Faecal contamination source identification using a combination of chemical and microbial biomarkers

Report to the
Water Research Commission

by

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EXECUTIVE SUMMARY

BACKGROUND

In South Africa, the probability of water supply interruptions was 47.6% in 2010 and there was a 61.8% increase in deaths from intestinal infectious diseases between 2001 and 2007, possibly partly due to intermittent water availability. Some households are forced to supplement their drinking water from water resources of inadequate microbial quality. Contamination of the water bodies occurs via surface runoff from sanitation infrastructure, defecation by livestock and laundry activities in the vicinity of the water bodies, or seepage from landfills and manure deposits.

Enumeration of *E. coli* forms the basis of the National Microbial Monitoring Programme. The analyses are performed using the Colilert®18 system at prioritised sampling sites. Advantages of this system include a fast availability of results, limited influence of environmental sources on the *E. coli* concentration and understanding of long-term trends of the surface-water related risks to public health. However, only 70% of relevant water resources were covered by the National Microbial Monitoring Programme in 2009 due to lack of accredited laboratories and skilled personnel and given financial limitations. At the same time, rates of false positive and negative results of Colilert®18 are unknown under South African conditions, and identification of sources of faecal contamination is not feasible at present.

Review of the South African and international literature led to the identification of four techniques which could be used to identify sources of faecal contamination, namely the enumeration of *Rhodococcus coprophilus,* enumeration of bifidobacteria on the YN-17 medium, the modified Beerens medium and the human-bifid sorbitol agar, measurement of the antibiotic-resistance spectra of *E. coli* and the quantification of the faecal sterols (corpostanol and stigmastanol). Bifidobacterial enumerations and the faecal-sterol quantification were investigated for their feasibility in this study.

AIMS

The following were the aims of the project:

1. To conduct a literature review of the methods for detection of presence/absence of the faecal contamination, and methods for identification of the sources of faecal contamination in South Africa;

2. To select appropriate methods for analyses of the sediment and water samples based on the literature review and feasibility in South Africa, and to collect information on practices around sampling sites.

METHODOLOGY

The concentration ratio of the sorbitol-utilising bifidobacteria (SUB) and the total bifidobacteria (TB), referred to as the tracking ratio, can be used to distinguish between animal and human sources of faecal water contamination based on a cut-off value. This needs to be calibrated at specific geographical locations. Seven sites with permanent faecal contamination were selected in South Africa based on overlap between enumerations of faecal coliforms (FC), *E. coli*, *Rhodococcus coprophilus* and the positive hydrogen-sulphide test. These were accomplished using the methods of Venkobachar et al. (1994), Genthe and Jagals (2003), Mara and Oragui (1983) and Isobe et al. (2002). The H_2S strip test medium of Venkobachar et al. (1994) was also modified according to Sobsey and Pfaender (2002) with addition of 0.5% (w/v) of deoxycholate to suppress non-specific growth. The sterol analysis was done on samples collected from the same 7 sites as

above. The method of Isobe et al. (2002) was followed, but only gas chromatography with flame ionisation detection was used due to equipment availability.

During the screening for calibration sites for the bifidobacterial tracking ratio, concentrations of faecal coliforms and *E. coli* ranged from 2 to above 16000 cells/100 mL, while concentrations of the sorbitol-utilising bifidobacteria ranged from 10-50000 cells/100 mL. Concentrations of 0-8000 cells/100 mL were the recorded for total bifidobacteria. The tracking ratio ranged from 0.10 to 6.25, but no clear cut-off value to distinguish between the human and animal sources of faecal contamination could be established.

Overgrowth of bifidobacteria by faecal streptococci and faster die-off of the total bifidobacteria versus their sorbitol-utilising counterparts are the two main obstacles to the applicability of bifidobacteria as a faecal-source tracking tool in South Africa. It was further hypothesised that the survival rates of bifidobacteria could be influenced by the water chemistry and that this could therefore the tracking ratio. Therefore bifidobacteria were monitored in all 7 selected/calibration sites from September 2010 to January 2011. Chemical parameters of the water column were measured and correlated with the SUB and TB. The modified-Beerens medium was used for the enumeration of total bifidobacteria.

The bifidobacterial survival seemed to be well correlated with temperature and concentrations of chlorides and sulphates from river data collected. The chemical ranges were as follows: pH 7.0-9.8; sulphate 0.5-90 mg/L; temperature 14-32°C; chloride 2-302 mg/L; hardness 0-403 dKh; phosphate 0-12 mg/L; conductivity 0-1539 ms/cm; ammonia 0-20 mg/L, the SUB concentrations 0-26512 CFU/100 ml, the TB concentrations 0-3600 CFU/100 mL, nitrates 0-47 mg/L, *E. coli* 0-80 CFU/100 ml, faecal coliforms 0-1600 CFU/100 mL. This data was used to identify possible key parameters controlling survival rates of bifidobacteria. The parameters were identified in descending order as follows: pH; sulphate; temperature; chloride; hardness; phosphate; conductivity; ammonia. The parameters used for the survival experiments were as follows, with ranges used in brackets pH (5.3-9.8); temperature (8-32°C); chloride (2-302 mg/L); protein (0-140 mg/L); nitrate (0-6.89 mg/L). These parameters were chosen according to physiological factors and nutrient requirements for the bacteria. Ammonia was not used as nitrate is needed for the bacteria and converted into ammonia. The other parameters of the water were kept constant. The survival tests for bifidobacteria were designed with a factorial design to minimise the number of experiments needed and to maximise the data from them. Factorial design is useful to identify relationships that depend on multiple factors simultaneously.

The pH of the incubation medium was found to decrease between 0.71 and 4.0 units of pH in 152 hours. The most likely cause was the production of lactic acid by the bacteria. Such vast changes in pH likely led to stress on the bifidobacteria and thus compromise the results of the survival experiments. The pH changes have been stabilised using calcium carbonate, which decreased the pH change amount to a maximum of 0.63, but the total bifidobacteria still died off before their sorbitol-utilising counterparts. A selected portion of the presumptive bifidobacterial colonies were re-plated onto the m-Enterococcus agar and grew as morphologically faecal streptococci. The portion of faecal streptococci present was equal to 81 ± 31% and not statistically different from 100%. Therefore up to 100% of the presumptive bifidobacteria were actually faecal streptococci, but genetic analyses will have to be performed to exclude the presence of mixed cultures. The bifidobacteria generally did not survive longer than 72 hours except in two cases where they survived for 152 hours. Stigmastanol concentrations ranged from below the detection limit to about 16.7 ng/mL. Coprostanol concentrations were below the detection limits of the method of Isobe et al. (2002).

CONCLUSIONS

Results of the project indicate that bifidobacterial and sterol analysis according to Leeming et al. (1996) are not feasible under South African conditions and other techniques will need to be investigated for this purpose. On the positive side, survival rates for faecal coliforms were measured for the first time in Eastern Cape and in more detail than is available in South African literature. Time periods for the discrepancies

between the H_2S strip test and the faecal coliform enumeration were investigated and it was found that this takes place after 22 to 29 hours after the faecal contamination event.

RECOMMENDATIONS

Further investigations will have to focus on the genetic diversity of bifidobacteria and faecal streptococci in South African water resources. The study results should be verified in a larger geographical area. For the broadening of the scope of NMMP, the antibiotic resistance spectra of the *E.coli* isolates from different faecal contamination sources could be considered. Databases could be created for this purpose and implemented into NMMP.

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Two reviewers who provided feedback on the original proposal	Unknown
Staff of the Kwandwe Game Reserve	Sampling site access on the Kwandwe game reserve

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ACRONYMS & ABBREVIATIONS

BM	Beerens medium
CFUs	Colony-forming units
COD	Chemical Oxygen Demand
DWA	Department of Water Affairs
DWAF	Department of Water Affairs and Forestry
FC	Faecal coliforms
HPC	Heterotrophic plate count
HSBA	Human bifid-sorbitol agar
MBM	Modified Beerens Medium
NDMW	Nutrient-depleted model water
NMMP	National Microbial Monitoring Programme
NRMW	Nutrient-rich model water
PFUs	Plaque-forming units
SDW	Sediment dry weight
TC	Total coliforms
TR	(Bifidobacterial) tracking ratio
VBNC	Viable but nonculturable state

CHAPTER 1: BACKGROUND

1.1 INTRODUCTION

Waterborne diseases include hepatitis E (Grabow et al., 1994), cryptosporidiosis (MacKenzie et al., 1994), and infections due to *E. coli* O157:H7 (Craun, 1991). Oncogenic viruses, e.g., papilloma and polyoma, can be present in drinking water thus posing considerable public health risks (Grabow, 1996). At the same time, viruses can be excreted into aqueous environments, e.g., by children or asymptomatic individuals, posing significant risks of infection in immunocompromised patients (Gerba and Hass, 1988). Incidence and prevalence of waterborne pathogens depend strongly on geographical factors (Grabow, 1996), as many pathogens are endemic to certain areas, but outbreaks are extremely rare, e.g., hepatitis E in South Africa (Grabow et al., 1994). Individuals most susceptible to waterborne diseases include HIV/AIDS patients, young children, the elderly, people with genetic predisposition to diabetes, and pregnant women. Public health risks, associated with water, originate mainly from the contamination/transmission by the faecal-oral route (WHO, 2006), or from the transmission of opportunistic pathogens (Grabow, 1996).

Even though many of the waterborne diseases are not fatal, they have severe socio-economic impact in low-income communities, and have severe implications in a country like South Africa (Grabow, 1996). Lack of drinking water infrastructure in some parts of South Africa forces people to turn to nearby streams to meet their demand for domestic uses of water. These water bodies serve as laundry stations, they are crossed by cattle during herd movement and watering of livestock, and they are also freely accessible to other living organisms. These activities put humans using the stream as a source of drinking water, at risk from waterborne diseases due to faecal contamination.

Risk of faecal contamination of surface waters depends strongly on land uses and water uses (Venter et al., 1998). The land uses include building dwellings with inadequate sanitation infrastructure in the vicinity of streams, poorly operated and maintained sanitation infrastructure, dense settlements in areas prone to surface runoff, and intensive livestock farming (Venter et al., 1998). The water uses include drinking of untreated or partially treated surface water, contact recreation and irrigation of crops for raw consumption with inadequately treated waters (Venter et al., 1998). The danger of a waterborne disease outbreak also increases if disinfection and filtration modules of wastewater treatment plants are not operated properly, or when absent in the design of these plants (Venter et al., 1998).

This project arose out of a perceived need to examine the possibility of using faecal source tracking in South Africa to help establish the sources of faecal contamination in environmental waters. This would permit more focused management of faecal contamination because information on the source of the contamination (e.g. animal or human) would be available.

1.2 RESEARCH PROCESS

1.2.1 Choice and characterisation of sites

On the basis of a literature review of the techniques reported in South African and international literature, we aimed to develop a strategy for the detection of faecal pollution and identification of the respective source(s). We aimed to achieve this goal by first screening the established DWAF and Institute for Water Research sampling sites on the Bloukrans and Buffalo Rivers for persistent faecal pollution. The detection of the

presence of faecal pollution was based on the H_2S strip test, and the enumeration of faecal coliforms/*E. coli*. Once sampling sites where faecal contamination had been proven by at least three independent tests on three randomly chosen sampling occasions, identification of the faecal contamination sources was attempted. Firstly, information on the different water and land uses of the stream in the area were collected. Sites used for laundry washing, livestock related activities, and those where faecal pollution can originate from dogs and sheep were targeted. After all the relevant information had been gathered, the sites were used for calibration of the approach in a wider area.

1.2.2 Basis of source tracking

The main method investigated in this work was based on bifidobacteria. These are Gram-positive and obligate anaerobic bacteria which colonise the guts of human and ruminants (Bonjoch et al., 2005). When grown on human bifido sorbitol agar, the sorbitol-fermenting bifidobacteria can be enumerated, while the total bifidobacteria can be enumerated on the YN-17 medium (Mara and Oragui, 1983; Bonjoch et al., 2005). The ratio of the concentration of the sorbitol-fermenting bifidobacteria to the total bifidobacteria, i.e. the tracking ratio (TR), is calculated. For this ratio, a cut-off value has been shown to exist based on whether the bacteria have been shed in the faeces of human or ruminants (Bonjoch et al., 2005). If the ratio for a particular water sample is higher in value than the cut-off value, then the faecal pollution is of human origin (Bonjoch et al., 2005). If the ratio has a value lower than the cut-off, then the faecal pollution is of animal/ruminant origin (Bonjoch et al., 2005). The cut-off value has been shown to be specific to a geographical area, and so would have to be determined under South African conditions, using the calibration sites. After calibration of the cut-off point has been conducted, the second group of sampling sites can be used to acertain the validity of the bifidobacteria in the identification of faecal source tracking.

The concentrations of certain sterols were also measured because these can also indicate the source of faecal contamination.

1.3 PROJECT AIMS

The following were the aims of the project:

1. To conduct a literature review of the methods for detection of presence/absence of the faecal contamination, and methods for identification of the sources of faecal contamination in South Africa;

2. To select appropriate methods for analyses of the sediment and water samples based on the literature review and feasibility in South Africa, and to collect information on practices around sampling sites.

1.4 SCOPE AND LIMITATIONS

This project is aimed at investigating the potential to broaden the scope of the National Microbial Monitoring Programme (NMMP) for surface water in South Africa by critical analysis of the existing framework, implementation faecal source tracking and assessment of the monitoring capacity in the rural areas. Faecal source tracking usually requires large-scale calibrations and so the study is limited due to the localised nature of the results obtained.

1.5 PROJECT STRATEGY

Based on the literature review and availability of equipment at Rhodes University, the following strategy was chosen for the project. Calibration of TR derived from the concentration of the sorbitol-utilising bifidobacteria and the total bifidobacteria was attempted. This was based on the derivation of the cut-off value between the human and animal sources of faecal contamination. For the chemical indicators, the sterols calibration was based using the method of Leeming et al. (1996). At the same time, the influence of survival rates on the detection of faecal contamination in South Africa was investigated.

CHAPTER 2: LITERATURE REVIEW

2.1 INTRODUCTION

Historically, the degree of development of drinking water infrastructure has varied significantly among different geographical areas in South Africa (Schreiner and Van Zyl, 2006). Prior to 1994, the majority of the lack population was confined to homelands and townships with limited if any infrastructure (Department of Water Affairs and Forestry – DWAF, 1994). This resulted in the development of large settlements with no drinking water infrastructure. After 1994, the South African government started to address these issues and major improvements have been achieved. In many areas, drinking water is supplied from communal taps (Jagals et al., 2004), and people are often forced to walk long distances to fetch potable water. These taps are often not maintained properly (Haarhoff et al., 2009) leading to interruptions of water supply. A similar situation is observed even if drinking water connections are installed in the households, as pipe bursts are common (PMG, 2009).

In 2009, it was estimated that up to 5 million South Africans were still without access to any potable/clean water sources (IRIN, 2009). Because of these factors, a significant part of the population in South Africa relies on water resources of inadequate microbial quality to meet their domestic water needs (Momba et al., 2006a; Tandlich and Muller, 2008). Such water resources include streams, springs, rivers and boreholes. Raw water can be extracted from these resources for potable use but it has to undergo a minimum treatment, such as boiling, addition of bleach or sand filtration, before human consumption (Murray et al., 2004). Processes such as boiling of raw water before consumption can, however, be time-consuming at the household level and bleach has proved too expensive for poor households (Monyai, 2004). Water treatment works maintenance and operation is often not of the required standard (e.g. Momba et al., 2006). A combination of these factors increases the chances of the waterborne disease outbreaks.

Waterborne diseases include hepatitis E (Grabow et al., 1994), cryptosporidiosis (MacKenzie et al., 1994; Jamieson et al. 2004), *Escherichia coli* (*E. coli*) O157:H7 infections (Craun, 1991; Heijnen and Medema, 2006), acute gastroenteritis and shigellosis (Pedley and Howard, 1997), cholera (Paulse et al., 2009), and/or infections by enterovirus, rotavirus, reovirus and hepatitis A (US EPA, 2001). In South Africa, epidemics/outbreaks of waterborne diseases have been reported recently, e.g. outbreaks of cholera in the provinces of Mpumalanga and Gauteng in 1998 (Dalsgaard et al., 2001) and Limpopo in 2008 and 2009 (Mail and Guardian, 2008; Digital Journal, 2009). Causative agents of waterborne diseases can be excreted in faeces of children (Taylor et al., 1993), asymptomatic individuals (Martin et al., 1988) and healthy adult individuals (Degener et al., 1983). This chapter provides a review of faecal contamination of potable water resources in South Africa. Detailed analysis of the sources and detection methods is provided using a combination of international and South African literature sources. Using this approach, the knowledge gaps and drawbacks of the current strategy are identified and solutions are proposed.

2.2 CLINICAL AND ECONOMIC RELEVANCE OF WATERBORNE DISEASES

In 2001, the direct costs of treating diarrhoeal diseases reached 5 billion ZAR in South Africa (approximately 670 million USD; SIA 2001) and the total cost to the economy was estimated at 15 billion ZAR (approximately 2 billion USD; SIA 2001). In 2003, there were 43000 deaths due to waterborne diseases in South Africa and an estimated 3 million infections reported for the same period (Mackintosh and Colvin, 2003). In 2009, there were 1.5 million child cases of diarrhoea reported in South Africa (DWAF, 2009b).

Waterborne diseases are likely to result in a significant loss of economic productivity and impose high health costs on the South African economy. To prevent these outbreaks, regular monitoring of microbial water quality is required in areas that are at risk of faecal contamination or any areas where drinking water supply is supplemented from potentially unsafe sources. In the following sections, data from international and South African literature are used to critically evaluate the current status of microbial water quality monitoring in South Africa. Strategies, based mainly on indicator microorganisms, are outlined and analysed to identify crucial knowledge gaps and drawbacks of the current strategies. Results of this analysis are then combined with the global knowledge in the fields of detection and source identification of faecal contamination to propose improvements to the existing monitoring framework.

2.3 SOURCES OF FAECAL CONTAMINATION

Laundry activities are performed directly at many water resources, e.g. at the banks of streams (Venter et al., 1998). Livestock can cross streams during herd movement and grazing, which can lead to presence of animal faeces in the vicinity of water collection sites (Venter et al., 1998) or directly in water resources (Hubbard et al., 2004). A lot of these water resources are freely accessible to wild animals, and such free access can result in faecal contamination as demonstrated in international literature for waterfowl (Ksoll et al., 2007). New informal settlements with inadequate or non-existent sanitation infrastructure are often built in the vicinity of streams and boreholes in South Africa, leading to faecal contamination of these water resources by surface runoff (Venter et al., 1998; Carden et al., 2007). In this context, surface runoff can occur in the form of greywater and/or sewage. Greywater and sewage are discharged into the water bodies, leading to faecal contamination (Mathee et al., 1999) caused by faecal matter in bathroom and laundry greywater (Eriksson et al., 2002) and pathogens in sewage (Stamper et al., 2008).

Rainwater also causes surface runoff. In the informal and peri-urban settlements in South Africa, stormwater pipes generally discharge directly into the nearby streams (Barnes, 2003). Stormwater has been shown to contain non-zero concentrations of *E. coli* and faecal coliforms (FC), indicating potential presence of faecal contamination (Pitkänen et al., 2008). Samples of stormwater discharged into the Plankenburg River contained concentrations of *E. coli* of 2.44 x 10^9 colony-forming units (CFUs)/100 mL (Barnes, 2003). Jagals et al. (1995) found that concentrations of FC reached 4.4 x 10^6 CFUs/100 mL in stormwater runoff from an informal settlement near Bloemfontein, South Africa. The concentrations of FC in surface water resources have been shown to increase substantially during rainfall events (Shehane et al., 2005). *E. coli* O157 was isolated from cattle dung and surface water samples in South Africa and Swaziland during a diarrhoeal outbreak in 1992 (Effler et al., 2001). In the same study, the authors reported a significant increase in the number of diarrhoea cases at the end of a drought period with the onset of heavy rains.

Paulse et al. (2009) found that concentrations of FC and *E. coli* in the Plankenburg River near the peri-urban settlement of Kayamandi ranged from 10 cells/100 mL up to approximately 3.5 x 10^6 cells/100 ml. The FC concentration ranged from 1.7 x 10^2 CFUs/100 mL to 3.5 x 10^7 CFUs/100 ml, while concentrations of *E. coli* ranged from 3.6 x 10^1 CFUs/100 mL to 1.7 x 10^7 CFUs/100 mL in water samples from the Berg River in another study (Paulse et al., 2007). An informal settlement located on the banks of the Modder River, approximately 60 km west of Bloemfontein, contained concentrations of FC which ranged from 0 to 8.4 x 10^5 CFUs/100 mL of FC (Jagals and Grabow, 1996). Similar observations were made for samples taken in the Jukskei River near Johannesburg. Background concentrations of *E. coli* were equal to 1.5 x 10^3 cells/100 mL upstream of the Alexandra settlement (De Wet et al., 2000). These increased to 3.7 x 10^5 cells/100 mL in the stretch of the river passing through the settlement, and the concentrations were also significantly elevated downstream of the settlement, where the concentrations of *E. coli* reached 1.3 x 10^5 cells/100 mL (De Wet et al., 2000).

Surface runoff and land uses in the vicinity of surface water bodies affect the extent of faecal contamination of these water bodies (Muirhead et al., 2006). Land uses have an impact on chemical composition of soils (Solomon and Lehman, 2000; Helfrich et al., 2006) and physical properties of soils (Batey, 2009) in the vicinity of streams. Increased soil compaction, i.e. decreased pore volume of soil, can be observed in the vicinity of surface water resources when these are crossed by agricultural animals during grazing. The animals trample on the soil surface and their hooves exert pressure on the soil surface. As a result, the surface horizons of the soil profile are compressed, leading to soil compaction (Betteridge et al., 1999; Broersma et al., 1999). The international literature contains data describing the extent of such effects for cattle grazing on coarse-textured soils (Mulholland and Fullen, 1991) and for sheep grazing on clay loam (Proffitt et al., 1995). Data for sheep are available in the South African literature (Du Toit et al., 2009). Increased soil compaction leads to decreased infiltration rate of water into the soil profile (Du Toit et al., 2009), which in turn results in increased surface runoff (Proffitt et al., 1995). The extent of the surface runoff will depend on the density of the grazing animals and the vegetation cover of the soil near the given water resource (Hubbard et al., 2004). The highest extent of soil compaction is observed in the shaded areas, because grazing animals tend to congregate in these areas (Hubbard et al., 2004).

Cattle, sheep and goats are commonly used in subsistence and commercial agriculture in South Africa. Defecation by these animals occurs during grazing in the vicinity of surface water bodies, and the dung of cattle (Muirhead et al., 2005), sheep (McDowell, 2006) and goats (Ufnar et al., 2007) have been shown to lead to faecal contamination of surface water resources. If the intensity of the surface runoff increases due to trampling by these animals on the banks of water resources, there is an increased probability that their faeces will be transported into the surface and adjacent water resource by surface runoff (McDowell, 2006). If raw water from this resource is used for potable purposes without adequate treatment, then human consumption of such water will result in a higher risk of a waterborne disease outbreak among the human population. In the South African context, risks of such outbreaks will be relevant in provinces of the Eastern Cape, the Free State, KwaZulu Natal and Limpopo.

Many informal settlements in South Africa are built below the flood line, such as the settlement of Alexandra in the vicinity of the Jukskei River near Johannesburg (De Wet et al., 2000). The surface water bodies can be faecally contaminated upon flooding of such settlements and through contact of river water with faeces of agricultural animals. This has been demonstrated around the Topehaehae Stream in New Zealand, where the concentration of *E. coli* increased from 1.0×10^2 cells/100 mL to $1.3\text{-}4.0 \times 10^4$ cells/100 mL during natural and artificial floods (Nagels et al., 2002). Similar observations have been reported from Germany (Kistemann et al., 2002). Flooding of informal settlements is likely to be a significant source of faecal contamination of surface water bodies in South Africa. If raw water from such resources is used for potable purposes without adequate treatment, then its consumption will result in a higher risk of a waterborne disease outbreak among the human population.

Wastewater treatment plants are used to treat municipal wastewater and sewage containing faecal contamination. Different technologies can be used to remove indicator and pathogenic microorganisms. The treatment technology (Shengji et al., 2004; Hassett et al., 2007; Devi et al., 2008; Tandlich et al., 2009) and functionality of the disinfection and filtration modules (Venter et al., 1998) control the microbial water quality of the final effluent from the wastewater treatment plant. If the treatment is insufficient due to technological limitations and/or improper operation, then discharge standards can be exceeded with respect to microbial water quality in the final effluent. Final effluents from wastewater treatment plants in South Africa are often discharged into the nearby streams (Odjadjare and Okoh, 2010). Therefore the water in these resources will exceed the microbial quality for potable purposes due to inadequate microbial quality of the discharged final effluent (Momba et al., 2006b; Momba et al., 2009). If raw water from such resources is used for potable purposes without adequate treatment, then its consumption will result in increased risk of a waterborne disease outbreak among the human population.

Odjadjare and Okoh (2010) reported that 96% of all surface water samples from the Tyume River near the town of Alice in the Eastern Cape Province, South Africa, contained concentrations of *Listeria spp.* in excess of 0 colony-forming units (CFUs)/mL. Concentrations of the bacterium were measured in the final effluent from the wastewater treatment plants after chlorination, as well as in water samples taken upstream and downstream of the final effluent discharge point into the Tyume River (Odjadjare and Okoh, 2010). Infections due to *Listeria spp.* lead to gastroenteritis and perinatal infections which can result in miscarriages in humans and livestock (Siegman-Igra et al., 2002). Even though such infections are rare (Lyautey et al., 2007) the mortality rates range from 20 to 50% of the infected patients/animals (Odjadjare and Okoh, 2010). There are no water quality regulations or guidelines for the concentrations of *Listeria spp*. However, high mortality rates among infected patients and the potential consumption of river water as potable water, without adequate treatment, give reasons for serious public health concerns. The problem of final effluent discharge is made even more significant because of the findings of Momba et al., (2006b) who made similar observations for *Salmonella spp.*, *Shigella spp.*, and *Vibrio cholerae* in the final effluents of the wastewater treatment plants in the Buffalo City and the Nkokonbe Municipalities of the Eastern Cape Province, South Africa.

Samie et al., (2009) investigated the efficiency of the removal of indicator microorganisms in 14 sewage/wastewater treatment plants in the province of Mpumalanga, South Africa. Ninety three percent of the final effluents were chlorinated, yet they contained concentrations of FC ranging from 3 to 3.6 x 10^5 CFUs/100 ml. At the same time, strains of *Vibrio spp.* were isolated from 36% of the final effluent samples, while strains of *Campylobacter spp.* were isolated from 57% of these samples (Samie et al., 2009). Both of these genera contain strains that are causative agents of waterborne diseases. If raw water is abstracted downstream of the discharge point of the final effluent and the minimum treatment is not conducted, then an outbreak of waterborne disease can take place in the population using the stream as a potable water source. Similar health risks can be expected if the water from such a water resource is used to recharge groundwater resources of potable water (see below).

Surface water bodies receiving improperly treated wastewaters/effluents are often used as sources of irrigation water in South Africa (Venter et al., 1998) after a licence has been obtained from DWAF (DWAF, 2009a). Irrigation with non-chlorinated effluents can transport coliform bacteria through the atmosphere in aerosols as far as 200 to 1800 m from the site of irrigation (Sorber et al., 1976). Outbreaks of various waterborne diseases have been reported in the vicinity of irrigation sites under favourable wind speeds and humidity (Chaikaew et al., 2009). Therefore irrigation with faecally-contaminated surface water may put the health of many people at risk. Groundwater constitutes a considerable source of potable water in South Africa (Murray et al., 2004). Pathogenic microorganisms can enter boreholes used for groundwater extraction after surface flooding from rivers (Levine et al., 1990; Barwick et al., 2000), through surface runoff (Kramer et al., 1996), or when sanitation infrastructure in a settlement is compromised and sewage enters the boreholes (Kramer et al., 1996; Barwick et al., 2000). Other contamination routes include seepage from the leaking septic tanks and pit-latrines (Kramer et al., 1996; Pedley and Howard, 1997), seepage from feedlots and manure deposits (Colvin 1999; Barwick et al., 2000), seepage from graveyards (Engelbrecht, 1997), or seepage from broken underground sewer pipes (Levine et al., 1990; Pedley and Howard, 1997).

If water used for artificial recharge of groundwater is faecally contaminated, then groundwater resources can become faecally contaminated (Pedley and Howard, 1997; Schijven, 2001). If the (clay) liners in landfills are faulty, then groundwater resources can also become contaminated by landfill leachates, which often carry finite concentrations of FC and potentially pathogenic microorganisms (Pedley and Howard, 1997). The probability and extent of faecal contamination of groundwater will strongly depend on the size and surface properties of viruses and bacteria, physical properties and chemical composition of the soil above the aquifer and the temperature of the environment (Schijven, 2001; Murray et al., 2004). Individuals that are the most susceptible to waterborne diseases include young children (Nwachuku and Gerba, 2004), the elderly, people with genetic predisposition to diabetes, and pregnant women (Gerba et al., 1996). The public health burden from waterborne diseases and faecal contamination of water resources is amplified by HIV/AIDS. High rates

of the HIV/AIDS infections are common among the population in many parts of South Africa (Avert, 2009), thus increasing the numbers of immunocompromised patients. Compromised immunity in these patients leads to high susceptibility to diarrhoeal diseases (Obi et al., 2006). Obi and Bessong (2002) found that 60% of patients with chronic diarrhoea were HIV-positive in the Limpopo province and the causative agents of diarrhoea in those patients included *Campylobacter jejuni, Campylobacter coli, Plesiomonas shigelloides, Aeromonas species, Shigella spp., Salmonella spp., Escherichia coli, Yersinia enterocolitica* and *Vibrio cholerae*. Similar observations were made by Obi et al., (2003a, b).

Sources of infection differ among these bacterial species, and they include consumption of mishandled and undercooked food and/or poultry products, along with sexual intercourse and the faecal-oral route, as demonstrated for *Campylobacter spp.* (Quinn, 1997). On the other hand, some of these pathogens can be waterborne, e.g., *Salmonella spp.* and *Vibrio spp.* (Momba et al., 2006b). Literature data indicates that the faeces of HIV/AIDS patients are likely to contain high levels of pathogens associated with waterborne diseases. In South Africa, the rates of the HIV/AIDS infections are high in provinces like KwaZulu-Natal, Gauteng and the Eastern Cape (National Department of Health of South Africa – NDOH, 2008). There large parts of the population live in settlements where the sanitation and drinking water infrastructures are underdeveloped or lacking (Obi et al., 2006). Therefore defecation in the vicinity of surface water bodies by HIV/AIDS sufferers, combined with surface runoff from the settlements, and settlement flooding, are likely to constitute a significant source of faecal contamination of the water resources in the vicinity of settlements. If the contaminated resources are used as sources of raw water and the minimum treatment does not take place before potable use, then risk of waterborne disease outbreaks will increase significantly. Similar health risks can be expected if groundwater wells/boreholes for extraction of drinking water are flooded and/or contaminated through surface runoff. These issues are of major concern to public health in South Africa.

2.4 INDICATOR ORGANISMS

The prohibitive cost of testing for all known pathogens has led to the use of a surrogate approach, i.e. the use of indicator microorganisms as the routine tool to examine microbial water quality and to make public health decisions. Indicator microorganisms include faecal coliforms (FC), *E. coli*, total coliforms (TC), enterococci/faecal streptococci and the heterotrophic plate count (HPC; Tandlich and Muller, 2008). If the concentrations of the above bacteria surpass the targets set in the microbial water quality guidelines and regulations, then it indicates the following problems with the potable water: possible faecal contamination of the water resource, the risk of the concomitant presence of pathogenic microorganisms and/or inadequate treatment (DWAF, 1996; Grabow, 2001; SABS, 2006).

Faecal contamination detection requires that the indicator microorganism is a member of the microflora of human and animal intestinal tract(s). It must be present in a water sample when faecal contamination is present (Tandlich and Muller, 2008). With regard to detection of waterborne pathogens, the indicator microorganism must fulfil the following criteria: it must be present in a water sample when the pathogen in question is present; it must be absent when the pathogen is absent in a given water sample; and lastly the indicator microorganism must have similar survival characteristics in the environment to the pathogen in question (Genthe and Franck, 1999). The most common groups of indicator microorganisms are discussed in the following sections. Some of these microorganisms are used in routine microbial water quality monitoring in South Africa and to define the microbial water quality guidelines and regulations in South Africa.

2.4.1 The coliform group

Coliforms are Gram-negative rods, which ferment lactose to produce acid/aldehyde and gas after incubation at 35-37°C for TC and *E. coli*, and/or 44.5°C for *E. coli* and FC (APHA, 1998; Murray et al., 2004). In most

instances, coliforms are not the causative agents of disease. However, they are easy to culture and their presence indicates that pathogenic organisms of faecal origin may be present in a water sample (Grabow 1996). Many strains and genera of the coliform group are members of normal microflora of the intestinal tract of humans and several mammals such as cattle. These strains are excreted in the faeces of humans and animals, and they fulfil the first criterion for an indicator microorganism of faecal contamination as stated in the previous paragraph.

The concentration of FC is one of the two most common criteria used to define the regulatory standards/guidelines for microbial water quality for human health (DWAF, 1996; SABS, 2006). The ability of FC to grow at 44.5°C separates them from other coliform bacteria. Advantages of using FC to detect faecal contamination in water include the availability of the standardised cultivation media (APHA, 1998; Garcia-Armisen et al., 2007) and wide acceptance by the regulatory community, municipal officials and public/environmental health professionals in South Africa (Genthe and Jagals, 2003). FC can be enumerated by several techniques. These include the membrane-filtration (MF) technique or the spread-plate technique with classical Petri dishes and the m-FC agar (Merck, Ltd., RSA), or the triphenyl 2, 3, 5-tetrazolium chloride-Tergitol agar (TTC agar; Garcia-Armisen et al., 2007). On the m-FC agar, FC produce blue colonies (Wutor et al., 2009), while on the TTC agar FC grow as orange colonies with a yellow halo under the membrane filter (Garcia-Armisen et al., 2007). Incubations are conducted at 44.5°C for 24 hours, and the results are expressed as CFUs/100 mL of water sample. Recent international developments in cultivation equipment include development of the 3M^{TM} Petrifilm plates (Schraft and Watterworth, 2005). These plates are flat-sheet and they allow for more samples to be processed in a limited incubator space (Petrifilm, 2009). *E. coli* and FC can be quantified after MF of the water sample as blue colonies with gas bubbles, and red colonies with gas bubbles (Schraft and Watterworth, 2005), respectively.

The other technique for the enumeration of FC which is specified in the DWAF water quality guidelines is the multiple tube fermentation technique, i.e. the most-probable number (MPN) technique (DWAF, 1996). An example of such a technique can be the five-tube MPN technique with the A1 medium (APHA, 1998). Incubations are first conducted at 35°C for 3 hours (the resuscitation period), and then at 44.5°C for 18-21 hours (APHA, 1998), or at 44.5°C for 20-24 hours (DWAF, 1996). Sample dilution is often required and the dilution solutions such as 0.1% peptone water (Schraft and Watterworth, 2005). The FC concentration is determined by counting tubes with visible growth/turbidity and gas production in the Durham tubes (APHA, 1998). The above mentioned media are mostly used in the studies on microbial water quality in South Africa (Bezuidenhout et al., 2002; Tandlich and Muller, 2008). Practical aspects and drawbacks of using FC, as an indicator microorganism of faecal contamination, are complex and they are critically evaluated in a separate section (see sections 2.4.1.1-2.4.1.3.)).

Apart from FC, the other most common indicator of faecal contamination of potable water is *E. coli*. The rationale for using this organism is that the majority of FC excreted in faeces of healthy humans and warm-blooded animals, such cattle and pigs is accounted for by *E. coli* (Medema et al., 2003). It has also been reported that if a finite concentration of *E. coli* is measured in a particular water sample, then humans exposed to the water will suffer from gastroenteritis (Pruss, 1998). Using the A1 MPN procedure mentioned for the FC enumeration *E. coli* can be enumerated when the FC positive tubes are re-inoculated into 0.1% tryptone water and incubated at 44.5°C for 24 hours. *E. coli* is then enumerated using the five-tube MPN procedure using the positive indole test (Grabow, 1996; DWAF, 1996).

A microtitration MPN method for the enumeration of *E. coli* has been developed based on the *β*-glucuronidase activity of *E. coli*. Decimal dilutions of the tested water samples are prepared using 0.1% peptone water or another suitable dilution medium. Two hundred microlitres of each dilution/sample is inoculated into individual wells of a 96-well plate with the dehydrated form of the medium containing 4-methylumbellferyl-*β*-D-glucuronide (ISO, 1994a; Garcia-Armisen et al., 2007). After inoculation, the plates are incubated at 44.5°C for 36 hours, and positive wells which fluoresce at 366 nm in a UV chamber are counted. Concentrations of *E. coli* are calculated using the eight-tube MPN procedure and the software from

the manufacturer (Grabow, 1996). The main disadvantage of this technique is that the limit of detection is 15 cells of *E. coli*/100 mL (Garcia-Armisen et al., 2007). Any enumeration technique, with application to potable water, must have the ability to detect 1 cell of *E. coli*/100 mL (SABS, 2006). Therefore this MPN technique is unlikely to become widely used in microbial water quality testing in South Africa.

Cultivation techniques in Petri dishes for the enumeration of *E. coli* have been used in studies on South African water quality. The techniques include MF or spread-plating on chromogenic media such as CM1046, where *E. coli* grows in the form of purple colonies after incubation at 37°C for 24 hours (Wutor et al., 2009). MF techniques that are based on *β*-glucuronidase activity include cultivation on nutrient agar with the addition of 4-methylumbelliferyl-*β*-D-glucuronide, where *E. coli* colonies fluoresce under UV light at 366 nm (Hamilton et al., 2005). They also include cultivations on the m-TEC agar where *E. coli* forms either glossy yellow colonies or the yellow and grey domed shaped colonies (Hamilton et al., 2005). Surveying the manufacturers in South Africa, this agar has become very popular in the enumeration of *E. coli*, as suggested by high demand and long backlogs in delivery times. Confirmation can be performed by picking off a representative number of colonies on the plate and conducting the indole test on the bacteria (Hamilton et al., 2005). Practical aspects and drawbacks of using *E. coli* as an indicator microorganism of faecal contamination are less complex than with FC and will be critically evaluated in a separate section below (see the relevant validity section).

TCs are the oldest indicator of faecal contamination from the coliform group. The TC concentrations are commonly enumerated as red colonies on m-Endo agar (Wutor et al., 2009). Incubations are performed at 35-37°C for 24 hours (APHA 1998) or 20-24 hours (DWAF 1996). TCs can be a useful indicator of faecal contamination in certain cases of viral waterborne diseases, as shown in international literature by Craun et al. (1997), thus fulfilling the second criterion for an indicator microorganism. However, TC violates the first criterion as an indicator microorganism for faecal contamination, since a large number of the TC species originates from non-faecal sources (Ashbolt et al., 2001; Leclerc et al., 2001). As a result, detection of finite concentrations of TC in a water sample does not necessarily indicate the presence of faecal contamination. Practical disadvantages of the enumeration of TC on the m-Endo agar include interferences from the growth of oxidase-positive non-coliform bacterial strains of *Aeromonas spp.* and *Pseudomonas spp.* (Grabow, 1996). These interferences can be eliminated through the application of chromogenic media, where detection of coliforms is based on their high *β*-galactosidase activity (Grabow, 1996). Enumeration of FC and TC can also be biased by high HPC values (LeChavellier and McPeters, 1985). For these reasons, TC is not recommended for routine detection of faecal contamination and the microbial water quality monitoring of potable water in South Africa.

Specific and commercially available cultivation media/systems can be used for simultaneous enumeration of FC, TC and/or *E. coli*. These include the Colilert®18 rapid test, the Chromocult coliform agar, the Fluorocult LMX broth, and the m-ColiBlue24 broth (Murray et al., 2004). The Chromocult coliform agar can also be used to enumerate *Salmonella spp., Yersinia spp.* and *Shigella spp.* (Murray et al., 2004). The fastest test currently available on the market is the Colilert®18 test which can provide the quantitative information about faecal contamination after 18 hours of incubation (Murray et al., 2004). This is shorter than 24 to 48 hours required in other routine tests. The Colilert®18 is an MPN technique and it constitutes the basis of the National Microbial Monitoring Programme (NMMP) for surface water (Murray et al., 2004) and groundwater in South Africa (Murray et al., 2007). No data are available in South Africa about the rates of false positive and false negative results. In international literature, the rates of false positive results for this test were equal to 7.4% for *E. coli* and to 9.6% for TC, while the rates of false negative results were equal to 3.5% for *E. coli* and 6.3% for TC, respectively (Chao et al., 2004). However, the rate of false positive results was equal 36.4% for the same system in the analysis of tropical freshwaters (Chao, 2006). These figures should be verified under South African conditions to obtain a complete understanding about the NMMP results.

The water quality guidelines for domestic use and other regulations require that the concentration of *E. coli* or FC is below 0 CFUs or cells/100 mL based on the geometric mean of several sample results (DWAF,

1996; SABS, 2006). Qualitative tests, giving only presence/absence information could be used to assess microbial water quality and public health risk. This type of information can be obtained using the Clark's presence-absence test which is commercially available (Clark, 1969; Clark and El-Shaarawi, 1993). It has been used routinely in drinking water microbial monitoring in the Ontario province of Canada for many years (Pipes et al., 1986). However, it requires incubation periods ranging from 48 to 120 hours (Pipes et al., 1986), and confirmatory tests and/or multiple incubations must be conducted simultaneously to detect FC, TC and/or *E. coli* (Clark and El-Shaarawi, 1993). The performance of the test and the labour-intensive character would preclude the application of this test in South Africa. In this section, we discussed the equipment requirements and time of analyses for different techniques used to enumerate coliform indicator microorganisms. The drawbacks of TC as an indicator of faecal contamination have also been outlined. Use of FC and/or *E. coli* as indicators of faecal contamination will depend on the survival rates, environmental conditions and non-faecal sources of the bacteria. This information must be considered when using these indicator microorganisms and the individual factors are critically evaluated in the next section.

2.4.1.1 E. coli:faecal coliform ratio

If the concentrations of *E. coli* and FC are equal in a water sample, then this should be an indication of faecal contamination from a (healthy) human, and/or a (healthy) animal (APHA, 1998). Thus the ratio of the concentration of *E. coli* and the concentration FC in water sample, i.e. the EC/FC ratio, is approximately 100%. Garcia-Armisen et al. (2007) analysed 166 samples of river water in France. The concentrations of *E. coli* and FC ranged from 1.00×10^1 to 10^7 CFUs/100 ml, and the average EC/FC was equal to 77 ± 62% (Garcia-Armisen et al., 2007). The standard deviation accounts for 81% of the average EC/FC value, and so the percentage of FC accounted for by *E. coli* will be highly variable. Guidelines of the US EPA state that the EC/FC ratio value is 63% in faecally-contaminated water samples (US EPA, 2002). Hamilton et al. (2005) studied the values of the EC/FC ratio for freshwater samples from Nashville, Tennessee. Concentrations of FC were enumerated using m-FC agar, while *E. coli* was enumerated on either m-TEC agar, nutrient agar with 4-methylumbelliferyl-*β*-D-glucuronide, m-ColiBlue or Colilert®24 (Hamilton et al., 2005). The authors found that the EC/FC ratio values ranged from 68 to 104%, and the values were the closest to 100% when the Colilert®24 method was used for enumeration (Hamilton et al., 2005). The EC/FC ratio results were highly dependent on the enumeration technique used, and high standard deviations of the EC/FC ratio were observed for individual water samples. In the international literature, there have been isolated reports where the values of the EC/FC ratio exceeded 200% (US EPA, 1984). This is probably caused by the FC cells entering the viable-but-non-culturable cells (VBNC) state due to environmental stress.

Using the data of Paulse et al., (2007), the EC/FC ratio values ranged from 1 to 21% for water samples taken in the Berg River in the Western Cape Province. These are much lower than the EC/FC values from the international literature, but the above discussion clearly demonstrates that the EC/FC ratio values are highly variable. It will therefore be very difficult to establish a clear cut-off percentage of the FC concentration that is accounted for by *E. coli*, if faecal contamination of a water resource comes from human and/or animal sources. Below we therefore explore probable reasons for the high variability of the EC/FC ratio and examine the drawbacks of using either FC or *E. coli* alone in the detection of faecal contamination.

Other FC besides *E. coli* can be excreted in faeces of humans and animals such as cattle, sheep and goats. One such example is *Klebsiella pneumoniae* which is excreted in faeces of 5% of the adult human population (Degener et al., 1983) and in 80% of dairy cow faeces (Munoz et al., 2006). Different strains of *Klebsiella spp.* have been shown to survive in the environment in substantial concentrations for up to 30 days after faecal contamination occurred (Dowd and Pillay, 1997). Isolates of *Klebsiella spp*., that were not of environmental origin and caused infections in humans and agricultural animals, were able to grow in the pulp mill wastewaters at 12ºC (Knittel et al., 1977). The chemical composition of soil and water, flow and atmospheric conditions on-site and the nature of the source of faecal contamination will control survival rates and concentrations of FC and *E. coli* in the contaminated water resource (Jamieson et al., 2004; Foppen and

Schijven, 2006). Factors controlling survival rates will vary between geographical areas, due to the variations in climate, soil type, land uses, agricultural and industrial activities, and variations in the composition of the sediment (Howell et al., 1996).

Different species of FC survive outside the host's body and can be detected in a water sample for different time intervals after faecal contamination has occurred (Medema et al., 1997). This time interval has a strong influence on the values of the EC/FC ratio and may explain the high variability in the EC/FC ratio values measured in the studies referenced herein. Evaluation of the risk to public health is based on the measured concentration of *E. coli* and/or FC, and therefore the survival rates have a major impact on the determined level of risk. To be able to assess the effect of survival rates on the concentrations of *E. coli* and FC, these will have to be measured under local conditions, i.e. in a given province of South Africa. Only a few studies have examined the influence of survival rates on the concentrations of FC in South Africa, e.g., Geldenhuys and Pretorius (1989) in surface water resources. The survival rate data could be outdated due to changing temperatures (Hunter et al., 2003), rainfall patterns and concentrations of nutrients in the water resources (Patz et al., 2000). More data of this kind will be required in South Africa, because the lack of survival rate data compromises the reliability of FC/*E. coli* as indicators of faecal contamination of water resources in the country.

Strains of *Klebsiella pneumoniae* are often isolated from plant surfaces and soils (Abbott, 1997). Examples of plant surfaces include tree bark and needles, mosses, and fern leaves (Duncan and Razzell, 1972). The cells of *Klebsiella spp.* can be released from plant surfaces into soil by precipitation and then possibly carried into a water resource with soil particles during surface runoff (Butterworth and MacCartney, 1991). Survival of FC during surface runoff will depend on the chemical composition of soils (Bitton et al., 1976). Cells of *Klebsiella spp.* can be found in wastewaters from the paper industry (Caplenas et al., 1981). Therefore FC can be released into the surface water bodies in the final effluent from paper mills, which are located in the vicinity of water resources. Concentrations of TC in the effluents/wastewaters from different stages of pulp milling were shown to range from 10^2 to 10^4 CFUs/mL, and strains of *Klebsiella spp.* accounted for 30 to 100% of the TC concentration (Knittel et al., 1977). The temperatures of these wastewaters often reach 44ºC, which is very close to the cultivation temperature used for the enumeration of FC, i.e. providing an ideal environment for growth of FC/*Klebsiella spp*.

2.4.1.2 Environmental sources

Surface runoff from environmental sources and discharge of industry wastewaters into surface water bodies can contribute to the measured FC concentrations. Besides *Klebsiella spp.*, environmental sources have been shown to contribute to the measured concentrations of *Enterobacter spp.*, *Citrobacter spp.*, and *Serratia spp.* (Edberg et al., 2000). Therefore if the risk of waterborne diseases is defined and assessed as a function of the FC concentration, any increase in the measured FC concentration, e.g. pulp and paper industry wastewaters, will exaggerate the true risk of waterborne diseases and faecal contamination due to the contributions from non-faecal sources. Therefore public health officials will have to gather as much on-site information as possible about the relative contributions of non-faecal to the measured concentration and speciation of FC. Creation and maintenance of relevant databases will also be required to properly assess the public health risks of waterborne diseases in a given water resource. This will, however, complicate the use of FC as an indicator of faecal pollution of water resources in South Africa.

Soils are the only environmental source of *E. coli* (Hardina and Fujioka, 1991; Desmarais et al., 2002) and so less information will have to be gathered about the non-faecal contributions to the measured concentration of *E. coli* than for FC. At the same time, the concentration of *E. coli* will be more reflective of the true extent of faecal contamination of a water resource than the concentration of FC (Hamilton et al., 2005). As *E. coli* is one bacterial species, one will be able to make clearer conclusions about the effect of the environmental fate and stress on the culturability in comparison of cells, compared to the multi-species FC. Thus enumeration of

E. coli will provide a more realistic assessment of the public health risk from faecal contamination of water resources than the enumeration of FC.

2.4.1.3 Enumeration problems

A problem in the enumeration of *E. coli* is that environmental stress can lead to the formation of *E.* coli that is viable but not culturable (VBNC), thus a decreased concentration of the indicator microorganism is measured. This leads to an underestimation of the risk of waterborne diseases. Methods based on DNA molecular markers take VBNC into account and the sensitivity of these techniques is comparable to the plating techniques (APHA, 1998). Examples in South Africa include the technique for *E. coli* O157:H7 (Ateba and Bezuidenhout, 2008). In international literature, DNA methods have been based on the detection of the glucuronidase gene, the glucuronidase operator gene, and the *β*-galactosidase gene (e.g. Bej et al., 1990; Fricker and Fricker, 1994; Iqbal et al., 1997).

Low bacterial concentrations can be detected using DNA techniques, but there have been reports of false positive and false negative results in *E. coli* detection using the DNA-based techniques (Pletschke et al., 2007). Environmental contaminants have been shown to interfere with the DNA-based techniques (Leonard et al., 2003) and the DNA methods do not distinguish between viable and dead cells (Pletschke et al., 2007). Besides the formation of VBNC, other problems originate from the application of *E. coli* as an indicator microorganism for faecal pollution. The first one is that *E. coli* has been shown to survive in the environment for longer periods of time than pathogenic microorganisms that cause waterborne diseases (Hamilton et al., 2005). This observation will have to be verified under local conditions in South Africa. The second problem is the inability to identify the source of faecal contamination directly from the concentration of *E. coli* in a given water sample. These will be addressed in more detail below (Section 3.5).

2.4.2 Faecal streptococci

Besides the coliform group, the other commonly used group of indicator bacteria is the enterococci/faecal streptococci. These are several groups of related bacteria that are a part of the normal microflora of the human gut (Grabow, 1996). As a result, they fulfil the first criterion for an indicator microorganism of faecal contamination. *Enterococcus faecalis* and *Enterococcus faecium* are the most common species of faecal streptococci present in human faeces (Franz et al., 1999). Concentrations in human faeces range from 10^5 to 10^7 cells or CFUs/g faeces for *Enterococcus faecalis* and 10^4 to 10^5 cells or CFUs/g faeces for *Enterococcus faecium* (Fisher and Phillips, 2009). Other species commonly found in human and animal faeces include *Enterococcus durans* and *Enterococcus hirae* (Grabow, 1996).

The standard methods for enumeration of faecal streptococci are the MF technique or the spread-plate technique on m-Enterococcus agar, followed by incubation at 35 to 37°C for 48 hours (DWAF 1996; APHA 1998). Enterococci are counted as reddish-brown colonies (DWAF, 1996). An MPN was developed by the International Standardization Organisation (ISO, 1994b) and its principle is similar to that for *E. coli* (ISO, 1994a). Incubations are also conducted at 44.5°C for 36 hours, but enumeration is based on fluorescence of thallium acetate in the presence of nalidixic acid for faecal streptococci (ISO, 1994b). The commercially available Enterolert® system is similar to the Colilert®18 system for *E. coli* and coliform bacteria (Desmarais et al., 2002).

Potgieter et al. (2005) studied the concentrations of indicator bacteria in stored tap water and spring water in households in the Venda region of the Limpopo Province, South Africa. In the individual stored tap water samples, the geometric mean concentrations ranged from 4.9×10^2 to 5.8×10^3 CFUs/100 mL for TC, from 2.6×10^2 to 3.7×10^3 CFUs/100 mL for FC; and from 3.1×10^3 to 5.8×10^3 CFUs/100 mL for faecal streptococci. On the other hand, the geometric mean concentrations in the spring water were equal to 5.1×10^3 CFUs/100 mL for TC, 3.2×10^3 CFUs/100 mL for FC, and 5.1×10^3 CFUs/100 mL for faecal streptococci.

When water samples were taken from the Modder River, concentrations of faecal streptococci have been shown to range from 1.0 x 10^0 to 6.5 x 10^4 CFUs/100 ml, while the FC values ranged from 0 to 8.4 x 10^5 CFUs/100 mL (Jagals and Grabow 1996).

Kühn et al. (2003) studied the prevalence of enterococci in different samples from Denmark, Spain, UK and Sweden. In faeces from healthy humans, 75 to 87% of samples contained enterococci while 100% of faecal samples from hospitalised patients contained enterococci. The prevalence of enterococci in pig faeces ranged from 86 to 100%, depending on the geographic location (Kühn et al., 2003). For domestic sewage samples, 78 to 97% of all samples tested positive for enterococci, while the percentage of positive samples ranged from 79 to 100% for hospital sewage. Thirty percent of soil and crop samples from fields without prior manure application contained enterococci. However, the concentrations were much lower than in other samples analysed. Moneoang and Bezuidenhout (2009) studied the prevalence of *Enterococcus spp.* and *E. coli* in faeces of commercial and communal pigs from the North-West Province, South Africa. Fifty eight percent of the faecal samples contained *Enterococcus faecium* and 23% contained *Enterococcus gallinarum*. The prevalence of *E. coli* in the faeces of pigs ranged from 88.5 to 100%.

Jagals et al. (1995) used multiple indicator microorganisms to measure the extent, and identify sources of faecal contamination in selected water resources in South Africa. Samples that were faecally contaminated from human and animal sources contained faecal streptococci. The FC/faecal streptococci concentration ratio ranged from 3.5 to 4.7 directly at the entry point of human faecal contamination into the sampled stream and river (Jagals et al., 1995). This ratio varied from 0.8 to 1.7 for water resources receiving faecal contamination from livestock (Jagals et al., 1995). The FC/faecal streptococci concentration ratio has been suggested by several authors as a possible way to distinguish between different sources of faecal contamination (e.g. Csuros and Csuros, 1999). However, other authors have shown that this criterion is not reliable due to different survival rates of FC and faecal streptococci, dilution in the surface streams and attachment to sediment particles (Howell et al., 1996). The literature data indicates that faecal streptococci fulfil the first criterion for the indicator microorganism of faecal contamination. At the same time, the concentrations in non-faecal sources are low and therefore if faecal streptococci are detected in a water sample, then this most likely indicates faecal contamination of the water resource.

Members of *Enterococcus spp.* have been reported to survive in the temperature range of 10 to 45°C, salinities up to 6.5% of NaCl, and they can grow at pH values ranging from 4.4 to 9.9 (Hardie and Whiley, 1997; Morrison et al., 1997; Fisher and Phillips, 2009) with an optimum pH of 7.5 (Van der Berghe et al., 2006). The resistance in such a wide range of pH values is caused by the stability of the cytoplasmic membrane towards acids and alkali (Fisher and Phillips, 2009) and can be due to the membrane-bound H^+-ATPase (Nakajo et al., 2005). The stability of the cytoplasmic membrane decreases with increasing temperature (Ivanov et al., 1999), caused by decreasing concentrations of fatty acids (Fisher and Phillips, 2009). If *Enterococcus faecalis* is grown at optimum temperature of 37ºC, then the cells are not able to withstand the temperature shock upon release into the environment (Ivanov et al., 1999). Thus survival and culturability of enterococci/faecal streptococci will strongly depend on the temperature difference between the host's body and the water body receiving the faecal contamination. This will influence the measured concentration of faecal streptococci in the water sample, i.e. the measured estimate of faecal contamination.

Enterococci produce extracellular peptides with antimicrobial properties, so-called bacteriocins (Fisher and Phillips, 2009). Isolates producing these proteins include strains of *Enterococcus faecalis* which are members of the microflora of porcine intestines (Fisher and Phillips, 2009). To the best of the authors' knowledge, production of bacteriocins has not yet been demonstrated for enterococci isolated from water bodies with faecal contamination. Release into the environment from the host's body will lead to substantial environmental stress on the cells of enterococci, resulting from temperature shifts and competition from the autochthonous microflora. Therefore, it is likely that bacteriocins will be produced by enterococci in faecally-contaminated water resources. This will have a strong potential influence on the survival rates of faecal streptococci in the contaminated water resource. It can influence the survival rate of the faecal streptococci,

i.e. influencing the adherence to the criteria for indicator organisms noted above. The exact extent of this effect will have to be verified under local conditions in South Africa.

In some studies, it was shown that faecal streptococci do not undergo multiplication outside the host's body (Howell et al., 1996; WHO, 1997), but results from other studies indicate significant re-growth (Desmarais et al., 2002). The environmental sources of faecal streptococci include soils and some agricultural crops, but these sources only contribute around 5% to the faecal streptococci concentration in water resources (Kühn et al., 2003). Therefore a non-zero concentration of faecal streptococci in a water sample provides a more conclusive indication of faecal contamination than a non-zero concentration of FC. The proof is probably comparable to that of *E. coli*. Conversely, the infectious doses, i.e. the number of bacterial cells that have to be ingested by an individual for the development of a waterborne infection, are commonly based on the concentrations of FC or *E. coli* (e.g. Hamilton et al., 2005). Most of the water quality guidelines and regulations are defined using FC/TC/*E. coli* (DWAF 1996; SABS, 2006). Consequently, public health officials will more likely make decisions regarding the risk of a waterborne disease outbreak, based on the concentrations of FC or *E. coli* and not based on the concentration of faecal streptococci.

Presence of faecal contamination or lack thereof in a given water sample can be determined within 18-24 hours using *E. coli* or FC, while 36-48 hours are required based on the enumeration of faecal streptococci. However, if time allows both *E. coli* or FC and faecal streptococci enumeration should be conducted to obtain more conclusive proof of faecal contamination of a given water resource. Major gaps in literature data exist in South Africa in regard to the survival rates in surface water resources in different geographical areas, and transport properties of indicator microorganisms through the main soil types. Studies aimed at obtaining such data will have to be conducted in the near future, since the framework for the microbial water quality monitoring in South Africa is based and will be based for the foreseeable future on bacterial indicator microorganisms.

2.4.3 *Rhodococcus coprophilus*

Rhodococcus coprophilus is a noncardioform actinomycete, which is excreted in the faeces of cattle, chickens, ducks, geese, horses, pigs, sheep, and turkeys, and but not in human faeces (Oragui and Mara, 1983; Savill et al., 2001). Environmental sources of this actinomycete include plant surfaces (Long et al., 2002) and soils (Hamamura et al., 2006). If the concentration of *Rhodococcus coprophilus* is higher than 5000 CFUs/100 mL in a water sample from the selected sampling site, then the faecal contamination is from an animal source and the influence of the environmental sources on the concentration of *Rhodococcus coprophilus* is negligible (Long et al., 2002).

2.4.4 Viral biomarkers

The majority of research on identifying the sources of faecal contamination in South African water resources has been done using viral biomarkers (Grabow et al., 1998; Sundram et al., 2006; Momba et al., 2009). The two main groups of viral biomarkers investigated are coliphages, infecting *E. coli* and closely related bacteria, and bacteriophages of *Bacteroides fragilis* HSP40 (Grabow 2001). Somatic coliphages include members of families *Myoviridae*, *Siphoviridae*, *Podoviridae* and *Microviridae* (Grabow 2001), and infect *E. coli* and related bacteria through an interaction with receptor sites on the cell walls of host bacteria (Grabow et al., 1998). They can contain DNA or RNA as nucleic acids (Grabow et al., 1998). Somatic coliphages have been shown to attach to dead bacteria (Grabow 2001).

Male-specific coliphages infect their host through the interaction with the fertility (sex) fimbriae (F-fimbriae) of *E. coli* and related host bacteria (Grabow 2001). These fimbriae (sex-pili) are coded by the F-plasmid of *E. coli* K-12, which is related to the plasmids of the IncFincompatibility group (Havelaar and Pot-Hogeboom,

1988). If the viral receptor is located at the shaft of the F-fimbriae, then the virus generally contains RNA as the nucleic acid, and such male-specific coliphages are called F-RNA coliphages (Grabow et al., 1998). If the viral receptor is located at the tip of the F-fimbriae, then the virus generally contains DNA as the nucleic acid, and such male-specific coliphages are called F-DNA coliphages (Grabow et al., 1998). Both types of bacteriophages are shed in faeces of humans, thus are candidates for indicator microorganisms of faecal contamination of water resources (Grabow, 2001). However, only F-RNA coliphages have been used to track sources of faecal contamination in South Africa.

In the case of *E. coli* and related bacteria, the F-fimbriae are only produced when the temperature of the environment reaches 32 to 40°C, which are rarely reached in South African water resources (Grabow et al., 1998). Therefore no replication of F-RNA coliphages is expected in the water environments after a faecal contamination event. Based on serological typing and genotyping, F-RNA coliphages can be separated into 4 distinct serotypes/genotypes, namely I, II, III, and IV (Grabow, 2001). Strains of *Bacteroides spp.* are anaerobic and Gram-negative bacteria, which do not produce spores, as they die-off rapidly upon exposure to O_2 (Grabow, 2001). Bacteriophages of *Bacteroides spp.* are fairly resistant to unfavourable environmental conditions (Tartera and Jofre, 1987; Tartera et al., 1988). They have attracted considerable research attention as viral indicators of human origin of faecal contamination of water resources (Schaper and Joffre, 2000) as they are not found in animals faeces (Grabow, 2001).

Grabow et al., (1998) investigated the applicability of F-RNA coliphages, somatic phages and the phages infecting *Bacteroides fragilis* HSP40 as a tool for distinguishing between human and animal sources of faecal contamination in water resources in South Africa. The authors also measured the survival rates of these bacteriophages in the environment and compared the environmental fate of the bacteriophages to the environmental fate of indicator microorganisms and human viruses (Grabow et al., 1998). F-RNA coliphages were enumerated by the double-agar plaque assay (ISO 1995), with *Salmonella typhimurium* WG49, and *E. coli* HS(pFamp)R as bacterial hosts. In 34 samples from the Klip River, the *Salmonella typhimurium* WG49 host gave consistently higher counts of F-RNA coliphages than the *E. coli* HS(pFamp)R. Experimental problems of viral enumeration included the recovery of viral particles and cost of laboratory consumables (Grabow et al., 1998). The presence/absence test for bacteriophages had been recommended for routine testing of water samples (DWAF, 1996), but Grabow et al. (1998) found that this method is highly prone to contamination, making its applicability in routine faecal source tracking and monitoring questionable.

A comparative study was done on the application of F-RNA coliphages as biomarkers for faecal contamination from different sources in South Africa and Spain (Schaper et al., 2002). In South Africa, the samples included stool samples and wastewater samples from the Pretoria Academic Hospital (human sources of faecal contamination), samples of faeces of pigs, poultry and cows from several rural farms around the Gauteng province (animal sources of faecal contamination), settled sewage from the Zeekoegat and Baviaanspoort wastewater treatment plants in the vicinity of Pretoria (sources of faecal contamination from human dwellings but possibly from dogs, poultry, and maybe cattle), wastewater from developing communities in Atteridgeville, Soshanguwe and Botshabelo (human sources of faecal contamination due to seepage from pit latrines and septic tanks), raw wastewaters from the abattoirs (mainly animal faecal pollution sources, i.e. cattle and sheep), and secondary settled effluent from the wastewater treatment plant in Daspoort (domestic sewage of with possible mixed faecal inputs).

Ten percent of human faeces samples contained F-RNA coliphages, while 70% of the faecal poultry samples contained F-RNA coliphages. Sixty six percent of the pig faeces samples were positive for the presence of F-RNA coliphages, but only 33% of cattle faeces samples contained F-RNA coliphages (Schaper et al., 2002). The samples that contained F-RNA coliphages will henceforth be referred to as “positive samples”. The F-RNA coliphage concentrations in samples ranged from 6.5×10^2 PFUs/100 g faeces cattle samples to 1.0×10^5 PFUs/100 g faeces in poultry. For wastewater samples, the concentrations ranged from 2.7×10^3 PFUs/100 mL in the final effluent from wastewater treatment plants to 3.8×10^6 PFUs/100 mL in abattoir wastewaters. F-RNA specific coliphages accounted for 62.6 to 98.7% of the plagues detected on *Salmonella*

typhimurium WG49 plates. If human faeces were positive for F-RNA coliphages, then 90% contained serotype/genotype II F-RNA coliphages and 10% contained serotype/genotype III. Results for the presence of serotypes/genotypes in faeces from poultry confirmed previous reports that 80% of samples positive for F-RNA coliphages serotype/genotype I, while F-RNA coliphages of serotype/genotype IV were found in 20% of the positive samples. One hundred percent of F-RNA coliphages in the (positive) cattle faeces belonged to serotype/genotype I.

A major overlap between the F-RNA coliphages serotypes/genotypes was established between human faeces and animal faeces or sources of faecal contamination. For the positive samples of pig faeces, serotype/genotype I accounted for 53% of the F-RNA coliphages, while serotype/genotype IV accounted for 19% of all coliphages. Twenty eight percent of all F-RNA coliphages in pig faeces belonged to serotype/genotype II. Abattoir wastewater contained 2% of serotype/genotype II, while 6% of the isolates from hospital wastewater belonged to serotype/genotype I. The concentrations of individual serotypes/genotypes in wastewaters from developing communities and wastewater treatment plants showed substantial variations in concentrations of individual serotypes/genotypes (Schaper et al., 2002).

Sundram et al., (2006) studied the application of F-RNA coliphages to track faecal contamination sources in the Umgeni catchment in the province of KwaZulu-Natal, South Africa. The samples included wastewaters from a cattle feedlot, a piggery and from two hospitals in Pietermaritzburg (Sundram et al., 2006). Methodologies of Grabow et al. (1998) and Hsu et al. (1995) were followed in this study. The authors detected serotypes III and IV in the pig slaughter house wastewaters, and serotypes I, III and IV in chicken wastewaters (Sundram et al., 2006). Serotypes II and III have been isolated from pigs of different ages, which may be explained by the exposure to human faecal material during feeding (Hsu et al., 1995).

Momba et al., (2009) studied the concentrations of free residual chlorine, somatic and F-RNA coliphages in the final effluent from wastewater treatment plants in Alice, Fort Beaufort, Dimbaza and East London in the Eastern Cape, South Africa. The average concentrations of somatic coliphages ranged from 3.88×10^5 to 2.45×10^6 PFUs/100 ml, while the average concentrations of F-RNA coliphages ranged from 2.43×10^3 to 1.82×10^5 PFUs/100 ml. The overlap between the serotypes/genotypes of F-RNA coliphages that can be found in human and animal source of faecal contamination will preclude conclusive identification of the source of faecal contamination. Therefore application of these bacteriophages in routine detection and source identification of faecal contamination should be discouraged in South Africa. Maintenance of a viable inoculum of *Bacteroides fragilis* HSP40 was shown to be difficult under routine laboratory conditions and the MPN counting of phages can be problematic due to high variability of the measured plaque numbers (Grabow et al., 1998). The inoculum problem could be solved using the triple agar technique with oxyrase addition (Puig et al., 1998). Oxyrase is a mixture of mono- and dioxygenases which are produced by *E. coli* (Puig et al., 1999) and it allows for anaerobic cultivations to be conducted under aerobic conditions. However, the enzyme is only available from a US supplier and no local distributors are currently operating in South Africa. Thus the routine application of the Oxyrase modification is not feasible at present.

A PCR method developed for the detection of bacteriophages of *Bacteroides fragilis* HSP40 is more sensitive than the plaque assay (Grabow, 2001). A threshold concentration of the cells of *Bacteroides fragilis* HSP40 is required for the bacteriophage replication to take place, and the value of 104 CFUs/mL has been suggested (Goyal et al., 1987). The B40-8 type-specific phage for *B. fragilis* HSP40 has been shown to replicate at 22 or 30°C, but no replication has been reported in freshwater or sediment samples under aerobic or anaerobic conditions (Grabow, 2001).

F-RNA coliphages are isolated from only 15% of human faeces samples (Grabow et al., 1995). Bacteriophages of *Bacteroides fragilis* are excreted in faeces of 5 (Kai et al., 1985) to 10% (Tartera and Jofre, 1987) of the human population. Therefore a failure to detect F-RNA coliphages and/or bacteriophages infecting *Bacteroides fragilis* in a water sample does not preclude the presence of faecal contamination (Grabow et al., 1998). The overlap between the F-RNA coliphage serotypes/genotypes, found in human and

animal sources, will preclude conclusive faecal source tracking in South African water resources. Plaque assays require the use of complex growth media, supplemented with antibiotics, and strict anaerobic conditions must be maintained during the entire incubation for the enumeration of bacteriophages of *Bacteroides fragilis* (Grabow, 2001). This requires specialised training and highly experienced laboratory staff to perform the enumerations. A severe skills shortage will preclude the widespread application of viral indicators in microbial water quality monitoring in South Africa (Murray et al., 2007).

Some papers in the international literature proposed the substitution of bacteriophages infecting *Bacteroides fragilis* strain RYC2056 for bacteriophages of *Bacteroides fragilis* HSP40 for the detection of human faecal pollution, as the former is not found in human faeces in certain geographical areas (Puig et al., 1999; ISO 1998).

Based on the information presented above, the application of F-RNA coliphages and the bacteriophages of *Bacteroides fragilis* will be limited because of the limited excretion of the viruses in faeces of humans and agriculturally-relevant animals, and the overlap of serotypes between the different sources of faecal contamination. At the same time, there are not enough specialised laboratories in South Africa as noted above.

2.4.5 Bifidobacteria

Bifidobacteria are anaerobic and Gram-positive bacteria, which are abundant among the intestinal microbiota of humans and other warm-blooded animals (Mitsuoka 1990; Bonjoch et al., 2005). They are excreted in faeces in concentrations as high as 10^{10} cells/g in faeces (Tannock, 1997). No replication of bifidobacteria generally takes place in environmental water resources unless the water temperature exceeds 30°C (Carillo et al., 1985), which is uncommon in South Africa. The YN-6 medium was the first medium specifically designed for the enumeration of bifidobacteria in water samples (Resnick and Levin 1981a, b).

Resnick and Levin (1981a, b) studied the distribution of bifidobacterial strains in faeces of healthy human adults, chickens, dogs, cows, pigs, horses, cats, sheep, beavers, goats, and turkeys; as well as in samples of raw, primary, secondary and chlorinated sewage from two wastewater treatment plants, mainly processing domestic sewage, and in environmental water samples (Resnick and Levin 1981a,b). Later studies reported problems with the inhibition of the growth of certain bifidobacteria on YN-6 medium and overgrowth by faecal streptococci (Mara and Oragui, 1983). To increase specificity, the YN-17 medium and the human bifid sorbitol agar were developed (Mara and Oragui, 1983). The YN-17 medium can be used to enumerate total bifidobacteria, while the human bifido sorbitol agar can be used to enumerate sorbitol-fermenting bifidobacteria (Mara and Oragui, 1983).

Bifidobacterium longum and *Bifidobacterium adolescentis* are the most common strains of bifidobacteria in human faeces, with concentrations of individual strains ranging from 2×10^6 to 2×10^9 CFUs/1 g of wet faeces (Resnick and Levin, 1981a; Bonjoch et al., 2009). The species composition in raw sewage copies the species distribution of bifidobacteria in human faeces (Resnick and Levin, 1981a). Fewer strains and lower concentrations of bifidobacteria are detected in the samples of animal faeces than in human faecal samples. The total culturable bifidobacteria were shown to survive in river water for 9 hours, while *Bifidobacterium adolescentis* and *Bifidobacterium dentum* were detected in river water for up to 48 hours after inoculation, using quantitative PCR (Bonjoch et al., 2009). The die-off rates ranged from 1.44 to 3.81 day^{-1} (Ottoson, 2009). Therefore the enumeration of bifidobacteria will be mostly useful for the detection of recent faecal contamination from unknown sources. Survival rates have not been in South Africa.

Bonjoch et al. (2005) tested the applicability of the ratio of the sorbitol-fermenting bifidobacteria and the total bifidobacteria (TR) as a criterion to distinguish between the human and animal sources of faecal pollution. Twenty two samples of sewage from municipal wastewater treatment plants were taken. Eleven wastewater

samples were collected from pig, cattle and poultry slaughter houses and 18 samples from the animal slaughter house wastewaters (Bonjoch et al., 2005). Putative bifidobacteria were confirmed by genus-specific colony hybridisation by replica-plating from selective media. This was done using nylon membranes, and the genus-specific digoxigenin-labelled fluorescent probes (Nebra et al., 2003). The sequence of the probe was 5'-ACG TCA CGG TGG GAA CTC A-3' (target site 1132-1151 with numbering of the 16SrRNA gene *E. coli*) and the 5'-CCG GTT CAC AGG TGG T-3' (target site 1037-1053 with numbering of the 16SrRNA gene *E. coli*). The authors attempted to prepare one probe for specific detection of *Bifidobacterium dentium* which is of human origin. The other probe was designed for the detection of bifidobacteria from animal sources. The results showed that only the probe for *Bifidobacterium dentium* was specific.

The concentrations of sorbitol-fermenting bifidobacteria were always higher than 10^6 CFUs/100 mL in the human-faecally-polluted wastewaters, while the animal wastewaters always contained concentrations lower than this number (Bonjoch et al., 2005). The concentrations of the total bifidobacteria ranged from 10^6 CFUs/100 mL to 10^7 CFUs/100 ml, and there was no systematic difference between the values in human and animal wastewaters. The ratio of the sorbitol-fermenting bifidobacteria and the total bifidobacteria was always higher than 0.20 for the wastewaters with human faecal pollution, and always lower than 0.05 for the wastewaters with faecal pollution of animal origin. No mixed sources were examined. It is for this reason that the authors proposed that values of the ratio to be used to distinguish human and animal source of faecal contamination of surface water resources. Different PCR-based approaches for the detection of *Bacteroidales spp.* and *Bifidobacterium spp.* have been developed but the specificity ranged from 17 to 87% in environmental water samples (Dorai-Raj et al., 2009).

In the study by Blanch et al. (2006), the cut-off point for tracking ratio was 3.2. Based on this value, the authors were able to distinguish human and animal faecal pollution sources with a 95% certainty. Samples were taken across Europe, covering a wider geographical area and wider range of climatic conditions than in the study of Bonjoch et al., (2005). Local differences in the survival/die-off kinetics of the respective group of bifidobacteria can cause the sorbitol-fermenting bifidobacteria and the total bifidobacteria to die-off at different rates. At the same time, environmental stress is likely to cause greater inhibition of growth by bifidobacteria on the YN-17 medium, which contains higher concentrations of antibiotics than the HSBA (Mara and Oragui 1983). As a result, the cut-off value of the ratio of the sorbitol-fermenting bifidobacteria and the total bifidobacteria will have to be calibrated for the conditions in South Africa.

Jagals and Grabow (1996) measured the concentrations of the sorbitol-fermenting bifidobacteria, values of FC and the concentrations of faecal streptococci in the vicinity of an informal settlement on the banks of the Modder River in the Free State Province. Pit and bucket latrines and limited waterborne sewage management constituted the sanitation infrastructure in the settlement. A non-perennial stream flowed through the informal settlement and fed into the perennial river downstream of the settlement. Both of these water resources were sampled at five sampling sites during the dry and rainy seasons, as well as immediately after a thunderstorm. The dominant portion of the bifidobacteria was of human origin. During the dry season, the concentration of the sorbitol-fermenting bifidobacteria ranged from 1.00×10^0 to 3.60×10^3 CFUs/100 mL in the non-perennial stream just downstream of the settlement. The concentrations decreased to 1.00×10^0 to 4.70×10^2 CFUs/100 mL further downstream of the settlement at the junction between the two streams. Concentrations of bifidobacteria were below 0 CFUs/100 mL upstream of the informal settlement, i.e. no environmental sources of bifidobacteria were observed. During the rainy season, the concentrations of the sorbitol-fermenting bifidobacteria ranged from 6.00×10^1 to 9.20×10^4 CFUs/100 mL in the non-perennial stream and just downstream of the informal settlement. At the junction of the two rivers, the concentrations increased to between 1.30×10^2 and 2.17×10^4 CFUs/100 mL. The concentrations dropped to between 3.00×10^0 and 2.73×10^2 CFUs/100 mL further downstream of the informal settlement along the perennial river.

Based on the data of Bonjoch et al. (2005), the distinction between human and animal sources of faecal contamination might be possible based on the ratio of the concentration of sorbitol-fermenting bifidobacteria

and the concentrations of the total bifidobacteria. The cut-off value of this ratio will be determined for local conditions in South Africa as described above. Distribution of bifidobacteria between the sediment and water will have to be measured. Since 48 hours is required to obtain the value of the ratio of the sorbitol-fermenting bifidobacteria and the total bifidobacteria, the time frame is comparable to the current indicator organism tests, but more information about the source of the faecal contamination could be obtained.

2.5 CHEMICAL BIOMARKERS

Many chemical compounds have been used as indicators or biomarkers of faecal pollution (Field and Samadpour, 2007). Production of urobilin starts with the degradation of haemoglobin, which takes place in the reticulonedothelial cells of spleen, the bone marrow and the liver. This leads to the production of biliverdin, which is then converted into bilirubin. Bilirubin is excreted into blood plasma and circulated in the form the bilirubin-albumin complex. This can be deposited into the adipose tissues, in the skin, or sclera. Hepatocytes take up the bilirubin-albumin complex from the blood plasma, and bilirubin forms a complex with ligandinin. Bilirubin is then converted into bilirubin-glucuronide, and this then passes through the bile duct into the duodenum, and the small intestine. Bacteria present there convert it into urobilinogen and stercobilinogen. Daily 1-4 mg of urobilinogen is excreted in urine, and can undergo photoconversion into urobilin. The dominant portion of urobilinogen is converted into stercobilin and urobilin, which are excreted in faeces at a rate of approximately 260 mg/day (http://florey.biosci.uq.edu.au/GMC/GMCyr1L1.html). The molecular weight of urobilinogen is 592.726 g/mol (www.sigmaaldrich.com), while the molecular weight of urobilin is 590.710 g/mol (www.sigmaaldrich.com). This means the urobilin detected in the water and sediment samples, if contaminated from human faecal pollution sources, 98% will originate from faeces, and only 2% will be due to urine. It will originate from human and other higher mammals.

Miyabara et al. (1994a) sampled river sediments and river water from the urbanised and heavily populated areas of Tokyo (Japan), and from the Miura peninsula (Japan). These were stored in glass bottles and transported into the laboratory on ice. Concentrations of urobilin were determined using HPLC with fluorescent detection. Concentration of heterotrophic bacteria (bouillon agar) and the total coliforms (deoxycholate agar) were measured. The average sediment concentrations of urobilin in samples from the urbanised areas, i.e. Tokyo rivers, was equal to 14 470 ng/kg. The average concentration in the rural areas, i.e. the Miura River was 160 ng/kg. Concentrations of urobilin increased in heavily populated areas in comparison with less populated areas, but fluctuations occurred in rivers likely as a result of rainfall and other factors (Miyabara et al., 1994a). In a follow-up study, Miyabara et al. (1994b) studied the stability of urobilin in river waters, and the authors also tried to relate the concentrations of urobilin to the COD, ammonium concentration measured as NH_4^+-N, and TC/FC. They collected samples from the same area as in their previous study (Miyabara et al., 1994b). FC and TC were quantified using the MPN procedure with the lactose broth, the EC medium, and the brilliant green lactose bile broth (APHA, 1998). The stability of urobilin was tested by incubating selected water samples with different levels of urobilin, and the concentrations of urobilin, COD, NH_4^+-N, and indicator bacteria were measured at different times of incubation.

The TC and FC values increased by 3 to 4 orders of magnitude within 12 hours of incubation, while the concentrations of urobilin, COD, and NH_4^+-N remained constant. This is not surprising as the correlation between different indicators of faecal pollution is usually only qualitative (Tandlich and Muller, 2008). Urobilin levels in treated sewage were around 400 ng/l, while the concentrations in regular streams ranged from 4 to 850 ng/l (Miyabara et al., 1994b). In the case of discharge of raw sewage into the stream, the concentration of urobilin jumped to 6992 ng/l. The distribution of COD, NH_4^+-N, and coliforms were different from the concentration distribution of urobilin. Both humans and higher animals produce urobilin. Based on the data of Miyabara et al. (1994a,b), sample processing and analyses can be completed within 8 hours. If samples of sediment and/or water to be tested can be delivered into an analytical laboratory on the day of samples

collection, and this laboratory is equipped with a HPLC with fluorescence detection, then the presence/absence of faecal pollution can be ascertained within 12 hours. As a result, the measurement of urobilin concentrations could be applied as a rapid screening method for the presence/absence of faecal pollution, if the equipment of choice is HPLC with fluorescence detection.

Aminoacetone is produced from L-threonine in mammals (Davis and Austic, 1997). It is excreted in urine and faeces, i.e. can be used as indicator for sewage contamination, but can't provide information about the source of the faecal pollution. Taking the structure of aminoacetone, coprostanol and urobilin into account, the respective 1-octanol/water partition coefficients (expressed as log *P*) can be calculated using the hydrophobic fragment constant method (Rekker and Mannhold, 1992). The calculations were estimated for the purpose of this communication using the Kowwin programme (version 1.54; Meylan, 1993-1996). The respective log *P* values were as follows: -0.94 for aminoacetone, 6.85 for urobilin and 8.82 for coprostanol (see below). This indicates that aminoacetone will be present mostly in the dissolved phase and not associated with particulate matter. At the same time, coprostanol and urobilin will be mostly associated with particulate matter. In sea water, the concentrations decreased from 40 µmol/l to around 5 µmol/l in 25 days. Therefore detection of aminoacetone could provide indication that a faecal contamination event took place a sampling site within the last month. Dependence of the concentration of aminoacetone mainly on flow phenomena would have to be verified in freshwater streams and under South African conditions. However, if similar behaviour of this compound can be expected in freshwater as it was reported in the sea water, then aminoacetone could be used a biomarker for measurement of faecal plume spread using gas chromatographic equipment (Davis and Austic, 1997).

Most literature on chemical biomarkers of faecal pollution has focused on the application of cholesterol metabolites, namely faecal sterols and bile acids (Bull et al., 2002). Cholesterol is reduced in the intestinal tracts of certain higher mammals, and the resulting products include 5*β*-stanols (namely 5*β*-cholestan-3*β*-ol or coprostanol, and 24-ethyl-5*β*-cholestan-3*β*-ol or 24-ethylcoprostanol or 5*β*-stigmastanol), 24-methylcholest-5-en-3*β*-ol (campesterol), 24-ethylcholest-5-en-3*β*-ol (sitosterol), and 24-ethylcholest-5,22-dien-3*β*-ol (stigmasterol). Coprostanol, i.e. 5*β*-cholestan-3*β*-ol, accounts for up to 60% of the sterols in human faeces (Leeming et al., 1984), while 5*β*-stigmastanol (24-ethylcoprostanol) and 5*β*-campestanol have been shown to dominate among sterols in the faeces of ruminants (Bull et al., 2002). It has been suggested that coprostanol undergoes epimerisation to 5*β*-cholestan-3*α*-ol (epicoprostanol) upon excretion into the environment (Wardroper and Maxwell, 1978; McCalley et al., 1981). Coprostanol and other sterols are highly hydrophobic (see previous paragraph), and they are strongly associated with particulate matter in aquatic environments (Bull et al., 2002). Therefore the measurement of coprostanol concentrations in aquatic environments is generally focused on the analyses of the particulate matter.

Total concentrations of sterols in human faeces range from 3 to 8 mg/1 g wet weight (Nichols et al., 1996). Human faeces have been shown to contain mainly coprostanol, ethylcoprostanol, cholesterol, 5*β*-epistigmasterol, 24-ethylcholesterol, 24-ethylcholestanol. The concentrations of sterols in faeces of pigs have been shown to be lower, but the composition of the sterols was shown to be very similar to the human faecal sterol profile. Differences were observed in the concentrations of 24-ethylcholestanol, and the concentrations of C_{29} sterols were higher than the C_{27} sterols (Nichols et al., 1996). Bird faeces contained mostly 24-ethylcholesterol, 24-ethylcholestanol, 24-ethylcoprostanol, 24-methylcholesterol and cholesterol. Faeces of cows and sheep 24-ethylcholesterol, 24-ethylcholestanol, 24-ethylcoprostanol, 24-methylcholesterol, cholesterol and coprostanol. C_{29} sterol concentrations were again higher than the concentrations of C_{27} sterols. Bifidobacteria producing cholesterol is not present in the guts of dogs and certain species of birds (Leeming et al., 1996). As a result, 5*β*-stanols are not found in the faeces of these animals, and the detection of faecal pollution from these sources is based on bile salts (see below).

Distinguishing between human and non-human sources of faecal pollution is generally based on the detection of a selected number of sterols/steroid compounds (Bull et al., 2002). The ratio of the concentration of coprostanol to the sum of the concentrations of coprostanol and 5*β*-stigmastanol (24-ethylcoprostanol)

has been reported to differ between the faeces of humans and animals (Bethell et al., 1994). Evershed and Bethell (1996) reported that this ratio had a value of 0.25 in ruminant faeces, while the value of 5.5 was reported for the human faeces. Leeming et al. (1997) studied the concentration of coprostanol and 24-ethylcoprostanol in the suspended matter in water samples, and sediments from inland and coastal regions in Australia. Faeces of sheep, dogs, birds and humans were also analysed (Leeming et al., 1997). The authors derived a mathematical model for the calculation of the relative contributions of human and herbivore faecal inputs to the pollution of water bodies. The basis for this approach is summarised in Eqs. (2.5) and (2.6).

$$Y = 100 \times \frac{C(\text{coprostanol})}{C(\text{coprostanol}) + C(24\text{-ethylcoprostanol})} \quad (2.5)$$

$$\%\ \text{herbivore contribution} = 2.86 \times (73 - Y) \quad (2.6)$$

The samples are analysed, and the concentrations of coprostanol and 24-ethylcoprostanol are determined. During the analyses of the data, first calculations are made according to Eq. (2.5). If the value of *Y* was higher than 73%, then only human inputs are causing the faecal pollution (Leeming et al., 1997). On the other hand, if the *Y* value is lower than 38% only ruminant sources are responsible (Leeming et al., 1997). If a mixed situation was encountered, i.e. the *Y* value ranged from 38 to 73% then the percentage of the contribution of herbivore inputs was calculated according to Eq. (2.6). This method could be adapted to the conditions of South Africa and it would be most useful for identifying the source of faecal pollution/contamination in rural areas where the streams are used for watering of livestock during grazing, human drinking water needs and laundry activities. Application of chemical indicators such as faecal sterols, aminoacetone or urobilin might be usefull in faecal source tracking in areas where the NMMPs laboratories are not present but chemical analysis equipment is available.

In unchlorinated effluents, 96% of the initial coprostanol concentration has been eliminated at room temperature within 10 days (Kirchmer, 1971). Thirty percent of coprostanol was eliminated from several samples of chlorinated effluent within 2 days and the extent of biodegradation reached 55% after 8 days of incubation (Kirchmer, 1971). Switzer-Howse and Dutka (1978) measured a 99% removal of coprostanol after one week, when the compound was exposed to microbial community of sewage, lake water, as well as an enrichment bacterial culture, isolated from these sources. In all of the above mentioned cases, aerobic biodegradation was the major removal mechanism. Under anaerobic conditions, cholesterol and cholestanol (an isomer of coprostanol) were stable in lake sediment for 450 days (Nishimura and Koyama, 1978). This result was later corroborated by Hatcher and McGillivary (1979) and Muller et al. (1979). However, particular values of rate constants of coprostanol biodegradation/removal, under environmental conditions are scarce in the literature.

In South Africa, the only study on the coprostanol as an indicator of faecal pollution was conducted by Bartlett (1987). Incubations were conducted in marine research aquaria at 20°C. Four incubations were run simultaneously. The first one was with raw primary sewage sludge (a), and the second one was run with a mixture of primary sludge and sea water (1:10, v/v; b). These experiments were done as static incubations. The third incubation was run by incubating a five-centimetre deep layer of sand/sewage sludge mixture (4:1, v/v), and pumping seawater was continuously through the tank at 500 ml/min (c). The fourth incubation was conducted identically as the third one, but with a static water column (d; Bartlett, 1987). The initial concentration of coprostanol in the raw primary sewage sludge suspension (incubation a) was 40 µg/mL, and the concentration decreased by 85% during the first 29 days of incubation (Bartlett, 1987). The concentration further decreased to 9% of the initial concentration at the end of the experiment. In incubation (b), the initial concentration of coprostanol was equal to 4 µg/mL, and complete elimination was observed after 4 days of

incubation. In incubation (c) with the sand/sewage sludge mixture, the initial concentrations of coprostanol were 45 µg/1 g dry weight of sediment (static tank), and 52 µg/1 g dry weight of sediment (flow-through tank). Concentration of coprostanol in the sediment decreased by 40% in the static tank, but fluctuations were observed in the later stages of the experiment. In the flow-through tank (incubation d), a 35% drop in the sediment coprostanol concentration was recorded, and the fluctuations were less pronounced in the latter stages of the incubation. These trends could be explained by the 22% relative standard deviation that the author reported for internal reference material (Bartlett, 1987). Experiments similar to those of Bartlett need to be conducted under freshwater conditions and the degradation rates can then be used to estimate the time after which the faecal contamination has taken place.

Bile acids contain between 24 and 28 carbon atoms in the molecule, and are synthesised from cholesterol in the digestive system (Bull et al., 2002). Their function is two-fold. Firstly, they act as detergent molecules during the catabolism of dietary fats. Secondly, bile acids assist in the excretion of excess sterols in faeces, and so help maintain normal cholesterol concentrations in the body (Bull et al., 2002). Primary bile acids are formed in the liver. These are cholic acid, hyocholic acid, and chenodeoxycholic acid (Bull et al., 2002). Primary bile acids are conjugated with taurine and glycine (Elliot, 1985), and excreted into the gut through the gall bladder (Bull et al., 2002). In the intestine, primary bile acids are metabolised to secondary bile acids and these are deoxycholic acid and lithocholic acid (Hirano et al., 1981). Around 95% of the secondary bile acids are re-absorbed, and travel back to the liver via the enterohepatic circulation, but a small portion is excreted in faeces (Bull et al., 2002). If the lithocholic acid dominates the bile acids in an environmental sample, then the faecal pollution originates mostly from omnivores, i.e. human and dogs. If the deoxycholic acid dominates among the bile acids in an environmental sample, then the faecal pollution probably originates to a high degree from ruminants. Presence of hyocholic acid and absence of deoxycholic acid indicates porcine faecal pollution (Evershed and Bethell, 1996).

Increased levels of NH_4^+ have been linked to sewage contamination of water bodies (Barrett et al., 1999). Organic nitrogen in sewage is rapidly transformed to NH_4^+, and nitrogen species readily undergo redox reactions, i.e. the fingerprint of a source is likely to be compromised upon discharge and as a function of environmental conditions (Barrett et al., 1999). Stable isotopes of ^{18}O, ^{34}S, and ^{15}N have been reported as possible tracers of sewage pollution (Barrett et al., 1999). The ratio of the stable isotopes of nitrogen has been proposed as a fingerprint of a particular source, and it changes through metabolism, and physical processes, e.g., dilution (Rivers et al., 1996). The degree of fractionation of the isotopes can be defined in Eq. (2.7).

$$\delta^{15}N = 1000 \times \frac{\left(\frac{^{15}N}{^{14}N}\right)_{\text{sample}} - \left(\frac{^{15}N}{^{14}N}\right)_{\text{air}}}{\left(\frac{^{15}N}{^{14}N}\right)_{\text{air}}};\ ^{\circ}/_{\circ\circ} \qquad (2.7)$$

In Eq. (2.7), terms in the parentheses represent the concentrations of the respective isotope of nitrogen, while the subscripts identify the sample or the ratio of the isotope concentrations in air (Barrett et al., 1999). If the value of $\delta^{15}N$ is higher than 0, then the sample in question is isotopically heavy in comparison to air. At the same time, if the value of $\delta^{15}N$ is negative, then the sample in question is isotopically light in comparison to air (Barrett et al., 1999). The fingerprint based on nitrogen stable isotopes has been suggested as a possible method for tracing different sources of faecal pollution (Heaton, 1986). Based on the $\delta^{15}N$ value for nitrates, it has been suggested that a value higher than 10 $^{\circ}/_{\circ\circ}$ indicates sewage pollution in the water in question (Barrett et al., 1999).

Ratios similar to the one for nitrogen stable isotope can be defined for other elements, such as O and H(D). In South Africa, a method has been developed and the results reported in the tracing the sources of groundwater recharge using $\delta^{18}O$ and δD values (Butler and Verhagen, 1997). The authors clearly demonstrated the ability of these two ratios to trace the mains water in the distribution pipe network supplying the townships of Pretoria. Based on the above results, calibrations could be done by measuring the $\delta^{15}N$ values for different types of wastewater, e.g., domestic sewage and abattoir wastewater, and these could be then compared to the $\delta^{15}N$ values of the streams, and the source of the faecal contamination should theoretically be identifiable. A combination of microbiological faecal indicators and nitrogen isotopes data were found to give the best indication of the presence or absence of sewage recharge to groundwater (Barrett et al., 1999). This type of faecal source tracking could be applicable is both an isotopic mass spectrometer and microbiological laboratory equipment are available.

The problem with routine application is likely going to be the lack of skilled labour to perform the isotope analysis similar to the expertise required for the viral biomarker applications (see above).

CHAPTER 3: NATIONAL MICROBIAL MONITORNG PROGRAMME (NMMP)

3.1 SCOPE

With respect to water resource management, South Africa is divided into 19 Water Management Areas (WMAs; National Water Act – NWA 1998; Murray et al., 2004). The microbial water quality monitoring is the responsibility of various departments at all levels of government. The National Health Act of South Africa (NHA) defines the responsibilities of local and district municipalities by including water monitoring and environmental pollution monitoring among municipal services (NHA, 2008). The NWA states in chapter 14 part 1 paragraph 137 that the Minister is responsible for setting up a monitoring system for microbial water quality, among others (NWA, 1998; Murray et al., 2004). Given the structure of the national government, the term “Minister” applied to the Minister of Water Affairs and Forestry up to the end of April 2009. Currently, this is the Minister of Water and Environmental Affairs, which is the result of the re-arrangement of the government departments in May 2009. The monitoring system of microbial water quality applies to the NMMP for surface water (Murray et al., 2004) and groundwater (DWAF, 2000a; DWAF, 2006; Murray et al., 2007).

The NMMP for surface water was developed based on research by Du Preez et al., (1999; 2001; 2002). One of the aims of the NMMP was to create a central hub for microbial water quality data in South Africa (Murray et al., 2004). The Department of Water Affairs in the Ministry of Water and Environmental Affairs (DWA) administrates the programme, and also operates the national information system of microbial water quality, the Central Water Quality Database (Murray et al., 2004). The DWA is responsible for the appointment of the national coordinator for both NMMP for surface water (Murray et al., 2004) and the NMMP for groundwater (Murray et al., 2007). As of May 2009, the national coordinator has been appointed for the NMMP for surface water (DWAF, 2010). The appointment of an assistant national coordinator has been suggested to guarantee running of the programme in the event of resignation of the national coordinator, or during exceptional situations (Murray et al., 2004; Murray et al., 2007).

A regional coordinator should be appointed in each of the WMAs to oversee the running of the NMMP (Murray et al., 2007). Samples are supposed to be taken once a week or bi-weekly at identified sampling sites (Murray et al., 2004; Murray et al., 2007). Turbidity, pH and the concentration of *E. coli* are determined in all samples and data are to be communicated to the Central Water Quality database (Murray et al., 2004; Murray et al., 2007). Periodical reports are written every two months by the regional coordinator, summarising the main trends in the monitoring results. These reports are subsequently sent by the national coordinator to the Ministry of water and Environmental Affairs (MWEA), decision-makers in the DWA, the NDOH, relevant water resource authorities, and any stakeholder that expresses interest (Murray et al., 2004; Murray et al., 2007).

3.2 PRIORITISATION

There are 278 tertiary catchments in South Africa, and it is impossible to sample water in every catchment. Therefore prioritisation is the only way to assess the status of the faecal contamination, given the finite financial resources of DWA/DWAF (DWAF, 2000b). The priority sampling sites are generally selected by local staff, under leadership of the regional NMMP co-ordinator (Murray et al., 2004). During this process, several criteria are taken into account. The first criterion is the land uses that are most likely to function as

point or non-point sources of faecal contamination. These include informal settlements in the vicinity of the water resources in question and agricultural activities, such as livestock watering (Murray et al., 2004). The second criterion is the size of the population that is likely to come into contact with the water resource in question (Murray et al., 2004). The last criterion is the water uses in the area (Murray et al., 2004).

Prioritisation of catchments within a WMA starts with the collection of preliminary data. These data include identification of catchments that have experienced microbial water quality problems, gathering of information about areas with high prevalence of waterborne diseases, identification of areas in the catchment where the population is using untreated or partially treated surface water to meet domestic water demand, and location of informal settlements without adequate sanitation infrastructure (Murray et al., 2004). The sources of information for the prioritisation process are DWAF Water Supply and Sanitation Database, the Agricultural Research Council, the Regional Deputy Directors of DWA/DWAF and the Regional Water Quality Managers and Water User Associations.

In the case of the NMMP for groundwater, the sources of faecal contamination differ somewhat from those in surface water. The linking of changes in *E. coli* concentrations to a particular source of faecal contamination will depend on the situation in a particular priority area. Transport of the indicator microorganisms into the groundwater will depend on the concentration of organic matter and clay minerals in soil, pH and ionic strength of the soil solution, virus type which dictates the surface charge of the virions, geological composition of the layers overlying the aquifer (e.g., Schijven, 2001).

These characteristics will affect all sources of faecal contamination of groundwater, besides surface flooding. Interactions between surface water and groundwater have not been considered in the prioritisation process, but this is likely to change in the near future. Bacteria are non-conservative indicators due to the ability to multiply in the environment. In the case of viruses, this will depend on the presence of a host. Monitoring of the groundwater microbial water quality will be different from that of the surface water. Multiple wells will have to be drilled in a priority area (Murray et al., 2007). One of the wells will have to be drilled at the faecal contamination source, i.e. in the flow of the discharge of the final effluent from a wastewater treatment plant, while another will have to simulate the real well, i.e. it will have to located at a setback distance from faecal contamination source to simulate the elimination/filtering out of pathogens (Murray et al., 2007). Preliminary studies have been conducted in two aquifers in South Africa (Murray et al., 2007). Problems with the location of the monitoring wells proved to be the main challenge in the widespread roll-out of the NMMPs for groundwater (Murray et al., 2007).

3.3 IMPLEMENTATION

The implementation process of both NMMPs is being rolled-out in phases to identify and eliminate as many problems as possible (Murray et al., 2004). For the successful functioning of the NMMP, there must be sufficient capacity to carry out all of the required functions (Murray et al., 2004; Murray et al., 2007). The role of the different stakeholders should be clearly defined at the beginning of programme implementation. This is best accomplished if contracts are signed between the regional NMMP coordinator (a DWA employee) and all persons taking on functions in the programme before the start of the NMMP (Murray et al., 2004; Murray et al., 2007). Binding target dates for the delivery of results should be included in the contracts (Murray et al., 2004; Murray et al., 2007). Local stakeholders, i.e. waters users, must be involved in the programme from the beginning and must have access to the information, i.e. results of the analyses, to achieve programme's sustainability. The NMMP for surface water has so far been implemented in 14 out of 19 WMAs with sampling taking place in the priority catchments.

3.4 SHORTCOMINGS

Analytical laboratories are not available in many parts of South Africa. In the NMMP prioritisation strategy, the overall area rating values are set so that monitoring is focused into areas with high levels of faecal pollution (Murray et al., 2004; Murray et al., 2007). A combination of these and other factors left around 30% of all potable water resources around South Africa outside of any microbial water quality monitoring in 2009 (Rivett et al., 2009). This is caused by limited financial resources and lack of accredited laboratories and skilled laboratory personnel (Monyai 2004; Rivett et al., 2009). The logistical part of the problem must be dealt with urgently to enable the sustainability of both NMMPs and microbial water quality monitoring in all parts of South Africa. A solution could lie in granting a transitional period to WMAs, where the capacity for testing is either not optimum or is lacking completely. During the transitional period, regional coordinators could use unaccredited laboratories to gather data for the NMMPs, after minimum quality assurance and quality control regulations were met. More details are given in the next section.

Prioritisation saves monitoring costs, but it leaves a significant part of the South African population at potential risk of contracting waterborne diseases. For known contamination sources, data measured in the context of the NMMPs allows the regulatory community and the public health officials to assess the status and long-term trends of the faecal contamination of surface water resource(s) in a particular priority area. However, this data will not provide any information about faecal contamination from sources that were not considered during prioritisation and the ranking processes. In such cases, other techniques which allow for faecal source tracking will have to be added to the array of methods used. These will be addressed in a subsequent section.

3.5 RECOMMENDATIONS

3.5.1 In areas with limited monitoring infrastructure

Problems with the current microbial water quality monitoring framework in South Africa are related to the lack of sufficient number of accredited laboratory facilities and skilled personnel to perform the analyses. One solution to this problem could be to grant a transitional period to the WMAs where the capacity for testing is not optimum. During the transitional period, regional co-ordinators could use unaccredited laboratories to gather data in the context of the NMMPs. Minimum quality assurance and quality control criteria would be put in place to guarantee comparability of the results to the accredited laboratories used in other WMAs. These guidelines/regulations would be developed by DWA, so that regulatory acceptance of the results is guaranteed. During the transitional period, the laboratories used would be given financial and logistical support to achieve mandatory accreditation at the end of the transitional period. Achievement of accreditation could be mandated in the contracts signed to guarantee return on the financial and logistical investment by the WMA and the regional co-ordinator. The length of the transitional period could vary between WMAs, depending on the starting point, but it should not exceed 4 years. One year would be reserved for preparation of the implementation framework, and three years would be reserved in training the future staff.

The laboratory facilities required for the NMMP analyses are often present on rural university campuses in South Africa. These facilities are not accredited, but they do have the required equipment. The University of Fort Hare and Rhodes University in the Eastern Cape can be used as examples. The NMMP could be integrated into the microbiology or public health curricula at these universities to give students an opportunity to understand the programme, learn relevant techniques of analyses, and become familiar with the regulatory management and decision-making process. The university laboratories could be given the financial assistance directly to achieve accreditation and part of the laboratory facilities would become a dedicated NMMP facility. The DWA could put financial resources aside to allow new graduates from rural

universities to establish laboratory facilities in the WMAs where these have been absent. These initiatives would help guarantee constant supply of skilled personnel to sustain the NMMPs in all parts of South Africa.

Both NMMPs are based on the enumeration of *E. coli*, but its concentration does not provide any information about the sources of faecal contamination. Outside the priority areas and those that have not been considered in the prioritisation process, source identification might be difficult. This disadvantage can be overcome using microbial-source tracking methods (Stoeckel and Harwood, 2007). In the library-dependent methods, isolates of *E. coli* are obtained from different sources of faecal contamination and selected properties are measured, e.g. the carbohydrate fermentation spectrum or the antibiotic resistance analysis (Harwood et al., 2003). Databases of isolate properties are created and maintained for a given area of the country (Stoeckel and Harwood, 2007). For a particular water sample, the public health risk can then be assessed based on the concentration of *E. coli* and the faecal contamination source can be identified using the relevant database of isolate properties. The database identification can be based on different statistical methods, e.g. jack-knife analysis (Stoeckel and Harwood, 2007). The antibiotic resistance analysis of bacteria from faecally contaminated water resources has been conducted in South Africa, e.g. Said et al., (2005). It could easily be integrated into the NMMPs and used for identification of the sources of faecal contamination. At the same time, the antibiotic resistance analysis is likely to be understood by the regulatory community.

Research and monitoring undertaken by the expanded developed network of laboratories can be used to refine and amend the criteria that are measured for the NMMP. Implementation of these new criteria would allow for identification of an unknown source of faecal contamination. The enumeration of *Bifidobacterium spp.* (Section 2.4.5) is one such criterion.

3.5.2 In areas with no monitoring infrastructure

The above mentioned strategy will work if there is access to laboratory facilities and/or the respective water samples can be transported on ice into the laboratory within the mandatory six-hour period. However, many water resources that supply potable water to the population in remote areas of South Africa do not meet this criterion. In such cases, a different monitoring strategy to that of the NMMPs is required. It will have to be based on tests that can be performed on-site and with limited equipment. Two tests that are available on the market or from non-profit organisations are the H_2S strip test and the Aquatest. These will have to be used by trained professionals such as environmental health officers from the local municipalities in combination with questionnaire tools analogous to those of Mosley and Sharp (2005).

3.5.2.1 H_2S strip test

The H_2S strip test for the determination of the presence/absence of faecal contamination has been developed as a low-cost test for the detection of faecal contamination in remote areas (Manja et al., 1982). In the H_2S strip test, a water sample is mixed with a cultivation medium which is prepared in dehydrated form (Genthe and Jagals, 2003; Tandlich and Muller, 2008). The medium contains Na_2SO_3 and bacteria of faecal origin reduce it to H_2S, which subsequently reacts with ferric iron forming a black precipitate (Mosley and Sharp, 2005). The H_2S strip test bacteria will be referred to in subsequent text as bacteria producing a positive signal in the H_2S strip test. The positive reaction, i.e. detection of faecal contamination of the water body, is based on a visual detection of a colour change from the original brown colour into a black colour of ferric sulphide. Incubations are performed away from direct sunlight and at room temperature. There is no need for an incubator and a sample is considered faecally contaminated if the black colour develops within 72 hours (Sobsey and Pfaender, 2002; Tandlich and Muller, 2008).

Test kits can be obtained from a commercial supplier or they can be produced in one central facility and distributed by mail. The commercial version of the H_2S strip test is called Pathoscreen and it available from

the Hach Company (Mosley and Sharp, 2005). The sample collection and transport can then be performed on site with minimum processing and low labour intensity (Genthe and Franck, 1999; Sobsey and Pfaender, 2002). There are only two requirements that need to be addresses during incubation. Firstly, the samples must be kept away from direct sunlight (Genthe and Jagals, 2003). Secondly, they must be checked for colour development once every 12 hours, since black colour must be observed within 72 hours of sample collection in order to detect faecal contamination (Genthe and Jagals, 2003). Genthe and Jagals (2003) indicated that one major research question has not been answered, i.e. the influence of large temperature fluctuations on the colour development of the sulphide precipitate. This will be addressed in the next section.

Bacteria which produce a positive signal in the H_2S strip test include *Citrobacter spp.* (Ratto et al., 1989), *E. coli* (Sobsey and Pfaender, 2002), *Proteus mirabilis* (Manja et al., 1982), *Proteus vulgaris* (Nagaraju and Sastri, 1999), *Aeromonas spp., Clostridium spp.* and *Salmonella spp.* (Sobsey and Pfaender, 2002). The coliform group of bacteria has been reported to contain the following species: *Citrobacter spp., Enterobacter spp.*, *Escherichia spp.*, *Hafnia spp.*, *Klebsiella spp., Serratia spp.* and *Yersinia spp.* (APHA, 1998). Thus there is a partial overlap between the species composition of FC/TC/*E. coli* and the H_2S strip test bacteria. Most strains of the species *Citrobacter spp.* are commonly found in the intestinal tract of humans (Abbott, 1997), and certain strains have been isolated from the intestinal tract of dairy cows (Sawant et al., 2007). Environmental isolates have been reported from soil, water, tree bark and logging operations (Abbott, 1997), but these accounted for only 12% of the total concentrations in environmental water samples (Duncan and Razzell, 1972).

Proteus vulgaris and *Proteus mirabilis* have been reported to cause diarrhoeal diseases in infants (Proteus, 2009). *Clostridium perfringens* has been detected in the faeces of human suffering with food poisoning from undercooked meat or gravy (Shandera et al., 1983). Strains of *Salmonella spp.* have been reported to cause diarrhoeal infections in humans (Popoff and LeMinor, 1997). Defecation by healthy and/or asymptomatic individuals, and patients with diarrhoeal diseases in the vicinity of water resources/leakage from sanitation infrastructure will lead to the release of the H_2S strip test bacteria into the water resources used for drinking water as described in the introduction for FC/TC/*E. coli*. Such contamination will result in a positive signal in the qualitative H_2S trip test. This makes the H_2S strip test a useful tool in microbial water quality monitoring in remote areas in South Africa, with no or limited laboratory facilities. Partial overlap between the species of the coliform group and the H_2S strip test bacteria should provide a good basis for the acceptance of the H_2S strip test by the regulatory community.

Genthe and Franck (1999) examined and reported the rate of correspondence between the positive H_2S strip test and the detection of non-zero concentrations of FC in various types of environmental water samples. The water resources examined include surface streams, drinking water, fire hydrant water, and borehole water (Genthe and Franck, 1999). The overall rate of correspondence ranged from 86 to 94% with incubations conducted either at room temperature or at 37ºC after a 72 hour incubation period (Genthe and Franck 1999). However, the H_2S strip test lacked the ability to provide a positive signal when the concentrations of FC were below 10 cells/100 mL (Genthe and Franck, 1999). This could be the reason for observing the first type of discrepancy. This drawback can be eliminated by the addition of 0.5% taurocholate or deoxycholate into the H_2S strip test medium (Sobsey and Pfaender, 2002). Temperature fluctuations, during sample collection and transport, could lead to stress of the cells of indicator organisms (Genthe and Jagals, 2003). At the same time, the chemical composition of water sample in question could lead to toxic effects towards the H_2S strip test bacteria and/or FC/TC. These research questions were investigated as part of this project and results will be presented in section 3.

Genthe and Jagals (2003) conducted a workshop where environmental health officers from different parts of South Africa were introduced to the H_2S strip test based on the medium of Venkobachar et al. (1994). Most of the study participants found the test easy to use, and there was high acceptance of the test as a potential tool for routine monitoring of the microbial water quality. This supports the above-mentioned conclusion of the authors relating to acceptance by the regulatory community. Certain concerns about practical

applicability were, however, raised by the environmental health practitioners. The first concern originated from possible discrepancies between the results from the qualitative H_2S strip test and the enumeration of FC/TC (Genthe and Jagals, 2003). There are two possible cases where a discrepancy can occur. The first one occurs when the H_2S strip test is negative, but a non-zero concentration of FC/TC/*E. coli* is measured. In the second case, the H_2S strip test is positive, but the concentration of FC/TC/*E. coli* is below the detection limit of the method used. This could be solved in two ways. One of the two solutions is the addition of 0.5% (w/v) deoxycholate as stated in the previous paragraph. The second one is measurement of the survival rates of *E. coli* and FC, and the H_2S strip test bacteria. This will have to be considered in the context of the calibration of TR and so will be addressed elsewhere in this report.

As for the second discrepancy, environmental health professionals were concerned about the possible interference from NO_3^- and NO_2^- anions on the colour development. Weon et al. (2002) reported a twenty-percent inhibition of growth a phosphate-accumulating strain of *Acinetobacter spp.* at nitrite concentration of around 604 mg/L. Such conditions will not be encountered in South African water resources (as demonstrated by the DWAF monitoring data; the reader can access the data at the DWA website: www.dwaf.gov.za), and therefore the H_2S strip test results will not be affected by the presence of NO_3^- and NO_2^- in the sampled water body. The cost per sample with the H_2S strip test is around 10.00 ZAR (approximately 1.32 USD).

3.5.2.2 Aquatest

Other low-cost presence/absence tests include Aquatest which has a shorter shelf life and costs approximately 10.50 ZAR (approximately 1.50 USD). Marketing of Aquatest is currently starting in South Africa through the University of Cape Town. It is designed in the form of individual devices which can detect 1 CFUs or cells/100 mL of *E. coli* (Rivett et al., 2009). A portable incubator will be available at a cost of 150.00 ZAR (approximately 20.00 USD) which allows users to perform cultivation of *E. coli* under field conditions (Rivett et al., 2009). Aquatest provides results within 24 hours in the form of a qualitative assessment of low, moderate and high (Rivett et al., 2009). By comparison, it takes 72 hours to obtain results in the H_2S strip test.

Aquatest results can be interpreted as a presence/absence test, because if the test is negative than the concentration of *E. coli* is below 0 CFUs or cells/100ml, i.e. the water sample fulfils the South African microbial water quality regulations for drinking water (SABS 2006).

3.5.2.3 Conclusions

Both the H_2S strip test and the Aquatest could be performed in remote areas where no laboratory facilities are available to the regional coordinators of the NMMPs. Individuals could be accredited to perform the tests in a fashion similar to the South African Scoring System (SASS, 2010). This would allow for the detection faecal contamination according to the drinking water quality guidelines. The testing could be performed by environmental health professionals that are employed by every municipality in South Africa where water quality is part of their job description. Identification of sources of faecal contamination can be accomplished by compiling an inventory of the faecal contamination sources as outlined by Mosley and Sharp (2005).

CHAPTER 4: SITE SELECTION

4.1 OVERALL SELECTION PROCESS

The first task of the project to find sampling sites with permanent faecal contamination, i.e. the selected sampling sites for calibration of TR and the sterols criterion of Leeming et al. (1996). Research data on the water quality throughout the Eastern Cape was used as the starting point. When the original project proposal was submitted, it was suggested that a sampling site should be selected if a non-zero concentration of faecal coliforms (FC) and a positive hydrogen-sulphide test (referred to in subsequent text as the H_2S strip test) are recorded at that site on two separate occasions. The updated version of the literature review provided new information which required a change of this strategy. The review indicated that the FC test could be strongly influenced by non-faecal sources. On the other hand, the enumeration of *E. coli* was less prone to such influences and so the sampling site was included among the selected ones if the following criteria were met. The concentrations of FC and *E. coli* were both higher than 0 cells/100 mL and the H_2S strip test was positive at a given site on three randomly selected occasions. All three tests are mutually independent and the three sampling dates were chosen at random. Thus if the selection criteria are met, then the probability of permanent faecal contamination at the selected sampling site is over 99% and the contribution from non-faecal sources is below 0.5%. The enumeration of FC and *E. coli* and the H_2S strip test are referred to as the screening tests in the text below.

Only a limited number of the sampling sites were chosen on the Bloukrans River and no sites were selected on the Buffalo River. Sites in the following municipalities were also tested: Coega, Ndlambe and Ikwezi, but no sampling sites were selected in these areas. Thus sites outside of the originally proposed area were considered, namely at the Kwandwe Game reserve outside of Grahamstown and at dams around Grahamstown. Discrepancies between the results of the individual screening tests were detected at the selected sampling sites. Based on the literature, this could have been caused by chemical interferences and/or environmental stress. Experiments were designed and performed to investigate the extent of some of those influences. Information about land and water uses and sources of faecal pollution was collected from literature and the public domain. Concentrations of *Rhodococcus coprophilus* were measured at the selected sampling sites to confirm faecal contamination from animal sources. This data was then used to divide the selected sampling sites into those receiving human, animal or mixed faecal contamination. Chemical composition of the water was studied at the selected sampling sites in the late spring and the early summer in 2008.

Enumeration of the sorbitol-utilising bifidobacteria was successful in water samples from the selected sampling sites. However, the YN-17 medium proved unreliable for enumeration of the total bifidobacteria which prevented calibration of the tracking ratio. Literature research into an alternative cultivation media is completed and the Beerens medium (BM; Beerens, 1990) is currently being tested as a potential replacement for YN-17. Methods of Batta et al. (1999) and Marty et al. (1996) were considered for the analyses of sterols but proved irreproducible. The method of Isobe et al. (2002) was therefore adopted for the final analyses.

Several and relevant knowledge gaps were identified in the South African and international literature. The first one was the delayed positive response in the H_2S strip test when the concentrations of FC and *E. coli* were in the range of 0-10 cells or CFUs/100 ml. Secondly, there is no information on the influence of temperature fluctuations during sampling and transport on the results of the H_2S strip test. Thirdly, there is very limited information on the survival rates and storage of indicator microorganisms in the

sediment/particulate matter in South African water resources. Attempts were made to address these knowledge gaps in the context of the current project.

4.2 MATERIALS AND METHODS

4.2.1 Chemicals and Consumables

The following chemicals and consumables were purchased from Merck Ltd. (Pty; Johannesburg/Cape Town, South Africa): KH_2PO_4, Na_2HPO_4, KNO_3, NaCl, $Na_2S_2O_3.5H_2O$, $CaCO_3$, phenol, HCl (32% aqueous solution), H_2SO_4 (95-98% aqueous solution), $BaCl_2.2H_2O$, the chloride kit (catalogue number 1.14897.0001), the ammonium kit (catalogue number 1.14752.0001), the phosphate kit (catalogue number 1.14848.0001), the indole reagent, the anaerobic jars, m-FC agar, tryptic soy broth, peptone from casein and peptone from meat. The last two chemicals were used to prepare polypeptone, i.e. the one-to-one mixture (w/w) of both components (Polypeptone, 2009). The following chemicals were purchased from Sigma-Aldrich (Johannesburg, South Africa): $MgSO_4.7H_20$, $FeSO_4.7H_20$, $ZnSO_4.7H_2O$, sodium propionate, $MnSO_4.4H_20$, NaN_3, nalidixic acid, cycloheximide, bacteriological agar, polymyxin B (1 mg/mL aqueous solution), kanamycin sulphate, lactose, tryptone powder, salicin, Triton X-100, ferric ammonium citrate, L-cysteine, deoxycholate, lithocholic acid, sorbitol, yeast extract, bromocresol purple, bromocresol green, cystein hydrochloride, glucose, bovine serine albumin, Bradford's reagent, the indole reagent, toluene, cholesterol, coprostanol, 24-ethylcoprostanol, and Silprep components.

Hyocholic acid was purchased from Steraloids (New Port, RI, USA), while glycerol, acetone and absolute ethanol were purchased from Chemstores (Rhodes University, Grahamstown, RSA). The following consumables were purchased from EC Labs (Port Elizabeth, RSA): the nylon membrane filters (pore size 0.45 µm) for the MF determinations, Oxoid gas generating kits and the ninety-millimetre sterile Petri dishes. The eleven-micrometer glass fibre prefilters for the determination of the total suspended solids (TSS) and the glass fibre filters (pore diameter 1.2 µm) for the measurement of chlorophyll a (chla) were purchased Microsep (Port Elizabeth, RSA). The instrument grade Ar, N_2 and CO_2 were purchased from Afrox (Port Elizabeth, RSA), while casamino acids were purchased from BD (Pretoria, RSA). The purity of all chemicals was 99% or higher and all chemicals were used as obtained from the suppliers.

4.2.2 Experimental protocols

Concentrations of FC were enumerated using the most-probable number (MPN) technique with the A1 medium according to Standard Methods (APHA, 1998); or using the MF technique with m-FC agar with the nylon filters (pore size 0.45 µm; Wutor et al., 2009). Cells and the colony-forming units (CFUs) are used interchangeably in further text. The concentrations of *E. coli* were measured with the indole test performed on all MPN tubes that were positive for FC; or the representative number of FC colonies from the membrane filters (DWAF, 1996d). The H_2S strip test medium of Venkobachar et al. (1994) was modified by addition of 0.5% deoxycholate (Sobsey and Pfaender, 2002). This modification was implemented to improve the selectivity of the H_2S strip test and to shorten the time necessary for detection of a positive H_2S strip test at the concentrations of FC and *E. coli* from 0-10 cells/100 mL (Sobsey and Pfaender, 2002; Mosley and Sharp, 2005). Concentrations of *Rhodococcus coprophilus* were measured at the selected sampling sites using the spread-plate technique on the M3 medium (Mara and Oragui, 1981). Bifidobacteria were enumerated using the methods of Oragui and Mara (1983) as outlined by Tandlich and Muller (1998), or using the Beerens medium with pH adjusted to 5.7 (Beerens, 1990). All plates were kept in the anaerobic atmosphere 12-24 hours prior to sample analyses, when bifidobacteria were enumerated.

The H_2S strip test was performed on-site. All samples for the enumeration of FC, *E. coli* and *Rhodococcus coprophilus* were transported into the laboratory on ice and the holding times for microbiological analyses ranged from 24 to 48 hours. The Standard Methods specify holding times at 4°C of 6 hours (APHA, 1998). However, no significant changes in the counts of indicator bacteria were observed during storage at 4°C for up to 48 hours after sample collection (Sonzogni et al., 2002). At the same time, *Rhodococcus coprophilus* has been shown to survive at 5ºC for up to 7 days (Oragui and Mara, 1983). Therefore it can be concluded that the extended holding times did not decrease the concentrations of indicator microorganisms. The holding times for the enumeration of bifidobacteria ranged from 2-4 hours, as the die-off generally occurs within 9 hours of sample collection (Bonjoch et al., 2009).

The ambient temperatures span a wide range in South Africa (SAWS, 2010). The results of the H_2S strip are therefore likely to be influenced by large temperature fluctuations during sample collection and transport (Genthe and Jagals, 2003). During the screening for the selected sampling sites, the outside temperature ranged from 10 to 32ºC and the water samples were transported back into the laboratory within 5-12 hours of collection. Experiments were conducted to examine the potential effect of these conditions on the results of the H_2S strip test. Samples were collected at sampling sites 10 and 11 (see Table 4.1 for details) and they were transported back into the laboratory within 30 minutes of collection. Based on the concentrations of FC and *E. coli*, water samples from these two sites represent the extremes of the concentration ranges observed on the selected sampling sites (Table 4.1).

Five replicate samples for the H_2S strip test and one sample for enumeration of FC and *E. coli*, respectively, were collected at each sampling site. Upon arrival back into the laboratory, one H_2S strip test sample from each sampling site was incubated between 18 and 25ºC and served as a control for colour development. The second and third H_2S sample from each sampling site was incubated at 10ºC for either 5 or 12 hours. After 5 or 12 hours, these samples were incubated as the control between 18 to 25ºC until 72 hours after sampling. The same procedure was repeated for the fourth and fifth H_2S sample, but the initial incubation was conducted at 32ºC instead of 10ºC.

Attachment of FC, *E. coli* and *Rhodococcus coprophilus* at the selected sampling sites was tested on the samples of the suspendable particulate matter. Two or three average-sized stones with visible particulate matter deposits were handpicked with sterile gloves and placed aseptically into sterile Tupperware containers for transport into the laboratory on ice. In the laboratory, 75 mL of the one-quarter-strength Ringer solution (Tandlich and Muller, 2008) was added to the stones and the bacteria were detached from the particles by vortexing on the MP-19 Deluxe Vortex Mixer (Chiltern Scientific, Pretoria, South Africa) for 2 minutes. The particle-associated bacteria were enumerated in the extract using the same methodologies as mentioned for the water samples. The results were expressed as the cell concentration of FC or *E. coli* per 1 g of the particulate matter dry weight and as CFUs per 1 g of the particulate matter dry weight for *Rhodococcus coprophilus*. The particulate matter dry weight was determined by drying the samples to constant weight at 60ºC in the UFE 700 oven (Memmert, Schwabach, Germany).

All incubations were done in one of the following incubators: the Labcon incubator Model FSIM B (Labmark, Johannesburg, RSA), the TS 606/3-I incubator (WTW, Weilheim, Germany), the Labcon low temperature incubator LTIE 10 (Labmark, Johannesburg, RSA); and/or the Heraeus Model FT 420 (Heraeus Kulzer GmbH, Dormagen, Germany). All sterilisations were conducted using the Model RAU-53Bd REX MED autoclave (Hirayama Manufacturing, Tokyo, Japan).

Turbidity of the water samples was measured using the Orbeco Hellige Model 966 Portable Turbidimeter (Lasec, Port Elizabeth, South Africa). pH of the water samples was either measured in the laboratory using the Cyberscan 5000 pH meter (Eutech Instruments, Singapore) or on-site using the portable Cyberscan 300 pH meter (Eutech Instruments, Singapore). Electrical conductivity (EC) was measured either in the laboratory using the AMEL 160 conductivity meter (AMEL Electrochemistry, Italy) or on-site using the Cyberscan Con II portable conductivity meter (Eutech Instruments, Singapore). The dissolved oxygen

concentration (DO) was always measured on-site using the portable Cyberscan Waterproof Dissolved oxygen/C/F DO 300 data meter (Eutech Instruments, Singapore). Concentration of total carbohydrates was determined using the phenol-H_2SO_4 method (Dubois et al., 1956), while the protein concentrations were determined using the Bradford's method and the Sigma-Aldrich protocol (Sigma-Aldrich, Johannesburg, RSA).

For the determination of chla, 120 mL of the respective water sample was filtered on-site through the glass fibre filter (pore size 1.2 µm) using the Geotech hand pump (Geotech, Denver, CO, USA). After filtration, filters were folded and placed into test tubes with 8 mL of the ninety-percent aqueous solution of acetone. The test tubes were kept in the dark at 4°C and chla was extracted by diffusion for 12 to 24 hours. After extraction, the test tubes were centrifuged at 3000 rpm on the Roto-Uni 752 centrifuge (Hettich Instruments, Beverly, MA, USA). During all stages of the sample preparation, the test tubes were shielded from light to prevent photodegradation of chla. Finally, concentrations of chla were measured using fluorescence spectroscopy according to Bukin et al. (2008) on the 10-AU Fluorometer (Turner Designs, Sunnyville, CA, USA).

The concentration of SO_4^{2-} was measured using the US EPA method 375.4 (US EPA, 1978), while the concentration of NO_3^- was measured using the method of Velghe and Claeys (1983). The NO_2^- concentrations were determined using the US EPA method 354.1 (US EPA, 1971). Concentrations of Cl^-, NH_4^+, and PO_4^{3-} were measured using the Merck test kits. All spectrophotometric and turbidimetric measurements were done using the Shimadzu UV-1601 spectrophotometer (Shimadzu, Johannesburg, South Africa). The TSS concentrations were measured using the method of Zuma et al. (2009), while the total alkalinity (TA) was estimated using the Blu 52 portable kit (Blu 52, Midrand, South Africa). The HPC concentrations were enumerated on the yeast extract-glucose-tryptone agar (APHA, 1998). Light intensity was measured using the LX101 LUX light meter (DM Agencies, Cape Town, South Africa). All samples were stored at 4°C until analyses. Information about the physical and chemical properties of water samples from the selected sampling sites was used to prepare the model waters for survival experiments.

Survival experiments were conducted to examine how long after a faecal contamination event there will be discrepancies between the results of the FC enumeration and the H_2S strip test and between *E. coli* and the H_2S strip test. Unless otherwise stated, the term model water(s) will refer to the media used for survival experiments (see Table 4.4 for details). For nutrient-rich model water (NRMW), the respective inorganic salts and organic components were dissolved in 800 mL of MilliQ water (Millipore, Johannesburg, South Africa). The TSS concentration was set to 0.00 mg/L to simulate minimum light scattering and the maximum primary production by algae. The resulting solution was poured into a 1000 mL Schott bottle (Labotec, Midrand, South Africa) and the pH was adjusted to 7.71. Then 100 g of coarse pool filter sand (Sparrow Pools, Grahamstown, South Africa) was added to simulate the inert particulate matter (see Results and Discussion for details). For the nutrient-depleted model water (NDMW), the same procedure was used but without the additions of NO_3^-, NH_4^+, PO_4^{3-} and proteins. Afterwards, 100 mg/L of $CaCO_3$ was also added to simulate increased turbidity and decreased primary production. This increased the pH of the final solution to 7.87.

The model waters were sterilised at 121 kPa for 15 minutes and then allowed to cool down to room temperature. Subsequently, the NRMW and the NDMW were inoculated with inocula FC, HPC and algae. Inocula of HPC and FC were isolated as mixed cultures from the selected sampling sites and they were maintained in tryptic soy broth at 35°C; and re-inoculated once a week. The algal inoculum was obtained from the Institute for Water Research at Rhodes University as a seven-strain mixed culture which had been isolated from the rivers in the Eastern Cape and Limpopo. Duplicate samples and one sterile blank from each of the two model waters were incubated in the laminar flow hood at 21.5 ± 0.5ºC, with 12 hours of light and 12 hours darkness. The bottles were aseptically opened once a day for 2 hours, to allow for aeration of the model waters.

The YN-17 medium proved unreliable in the enumeration of the total bifidobacteria and so the BM (Beerens, 1990) with the pH value adjusted to 5.7 was tested as a replacement (referred to as modified Bareens medium (MBM) in subsequent text). An inoculum of the sorbitol-utilising bifidobacteria was prepared by picking off characteristic colonies from water samples, collected at the selected sampling sites. The colonies were aseptically transferred into the sterile tryptic soy broth which was deoxygenated with CO_2/N_2 and Ar prior to sterilisation. Bacteria were grown at 37ºC for 48 hours and then two concentrations of bifidobacteria were prepared by diluting the stock culture with physiological saline. Concentrations of bifidobacteria were then enumerated in four replicates using the spread-plate or the MF technique on the human bifid sorbitol agar (HSBA) and the BM.

The working hypothesis was that if the BM is a suitable replacement for the YN-17 medium, there should be no statistical difference between the concentrations of the sorbitol-utilising bifidobacteria measured using the HSBA and the BM. Statistical testing was done using the Statistica software package (version 8.0; StatSoft Southern Africa – Research (Pty) Ltd., Sandton, South Africa). Data was assumed to follow the normal distribution. Results of the testing proved the working hypothesis at the five-percent level of significance. Subsequently, all selected sampling sites were re-sampled and the bifidobacteria concentrations and the value of the tracking ratio were determined using the MF technique on the HSBA and the MBM.

4.3 RESULTS AND DISCUSSION

4.3.1 Screening

Results of the screening tests are summarised in Table 4.1. Paulse et al. (2009) found that concentrations of FC and *E. coli* ranged from 1.00 x 10^1 to 3.5 x 10^6 cells/100 mL in the Plankenburg River in the Western Cape, South Africa. In another study, the FC concentrations varied between 1.7 x 10^2 and 3.5 x 10^7 CFUs/100 ml, while the concentrations of *E. coli* ranged from 3.6 x 10^1 to 1.7 x 10^7 CFUs/100 mL in the Berg River, Western Cape, South Africa (Paulse et al., 2007). Wutor et al. (2009) reported the FC concentrations ranging from 2 x 10^6 to 1.1 x 10^7 CFUs/100 mL and concentrations of *E. coli* ranging from 2 x 10^6 to 1.3 x 10^7 CFUs/100 mL in the Bloukrans River in the Eastern Cape, South Africa. Germs et al. (2004) measured concentrations of FC ranging from 6 to 40.5 CFUs/100 mL in the Chune River in the province of Limpopo, South Africa. The Modder River contained from 0 to 8.4 x 10^5 CFUs/100 mL of FC in the vicinity of an informal settlement approximately 60 km westwards of Bloemfontein (Jagals and Grabow, 1996). Similar observations were made in the Jukskei River near Johannesburg, where the background concentration of *E. coli* was equal to 1.5 x 10^3 cells/100 mL (De Wet et al., 2000). It increased to 3.7 x 10^5 cells/100 mL in the stretch of the river passing through the Alexandra peri-urban settlement and slightly dropped to 1.3 x 10^5 cells/100 mL just downstream of the settlement (De Wet et al., 2000).

The FC concentration was below 0 cells/100 mL at site 3 on 15th April 2008 and site 8 on 4th August 2008. The concentration of E. coli was below 0 cells/100 mL at sites 1-7 and at site 8 conducted on 4th August 2008. The H_2S strip test was negative on site 7 on 5th June 2008. The minimum and non-zero concentration of FC was equal to 2 cells/100 mL at site 7 on 5th June 2008, at site 8 on 11th June 2008, at site 10 on 4th August 2008 and at site 15 on 5th August and 2nd September 2008. The maximum FC concentration was higher than 16000 cells/100 mL and it was measured at site 11 on 12th September 2008 and at site 12 on 1st August 2008; on 22nd August 2008; and on 12th September 2008. These values were not further refined due to the screening type of analysis. The FC concentrations are comparable to those of Jagals and Grabow (1996), but they span a narrower interval than the data of Wutor et al. (2009) and Paulse et al. (2007; 2009). On the other hand, the data spans a wider interval than the data of Germs et al. (2004).

Table 4.1. Results of screening for the selected sampling sites.

Site	Sampling date	GPS coordinates	FC (MPN cells/100 ml)	*E. coli* (MPN cells/100 ml)	H_2S strip test[a] (hours)	Site selected
1	15/04/2008	32° 54' 51.2"S 27° 24' 34.3"E	260	0	26	No
2	15/04/2008	32° 56' 56.7"S 27° 26' 58.1"E	130	0	26	No
3	15/04/2008	32° 57' 29.8"S 27° 31' 32.8"E	0	0	26	No
4	15/04/2008	33° 00' 21.3"S 27° 49' 31.7"E	3000	0	26	No
5	15/04/2008	32° 56' 40.9"S 27° 28' 24.7"E	170	0	21	No
6	15/04/2008	32° 48' 26.0"S 27° 28' 11.1"E	80	0	24	No
7	05/06/2008	33° 19' 13.9"S 26° 31' 14.8"E	2	0	Negative	No
8	05/06/2008	33° 19' 29.4"S 26° 25' 29.0"E	8	8	60	No
8	11/06/2008	33° 19' 29.4"S 26° 25' 29.0"E	2	2	60	No
8	04/08/2008	33° 19' 29.4"S 26° 25' 29.0"E	0	0	60	No
9	11/06/2008	33° 17' 40.0"S 26° 30' 54.2"E	30	30	48	Yes
9	04/08/2008	33° 17' 40.0"S 26° 30' 54.2"E	8	4	60	Yes
9	25/08/2008	33° 17' 40.0"S 26° 30' 54.2"E	8	8	60	Yes
10	29/05/2008	33° 19' 26.7"S 26° 31' 39.9"E	20	20	24	Yes
10	11/06/2008	33° 19' 26.7"S 26° 31' 39.9"E	13	4	24	Yes
10	04/08/2008	33° 19' 26.7"S 26° 31' 39.9"E	2	2	48	Yes
11	01/08/2008	33° 18' 51.9"S 26° 33' 05.5"E	1700	700	25	Yes
11	22/08/2008	33° 18' 51.9"S 26° 33' 05.5"E	5000	1300	24	Yes
11	12/09/2008	33° 18' 51.9"S 26° 33' 05.5"E	> 16000	> 16000	24	Yes
12	01/08/2008	33° 18' 46.7"S 26° 32' 29.3"E	> 16000	7600	14	Yes
12	22/08/2008	33° 18' 46.7"S 26° 32' 29.3"E	> 16000	110	24	Yes
12	12/09/2008	33° 18' 46.7"S 26° 32' 29.3"E	> 16000	> 16000	24	Yes

Site	Sampling date	GPS coordinates	FC (MPN cells/100 ml)	*E. coli* (MPN cells/100 ml)	H_2S strip test[a] (hours)	Site selected
13	05/08/2008	33° 11' 59.4"S 26° 29' 42.8"E	23	23	48	Yes
13	02/09/2008	33° 11' 59.4"S 26° 29' 42.8"E	300	300	40	Yes
13	16/09/2008	33° 11' 59.4"S 26° 29' 42.8"E	110	110	27	Yes
14	05/08/2008	33° 11' 49.5"S 26° 29' 28.0"E	13	13	48	Yes
14	02/09/2008	33° 11' 49.5"S 26° 29' 28.0"E	30	30	40	Yes
14	16/09/2008	33° 11' 49.5"S 26° 29' 28.0"E	170	110	27	Yes
15	05/08/2008	33° 11' 10.1"S 26° 29' 55.9"E	2	2	60	Yes
15	02/09/2008	33° 11' 10.1"S 26° 29' 55.9"E	2	2	40	Yes
15	16/09/2008	33° 11' 10.1"S 26° 29' 55.9"E	70	50	27	Yes

[a] If the H_2S strip test was positive for a particular water sample, then the period indicated was required for the first detection of black colour.

The minimum and non-zero concentration of *E. coli* was equal to 2 cells/100 mL at site 8 on 11th June 2008, at site 10 on 4th August 2008 and at site 15 on 5th August and 2nd September 2008. The maximum concentration of *E. coli* was higher than 16000 cells/100 mL and it was measured at site 11 and at site 12 on 12th September 2008. These values were not further refined due to the screening type of analysis. The concentrations of *E. coli* in this study are comparable to those of De Wet et al. (2000), but they span a narrower interval than those of Wutor et al. (2009) and Paulse et al. (2007; 2009). Positive H_2S strip test was observed after a minimum of 14 hours at site 12 on 1st August 2008 and after a maximum of 60 hours at site 8 on 5th June and 11th June 2008; and on 4th August 2008. The same period was required for the positive H_2S strip test at site 9 on 4th August 2008 and on 25th August 2008; and site 15 on 5th August 2008. Mosley and Sharp found that the H_2S strip test was positive after 96 hours in water samples containing 1 CFU/100 mL of *E. coli* (Mosley and Sharp, 2005). After the addition of deoxycholic acid, this period was shortened below 72 hours at the comparable concentration of 2 cells/100 mL (Table 4.1). Based on these results, sampling sites 9-15 were selected and used in the remainder of the study.

4.3.2 Possible explanations for discrepancies

4.3.2.1 Chemical interferences

The H_2S strip test was negative and the concentration of *E. coli* was below 0 cells/100 mL at site 7 on 5th June 2008. The FC concentration was equal to 2 cells/100 mL in the same sample. On the other hand, the concentrations of FC and of *E. coli* were below 0 cells/100 mL and the H_2S strip test was positive at site 3 on 15th April 2008 and site 8 on 4th August 2008. The screening tests used could be influenced by chemical interferences. It has been suggested in the literature that SO_4^{2-} anions can cause a false positive H_2S strip test in the absence of the H_2S strip test bacteria (Wetzel, 2001; Sobsey and Pfaender, 2002). The environmental health professionals from South Africa raised the possibility of the interference by NO_2^- and NO_3^- (Genthe and Jagals, 2003).

Possible interference from SO_4^{2-} was tested using sterile solutions of Na_2SO_4 with sulphate concentrations ranging from 14 to 4240 mg/L and selected samples are shown in Figure 4.1. As can be seen, no black colour was observed even at 4240 mg/L after 72 hours of incubation and so SO_4^{2-} ions did not interfere with the H_2S strip test results. The interference from NO_2^- and NO_3^- could occur through the growth inhibition of *Acinetobacter spp.* (one of the H_2S strip test bacteria) when the anion concentrations reach 604 mg/L (Weon et al., 2002). However, concentrations of NO_2^- and NO_3^- were lower than this value at all of the selected sampling sites (Table 4.4) and these concentrations are rarely if ever exceeded in South Africa. Thus neither of these two anions interfered with the results of the H_2S strip test.

a) b)

Figure 4.1. Examination of the potential SO_4^{2-} interference with the H_2S strip test at the sulphate concentrations of 54 mg/L (a) and 4240 mg/L (b).

4.3.2.2 Temperature fluctuations

The H_2S strip test was designed for application in countries like South Africa, where the ambient temperatures vary significantly with geographical location and with the season. Literature data indicate the relevant interval varies from -12ºC (Du Toit et al., 2009) to 50ºC (SAWS, 2010).

The temperature values ranged from 10 to 32ºC in the sampled area during this study. The influence of possible temperature fluctuations on the H_2S strip test results was studied and selected results are shown in Figure 4.2 and Figure 4.3. The H_2S samples were first checked for a positive response after 14 hours, which was the shortest time required for a positive H_2S strip test in Table 4.1. The FC concentration was equal to 3.60×10^1 CFUs/100 mL and the concentration of *E. coli* was equal to 3.00×10^1 CFUs/100 mL at sampling site 10. The respective concentrations were equal to 1.00×10^5 CFUs/100 mL and to 3.05×10^4 CFUs/100 mL at sampling site 11. Length of the sampling trip and exposure to a given temperature are used interchangeably in the following paragraph and in Figure 4.2 and Figure 4.3.

The five-hour exposure to 10 or 32°C did not influence the results of the H_2S strip test at site 10. For the twelve-hour exposure period, the positive H_2S strip test was observed 12 hours earlier than in the control after preincubation at 32°C and 12 hours later than the control after preincubation at 10°C (Figure 4.2). For the five-hour exposure to 10°C, the positive H_2S strip test was recorded 5 hours later than in the control at sampling site 11 (Figure 4.3). Exposure to the other three combinations of temperatures and preincubation periods did not influence the results of the H_2S strip test at sampling site 11. The positive H_2S strip test was recorded within the standard 72 hour period and so temperature fluctuations during sample collection and transport into the laboratory did not influence the H_2S strip test results. There are several explanations for the discrepancies in the results of the screening tests. One is that some of the indicator organism cells entered the VBNC state which led to a partial loss of culturability (Signoretto et al., 2002). The other possible

explanation is that the bacterial cells might have been attached to the particulate matter and this led to release/retention during water sampling. The latter point was investigated in more detail and the results are summarised in the next section.

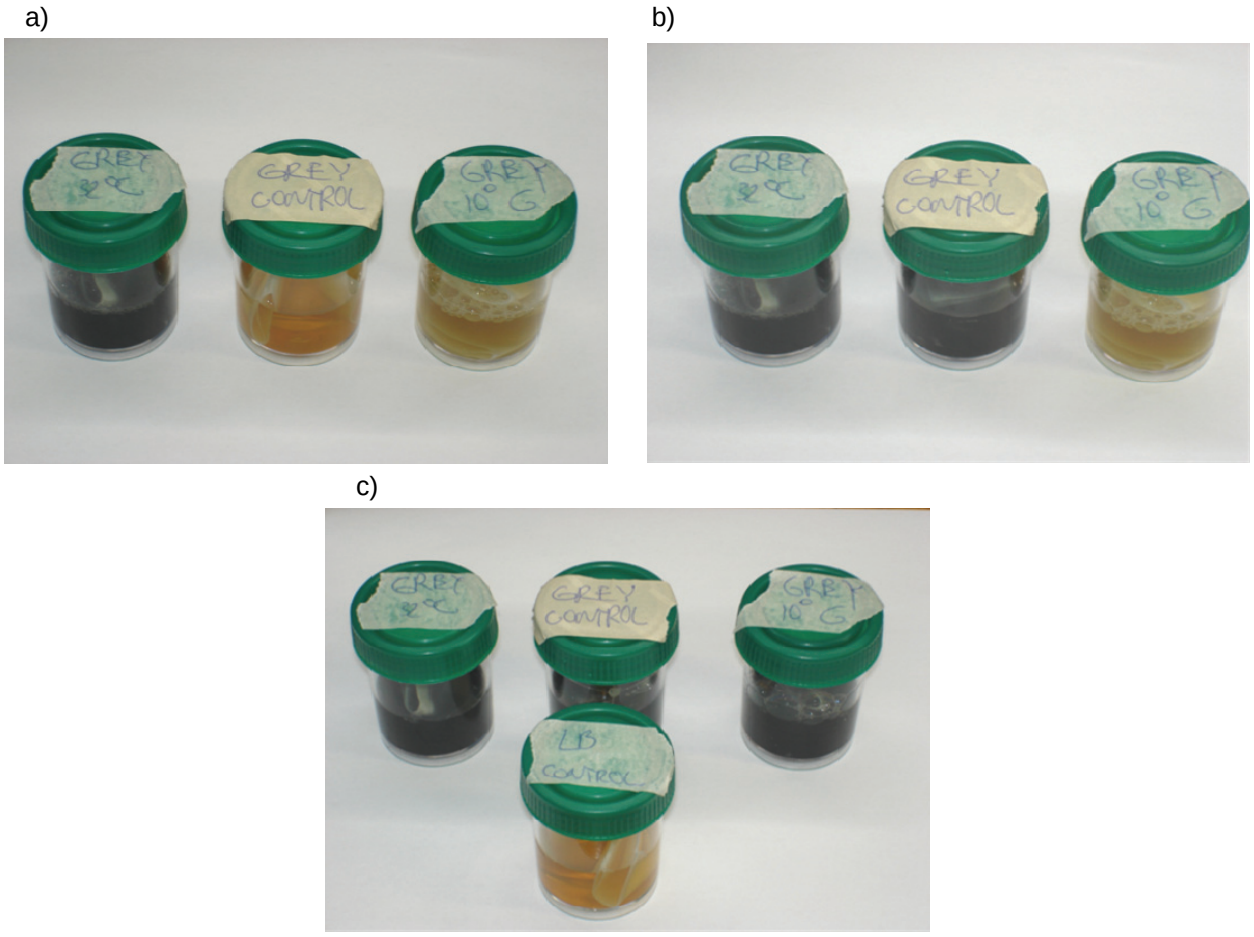


Figure 4.2. Influence of temperature fluctuations on the results of the H_2S strip test during the twelve-hour sampling trip at site 10 after 16 hours (a), 28 hours (b) and 40 hours (c) of incubation.

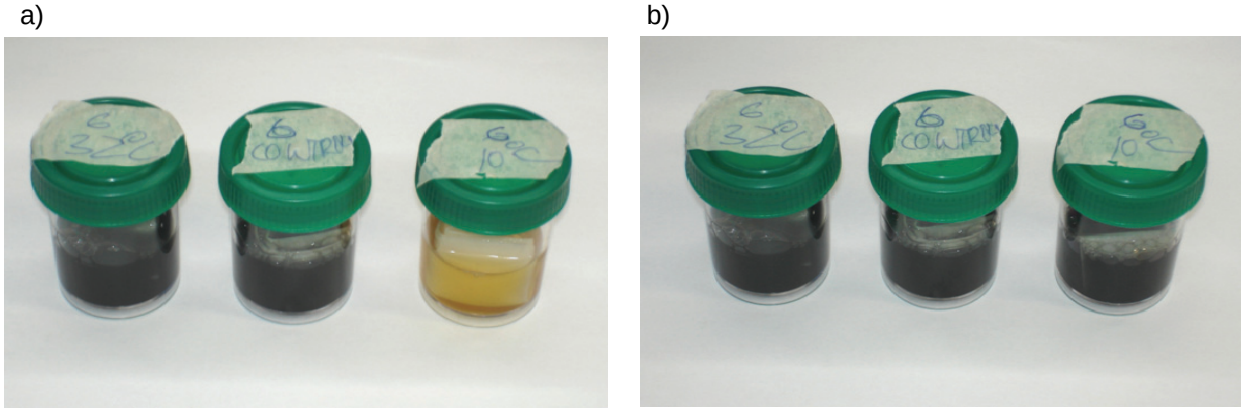


Figure 4.3. Influence of temperature fluctuations on the results of the H2S strip test during the five-hour sampling trip in water samples from site 11 after 16 hours (a) and 21 hours (b).

4.3.2.3 Influence of attachment to particulate matter

The extent of attachment of indicator microorganisms to particulate matter has not yet been studied in South African water resources. Re-suspension of such particles can influence the concentrations of indicator microorganisms during storms and other events (LaLiberte and Grimes, 1982). Thus the attachment was measured at the selected sampling sites. First, the concentration of FC or *E. coli* in the particulate matter was calculated using to Eq. (4.1).

$$X_{\text{Sediment}} = \frac{0.75 \times X_{\text{Extract}}}{SDW} \quad (4.1)$$

In Eq. (4.1), X_{Sediment} is the concentration of FC or *E. coli* in the particulate matter (FC_{Sediment} or $E.coli_{\text{Sediment}}$ in Table 4.2 in units of MPN cells/g sediment dry weight), while X_{Extract} is the concentration of FC or *E. coli* in the Ringer solution extract of the particulate matter (MPN cells/100 ml). *SDW* is the sediment dry weight (g). The coefficient 0.75 in the numerator of Eq. (1) represents the conversion of the X_{Extract} value to 75 mL of the Ringer solution extract. The results are summarised in Table 4.2.

Table 4.2. Influence of suspendable particulate matter on the concentrations of FC and E. coli.

Site	Date of sampling	FC_{Water} (cells/100 ml)	$E. coli_{Water}$ (cells/100 ml)	*SDW* (g)	$FC_{Sediment}$ (cells/g SDW)	$E.coli_{Sediment}$ (cells/g SDW)	FC median (%)	*E. coli* median (%)
9	10/12/2008	1600	1600	0.98	1230	1150	1.7	1.5
9	20/05/2009	31	17	1.25	170	170	11.8	21.5
10	10/12/2008	1600	1600	6.78	180	170	0.2	0.2
10	25/06/2009	4	0	0.01	0.00	0.00	0	ND[a]
11	15/12/2008	> 16000	> 16000	0.46	26100	24300	3.3	3.1
11	20/05/2009	920	84	0.37	150	130	0.4	3.3
12	20/12/2008	> 16000	> 16000	0.00	ND[a]	ND[a]	ND[a]	ND[a]
12	25/06/2009	2400	25	0.00	ND[a]	ND[a]	ND[a]	ND[a]
13	22/11/2008	70	70	0.44	120	110	3.7	3.4
13	15/07/2009	33	33	0.67	170	120	11.1	7.8
14	22/11/2008	130	130	0.06	2440	2280	40.4	37.7
14	15/07/2009	79	8	0.00	ND[a]	ND[a]	ND[a]	ND[a]
15	22/11/2008	31	31	0.04	380	370	26.4	25.7
15	15/07/2009	2	0	0.00	ND[a]	ND[a]	ND[a]	ND[a]

[a] ND – not determined

Concentrations of FC_{Sediment} ranged from 0 cells/g SDW at site 10 on 25th June 2009 to 2.61 x 10^4 cells/g SDW at site 11 on 12th December 2008. The respective values for *E. coli* ranged from 0 cells/g SDW at site 10 on 25th June 2009 up to 2.43 x 10^4 cells/g SDW at site 11 on 12th December 2008. No visible particulate matter deposits were observed at site 12 on 20th December 2008 and 25th June 2009 and therefore no X_{Sediment} could be calculated for the indicator microorganisms. A similar situation was observed at sites 14 and 15 on 15th July 2009. No value of $E.coli_{\text{Sediment}}$ was calculated for site 10 on 25th June 2009 since cells were detected in the particulate matter extract. The amount of suspendable particulate matter varied widely

with the season of sampling and between different sampling sites. However, no systematic trends were observed.

To examine the extent by which the water concentrations of FC and *E. coli* could change upon re-suspending particulate matter was estimated as follows. The median amount of particulate matter from the selected sampling sites was equal to 0.215 g. We assumed that upon re-suspension of the particulate matter the TSS concentration would increase by 0.215 g/l which is comparable to the increases in sediment load during storm events (Houser et al., 2006). We further assumed that all the microbial cells attached to the suspendable particulate matter could be enumerated using the MPN technique. Based on these assumptions, the possible changes in the concentrations of FC (FC_{median}) and *E. coli* ($E.\ coli_{median}$) were calculated using Eq. (4.2).

$$X_{\text{median}} = 100 \times \frac{0.215 \times X_{\text{Sediment}}}{10 \times X_{\text{Water}}} \qquad (4.2)$$

In Eq. (4.2), $X_{Sediment}$ has the same meaning as in Eq. (4.1), while X_{Water} is the concentration of the indicator microorganism in question in the water column at the selected sampling site (MPN cells/100 ml). Coefficient 100 is used to convert the ratio to percentage. The coefficient 0.215 is the median TSS change (g) and coefficient 10 is used to convert the concentration of indicator organisms into cells/100 mL. All calculated values are summarised in Table 4.2. The FC_{median} values ranged from 0 to 40.4% and the $E.\ coli_{median}$ values ranged from 0.2 to 37.7%. The maximum values were both recorded at site 14 on 22nd November 2008. Besides these two values, all other values were comparable to the coefficient of variation of the MPN technique (APHA, 1998). Therefore it can be concluded that the re-suspension would not have any influence on the concentration of FC and *E. coli* at the selected sampling sites.

Chemical water quality and its links to the health status of wild game have been studied in South Africa (Casey and Meyer, 2006). However, the microbial water quality has not been evaluated in the same context and no guidelines exist for the evaluation of the relevant animal health risks. Sampling sites 13-15 are used as drinking points by wild game and the microbial water quality at these sites is therefore evaluated in the context of the guidelines for livestock watering (DWAF, 1996c). If there are to be no adverse effects of the water quality on the health of livestock, then the concentration of FC must range from 0 to 200 CFUs or cells/100 mL and no more than 20% of the samples can contain between 0 and 1000 CFUs or cells/100 mL (DWAF, 1996c). Together 15 samples were taken at sites 13-15 and the FC concentrations ranged from 2-300 cells/100 mL (Table 4.1 and Table 4.2). One sample contained 300 cells/100 mL and so the data indicates that the health of wild game at the Kwandwe game reserve will not be adversely affected by water quality at sites 13-15. The FC concentrations measured at the Kwandwe game reserve are comparable to those in the Boteti River in Botswana which ranged from 0 to 48 CFUs/100 mL (Masamba and Mazvimavi, 2008).

4.3.2.4 Faecal coliform die-off

The chemical composition of the water and the concentration of indigenous microflora (enumerated as HPC) at the selected sampling sites were measured to prepare model waters and to study the survival of FC and bifidobacteria. The results are summarised in Table 4.3 with the maximum and minimum values shown in bold. Comparisons are made to literature data from South Africa. As can be seen, the values from this study are either comparable to the literature values or they span narrower intervals. No reference data is available for the concentrations of total saccharides and proteins in South Africa. In international literature, concentrations of monosaccharides and oligosaccharides are rarely reported to be higher than ten micrograms per litre in environmental water resources (Meon and Jüttner, 1999), due to their rapid assimilation by bacteria (Wetzel, 2001).

Table 4.3. Water composition at the sampling sites.

	Site 9	Site 10	Site 11	Site 12	Site 13	Site 14	Site 15	Literature range	Reference
pH	8.32	**6.21**	7.37	7.43	7.74	7.71	**8.62**	7.10-10.60 5.30-8.10 6.87-8.33 8.10-8.60 2.06-12.42	Harding (1992) Palmer et al. (1994) Faniran et al.(2001) Germs et al. (2004) DWAF (2008)
Turbidity (NTU)	**186**	**0.0**	5.8	18.5	14.8	23.0	3.8	0.59-85.6 0.50-2662	Faniran et al.(2001) DWAF (2008)
EC (mS/m)	38	**27**	**202**	160	111	114	122	72-133 50- 95 64-332 4.70-770	Harding (1992) Faniran et al.(2001) Germs et al. (2004) DWAF (2008)
DO (mg/l)	**8.88**	7.49	5.58	**2.50**	5.89	6.01	7.48	5.40-19.40 1.73-14.40 1.60-7.50	Harding (1992) Faniran et al.(2001) DWAF (2008)
Cl^- (mg/l)	64	**40**	207	**410**	145	154	186	95-1223 59-894 1.50-2470	Faniran et al.(2001) Germs et al. (2004) DWAF (2008)
T (ºC)	21.0	19.9	22.0	**24.0**	**18.5**	20.0	22.7	10.9-27.8 8.8-22.8 14-25 0.0-28.2	Harding (1992) Palmer et al. (1994) Faniran et al.(2001) DWAF (2008)
Light Intensity (Lux)	15400	**412-1760**	3500	5290	16000	14400	**75090**	NRD[a]	NRD[a]
HPC (CFUs/mL)	649000	16400	4250000	**5450000**	**600**	3000	3000	5.42×10^2-7.35×10^3 3.00×10^3-3.60×10^5	Germs et al. (2004) Paulse et al. (2007)
SO_4^{2-} (mg/l)	75	25	34	**58**	**53**	54	28	57-155 9.5-239 0.5-940.7	Faniran et al.(2001) Germs et al. (2004) DWAF (2008)
TSc (mg GE/l)b	5.3	39.4	6.0	**23.0**	**4.2**	5.4	5.0	<10 µg/l	Meon and Jüttner (1999)
Proteins (mg BSAE/l)d	5.7	<2	<2	**<2**	**<2**	<2	<2	NRDa	NRDa
PO_4^{3-} (mg/l)	<0.10	<0.10	0.78	**7.00**	**2.07**	1.70	0.56	0.01-1.29 0.05-0.09 <0.01 0.00-34.1	Harding (1992) Palmer et al. (1994) Germs et al. (2004) DWAF (2008)

	Site 9	Site 10	Site 11	Site 12	Site 13	Site 14	Site 15	Literature range	Reference
NH^{4+} (mg/l)	<0.10	<0.10	4.95	**<0.10**	**<0.10**	<0.10	<0.10	0.00-2.15e 0.66-26.78 e 0.40-0.78 e 0.00-215 e	Harding (1992) Faniran et al.(2001) Germs et al. (2004) DWAF (2008)
NO_3^- (mg/l)	4.28	<1.0	19.5	**<1.0**	**<1.0**	<1.0	<1.0	0.00-39.9f 0.04-31.9 f 0.31-42.9 0.00-241	Harding (1992) Faniran et al.(2001) Germs et al. (2004) DWAF (2008)
TSS (mg/l)	340	58	113	**130**	**0**	0	0	4-153 4.60-69.6 0.1-1583	Harding (1992) Faniran et al.(2001) DWAF (2008)
TA (mg $CaCO_3$/l)	0	0	80	**160**	**100**	120	70	82-270 3.30-688.7 2.0-1558	Harding (1992) Faniran et al.(2001) DWAF (2008)
Chla (µg/l)	5.6	5.7	104	**183**	**2.6**	2.8	2.9	69-796 0.5-1511	Harding (1992) DWAF (2008)

[a] No reference data is available in the literature
[b] Glucose equivalents
[c] Total saccharides
[d] Bovine Serum Albumin equivalents
[e] Expressed as NH_4^+-N in the original reference and recalculated into NH_4^+
[f] Expressed as NO_3^--N in the original reference and recalculated into NO_3^-

Faecal contamination leads to the presence of non-indigenous microorganisms, i.e. faecal indicator bacteria, in the water resource. This causes environmental stress and the cells of the indicator microorganisms will probably transfer from the vegetative state to the VBNC state. Release of monocarbohydrates, amino acids and proteins from bacterial cells has been demonstrated during this transfer (Arana et al., 2004). This could be one possible explanation for the finite water concentrations of saccharides and proteins detected in this study. Other possible sources of saccharides and proteins include dead plant biomass and loose skin from swimmers. Association of proteins with humic substances can occur in water resources (Brehm, 1967), making them highly resistant to biodegradation (Wetzel, 2001). Two types of model waters were prepared, namely the nutrient-rich model water (NRMW) and the nutrient-depleted model water (NDMW). Compositions of the NRMW and NDMW are shown in Table 4.4. The light intensity was set approximately to the minimum values measured at the selected sampling sites due to the equipment limitations. Other parameters were set to values inside the intervals measured at the selected sampling sites.

Table 4.4. Composition of the chosen model waters.

Parameter	NRMW	NDMW
pH	7.71	7.87
Turbidity (NTU)	23	87
Salinity (mS/m)	82	82
DO (mg/l)	6.01	6.01
Cl^- (mg/l)	77	77
T (ºC)	21.5	21.5
Light Intensity (lx)	386-1760	386-1760

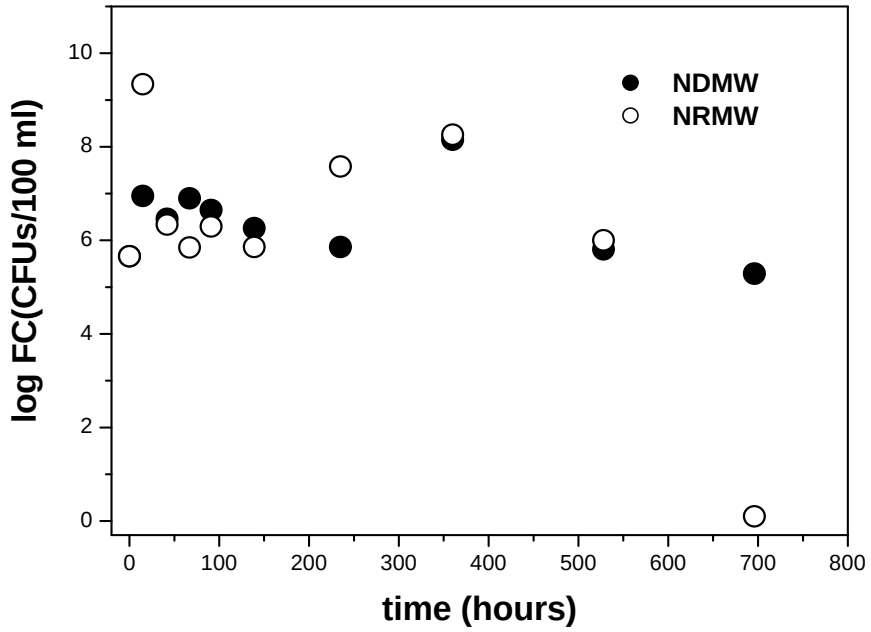
HPC (CFUs/mL)	48000	48000
SO_4^{2-} (mg/l)	43	43
Saccharides (mg GE/l)	5.7	5.7
Proteins (mg BSAE/l)	5.7	0
PO_4^{3-} (mg/l)	1.49	0
NH_4^+ (mg/l)	1.08	0
NO_3^- (mg/l)	11.7	0
TSS (mg/l)	0	50
TA (mg $CaCO_3$ /l)	60	100
Chla (µg/l)	2.9	2.9

Results of the survival experiments are shown in Figure 4.4. The initial concentration of faecal coliforms (FC) was set to 4.60 x 10^5 CFUs/100 ml, while the initial heterotrophic plate count (HPC) concentration was to 4.80 x 10^4 CFUs/mL. During the initial 15 hours of incubation, the FC concentration increased to 9.00 x 10^6 CFUs/100 mL in the NDMW and to 2.21 x 10^9 CFUs/100 mL in the NRMW. This increase probably originated from the production of new bacterial biomass after biodegradation of glucose and the proteins took place in the model waters. The peak concentration in the NRMW is higher than in the NDMW because both proteins and saccharides were present there at the beginning of the experiment. There was no systematic trend of the FC concentration with time from 15 until 235 hours of incubation in the NRMW and from 15 until 139 hours of incubation in the NDMW. Successively, the FC concentration started increasing again reaching 1.40 x 10^8 CFUs/100 mL in the NDMW and 1.80 x 10^8 CFUs/100 mL in the NRMW after 360 hours of incubation. This spike could be explained through the release of newly produced saccharides from the algal biomass and production of new bacterial biomass upon their biodegradation.

If this was the case, then all the glucose and proteins, which were initially added to the NRMW would have been depleted prior to the assimilation of the saccharides from algal sources. As a result, the second peak concentrations of FC would be comparable in both the NDMW and the NRMW. The data in Figure 4.4 indicates that this was indeed the case. After 360 hours, the concentration of FC decreased to the final values of 1.96 x 10^5 CFUs/100 mL in the NDMW and 0 CFUs/100 mL in the NRMW. The experiment was terminated after 696 hours. The HPC concentration increased from the initial value to 8.80 x 10^7 CFUs/mL in the NDMW, but it remained practically unchanged in the NRMW during the initial 91 hours of incubation (Figure 4.4 b). Then the HPC concentration decreased as a function of time to reach 0 CFUs/mL in the NDMW after 696 hours.

In the NRMW, the HPC concentration increased to 4.12 x 10^6 CFUs/mL after 235 hours and it dropped to 0 CFUs/mL after 696 hours of incubation. No explanation can be provided for this observation at present. The H_2S strip tests were positive in both model waters at the beginning of the survival experiment and after 235 and 528 hours of incubation. However, all tests were negative after 696 hours. Cells of the H_2S strip test bacteria probably entered the VBNC state or died off and thus no culturable H_2S strip test bacteria could be detected. Results of the survival experiments indicate that discrepancy between the results of the FC enumeration and the H_2S strip will occur between 22 and 29 days after the faecal contamination. This could provide and explanation for some of the discrepancies observed in Table 4.1.

a)



b)

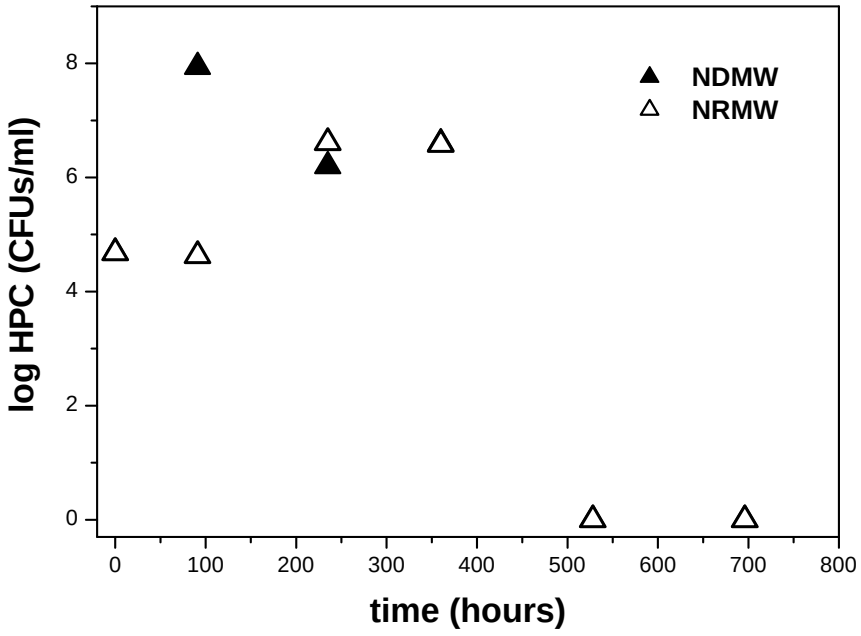


Figure 4.4. Concentrations of FC (a) and HPC (b) as a function of time during the survival experiments in model waters.

The survival data for FC did not follow a first-order decay model (Foppen and Schijven, 2006), because the respective R^2 values ranged from 0.0902 to 0.1009 (data not shown). More than one factor probably controls the survival of FC at the selected sampling sites, with the protein concentration probably playing a crucial role. Data presented here should be treated as preliminary. Further investigations are recommended into the combined effect of the physical and chemical factors on the survival of FC and the H_2S strip test bacteria in the Eastern Cape and other provinces of South Africa.

4.3.3 Faecal source identification using *Rhodococcus coprophilus*

Enumeration of *Rhodococcus coprophilus* was conducted in two water samples and one sample of the particulate matter at all selected sampling sites. This helped in the division of the selected sampling sites into those receiving human, animal or mixed type of faecal contamination.

The concentration of *Rhodococcus coprophilus* in the particulate matter and the associated potential change in the concentration of *Rhodococcus coprophilus* were calculated using Eqs. (4.1) and (4.2). Concentrations of *Rhodococcus coprophilus* at the selected sampling sites are shown in Table 4.5. No *Rhodococcus coprophilus* was detected in water samples from site 10 on 10th December 2008 and 25th June 2009; and at site 12 on 25th June 2009. The maximum concentration of *Rhodococcus coprophilus* was equal to 8.18 x 10^6 CFUs/100 mL at site 11 on 15th December 2008. The results from this study are comparable to those of Jagals et al. (1995). In the suspendable particulate matter, concentrations of *Rhodococcus coprophilus* ranged from 1.80 x 10^2 to 4.69 x 10^6 CFUs/g SDW. No reference values were found in South Africa and the concentrations in many samples were below the detection limit of 500 CFUs/100 mL of the spread-plate technique. The MF technique with the M3 medium was tested but results suffered from large variability and so it was not used in this study.

Results of the enumeration of *Rhodococcus coprophilus* indicate that sampling sites 9 and 10 receive only faecal contamination from human sources. Sampling sites 11 and 12 receive mixed faecal contamination from human and animal sources, predominantly cattle. Sampling site 13 received mixed faecal contamination from humans and wildlife, while sampling sites 14 and 15 receive faecal contamination from only wildlife sources.

Table 4.5. Concentrations of Rhodococcus coprophilus (RC) at the sampling sites.

Site	Date of sampling	RC_W (CFUs/100 ml)	*SDW* (g)	$RC_{sediment}$ (CFUs/ g SDW)	RC_{median} (%)	Contamination source
9	10/12/2008	3500	0.98	ND[a]	ND[a]	Human
9	20/05/2009	4500	1.25	14100	6.7	Human
10	10/12/2008	0	6.78	180	ND[a]	Human
10	25/06/2009	0	0.01	4688000	Infinity	Human
11	15/12/2008	8180000	0.46	ND[a]	ND[a]	Mixed
11	20/05/2009	150000	0.37	80000	1.1	Mixed
12	20/12/2008	0	0.00	ND[a]	ND[a]	Human
12	25/06/2009	7500	ND[a]	ND[a]	ND[a]	Mixed
13	22/11/2008	26300	0.44	ND[a]	ND[a]	Wild Game
13	15/07/2009	3300	0.67	36900	24.0	Unknown
14	22/11/2008	17000	0.06	ND[a]	ND[a]	Wild Game
14	15/07/2009	5000	0.00	ND[a]	ND[a]	Wild Game
15	22/11/2008	13000	0.04	ND[a]	ND[a]	Wild Game
15	15/07/2009	15000	0.00	ND[a]	ND[a]	Wild Game

[a] ND – not determined

4.4 SAMPLING SITE DESCRIPTIONS

Sampling site 9 (Figure 4.5) is the Gowie dam in a suburb of Grahamstown and it is used for swimming and livestock watering by the residents. Bathroom greywater has been shown to contain non-zero concentrations of *E. coli* and FC (Zuma et al., 2009). Thus swimming is likely to be one of the faecal contamination sources of human origin at this sampling site. Visual examination of the dam banks indicated seepage of water through them. Sanitation infrastructure from the residential area is located in the vicinity of the dam and contamination could take place if pipes burst. Sewage could then flow into the dam by the matrix flow and the pipe flow through the soil structure (Atkinson, 1979). The matrix flow is defined as water movement through the intergranular pores, while the pipe flow is defined as water movement through large voids, larger than capillary pores, in the soil structure (Atkinson, 1979). The maximum concentrations of FC and *E. coli* were equal to 1600 cells/100 mL in the late spring and early summer, while the HPC concentration was equal to 6.49 x 10^7 CFUs/100 mL (Table 4.3). This indicates that the faecal indicator bacteria were outcompeted by the indigenous microflora, leading to the low concentrations of the indicator bacteria observed at site 9. Cattle dung was observed on the banks of the dam, but the vegetation cover is well developed and transport of cattle faeces into the dam with surface runoff is probably not taking place. Results of the enumeration of *Rhodococcus coprophilus* indicated absence of faecal contamination from animal sources on two separate occasions and during two different seasons. Thus sampling site 9 will be classified as receiving faecal contamination from human sources.

Figure 4.5. Sampling site 9 at the Gowie dam in Grahamstown.

Sampling site 10 (Figure 4.6) is the Grey dam which is located on the outskirts of Grahamstown, close to the N2 highway between Grahamstown and Port Elizabeth. This dam, built in 1860, is one of the oldest reservoirs of raw water in Grahamstown (Mullins, 2009). Water from this dam is pumped into the water treatment works for production of the city's drinking water. It is also used for recreational swimming and dogs are walked on its banks. Swimming in the dam can lead to faecal contamination from humans based on the same reasoning as at sampling site 9. Dog faeces were observed on the banks of the dam, but the vegetation cover was well developed in the form of high grass. Their transport through surface runoff into the dam is unlikely under such conditions. The enumeration of *Rhodococcus coprophilus* indicates absence of faecal contamination from animal sources. Thus sampling site 10 will be classified as receiving faecal contamination from human sources.

Figure 4.6. Sampling site 10 at the Grey Dam in Grahamstown.

Sampling site 11 (Figure 4.7) is located on the Bloukrans River in Grahamstown-East, close to the municipal sewage treatment works. Banks of the river are very steep in this area and pit latrines, which are often not maintained, constitute the sanitation infrastructure in the houses located on these banks. Under such conditions, site 11 can be contaminated by seepage from the pit latrines by the matrix flow and the pipe flow through the soil structure as described on site 9 (Atkinson, 1979). The river is crossed by cattle during grazing and defecation by livestock has been observed at this sampling site on multiple occasions. The enumeration of *Rhodococcus coprophilus* supports faecal contamination from animal sources. Thus sampling site 11 will be classified as receiving faecal contamination from human sources and animal sources, mostly cattle.

Figure 4.7. Sampling site 11 at the Bloukrans River near the sewage treatment work in Grahamstown.

Sampling site 12 (Figure 4.8) is located at the sewage outflow from the Fingo Village (Grahamstown-East) into the Bloukrans River. The vegetation cover is unevenly distributed at this sampling site. The sewage is a source of faecal contamination of human origin, while cattle dung observed on the river banks can lead to faecal input from livestock due to surface runoff and precipitation. The enumeration of *Rhodococcus coprophilus* indicates faecal contamination from animal sources at this site. Thus sampling site 12 can be classified as receiving faecal contamination from human sources and animal sources, mostly cattle.

Figure 4.8. Sampling site 12 is located at the sewage outflow from the Fingo Village (Grahamstown-East) into the Bloukrans River.

Sampling site 13 (Figure 4.9) is located on the Botha's River on the southern edge of the Kwandwe game reserve near Grahamstown. The Grahamstown military base is located just upstream of this sampling site but the contribution to the faecal contamination from this source is unknown. Animals from the reserve cross this sampling site during herd and pack movement. The animals include lions, zebra and several species of antelope, rhinos, elephants and hippopotamus. Defecation by these animals on the banks of the river, as well as in the river, is possible and so the site is likely to receive faecal contamination from animal sources. The enumeration of *Rhodococcus coprophilus* indicates intermittent faecal contamination from animal sources, but it also points to the influence from other sources. For the time being, sampling site 13 will be classified as receiving faecal contamination from wildlife sources. However, further investigation about the source of faecal contamination will have to be conducted in the future.

Figure 4.9. Sampling site 13 on the Botha River in the Kwandwe Nature Reserve.

Sampling site 14 (Figure 4.10) is located downstream from site 13 and just after the ephemeral Brak River flows into the Botha's River. At this sampling site, the Botha River also flows into a retention dam, which is used as a watering hole for all animals from the Kwandwe Game Reserve.

Figure 4.10. Sampling site 14 at the entry of the Botha's River into the retention dam at the Kwandwe game reserve.

Sampling site 15 (Figure 4.11) is located at the retention wall of this dam. Defecation by the animals directly in the dam water, or contamination from faeces on the banks due to surface runoff, are likely to be the only sources of faecal contamination at these two sites. The enumeration of *Rhodococcus coprophilus* supports the presence of faecal contamination from animal sources. Thus sampling sites 14 and 15 will be classified as receiving only faecal contamination from wildlife sources.

Figure 4.11. Sampling site 15 at the retention dam wall at the Kwandwe game reserve.

CHAPTER 5: RESULTS AND DISCUSSION

5.1 CALCULATED TRACKING RATIOS

5.1.1 Bifidobacteria enumeration

Bifidobacteria were first enumerated using the method of Mara and Oragui (1983) and the respective results are shown in Table 5.1. Concentrations of the sorbitol-utilising bifidobacteria (SUB) ranged from 1.00 x 10^1 CFUs/100 mL at site 11 on 12th February 2009 to 5.00 x 10^4 CFUs/100 mL at site 12 on 20th December 2008. At the same time, concentrations of the total bifidobacteria (TB) ranged from 4 x 10^0 CFUs/100 mL at site 10 on 15th February 2009 to 8.00 x 10^3 CFUs/100 mL at site 12 on 20th December 2008.

Jagals and Grabow (1996) measured the concentrations of the sorbitol-utilising bifidobacteria in the Modder River in the vicinity of an informal settlement about 60 km westwards of Bloemfontein, South Africa. During the dry season, the concentration of the sorbitol-fermenting bifidobacteria ranged from 0-3.60 x 10^3 CFUs/100 mL and these increased to between 6.00 x 10^1 and 9.20 x 10^4 CFUs/100 mL during the rainy season (Jagals and Grabow, 1996)

It can be seen that the concentrations of the sorbitol-utilising bifidobacteria from this study are comparable to those of Jagals and Grabow (1996), but in contrast there was no systematic trend in the concentration data as a function of the rainfall intensity. No reference data were found for the total bifidobacterial concentrations in South African water resources.

To distinguish between the sources of faecal contamination, the tracking ratio (*TR)* was calculated according to Eq. (5.1).

$$TR = \frac{SUB}{TB} \qquad (5.1)$$

The calculated values are shown in Table 5.1, and they ranged from 0.10 at site 11 on 12th February 2009 to 6.25 at site 12 on 20th December 2008. Bonjoch et al. (2005) stated that the tracking ratio should be higher than 0.20 at sites with only human faecal contamination and below 0.20 at sites receiving animal or mixed faecal contamination. Blanch et al. (2006) reported a value of 3.2 as the threshold for distinguishing between the different sources of faecal contamination.

The YN-17 contains higher concentrations of antibiotics than the human-bifid sorbitol agar (HSBA) (Mara and Oragui, 1983) and bifidobacteria are highly sensitive to O_2 (Tandlich and Muller, 2008). A combination of these factors could increase the environmental stress and decrease the culturability of the bifidobacteria on the YN-17 medium. This could be the explanation for higher concentrations of the sorbitol-utilising bifidobacteria than the measured concentrations of the total bifidobacteria in this study and the study of Blanch et al. (2006). This in turn might be the reason for the TR values being higher than 1.00

No threshold TR value could be determined based on the methodology of Mara and Oragui (1983), as overlap was observed for the different sources of faecal contamination (Table 5.1). At the same time, the total bifidobacterial concentrations were below 0 CFUs/100 mL or were overgrown by faecal streptococci in

33% of the samples. Both of these observations prevent the calibration of the threshold value of the tracking ratio for the water resources sampled. The main problem seems to be the culturability of the total bifidobacteria on the YN-17 medium. Thus a literature search and laboratory testing for an alternative was therefore conducted. The BM (Beerens, 1990) was chosen as the replacement for the YN-17 after the pH was optimised at 5.70 and this is referred to as modified Bareens medium (MBM).

Table 5.1. Concentrations of bifidobacteria using the method of Mara and Oragui (1983), tracking ratio (TR) and faecal source identification

Site	Sampling date	Rainfall	Sorbitol-utilising bifidobacteria (CFUs/100 ml)	Total bifidobacteria (CFUs/100 ml)	Faecal streptococci (CFUs/100 ml)	TR	Faecal contamination source (Table 4.5)
9	12/02/2009	Sampling during a ten-day drought	510	Overgrown by FS	> 300	ND[a]	Human
9	15/02/2009	Sampling during a storm	44	73	0	0.60	Human
10	15/02/2009	Sampling during a storm	18	4	0	4.50	Human
11	15/12/2008	Sampling during a seven-day drought	1000	Overgrown by FS	5500	ND[a]	Mixed
11	12/02/2009	Sampling during a ten-day drought	10	100	0	0.10	Mixed
12	20/12/2008	Sampling 2-3 days after rain	50000	8000	90000	6.25	Mixed
13	13/03/2009	Sampling 2-3 days after rain	159	50	0	3.18	Wildlife
14	13/03/2009	Sampling 2-3 days after rain	49	14	0	3.50	Wildlife
15	13/03/2009	Sampling 2-3 days after rain	64	0	0	ND[a]	Wildlife

[a] Not determined

The suitability of MBM for the enumeration of total bifidobacteria was tested by enumerating two concentrations of sorbitol-utilising bifidobacteria on the HSBA and the MBM at two concentration levels. Experimental data and the results of the statistical testing are shown in Table 5.2. The concentrations of the sorbitol-utilising bifidobacteria were not statistically different at the five-percent level of significance. Plating onto m-Enterococcus agar did not show any growth, i.e. faecal streptococci did not grow on the HSBA or the MBM. Thus the MBM was considered a suitable replacement for the YN-17 medium in the enumeration of the total bifidobacteria and all the selected sampling sites were re-sampled and the tracking ratios re-calculated. The results are summarised in Table 5.3.

Table 5.2. Comparison of human-bifid sorbitol agar (HSBA) and modified Bareens medium (MBM).

	HSBA (CFUs/100ml)	MBM (CFUs/100 ml)	*p*-value
Concentration level 1	$(4.88 \pm 0.38) \times 10^2$	$(4.78 \pm 0.23) \times 10^2$	0.1240
Concentration level 2	$(3.67 \pm 2.88) \times 10^6$	$(1.38 \pm 0.86) \times 10^6$	0.1772

Table 5.3. Concentrations of bifidobacteria using both HSBA and MBM, calculated tracking ratios and faecal source identification.

Site	Sampling date	Sorbitol-utilising bifidobacteria[a] (CFUs/100 ml)	Total bifidobacteria[b] (CFUs/100 ml)	Tracking ratio	Source of faecal contamination (Table 4.5)
9	27/03/2010	55	36	1.53	Human
10	27/03/2010	0	7	0	Human
11	27/03/2010	23000	8000	2.88	Mixed
12	27/03/2010	0	2500	0	Mixed
13	31/03/2010	123	81	1.52	Wildlife
14	31/03/2010	24	39	0.61	Wildlife
15	31/03/2010	95	0	ND[c]	Wildlife

[a] Using human-bifid sorbitol agar
[b] Using modified Bareens medium
[c] Not determined

The sorbitol-utilising bifidobacteria values ranged from 0 CFUs/100 mL at site 10 and at site 12 to 2.30×10^4 CFUs/100 mL at site 11, while the total bifidobacteria values ranged from 0 CFUs/100 mL at site 15 to 8.00×10^3 CFUs/100 mL at site 11. No tracking ratio could be determined at site 15. Tracking ratios at the other selected sampling sites ranged from 0 at site 10 to 2.88 at site 11.

5.1.2 Conclusions

The substitution of the MBM for the YN-17 did not lead to a significant improvement in the calibration of the tracking ratio and so the use of bifidobacteria in faecal source tracking may strongly depend on the survival rates.

5.2 BIFIDOBACTERIA SURVIVAL

5.2.1 Methods

All sampling sites were re-sampled and the chemical parameters and bacterial enumeration were repeated using the same methodologies as above. This round of bifidobacterial sampling took place at all seven sampling sites from September 2010 to January 2011.

The model water was made freshly before the experiment began and autoclaved for 10 minutes. The model water was decanted into test tubes (20 mL) and the water was sampled at the following times: 0, 1, 6, 12, 32, 72, 152, 336 hours. These follow an exponential curve to help identify changes in log values. At the given times, the samples were membrane filtered (10 mL) and spread plated (100 µL) and the bacterial concentration enumerated. Membrane filtration is used experimentally in identifying the *bifidobacterial* concentration of river water so it was used here. Spread plating has been reported to decrease the concentration of enumerated bacteria, as it could cause damage to bacteria if they come into direct contact with the selective media (Rhodes and Kator, 1999). The *bifidobacteria* were incubated under anaerobic conditions at 37℃ for 48 hours on HBSA and the MBM. Subsequently the concentrations were counted. The river water quality tended to change drastically over these 4 months especially due to heavy rainfall. The results from the chemical analysis were analysed using Primer 6 + PERMANOVA add on. The statistical test is DISTLM and was done using Bray Curtis and AIC to be least reliant on linearity of data.

The environmental water samples were collected in sterile 5 litre polyethylene bottles and then filtered through 0.7 µm glass fibre filters (Whatman, GF/F) immediately on arrival at the laboratory. The filters were placed into glass tubes and frozen at -20℃ until analysis. All glassware was HCl acid washed and then rinsed with methanol, acetone and hexane before use. The method by Isobe et al., (2002) was followed as stated below. The filters were placed into a clean 40 mL Pyrex centrifuge tube. The sterols were then ultrasonically extracted using a sonicator for 1-5 minutes per solvent mixture with 30 mL each of methanol, methanol/dichloromethane (DCM) (1:1, v/v) and then DCM consecutively. After sonication the filters were centrifuged (rotoUni) at 2000 rpm for 5 minutes and the solvent was transferred to 100 mL round bottom flask. The solvent was evaporated on a rotovamp (Buchi Rotovapor R215) at 30℃ and 100 rpm. The different solvent mixtures were combined into the same round bottom flask. The resultant extract was re-dissolved in 1mL hexane/DCM (3:1, v/v) and pipetted onto the top of a silica gel column. The silica gel column was prepared in a muffle furnace by baking at 380°C for 4 h to remove organic contamination, activated at 200°C for 5 h and stored in hexane until use. The first of three fractions was separately collected and the second two which should contain the sterols were combined. The solution was evaporated to approximately 0.5 mL then transferred to a vial and dried under a nitrogen stream. The three solvent mixtures in order were 20 mL of hexane/DCM (3:1, v/v), 40 mL of DCM and 30 mL of acetone/DCM (3:7, v/v).

The sterols were derivatised using a solution of pyridine/acetic acid (1:1, 50 uL) and allowed to run to completion overnight after vigorous shaking to ensure mixing. The mixture then had 0.2 mL of 4 M HCl added with 0.5 mL hexane and was shaken by hand for 30 seconds before centrifuging for 1 min at 200 rpm. The upper hexane layer was pipetted into an anhydrous sodium sulphate column. The mixture was then dried under nitrogen before adding 1 mL hexane and freezing at -20℃ until GC analysis. The conditions were set according to (Isobe et al., 2002). Sterols were analysed on an Agilent GC system 7890A. A silica capillary column was used with helium as the carrier gas at 100 kPa. The injection port was maintained at 300°C in the splitless mode followed by a 1-min purge after the injection. The auto sampler injected the sample. The column temperature was held at 70°C for 1 min, then set to increase by 20°C/min to 250°C and then by 4°C/min to 310°C, and held for 20 min.

5.2.2 Results and discussion

Table 5.4 and Table 5.5 show the measured chemical parameters and bacterial enumeration for the samples taken from September 2010 to January 2011.

Table 5.4. Chemical concentrations used to establish the key parameters to which bifidobacteria survival is sensitive.

			Cl	NH_3	PO_4	NO_3	SO_4	EC	Hardness	T
Site	Date	pH	(mg/L)						(dkh)	(°C)
9	2010/09/29	8	197.75	17.93	12.14	0	63.8	0.9	30	18.9
9	2010/11/03	7.37	64.88	0	0	0.02	41.34	0.386	80	19
9	2010/11/25	7.32	8.66	0.4	4.28	45.85	0.56	0.36	57	19
9	2011/01/26	8.06	172.69	0.2	1.09	1.6	18.74	0.34	57.28	32.3
10	2010/09/29	8.99	151.5	20.21	7.36	0	14.38	0.99	10	18
10	2010/11/03	7.47	24.5	0.04	0	0.02	43.57	0.28	80	19
10	2010/11/25	7.06	2.03	0.1	2.93	47.3	26.29	0.31	11	18.8
10	2011/01/26	9.27	37.56	0.02	0.18	0	2.96	0.2	10.74	26.5
11	2010/09/29	7.86	235.13	1.51	0	4.87	90.01	0.09	240	13.5
11	2010/11/03	7.39	73.19	4.31	0.73	0.02	4.98	0.282	0	19
11	2010/11/25	7.89	6	0.7	6.12	0.54	34.57	1.38	206	18.3
11	2011/01/26	8.27	161.85	12.17	4.15	41.5	64.96	1.26	239.86	28.6
12	2010/09/29	7.72	228.5	0.2	0.54	0	82.55	2.38	240	17.3
12	2010/11/03	7.24	63.19	5.67	1.67	0.01	30.84	0.668	0	19
12	2010/11/25	7.91	7.34	1.01	7.12	3.3	28.81	1.26	206	18.2
12	2011/01/26	8.04	283.19	12.11	7.85	45.82	59.35	1.17	239.86	27.2
13	2010/10/01	8.59	219.38	0.17	4.65	0	55.86	1539	160	21
13	2010/11/08	8.01	193.19	0.22	7.4	3.87	47.6	1.46	240	19
13	2010/12/01	8.4	225.06	0.31	6.34	0	44.64	0.99	403.52	21
13	2011/01/26	8.78	57.4	0.08	6.21	0	21.75	1.06	332.94	22.2
14	2010/10/01	9.66	233.81	0.2	1.76	0	37.81	1455	160	20.4
14	2010/11/08	9.28	276.44	0	2.73	0	48.63	1.49	240	22
14	2010/12/01	8.39	252.25	0.15	2.39	0	25.37	1.01	253.2	20.8
14	2011/01/26	8.57	202.81	0	2.45	0	16.84	1.08	343.68	21
15	2010/10/01	9.14	208	0.19	4.95	0	27.55	1262	160	26
15	2010/11/08	8.33	227.25	0.22	7.62	2.11	47.6	1.27	240	20
15	2010/12/01	9.83	178.75	0.19	5.86	0	34.2	1.81	357.12	26
15	2011/01/26	9.85	179.52	0.11	4.32	0	28.84	1.66	400.96	25.6
16	2010/11/08	8.25	302.69	0.24	7.58	2.3	43.13	1.4	20	20

Table 5.5. Bacterial concentrations and H_2S strip test results used to establish the key parameters to which bifidobacteria survival is sensitive.

	SUB	TB	E. coli	FC	H_2S
Site			CFUs/100 mL		(Hours)
9	38	2	0	22	40
9	20	8	4	34	24
9	43	21	2	30	24
9	31	25	0	50	22
10	15.5	0	0	30	19
10	47	44	0	4	24
10	0	2	0	0	24
10	11	14	2	8	24
11	0	62	0	1600	18
11	15900	3600	2	1600	12
11	9517.5	4	80	1600	12
11	4000	3200	26	350	12
12	0	0	0	1600	18
12	12200	1700	8	1600	12
12	26512	17	4	1600	12
12	8	19	14	900	12
13	50.5	23	0	0	24
13	50	94	4	170	12
13	14	49.5	0	30	18
13	0	0	17	900	12
14	14	14	0	80	48
14	42	9	8	79	12
14	14	47.5	0	50	18
14	76	64	6	240	12
15	25.5	14	0	0	48
15	38	80	2	79	12
15	0	14	0	30	18
15	13	0	4	23	12
16	142	150	4	220	12

The results in the above tables were analysed using Primer 6+ Permanova DistLM (Plymouth Routines in Multivariate Ecological Research) obtained from PRIMER-E Ltd, Plymouth, UK, by Clarke KR, Gorley RN and Anderson MJ (2008). PRIMER was used to do distance-based linear models or PERMANOVA permutations. This allowed the most correlated parameters to be tested, i.e. to establish the chemical parameters that affected the concentration of bifidobacteria the most.

The analysis was also run with Bray Curtis AIC as this decreased the problem of possible non-linearity of data. The results are shown Figure 5.1. It shows the Euclidean distance information which is used to generate the survival experiments. The survival experiments were designed to identify which environmental factors which were affecting the survival of the bacteria. However, this does not take into account all the different bacterial populations found in river water or the changes in dissolved oxygen from stream flow which may have a significant effect.

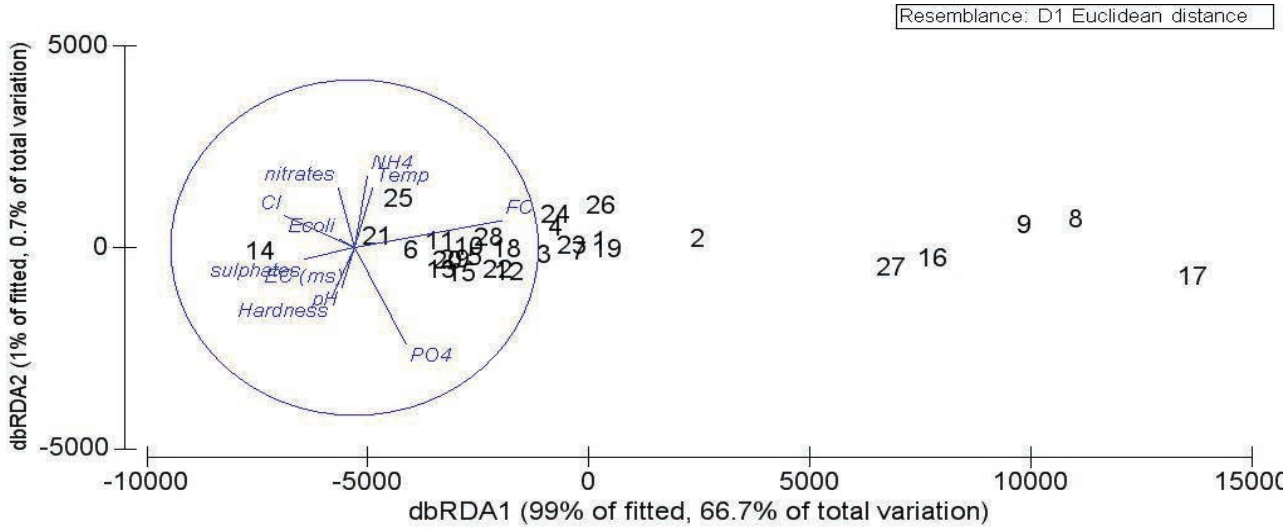


Figure 5.1. The correlation between river water parameters and the concentration of bifidobacteria.

The DISTLM analysis had chemical ranges as follows: pH 7.0-9.8; sulphate 0.5-90 mg/L; temperature 14-32°C; chloride 2-302 mg/L; hardness 0-403dKh; phosphate 0-12 mg/L; conductivity 0-1539 ms; ammonia 0-20 mg/L, *bifidobacteria* from HSBA 0-26512 CFU/100ml, *bifidobacteria* from Beerens media 0-3600 CFU/100ml, nitrates 0-47 mg/L, *E. coli* 0-80 CFU/100ml, faecal coliforms 0-1600 CFU/100ml. This data was used to identify possible key parameters for the survival tests.

The parameters were identified in descending order as follows: pH; sulphate; temperature; chloride; hardness; phosphate; conductivity; ammonia. The parameters used for the survival experiments were as follows, with ranges used in brackets: pH (5.3-9.8); temperature (8-32°C); chloride (2-302 mg/L); protein (0-140 mg/L); nitrate (0-6.89 mg/L). The actual values are shown in Table 5.6. The bifidobacterial survival data for these chemical parameters are shown in Table 5.7.

Table 5.6. Values of chemical parameters used in bifidobacterial survival experiments.

Design number	pH	T (ºC)	NO_3^- (mg/L)	Cl^- (mg/L)	Proteins (mg/L)
5	9.80	8	6.89	302	0
6	5.3	8	6.89	302	0
7	9.80	32	0	0	140
8	5.30	8	0	0	0
9	5.30	8	6.89	0	0
10	5.30	32	6.89	302	0
11	9.80	32	6.89	302	0

Table 5.7. The average sorbitol-utilising bifidobacteria (SUB) and total concentration (CFU/100 mL) as a function of time during survival experiments (no buffering).

	Hours												
Design	**0**		**1**		**3**		**24**		**72**		**152**		**Final**
Number	**SUB**	**total**	**SUB**	**total**	**SUB**	**total**	**SUB**	**total**	**SUB**	**total**	**SUB**	**total**	**pH**
5	2760	1288	1110	700	1130	433	1100	1000	1050	0	overgrown	0	3.01
6	2355	1320	500	700	300	680	295	1085	250	0	15	0	3.56
7	1840	2118	1165	2100	1150	2000	255	1493	180	0	0	0	6.4
8	3630	2000	2630	693	1150	1567	405	775	750	210	0	0	2.93
9	1490	2280	975	1830	500	1430	120	1250	50	0	0	0	3.33
10	1475	2285	1350	2035	665	1745	375	1380	0	0	0	0	4.59
11	1480	1500	1415	1445	880	1000	670	770	0	0	0	0	6.73

Most bifidobacteria died off after 72-152 hours and once again the concentrations of sorbitol-utilising bifidobacteria were higher than the total bifidobacterial concentrations. Of concern was that the pH of the medium dropped to very acidic values in many cases, e.g. 3.01 in experiment 5. Bifidobacteria are not able to survive below pH 5.0, as was established during the optimisation of the MBM. Thus buffering with $CaCO_3$ was attempted as a remedy. The effect of different concentrations of calcium carbonate is shown in Table 5.8.

Table 5.8. The buffering effect of different calcium carbonate concentrations while bacteria still viable (starting pH = 7.20).

Concentration of Calcium Carbonate	Days						Final change in
(g/L)	0	1	2	4	7	11	pH
0	7	6.4	6.3	6.3	6.4	6.2	0.63
0.1	7.15	6.69	7.07	7.09	6.81	7.27	0.46
0.2	8.18	7.63	7.45	7.64	7.54	7.7	0.55
1	6.56	7.2	7.37	7.86	7.98	8.08	-0.64
2	7.87	8.02	7.44	7.91	8.08	8.2	-0.15

As can be seen, additions between 0.1 and 0.2 g/L were sufficient to maintain the pH near the original values. To double-check the portion of the presumptive bifidobacterial colonies which would grow on the m-Enterococcus agar, a selected number of colonies were re-streaked onto from HSBA and the MBM onto the m-Enterococcus agar. The proportion of the colonies that grew as faecal streptococci was equal to 81 ± 31 % and this was statistically not significantly different from 100%. Based on the results referred to in the literature review, this contradicts the laboratory testing of the MBM viability where no faecal streptococci were detected (see section 5.1.1 for details). The faecal streptococci are able to survive under highly acidic pH values which were observed during the survival experiments (Ivan et al., 1999).

5.2.3 Conclusions

The results of the bifidobacterial experiments point to major issues with routine application of the TR in faecal source tracking in South Africa. Therefore further studies should be conducted on the genetic identity of the presumptive bifidobacterial isolates from both HSBA and MBM. This is because the bifidobacteria might have been cultured together with faecal streptococci as mixed cultures or they might have been absent. The sensitivity of bifidobacteria to acidic pHs is higher than that of faecal streptococci. It is therefore possible that the faecal streptococci overgrew the bifidobacterial cells on HSBA and MBM.

Further survival experiments are currently under way in buffered systems. It will also be necessary to identify the conditions and chemical composition of surface water under which bifidobacteria is likely to survive *in-situ*. Consideration could be given to testing the findings of this study in other geographical regions in South Africa.

5.3 CHEMICAL BIOMARKERS

5.3.1 Results

The sterol method of Isobe et al. (2002) and the sampling took place between August and October 2011. No coprostanol or stigmastanol was detected at sites 9, 10, 13, 14 and 15. At sites 11 and 12, no coprostanol was detected although the concentrations of stigmastanol were 2.9 and 16.7 µg/mL respectively.

5.3.2 Conclusions

Calibration of the Leeming method of faecal source tracking will apparently not be feasible under South African conditions.

CHAPTER 6: CONCLUSIONS & RECOMMENDATIONS

6.1 CONCLUSIONS

Results of the project indicate that bifidobacterial and sterol analysis according to Leeming et al. (1996) are not feasible under South African conditions and other techniques will need to be investigated for this purpose. On the positive side, survival rates for faecal coliforms were measured for the first time in Eastern Cape and in more detail than is available in South African literature. Time periods for the discrepancies between the H_2S strip test and the faecal coliform enumeration were investigated and it was found that this takes place after 22 to 29 hours after the faecal contamination event.

6.2 RECOMMENDATIONS

Further investigations should focus on the genetic diversity of bifidobacteria and faecal streptococci in South African water resources. This study's results should be verified in a larger geographical area. For the broadening of the scope of NMMP, the antibiotic resistance spectra of the *E. coli* isolates from different faecal contamination sources could be considered. Databases could be created for this purpose and implemented into NMMP.

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