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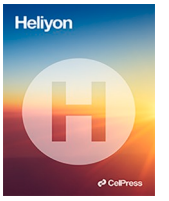

Research article

Tracking enteric pathogen contamination from on-site sanitation facilities to groundwater in selected rural areas of Vhembe District Municipality, Limpopo Province, South Africa

Jeridah Matlhokha Sekgobela [a,*], Colette Mmapenya Khabo-Mmekoa [b], Maggy Ndombo Benteke Momba [a,**]

[a] Tshwane University of Technology, Department of Environmental, Water and Earth Sciences, Arcadia Campus, P/B X 680, Pretoria, 0001, South Africa

[b] Tshwane University of Technology, Department of Biomedical Sciences, Arcadia Campus, P/B X 680, Pretoria, 0001, South Africa

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ABSTRACT

Groundwater is valued as a source of potable water, although it is vulnerable to environmental pollution. The aim of this study was to track enteric pathogen contamination from on-site sanitation (OSS) facilities to 70 household boreholes used by four villages of the Vhembe District Municipality. Two objectives were pursued: to measure the lateral distance between the borehole and the sanitation facilities in household yards, and to track the enteric pathogens. The borehole abstraction point and OSS system distance were determined using a steel measuring tape. Real-time PCR was used to track *Shigella flexneri*, *Salmonella* typhimurium, *Campylobacter jejuni*, and enterotoxigenic *Escherichia coli* (ETEC) from Wastewater (WW) from domestic septic tank and sludge from pit latrines to boreholes. *Escherichia coli* was used as an indicator of faecal contamination. Results showed that 25% of households kept a distance of ≥50 m between the OSS facilities and the boreholes. In total, 87.5% of household boreholes in the rainy season and 72.5% in the dry season were contaminated with *E. coli* and pathogenic bacteria: *Shigella flexneri*, *Salmonella typhimurium*, and ETEC. The concentrations of the pathogens ranged from 2.03 to 2.12 LogEGC/100 mL. A very weak ($r = -0.093$) to moderate ($r = -0.541$) association was found between pathogens in groundwater and on-site sanitation systems (WW from septic tank and sludge from pit latrine). This suggests that the pathogens were not present in the sanitation compartment when they were found in the groundwater and vice versa. Moreover, a very weak ($r = 0.007$) to moderate ($r = 0.525$) association was found between the detected contaminants in groundwater and the lateral distance between the OSS facilities and the boreholes. The pathogens detected in all samples showed consistent concentrations, suggesting potential contamination from OSS systems' waste, possibly in groundwater, indicating potential contamination. The siting of OSS facilities at the yards in this study appeared to have a slight influence on the contaminants detected in groundwater. This study calls for an education program to be implemented by the Water and Sanitation Services Authorities to prevent contamination of groundwater and the risk of waterborne diseases.

* Corresponding author.
** Corresponding author.
E-mail addresses: sekgobelajeridah@gmail.com, 211140178@tut4life.ac.za (J.M. Sekgobela), MmekoaKCM@tut.ac.za (C.M. Khabo-Mmekoa), momba1958@gmail.com (M.N.B. Momba).

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1. Introduction

Access to continuous water piped water from the treatment plant is not always available, there are often water cuts due to treatment plant not working. Groundwater is thought to be an important source of drinking water, particularly in places where access to clean water is scarce. In 2020, globally, 771 million people did not have access to services that provided safe drinking water, and 80% lived in rural areas of which over 50% reside in sub-Saharan Africa [1]. The use of groundwater from household boreholes is increasing in developing countries [2]. South Africa (SA) is a water-stressed country and groundwater is a valuable and essential water source. According to data from Stats SA [3], 20 378 households in the Vhembe District Municipality (VDM) relied on groundwater from household boreholes as their primary drinking water source. Groundwater from household boreholes is considered of exceptional quality and an independent water supply, making it accessible and convenient for household members. Groundwater is currently the most favored source of drinking water worldwide [4] and it is regarded as one of the enhanced supplies of drinking water [5]. Despite all the benefits listed above, environmental hazards such as fertilisers, poor sanitation, inadequate sewerage infrastructure, on-site sanitation (OSS) systems located in close proximity of boreholes, and surface water-groundwater interactions as well as improperly sealed, abandoned household water wells and lead to the deterioration of its quality [6–10].

There have been numerous reports from earlier and more recent studies on the contamination of water sources within the VDM region. In a microbiological study of the river water sources used by the Venda rural community [11], it was found that the river water contained microorganisms such as *Escherichia coli, Shigella* spp. And *Salmonella* spp., and that the water is not safe for human consumption. Another study in the Tshitale-Hlanganani region detected *E. coli* in groundwater from the borehole used by communities for drinking [12]. In 2006, between June and July, there was an outbreak of diarrhea in Tshikuwi (a rural area in Venda) [13]; the authors also indicated that most of the river water and groundwater used by households in the rural areas of the VDM is of poor microbial quality. Taonameso and co-workers [14] also pointed out that groundwater from the boreholes used in Dididi village (in VDM) tested positive for enteropathogenic *Escherichia coli* (EPEC) and enterotoxigenic *Escherichia coli* (ETEC). In Muledane (in Thulamela Local Municipality, VDM) [15], detected high faecal coliform bacteria (*E. faecalis* and *E. coli*) in groundwater from boreholes. In a separate study conducted by Ref. [16], *Salmonella* Typhimurium, *Shigella flexneri,* and *E. coli* were detected in groundwater used by school children in the Vhuronga 1 Circuit of the VDM. Based on these findings, monitoring of microbial contamination in groundwater within the VDM sites is critical.

A report by Ref. [17] pointed out that on-site sanitation systems are used by more people in the sub-Saharan Africa region (44%) than those having sewer connections (7%) in 2020. Pit latrines are one of the world's most common primary types of improved on-site sanitation systems in this region, and in South Africa, a ventilated improved pit (VIP) latrine is the minimum acceptable level of sanitation [18]. A recent study by Ref. [19] has highlighted that the majority of households in the VDM use on-site sanitation facilities in the form of pit latrines and septic tanks. These sanitation systems are prevalent due to the country's overall relative lack of water and frequent water outages. Additionally, these OSS systems are used in areas where housing density is low and thus centralised wastewater treatment plant (WWTP) is not economically feasible; or where resource limitations do not permit centralised wastewater treatment. On-site sanitation is a much-overlooked source of faecal contamination in groundwater; however, these systems pose a serious threat to groundwater because faecal matter accumulates in one location, and contaminants may seep into the subsurface [20]. The waste generated by these OSS systems is underground; therefore, it is easy to ignore and not put a proper management system in place. However, what goes down the drain and into the ground is not gone forever; these OSS systems have an impact on the environment. If the waste generated by these on-site sanitation systems is not adequately managed, groundwater sources will be at risk of contamination.

Recommendations for the siting of pit latrines differ across countries; they range from 15 to 75 m between the groundwater source and the sanitation unit. Ensuring an adequate distance between wastewater disposal facilities and drinking water wells is crucial to safeguard the water sources against microbial contamination [21]. To our knowledge, no research has been done to link on-site sanitation facilities to the faecal contamination of boreholes that are located on the same household premises at the specific sites investigated in the present study. Assessing groundwater quality in boreholes is crucial, especially in challenging water supply areas with frequent contamination issues. This study aimed to track the selected enteric pathogens from OSS facilities to household boreholes used as the main water sources by four selected villages of the Vhembe District Municipality; as well as to establish the lateral distances between the sanitation facilities and the boreholes where groundwater samples were collected, and to determine the relationship between the OSS facilities and the contaminants in the groundwater. The following objectives were set to achieve the aim of the study: The first step was to ascertain whether the communities apply the National Norms and Standards for Domestic Water and Sanitation Services [18], by measuring the lateral distance between the borehole and the sanitation facility in household yards across the four villages; and the second step was to track the presence of the selected pathogens from WW from septic tank and from sludge from pit latrines to groundwater abstracted from the household boreholes.

2. Methods

2.1. Description of study sites and ethical approval

The current investigation was carried out for a total of 28 days between March 2021 and August 2021 in the Limpopo Province's Vhembe District Municipality (VDM), which is situated in the far north of South Africa. The study was conducted in four (4) villages: Tshilapfene-Village A, (Tsianda-Village B, Ha-Mutsha-Village C, and Njhakanjhaka-Village D (Fig. 1). Information from the South

African Geomatics Council was used to determine the geology of the study sites (Table 1). Although Village B and Village C share the same geology and are separated by a road, Village B village stands out due to its steep slopes. The study was reviewed and approved by the Tshwane University of Technology's Faculty of Science Research Ethical Committee, with the approval number: [2019/09/017 (FCPS 03) (SCI)] and after describing the project's goals to the municipal committee, access to VDM villages was granted. All participants provided informed consent to participate in the study.

2.2. Selection of study households and sampling points

A systematic sampling criterion was used for the selection of study households (HHs). Before the study was conducted, a survey was conducted to identify villages and HHs that fall within the study criteria. The study aimed at assessing the impact of OSS systems on the quality of groundwater. The study households were selected based on the following criteria: (i) Use groundwater from drilled electricity borehole (ii) Use on-site sanitation facility and (iii) Both the borehole and sanitation facility are located in the yard. To make it easier to generalize the findings to the entire village population while reducing the need to sample all the HHs, the number of households selected per village represents 5% of the households in that village that fall under the study criteria.

2.3. Outline of the methodology

Fig. 2 provides a detailed layout of the research method followed in this study. The distance between the Submerged electricity borehole (drilled holes in the ground that access groundwater, with the added feature of electrical equipment submerged within the borehole) and the on-site sanitation facility was measured, followed by the collection of samples. Groundwater samples were collected at 70 household boreholes, while wastewater was collected from 18 household septic tanks and human waste from 52 pit latrines. *Escherichia coli* was quantified using a culture-based method and quantitative polymerase chain reaction (qPCR) was used to track the target pathogenic bacteria.

2.4. Measurement of the distance

The boreholes in the study area were equipped with electric submersible pumps. The lateral distance between the borehole abstraction point and the OSS system (pit latrine or a septic tank system) was determined using a steel measuring tape as used in previous studies [22,23]. The distance between OSS facilities and boreholes was measured at 70 HHs in the selected villages. Ten HHs were chosen for Village D village, while twenty HHs were taken into consideration for each of the following three villages: Village A, Village B, and Village C. The measurements were recorded in metres.

2.5. Collection of samples

Samples were collected from a total of 70 HHs, during the rainy season (March 2021 and April 2021) and the dry season (June and

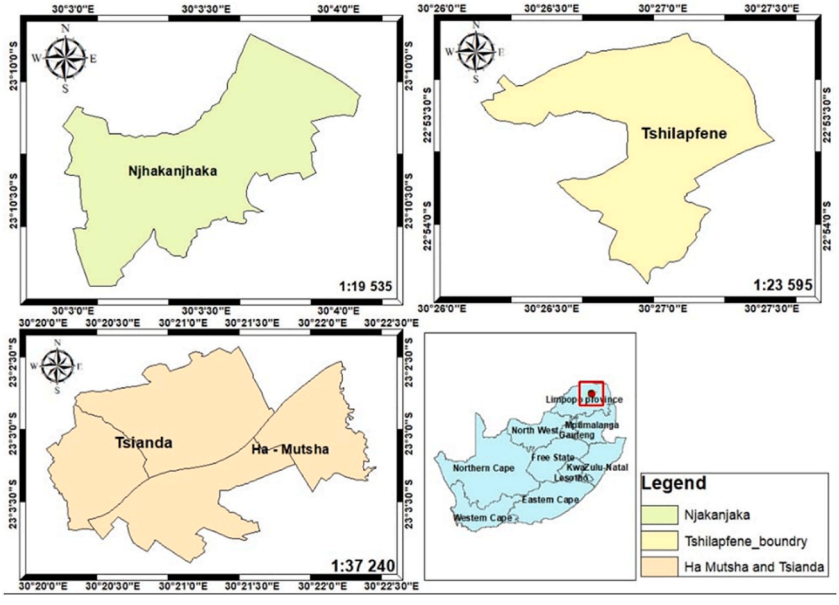

Fig. 1. A map indicating the sites where the study was conducted. This figure indicates the four villages, namely: Village A, Village B, Village C, and Village D.

Table 1
Geological data of the sampling sites.

Villages	Geology
Village A	- Basaltic and andesitic lavas with subordinate interbedded pyroclastic and clastic sedimentary rocks - Diabase - Sandstone (locally quartzitic), subordinate conglomerate, basaltic lava, tuff, shale, and siltstone
Village B & Village C	- Basaltic and andesitic lavas with subordinate interbedded pyroclastic and clastic sedimentary rocks. - Conglomerate, quartzitic or feldspathic sandstone, greywacke, shale - Leucocratic, tonalite-trondhjemite-granodiorite (TTG) gneisses.
Village D	- Leucocratic, tonalite-trondhjemite-granodiorite (TTG) gneisses. - Metapelite

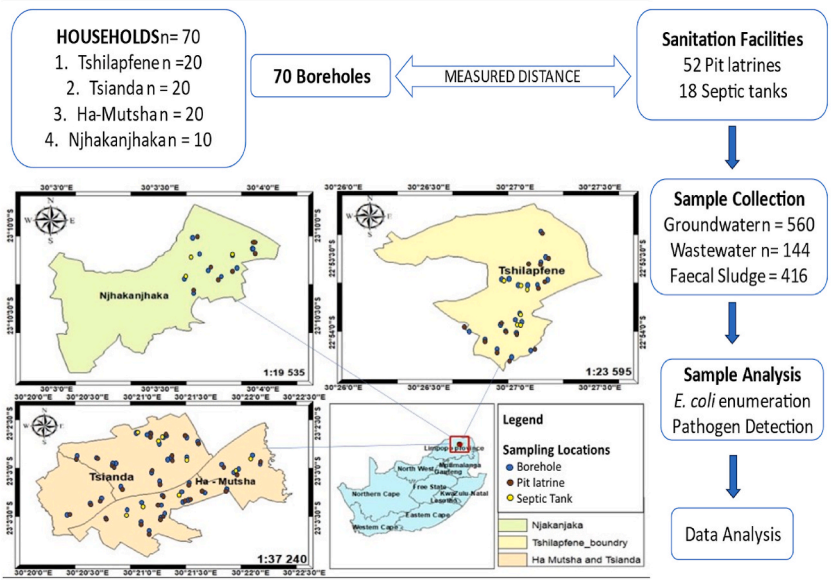

Fig. 2. Research methodology flow chart. The figure indicates the study site's sampling points and the number of boreholes, pit latrines and septic tanks sampled as well as the total number of samples collected. The figure further shows the methods used for the analysis of the samples.

Table 2
Borehole groundwater, WW from septic tank, and sludge from pit latrine samples collected during the study period.

Villages Households	Number of boreholes, septic tanks and pit latrines sampled	Number of groundwater, wastewater and human waste samples collected					
		Groundwater		Wastewater		Human waste	
		Wet	Dry	Wet	Dry	Wet	Dry
Village A 20 HHs	20 BHs 6 STs 14 PLs	80	80	24	24	56	56
Village B 20 HHs	20 BHs 4 STs 16 PLs	80	80	16	16	64	64
Village C 20 HHs	20 BHs 5 STs 15 PLs	80	80	20	20	60	60
Village D 10 HHs	10 BHs 3 STs 7 PLs	40	40	12	12	28	28
Total	70 BHs 18 STs 52 PLs	280	280	72	72	208	208
Grand Total		560		144		416	

Boreholes (BHs); pit Latrines (PLs); septic tanks (STs).

August 2021). In each HH there were two sampling points, namely OSS compartment and the borehole. For each sampling point, samples were collected four times per season. In each season sample collection took place for two months (8 weeks). Samples were collected weekly (Mondays)/once per week. On each sampling day we collected samples from 35 households/week). To minimize contamination samples were all collected using sterile bottles. The total number of sampling days was 32, 16 days for each season. Sample collection took place twice a week (a total of 8 weeks per season). Table S1 (Supplementary Table 1) represents the geological coordinates (latitude, longitude) associated with a specific location of the sampling points. Table 2 provides a summary of the total number of samples collected for the study during the wet and dry seasons. Before collecting the groundwater samples, the tap was run for a few minutes to clear the plumbing system of any standing water. In total, 560 groundwater samples were collected from 70 household boreholes, namely 20 boreholes in each of the following villages: Village A, Village B and Village C and 10 boreholes in Village D using standard methods [24]. A total of 144 wastewater samples were collected from 18 household septic tanks (ST) in Village A (n = 6 STs), Village B (n = 4 STs), Village C (n = 5 STs), and in Village D (n = 3 STs). A modified method described by Ref. [25] was used to collect the wastewater samples. The collection of wastewater samples is described in our previous study [26]. For the sampling of human waste from pit latrines, 416 faecal sludge samples were collected from 52 household pit latrines (PLs) distributed across four villages: Village A (n = 14 PLs), Village B (n = 16 PLs), Village C (n = 15 PLs), and Village D (n = 7 PLs). Human waste samples were collected from pit latrines using a modified method described by Strande et al. (2014). Briefly, a sterile stool collection cup was firmly attached to the metal rod and inserted into the drop hole in the floor connected to the toilet seat to obtain faecal sludge. The sludge was collected multiple times at different sites within the pit, mixed, and added to a stool collection tube. To prevent the growth and degradation of the microorganisms during transport to the laboratory, all samples were kept cold inside the cooler box containing ice packs. All samples were clearly labelled with unique identification number. Human waste samples were transported to the University of Venda in the Parasitology Laboratory for DNA extraction. To maintain the viability and slow down the metabolic activity of any present microorganisms, groundwater and wastewater samples were kept cool (between 2 and 8 °C) on ice and transported to the Microbiology Laboratories at the Tshwane University of Technology in Pretoria and analysed within 24 h. To ensure that the sample temperature was not affected during transportation, upon arrival at the laboratory the temperature of the samples was measured aseptically using a liquid-in-glass thermometer. A portion of the water samples originally held in the sample bottles was transferred into the sterile sample container for the purpose of temperature analysis. The liquid-in-glass thermometer was rinsed with distilled water and immersed into the water sample without touching the sides or bottom of the container.

2.6. Detection and enumeration of Escherichia coli

The membrane filtration technique was used for the detection and enumeration of *E. coli* according to the standard methods [24] using Chromocult® Coliform Agar (CCA) (Merck, Darmstadt, Germany). All of the equipment needed for the membrane filtering process, including the forceps, collecting jars, and filtration apparatus, was sanitized to prevent contamination and guarantee accurate results. The filtration gear was cleaned with sterile water before filtering to get rid of any leftover impurities. Groundwater (100 mL) was filtered through a sterile 0.45 μm pore size membrane filter (47 mm diameter, Sartorius Stedim Biotech GmbH, Göttingen, Germany). The membrane filter was taken out of the equipment and placed into an agar plate using sterile forceps. To rule out contamination, blank samples (sterile distilled water) were also filtered and plated onto CCA. The agar plates were prepared according to the manufacturer's instructions. Incubation of the agar plates was performed at 36 ± 1 °C for 18–24 h. Plates were always prepared in triplicate. Following incubation, quantification of *E. coli* was performed by counting household dark blue/violet colonies [27] which were recorded as colony-forming units (CFU/100 mL). A mean value of the three replicates from all samples was obtained. Furthermore, *E. coli* counts (ECC) were averaged over the four sampling cycles for each season and borehole.

2.7. DNA extraction

Before DNA extraction the equipment and instruments, such as pipettes, centrifuges, and thermocyclers were calibrated. Modified methods from previous studies were used for DNA extraction [28,29]. For the groundwater samples and the WW from septic tank samples, DNA was obtained using a combination of two concentration methods (membrane filtration and centrifugation) as well as a pre-enrichment step. Membrane filtration and centrifugation were used to concentrate the groundwater and WW from septic tank samples and increase the detection sensitivity, while the pre-enrichment step was used to resuscitate the cells and allow them to proliferate. Sample volumes of 1000 mL of groundwater and 90 mL of wastewater were filtered through a 0.22 μm pore size membrane filter (47-mm diameter, Sartorius). The filters were transferred into 15 mL screw-cap tubes containing *Campylobacter* enrichment broth (Bolton Broth) (Merck, 67454), which supports the growth of *Campylobacter* species, and Gram Negative (GN) enrichment broth (Separations, 1248-CONDA) (developed by Hajna, 1955), which supports the growth of *Salmonella* spp., *Shigella* spp. As well as *Escherichia coli*. The filters were placed such that the broth covered the filter; this was followed by incubation at 37 °C for 24 h (GN Broth) and the Bolton Broth was firstly incubated at 37 °C for 6 h and then for another 24 h at 42 °C. Following incubation, 500 μL of each enrichment culture was transferred to a microcentrifuge tube. The tubes were centrifuged for 20 min at 13 000 rpm at 4 °C to obtain a pellet; centrifugation was repeated two to three times until desired/some visible pellet was obtained. The supernatant was discarded, and the pellet was re-suspended in 2 mL of sterile distilled water. The DNA was extracted from this re-suspended pellet using the ZR Soil Microbe DNA MiniPrep™ Kit (Zymo Research, USA), following the manufacturer's protocol. For DNA extraction from the human waste samples, a bead-beating step was included, following a modified method as described by Ref. [30]. Approximately 200 mg of each human waste sample was used for DNA extraction (the extracted DNA was frozen at −80 °C and transported to Tshwane University of Technology, Microbiology Laboratory for further analysis). The elution volume in DNA extraction was 35 μL. Finally, a

NanoDrop 2000 spectrophotometer (Thermo Scientific, South Africa) was used to determine the quantity and quality of the DNA using the (A260/A280 ratio of 1.8–2.0) of the extracted DNA. All the DNA samples were stored at −80 °C until further processing.

2.8. Molecular analysis

The primers and probes of the target pathogens and the target genes are indicated in Table 3. All the target genes are virulence genes and species-specific, except for ipaH gene in *Shigella flexneri*, which is also found in Enteroinvasive *E. coli*. Internal positive and negative controls were used to identify contamination from PCR reagents and DNA extraction. Positive controls included *Campylobacter jejuni* (ATCC33291), *Shigella flexneri* (ATCC12022), ETEC (ATCC35401), and *Salmonella typhi* (ATCC13311) bacterial strains. These strains were grown and maintained in culture media, and genomic DNA was extracted using a Zymo DNA extraction kit (Zymo Research, USA). Positive controls were added to the PCR reaction mix as a DNA solution. Negative controls included PCR (nuclease-free) water, a no template control (NTC), and an extraction blank. To ensure that the primers and probes bind to and amplify the target sequences in real-time, standard curves were created using positive controls of target pathogens, serially diluted ten times. The efficiency of all PCR assays was determined using the formula E = [10 (−1/M)]-1 [31], where M is the slope and E is the assay efficiency and ranged between 106% and 110% (Table S2).

The stock solution for all the primers and probes was 100 μM. From the stock solution, a 10 μM working stock for PCR reactions was prepared. This was achieved by preparing a 10-fold dilution and doing a 1:10 dilution (one part of the stock solution was mixed with nine parts of sterile PCR water). The PCR amplification reactions were performed using the Bio-Rad CFX96 with a 96-well design Touch Deep Well Real-time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). All the nucleic acid extracts were analysed via a multiplex qPCR for the detection of *Shigella flexneri*, *Campylobacter jejuni* and *Salmonella* typhimurium. Each PCR reaction was performed in a volume of 25 μL consisting of 12.5 μL of GoTaq® Probe qPCR Master Mix [contains GoTaq® Hot Start Polymerase, $MgCl_2$, dNTPs and a proprietary reaction buffer (Promega)], 3 μL of PCR/nuclease-free water, and 2.0 μL of DNA template. For each of the three target genes, 1 μL of forward primer, 1 μL of reverse primer and 0.5 μL of the probe were used. Amplification was performed using the cycling conditions outlined in Table 3. For the detection of ETEC, the singleplex assay was employed. Amplification reactions were performed in a total of 25 μL, which contained 12.5 μL of GoTaq® Probe qPCR Master Mix, 3 μL of PCR nuclease-free water, 2.0 μL of DNA template, 1 μL of forward primer, 1 μL of reverse primer, and 0.5 μL of the TaqMan probe. To maintain the stability of the reagents and minimize the risk of non-specific amplification or contamination, all the PCR master mixes were prepared on ice. To ensure the reliability and reproducibility of your results, all PCR reactions were run in duplicates. Following amplification, the data were processed using Rotor-Gene 6000 software, which automatically interprets the data and generates cycle threshold (Ct) values and fluorescence curves. The Ct values were compared to the positive controls of each target. Samples that tested positive for the target genes were noted and recorded.

2.9. Data analysis

The data were analysed using a combination of data analysis tools on Excel (365) and IBM SPSS statistics (Version 28.0.1.1 (15)). General descriptive statistics were used to obtain summary statistics. For every sample, quantification of every pathogen was carried out by calculating the mean Ct value by averaging the Ct values from duplicate wells. The equivalent genome copies (EGC) were calculated by interjecting the mean Ct value to the standard curve for all target pathogens and the dilution factor of the PCR assay. For the groundwater and wastewater samples, quantification was given as log10 EGC per 100 mL, and for the faecal sludge samples, log10 EGC per gram. A Pearson's correlation test was used to measure the degree of association between (i) the presence/absence of pathogens and lateral distance between boreholes and the OSS facility, and (ii) the linear association between *E. coli* concentration in

Table 3
Primer and probe sequences of the target pathogens.

Pathogen (Gene)	Primer	Sequence 5′ to 3′	Cycling conditions	Reference
Campylobacter jejuni subsp. *jejuni* (GyrA)	366F 614R 409P	CTA TAA CAA CTG CAC CTA CTA AT ATG AAA TTT TTG CCA GTG GTG Fam-CTT AAT AGC CGT CAC CCC AC-Tam	initial incubation at 95 °C for 1 min, 45 cycles for 15 s at 94 °C, 20 s at 50 °C, 30 s at 72 °C and a final extension at 72 °C for 30 s	[49]
Shigella flexneri (ipaH)	1635F 1804R 1747P	CAG AAG AGC AGA AGT ATG AG CAG TAC CTC GTC AGT CAG ROX-ACA GGT GAT GCG TGA GAC TG-BHQ2		[50]
Salmonella enterica subsp. *enterica* serovar typhimurium LT2 (TtrC)	4136F 4315R 4163P	AAT TAG CCA TGT TGT AAT CTC ATT GTT GAT TCA GGT ACA AAC JOE-CAA GTT CAA CGC GCA ATT TA-BHQ1a		[51]
Enterotoxigenic *Escherichia coli* (STh)	F R P	GCTAAACCAGYAGRGTCTTCAAAA CCCGGTACARGCAGGATTACAACA Quasar-705 TGGTCCTGAAAGCATGAA-BHQ2	3 min at 95 °C for initial denaturation, 45 cycles at 95 °C for 10 s, and at 60 °C for 1 min.	[52]

Forward (F); Reverse (R); Probe (P); Primer (').

groundwater and the lateral distance between the boreholes and OSS facilities. A point-biserial correlation test was used to find the association between the detected pathogens in groundwater and those in on-site sanitation systems (wastewater and sludge from pit latrine). To determine significance, an alpha value of ≤0.05 was used. Pearson's r was used to indicate the strength of the associations, where values of r = 0–0.19 were regarded as very weak, r = 0.2–0.39 as weak, r = 0.40–0.59 as moderate, r = 0.6–0.79 as strong, and r = 0.8–1 as a very strong correlation. Statistical analysis was not calculated where there were low or no detection rates.

3. Results

3.1. The lateral distance between OSS facilities and boreholes

The lateral distance between borehole and OSS facility for each of the 70 households distributed across four villages is indicated in Table S3. Table 4 indicates the summary statistics of the measured distance between boreholes and sanitation facilities per village. The findings revealed that throughout the four villages, the lateral distance between the OSS facilities and household boreholes located on the same properties ranged from 11 to 81 m. In Village A, the measured distance ranged from 15 to 75 m, for Village B from 11 to 66 m, for Village C from 22 to 81 m, and for Village D from 12 to 55 m. The overall mean distance was 38.1 m and a total of 22.9% (n = 16) of HHs exhibited a distance of ≥50 m between the OSS facility and the borehole. These include 7 HHs in Village C, 5 HHs in Village A and 2 HHs in Village B, and another 2 HHs in Village D. The results showed that the household with the longest measured distance between the sanitation facility and the borehole was recorded in Village C village; the average mean was 44.75 m. The findings also revealed that in all four villages, the standard deviations were less than their respective means.

3.2. Prevalence of pathogens

The study found that 87.5% of boreholes had detectable *E. coli* during the rainy season and 72.5% during the dry season. *Escherichia coli* was detected in groundwater samples from all BHs during the rainy season, except for Village A village, where only 50% of BHs tested positive for *E. coli* in both dry and rainy seasons. The WHO states that drinking water should not contain any detectable *E. coli*. The prevalence of pathogens in sludge from pit latrine (HW) (Fig. 3) and WW from septic tank (Fig. 4) from the on-site sanitation systems across the four villages. Overall, ETEC was the most common pathogen in both faecal sludge and wastewater and *Shigella flexneri* was the least prevalent pathogen. *Campylobacter* was not detected in any of the sites, while in Village D village, all the target pathogens were not detected in both faecal sludge and WW from septic tank. The highest ETEC incidence was found in faecal sludge from Village C throughout both the wet (75%) and dry (40%) seasons. The WW from septic tank from Village A exhibited the highest ETEC incidence in the wet (66.7%) and the dry (45.8%) seasons among the target pathogens. *Shigella flexneri* and *Salmonella* Typhimurium were only detected at low rates in human waste (6.7% & 1.7%) and WW from septic tank (5% & 10%) from Village C during the rainy season. The presence of the target pathogens in groundwater differed across the four villages (Fig. 4). Except for *Campylobacter jejuni* which was not detected in any of the groundwater samples from all four villages, these water sources displayed all the target pathogens, with ETEC and *Salmonella* typhimurium being the most prevalent pathogens. The prevalence of pathogens in groundwater samples from the Village A and Village B villages was low. In Village A, *Salmonella* typhimurium was detected in 21.3% of groundwater samples in the dry and ETEC was detected in 7.5% of groundwater samples in the wet and 6% of groundwater samples in the dry season. *Salmonella* typhimurium was the only pathogen that was detected in Village B village in 25% of the groundwater samples during the dry season. *Salmonella* typhimurium, *Shigella flexneri* and ETEC were detected in groundwater samples from Village C and Village D villages and they were mainly prevalent during rainy seasonal conditions. The findings further demonstrated that the target pathogens were not detected in all of the sampled boreholes (Table 5); in fact, none of the target pathogens were present in several boreholes sampled in the Village B and Village A villages. The overall results showed that *Shigella flexneri* was detected in 21.4% (n = 15) of the boreholes in the rainy season and 2.9% (n = 2) in the dry season. *Salmonella* typhimurium was detected in 22.7% (n = 18) of the boreholes in the wet and 18.6% (n = 13) in the dry season. At the same time, ETEC was detected in 24.3% (n = 17) of the boreholes in the wet and 5.7% (n = 4) in the dry season. A summary of the concentration of the detected pathogens in groundwater, faecal sludge and wastewater is displayed in Table 6 as log copies per 100 μL or per gram. The concentration of the detected pathogens was the same across all samples. Overall, the concentrations ranged from 1.99 to 2.11 EGC/gram in faecal sludge; 2.05 to 2.11 EGC/100 mL in wastewater and 2.03 to 2.12 EGC/100 mL in groundwater.

Table 4
Summary statistics of the measured lateral distance (m) between on-site sanitation facilities and boreholes.

	Village A 20 HHs	Village B 20 HHs	Village C 20 HHs	Village D 10 HHs	Overall 70 HHs
Minimum	15	11	22	12	11
Maximum	75	66	81	55	81
Mean	41.25	32.4	44.75	33.9	38.1
≥50 m	5 (25%)	2 (10%)	7 (35%)	2 (10%)	16 (22.9%)
SD	13.68	13.92	15.24	13.84	14.17

Standard deviation (SD); metre (m); greater or equal to (≥).

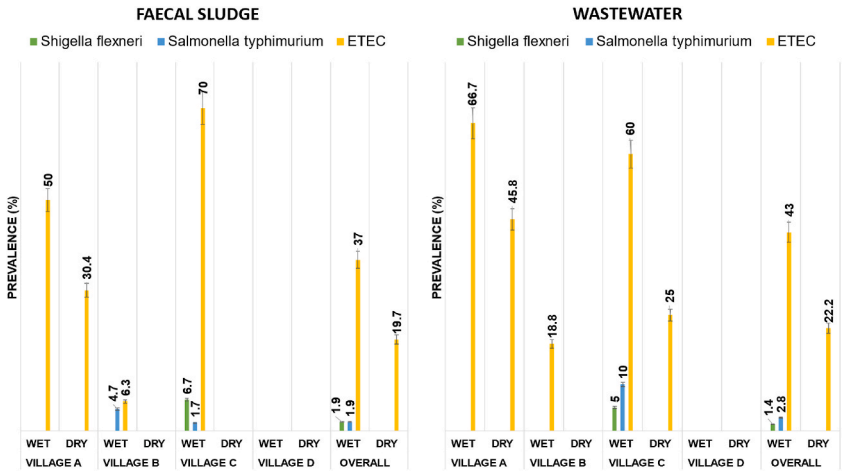

Fig. 3. Prevalence of pathogens (Namely: *Salmonella* typhimurium, *Shigella Flexneri,* and ETEC) in human waste from pit latrines and wastewater from septic tanks across four villages for the wet and dry seasons. The bar charts are displayed with percentage error bars.

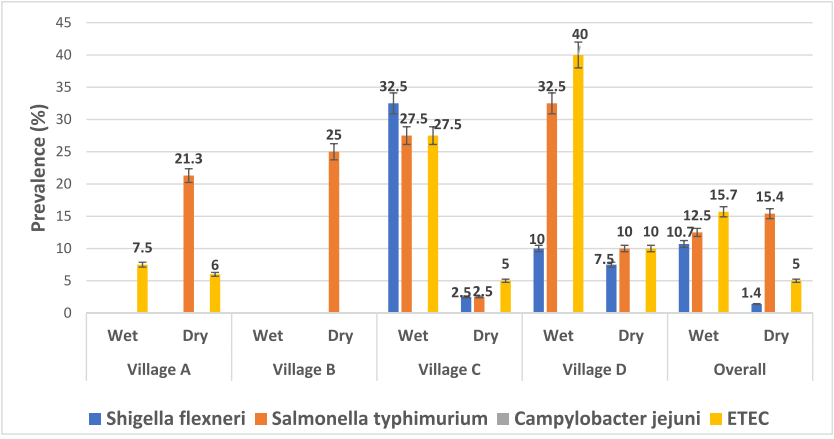

Fig. 4. Prevalence of pathogens (Namely: *Salmonella* Typhimurium, *Shigella Flexneri*, and ETEC) in groundwater samples from household boreholes during wet and dry seasons. The bar charts are displayed with percentage error bars.

Table 5
Overall number of boreholes contaminated with the target pathogens.

Target pathogen	Season	A 20 BHs	B 20 BHs	C 20 BHs	D 10 BHs	Overall 70 BHs
Shigella flexneri	Wet	0	0	14 (70%)	1 (5%)	15 (21.4%)
	Dry	0	0	1 (5%)	1 (5%)	2 (2.9%)
Salmonella typhimurium	Wet	0	0	14 (70%)	4 (20%)	18 (25.7%)
	Dry	5 (25%)	6 (30%)	2 (10%)	0	13 (18.6%)
ETEC	Wet	2 (10%)	0	10 (50%)	5 (25%)	17 (24%)
	Dry	2 (10%)	0	1 (5%)	1 (5%)	4 (5.7%)

Note: Village A (A); Village B (B); Village C (C); Village D (D); boreholes (BHs); enterotoxigenic *E. coli* (ETEC).

3.3. Relationship between pathogens in boreholes and sanitation systems

The results of a point-biserial correlation analysis between the presence/absence of pathogens in boreholes and OSS facilities (wastewater and human waste) ranged from very weak ($r = -0.093$) to moderate ($r = -0.358$) correlation (Table 7). In Village A, a negative moderately significant correlation was established for the presence of ETEC in the wet ($r = -0.514$; $p = 0.020$*) and a weak negative correlation in the dry season ($r = -0.333$; $p = 0.151$). In Village B, no significant correlation was established between boreholes and sanitation facilities in terms of *Salmonella* typhimurium ($r = 0.145$; $p = 0.541$). In Village C, low negative correlations

Table 6
Concentrations of pathogens (equivalent genome copies (EGC) per 100 mL or per gram) in groundwater, faecal sludge, and wastewater.

Village		*Shigella flexneri*		*Salmonella* typhimurium		Enterotoxigenic *Escherichia coli*	
		Mean (std)		Mean (std)		Mean (std)	
Village A	**Total (n)**	**Wet**	**Dry**	**Wet**	**Dry**	**Wet**	**Dry**
Groundwater	80	0	0	0	2.08 (0.04)	2.12 (0.01)	2.09 (2.02)
Faecal Sludge	56	0	0	0	0	2.06 (0.02)	2.07 (2.02)
Wastewater	24	0	0	0	0	2.07 (0.02)	2.06 (0.02)
Village B							
Groundwater	80	0	0	0	0	2.03 (0.04)	0
Faecal Sludge	64	0	0	0	0	2.11 (0.005)	0
Wastewater	16	0	0	2.05 (1.02)	0	2.11 (0)	0
Village C							
Groundwater	80	2.14 (0.17)	2.09 (0)	2.90 (3.95)	2.10 (0)	2.09 (0.007)	2.09 (0)
Faecal sludge	60	2.05 (0.01)	0	1.99 (0)	0	2.02 (0.05)	2.06 (0.03)
Wastewater	20	2.08 (0)	0	2.06 (0.04)	0	2.22 (0.30)	2.09 (0)
Village D							
Groundwater	40	2.04 (0.01)	2.02 (0.02)	2.05 (0.03)	2.08 (0.01)	2.10 (0.42)	2.10 (0.01)
Faecal sludge	28	0	0	0	0	0	0
Wastewater	12	0	0	0	0	0	0

for *Shigella flexneri*, *Salmonella* typhimurium, and ETEC were determined in the rainy season. A similar result was also observed in Village D village, where no relationship was established between the pathogens detected in groundwater and those in household WW from septic tank and human waste from the pit latrines.

3.4. The relationship between contaminants in groundwater and the lateral distance

In this study, *E. coli* was used as an indicator bacterium for the presence of faecal pollution, the results of average *E. coli* counts for each borehole are displayed in (Table S4). The results of Pearson's correlation between *E. coli* concentrations in groundwater and the measured distance between OSS facilities and boreholes are displayed in Table 8. All the correlations were positive except for one, namely Village A in the dry season ($r = -0.1155$). The results ranged from very weak to moderate positive correlations. In general, no correlation was recorded ($r = 0.1642$, $p = 0.1744$) for the rainy season, while statistically, a significant difference between the two parameters was found, although the correlation was not established ($r = 0.2504$, $p = 0.0365$*) for the dry season. In Village C, a significant moderate correlation was established for the dry season ($r = 0.5481$, $p = 0.0123$*).

Figs. 5–8 displays the association between quantities of *E. coli* in groundwater during the wet and dry seasons and the lateral distance between the borehole and the on-site sanitation facility in the yards of households located in the four villages. Overall, variations were observed in lateral distances between the boreholes and the sanitation facilities located across the four villages. The results also showed that the concentration of *E. coli* was high in groundwater samples even though the household sanitation facility was located far away from the borehole. For example, in Village D (Fig. 8), borehole BH7 was found to have the highest *E. coli* counts (117 CFU/100 mL), while the measured distance between the OSS and the borehole was 55 m. The same was evident in Village C (Fig. 7), where BH14 was found to have the highest *E. coli* concentration (169 CFU/100 mL) during the rainy season and the measured distance was 81 m. However, the opposite was observed for BH9 in Village A (Fig. 5), where the measured distance was 75 m and the *E. coli* concentration was zero for the dry season and very low (10 CFU/100 mL) for the rainy season.

The overall results of the association between the presence/absence of pathogens in groundwater and the measured distances of on-site sanitation facilities to boreholes at the abstraction point ranged from very weak ($r = 0.0071$) to moderate ($r = 0.5258$) correlations (Table 9). The correlation in Village A and Village B villages was not calculated because most of the target pathogens were not detected in groundwater samples. In Village D, weak correlations were established between most pathogens and the borehole distance to

Table 7
Correlations between the presence/absence of pathogens in boreholes and OSS systems (wastewater and sludge from pit latrine).

	Shigella flexneri		*Salmonella* typhimurium		ETEC	
	Wet	Dry	Wet	Dry	Wet	Dry
Village A	NC	NC	NC	NC	$r = -0.514$ $p = 0.020$*	$r = -0.333$ $p = 0.151$
Village B	NC	NC	NC	$r = 0.145$ $p = 0.541$	NC	NC
Village C	$r = -0.096$ $p = 0.686$	NC	$r = -0.111$ $p = 0.641$	NC	$r = -0.229$ $p = 0.331$	NC
Village D	NC	NC	NC	NC	NC	NC
Overall	$r = -0.093$ $p = 0.429$	NC	$r = -0.111$ $p = 0.360$	$r = 0.145$ $p = 0.231$	r = -0.358 p =<0.01*	r = -0.333 p = 0.004*

Pearson's correlation (r); p-value (p); significant (*); not calculated (NC) due to low pathogen numbers or pathogens not detected.

Table 8
Correlations between *E. coli* concentration in groundwater and distance between OSS facilities and boreholes in household yards across the four villages.

Villages	Season	Correlation
Village A	Wet	r = 0.08833, p = 0.7112
	Dry	r = −0.1155, p = 0.6277
Village B	Wet	r = 0.279, p = 0.233
	Dry	r = 0.2981, p = 0.2018
Village C	Wet	r = 0.1015, p = 0.6703
	Dry	r = 0.5481, p = 0.0123*
Village D	Wet	r = 0.5452, p = 0.1031
	Dry	r = 0.288, p = 0.419
Overall	Wet	r = 0.1642, p = 0.1744
	Dry	r = 0.2504, p = 0.0365*

Pearson's correlation (r); p-value (p); *significant.

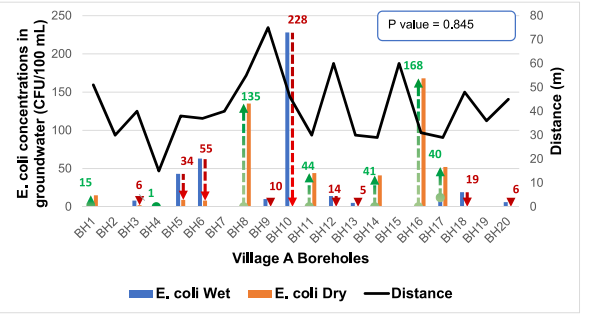

Fig. 5. Concentrations of *E. coli* (CFU/100 mL) in groundwater samples during the wet and dry seasons and the measured distance between OSS facilities and boreholes in household yards in Village A village. This figure displays the association between the *E. coli* concentration in groundwater and the measured distance between the OSS facility and borehole for each sampled household. The concentration differences are also indicated.

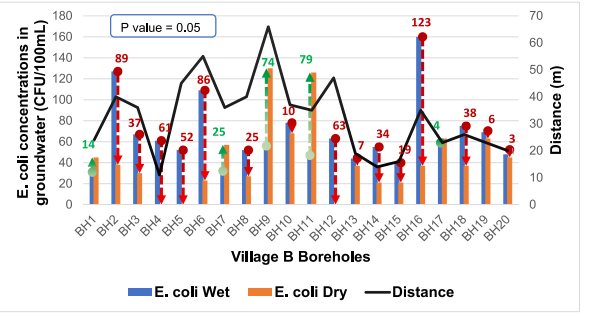

Fig. 6. Concentrations of *E. coli* (CFU/100 mL) in groundwater samples during the wet and dry seasons and the measured distance between OSS facilities and boreholes in household yards in Village B village. This figure displays the association between the *E. coli* concentration in groundwater and the measured distance between the OSS facility and borehole for each sampled household. The concentration differences are also indicated.

sanitation facilities, while in Village C, the correlation was recorded to be moderately significant, especially for *Shigella flexneri* in the dry season (r = 0.5458; p = 0.0128).

4. Discussion

Pit latrines and flush toilets connected to septic tanks are effective in reducing disease spread, but improper construction can lead to groundwater contamination. The minimum recorded distance between the OSS facility and the borehole was 11 m, which was in line with the findings of a study conducted by Ref. [15] in Muledane. The measured distances in homes were clustered around the mean, as the standard deviations were less than their respective means for all four villages. Furthermore, the results revealed that overall, 22.9% of HHs complied with the national norms and standards of SA. The yards in Village C households were oversized compared to other villages, 35% of households could maintain a ≥50 m distance. Households with two to three yards and farms have sufficient space to maintain distance between boreholes and OSS facilities. Maintaining safe spaces is crucial to protect groundwater sources from faecal

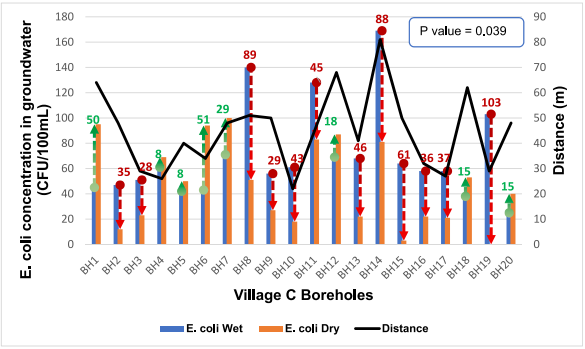

Fig. 7. Concentrations of *E. coli* (CFU/100 mL) in groundwater samples during the wet and dry seasons and the measured distance between OSS facilities and boreholes in household yards in Village C village. This figure displays the association between the *E. coli* concentration in groundwater and the measured distance between the OSS facility and borehole for each sampled household. The concentration differences are also indicated.

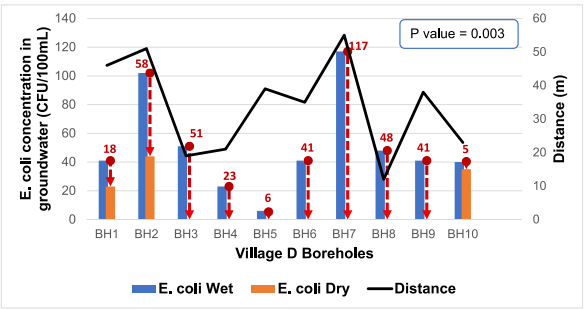

Fig. 8. Concentrations of *E. coli* (CFU/100 mL) in groundwater samples during the wet and dry seasons and the measured distance between OSS facilities and boreholes in household yards in Nkhakanjhaka village. This figure displays the association between the *E. coli* concentration in groundwater and the measured distance between the OSS facility and borehole for each sampled household. The concentration differences are also indicated.

Table 9
Correlations between the presence/absence of pathogens in groundwater samples and the distance between the borehole and OSS facility in household yards across the four villages.

Pathogen	Season	Village A	Village B	Village C	Village D	Overall
Shigella flexneri	Wet	NC	NC	r = −0.3186 p = 0.1709	r = 0.291 p = 0.414	r = −0.014 p = 0.908
	Dry	NC	NC	r = 0.5458 p = 0.0128*	r = 0.5083 p = 0.1336	r = 0.5258 p = <0.0001*
Salmonella typhimurium	Wet	NC	NC	r = 0.0465 p = 0.8455	r = 0.316 p = 0.374	r = 0.1812 p = 0.1333
	Dry	r = 0.4284 p = 0.0595	r = 0.5441 p = −0.1442	r = 0.1477 p = 0.5344	NC	r = 0.3734 p = 0.0014*
ETEC	Wet	r = 0.1645 p = 0.4883	NC	r = −0.3380 p = 0.1450	r = 0.195 p = 0.589	r = 0.0071 p = 0.9534
	Dry	r = 0.1767 p = 0.4562	NC	r = 0.2597 p = 0.2688	r = 0.412 p = 0.237	r = 0.2828 p = 0.017*

Not calculated (NC); Pearson's correlation (r); p-value (p); *moderately significant.

contamination. The WHO states that water intended for drinking should not contain any detectable *E. coli*. Only 45 samples in the rainy and 90 samples in the dry seasons met the WHO standard for drinking water. In Village A, 50% of BHs had zero *E. coli* counts, with a 41 m lateral distance between boreholes and OSS systems, ranging from 15 to 75 m. The presence of *E. coli* in boreholes indicates that groundwater from these specific sites is at greater risk of possible enteric pathogens being present [32]. The results showed that one or more of the following pathogens were detected in groundwater: *Shigella flexneri*, *Salmonella typhimurium* and ETEC. The findings are comparable to those of [16], in which the same pathogens were detected in groundwater samples from boreholes used by school-children in the Vhuronga 1 Circuit in the VDM region.

Evidence suggests that during the rainy season, faecal contamination is frequently detected at higher concentrations [33,34]. The

study found a higher prevalence of *Salmonella typhimurium*, *Shigella flexneri* and ETEC in Village C and Village D during the rainy season, while *Salmonella typhimurium* was detected in Village A and Village B villages during the dry season. Enterotoxigenic *Escherichia coli* and *Salmonella typhimurium* were the most prevalent pathogens in groundwater. *Shigella flexneri* was the least prevalent pathogen; this finding agrees with the results of a study by Ref. [35] where Shigella was found to be the least dominant pathogen in groundwater from boreholes. *Shigella flexneri* is among the most common diarrhoeagenic pathogens in groundwater in the rural districts of the Limpopo Province [36]. In a study conducted by Ref. [14], ETEC was isolated from groundwater in the VDM region. Despite using a probe-based real-time PCR assay, there was no presence of *Campylobacter jejuni* in any of the borehole groundwater samples. *Campylobacter* concentrations in contaminated drinking water are typically low, with less than ten CFU per litre. Due to their microaerophilic properties, they have a low environmental survival probability. In Village B, pathogens were found in 6 boreholes during the dry season, while in Village A, they were found in 2 and 7 boreholes during the wet and dry seasons, respectively. The presence of pathogens in groundwater raises concerns about its suitability for drinking, as it is often used without treatment, potentially posing health risks to community members, particularly children and immunocompromised households. Many believe it requires little treatment before consumption [37]. According to data from our previous study [26] most households (92.9%) in these sites do not treat water before use, and 34.3% are unaware of household water treatment methods.

The study revealed significant variations in pathogen presence in sludge from pit latrines and WW from septic tanks. The most prevalent pathogen was ETEC in both human waste (75%) and wastewater (66.7%), and the least prevalent pathogen was *Shigella flexneri* in human waste (1.7%) and wastewater (1.4%). Enterotoxigenic *Escherichia coli* was prevalent primarily in wastewater and human waste from Village A and Village C villages. Sludge characteristics in pit latrines vary significantly within the same municipality or town [38]. The difference depends on user practices, such as diets and anal cleansing products [39]. The study reveals that pathogens detected in human waste from pit latrines and septic tanks in the VDM region are common or endemic, as confirmed by studies on diarrheal diseases and enteric pathogen infections. For example, in a cross-sectional study by Ref. [40], ETEC was detected in 27.9% of stool specimens, and it was concluded that enteric pathogen co-infection is the major cause of diarrhea in children in the VDM region. Enterotoxigenic *Escherichia coli* is a pathogenic agent causing acute diarrhea in children under five years in underdeveloped nations [41].

The study found a very weak ($r = -0.093$) to moderate ($r = -0.541$) association between pathogens in groundwater and on-site sanitation systems. This implies that when the pathogens were detected in groundwater, they were absent in the sanitation compartment and vice-versa. For ETEC, there was a statistically significant moderate negative correlation in the rainy season ($r = -0.358$; $p = 0.01$*), implying that there was some relationship between detected ETEC in groundwater and OSS systems, though it was not particularly strong. Consequently, the detected pathogens in groundwater samples from the boreholes could be directly originating from the waste generated by the OSS systems. Nevertheless, in Village D village, none of the target pathogens were detected in wastewater and human waste, and yet the pathogens were detected in groundwater samples. In our previous research [26] the faecal pollution sources found in the groundwater of Village D village were (*Cytb*-Chicken, *BacCan*-Dogs as well as *Pig-2Bac-Pig*). None of the human markers were detected. This implies that animals are the sources of faecal contamination in groundwater from this village and that the OSS systems in Village D village might not have an impact on the quality of groundwater. There was no association between the pathogens in boreholes and those in OSS systems in this village.

Regarding the relationship between *E. coli* in groundwater and the lateral distance between OSS facilities and boreholes, the statistical analysis indicated a very weak non-significant association ($r = 0.1642$, $p = 0.1744$) for the rainy season and a weak significant positive correlation ($r = 0.2504$, $p = 0.0365$) for the dry season. In Village C, a significant moderate correlation was established for the dry season ($r = 0.5481$, $p = 0.0123$*). The positive correlation indicates that as the borehole distance from the OSS increases, so do the levels of contaminants, and vice versa. Despite the household sanitation facility's distance from the borehole, some boreholes had high *E. coli* concentrations in groundwater samples (Fig. 5). The concentration of *E. coli* in groundwater was not strongly influenced by the measured distance between the OSS facility and the borehole. The literature suggests that the geological structure of water points, well design, and proximity to OSS facilities are the primary factors influencing *E. coli* occurrence [42]. The study found that the distance between OSS facilities and boreholes in the same yard did not significantly impact the presence of *E. coli* in groundwater samples from the boreholes, suggesting that the concentration of *E. coli* does not significantly depend on the distance. The study hypothesized that on-site sanitation facilities might have an impact on the groundwater quality, i.e. the shorter the distance between the borehole and the OSS facility, the higher the *E. coli* concentrations. The study results showed that pathogens were detected in groundwater even when the OSS facility was located far away from the borehole abstraction point. This was evident in Village C, where the distance between OSS facilities and boreholes was measured to be $\geq$ 50 m in 60% of HHs; however, most of the target pathogens were detected in the borehole water samples.

The study found no significant correlation between the distance between OSS facilities and boreholes and pathogens' existence or lack in groundwater. The results align with previous research in Zambia, where [43] found no strong relationship between the distance from a borehole to a soakaway and groundwater quality. In another study in Zambia [44], found no distinct relationship between the distance from the borehole to the septic tank and the quality of borehole water in Kitwe West Township. These findings suggest that groundwater quality is not significantly influenced by the distance between boreholes and septic tanks. Conversely [45], found a positive correlation between mean length (23.48 m) and *E. coli* in dry and rainy seasons. Contaminants were found to increase with distance from septic tank systems. A study by Ref. [46] found a moderate negative correlation between distance from the latrine and coliform count. While [47] found a significant increase in TCC and FCC with a decrease in distance between wells and latrines. In South Africa, the national norms and standards released by the Department of Water and Sanitation in 2017, mandate sanitation facilities to be at least 50 m away from any groundwater source. Groundwater contamination is more common in sedimentary [48]. Village B and Village C villages share similar geology, however, pathogen detection in groundwater differed. Pathogens in Village B's groundwater

were low compared to those in Village C, despite the average lateral distance between the borehole and sanitation facility being 32.4 m, which is significantly shorter than the measurement in Village C (44.75 m). The study suggests neither geology nor distance significantly affects pathogen presence/absence.

5. Conclusion

This study aimed to track enteric pathogens from OSS facilities to boreholes and assess the sitting of OSS facilities in relation to boreholes. The findings showed that most households were unable to comply with national norms and standards regarding the distance between OSS facilities and groundwater sources. The lowest measured distance between the borehole and the OSS facility was 11 m. Proper management and maintenance of OSS facilities and waste are crucial to protect groundwater sources. Groundwater samples from boreholes tested positive for *E. coli*, *Shigella flexneri*, *Salmonella* typhimurium, and ETEC, but not all the boreholes. The concentration of the detected pathogens was similar across all samples. The presence of the same concentration of the pathogen in groundwater, fecal sludge and wastewater could indicate a potential contamination source. This suggests that the pathogens detected in groundwater could be from the waste generated by the OSS systems. The study recommends strategic measures to protect groundwater sources and education of community members on safe management of sanitation facilities and groundwater to counteract public health risks.

5.1. Study limitations

The current study focused on microbiology and molecular analysis, by detecting pathogens in groundwater, septic tank WW and faecal sludge. There was no data on the depth of the sampled boreholes and geohydrology of the study sites. For future studies, there should be a collaboration between microbiologists, hydrogeologists, and environmental engineers to have more comprehensive data on groundwater contamination assessment.

Data availability statement

The data associated with this study has not been deposited into a publicly available repository. All the data that support the findings of this study are available on request, from the corresponding author.

CRediT authorship contribution statement

Jeridah Matlhokha Sekgobela: Writing – review & editing, Writing – original draft, Methodology, Formal analysis. **Colette Mmapenya Khabo-Mmekoa:** Writing – review & editing, Supervision. **Maggy Ndombo Benteke Momba:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

We can disclose that we have no conflicts of interest. The study was conducted based on the requirement of the ethics clearance approved by the Faculty of Science Research Ethics Committee (FCRE) at the Tshwane University of Technology (TUT) (FCRE 2019/09/017 (FCPS 03) (SCI)).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e27271.

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