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Research Article

Characterization and Molecular Identification of Some Microorganisms Isolated from Soil Exposed to Leachate from Septic Tanks

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ABSTRACT

This study was undertaken to access the microbiological quality of soil exposed to leachate from septic tanks in International Secondary School, Rivers State University, Port Harcourt, Nigeria. This was achieved by characterization and molecular identification of microorganisms in the soil samples. A total of 128 soil samples were aseptically collected from four sides of two septic tanks at vertical depths (0.0m, 0.5m, 1.0m and 1.5m) and at horizontal distances (0.5m, 1.0m, 1.5m and 2.0m) away from the septic tanks. Samples from same depth of each septic tank were bulked to form composite sample. Soil samples not exposed to septic tank leachate served as control. Collected samples were immediately taken to the laboratory for analysis using standard microbiological and molecular techniques. Results revealed mean counts of Total Heterotrophic Bacteria was 3.66 x 10^7 ± 2895cfu/g, Total Fungi 1.225 x 10^4 ± 0.83cfu/g, Total Vibrio 0.725 x10^4 ± 0.901cfu/g, Total Coliform 1.922 x10^4 ± 0.689cfu/g, Total *Escherichia coli* 2.05x10^4 ± 4.85cfu/g, and Anaerobic Plate Count 0.53 x 10^4 ± 0.968cfu/g. Mean counts of corresponding controls were lower. THB had the highest population which was above FAO/WHO maximum permissible limit. Results showed that highest population occurred at 0.0m depth (surface). Physiological characteristics of isolates showed presence of six bacterial genera; *Bacillus, Pseudomonas, Micrococus, Kluyvera, Escherichia* and *Staphylococcus* and eight fungal genera; *Candida, Rhizopus, Penicillium, Mucor, Fusarium, Alternaria, Trichoderma* and *Aspergillus*. Molecular sequencing identified *Bacillus thuringiensis* (strain Bi54 and strain HPPD), *Bacillus firmus* (strain T1), *Bacillus megaterium* (strain A20 and strain 3.5), *Myriodes odoratimimus* (strain F1), *Bacillus altitudinis* (NIOER258) and *Bacillus cereus* (CUMB CDB-09). This study detected microbes capable of causing diseases in soils exposed to septic tank leachate. Therefore, to protect human, animal and environmental health, implementation of modern sewage management system to avoid leaching of septic tank effluent should be vigorously pursued.

Keywords: Septic tank leachate, soil, gene sequencing, pathogens, *Bacillus, Myriodes, Aspergillus*.

Introduction

Septic leachate is the liquid that remains after the waste water typically originating from toilet, bathrooms and kitchen flushings has emptied into underground septic tanks, and drained through septic solids. It is that effluent that empties into the soil absorption area for further treatment. Leachate composition varies widely and may include microorganisms, fats and oils, protozoa, heavy metals and inorganic macro-components such as magnesium, calcium, sodium, potassium, iron, chloride, sulfate (Oyem *et al.*, 2020; Ugbebor and Ntesat, 2019) depending on such diverse factors as soil properties, rate at which effluents from the septic tank infiltrate the soil absorption area and frequency of the use and desludging of the septic tank.

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According Rim-Rukeh and Agbozu (2013), this rich cocktail of sewage constituents encourage the growth of many different types of heterotrophic bacterial colonies including *Micrococcus, Escherichia coli, Pseudomonas, Enterobacter* and fungi such as yeasts and *Mucor*. Oyem *et al.* (2020) confirmed the presence of methane-producing and sulfur bacteria in sewage samples from septic systems in Delta and Edo States of Nigeria. Nwuba and Phillips (2015), reported that many of such bacteria from raw sewage or leachates succeed in being carried away due to incomplete degradation or sorption/filteration by soil adsorption systems and end up in groundwater or in surface water (Standley *et al.*, 2008; Doherty *et al.*, 2010). In this way, septic systems become a significant contributor of fecal pollution of diverse land and water bodies, and result in such outcomes as endemic diarrheal illnesses especially in areas with high septic system density ((Borchardt *et al*., 2003; Bremer and Harter, 2012; Nduka and Orisakwe, 2009; Dorsey and Rasmussen, 2012; Dorsey, 2015). Poorly designed or failing septic systems have also directly contributed to the microbial contamination of soils, surface and well waters in some situations leading to norovirus outbreaks in human habitations (Anderson *et al*., 2003; Gunnarsdottir *et al*., 2013). In worrying dimensions, even newly installed septic tanks which are operating below their name plate capacities were indicted as microbial feed to the contaminated well water associated with the norovirus outbreaks in the United States (Borchardt *et al*., 2011; Kauppinen *et al*., 2018). Therefore, minimizing the risk of contamination of soil ecosystems and water reservoirs and lowering or eliminating environmental and health hazards traceable to septic tanks and its leachate, hinges on the brilliance of the site selection, system design and maintainence as well as cognisance of the septic tanks density per area (Olatunde *et al.*, 2021).

Sadly, most septic tanks will fail eventually. Worse still, the common remedy is abandonment of the old or failed systems. However, a change of the septic tank system after 25-30 years is generally accepted as best practice, because the mortar and steel construction materials will eventually succumb to corrosion. Therefore, it is necessary to generate data on the profile of microorganisms of soils into which septic leachate plumes are pumped from several closely sited septic tanks. As such data information will provide viable options for human health and environmental management.

Materials and Methods

Study Area

The study area is the International Secondary School (ISS) situated within the premises of Rivers State University, Nkpolu Oroworukwo, Port Harcourt (RSU). ISS lies within the following projected coordinates Universal Transverse Mercator, (UTM) Zone 32N, World Geodetic System (WGS) 84; 276010.902m & 531656.115m; 276315.407m & 531687.070m; 276312.875m & 531522.450m and 276011.803m & 531520.308m (Eastings and Northings respectively). Port Harcourt City is a metropolitan, oil and gas, banking and manufacturing hub with a population of approximately 3 million siting on approximately 181km^2 of land with a sub-equatorial wetland climate, a rainfall of about 2000mm and annual mean temperature of about 29ºC (Yakubu, 2017; NMS, 1998). Its surface cover geography is coastal plain sands (Ideriah and Ikoro, 2015) arising from a Miocene-Recent Benin Formation about 2100km thick at its center, (NMS, 1998); resulting in a porous and permeable soil surface during the rainy season which allows waste leachates access to the underlying soil layers as well as bedrock ground water levels (Ideriah and Ikoro, 2015).
The septic tanks of interest within the study area of this research, from around which soil samples exposed to septic tank leachate were collected had details as listed out in Table 1 below. While the Map of the study area is as shown in Figure 1.

Table 1: Relevant information on the Septic Tanks of the study

Septic Tank ID	Colour on map	Status	Dimensions				Geo-location	
			Length	Breadth	Perimeter	Area	Northings	Eastings
1	Green	Historical	5.47m	2.75m	16.44m	15.04m^2	531666m	276250m
2	Blue	Active	5.45m	1.95m	14.8m	10.63m^2	531663m	276253m

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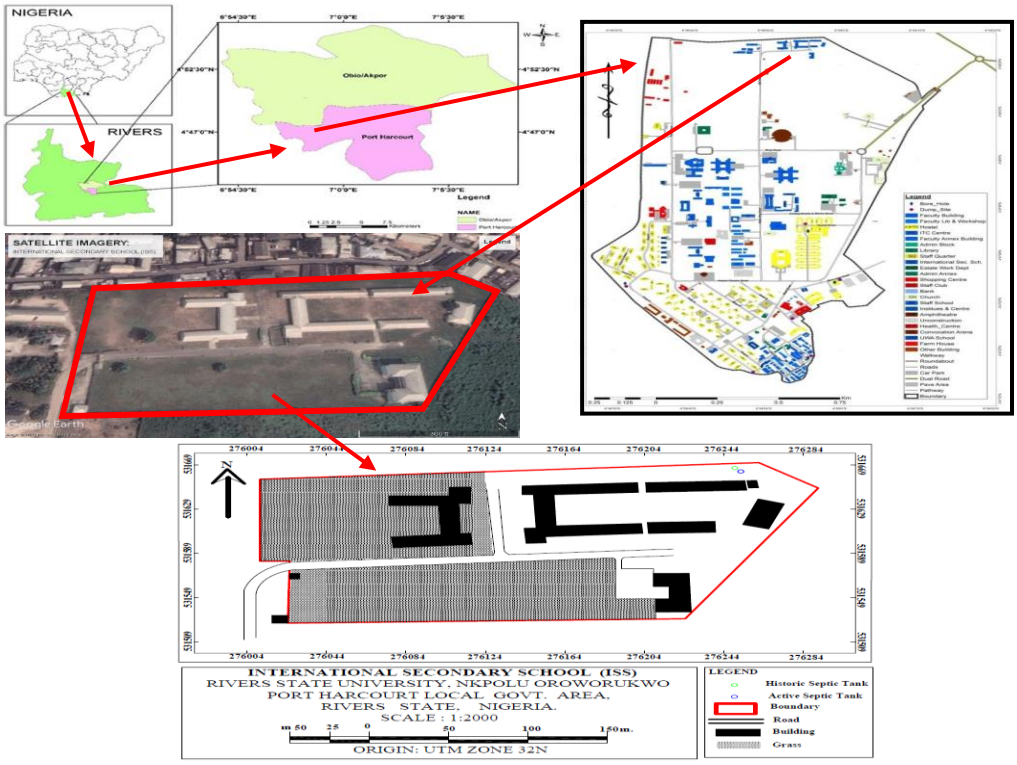

Fig. 1: Map of the study area showing the soakaways at the International Secondary School

Soil sample collection

Soil samples were collected on 7th September 2020 from 6.30am to 7pm. A modified hand soil auger was used to collect samples from the sides of the soakaways. The auger was sterilized using absolute ethanol before use. Soil samples were aseptically collected from the four sides of two separate septic tanks at vertical depths of 0.0m (surface or top soil), 0.5m (sub soil), 1.0m (deep subsoil) and 1.5m (Maxsub depth soils) and at horizontal distances of 0.5m, 1.0m, 1.5m and 2.0m, away from the from each side of two septic tanks. In all, A total of one hundred and twenty-eight (128) soil samples (4×4×4×2 = 128) exposed to septic tank leachate were collected. Thereafter, soil samples from same depth and distance from each septic tank were bulked and mixed thoroughly to form a composite sample into sterile pre-labelled 50ml sampling bottles and the caps returned. Soil samples collected from same depths and distances but not exposed to septic tank leachate served as control.

Collected soil samples were immediately stored in ice packed coolers and transported to the laboratory for further treatment and analysis.

Microbiological Analysis

Media Preparation

Three microbiological media were used for the primary isolation and culturing of the isolates. They include Nutrient agar, Sabouraud dextrose agar and MacConkey agar. Other media used were Eosin methylene blue agar, Salmonella Shigella agar, and Thiosulfate Citrate Bile Salt agar. These media were all prepared according to manufacturers' instruction before use (Cruickshank *et al*., 1975). The diluent used for serial dilution was normal saline because it helps to reactivate microorganisms. Serial dilution was made serially in one –tenth stepwise for each of the composite soil samples up to 10^{-5} dilution (Obire and Deeyah, 2021).

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Cultivation of Bacteria and Fungi

All glass ware used for sample handling were sterilized using standard methods. Ten fold serial dilution of samples was performed and enumeration carried out using the spread plate method From the dilutions of 10^{-3} and 10^{-5} of each soil sample, 0.1ml (aliquot) was transferred aseptically onto freshly prepared SDA plates and Nutrient agar plates respectively, using the spread plate method with the aid of a sterile glass bent rod (Obire and Deeyah, 2021). Inoculated nutrient agar plates were also introduced into anaerobic jar into which was placed an AnaeroGen Pack (3.5 L AN0035A Oxiod) for the generation of anaerobic condition in the anaerobic jar. This encouraged the growth of colonies of only anaerobes that were recorded for the anaerobic plate count (APC). The inoculated plates were inverted and labeled accordingly and incubated at 37^0C for 24 hours for Nutrient agar plates for cultivation of bacteria and at 28^0C for 5-7 days for SDA plates for the cultivation of fungi. The discrete colonies which developed were counted and the average counts for duplicate cultures were recorded as total viable aerobic heterotrophic bacteria and fungi in the sample with regards to size of colony, colour, and edge.

Isolation and Enumeration of Bacteria and Fungi and Preparation of Stock Culture

Pure cultures of bacteria were obtained by aseptically streaking representative colonies of different morphological types which appeared on the cultured plates into freshly prepared Nutrient agar plates which were incubated at 37^0C for 24 hours. Pure cultures of fungi were obtained by subculturing discrete colonies onto freshly prepared Sabouraud dextrose agar plates and incubated at 28^0C for 5-7 days. The following standard characterization tests were performed: Macroscopic examination of fungal growth was carried out by observing the colony morphology – colour (pigmentation), texture and surface appearance. Microscopic examination was done by using the wet mount technique (Harrigan and McCance, 1990; Obire and Deeyah, 2021) and observing them under the microscope. The complete identification of fungal isolates was done by comparing the result of their cultural and morphological characteristics with those of known Taxa (Harley and Callaway, 1978; Olds, 1983).

Glycerol solution (10%) was prepared and dispensed in McCartney bottles and autoclaved at 121^0C for 15minutes, allowed to cool, then the pure cultures were inoculated into each McCartney bottle, till the clear colourless solution turns turbid and stored in the refrigerator. This serves as pure stock cultures for subsequent characterization (Obire and Deeyah, 2021). The following standard characterization tests were performed: Gram's staining reaction, Motility test, Catalase test, Coagulase test, Starch hydrolysis, Indole, Methyl red, Voges Proskauer, and Carbohydrate fermentation test (Glucose, Fructose, Sucrose, Lactose and Mannitol). The organisms were tentatively identified to the genera level using identification manuals (Collins and Lyne, 1979).

Microscopic Examination of Fungi Using Wet Mount

The wet mount method as described by (Cheesebrough, 2005; Obire and Deeyah, 2021) was used. A small portion of the isolate was picked with a sterile needle and teased out in a drop of water on a clean microscopic slide using wire loop to emulsify the smear. A drop of lactophenol cotton blue was added to the smear and emulsified, covered with a cover slip and was examined under microscope, starting with ×10 objective and highest power objective, ×40 for better field and magnification. The microscopic examination included sexual and asexual reproductive structures like conidia, conidiophores reliable characters for specie recognition.

Molecular Analysis

DNA extraction using ZR fungal/bacterial DNA MINIPREP (Manufactured by Zymo Research)

The steps below were followed for the extraction of DNA from the isolates

1. Add 2mLs of bacterial cells broth to a ZR BashingTM Lysis Tube. Add 750ul Lysis Solution to the tube.
2. Secure in a bead fitted with 2 ml tube holder assembly and process at maximum speed for > 5 minutes.
3. Centrifuge the ZR Bashing BeadTM Lysis Tube in a microcentirifuge at > 10,000 x g for 1 minute.

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4. Transfer up to 400 µl supernatant to a Zymo-SpinTM IV Spin Filter (orange top) in a Collection Tube and centrifuge at 7,000 x g for 1 minute.
5. Add 1,200 µl of Fungal/Bacterial DNA Binding Buffer to the filtrate in the Collection Tube from Step 4.
6. Transfer 800 µl of the mixture from Step 5 to a Zymo-SpinTM IIC Column in a Collection Tube and centrifuge at 10,000 x g for 1 minute.
7. Discard the flow through from the Collection Tube and repeat Step 6.
8. Add 200 µl DNA Pre-Wash Buffer to the Zymo-Spin TM IIC Column in new Collection Tube and centrifuge at 10,000 x g for 1 minute
9. Add 500 µl Fungal/Bacterial DNA Wash Buffer to the Zymo-SpinTM IIC Column and centrifuge at 10,000 x g for 1 minute
10. Transfer the Zymo-SpinTM IIC Column to a clean 1.5 ml microcentrifuge tube and add 100ul (35 µl minimum) DNA Elution Buffer directly to the column matrix. Centrifuge at 10,000 x g for 30 seconds to elute the DNA.

Electrophoresis for DNA and PCR

1. Measure 1 g of agarose (for DNA) ; 2g of agarose for PCR
2. Mix agarose powder with 100 mL 1xTAE in a microwavable flask.
3. Microwave for 1-3 min until the agarose is completely dissolved (but do not over boil the solution, as some of the buffer will evaporate and thus alter the final percentage of agarose in the gel.
4. Let agarose solution cool down to about 50 °C (about when you can comfortably keep your hand on the flask), about 5 mins.
5. Add 10µL EZ vision DNA stain. EZ vision binds to the DNA and allows you to visualize the DNA under ultraviolet (UV) light.
6. Pour the agarose into a gel tray with the well comb in place
7. Place newly poured gel at 4 °C for 10-15 mins OR let sit at room temperature for 20-30 mins, until it has completely solidified.

Sample Code for Extracted DNA

The sample Codes for the Extracted DNA of the eight (8) bacterial isolates were, M1, M3, M7, M10, M14, M15, M17, and M18.

Loading Samples and Running an Agarose Gel

1. Add loading buffer to each of your DNA samples or PCR products
2. Once solidified, place the agarose gel into the gel box (electrophoresis unit).
3. Fill gel box with 1xTAE (or TBE) until the gel is covered.
4. Carefully load a molecular weight ladder into the first lane of the gel.
5. Carefully load your samples into the additional wells of the gel.
6. Run the gel at 80-150 V for about 1-1.5 hours
7. Turn OFF power, disconnect the electrodes from the power source, and then carefully remove the gel from the gel box.
8. Visualize DNA fragments or PCR product under UV transilluminator.

PCR Mix Components

The PCR mix is made up of 12.5µL of Taq 2X Master Mix from New England Biolabs (M0270); 1µL each of 10µM forward and reverse primer; 2µL of DNA template and then made up with 8.5µL Nuclease free water.

Primer Sequences

27F: AGAGTTTGATCMTGGCTCAG
1525R: AAGGAGGTGWTCCARCCGCA

Cycling Conditions

Initial denaturation at 94˚C for 5mins, followed by 36 cycles of denaturation at 94˚C for 30sec, annealing at 56˚C for 30secs and elongation at 72˚C for 45sec. Followed by a final elongation step at 72˚C for 7 minutes and hold temperature at 10˚C forever.

Sequencing

The amplified fragments were sequenced using a Genetic Analyzer 3130xl sequencer from Applied Biosystems using manufacturers' manual while the sequencing kit used was that of BigDye terminator v3.1 cycle sequencing kit. Bio- Edit software and MEGA 6 were used for all genetic analysis.

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Evolutionary relationships of taxa

The evolutionary history was inferred using the UPGMA method. The optimal tree with the sum of branch length = 1.42612523 is shown below.
The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. This analysis involved 5 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1184 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

Results

Results of the variation of mean population (log cfu/g +1)of total heterotrophic bacterial (THB), total Total coliform count (TCC), *E. coli* count (TEC), total *Vibrio* count (TVC), total heterotrophic fungi (THF), and anaerobic plate count (APC) are shown in Figure 2 below. The Log10 (CFU/g + 1) means of the various microbial populations in the vertical depths of the soil samples exposed to septic tank leachate is also as shown in Figure 3 below

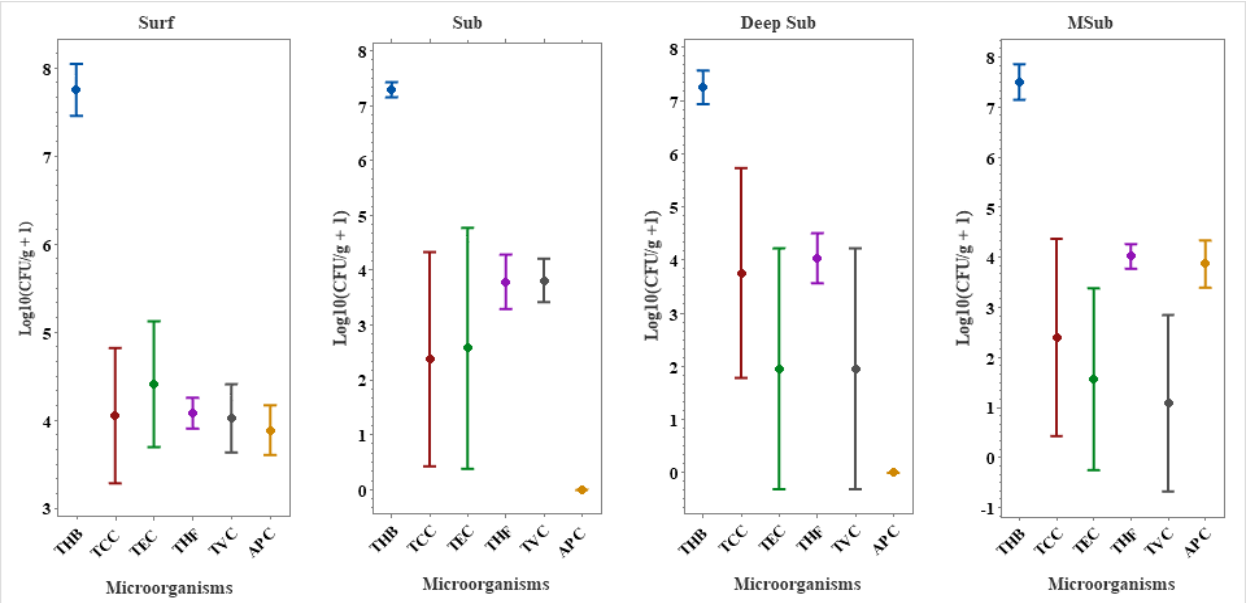

Figure 2: Variation of mean population (log cfu/g +1) of the microorganisms

Key: THB = total heterotrophic bacterial, TCC = total coliform count, TEC = *E. coli* count, TVC = total *Vibrio* count, THF = total heterotrophic fungi, APC = anaerobic plate count.

Following the results of the Gram's staining reaction, morphological, cultural and Biochemical characteristics and comparison with known Taxa, the isolated bacteria were identified as; *Bacillus mycoides, Bacillus smithii, Bacillus thuringiensis, Bacillus anthracis, Bacillus carboniphilus, Pseudomonas* sp, *Micrococcus* sp, *Kluyvera ascorbate, Bacillus endophiticus, Escherichia coli, Staphylococcus* sp., *Bacillus* subtilis, *Bacillus* sp

Following the results of the Macroscopic, cultural and microscopic observations and comparison with known Taxa, the isolated fungi were identified as; C*andida* sp., *Rhizopus* sp., *Penicillium* sp., *Mucor* sp., *Fusarium* sp., *Alternaria* sp., *Trichoderma* sp., *Aspergillus* ., and *Aspergillus fumigatus*.

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Percentage Occurrence of the Isolates

The occurrences (%) of the isolated bacteria and fungi are presented in Figure 4 and in Figure 5 respectively. The results shows *Bacillus endophiticus* (13.4%) and *Bacillus sp / Escherichia coli* (2.4%) bacteria had the highest and lowest occurrences respectively while *Aspergillus fumigalus* (15.9%) had the highest occurrence and *Mucor sp / Alternaria sp* (6.8%) the lowest for the fungi.

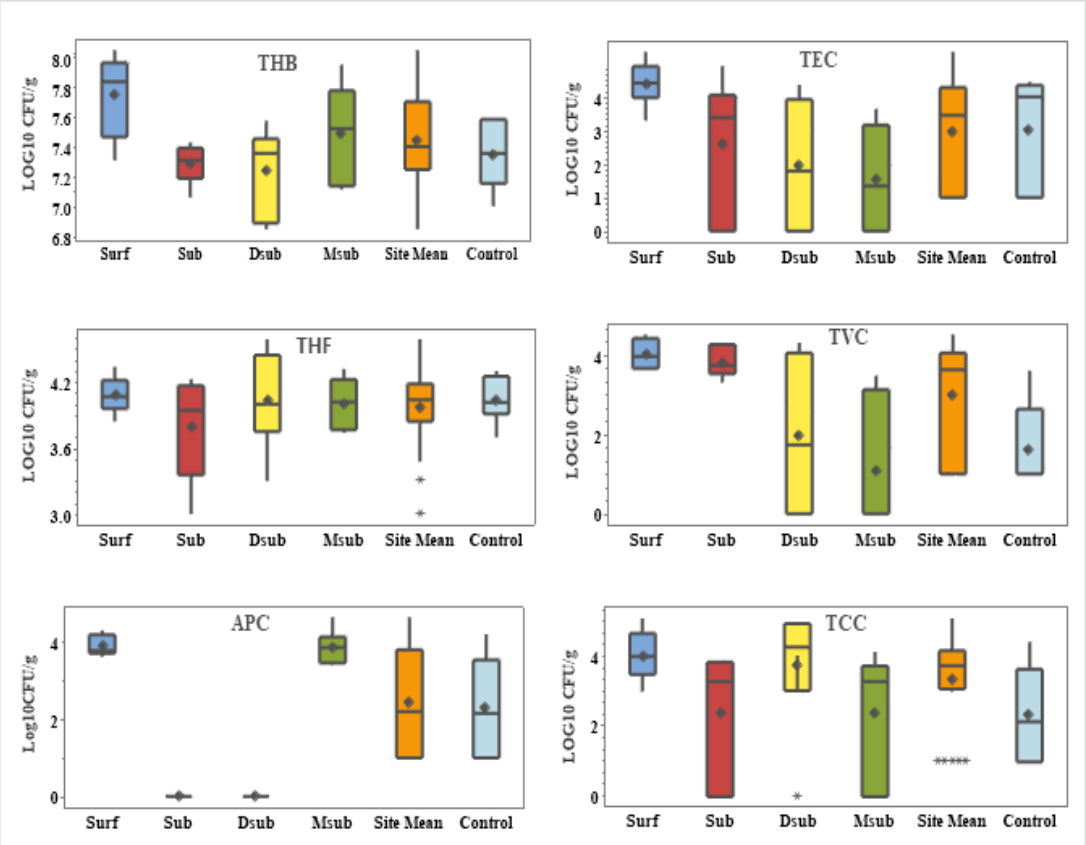

Figure 3: Means of the various microbial populations in depth of soil exposed to septic tank leachate

Key: THB = total heterotrophic bacterial, TCC = total Total coliform count, TEC = *E. coli* count, TVC = total *Vibrio* count, THF = total heterotrophic fungi, APC = anaerobic plate count.

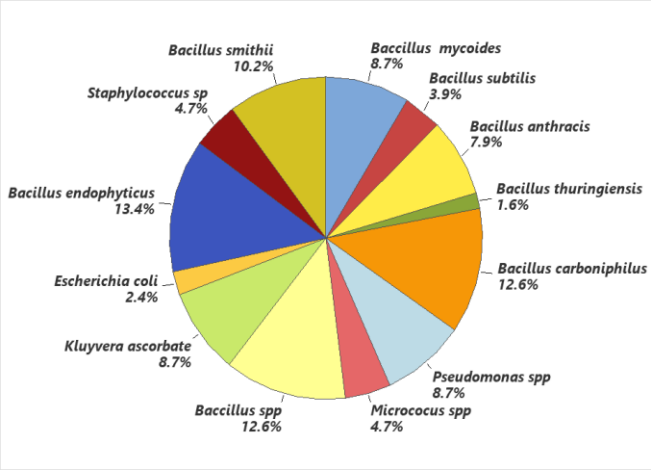

Figure 4: Occurrences (%) of bacteria isolated from soils exposed to septic tank leachate

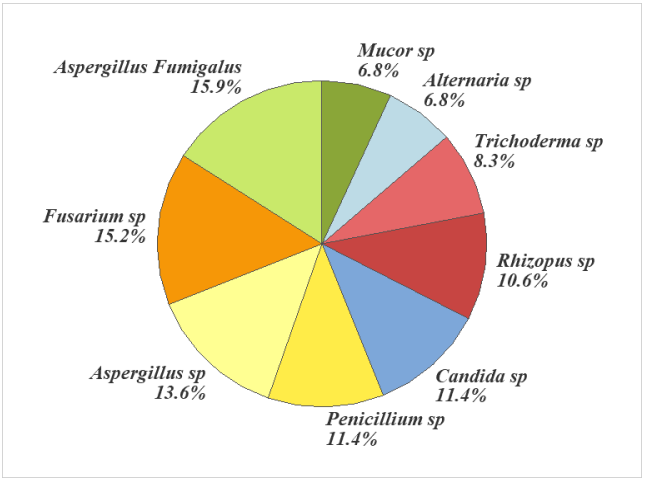

Figure 5: Occurrences (%) of fungi isolated from soils exposed to septic tank leachate

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The frequency distribution of the bacterial isolates at the various depths of soils exposed to septic tank leachate are presented in Figure 6. While the frequency of distribution of the fungal isolates at the various depths of soils exposed to septic tank leachate are presented in Figure 7.

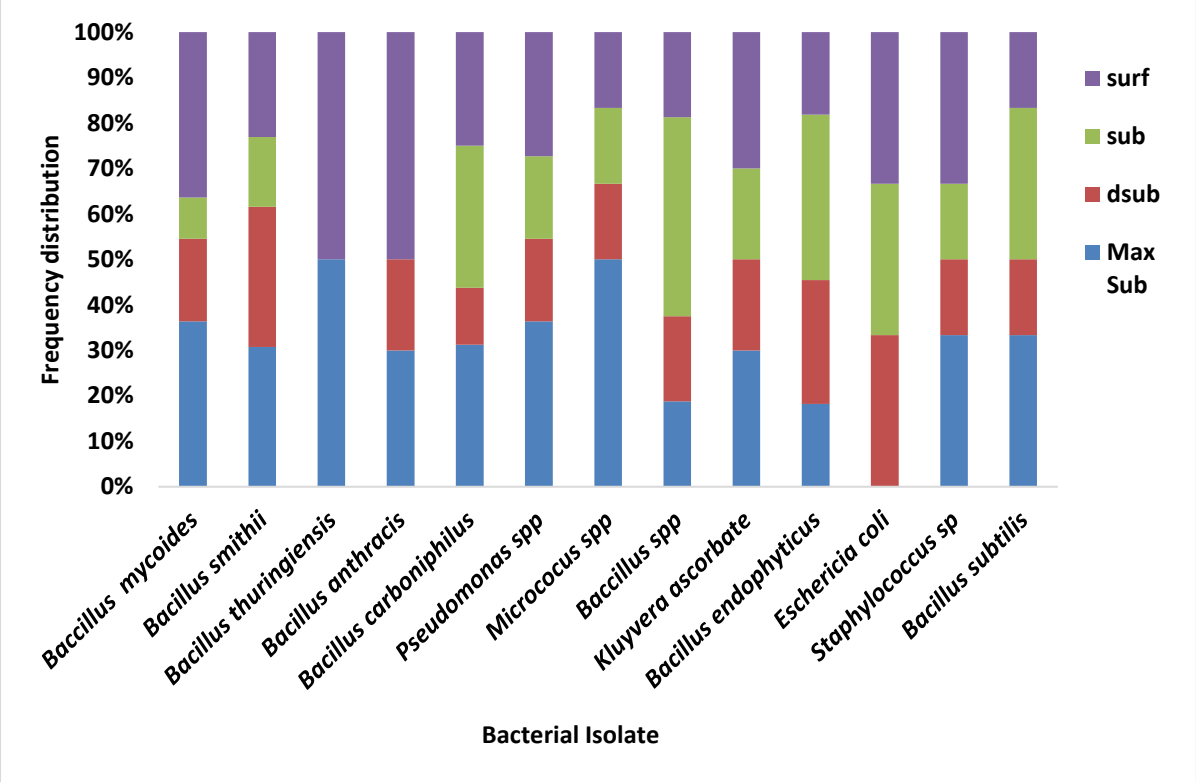

Figure 6: Frequency distribution of bacterial isolates with respect to soil depths

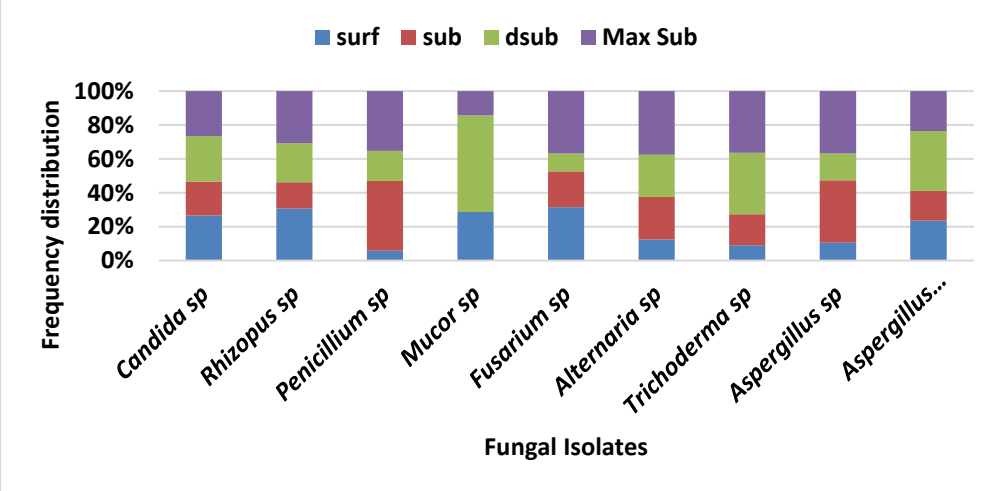

Figure 7: Showing frequency distribution of fungal isolates with respect to soil depths

Correlation

The Pearson's Correlation Coefficient and Matrix Plot Analysis (Correlogram) on the interrelatedness amongst the microorganisms and the extent of the relationship at the vertical soil depths are presented in Table 2.

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Table 2: Correlogram showing correlation of the various groups of microorganisms at various depths of soils exposed to septic tank leachate.

	THBs	THB su	THB ds	THB msub	TCCs	TCC su	TCC ds	TCC msub	TECs	TEC surf	TEC ds	TEC Msub	THF s	THF su	THF ds	THF msub	TVC s	TVC su	TVC ds	TVC msub	APC s	APC su
THBsu	-0.29																					
THBds	0.57	-0.52																				
THBMsub	-0.43	-0.55	0.28																			
TCCs	-0.28	0.62	0.04	-0.27																		
TCCsu	-0.49	-0.18	0.34	0.88	0.13																	
TCCds	-0.46	0.38	-0.32	0.38	-0.01	0.51																
TCCMsub	-0.75	0.79	-0.62	-0.02	0.56	0.26	0.69															
TECs	-0.18	-0.21	-0.67	-0.07	-0.54	-0.45	0.03	-0.02														
TECsu	-0.44	-0.37	0.35	0.96	-0.08	0.98	0.46	0.11	-0.31													
TECds	-0.76	0.51	-0.13	0.32	0.70	0.66	0.43	0.77	-0.48	0.50												
TECMsub	-0.32	-0.12	0.07	0.59	-0.34	0.62	0.36	0.04	-0.29	0.66	0.34											
THFs	-0.74	0.64	-0.88	-0.20	0.20	-0.11	0.28	0.72	0.30	-0.17	0.48	0.20										
THFsu	0.39	0.04	0.22	-0.06	0.05	-0.02	0.40	0.06	0.03	-0.05	-0.24	-0.50	-0.52									
THFds	-0.73	-0.20	-0.52	0.36	-0.02	0.13	0.02	0.28	0.62	0.20	0.22	-0.09	0.45	-0.31								
THFMsub	0.43	-0.14	0.14	-0.51	0.26	-0.60	-0.61	-0.31	0.23	-0.60	-0.43	-0.95	-0.36	0.35	0.10							
TVCs	0.62	-0.21	-0.12	-0.57	-0.37	-0.84	-0.42	-0.47	0.60	-0.75	-0.88	-0.72	-0.28	0.38	-0.06	0.72						
TVCsu	0.39	-0.24	-0.31	-0.54	-0.38	-0.87	-0.55	-0.44	0.73	-0.75	-0.79	-0.63	-0.03	0.04	0.22	0.68	0.93					
TVCds	0.75	-0.48	0.10	-0.40	-0.59	-0.73	-0.51	-0.74	0.50	-0.58	-0.99	-0.48	-0.46	0.25	-0.16	0.57	0.93	0.86				
TVC msub	0.37	-0.47	-0.01	0.19	-0.80	-0.13	0.29	-0.35	0.56	0.02	-0.70	-0.05	-0.39	0.56	-0.06	0.01	0.55	0.38	0.62			
APCs	-0.81	-0.03	-0.07	0.58	0.48	0.65	0.15	0.46	-0.10	0.60	0.76	0.13	0.28	-0.27	0.70	-0.10	-0.63	-0.45	-0.70	-0.50		
APC su	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
APCds	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
APCmsub	-0.04	-0.90	0.50	0.85	-0.47	0.58	-0.06	-0.49	0.07	0.73	-0.13	0.33	-0.52	-0.03	0.32	-0.13	-0.17	-0.14	0.08	0.35	0.35	-

Key: THB = total heterotrophic bacterial, TCC = total Total coliform count, TEC = *E. coli* count, TVC = total *Vibrio* count, THF = total heterotrophic fungi, APC = anaerobic plate count. S = surface (0.0m), su = subsurface (0.5m), ds = deep subsurface (1.0m), Msub = Max subsurface (1.5m).

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The Gel electrophoresis result of the amplified 16S rRNA of eight (8) bacterial isolates from soil exposed to septic tank leachate that were subjected to molecular analysis is shown in Plate 1 below. While the Phylogenetic identification of amplified sequences of 16S rRNA of the isolates from soil samples is shown in Figure 8. The molecular sequencing examination from the DNA and 16S PCR examinations identified the presence of *Bacillus thuringiensis, Bacillus firmus, Bacillus megaterium, Myriodes odoratimimus, Bacillus altitudinis* and *Bacillus cereus.* The ancestry tree of the bacteria isolated from soils exposed to septic tank leachate is divided into three major groups.

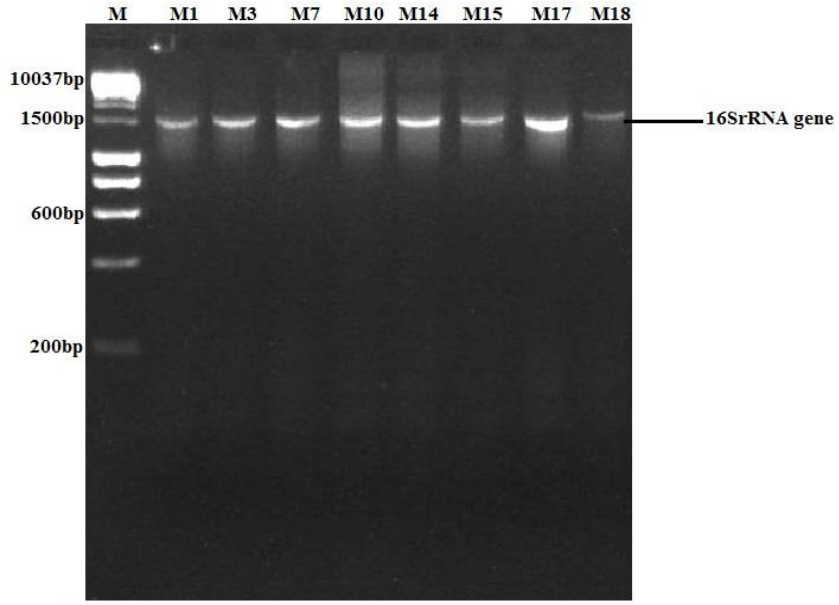

Plate 1: Gel electrophoresis of 16SrRNA gene amplification of isolates at about 1500bp: M is Hyper Ladder 1kb bioline

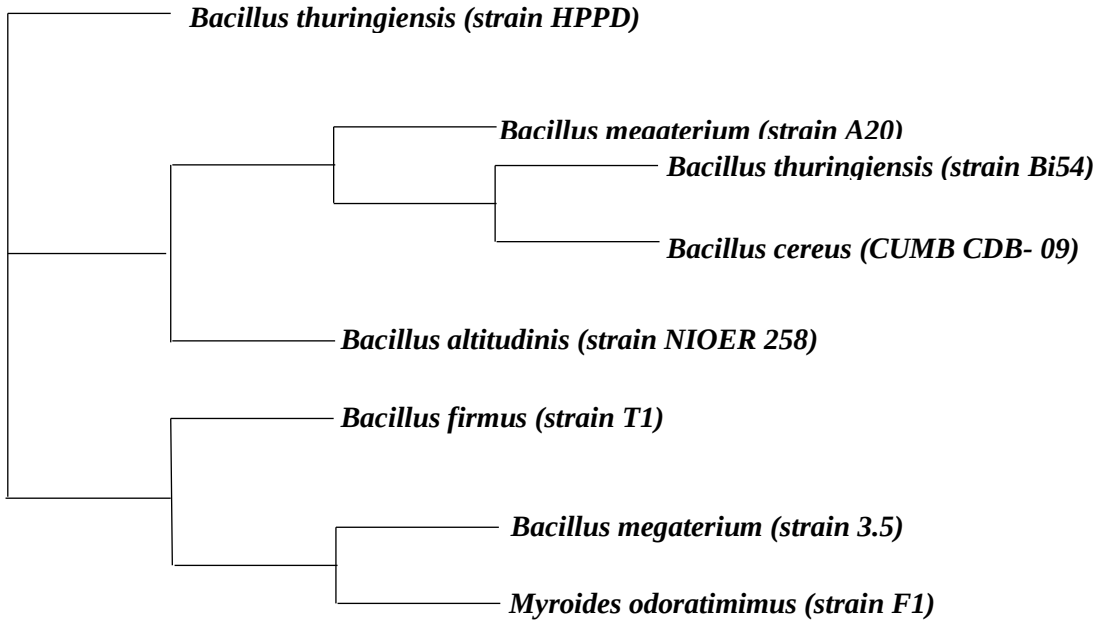

Figure 8: Ancestry tree of the bacteria isolated from soils exposed to septic tank leachate

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Discussion

This present study has revealed the microbiological quality of soils exposed to leachate from septic tanks with respect to the population of bacteria and fungi and to the types of these microbes in these soils. The highest colony growth of THB, TEC and TVC occurred at the surface soils, indicating that these microbes benefitted from the combination of organic nutrients and aeration which were probably richer at surface soils. On the other hand, THF and TCC recorded the highest mean colony populations at the 1.0m (deepsub depth). This indicates that the fungi may have benefited from a likely acidic soil pedon while the high coliform shows it's resilience to adsorption within the biomat. The occurrence of the highest APC population at the Msub depth (1.5m) is in agreement with the known ability of anaerobic bacteria to thrive in regions of very low aeration. The results further showed that THF thrived least at the sub soil profile. The statistical summary obtained by use of Minitab version 20 software, on the results of microbial colony counts and the variation of the mean total counts showed that the least colony growth was recorded for anaerobic plate count (APC), 0.53 $x10^4 \pm 0.968$ cfu/g for sub and deep sub soils , while the highest was THB 3.66 x $10^7 \pm 2894$ cfu/g for the surface soil while the colony forming units variation showed the decreasing order of microbial population was; THB > TEC > TCC > THF > TVC > APC.

The Total Heterotrophic Bacteria (THB) colony count obtained ranged from 0.700 - 11.35 x 10^7 CFU/g, while the mean counts were 6.53 x 10^7 CFU/g, 2.01 x 10^7 CFU/g, 2.08 x 10^7 CFU/g and 4.00 x 10^7 CFU/g for the surface soil, sub, deepsub and Msub depths respectively showing a decreasing order of surface > Max subsurface > deep sub and sub surface. The site average, 3.66 x 10^7 CFU/g is higher than 1.17 x 10^6 CFU/g and 8.57 x 10^6 CFU/g reported by Fatunla *et al.*, (2017) and 2.08 x 10^6 CFU/g previously reported for sewage by Enerijiofi *et al.* (2018). It is also higher than the WHO/FAO/DPR permissible limit for heterotrophic bacteria population in septic tank effluent or leachates.

For *Escherichia coli* , counts obtained were 5.95 $x10^4$ cfu/g, 1.62 $x10^4$ cfu/g, 0.55 $x10^4$ cfu/g and 0.11 $x10^4$ cfu/g for surf, sub, deepsub and Msub depths respectively. This translated to a 98.32 % decrease of *E. coli* population (or pathogen removal efficiency) across the vertical depths, from surface to Msub depth soils. The order was accordingly surface > sub surf > deep sub > Msub which is expected of a septic system functioning effectively as a pathogen remover. Balogun & Ogwueleka, (2021) reported a similar pathogen removal efficiency in their study on the Wupa waste water treatment plant in Abuja.

The Total Heterotrophic Fungi (THF) count from the study ranged from 0.1 x 10^4cfu/g - 3.9 $x10^4$cfu/g which is consistent with those of Fatunla *et al.*, (2017). They were however lower than (0.10 x 10^6 – 4.94 x 10^6) cfu/g reported by Enerijiofi *et al.*, (2018) for sewage sludge in their studies. The highest THF count, 3.9 $x10^4$cfu/g, was recorded at the 1.0m (deepsub) depth while the lowest was at the 0.5m (sub) soil depth. This shows that fungal colony growth was higher at the deepsub depth than at the sub soil depth (3.9 x 10^4cfu/g compared to 0.1 x 10^4 cfu/g), a finding which is very consistent with the ability of fungi to thrive or survive at lower soil pH (or higher acidity). Furthermore, the results of the mean counts at 0.0m, 0.5m, 1.0m and 1.5 m depths 1.29 x 10^4cfu/g, 0.88 x 10^4cfu/g, 1.57 x 10^4cfu/g and 1.16 x 10^4cfu/g respectively narrowly confirm the net benefit of increasing acidic soil conditions to the fungal population or colony growth or survival. However, fungal growth reduction was somewhat low from the surface through to the Msub depth (21.77%) compared to the 60.05% removal of THB achieved by the soil.

For the total *Vibrio* counts, surf, sub, deepsub and Msub depth levels were 1.42 x 10^4 cfu/g, 0.88 x 10^4 cfu/g, 0.53 x 10^4 cfu/g and 0.07 x 10^4 cfu/g respectively. This showed that the *Vibrio* bacteria was progressively filtered or attenuated by the soil biota with increasing depth and followed the order surf > sub > deepsub > Msub. The results also translated to a 93.06% vibrio bacteria removal efficiency by the study soils. This manifested as a 3.72 Log10 (x + 1) reduction of *vibrio* bacteria between the surface and the Msub soils which also showed that *vibrio* bacteria were better attenuated by the soil than *Escherichia coli* and Total coliforms, which were 2.83 and 1.69 times reduced respectively. The highest count was 3.3 x 10^4cfu/g obtained for the surf soils while all the depths recorded zero growths at some sampling points in the study for unclear reasons.

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The Anaerobic Plate Count (APC) ranged from (0.0 – 3.9 x 10^4) cfu/g, with the highest value occurring at the surface soil (0.0m). The mean APC counts at the 0.0m, 0.5m, 1.0m and 1.5m were 1.92 x10^4 cfu/g, 0.0 cfu/g, 0.0 cfu/g and 1.21 x 10^4cfu/g respectively which yields the decreasing order of APCsurf > APCMsub > APCsub and APCdeepsub. Although, the sub surface and deepsub depth soils recorded no detectable counts or responses, there was significant growth at the 1.5m (Msub) depth. This high count at a lower soil depth may be an indication of bacterial sedimentation due to gravitational pull toward a depth where there is more available and richer organic matter (Igbinovia *et al.,* 2016) while the high counts at the surface soils may have been due to resuspension of bacteria at the surface soil layers. The mean count across all depths (0.95 x 10^4cfu/g) was higher than the EPA limit of 5.2 x 10^2cfu/g.

The mean Coliform Count obtained, 3.15 x 10^4 cfu/g, 0.3 x 10^4 cfu/g, 3.87 x 10^4 cfu/g and 0.37 x 10^4 cfu/g for surf, sub, deepsub and Msub depths respectively indicated the increasing order of sub < Msub < surf < dsub. The occurrence of the highest Coliform count at the deep sub depth (1.0m) may be attributed to stronger adhesion to a soil pedon which consisted of more clay particles that provided several nutrient rich biotic and abiotic surfaces compared to the surface and sub surface profiles (Shikuma and Hadfield, 2010) or by the formation of biofilms (Boyd and Waldor, 2002; Aagesen and Hase, 2012; Nalin *et al.,* 1979). This aligns with the findings of Howell *et al.,* (1996) that fecal coliform survived significantly more in the presence of saturated clays in comparison with silt or sand particles. Furthermore, coliform count range for the study site (0.0 -12.65 x 10^4) cfu/g was similar to (0 - 12.0 x 10^5) cfu/g range for septic effluent in Ado Ekiti and (0 - 58.2 x 10^5) cfu/g for sewage from Esan land reported by Oluwasola *et al.,* (2017) and Enerijiofi *et al.,* (2018) respectively. Despite the significant coliform presence at the 1.0m depth, overall coliform removal efficiency between the surface and Msub depths was about 85.29%.

In general, the surface soil showed the highest colony growth of THB, TEC and TVC indicating that these microbes benefitted from the combination of organic nutrients and aeration which were probably richer at surface soils. THF and TCC recorded the highest mean colony populations at the 1.0m (deepsub depth). This indicates that the fungi may have benefited from a likely acidic soil pedon while the high coliform shows it's resilience to adsorption toward within the biomat. The occurrence of the highest APC population at the Msub depth (1.5m) is in agreement with the known ability of anaerobic bacteria to thrive in regions of very low aeration. The results further showed that THF thrived least at the sub soil profile. Figure 2 showed the order of decreasing microbial population as THB > TEC > TCC > THF > TVC > APC.

The Pearson's Correlation Coefficient and Matrix Plot Analysis (Correlogram) on the interrelatedness amongst the microorganisms and the extent of the relationship at the vertical soil depths presented in Table 2 showed that, at the surface depths, both THF and APC, were strongly and negatively correlated with THB (r = -0.74 and r = -0.81 respectively). TVC was the only positive correlation that was recorded of all the studied organisms versus THB (r = 0.62), which may suggest that the vibrio bateria surveillance may be an indicator of microbial contamination at the surface depths and are probably of a similar origin. Similarly, TVC was positively correlated with TEC, while APC vs TVC was negatively correlated (r = - 0.63).

The sub soil pedon (0.5m depth) revealed strong correlation between TVC vs TCC (r = 0.87) and TVC vs TEC (r = - 0.75) compared to the strength of their relationships (and direction for TVC vs TEC) at the surface depth. Furthermore, there was near linear correlation between *E. coli* and *coliforms* (r = 0.97) indicating very similar influencing factors. At the deepsub depth, the correlation between TVC vs TEC became stronge, almost being perfectly linear (r = - 0.99).

At the Msub soil depth, APC and THB were very strongly correlated (r = 0.85), and were slightly greater in strength than they were at the surface soil depth (r = - 0.81), although the direction was opposite, indicating that correlation improved with soil vertical depth. Further more THF and TEC were strong and negatively correlated (r = - 0.95). However, it was observed that at this surface soils, THB was negagively correlated with all the microorganisms as also occurred at the sub surface soils except with TVC with which it was positively correlated. Similarly, all organisms were

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negatively or very insignificantly correlated one with the other except TEC vs TCC at the subsurface depths.

The results for the frequency distributions of the bacterial isalates presented in Figure 6 showed that *Bacillus thuringiensis* and *Bacillus anthracis* thrived most at the surface (0.0m) depths which may be closely linked to the rich organic and mineral matter at the surface soils. In contrast, *Micrococus* had its highest occurrence at the Max sub depth which suggests amongst other things that they are anaerobic bacteria or are facultative organisms. Furthermore, while *Bacillus thuringiensis* recorded no growth at the sub and deep sub depths, *Escherichia coli*, similarly recorded no significant occurrences after the dsub depth. In like manner, *Bacillus anthracis* showed zero growth at the sub surface soil (0.5m) depths. These observations may be attributed to adsorption or filteration of the microorganisms by soil constituents at these soil depths.

In general, the *Bacillus* genera were able to thrive both at the Max sub and surface soil depths. In congruence with this study, several researchers have reported similar isolates in their septic systems. Santhiya *et al.* (2011), reported the presence of *Pseudomonas* in their studies on discharged treated sewage in Morocco. Similarly, Ibenyassine *et al*. (2007) and Al- Jaboobi *et al.* (2013), revealed the presence of *E. coli* and *Staphylococcus* in their research. *Bacillus* spp, *Escherichia coli*, *Pseudomonas* spp, and *Staphylococcus* were reported by Obire and Deeyah (2021) in soil samples exposed to cassava effluent.

Likewise, Iyerite, Obire and Douglas (2021), reported a rich spectrum which included *Enterobacter*, and *Micrococcus* amongst others in their studies which are similar to those of this present study. However, in contrast, the soils of this study did not reveal the presence of either *Salmonella* sp. or *Shigella* sp. which were common in the aforementioned.

For the fungal frequency distribution (Figure 7), the obtained fungi spectrum in the studies were in very strong agreement with those reported by Iyerite, Obire and Douglas (2021). *Aspergillus* spp, *Penicillium* spp, *Fusarium* spp, *Mucor* spp. were also reported by Obire and Deeyah (2021) in soil samples exposed to cassava effluent.

This study also showed that whereas *Candida* sp. and *Rhizopus* sp. showed zero presences at the Max sub soil depth, *Alternaria*, *Trichoderma* and *Aspergillus* sp. all showed increases in occurrence with increasing soil depth.

Also, the fungi seemed to have thrived better at the Max sub soil depths possibly pointing to a more acidic soil pedon as fungi are generally known to grow well in soils of low pH. It is not immediately clear if the higher occurrence of *Mucor* sp., at the Deep sub level compared to the Max sub depth is related to such factors as aeration, moisture content and/or clayey fractions.

This present study has also revealed the molecular identity of some bacteria isolated from the soils exposed to septic tank leachate. The 16SrRNA gene sequence of the eight (8) bacteria analyzed showed that, isolate M1 has 86.5% pairwise identity with *Bacillus thuringiensis* strain HPPD which has NCBI accession number MT125885. The e value is 3.98E-66. Isolate M3 has 83.4% pairwise identity with *Bacillus firmus* strain T1 which has NCBI accession number MG593547. The e value is 2.95E-43. Isolate M7 has 94.1% pairwise identity with *Bacillus megaterium* strain A20 which has NCBI accession number MT597980. The e value is 0. Isolate M10 has 89% pairwise identity with *Bacillus megaterium* strain 3.5 which has NCBI accession number MH973228. The e value is 0. Isolate M14 has 92.6% pairwise identity with *Bacillus thuringiensis* strain Bi54 which has NCBI accession number HQ336297. The e value is 0. Isolate M15 has 87.8% pairwise identity with *Myroides odoratimimus* strain F1 which has NCBI accession number KJ197176. The e value is 3.17E-123. Isolate M17 has 93.3% pairwise identity with *Bacillus altitudinis* strain NIOER258 which has NCBI accession number MG205975. The e value is 0. While isolate M18 has 94.7% pairwise identity with *Bacillus cereus* strain CUMB CDB – 09 which has NCBI accession number MH899083. The e value is 0.

The ancestry tree of the bacteria isolated from soils exposed to septic tank leachate that were molecularly analyzed is divided into three major groups. Group A consist of only isolate *Bacillus thuringiensis* (strain HPPD) which is more diverse from other organisms in Groups B and C. Group B is further divided into two sub-groups. *Bacillus thuringiensis* (strain Bi54) and

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Bacillus cereus (CUMB CDB- 09) are closely related having *Bacillus megaterium* (strain A20) related to them. *Bacillus altitudinis* (strain NIOER 258) is related to organisms in the first division. Group C is further divided into two sub-groups. Isolates *Bacillus megaterium* (strain 3.5) and *Myroides odoratimimus* (strain F1) are closely related having isolate *Bacillus firmus* (strain T1) in the first division related to them.

The results of this study highlight the ubiquity of the *Bacillus* genera in septic leachate contaminated soil environments. A common presence echoed in several findings from similar research of Obire and Aguda, (2002) and Chukwuma *et al.* (2022) who reported occurrences of 26.06% and 20% respectively. However, it is observed that whereas the reported occurrences for *Escherichia coli* by Obire and Aguda, (2002) was two-fold more than those of this present study (4.35% compared to 2.40%), the occurrences for *Pseudomonas* in this present study is about twofold higher than those of the authors (8.7% vs 4.35%).

The predominance of *Aspergillus* in the present study resonates very closely with the findings of Enuneku *et al.,* (2020) in which they reflected *Aspergillus* as prevalent in almost all the soils of the sites they studied. They also reported the presence of same organisms. However, in contrast to the findings of this present study, the authors reported *Fusarium sp* and *Penicillin* to have had the lowest occurrence. The dominance of *Aspergillus* in this study may infer that the composting of dead plant matter and other materials for top soil organic layer formation may have provided temperatures best suited for their flourish.

This study has demonstrated that soakaway septic tank system does not treat sewage sufficiently to an extent that ensures pathogenic bacteria and fungi are not carried in its leachates into the surrounding soil because several microorganisms capable of causing diseases and illnesses were detected. *Bacillus* genera boasts a plethora of organisms that are pathogenic to human and animal lifeforms such as *Bacillus subtilis* which has been implicated in pneumonia and endocarditis; *Bacillus cereus* causes gastrointestinal illnesses, and *Bacillus anthracis* which causes anthrax in animals, and humans who sufficiently come in contact with its carrier animals. *Pseudomonas* has been implicated in endocarditis, pneumonia and meningitis.

Aspergillus causes infections that impair the lungs or sinuses. *Aspergillus fumigatus* causes aspergillosis, and is currently one of three pathogens on the watch list of the United States Centre for Disease and Control, (CDC) because of its increasing resistance to triazoles used in the treatment of the infection it causes. *Escherichia coli* sets off urinary tract infections while *Staphylococcus* cause abscesses (boils) (Obire and Aguda, 2002). *Myroides odoratimimus* is a low grade opportunistic pathogen that causes soft tissue infections (Sophia *et al*., 2012). As leachates seep into lower pedons of soil, they will eventually come in contact with the ground water geology and contaminate ground and well waters and their courses. They may also be carried by rain or runoff surface water and get into rivers and other water bodies that serve human and animal needs and cause serious health problems. In the immediate, the educational or academic community around will be at great risk. Productive school hours could be lost through possible illnesses. Therefore, human and animal health forms around the vicinity should be protected through additional health and environmental management interventions and accelerated planning towards implementation of a more modern sewage management system vigorously pursued to avoid leaching of septic tank effluent.

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