

**INVESTIGATION INTO METHODS OF RECOVERING
CAMPYLOBACTER SPP. FROM RIVER WATER SAMPLES**

Thesis submitted in the fulfilment of the requirements of the degree.

**Master of Science (Water Resource Sciences)
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by

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ABSTRACT

Campylobacter species are slender, gram-negative, rod-shaped, spiral- or curved-shaped with single or pairs of flagella. They are the leading cause of diarrheal disease globally, consumption of and contact with water contaminated by faeces is a major risk factor for transmission of these organisms to humans. Rivers used for recreation and domestic and agricultural activities represent all the risk factors for *Campylobacter* spp. pollution and human exposure. *Campylobacter* spp. However, effective methods to recover *Campylobacter* spp. from river water samples are lacking, indicating the need for the development of more efficient methods of detection and isolation of these organisms from environmental water samples. *Campylobacter* detection in a water sample is critical to ascertain potential risks to humans. The aim of this study was to determine a suitable method for the detection of *Campylobacter* spp. from river water samples and the objectives were to (i) to evaluate the performance of different methods used for the recovery of *Campylobacter* spp. from environmental water samples based on *Campylobacter* colony count and PCR identification results, (ii) isolate and enumerate *Campylobacter* cells from river water samples, and (iii) identify *Campylobacter* spp. in river water samples.

The Bloukrans River was chosen for this study because it is suspected to be contaminated by faecal inputs from nearby informal settlements without adequate sanitation, as well as untreated/insufficiently treated effluents from nearby wastewater treatment plants. First, the physicochemical quality of the river water and the presence of faecal contamination were assessed to confirm suitability for *Campylobacter* spp. survival and presence. Then different approaches to sample, concentrate and recover *Campylobacter* spp. from river water samples were assessed. The different methods assessed were (i) direct enrichment of water samples without prior concentration, (ii) prior concentration of water samples by centrifugation followed by membrane filtration of supernatant, and after that, pooling the residue and pellet together for enrichment, (iii) sampling by the Moore Swab technique. For all three methods, enrichment in Bolton broth supplemented with Bolton antibiotics was conducted. This was followed by plating on modified cefoperazone charcoal deoxycholate agar (mCCDA) and incubation under a microaerophilic atmosphere at 42°C for 48 h. Colony morphology, Gram staining and polymerase chain reaction (PCR) were used to identify and characterize the microorganisms.

The growth of blue colonies on the mFc agar surface confirmed presence and faecal pollution of the Bloukrans River. The physicochemical properties, based on the range of pH measured

at different sites of the river (between acidic 3.45 to 6.42 and alkaline 7.2 to 8.74) indicate that *Campylobacter* spp. can thrive in the river. Based on the results from enumeration and sequencing of colonies recovered by each method, it was discovered that the most suitable method to recover *Campylobacter* spp. from river water samples is by prior centrifugation ($14,000 \times g$ for 30 minutes) followed by membrane filtration of the supernatant, and subsequent pooling of the residue and pellet. The pooled residue and pellet might have increased *Campylobacter* spp. concentrations aiding more growth during the enrichment of *Campylobacter* spp. from the river water samples. Results from enumerating *Campylobacter* spp. cells from river water samples indicate that *Campylobacter* spp. are present in Bloukrans River. The sequence obtained from the PCR product indicates that the species found were *Campylobacter jejuni* (96% homology as evaluated by BLAST). This study provided a procedure effective for obtaining a satisfactory quantitative recovery of *Campylobacter* spp. from environmental waters, a critical need for quantitative microbial risk assessment studies.

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ACRONYMS & ABBREVIATIONS

µl	micro litre
Bp	base pairs
CFU/mL	Colony-forming units per millilitres
<i>C. coli</i>	<i>Campylobacter Coli</i>
<i>C. jejuni</i>	<i>Campylobacter jejuni</i>
<i>C. showae</i>	<i>Campylobacter showae</i>
°C	Degrees Celsius
dH ₂ O	Distilled water.
DNA	Deoxyribonucleic Acid
DO	Dissolved Oxygen
EC	Electrical Conductivity
et al.,	<i>et alia</i> (and others)
h	Hour
MAE	microaerophilic environment
mCCDA	Modified charcoal cepharazone deoxycholate agar.
MKA	Modified Karmali agar.
mg	milligram
mL	millilitres
mm	millimetre
MPN	Most Probable Number
NCBI	National Center for Biotechnology Information
NICD	The National Institute for Communicable Diseases
NTU	Nephelometric Turbidity unit
UV	Ultraviolet
PCR	Polymerase chain reaction
pH	Acidity and Alkalinity
qPCR	Qualitative Polymerase chain reaction
WHO	World Health Organisation
WWTP	Wastewater Treatment Plant.

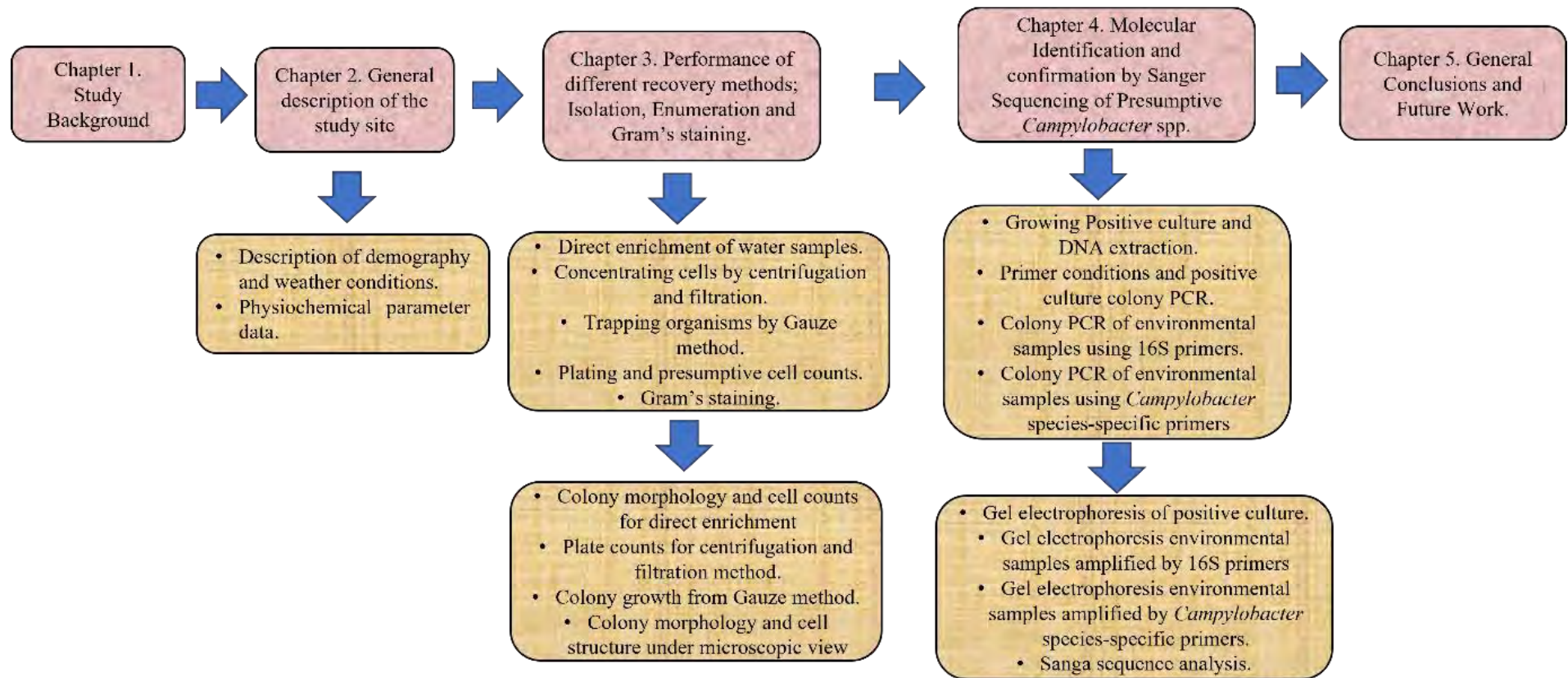


Figure 1.1: Thesis structure/layout.

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW.

1.1 DESCRIPTION OF CAMPYLOBACTER

Campylobacter spp. are Gram-negative slender rod or spiral and curved-shaped bacteria with single or paired flagella. The paired flagella are mostly observed in *C. showae*, whereas other species such as *C. gracilis* are non-motile (Acke, 2018). They are indole negative, oxidase-positive, hippurate positive, catalase-positive, nitrate positive, and glucose utilization negative (Igwaran & Okoh, 2019). They cause both human and animal infections. Infection requires a dose of 500–800 cells to be ingested to occur (El-gendy et al., 2007; Kaakoush, et al., 2015) . However, it has also been noted that 100 cells are sufficient for an infection to occur (Tribble et al., 2010).

Campylobacter spp. infection mainly causes acute diarrhoea (Igwaran & Okoh, 2019). However, post infection complications caused by these bacteria can include Guillain-Barré syndrome (Heikema et al., 2015), septic thrombophlebitis (Morishita & Fujiwara, 2013), bloodstream infections (BSIs) (Morishita & Fujiwara, 2013), acute colitis of inflammatory bowel disease and acute appendicitis (Lagler et al., 2016).

1.2 RISK FACTORS FOR CAMPYLOBACTERIOSIS

Consumption of water contaminated by faecal waste is a major risk factor for Campylobacteriosis (Pitkanen and Hanninen 2017). In studies which have provided evidence of Campylobacteriosis outbreak resulting from the consumption of unclean drinking water, water samples were sourced from groundwater, rivers or lakes most likely contaminated by sewage (Vogt et al., 1982; O'Reilly et al., 2007; Pitkanen and Hanninen 2017), or by manure from livestock from nearby agricultural farms (Vogt et al., 1982; Clark et al., 2003). In addition, faecal shedding among domestic and wild animals at rates of between 9–20% has been implicated in previous studies, as well as by avian species and poultry, which are perfect hosts for *Campylobacter* (Whiley, et al., 2013). Livestock farms, sewage effluents, wild and domestic animals, poultry, and avian species are important reservoirs (Abulreesh et al., 2017; Rechenburg & Kistemann, 2009; Jokinen et al., 2010; Anderzejewska et al., 2013; Rodrigues et al., 2015; Denis et al., 2011; Mughini-Gras et al., 2016; Abulreesh et al., 2006. Seliwiorstow et al., 2015; Magoto et al., 2018; Iversen et al., 2012; Vandaplas et al., 2010; Ellerbroek et al., 2009; Mughini-Gras et. al., 2016; Huang et. al., 2015 and Medema et al., 2015). Moreover, pets transmit an estimated 6% of human enteric *Campylobacter* (Levican et al., 2019).

Table 1.1 Different known *Campylobacter* spp.(Kaakoush et al., 2015; Acke, 2018)

<i>Campylobacter avium</i>
<i>Campylobacter canadensis</i>
<i>Campylobacter coli</i> (including the proposed intrasubspecific designation 'hyoilei')
<i>Campylobacter concisus</i>
<i>Campylobacter cuniculorum</i>
<i>Campylobacter curveus</i>
<i>Campylobacter fetus</i> subsp. <i>fetus</i>
<i>Campylobacter fetus</i> subs. <i>venerealis</i>
<i>Campylobacter fetus</i> subs. <i>venerealis</i> biovar <i>intermedius</i>
<i>Campylobacter graciosus</i>
<i>Campylobacter hominis</i>
<i>Campylobacter helveticus</i>
<i>Campylobacter hyointestinalis</i> sub. <i>hyointestinalis</i>
<i>Campylobacter hyointestinalis</i> sub. <i>lawsonii</i>
<i>Campylobacter insulaenigae</i>
<i>Campylobacter jejuni</i> subsp. <i>doylei</i>
<i>Campylobacter jejuni</i> subsp. <i>jejuni</i>
<i>Campylobacter lanienae</i>
<i>Campylobacter lari</i> subsp. <i>concheus</i>
<i>Campylobacter lari</i> subsp. <i>lari</i>
<i>Campylobacter mucosal</i>
<i>Campylobacter peridis</i>
<i>Campylobacter rectus</i>
<i>Campylobacter showae</i>
<i>Campylobacter sputorum</i> biovar <i>feacalis</i>
<i>Campylobacter sputorum</i> biovar <i>paraurolyticus</i>
<i>Campylobacter sputorum</i> biovar <i>sputorum</i>
<i>Campylobacter subantarcticus</i>
<i>Campylobacter troglotis</i>
<i>Campylobacter upsaliensis</i>
<i>Campylobacter ureolyticus</i>
<i>Campylobacter volucris</i>
<i>Campylobacter</i> sp. Dolphin DP (Provisional)
<i>Campylobacter</i> sp. Prairie Dog (Provisional)

1.3 EPIDEMIOLOGY OF *CAMPYLOBACTER* SPP.

Globally, *Campylobacter* is a leading aetiological agent of bacterial diarrheal disease in humans (Lurchachaiwong et al., 2020). In 2019, globally, *Campylobacters* spp. infection accounted for an estimated 96 million cases of human gastroenteritis annually (Asuming-Bediako et al., 2019). *Campylobacter* gastrointestinal disease is the most frequently recorded enteric pathogen in some European countries (Schielke et al., 2014). In the United States, *Campylobacter* infection is the second most common cause of bacterial enteritis following salmonellosis. A 2010 CDC report noted that an estimated 2.4 million cases were being recorded yearly (CDC, 2010). In 2014, the US attributed 13% of infection cases and 0.2% of deaths to *Campylobacter* spp. (Tack et al., 2019); the U.S experienced an increase of 10% in *Campylobacter* spp. infections from 2004–2012 (Geissler et al., 2017).

The Sub Sahara region in Africa reports a 3.8 million diarrheal cases of which 25% of these results to deaths which occur in children under five (Sigaúque, 2020; Duan & Opolot, 2020). In South Africa, diarrheal disease is the third leading cause of death, and the most common pathogen linked to the cases includes Retroviruses and *Shingella* ssp. (Omatola & Ogunsakin, 2021; NICD 2017/18 & NICD 2019/20).

Campylobacter infection is more prevalent in developing countries, including Asia, Middle East, and Africa, where it is the foremost enteric bacterial pathogen (Kaakoush et al., 2015). Infections in these regions have been variously attributed to a lack of or poor sanitation, poor hygiene and living close to the vicinity of animals. In Bangladesh, *C. jejuni* diarrhoea is the fourth most frequent disease in the first year of life, whereas in Karachi and Pakistan, it is the fifth highest disease burden in the first year of life. Other species of non-jejuni/coli species of *Campylobacter*, such as or *C. troglodytis* and *C. hyointestinalis* subsp. *lawsonii* have been identified in Bangladesh, Tanzania, and Peru, comprising about one-third of detections (Platts-Mills et al., 2014).

In Africa, of all pathogens, *Campylobacter* spp. is most frequently related to diarrhoea in children under the age of five years (Tafa et al., 2014; Nguyen et al., 2016; Asuming-Bediako et al., 2019). Symptomatic infections in young individuals are more prevalent than those in asymptomatic and adult individuals (Komba et al., 2015).

In Malawi, *Campylobacter jejuni/coli* were detected in 21% of diarrheal episodes. *C. coli* comprises more than one-third of bacteria detected in diarrhoea samples (Mason et al., 2013). *Campylobacter* was detected in 16.7% of 227 stools of diarrhoeal children under the age of five years in Ethiopia

(Tafa et al., 2014b). In another study, *Campylobacter* was detected 15.4% of 285 diarrhoeal children undergoing treatment at the University of Gondar Hospital in northwest Ethiopia (Lengerh et al., 2013). In Tanzania, a cross-sectional study of 300 children with acute watery diarrhoea indicated that 9.7% of the stool samples were *Campylobacter* positive (Deogratias et al., 2014). In Egypt, from 1995 to 2003, *Campylobacter* was isolated from 9.37% of 6562 stools sampled from 1057 children (ElGendy et al., 2018). In another study in Gharbia, Egypt, an incidence of 2.8% and 12.3% for *C. coli* and *C. jejuni*, respectively, was reported in samples for 106 children (ElGendy et al., 2018). In Nigeria, *Campylobacter* was detected in 8.2% of the stool samples from 306 diarrhoeal children under the age of 2 in Ilorin in the middle belt zone of Nigeria (Samuel et al., 2006).

Locally in South Africa, literature on the epidemiology of *Campylobacter* is scarce (Thobela, 2017). Therefore, it is difficult to estimate the percentage to which *Campylobacter* currently contributes to the disease burden in the country. In a study by Mason et al., 2013), out of 225 samples, *C. jejuni* (12%) and *C. coli* (7.5%) were present in stool specimens in children and adults suffering from diarrhoea in South Africa. In Venda, Limpopo province, out of 322 stool samples *C. jejuni* was slightly less prevalent (10.2%), followed by *C. coli* (6.5%) (Samie et al., 2007). In a Rotavirus surveillance project, 13% of isolates in stool specimens from children (< 5 years of age) with acute diarrhoea in South Africa were *Campylobacter* (NICD annual review, 2014). In Cape Town, South Africa, 5 443 strains of *Campylobacter* were isolated from the stools of children suffering from diarrhoea (Lastovica, 2009). Additionally, in a *Campylobacter* Surveillance Program in South Africa, 801 of 848 isolates submitted by GERMS-SA (Group for Enteric, Respiratory and Meningeal Surveillance in South Africa) laboratories from different provinces in South Africa were positive for species of *Campylobacter* (Thobela, 2017). *C. jejuni* was the most predominant (80%) specie of *Campylobacter* followed by *C. coli* (6%).

Campylobacter spp. outbreaks have also been documented in middle- and high-income countries such as India (Mukherjee et al., 2013), Norway (Jokinen et al., 2011), New Zealand, (Bartholomew et al., 2014), the Netherlands (Weinberger et al., 2013), China (Chen et al., 2011), Japan (Kubota et al., 2011), Sweden (Mukherjee et al., 2013), United States (Crim et al., 2015, Mukherjee et al., 2013), Canada (Ravel et al., 2016a), Australia (Kaakoush, et al., 2015), and Belgium (Braeye et al., 2015). Overall, the global burden of *Campylobacter* infection is not fully understood due to a lack of improved and consistently applied assays which could contribute towards our understanding of the epidemiology of various *Campylobacter* species. In Africa, particularly, there is a lack of established programs which focus on *Campylobacter* surveillance in Africa (Thobela, 2017 and Tauxe et al., 1988).

1.4 RESERVOIR AND TRANSMISSION PATHWAYS

Campylobacter spp. occur in the environments as the result of contamination from their natural hosts, warm-blooded animals, for example, wild birds, cattle (Shrestha et al., 2019; Medema et al., 2015), and domestic pets such as dogs and cats (Anderzejewska et al., 2013; Rodrigues et al. 2015). The transmission cycle begins with an animal reservoir such as cattle, poultry, wildlife, and waterfowl, which contaminate water. It then travels to humans in the form of drinking water, recreational water, or irrigation water (Kaakoush et al., 2015). Water has been implicated to be the main source of *Campylobacter* spp. infections and has led to outbreaks in low-income countries such as Malawi (Mason et al., 2013) and Kenya (Swierczewski et al., 2013; Igwaran & Okoh, 2019). The link between contact with environmental water and *Campylobacter* infection is well established (Mughini Gras et al., 2012; Ravel et al., 2016b; Schönberg-Norio et al., 2004), in outbreak situations (Dale et al., 2010; Harder-Lauridsen et al., 2013). In Denmark, up to 4% of sporadic infections is caused by recreational water (Gaardbo Kuhn et al., 2018). Both freshwater and sea water can harbour *Campylobacter* spp. (Kovanen et al., 2016; Moore et al., 2001). Humans become infected by the bacteria and then transfer it to each other. Person-to-person transmission (faecal-oral route) is possible (Kaakoush et al., 2015). Furthermore, humans may excrete the bacteria through faeces, which then moves back into the environment in the form of sewage (Kaakoush et al., 2015; Pitkanen & Hanninen, 2017). Environmental waters can become infected because of sewage effluent and surface run-off of faeces from wildlife, domestic animals, or manure-fertilized field (Rechenburg & Kistemann, 2009; Sterk et al., 2016).

1.5 *CAMPYLOBACTER* SPP. SURVIVAL IN WATER

Campylobacter spp. organisms have been found to survive in water for a period 2–4 weeks. In some cases, survival of *Campylobacter* spp. organisms can even last up to four months (Pitkänen, 2013). However, their prolonged survival in environmental water requires temperatures around 4°C (Schang et al., 2016).

The survival of these organisms in environmental waters has been associated with risks to humans' health (Kistemann et al., 2016). For example, pollution of river water with these organisms is a major public health hazard (Adeniji et al., 2019). Their survival in water is critical for transmission to humans through the ingestion of contaminated water. Factors affecting the concentrations of these organisms in environmental waters include interference from other bacteria (Schang et al., 2016a), slow growth rates, dissolved oxygen concentrations, the amount of rainfall before sampling, (Vereen

et al., 2013), temperature, biotic interactions, and nutrient concentrations, (Hokajärvi et al., 2013). It is important to monitor the presence of the bacteria in environmental waters, especially since transmission from environment to humans and animals is possible. Environmental monitoring and consequently early detection of the bacteria is necessary for outbreak prevention.

1.6 METHODS USED TO STUDY THE OCCURRENCE OF *CAMPYLOBACTER* SPP. IN ENVIRONMENTAL WATER SAMPLES

Several studies have been conducted to assess the occurrence of *Campylobacter* spp. in environmental waters (Meinersmann et al. 2020 ; Pitkänen 2013b ; Henry et al. 2015 & Van Dyke et al. 2010a). *Campylobacter* spp. occur in incredibly low numbers, and are unevenly distributed in the environmental waters, which makes them very difficult to detect, and makes a meaningful study of their occurrence difficult (Kaakoush et al., 2015). To appropriately conduct occurrence experiments, it is critical to review earlier studies to understand the factors that should be considered when researching the occurrence of *Campylobacter* spp. in environmental water samples.

1.6.1 Measuring the physicochemical characteristics of water

The physicochemical characteristics such as temperature, dissolved oxygen, turbidity, electro-conductivity, and pH of a site influence the concentrations and occurrence of *Campylobacter*. Turbidity is a measurement of the cloudiness of water. High levels of turbidity are an indication of dissolved organics, such as humic acids and inorganic materials. *Campylobacter* spp. is frequently found in faecal-contaminated environmental water. If the physicochemical characteristic of a site is conducive for *Campylobacter* survival, the concentration becomes high enough to allow detection, thereby allowing measurements.

1.6.2 Sampling methods

Numerous sampling methods can be applied to enhance the detection of *Campylobacter* spp. in environmental waters.

1.6.3 Grab and composite sampling (directly collect a known volume of water sample)

Grab and composite sampling are the traditional methods applied in environmental water surveillance. Grab sampling involves filling a vessel with water at a single point in time. This method is easy, affordable and less time consuming, but is limited because only a snapshot at a moment is

represented. Alternatively, composite sampling involves collecting several individual samples at frequent intervals, and thereafter the individual samples are combined. Composite sampling is considered a more representative method than grab sampling, but is limited because it is expensive and time-consuming, as it requires the sampler to return to the same site multiple times over a period (Liu et al., 2020; Johannessen et al., 2012). Given the sporadic distribution of *Campylobacter* spp. in rivers it is more appropriate to collect numerous samples, and so composite sampling is more desirable.

1.6.3.1 Concentration of *Campylobacter* spp. from Environmental water samples

As *Campylobacter* spp. occur in very low numbers in environmental waters, a high volume of water needs to be collected to ensure the detection of *Campylobacter* species, and thereafter the sample needs to be concentrated to a volume that can be examined easily in the laboratory. In the past different approaches have been employed to concentrate *Campylobacter* spp. from environmental water samples, including membrane filtration (Chukwu et al., 2019) and centrifugation of water samples (Kim et al., 2016).

1.6.3.2 Concentration of *Campylobacter* spp. from water samples by centrifugation

The centrifugation technique is a method used to separate molecules with different densities by spinning them in solution around an axis (in a centrifuge rotor). *Campylobacter* can be concentrated from water samples by centrifugation and the pellets can then resuspended in broth for culturing (Kim et al., 2016). *Campylobacter* cells were recovered from river water samples by using centrifugation at $14,000 \times g$ for 20 minutes to concentrate 500 mL of water samples (Khan et al., 2013).

1.6.3.3 Concentration of *Campylobacter* from water samples by membrane filtration (MF)

Filtration is a mechanical or physical process used for the separation of one substance from another, such as solids, liquids, and gases, using an interposing medium (filter). Solids can be separated from a liquid suspension via a porous medium, or membrane, of a specific size according to the size of the cells to be trapped, that permits the liquid to pass through while retaining the solids/cells.

Most studies have applied membrane filtration as a method of concentrating *Campylobacter* spp. cells from river water samples, as indicated in Table 1.2. In membrane filtration a known volume of river water sample such as 1 mL, 10 mL, 100 mL, 140 mL, 250 mL, 500 mL, 700 mL, 1000 mL, and 2000 mL is filtered directly through a cellulose-based filter membrane of a specific pore size such as 0.45

μm and $0.22 \mu\text{m}$. Thereafter, the filter membrane with the trapped microorganisms is used for culturing (Rechenburg and Kistemann, 2009; Carter et al., 2009; Khan et al., 2009; Jokinen et al., 2010; Van Dyke et al., 2010; Denis et al., 2011; Lévesque et al., 2011; Hu and Kuo, 2011; Khan et al., 2013; Denis et al., 2011; Vereen et al., 2013; Hokajärvi et al., 2013; Mughini-Gras et al., 2016; Guy et al., 2018). Studies which filtered small volumes of river water samples such as 1 mL, 10 mL, 100 mL, 140 mL and 250 mL were able to recover *Campylobacter* spp. (Hokajärvi et al., 2013).

Previous studies suggest that large volumes of water samples up to 2000 ml give a chance of greater recovery, and therefore this volume should be used for concentrating *Campylobacter* spp. (Lévesque et al., 2011). Differences in physio-chemical characteristics may affect the success of the approach that is used to recover *Campylobacter* spp. cells from respective water samples (Abulreesh et al., 2017). A key factor that affects the recovery of *Campylobacter* spp. from river water samples by membrane filtration is the turbidity of the water sample. Water samples with high turbidity contain organic and inorganic materials that block the membrane filter and limit the volume of sample that can be filtered. The presence of suspended material, slugs and sediment can easily block the filter before an adequate volume of water can pass through the membrane filter, making the technique unstable (UNEP/WHO, 1996). This is particularly a problem when working with large volumes of environmental water samples because filtration can be limited by the presence of very high levels of suspended material.

Some researchers, such as Van Dyke et al., 2010, have used a sequential membrane filtration technique to concentrate *Campylobacter* spp. in cases of high turbidity. The technique involves filtering water samples through a $0.45 \mu\text{m}$ membrane first, followed by a $0.22 \mu\text{m}$ membrane. In one study where river water sample was very turbid the resolution to blockage of membrane filters was pre-filtration through a $1.5 \mu\text{m}$ (VWR International) pore size membrane filter before filtering through the $0.45 \mu\text{m}$ pore size (Lévesque et al., 2011). However, no conclusions have been made about the effectiveness of this pre-filtration step, as investigators have indicated that this step has not been formally investigated. Although Lévesque et al., (2011), recorded no values for the turbidity of the water samples, their success in finding *Campylobacter* spp. may have been due to the large volume (2000 mL) of water they analysed. After pre-filtration through the $0.45 \mu\text{m}$ membrane both this and the $1.5 \mu\text{m}$ filter were analysed, as it was possible that some *Campylobacter* spp. could have been trapped in the pre-filter membrane (Lévesque et al., 2011).

In general, membrane filtration procedure is the most frequently used method, as indicated in Table 1.2. It is a suitable method for most types of water samples except for those with high turbidity. River

water samples with turbidity from 8.4555 NTU and above can lead to low recovery of *Campylobacter* using the membrane filtration method (Hokajärvi et al., 2013).

Filtration of larger volumes (from 1000 mL and above) of water samples with high turbidity or heavily contaminated sewage effluents can produce false-negative results. This is due to the presence of high quantities of background heterotrophic bacteria and coliforms, which can overtake the growth of *Campylobacter* spp. due to its very slow metabolism during the enrichment stage, preventing growth to a detectable level (Abulreesh et al., 2017). This occurs because during the lag to the exponential growth phase there is high competition for available nutrients and space on the growth media between *Campylobacter* spp., heterotrophic bacteria, and coliforms. It is suggested that with turbid surface waters, a volume of less than 1000 mL (100 to 500 mL) should be filtered. With this smaller volume enrichment will favour the growth of *Campylobacter* spp. (Jokinen et al., 2010). Thereafter the individual enriched volumes can be pooled before plating.

1.6.3.4 Comparison of centrifugation and membrane filtration (MF) methods of concentrating *Campylobacter* from water samples

No conclusion could be drawn from the existing literature about the best method of concentrating *Campylobacter* spp. from environmental water samples. A study by Khan et al., (2009) compared the filtration method using a 0.45µm filter membrane against the centrifugation method running at 14,000 × g for 20 minutes to concentrate *Campylobacter* species present in surface water samples collected from agricultural watersheds across Canada. The centrifugation method produced better results than the filtration technique in concentrating bacterial cells from environmental water samples. Centrifugation isolated significantly higher numbers of *C. coli* and other species than membrane filtration. However, both methods detected a very low probable number of *Campylobacter* species. Each method is sensitive towards a particular specie of *Campylobacter*. Membrane filtration detected more *C. jejuni* when centrifugation was negative. Although the centrifugation method produced better results, the superiority of this method over membrane filtration could not be established as the researchers processed different water volumes using the two methods and used different incubation periods and selective agar.

Table 1.2 Different methods used for concentrating *Campylobacter* spp. from Water Samples

Methods of recovery	Water sample volume	References	Isolation rates (Number of positive samples) Study Findings and Conclusions
Membrane filtration (0.45 µm).	100 mL, 10 mL and 1 mL	Rechenburg & Kistemann, (2009)	In 101 collected river water samples. 86% (n = 87) were positive for <i>Campylobacter</i> sp. and 14% (n = 14) were negative.
	500 mL	Jokinen et al., (2010)	372 samples were collected. 42% (n=156) were positive for <i>Campylobacter</i> spp.
	1000 mL	Hu & Kuo, (2011)	For the 75 collected samples, <i>Campylobacter</i> spp. was positive in 2.6% (n=2).
	1000 mL	Van Dyke et al., (2010a)	344 samples were collected and 65% (n=223) were positive for <i>Campylobacter</i> spp.
	500 & 2000 mL	Levesque et al., (2011)	27 samples were collected, 93% (n=25) were positive for <i>Campylobacter</i> spp.
	250 mL	Vereen Jr et al., (2013)	For the collected 156 samples 62% (n=96) were <i>Campylobacter</i> spp. positive.
	10, 100, 1000, 0.1 & 1 mL	Hokajärvi et al., (2013)	84 samples were collected of which 58% (n=45) were positive for <i>Campylobacter</i> spp.
	1000 mL	Guy et al., (2018).	Only 33.9% were positive for <i>Campylobacter</i> spp.
	500 mL	Chukwu et al., (2019).	4 river samples were collected, and they were all negative for <i>Campylobacter</i> spp.
Centrifuged at 14,000 x g for 20 min	100 mL	Khan et al., (2013).	Collected samples not specified. Positive <i>Campylobacter</i> spp. 28.5%
Direct enrichment	500 mL	Denis et al., (2011).	60 samples were collected, 50% (n=30) was positive for <i>Campylobacter</i> spp.
Centrifugation (14,000 × g for 20 min). Membrane filtration (0.45 µm).	1000 mL	Khan et al., (2009).	699 water samples were collected, 33% (n=230) positive by Membrane filtration. 49% (n=341) positive by centrifugation.
	500 mL		
Moore swab	Continuous flowing surface water	Fernandez et al., (2003).	(44/181) 24.3% of water samples from different points of the river were positive for <i>Campylobacter</i> spp.

1.6.3.5 Direct enrichment of water samples without prior concentration

Alternatively, *Campylobacter* spp. can be enriched from water samples directly, without prior concentration. This method is recommended for high turbid samples such as river water samples (Denis et al., 2011). Denis et al., (2011) used this approach to enrich 500 mL river water samples in 500 mL of Bolton Broth, in a microaerobic (5% O₂, 10% CO₂, 85% N₂) atmosphere, and at a temperature of 37°C for 48h. Of his 60 collected samples 30 (50%) were positive, and two hundred and ninety-nine isolates were recovered successfully. A major disadvantage of this method is that growth might be hindered by the proportion of river water to the broth medium; a greater volume of the enrichment broth (10% of sample to 90% of medium) than the processed turbid water sample is ideal for the direct enrichment method (Lévesque et al., 2011).

Overall, these grab sampling-based methods appear to provide improved detection sensitivity for these pathogens, but there are several issues to be considered when using these methods.

1.6.4 Moore Swab Method for capturing *Campylobacter* in river water.

With grab or even composite sampling methods only a certain volume of water can be assayed. This is a problem because of the non-uniform distribution, intermittent discharge from reservoirs and relatively low number of *Campylobacter* spp. in environmental water samples. *Campylobacter* spp. cells can be missed during a grab and catch water sampling event. To overcome these challenges, there is an alternative method which can continuously trap, over extended periods of time, the microbial contents of flowing surface water as they enter. This is called Moore Swab Method (Sikorski and Levine, 2020).

The Moore swab method has been in use for years by public health experts, globally for the detection and isolation of enteric pathogens from water. It is an environmental surveillance method that involves the use of a strip of gauze tied with string floating in flowing water. The gauze acts as a filter, enabling the collection of microbes over an extensive period. The Moore swab method was first suggested in 1948 by Brendan Moore for tracing *Salmonella Paratyphi B* from discharged sewage effluent water in North Devon, England, to establish the sources of infection liable for sporadic outbreaks of paratyphoid fever (Sikorski and Levine, 2020) (Liu et al., 2020). Since then, this method has been used to detect numerous faecal borne pathogens in water and has been extended to environmental surveillance and outbreak investigation as well as the enumeration of bacteria in surface water, including *Campylobacter* (Ernandez et al., 2003). When Moore Gauze was compared to the grab sampling methods to isolate *Campylobacter* spp. from environmental samples, the method

yielded 9% positive samples while the grab sampling method yielded 31% (Meinersmann et al., 2020). This method is used in situ, whereby the flowing surface water is filtered through a gauze immersed in the flowing water body. The gauze pad is left for a desirable period, which can be 24 to 48 hours (Callahan et al., 2019) and up to 2–3 days (Berrang et al., 2019). The Moore swab method is recommended for the isolation of *Campylobacter* spp. from environmental water samples, as it is simple, low cost and reliable (Meinersmann et al., 2020).

1.7 CULTIVATION APPROACHES FOR STUDYING *CAMPYLOBACTER* SPP. IN ENVIRONMENTAL WATER SAMPLES

The current understanding of the diversity of pathogenic *Campylobacter* spp. is based on the application of highly selective cultivation approaches. This section will review the different selective media, enrichment media and conditions required for culturing *Campylobacter* spp. from river water samples.

1.7.1 Enrichment

In environmental water samples, *Campylobacter* spp. occur in low numbers. Therefore, pre-enrichment in broth is recommended to provide nutrients and a conducive environment to encourage the growth of any *Campylobacter* spp. present (Lisa et al., 2009). Different volumes of enrichment broth, such as 10 mL, 20 mL, 25 mL, 90 mL, and 100 mL have been used for *Campylobacter* spp. isolation from river water samples (Rechenburg and Kistemann, 2009; Khan et al., 2009; Hu and Kuo, 2011; Levesque et al., 2011). Several types of enrichment broth have also been used, including the Preston, Exeter, and Bolton broth (Carter et al., 2009). All these enrichment media rely only on the antibiotics added to the medium's selective supplements, which suppress growth of all other competing organisms (Kashappanavar et al., 2018). The Bolton Broth selective supplement consists of a gram-positive inhibitor trimetropin. Gram-negative competitors are suppressed by Vacomycin, and Cephoperozone and Cycloheximide are used for mould inhibition. Some authors such as Williams et al. 2012 altered the medium by substituting cycloheximide with amphotericin B. Preston broth uses Polymyxin, Rifampicin, Trimethoprim and Cycloheximide. Exeter broth uses Trimethoprim, Rifampicin, Polymixin B, Cefoperozone and Amphotericin B.

Incubation of the broth is carried out in microaerophilic atmosphere, a growth condition that requires an atmosphere of reduced O₂ and increased CO₂ (Meurer et al., 2020). Different incubation temperatures and durations have been applied. These include 37°C for 24±4 h (Rechenburg and

Kistemann, 2009), 42°C for 24 h (Khan et al., 2009), 42°C for 48±2 h (Vereen Jr et al., 2013), 37±1°C for 44±4 h (Hokajärvi et al., 2013), 42°C at 48 h (Mughini-Gras et al., 2016), 37 ±1°C for 44+/-4 h (Hokajärvi et al., 2013), 42±2°C for 44 h (Guy et al., 2018), and 37°C for 48 h (Abulreesh et al., 2017). Incubation temperature of 37°C or 42°C are most frequently used. More *Campylobacter* have been cultured from river water samples at an incubation temperature of 42°C (Khan et al., 2009; Dyke et al., 2010; Jokinen et al., 2010; Levesque et al., 2011, Vereen Jr et al., 2013; Khan et al., 2013; Mughini-Gras et al., 2016 and Kim et al., 2016).

However, it is notable that incubations at 42°C may lead to reduced specificity, whereby other competing organisms may grow alongside *Campylobacter* spp. during the enrichment stage (Oakley et al., 2011). It has been noted certain species of *Campylobacter* such as *C. jejuni* and *C. lari* grow better at a temperature of 42°C (Khan et al., 2013). The duration of incubation is usually between 24 to 48 hours. Bolton broth is preferred for the enrichment of *Campylobacter* spp. at 42°C in a controlled atmosphere by using CampyGen sachets (Oxoid) for 48 h (Mughini-Gras et al., 2016). This enrichment condition has been reported to have the best balance between *Campylobacter* spp. recovery and inhibition of competitor organisms (Carter et al., 2009). This explained why Chukwu et al., (2019) were not able to recover *Campylobacter* spp. from river water samples incubated for 24 h instead of 48h.

The enrichment period greatly influences *Campylobacter* spp. detection. Enrichment for just 24 h is too short and can negatively affect growth during the enrichment stage. *Campylobacter* spp. cells might not reach their exponential phase due to their slow metabolism, resulting in very low concentrations (Jokinen et al. 2010; Chukwu et al., 2019). Exclusion of the enrichment phase during the isolation of *Campylobacter* spp. also results in low detection as was encountered by (Mughini-Gras et al., 2016).

1.7.1.1 Selective growth media (agar) and incubation conditions

Following enrichment, a suspension of *Campylobacter* cells in broth is plated on selective growth medium incorporated with various combinations of antibiotics, nutrients, and oxygen quenching agents. Plating is carried out by dispensing a known volume of the enrichment broth onto the surface of the agar-based nutrient medium. The antibiotics are added to restrain the growth of other competitors. Antibiotics such as cefoperazone vancomycin, trimethoprim, polymyxin B, and rifampicin are added into the *Campylobacter* media to eliminate competitive organisms (Kim et al., 2016). Different selective media, including Karmali agar (Rechenburg and Kistemann, 2009);

Campy-Line agar (CLA)(Sylte et al., 2018), modified cefoperazone charcoal deoxycholate agar (mCCDA) (Khan et al., 2009); Preston and Exeter (Carter et al., 2009), *Campylobacter* agar plates CAP (Kiess et al., 2010) and campy-cefex agar (CCA) have been used for *Campylobacter* isolation from river water samples. These media contain common nutrients, which include sodium glutamate, sprat hydrolysate. They also contain additives which quench oxygen, such as ferric sulfateoxide, sodium pyrosulfite and sodium pyruvate (Vaz et al., 2014). CAP, CCA and mCCDA all contain Casein hydrolysate. CLA is different because it contains α -Ketoglutaric acid Casein hydrolysate (Kiess et al., 2010). mCCDA is distinguished by bacteriological charcoal, and *Campylobacter* spp. have shown the best growth in mCCDA agar. The agar is a blood free medium. mCCDA is the most frequently medium used for *Campylobacter* isolation from river water samples (Khan et al., 2009; Carter et al., 2009; Dyke et al., 2010; Jokinen et al., 2010 ; Hu and Kuo, 2011; Denis et al., 2011; Hokajärvi et al., 2013; Abulreesh et al., 2017). The combination of activated charcoal and cefoperazone in mCCDA has been acknowledged to be an effective combination which produces high recovery rates of *Campylobacter* spp. from water, environmental food, and animal feed samples (Smith et al., 2015). Furthermore, mCCDA is endorsed by the International Organisation for Standardization (ISO) for the selective isolation and enumeration of *Campylobacter* from environmental, water, animal feed and food samples (Smith et al., 2015). Modified charcoal-cefoperazone-deoxycholate agar (mCCDA) contains two antibiotics, cefoperazone and amphotericin (Hu & Kuo, 2011). The oxygen quenching agents in the medium create a microaerophilic atmosphere and thereby limit the growth of other members such as obligate aerobes (Lai & Chu, 2008), and provide a favourable environment for *Campylobacter* spp., which are oxygen intolerant organisms (Akosua et al., 2016).

Different incubation temperatures and durations have been applied, but previous studies suggest that a combination of Bolton broth enrichment and subsequent plating on mCCDA provides better recovery of *Campylobacter* spp. growth in a laboratory, under microaerophilic conditions at 42°C for 24–48 h (Khan et al., 2009; Carter et al., 2009).

1.8 STUDY JUSTIFICATION

Campylobacter infections remain a leading causative agent of diarrhoeal disease, with incidences higher than those caused by *Shigella* and *Salmonella* (Kaakoush, Castaño-Rodríguez, et al., 2015). In South Africa, diarrhoeal diseases are the third leading cause of death (Chola et al., 2015). Other pathogens linked to diarrhoeal outbreaks in South Africa include *Shigella* spp., non-typhoidal *Salmonella* spp., *Escherichia coli*, *Staphylococcus aureus*, enterotoxigenic *Bacteroides fragilis*,

several viruses (norovirus, rotavirus, and adenovirus) and parasites (e.g., *Entamoeba* spp and *Giardia lamblia*) (Pfeiffer et al. 2012; Kullin et al. 2015).

River water is often contaminated with faecal waste from infected humans or animal reservoirs, which makes these waters a risk factor for transmission to humans. South Africa is a water-scarce country, and depends on available freshwater systems, including rivers, for irrigation, domestic purposes as well as recreational activities.

Grahamstown (Makhanda) has a sewage treatment crisis and a high number of leaks that occur in pipelines, that can convey faeces-borne pathogens into the Bloukrans River. The Bloukrans River is formed by streams in Grahamstown and flows past livestock farms, and therefore the possibility of contamination by manure from livestock is very high.

It is a challenge to isolate and detect *Campylobacter* spp. in river water samples using traditional culture methods because they are mostly present in low numbers. Concentration either by filtration using membranes of known materials (polycarbonate) and pore sizes (between 0.8–0.1 μm) or by centrifugation are the two standard methods for amassing microbial cells from water samples. Previous studies comparing concentration by membrane filtration and centrifugation do not definitively prove which method is better for recovering *Campylobacter* from water samples.

Alternatively, *Campylobacter* can be enriched from water samples directly, without prior filtration or centrifugation of water samples. However, the proportion of river water to the broth medium can affect the recoverability of *Campylobacter* using this method. The low and sporadic distribution of *Campylobacter* in rivers therefore mean that the Moore Swab method is preferable for trapping *Campylobacter* (Bisha et al., 2011; Sikorski & Levine, 2020) An established protocol to recover *Campylobacter* spp. from water samples is lacking.

1.9 AIM AND OBJECTIVES

1.9.1 Overall aim

To determine the most suitable method to be used to recover *Campylobacter* spp. from river water samples.

1.9.2 Specific Objectives

- To evaluate the performance of different methods used for the recovery of *Campylobacter* spp. from environmental water samples.
- To isolate and enumerate *Campylobacter* spp. cells from environmental water samples.
- To identify *Campylobacter* spp.

1.10 HYPOTHESIS

The study hypothesizes that Bloukrans River is contaminated with *Campylobacter* spp. and cell recovery rates will vary depending on the recovery method used.

CHAPTER 2: STUDY AREA DESCRIPTION

This chapter describes the socio-economic characteristics of the study area, as well as land uses and anthropogenic activities in the area that might play a significantly role in the *Campylobacter* spp. contamination of the river system. It also describes the climate conditions of the study area and its influence that such conditions may play on the growth and survival of *Campylobacter* spp. in river waters.

2.1 CHARACTERISTICS OF THE STUDY AREA (MAKHANDA).

Makhanda is a medium-sized town with an estimation of population of about 70,000, located 33°18'36"S; 26°31'36" E in the Sarah Baartman District of the Eastern Cape Province of South Africa (Palma et al., 2021) (Figure 2.1). The town's geographical arrangement follows the old apartheid spatial setting. The Western part is occupied by high-income households, this part is expected to contribute least in pollution of environment with *Campylobacter* spp., while the Eastern part (the township sections) is made up of low income households populated by individuals affected by high unemployment, and small homesteads and houses (Mutumbi et al., 2021), this part is assumed to be the one amongst other major contributors to pollution of the river due to activities (littering, open defecation, movement of livestock) occurring in this area.

An inaccurate population estimation of the Cacadu District (now Sarah Baartman District) by Census 2001 affected the Makana local Municipality budget allocation, which then resulted in a sanitation backlog (Roodt, n.d), consequently deposition of active sewage to the environment due to the malfunctioning decaying drainage system can be observed within the town.

The Belmont Valley sewerage treatment plant services the entire CBD and Western side, and 60% of the Eastern side of Makhanda. This plant is currently exceeding work capacity and plans to increase its capacity was promised to be implemented by 2014. In 2023 this has not yet occurred, and the plant remains in the same condition (Makana-Final-IDP 2021–2022), this means the plant release effluent that is not properly treated to the Bloukrans River. In addition to the mentioned factors, 2013 Stats SA collected data on the social welfare conditions of Makhanda and identified certain households within the population (in the Township area in particular) that were still using the bucket toilet system. This demonstrates poor infrastructure which may contribute to the pollution of surface water by human faeces, which can be deposited in the river through surface run-offs (Eastern Cape Socio-Economic Council, n.d). The Makana 2021–2022 integrated Development plan highlighted that, in 2009, the Municipality had adopted a Water Service Development Plan; however, this plan has not been updated since its inception. This lack of implementation of the plan is another factor that could

negatively affect the riverine system and contribute to the contamination of recreational water in the study area.

All the above-mentioned factors are playing a role in the possibilities of pollution of the study area by several pathogens which include *Campylobacter* spp. making the area an adequate environment for the study.

2.2 ANTHROPOGENIC ACTIVITIES AROUND THE STUDY AREA

Urban activities, including medical services, can contribute to water pollution through the run-off of waste into the environment. Makhanda has a malfunctioning sewage drainage system that frequently discharges into the streets, which could pollute the river through surface runoff. Inadequate sanitation in townships has been known to motivate residents to practice open defecation, increasing the chances of contaminating the river through surface runoff of organic matter, and farming around the study area also has many possibilities of pollution by animal manure runoff. The Makana Local Municipality Water and Sanitation By-Law document describes the riverine system (including Bloukrans River) within this region as environmental parts which need protections from pollution from different activities. However, due to the notable fading infrastructure in Makhanda, contamination of these rivers by microbial populations from the broken pipe system, including pathogenic organisms such as *Campylobacter* spp., is possible.

2.3 COMMON WEATHER CONDITIONS IN MAKHANDA

Similar to other towns within South Africa, Makhanda is characterized by a highly variable climate with four seasons per year, namely autumn, winter, spring, and summer. The climate conditions of Makhanda are characterized according to the four seasons of the year. The town is in the southern hemisphere, and at altitude +/- 550 m, the town has a warm and temperate climate and is highly susceptible to drought, with evidence of widespread occurrence of droughts and consistent drying conditions (Botai et al., 2018). Makhanda is negatively impacted by persistent droughts, which has undoubtedly affected the poor infrastructure and development of this town. This location is classified as Cfb (a climate where the coldest month is warmer than -3°C but colder than +18°C) by Köppen and Geiger. According to Makhanda climate condition records the warmest month of the year is February, with temperature averages ranging from 20.9 °C-69.6 °F; June is noted to be the coldest month, with temperature averages ranging from 12.5 °C-54.4 °F (Climate data, n.d). These temperatures/ climate conditions suggests that *Campylobacter* spp. can survive in this town since it is characterized by low temperatures.

2.4 DESCRIPTION OF SAMPLING SITES

The Bloukrans River (Figure 2.1) is a Kowie tributary river formed from streams running through the Makhanda town. The river rises in areas near the Vukani informal settlement and flows down to where it receives effluent from the Belmont Valley Wastewater Treatment plant and then flows in a south-easterly direction to later join the Kowie River. The Bloukrans River is of interest because it receives effluent from the Belmont Valley treatment plant, which services the CBD, Western side and 60% of the eastern side of the town and is the only treatment plant which deals with industrial effluent in the town. Currently the plant does not meet the set discharge standards and needs to be upgraded and refurbished. It is overloaded, overflowing, and running at 150% of its capacity (Makana Development 2021–2022). The Bloukrans River system was selected for this study due to the potential contributors to pollution in water sources surrounding it. Six study sites indicated by red dots in Figure 2.1 were selected for this study.

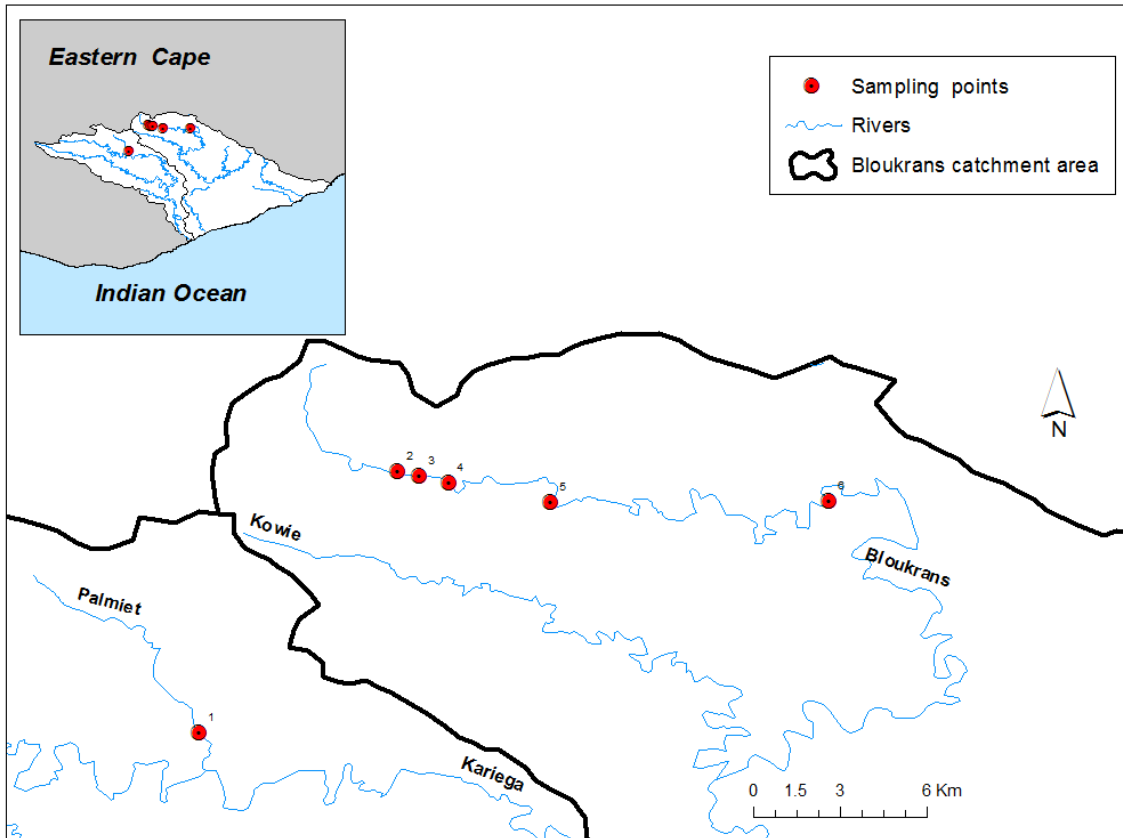


Figure 2.1. A map indicating the location of Makhanda in the Eastern Cape province of South Africa, with sampling points indicated by red dots labelled 1–6. Site 1 is the control, Palmiet River. Site 2 is a site near the Vukani informal settlement. Site 3 is 500 m downstream. Site 4 is the Belmont valley WWTP discharge point. Site 5 is near a commercial farm and Site 6 is the last point where pollution is expected to be from human practises.



Figure 2.2 Physical appearance of the 6 sampling points. Site 1 is the control site. Site 2 is the sampling site near the Vukani Informal Settling. Site 3 is 500 m downstream from the Vukani site with water displaying the same physical features. Site 4 is the Wastewater Treatment Discharge Point. Site 5 is the site which was expected to be contaminated by nearby farm surface run-off. Site 6 shows the typical physical characteristics of the site.

Site 1 is the control site, the Palmiet River located southwest of Grahamstown. This river was selected as a control site because it is within the same ecoregion as the Bloukrans River, and is pristine, given that it is less affected by anthropogenic activities which can contribute to contamination of surface water with *Campylobacter* spp.

Site 2 is located near the urban area of Grahamstown and the informal settlement of the Vukani area. It is influenced by human activities such as open defecation by residents with inadequate sanitation. Site 3 is approximately 500 m downstream of Site 2 and could contain the same pollution as in Site 2, particularly runoff from the nearby Vukani settlement and subsequent the microbial pollution of the river.

Site 4 is the effluent discharge point (EDP) of the Belmont Valley Wastewater Treatment Plant (WWTP). In addition to the effluent from the WWTP, this site is also impacted by livestock manure contamination.

Site 5 is about 5 km downstream of the WWTP effluent discharge point. This site was expected to be mainly impacted by pollution from nearby farms and run-offs from surrounding gravel roads.

Site 6 is situated in an area where the river is clearer and cleaner, which makes it desirable for human activities such as swimming, baptism, and ritual performances by locals.

CHAPTER 3: INVESTIGATING THE EFFICACY OF DIFFERENT METHODS OF RECOVERING *CAMPYLOBACTER* SPP. FROM RIVER WATER

3.1 INTRODUCTION

Different sampling methods have been used to recover *Campylobacter* spp. from river water samples. One of such methods is the catch and grab method (Meinersmann et al., 2020) whereby water is collected using a sterile container, and then concentrated or enriched without prior concentration to recover *Campylobacter* spp. The other method, Moore swab involves filtering surface water through a gauze immersed in the flowing water body to collect microbes over an extensive period (Sikorski & Levine, 2020). The Moore Swab is a continuous filtration method and involves the deployment of Moore swabs that were prepared from cotton gauze strips so that the continuous filtration of a nonfixed volume of water for at least 48 h period is allowed. The swabs act as continuous filters to “trap” microorganisms that are present in the river. Thereafter, the microorganisms will be isolated and confirmed using appropriate laboratory methods. However, it is unclear as to which method performs the best in recovering *Campylobacter* spp. from water samples. This chapter compares the performances of the grab method with and without prior concentration, and Moore swab (MSM) for the recovery of *Campylobacter* spp. from water samples obtained from the Bloukrans River.

The physicochemical quality of the water and the presence of faecal contamination influence the occurrence and survival of pathogens such as *Campylobacter* spp. in rivers. Higher oxygen levels impair *Campylobacter* spp. growth (Schang et al., 2016b). Different pH levels impact *Campylobacter* spp. growth differently. Lower pH (2-3.5) lowers growth of these organisms, however species such as *Campylobacter jejuni* have displayed resistances towards very acid environment and grew in at low pH levels (Axelsson-Olsson et al., 2010). While on another hand pH 7 have been general found to accelerate their growth (Ma et al., 2015). Higher temperatures and solar radiation inactivate *Campylobacter* spp. in water (Meurer et al., 2020). The mentioned evidence about the impact of physiochemical properties of the water samples can be used as indicators for whether *Campylobacter* spp. can thrive in the river or not. Therefore, faecal coliforms (for faecal contamination), and parameters such as pH, temperature (°C), dissolved oxygen (mg/L), turbidity (NTU) and electro conductivity ($\mu\text{S}/\text{cm}$) had to be measured at the different sampling points.

3.2 MATERIALS AND METHODS

3.2.1 Measurement of physicochemical parameters of the river

Physicochemical parameters such as pH, temperature (°C), dissolved oxygen (mg/L), turbidity (NTU) and electro conductivity ($\mu\text{S}/\text{cm}$) were measured at the different sampling points, on-site, using the SL1000 Portable Parallel Analyser (PPA) with probes, before collection of samples.

3.2.2 River water sampling (Grab method)

Water samples were collected by immersing sterile 5 L containers 5 cm beneath the surface of the river. The collected samples were stored in cooler boxes containing ice and transported to the Rhodes University IWR research lab. In total 36 (6 samples per-site) water samples were collected for this project. Out of the 36 samples, 24 were collected by the catch and grab method and 6 were sampled by continuous filtration, the Moore Swab technique (See section 3.5). These samples were collected in October 2020, and June and September 2021.

3.2.3 Enumeration of faecal coliform concentrations in water samples

The level of faecal coliforms was too great to be counted directly. Therefore, each water sample was serially diluted. To do this 10 mL of a water sample was diluted by factors of 10. 10 mL of a 1/1,000 dilution of the water samples was filtered onto a 0.45 μm cellulose-nitrate membrane filter (Sartorius Stedim Biotech GmbH, Goettingen, Germany) using a Millipore manifold filtration system (EZ-Fit™ Manifold, Merck KGaA, Darmstadt, Germany). mFC plates were prepared by dissolving 52 g of the mFC powder into 1 L of dH_2O water. The solution was boiled for one minute to ensure that it was completely dissolved. Thereafter, 20–25 mL of this solution was poured into each petri-dish and left to solidify. The filter was placed on the surface (at the centre) of prepared mFc agar plates, and thereafter incubated at 44°C and observed for growth after 22 h (Berthe et al., 2013). Respective water samples were inoculated in triplicate, for each of the sampling sites. Observed blue colonies were counted and recorded as colony forming units per mL.

3.3 DIRECT ENRICHMENT OF RIVER WATER SAMPLES WITHOUT PRIOR CONCENTRATION

The technique described by Denis et al. (2011) was applied in this study to isolate *Campylobacter* spp. from the river water samples. Denis and colleagues directly enriched environmental water samples using equal volumes (500 mL, respectively) of the water sample and Bolton Broth enrichment medium and incubated the resulting volume under microaerobic conditions at 37°C for 48 hours. For this study slight changes were made. Briefly, the sample to broth media proportion was changed to 10 mL: 90 mL, this decision was based on the conclusion that Denis's team made, which suggested that using an equal volume of sample and enrichment broth, and a volume as large as 500 mL during the enrichment stage, might lead to excessive background growth. The microaerobic condition was created by using CampyGen compact sachets. Another change was the temperature used for incubation. The incubation temperature for this study was 42°C, but the incubation period remained the same 48 hours used by Denis et al. (2011). Our broth was prepared following the manufacturer's instructions and supplemented with 5% lysed horse blood bought from Food Chain Laboratories in Gqeberha.

3.4 CONCENTRATING *CAMPYLOBACTER* SPP. USING COMBINED CENTRIFUGATION AND FILTRATION

A consensus on the superiority of centrifugation method over filtration method (vice versa) has not been reached (Khan et al., 2009). Therefore, for this study each water sample was processed by combining both methods in a single set-up to concentrate *Campylobacter* spp. cells from water samples. As the river water samples of this study contained suspended materials, filtration volume less than 1000 mL (500 mL) was adopted, as recommended by Abulreesh et. al., 2017.

The total volume 500 mL of each water sample was measured using a 500 mL measuring cylinder and transferred to a sterile centrifuge tube. The Precisa: BJ 1000C Precision Balance, 1020 grams x 0.01 grams, was used to balance samples before they were concentrated. The 500 mL was first centrifuged using a Beckman Coulter Avent® J-E 14000 centrifuge (rotter JSE 06A08) at 14,000×g for 30 minutes (Khan et. et al., 2009). Then the supernatant was decanted and collected in a sterile conical flask to separate it from the pellet. The pellet believed to be containing bacteria cells, including *Campylobacter* cells attached to solids was mixed by vortexing using 10 mL of sterile distilled water. The collected supernatant was thereafter filtered through a 0.45µm pore size (47 mm diameter) filter membrane (Millipore) to trap any suspended bacterial cell that was not pelleted

(Vereen Jr. et al., 2013). Both the pellet and filter membrane were used for enrichment. The resuspended pellets were placed together with the filter membranes in 100 mL flasks containing 90 mL of Bolton broth (BB) (UK Environmental agency 2018) and incubated at 42°C in a microaerobic environment for 21 h (Pitkänen et al., 2009a). The inoculum was observed for growth. Because there was no indication of growth after 21 h, incubation period was extended to +/-48 h. A microaerobic atmosphere was created by placing growth media containing both the filter membrane and the solution of the resuspended pellet in an OXOID Anaerobic Jar (2.5L), and CampyGen compact was used in a proportion of one is to two plates/flasks.

3.5 MOORE SWAB SAMPLING METHOD

Moore swabs were prepared in the laboratory using cotton gauze strips following the method described by Sikorski and Levine (2020). Briefly, a 7.5 x 7.5 cm gauze swab was enlarged to rectangular shapes then folded eight times until it formed an 8-ply square pad. The 8-ply square was tied with string, placed in a 150 mL beaker capped by aluminium foil, and autoclaved at 120°C for 15 minutes. The sterilised six swabs were deployed on the sampling sites and the string was used to tie the gauze swabs onto objects (sticks and rocks) heavy enough to prevent the swabs from being washed away by the flowing river. The deployed gauze was left at the sites for 72 h, and then collected by placing into sterile 100 mL flasks with site labels. Flasks were placed in a cooler box containing ice and transported to our lab.

3.5.1 Culturing of *Campylobacter* spp. from Moore swab method

Campylobacter spp. cells were cultured from the swab samples by adding 90 mL of Bolton Broth to the flasks containing the gauze swabs. These were then incubated under microaerobic conditions at 42°C for 48 h as explained previously.

3.6 ENUMERATION OF PRESUMPTIVE *CAMPYLOBACTER* SPP.

The presumptive colonies were enumerated using one quantification method, the plate counts (Truchado et al., 2020). For direct enrichment without prior concentration of water samples, after enrichment 100 µL of enrichment broth culture was collected by pipetting from just below the surface layer of the broth media. The liquid cultures were spread across the whole surface of an mCCDA agar plate by rubbing back and forth using a glass spreader while rotating the plate around four times in different directions. These plates were incubated using the same incubation conditions (microaerobic

at 42°C for 48 h) as during the enrichment stage, and observed for colony growth, at which time suspected colonies were enumerated and recorded (Lévesque et. al., 2011; Diergaardt et. al., 2004; Mughini-Gras et. al., 2016).

For the centrifugation combined with filtration method, the resulting growth from the plate spread of 100 µL broth cultures could not be used for plate counts due to overgrowth of the cultures. Therefore, three-fold dilution factors (10^{-1} , 10^{-2} and 10^{-3}) were prepared by diluting broth cultures using 1 mL of the enrichment cultures. This volume of enrichment cultures was transferred into a test tube containing 9 mL of distilled water, then a second dilution factor was prepared by pipetting 1 mL from the first dilution and transferring this to another test tube with 9 mL distilled water. The last dilution was prepared following the procedures used in the first two dilution factors. All these dilution factors were then used for spreading by pipetting 100 µL of the resulting solution onto the surface of Modified charcoal cefoperazone deoxycholate agar (mCCDA) agar plates, spreading across the whole surface of a plate using a glass spreader. The plates were incubated using the same conditions (microaerobic environment at 42°C for 48 h).

For the Moore Swab method, the resulting growth was used for colony counts without having to further dilute the broth cultures. The diluted broth culture produced no growth so it was decided to directly plate the enrichment cultures without diluting prior plating out.

mCCDA agar plates indicating statistical reliability (plates with colonies not less than 30) and those that were not difficult to accurately count individual colonies (not greater than 350 colonies) (Ben-David & Davidson, 2014) were considered for enumeration of presumptive *Campylobacter* spp.. Only 10^{-1} and 10^{-2} factors could produce growth that can be considered in enumeration of colonies. The counts were then presented as colony-forming unit per millilitre (CFU/mL). These colonies were then sub-cultured in prepared fresh mCCDA and Colombia Agar Base European Pharmacopoeia, USP (ISO 10272) to obtain pure cultures. Thereafter, suspected *Campylobacter* spp. colonies were subjected to identification by Gram's staining.

3.7 GRAM'S STAINING

Smears of bacterial cultures were prepared for Gram's staining by using a sterile metal inoculating loop to pick one to three colonies growing on the surface of the mCCDA, Colombia Agar Base European Pharmacopoeia, USP (ISO 10272) agar. The loop was first sterilized by dipping the wire part of the inoculating loop into 75% prepared ethanol and burning using a Bunsen burner. The

loopful of colonies was placed on a slide with a drop of sterile distilled water. The Gram's procedures were performed using Gram's staining reagents on the prepared smears. The structure of cells present in the prepared smear were then examined using light microscope using the 100X object (Mughini-Gras et. al., 2016) and pictures taken using an Olympus BX51 microscope.

3.8 STATISTICAL ANALYSIS

Two-factor ANOVA without replication with alpha level (0.05) was conducted where the factors were the recovery methods (with 3 levels) and sampling sites (with 6 levels). Using the two-factor ANOVA, differences in means due to the recovery method, sampling sites or whether there was a difference in outcomes by the combination or interaction of recovery methods and sampling sites, were assessed. The impact of physiochemical parameters on each method was analysed using the same test (Otero et al., 2007). Correlations coefficients were computed with the Spearman rank correlation tests to measure the relationship between recovered *Campylobacter* (Cfu / mL) and the different physiochemical parameters, and the significance level (P-value) of 0.05 was used. Statistical analysis was performed using Microsoft Excel 2016.

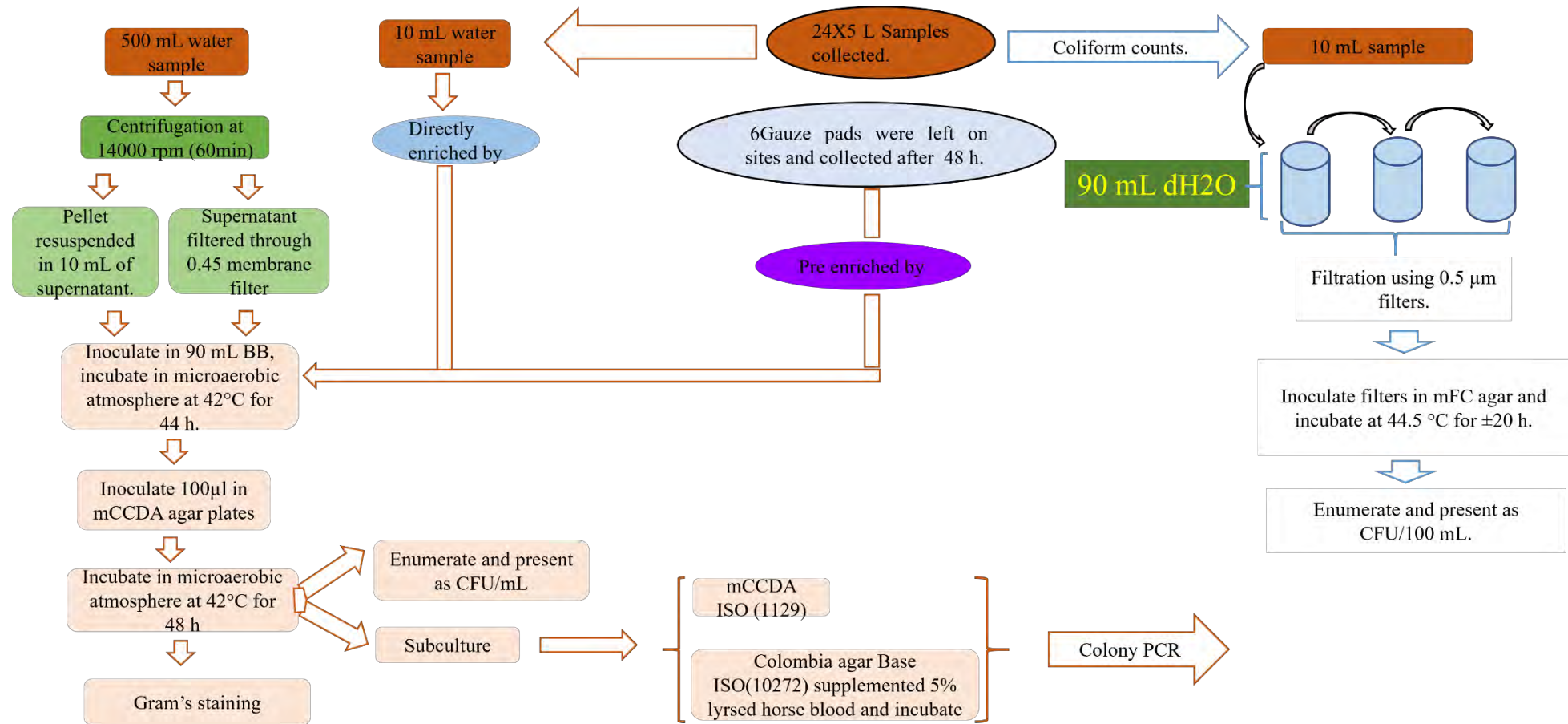


Figure 3.1 Flow diagram demonstrating isolation of *Campylobacter* spp. from river water samples. Samples were collected by grab and catch and continuous flow filtration (Moore Swab) methods. Catch and grab samples were analysed by concentrating and enriching directly to Bolton Broth. Moore Swabs (peds) were enriched by the same conditions used for grab and catch samples. Coliform counts were enumerated from the collected sample

3.9 RESULTS

3.9.1 Measurement of physicochemical parameters of the river

Figure 3.2 shows the physicochemical characteristics and faecal coliform concentrations of water samples. A high conductivity (EC) was observed for all sampled sites except for Site 1. Regarding Turbidity (NTU), Site 6 had the highest recorded value at 8.5, followed by Site 5. A high turbidity was also recorded at Site 2 (303) followed by Site 3 (287). The control site, Site 1, had low turbidity compared to all the experimental sites. Turbidity of the river ranged from 2.48 to 303 NTU. The highest temperature recorded in this study was measured at Site 3 (24.1°C), while the lowest temperature was 11.9 °C in two sites (Site 2 and the control Site 1).

Table 3.1 Physicochemical Parameter values of Bloukrans River measured during the sample collection.

Physicochemical parameter	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6
EC (µS/cm)	1234 - 1961	1043 - 1638	1226 - 1663	1272 - 1776	1281 - 1835	1541 - 1980
Turb (NTU)	2.48 - 15.8	9.4 - 303	5.6 - 287	4.74 - 20.3	5.09 - 14.86	2.6 - 8.5
Temp (°C)	11.9 - 20.5	11.9 - 21.3	12.4 - 24.1	13.1 - 22.4	12.7 - 22.6	14.2 - 20.2
pH	5.7 - 8.33	5.77 - 8.37	3.45 - 7.4	5.8 - 7.4	6.42 - 7.79	6.1 - 7.2
DO (mg/L)	3.4 - 9.98	3.4 - 5.3	0.68 - 4.98	0.63 - 4.98	1.59 - 9.62	6.3 - 11
Coliform (CFU/mL)	0 - 0	22 - 165	42 - 362	113 - 285	15 - 242	54 - 245

Note* The measured physicochemical values are recorded from a lowest-to-highest value that was measured per site.

The most acidic value (3.4) was recorded at Site 3. The most alkaline value was recorded at Site 2 (8.7). This is followed by Site 1 (8.3). Dissolved Oxygen (DO) is the measurement of the quantity of the dissolved oxygen in the water. The site with the highest concentrations of dissolved oxygen was Site 6 (6.3–11 mg/L) and the lowest concentrations were observed at Site 4. The overall range of dissolved oxygen concentration for the river was 0.63–11(mg/L). In terms of faecal coliform, Site 3 had the highest counts (362 x10⁻³ CFU/mL) followed by Site 4 (285 x10⁻³ CFU/mL), Site 6 (245 x10⁻³ CFU/mL), Site 5 (242 x10⁻³ CFU/mL) and Site 2 (165 x10⁻³ CFU/mL). The control site had no growth. Faecal coliform counts for the studied river ranged from 22x10⁻³ to 362x10⁻³ CFU/mL.

3.9.2 Colony morphology and counts of mCCDA plates obtained by direct enrichment of water samples to recover *Campylobacter* spp. from Bloukrans River.

The mCCDA agar plates inoculated with broth cultures grew distinct round moist non-metallic grey colonies and exhibited a medium growth, as shown in Figure 3.3. Counts were presented as colony-forming units per millilitre (CFU/mL) to estimate the number of viable cells present in each tested sample.



Figure 3.2 Photograph of a mCCDA agar plate demonstrating colony morphology of the growth recovered from the first attempt at plating out of broth cultures grown from directly enriched isolation. Grey moist raised non-metallic colonies were observed.

3.9.3 Colony morphology and counts from mCCDA plates obtained by concentrating water samples through centrifugation followed by filtration to recover *Campylobacter* spp. from Bloukrans River.

The growth that resulted from plating out directly from the enrichment broth for this method was too numerous to count (Figure 3.4). However, following serial dilutions, it became possible to produce statically reliable number of colonies which were countable and usable for enumeration (Figure 3.5). Round moist white cream/yellow colonies were observed, counted, and presented as forming units per millilitre (CFU/mL).



Figure 3.3 Photograph of a mCCDA plate demonstrating colony growth which resulted from first attempt at plating out of broth cultures of centrifugation prior to the supernatant filtration method. The growth exhibits a very highly concentrated growth, no distinct colonies were formed.

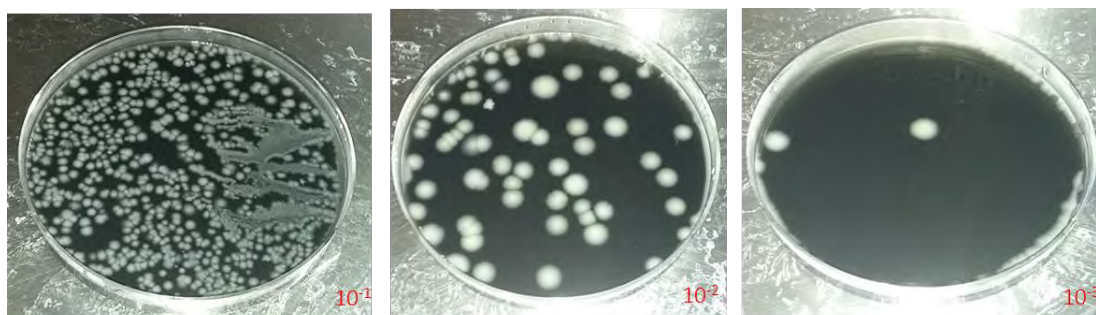


Figure 3.4 Photograph of mCCDA agar plates demonstrating colony growth after plating out using a diluted broth culture on mCCDA agar. Distinct raised, moist, white cream/yellow colonies were observed after broth cultures were diluted. From left is the highest dilution (10^{-1}), second dilution (10^{-2}) and the lowest dilution factor (10^{-3}).

3.9.4 Colony morphology and count for mCCDA plates obtained using Moore Swab sampling method to recover *Campylobacter* spp. from Bloukrans River water.

Figure 3.4 shows the morphology of the colonies obtained using the Moore Swab method. Results demonstrate that morphology of the colonies differed from site to site. Site 2 colony morphology is moist, grey, irregular, and flat, while Site 3 morphology shows raised, moist, cream colonies. Site 6, produced raised, moist, cream white colonies.



Figure 3.5 shows a photograph of mCCDA agar plates demonstrating morphology and different *Campylobacter* spp., growth concentrations obtained using the Moore Swab method. The numbers on the photos are the site numbers from which the samples were collected. Only photographs of plates that exhibited statically reliability are shown.

3.9.5 Comparative results of colony count for mCCDA plates obtained using different methods to recover *Campylobacter* spp. from Bloukrans River water.

As demonstrated in Figure 3.7, the results from data analysis illustrate that each *Campylobacter* spp. recovery method differed by site. The results for Sites 2, 4 and 6, shows that the method of recovering *Campylobacter* cells by concentrating the river water by centrifugation to collect the pellets, followed by filtering the supernatant to trap any remaining suspended cells yielded the highest count of presumptive *Campylobacter* colonies (265, 273 and 341 CFU/mL), compared to Sites 3 and 5 (100 and 245 CFU/mL). However, for Sites 3 and 5, the method of recovering *Campylobacter* spp. cells by direct enrichment method yielded a higher number (167 and 252 CFU/mL) of presumptive *Campylobacter* colonies than the centrifugation combined with centrifugation method used (167 and 252 CFU/mL).

At all the studied sites, the Moore Swab Method yielded the lowest count (47, 49 and 161 CFU/mL) of presumptive *Campylobacter* colonies, compared to the other methods. No growth was observed on mCCDA plates for water samples collected from Site 1 (control site), which was not surprising given that it is pristine and without *Campylobacter* contamination.

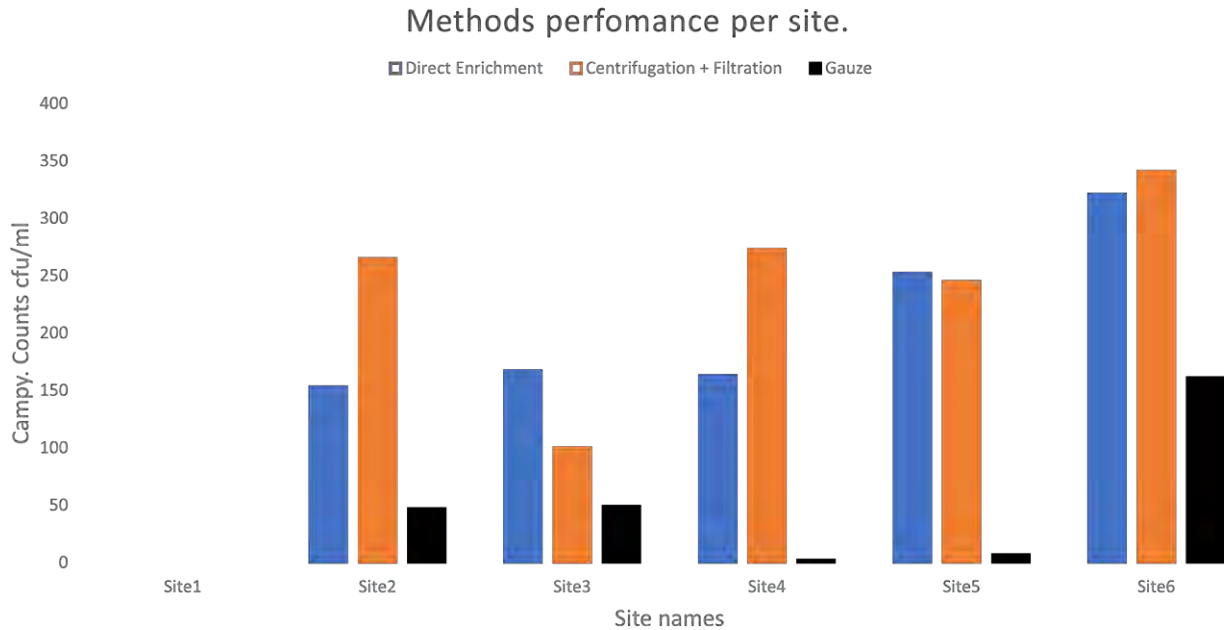


Figure 3.6 Graphical representation of *Campylobacter* spp. counts (CFU/mL) in the Y-axis of all sampled sites for both the directly enriched (indicated by Blue Bars), centrifugation prior to filtration of supernatant (represented by Orange Bars) and Moore Swab Method (represented by Grey Bars) vs the site names on the X-axis from which samples were collected. The graph was plotted using averaged *Campylobacter* spp. counts recovered by each method per sampling site.

The two factor ANOVA without replicate outcome indicates that the performance of investigated methods is significantly different to each other per site with a P-value 0.008 which is lesser than the alpha level (0.05). Furthermore, Spearman's rank correlation analysis showed that water temperature is moderately, negatively ($r = - 0.58$) but not significantly ($P < 0.05$) correlated to the recovered *Campylobacter* CFU/mL in water samples (Table 3.2).

Table 3.2 Correlation of different physiochemical parameters with the actual recovered *Campylobacter* spp. CfU/mL

	Turb (NTU)	Temp (°C)	pH	EC (µS/cm)	DO (mg/L)	Average <i>Campylobacter</i> recovered
Turb (NTU)	1					
Temp (°C)	0.402791	1				
pH	-0.3488	-0.51202	1			
EC (µS/cm)	-0.99249	-0.50083	0.382707	1		
DO (mg/L)	-0.67837	-0.601	0.277657	0.737308038	1	
Average <i>Campylobacter</i> recovered	-0.13677	-0.58089	-0.04385	0.167909565	0.133183	1

On the other hand, Spearman's rank correlation analysis showed that the other physiochemical parameters, including Turbidity, pH, DO and EC weakly correlated to the actual recovered *Campylobacter* CFU/mL in water samples ($r < 0.25$).

3.9.6 Isolation rates using the different recovery methods.

For each of the methods, the percentage of the total number of collected water samples which tested positive for presumptive *Campylobacter* spp. based on the colony morphology, cell counts is illustrated in Figure 3.8. Isolation rates using the different methods were different. 11% of the samples tested were positive for *Campylobacter* spp. using the Moore Swab, 42% using direct enrichment, and concentration by centrifugation followed by filtration yielded 47%. This means that the centrifugation combined by filtration method is more effective in the isolation of *Campylobacter* spp. from river water samples.

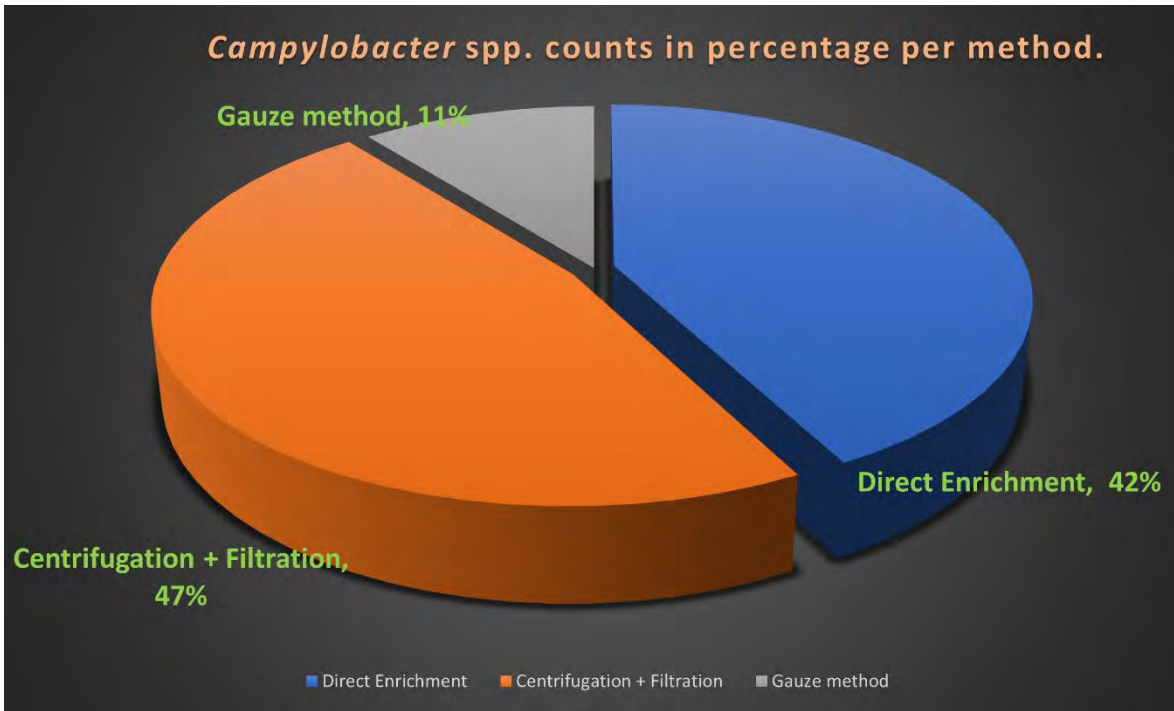


Figure 3.7 Comparison of *Campylobacter* spp. detection rates for the different methods that were used for recovery of *Campylobacter* spp. from water. The lowest of *Campylobacter* spp. concentrations was observed from Moore Swab and centrifugation prior filtration outperformed the three methods, followed by direct enrichment.

3.9.7 Results from seeding presumptively identified *Campylobacter* spp. colonies on the primary selective media to obtain pure cultures.

Figure 3.9 shows an mCCDA agar plate of pure *Campylobacter* spp. culture obtained by streaking technique by picking distinct colonies and streaking on a freshly prepared mCCDA agar plate. Single or individual round moist white colonies could be achieved by streaking on all the quadrants of the petri dish.

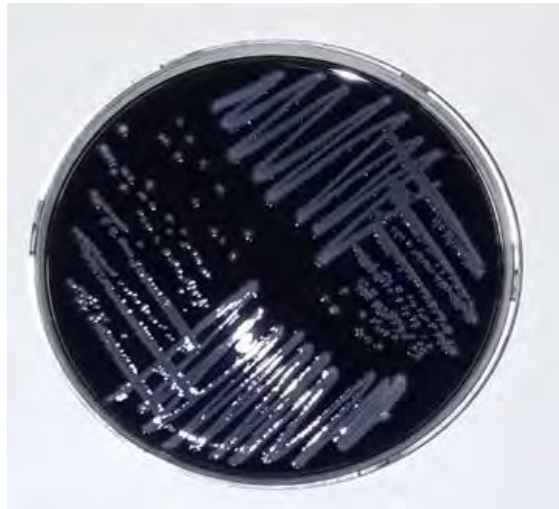


Figure 3.8 Photograph of *Campylobacter* spp. colonies subcultured by discontinuous streaking on a prepared mCCDA agar plate. Pure distinct moist single grey-white colonies grew on the second quadrant of the petri dish.

On the other hand, Figure 3.9 shows a plate of pure *Campylobacter* spp. culture obtained by the streaking technique by picking distinct colonies from an mCCDA agar plate and streaking on a freshly prepared Columbia base agar plate supplemented with lysed horse blood. Single or individual round moist cream colonies could be achieved by streaking on the first quadrant of the petri dish, whereas no growth was observed on the second or the third quadrant of the petri dish. The resulting cultures from both plates are amenable for downstream analyses.



Figure 3.9 Photograph of *Campylobacter* spp. colonies subcultured by discontinuous streaking on prepared Columbia Agar Base plate supplemented with lysed horse blood demonstrating presumptive *Campylobacter* spp. Distinct round, moist, cream colonies grew on the first quadrant of the petri dish. No growth was observed on the second and third quadrants.

3.9.8 Results from Gram staining identification of suspected *Campylobacter* spp. colonies

Pink rods, spiral, and curved *Campylobacter* spp. cell structures were observed for colonies picked from plates of different methods used to recover *Campylobacter* spp. from water samples. The tested samples contained *Campylobacter* spp., and this means that the tested methods were successful in detecting *Campylobacter* spp. (Figure 3.11).

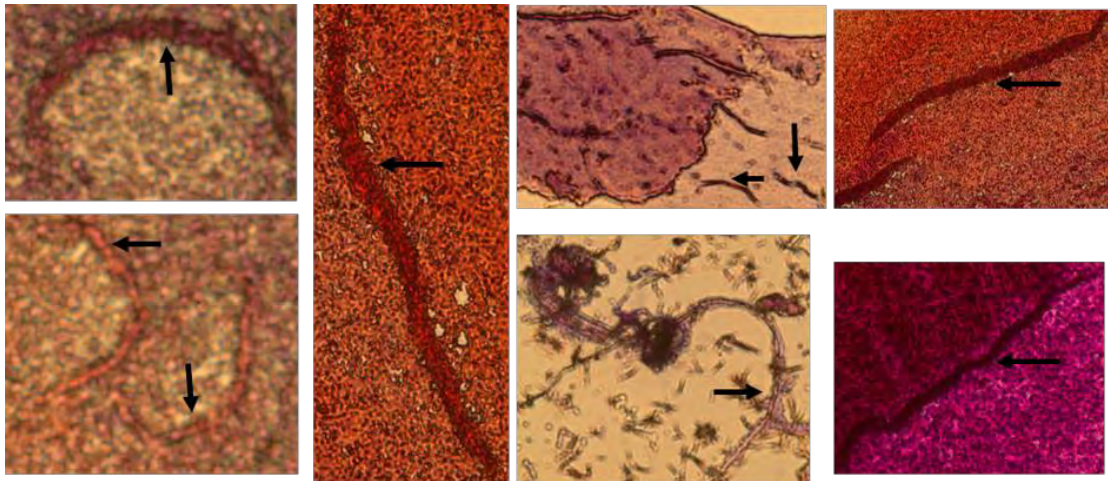


Figure 3.10 Microscopic image of Gram stain for randomly selected colonies from mCCDA plates, obtained by culturing *Campylobacter* cells from water samples processed by the three different recovery methods. Magnification:1,000.

3.10 DISCUSSION AND CONCLUSION.

The physiochemical parameter data collected for the study show that the pH of water samples collected from Bloukrans River ranged between acidic (3.45 to 6.42) and alkaline (7.2 to 8.74) across the sampled sites in this study. This result suggests that *Campylobacter* spp. should be able to thrive in this river, that is given that the range of pH measured at different sites of the river can be tolerated by *Campylobacter* spp. *Campylobacter* bacteria require pH 4.9 -9.0 to survive. But this pathogen has acquired the ability to adapt to severe environmental conditions. Besides, *Campylobacter* spp. exhibit differences in survival patterns, attributed to the high genetic diversity among *Campylobacter* spp, and consequently interspecies differences in stress response genes (Elmonir et al., 2022). For example, *C jejuni* are less severely affected under extreme acidic condition and can survive up to 3 days, compared to *C upsaliensis*. Besides, it has been suggested that in water related environments, water acts as buffers increasing the survival of pathogenic *Campylobacter* in extremely acid conditions (Elmonir et al., 2022; Koenraad et al., 1997).

It was expected that Turbidity as high as 303 NTU and 287 NTU, which were measured at Sites 2 and 3, respectively, should have led to higher isolation of *Campylobacter* spp. at these sites. This is because turbidity results from a high concentration of dissolved particles, which in turn provides nutrients for the growth of microorganisms/bacteria present in the river (Cai et al., 2021). However, more *Campylobacter* spp. was recovered at Site 6, a site with low turbidity average value of 5.5517 and high oxygen average value of 8.2917. An explanation for this is that the growth requirements of the organism could vary under environmental conditions.

DO values (0.63–9.98 mg/L) obtained from river shows that the dissolved oxygen concentrations were very low except for Site 6 which recorded range of 6.3–11(mg/L). *Campylobacter* spp. is known for its oxygen intolerance (Oh et al., 2015). Their growth is negatively impacted by the presence of high oxygen concentrations in their environment (Kaakoush et al., 2007). This is why growing *Campylobacter* spp. requires a controlled oxygen environment. Interestingly, Site 6 which had highest ranges of dissolved oxygen concentration compared to any other sampled sites contained higher concentrations of *Campylobacter* spp. for all collected samples and all three different isolation methods. Site 6, which is located downstream, is mostly contaminated by human activities and wild birds. This might be an indication that most *Campylobacter* spp. contamination at Bloukrans River is by humans and wild birds.

Organisms living in water, including bacterial population, cannot tolerate high levels of conductivity (Banihashemi et al., 2017). Conductivity of 200 to 1000 $\mu\text{S}/\text{cm}$ is the normal level for rivers. Conductivity of 1000 to 10,000 $\mu\text{S}/\text{cm}$ is very high, indicating saline conditions. Industrial pollution is the major contributor to high conductivity in water. This study recorded very high conductivity for all sampled sites except for the control site. This means that the microbial population of the river, which includes *Campylobacter* spp., would have been affected by the presence of these high salts. The *Campylobacter* spp. counts for all sampled sites was not influenced by physiochemical parameters. No relationship was observed between *Campylobacter* spp. occurrence and the water quality parameters. These results comply with those found by Jokinen et al., 2019 and Van Dyke et al., 2010a. In their studies *Campylobacter* spp. occurrence was not related to physiochemical parameters. This observation suggests that their presence might have been influenced by other factors known to impact the presence of *Campylobacter* spp. in water. These other factors include precipitation before sampling, and time between depositing in the river and sampling period (Rechenburg & Kistemann, 2009 ; Guy et al., 2018).

The moderate negative correlation (Spearman coefficient of -0.5) between *Campylobacter* and water temperature, observed in this study is in agreement with previous studies (Thomas et al. 2002; Vereen et al. 2007; Rodríguez and Araujo 2010). On the other hand, observed weak correlation ($r < 0.25$) between the other physiochemical parameters, turbidity, pH, and DO and EC and the actual recovered *Campylobacter* CFU/mL in water samples cannot be generalised. Several studies have reported conflicting results regarding the correlations between the *Campylobacter* in rivers and these parameters. Some studies have found a correlation between dissolved oxygen and presence of *Campylobacter* in environmental waters (Rollins and Colwell 1986; Shagieva et al. 2021) while others have not (Shagieva et al. 2021). In the same way, some studies have found strong positive correlation between turbidity and presence of *Campylobacter* in environmental waters (Azhdarpoor et al. 2019; Olalemi et al. 2020, 2021), which is opposite of findings in this study ($r = -0.14$).

It is notable that *Campylobacter* spp. recovery for each method differed by site. This suggests that each site sampled possessed different levels of pollution, and that the *Campylobacter* counts from each method can be impacted by the level of *Campylobacter* pollution at a river site.

The method of recovering *Campylobacter* cells by concentrating samples by centrifugation to collect the pellets, followed by filtering the supernatant to trap any remaining suspended cells, produced the highest percentage recovery of collected water samples. This could be because the method combined two isolation techniques, centrifugation and filtration in one procedure. The principle of centrifugation involves pelleting down particles based on their molecular weight (Frohlich et al., 2012). Therefore, based on their molecular weight, certain bacterial cells would be forced down alongside with solid particles. The solid particles may have also had other bacterial cells trapped or attached to them in the river. However, some bacteria might remain in the liquid phase of the sample, which could also be collected as a residue on a filter membrane using filtration. Filtration separates particles based on their size and the pore size of the used membrane (Ghugare et al., 2017). Altogether, based on the principle of these two methods, it becomes possible to trap the cells both in the pellets and in the supernatant as residue on a membrane filter. Therefore, by using both the pellet from centrifugation and the residue on the filter membrane for enrichment, the number of recoverable cells increased. This explained why this method recovered more *Campylobacter* spp. from the water samples.

It is not surprising that at all the sites, the Moore Swab Method yielded the least count (47, 49 and 161 CFU/mL) presumptive *Campylobacter* colonies, compared to the other methods. According to the literature, this method did not perform well in isolating *Campylobacter* spp., when compared to the filtration method (El-Sherbeeney et al., 1985). The isolation rate of 11% for all samples collected

using this method is in line with the results obtained by Meinersmann et al., 2020, whose work yielded 9% *Campylobacter* spp. These results also concur with those of Fernandez et al., (2003), who found few of the samples collected using this method to be *Campylobacter* positive.

Based on the results from this chapter, it can be concluded that processing river water samples by centrifugation followed by membrane filtration is the most promising method for recovering *Campylobacter* spp. from river water samples, and that the sampling site at the river influences *Campylobacter* spp. recovery by each method.

CHAPTER 4: MOLECULAR IDENTIFICATION AND CONFIRMATION BY SANGER SEQUENCING OF PRESUMPTIVE *CAMPYLOBACTER* COLONIES

4.1 INTRODUCTION

The standard method of detection for *Campylobacter* spp. in water is enrichment in a selective liquid medium, with different incubation temperatures and duration (Rechenburg and Kistemann, 2009; Vereen Jr et al., 2013; Hokajärvi et al., 2013; Mughini-Gras et al., 2016; Abulreesh et al., 2017; Guy et al., 2018). This is followed by microaerobic incubation at 42°C for at least 48 h to enable recovery, then subculture onto selective solid media, and assessment for colonies deemed to be typical of *Campylobacter* spp. Confirmation of the colonies as *Campylobacter* species is conducted using morphological, biochemical and growth property tests. However, morphological, biochemical and growth property tests present disadvantages, such as frequent false positive results. In addition, it is common for other contaminating bacteria to grow simultaneously alongside the target *Campylobacter* spp, on the selective solid media (Kim et al., 2016).

Molecular techniques are more specific and sensitive than the conventional morphological, biochemical and growth property tests for confirmation of presumptive *Campylobacter* spp. (Nwankwo et al., 2017 ; Nwankwo et al., 2016), and reduce the occurrence of false positives. Specifically, DNA-based methods for the detection and confirmation of different bacteria are now available. Polymerase chain reaction (PCR) is a specific nucleic acid amplification technique for the detection and confirmation of bacterial pathogens. Many PCR methods have been developed for the detection and confirmation of *Campylobacter* spp. These are based on 16S rRNA, or the target gene such as flagellin (flA), or 16S/23S intergenic space regions (Elbrissi et al., 2017 ; Delpiazzo et al., 2021). The different PCR methods include conventional PCR (Nwankwo et al. 2017), nested and semi-nested PCR (Bharathi et al., 2013), reverse transcriptase RT(Sails et al., 1998), real-time / quantitative PCR (Lanzl et al., 2022) and multiplex qPCR (Frasao et al., 2017).

Chapter 3 described how presumptive colonies were obtained by plating *Campylobacter* cells using different recovery methods. This chapter outlines the confirmation of the presumptive *Campylobacter* spp. colonies using conventional PCR. The presumptive colonies assayed here were obtained using the method of recovering *Campylobacter* cells by concentrating the river water by centrifugation to collect the pellets, followed by filtering the supernatant to trap any remaining suspended cells. The

PCR method is based on the genotypic characteristics of bacteria, which is not based on the expression on different culture media. Therefore, the method can be used without any subculturing. To speed up the confirmation step, the PCR was conducted for the confirmation of colonies directly from selective plating media (mCCDA). The presumptive *Campylobacter* spp. colonies grown for +/-48 h on mCCDA agar were analysed. PCR was conducted to confirm colonies subculture in Columbia Agar. A positive control was also prepared for the optimization of PCR conditions and to test for the efficiency of primers to be used in the identification of colonies in river water samples. The positive control used was an enrichment broth inoculated with an already identified organism (*Campylobacter jejuni*), from the Food Chain Laboratories in Gqeberha, Eastern Cape.

4.2 METHODS AND MATERIALS

4.2.1 Isolation of *Campylobacter* spp. from the positive culture purchased from Food Chain Laboratories

100 µL of the positive culture, an enrichment broth already inoculated with *Campylobacter jejuni*, was spread on the surface of our prepared CCDA agar plate and incubated using a Labcon Low Temperature incubator under the same conditions used for growing *Campylobacter* spp. from the river water samples. This was then incubated under microaerobic conditions at 42°C for 48 h using Oxoid CampGen (the final atmosphere reduced to 6% oxygen and 14% carbon dioxide).

4.2.2 Colony PCR of presumptively identified colonies.

Colonies grown on mCCDA agar plates were picked for this preparation by means of a sterile wooden toothpick. One distinct colony grown on mCCDA agar plates was picked and transferred into 100 µl of sterile distilled water in a sterile 1.5 mL Eppendorf tube. Then using a Labnet's Vortex Mixer VX-200, the mixture was vortexed to completely suspend colonies in water. This was followed by boiling for 10 min at 100°C on a heating block and the solution was allowed to cool. Thereafter, the suspensions were micro-centrifuged at 12,800 rpm for 5 min using an Eppendorf Centrifuge MiniSpin®, 230V/50-60Hz with lid. The supernatant was collected into new sterile 1.5 mL Eppendorf tubes. The quantity (A260) and quality (ratio of A260/A280) of DNA was measured using a Thermo Scientific NanoDrop 2000 spectrophotometer, and concentrates were stored at -20°C for use. The supernatant was used as template for PCR.

A 5 µl aliquot of the boiled bacterial supernatant was used for the PCR assay. Reactions were carried out in a 50 µL reaction total volume (5 µL of the DNA template, 25 µL master mix, 2 µL of the forward and reverse primers, and 16 µL of nuclear free water), using a SimpliAmp Thermo Cycler™ (Applied Biosystem™). PCR conditions were, initial denaturation at 94°C for five minutes, 30 amplification cycles of denaturation (one minute at 94°C), annealing (one minute at 58°C), and extension (one minute at 72°C), followed by a final extension step for 10 minutes at 72°C (Savill et al., 2001). Since the colony PCR was positive for 16S primers (results not shown), PCR primer HIP400F 5'-GAA GAG GGT TTG GGT GGT G-3'; HIP1134R 5'-AGC TAG CTT CGC ATA ATA ACT TG-3' targeting the 735 bp of the hip gene specific to *C. jejuni* were used to test for the presence of *C. jejuni*. The justification for using this *C. jejuni* specific primers is due to their predominance as a waterborne pathogen compared to other species and their ability to survive better in aquatic environments (Reddy & Zishiri, 2018). Besides, *Campylobacter jejuni* is commonly isolated in human infection cases, and given that humans interact with the study river, it was expected to be found in the river. The presence and expression of the N-benzoylglycine amidohydrolase (hippuricase, hipO) enzyme is used to differentiate *Campylobacter coli* from *Campylobacter jejuni*, as only *Campylobacter jejuni* express the gene (Caner et al., 2008). For each PCR run, a negative control (5 µl of distilled water) was prepared.

4.2.3 Gel electrophoresis of the colony PCR Amplicons and Sanger sequencing

The verification of the PCR products through gel electrophoresis was conducted to test for the presence of DNA fragments of targeted size. This was carried out by resolving them in 1% agarose gel stained with 5 µL of 10 mg/µL ethidium bromide and inspecting under a short-wavelength UV light source. DNA fragments migrated through the gel as separate bands of varied sizes.

Agarose gel electrophoresis was prepared by weighing a 1 g mass of Agarose powder, transferred into a 500 mL flask containing 50 mL of 100 mL (1X TAE) mixing by gently shaking. The remaining 50 mL (1X TAE) was added to make a 100 mL total volume. The contents were heated up in a microwave for 2–3 minutes until the powder had dissolved and was transparent. Thereafter the

solution was allowed to cool to at least 42°C, and then 6 µl of Ethidium bromide (10 mg/mL) was added to the solution and poured into a gel dock, with a comb in place. After the solution set, the comb was removed, and the gel was placed in a gel station. The gel station was filled with 1X TAE solution, 2 cm above the gel. A mixture of the 5 µl PCR product and 1 µl 6x loading dye were added into the wells. 6 µL control solution and 6 µL of the ladder solution (Quick load purple 1 kb DNA Ladder) were also added in respective wells. The Electrophoresis machine was run for 45 minutes at 90 volts. Thereafter, the gel was placed in a UV machine (molecular imager ChemiDoc™ XRS+, supplied by BIO-RAD) to capture its image.

The DNA bands of expected size +/-735 bp, which is the size of the target gene, were cut from gel slabs and purified using a NucleoSpin® Gel and PCR Clean-up Kit, following the kit instructions, to obtain quantity and quality DNA for sequencing by eliminating salts and other PCR reagents. Thereafter, 15 µl of the purified amplicons was sent to Inqaba Biotec (Pretoria, South Africa) for Sanger sequencing to determine sequence of the DNA. The sequences generated from Sanger sequencing were arranged using the ABI base recall web program link: https://pasteurmaroc.shinyapps.io/abi_base_recall_app/ (Elyazghi et al., 2017). This program covers automatic end clipping options, alignment against reference sequences, and final BLAST in a series of steps. The programme is designed in such a way that for each chromatogram, the ends are clipped based on Phred quality scores, and N peaks are corrected by finding the local maxima at each N site. The advantages of this program are that it increases the rate of identity and coverage. It also minimizes the number of mismatches and gaps, and so provides a solution to sequencing ambiguities and saves time and labour encountered when using other, more manual methods.

The recovered sequence(s) identity was further tested using a phylogenetic tree analysis method to indicate their phylogeny relationship with the reported ones.. The multiple sequence alignment and neighbor joining phylogenetic tree were performed using Unweight Pair Group method with Arithmetic Mean (UPGMA tree) in MEGA 11 version 11.0.13 (Huda, 2016).

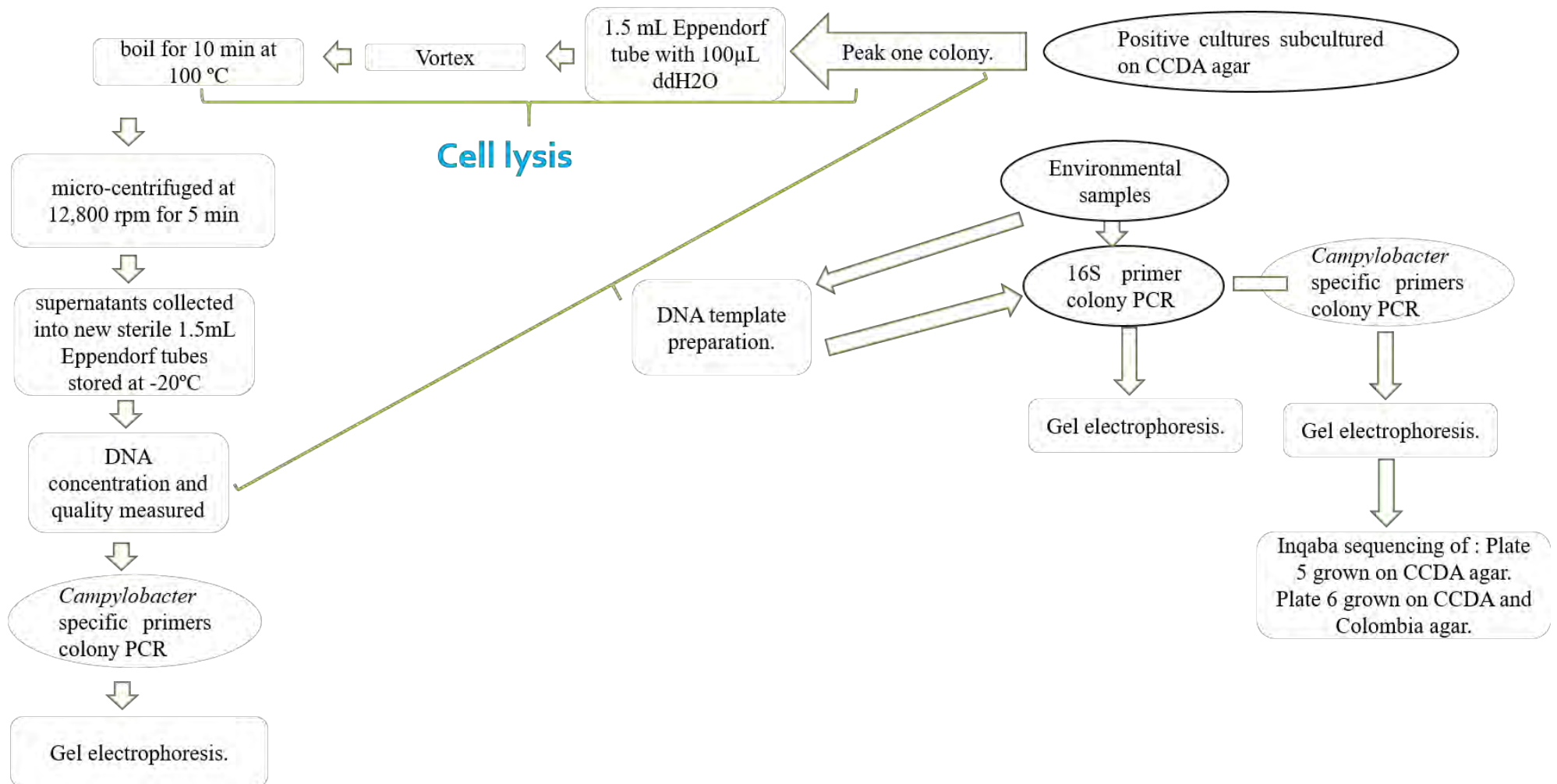


Figure 4.1 The flow diagram demonstrating methods used by this study in DNA extraction, PCR optimisation using positive culture, and use of 16S and *Campylobacter* species specific primers to identify presumptive colonies using colony PCR.

4.3 RESULTS

4.3.1 Colony morphology of the positive culture

Grey metallic round moist colonies were observed on mCCDA plates spread with the positive culture of enrichment broth inoculated with *Campylobacter jejuni* (Figure 4.2).



Figure 4.2 Morphology of colonies on mCCDA plates, obtained by culturing *Campylobacter* cells from positive cultures purchased from Food Chain Laboratories on an mCCDA agar plate surface. Round grey metallic colonies grew on the surface of the agar plate.

4.3.2 Gel electrophoresis of the positive culture

Visible bands were observed after electrophoresis in 1% gel (Figure 4.3). The band size obtained is equivalent to the expected *C jejuni* PCR product (± 735 bp). This means that the primers and PCR conditions used were satisfactory for amplification of *C. jejuni*. It also suggests these primers and PCR conditions are suitable for detecting *Campylobacter* spp. in the river water colonies.

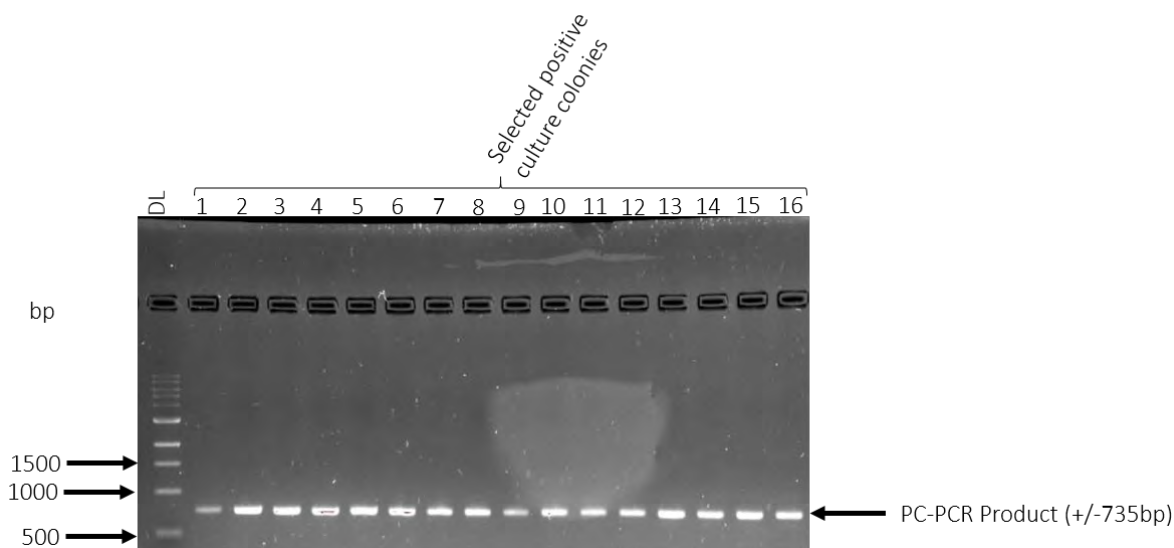


Figure 4.3 Photograph of UV illuminated plate stained with ethidium bromide 1% Agarose gel displaying Positive Culture Amplicons amplified by *Campylobacter jejuni* HIP400F and HIP113R primers. Lane DL: Quick-Load Purple 1kb DNA ladder. Lane 1-16 selected positive culture colonies

4.3.3 Gel electrophoresis of river water colonies using *Campylobacter* species-specific primers

Considering that the colony PCR of the positive culture colonies using *C. jejuni* specific primers produced visible single bands of appropriate size (desirable results), the same conditions were applied to amplify the river water colonies. As represented in Figure 4.4, visible bands were observed for all the colonies directly from the selective plating medium (mCCDA) without subculturing. This is true for water samples from all the experimental sites (Sites 2, 3, 4, 5 and 6). It is notable that migration of multiple bands was visible. There are number of factors that lead to migration of DNA into multiple bands: In some cases, multiple bands would mean that isolated colony and used for the PCR was not a pure culture but mixed and the genus primers was employed to amplify the DNA template; used primers contain very short sequences giving it many possibilities to find and bind to many complimentary bases in multiple regions within the genome thereby producing diverse copies at the same time. In our case, as it is reported in Section 4.3.2, PCR conditions were optimised by trying different annealing temperatures using the positive control. The optimization was done using a positive culture (*Campylobacter jejuni*) and it was concluded that both the primers and PCR conditions would be suitable for amplification of environmental samples. The observed multiple bands from the results of environmental samples can be the indication for the presence of the two *Campylobacter jejuni* subsp. the *Campylobacter jejuni* subsp. *doylei* and *Campylobacter jejuni* subsp. *Jejuni* in the sample. The other factor could be that the used DNA template contained impurities that

can interfere with the PCR reactions. The third factor can be accountable for the size of the primer, the forward primer sequence base is lower than 20 bases making it eligible to binding in many regions within the genome.

Visible single bands were observed for the colonies following subculturing on Columbia agar, as shown in Figure 4.5. It could be that conducting discontinuous streaking of the colonies on Columbia agar gave rise to individual colonies. This could explain why single bands were visible in Figure 4.5.

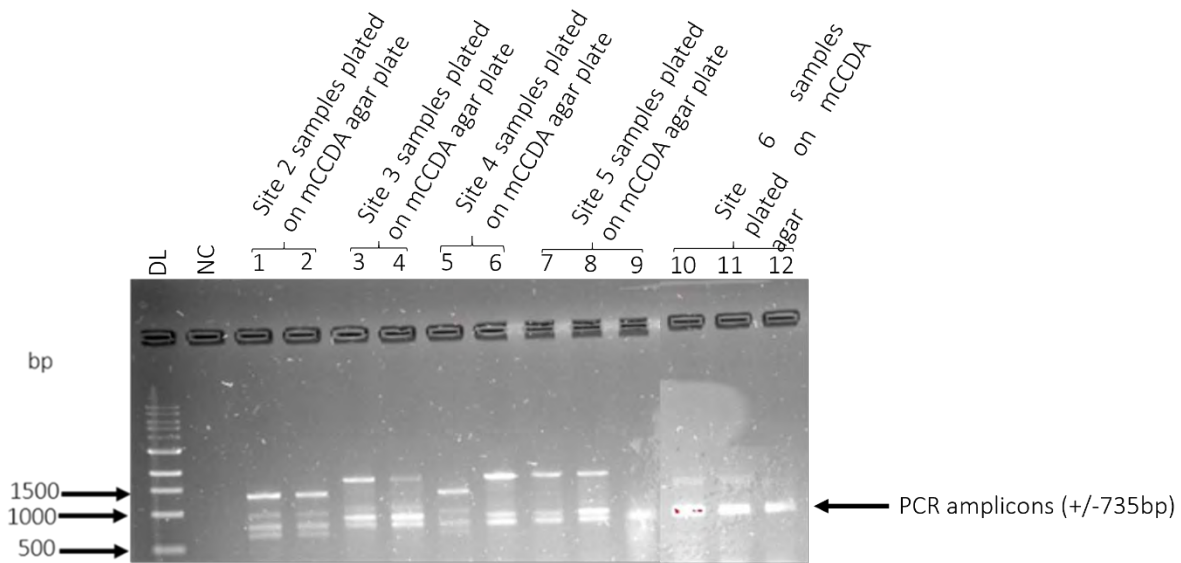


Figure 4.4 Photograph of UV illuminated, stained 1% Agarose gel displaying visible bands for all the colonies directly from the selective plating media (mCCDA) without subculturing. The *Campylobacter Jejuni* HIP400F and HIP113R primer was used. (From Left to Right) Lane 1: Quick-Load Purple 1kb DNA ladder. Lane 2: Negative Control. Lanes 3–14: encompass river water colonies directly from the selective plating media (mCCDA) without subculturing.

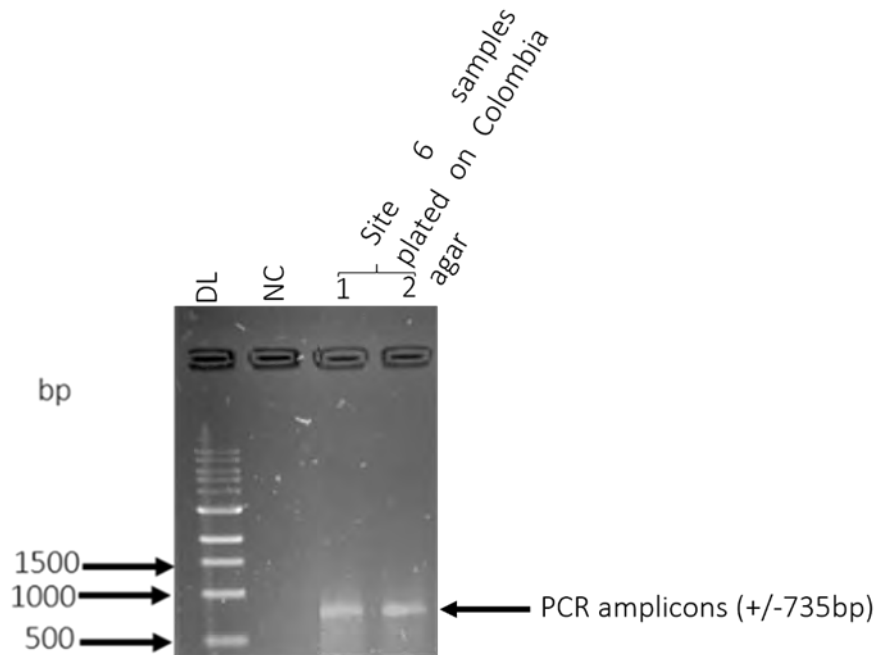


Figure 4.5. Photograph of UV illuminated, stained 1% Agarose gel displaying visible bands observed for the colonies following subculturing on Colombia agar. The *Campylobacter Jejuni* HIP400F and HIP113R primer was used. (From Left to Right) Lane DL: Quick-Load Purple 1kb DNA ladder. Lane ND: Negative Control. Lanes 1–2 show Site 6 river water colonies subcultured on Columbia agar.

4.3.4 Sanger sequence(s) analyses and Sequence Confirmation.

Results were achieved after sequencing the PCR product (+/- 735 bp) of all samples. The test was positive for all samples that were sent for sequencing except for one Site six sample which failed QC and could not be processed (sequenced by Inqaba). In total three samples were sent for sequencing, two from site six (colonies grown on both the mCCDA and Colombia Base agar) and one from site five (mCCDA grown colony). The blast similarity results illustrated that all sequences were comparable to *Campylobacter jejuni* strains. The lowest observed similarity score was from a Site 6 sequence (colony grown on mCCDA), which was 75.5%, and both Site 6 sequences had a similarity of score of 98%.

Figure 4.6 presents the *abi1* sequencing chromatograms of the PCR product of one of the colonies isolated directly from the selective plating media (mCCDA) without subculturing. Chromatograms with sharp separated peaks and no background noise chromatograms were observed. This colony was grown from the Site 6 (mCCDA grown) water sample. The other chromatograms are not shown here because the peaks were barely separated, with possibilities of ambiguities at any point within the sequence and too much background noise (data not shown).

Figure 4.6 is the sequence chromatogram of the PCR product of the colonies isolated following subculturing onto Columbia Base agar by streaking. It can be observed that the sequence and chromatogram is cleaner, with sharper distinct peaks and almost no background noise, compared to those obtained with colonies isolated directly from selective plating media (mCCDA) without subculturing.

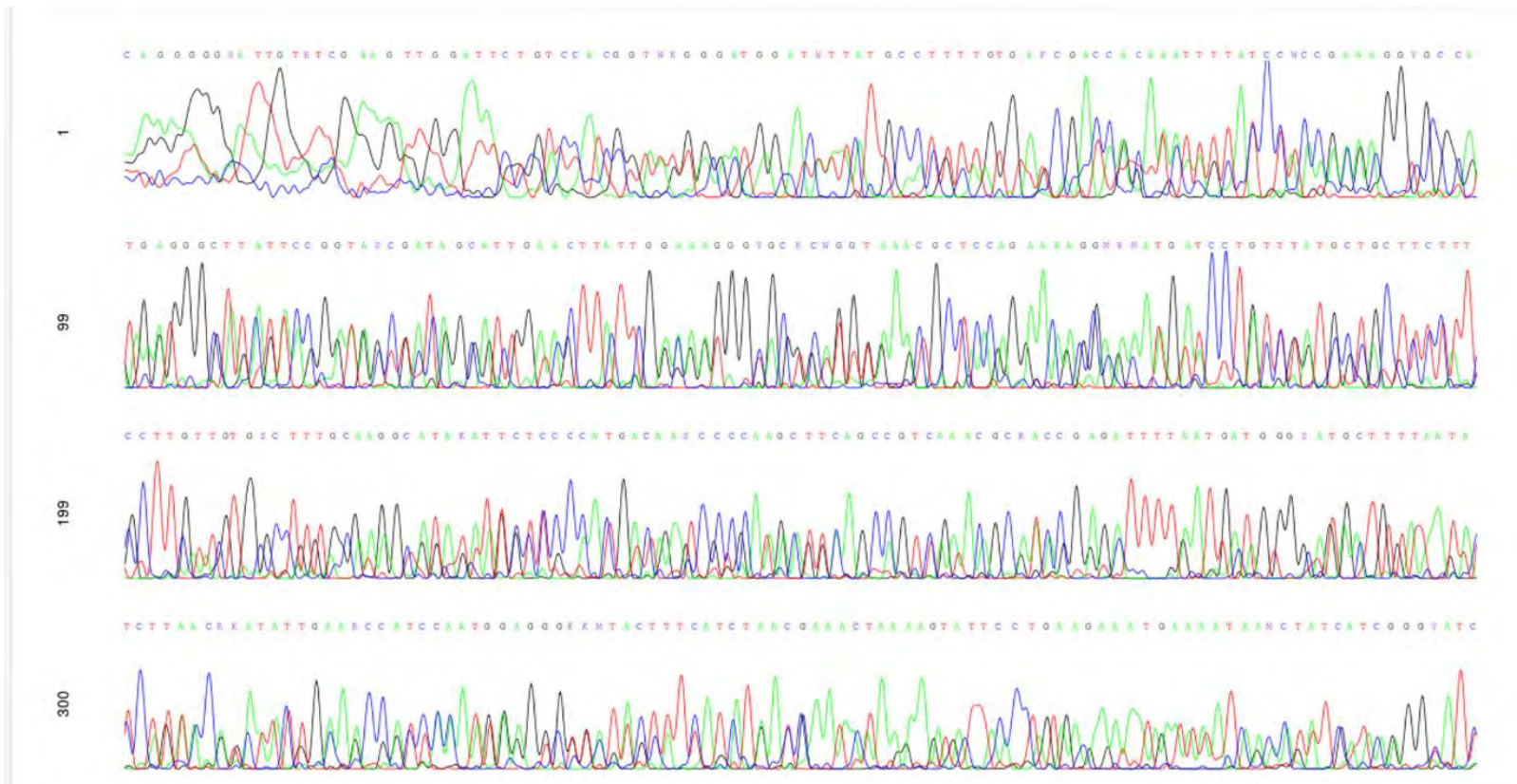


Figure 4.6 The image of ABI Base Call program indicating *abi.1* sequence chromatogram of site six PCR product of the colonies isolated directly from the selective plating media (mCCDA) without subculturing.

The sequence obtained for the PCR product of the colonies isolated directly from the selective plating media (mCCDA) without subculturing showed at least 75% homology with *C. jejuni*, as evaluated by NCBI BLAST. On the other hand, the sequence obtained for the PCR product of the colonies isolated following subculturing onto a Colombia Base by discontinuous streaking showed at least 98% homology with *C. jejuni* as evaluated by NCBI BLAST (Figure 4.8)

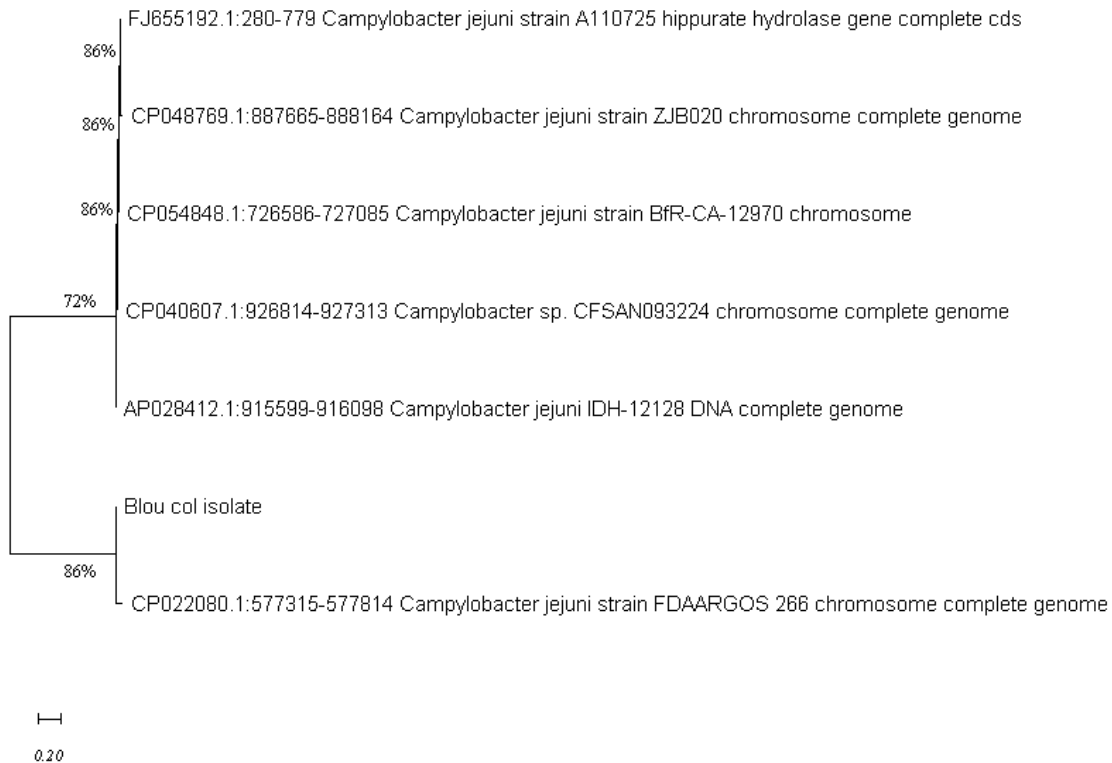


Figure 4.8: The phylogenetic tree analysis of the sequence obtained for the PCR product of the colony (Blou Col isolate) isolated following subculturing onto a Colombia Base agar media using unweight pair group method with arithmetic mean (UPGMA tree) in (MEGA 11 version 11.0.13).

4.4 DISCUSSION AND CONCLUSIONS.

This chapter shows the confirmation of the presumptive *Campylobacter jejuni* colonies that were grown in both the primary selective agar media mCCDA and secondary selective growth agar Columbia Base agar using conventional PCR.

The PCR was used for the confirmation of colonies directly from selective plating media (mCCDA) and those sub-cultured on Columbia Agar. The results from both the positive culture (Figure 4.2) and environmental samples (Figure 4.4) were positive for PCR. The resolution of positive culture DNA segments through the gel indicated the efficacy of the used primers and their conditions, single clearly visible bands were situated where the size of the end product is expected to be found. This was treated as the indication for proper functioning of primers and their conditions. Thus, all the conditions were used for the environmental samples. However, the results shown by the environmental samples were different from what was produced by colony PCR of the positive culture, the multiple band (s) migration of environmental samples DNA was surprising considering that the optimization was considered successful due to results of the test, and that conditions were kept constantly when running the environmental samples.

Both colonies directly peaked from the selective plating media (mCCDA) without subculturing (Figure 4.4), as well as those sub-cultured using Columbia Agar (Figure 4.5) produced visible bands. However, unlike for the colonies from Columbia Agar, multiple bands are visible on the gel for the colonies directly from the selective plating media (mCCDA) without subculturing.

The outcome observed on mCCDA agar grown samples could have been the emphasis on the need for subculturing the colonies more-than once before using them in PCR. This argument can be supported by result observed on colonies where conducting discontinuous streaking of the colonies on Columbia agar before picking a colony for PCR gave rise to individual colonies with a much pure DNA. It is known that sterilizing the loop by flaming it after streaking each quadrant reduces the bacterial population, and that inoculation at the fourth quadrante is done by a more diluted population which produced single colonies with a clean DNA (Sanders, 2012). Also, it is worth noting that with direct PCR using colonies that were not subjected to subculturing for at least more-than once, secondary products as well as high background can be expected (Williams et al., 2017).

Overall, PCR experiment of this work confirmed all the isolated presumptive colonies to be *Campylobacter jejuni*.

CHAPTER 5: GENERAL DISCUSSION, CONCLUSIONS AND FUTURE WORK

It was explained in Chapter 1 that this study was motivated by the lack of an established method to recover *Campylobacter* cells from environmental water samples, which is a significant problem in investigating their occurrence. *Campylobacter* spp. infection is a leading causative agent of diarrhoeal disease, and consumption of faecal-contaminated water is a major risk factor. Environmental water, such as river water, is often contaminated with faecal waste from infected humans or animal reservoirs, which makes these waters a risk factor. Transmission to humans is very possible (Kaakoush, Castaño-Rodríguez, et al., 2015). Effective recovery of *Campylobacter* cells from water samples is critical for enumeration and quantifying, which is necessary for risk assessment. Therefore, the overall aim of this study was to investigate different methods of recovering *Campylobacter* spp. from river water to find the most suitable one. The study hypothesized that the Bloukrans River is contaminated with *Campylobacter* spp. and cell recovery rate would vary depending on the recovery method used.

In the first instance, results of this study confirm that *Campylobacter* is detectable in the Bloukrans River and the applied method in detection is efficiently for river water samples with high turbidity, a finding that also highlights the importance of this river as a site suitable for this type of study. This was expected because it represents all the risk factors necessary for *Campylobacter* pollution, such as contamination by sewage (Vogt et al., 1982; O'Reilly et al., 2007; Pitkanen and Hanninen, 2017), manure from livestock from nearby agricultural farms (Vogt et al., 1982; Clark et al., 2003), and wild and domestic animal run-off (Levicán et al., 2019). These findings also highlight the apparent potential risk of disease caused by *Campylobacter jejuni* for populations exposed to the river.

It is difficult to accept or reject the hypothesis that cell recovery rate will vary depending on the recovery method used. Based on the results described in this work, several conclusions can be drawn. The performance of each *Campylobacter* spp. recovery method differed by the sampling site. This was most due to the *Campylobacter* spp. distribution and pollution at each site (Bradshaw et al., 2016). The Moore Swab method yielded the lowest count at all sites. This was expected given that the literature documented low detection of *Campylobacter* spp. by this method (Meinersmann et al., 2020). According to a study by Meinersmann et al. (2020) *Campylobacter* spp. could only be isolated from 9% of water samples investigated. However, this study will allow conclusions to be drawn regarding the most suitable method of concentrating *Campylobacter* spp. from environmental water samples.

Our results show that concentrating the river water by centrifugation to collect the pellets, followed by filtering the supernatant to trap any remaining suspended cells, produced the highest percentage recovery of *Campylobacter* cells in the collected water samples. This method of recovery is highly promising and potentially applicable to concentrate and recover *Campylobacter* spp. from environmental water samples. This study is the first to attempt combining two previously used methods for concentrating *Campylobacter* spp. from environmental water samples in one setup.

Generally, a major observation while conducting this study is that it is difficult to culture *Campylobacter* spp. from the water samples, regardless of the recovery method used. The low counts generally obtained from the water samples can attest to this. This is because they are mostly present in low numbers (Jiang et al., 2015). Detection depends on the time and site of sampling. *Campylobacter* is most likely to be detected in sites with fresh contamination, and competitive contaminants can grow on the selective media (Enrichment et al., 2000). Adaptation to in vitro conditions for growth is also difficult (Acheson and Allos, 2001). In addition, *Campylobacter* that have been exposed to hot temperatures in the study area for a prolonged period may become unculturable. This can influence enumeration results following recovery. Therefore, future studies must consider these factors during planning.

A major limitation of this study is the sample size, the rate of sampling time could have affected detection at some of the sites because *Campylobacter* spp. life span depends mostly on time between deposition in the river and sample collection. Biweekly collection is ideal for growing *Campylobacter* spp. Also, sampling for this project was conducted during the COVID-19 pandemic period, and so sampling was disrupted by COVID-19 regulations thus affecting sample plans. This work recommends that future works apply our successful recovery method to larger sample size. A seasonal data collection for the purpose of learning the season impact on recovery concentrations would also contribute greatly to a future study. The other factor to be recommended is subculturing of presumptive colonies on Colombia Base Agar to dilute population density before DNA extraction and PCR. In case where PCR results produces multiple bands sizes, sending different band sizes for sequencing would assist in concluding on the primer specificity and the identity of the isolated organisms. Primers can then be designed in way that they are more specific to the organisms.

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APPENDIX A: ENVIRONMENTAL BROTH CULTURE.

The pellicle cell
growth pattern .



Figure: A 1. The photograph of 100mL flask with a growth pattern observed after enrichment of samples in Bolton Broth medium. Growth Characterised by the formation a thick layer covering the surface of the media.

APPENDIX B: MAKHANDA WEATHER CONDITIONS.

Table 1A: Makhanda general weather conditions | Average, minimum and maximum Temperatures | Precipitation| Humidity | Rainy days

	January	February	March	April	May	June	July	August	September	October	November	December
Avg. Temperature °C (°F)	20.7 °C (69.3) °F	20.9 °C (69.6) °F	19.8 °C (67.6) °F	17.2 °C (62.9) °F	15.1 °C (59.2) °F	12.8 °C (55) °F	12.5 °C (54.4) °F	13.3 °C (56) °F	14.4 °C (57.9) °F	16.1 °C (61.1) °F	17.5 °C (63.5) °F	19.5 °C (67.1) °F
Min. Temperature °C (°F)	15.9 °C (60.6) °F	16.3 °C (61.3) °F	15 °C (59) °F	12.2 °C (54) °F	9.8 °C (49.6) °F	7.1 °C (44.8) °F	6.7 °C (44.1) °F	7.5 °C (45.4) °F	8.7 °C (47.7) °F	10.8 °C (51.4) °F	12.3 °C (54.1) °F	14.5 °C (58.2) °F
Max. Temperature °C (°F)	26.8 °C (80.2) °F	26.9 °C (80.4) °F	26 °C (78.7) °F	23.4 °C (74.2) °F	21.7 °C (71.1) °F	19.5 °C (67) °F	19.4 °C (66.9) °F	20.4 °C (68.7) °F	21.5 °C (70.6) °F	22.6 °C (72.6) °F	23.7 °C (74.6) °F	25.6 °C (78.1) °F
Precipitation / Rainfall mm (in)	66 (2.6)	67 (2.6)	63 (2.5)	52 (2)	25 (1)	24 (0.9)	24 (0.9)	38 (1.5)	43 (1.7)	60 (2.4)	62 (2.4)	66 (2.6)
Humidity(%)	71%	73%	71%	69%	63%	57%	55%	59%	64%	68%	69%	69%
Rainy days (d)	7	8	7	6	4	3	5	5	7	7	7	8
avg. Sun hours (hours)	7.3	6.8	7.1	7.1	7.6	7.5	7.6	7.7	7.7	7.4	7.6	7.3

The table shows Makhanda weather conditions on a year basis. February is known to be hottest month of year. Coldest months are June–August. More rain is expected in January–March and from October–December every year (Climate Data, n.d).

APPENDIX C: THE OBTAINED SEQUENCES.

APENDIX C1: The Sequence obtained from a site 5 water sample colony grown on mCCDA agar accession number CP066243.1.

ATGTTGGATTCAACAATGTGCCTTAGCGGCGAGAATGACGGATATCACAAACCGTGGGGA
GGGTGGCTTCTTCGGAGAGCGATAGGATTGAAGTTCTTTGCAGGTTGTTTCAGGGGGGGCT
ACAGAAGGGGCGGAAGAGCCTGGGGAGCTGCGACTTTTCTGGGCTGCGTTGGCGAAGGGA
GCAACTCGTGTGCCGTTCCCGGAGACCAGCGGGTGATCCAAGGGAGCGTTTAATGCCACT
TTTCGTAATAATGATTCCAGCTAAGGCAAGGTATCATATGAAGGGAGAGGATCGGCAGGA
GCGAGAAAGTAACTGAGGAATGTTGTAAGCTTGACATCACCTTCACAGGATATGGATAT
GGAGGTGAAAGCCAATATTGCGCCGATGCACCGCGAGTATCAAGAACGATACACCTGCGA
CTCTGCTGGGGAGGGCGAAGGGAATTATTTGGCGAGAACACACGGATTTAAAGTGGGCTT
TGATTCCAGGGAGGATTAATGCTATAACTATCCGAAGAAGCCATCATCGCACCTTTTTAA
GATAAAATTTCTTATCGCTACCAAAAAGGCATATT

APENDIX C2: Site 6 sequence obtained from a colony grown subcultured using Colombia Base Agar with FJ655192.1

GATAGTGATTATGTTTTGGATGGCACAATATGCCTTTTGGTACCGATAAGAAATTTTAT
CTTAAAAAAGGTGCGATGATGGCTTCTTCGGATAGTTATAGCATTGAAGTTATTGGAAGG
GGTGGTCATGGAAGTGCTCCAGAAAAGGCAAAAAGATCCTATTTATGCTGCTTCTTTGCTT
GTTGTGGCTTTGCAAAGTATAGTATCTCGCAATGTTGATCCCCAAAATTCAGCAGTTGTA
AGCATAGGAGCTTTTAATGCAGGGCATGCTTTTAATATCATTCCAGATATTGCAACGATT
AAAATGAGTGTTAGAGCATTAGATAATGAACTAGAAAGCTAACTGAAGAAAAAATTTAT
AAAATTTGTAAAGGTATTGCACAGGCTAATGATATAGAGATTAATCAATAAAAATGTT
GTTGCACCAGTGACTATGAATAACGATGAAGCTGTGGATTTTGCTAGTGAGGTTGCAAAA
GAATTATTTGGCGAAAAAAA

APPENDIX D: PUBLICATION(S) EMANATING FROM THIS STUDY

Nnadozie CF, Ngoni N. A method of recovering the very low concentration of pathogens in river water by combining centrifugation and membrane filtration. *MethodsX*. 2023 Jul 23;11:102291. doi: 10.1016/j.mex.2023.102291. PMID: 37560404; PMCID: PMC10407429.