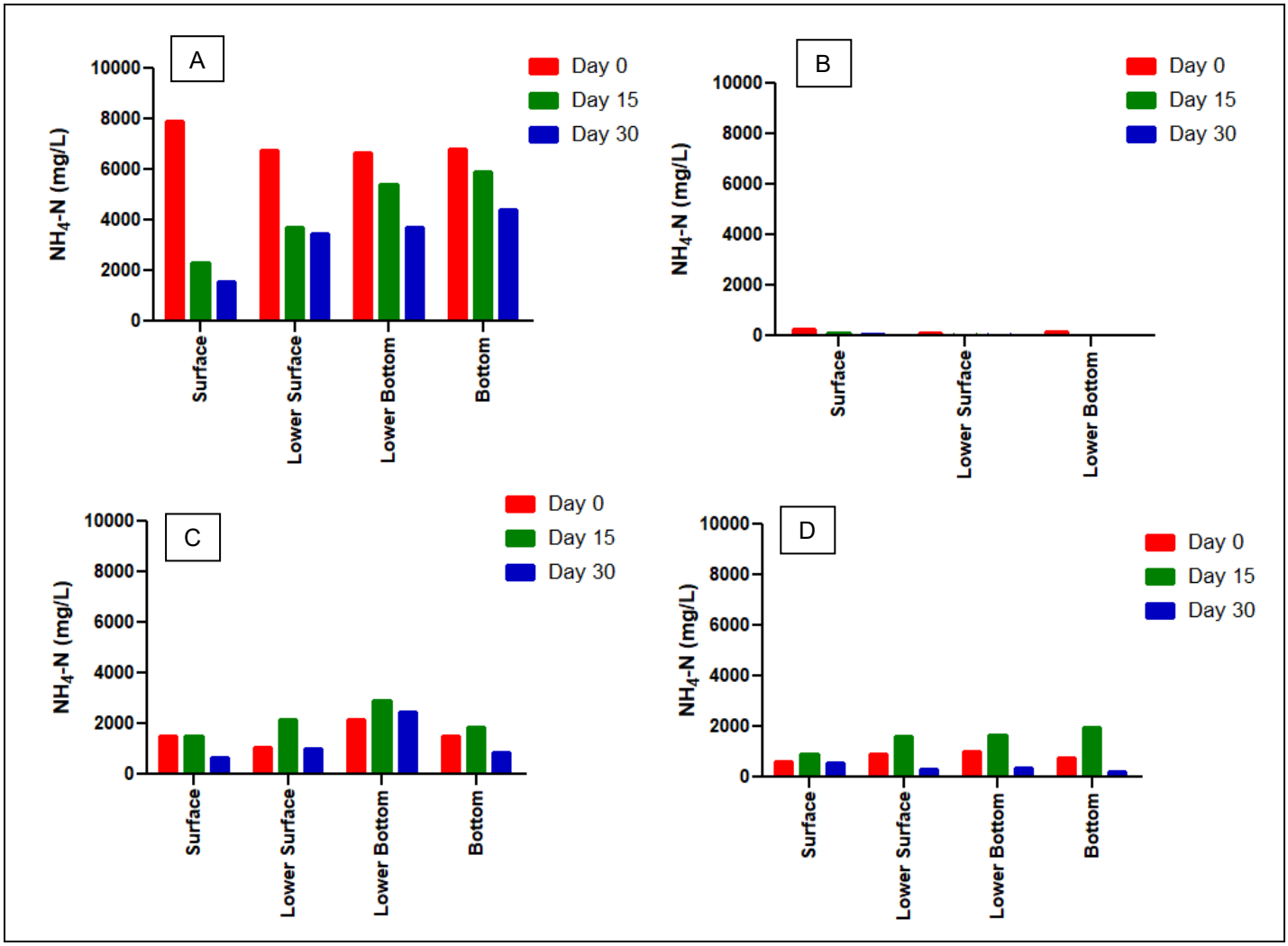


Figure 4-10: The ammonium (NH_4-N) in faecal sludge from different layers in VIP latrines for 30 days in honey jars at room temperature. A – VIP 1, B – VIP 2, C – VIP 3, C – VIP 4

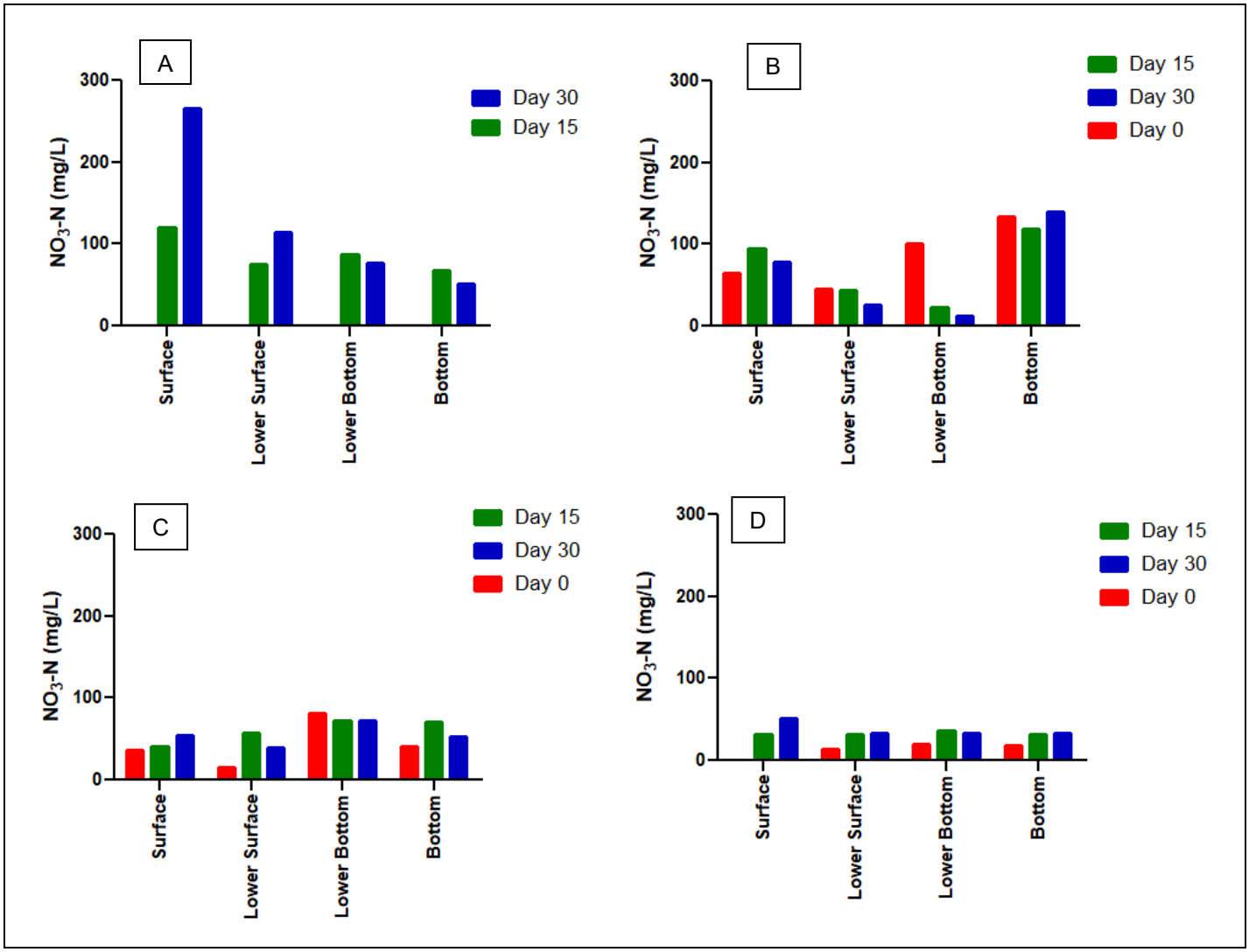


Figure 4-11: The nitrate (NO_3-N) in faecal sludge from different layers in VIP latrines for 30 days in honey jars at room temperature. A – VIP 1, B – VIP 2, C – VIP 3, C – VIP 4

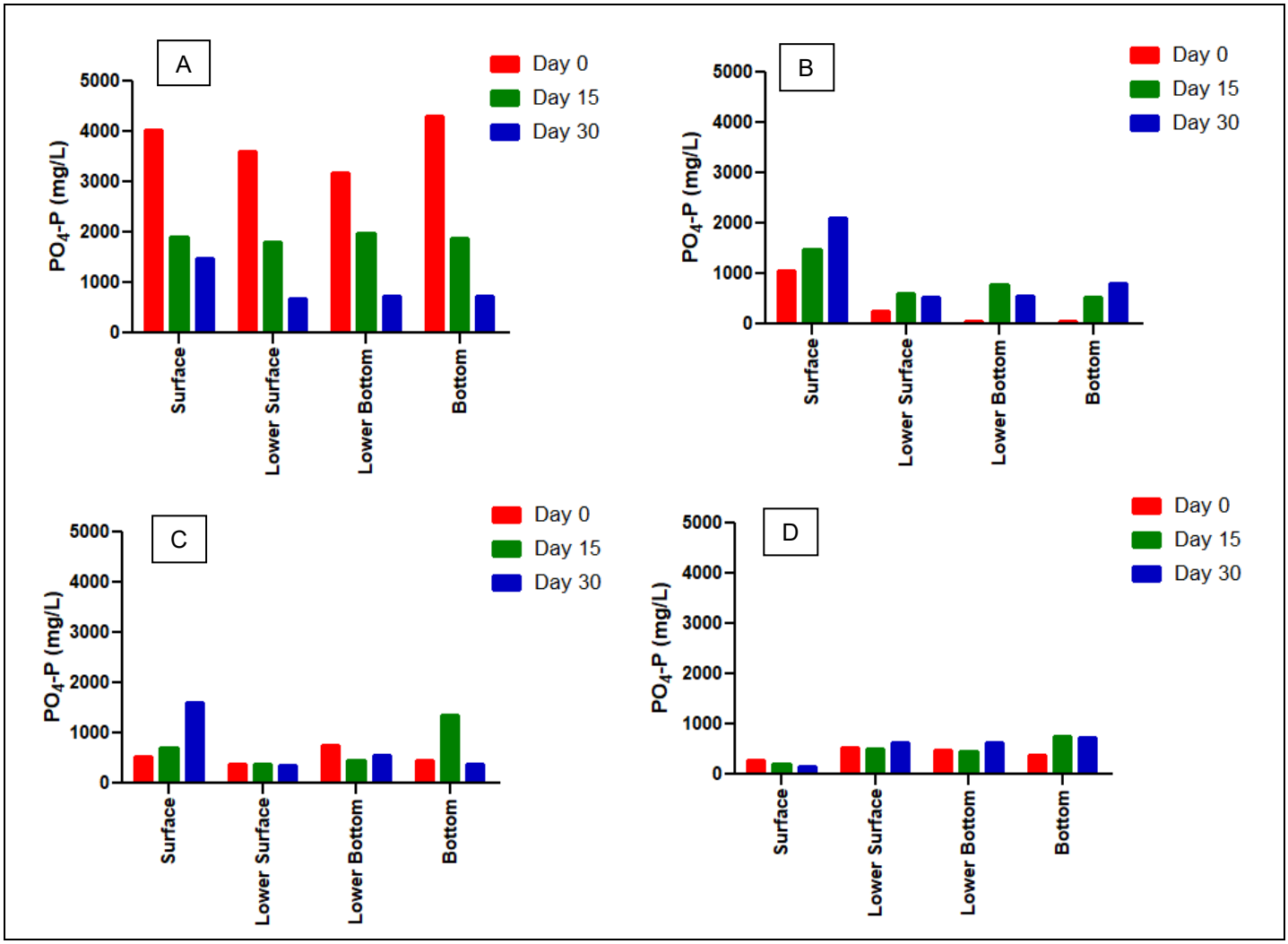


Figure 4-12: The orthophosphate (PO_4-P) in faecal sludge from different layers in VIP latrines for 30 days in honey jars at room temperature. A – VIP 1, B – VIP 2, C – VIP 3, C – VIP 4

4.2 Mass reduction of faecal sludge

As the faeces enter the pit, it undergoes a series of reactions resulting in products that are released from the pit and non-biodegradable matter that settle at the bottom of the pit. The processes occurring in the faecal sludge reduce the mass within the pit. Therefore, the change in the mass of faecal sludge was observed over 30 days. This is depicted in Figure 4-13 and Table 4-1.

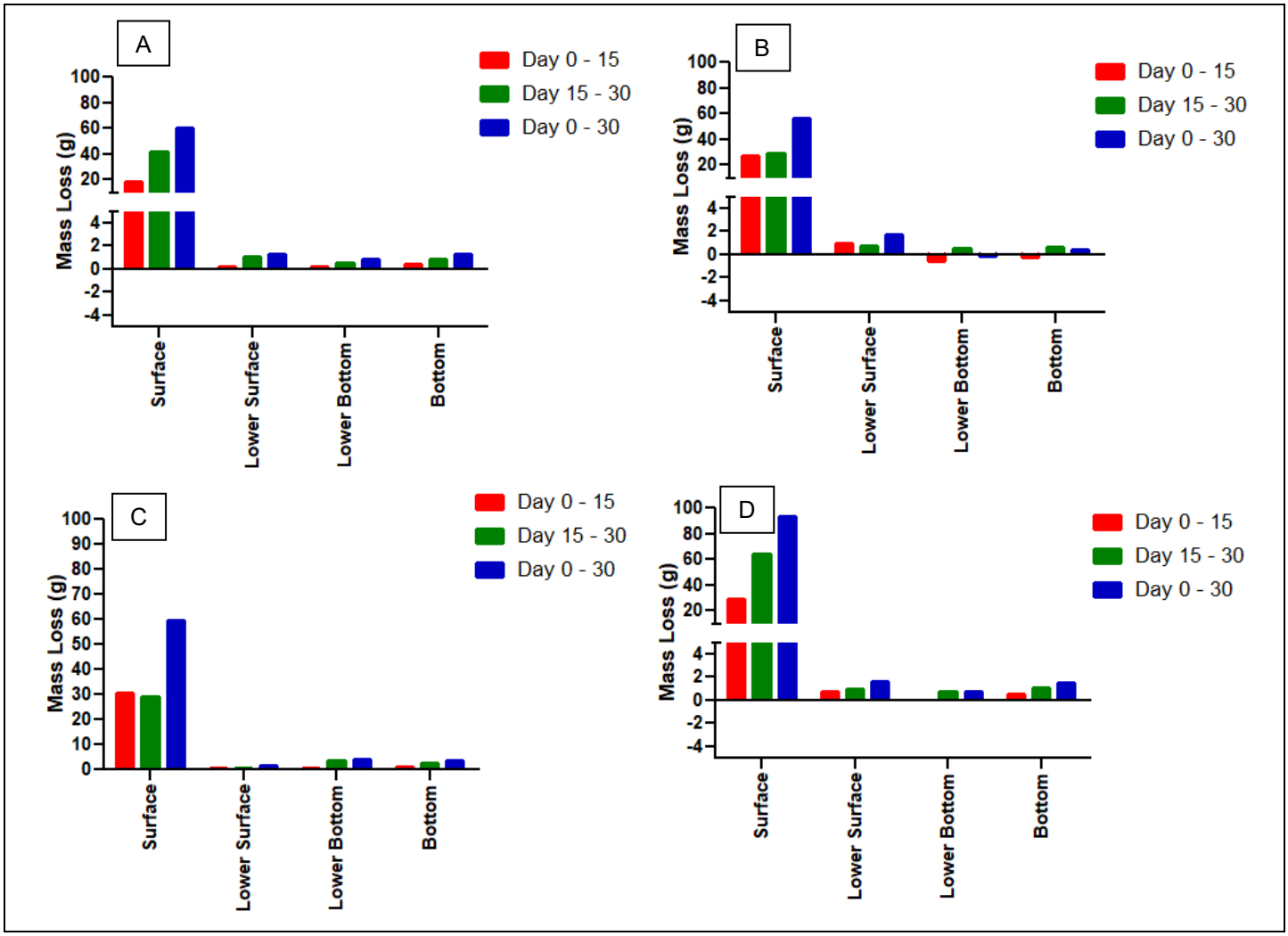


Figure 4-13: The mass loss of faecal sludge from different layers of the VIP latrines pit for 30 days in honey jars at room temperature. A – VIP 1, B – VIP 2, C – VIP 3, C – VIP 4

Table 4-1: % Mass loss of faecal sludge after 30 days incubation from different layers of VIP latrines

PIT LAYERS	% MASS LOSS			
	VIP 1	VIP 2	VIP 3	VIP 4
TOP	18.27	17.04	18.01	28.25
LOWER SURFACE (LAYER 2)	0.39	0.50	0.47	0.49
LOWER BOTTOM (LAYER 3)	0.25	-0.02	1.16	0.21
BOTTOM	0.38	0.13	1.07	0.46

4.3 Enumeration of Total coliforms and *E. coli* in faecal sludge

Microbes excreted from the gut may play a leading role in the initial formation of microbial communities in the faecal sludge in pit latrines. Faecal coliform bacteria are considered to be specifically present in the human or animal gut, while total coliforms include various bacteria not related to the gut environment. *E. coli* is considered to be the species of coliform bacteria that is the best indicator of faecal pollution. Therefore, total coliforms and *E. coli* were enumerated from the faecal sludge samples from different depths of the VIP latrines. These are shown in Figure 4-14 and Figure 4-15.

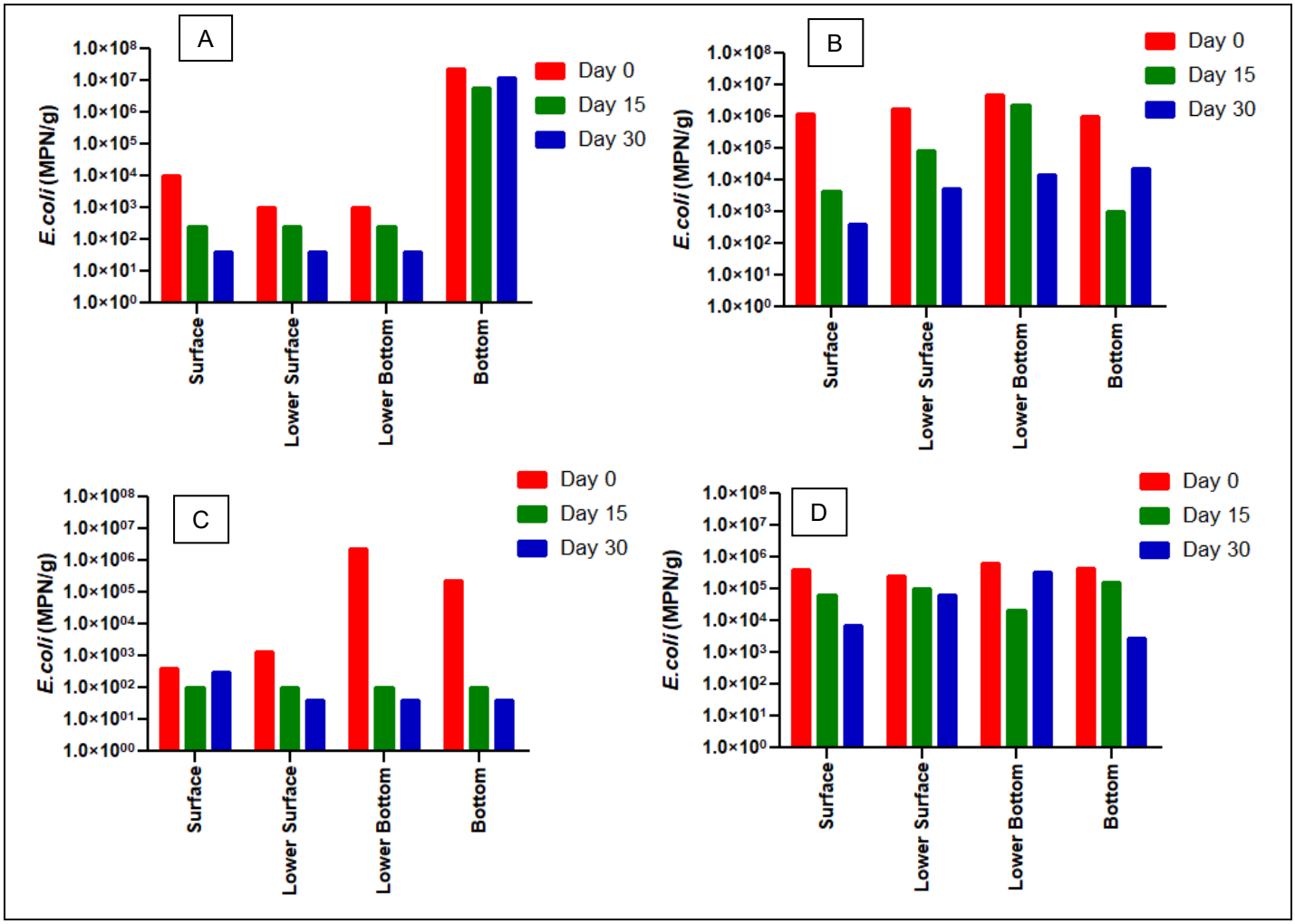


Figure 4-14: Log MPN/ g of *E. coli* present in faecal sludge from different layers for 30 days in honey jars at room temperature. A – VIP 1, B – VIP 2, C – VIP 3, C – VIP 4

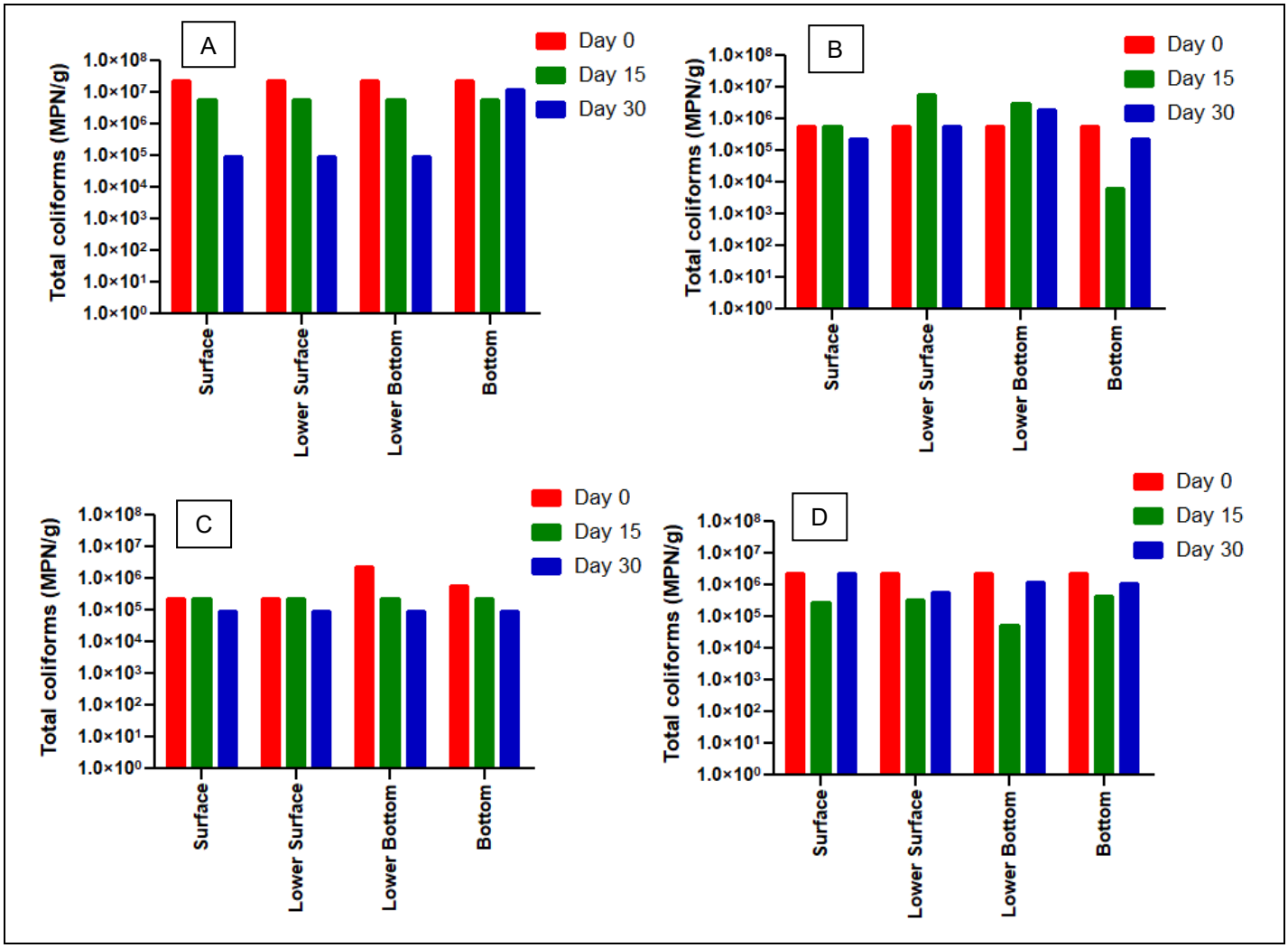


Figure 4-15: Log MPN/ g of total coliforms present in faecal sludge from different layers for 30 days in honey jars at room temperature. A – VIP 1, B – VIP 2, C – VIP 3, C – VIP 4

4.4 Microbiome Analysis

Microbes present in the faecal sludge play a key role in the biodegradation of faecal sludge with the pit latrines. The microbial community structure and abundance of community members can vary based on environmental factors. Therefore, samples were collected from different depths from pit latrines, prepared and sub-sampled during the laboratory experiments, and were analysed using Next Generation Sequencing (NGS) of 16S rRNA gene amplicons. The sequences were analysed at the Earth and Environmental Engineering department in Columbia University (USA) as outlined in Chapter 5.

4.5 Discussion of laboratory results

The results obtained in this study indicate that the biological, chemical, and physical characteristics of faecal sludge vary greatly from pit to pit, even within the same community. The faecal sludge collected from VIP 1 at different layers generally had a higher range of volatile solids, total COD, soluble COD, ammonium, orthophosphate and total coliforms compared to the faecal sludge collected from the other VIP's. Several studies have reported on the variation of physicochemical

characteristics of faecal sludge, and they attributed this variation to different household diets, lifestyles, and behaviour (Niwagaba et al., 2014b; Zziwa et al., 2016; Doglas et al., 2021).

Analysis of faecal sludge collected from different layers from each pit revealed that there were variations in the physicochemical characteristics of the faecal sludge analysed at Day 0. For example, properties such as pH ranged from 6.62 (VIP 2 layer 3) to 8.4 (VIP 1 layer 4) (Figure 4-1) and moisture content ranged from 82.62% (VIP 1 layer 4) to 63.76% (VIP 4 bottom) (Figure 4-3). In general, for all four VIP's sampled, the total solids, volatile solids, total COD and soluble COD decreased with an increase in depth. These observations match a previous study by (Zziwa et al., 2016).

Overall, across the four VIP's, pH appears to increase with time over the 30-day period, peaking at day 30 for the first two layers and at day 15 for the lower two layers (Figure 4-1). This pattern is not seen for the EC (Figure 4-2) with wildly varying peaks for each VIP, although the trend shows an increase in the EC for each pit over time, generally peaking at day 30. A decrease in EC indicates a decrease in dissolved salts and inorganic matter, therefore, the increase in EC can potentially indicate the metabolic conversion of organic matter, into inorganic matter, resulting in an increase in the salts concentration of the material. At high levels, EC can retard the growth of microorganisms by coagulating cell content (Thornton, 1912).

The moisture content was consistent over the 30-day period, especially for VIP 2 which was the driest material at the start (Figure 4-3). The moisture content for VIPs 3 and 4 fluctuated over time and between layers, being the lowest at layers 3 and 4, while the moisture content for VIP 1 remained fairly consistent over the 30-day period.

The total solids were the most stable for VIP 2 (being the driest) (Figure 4-4), with the highest total solids after the 30 days being for VIP 3 at the lower bottom (third layer) with 41.87%. The anaerobic process occurring within the pit stabilises the organic matter in the faecal sludge, reducing total solids quantity by converting part of the volatile solids fraction to gases (Gao et al., 2020). This was observed for VIP 2 with very similar total solids data throughout the pit layers and for the volatile solids (Figure 4-5) where the deeper layers had a lower percentage of volatile solids compared to the upper layers, potentially indicating pit layer stabilisation.

The ash content for VIP 1 was the lowest of all the sampled VIP's (Figure 4-6). This could indicate that the biological activity throughout the pit is still continuing as the state of the pit has not been flooded with stable inorganic material. VIPs 3 (31.09% lower bottom day 30) and 4 (23.38% bottom day 30) showed the highest percentage ash compared to the other pits, with these layers potentially being the most stable based on ash content. This could be due to non-biodegradable matter that stabilises at the bottom of the pit (Zziwa et al., 2016).

The physical characteristics determined for all the sampled VIP's would be considered favourable for microbial processes as overall, the pH ranges from ±6.35-8.91, with the optimal pH being 6.8-7.4, and a general moisture content of >60%.

Higher COD levels indicate a greater amount of oxidisable organic matter in the faecal sludge and the need for higher concentrations of dissolved oxygen that would be required for chemical reactions. Overall, the total (Figure 4-7) and soluble COD (Figure 4-8) decreased with increasing depth, however, there appeared to be little variation between the 2nd and 3rd layers within each of the pits. The volatile solids content and COD can both be used to determine the organic matter content of the faecal sludge. The fresh faeces entering the pit contain high amounts of organic matter resulting in high levels of COD and volatile solids at the top surface of the faecal sludge. A study by Bakare et al. (2012) observed a decreasing concentration of COD from the surface layer to the bottom and implied that an additional degradation or stabilisation occurs down the depth of the pit.

Ammonium concentration was highest at the top layer of VIP 1 (77929.83 mg/L), while VIP 2 had the lowest concentration overall, with the bottom layer being below the detection range of analytical kit used (Figure 4-10). VIP's 1, 3 and 4 were still actively used while VIP 2 was rarely used. This would explain the very low levels of ammonium, as the top layer in the pit receives urine which is composed of 0.05% ammonia. Nitrate in faecal sludge from VIP 1 was below the detectable level on day 0 (Figure 4-11), while for all other VIP's, the range was variable at each layer, but appeared to peak

between day 15 to 30. Orthophosphate for VIP 1 was significantly higher (almost double) the highest level from any other pit sampled (Figure 4-12), which was rapidly reduced from day 0 to 15, and again from day 15 to 30, indicative of very active microbial use as phosphate is a limiting nutrient. This pattern was not seen with the other pits, where the orthophosphate levels generally increased over the 30-day experiment.

E. coli, a faecal coliform bacterium that is typically present in the intestinal tract of warm-blooded animals, was detected in high concentrations in faecal sludge collected from the bottom of VIP 1 (Figure 4-14). This was unexpected as higher concentrations of *E. coli* are usually found within the freshly deposited material at the surface. *E. coli* was detected within all four layers for all four sampled VIP's at various levels of concentrations, with VIP 3 showing the lowest overall concentrations within the lowest three layers after the 30 days. This was potentially due to the addition of detergents and additives which are used for odour reduction and sludge stabilisation, and which could impact the number of *E. coli* in the faecal sludge due to their inhibitory features. The presence of bacterial predators such as protists and bacteriophages may also contribute to low levels of *E. coli* given that lytic coliphages are naturally present in human faeces. The presence of antimicrobial compounds in the faecal sludge (highly probable given the abundance of citizens receiving antibiotic treatments in communities using pit latrines) can greatly reduce microbial abundance and activity in the pit. In addition, the age of the pit can also be a factor in the microbial load as the organic matter may have been insufficient to support and maintain microbial communities. This pattern was not seen for the total coliforms with each layer of each VIP being consistent in their numbers, but varying by depth and day (Figure 4-15).

The breakdown of organic and inorganic matter in faecal sludge through a series of reactions results in products that are released out of the pit, consequently reducing the mass and/or volume of pit contents while non-biodegradable matters stabilise at the bottom of the pit. When the faecal sludge was monitored in honey jars over 30 days, only faecal sludge from the top surface of all VIPs showed substantial mass reduction compared to the lower three layers (Figure 4-13). This might be expected as the top layer of sludge is exposed to air where the air/sludge interface enables aerobic processes to occur, but also allows for a far greater rate of evaporation Aerobic processes are rapid reaction sequences resulting in the rapid breakdown of faecal sludge. Moreover, gases that are produced during aerobic processes are released from the faecal sludge and water evaporates; this was evident when the faecal sludge was slightly drier at day 30 than it was at day 0, and the top surface faecal sludge had the highest loss of moisture content (Figure 4-13).

Biodegradation processes occurring in the pit can change the characteristics of the faecal sludge due to products from chemical and biological reactions. As observed with the faecal sludge from VIP 1, properties from different layers such as pH increased to a range between 8.5 and 8.9, while there was a slight change of 0.5 to 4% in moisture content, total solids, volatile solids, and ash content. Microbial processes in faecal sludge can sequentially oxidise nitrogen compounds such as ammonium to hydroxylamine, nitrite and nitrate; thus, ammonium in faecal sludge from different layers decreased while nitrate had increased after 30 days of incubation (Figure 4-10, and Figure 4-11). The oxidation and hydrolysis of phosphorous produce phosphates which combine with available water producing a weak phosphoric acid that can cause a decrease in orthophosphate concentrations (Figure 4-12) and a change in the pH at 4 levels (Figure 4-1). However, orthophosphate from the faecal sludge of VIP 2 increased by a factor of 2 at the top and second layer sludge after 30 days and by more than 100 times at the third and bottom layer (from 43 to 804 mg/L, Figure 4-12).

The MPN values for total coliforms and *E. coli* present in the faecal sludge decreased over 30 days (Figure 4-14). The decrease in the bacterial numbers could be due to the change in the physical and chemical properties of faecal sludge taking place over 30 days. High pH levels (pH > 8.5) are known to cause a decline in coliform loads in faecal sludge (Appiah-Effah et al., 2020). Although the experimental setup mimicked the conditions of different layers of faecal sludge in pit latrines, i.e. maintaining aerobic conditions for the top layer sludge and anaerobic conditions for 2nd to 4th layer in the pit, the removal of faecal sludge from the cold room, then into the experimental honey jars may cause slight disturbance of the microbial communities.

5 CHAPTER 5: 16S rRNA AMPLICON NEXT GENERATION SEQUENCING

5.1 Background

This project also included a sequenced based analysis of the bacterial diversity of sludge across the sampled VIPs, and across the depths and days during the running of the experiments. The main aim was to enable the matching of the physicochemical properties and bacterial diversity using the 16S rRNA amplicon sequencing data generated at the Earth and Environmental Engineering department in Columbia University (USA), and to incorporate this data into a VIP reactor model in Chapter 6.

5.2 Methodology

Samples were prepared as described in Section 3.5.1. A total of 48 samples were sent for 16S rRNA amplicon sequencing analysis, using the general purpose bacterial primers 27f and 1492r to generate the amplicons for NGS analysis. After sequencing, the 16S nextflow pipeline from Epi2Me (Bioinformatics resources from Oxford Nanopore) was used for trimming and the read alignment. The Kraken2 classifier pipeline was used with the ncbi_16S_18S as reference database. The minimum read length was 1000, the maximum was 2000 with the minimum read quality score of 10. This generated bacterial classifications to the genus level with usable reads and relative reads data for the 16S rRNA sequences identified in the samples. Only the relative reads data was used for analysis.

Species level classification was attempted using minimap2 as the classifier, but for the requirements of this project, only the initial genus level data will be reported on, as the confidence level of the species data is much lower than that of the genus data especially for genera showing highly similar 16S rRNA gene sequences. Please note that the old bacterial terminology is being used here as this was based on the National Centre for Biotechnology Information database.

Bacterial functions were matched, where possible, with the MiDAS 4 field guide database based on the genus level classification (Dueholm et al., 2022; Dueholm et al., 2023). Table 5-1 contains a summarised description of the functions terminology used in the MiDAS field guide (MiDAS 2024) which will be used for the bacterial genus functions.

Table 5-1: Brief description of the functions / metabolisms from the MiDAS 4 field guide (MiDAS 2024)

Aerobic	Organisms able to grow optimally in the presence of oxygen, usually by using oxygen as electron acceptor.
Ammonia oxidizing bacteria (AOB)	The AOB are chemolithoautotrophic organisms, which oxidize ammonia to hydroxylamine using oxygen as an electron acceptor (aerobic process).
Nitrite oxidizing bacteria (NOB)	The NOB are chemolithoautotrophic organisms, which oxidize nitrite to nitrate using oxygen as an electron acceptor (aerobic process).
Polyphosphate accumulating organisms (PAO)	The PAOs are enriched for in wastewater treatment systems configured for enhanced biological phosphorus removal (EBPR). This is an aerobic process when they consume phosphates.
Glycogen accumulating organisms (GAO)	The GAO are well adapted to the dynamic conditions of EBPR where they compete with the PAO for resources. Under starvation conditions they can utilize stored glycogen aerobically or anaerobically as carbon or energy source. Some of these can synthesize polyhydroxyalkanoates (PHAs). This process can take place aerobically and anaerobically.
Nitrite reducing bacteria	The denitrifiers are usually facultative-anaerobic heterotrophic organisms utilizing nitrate and/or nitrite as electron acceptors in the absence of oxygen. This is a facultative anaerobic process.
Sulfate reducing bacteria	These organisms utilize sulfate as a terminal electron acceptor producing hydrogen sulphide. This is an anaerobic process.

Aerobic	Organisms able to grow optimally in the presence of oxygen, usually by using oxygen as electron acceptor.	
Fermenter	During fermentation, organic compounds act as both the electron acceptor and donor. This is an anaerobic process.	
Acetogen	Acetogenesis is a term routinely applied to broadly describe the biological synthesis of acetate. This is an anaerobic process.	
Substrate assimilation	Short chain fatty acids (SCFA)	Lipids/fatty acids
	Sugars	Carbohydrates/sugars
	Proteins and amino acids	Proteins/amino acids

5.3 Results and Conclusions

The results from the analysis/es are presented in this section. Averaged across all the sampled VIPs, the lowest read-based abundance of genera was observed for the lower surface at day 30 with about 619 (± 210) identified genera, and the highest for the lower surface at day 0 with about 1131 (± 697) genera (Figure 5-1). In total, summing all identified bacterial genera (including duplicates based on reads) there were 40 040 bacterial genera, with an average of 44% (

Table 5-2) matching with the MiDAS database for functional metabolic groups.

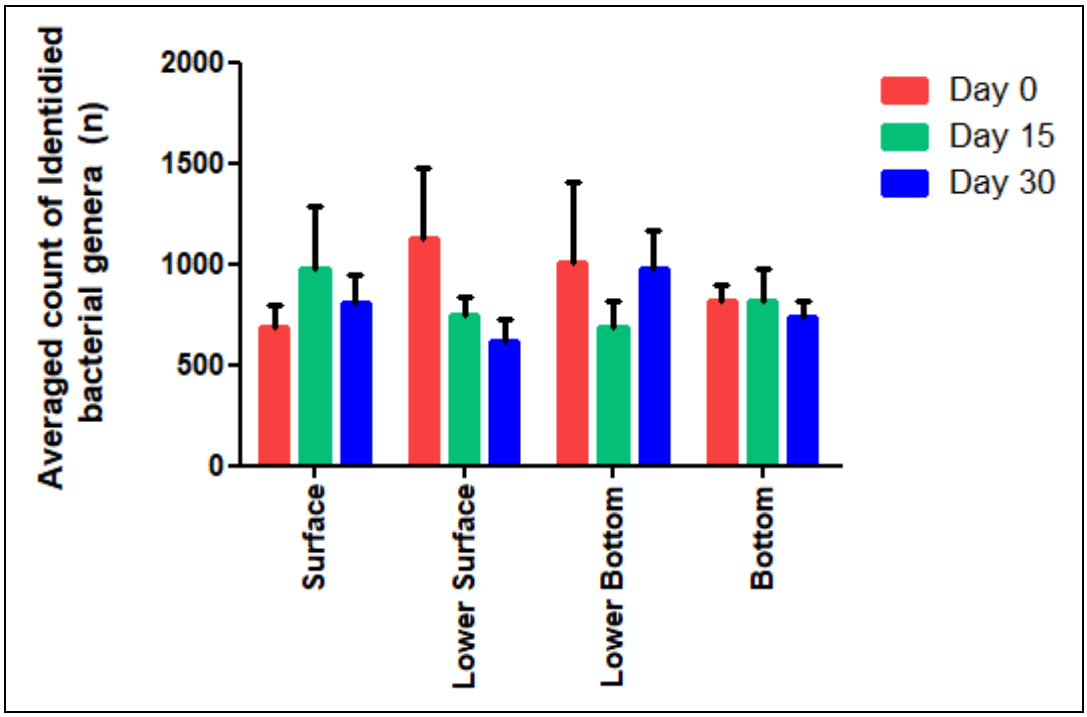


Figure 5-1: Averaged count of genera (n) across all sampled VIPs with at least 1 read from the 16S rRNA gene sequencing.

Table 5-2: Summed total count of all identified genera from the 16S rRNA sequencing across all sampled VIPs, depths and days, with the percentage of this data matched with the MiDAS database for metabolic functional groups.

	Sum	Average	Std. Dev.
Bacterial genera identified	40 040	834,17	392,92
% MiDAS matched		44,14	4,10

Figure 5-2 shows the percentage of total bacterial abundance at genus level based on the 20 genera with the highest abundance identified within each layer and at each day. The lowest values is for the bottom layer at day 30 with about 54 (± 15%) identified bacterial genera, whilst the highest number of bacterial genera was observed for is the lower bottom layer at day 0 with about 72 (± 14%). The 20 most abundant bacterial genera accounted for at least >52% of the total abundance based on relative reads.

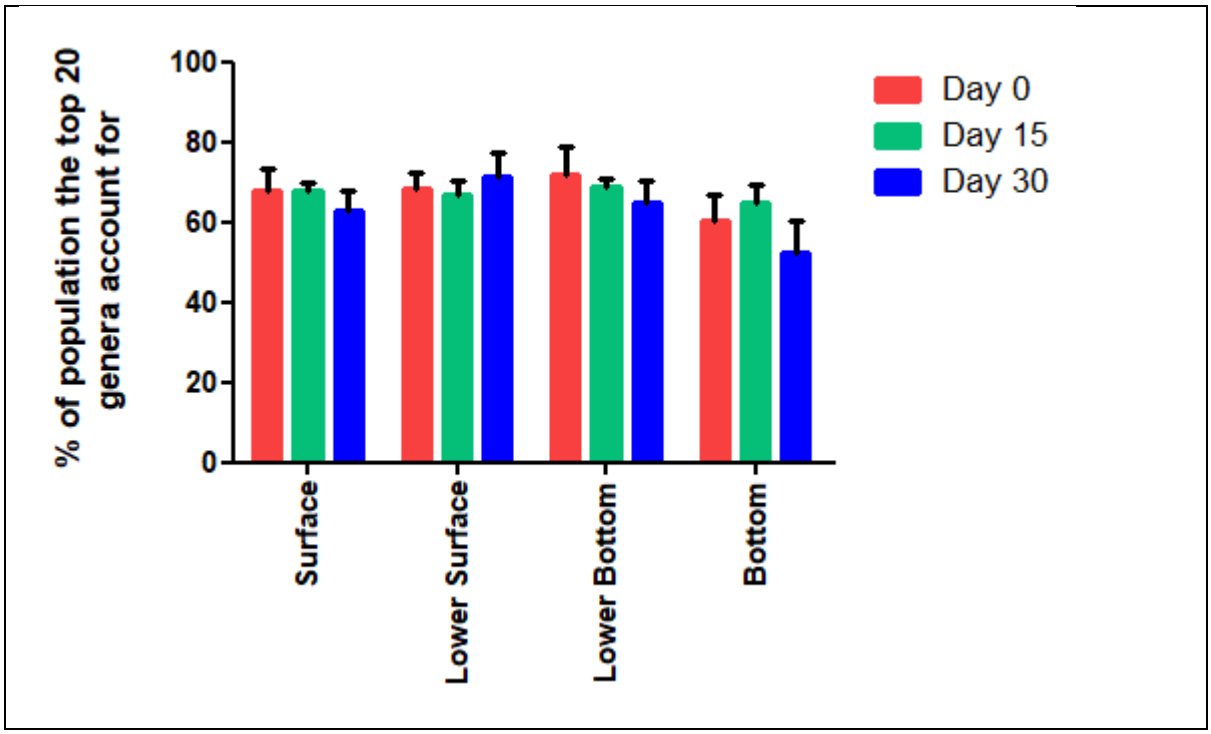


Figure 5-2: Relative abundance of bacterial genera based on the top 20 genera identified.

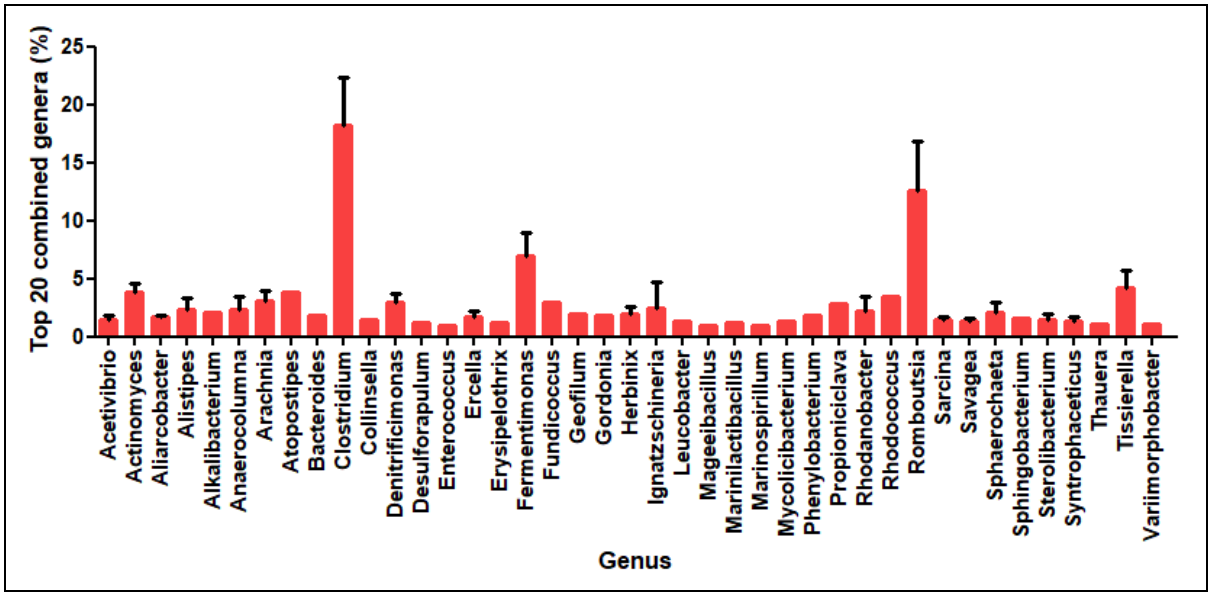


Figure 5-3: The top "20" identified bacterial genera, irrespective of sampled VIP, day or layer.

The sequence-based analysis of bacterial abundance across all samples revealed the dominance of members of the family Clostridiaceae (Figure 5-3), with "Clostridium" and Romboutsia" accounting for more than 25% of the 41 bacterial genera that are present within the "top 20" genera from each grouping. The sampling location common 7 bacterial genera account for more than 65% of the genera based abundance (Table 5-3).

Table 5-3: The common 7 genera from top 20 combined genera with basic information

	Genus	%	Metabolism	Energy source	Main metabolite	Pathogenic?
1	Clostridium	24,62	Anaerobic	Various organic matter (carbohydrates, proteins)	Acids, hydrogen	This genus contains pathogenic and non-pathogenic species.
2	Romboutsia	17,02	Anaerobic	Carbohydrates	Acids	Most current validly reported species (5) are considered as non-pathogenic.
3	Fermentimonas	9,42	Facultative anaerobic	Carbohydrates	Acids	One validly published species isolated from biogas reactors. (family Porphyromonadaceae), unknown.
4	Tissierella	5,65	Anaerobic/aerotolerant	Various organic materials.	Acids	6 validly published species, some clinical isolates regarded as potential pathogens.
5	Denitrificimonas	3,96	Facultative anaerobic	Organic materials	Nitrite, Nitrogen gas, acids	Only one validly published species (basonym=Pseudomonas), unknown.

	Genus	%	Metabolism	Energy source	Main metabolite	Pathogenic?
6	Anaerocolumna	3,09	Obligate anaerobic	Carbohydrates (e.g. cellulose), proteins, amino acids	Ethanol, acids	6 validly published species, family Lachnospiraceae, unknown
7	Ercella	2,23	Anaerobic	Glycerol	Succinate	Only one validly published species, unknown.

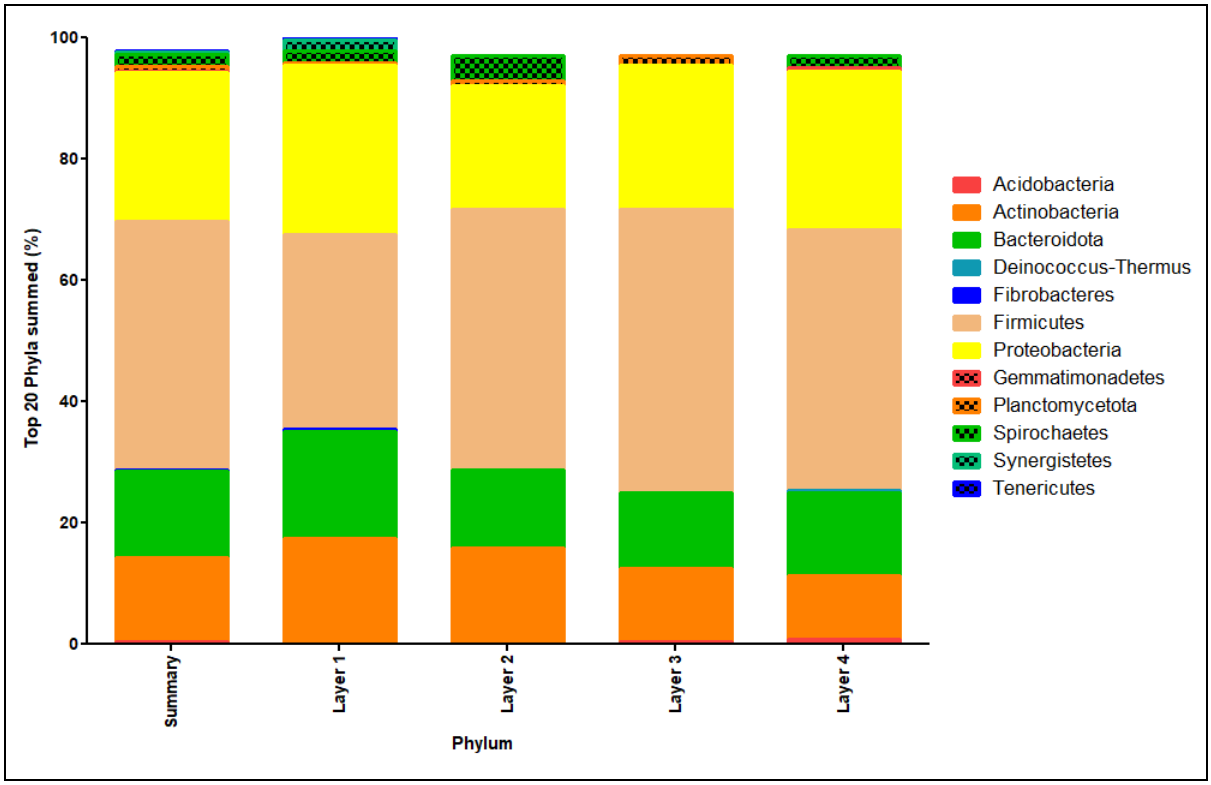


Figure 5-4: Phyla hosting the top 20 bacterial genera identified.

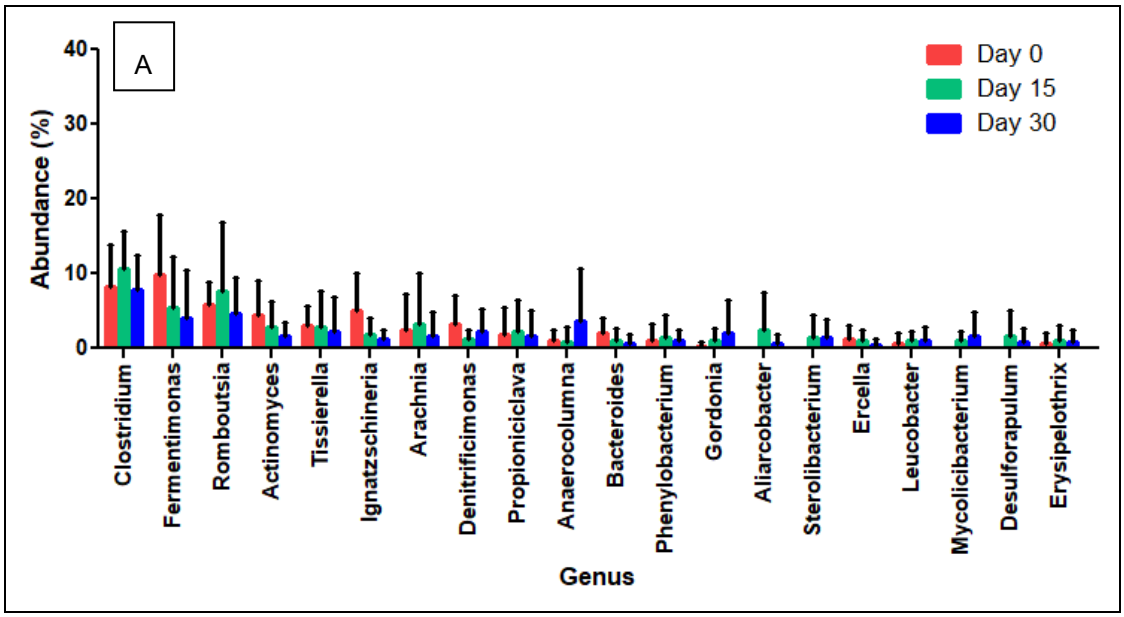
The 12 bacterial phyla that make up the top 20 bacterial genera (from Figure 5-3) account for about 98% of the phyla across all samples (Figure 5-4), while just the 5 most abundant phyla (

Table 5-4) account for about 96% of the phyla abundance. Four of our five top phyla fall within the six major phyla found globally within the human gut microbiota (Bliss and Whiteside, 2018; King et al., 2019). These results match those phyla found in other studies for a pour flush pit (Byrne et al., 2017), and other VIPs (De los Reyes III et al., 2017; Beukes, 2019) in South Africa.

Table 5-4: Top 5 most abundant phyla representing the top 20 bacterial genera and their possible human gut function

	Phylum	%	Main gut function
1	Firmicutes (now Bacillota)	41,14%	Carbohydrate, fat and protein metabolism, generating substrates to feed other members of the anaerobic food chain
2	Proteobacteria (now Pseudomonadota)	24,58%	Metabolically highly versatile phylum, can prepare the gut for colonization for strict anaerobes by consuming O_2
3	Bacteroidetes (now Bacteroidota)	14,16%	Carbohydrate fermentation to volatile fatty acids that can be used by the host or accompanying gut bacteria
4	Actinobacteria (now Actinomycetota)	13,95%	A number of species can produce secondary metabolites useful to the host
5	Spirochaeta (now Spirochaetota)	2,08%	Contains genera and species that can cause diarrhoea and bleeding

Six of the seven most abundant bacterial genera reported in Table 5-3 appear within all the pit latrine layers with varying abundance, but are not always present at each sampling occasion. The most common, abundant and prevalent genera appear to be Clostridium and Romboutsia, which are both present within every individual sampled VIP, within every layer, and found on each sampling occasion, which appear to match similar taxonomic data by Ijaz et al. (2022).



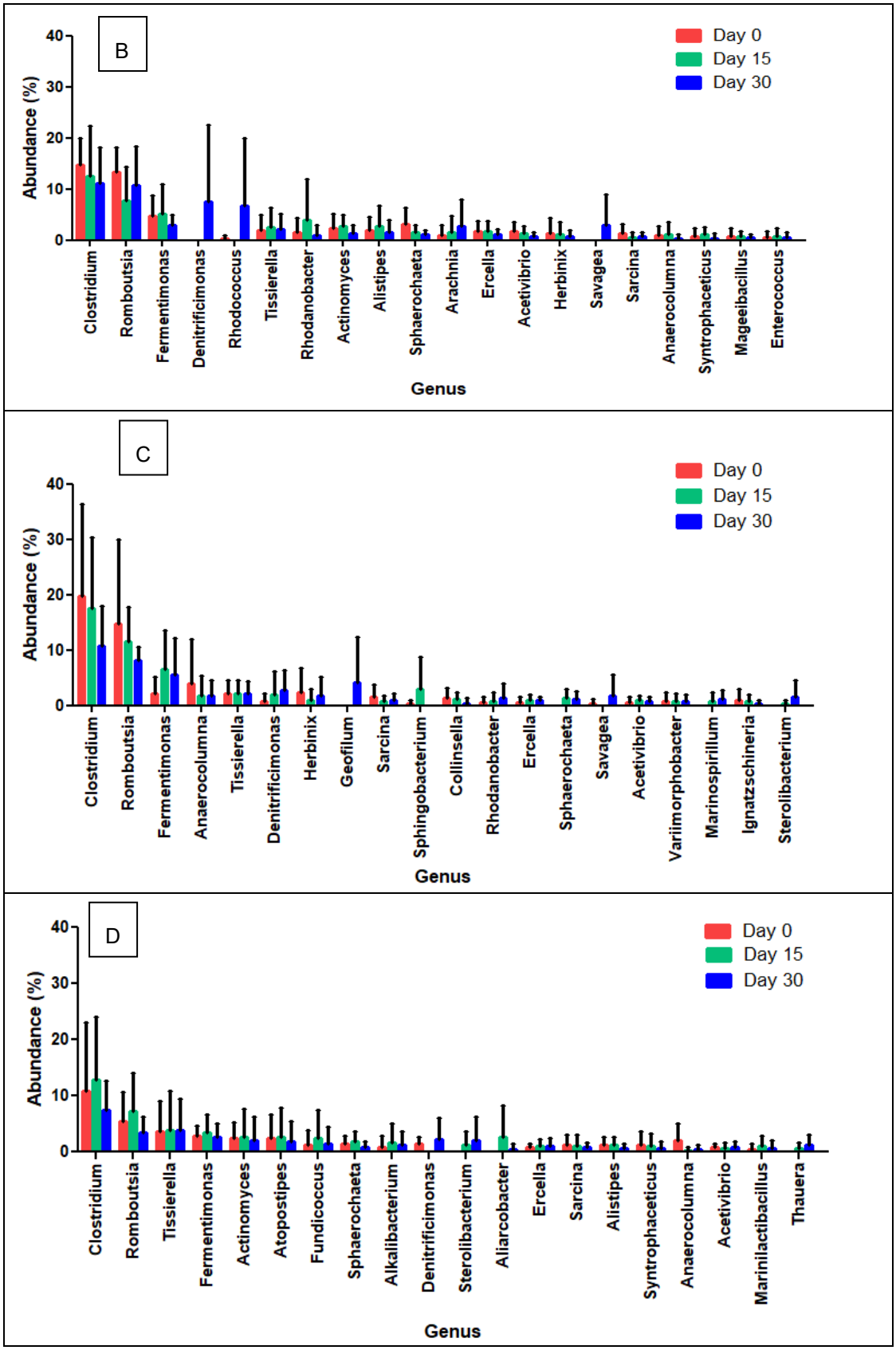


Figure 5-5: Average abundance (%) from all the combined VIPs for the top 20 genera for each sampled layer by day. Surface (A), lower surface (B), lower bottom (C) and bottom (D).

For the surface layer functional groups, about 44% (± 4.38%) were matched against the MiDAS 4 database. Over the 30 days the proportion of aerobes remained stable (Figure 5-6), with the same applying to AOBs and NOBs. The sugar consuming genera declined over time marginally from about 20% down to about 18%, indicating possible substrate limitation or increasing competition for a food source. The proportion of fermenters showed a similar trend (from about 14% down to about 10%) (Figure 5-6).

Of the lower surface layer functional metabolic bacterial groups identified, about 45% matched the MiDAS 4 database, the highest match for the sampled layer data. The lower surface layer is the boundary between being aerobic and anaerobic conditions, with already limited access to oxygen. The presence of both autotrophic aerobes and aerobic heterotrophs from day 0 to day 30 indicates the availability of oxygen within this layer (Figure 5-7), even though facultative anaerobes might take over by switching to alternative electron acceptors or by switching to fermentation. That said, the fermenters increased from 10.71% (Day 0) to 12.22% (Day 30), with categorized anaerobes increasing by 2.49% over 30 days for the lower surface layer.

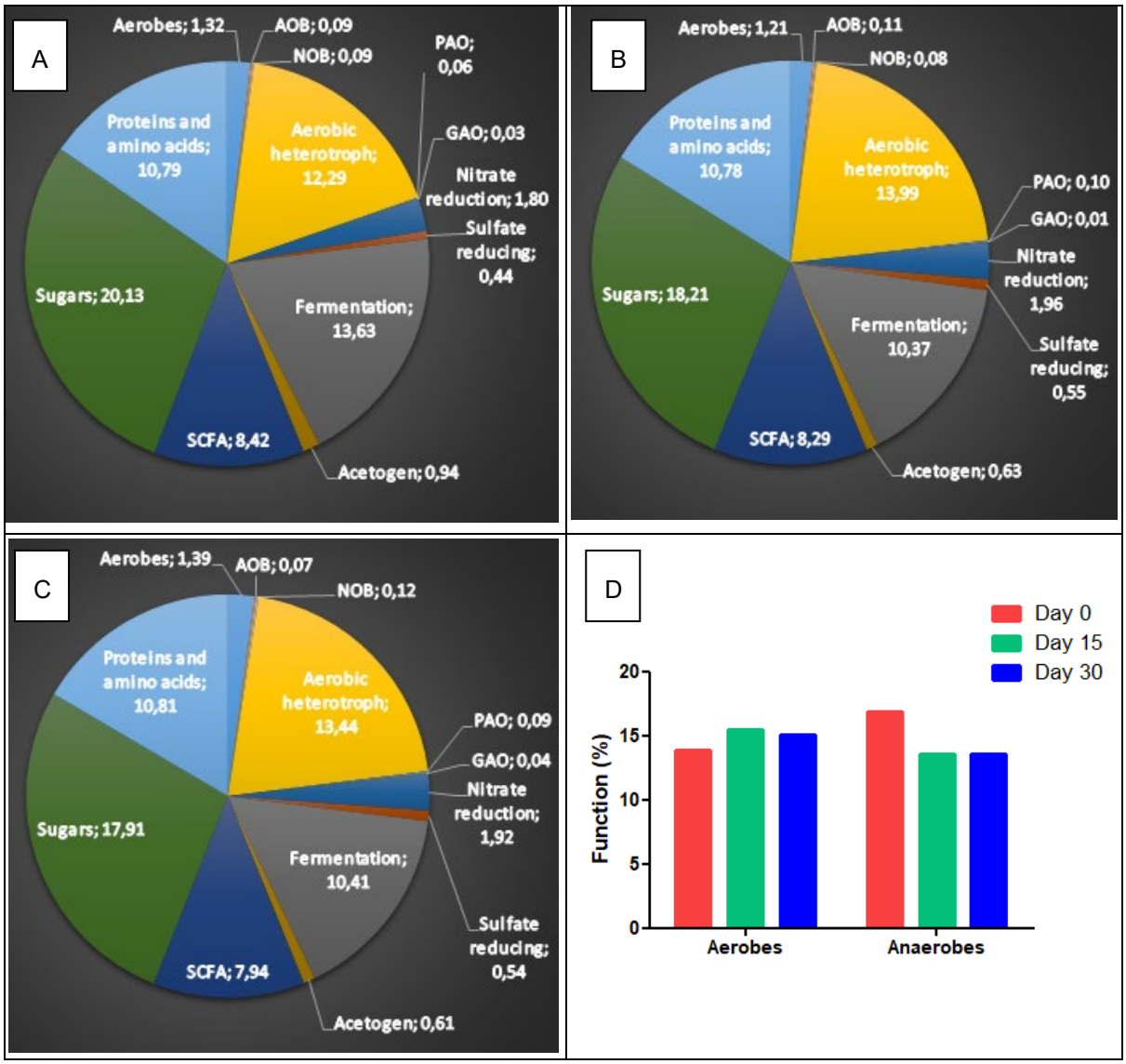


Figure 5-6: Averaged surface layer functional groups (%) across all sampled VIPs, for the entire experimental dataset, for Day 0 (A), Day 15 (B), Day 30 (C) and aerobes and anaerobes (D, excluding substrate assimilation functions), for the positive matches against the MiDAS 4 database.

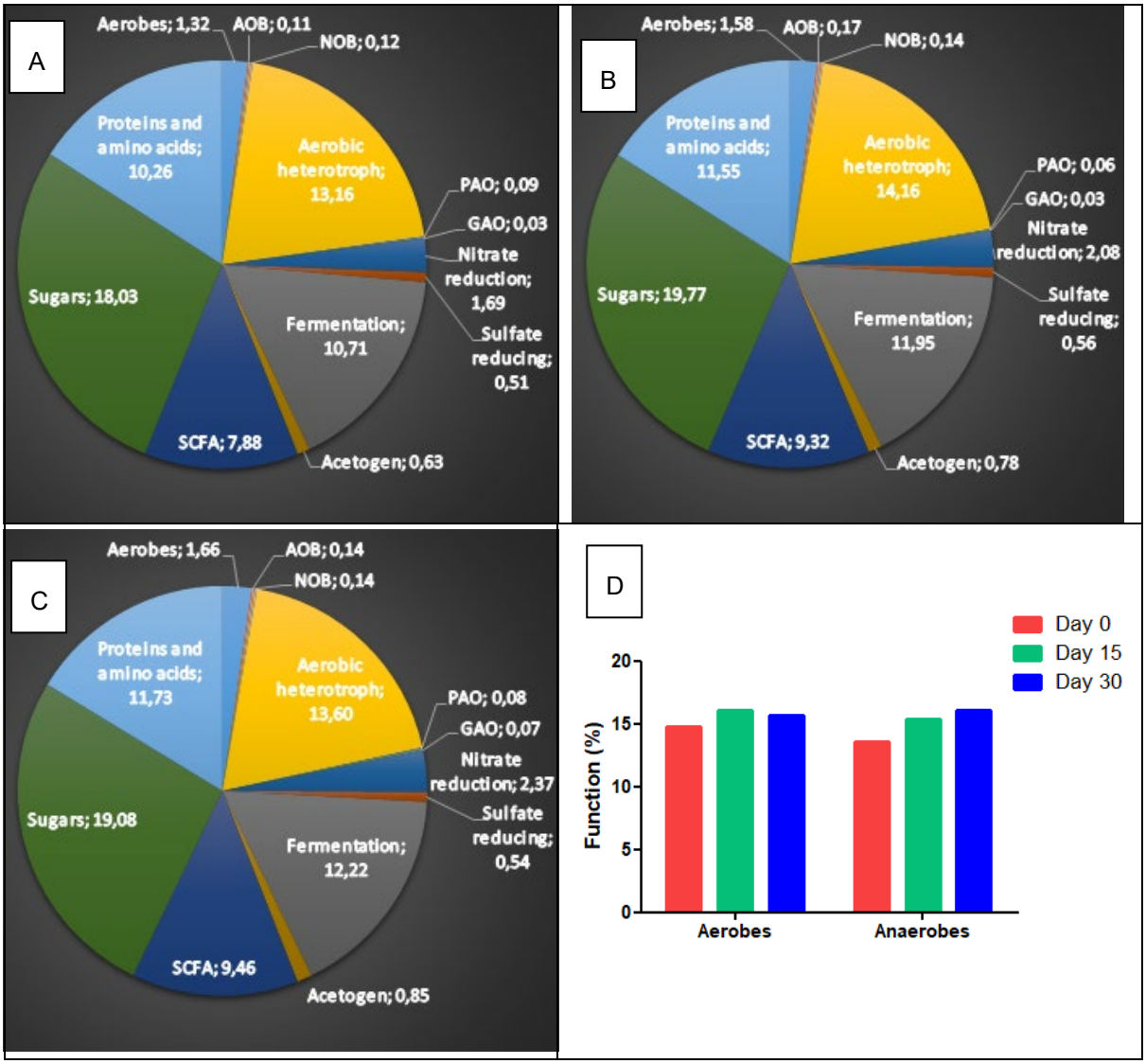


Figure 5-7: Averaged lower surface layer functional groups (%) across all sampled VIPs, for the entire experimental dataset, for Day 0 (A), Day 15 (B), Day 30 (C) and aerobes and anaerobes (D, excluding substrate assimilation functions), for the positive matches against the MiDAS 4 database.

For the lower bottom layer, bacterial functional metabolic groups matched to about 43% the MiDAS 4 database. This layer is completely anaerobic (Buckley et al., 2008) unless mixing and gas exchanges takes place, with anaerobes are about 2% more abundant than aerobes based on matched metabolic function, after 30 days the aerobes had increased by 2.06%, while the anaerobes decreased by 1.98% (Figure 5-8 D). However, after 30 days anaerobic processes appear to be dominating.

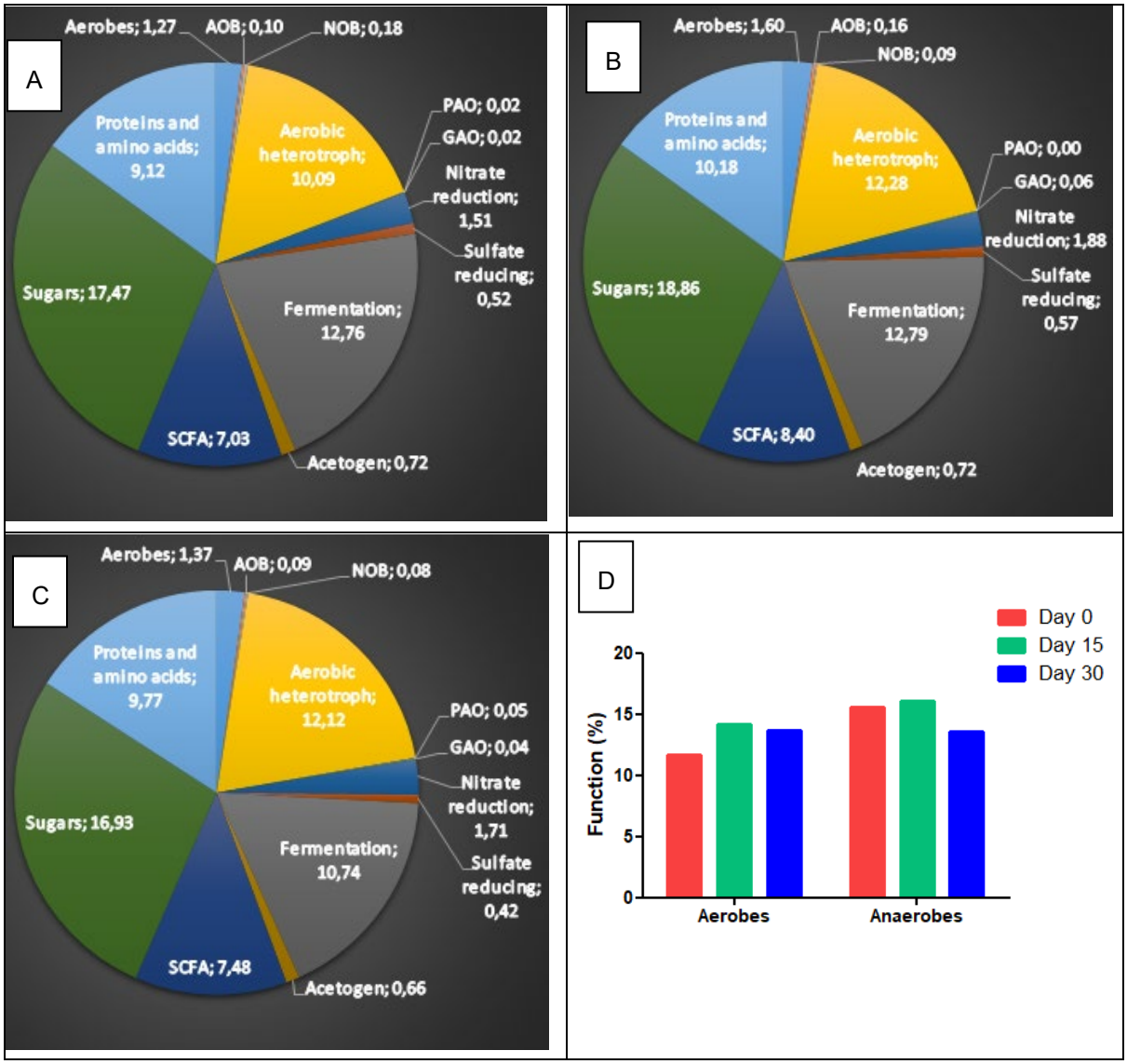


Figure 5-8: Averaged lower bottom layer functional groups (%) across all sampled VIPs, for the entire experimental dataset, for Day 0 (A), Day 15 (B), Day 30 (C) and aerobes and anaerobes (D, excluding substrate assimilation functions), for the positive matches against the MiDAS 4 database.

The final and deepest layer of the VIP (Figure 5-9), again while meant to be strictly anaerobic, still appears to contain bacteria categorized as aerobes (Figure 5-9 D). However, this might be due to the matching percentage to the MIDAS database was always below 50% and the category aerobes might contain members such as Pseudomonadota, many of which can switch from aerobic metabolism to anaerobic respiration of even fermentation such as many Enterobacterales. In addition, disturbance of the sludge pile introducing oxygen supporting strictly aerobic bacteria cannot be excluded.

Overall, the dominance of bacteria assigned to the genera Clostridium and Romboutsia is striking, even though these are typical gut inhabitants and are involved in the anaerobic food chain. Nevertheless, the proportion of bacteria assigned to metabolic categories using the MIDAS database appears to be fairly stable. However, the lower surface showed an increase in anaerobic functions as would be expected on microbiological grounds. Similarly, fermentation did appear to increase with increasing depth but with limited variation observed on the different sampling occasions, results differing from Smith et al. (2023) when sampling Malawian VIPs.

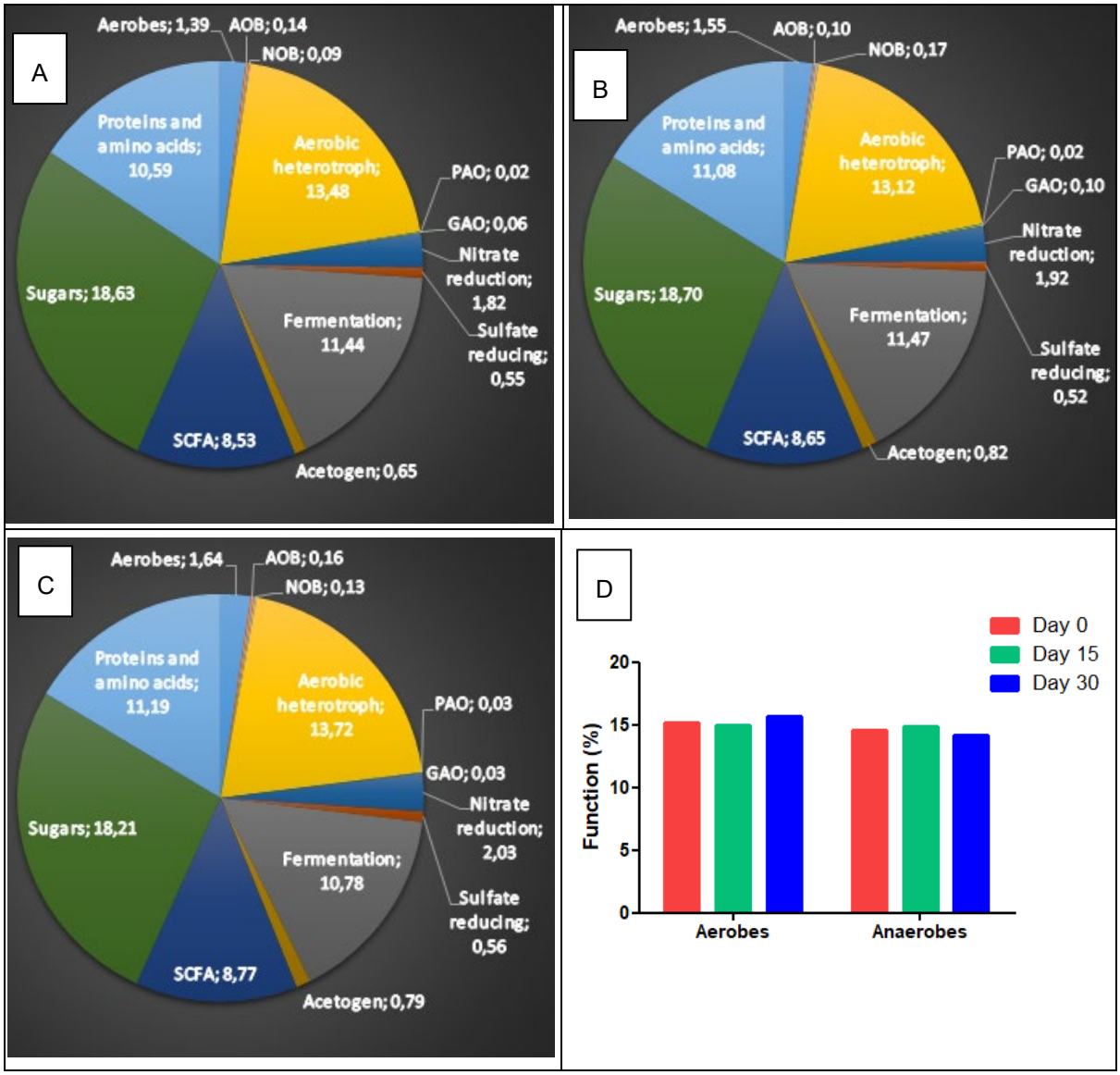


Figure 5-9: Averaged bottom layer functional groups (%) across all sampled VIPs, for the entire experimental dataset, for Day 0 (A), Day 15 (B), Day 30 (C) and aerobes and anaerobes (D, excluding substrate assimilation functions), for the positive matches against the MiDAS 4 database.

6 CHAPTER 6: DEVELOPMENT OF A REACTOR MODEL

6.1 Background

The study of the accumulation of material in VIP latrines has been the subject of several papers (Nakagiri et al., 2017; Gudda et al., 2019; Mubatsi et al., 2021). The modelling of the filling rate of VIP latrines is important in terms of understanding the service life of the pit, and for developing strategies for emptying such pits and extending the useable life. There are several complexities encountered when developing a model for this system. Firstly, the material flow into the pit and the contents of the pit have varying characteristics. Often it is not only the human excreta and anal cleansing materials that are extant, but also a range of foreign matter (trash). Secondly, the biological processes that are underway inside the pit are not uniform, and vary depending on the location within the pit. Typically, the pit has an aerobic surface layer, which comprises newly added material. Deeper into the pit the biological processes are mostly anaerobic.

Brouckaert et al. (2013) developed a sludge accumulation model for VIP latrines, based on conventional residence time distribution theory. The basis of the model was a macro-scale fractionation of material in the pit, based on biodegradability. This is shown in Figure 6-1.

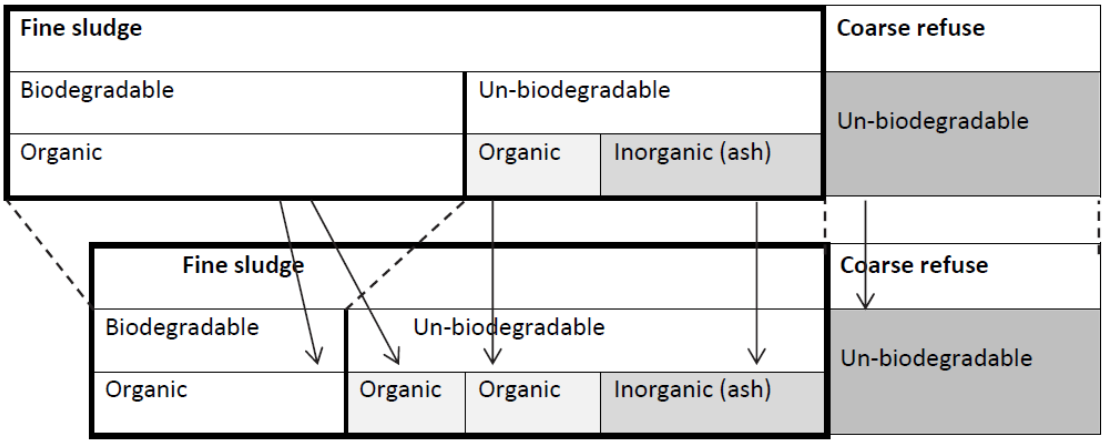


Figure 6-1: Fractionation of the material in a VIP latrine, showing transformations over time (Brouckaert et al., 2013)

Assuming fixed rates of biodegradation of organic matter into non-biodegradable material (in a single anaerobic layer), and considering the flow and age of material within the pit according to the segregated flow model, the authors were able to establish a relationship between the combined volume of material (both biodegradable and non-biodegradable) and time (Equation 2).

$$V(t,T) = R_u\left[\left(1 + k\frac{v_{b0}}{v_{u0}}\right)(T-t) + \left((1-k)\frac{v_{b0}}{v_{u0}}\right)\frac{(e^{-rt}-e^{-rt})}{r}\right] \qquad \textbf{(Equation 2)}$$

where V is the volume of the material in the pit, t is the time, T is the total age of the pit, r is the first order rate constant for biodegradation, v_{b0} is the initial volume of biodegradable material, v_{u0} is the initial volume of non-biodegradable material and k is the new non-biodegradable material.

The model has certain limitations, i.e. it considers all bio-degradable material to have the same rate of transformation and assumes that the feed addition rate is constant. Nevertheless, the model showed satisfactory agreement with a range of temporal data representing the physicochemical characteristics of the pit (e.g. ash content and COD). The model was also used to predict the fill rate for pits with varying input values for the non-biodegradable material (trash). Unsurprisingly, the higher the latter, the quicker the fill rate of the pit and the lower the utilization (Figure 6-2 and Figure 6-3).

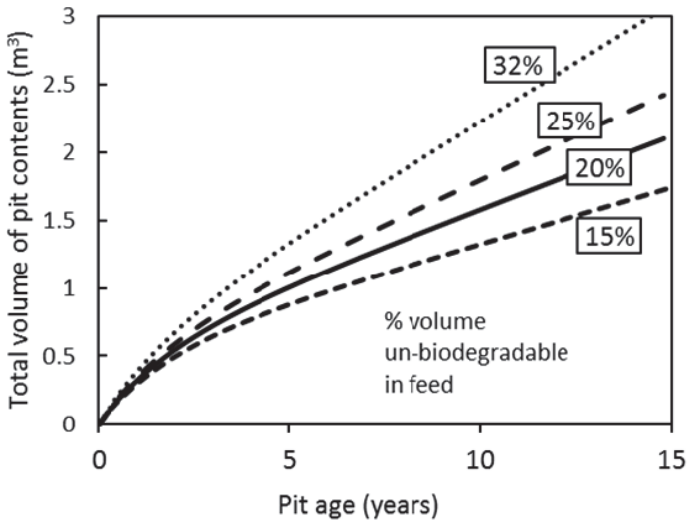


Figure 6-2: Total volume of pit contents versus time with varying input fractions of non-biodegradable material (trash), according to the sludge accumulation model of Brouckaert et al. (2013)

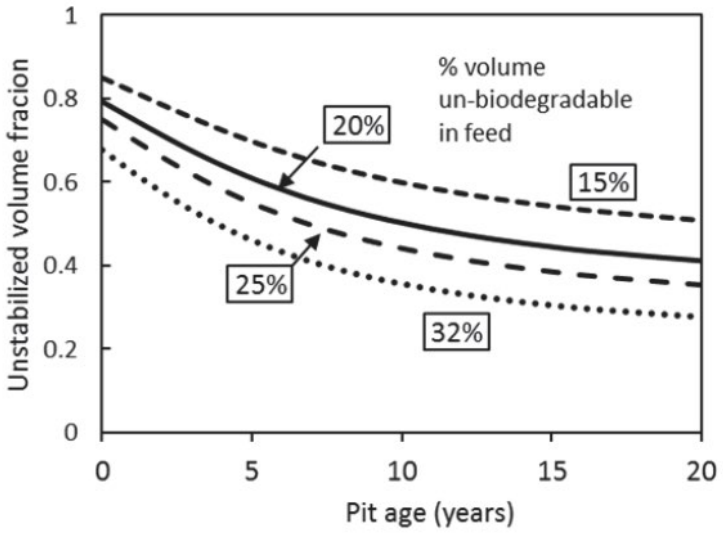


Figure 6-3: Utilized volume fraction versus time with varying input fractions of non-biodegradable material (trash), according to the sludge accumulation model of Brouckaert et al. (2013)

Todman et al. (2015) modified the model of Brouckaert et al. (2013) for pit latrines in Tanzania. These pits were characterized by low trash volumes and hence the model neglected the accumulation of the trash fraction. The model provided reasonable agreement with average experimental fill rates, but could not accurately predict fill rates of individual pits (Figure 6-4). Moreover, the authors concluded that the flow and accumulation of water in the latrine has an important effect on the fill rate.

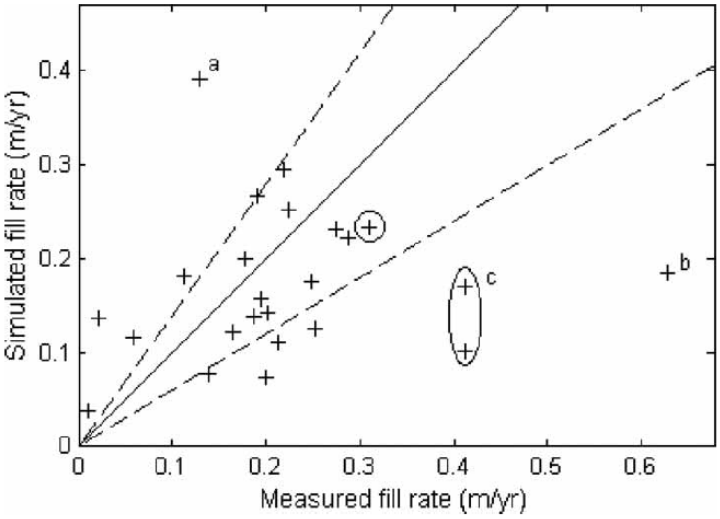


Figure 6-4: Parity plot of simulated and measured filled rates according to the model of Todman et al. (2015), with the majority of data within 40% error

Ifeuzu and Chukwuemeka (2021) utilized the sludge accumulation model of Brouckaert et al. (2013) for analysing the filling rate of pit latrines in Auk, Nigeria. Following reparameterization and model identification, the authors were able to achieve predictive fits of greater than 93% of the data collected from 15 pits and 100 households in the area. The empiricism of the final model, however, implies that the results cannot easily be extrapolated to other cases.

The macro-models of filling rate of VIP latrines ignore the variation in microbiological activity within the various layers of the pit (according to oxygen availability and the prevailing microbiological community). Buckley et al. (2008) provided a succinct description of the various layers and the type of biological processes occurring therein. Figure 6-5 shows four distinct regions. The first layer comprises material freshly introduced to the pit, where the biological processes are akin to the human gut. It is almost impossible to sample this from the pit in practice. The next layer is sufficiently close to the surface and with enough oxygen to be characterized by aerobic degradation of hydrolysable organic material. In the third layer, the amount of oxygen is limited by the material above, so the prevailing process is anaerobic digestion. In the last layer, theoretically no further degradation is extant.

Bakare et al. (2012) confirmed this hypothesis by conducting a comprehensive testing campaign on the sludge contents of VIP latrines. Amongst the various characterization data, the authors found that the volatile solids, COD and biodegradability were all dependent on the location within the pit. The amount of organic material decreased with depth, which is congruent with the hypothesis and the rates and types of biodegradation that are prevalent in the various layers.

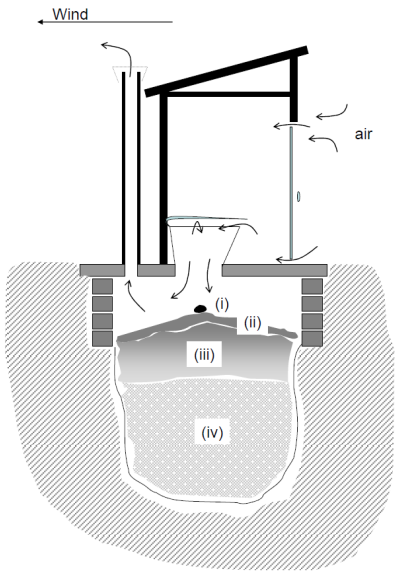


Figure 6-5: Schematic of a VIP latrine, showing the different layers present (Buckley et al., 2008)

Although the macro-scale physicochemical factors, e.g. COD, are useful in tracking the overall transformations of organic material within the pit, these are merely response variables. The biological processes are driven by the microbiota. The microbial community within the pit may be dependent on both the gut microbiome of the users and environmental factors. Ijaz et al. (2022) conducted a comprehensive study of the microbial community within different layers of pit latrines. Microbes can enter the pit from the human gut, as well as from the surrounding environment (air, soil, water). They found a clear sample depth gradient within the pits, with gut-derived microbes associated with the upper layers and environmentally-associated microbes with the lower layers. Importantly, these spatial differences also correlated with the pit fill rates. Torondel et al. (2016), in an earlier and related study, investigated the prevalence of these two types of microbes (i.e. those derived from human faeces and those from the environment) in pit latrines at various locations around the world. There were intrinsic differences in the bacterial communities, not only between regions but also between different pits. Nevertheless, these two papers show that the nature of the microbial community does play a critical role in the transformation of organic material within the various layers of the pit.

6.2 Model Development

The new model of filling rate within the VIP latrine should consider the variability in the biological transformations at various levels within the pit as well as the microbial community extant within these strata. A diagrammatic representation of the new model is shown in Figure 6-6.

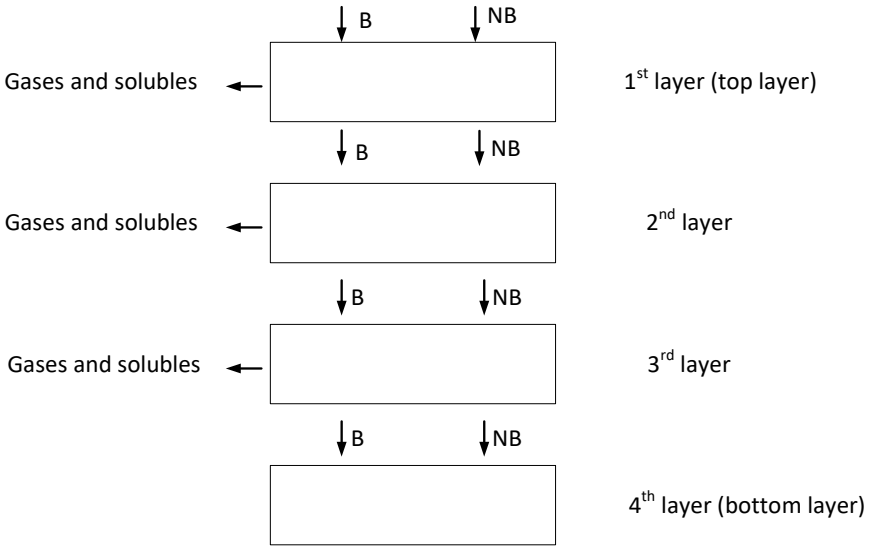


Figure 6-6: Basic representation of the proposed microbiome-based filling rate model

Based on the data of the physicochemical characterisation of sludge at various depths, four distinct layers are present in the pit. These are distinguished by, amongst other variables, the COD, volatile solids and micro-elemental composition. The total volume of the pit is the sum of the volume of these individual layers. The material that enters the pit is comprised of biodegradable material (faecal matter) and non-biodegradable material (trash). The pit is modelled as a series of semi-batch reactors. Within each layer/reactor the biodegradable fraction is consumed through the action of the microbial community present in that layer. The products of this transformation include non-biodegradable material, gases and soluble material and can be represented by Equation 3:

$$\frac{dN_{B,i}}{dt} = B_{i,in} - B_{i,out} - R_{B,i}V_i \qquad \textbf{(Equation 3)}$$

Where N_B is the mass of biodegradable material, B_{in} and B_{out} are the flowrates of biodegradable material in and out, R_B is the rate of transformation of the biodegradable material into products and V is the volume of the layer i.

There is a corresponding balance for the non-biodegradable material (Equation 4):

$$\frac{dN_{NB,i}}{dt} = NB_{i,in} - NB_{i,out} + F_{NB}R_{B,i}V_i \qquad \textbf{(Equation 4)}$$

Where N_{NB} is the mass of non-biodegradable material, F_{NB} is the fraction of biodegradable material converted to non-biodegradable material, and NB_{in} and NB_{out} are the flowrates of non-biodegradable material in and out.

The total volume in each layer is related to the mass of each fraction, and their respective densities are calculated from Equation 5:

$$V_i = \rho_B \times N_{B,i} + \rho_{NB} \times N_{NB,i} \quad \textbf{(Equation 5)}$$

The height of the layer is calculated from the cross-sectional area of the pit A_x is given by Equation 6:

$$H_i = \frac{V_i}{A_x} \quad \textbf{(Equation 6)}$$

The rate of transformation of biodegradable material is assumed to be first order and dependent on the mass concentration of biodegradable material in the layer, as well as a rate constant (Equations 7 and 8):

$$R_{B,i} = k_i C_{B,i} \quad \textbf{(Equation 7)}$$

$$C_{B,i} = \frac{N_{B,i}}{V_i} \quad \textbf{(Equation 8)}$$

The rate constant is not assumed to be fixed, as in previous filling rate models, but is itself a function of the active microbes (*MB*) for the type of transformation extant in that layer and is given by Equation 9:

$$k_i = f(MB_i) \quad \textbf{(Equation 9)}$$

In the simplest form, this will be a linear relationship (Equation 10):

$$k_i = \alpha \times MB_i \quad \textbf{(Equation 10)}$$

where α is a proportionality constant. For example, when the pit is initially empty and beginning to be filled, the dominant process is aerobic degradation of hydrolysable organic material. This will be driven by obligate and facultative aerobes present in the sludge. As more material enters the pit over time, this initial layer is covered, and in the absence of sufficient oxygen the obligate aerobes will decrease in number, transition to endospores and even die off, whereas the facultative aerobes will continue to function under the anaerobic conditions. There may be a transformation of the microbial community to the obligate anaerobes such as clostridia, which are well known for their involvement in organic matter hydrolysis in the anaerobic food chain.

Overall, there is a transformation of the microbial community over time, which can be represented by the simple expression given in Equation 11:

$$\frac{dMB_i}{dt} = R_{MB,i}V_i \qquad \textbf{(Equation 11)}$$

In each layer there is gradually a more anaerobic and less active microbial community.

The model is initialized by considering an empty pit with an initial input rate for both biodegradable and non-biodegradable material. This constitutes the first layer in the pit. The change in the amount of material (hence volume and height of material in the pit) is calculated from the integrated form of equations 2 and 3 (and associated expressions). In order to determine when the height of this initial layer has reached a critical value, such that another distinct layer is present above it, a decision variable is required. This decision variable is related to the active microbial community within the layer (MB_i). When the level and type of anaerobic microbes has reached a specific range, a new layer is added to the model. This process is repeated until the four layers are present, and the model is terminated when a specific height is reached for all layers combined, or when a specific age of the pit is reached.

In terms of model identification/parameter regression, the input data to the model will include the qualitative and quantitative description of the microbial community within each layer based on the Next Generation Sequencing (NGS) of 16S rRNA Genetic Sequencing results obtained after trimming and processing of reads and taxonomic assignment. A result of similar analyses presented in the literature is shown in Figure 6-7, extracted from the study of Ijaz et al. (2022).

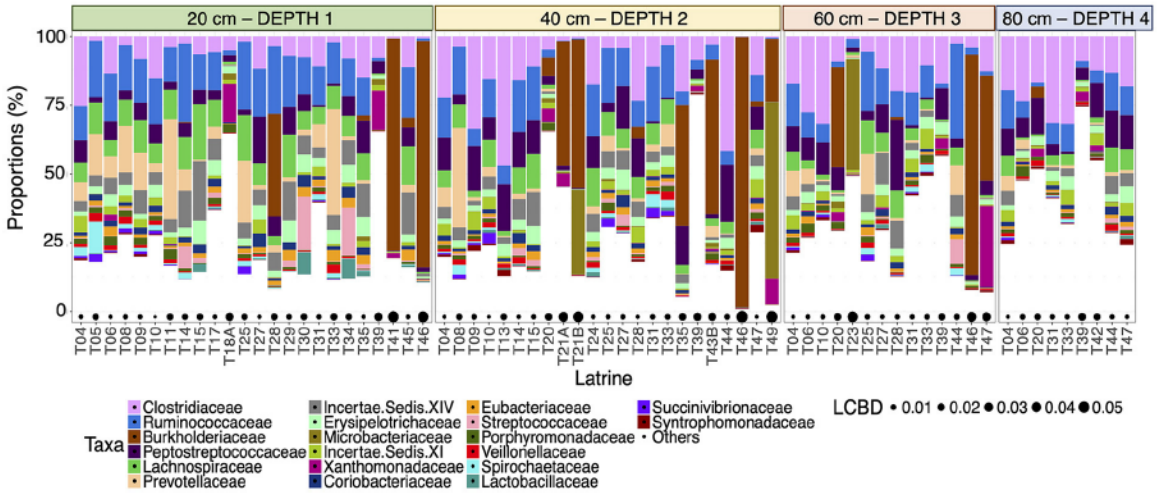


Figure 6-7: Bar plot of proportionally abundant family-level taxa at different depths within pit latrines (Ijaz et al., 2022)

Other inputs to the model will be the COD data and volatile solids, which represent a measure of the biodegradable material within each layer. the model may also include fluorescein diacetate (FDA) hydrolysis activity data. The two fitting variables will be the proportionality constant α (relating the active microbe composition and the rate of degradation) and the lumped rate of transformation of active microbes $R_{MB,i}$ (which should be different for each layer).

6.3 Model identification

The NGS / 16S rRNA data for the sludge samples from different layers in the pits, and for different days exposed to both aerobic and anaerobic conditions, were obtained from the University of Columbia Genome Centre. These data were used, along with the physicochemical data, to identify various parameters within the new model. The model can then be used to carry out predictive calculations of fill rate for VIP latrines, considering variations in the input rates for biodegradable and non-biodegradable material.

6.3.1 Modelling assumptions and approximations

The pit is assumed to be square with a width of 1 m on each side, giving a cross sectional area of 1 m^2. Other input parameters were taken from Brouckaert et al. (2013) and are shown in Table 6-1.

Table 6-1: Model input parameters

Parameter	symbol	value	unit
Cross sectional area	A_x	1	m^2
Addition rate of biodegradable material	$B_{i,in}$	4.782	kg/month
Addition rate of non-biodegradable material	$NB_{i,in}$	2.001	kg/month
Density of biodegradable fraction	ρ_B	1000	kg/m^3
Density of non-biodegradable fraction	ρ_{NB}	1000	kg/m^3

The non-biodegradable material consists of organic, inorganic and coarse refuse. Another important input parameter is F_{NB}, the fraction of biodegradable material converted to non-biodegradable material. The parameter was initially set to 0.9, assuming that 10% of the biodegradable material is converted to gases and solubles through biodegradation.

A representation of the active microbial community in each layer of the pit (MB_i) is necessary for the solution of the model. For the initial demonstration of the model, data from Smith et al. (2023) was used. Although detailed taxonomic analyses are available, an aggregation of such analyses by metabolic function is necessary for practical application in the model. Figure 6-8 shows the summarized metabolic functions of the microorganisms in different layers of pits located in Malawi. The most significant changes evident are with the methanogens and fermenters. The microbial populations present suggest that sugar metabolism and fermentation occur mostly at the upper layers and decrease with depth (Smith et al., 2023). These aerobic processes become less significant as depth in the pit increases, and the anaerobic digestion pathways becomes dominant.

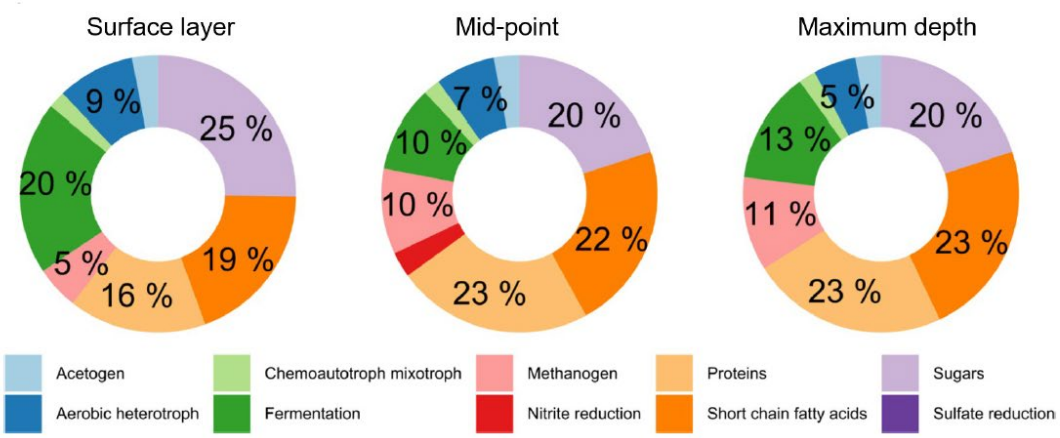


Figure 6-8: Summary of microbial metabolic functions for 55 sampled pit latrines in Malawi (Smith et al., 2023)

Unfortunately, the aggregated data for the South African pits considered in this study, summarized in Figures 5.6 to 5.9, do not show any significant changes in functional groups across the different layers (the average data for Day 0 were compared, which is representative of their condition upon extraction from the pit). This may have been due to the sampling technique and the introduction of oxygen into the sludge samples extracted from the lower layers of the pits.

The microbial activity function MB_i does not completely dissipate even at the lowest level of the pit. Thus, a linear relationship between MB_i and H_i is unlikely. An exponential decay was assumed rather, which has the following form (Equation 12):

$$\boldsymbol{MB_i = exp(-\beta \times H_i)} \qquad \textbf{(Equation 12)}$$

where β is an exponential constant (the same for each layer). Since H_i is a function of time (based on filling rate and degradation), it follows that MB_i will also experience temporal changes in each layer. For practical application, the value of MB_i is normalized to range between 0 and 1 (with the maximum activity extant when the material is freshly deposited in the pit).

6.3.2. Identification of model parameters

The fitting parameters for the model are shown in Table 6-2: Fitting parameters for the model. Knowledge of the metabolic functions would inform the selection of β values. The degradation constant α would depend on the final pit and layer depths. The fraction of biodegradable material converted to non-biodegradable material, F_{NB}, was also found to be critical fitting parameter for proper model identification, and was included.

Table 6-2: Fitting parameters for the model

Parameter	symbol	Initial value	unit
Degradation constant (Equation 10)	α	1	month^{-1}
Microbial activity constant	β	5	m^{-1}
Fraction of biodegradable material converted to non-biodegradable material	F_{NB}	0.9	-

The response function for the parameter fitting was the sum residuals of the ratio of biodegradable to non-biodegradable material left in each layer at the end of the time period (i.e. 120 months). These residuals were calculated as the absolute differences between predicted ratios and those obtained from the physicochemical analyses of the pit samples. Since no BOD analyses were conducted, this ratio was estimated from the volatile solids and ash present in each layer (see Figures 4.5 and 4.6).

The model was implemented in Microsoft Excel, through solution of Equations 3 to 12 using the explicit Euler integration method. A step size of Δt = 2 months was used, and the integration was performed over a period of 120 months (10 years, the final age of the pit). To provide a rational value of $C_{B,i}$ (Equation 8) at the first step, a small finite value for V_i was used. The calculation was initialized with layer 4, which represents the lowest layer in the pit. For the purposes of the parameter fitting, a 50% reduction in microbial activity was selected as the decision variable for transition to the next layer (i.e. the start of layer 3). The fitted parameters and residuals are shown in Table 6-3 and Table 6-4, respectively.

Table 6-3: Final fitted parameters for the model

Parameter	symbol	Final value	unit
Degradation constant (Equation 10)	α	0.029	month^{-1}
Microbial activity constant (Equation 12)	β	3.032	m^{-1}
Fraction of biodegradable material converted to non-biodegradable material	F_{NB}	0.95	-

Table 6-4: Residuals for the model

Parameter	symbol	Predicted value	Actual value*	Absolute residual
Ratio of biodegradable to non-biodegradable material in layer 1 (surface layer)	$\frac{N_{B,1}}{N_{NB,1}}$	1.7	2.0	0.33
Ratio of biodegradable to non-biodegradable material in layer 2 (Lower surface layer)	$\frac{N_{B,2}}{N_{NB,2}}$	0.84	0.90	0.06
Ratio of biodegradable to non-biodegradable material in layer 3 (Lower bottom layer)	$\frac{N_{B,3}}{N_{NB,3}}$	0.53	0.64	0.11
Ratio of biodegradable to non-biodegradable material in layer 4 (Bottom layer)	$\frac{N_{B,4}}{N_{NB,4}}$	0.43	0.4	0.03

*Estimated from the experimental data for volatile solids and ash

For the assumed parameter values, the transition to layer 3 occurred at a depth of 23 cm and after 34 months. At this point, the reduction of microbial activity accelerated. There was only a marginal change in the layer depth after this point, possibly due to the low conversion to gases and solubles, and the equivalent density of biodegradable and non-biodegradable fractions.

Similar trends were observed with layers 1 to 3. The overall depth of the pit after 120 months was 80 cm, and the temporal depth profile is shown in Figure 6-9.

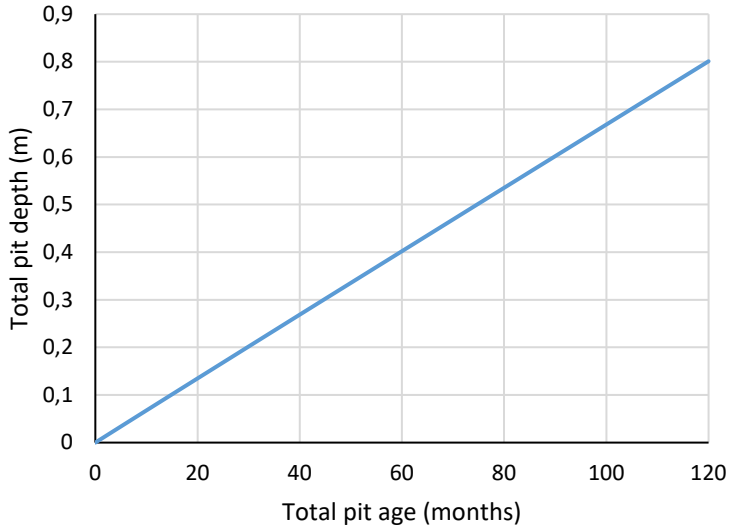


Figure 6-9: Temporal depth profile for pit based on assumed parameter values

The model shows that the deepest layer of the pit contains less than 30% of biodegradable material after 120 months. Each layer contains about 240 kg of nonbiodegradable material after maturation. The value of the degradation constant α is limited to below 1, higher values resulted in rapid and unrealistic consumption of biodegradable material. The layer transition point is strongly dependent on the microbial activity constant, which governs both the period before transition and the final depth of each layer. The model can be used to probe different input conditions, such as varying the ratio of non-biodegradable to biodegradable material fed to the pit.

An extract from the model is provided in **Appendix 1** and as a separate Excel file.

7 CHAPTER 7: CONCLUSIONS AND RECOMMENDATIONS

Physicochemical analyses of the different pit layers have shown statistically significant differences in the amount and type of organic and inorganic material present. These are related to the dominant metabolic pathways extant in each layer of the pit. The data is congruent with the hypothesis that the pits are segregated according to different layers with different microbial communities and functions. In the South African context, there is little to no transferral of material between layers, i.e. these can be modelled as separate batch reactors.

Whereas previous pit fill-rate models have considered the entire pit as a continuum, in this work a comprehensive model of the pit has been developed which accounts for the different layers and type and rate of degradation present. Rather than a single overall equation, the model consists of a series of interrelated material balances. The model in the current form is able to predict the height of each layer and the total height of the pit, based on the overall degradation rate and aggregated change in the microbial functions. This may be useful for design of sanitation systems based on pit latrines.

The adjustable parameters in the model were the degradation constant (accounting for the transformation of biodegradable material), microbial activity constant (accounting for the change in the transformation rate in the lower layers of the pit) and the fraction of biodegradable material transformed into non-biodegradable material. The fitted model was able to adequately predict the final ratios of biodegradable to non-biodegradable material in the various layers of the pit, as well as the overall height of the pit after maturation. For model fitting, characterization of the unmanipulated sludge is more valuable, as it represents the true nature of the functional groups, and corresponding microbial activity, in each layer.

8 CAPACITY BUILDING REPORT

Together with the project funded by Unilever, India, provision was made for the recruitment of a post-doctoral researcher, a research assistant and a Masters student. However, due to the delays caused by the COVID-19 pandemic, this research took longer than expected, resulting in insufficient funds to recruit a full-time Masters student. Therefore, only the post-doctoral researcher and the research assistant were assigned to this project.

The research assistant was responsible for conducting the majority of the experimental work in the laboratory and reporting on the results of the physical, chemical and biological results. This research assistant also conducted the experimental work required for the project funded by Unilever India. Due to the experience gained on this project, the research assistant was recruited to undertake similar experimental research for another WRC project awarded to WITS University.

The post-doctoral researcher has a background in microbiology and was first recruited as a graduate assistant while awaiting the awarding of her PhD degree. Once this had been finalised, the appointment was upgraded to that of post-doctoral researcher. Her role was to provide input into the microbiological aspects and analysis of the genetic results.

The details of these researchers are provided in Table 8-1, and Appendix 2 contains the signed declaration of the involvement of the post-doctoral student.

Table 8-1: Capacity building on the project

Name	Position	Gender	Highest qualification
Rendani Bulannga	Post-Doc	Female	PhD (Microbiology)
Travis Kunnen	Research Assistant	Male	MSc (Life Science)

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APPENDIX 1: SPREAD SHEET MODEL

A print out of the spread sheet model for pit layer 4 (lowest layer) is shown below.

	Layer 4									
t	V_i	H_i	MB_i	k_i	N_{Bi}	C_{Bi}	R_{Bi}	dN_{Bi}	dN_{NBi}	N_{NBi}
0	1.00E-19	1.00E-19	1	0.029027	0	0.00E+00	0.00E+00	9.56E+00	4.00E+00	0
2	1.36E-02	1.36E-02	0.959702	0.027857	9.56E+00	7.05E+02	1.96E+01	9.0E+00	4.51E+00	4.00E+00
4	2.71E-02	2.71E-02	0.921102	0.026736	1.86E+01	6.86E+02	1.83E+01	8.57E+00	4.95E+00	8.51E+00
6	4.06E-02	4.06E-02	0.884116	0.025663	2.72E+01	6.69E+02	1.72E+01	8.2E+00	5.33E+00	1.35E+01
8	5.41E-02	5.41E-02	0.848667	0.024634	3.53E+01	6.53E+02	1.61E+01	7.82E+00	5.66E+00	1.88E+01
10	6.76E-02	6.76E-02	0.814683	2.36E-02	4.32E+01	6.38E+02	1.51E+01	7.5E+00	5.94E+00	2.44E+01
12	8.11E-02	8.11E-02	0.782094	0.022702	5.07E+01	6.25E+02	1.42E+01	7.26E+00	6.19E+00	3.04E+01
14	9.45E-02	9.45E-02	0.750839	0.021794	5.79E+01	6.13E+02	1.34E+01	7.04E+00	6.40E+00	3.66E+01
16	1.08E-01	1.08E-01	0.720858	0.020924	6.50E+01	6.02E+02	1.26E+01	6.84E+00	6.59E+00	4.30E+01
18	1.21E-01	1.21E-01	0.692094	0.020	7.18E+01	5.92E+02	1.19E+01	6.68E+00	6.74E+00	4.96E+01
20	1.35E-01	1.35E-01	0.664494	0.019288	7.85E+01	5.82E+02	1.12E+01	6.54E+00	6.88E+00	5.63E+01
22	1.48E-01	1.48E-01	0.638009	0.018519	8.50E+01	5.74E+02	1.06E+01	6.41E+00	6.99E+00	6.32E+01
24	1.62E-01	1.62E-01	0.612591	0.017781	9.15E+01	5.66E+02	1.01E+01	6.31E+00	7.09E+00	7.02E+01
26	1.75E-01	1.75E-01	0.588194	0.017073	9.78E+01	5.59E+02	9.54E+00	6.23E+00	7.17E+00	7.73E+01
28	1.88E-01	1.88E-01	0.564777	0.016394	1.04E+02	5.52E+02	9.05E+00	6.15E+00	7.24E+00	8.44E+01
30	2.02E-01	2.02E-01	0.542298	0.015741	1.10E+02	5.46E+02	8.59E+00	6.10E+00	7.30E+00	9.17E+01
32	2.15E-01	2.15E-01	0.520718	0.015115	1.16E+02	5.40E+02	8.16E+00	6.05E+00	7.34E+00	9.90E+01
34	2.29E-01	2.29E-01	0.5	0.014513	1.22E+02	5.35E+02	7.76E+00	6.01E+00	7.37E+00	1.06E+02
36	2.42E-01	2.42E-01	0.480109	0.013936	1.28E+02	5.30E+02	7.39E+00	-3.58E+00	3.40E+00	1.14E+02
38	2.42E-01	2.42E-01	0.461011	0.013382	1.25E+02	5.16E+02	6.90E+00	-3.3E+00	3.17E+00	1.17E+02
40	2.42E-01	2.42E-01	0.442693	0.01285	1.21E+02	5.02E+02	6.46E+00	-3.12E+00	2.96E+00	1.20E+02
42	2.41E-01	2.41E-01	0.425119	0.01234	1.18E+02	4.90E+02	6.04E+00	-2.92E+00	2.77E+00	1.23E+02
44	2.41E-01	2.41E-01	0.408254	0.01185	1.15E+02	4.78E+02	5.66E+00	-2.7E+00	2.60E+00	1.26E+02
46	2.41E-01	2.41E-01	0.392068	0.01138	1.13E+02	4.67E+02	5.31E+00	-2.6E+00	2.44E+00	1.29E+02
48	2.41E-01	2.41E-01	0.376531	0.010929	1.10E+02	4.57E+02	4.99E+00	-2.4E+00	2.29E+00	1.31E+02
50	2.41E-01	2.41E-01	0.361615	0.010496	1.08E+02	4.47E+02	4.69E+00	-2.3E+00	2.15E+00	1.33E+02
52	2.41E-01	2.41E-01	0.347295	0.010081	1.05E+02	4.38E+02	4.41E+00	-2.1E+00	2.02E+00	1.35E+02
54	2.41E-01	2.41E-01	0.333544	0.009682	1.03E+02	4.29E+02	4.15E+00	-2.0E+00	1.90E+00	1.37E+02
56	2.41E-01	2.41E-01	0.32034	0.009298	1.01E+02	4.21E+02	3.91E+00	-1.9E+00	1.79E+00	1.39E+02
58	2.41E-01	2.41E-01	0.30766	0.00893	9.94E+01	4.13E+02	3.69E+00	-1.8E+00	1.69E+00	1.41E+02
60	2.40E-01	2.40E-01	0.295482	0.008577	9.76E+01	4.06E+02	3.48E+00	-1.7E+00	1.59E+00	1.43E+02
62	2.40E-01	2.40E-01	0.283787	0.008237	9.59E+01	3.99E+02	3.29E+00	-1.6E+00	1.50E+00	1.44E+02
64	2.40E-01	2.40E-01	0.272554	0.007911	9.44E+01	3.93E+02	3.11E+00	-1.5E+00	1.42E+00	1.46E+02
66	2.40E-01	2.40E-01	0.261765	0.007598	9.29E+01	3.87E+02	2.94E+00	-1.4E+00	1.34E+00	1.47E+02
68	2.40E-01	2.40E-01	0.251402	0.007297	9.14E+01	3.81E+02	2.78E+00	-1.3E+00	1.27E+00	1.49E+02
70	2.40E-01	2.40E-01	0.241449	0.007008	9.01E+01	3.75E+02	2.63E+00	-1.3E+00	1.20E+00	1.50E+02
72	2.40E-01	2.40E-01	0.231888	0.006731	8.89E+01	3.70E+02	2.49E+00	-1.2E+00	1.14E+00	1.51E+02
74	2.40E-01	2.40E-01	0.222704	0.006464	8.77E+01	3.65E+02	2.36E+00	-1.1E+00	1.08E+00	1.52E+02
76	2.40E-01	2.40E-01	0.213892	0.006209	8.65E+01	3.61E+02	2.24E+00	-1.1E+00	1.02E+00	1.53E+02
78	2.40E-01	2.40E-01	0.205434	0.005963	8.54E+01	3.56E+02	2.12E+00	-1.0E+00	9.68E-01	1.54E+02
80	2.40E-01	2.40E-01	0.197315	0.005727	8.44E+01	3.52E+02	2.02E+00	-9.7E-01	9.19E-01	1.55E+02
82	2.40E-01	2.40E-01	0.189519	0.005501	8.35E+01	3.48E+02	1.92E+00	-9.2E-01	8.72E-01	1.56E+02
84	2.40E-01	2.40E-01	0.182035	0.005284	8.25E+01	3.44E+02	1.82E+00	-8.7E-01	8.29E-01	1.57E+02
86	2.40E-01	2.40E-01	0.174847	0.005075	8.17E+01	3.41E+02	1.73E+00	-8.3E-01	7.88E-01	1.58E+02
88	2.40E-01	2.40E-01	0.167944	0.004875	8.08E+01	3.37E+02	1.64E+00	-7.9E-01	7.49E-01	1.59E+02
90	2.40E-01	2.40E-01	0.161313	0.004682	8.01E+01	3.34E+02	1.56E+00	-7.5E-01	7.12E-01	1.60E+02
92	2.40E-01	2.40E-01	0.154945	0.004498	7.93E+01	3.31E+02	1.49E+00	-7.1E-01	6.78E-01	1.60E+02
94	2.40E-01	2.40E-01	0.148828	0.00432	7.86E+01	3.28E+02	1.42E+00	-6.8E-01	6.45E-01	1.61E+02
96	2.39E-01	2.39E-01	0.142952	0.004149	7.79E+01	3.25E+02	1.35E+00	-6.5E-01	6.14E-01	1.62E+02
98	2.39E-01	2.39E-01	0.137307	0.003986	7.73E+01	3.23E+02	1.29E+00	-6.2E-01	5.85E-01	1.62E+02
100	2.39E-01	2.39E-01	0.131884	0.003828	7.66E+01	3.20E+02	1.23E+00	-5.9E-01	5.58E-01	1.63E+02
102	2.39E-01	2.39E-01	0.126675	0.003677	7.61E+01	3.18E+02	1.17E+00	-5.6E-01	5.31E-01	1.63E+02
104	2.39E-01	2.39E-01	0.121671	0.003532	7.55E+01	3.15E+02	1.11E+00	-5.3E-01	5.07E-01	1.64E+02
106	2.39E-01	2.39E-01	0.116863	0.003392	7.50E+01	3.13E+02	1.06E+00	-5.1E-01	4.83E-01	1.64E+02
108	2.39E-01	2.39E-01	0.112244	0.003258	7.45E+01	3.11E+02	1.01E+00	-4.9E-01	4.61E-01	1.65E+02
110	2.39E-01	2.39E-01	0.107807	0.003129	7.40E+01	3.09E+02	9.67E-01	-4.6E-01	4.40E-01	1.65E+02
112	2.39E-01	2.39E-01	0.103548	0.003006	7.35E+01	3.07E+02	9.24E-01	-4.4E-01	4.20E-01	1.66E+02
114	2.39E-01	2.39E-01	0.09946	0.002887	7.31E+01	3.05E+02	8.82E-01	-4.2E-01	4.01E-01	1.66E+02
116	2.39E-01	2.39E-01	0.095535	0.002773	7.26E+01	3.04E+02	8.42E-01	-4.0E-01	3.83E-01	1.67E+02
118	2.39E-01	2.39E-01	0.091767	0.002664	7.22E+01	3.02E+02	8.05E-01	-3.8E-01	3.66E-01	1.67E+02
120	2.39E-01	2.39E-01	0.088148	0.002559	7.19E+01	3.00E+02	7.69E-01	-3.7E-01	3.49E-01	1.67E+02

APPENDIX 2: CAPACITY BUILDING

Name	Position	Gender	Highest qualification
Rendani Bulannga	Post-Doc	Female	PhD (Microbiology)
Travis Kunnen	Research Assistant	Male	MSc (Life Science)