Research Article

# Assessment of Vibrio Cholerae and Its Biotypes Associated With Wastewater, Sludge, River Water and Riverbed Sediment: A Case Study in Limpopo Province

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In South Africa, the Limpopo Province houses 63 wastewater treatment plants, and findings of the Green Drop assessment indicated that most of these plants are in the high risk and critical risk space, except for four plants, which are still in the low and medium risk space. The current study, therefore, investigated the presence of Vibrio cholerae and its biotypes/serotypes in selected wastewater treatment plants (WWTP) of the Limpopo Province (Burgersfort Wastewater Treatment Plant, Paarl Wastewater Treatment Plant, Thohoyandou Wastewater Treatment Plant, Makhado Wastewater Treatment Plant, and Ga-Kgapane Wastewater Treatment Plant). The selection was based on their non-compliance between 2009 and 2013 as reported in the Green Drop statistics by the Department of Water and Sanitation. Furthermore, these facilities have been rated as being in high risk and critical risk positions based on the wastewater risk rating analysis and previous cholera outbreaks. For the purpose of this study, four different matrices were used: influent and effluent wastewater, sewage sediments, receiving water bodies (river water), and riverbed sediments. Culture-based methods and serotyping methods were used to identify the V. cholerae and their biotype/serotype groups. Between July and December 2017, the results revealed that 76.32% (87) of isolates were identified as V. cholerae and 23.68% (27) belonged to other Vibrio species and bacterial species. Among the Vibrio species, 13.16% (15) were V. mimicus and 4.38 % (5) V. vulnificus. Bacterial species [6.14% (7)] included Achromobacter xylosoxidans, Plesiomonas shigelloides, Flavobacterium species group IIb, a rare biotype, and Pasteurella multocida. Using the serum tests (Vibrio cholerae Ogawa Sera, Vibrio cholerae Polyvalent Agglutinating sera, and the Vibrio cholerae Inaba Sera), all 87 isolates identified as V. cholerae, tested positive for V. cholerae O1 Ogawa serum. During the study period, between 50% and 100% of all matrix samples were positive for Vibrio cholerae O1 Ogawa, with the highest percentage found in all sewage sludge matrices (100% of samples), except for Paarl WWTP, in which 66.7% of sludge samples tested positive. Of the five wastewater treatment plants, there were only two plants (Burgersfort WWTP and Ga-Kgapane WWTP) which had accessibility for river water and riverbed sampling points. The riverbeds of their receiving water bodies (Spekboom River and Molototsi River, respectively) displayed 50% of samples with the presence of V. cholerae O1 Ogawa. The high percentage occurrence of V. cholerae O1 Ogawa in sewage sludge and riverbed sediment is a matter of great concern, as these solid matrices could be the potential reservoirs of V. cholerae and its serotypes causing cholera outbreaks in the Limpopo Province. This study therefore calls for immediate remedial measures, which may include these solid matrices in the monitoring programme of V. cholerae in endemic areas.

## INTRODUCTION

Vibrio cholerae continues to be classified as one of the world's most deadly diarrhoea-causing agents, which has been at the epicentre of many epidemic and pandemic outbreaks of cholera, especially in the developing world. To date, seven pandemic cholera outbreaks have been reported, and approximately 3.5 million people have been infected, and it has been estimated that between 100 000 and 120 000 deaths occur each year in the developing countries [3]. Recurrent cholera outbreaks were recorded in the Democratic Republic of Congo, Cameroon, Kenya, Tanzania, Mozambique, Zambia, and Zimbabwe in 2017 and 2018.

V. cholerae has been found to have a diverse range of strains and biotypes capable of receiving and transmitting toxin genes. Vibrio cholerae also has cellular functions such as colonisation factors, and antibiotic resistance characteristics in both environmental and human settings. The serotype of V. cholerae has been determined by analysing surface antigens such as the O antigen capsule. According to Bik, there are more than 200 serotypes of V. cholerae based on its O antigen, with only two V. cholerae serogroups (O1 and O139) known to cause epidemic and pandemic cholera outbreaks. Serogroup O1 and O139 pandemic strains have been reported to be natural inhabitants of aquatic habitats, making them facultative human pathogens. V. cholerae serogroup O1 has two biotypes (classical and El Tor) and three serotypes, as opposed to the serogroups O1 and O139 that cause pandemic cholera (Ogawa, Inaba and Hikojima).Ogawa is the most common serotype, but Hikojima is extremely rare and unstable in nature. The ability to manufacture cholera toxin, which is encoded by the ctx gene, distinguishes and classifies the distinct serotypes of V. cholerae. The ctx gene has been utilized to detect choleragenic V. cholerae in environmental samples with pinpoint accuracy.

While studies have mostly focused on serological and biotype characteristics of V. cholerae, new pathogenic variants of V. cholerae have been spread around the world. One of the neighbouring countries of South Africa, Mozambique, had shown these variants as atypical El Tor strain harbouring CTXϕCla. Tests such as V. cholerae agglutination tests have helped to detect non-choleragenic from the choleragenic strains of the species through serological classification of V. cholerae. Serogroups which do not conform to the agglutination test are known as non-O1 and non-O139 V. cholerae, also called non-agglutinating (NAG) vibrios, and the NAGs mostly lack the cholera toxin gene. To distinguish the two serotypes from each other, the laboratory detection method for O1 and non-O1 is not yet clear. Because of longer exposure to V. cholerae in endemic areas, the population becomes immune to cholera reinfection as these endemic areas have the potential to develop a slow rate of V. cholerae in the environment or exponential growth in different seasons of the year. This will still allow cholera to be transmitted to humans even in unfavorable conditions.

In past research studies in South Africa, the residence time of toxigenic V. cholerae in the environment was considered short as they showed steady

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growth in the environment with ongoing epidemiology studies and microorganism occurrence. In addition, most cases of reported outbreaks of cholera have been caused by faecal-oral transmission .Vibrio cholerae has been found to survive in drinking water and wastewater for a long time, maintaining a viable yet non-culturable condition. The findings of Li and coworkers led to the formulation of a novel hypothesis, namely that V. cholerae environmental reservoirs are responsible for endemic cholera, and a cholera epidemiology framework that includes a V. cholerae environmental reservoir. However, as potential natural reservoirs of V. cholerae, sampling areas that include riverbed sediment and sewage sludge have been overlooked.

During the sixth cholera pandemic, the serological classification of the V. cholerae and other Vibrio species was established. Traditional identification for V. cholerae serotype and biotype uses methods such as phage typing to identify and classify V. cholerae. These methods have been used for many years in the characterisation and classification of V. cholerae. However, because of their low discriminatory traits, these methods have major challenges.

In this study, traditional culture-based methods were used to selectively culture V. cholerae isolates on CHROMagar™ Vibrio (Merck KGaA, Darmstadt, Germany), followed by the use of the RapID NF Plus test kit (Thermo Fisher Scientific), which contains a variety of biochemical tests to further distinctly classify V. cholerae strains. Furthermore, modern and time efficient biotyping and serotyping methods were used for the identification and characterisation of V. cholerae strains in wastewater, sewage sludge, receiving water body, and riverbed sediments.

## MATERIALS AND METHODS

### Site description

Wastewater samples were collected on a monthly basis between July and December 2017 from the following sites: Burgersfort WWTP, Paarl WWTP, Thohoyandou WWTP, Makhado WWTP, and Ga-Kgapane WWTP. These sites are within the following local district municipalities, namely Sekhukhune, Waterberg, Vhembe, and Mopani District Municipalities, respectively (Table 2.1; Figure 2.1). These WWTPs were selected based on their non-compliance as reported in the Green Drop Report Card. Furthermore, these districts have been rated into high and critical risk positions based on the wastewater risk rating analysis and previous cholera outbreaks

### Sample collection and preparation

Prior to sample collection, 1 L Schott sampling bottles were soaked in 10% nitrite acid for 24 h, rinsed thoroughly with distilled water and autoclaved at 121 °C for 15 minutes as previously described by Teklehaimanot. Ethanol (70% v/v) was prepared and used in between each sampling point to disinfect the sample bottles before moving onto the next sampling point. Sterile Milli-Q water was used to rinse the sample bottles prior to use. Five sampling sites, which contained three to five sampling points, were selected. Samples of wastewater (influent and effluent) and receiving water bodies (river water) were collected in 1 L sterile Schott bottles with screw caps. For solid matrices, samples (sewage sludge from secondary digester and riverbed sediments) were aseptically scooped 5 cm from the surface and carefully transferred into sterile Schott bottle as previously described by Abia. Surface water and riverbed sediment samples were collected only from wastewater treatment plants and rivers where the sampling sites were accessible. These included Burgersfort WWTP and Ga-Kgapane WWTP. Samples were stored in a cooler box at 4 °C and transported to Tshwane University of Technology (TUT) Water Research Unit laboratory for analyses within 24 h. It should be mentioned that the research team worked hand in hand with the respective municipalities (Table 3.1) and necessary permissions were obtained from the water services authorities of the respective district municipalities prior to the collection of samples.

**Table1**: GPS coordinates of selected wastewater treatment plants located in Limpopo Province and used during the study period

| District municipalities | Local municipalities | Border | WWTPs and rivers | Coordinates |
|---|---|---|---|---|
| Waterberg | Lephalale | Botswana | Paarl WWTP | 23°43'11.6"S 27°41'51.2"E |
| Mopani | Greater Letaba | Mozambique | Ga-Kgapane WWTP, Modjadjiskloof | 23°37'56.6"S 30°12'58.0"E |
| | | | Molototsi River | 23°37'56.6"S 30°12'58.0"E |
| Vhembe | Makhado - Louis Trichardt | Zimbabwe | Makhado WWTP (old plant) | 23°03'27.2"S 29°53'51.4"E |
| | Thulamela | Zimbabwe | Thohoyandou WWTP (Muludani) | 23°00'09.0"S 30°28'31.2"E |
| Sekhukhune | Greater Tubatse | Mpumalanga and parts of Mozambique | Burgersfort WWTP | 24°39'51.2"S 30°20'15.3"E |
| | | | Spekboom River | 24°39'51.2"S 30°20'15.3"E |

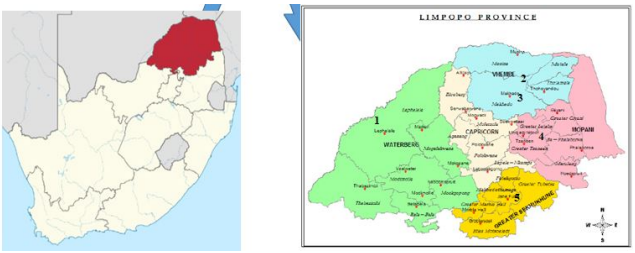


**Figure1**: Map indicating the location of sampling sites across Limpopo Province. 1) Paarl WWTP located in Lephalale; 2) Thohoyandou WWTP, Thohoyandou; 3) Makhado WWTP, Makhado; 4) Ga-Kgapane WWTP, Modjadjiskloof; 5) Burgersfort WWTP, in Burgersfort.

### Sample analysis for detection of Vibrio cholerae and its biotypes and serotypes

Detection of presumptive Vibrio cholerae isolates

For wastewater and river water samples, 100 mL of water was concentrated onto a 0.45 µm nitrocellulose membrane filter paper (Whatman, Merck). These membrane filter papers were transferred into 10 mL of alkaline peptone water (APW) (Merck) broth for enrichment of V. cholerae and incubated at 37 °C for 6-8 h. Thereafter, the quadrant streak technique was used on CHROMagarTM Vibrio (MEIDA-MAGE) media plates (in duplicate) for isolating single colonies from the enriched samples. Furthermore, plates were incubated at 37 °C for 18-24 h. The analysis of the solid matrix samples (riverbed sediment and sewage sludge) was done using the displacement method previously described by Abia [38]. Briefly, a 100 cm3 aliquot of wet riverbed sediment or sewage sludge was suspended in 900 mL of sterile 1X phosphate buffer solution (PBS) (containing 137 mmol/L NaCl; 2.7 mmol/L KCL; 10 mmol/L Na2HPO4 and 1.8 mmol/L KH2PO4). The solution was then shaken vigorously for 2 minutes to dislodge the microorganisms from the solid matrixes. A volume of 100 mL supernatant of the solution was measured out into a sterile glass cylinder tube, and then filtered the same way as the wastewater or river water samples. All samples were incubated in APW at 37 °C for 6-8 hours and the same method was followed as mentioned above. Based on their morphological characteristics, presumptive V. cholerae colonies detected on the CHROMagarTM Vibrio (MEIDA-MAGE) media plates were randomly picked and subjected to a series of biochemical analysis. Firstly, the oxidase test using the BactiDrop™ oxidase test kit (Thermo Fisher Scientific) was

performed. Bacterial isolates, which tested oxidase positive through the indication of a purple colour on a filter paper, were subjected to the RapID™ NF PLUS System (Thermo Fisher Scientific). This is an identification system based on enzyme technology, which is aimed to identify oxidase-positive, Gram-negative bacilli, including Vibrio species. The test was performed according to the manufacturer's instructions. The incubation time for the RapID™ NF Plus test kit was 4 h at an incubation temperature of 37 °C. The RapID™ software was used to generate a microcode for the identification and classification of the different Vibrio species

Serotyping of presumptive V. cholerae isolates

Presumptive V. cholerae colonies identified from morphology studies on the culture media were used to determine the serotype and biotype of this bacterial species. Three agglutination serum test kits namely Vibrio cholerae Ogawa Sera, Vibrio cholerae Polyvalent Agglutinating sera, and the Vibrio cholerae Inaba Sera were used for the identification of Vibrio cholerae serogroups. Should the colonies agglutinate to the test serums, they would represent the toxigenic strains of V. cholerae for that specific serogroup; however, should the opposite happen, then the colonies would be classified as non-toxigenic V. cholerae strains. The V. cholerae isolates were preserved in 1 mL of 20% glycerol at -80 °C for DNA isolation and further use for analysis and identification of virulence genes.

## RESULTS

### Isolation and detection of presumptive Vibrio cholerae in aquatic environments

Using culture-based methods, colonies presenting green blue to turquoise blue pigment on CHROMagarTM Vibrio media were randomly picked and subjected to further testing. Overall, there were 114 isolates with these morphological characteristics. The first line of testing was through BactiDrop oxidase testing in which all 114 (100%) isolates tested positive and were classified as Gram-negative. Table 2.2 depicts the results of RapID™ NF PLUS System for identification of V. cholerae in wastewater, receiving water bodies, the riverbed sediments, and the sewage sludge. These results revealed that 76.32% (87) of isolates were identified as V. cholerae and 23.68% belonged to other Vibrio species and bacterial species (in Table 3.2, Figure 2.2). Among the Vibrio species, 13.16% were V. mimicus and 4.38% V. vulnificus. Beside Vibrio species, 6.14% of the isolates included other bacteria: Achromobacter xylosoxidans, Plesiomonas shigelloides, Flavobacterium species group IIb, a rare biotype, and Pasteurella multocida.

As can be seen in Table 2.2 and Table 2.3, samples were collected once a month over a six-month period from July to December 2017. During this period, V. cholerae was found to be present in all the five different matrices of the selected aquatic environments (wastewater influents, effluents, sludge and accessible river water and riverbed sediment sampling sites) (Table 3.3). When observing individual wastewater treatment plants, Vibrio cholerae showed up in all the influent samples of Ga-Kgapane WWTP during the entire study period (100%), while it appeared in 83.3% of influent samples from Burgersfort WWTP and in 66.7% of influent samples from Thohoyandou WWTP and in 50% of influent samples from Paarl WWTP. In terms of the prevalence of Vibrio cholerae in WWTP effluents, 66.6% of effluent samples from all the WWTPs tested positive, with the exception of Makhado WWTP where 50% of the effluent samples tested positive. In terms of sludge samples, 100% of WWTP sludge samples were found to be positive for V. cholerae during the entire study period, with the exception of Paarl WWTP where 66.7% of sludge

Samples were positive. River water and riverbed sediment sampling points were accessible only at Burgersfort WWTP and at Ga-Kgapane WWTP. For Burgersfort WWTP, 83.3% of river water samples and 50% of riverbed sediment samples (Spekboom River) were found to be positive for V. cholerae, while for Ga-Kgapane WWTP, 100% of the river water samples and 50% of riverbed samples (Molototsi River) were found to be positive for V. cholerae (Table 3.3).

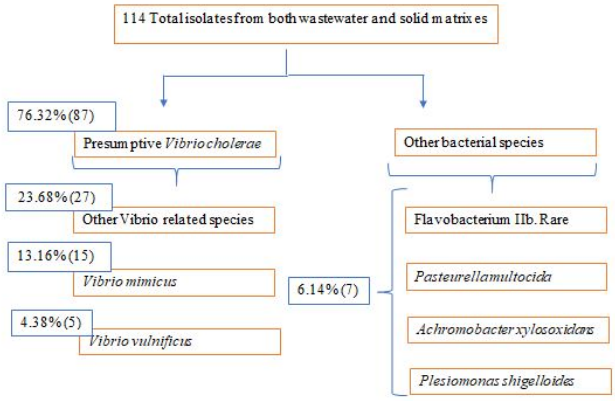


**Figure2**: Diagram illustrating presumptive V. cholerae strains and other bacterial species detected in wastewater, river water, and solid matrices in Limpopo Province wastewater treatment plants

**Table2**: Percentage occurrence of Vibrio cholerae and other bacteria in sample matrices from July to December 2017

| Matrices | Paarl WWTP | Makhado WWTP | Thohoyandou WWTP | Burgersfort WWTP | Ga-Kgapane WWTP |
|---|---|---|---|---|---|
| Influents | (3)50% Vibrio cholerae<br>(1)16.7% Flavobacterium IIb<br>(1)16.7% Vibrio vulnificus<br>(1)16.7% Vibrio mimicus | (4)66.7% Vibrio cholerae<br>(1)16.7% Vibrio mimicus<br>(1)16.7% Achromobacter xylosoxidans | (4)66.7% Vibrio cholerae<br>(1)16.7% Vibrio vulnificus<br>(1.)16.7% Pasteurella multocida | (5)83.3% Vibrio cholerae<br>(1)16.7% Achromobacter xylosoxidans | (6)100% Vibrio cholerae |
| Effluents | (4)66.7% Vibrio cholerae<br>(2)33.3% Vibrio mimicus | (3)50% Vibrio mimicus<br>(3)50% Vibrio cholerae | (4)66.7% Vibrio cholerae<br>(1)16.7% Vibrio vulnificus<br>(1)16.7% Vibrio vulnificus | (4)66.7% Vibrio cholerae<br>(2)33.3% Vibrio mimicus | (4)66.7% Vibrio cholerae<br>(2)33.3% Vibrio mimicus |
| Sewage sludge | (4)66.7% Vibrio cholerae<br>(2)33.3% Vibrio mimicus | (6)100% Vibrio cholerae | (6)100% Vibrio cholerae | (6)100% Vibrio cholerae | (6)100% Vibrio cholerae |
| River water | Not accessible | Not accessible | Not accessible | (1)16.7% Vibrio mimicus<br>(5)83.3% Vibrio cholerae | (6)100% Vibrio cholerae |
| Riverbed sediment | Not accessible | Not accessible | Not accessible | (3)50% Vibrio cholerae<br>(2)33.3% Achromobacter xylosoxidans | (3)50% Vibrio cholerae<br>(1)16.7% Plesiomonas shigelloides<br>(2)33.3% Vibrio vulnificus |

| | |
|---|---|
| | (1)16.7% Vibrio vulnificus |

## Identification of Vibrio cholerae biotype and serotype

Results of the three Vibrio serum agglutination tests (Vibrio cholerae Ogawa Serum, Vibrio cholerae Polyvalent Agglutinating sera, and the Vibrio cholerae Inaba Serum) are illustrated in Figure 3.3. Of these serum tests, only the Vibrio cholerae Ogawa Serum resulted in the agglutination of the isolates identified as V. cholerae. In other words, all 87 isolates tested negative for Vibrio cholerae Polyvalent Agglutinating sera and Vibrio cholerae Inaba Sera. All presumptive V. cholerae isolates (Table 3.3) belonged to the serogroup O1 of V. cholerae, which could be categorised into classical or E1 Tor biotypes, and were classified to one serotype, namely Ogawa. As can be seen in Figure 3.3, V. cholerae Ogawa predominated at 76.32% in aquatic environments of the selected WWTPs compared to other bacterial species.

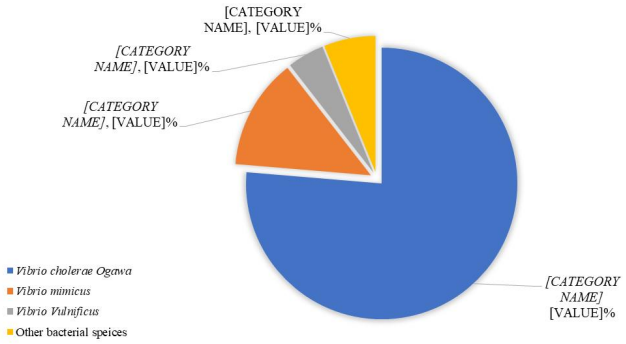


**Figure3**: Overall distribution of V. cholerae Ogawa and other bacterial species in aquatic environments of selected wastewater treatment plants of Limpopo Province, South Africa

Considering the distribution of bacterial species in aquatic environments, there was a high number of Vibrio cholerae Ogawa in samples collected across all the matrices during the study period. All 87 colonies identified as V. cholerae, tested positive for Vibrio cholerae for Ogawa serum (Table 2.3). Therefore, the percentage occurrence of Vibrio cholerae Ogawa ranged from 50% to 100% between July and December 2017 for each matrix. Among all the matrices, Vibrio cholerae Ogawa showed up in all wastewater matrices (100%) collected from all WWTPs, except for Paarl WWTP, which displayed Vibrio cholerae in 66.7% of sludge samples. Of the five wastewater treatment plants, there were only two plants (Burgersfort WWTP and Ga-Kgapane WWTP), which had accessible river sampling points. For the riverbeds of their receiving water bodies (Spekboom River and Molototsi River, respectively), 50% of the samples were positive for Vibrio cholerae Ogawa. DISCUSSIONS

The ability of V. cholerae to survive and reproduce in the environment has a significant impact on the severity of the cholera outbreak. Cholera is endemic in areas where socioeconomic conditions are poor, sanitary systems and public hygiene are basic, and safe drinking water is scarce, particularly during floods. In places where there is a scarcity of fuel for boiling water, such situations are considerably more detrimental to human health.. Between November 2008 and April 2009, 720 Vibrio cholerae O1 strains caused an outbreak of cholera in South Africa. Because V. cholerae may live for a long period in drinking water and wastewater, assuming a viable but non-culturable condition, this epidemic demonstrated that cholera is endemic in South African water resources.Results of the present study are in agreement with previous findings in 2009 as Vibrio cholerae O1 Ogawa was found in river water of both the Burgersfort WWTP and the Ga-Kgapane WWTP between July and December 2017. For this study, one of the main objectives was to investigate these WWTPs in order to gain an understanding of the sources of V. cholerae and its biotypes and serotypes in the rivers (Spekboom River and Molototsi River), which receive the effluents from these WWTPs. Results of this study have revealed that the selected five WWTPs of the Limpopo Province produced inadequately treated effluents. During the study period, up to 66% of the effluent samples displayed the presence of V. cholerae serogroup 01 and its serotype Ogawa. Inadequate treatment of wastewater by the five WWTPs has been previously reported in Green Drop Report statistics by the Department of Water and Sanitation [31-36]. Because of their non-compliance, these WWTPs have been rated into high and critical risk positions. The present study, therefore, confirms that the effluents discharged by these plants are the sources of V. cholerae in both these rivers. Previous cholera outbreaks in the province might be linked to these water sources, which can also lead to future cholera outbreaks if urgent actions are not taken. As a result of the non-compliance to standards set by the National Water Service Authority, Burgersfort WWTP, Paarl WWTP, Thohoyandou WWTP, Makhado WWTP, and Ga-Kgapane WWTP continued to produce effluents of poor quality, and V. cholerae and its serotype V. cholerae Ogawa were found to occur in sewage sludge and accessible riverbed sediments (Tables 3.2 – 3.3; Figures 3.2 – 3.3). All the sewage sludge samples (100%) collected from the five WWTPs displayed V. cholerae and its serotype Ogawa, with the exception of Paarl WWTP, in which 66.7% of the collected sewage sludge samples exhibited these organisms. Out of 114 randomly selected isolates, 76.32% were found to be V. cholerae Ogawa (Figure 3.2). These results revealed that solid matrices in aquatic environments are the hotspots of V. cholerae and its biotypes, especially V. cholerae O1 Ogawa.

These authors pointed out that the serotype Ogawa is endemic in many part of Africa including South Africa and are usually associated with cholera outbreaks on the continent. In the present study, V. cholerae O139 strains were not identified in any of the matrices under investigation. This result corroborates the findings of previous investigators, who stated that V. cholerae O139 is not endemic in Africa, and has only been reported from limited aquatic ecosystems. These authors have also concluded that serogroup O139 is steadily dwindling in number and now virtually non-existent. In the past cholera outbreaks reported in South Africa, V. cholerae O1 was responsible for most of the cases within the country as it was introduced from Mozambique. Furthermore, in the present study, among the 114 isolates observed in the aquatic environment from July 2017 to December 2017, the percentage occurrence of Vibrio cholerae O1 Ogawa (76.32%) was the highest among the Vibrio species detected. Previous studies also have highlighted the existence and persistence of this V. cholerae strain in the environment especially in water sources such as rivers and lakes. The existence of Vibrio cholerae O1 Ogawa in these water sources potentially causes public health concerns as these water sources are primarily used for drinking by nearby communities. Additionally, According to Smith and colleagues, cross-border migration, environmental reservoirs, socioeconomic variables, climatic change, and political instability all contribute to cholera epidemics in Africa. It is also important to point out that the persistence of V. cholerae Ogawa in both wastewater effluents and sewage sludge of all the five wastewater treatment plants has the potential to trigger epidemic episodes with pandemic potential. According to McMichael. V. cholerae O1 has the ability to affect other environmental settings such as agriculture. In addition to V. cholerae and its serotype Ogawa, other Vibrio species (V. mimicus and V. vulnificus) and bacterial species (Achromobacter xylosoxidans and Plesiomonas shigelloides) were detected in some of the aquatic matrices such as effluents and/or sewage sludges or river water and/or riverbed sediments (Tables 3.2 and 3.3, Figures 3.2 and 3.3) across wastewater treatment plants of the Limpopo Province. Vibrio mimicus and V. vulnificus are also known to cause severe watery diarrhoea once a person is infected. In addition, A. xylosoxidans, considered to be a waterborne bacterium, was found in river waters of Makhado WWTP and Burgersfort WWTP. Amoureux have highlighted the persistence of A. xylosoxidans in hospital, domestic and outdoor environment samples, which have highly affected public health. These authors have also indicated that this species can be considered as an emerging pathogen. The detection of this microorganism in river waters of Makhado sewage plant and Burgersfort WWTP in this study calls for immediate attention as this is still yet an emerging waterborne pathogen, which could potentially escalate infection, placing public health in danger as communities still use river water as their main water source for everyday activities. Findings of this study have also revealed the presence of P. shigelloides in river water at the Ga-Kgapane WWTP. Many questions

remain unanswered about the detection and epidemiology of P. shigelloides in the environment. The pathogen continues to remain unstable in its detection and classification. This issue raises many concerns about potential enteropathogenic mechanisms and the prevalence of P. shigelloides (gastroenteritis) varies dramatically in relationship to geographic location. This could explain the reason for detecting this microorganism only in one wastewater treatment facility (Ga-Kgapane WWTP). It could potentially be that sanitary conditions (the Ga-Kgapane area struggles tremendously with high pollution of the environment), or environmental factors and stimuli play an important role in determining the global incidence of this disease. Although Flavobacterium species group IIb, a rare biotype, was detected only in the influent of Paarl WWTP, its ability to adapt easily in the environment could alert authorities to control the growth of this microorganism in wastewater. Lastly, Pasteurella multocida was among the additional bacterial species detected and isolated in Thohoyandou WWTP. These additional bacterial species other than Vibrio species detected equally pose a high risk to human health based on their toxicity within the aquatic environment, more especially in river water. These microorganisms, which have been detected in different sample matrices, have been reported to have detrimental health implications once an individual is infected. With high numbers of microorganisms, particularly Vibrio cholerae O1 Ogawa, being isolated from wastewater treatment plants, a thorough understanding of recent V. cholerae detections and new recombinant V. cholerae biotypes and serotypes, as well as evolution and spread, is required to forecast future developments.

## CONCLUSIONS

The inability of the selected wastewater treatment facilities of the Limpopo Province to produce effluents of high quality has resulted in the presence and persistence of V. cholerae Ogawa in the effluents, sludge, receiving water bodies, and riverbed sediments. Beside the presence of this waterborne agent responsible for cholera outbreaks in this province, other Vibrio species such as V. mimicus and V. vulnificus and bacteria species such as Achromobacter xylosoxidans and Plesiomonas shigelloides were apparent and persisting in some of these environmental settings. Furthermore, percentage occurrence of 50% and 100% of V. cholerae Ogawa in both riverbed sediments and sewage sludge, respectively, is a clear indication that these matrices potentially serve as reservoirs for this bacterial species and may also contribute to cholera outbreaks in the province. This study suggests that V. cholerae Ogawa still presents a potential health concern in South Africa and particularly in the Limpopo Province. Further studies on the seasonal occurrence of V. cholerae Ogawa are needed. The findings of this study may be useful to Limpopo Province's health agencies and local municipalities in preventing future Vibrio outbreaks.. For this reason, in the next chapter, we investigated the seasonal distribution of V. cholerae Ogawa within various matrices of the five selected wastewater treatment plants in Limpopo Province.

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