AN INDEPENDENT INVESTIGATION AND ADVISORY ON THE ROLE OF WATER, SANITATION AND HYGIENE IN THE 2023 CHOLERA OUTBREAK IN HAMMANSKRAAL, SOUTH AFRICA

WORK PACKAGE 5: WATER QUALITY AND FOOD SAFETY ASSESSMENT

Final Report to the
Water Research Commission

by

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EXECUTIVE SUMMARY

The main aim of the solicited WRC project entitled: "*An independent investigation and advisory on the role of water, sanitation and hygiene in the current cholera outbreak in South Africa Work package 5: Water quality and food safety assessment"* is to evaluate the occurrence and dissemination of pathogenic *Vibrio* spp. (focusing on *V. cholerae*) in irrigation water and fresh produce from selected farms within the designated outbreak areas. Moreover, the project aligns with University of Pretoria's research activities over the past 12 years, which focused on determining the presence, characteristics and dissemination of potential human pathogenic and multidrug resistant bacteria in the water-plant-food-public health interface. The project was incorporated into the current WRC funded research project 2022/2023-00885 "Development of a fit-for-purpose water microbiological quality guideline for smallholder farmers and informal food traders".

The terms of reference for the solicited WRC project are as follows:

BUSINESS UNIT: **Research, Development and Innovation**
THEMATIC AREA: **Water Quality and Health**

Background and Rationale

Water, sanitation, hygiene (WASH), and health are closely interconnected and play a crucial role in promoting public health and preventing the spread of diseases. Cholera is an acute diarrheal disease caused by the bacterium Vibrio cholerae. It is primarily transmitted through the ingestion of contaminated water or food, often due to poor sanitation and inadequate hygiene practices. In recent years, there has been increased incidences of confirmed cholera cases in the Southern African region. According to the World Health Organisation, the number of reported cholera cases has been on the rise globally since 2021, suggesting that the seventh cholera pandemic has been ongoing since 1961. Since February 2023, a total of 22 confirmed cholera cases were reported, of which 15 are in Gauteng, and seven cases in the Free State Province. The aim of this investigation is to determine the source of contamination and possible transmission pathways contributing to the spread of cholera within the designated areas where cases have been confirmed.

Work package 5: Water quality and food safety assessment

Scope of work:

- Assess the current food gardening practices and identify risks for transmission within the designated outbreak areas.
- Assess the fresh produce food value chains and identify contamination points within the designated outbreak areas.
- Output and timeframes: 2 weeks for the WRC to commission public health experts to undertake WP5 and generate a preliminary report in 2 weeks.

AIMS

The following were the aims of the project:

1. **Main aim:** To evaluate the occurrence and dissemination of know pathogenic *Vibrio* spp. (including *Vibrio cholerae*) in irrigation water and fresh produce from selected farms within the designated outbreak areas.
2. To evaluate farming practices, i.e. irrigation water sources, irrigation method (overhead/drip/flooding), manure amendment, supply chain mapping via observation/short questionnaires on selected farms

(current WRC-funded project selected farms and one/two additional farms) to identify potential risks that could facilitate transmission within the designated outbreak areas.

3. To sample irrigation water and fresh produce (vegetables) to assess the produce food value chains and identify contamination points with *Vibrio* spp. (including *V. cholerae*) on selected farms within the designated outbreak areas.

Background Water Quality and Food Safety Assessment (WP5):

In South Africa, irrigated agriculture is the main user of surface and groundwater, with an estimated 53% to 61% of all available water sources used for irrigation purposes (Bonthuys, 2018). Irrigation water sources in SA include surface water, borehole water (groundwater) and potable or rainwater, while untreated or treated wastewater is not routinely used (Iwu and Okoh, 2019). However, the microbiological quality of water sources, especially surface water, have been seriously compromised by municipal wastewater discharge, sewage from informal settlements with inadequate sanitation, wastes from animal husbandry, industrial companies, hospitals effluents and the mining sector (Adesifoye and Okoh, 2017; Verlicchi and Grillini, 2020). Moreover, the use of polluted water sources for irrigation purposes has been proven to pose a risk of transferring potential pathogenic microorganisms including shiga-toxin producing *Escherichia coli*, *Salmonella* spp. and *Listeria monocytogenes* amongst others onto crops, especially those which undergo minimal post-harvest processing and are usually consumed raw (Uyttendaele et al., 2015; Self et al., 2019; CDC, 2020). In the post-harvest stages produce can be contaminated by contact with dirty transport containers, polluted process water, conveyor belts and human handlers (Althaues et al., 2012). This has implications for compliance with local and international food safety regulations (DWAF, 2008; FDA, 2021) and is critical given the high proportion of more susceptible consumers within the South African society (e.g. the so-called YOPIs). Further to this, the increasing use of antimicrobials in the healthcare system and intensive livestock farming led to increased levels of antibiotic resistance in environmental microbial populations, thus exerting selection pressures and inducing the transfer of antibiotic resistance genes to potential human pathogenic bacteria (Manyi-Loh et al., 2018). Contaminated environmental resources, especially irrigation water, are known to play an important role in the increased prevalence and dissemination of potential human pathogenic and multidrug resistant bacteria (Larsson et al., 2018). These pathogens have been identified in health care systems, agroecosystems including surface and wastewater, irrigation water, soil, vegetable crops and animal husbandry (Richter et al., 2019; Richter et al., 2020; Ebomah and Okoh, 2020; Iwu et al., 2021).

The fact that human pathogens can survive on fresh produce for extended periods of time, coupled with the presence of an unacceptably high microbial load in irrigation water, represent a potential microbial hazard that could pose a food safety risk. World-wide an increase in the number of foodborne disease outbreaks have been linked with the increased demand and consumption of fresh produce (fruit and vegetables). During 2008 the World Health Organization (WHO) and Food and Agriculture Organization (FAO) prioritised fresh produce with the highest food safety risk in terms of microbial hazards associated with the product (FAO/WHO 2008).

The recent *V. cholerae* outbreak in the Hammanskraal area is a serious environmental and human health concern (Sakib et al., 2018). The disease is appropriately named as cholera literally means “gutter” and it has been associated with contaminated water sources (Lekshmi et al., 2018). According to Lekshmi et al. (2018) there have been seven pandemics of cholera in all major continents including Asia, America, Europe and Africa. The role of the environment and humans in the development and spread of *V. cholerae* pathogenic traits were reviewed extensively by Sakib et al. (2018). These include virulence factors that are involved in colonization ability, motility, adhesion, biofilm formation, quorum sensing (QS), and toxin secretion of the pathogen. The infectious dose of *V. cholerae* from ingesting contaminated water was reported as 10^3-10^6 colony forming units (CFU’s) to cause disease, while a lower dose of 10^2-10^4 is required from food Adesifoye and Okoh (2017). Risk factors in contracting the cholera disease included a history of travelling, not washing fruit and vegetables and no access to clean water in a case-control cholera study in Yemen (Dureab et al., 2019). Dinede et al. (2020) concluded that the consumption of raw vegetables as well as drinking holy water was linked to a *V. cholerae* related outbreak in Ethiopia. More recently Osunla et al. (2021) urged that *Vibrio*

spp. as emerging pathogens, be included as water quality indicators by the South African Department of Water Affairs (DWAF). This follows as they isolated six medically important *Vibrio* species from wastewater treatment effluents and surface water downstream of the wastewater works.

Chapter 1 includes the literature review and more detailed bibliometric analysis of current literature around *V. cholerae* in relation to food safety. Furthermore, research results of the microbiological quality and safety in formal and informal fresh produce supply chains in South Africa were summarised in Chapter 1.

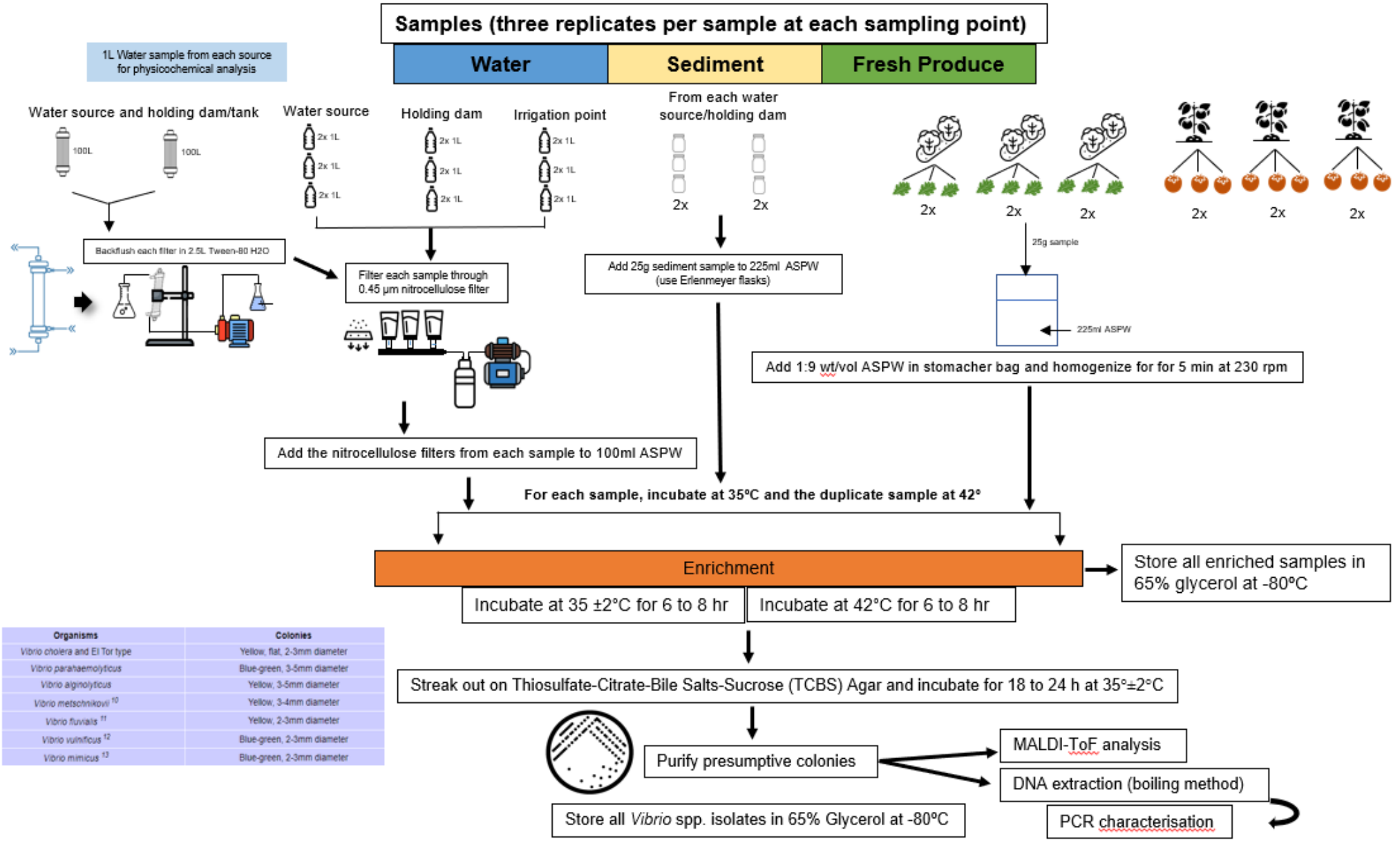
METHODOLOGY

The sampling sites selected, irrigation water sources used, irrigation method, fresh produce sampled, observations and information regarding farming practices on each of the respective farms (Farm A, D G and H) as well as water from the Kaalplaasspruit and Apies River sampling points were summarised in Table 1. Additional sampling site details, sample collection, processing and analysis were included in Chapter 2.

Table 1: Summary of the sampling sites selected, water sources used, irrigation method, fresh produce sampled, additional on-farm observations and information.

Sapling sites		Water source/s	Irrigation method	Fresh produce sampled	Additional observations and information
Farm A	Gauteng	Borehole water Aquaculture system used for nutrient enrichment before irrigation	Overhead irrigation (hosepipe)	Mustard (leafy greens)	Aquaculture farming (Tilapia). Vegetables – hydroponic production as well as in soil during 2022. No hydroponically grown vegetables on sampling day in June 2023.
Farm D	North-West	River – canal system	Overhead irrigation	Celery and rocket	Supplied retailers and exported selected produce. GLOBAL'GAP certified.
Farm G	Gauteng	Dam (earth without a lining)	Flood irrigation	Mustard (leafy green)	The source water from earth dam pumped into a lined 2 dam. Supply informal traders Tshwane City Centre, Ga Rankuwa and Marabastad,
Farm H	Gauteng	Spruit/small tributary	Overhead sprinklers	Peas	Routinely uses Kaalplaasspruit water for irrigation, but sometimes supplements with a hole dug in the ground ground. Soil amendment: Composted chicken manure and sawdust cow manure as well as 2:3:4 fertiliser Tshwane Market
Kaalplaasspruit downstream of Soshanguve and upstream of Farm H					Burst sewage pipe upstream flowing into the Kaalplaasspruit
Apies River site downstream of Daspoort Wastewater Works					

A schematic diagram of the sample collection, processing and analysis are shown in Figure 1 below. Additional details of the Experimental Procedures were described in Chapter 2.



Organisms	Colonies
Vibrio cholera and El Tor type	Yellow, flat, 2-3mm diameter
Vibrio parahaemolyticus	Blue-green, 3-5mm diameter
Vibrio alginolyticus	Yellow, 3-5mm diameter
Vibrio metschnikovii [10]	Yellow, 3-4mm diameter
Vibrio fluvialis [11]	Yellow, 2-3mm diameter
Vibrio vulnificus [12]	Blue-green, 2-3mm diameter
Vibrio mimicus [13]	Blue-green, 2-3mm diameter

RESULTS AND DISCUSSION

To evaluate the occurrence and dissemination of know pathogenic *Vibrio spp*. (including *V. cholerae*) in irrigation water and fresh produce, four farms were selected which included Farm A, D, G and H. In addition, water samples were collected from the Apies River (downstream of the Daspoort wastewater works) and from the Kaalplaasspruit (downstream of the Soshanguve wastewater works) where a sewage pipe had burst and was flowing into the Kaalplaasspruit. A map of the of the sampling sites was included in Chapter 2 (Figure 2.1). Additional farming practices, i.e. irrigation water sources, irrigation methods (overhead/drip/flooding), manure amendment, supply chain mapping via observation on selected farms to identify potential risks that could facilitate transmission within the designated outbreak areas were summarised in Table 2.1.

Sampling sites and sample collection: All the field trips and sample collection (irrigation water and fresh produce) from selected farms were completed from Farm A (21 June), Farm D (27 June), Farm G (29 June) as well as Farm H and the water samples from the Kaalplaasspruit and the Apies River (4 July). A total of 170 samples (water, sediment and fresh produce). *These included the following number of samples on each of the farms:*
Farm A, a total of 39 samples (water, n=21; fresh produce, n=12 and sediment, n=6) were collected (Chapter 2, Figure 2.2).
Farm D, a total of 76 samples (water, n=40; fresh produce, n=12 and sediment, n=24) were collected (Chapter 2, Figure 2.3).
Farm G, a total of 25 samples (water, n=13; fresh produce, n=6 and sediment, n=6) were collected (Chapter 2, Figure 2.4).
Farm H, a total 28 samples (water, n=16; fresh produce, n=6 and sediment, n=6) were collected (Chapter 2, Figure 2.5).
Kaalplaasspruit upstream of Farm H Figure 2.6 (n=1).
Apies river site downstream of Daspoort wastewater works Figure 2.7 (n=1).

Microbiological analysis: Following sample processing, selective enrichment and streaking onto selective chromogenic media as described in Chapter 2, presumptive positive *V. cholerae* isolates were obtained Farm A (n=41), Farm D (n=179), Farm G (n=34) and Farm H:(n=43). Identities of all isolates were confirmed using MALDI-TOF analysis. The isolates predominantly included *Aeromonas* spp. from all the farms. Janda and Abott (2010) reported that the main reservoir of Aeromonas is the aquatic environment (fresh and brackish water). Moreover, Aeromonas bacteria were also found in food products, vegetables, and animal faeces and in the digestive tract microbiota (Janda and Abbott, 2010). More recently Canellas et al. (2023) reported that *Aeromonas* spp. can be found in a wide range of habitats and are able to thrive under adverse conditions, including in highly polluted waters. Moreover, the research focus on the *Aeromonas* genus has increased due to it being pathogenic to aquatic organisms and humans (Canellas et al., 2023). *Aeromonas* spp. most frequently implicated in human infections include *A. caviae*, *A. dhakensis*, *A. veronii*, and *A. hydrophila*. Sadique et al. (2021) reported that *V. cholerae* frequently causes outbreaks of diarrhoea in coastal Bangladesh. Other studies have shown that *V. cholerae* can survive on crops and that waterborne pathogens (including *V. cholerae*) can be transferred to crops (Hounmanou et al., 2016; Buiman et al., 2022). Interestingly, Tagliavia et al. (2019) reported that using thiosulfate-citrate-bile salts sucrose as selective medium for isolation of *Vibrio* spp. may be hampered by the variable adaptability of different taxa to the medium. This agrees with findings of this study as *Aeromonas* spp. were predominantly isolated. *V. cholerae* isolated from a selectively enriched 1 L water sample from the Kaalplaasspruit used for irrigation of peas on Farm H. Most importantly this spruit flows into the Apies River further downstream in the Hammanskraal area. It was determined that the isolate from the Kaalplaasspruit is *V. cholerae* non-O1; PCR-negative for the toxin gene (*ctxA*) and that a public health response is not required by the National Institute of

Communicable diseases. The results from the current study correspond to a previous study in Kwazulu-Natal where Ntema et al. (2014) reported the presence of non-toxigenic *V. cholerae* non-O1/O139 in the Msunduzi, Umlazi and Isipingo Rivers. Globally, non-toxigenic *V. cholerae* have been increasingly implicated in sporadic human infection (Dowling et al., 2021; Wang et al., 2020). Moreover, Dowling et al. (2021) reported the that the isolation of the non-O1/0139 V. cholerae from water sources in South Africa have been increasing.

Molecular characterisation*:* PCR analysis for all samples for the genotypic detection of the specific target outer membrane protein (*ompW*) of *V. cholerae* has been completed. Overall, 21 samples (12.5%) were PCR-positive for the *ompW* gene as well as the 16s *V. cholerae* gene, while three of these samples tested positive for the *toxR* gene. This included 5 fresh produce samples (Farm A, n=2 and Farm G, n=3), 5 sediment samples (Farm A, n=4, Farm G, n=1), and 11 water samples (Farm A, n=9 and Farm H, n=2). These samples were regarded as presumptive positive for the presence of *V. cholerae* (Takahashi et al., 2021). Subsequently, the samples were screened for the presence of the *toxR* regulatory gene and the *hlyA* hemolysin gene. Overall, 38.1% (8/21) showed presumptive positive PCR results. PCR analysis of the *ctxA* gene is in progress after which all presumptive positive results will be confirmed with sequencing. Furthermore, all presumptive positive samples will be restreaked on TCBS following the second enrichment, purified, and identified with MALDI-TOF, to ensure that all possible colonies have been isolated. Additionally, the MALDI-ToF identified *V. chole*rae isolate from Farm H (H21) was also PCR-positive for *ompW, toxR,* and *hlyA*. However, the *V. cholerae* isolate was sent to the NICD where it was confirmed that the isolate was not positive *ctxA,* indicating that the isolate is a non-O1/non-O139 *V. cholerae* strain.

In addition to the physicochemical analyses (summarised below), a replicate of all water samples used for irrigation on the respective farms were analysed for the presence of enterotoxigenic *V. cholerae* at an external laboratory (Waterlab). The results for all the water samples indicated that enterotoxigenic *Vibrio cholerae* was not detected, which agrees with the culture dependant microbiological analysis results obtained in this scoping study.

Physicochemical analysis*:* The pH of the irrigation water from all the farms ranged between pH 6.2-8.4 and were all acceptable according to the DWAF guideline of pH 6-9 (DWAF, 1996). The dissolved oxygen (DO) levels ranged between 7.2 and 10.6 mg/L, which is also acceptable according to the guideline of >3 mg/L. Dissolved oxygen is a measure of water quality and an indicator of a water resource's ability to sustain aquatic life. The required electrical conductivity of irrigation water should be ≤40 mS/m. The values on Farm A (aquaculture farm) were the lowest ranging between 12.2 and 18.5 mS/m (milli-Siemens per metre). Interestingly the North and South canals (fed by the Skeerpoort River) on Farm D, although within the acceptable limit, were quite high ranging between 31.0 and 31.3 mS/m. Elevated electrical conductivity can indicate that pollution has entered the river and that the water is unsuitable for irrigation purposes. (https://wq.epa.gov.tw/EWQP/en/Encyclopedia/NounDefinition/Pedia_48.aspx#:~:text=Electrical%20conductivity%20(EC)%20is%20a,more%20electrolytes%20in%20the%20water, accessed 19-7-2023).

According to the US-EPA (United States Environmental Protection Agency, 2012) guidelines for water reuse, the turbidity levels of irrigation water should be <5 NTU (nephelometric turbidity units). Suspended solids give rise to turbidity in water. The relationship between the amount of suspended solids and the turbidity measurement is dependent on the nature and particle size distribution of the suspended matter. On Farm A the values ranged from 0.5 NTU [borehole] to 24 NTU [irrigation pipe]; Farm D from 1.9 NTU [source water] and 6.7 [irrigation pivot point]; Farm G [earth dam used for irrigation] was 87 NTU and the holding dam 63 NTU, which is totally unacceptable according to the USEPA (2012) guidelines. On Farm H the turbidity of the water from the hole dug next to the fresh

produce field was unacceptable at 52 NTU. Interestingly, the electrical conductivity of the Kaalplaasspruit sampled on Farm H was 56.4 mS/m, which was much higher that the allowed ≤ 40 mS/m and this is the water sample from which the *V. cholerae* non-O1 isolate was obtained.

GENERAL

The aims of WP5 have been completed. The results of this study will form the basis of future research proposals and post graduate studies.

CONCLUSIONS

A non-toxigenic *V. cholerae* isolate was shown to be present in a water sample used for irrigation purposes on one of the small-scale farms chosen in the Hammanskraal area. Although it was confirmed to be *V. cholerae* non-O1, it is still a reason for concern as diarrheal disease has increasingly been reported due to these organisms (Wang et al., 2020). The compromised microbial and chemical water quality of water sources used for fresh produce production impacts food safety and security negatively. Moreover, it is an additional hurdle as far as adhering to regulatory requirements regarding the microbiological quality of fresh produce is concerned. If these requirements are not met market access is impacted negatively. Our reputation globally is also affected negatively as it raises the question whether we can produce safe food.

RECOMMENDATIONS

- Expand surveillance of the physico-chemical and microbiological quality of informally produced fruit and vegetables sold in the peri-urban townships and settlements in production areas where the quality of the water sources is known to have been compromised.
- Regular feedback to farmers and local authorities (i.e. municipalities, Environmental Health Protection Officers).
- Creating food safety awareness and training on safe practices for farmers, farm workers, food handlers and informal vendors.
- To develop and implement mitigation strategies to improve food safety and to develop policies to assure safe quality water and food.

ACKNOWLEDGEMENTS

The project team wishes to thank the following people for their contributions to the project.

Name	**Affiliation**
Cholera Advisory Panel	Various Institutions
Dr Eunice Ubomba-Jaswa (Research Manager)	Water Research Commission
Dr Anthony Smith	National Institute of Communicable Diseases
Miss Zama Zulu	Department Plant and Soil Sciences, University of Pretoria
Miss Michelle Adams	Department Plant and Soil Sciences, University of Pretoria
Community and Farmers	Hammanskraal

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CHAPTER 1: BACKGROUND

1.1 INTRODUCTION

The main aim of the solicited WRC project entitled: “An independent investigation and advisory on the role of water, sanitation and hygiene in the current cholera outbreak in South Africa Work package 5: Water quality and food safety assessment” is to evaluate the occurrence and dissemination of pathogenic *Vibrio* spp. (focusing on *V. cholerae*) in irrigation water and fresh produce from selected farms within the designated outbreak areas. Moreover, the project fits into our main research over the past 12 years, which focused on determining the presence, characteristics and dissemination of potential human pathogenic and multidrug resistant bacteria in the water-plant-food-public health interface. The project was incorporated into the current WRC funded research project 2022/2023-00885 “Development of a fit-for-purpose water microbiological quality guideline for smallholder farmers and informal food traders”, which will be expanded to include *Vibrio* spp., including *V. cholerae* specifically.

BUSINESS UNIT: **Research, Development and Innovation**
THEMATIC AREA: **Water and Health**

Background and Rationale
Water, sanitation, hygiene (WASH), and health are closely interconnected and play a crucial role in promoting public health and preventing the spread of diseases. Cholera is an acute diarrheal disease caused by the bacterium Vibrio cholerae. It is primarily transmitted through the ingestion of contaminated water or food, often due to poor sanitation and inadequate hygiene practices. In recent years, there has been increased incidences of confirmed cholera cases in the Southern African region. According to the World Health Organisation, the number of reported cholera cases has been on the rise globally since 2021, suggesting that the seventh cholera pandemic has been ongoing since 1961. Since February 2023, a total of 22 confirmed cholera cases were reported, of which 15 are in Gauteng, and seven cases in the Free State Province. The aim of this investigation was to determine the source of contamination and possible transmission pathways contributing to the spread of cholera within the designated areas where cases have been confirmed.

Work package 5: Water quality and food safety assessment
Scope of work:

- Assess the current food gardening practices and identify risks for transmission within the designated outbreak areas.
- Assess the fresh produce food value chains and identify contamination points within the designated outbreak areas.
- Output and timeframes: 2 weeks for the WRC to commission public health experts to undertake WP5 and generate a preliminary report in 2 weeks.

Reporting and project review mechanism
An independent advisory panel will be appointed to guide the outcomes from this investigation. The envisaged reporting is as follows:

- Progress report – weekly
- Preliminary report – 2 weeks after start of project
- Final report.

1.2 PROJECT AIMS

1. **Main aim:** To evaluate the occurrence and dissemination of know pathogenic *Vibrio* spp. (including *Vibrio cholerae*) in irrigation water and fresh produce from selected farms within the designated outbreak areas.
2. To evaluate farming practices, i.e. irrigation water sources, irrigation method (overhead/drip/flooding), manure amendment, supply chain mapping via observation/short questionnaires on selected farms (current WRC funded project selected farms and one/two additional farms) to identify potential risks that could facilitate transmission within the designated outbreak areas.
3. To sample irrigation water and fresh produce (vegetables) to assess the produce food value chains and identify contamination points with *Vibrio* spp. (including *V. cholerae*) on selected farms within the designated outbreak areas.

1.3 SCOPE AND LIMITATONS

Water Quality and Food Safety Assessment (WP5):

In South Africa, irrigated agriculture is the main user of surface and groundwater, with an estimated 53% to 61% of all available water sources used for irrigation purposes (Bonthuys J, 2018). Irrigation water sources in SA include surface water, borehole water (groundwater) and potable or rainwater, while untreated or treated wastewater is not routinely used (Iwu and Okoh, 2019). However, the microbiological quality of water sources, especially surface water, have been seriously compromised by municipal wastewater discharge, sewage from informal settlements with inadequate sanitation, wastes from animal husbandry, industrial companies, hospitals effluents and the mining sector (Adesifoye and Okoh, 2017; Verlicchi and Grillini, 2020).

Moreover, the use of polluted water sources for irrigation purposes has been proven to pose a risk of transferring potential pathogenic microorganisms including shiga-toxin producing *Escherichia coli*, *Salmonella* spp. and *Listeria monocytogenes* amongst others onto crops, especially those which undergo minimal post-harvest processing and are usually consumed raw (Uyttendaele et al., 2015; Self et al., 2019; CDC, 2020). In the post-harvest stages produce can be contaminated by contact with dirty transport containers, polluted process water, conveyor belts and human handlers (Althaues et al., 2012). This has implications for compliance with local and international food safety regulations (DWAF, 2008; FDA, 2021) and is critical given the high proportion of more susceptible consumers within the South African society (e.g. the so-called YOPIs). Further to this, the increasing use of antimicrobials in the healthcare system and intensive livestock farming led to increased levels of antibiotic resistance in environmental microbial populations, thus exerting selection pressures and inducing the transfer of antibiotic resistance genes to potential human pathogenic bacteria (Manyi-Loh et al., 2018). Contaminated environmental resources, especially irrigation water, are known to play an important role in the increased prevalence and dissemination of potential human pathogenic and multidrug resistant bacteria (Larsson et al., 2018). These pathogens have been identified in health care systems, agroecosystems including surface and wastewater, irrigation water, soil, vegetable crops and animal husbandry (Richter et al., 2019; Richter et al., 2020; Ebomah and Okoh, 2020; Iwu et al., 2021).

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vegetables). During 2008 the World Health Organization (WHO) and Food and Agriculture Organization (FAO) prioritized fresh produce with the highest food safety risk in terms of microbial hazards associated with the product (FAO/WHO 2008).

The recent *V. cholerae* outbreak in the Hammanskraal area is a serious environmental and human health concern (Sakib et al., 2018). The disease is appropriately named as cholera literally means “gutter” and it has been associated with contaminated water sources (Lekshmi et al., 2018). According to Lekshmi (et al., 2018) there have been seven pandemics of cholera in all major continents including Asia, America, Europe and Africa. The role of the environment and humans in the development and spread of *V. cholerae* pathogenic traits were reviewed extensively by Sakib et al. (2018). These include virulence factors that are involved in colonization ability, motility, adhesion, biofilm formation, quorum sensing (QS), and toxin secretion of the pathogen. The infectious dose of V. cholerae from ingesting contaminated water was reported as 10^3-10^6 colony forming units (CFU’s) to cause disease, while lower dose of 10^2-10^4 is required from food Adesifoye and Okok (2017). Risk factors in contracting the cholera disease included a history of travelling, not washing fruit and vegetables and no access to clean water in a case-control cholera study in Yemen (Dureab et al., 2019). Dinede et al. (2020) concluded that the consumption of raw vegetables as well as drinking holy water was linked to a *V. cholerae* related outbreak in Ethiopia. More recently Osunla et al. (2021) urged that *Vibrio* spp. as emerging pathogens, be included as water quality indicators by the South African Department of Water Affairs. This follows as they isolated six medically important *Vibrio* species from wastewater treatment effluents and surface water downstream of the wastewater works.

The global literature about *V. cholerae* in fresh produce supply chains was scanned in the Web of Science (WOS) core collection database (https://www-webofscience-com), accessed 14 June 2023. The topic search (which searches the title, abstract, author keywords, and Keywords Plus) were used to identify the closest matching publications included the following search terms: “*Vibrio cholera*e*" and ("fresh produce" or "fruit" or "vegetable*") or "contamination" or "food safety" or "detection" and (*V. cholerae** and "irrigation water quality"). Subsequently, a bibliometric analysis was done using VOSViewer (Van Eck and Waltman, 2017), to highlight the co-occurrence (threshold of a minimum of 20 words) of all relevant keywords (Figure 1.1). The network analysis indicated that food safety was the keyword predominantly included, furthermore, that most of the food safety publications were found between 2016-2018. Of note was that the studies predominantly included the detection of common potential foodborne pathogens (not *Vibrio* spp.) including *Salmonella* spp. and *Staphylococcus aureus* and more recently *L. monocytogenes* and *E. coli* in relation to fresh produce and irrigation water. The network analysis further indicated that the inclusion of antimicrobial resistance surveillance in food safety, especially linked to foodborne pathogen occurrence in irrigation water and water quality has become a food safety research focus of note from 2019 onwards (Figure 1.2). Focussing on *V. cholerae*, it was shown that a few studies have linked this pathogen to food safety studies as well as food, water and more recently fruit and vegetables (Figure 1.3). However, the occurrence of *V. cholerae* in fresh produce supply chain food safety-related publications have not been as evident as other pathogens like *Salmonella* spp. for example.

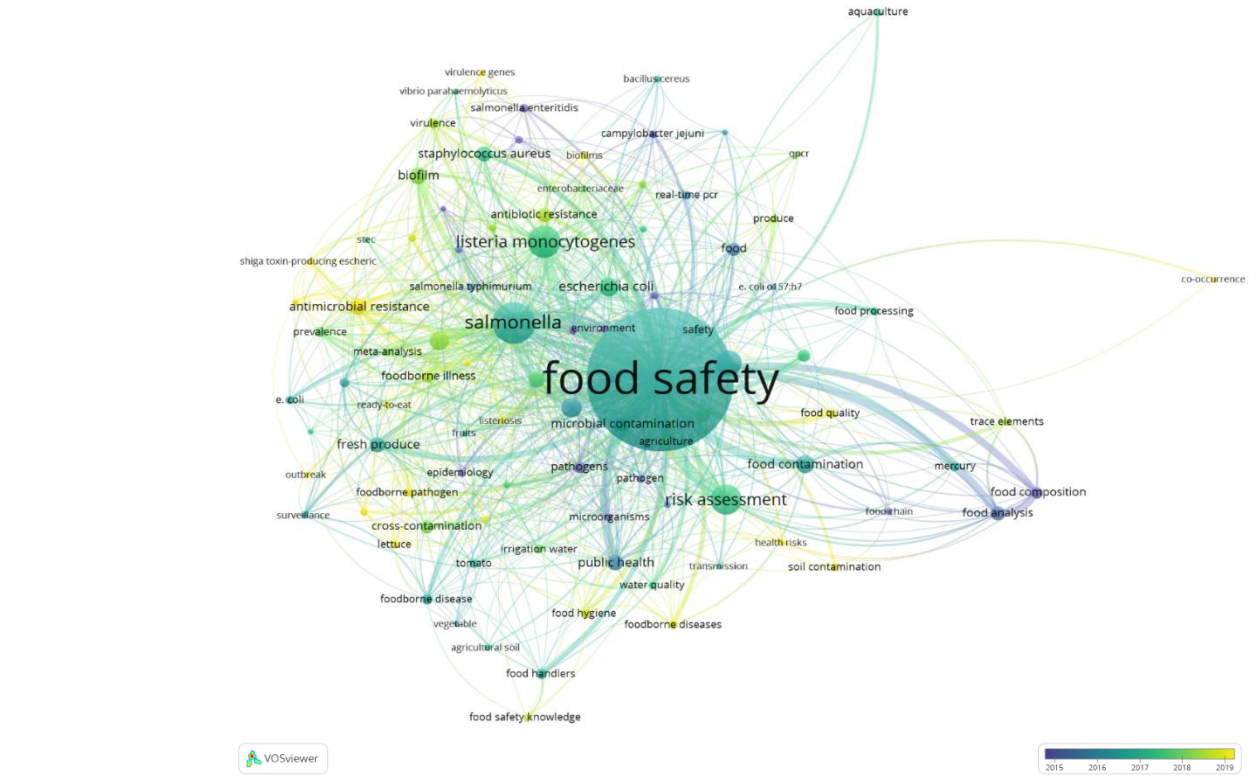

Figure 1.1: The keywords co-occurrence network of *Vibrio cholerae* in fresh produce supply chains-related publications. The colour indicated the publication year while the circles are sized according to keyword co-occurrence.

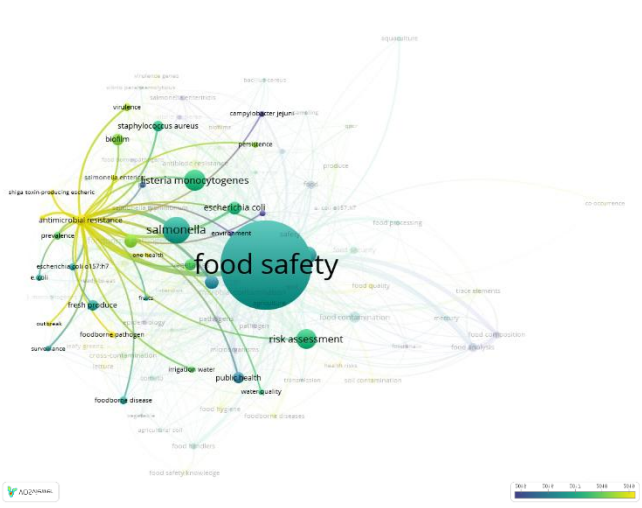

Figure 1.2: The keywords co-occurrence network of *Vibrio cholerae* in fresh produce supply chains-related publications. The keyword "antimicrobial resistance" was highlighted to indicate the increased co-occurrence of foodborne pathogen and antimicrobial resistance keywords in water and fresh produce related publications. The colour indicated the publication year while the circles are sized according to keyword co-occurrence.

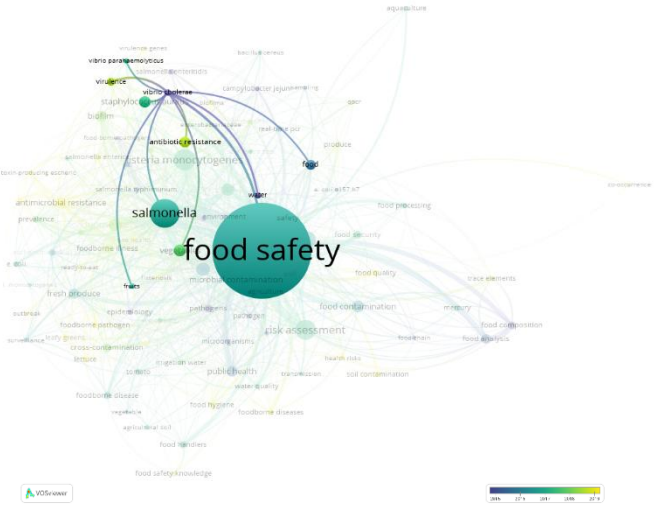

Figure 1.3: The keywords co-occurrence network of *Vibrio cholerae* in fresh produce supply chains-related publications. The keyword "Vibrio cholerae" was highlighted to indicate the co-occurrence of *Vibrio cholerae* and food safety in water and fresh produce related publications. The colour indicated the publication year while the circles are sized according to keyword co-occurrence.

Microbiological quality of fresh produce and irrigation water – recent South African scientific evidence (unpublished)

Authors: Richter, L., du Plessis, E., Duvenage, S. and Korsten, L

The microbiological safety of fresh produce at retail has been studied globally with the focus mainly on assessing indicator bacteria levels and detection and characterisation of foodborne pathogens (*E. coli*, *Salmonella* spp., and *L. monocytogenes*) (Denis et al., 2016b; du Plessis et al., 2017a; Roth et al., 2018; Vital et al., 2014). The lack of consensus in guidelines regarding acceptable hygiene indicator bacteria levels on ready-to-eat (RTE) fresh produce renders compliance according to different countries difficult. In the South African context, adding complexity to integrated fresh produce safety surveillance, is the dualistic fresh produce food supply system. Both commercial and small-scale farmers supply fresh produce to the public, with distribution channels that go through a formal (regulated) or an informal (unregulated) system. A few SA studies have focused on the microbiological quality of leafy green vegetables at different points throughout the supply chain, predominantly in the formal sector. This follows as leafy greens have previously been prioritized as the highest level of concern in terms of fresh produce safety from a global perspective (WHO, 2008). Other studies have also focused on different fresh produce product types in selected informal markets at the point of sale, predominantly in Gauteng Province, the most densely populated province in SA (Table 1) (Baloyi, 2021; du Plessis et al., 2017a; Richter et al., 2021a). Microbial analysis of fresh vegetables (spinach and tomatoes) from street vendors, trolley vendors, farmers' markets and formal retailers showed that the hygiene indicator bacteria counts were mostly not significantly different between formal and informal markets, with exceptions noted on occasion (Richter et al., 2021a). Similarly, coliform counts on cabbage and spinach from street vendors and retailers were not significantly different, however spinach from street vendors had significantly higher mean *E. coli* counts than spinach from retailers in another Gauteng scoping study (du Plessis et al., 2017a). Coliforms form part of the natural microflora of fruit and vegetables, therefore, testing for total coliforms in fresh produce food safety practices is not intended to detect faecal contamination, but rather to reflect general hygiene during food production or handling as the natural occurrence is expected (CFS, 2014). However, most international guidelines omit the coliforms and Enterobacteriaceae criteria completely for fresh fruit and vegetables due to the natural high bacterial load on these products (FPSC A-NZ, 2019; FSAI, 2016; Health Canada, 2010; Health Protection Agency, 2009). The main hygiene indicator used in fresh produce safety being E. coli, with varied acceptable limits in different countries.

Acceptable *E. coli* limits for retailed fresh produce is specified in the UK as 20 to 100 CFU/g, Australia as 3 to 100 CFU/g, and Canada as 100 MPN/g, while the SA Department of Health (DoH) guidelines which are currently under revision proposed zero *E. coli* per gram for raw fruit and vegetables (DoH, 2000; FSANZ, 2001; Health Canada, 2010; Health Protection Agency, 2009). The EU guidelines for E. coli limits on RTE pre-cut fruit and vegetables state that levels <100 CFU/g are satisfactory, *E. coli* levels between 10^2-10^3 CFU/g are borderline and samples with *E. coli* >103 CFU/g are unsatisfactory (EC, 2007). Fresh produce (lettuce, parsley, spinach and carrots) supplied by different smallholder farmers in KwaZulu Natal, SA as well as irrigation water samples representative of water sources frequently used for irrigation, was recently analysed for hygiene indicator bacteria and presence of potential pathogens (Beharielal et al., 2018). The safety and quality of the irrigation water used on these smallholder farms were regarded as unsuitable, as the fecal coliform counts of most samples exceeded the WHO recommendation of faecal coliforms <1000/100 ml of irrigation water used in the production of minimally processed fresh produce (Table 2) (Beharielal et al., 2018). Furthermore, coliform and *E. coli* levels on the fresh produce exceeded the current SA Department of Health (DoH) guidelines (Table 2) (Beharielal et al., 2018).

Table 1.1: The microbiological quality of whole and fresh-cut ready-to-eat (RTE) vegetables that have recently (2015-2021) been studied in South Africa at harvest or the point of sale in formal or informal markets

Analysis Year	Province	Vegetable type	Whole/RTE bagged and cut	Sampling site	Sample size	Sample weight (g)	Microbiological quality analysis (Min-Max Range, log CFU/g) *			Detection of foodborne pathogens (1=detected; 0=not detected)			Reference
							Coliform counts	*E. coli* counts	Enterobacteriaceae counts	*E. coli*	*Salmonella* spp.	*Listeria* spp.	
2012	Limpopo	Onions	Whole	At harvest	12	25	2,37-2,96	0,00-0,09	-	1	0	0	(Du Plessis et al., 2015)
	Limpopo	Onions	Whole	Market	12	25	1,11-2,05	0,00-0,09	-	1	0	0	
2014	Limpopo	Tomato	Whole	At harvest	45	25	3,2	<1	-	0	0	-	(van Dyk et al., 2016)
	Limpopo	Tomato	Whole	Informal market	45	25	1,9-6,2	0,00-0,00	-	0	0	-	
	Limpopo	Tomato	Whole	Retailers	45	25	<1-6,6	0,00-0,00	-	0	0	-	
2015	Gauteng	Cabbage	Whole	Informal street vendors	45	25	2,78-5,73	0,00-0,15	-	1	0	0	(du Plessis et al., 2017a)
	Gauteng	Cabbage	Whole	Retailers	45	25	0,95-5,99	0,00-1,5	-	1	0	0	
	Gauteng	Spinach	Bunch	Informal street vendors	45	25	1,39-5,89	0,00-4,34	-	1	0	0	
	Gauteng	Spinach	Bunch	Retailers	45	25	2,64-5,74	0,00-1,85	-	1	0	0	
2016-2018	Gauteng	Spinach	Bunch/RTE fresh cut	Retailers	50	50	2.90-7.17	0.00-3.42	2.78-8.16	1	0	0	(Richter et al., 2021a)
2016-2018	Gauteng	Spinach	Bunch	Informal street vendors	50	50	0.70-7.60	0.00-2.08	0.00-6.99	1	0	0	
2016-2018	Gauteng	Spinach	Bunch	Trolley vendors	50	50	0.59-7.04	0.00-1.29	0.00-7.27	1	0	0	
2016-2018	Gauteng	Spinach	Bunch	Farmers' market vendors	50	50	3.76-8.10	0.00-5.88	4.03-7.88	1	0	0	
2016-2018	Gauteng	Tomato	Whole	Retailers	50	150	0.48-8.04	0.00-0.89	2.40-8.10	1	0	0	
2016-2018	Gauteng	Tomato	Whole	Informal street vendors	50	150	2.00-8.21	0.00-2.30	0.00-7.82	1	0	0	
2016-2018	Gauteng	Tomato	Whole	Trolley vendors	50	150	0.00-6.36	0.00-3.60	0.00-7.94	1	0	0	
2016-2018	Gauteng	Tomato	Whole	Farmers' market vendors	50	150	3.15-7.89	0.00-5.10	1.49-7.75	1	0	0	
2017-2018	Gauteng	Lettuce	Whole/RTE bagged and cut	Farmers' market vendors	50	50	3.58-7.82	0.00-3.31	4.18-8.26	1	0	0	
2017-2018	Gauteng	Cucumber	Whole	Farmers' market vendors	45	150	0.00-6.48	0.00-3.78	0.00-6.45	1	0	0	
2017-2018	Gauteng	Green beans	Whole/Bagged	Farmers' market vendors	50	150	0.70-6.77	0.00-4.78	0.00-6.71	1	0	0	
2016-2018	Gauteng	Apples	Whole	Informal street vendors	75	150	0,00-4,65	0,00-0,00	0,00-3,57	0	0	0	(Baloyi, 2021)
2016-2018	Gauteng	Cabbage	Whole	Informal street vendors	75	50	0,00-8,02	0,00-2,80	3,70-7,61	1	0	0	

2016-2018	Gauteng	Carrots	Whole	Informal street vendors	75	150	0,00-7,78	0,00-5,3	0,00-6,74	1	0	0	
2016-2018	Gauteng	Spinach	Bunch	Informal street vendors	75	50	0,00-9,06	0,00-5,24	0,00-9,20	1	1	0	
2016-2018	Gauteng	Tomato	Whole	Informal street vendors	75	150	0,00-8,51	0,00-7,34	0,00-6.78	1	0	0	
2017-2018	Gauteng	Lettuce	Whole	At harvest	20	50	3,61-4,61	0,00-2,68	0,00-4,56	1	0	0	
2017-2018	Gauteng	Lettuce	Whole	Retailers	16	50	2,55-4,00	0,00-2,70	2,80-4,07	1	0	0	
2017-2018	North-West Province	Lettuce	Whole	At harvest	20	50	3,81-4,65	0,00-1,66	3,12-4,29	1	0	0	
2017-2018	North-West Province	Lettuce	Whole	Retailers	16	50	3,28-4,63	0,00-0,00	2,89-3,95	1	0	0	(Ratshilingano et al., 2021)
2017-2018	Gauteng	Spinach	Bunch	At harvest	20	50	3,24-4,58	0,00-3,16	2,89-4,07	1	0	0	
2017-2018	Gauteng	Spinach	Bunch	Retailers	16	50	4,51-4,64	0,00-2,74	3,46-4,52	1	0	0	
2017-2018	North-West Province	Spinach	Bunch	At harvest	20	50	3,56-4,71	0,00-2,36	2,90-4,29	1	0	0	
2017-2018	North-West Province	Spinach	Bunch	Retailers	16	50	3,54-4,23	0,00-2,36	2,43-4,27	1	0	0	
2017-2018	Cape Town	Lettuce	Whole	Informal street vendors	75	25	6,85	1,60	7	1	0	0	
2017-2018	Cape Town	Cabbage	Whole	Informal street vendors	20	25	4,89	-	6,3	0	0	0	
2017-2018	Cape Town	Spinach	Whole	Informal street vendors	20	25	2,43	3,60	5,8	0	0	0	
2017-2018	Cape Town	Green pepper	Whole	Informal street vendors	10	25	3,04	-	3,9	0	0	0	(Laubscher, 2019)
2017-2018	Cape Town	Tomato	Whole	Informal street vendors	15	25	-	-	3,7	0	0	0	
2017-2018	Cape Town	Green beans	Whole	Informal street vendors	10	25		-	5,6	0	0	0	
2017-2018	Gauteng	Spinach	Bunch	At harvest	30	50	0,00-,01	0,00-3,7	3,28-7,07	1	0	0	
2017-2018	Gauteng	Spinach	Washed, RTE, bagged	Retailers	30	50	3,56-6,33	0,00-1,7	3,56-6,52	1	0	0	(Richter et al., 2022)
2017-2018	Gauteng	Spinach	Unwashed bunch/ bagged	Retailers	30	50	3,69-6,85	0,00-2,00	3,92-6,78	1	0	0	
Not specified	KwaZulu-Natal	Carrot	Whole	Market (Agri-hub)	Not specified	25		1,5	-	0	0	0	
Not specified	KwaZulu-Natal	Lettuce	Whole	Market (Agri-hub)	Not specified	25		1,69	-	0	0	0	(Beharielal et al., 2018)
Not specified	KwaZulu-Natal	Spinach	Whole	Market (Agri-hub)	Not specified	25		1,5	-	0	0	0	
Not specified	KwaZulu-Natal	Parsley	Whole	Market (Agri-hub)	Not specified	25		-	-	0	0	0	

* Where a range was not reported, mean counts are given.

Table 1.2: Enumeration of microbial indicators and prevalence of foodborne pathogens in water used to irrigate fresh produce in South Africa

Province	Produce	Water Source	Microbiological quality analysis (Min-Max Range, log MPN/100 ml) *			Detection of foodborne pathogens (1=detected; 0=not detected)			Reference
			Coliform counts	*E. coli* counts	Enterobacteriaceae counts	*E. coli*	*Salmonella* spp.	*Listeria* spp.	
Gauteng	Spinach	River water	3,38-4,76	2,20-2,64	2,84-3,20	1	1	0	(Richter et al., 2022)
Gauteng	Spinach	Borehole water	0,00-5,44	0,00- 0,61	0,00-2,49	1	0	0	(Richter et al., 2022)
Gauteng	Lettuce	River water	3,43-4,09	0,00-3,53	3.46-3,64	1	1	1	(Ratshilingano et al., 2021)
North-West	Lettuce	Dam	2,72-4,16	0,00-2,93	3,37-4,04	1	0	0	(Ratshilingano et al., 2021)
Gauteng	Spinach	River water	3,67-4,29	0,00-3,53	3,06-3,60	1	1	1	(Ratshilingano et al., 2021)
North-West	Spinach	Dam	4,02-4,27	0,00-2,93	2,53-3,62	1	0	0	(Ratshilingano et al., 2021)
North-West	African leafy greens	Borehole water	0,00-4,81	0,00-3,03	0,00-4,4	1	1	1	(du Plessis et al., 2021)
KwaZulu-Natal	Carrots and leafy greens	River water	3,4	2,36	-	1	0	0	(Beharielal et al., 2018)

Globally, limited quantitative data is available and a lack of understanding regarding the behaviour and persistence of microbial hazards introduced via irrigation water, and the interaction of water with different fresh produce products in varied environments at different steps along the supply chain remains (FAO and WHO, 2019). Only once sufficient data is generated, risk assessments for commodity specific microbiological hazards within fresh produce supply chains can be initiated. This scoping study will therefore contribute to the existing knowledge base regarding the presence of *V. cholerae* in the environment (irrigation water sources) and on fresh produce producing small-scale farms. However, this study evaluated a limited number of farms selected given the short time frame allocated to the research project.

CHAPTER 2: EXPERIMENTAL PROCEDURES

2.1 SAMPLING SITES

Four farms were selected (three in the Hammanskraal area and another in the North-West Province where the microbiological quality of the irrigation water was determined to be severely compromised. Additionally, potential human pathogenic (including *Salmonella* spp.) and multidrug resistant bacteria were also isolated. Water was also sampled from the Kaalplaasspruit (downstream of Soshanguve and upstream of Farm H) and from the Apies River (downstream of the Daspoort Wastewater Works). The sampling site locations can be viewed in the map below (Figure 2.1), while additional details of the sites were summarised in Table 2.1 below.

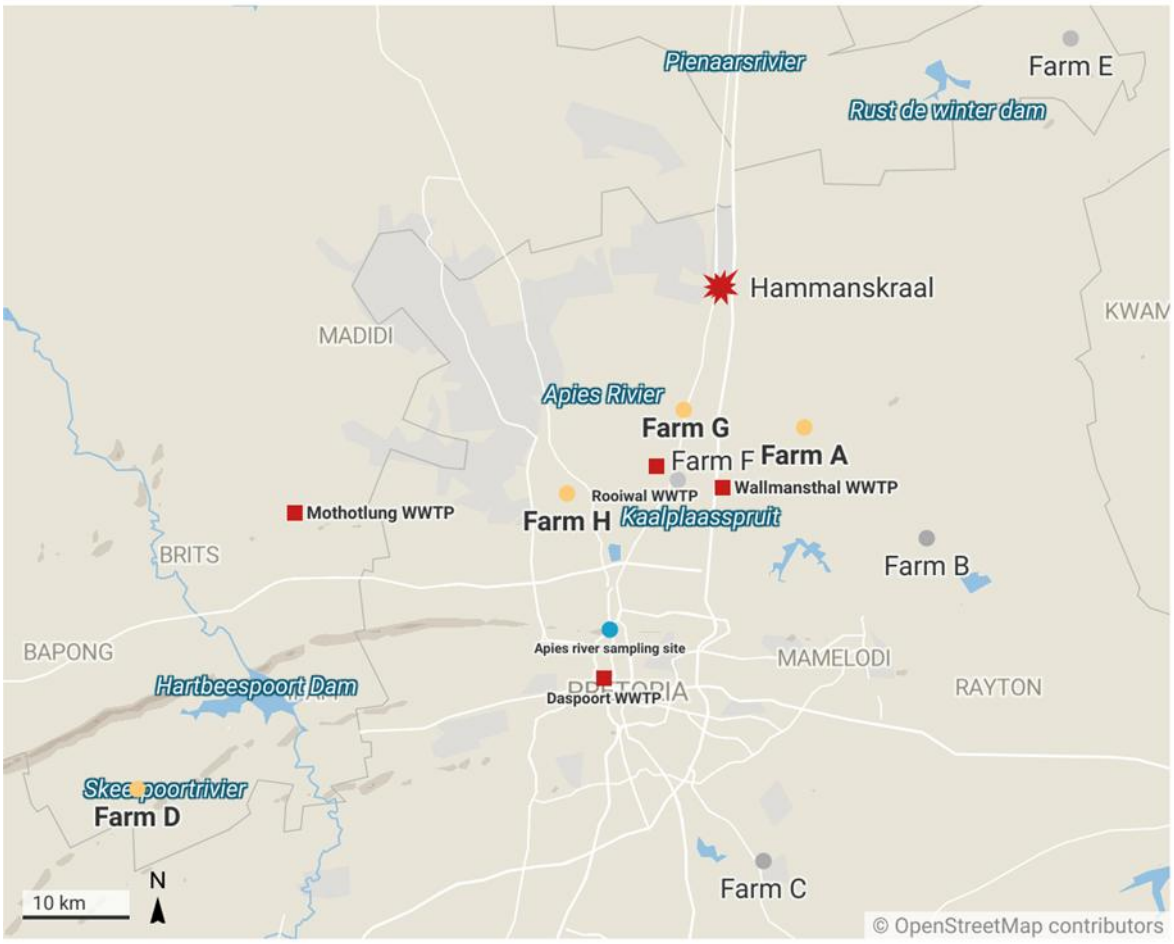

Figure 2.1: Map indicating the sampling sites including Farm A, Farm D, Farm G and Farm H (in bold with yellow dots) where water, fresh produce and sediment samples were taken for analysis and detection of *Vibrio* spp. The Apies River sampling site is indicated with a blue dot.

Table 2.1: Summary of the sampling sites selected, water sources used, irrigation method, fresh produce sampled, additional on-farm observations and information.

Sapling sites		Water source/s	Irrigation method	Fresh produce sampled	Additional observations and information
Farm A	Gauteng	Borehole water Aquaculture system used for nutrient enrichment before irrigation	Overhead irrigation (hosepipe)	Mustard (leafy greens)	Aquaculture farming (Tilapia) Vegetables – hydroponic production as well as in soil during 2022 No hydroponically grown vegetables on sampling day in June 2023
Farm D	North-West	River – canal system	Overhead irrigation	Celery and rocket	Supplied retailers and exported selected produce GLOBAL'GAP certified
Farm G	Gauteng	Dam (earth without a lining)	Flood irrigation	Mustard (leafy green)	The source water from earth dam pumped into a lined 2 dam. Supply informal traders Tshwane City Centre, Ga Rankuwa and Marabastad,
Farm H	Gauteng	Spruit/small tributary	Overhead sprinklers	Peas	Routinely uses Kaalplaasspruit water for irrigation, but sometimes supplements with a hole dug in the ground. Soil amendment: Composted chicken manure and sawdust cow manure as well as 2:3:4 fertiliser Tshwane Market
Kaalplaasspruit downstream of Soshanguve and upstream of Farm H					Burst sewage pipe upstream flowing into the Kaalplaasspruit
Apies River site downstream of Daspoort Wastewater Works					

2.2 SAMPLE COLLECTION

Water: Water samples (1 L and 100 L) were collected from each farm at each point in the irrigation system, i.e. the water source and/or holding dam and irrigation point in the field. The 1 L water samples were collected in ethanol-sterilized, air-dried plastic bottles at each respective site. The 100 L water samples were collected by filtering water at each water source site through a kidney dialysis filter (FDA, 2021). All samples were stored and transported in a cooler box at 4 ºC until analysis within 24 hours.

Fresh produce: Based on availability, at least one to two different types of fresh produce samples were collected at harvest at each respective farm. Of each fresh produce type, three replicates, each consisting of at least three leaves from the leafy greens or a composite of at least three tomatoes, or any other produce

type other than leafy green vegetables that are available, e.g. green pepper, onion, carrots, radishes, etc. were collected in paper bags in an unbiased random manner across the fields using a 70 % ethanol sterilized knife. All samples were transported to the University of Pretoria Food safety laboratories in cooler boxes as described for the water samples.

Sediment: Where possible for the respective water sources, grab sediment samples were scooped from the dam/storage tank/riverbed directly below the point at which water samples were collected and transferred into sterile plastic cups with lids. All samples were transported to the laboratory at 4°C in a cooler box as described for the water samples.

Summary of the water sampling points, sediments and fresh produce samples

A total of 170 samples have been collected from four small-scale farms including Farm A, Farm D, Farm G and H as well as the Kaalplaasspruit and Apies River water samples. This included 90 water samples, 36 fresh produce samples, and 42 sediment samples.

From Farm A, a total of 39 samples (water, n=21; fresh produce, n=12 and sediment, n=6) were collected (Figure 2.2).

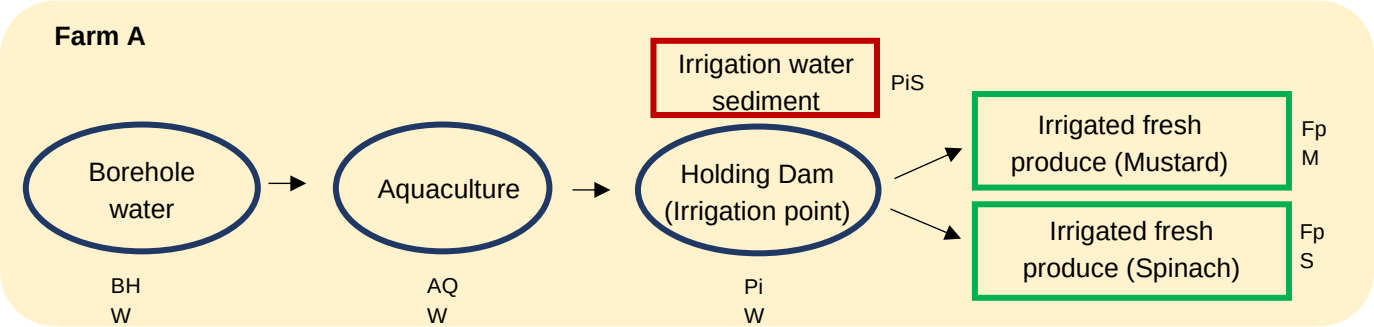

Figure 2.2: Sampling points for water, fresh produce and sediment samples from Farm A.

From Farm D a total of 76 samples (water, n=40; fresh produce, n=12 and sediment, n=24) were collected (Figure 2.3).

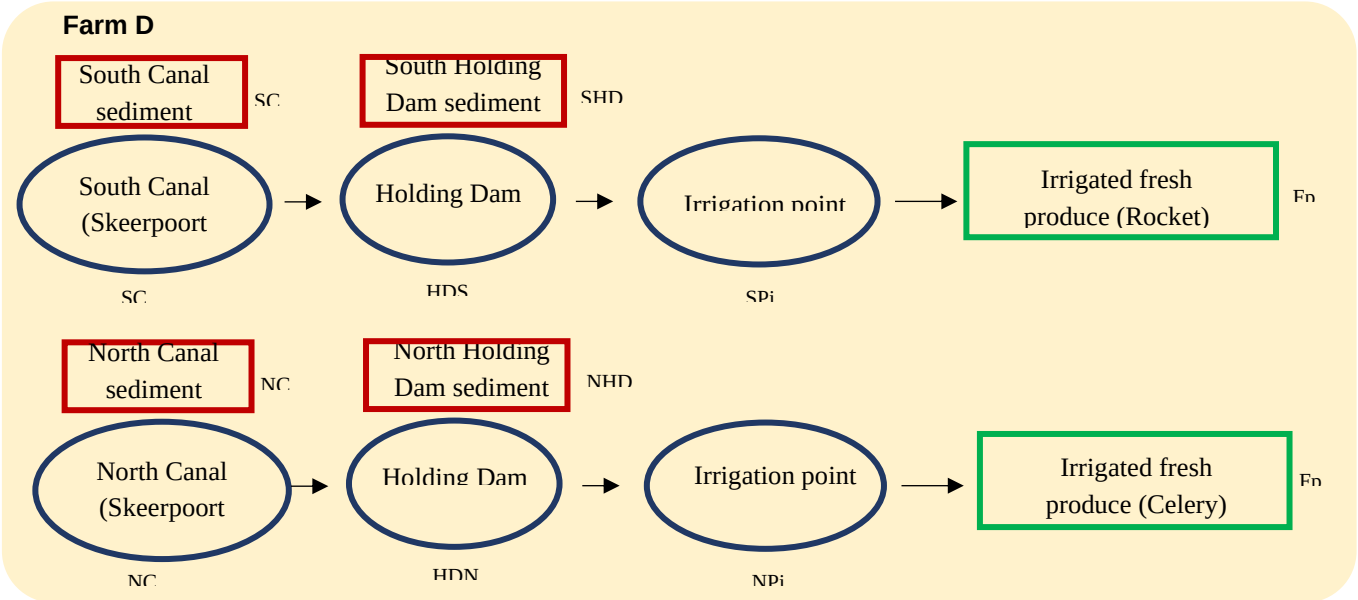

Figure 2.3: Sampling points for water, fresh produce and sediment samples from Farm D.

From Farm G, a total of 25 samples (water, n=13; fresh produce, n=6 and sediment, n=6) were collected (Figure 2.4).

Risk Assessment Findings

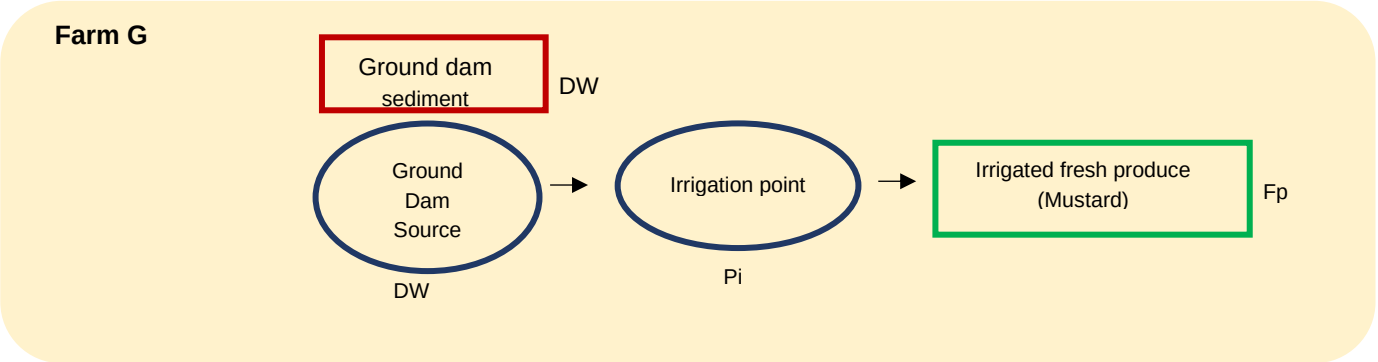

Figure 2.4: Sampling points for water, fresh produce and sediment samples from Farm G.

From Farm H, a total 28 samples (water, n=16; fresh produce, n=6 and sediment, n=6) were collected (Figure 2.5). The Kaalplaasspruit sampling point of water used on Farm H for irrigation of peas is indicated in Figure 2.6.

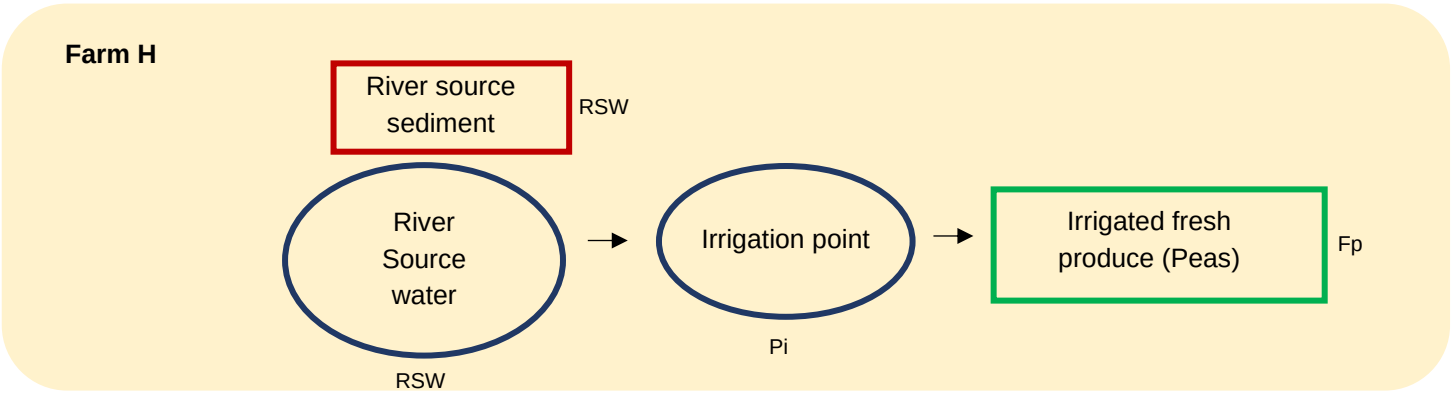

Figure 2.5: Sampling points for water, fresh produce and sediment samples from Farm H.

Figure 2.6: The Kaalplaasspruit sampling point of water used on Farm H for irrigation of peas indicated with a red dot.

River and spruit water samples:
Additionally, samples were also collected from the Apies River (n=1) site downstream of Daspoort wastewater works as well as from the Kaalplaasspruit (n=3) upstream of Farm H (Figure 2.7).

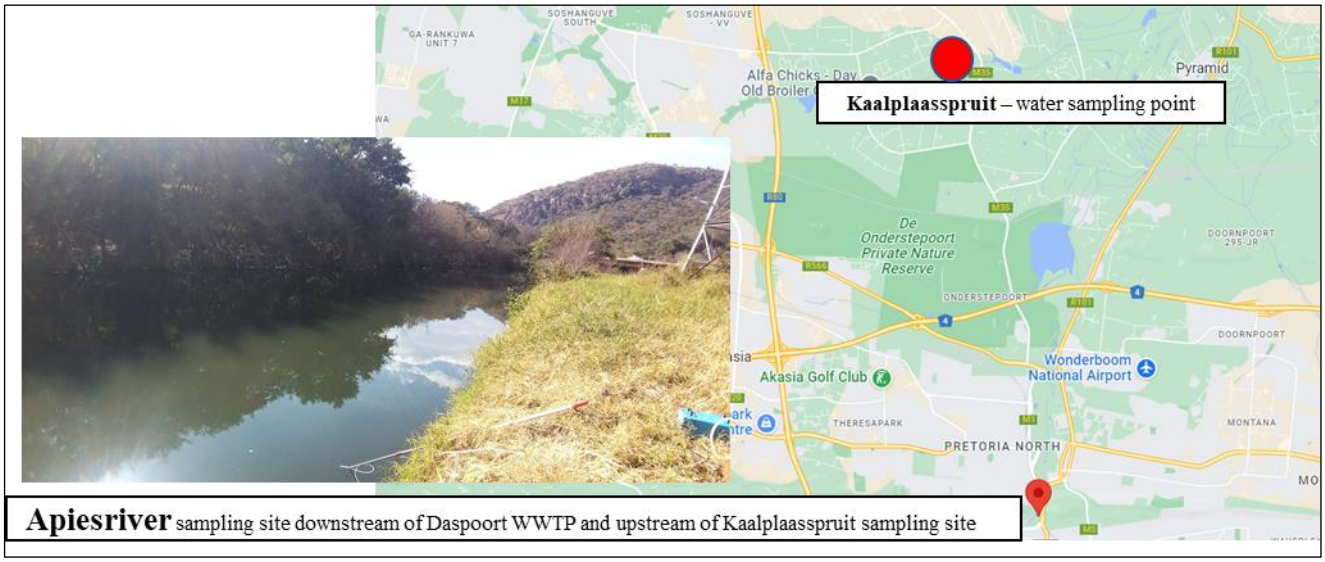

Figure 2.7: Apies River (n=1) site downstream of Daspoort wastewater works and Kaalplaasspruit water sampling point.

2.3 SAMPLE PROCESSING

Water:
For the 1 L water samples, the samples were filtered through nitrocellulose membranes (Sartorius, Gottingen, Germany). The membrane will subsequently be placed into 100 mL Alkaline Saline Peptone Water (ASPW) (ISO) (Thermofisher Scientific) and incubated at 35 ±2°C for 6 to 8 hr. ASPW provides suitable enrichment for

incubation periods of 6 to 8 h, but other competing microflora may overgrow *V. cholerae* during longer enrichment periods for certain types of samples FDA BAM). The kidney dialysis filter membranes, used to filter the 100 L water samples, were back flushed with 2.5 L Tween-80 dH20 and the subsequent back-flushed liquid were filtered through nitrocellulose membranes like the 1 L water samples.

Fresh Produce:
A 25 g composite sample of each respective fresh produce product type were aseptically cut and subsequently placed into a pre-labelled sterile polyethylene strainer stomacher bag containing 225 mL of ASPW, in a 1:9 weight to volume ratio. Each sample were homogenized using a Stomacher® 400 Circulator paddle blender (Seward Ltd.) All samples were incubated, without shaking, for 8 ± 1 hr at 35 ± 1°C or 42 ± 1°C for enrichment purposes.

Sediment: The samples were transferred to 50 ml Falcon tubes and centrifuged at maximum speed in a Sigma 2-16 K centrifuge for 1 hour. After discarding the supernatant, the samples were weighed, and a 1:1 ratio of ASPW was added and processed as described for the fresh produce.

2.4 MICROBIOLOGICAL ANALYSIS

Isolation of presumptive Vibrio cholerae
Following enrichment, all samples were streaked onto Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) Agar. Briefly, a 3-mm loopful from the surface pellicle of ASPW culture were transferred to the surface of a dried TCBS plate and streaked in a manner that yielded single colonies. The TCBS agar plates were incubated overnight (18 to 24 h) at 35°±2°C. Colonies of *Vibrio spp.* with typical colony morphology were further purified for further identification and characterisation purposes. All presumptive positive *Vibrio* spp. isolates including *V. cholerae* and WI Tor type (yellow, flat, 2-3 mm); *V. parahaemolyticus* (blue-green, 3-5 mm); *V. alginolyticus* (yellow, 3-5 mm); *V. metschnikovii* (yellow, 3-4 mm); *V. fluvialis* (yellow, 2-3 mm) *V. vulnificus* (blue-green, 2-3 mm) and *V. mimicus* (blue-green, 2-3 mm) were identified using matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) (Bruker, Bremen, Germany) to species level as described by Standing et al. (2013) and AOAC-OMA#2017.09. All presumptive positive pure cultures were also preserved in 65% glycerol and stored as part of the University of Pretoria food safety culture collection.

Molecular analysis of presumptive Vibrio isolates

Of each of the enriched water, fresh produce, and sediment samples (n=168), 1 ml was added to 10 ml ASPW for a second enrichment and incubated for 18 hours at 42°C (ISO/TS 21872-1:2007). Subsequently, the DNA of all samples (n=168) was extracted using the boiling method as described by Queipo-Ortuño et al. (2008) with slight modifications. Briefly, 1 ml aliquots of each sample were centrifuged for 20 min at 10 000 g. The supernatant was discarded, and the pellet was resuspended in 400 µl double distilled water (ddH_2O) and centrifuged for 15 min at 10 000 g. After discarding the supernatant, the pellet was resuspended in 100 µl ddH_2O. All samples were subsequently boiled for 10 min at 100°C, cooled on ice, and centrifuged for 30s at 10 000 g. The DNA concentrations of the supernatants were determined using the Qubit dsDNA Broad Range Assay and a Qubit 2.0 fluorometer (Life Technologies). PCR was performed using 1× DreamTaq Green PCR Master Mix (ThermoFisher Scientific), with specific primers, and thermocycling conditions for each of the genes as described in Table 2.1.

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Table 2.2: List of primers used for species specific detection of Vibrio as well as detection of associated toxigenic genes

Species /Toxigenic gene of interest	Sequence	Size bp	Thermocycling conditions	References
V. cholerae	F: 3'-CAC CAA GAA GGT GAC TTT ATT GTG-5'			
	R: 3'-AGG ATA CGG CAC TTG AGT AAG ACTC-5'	304		Abioye et al., 2021
V. parahaemolyticus	F: 3'-GCA GCT GAT CAA AAC GTT GAG T-5'			
	R: 3'-ATT ATC GAT CGT GCC ACT CAC-5'	897		Abioye et al., 2021
V. vulnificus	F: 3'-GTC TTA AAG CGG TTG CTG C-5'			
	R: 3'-CGC TTC AAG TGC TGG TAG AAG-5'	410	93°C, 15min; 35 cycles of 92°C, 40 s, 57°C, 1min, 72°C, 1.5min; 75°C, 7min	Abioye et al., 2021
V. fluvialis	F: 3'-GAC CAG GGC TTT GAG GTG GAC GAC-5'			
	R: 3'GGT TTG TCG AAT TAG CTT CAC C-5'	217		Abioye et al., 2021
V. mimicus	F: 3'-GGTAGCCATCAGTCTTATCACG-5'			
	R: 3'-ATCGTGTCCCAATACTTCACCG-5'	390		Abioye et al., 2021
V. alginolyticus	F: 3'-GAGAACCCGACAGAAGCGAAG-5'			
	R: 3'-CCTAGTGCGGTGATCAGTGTTG-5'	337		Abioye et al., 2021
toxR	F: 3'-CCTTCGATCCCCTAAGCAATAC-5'	779	95°C, 50s, 40 cycles of 95°C, 10s, 55°C, 15s, 72°C, 25s; 72°C, 5min	Abia et al., 2017
	R: 3'-AGGGTTAGCAACGATGCGTAAG-5'			
ctxA	F: 3'-CGGGCAGATTCTAGACCTCCTG-5'	564	95°C, 1min, 40 cycles of 95°C, 1min, 56°C, 1min, 72°C, 1min; 72°C, 10min	Saleh et al., 2011
	R: 3'-CGATGATCTTGGAGCATTCCCAC-5'			
ompW	F: 5'- CACCAAGAAGGTGACTTTATTGTG-3'	587	94°C, 4min; 35 cycles of 94°C, 1min, 50°C, 1min, 72°C, 1min; 72°C, 5min	Ismael et al., 2021
	R: 5'-GAACTTATAACCACCCGCG-3'			
hlyA	F: 5'-GTGCGTATCAGCCTAGATGA-3'	216	95°C, 50s, 40 cycles of 95°C, 10s, 55°C, 15s, 72°C, 25s; 72°C, 5min	Abia et al., 2017
	R: 5'-CCAAGCTCAAAACCTGAAA-3'			

2.5 RESULTS AND DISCUSSION

Farm A:

After enrichment and streaking onto TCBS selective media, a total of 41 presumptive positive isolates of *Vibrio* spp. were obtained. Overall, presumptive positive yellow colonies were obtained from the aquaculture and irrigation point water samples as well as sediment samples from the irrigation water holding dam and no isolates were obtained from any of the fresh produce samples (Figure 2.8).

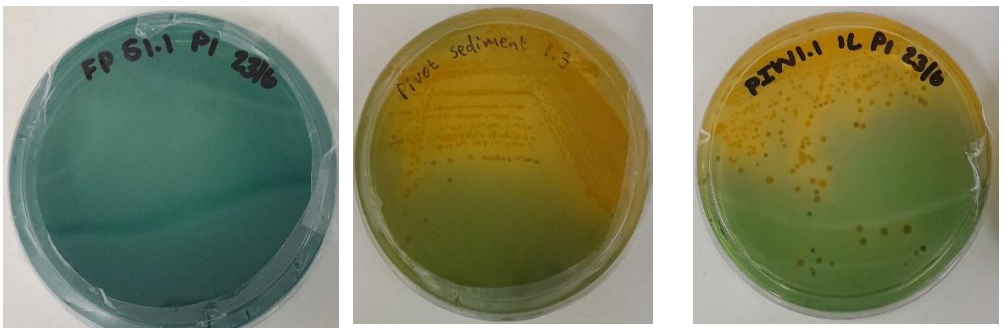

Figure 2.8: Examples of plates where enriched water, sediment and fresh produce samples were streaked out on TCBS for detection of *Vibrio* spp. A: no growth observed; B: Yellow colonies observed which were morphologically presumptive positives of *Vibrio* spp. including *V. cholerae*; C: Yellow and blue-green colonies observed which were morphologically presumptive positive for *Vibrio* spp. including *V. cholerae* and *V. parahaemolyticus*.

Subsequently, all purified isolates were identified using MALDI-TOF analysis (Table 2.3). MALDI-TOF analysis showed that the presumptive *V. spp.* isolated from the water samples (aquaculture and holding dam) and the single isolate from spinach belonged to the *Aeromonas* spp. These results concurred with the observation of Tagliavia et al. (2019) that using thiosulfate-citrate-bile salts sucrose as selective medium for isolation of *Vibrio* spp. may be hampered by the variable adaptability of different taxa to the medium. Janda et al. (2010) reported that the main reservoir of Aeromonas is the aquatic environment (fresh and brackish water). Moreover, Aeromonas bacteria were also found in food products, vegetables, and animal faeces and in the microbiota of the digestive tract (Janda and Abbott, 2010). More recently Canellas et al. (2023) reported that bacteria can be found in a wide range of habitats, are able to thrive under adverse conditions, including in highly polluted waters. Moreover, the research focus on the Aeromonas genus has increased due to it being pathogenic to aquatic organisms and humans (Canellas et al., 2023). *Aeromonas* spp. most frequently implicated in human infections include *A. caviae*, *A. dhakensis*, *A. veronii*, and *A. hydrophila*. Sadique et al. (2021) reported that frequently causes outbreaks of diarrhoea in coastal Bangladesh. The borehole water samples all belonged to the Enterobacterales *family (Enterobacter asburiae*, *E. cloacae*, *Enterococcus faecalis* and *Exiguobacterium* spp.). From the 100L sample (larger volume used to increase the likelihood of finding low levels of potential human pathogenic bacteria (*Vibrio* spp.) collected from the Irrigation water holding dam were identified as *Pleisomonas shigelloides*.

Table 2.3: Bacterial identities of isolates from water, fresh produce and sediment sampled on Farm A

Sample point	MALDI Code	Sample Code	Isolate identity
Aquaculture water	A10	AQW 1.1	*Aeromonas caviae*
	A4	AQW 1.2	*Aeromonas hydrophila*
	A5	AQW 1.2	*Aeromonas veronii*
	A11	AQW 1.3	*Aeromonas veronii*
	A20	AQW 100L	*Aeromonas veronii*
	A21	AQW 100L	*Aeromonas hydrophila*
	A12	AQW 2.1	*Aeromonas veronii*
	A2	AQW 2.2	*Aeromonas jandaei*
	A3	AQW 2.2	*Aeromonas veronii*
	A1	AQW 2.3	*Aeromonas veronii*
	A40	AQW 2.3	*Aeromonas caviae*
Borehole water source	A36	BHW 1.1	*Enterobacter asburiae*
	A38	BHW 1.1	*Enterococcus faecalis*
	A41	BHW 1.1	*Enterobacter cloacae*
	A13	BHW 1.2	*Exiguobacterium spp*
	A37	BHW 1.3	*Enterobacter cloacae*
	A39	BHW 1.3	*Enterobacter cloacae*
Fresh produce	A7	FpS 1.2	*Aeromonas veronii*
Irrigation holding dam sediment	A6	PiSed 1.1	*Aeromonas caviae*
	A14	PiSed 1.2	*Aeromonas jandaei*
	A15	PiSed 1.2	*Aeromonas veronii*
	A16	PiSed 1.3	*Aeromonas caviae*
	A17	PiSed 1.3	*Aeromonas caviae*
	A9	PiSed 2.1	*Aeromonas veronii*
	A8	PiSed 2.2	*Aeromonas caviae*
	A18	PiSed 2.3	*Aeromonas caviae*
	A19	PiSed 2.3	*Enterobater cloacae*
	A24	PiW 1.1	*Aeromonas jandaei*
	A25	PiW 1.1	*Aeromonas veronii*
	A28	PiW 1.2	*Aeromonas caviae*
Irrigation Holding Dam	A29	PiW 1.2	*Aeromonas jandaei*
	A26	PiW 1.3	*Aeromonas veronii*
	A27	PiW 1.3	*Aeromonas veronii*
	A22	PiW 100L	*Aeromonas veronii*
	A23	PiW 100L	*Plesiomonas shigelloides*
	A34	PiW 2.1	*Aeromon caviae*
	A33	PiW 2.2	*Aeromonas caviae*
	A30	PiW 2.3	*Aeromonas veronii*
	A31	PiW 2.3	*Aeromonas veronii*
	A32	PiW 2.32	*Aeromonas caviae*
	A35	PiW2.2	*Aeromonas hydrophila*

Farm D results:

After enrichment and streaking on TCBS agar, a total of 179 presumptive positive Vibrio spp. isolates were obtained (Figure 2.9).This included 66 colonies (of which 35 were yellow colonies) from 34 of the water samples, 12 colonies (of which 6 were yellow colonies) from 6 of the fresh produce (all rocket) samples, and 15 colonies (of which 6 were yellow colonies) from 5 of the sediment samples. Subsequently, all purified presumptive isolates were identified using MALDI-TOF analysis (Table 2.4). Like Farm A, most of the presumptive positive *V. cholerae* isolates from the irrigation water and the sediment were confirmed to be

Aeromonas spp. *Enterobacter cloacae* was isolated from both celery and rocket, while *Enterococcus faecalis* was isolated from rocket only. *E. faecalis* is regarded as an indicator of faecal contamination.

Figure 2.9: Examples of presumptive positive *Vibrio* spp. including *V. cholerae* (yellow) and other *Vibrio* spp. including *V. parahaemolyticus* (blue-green) isolated from the enriched water, fresh produce and sediment samples from Farm D.

Table 2.4: Bacterial identities of isolates from water, fresh produce and sediment sampled on Farm D

Sample point	MALDI Code	Sample Code	Isolate identity
North Canal source water	D81	NCW 1.1	*Aeromonas enteropelogenes*
	D82	NCW 1.2	*Aeromonas veronii*
	D83	NCW 1.2	*Aeromonas enteropelogenes*
	D84	NCW 1.3	*Aeromonas hydrophila*
	D85	NCW 1.3	*Aeromonas enteropelogenes*
	D86	NCW 2.1	*Aeromonas enteropelogenes*
	D87	NCW 2.1	*Aeromonas enteropelogenes*
	D88	NCW 2.2	*Aeromonas enteropelogenes*
	D89	NCW 2.2	*Aeromonas enteropelogenes*
	D90	NCW 2.3	*Aeromonas enteropelogenes*
	D91	NCW 2.3	*Aeromonas veronii*
	D92	NCW 100L	*Aeromonas veronii*
	D93	NCW 100L	*Aeromonas veronii*
North Holding Dam	D94	HDNW 2.2	*Aeromonas veronii*
	D107	HDNW 1.1	*Aeromonas hydrophila*
	D108	HDNW 1.1	*Aeromonas ichthiosmia*
	D109	HDNW 1.2	*Aeromonas veronii*
	D110	HDNW 1.2	*Aeromonas hydrophila*
	D111	HDNW 1.3	*Aeromonas veronii*
	D112	HDNW 1.3	*Aeromonas enteropelogenes*
	D113	HDNW 2.1	*Aeromonas veronii*
	D114	HDNW 2.1	*Aeromonas enteropelogenes*
	D115	HDNW 2.1	*Proteus vulgaris*
	D116	HDNW 2.3	*Aeromonas enteropelogenes*
	D137	HDNW 100L	*Aeromonas veronii*
	D138	HDNW 100L	*Aeromonas jandaei*
North Irrigation pivot water	D96	NPIW 1.3	*Aeromonas veronii*
	D97	NPIW 1.1	*Aeromonas veronii*
	D98	NPIW 1.1	*Aeromonas enteropelogenes*
	D99	NPIW 1.2	*Aeromonas media*
	D100	NPIW 1.2	*Aeromonas enteropelogenes*
	D101	NPIW 2.1	*Aeromonas enteropelogenes*
	D102	NPIW 2.1	*Aeromonas jandaei*
	D103	NPIW 2.2	*Aeromonas enteropelogenes*
	D104	NPIW 2.2	*Aeromonas enteropelogenes*
	D105	NPIW 2.3	*Enterobacter cloacae*
	D106	NPIW 2.3	*Aeromonas enteropelogenes*
North Canal Sediment	D117	NCS 1.1 Sed	*Aeromonas veronii*
	D118	NCS 1.1 Sed	*Aeromonas hydrophila*
	D119	NCS 1.2 Sed	*Aeromonas hydrophila*
	D120	NCS 1.2 Sed	*Aeromonas caviae*
	D121	NCS 1.3 Sed	*Aeromonas veronii*
	D122	NCS 1.3 Sed	*Aeromas veronii*
	D123	NCS 2.1 Sed	*Aeromonas ichthiosmia*
	D124	NCS 2.1 Sed	*Plesiomonas shigelloides*
	D125	NCS 2.2 Sed	*Aeromonas veronii*
	D126	NCS 2.2 Sed	*Plesiomonas shigelloides*
	D95	NCS 2.3 Sed	*Aeromonas enteropelogenes*

Sample point	MALDI Code	Sample Code	Isolate identity
Holding Dam Sediment	D131	HDNW 2.3 Sed	*Exiguobacterium artemiae*
	D132	HDNW 2.3 Sed	*Citrobacter braakii*
	D133	HDNW 2.2 Sed	*Aeromonas hydrophila*
	D134	HDNW 2.2 Sed	*Aeromonas caviae*
	D135	HDNW 1.1 Sed	*Aeromonas veronii*
	D136	HDNW 1.1 Sed	*Aeromonas caviae*
	D139	HDNW 1.2 Sed	*Aeromonas media*
	D140	HDNW 1.2 Sed	*Aeromonas enteropelogenes*
	D141	HDNW 1.3 Sed	*Aeromonas veronii*
	D142	HDNW 1.3 Sed	*Aeromonas veronii*
	D143	HDNW 2.1 Sed	*Aeromonas caviae*
	D144	HDNW 2.1 Sed	*Aeromonas veronii*
South Canal Source water	D1	SCW 1.1	*Aeromonas enteropelogenes*
	D2	SCW 1.1	*Aeromonas veronii*
	D3	SCW 1.2	*Aeromonas veronii*
	D4	SCW 1.2	*Aeromonas hydrophila*
	D5	SCW 1.3	*Aeromonas enteropelogenes*
	D6	SCW 1.3	*Aeromonas enteropelogenes*
	D7	SCw 2.1	*Aeromonas enteropelogenes*
	D8	SCW 2.1	*Aeromonas veronii*
	D9	SCW 2.2	*Aeromonas veronii*
	D10	SCW 2.2	*Proteus vulgaris*
	D11	SCW 2.1	*Aeromonas enteropelogenes*
	D12	SCW 2.3	*Aeromonas enteropelogenes*
	D13	SCW 2.3	*Aeromonas veronii*
	D14	SCW 100L	*Aeromonas veronii*
	D15	SCW 100L	*Aeromonas hydrophila*
South Holding Dam	D39	HDSW 100L	*Aeromonas veronii*
	D40	HDSW 100L	*Aeromonas hydrophila*
	D41	HDSW 2.2	*Aeromonas veronii*
	D42	HDSW 2.2	*Acinetobacter haemolyticus*
	D43	HDSW 2.1	*Aeromonas veronii*
	D44	HDSW 2.1	*Aeromonas ichthiosmia*
	D45	HDSW 1.2	*Acinetobacter haemolyticus*
	D46	HDSW 2.1	*Aeromonas veronii*
	D47	HDSW 1.2	*Aeromonas veronii*
	D48	HDSW 1.2	*Aeromonas veronii*
	D49	HDSW 1.1	*Aeromonas caviae*
	D50	HDSW 1.1	*Citrobacter freundii*
	D51	HSW 1.3	*Aeromonas caviae*
	D52	HDSW 2.2	*Aeromonas veronii*
	D28	HDSW 2.3	*Aeromonas veronii*
	D29	HDSW 2.3	*Aeromonas veronii*
	D30	HDSW 2.3	*Aeromonas enteropelogenes*
South Irrigation Pivot Water	D16	SPIW 2.1	*Aeromonas enteropelogenes*
	D17	SPIW 2.1	*Aeromonas enteropelogenes*
	D18	SPIW 2.3	*Aeromonas veronii*
	D19	SPIW 2.3	*Aeromonas veronii*
	D20	SPIW 2.2	*Aeromonas veronii*
	D21	SPIW 1.3	*Aeromonas hydrophila*
	D22	SPIW 1.3	*Aeromonas enteropelogenes*

	D23	SPIW 1.3	*Aeromonas enteropelogenes*
	D24	SPIW 1.2	*Aeromonas enteropelogenes*
	D25	SPIW 1.2	*Aeromonas veronii*
	D26	SPIW 1.1	*Aeromonas enteropelogenes*
	D27	SPIW 1.1	*Aeromonas enteropelogenes*
South Canal Sediment	D65	SCS 1.1	*Aeromonas veronii*
	D66	SCS 1.1	*Aeromonas enteropelogenes*
	D67	SCS 1.2	*Citrobacter freundii*
	D68	SCS 1.2	*Aeromonas caviae*
	D69	SCS 1.2	*Aeromonas enteropelogenes*
	D70	SCS 1.3	*Citrobacter freundii*
	D71	SCS 1.3	*Aeromonas caviae*
	D72	SCS 1.3	*Aeromonas veronii*
	D73	SCS 2.1	*Citrobacter freundii*
	D74	SCS 2.1	*Aeromonas enteropelogenes*
	D75	SCS 2.1	*Aeromonas enteropelogenes*
	D76	SCS 2.2	*Aeromonas enteropelogenes*
	D77	SCS 2.2	*Aeromonas enteropelogenes*
	D78	SCS 2.3	*Aeromonas enteropelogenes*
	D79	SCS 2.3	*Aeromonas enteropelogenes*
South Holding Dam Sediment	D31	HDSW Sed 2.1	*Aeromonas veronii*
	D32	HDSW Sed 2.1	*Aeromonas enteropelogenes*
	D33	HDSW Sed 1.3	*Aeromonas veronii*
	D34	HDSW Sed 1.3	*Aeromonas enteropelogenes*
	D35	HDSW Sed 1.2	*Aeromonas veronii*
	D36	HDSW Sed 1.2	*Aeromonas enteropelogenes*
	D37	HDSW Sed 1.1	*Aeromonas ichthiosmia*
	D38	HDSW Sed 1.1	*Aeromonas hydrophila*
Fresh Produce (Celery)- North Field	D145	N FpC 1.1	*Exiguobacterium sp*
	D146	N FpC 1.1	*Exiguobacterium artemiae*
	D147	N FpC 1.2	*Exiguobacterium artemiae*
	D148	N FpC 1.2	*Exiguobacterium artemiae*
	D127	FpC 2.2	*Enterobacter cloacae*
	D128	FpC 2.2	*Enterobacter cloacae*
	D129	FpC 2.3	*Exiguobacterium sp*
	D130	FpC 2.3	*Exiguobacterium artemiae*
Fresh Produce (Rocket) - South Field	D53	SFpR 1.1	*Bacillus pumilus*
	D54	SFpR 1.2	*Morganella morganii*
	D55	SFpR 1.2	*Enterobacter bugandensis*
	D56	SFpR 1.2	*Pseudomonas flavescens*
	D57	SFpR 1.3	*Enterococcus faecalis*
	D58	SFpR 1.3	*Exiguobacterium artemiae*
	D59	SFpR 1.3	*Exiguobacterium artemiae*
	D60	SFpR 2.1	*Staphylococcus xylosus*
	D61	SFpR 2.1	*Staphylococcus xylosus*
	D62	SFpR 2.1	*Exiguobacterium artemiae*
	D63	SFpR 2.2	*Enterobacter cloacae*
	D64	SFpR 2.3	*Exiguobacterium artemiae*

Farm G results:

Following enrichment, blue-green colonies were observed from the enriched irrigation point water, dam water, and dam water sediment and fresh produce (mustard leafy green) samples. All presumptive positive isolate identities (n=34) were confirmed using MALDI-TOF analysis (Table 2.5). Similar to Farm A and D, most of the presumptive positive *V. cholerae* isolates from the irrigation water and the sediment were confirmed belong to *Aeromonas* spp. *Enterococcus faecalis* was isolated from the Chinese spinach (chinensis) as was from rocket on Farm D.

Table 2.5: Bacterial identities of isolates from water, fresh produce and sediment sampled on Farm G

Sample point	MALDI Code	Sample Code	Isolate identity
Dam Source Water	G9	DW 100L	*Aeromonas veronii*
	G10	DW 100L	*Aeromonas veronii*
	G27	DW 1.1	*Aeromonas caviae*
	G28	DW 1.2	*Aeromonas jandaei*
	G29	DW 1.3	*Aeromonas caviae*
	G30	DW 1.3	*Aeromonas veronii*
	G31	DW 2.1	*Aeromonas veronii*
	G32	DW 2.3	*Aeromonas veronii*
	G33	DW 2.2	*Aeromonas veronii*
	G34	DW 2.2	*Aeromonas veronii*
Holding Dam Water	G7	PiW 2.2 1L	*Exiguobacterium artemiae*
	G8	PiW 2.1 1L	*Aeromonas veronii*
	G11	PiW 1.1	*Aeromonas caviae*
	G12	PiW 1.1	*Aeromonas veronii*
	G13	PiW 1.2	*Aeromonas veronii*
	G14	PiW 1.2	*Aeromonas veronii*
	G15	PiW 1.3	*Aeromonas hydrophila*
	G16	PiW 1.3	*Aeromonas veronii*
	G17	PiW 100L	*Aeromonas veronii*
	G18	PiW 100L	*Aeromonas veronii*
	G19	PiW 2.3	*Aeromonas veronii*
	G20	PiW 2.3	*Exiguobacterium sp*
Dam Source water Sediment	G1	DW 1.2 Sed	*Aeromonas veronii*
	G2	DW 1.3 Sed	*Aeromonas veronii*
	G3	DW 1.1 Sed	*Aeromonas veronii*
	G4	DW 2.2 Sed	*Aeromonas veronii*
	G5	DW 2.3 Sed	*Aeromonas veronii*
	G6	DW 2.1 Sed	*Exiguobacterium sp*
Fresh Produce (Mustard)	G21	FpM 2.1	*Enterobacter asburiae*
	G22	FpM 2.1	*Exiguobacterium artemiae*
	G23	FpM 2.2	*Exiguobacterium artemiae*
	G24	FpM 2.2	*Exiguobacterium artemiae*
	G25	FpM 1.1	*Enterobacter cloacae*
	G26	FpM 1.1	*Enterobacter cloacae*

Farm H results:

A total of 43 presumptive positive *V. cholerae* were isolated and the identities were confirmed using MALDI-TOF analysis (Table 2.6). Like Farm A, D and G most of the isolates from irrigation water and the sediment samples belonged to the *Aeromonas* spp. However, from the Kaalplaasspruit one *V. cholerae* isolate was obtained. The *V. cholerae* isolate was sent to the NICD where it was confirmed that the isolate was not positive

ctxA, indicating that the isolate is a non-O1/non-O139 *V. cholerae* strain. No presumptive positive *V. cholerae* isolates were obtained from the peas following selective enrichment and streaking onto selective media.

Table 2.6: Bacterial identities of isolates from water, fresh produce and sediment sampled on Farm H

Sample point	MALDI Code	Sample Code	Isolate identity
Dug-out-dam on Farm H	H7	Dug-out-dam 100L	*Aeromonas hydrophila*
	H8	Dug-out-dam 100L	*Aeromonas veronii*
	H3	Dug-out-dam 2.3	*Providencia alcalifaciens*
	H28	Dug-out-dam 1.3	*Aeromonas hydrophila*
	H29	Dug-out-dam 1.3	*Aeromonas hydrophila*
	H30	Dug-out-dam 1.2	*Enterococcus faecalis*
	H31	Dug-out-dam 1.2	*Aeromonas hydrophila*
	H32	Dug-out-dam 1.2	*Aeromonas hydrophila*
	H33	Dug-out-dam 1.1	*Enterococcus faecalis*
	H34	Dug-out-dam 1.1	*Aeromonas hydrophila*
	H35	Dug-out-dam 1.1	*Aeromonas hydrophila*
Sediment from dug-out-dam	H36	Dug-out-dam 2.3 Sed	*Aeromonas veronii*
	H37	Dug-out-dam 2.3 Sed	*Aeromonas enteropelogenes*
	H38	Dug-out-dam 2.2 Sed	*Aeromonas veronii*
	H39	Dug-out-dam 2.2 Sed	*Aeromonas veronii*
	H40	Dug-out-dam 2.1 Sed	*Aeromonas veronii*
	H41	Dug-out-dam 2.1 Sed	*Aeromonas veronii*
	H42	Dug-out-dam 1.2 Sed	*Aeromonas hydrophila*
	H43	Dug-out-dam 1.2 Sed	*Aeromonas hydrophila*
	H4	Dug-out-dam 1.1 Sed	*Aeromonas enterpelogenes*
	H5	Dug-out-dam 1.3 Sed	*Aeromonas hydrophila*
Kaalplaasspruit irrigation point	H1	River 1.3	*Aeromonas veronii*
	H2	River 2.1	*Providencia alcalifaciens*
	H6	River 1.2	*Aeromonas enteropelogenes*
	H12	River 3 100L	*Aeromonas veronii*
	H13	River 3 100L	*Aeromonas veronii*
	H14	River 3 100L	*Aeromonas veronii*
	H18	River 2.3	*Aeromonas enteropelogenes*
	H19	River 2.3	*Aeromonas enteropelogenes*
	H20	River 2.2	*Aeromonas veronii*
	H21	River 2.2	***Vibrio cholerae***
	H22	River 2.2	*Aeromonas ichthiosmia*
	H23	River 2.1	*Escherichia coli*
	H24	River 2.1	*Aeromonas enteropelogenes*
	H25	River 2.1	*Aeromonas enteropelogenes*
	H26	River 1.1	*Aeromonas veronii*
	H27	River 1.1	*Aeromonas veronii*
Kaalplaasspruit downstream of sewage burst pipe	H9	Kaalplaasspruit 100L	*Aeromonas veronii*
	H10	Kaalplaasspruit 100L	*Aeromonas veronii*
	H11	Kaalplaasspruit 100L	*Aeromonas veronii*
Apies River	H15	Apies river 100L	*Aeromonas hydrophila*
	H16	Apies river 100L	*Aeromonas caviae*
	H17	Apies river 100L	*Aeromonas caviae*

Table 2.7: PCR analysis of key genes associated with *V. cholerae* in samples isolated from water, fresh produce and sediment.

Farm	Source	Sample code	Genes				
			ompW	***16S (V. cholerae)***	***toxR***	***hlyA***	***ctxA***
Farm A	Irrigation water	PiW1.2	+	+	-	-	-
	Irrigation water	PiW2.2	+	+	-	-	-
	Irrigation water	PiW100L	+	+	-	-	-
	Aquaculture water	AQW2.1	+	+	-	-	-
	Aquaculture water	AQW2.2	+	+	-	-	-
	Aquaculture water	AQW2.3	+	+	-	-	-
	Borehole water	BHW1.1	+	+	-	-	-
	Borehole water	BHW1.2	+	+	-	-	-
	Borehole water	BHW1.3	+	+	-	-	-
	Borehole water	BHW2.1	+	+	-	-	-
	Fresh Produce (spinach)	SFpS1.1	+	+	+	-	-
	Fresh Produce (spinach)	SFpS1.3	+	+	-	-	-
	Irrigation water sediment	PiS1.1	+	+	-	+	-
	Irrigation water sediment	PiS2.1	+	+	-	-	-
	Irrigation water sediment	PiS2.2	+	+	-	-	-
	Irrigation water sediment	PiS2.3	+	+	-	-	-
Farm D	Holding dam	SHDS1.1	+	+	-	-	-
	Fresh Produce (mustard)	SFpM1.1	+	+	-	+	-
	Fresh Produce (mustard)	SFpM1.2	+	+	-	+	-
	Fresh Produce (mustard)	SFpM2.2	+	-	-	-	-
Farm G	Dam water	DWS2.2	+	+	-	-	-
	Irrigation water	PiW2.2	+	+	+	+	-
Farm H	Dug-out-dam	DUDS2.3	+	-	+	+	-

Molecular characterisation of samples collected from the selected farms

PCR analysis for all samples for the genotypic detection of the specific target outer membrane protein (*ompW*) and the 16s rDNA gene of *V. cholerae* have been completed. Overall, 21 samples (12.5%) were PCR-positive for the *ompW* gene as well as the 16s rDNA *V. cholerae* gene. This included 5 fresh produce samples (Farm A, n=2 and Farm G, n=3), 5 sediment samples (Farm A, n=4, Farm G, n=1), and 11 water samples (Farm A, n=9 and Farm H, n=2). These samples were regarded as presumptive positive for the presence of *V. cholerae* (Takahashi et al., 2021). Subsequently, the samples were screened for the presence of the *toxR* regulatory gene and the *hlyA* hemolysin gene. Two irrigation water samples from Farm H tested positive for the *toxR* and *hlyA* genes. The non-O1 *V. chole*rae isolate from Farm H (H21) was isolated from the irrigation water sample n Farm H

Physicochemical analysis of irrigation water

The results for the physicochemical analysis of irrigation water from three of the four farms were summarised in Table 2. The pH of the water sources from Farm's A, D, G and H ranged between pH 6.2-8.4 and were all acceptable according to the DWAF, 1996 guideline of pH 6-9. The dissolved oxygen (DO) levels ranged between 7.2 and 10.6 mg/L which is also acceptable according to the guideline of >3 mg/L. DO is a measure of water quality and an indicator of a water resource's ability to sustain aquatic life. The required electrical conductivity of irrigation water should be ≤40 mS/m. The values on Farm A (aquaculture farm) were the lowest ranging between 12.2 and 18.5 mS/m (milli-Siemens per metre). Interestingly the North and South canals (fed

by the Skeerpoort River) on Farm D, although within the acceptable limit, were quite high ranging between 31.0 and 31.3 mS/m. Elevated electrical conductivity can indicate that pollution has entered the river and indicates is unsuitable for irrigation purposes. (https://wq.epa.gov.tw/EWQP/en/Encyclopedia/NounDefinition/Pedia_48.aspx#:~:text=Electrical%20conductivity%20(EC)%20is%20a,more%20electrolytes%20in%20the%20water, accessed 19-7-2023). According to the US-EPA (United States Environmental Protection Agency, 2012) guidelines for water reuse the turbidity levels of irrigation water should be <5 NTU (nephelometric turbidity units). Suspended solids give rise to turbidity in water. The relationship between the amount of suspended solids and the turbidity measurement is, however, dependent on the nature and particle size distribution of the suspended matter. On Farm A the values ranged from 0.5 NTU [borehole] to 24 NTU [irrigation pipe]; Farm D from 1.9 NTU [source water] and 6.7 [irrigation pivot point]; Farm G earth dam [irrigation source] was 87 NTU and the holding dam 63 NTU, which is totally unacceptable according to the USEPA (2012) guidelines.

Table 2.8: Physicochemical analyses of water samples from smallholder farms around the cholera outbreak area in South Africa as well as the physicochemical guidelines for irrigation water recommended by DWAF.

Reference – recommended guidelines		pH	Dissolved Oxygen (mg/L)	Electrical conductivity (mS/m)	Turbidity (NTU)
Department of Water Affairs (DWAF), 1996 and US-EPA 2012		6-9	>3	≤40	<5
Farm	**Sample**	**pH**	**Dissolved Oxygen (mg/L)**	**Electrical conductivity (mS/m)**	**Turbidity (NTU)**
A	Borehole water	6.2	9.8	12.2	0.5
	Aquaculture water	8.5	10.5	17.7	2.7
	Irrigation point water	7.3	9.6	18.5	24
D	North canal source water	8.2	8.2	31.5	1.9
	North holding dam water	8.4	8.0	31.3	4.6
	North Irrigation pivot point	8.4	7.9	31.3	6.7
	South canal source water	8.3	8.1	31.0	1.4
	South holding dam water	8.2	8.1	31.1	1.6
	South Irrigation pivot point	8.4	8.1	31.2	7.8
G	Dam source water	7.2	7.2	37.6	87
	Holding dam water	7.5	7.7	37.5	63
H	Dam source water (hole dug next to fresh produce field)	6.5	8.1	6.1	52
	Tributary/Kaalplaasspruit used for irrigation	7.8	7.8	56.4	3.1
	Upstream tributary point, downstream of Shoshanguwe sewage burst pipe	7.7	7.9	35.7	5.7

CONCLUSIONS

A non-toxigenic *V. cholerae* isolate was shown to be present in a water sample used for irrigation purposes on one of the small-scale farms chosen in the Hammanskraal area. Although it was confirmed to be *V. cholerae* non-O1, it is still a reason for concern as diarrheal disease has increasingly been reported due to these organisms (Wang et al., 2020). The negative impact of the compromised microbial and chemical water quality of water sources used for fresh produce production impacts food safety and security. It is an additional hurdle as far as adhering to regulatory requirements regarding the microbiological quality of fresh produce is concerned. If these requirements are not met market access is impacted negatively. Our reputation globally is also affected negatively as it raises the question whether we can produce safe food.

RECOMMENDATIONS

- Expand surveillance of the physico-chemical and microbiological quality of informally produced fruit and vegetables sold in the peri-urban townships and settlements in production areas where the quality of the water sources are known to have been compromised.
- Provide regular feedback to farmers and local authorities (i.e. municipalities, Environmental Health Protection Officers) regarding research findings, potential mitigation strategies and implementation thereof to address water and food safety issues
- Creating food safety awareness and training on safe practices for farmers, farm workers, food handlers and informal vendors.

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