HEALTH RISK ASSESSMENT IN CONNECTION WITH THE USE OF MICROBIOLOGICALLY CONTAMINATED SOURCE WATERS FOR IRRIGATION

Report to the

WATER RESEARCH COMMISSION

by

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EXECUTIVE SUMMARY

1. Part A: Presence, antibiotic susceptibility patterns and chlorination resistance of faecal pollution from a dense settlement and the possible impact of polluted water on downstream water users

1.1 Background and motivation

The presence of dense settlements on the river banks in the Western Cape give rise to water pollution of nearby rivers and severely affect the water quality downstream. Most of the water pollution can be attributed to inadequate sanitation in these settlements, severe overcrowding, as well as failing sewerage systems. The resultant burden of disease, loss of productivity and health costs should make the reduction of pollution and improvement of sanitation in Kayamandi a priority for the local authorities. The study site chosen was the Plankenbrug River running through Stellenbosch and the dense settlement of Kayamandi on its banks.

1.2 Objectives

The objectives of this section of the investigation were:

i) Description of the exposed population in the dense settlement of Kayamandi;
ii) Quantification of faecal pollution in the Plankenbrug River using *Escherichia coli* as an indicator organism;
iii) Identification of the most likely pathogens involved in the Plankenbrug River by additional analyses of water samples;
iv) Preliminary tests of antibiotic resistance of organisms in the river water; and
v) Preliminary tests on chlorination resistance of organisms on the river water

1.3 Summary of Results

1.3.1 Exposed population

There are many more persons living in the settlement than previously acknowledged. At least 25 000 persons live in the area, 80% of them in shack housing. Throughout the year there is a high prevalence of diarrhoea in the area.

1.3.2 Bacteriological analysis of water samples and biofilms

The faecal pollution in the river over the past four years reached a high count of 12 million *E. coli* per 100 ml water on one occasion, but frequently reached the million mark. It was above the recommended limit of 2000 *E. coli* per 100 ml water for 97% of the sampling occasions over the four years. Many pathogens carrying considerable health risks were identified in the water. Some, like β haemolytic streptococcus Group A, were an unusual find in free-flowing water. A number of the organisms in

the water and in the biofilms on stones in the river, exhibited signs of antibiotic resistance to some commonly used antibiotics and also resistance to chlorination. A further complication was that some of the organisms surviving the chlorination experiments showed enhanced antibiotic resistance.

1.3.3 Virological analysis of water samples and associated biofilms

Three sets, i.e. before and after the source of pollution, of water samples (2 litres) from the Plankenbrug River were referred for virological analysis between April and June 2002. In addition biofilms from recovered stones within the river were included with one of the sample sets. Viruses were recovered from the water samples, inoculated onto a variety of cell cultures, and then the concentrated sample and associated cell cultures analysed for enteroviruses, hepatitis A virus (HAV), human astrovirus (HAstV), human caliciviruses (HuCV) and human rotaviruses (HRV) by reverse transcriptase-polymerase reaction (RT-PCR).

Enteroviruses, namely polioviruses and an untypable enterovirus, were isolated from and HAstVs detected in water samples drawn from the Plankenbrug River below Kayamandi in April 2002. Enteroviruses were also detected in water drawn from a site further downstream on the same date. Water samples drawn from the Plankenbrug River below Kayamandi in May 2002 yielded adenoviruses and enteroviruses were detected by RT-PCR. HRVs, adenoviruses, enteroviruses and HAstVs were detected in samples collected in June 2002. Although the virological analysis was qualitative these data suggest that the Plankenbrug River below Kayamandi is heavily polluted with human faecal material.

1.4 Conclusions

The risks to health, environmental damage and the problems foreseen with economic activities downstream from the Plankenbrug River make it imperative that attention be given to the sanitation situation in Kayamandi, the state of the sanitation system in the whole of the town of Stellenbosch and assistance rendered to the local authority to start remedial action without delay.

1.5 Recommendations and Future Research

Several aspects of the present study should be investigated on a larger scale:

i) There is an urgent need for research into the feasibility and practical applications of monitoring water quality in natural watercourses by means of microbiological determinations.

ii) The antibiotic resistance of organisms in natural water sources and the possible impact of such resistance on health in the area need to be assessed;

iii) Additional research regarding chlorination resistance of organisms reaching natural waters, either in a treated or untreated state is required, especially the link between chlorination resistance and antibiotic resistance ought to receive urgent attention.

iv) Research into the links between community behaviour, availability of sanitation services and receptivity to educational programmes should be expanded, especially for the local populations of impoverished people in dense settlements.

2. Part B: Microbiological analysis of irrigation waters and food crops

2.1 Background and Motivation

Despite major advances in preventative health foodborne illnesses remain a widespread and growing public health problem in the developed and developing world (Satcher, 2000), and the burden of infection is grossly underestimated (Marx, 1997; Koopmans *et al*., 2002). Many factors, including changing lifestyles and demographics, faster and more frequent travel, decreasing water supplies in certain countries and enhance importation of foods have contributed to the increase in food- and waterborne infections (Cuthbert, 2001; Koopmans *et al*., 2002). The threat of foodborne viral diseases has triggered worldwide interest in the fate of viruses on fresh produce irrigated with sludge or sewage effluents (Petterson and Ashbolt, 2001). Minimally processed foods (MPFs) such as salads, vegetables, fruits and other fresh produce that require only minimal processing before consumption are usually contaminated through human contact during harvesting or processing but contamination via wastewater and sludge, used for crop irrigation and fertilization, has also been documented (Petterson and Ashbolt, 2001). The use of wastewater for irrigation purposes has been responsible for many disease outbreaks caused by bacteria, protozoa, parasitic helminths and viruses (Bitton, 1980). The World Health Organization (WHO) estimates that 70% of diarrhoeal episodes are caused by biologically contaminated food (Satcher, 2000).

A large number of viruses are found in the human intestinal tract, with three disease categories being associated with food- and waterborne viruses, namely:

i) Gastroenteritis: caused by HRV, HuCV which include the “Norwalk-like viruses” (NLVs) or noroviruses, and the “Sapporo-like viruses” (SLVs) or sapoviruses, HAstV and the enteric adenoviruses;

ii) Hepatitis: caused by the faecally transmitted hepatitis viruses, namely HAV and hepatitis E virus (HEV); and

iii) Other severe illnesses such as myocarditis: caused by enteroviruses which include polioviruses, coxsackie A and B viruses, echoviruses and enteroviruses 68-71 (Koopmans *et al*., 2002).

HAV is a major cause of morbidity associated with faecally contaminated food and water (Mead *et al*., 1999). HuCVs (Parashar and Monroe, 2001; Koopmans *et al*., 2002) and HAstVs (Glass et al., 1996; Ferrari and Torres, 1998; Walter and Mitchell, 2000) are increasingly being identified as important foodborne viruses. Viruses are strict intracellular parasites and cannot replicate in food and water. Viral infection via contaminated food therefore depends on viral stability, amounts of virus shed by an infected person, method of processing of the food or water, infective dose and susceptibility of the host (Koopmans *et al.,* 2002). Most of the food- and waterborne viruses are non-enveloped and are resistant to heat, disinfection and pH changes. Many of these viruses cannot be isolated or detected by conventional routine laboratory techniques and molecular methods for the detection of HAV, HuCV and HAstV have, until recently, had limited applicability in food and environmental virology (Richards, 1999).

There is very little data on the presence of HAV, HuCV and HAstV in food sources and domestic, agricultural and recreational water sources in SA. This is partly due to the lack of infrastructure for the detection and recording of such infections (Grabow, 1996). There is no reason to believe that risks of food- and waterborne disease in SA are any different from those in the rest of the world. Escalating demands and pollution of already limited water sources, particularly in rural and developing communities, may even elevate risks (DAWN, 2001). Common-source viral foodborne outbreaks, such as SRSV-associated gastroenteritis, have been described in SA (Taylor *et al.,* 1993), but to date, no food- and waterborne outbreaks of HA or HAstV have been documented due to the relative underreporting of these diseases. HAV and HAstV have been detected in raw and treated water sources (Marx *et al.*, 1998; Grabow *et al.*, 2001; Taylor *et al*., 2001) confirming the risk of waterborne transmission of these viruses. In view of these facts better approaches to prevent the contamination of foods with potentially pathogenic viruses via irrigation or processing water, food handlers and sewage are needed. In addition, more effective techniques for the recovery and detection of these viruses in food and water will be beneficial (Keddy, 1998).

2.2 Objectives

The objectives of this investigation were:

i) To establish recovery and molecular detection and characterisation techniques for HuCV, HAstV, and HAV in raw and partially treated irrigation waters and selected food crops.

ii) To monitor selected irrigation waters and associated fruit and vegetable crops for the following pathogens which can be waterborne and associated with foodborne disease, namely HuCV, HAstV and HAV, as well as selected indicator organisms.

2.3 Summary of Results

2.3.1 *Optimisation of methods for the recovery of viruses and bacteria from food and water sources*

Irrigation water: A glass wool adsorption-elution method described by Grabow and Taylor (1993) was optimised and used for the recovery of viruses from water samples >2L. For sample volumes <2L, and for the eluate from the glass wool adsorption-elution method, viruses were recovered in a final volume of 6 ml phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA) by precipitation using the NaCl/polyethylene-glycol method described by Minor (1985).

Heavily polluted water: Three different methods, namely glass wool adsorption-elution, NaCl/PEG precipitation and a SiO_2 method described by Baggi and Peduzzi (2000) for the concentration of rotaviruses from surface water and raw sewage were compared for the recovery of viruses from heavily polluted water. Of these three methods, the NaCl /PEG precipitation was shown to be the most cost-effective and efficient for the recovery of viruses from the heavily polluted water samples.

Minimally processed foods: For the purposes of this investigation the methods described by Bidawid *et al*. (2000) for the recovery of HAV from lettuce and strawberries, and Schwab *et al*. (2000) for the recovery of NLVs and HAV from delicatessen foods were adapted and optimised for the recovery of viruses and bacteria from MPFs. Three buffer systems, namely PBS pH 7.4, Tris-EDTA pH 8.0 and minimal essential medium (MEM) pH 7.2 were compared for the recovery of viruses from MPFs with rough (e.g. strawberries) and smooth (grapes, cherry tomatoes) surfaces. PBS was found to be the most efficient recovery buffer. For field analyses, ± 100 gm MPF was washed overnight at 4ºC in 50 ml PBS. Bacteriological analysis was done directly on the PBS washing while the viruses were recovered from the PBS washing in a final volume of 6 ml PBS containing 1% BSA by NaCl/ polyethylene-glycol precipitation.

2.3.2 *Molecular detection of viruses*

RNA extraction: Two extraction procedures, namely the QIAGEN QIAamp viral RNA extraction kit and TRIZOL® reagent, were compared to ascertain which method was the most suitable for the extraction of viral RNA from concentrates from fruit washings, water and sewage samples and cell culture extracts. The procedure using the TRIZOL® reagent proved to be the most suitable for the type of samples used. Subsequently a newer more effective RNA extraction kit, the QIAGEN QIAamp UltraSens Virus Kit, which uses larger volumes of sample, was used.

***Hepatitis A virus**:* The RT-PCR oligonucleotide probe assay (Taylor *et al*., 2001) was used for the detection of HAV.

Human astroviruses: The RT-PCR oligonucleotide probe assay as described by Marx *et al*. (1998) and modified by Taylor *et al*. (2001) was used for the detection of HAstVs.

***Human caliciviruses**:* A sensitive and specific RT-PCR was developed and optimised for the detection of HuCVs using the primer pair described by Jiang *et al*. (1999) for the detection of both noro- and sapoviruses.

***Multiplex RT-PCR for the simultaneous detection of HAV and HuCVs**:* A multiplex RT-PCR, described by Schwab *et al*. (2000), was assessed for the detection of HAV and HuCVs. This multiplex RT-PCR was found to be less sensitive than the single RT-PCRs, i.e. using the multiplex RT-PCR no HuCVs could be detected and HAVs were only detected up to a dilution of 1:8 compared to detection at dilutions of 1:2 and 1:32 respectively for the individual RT-PCRs.

2.3.3 *Molecular characterisation of viruses*

A dideoxynucleotide chain termination sequencing method was established for the direct sequencing of PCR amplicons. For the characterisation of the HAV, HuCV and HAstV sequences of field isolates were then compared to sequences from well-described reference strains using the CLUSTAL X program. HRVs and human adenoviruses were typed by specific RT-PCRs and restriction enzyme analysis respectively.

2.3.4 *Analysis of irrigation water and minimally processed food samples.*

***Commercially available MPFs**:* Six samples of packaged strawberries, from two different sources, and two of cherry tomatoes were analysed for HAV, HAstV and HuCVs. No viruses were detected on the surfaces of any of these MPFs.

***Irrigation water and associated MPFs*:** Samples of MPFs and associated irrigation water were obtained from two rural areas, namely Letaba and Venda. The MPF samples from Venda were washed on site, where possible, in 25 ml PBS/50 g sample. The MPF-PBS was analysed at the University of Venda for indicator organisms and pathogenic bacteria and the remaining MPF-PBS wash was forwarded to the University of Pretoria for virological analysis. Two litres samples of irrigation water, i.e. either borehole or river, were forwarded to the University of Pretoria for virological analysis while at the University of Venda bacteriological analysis was performed on simultaneously water drawn samples.

Letaba: A single river sample and associated MPF (cabbage) was analysed for HAV and HAstV, but no viruses were detected.

Venda: A total of nine sets of samples, i.e. irrigation and one or more associated MPFs, were received from Venda, five in 2001 and four in 2002. Analysis for bacteria, indicator organisms and selected pathogens was performed on the samples. Viruses were recovered from both irrigation water and associated MPFs, inoculated onto a variety of cell cultures and then the sample and associated cell cultures analysed for HAV, HAstV, HuCV and HRV by RT-PCR.

Faecal coliform counts of >1000 per 100 ml were evident in a number of the irrigation water samples. This water should therefore not be used for any produce in contact with humans (Department of Water Affairs and Forestry, 1996). In addition pathogenic bacteria such as *Salmonella, Shigella* and *E. coli* were detected in a number of irrigation water and MPF samples. HRVs were detected in one irrigation water sample and from one MPF, namely tomatoes.

2.4 Conclusions

The objectives of the project have been met and the following conclusions drawn:

i) Techniques for the recovery, detection and characterisation of potentially pathogenic food- and waterborne viruses in raw and partially treated irrigation water and associated MPFs have been established.

ii) Although a multiplex RT-PCR may be an attractive cost-effective alternate to individual RT-PCRs for the detection of selective viruses, the multiplex RT-PCR was found not be as sensitive as individual RT-PCRs for the viruses assessed.

iii) No HAVs, HAstVs or HuCVs were detected in any of the irrigation water samples or associated MPFs from Venda. However, HRVs were detected in one of the irrigation water samples, namely Tshikuwi River sample, drawn on 2002/07/09. This finding is highly significant as the peak incidence of HRV infection is in the winter months. The occurrence of HRV in this water sample correlates with the high thermotolerant or faecal coliform count indicating faecal pollution of the water. However no HRVs were detected on any of the associated MPFs for that particular sampling date. HRVs were however detected on a MPF (tomato) on a previous sampling date where no HRVs were detected in the associated irrigation water. Characterisation data on the HRV isolates are still outstanding

iv) The isolation and detection of enteroviruses, human adenoviruses, HRVs and HAstVs from a river used for domestic purposes and as irrigation water suggests that this water could pose a potential health risk, but more data are required to quantify the risk.

2.5 Future research

Future research should focus on the following:

i) The use of larger volumes of irrigation water for the recovery and detection of potentially pathogenic viruses
ii) Analysis of irrigation water and associated MPFs for pathogenic protozoan parasites
iii) The development of quantitative real-time RT-PCRs to quantify the viruses present in field samples
iv) Determine whether there are seasonal fluctuations in the type of micro-organisms present in irrigation water and associated MPFs.

3. Collaboration

Part A: Invaluable collaboration was built up in this project with the following organisations:

i) Division of Agrimeteorology of the Agricultural Research Council for the present and future analyses of pollution patterns in relation to weather data.
ii) Department of Microbiology, University of Stellenbosch for the exchange of knowledge and experience in the field of environmental pollution.
iii) Department of Water Affairs and Forestry, Western Cape for the sharing of samples and sample data as well as co-operation in many spheres of knowledge of water sources in the province.
iv) Prof EP Jacobs, Chief Researcher, Institute for Polymer Science, University of Stellenbosch for discussions and collaboration on membrane technology used to remove pathogens from water.
v) The Kayamandi Steering Committee and the greater community of Kayamandi itself with whom valuable bonds have been established and from most of whom good co-operation had been received.
vi) The Wildlife and Environment Society of South Africa, and especially Mr Stephen Finnemore, who shared previous data and correspondence on the river.

Part B: Prof JE Walter and Prof DK Mitchell, Center for Pediatric Research, Eastern Virginia Medical School, Norfolk, Virginia gave advice with regard to the molecular detection and characterisation of HAstVs and supplied valuable references viruses

4 Technology transfer

Part A: Technology used in the investigations of river water has been shared with other laboratories as well as the Department of Water Affairs and Forestry and the Stellenbosch Municipality

Part B: Technology developed during the course of this project has been transferred to personnel from other institutions, namely University of Venda, Medical University of Southern Africa, Noguchi Memorial Institute for Medical Research, University of Ghana (Dr G Armah), and University of Botswana (Ms M Kasule).

CAPACITY BUILDING

Part A:

i) Ms Wesaal Khan, a PhD student of the Department of Microbiology, Faculty of Science, University of Stellenbosch used some of the organisms isolated during the course of this study for further investigation

ii) Several community workshops involving various segments of the Kayamandi community, DWAF, the Stellenbosch Municipality and other stakeholders.

Part B:

The work was carried out by post-graduate students and the project therefore contributed to the training of manpower in advanced technology for the microbial analysis of water and minimally processed foods.

i) This project formed the basis for the practical training of Ms T Naus for the degree BSc (Hons) Medical Virology;

ii) University of Pretoria students Ms S Nadan and Ms JME Venter working on HAstVs and HAV respectively participated in the project as part of their investigations for their MSc degrees;

iii) Student assistants from the University of Venda gained experience in the bacteriological analysis of irrigation waters and associated minimally processed foods. Neither student chose to continue with further post-graduate studies.

iv) Two PhD students from the Medical University of Southern Africa gained experience with regard to the typing of environmental viral isolates;

v) Post-doctoral fellow, WB van Zyl, gained experience with the virological analysis of irrigation waters and associated minimally processed foods

RESEARCH OUTPUTS

Reports

Barnes JM, Taylor MB, Slabbert M, Huisamen M and Post-graduate students. Part A: Presence, antibiotic susceptibility patterns and chlorination resistance of faecal pollution from a dense settlement and the possible impact of polluted water on downstream water users. Water Research Commission

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PhD (Community Health)

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Barnes JM. SA FM: Interview on paper presented to Infectious Diseases Conference. 2001 December 7;18h15 (Repeated Sunday 2001 December 9).

Barnes JM. SA FM: Interview with Sue Valentine 2002 June 19. Broadcast in three parts on SA FM (dates not known)

Barnes JM. Matie FM Radio: Interview about the pollution in the Plankenbrug River. 2002 September 12; 18h30.

Conference presentations
International : None

Conference presentations
National/Regional

Barnes JM, De Villiers AS. Complex interaction between river pollution and a dense settlement along the Plankenbrug River. 44th Academic Day, Faculty of Medicine, University of Stellenbosch, 24 August 2000: Tygerberg.

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Venter JME, Grabow WOK, Taylor MB. Comparison of methods for the isolation and detection of hepatitis A virus in water samples [Presentation]. "Microbial Diversity" 12th Biennial Congress of the South African Society for Microbiology, Faculty of Health Sciences, University of the Free State, 2-5 April 2002: Bloemfontein.

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xv) Colleagues and collaborators for invaluable advice and assistance (Part B);
xvi) The Steering Committee:

Ms APM Moolman	Water Research Commission
Dr NP Mjoli	Water Research Commission
Mr OCE Hempel	Water Research Commission (Secretary)
Dr JM Barnes	University of Stellenbosch
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Mr PF Cilliers	ARC: Inst for Agricultural Engineering
Ms E Pretorius	Technikon Free State
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Prof WH van Zyl	University of Stellenbosch
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PART A

PRESENCE, ANTIBIOTIC SUSCEPTIBILITY PATTERNS AND CHLORINATION RESISTANCE OF FAECAL POLLUTION FROM A DENSE SETTLEMENT AND THE POSSIBLE IMPACT OF POLLUTED WATER ON DOWNSTREAM WATER USERS

by

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CHAPTER 1

1. BACKGROUND TO STUDY SITE AND ENVIRONMENT

1.1 Historical perspective

During the past decade of major changes in South Africa, the process of urbanization of the rural poor took place at an accelerated pace. As a result, dense informal settlements developed on the riverbanks at the outskirts of many towns and villages in the Western Cape. Pollution from these areas reach the rivers in very large quantities and this has a severe impact on the health of the population coming into contact with such polluted water, as well as the down-stream use of such water for human activities and animal consumption.

In many of the Western Cape streams and rivers, the water flow slows to a trickle during extended dry periods. This contributes to the severe problems with water quality. The first rains after a prolonged dry spell washes the accumulated pollution on the banks into the river at very high concentrations. With the slow flow of the water during most of the year, rivers do not flush clean and visible pollution of plastic bags; tins, etc accumulate in the riverbed. The periodic fast flows or winter floods simply distribute this visible pollution higher up the banks down-river. The banks and riverbed are choked with alien vegetation that contributes to slow flow and causes even lower water levels.

The visible pollution in the form of tins, plastic bags, etc is what concerned members of the public most often notice. The micro-organisms originating from sewage that pollute the natural watercourses are however far more dangerous, but unfortunately invisible to the naked eye. The traditional ways employed by local authorities cannot cope with the pollution loads created by the rapid increase of inhabitants in unplanned housing communities. In most of these cases, water from these rivers is used down-stream for farming activities. Water that is highly contaminated with micro-organisms such as intestinal bacteria, viruses, and protozoa are dangerous to the health of farm inhabitants who come into contact with such irrigation water and can be dangerous to the health of people who use the river and its environment for recreation.

The Plankenbrug River runs through Stellenbosch past the dense settlement of Kayamandi and joins the Eerste River on the outskirts of the town. The pollution levels inside Kayamandi and the contamination of the environment, especially the Plankenbrug River are cause for grave concern. The inhabitants of Kayamandi are suffering from infectious diseases arising from the contaminated surroundings. This contamination mainly originated from human faeces, wastewater and solid waste not disposed of into the sewage systems or from some areas of the township that are without sewage systems at all.

1.2 The DWAF/DANCED Test Case Project

The present project funded by the Water Research Commission produced results that dovetailed with the previous work done for the DWAF/DANCED Test Case project. To promote the wider dissemination of the findings as well as the good that can come from placing results in proper context, some of the major findings of the previous research studies are summarised below.

The South African Department of Water Affairs and Forestry (DWAF) developed a National Strategy to manage the water quality effects of densely populated settlements. This project is funded by the Danish Government via their DANCED programme. The strategy aims to find the appropriate balance between protection of the water resource, the levels of services and the density of the settlement. The strategy is based on a *structured-facilitated* process, whereby DWAF, local authorities and communities jointly identify the main causes of pollution from a settlement and outline interventions for addressing these problems.

Kayamandi was selected as the test case for the Western Cape. The Kayamandi Test Case Steering Committee (KTCSC) was established in August 1999 and consists of councillors for Kayamandi serving on the Stellenbosch Municipal Council and officials of the Stellenbosch Municipality, members of the Kayamandi community, including the schools, clinic, churches and an official from DWAF (Wilna Kloppers) as well as a lecturer (JM Barnes) from the Faculty of Health Sciences of the University of Stellenbosch at Tygerberg.

1.3 Main findings of baseline community survey carried out during February 2000

The community survey was undertaken to determine the level of basic services in the community and to determine the level of awareness in the community about pollution and the related impacts. A total of 528 dwellings were surveyed and 2196 inhabitants were recorded as living in those dwellings. As this was done according to a strict 10% sample of all the dwellings, it meant that the population in Kayamandi was estimated to be 22 000 persons, excluding the hostel dwellers.

A group of 14 volunteer community workers from Kayamandi were trained to gather the data by means of a questionnaire. Interviews were conducted with the inhabitants of 10% of all the dwellings in the township obtained by means of a strict systematic sample with random starting points. For the purpose of this survey, the hostels were excluded.

Some of the results obtained from the survey:

i) The survey indicated a population living in Kayamandi that is considerably higher than previous estimates of 9 500 and 15 500 respectively.

ii) *There is a predominance of children in Kayamandi - 33,5% of the inhabitants in Kayamandi are under the age of 15 years*. There is also a marked under-representation of the older age groups (people over 55 years). This means that the older role models and mentors of the youth of Kayamandi are virtually absent.

iii) The poverty of the community is reflected in the fact that, of those who did reveal the total income of the household, 49% reported earning less than R500.00 per month, while 37% reported total earnings between R500.00 and R1000.00

iv) *Of the **dwellings in the survey, 18% were brick houses and 81% were shacks.***

v) Eighteen percent of dwellings (almost all of those brick houses) had toilets inside the house, 12% had toilets outside the house but directly next door (mainly those shacks situated directly next to toilet facilities), 64% used communal facilities away from the dwelling and 6,4% reported no toilet available within easy walking distance.

vi) The reported average distance to the nearest toilet was about 67 paces; the distance to the nearest tap was about 70 paces while the distance to the nearest waste skip was about 66 paces.

vii) There is a tap with clean water available inside the house for 17,7% of the respondents (those with brick houses); on the property or next to the dwelling for 5,7% while for 76,6% there was no tap directly near.

viii) For the shack dwellers as a separate group, 86,4% said that they disposed of dirty wash water into the drain - both storm water and sewage drains. A further 12,2% said that they disposed of wash water (grey water) in the street or on the ground. The storm water drains lead directly to the nearby Plankenbrug River. Most inhabitants also use a container to collect excreta overnight and empty those containers into the storm water drains since the communal toilets are locked overnight.

1.4 Education campaigns and findings of the 'before' and 'after' survey

With the findings of the previous survey in mind, a door-to-door education campaign was planned to improve sanitation knowledge of the inhabitants of Kayamandi. This campaign was carried out in July 2001. During the first visit, baseline information was gathered by means of a questionnaire, followed by an education session. A pamphlet in Xhosa containing the message in words as well as illustrations was handed to each house. A second visit a few days later was carried out to all participating dwellings to assess the short term retention of the message and to see whether the information pamphlet was still available in each home. It was hoped that the improved knowledge will also in the long run translate into improved sanitation behaviour, but this could obviously not be assessed over such a short term.

The basic messages in this campaign were simple but important:

1. Who is launching this campaign? (Kayamandi Test Case Steering Committee)
2. Important message: "Let us keep Kayamandi clean!"
3. Please do not throw rubbish in the toilet. It will block.
4. Please accompany children to the toilet so that they can learn to use it properly.
5. Please soften the newspaper used as toilet paper by rubbing it so that it does not block up the toilet and pipes.
6. Please wash your hands after you have used the toilet. This will help to protect you from illness.
7. Please report any blockages to Mr Sipho Menziwa, Kayamandi Administration Building.

The pamphlet was designed by Jo Barnes for distribution to each household and was translated into Xhosa. Posters with the same illustrations and messages were made and put up at each toilet block, at the Kayamandi Administration Building and at some other key points such as the library, the police station and some shops.

Volunteer community health workers were trained for the survey. For the education campaign and survey, all dwellings in Zones L, K and A were selected. These zones were indicated by the Kayamandi Test Case Steering Committee as good areas to test the education campaign. A total of 1027 dwellings housing 3568 persons participated in this education campaign. The rest of the shack areas (80% of dwellings in the township) all received the education sessions and pamphlet, but did not participate in the survey.

1.5 Results of the campaigns

It should be noted at the outset that the second education campaign and therefore the survey were conducted in the middle of one of the wettest winters in decades. This implied that the environment had been washed fairly clean and that food spoilage would have been lower than during the heat of summer. It should also be kept in mind that the community of Kayamandi differs from the usual rural situation in that it receives clean municipal water, albeit from communal taps in most cases. The results from this survey therefore represent the *minimum* levels of diarrhoea in the community of Kayamandi due to the time of the year the survey was undertaken and at the prevailing weather conditions.

The following are a few of the most important findings:

i) During the first visit 107 of households (10,1%) reported one or more cases of diarrhoea during the preceding week. After the education session and the passing of 3 or 4 days, a further 38 households (3,9%) reported one or more cases of diarrhoea (total 14,5%). This is very high given the presence of circumstances that should

have decreased this figure. This high prevalence is a cause for concern. *The cost in human suffering, loss of productivity and health costs should make the reduction of pollution and improvement of sanitation in Kayamandi a priority for the Stellenbosch Municipality.*

ii) Of the households represented in the survey, 55% said that the toilet available to them is often broken or not working. It was heartening to note that 92% said that there is a tap nearby to wash their hands after they have been using the toilet. No question was asked about the state of repair of the taps though. There was quite an acceptable level of knowledge regarding some aspects of sanitation already present in the households participating in the survey: 95% before the education session and 99% afterwards said that one can get ill from not washing one's hands after using the toilet, while virtually the same percentages said that one can get ill from dirt and rubbish in the street and the environment. Almost all the respondents (97%) said a clean toilet is important.

iii) The education campaign showed some important improvements when some of the answers were compared before and after the education session. After the education session, knowledge of where to report blockages improved from 37% of respondents to 96,6% while 94% of dwellings could still produce the information pamphlet containing all the necessary information handed out at the time of the education session at the follow-up visit.

CHAPTER 2

2. LITERATURE REVIEW OF GENERAL SANITATION PROBLEMS ASSOCIATED WITH DENSE SETTLEMENTS

Access to safe water supply and sanitation is a fundamental human need and has been recognised as a human right by the World Health Organisation (WHO) (Brundtland and Bellamy, 2001) and is supported as such by many countries, including South Africa (Water Services Act of 1997).

Safe water supplies and adequate sanitation systems are essential elements of human development and poverty alleviation and constitute an indispensable component of primary health care (Brundtland and Bellamy, 2001). There is evidence that provision of adequate sanitation services, safe water supply and hygiene education represents an effective health intervention that reduces the mortality caused by diarrhoeal disease by an average of 65% and related morbidity by 26% (Brundtland and Bellamy, 2001). Inadequate sanitation, hygiene and water supply result not only in more sickness and death, but also in higher health costs, lower worker productivity, lower school enrolment and lower retention rates of girl pupils. Most important of all, according to the Joint Report of the WHO and UNICEF on Global Water Supply and Sanitation Assessment 2000 (Brundtland and Bellamy, 2001), inadequate water supplies and sanitation services also deny people the right to live in dignity.

2.1 Provision of Basic Services

2.1.1 *Situation Worldwide*

According to the Global Water Supply and Sanitation Assessment 2000 of the WHO (Water Supply Collaborative Council, 2001a), the percentage of people with access to some form of improved water supply rose from 79% (4,1 billion people) in 1990 to 82% (4,9 billion) in 2000. Over the same period, the proportion of the world's population with access to excreta disposal facilities increased from 55% (2,9 billion people served) to 60% (3,6 billion). At the beginning of 2000, 16.6% of the world's population (1,1 billion people) was without improved water supply and 40% (2,4 billion) lacked access to improved sanitation. The majority of these people live in Asia and Africa, where fewer than half of all Asians have access to improved sanitation and two out of every five Africans lack an improved water supply.

Globally, rural services lag far behind urban services (Water Supply Collaborative Council, 2001a). Sanitation coverage in rural areas, for example, is less than half of that in urban settings, even though 80% of those lacking adequate sanitation (2 billion people) live in rural areas - some 1,3 billion in China and India alone. This is even more alarming since

during the last two decades of the previous century, concerted efforts were made by the WHO and considerable publicity was spent on the need to improve sanitation coverage.

Access to services improved considerably between 1990 and 2000, with approximately 816 million additional people gaining access to water supplies and 747 million additional people gaining access to sanitation facilities (Water Supply Collaborative Council, 2001a). Unfortunately, the *percentage* increases in access were modest. This is because the global population growth during that time outstripped the gains in coverage by a considerable factor.

In 2000, the Water Supply and Sanitation Collaborative Council of the WHO (Water Supply Collaborative Council, 2001a) started with Vision 21, a strong consensus not to rely only on governments, but also to support people's own energy and initiatives to bring about rapid and lasting improvements. Thus, the Council plans to focus on users of services, not providers.

The global water supply and sanitation services will face enormous challenges in the coming decades. The populations of Africa, Asia, Latin America and the Caribbean are expected to increase dramatically (Water Supply Collaborative Council, 2001a). The urban populations of Latin America and the Caribbean are expected to increase by almost 50% in the next 25 years. The population growth in Africa is almost double the global average. The combination of fast population growth with accelerated urbanization, and low levels of water supply and sanitation coverage make Africa especially vulnerable to the risk of water-related diseases (Water Supply Collaborative Council, 2001a).

The health hazards of poor water supply and sanitation are manifold, but one of the major indicators used worldwide is the incidence of diarrhoea. According to the WHO Global Health Statistics (Harvard School of Public Health, 1996), an incidence rate of 77 344 cases of diarrhoea per 100 000 occurred worldwide in 1990, which caused 2,9 million deaths or a mortality rate of 56 per 100 000, mostly among children under the age of 5 years. These deaths represent approximately 15% of all child deaths under the age of 5 years in developing countries. Water, sanitation and hygiene interventions reduce diarrhoeal disease on average by between 25% and 33,3% (Esrey *et al*., 1991).

Inadequate water supply and poor sanitation therefore carries a considerably increased morbidity and mortality, while improvement of those services will not only bring social and economic improvement, but also specifically help with poverty alleviation.

2.1.2 *Situation in South Africa*

A District Health Information System has been put into place in South Africa, but there appears to be inadequate resources, both human and financial, to ensure that the system can fulfil its potential to assist with planning and provide information (Day and Gray, 2002). Obtaining comprehensive health information in South Africa regarding sanitation, water supply and waterborne diseases is therefore not easy. Day and Gray (2002) recently collated the available data from the various State and non-governmental sources and publications in South Africa.

According to the synopsis of this data in the South African Health Review 2001 (Day and Gray, 2002), the percentage of households with no toilet was 12,4% in 1996, but was reduced to 9,4% in 1999. In the Western Cape, the percentage of households with no toilet was 5,4% in 1996, but was reduced to 3,8% in 1999. These data were obtained[6] from the October Household Survey of Statistics SA. The percentage of households with piped water inside was 44,7% for the country as a whole in 1996, but reduced to 38,7% in 1999. The corresponding percentage of households with piped water inside for the Western Cape was 76,4% in 1996, increasing slightly to 76,7% in 1999.

When the data were collated in racial groupings (Day and Gray, 2002), the percentages of households in 1999 with no toilet were 12,1% for Africans, 4,8% for Coloureds, 0% for Indian/Asians, 0% for Whites (9,4% overall for all South Africans). The percentages of households with piped water inside were 21,1% for Africans, 73,6% for Coloureds, 95,9% for Indian/Asians, 97,1% for Whites (38,7% overall for all South Africans.

Diarrhoeal disease is one of the leading causes of infant mortality, and is closely related to both socio-economic situation and environmental health issues such as access to clean water. The incidence of diarrhoea is used as an indicator to determine the health status of children and identify possible environmental hazards (e.g. contamination of water sources). The South African Health Review 2001 (Day and Gray, 2002) gives data on incidence of diarrhoea in children under 5 years old per 1000 children in the target population (Table 2.1). Diarrhoea was formally defined as 3 or more watery stools in 24 hours, but "any episode and/or treated as diarrhoea after an interview with the adult accompanying the child" was also counted (Day and Gray, 2002). Although no doubt useful for planning purposes, the data were however collected in a format that does not make it easy to compare epidemiologically.

As can be seen from the footnotes below Table 2.1, comparing the two rows of data depend on acceptance of the stated assumption that the data for 2000 is about half of the actual incidence. Thus, the data for 2000 should be doubled before comparing it to that of 1998**.** This is problematic from an epidemiological point of view.

Table 2.1: Incidence of diarrhoea per 1000 in children under 5 years of age [Extract from Table in South African Health Review 2001 (p. 314 Day and Gray, 2002)]

Year	Eastern	Free	Gauteng	KwaZulu	Mpuma	Northern	Northern	North	Western	South

	Cape	State		Natal	-langa	Cape	Province	West	Cape	Africa
1998[1]	275.6	197.5	204.0	236.3	351.5	225.7	316.8	264.7	214.8	286.4
2000[2]	145.7	84.2	67.7	292.2	107.2	135.5	217.6	241.3	99.7	174.3

The following two footnotes were presented with the data:

1. The incidence rates per 1000 given for 1996 were calculated from the percentage of children in the previous two weeks reported in the SADHS 1998 (Greenwood *et al*., 1997) to approximate an annual incidence.
2. This [data] refers to the number of children seeking treatment for diarrhoea from primary health care facilities. This is about half the number of cases expected, based on the number of cases reported in the SADHS 1998 survey to have experienced diarrhoea in the 2 weeks prior to the survey. Thus, this figure has to be doubled before comparison with the preceding year.

It is not possible to determine whether differences thus observed are due to actual changes in the incidence of diarrhoea or to erroneous assumptions about the number of cases in the population represented by the number of cases reporting at primary health care facilities. More reliable data on the burden of disease caused by diarrhoea and the associated mortality are urgently needed for the full impact of the problem to be determined.

2.2 Public Health Problems Related to Poor Sanitation

The benefits of improved water and sanitation include both health and non-health effects (Greenwood *et al*., 1997). The direct health benefits are related to two contrasting roles of water: that of disease vector when it carries pathogens and that of health mediator through its use in personal and domestic hygiene. Indirect effects related to health include, for example, improved quality of life and decreased expenditure on medical expenses. Non-health effects include time-saving for productive activity or education.

Clean water is necessary for drinking, food preparation and washing purposes as well as growing food crops and the safe watering of livestock. Qualitatively, water can become unfit for human consumption due to contamination from human or animal excreta, toxic industrial effluent or contaminants derived from the surrounding geological formations (less frequent than the first two). Contaminants include waterborne pathogens, viruses, parasites, chemical compounds and heavy metals, all of which may cause disease, with intestinal illnesses being the most frequent (Ranson, 1999).

The collection and sanitary disposal of domestic wastewater are neglected in substantial areas of many countries (Water Supply Collaborative Council, 2001a). As was shown in the previous section, the global provision of improved water supplies to many communities have increased over the previous decade (Water Supply Collaborative Council, 2001a), but this reported increase did not take into account how many of the facilities were functioning at all or functioning only intermittently. It is also ironic that provision of piped water to a previously unserviced area *commonly causes deterioration in existing health conditions until adequate waste disposal facilities have been installed* (Water Supply Collaborative Council, 2001a). Inadequately disposal of wastewater can cause flooding of roads,

housing, and communal areas where large numbers of people pass through, thus creating further health hazards. To control this, wastewater drainage systems should be installed as well as toilet facilities of such a nature as to minimise the danger of transmission of disease. Unsanitary toilet facilities encourage the spread of enteric diseases through the faecal-oral route or by flies. Where toilets are shared, the health risks increase (Water Supply Collaborative Council, 2001a).

Apart from direct transmission of disease at the point where the waste is generated, inadequate disposal can also cause pollution of water courses, land and food crops. In general, control measures include installation of piped, clean water systems, and adequate toilet facilities and sewage systems (Ranson, 1999). In some cases ventilated improved pit latrines may be an appropriate option. Different solutions may be needed in urban versus rural areas.

The hygienic storage, timely collection and safe disposal of household refuse are essential requirements of public health, as well as being aesthetically and environmentally desirable (Ranson, 1999; Water Supply Collaborative Council, 2001a). Health effects associated with poorly managed solid waste systems are related to disease transmission by flies, cockroaches, rats and mice, general pollution of the ground around waste disposal containers from the seepage out of such bins, increased fire hazards, injuries from broken glass, tins etc. (Ranson, 1999). Open communal waste containers (so-called "skips") for solid household waste placed at convenient intervals in poor areas are open to the elements and run-off from the skip distribute heavily polluted seepage water over the surrounding ground. Wind also redistributes any waste that can become airborne such as paper, plastic and other contaminated packaging material, feathers and contaminated dust, etc. over the surrounding environment. Poorly managed domestic solid waste in areas lacking sanitation become mixed with excreta, contributing even further to the spread of disease. The urban poor suffer most in this regard, as they often live in areas with poorly functioning waste removal or near waste dumps and their children are the waste pickers. Uncollected solid waste is further the most common cause of blocked urban drainage systems, increasing the risk of flooding and waterborne diseases (Ranson, 1999).

The importance of hygiene behaviour in individuals and the proper management of sanitation have only recently returned to the forefront of attention, mainly as a result of the United Nations International Drinking Water Supply and Sanitation Decade (IDWSSD)(Water Supply Collaborative Council, 2001b). Concerns about hygiene and the use of toilets, rather than simply their construction, are not new. ***What is new is the rapid increase of epidemiological evidence pointing to the importance of relatively small behavioural changes in protecting families and communities from faecal-oral disease*** (Water Supply Collaborative Council, 2001b).

A comprehensive overview, with many case studies, of the unequal human impacts of environmental damage was presented in the Human Development Report 1998 of the United Nations (United Nations Development Programme, 1998). It also presented case studies as well as data of the important improvements in hygiene and health that can be effected when the creative solutions of the affected communities themselves are harnessed to solve problems. Communication, community participation, and more specifically education are at the heart of all these successes.

The evidence of a causal link between hygiene and infections was reviewed by Alello and Larson (2002). They reviewed 30 interventional and 24 observational studies published from January 1980 to June 2001. They restricted inclusion of interventional studies to only those with an experimental design (formally randomised) or quasi-experimental design (non-randomised intervention assignment). All studies without implementation of an intervention was considered observational.

Of the interventional studies in this review hygiene education was the most common intervention (77%) followed by various hand washing practices (20%)(Alello and Larson, 2002). Infrastructure interventions in combination with either education or hand washing comprised only 17% of the interventional studies. Less than half of the studies examined diarrhoea or gastrointestinal illness as at least one of the main outcomes. Many other symptoms and signs such as respiratory infections, skin infections, flu-like symptoms, otitis, sinusitis, absence from school due to illness, etc. were also employed in these studies. In general, the reductions in all infectious disease symptoms and infections were appreciable, more than 20% for most hygiene interventions. Only two (7%) studies found no reduction in diarrhoea illness after implementation of hygiene education intervention.

The observational studies included in the review were overwhelmingly carried out in developing countries (96%)(Alello and Larson, 2002). Most of the studies created 'hygiene indicator variables' that encompassed behaviour (e.g. hand washing, infant feeding practices), knowledge (e.g. transmission routes, methods of prevention) and/or personal and environmental cleanliness (e.g. refuse disposal, food handling, general household hygiene). Diarrhoeal illness was the most common outcome studied (79%), followed by various other illnesses such as trachoma, respiratory illness and helminth infection. All but two of the studies (92%) found a correlation between hygiene variables and a reduction in infection.

Esrey and co-workers (1991) carried out an analysis of 144 studies to examine the impact of improved water supply and sanitation facilities on ascariasis, diarrhoea, dracunculiasis, hookworm infection, schistosomiasis, and trachoma. These diseases were selected because they are widespread and illustrate the variety of mechanisms through which improved water and sanitation can protect people or promote health. The authors divided the studies under review into two categories: "rigorous" if the were free of one single major design flaw or several known or suspected minor flaws that could have biased the results, and "all studies"

meeting their inclusion criteria. The results of the assessment of these studies are given in Table 2.2

Table 2.2: Expected reductions in morbidity and mortality from improved water and sanitation for selected diseases (Esrey *et al.,* 1991)

Diseases	All studies		Rigorous studies	
	n	Median % reduction (range)	n	Median % reduction (range)
Ascariasis	11	28 (0-83)	4	29 (15-83)
Diarrhoeal diseases:				
Morbidity	49	22 (0-100)	19	26 (0-68)
Mortality	3	65 (43-79)	–	– (–)
Dracunculiasis	7	76 (37-98)	2	78 (75-81)
Hookworm infection	9	4 (0-100)	1	4 (–)
Schistosomiasis	4	73 (59-87)	3	77 (59-87)
Trachoma	13	50 (0-91)	7	27 (0-79)
Child mortality	9	60 (0-82)	6	55 (20-82)

This review indicated that improvements in one or more components of water supply and sanitation could substantially reduce the rates of morbidity and severity of the diseases used as indicator conditions (Esrey *et al.,* 1991). Despite the mix of both positive and negative studies, the overwhelming evidence was in favour of positive impacts, with the exception of hookworm infection for which the impact was negligible.

Studies that reported reductions for one disease in isolation most probably underestimated the total effect of water and sanitation on improving health (Esrey *et al.,* 1991). This was particularly true if several diseases affected by water and sanitation were present in the intervention area. In addition to reducing the incidence or prevalence of disease, improvements in water and sanitation can be expected to affect other aspects of health as well. When infection rates were reduced by chemotherapy, improved water and sanitation facilities in the studies under review prevented infection from increasing back to pre-treatment levels. Furthermore, the severity of infection was often reduced more than the incidence or prevalence.

The following recommendations were made after the assessment of these studies (Esrey *et al.,* 1991).

i) To achieve broad health impact, greater attention should be given to safe excreta disposal and proper use of water for hygiene than to drinking water quality.

ii) Sanitation facilities should be installed at the same time as water distribution facilities when faecal-oral diseases are present.

iii) Access to the water supply should be as close to the home as possible, in order to foster the use of water for hygiene practices.
iv) Sanitation facilities should be culturally appropriate to ensure their use.
v) Water and sanitation programmes should complement those in other sectors (e.g. chemotherapy) to reduce disease rates.

These recommendations offer valuable pointers to remedial action for the dire sanitation crisis in the present study site (Kayamandi).

Limitations in all comparative studies on sanitation and hygiene education include:
i) The great difficulty in employing blinding in assessing the outcome;
ii) The difficulty in employing randomisation when selecting participants in the intervention studies;
iii) The enormous difficulty in controlling for bias and confounding factors as well as effect modifiers;
iv) The difficulty in selecting two comparable sites so that the baseline disease rates and many other factors are the same before the intervention starts
v) Lack of statistical power in some situations or designs (Blum and Feachem, 1983).

2.3 Environmental impacts and contamination

Human beings can only be healthy in a healthy environment. We cannot isolate ourselves from the air we breathe, the water we drink, the food we eat and the dwellings and landscapes we inhabit. Most human activities impact on the environment. Therefore, sanitation systems for disposal and treatment of wastes are essential for minimising human impact and creating a sustainable environment. A lack of adequate sanitation, or inadequately maintained or inappropriately designed systems can therefore constitute a range of health risks to humans and animals and pollution risks to the environment, especially the contamination of surface and ground water sources.

Most faecal-oral infections are transmitted on hands and during food preparation, rather than through drinking contaminated water directly. Faeces also provide a fertile environment for many organisms that cause diseases in humans apart from those organisms from the normal gut flora already present in the faeces. Any action that prevents faeces from getting onto or into human bodies will help to break the cycle of infection.

Sanitation programmes can have dramatic health benefits because many of the infective organisms that are spread from hand-to-mouth or from hand-to-food-to-mouth rather than through drinking contaminated water (Alello and Larson, 2002). Improving hygiene practices and providing sanitation facilities could have a direct influence on a number of important public health problems besetting South Africa. Thus, understanding how

infections are transmitted and how to break the cycle of infection are important public health messages.

Significant investments are being made in the provision of safe water supplies for all inhabitants of South Africa. However, the health benefit of this investment is limited where inadequate attention is paid to sanitation and to health and hygiene promotion. Once people's basic needs are met (especially the provision of clean water), sanitation improvements together with health and hygiene promotion result in the most significant impact on their health (Ranson, 1999).

Although natural water systems such as wetlands are able to tolerate and "clean up" a certain small degree of pollution, there is a limit to the amount that can be assimilated without causing the water quality to deteriorate to such an extent that the water cannot be used.

Factors listed in the White Paper on Basic Household Sanitation (2001) that affect the impact of sanitation systems on water quality are:

i) Size and density of the settlement being served;
ii) Sensitivity (or class) of the receiving water resource;
iii) Type of sanitation system;
iv) Capacity of the service provider to manage the system; and
v) Depth to ground water table and the soil type.

Absent from this list is the slope of the ground to the nearest watercourse. Settlements on steep hillsides can cause significant run-off into rivers and watercourses without even penetrating the intervening ground to any great extent**.**

An aim of the national sanitation policy is to promote the environmental sustainability of sanitation systems to ensure that sanitation systems are designed, constructed and operated in such a way that contamination caused by the system is restricted to acceptable levels throughout the life cycle of the system, regardless of the chosen technology option (White Paper on Basic Household Sanitation, 2001).

Environmental damage almost always hits those living in poverty the hardest (United Nations Development Programme, 1998). The overwhelming majority of those who die from air and water pollution are poor people living in developing countries (Water Supply Collaborative Council, 2001a, 2001b; United Nations Development Programme, 1998). All over the world poor people generally live nearest to dirty factories, busy roads and waste dumps. According to the exhaustive review contained in the United Nations Development Programme's Human Development Report, there is an irony in these statements (United Nations Development Programme, 1998). Even though poor people bear the brunt of environmental damage, they seldom are the principal creators of that damage. The affluent generate far more waste and consume far more resources (United Nations Development

Programme, 1998). Yet, there are also environmental challenges that stem from growing poverty, not growing affluence. As a result of increasing impoverishment and the absence of alternatives, a swelling number of poor and landless people migrating to peri-urban areas are putting unprecedented pressure on the natural resource base as they struggle to survive.

Poor people and environmental damage are often caught in a downward spiral (United Nations Development Programme, 1998). Past degradation of resources deepens today's poverty, while the poverty of today makes it very hard to care for or restore the agricultural resource base, to find alternatives for deforestation, to control erosion and to replenish soil nutrients. Poor people are forced to deplete resources to survive and this degradation further impoverishes people. When this downward spiral becomes extreme, poor people are forced onto marginal land and fragile ecosystems in ever increasing numbers. About half of the world poorest people (about 500 million people) live on marginal lands (United Nations Development Programme, 1998).

The issue of the link between poverty and environmental damage is complex and explaining the observed phenomena only in terms of income is oversimplistic (United Nations Development Programme, 1998). The following also impact on the relationship:

i) Ownership of natural resources – often ill-defined under a social system no longer in operation, especially in developing countries
ii) Access to common resources – ill-defined in poor communities
iii) The strength or weakness of communities and local institutions
iv) The way information about poor people's rights to resources is shared with them
v) The way people cope with risk and uncertainty
vi) The way in which people allocate their scarce time in order to survive – nearest sources are over-exploited first (United Nations Development Programme, 1998).

Some kinds of environmental degradation are truly global concerns, such as global warming and the depletion of the ozone layer. Others are international such as acid rain and the state of the oceans. Yet others are more localized, although they may occur worldwide – water pollution and river and soil degradation are such issues (Water Supply Collaborative Council, 2001a, 2001b; United Nations Development Programme, 1998). Regardless of categorization, the costs of environmental degradation are enormous.

Especially in developing countries, local or indigenous institutions that could have controlled the wise use of such resources reflecting consensus of ownership and that were once effective, have eroded (United Nations Development Programme, 1998). It is ironic that the institutions and powerful lobby groups who are able to make changes to the way common property such as rivers and water sources are managed (e.g. politicians, local officials), are not the first to feel the effects of degradation personally. The big time lags built into political systems demand that medium- and long-term action to prevent

environmental damage receive attention before it is too late (United Nations Development Programme, 1998).

CHAPTER 3

3. QUANTIFICATION OF FAECAL POLLUTION IN THE PLANKENBRUG RIVER USING *Escherichia coli* AS INDICATOR ORGANISM

3.1 Introduction

The Plankenbrug River has been monitored for the presence of faecal coliform bacteria and levels of *E. coli* since 4 May 1998. This was done as a long-term study of the seasonal variation of the level of faecal pollution in the river as well as a way of monitoring the results of various interventions in the dense settlement along the riverbank from whence a large proportion of the pollution originated. Thus, *E. coli* levels were used in its traditional role as an indicator of faecal pollution, but also as a monitoring index of eventual environmental effects of sanitation education and interventions into the functioning of the sanitation systems in the township.

Enumeration of the coliform group of bacteria and specifically faecal coliforms and *E. coli* is the most widely used method in the estimation of health-related water quality (Rompre *et al.*, 2002). Enumerating the faecal coliform levels and levels of *E. coli* could therefore also serve as bacteriological indicators of the fitness-for-use of the water downstream from the sources of pollution in the same river drainage system. Water use in this sense is defined

broadly as also including irrigation uses, especially on edible crops and crops consumed by animals. Since the Plankenbrug River runs through some very ecologically, agriculturally and recreationally sensitive areas, this indication of the health of the river is very important in coping with the deleterious effects of the large quantities of pollution.

The aim of this substudy was to quantify the levels of faecal pollution present in the Plankenbrug River.

3.2 Materials and Methods

3.2.1 *Collection of samples*

From 4 May 1998 to 25 November 2002, samples were drawn approximately every six weeks in order to do a prospective study of the seasonal variation of the pollution levels in the river. For the first two years, samples were drawn from eight sites along the entire length of the Plankenbrug River (see map: Appendix A). Three of these samples in the upper reaches (before the urban developments of Stellenbosch Town) were situated in agricultural land, mainly vineyards and fruit orchards. These three sample sites were not used after 27 March 2000 due to consistently low *E. coli* counts.

The samples were collected in two litre autoclaved plastic sample bottles according to the guidelines set out by the South African Bureau of Standards (SABS)(1984), that incorporates the standard methods set out by the American Public Health Association (American Public Health Association, 1992), American Water Works Association (AWWA)(American Public Health Association, 1995) and the Water Environment Federation (American Society for Microbiology, 1997). Samples were taken mid-stream, as far as possible. Whenever the water depth allowed, the samples were taken at 30 cm depth. During low flow conditions the samples were taken at the halfway point between the water surface and the riverbed. All samples were taken between 07h00 and 09h00 and immediately transported on ice to the laboratory, a period of approximately 30 minutes.

3.2.2 *Coliform and E. coli counts*

E. coli counts were determined using the multiple tube fermentation method. Water was inoculated into tubes containing laurel tryptose broth (Oxoid) and incubated aerobically for 48 h. Production of gas after 48 h of incubation constituted a positive presumptive reaction. All tubes with gas formation were taken to be positive and the presumptive coliform count were read off from the De Mans tables (American Public Health Association, 1992, 1995). All tubes with a positive presumptive reaction were subsequently subjected to a confirmation test in a brilliant green lactose bile broth (Biolab) and indole tryptone water (Oxoid) according to the guidelines set out by the SABS (1994). Those tubes showing positive on brilliant green broth provided a faecal coliform count and those showing gas formation on indole tryptone water provided an enumeration of *E. coli* count in 100 ml of the sampled water.

Five dilutions were used except on a few occasions when up to seven or eight dilutions were used for the heavily contaminated sampling sites during summer (peak counts) in order to verify the extraordinarily high values obtained. The analyses were performed in triplicate for the first three years after which the analyses were performed in duplicate.

3.2.3 *Statistical analyses of meteorological data*

The relevant meteorological data was obtained from the Division of Agrimeteorology and the analyses were carried out in co-operation with Mr Pieter Haasbroek, Agrimeteorologist from the Agricultural Research Council. The average maximum air temperature for the three days preceding sampling and total rainfall over the preceding seven days for the first four years of the study were included in the model. Two models were constructed: one where temperature and rainfall were taken into account to calculate expected *E. coli* levels (3D model), and one where only temperature was used (2D model). The model was constructed with the aid of Tablecurve 3D, Version V3.12 from Cystat Software Inc, United States of America (USA).

At the last two sampling sessions of 2002, the logarithmic version of the model was used to predict what the expected load would have been if the *E. coli* status of the river remained the same as for May 1998 to May 2002.

3.3 Results

The levels of pollution, as reflected in the *E. coli* counts, were dangerously high, especially during summer when peak water extraction from the Eerste River Catchment system occurs. An overview of the *E. coli* counts for the two sampling points "Below Kayamandi" and "Before Gilbeys" (the next one lower down the river course) are depicted in Figure 3.1.

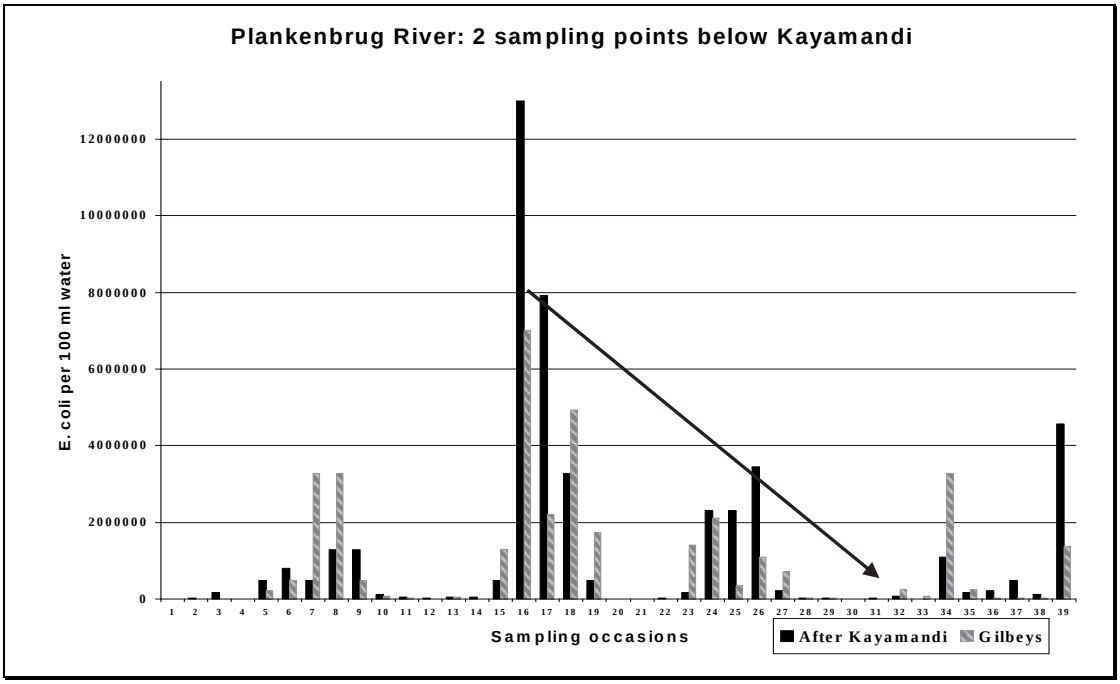

Fig 3.1: *E. coli* counts from May 1998 to September 2002 for the two sampling points, namely "After Kayamandi" and "Before Gilbeys"

Selected results of the Faecal coliform and *E. coli* counts at various sampling sites on the Plankenbrug river are given in Table 3.1

Table 3.1: Selected results of the microbial monitoring of the Plankenbrug River.

1998

Sampling point	**Faecal coliforms per 100 ml**	***E. coli* organisms per 100 ml**
4 May 1998 (start of project):		
Elsenburg Road	920	920
Nooitgedacht	92 000	92 000
Weltevreden	220	220
Before Kayamandi	12 000	6 300
After Kayamandi	16 000	16 000
Gilbeys (±1 km down-stream)	3 500	3 500

Adam Tas Bridge	17 000	11 000
Die Boord (before confluence)	11 000	7 000
17 August 1998 (winter):		
Elsenburg Road	792	792
Nooitgedacht	792	792
Weltevreden	2 110	2 110
Before Kayamandi	329	329
After Kayamandi	172 300	172 300
Gilbeys (±1 km down-stream)	14 060	6 990
Adam Tas Bridge	3 290	3 290
Die Boord (before confluence)	4 930	3 290
7 December 1998 (summer):		
Elsenburg Road	329	329
Nooitgedacht	1 406	1 406
Weltevreden	456	456
Before Kayamandi	6 310	6 310
After Kayamandi	792 000	792 000
Gilbeys (±1 km down-stream)	792 000	493 000
Adam Tas Bridge	17 240	10 860
Die Boord (before confluence)	10 860	7 920

Table 3.1: Selected results of the microbial monitoring of the Plankenbrug River.

1999

Sampling point	Faecal coliforms per 100 ml	*E. coli* organisms per 100 ml
25 January 1999 (summer):		
Elsenburg Road	329	231
Nooitgedacht	792	792
Weltevreden	493	493
Before Kayamandi	347	347
After Kayamandi	493 000	493 000
Gilbeys (±1 km down-stream)	3 290 000	3 290 000
Adam Tas Bridge	792 000	129 900
Die Boord (before confluence)	4 930	4 930
22 June 1999 (winter):		
Elsenburg Road	4 930	4 930
Nooitgedacht	3 290	3 290
Weltevreden	4 560	4 560
Before Kayamandi	10 860	10 860
After Kayamandi	49 300	49 300
Gilbeys (±1 km down-stream)	12 990	12 990
Adam Tas Bridge	22 120	14 060
Die Boord (before confluence)	10 860	10 860
6 December 1999 (summer):		
Elsenburg Road	130	130
Nooitgedacht	329	329
Weltevreden	2 781	2 781
Before Kayamandi	329	329
After Kayamandi	4 930 000	4 930 000
Gilbeys (±1 km down-stream)	1 724 000	1 299 000
Adam Tas Bridge	490 000	490 000
Die Boord (before confluence)	172 400	108 600

Table 3.1: Selected results of the microbial monitoring of the Plankenbrug River.

2000

Sampling point	**Faecal coliforms per 100 ml**	***E. coli* organisms per 100 ml**
24 January 2000 (summer):		
Elsenburg Road	10 860	6 990
Nooitgedacht	221	221
Weltevreden	79	79
Before Kayamandi	130	130
After Kayamandi	17 420 000	12 990 000
Before Gilbeys (±1 km down-stream)	10 860 000	6 990 000
Adam Tas Bridge	944	944
Die Boord (before confluence)	10 860	10 860
26 June 2000 (winter):		
Before Kayamandi	493	221
After Kayamandi	2 640	1 660
Before Gilbeys (±1 km down-stream)	792	493
Adam Tas Bridge	6 700	6 700
Die Boord (before confluence)	1 406	631
4 December 2000 (summer):		
Before Kayamandi	493	493
After Kayamandi	3 290 000	2 310 000
Before Gilbeys (±1 km down-stream)	2110000	2110000
Adam Tas Bridge	10 860	7 000
Die Boord (before confluence)	10 860	10 860

Table 3.1: Selected results of the microbial monitoring of the Plankenbrug River.

2001

Sampling point	**Faecal coliforms per 100 ml**	***E. coli* organisms per 100 ml**
23 January 2001 (summer):		
Before Kayamandi	3 290	3 290
After Kayamandi	3 290 000	2 310 000
Before Gilbeys (±1 km down-stream)	329 000	329 000
Adam Tas Bridge	79 200	79 200
Die Boord (before confluence)	12 990	12 990
23 July 2001 (winter):		
Before Kayamandi	278	278
After Kayamandi	32 900	23 100
Before Gilbeys (±1 km down-stream)	12 990	12 990
Adam Tas Bridge	16 600	6 800
Die Boord (before confluence)	9 200	4 930
11 December 2001 (summer):		
Before Kayamandi	221	221
After Kayamandi	69 900	69 900
Before Gilbeys (±1 km down-stream)	231 000	231 000
Adam Tas Bridge	49300	49 300
Die Boord (before confluence)	264 000	264 000

Table 3.1: Selected results of the microbial monitoring of the Plankenbrug River.

2002

Sampling point	**Faecal coliforms per 100 ml**	***E. coli* organisms per 100 ml**
22 January 2002 (summer):		
Before Kayamandi	493	493
After Kayamandi	17 500	9 440
Before Gilbeys (±1 km down-stream)	79 200	79 200
Adam Tas Bridge	9 440	9 440
Die Boord (before confluence)	3 290	3 290
24 June 2002 (winter):		
Before Kayamandi	3 454	3 454
After Kayamandi	493 000	493 000
Before Gilbeys (±1 km down-stream)	23 100	23 100
Adam Tas Bridge	3 290	3 290
Die Boord (before confluence)	14 060	10 080
14 October 2002 (end of project):		
Before Kayamandi	1 300	1 300
After Kayamandi	129 000	129 000
Before Gilbeys (±1 km down-stream)	23 100	13 000
Adam Tas Bridge	7 000	4 560
10 February 2003 (final assessment):		
Before Kayamandi	329	329
After Kayamandi	10 860 000	4 560 000
Before Gilbeys (±1 km down-stream)	1 370 000	1 370 000
Adam Tas Bridge	4 930	3 290
Die Boord (before confluence)	329	130

The percentage of samples with *E. coli* counts above 2000 organisms per ml are given in Figure 3.2.

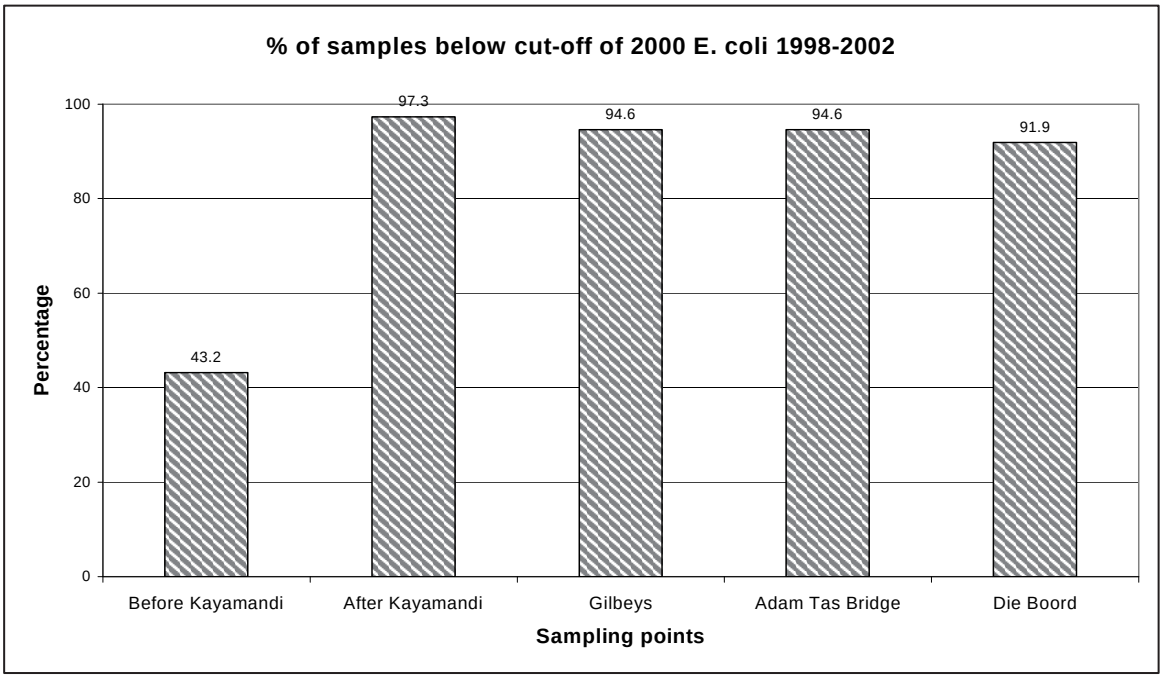

Fig. 3.2: Percentage of samples at all 5 sampling points above the cut-off level of 2000 *E. coli* per 100 ml water

As can be seen from these results, Kayamandi is by no means always the only source of *E. coli* organisms. At times it was not even the largest contributor. Other sampling dates with anomalous results indicating sewage intrusion from sources other than Kayamandi are illustrated in Figures 3.3, 3.4 and 3.5.

Sampling points on the following graphs are as follows:
Point 1 - Above Kayamandi
Point 2 - Below Kayamandi
Point 3- Before the Gilbeys property, just above the cement causeway
Point 4 - Under Adam Tas Bridge
Point 5 - Just before the confluence with the Eerste River

3.3.1 *"Expected" pattern*

The following pattern (Figure 3.3) is typical of the pattern one would expect if Kayamandi were the only source of pollution (so-called "point source pollution"). It showed a big increase from before Kayamandi (sampling point 1) to after Kayamandi (sampling point 2).

After this, there is a gradual, steady decline. This pattern occurred only a few times over the four years of microbiological sampling.

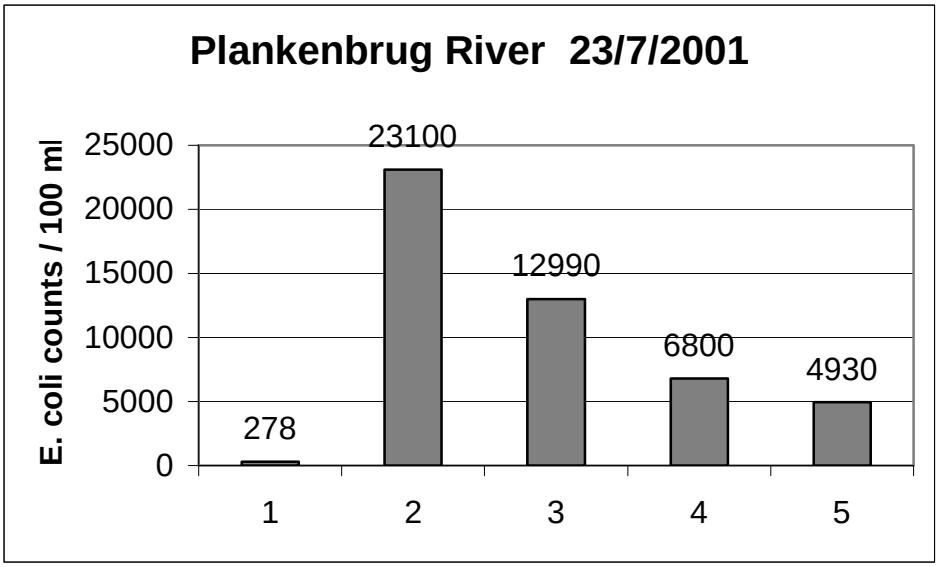

Fig. 3.3: *E. coli* counts on 23 July 2001

3.3.2 *Unexpected patterns indicating sewage intrusion from other sources*

In the following situation there is a 250% increase from the *E. coli* count after Kayamandi to the next sampling point (before Gilbeys) (Figure 3.4). The sharp drop after point no. 3 is rather a factor of other pollution such as wine cellar waste entering the river than an improvement in the *E. coli* and therefore in the faecal pollution.

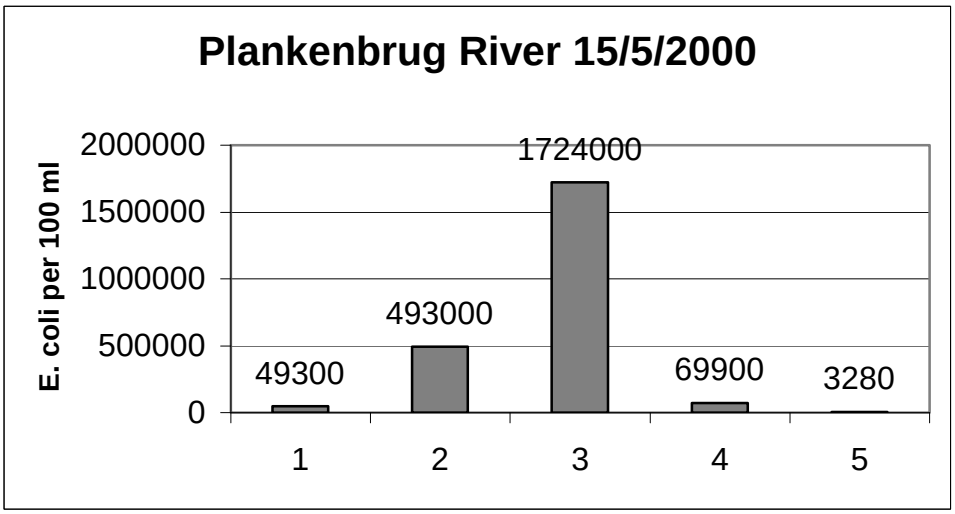

Fig. 3.4: *E. coli* counts on 15 May 2000

On this sampling occasion, there was an increase (2497%) from after Kayamandi (point 2) to the next point (just before Gilbeys). A sharp drop (2366%) after that seems to indicate the presence in the water of a competing pollutant. It is known that at that point there is periodic intrusion of wine cellar effluent into the river, which could account for the precipitous drop in *E. coli* counts.

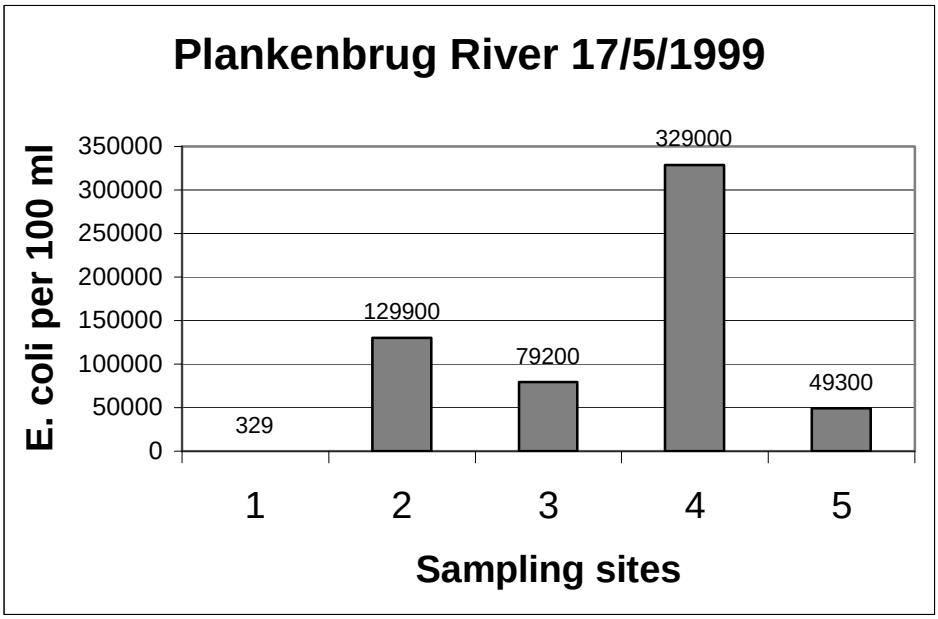

Fig. 3.5: *E. coli* counts on 17 May 1999

The numbers of *E. coli* organisms were generally lower in late autumn and winter due to rain diluting the pollution (Figure 3.5). It is however important to note the sharp increase seen under the Adam Tas Bridge (sampling point 4). The next graph (Figure 3.6) shows an elevated level of *E. coli* organisms along the entire lower stretch of the Plankenbrug. This increased level is much higher than the contribution coming from Kayamandi (point 2).

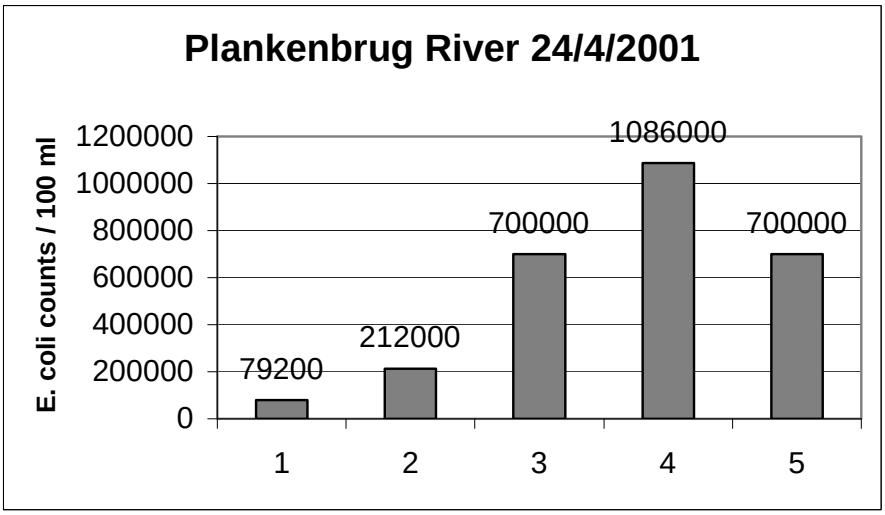

Fig. 3.6: *E. coli* counts on 24 April 2001

Other possible sources of faecal pollution have received scant attention in the past because it was always assumed that Kayamandi is the "obvious" source of all pollution. The above

patterns (Figs. 3.3 to 3.6) show that Kayamandi is by no means the only source of pollution entering the Plankenbrug River. In some instances, the estimated load of sewage entering the river is several times the size of that emanating from Kayamandi.

3.3.3 *Model for assessment of change in pollution levels*

The model developed for assessment of change in pollution levels is given in Figure 3.7

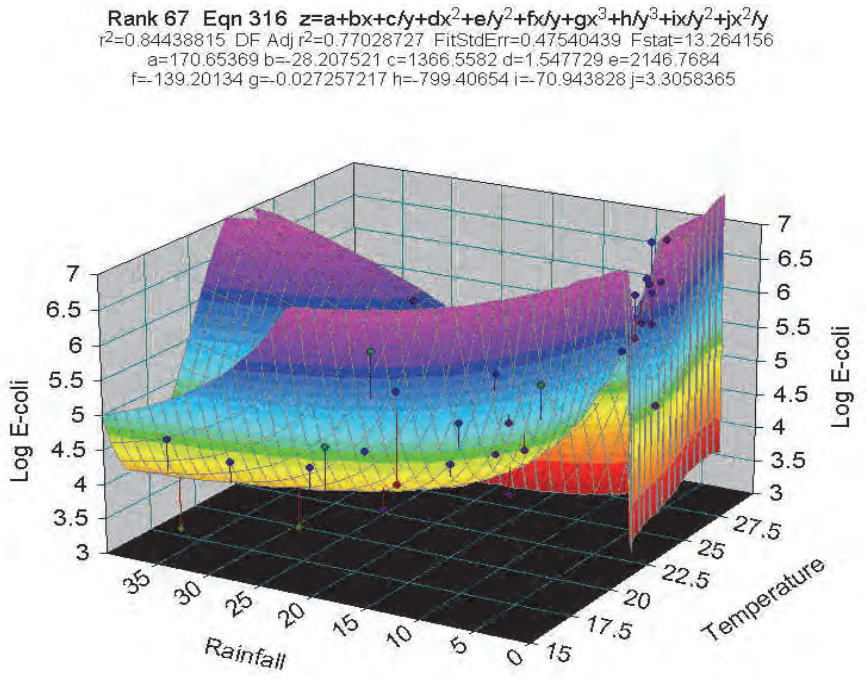

Fig. 3.7: Relationship between rainfall in the previous 48 h, air temperature at the sampling site and *E. coli* levels

Fig. 3.7 depicts the statistical relationship between rainfall in the previous 48 hours, air temperature at the sampling site "Below Kayamandi" and *E. coli* levels. The equation on which this graphic depiction is based, is:

$z = a+bx+c/y+dx^2+e/y^2+fx/y+gx^3+h/y^3+ix/y^2+jx^2/y$ where

a = 170.65369
b = 28.207521
c = 1366.5582
d = 1.547729
e = 2146.7684
f = 139.20134
g = 0.027257217
h = 799.40654
i = 70.943828
j = 3.3058365

The correlation coefficient is r = 0.9189

This is one of a series of a Chebychev polynomials (Schulz and Griffin ,1999), chosen to give the best fit of the data coupled with the best stability under conditions of small data changes. This is not a statistical model in the classic sense, where theoretical values are often generated in order to predict results under different conditions. This is an accurate

reflection of how the *E. coli* levels varied at the sampling point "below Kayamandi" in relation to its two greatest contributing variables, average maximum air temperature for the preceding 3 days and total rainfall over the preceding 7 days for the first four years of the study. We constructed two models: one where temperature and rainfall were taken into account to calculate expected E. coli levels (3D model) and one where only temperature was used (2D model). This statistical tool can now be used in subsequent sampling in order to determine whether the pollution loads observed are getting worse (given the temperature and/or rainfall).

The previous two sampling sessions the logarithmic version of the model was used to predict what the expected load would have been if the *E. coli* status of the river remained the same as for May 1998 to May 2002. The following results were obtained: On 24/6/2002 the range predicted was 31 623 - 38 904 *E. coli* per 100 water and the actual observation was 493 000 organisms. On 19/8/2002 the predicted range was 7079 - 15849 *E. coli* organisms while the observed value was 79 000 *E. coli* organisms.

3.4 Discussion

As can be seen from the brief overview of microbial data shown above, the levels of pollution reached in the Plankenbrug River are a source of great concern. In Fig, 3.1 the expected fluctuations between winter and summer can be seen. In early summer 2000, the *E. coli* counts reached a peak of almost 13 million organisms per 100 ml water (sampling occasion no. 16 in Fig. 3.1). The reductions seen after that date was due to the interventions in education and maintenance of sanitation systems during the DANCED project. As can be seen, the levels started rising as soon as the project came to an end (sampling occasion no. 33 in Fig. 3.1).

Kayamandi is by no means always the major source of *E. coli* organisms. Other sampling dates with anomalous results indicating sewage intrusion from sources other than Kayamandi were illustrated in Figs. 3.3 to 3.6. This situation needs urgent attention, as systems failures such as these results suggest, should be rectified as soon as possible. Upgrading of the sewage system (if needed) as well as much more frequent monitoring of main sewage lines crossing the river should be instituted without delay to avoid future contamination.

Drinking water should contain no *E. coli* organisms (SABS, 1994). For irrigation purposes, internationally the requirement is that the level should not rise above 2000 organisms per 100 ml water (American Public Health Association 1992, 1995). Above that level, the hazards increase with increasing organism count and no direct contact with humans, animals or crops should occur. Such water could be dangerous to health. High levels of *E. coli* also indicate that there is highly likely to be other disease-causing organisms present. *E. coli* does not occur in high numbers on its own. If the sanitation systems along the

Plankenbrug River continue to fail on such a large scale, it is reasonable to assume that the risk of the river eventually being contaminated by organisms such as cholera will be increasing. This will have a huge impact on the health status and economic activity of the region since tourism, agriculture and tertiary education are the three major activities sustaining the immediate district.

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CHAPTER 4

4. IDENTIFICATION OF THE POTENTIAL PATHOGENS ISOLATED FROM THE PLANKENBRUG RIVER

4.1 Introduction

Testing for every pathogenic organism in order to monitor water quality is an expensive and technically problematic proposition. Thus, the concept of an indicator organism is used to indicate the possible presence of other disease-causing constituents. The coliform group of bacteria and specifically *E. coli* are used as such indicators (Rompre *et al*., 2002). Once substantial numbers of *E. coli* are detected, it is prudent to assume that such water has been contaminated with faecal waste and that other, harmful pathogens are also present in significant numbers. Thus, the high *E. coli* numbers observed in the Plankenbrug River over the previous four years send a clear health warning.

The elevated numbers of *E. coli* organisms led to the need to find out exactly what other pathogens were present in the river, so as to be better prepared for any health crises that may ensue. In other words, what other pathogens occur in the Plankenbrug River that *E. coli* acts an indicator for?

Besides the water in the river, which had already been shown to carry huge bacterial loads, the biofilms on the stones and other surfaces in the riverbed were also of epidemiological and clinical importance. Biofilms can be defined as communities of micro-organisms attached to a surface (O'Toole *et al.*, 2000). Micro-organisms undergo profound changes during their transition from planktonic organisms to cells that are part of a complex, surface-attached community. It is known that biofilm bacteria are significantly less susceptible to biocides than planktonic cells (Eginton *et al*., 1998). Micro-organisms growing in a biofilm are highly resistant to antimicrobial agents by one or more mechanisms (Donlan and Costerton, 2002). Biofilm-associated micro-organisms have also been shown to be associated with several human diseases such as native valve endocarditis and cystic fibrosis (Donlan and Costerton, 2002). It also provides a niche for the generation of resistant organisms (De Lancy and Pulcini, 2001; Donlan and Costerton, 2002). Biofilms can form rapidly in raw or treated potable water, even in the presence of 1 to 2 mg/litre free chlorine residual (Norton and LeChevallier, 2000).

For these reasons, water and stones with visible biofilms were subjected to preliminary identification procedures in order to screen for most of the clinically important genera present in the samples. Where possible, the identification was taken down to species level.

4.2 Materials and Methods

4.2.1 *Bacteriological analysis*

Water samples were taken on two occasions in the same manner as for enumeration of *E. coli* as described previously (Section 3.2.1). The samples were obtained at the sampling points called "Below Kayamandi" and "Before Gilbeys" on 19 March 2002 and 15 April 2002. The sample for 11 February 2002 was collected only from
"Below Kayamandi" and was used as an initial pilot study.

Below the water surface stones with visible biofilms were also collected at the same points as where the free-flowing water samples were taken on 19 March and 15 April 2002. The water was so polluted with untreated sewage at these sampling points that all objects below the water surface (tins, branches, pieces of cement and brick and other rubbish) that were present for any length of time were covered in a grey slime. Only non-porous natural stones were collected, as the highly alkaline composition of lumps of cement (for example) may have altered the bioslime community growing on the surface. The stones were placed in airtight new plastic bags and sealed until sonication.

The water samples and the bagged stones were transported in cooler bags and on ice. At the Dept of Microbiology, Stellenbosch the stones were sonicated within 15 minutes of collection for 6 minutes in approximately 100 ml sterile deionised water to loosen the biofilm and bring it into resuspension. The resultant water was carefully decanted into autoclaved sample bottles and immediately transported on ice to the Department of Microbiology, Tygerberg Hospital.

The water samples were thoroughly mixed and a mini-MPN (most probable number determination), a faecal coliform determination and a confirmatory test for *E. coli* were carried out on all samples. After vigorous shaking of the water sample bottles, the following plates and nutrient broths were inoculated:

i) directly from the sample, and
ii) after 48 h of incubation from the positive laurel tryptose broth tubes.

4.2.1.1 *Blood agar plates:* [Tryptose blood agar, Base: Biolab (Merck) to which horse blood was added]

- i) Incubated aerobically (35º - 37ºC)
- ii) Incubated anaerobically (35º - 37ºC)
- iii) Incubated in CO_2 incubator (35º - 37ºC)

4.2.1.2 *McConkey agar plates* [McConkey agar base: Biolab (Merck) without crystal violet and with salt]: To screen for *Enterobacteriacea* spp., *Enterococcus* spp. and coagulase negative *Staphylococcus* spp.

4.2.1.3 *Campylobacter plates* [Columbia agar base (Oxoid) to which horse blood was added]: To screen for *Campylobacter* spp. Incubated micro-aerophyllic in an Oxoid plastic anaerobic jar at 37ºC

4.2.1.4 *Salmonella and Shigella agar plates* [SS agar Biolab (Merck)]:To screen for *Salmonella* spp and *Shigella* spp.

4.2.1.5 *XLD agar plates* [Xylose lysine desoxycholate agar, Biolab (Merck)]:To screen for *Salmonella* and *Shigella* spp

4.2.1.6 *Brain heart infusion broth* [Biolab (Merck)]

4.2.1.7 *Tryptone Tetrathionate Broth* [Tetrathionate broth base (Oxoid)]: To screen for *Salmonella* and *Shigella*

4.2.1.8 *Mycobacterium*: Aliquots of water were taken directly from the sample bottles. These were centrifuged for 20 minutes at 3000 rpm. The samples were decontaminated with 4% NaOH + 4% $NaSO_3$ v/v and left for 20 minutes in an incubator at 37ºC. The samples were then neutralized with PO_4 buffer (equal volumes) and immediately centrifuged for 20 minutes at 3000 rpm. The supernatant was decanted and 0,5 ml of the deposit was inoculated into a Bactec 12B mycobacteria (Middlebrook 7H12) medium for culturing mycobacteria. The samples were then incubated at 37ºC and readings in the Bactec machine were done once a week until the samples were positive. When a positive reading was obtained, a Ziehl-Nielsen stain, positive for acid-fast bacilli, was done. The positive sample was then sent to Groote Schuur Hospital for identification by means of PCR. This procedure was carried out on two sampling occasions (11 February 2002 and 19 March 2002).

4.2.1.9 *Identification:* Bacterial isolates were identified by routine microbiological methods.

4.2.2 *Virological analysis*

Three sets of water samples (Table 4.6) were referred for virological analysis. Two litre samples of water were taken at the relevant sites and sent, on ice, to the Department of Medical Virology, University of Pretoria for analysis. Samples of the biofilm washes were also referred for virological analysis.

4.2.2.1 *Recovery of viruses from the water samples*

Viruses were recovered in a final volume of 10 ml phosphate buffered saline (PBS)(Sigma) from the water and biofilm samples using the PEG/NaCl viral recovery method (See Section 9.1.2). RNA was extracted from the viral concentrate, for viral detection, using the TRIzol®(GIBCOBRL) reagent.

4.2.2.2 *Isolation and propagation of viruses in cell culture*

Cell culture flasks ($25cm^2$) were seeded with the appropriate volume of 10^5 cells/ml and incubated for 24-48 h at 37ºC in a humidified 5% CO_2 incubator to provide a semi-confluent (70-80%) monolayer. Prior to infection with viruses recovered from water or biofilm samples, the growth medium was withdrawn from and the cells were starved for 1 h at 37ºC in prewarmed serum-free MEM. After withdrawal of the starvation medium, the appropriate volume of virus or treated sample was left to adsorb to the cells for 1 h at 37ºC, with gentle swirling every 15 min.

For the isolation and propagation of HAV the appropriate volume of maintenance media was then added to the FRHk-4R cells followed by incubation at 37ºC for 21 days, with the replacement of the maintenance medium 10 days prior to harvesting. For HAstV and HRV, the appropriate volume of serum-free medium containing 10µg per ml trypsin (Trypsin 250; DIFCO, Detroit, MI) was added to each flask of PLC/PRF/5 and Caco-2 cell cultures and the infected cells incubated at 37ºC for 7 days, after which they were blind-passaged and the cells harvested after a further 7 days incubation at 37ºC.

Cell cultures were examined daily for a cytopathic effect. Cytopathogenic viruses were identified by identification of virus specific inclusion bodies in haematoxylin-eosin-stained slides.

4.2.2.3 *Molecular detection of viruses*

Cell cultures were examined for cytopathic effect prior to being harvested and analysed for hepatitis A virus (HAV), human astrovirus (HAstV), human rotavirus (HRV) and human caliciviruses (HuCVs) by reverse transcriptase polymerase chain reaction (RT-PCR) (see Section 9.1.3)

4.2.2.4 *Typing of isolates*

Isolates of HAstVs were typed by partial sequence analysis of the 3' end of ORF2; HRVs were typed by type-specific RT-PCRs, adenoviruses by restriction enzyme analysis and enteroviruses were typed by a combination of fluorescent antibody assays, type-specific RT-PCRs and restriction fragment length polymorphisms (RFLPs).

4.3 Results

4.3.1 *Bacteriological analysis*

Table 4.1: Organisms isolated from the water sample of 11 February 2002

Water sampled on 11 February 2002
Water sample "Below Kayamandi"
β haemolytic *Streptococcus* Group A* α haemolytic s*treptococci* Viridans group of streptococci *Enterococcus faecalis* ***Mycobacterium intracellulare*** Coagulase negative staphylococcus *Klebsiella* spp. amongst which *K. pneumoniae, K. ozaenae* *Citrobacter* spp. *Escherichia coli* (4 morphologically distinct types) *Acinetobacter* spp. *Pseudomonas* spp. amongst which *P. aeruginosa* ***Proteus mirabilis, P. vulgaris*** Providencia rettgeri

* The identification of these organisms was confirmed by Prof A Forder, emeritus Professor of Medical Microbiology, University of Cape Town, who is now working part-time at the University of Stellenbosch.

****** Identified by means of PCR by the Dept of Microbiology, University of Cape Town

Table 4.2: Organisms isolated from the water samples of 19 March 2002

Water sampled on 19 March 2002	
Water sample "Below Kayamandi"	**Water sample "Before Gilbeys"**
β haemolytic *Streptococcus* Group B*****	*Enterococcus faecalis*
Streptococcus agalactiae	*Staphyloccocus epidermidis*
*Mycobacterium intracellulare***	*Enterobacter* spp.
Viridans group of *streptococci*	*Providencia* spp.
Enterococcus faecalis	*Klebsiella* spp.
Coagulase negative *Staphylococcus* spp.	*Escherichia coli*
Enterobacter spp.	*Citrobacter* spp.
Klebsiella spp.	*Proteus* spp. including *P. mirabilis*
Escherichia coli (4 morphologically distinct types)	*Providencia* spp. *(Yersinia?)*
Citrobacter spp.	*Acinetobacter* spp.
Acinetobacter spp.	
Proteus spp. including ***P. mirabilis***	

***** These organisms were identified in two different cultures.

****** Identified by means of PCR by the Dept of Microbiology, University of Cape Town

Table 4.3: Organisms were isolated from biofilms on stones sampled on 19 March 2002

Biofilms on stones sampled on 19 March 2002	
Stone sample "Below Kayamandi"	**Stone sample "Before Gilbeys"**

α haemolytic *Streptococcus* spp. Viridans group of streptococci ***Enterococcus faecalis*** Coagulase negative *Staphylococcus* spp. amongst which *S. epidermidis* *Providencia* spp. *Enterobacter* spp. *Klebsiella* spp. *Escherichia coli* *Citrobacter* spp. *Acinetobacter* spp. ***Proteus*** spp**.** ***Yersinia*** spp**.**	*Staphylococcus epidermidis* (in large numbers) *Pseudomonas* spp. *Escherichia coli* *Citrobacter* spp. *Proteus* spp.

Table 4.4: Organisms were isolated from the water samples of 15 April 2002

Water sampled on 15 April 2002	
Water sample "Below Kayamandi"	**Water sample "Before Gilbeys"**
Streptococcus faecalis Coagulase negative *Staphylococcus* spp. - not *S. aureus* *Enterobacter cloacae* *Klebsiella pneumoniae* *Klebsiella oxytoca* *Escherichia coli* *Citrobacter* spp. *Acinetobacter* spp. amongst which *A. lwoffii* ***Proteus*** spp. ***Bacillus*** spp. including ***B. subtilis***	*Streptococcus* spp.Coagulase negative *Staphylococcus* spp. - not *S. aureus* *Enterobacter cloacae* *Klebsiella ozaenae* *Escherichia coli* *Citrobacter* spp. *Proteus* spp. *Acinetobacter baumanii* Gram-positive bacillus cultured anaerobically, but could not be identified as Clostridium

Table 4.5: Organisms isolated from biofilms on stones sampled on 15 April 2002

Biofilms on stones sampled on 15 April 2002	
Stone sample "Below Kayamandi"	**Stone sample "Before Gilbeys"**
α haemolytic *Streptococcus* spp. Viridans group of streptococci ***Streptococcus*** spp. ***Enterococcus faecalis***	Coagulase negative *Staphylococcus* spp., not *S. aureus* ***Streptococcus*** spp**.** ***Enterococcus faecalis***

Coagulase negative *Staphylococcus* spp. amongst which *S. epidermidis* *Providencia* spp. *Enterobacter* spp. including *E. aerogenes* *Klebsiella* spp. including *K. pneumoniae* & *K. oxytoca* *Escherichia coli* *Citrobacter* spp. *Alcaligenes* spp. *Acinetobacter* spp. including *A. lwoffii* ***Proteus*** spp. ***Bacillus subtilis***	*Escherichia coli* *Citrobacter* spp. ***Edwardsiella*** spp. ***Klebsiella*** spp**.** ***Enterobacter*** spp. ***Acinetobacter baumanii*** ***Bacillus*** spp. including ***B. subtilis***

4.3.2 *Virological analysis*

Results of the virological analyses of the water samples and biofilms are summarised in Table 4.6

Table 4.6: Source of water samples and viruses detected in each sample

Sample collection date	Sampling site	Viruses isolated/detected
2002-04-15	Below Kayamandi	Enterovirus; HAstV
	Below Kayamandi biofilm	Negative
	Gilbeys	Enterovirus; HAstV
	Gilbeys biofilm	
2002-05-06	Before Kayamandi	
	Below Kayamandi	Enterovirus; HAstV,
2002-06-24	Before Kayamandi	Enterovirus
	Below Kayamandi	HRV; HAstV; Adenovirus

Multiple strains of viruses were identified in a single water sample.

Enteroviruses: The Sabin vaccine strain of poliovirus type 3 and a non-polio enterovirus were identified in the Plankenbrug River water drawn below Kayamandi on 2002-04-15. The enteroviruses detected in water drawn on 2002-05-06 were also Sabin vaccine strains of poliovirus type 3. Non-polio enteroviruses can be associated with infections ranging from non-specific febrile illness to life-threatening myocarditis.

HAstV: The HAstV isolates were identified as HAstV type 1. These viruses have been associated with sporadic and epidemic diarrhoea in children in South Africa.
Adenoviruses: Types D, B1 and B2 were identified in the water drawn at the "After Kayamandi" sampling site. These viruses are typically associated with keratoconjunctivitis (type D), respiratory infections (B12) and urinary tract infections (B2).
Human rotaviruses: The HRV isolates identified in the water samples are typically associated with paediatric gastroenteritis.

4.4 Discussion

Many of the bacteria isolated also occur in the natural environment and are known to be able to survive in free-flowing surface water. The numbers of these organisms in the Plankenbrug River were however greatly elevated above the levels normally encountered. Only the organisms that are potential human pathogens were listed and not even all of these could be pursued to full identification. The range of potentially dangerous pathogens identified is another indication that this river is severely polluted and constitutes a health hazard as well as a danger to the environment.

One of the organisms isolated in February 2002, namely β haemolytic *Streptococcus* Group A, was a significant find. After an exhaustive search of the Medline database of 11 million research articles, only 9 references to haemolytic streptococci in association with water could be found. All of these except one were related to water in treatment apparatus such as inhalation systems. Only one made mention of free flowing water in the environment (Likitnukul *et al*., 1994). The authors reported children with streptococcal disease from poor areas in Thailand who were exposed to dirty water (Likitnukul *et al*., 1994). No direct connection was made between the water and the infections and the organisms were not isolated from the water in that study. The fact that this group of organisms has been isolated in a viable state from river water below Kayamandi is scientifically remarkable and its implications cannot be ignored. The improvement of the sewage disposal and the construction of informal slaughtering places to prevent sewage and blood from reaching the river in large quantities should receive urgent attention.

It was interesting to note the presence of *Bacillus subtilis* in the biofilm on the stones below Kayamandi on 15 April 2002. *Bacillus* has very recently been proposed as a tool for the detection of genotoxic substances in the water environment (Takigami *et al*., 2002). The *Bacillus* rec-assay has been specially developed to detect genotoxicity in environmental water samples. Genotoxic pollutants seem to pass through conventional water treatment processes and the presence of *Bacillus* offers the chance to monitor that.

From the virological analyses it is evident that potentially pathogenic viruses, namely enteroviruses, HAstVs, HRVs and adenoviruses, could be detected in the waters analysed. However the data reported are purely qualitative and quantitative data are required to

determine the health risk posed to people using these waters for domestic or irrigation purposes.

CHAPTER 5

5. PRELIMINARY TESTS ON ANTIBIOTIC RESISTANCE OF ORGANISMS IN THE PLANKENBRUG RIVER WATER

5.1 Introduction

Antibiotic resistance is being found with increasing frequency in both pathogenic and commensal bacteria of humans and animals (Davison *et al.,* 2000). Antimicrobial resistance has become a major medical and public health problem (Urassa et al., 1997; Davison et al., 2000). One traditional school of thought had been that only improper use of antibiotics has led to the phenomenon that many gram positive and gram negative bacteria continuously develop resistance to antibiotics in regular use (Urassa *et al.,* 1997). However, the role of increased antibiotic resistance occurring in organisms surviving disinfection is realised more and more (Russel, 2002). This constitutes a second group of causative factors in the development of resistance quite separate from the use of medication, injudicious or otherwise.

Antibiotic resistance is widespread in the environment because of uncontrolled discharge of urban and agricultural waste water (Le Dantec *et al.,* 2002). Sewage treatment can significantly reduce the number of organisms in wastewater, but also cause an increase in the proportion of resistant strains occurring because of phenomena that are not always well understood (Meckes, 1982; Le Dantec *et al.,* 2002). Surviving, chlorine-tolerant bacteria seem to be antibiotic resistant in higher percentages than the chlorine-sensitive ones and thus significant increases of antibiotic resistance and multiresistance are observed in chlorinated effluent (Meckes, 1982; Morozzi et al., 1982; Bloomfield, 2002; Le Dantec *et al.,* 2002).

Bacteria with intrinsic resistance to antibiotics are found in nature (Ash *et al*., 2002). Such organisms may acquire additional resistance genes from bacteria introduced into soil or water and the resident bacteria may be the reservoir or source of widespread resistant organisms found in many environments (Ash *et al*., 2002). According to a survey of 16 United States rivers at 22 sites, over 40% of the bacteria resistant to more than one antibiotic had at least one plasmid (Ash *et al*., 2002). Ampicillin resistance genes, as well as other resistance traits, were identified in 70% of the plasmids. The most common resistant organisms in that survey were found to belong to the following genera: *Acinetobacter, Alcaligenes, Citrobacter, Enterobacter, Pseudomonas* and *Serratia*.

A survey of antibiotic resistance can be seen as one of the more sophisticated measures of water quality as well as an indication of the impact of health-related organisms in environmental waters. With this in mind, surveys of antibiotic resistance were carried out

on three different occasions on organisms obtained from the Plankenbrug River. On one of the occasions the organisms surviving the chlorine treatment during the study on chlorine resistance were tested for antibiotic resistance as well.

5.2 Materials and methods

One hundred *E. coli* organisms collected at various sampling occasions prior to May 1999 from the two sampling sites "Below Kayamandi" and "Before Gilbeys" were selected for antibiotic sensitivity testing. In addition, some of the organisms identified during the sampling sessions of 19 March 2002 and 15 April 2002 were also subjected to tests to determine the patterns of sensitivity to various antibiotics. One hundred *E. coli* colonies were also subjected to sensitivity analyses during the March/April 2002 round of experiments as well as during the August 2002 sampling round. During the determinations for chlorine resistance in June 2002, the *E. coli* organisms surviving the chlorine treatment were also subjected to antibiotic sensitivity analyses.

Sensitivity testing was performed using Kirby-Bauer disk diffusion Harley and Prescott, 1996). Colonies of the selected organisms were inoculated into Mueller-Hinton broth and diluted with Mueller-Hinton broth to 0,5 MacFarland. The colonies were planted out on Mueller-Hinton agar and paper disks impregnated with the antibiotics were placed onto the seeded agar plates with sterile forceps. The seeded plates were allowed to dry for a few minutes with the tops in place and then incubated overnight at 35°C. The zones of inhibition were measured after incubation and the sensitivity/resistance determined using the tables of inhibition zones. The scoring was double-checked by a senior specialist in Medical Microbiology.

5.3 Results

The susceptibility patterns of the 100 *E. coli* organisms collected at the "Below Kayamandi" and "Before Gilbeys" sampling points are given in Table 5.1, while Table 5.2 summarizes the antibiotic susceptibility patterns of *E. coli* isolated during March/April 2002, June 2002 (chlorine resistant isolates) and August 2002. The partial sensitivity patterns for β haemolytic *Streptococcus* Group A, isolated from a water sample drawn from the "Below Kayamandi" sampling point on 19 March 2002
are given in Table 5.3

Table 5.1: The susceptibility patterns of the 100 *E. coli* organisms collected at "Below Kayamandi" and "Before Gilbeys"

Antibiotic	Below Kayamandi % resistant	Before Gilbeys % resistant
Amoxycillin	48%	44%
Cefuroxime	37%	9%
Co-trimoxazole	36%	42%
Gentamicin	4%	1%
Chloramphenicol	14%	14%
Ofloxacin	0%	0%

Table 5.2: Summary table of susceptibility patterns for *E. coli* sampled during March/April 2002, June 2002 (chlorine resistant organisms) and August 2002

Antibiotic	March/April 2002 % resistant	August 2002 % resistant	June 2002 (Survivors of chlorine treatment) % Resistant
Amoxycillin	34%	34%	65,2%
Cefuroxime	0%	0%	0%
Co-trimoxazole	26%	20%	30,4%
Gentamicin	2%	0%	0%
Ofloxacin	0%	4%	-
Siprofloxacin	-	-	13%
Amoxicillin/Clavulanic acid	21%	16%	9%
Ceftazidime	0%	0%	0%
Cefazolin	15%	6%	0%

Table 5.3: Partial determination of sensitivity patterns for β haemolytic *Streptococcus* Group A as isolated from water sample "Below Kayamandi" on 19 March 2002.

β haemolytic *Streptococcus* Group A	
Antibiotic	Sensitivity
Penicillin	Sensitive
Cefazolin	Sensitive
Co-trimoxazole	Resistant
Tetracyclin	Sensitive
Erythromycin	Resistant
Vancomycin	Sensitive
Clindamycin	Sensitive
Cefuroxime	Sensitive
Total % resistant = 25%	

The partial sensitivity patterns of a variety of potentially pathogenic bacterial isolates from a water sample drawn at sampling site “Below Kayamandi” and the associated biofilm are given in Tables 5.4 and 5.5, respectively.

Table 5.4: Partial determinations of sensitivity patterns for various organisms as isolated from water sample "Below Kayamandi" on 15 April 2002.

Antibiotic	***S. viridans***	***K. pneumoniae***	***A. lwoffii***	***K. oxytoca***
Ampicillin	R	R	R	R
Cefazolin	S	R	R	S
Co-trimoxazole	S	S	S	S
Gentamicin	S	S	S	S
Amikacin	S	S	S	S
Piperacillin	S	S	S	S
Cefuroxime	S	S	S	S
Ciprofloxacin	S	S	S	S
Ceftazidime	S	S	S	S
Cefoxitin	S	R	S	S
Cefotaxime	S	S	S	S
Amoxicillin/Clavulanic acid	S	R	S	S
Total % resistant = 12,5%				

S=sensitive, R=resistant

Table 5.5: Partial determination of sensitivity patterns for various organisms as isolated from biofilm on stone sample "Below Kayamandi" on 15 April 2002.

Antibiotic	***S. viridans***	***K. pneumoniae***	***A. lwoffii***	***K. oxytoca***
Ampicillin		R	S	R
Penicillin	S			
Cefazolin	S	S	S	S
Co-trimoxazole	R	S	S	S
Tetracycline	S			
Erythromycin	R			
Vancomycin	S			
Clindamycin	S			
Gentamicin		S	S	S
Amikacin		S	S	S
Piperacillin		S	S	S
Cefuroxime		S	R	S
Ciprofloxacin		S	S	S
Ceftazidime		S	S	S
Cefoxitin		S	S	S
Cefotaxime	S	S	S	S
Amoxicillin/Clavulanic acid		S	S	S
Total % resistant = 10,4%				

S=sensitive, R=resistant

The partial sensitivity patterns of a variety of potentially pathogenic bacterial isolates from a water sample drawn at sampling site “Before Gilbeys” and the associated biofilm are given in Tables 5.6 and 5.7, respectively.

Table 5.6: Partial determinations of sensitivity patterns for various organisms as isolated from water sample "Before Gilbeys" on 15 April 2002.

Antibiotic	***E. cloacae***	***A. baumanii***
Ampicillin	R	R
Cefazolin	R	R
Co-trimoxazole	S	R
Gentamicin	S	S
Amikacin	S	S
Piperacillin	S	S
Cefuroxime	S	
Ciprofloxacin	S	S
Ceftazidime	S	S
Cefoxitin	R	R
Cefotaxime	S	S
Amoxicillin/Clavulanic acid	R	R
Total % resistant = 37,5%		

S=sensitive, R=resistant

Table 5.7: Partial determination of sensitivity patterns for various organisms as isolated from biofilm on the stone sample from "Before Gilbeys" on 15 April 2002.

Antibiotic	***A. baumanii***
Ampicillin	R
Cefazolin	R
Co-trimoxazole	R
Gentamicin	S
Amikacin	S
Piperacillin	S
Cefuroxime	R
Ciprofloxacin	S
Ceftazidime	S
Cefoxitin	R
Cefotaxime	S
Amoxicillin/Clavulanic acid	R
Total % resistant = 50%	

S=sensitive, R=resistant

5.4 Discussion

The resistance patterns for the *E. coli* organisms collected over a period of time shows that there are definite subpopulations of organisms freely occurring in surface water or biofilms

in the Plankenbrug River that are resistant to some of the commonly available antibiotics. In this respect such sensitivity surveys of organisms occurring in free-flowing water is often seen in environmental epidemiology as an oblique indicator of degradation of the environment due to human impact. This impact can come either from humans themselves or from animal husbandry. Given the very low numbers of *E. coli* found above Kayamandi for almost the entire duration of the study, animal husbandry could not have been a very large contributor to the observed resistance patterns.

Enquiries were made about the possibility to use information on antibiograms requested for patient treatment at the Stellenbosch provincial hospital (as was originally envisaged as an aim for this study). It was revealed that antibiograms are almost never requested because of cost constraints. The hospital does not have its own laboratory any more and all samples have to be sent to Hottents-Holland provincial hospital. Apparently, patients are treated by broad spectrum antibiotics in the absence of precise diagnoses of organisms involved in specific infections. A doctor from Stellenbosch Hospital also visits the Kayamandi clinic on a regular basis, implementing the same policies as at Stellenbosch Hospital. Since by far the majority of the inhabitants of Kayamandi make use of these two health service facilities, this diagnostic/antibiotic policy will definitely pertain to most of the inhabitants needing treatment for bacterial infections. It is not known to what extent one can see the antibiotic resistance patterns in the river as indirectly related to what occurs in the community of Kayamandi, but this is also an aspect needing clarification. If such links are found, then the diagnostic and treatment policies of the health authorities need to be revised.

This kind of survey needs to be carried out on a much wider scale in the rivers of the Western Cape that are contaminated by untreated sewage. It is an essential part of the picture of health impacts of poor sanitation and has been neglected in this region in the past. At present the impact of human activity in general on rivers and water supplies are very narrowly defined. In most cases, it reduces to the monitoring of a few chemical parameters. Without other sensitive indications such as antibiotic resistance of organisms in rivers, the consequences of such wide-ranging activities as agriculture, health services and sanitation, remedial action cannot be taken in time before widespread damage sets in.

CHAPTER 6

6. PRELIMINARY TESTS ON CHLORINATION RESISTANCE OF ORGANISMS IN THE RIVER WATER

6.1 Introduction

Chlorination remains the most affordable inactivation of micro-organisms in water supplies. The levels of chlorine resistance have not been done in any of the river systems in the catchment area. This is a sensitive area from the standpoint of tourism, agriculture and contact of the local population with the river water. The health implications of such resistant organisms are considerable. There is increasing concern about the ability of local authorities in the Western Cape to treat raw water to prevent chlorine resistance from developing. Should chlorine resistance be present to any significant degree in organisms occurring in free-flowing waters, this will imply that the ability to disinfect raw water from such polluted rivers is severely impaired.

6.2 Materials and methods

6.2.1 *Indicator organism*

E. coli was chosen as indicator organism.

6.2.2 *Chlorine resistance assay*

The river was sampled at downstream from Kayamandi ("Below Kayamandi"). The samples were collected in autoclaved sample bottles according to the guidelines set out by the SABS, 1984. The standard methods to assess the resistance of organisms isolated from river water to chlorination disinfection were followed (Maurer, 1978; Harley and Prescott, 1996). The samples were mixed thoroughly, filtered and divided in 5 equal samples of 225 ml each into clean sterile bottles. The rest of the water in the original sample bottle was used as a control sample with no chlorine added and was tested as usual for faecal coliforms and *E. coli*.

Freshly manufactured Medisure® chlorine powder (Medichem, Tokai, South Africa) was used. The powder was weighed to four decimals of a gram and added to the sample water in the bottles so that initial concentrations formed a range: 0,1 mg/l; 0,3 mg/l; 0,4 mg/l, 0,5 mg/l and 0,6 mg/l. This range of initial concentrations was decided upon after consultation with the Chief Superintendent of the Cape Town water treatment works. The calculations of the weight of chlorine powder needed to make up the various concentrations to the desired available chlorine were verified by testing a sample of the 0,1 mg/l concentration in sterile deionised water with a Hach 2000 spectrophotometer.

After 30 minutes, 1 ml of the contents of each of the bottles of sample water from the river plus chlorine were added to 9 ml non-ionic ethoxylated sorbitan ester (Tween 80) plus nutrient broth. These were left covered on the laboratory bench for 30 minutes to recover. The standard determinations for faecal coliforms and *E. coli* were then carried out.

River water and chlorine mixture from each bottle were plated out on MacConkey agar, bacitracin agar and trypticase soy agar. These were incubated overnight. From the river water and chlorine mixture, 1 ml was added to 9 ml serum broth and also incubated overnight. This was done to verify by means of another method whether any *E. coli* survived the various concentrations of chlorine. Selected organisms cultured in this manner were also subjected to antibiotic resistance testing (Section 5).

The rest of the river water and chlorine mixture was left for a further hour on the bench (total exposure time 90 minutes). These bottles were agitated from time to time. After the extra hour, 1 ml of the contents of each of the bottles of sample water from the river plus chlorine were added to 9 ml Tween 80 plus nutrient broth. These were left covered on the laboratory bench for 30 minutes to recover. The standard determinations for faecal coliforms and *E. coli* were then carried out.

River water and chlorine mixture from each bottle were again plated out on MacConkey agar, bacitracin agar and trypticase soy agar. These were also incubated overnight. From the river water and chlorine mixture, 1 ml was added to 9 ml serum broth and also incubated overnight.

6.3 Results

6.3.1 *After 30 minutes contact with chlorine*

Table 6.1: Results obtained from organisms extracted from water sampled "Below Kayamandi" on 11 June 2002 after treatment with various chlorine concentrations for 30 minutes

Total chlorine concentration	**Faecal coliforms per 100 ml surviving**	***E. coli per* 100 ml surviving**
0 (control)	27 810	27 810
0.1 mg/*l*	4 930	4 930
0.3 mg/*l*	792	792
0.4 mg/*l*	3 290	3 290
0.5 mg/*l*	0	0
0.6 mg/*l*	2	2

The results from the cultures of organisms that had the chlorine neutralised by Tween 80 or without neutralising both support the above observations. In fact, the organisms were so vigorous that neutralising of the chlorine with Tween 80 made almost no difference to the number of surviving colonies. Those plated out without Tween 80 treatment survived almost as well as their counterparts for whom the chlorine was neutralised and the organisms revived with the aid of nutrient broth.

The two *E. coli* organisms that survived the highest chlorine concentration after 30 minutes were frozen and are available for further research.

6.3.2 *After 90 minutes contact with chlorine*

Results obtained from organisms extracted from water sampled "Below Kayamandi" on 11 June 2002 after treatment with various chlorine concentrations as used in the previous experiment, but after a further 60 minutes (one-and-a-half hours in total) (Table 6.2).

Table 6.2: *E. coli* organisms surviving various chlorine concentrations after 90 minutes

Total chlorine concentration	**Fecal coliforms per 100 ml surviving**	***E. coli* per 100 ml surviving**
0.1 mg/*l*	24	24
0.3 mg/*l*	12	7
0.4 mg/*l*	6	6
0.5 mg/*l*	0	0
0.6 mg/*l*	0	0

These results were also supported by the outcome of the plated out organisms (with and without Tween 80).

6.4 Discussion

An anomaly occurred in the results of the number of organisms surviving after 30 minutes of contact with the chlorine. At 0,3 mg/l chlorine, 792 *E. coli* organisms survived, while at 0,4 mg/l 3 290 organisms survived - an increase of 315% over the previous concentration rather than the expected reduction. This was double-checked. We do not know why more organisms survived at the higher concentration. It may have simply been a factor of coincidental selection of more resistant organisms. Even though the number of organisms surviving contact with the chlorine reduced after the extra 60 minutes of contact time compared with the survival after 30 minutes, some organisms still survived even up to 0,4 mg/l. If one keeps in mind that only 225 ml of river water was exposed to each concentration of chlorine, then the number of organisms that would have survived

conventional chlorination of bulk water supplies sourced from the river would have been considerable.

Payment (1999) evaluated transient failures or reductions in treatment efficacy in two water distribution systems in Canada. The experiments evaluated the survival, inter alia, of *E. coli* up to 0,7 mg/l total chlorine. His results suggested that the maintenance of a free residual concentration of chlorine in a distribution system did not provide a significant inactivation of pathogens, especially those represented by intrusions into the distribution system, or regrowth of organisms in biofilms along the distribution line. Cross-connections and back-flows can also introduce pathogens into the distribution system. Typically those failures would result in infecting clusters of individuals, without eliciting the usual signs accompanying large outbreaks and thus remain undetected.

Payment (1999) found *E. coli* organisms surviving various concentrations of total and free residual chlorine up to 100 minutes after introduction of the pathogens into the chlorinated water. The results from the present pilot study indicate that organisms can survive after 30 minutes even at the highest concentration used while some organisms even survived after 90 minutes in concentrations up to 4 mg/l. Chlorine concentrations vary considerably in water distribution lines and the users at the furthest ends of the line often receive water with low concentrations of chlorine. Especially those users may be more likely to receive water with pathogens surviving the chlorination treatment of the water. At the lower concentration ranges used in the present study, there were definite signs of resistant *E. coli* organisms present in the sampled water.

Antiseptics and disinfectants are extensively used in e.g. health care settings for the disinfecting of a variety of topical and hard-surface applications. Although there is a wide variety of active ingredients found in these products, chlorine figures prominently amongst them. The widespread use of such agents have prompted speculation on the development of microbial resistance, in particular whether antibiotic resistance is induced by disinfectants (McDonnell and Russell, 1999). In the light of the increased chlorine resistance noted in the data above and the increased antibiotic resistance to some antibiotics noted in the organisms surviving the chlorination treatment (Table 5.2), the present preliminary studies found support for these concerns. This is a matter of great concern for all health authorities.

CHAPTER 7

7. GENERAL DISCUSSION AND CONCLUSIONS

The complete picture emerging from the large amount of data available on this seriously polluted river is one of high health risk and grave environmental degradation. Apart from the serious shortcomings in the provision and maintenance of sanitation to the dense settlement of Kayamandi, the periodic failing of he sanitation system of the rest of the town of Stellenbosch also provided sporadic sewage intrusions into the river. The latter situation occurred immediately prior to at least a third of the sampling occasions, thus leading to the conclusion that the extent of the pollution load, apart from what originates in Kayamandi, is large.

The role of the authorities in dealing with the situation has been problematic so far and the impasse around local authorities' transgressions of the various pieces of legislation safeguarding our natural water sources should be sorted out without delay.

On the whole, regular monitoring of microbiological water quality is seen as a routine action with very few research benefits. This study has however shown the very opposite. If it were not for the monitoring over several seasons, the full picture of the sources and impact of this pollution would never have become known. The dense settlement of Kayamandi would have been viewed as the obvious source of all the pollution, which is not the case. This would have resulted in inadequate measures to combat the pollution, trailing problems into the future.

The information obtained on the extent of the antibiotic resistance and resistance to chlorination should be viewed as only the beginning of such monitoring in the Eerste River Catchment Area. Such indicators of water quality and the impact of pollution are much neglected - indeed absent from all previous efforts to monitor the impact of human waste on the catchment area.

Great efforts were made to trace as many previous studies done on the Plankenbrug River or indeed the Eerste River Cathment System, as possible. Some were traced, but they all turned out to be small studies carried out by students for coursework and they were not of the extent or sophistication that provided credible conclusions. Many of these studies suffered from severe methodological flaws. Among the few that were done on a higher level, the one by Rossouw (1999), can serve as an example. None of the studies however, considered any health impacts and the few studies mentioning the determination of *E. coli*, only used it as an indicator of pollution. No studies reported investigating any other pathogenic organisms, nor their antibiotic resistance or chlorination resistance.

There is reason for concern regarding the organisms identified as present in the river system. Although some of them are known to occur in free-flowing water even in pristine areas, the numbers of those organisms seen in the water at times resembled the situation in untreated sewage.

Antibiotic sensitivity in broad terms represent the ability of the health services to treat infections resulting form these organisms. It has been mentioned before the study started that there would probably not be very many organisms in free-flowing water exhibiting antibiotic resistance, but this turned out not to be the case. Further studies are needed to link the drug policies of the local health service points to the resistance patterns observed in the environment with a view to effecting changes if need be.

Chlorination resistance is of equal importance, but to a different sector of society. Water for both human consumption and agricultural use is becoming scarce and no source of water can be viewed as not needed or not important. Chlorination remains one of the easiest and cheapest options for bulk disinfection of large quantities of raw water. The fact that organisms survived the contact with chlorine even after 90 minutes of contact time is cause for concern. The further disquieting indication that those organisms surviving chlorine treatment may have a tendency towards increased resistance to some antibiotics at least carry serious implications for bulk water providers as well as the disinfection of hospitals, food manufacturers, etc.

Although the undertaking of community surveys and sanitation education programmes on a scale done in this study is rare and a luxury, the value of the information obtained shows that some efforts at enumeration of the community (e.g. a properly designed sample survey and community diagnosis) could be of immense value. The authors were told by many so-called experts before the study started that they would not be able to see any difference in the pollution levels in the river before and after the education campaigns and toilet repair campaigns. Dramatic reductions in the levels were in fact recorded, and to add to the credibility of the reduction, those levels started rising again immediately after the campaigns and repairs were stopped. Regular determination of microbiological water quality is therefore a sensitive enough indicator in this scenario to monitor the interventions in sanitation and services in Kayamandi that are sure to follow on this study.

PART B

MICROBIOLOGICAL ANALYSIS OF IRRIGATION WATERS AND FOOD CROPS

by

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CHAPTER 8

8. LITERATURE REVIEW

8.1 General introduction

Hepatitis A virus (HAV) is a major cause of morbidity associated with faecally contaminated food and water (Mead *et al.,* 1999). Epidemiological studies suggest that astroviruses (AstV) are a more common agent of gastroenteritis than previously recognised (Herrmann *et al.,* 1991; Greenberg and Matsui, 1992; Marx *et al*., 1998a; Steele *et al*., 1998) and are increasingly being identified as an important source of foodborne disease (Ferrari and Torres, 1998; Walter and Mitchell, 2000). Outbreaks have been associated with food and water-related incidents (Kurtz and Lee, 1987; Oishi *et al.,* 1994; Glass *et al.,* 1996). HAV and human astroviruses (HAstV) are primarily transmitted via the faecal-oral route with person-to-person spread being the most common route of infection (Midthun *et al.,* 1993; Cuthbert, 2001). Many factors, including changing lifestyles and demographics, faster and more frequent travel, decreasing water supplies in certain countries and enhanced importation of foods may be behind the increase in food- and waterborne infections (Cuthbert, 2001). HAV and HAstV are also responsible for considerable economic losses worldwide (Marx, 1997; Cuthbert, 2001). However, due to the prevalence of many asymptomatic or mild infections, the true clinical and economic impact of food- and waterborne HA and HAstV infection may be underestimated (Marx, 1997). Molecular methods are available for the detection of HAV and HAstV but have, until recently, had limited applicability in food and environmental virology (Richards, 1999).

In view of these recent findings, better approaches to prevent the contamination of foods with HAV, HuCV and HAstV via irrigation and processing water, food handlers and sewage are needed. In addition, more effective techniques for the recovery and detection of these viruses in food and water will be beneficial (Keddy, 1998).

8.2 Hepatitis A virus

Early literature, dating as far back as the 8th century AD, describes various types of jaundice-like diseases. Symptoms of hepatitis A (HA) were only observed accurately by Blumer in 1923 (Blumer, 1923). It would be half a century later before HAV was unequivocally identified for the first time by immune electron microscopy (IEM)(Feinstone *et al*., 1973).

HAV is a member of the *Picornaviridae* family (Ticehurst *et al.,* 1986; Cohen, 1989; Melnick, 1992) and due to its unique biochemical and biophysical characteristics has been placed into its own genus, *Hepatovirus* (Minor, 1991). HAV is a non-enveloped,

hepatotropic virus, 27 - 32nm in diameter and appears roughly spherical by electron microscopy (EM) (Feinstone and Gust, 1988).

The nucleotide sequence of HAV, while relatively well conserved among different isolates, is also widely divergent from those of the other picornaviral genera (Beard and Lemon, 1999). Human HAV isolates show no evidence of antigenic variability and share no antigenic determinants with any of the other picornaviruses or human hepatitis agents (Beard and Lemon, 1999). There are two biotypes (King *et al*., 2001) within a single serotype of HAV (Rakela *et al.,* 1977; Dawson *et al.,* 1984; Koff, 1998; King *et al*., 2001). On the basis of their nucleic acid sequence heterogeneity, HAV isolates could be differentiated into seven unique genotypes. A genotype is defined as a group of viruses that differ at no more than 15% of base positions (Robertson *et al.*, 1992). Human isolates of HAV are categorised within four genotypes. In addition there are three genotypes exclusively associated with Old World monkeys (Robertson *et al.*, 1992). In some geographical regions, related isolates cluster suggesting endemic spread of the virus, while in other regions several genotypes circulate (Robertson *et al.,* 1992).

8.2.1 *Biochemical and biophysical characteristics*

A major distinguishing feature of HAV among the picornaviruses is its exceptional stability (Siegl and Weitz, 1996). HAV has been found to resist heating to 60°C at a neutral pH for 60 min (Parry and Mortimer, 1984). At higher temperatures (80°C) infectivity is lost rapidly in a physiological, isotonic environment. However, in the presence of 1M magnesium chloride, HAV retains its structural integrity and biological functions even at temperatures of up to 80°C (Hollinger and Ticehurst, 1996). HAV has a buoyant density of 1,32 – 1,34 g/ml in caesium chloride (Hollinger and Ticehurst, 1996). A variation in pH between three and ten has no demonstrable effect on the stability of the virus.

Survival of HAV in the environment is favoured at low relative humidity (Mbithi *et al.*, 1991). HAV is not inactivated by chloramine-T (1 g/L for 15 min at 20°C or perchloracetic acid (300 mg/L for 15 min at 20°C)(Cuthbert, 2001). HAV is inactivated by autoclaving (121°C for 20 min), by ultraviolet radiation (1,1 W at a depth of 0,9 cm for 1 min), by formalin (8% for 1 min at 25°C) or by chlorine (10 – 15 ppm residual chlorine concentration after 30 min; free residual chlorine concentration of 2,0 to 2,5 mg/L for 15 min) or chlorine containing compounds (sodium hypochlorite) (Hollinger and Ticehurst, 1996; Feinstone and Gust, 1997). Most studies indicate that HAV may be more chlorine-resistant than other picornaviruses (Siegl, 1984; Mbithi *et al.*, 1991; Feinstone and Gust, 1997).

8.2.2 *Clinical manifestations*

The incubation period of HA ranges from two to seven weeks, with an average of 28 - 30 days (Cuthbert, 2001), and is dependent on the infective dose (Hollinger and Ticehurst,

1996). Symptoms include fever, dark urine, malaise, stomach cramps and diarrhoea, all of which precede the onset of jaundice. Diarrhoea, nausea and vomiting are more frequent in children than in adults (Lemon, 1985). In some cases, the diarrhoea may be the result of another enteric infection acquired from the same source (Mason and McLean, 1962; Halliday *et al.,* 1991). Prior to the onset of clinical symptoms, HAV appears in blood and stool (Hollinger *et al.*, 1991). Maximal shedding of the virus in the faeces occurs three to ten days prior to the onset of clinical symptoms or jaundice, i.e. near the time when the alanine aminotransferase (ALT) level initially becomes elevated (Dienstag *et al.,* 1975). HAV RNA has been detected in the stool specimens of infected children for up to 10 weeks after the onset of symptoms (Robertson *et al.,* 2000), but infectivity studies in tamarins indicate that stools where HAV is only detectable by reverse transcriptase polymerase chain reaction (RT-PCR) have a lower potential for infectivity when compared to stools where HAV is detectable by enzyme immunoassay (EIA)(Polish *et al.,* 1999). Viremia has been documented during the incubation period and acute phase of the disease (Hollinger and Ticehurst, 1996). Recent studies indicate that viremia is detectable 19 days before the ALT peak and can persist for an average of 95 days thereafter (Bower *et al.,* 2000). However, the concentration of HAV was found to be the lowest during the convalescent period (Bower *et al.,* 2000). Relapsing or biphasic HA has been reported, with HAV RNA present in the serum during the initial rise in ALT and which becomes undetectable once the ALT levels drop, and reappears during the second ALT peak (Bower *et al.,* 2000). Although the incidence is higher in adults than children (Willner *et al.,* 1998) fulminant disease does occur in childhood (Debray *et al.,*1997). The spontaneous recovery rate for patients with acute fulminant HA in a retrospective study in the United States of America (USA), which included all age groups, was 35% compared to 39% in a French paediatric population (Debray *et al.,* 1997). The mortality rate for HA is generally low, quoted as <1,5% of all hospitalized icteric cases (Hollinger and Ticehurst, 1996). With approximately 30 000 cases reported yearly in the USA, the estimated fatality rate is 1,3%, likely a maximum rate because of relative underreporting of nonfatal disease (Cuthbert, 2001).

8.2.3 *Epidemiology*

HAV is prevalent worldwide (Cuthbert, 2001). In temperate climates seasonal peaks in autumn or early winter have been observed (Feinstone and Gust, 1997) and the incidence of HA infections may fluctuate widely in cycles extending over several decades (Cuthbert, 2001). HAV is primarily transmitted via the faecal-oral route with person-to-person spread the most prominent mode of transmission (Cuthbert, 2001). At greatest risk are staff and children in child care centres (Hadler *et al.,* 1986), military personnel (Tilzey *et al.,* 1992), household contacts of infected persons and patients in care and rehabilitation centres. Nosocomial outbreaks of HAV have also been reported amongst neonates in hospitals (Azimi et al., 1986). Linked to the faecal-oral transmission of HAV are common source outbreaks of HA due to faecally contaminated food and water (Cuthbert, 2001), the sexual transmission reported among homosexual men and infection in sewage workers (Tilzey *et al.,* 1992).

The prevalence of antibodies to HAV in a community is an excellent reflection of current and past standards of hygiene and sanitation (Feinstone and Gust, 1997). HA is most common in underdeveloped communities, where hygiene and sanitation standards are low. In these circumstances, childhood infections are frequent, usually asymptomatic and confer lifelong immunity (Martin, 1992). In developing countries with better hygiene standards, adults and adolescents are more susceptible to symptomatic infection (Martin, 1992). HA is endemic in South Africa (SA) (Robertson *et al.*, 1992) and the epidemiology is in a transitional phase with epidemiological patterns of both developed and developing communities (Martin, 1992). Studies in Kwa-Zulu Natal have demonstrated that the seroepidemiology of HAV infection in SA has not altered substantially from that reported in previous studies (Martin *et al*., 1994). It has been speculated that, with the changing socioeconomic conditions of the urban black population accompanied by an improvement in water supply and sewage disposal, a large pool of susceptible individuals may be accumulating (Martin, 1992).

8.2.4 *Prevention*

The prevention of HA on a worldwide basis is primarily a political, social, economic and engineering problem (Cuthbert, 2001). Providing a population with clean water, proper waste disposal and improved living conditions plays an essential role in the prevention of HA (Beard and Lemon, 1999).

Passive immunoprophylaxis by means of pooled immunoglobulin (Ig) prevents or attenuates disease in exposed individuals but does not provide long term protection (Martin, 1992). If administered prior to exposure, passive immunization can reduce the incidence of HA by up to 90% (Winikur *et al*., 1992). Although the minimum effective titre of Ig is not known, it appears that very low levels of passively acquired antibodies provide protection (Feinstone and Gust, 1996). Vaccination (active immunoprophylaxis) with predicted long-term protection against and future eradication of this disease is now possible (Flehmig *et al*., 1990, Cuthbert, 2001). The advent of an efficacious inactivated vaccine to prevent HA solves some problems, from decreasing titres of anti-HAV antibody to increasing numbers of susceptible persons in a population. Recent recommendations for prevention of HA include active immunization of travellers, men who have sex with men, users of illegal drugs, persons who are at occupational risk for infection, persons who have clotting factor disorders, and persons with chronic liver disorders (Cuthbert, 2001).

8.3 Astroviruses

In 1975, HAstVs were first described when small round viruses (SRVs) were detected by electron microscopy (EM) in the stools of infants during an outbreak of mild diarrhoea and vomiting in a maternity ward (Appleton and Higgins, 1975). Besides humans, AstV have been detected in stool samples from a variety of vertebrates including cats, dogs, pigs,

lambs calves and turkeys (Kurtz, 1994). AstV infection appears to be species-specific (Matsui and Greenberg, 1996) and to date no interspecies transmission has been documented (Matsui and Greenberg, 2001).

HAstV are classified in the family *Astroviridae*, and the genus *Astrovirus* (Monroe, 1999). AstV are non-enveloped, small, round virions, 28 – 30 nm in diameter, with a smooth edge and a five to six pointed star- shaped surface (Appleton and Higgins, 1975; Madeley and Cosgrove, 1975; Madeley, 1979; Caul and Appleton., 1982; Matsui and Greenberg, 1996). HAstV can be classified into eight serotypes that correlate with genotypes (Noel *et al.*, 1995; Taylor *et al.*, 2001a). HAstV-1 has been reported to be the most prevalent serotype detected in the United Kingdom (Noel and Cubitt, 1994), Australia and Japan. HAstV-2 to HAstV-5 seem to be less common and HAstV types 6 and 8 are seldom detected (Glass *et al.*, 1996). Although there is very little data available on HAstV serotypes in SA, recent studies indicate that HAstV-1 predominates in the Pretoria area (Nadan *et al*., 2001, Taylor *et al.*, 2001b).

8.3.1 *Biochemical and biophysical characteristics*

HAstV is acid stable at pH 3 (Kurtz and Lee, 1987). HAstV can withstand the action of chloroform, lipid solvents and non-ionic, anionic and zwitterionic detergents (Kurtz and Lee, 1987). Ethanol and methanol, at 90% concentrations, reduce HAstV titres, with methanol being more effective than ethanol. The virus is capable of surviving temperatures of 60°C for five but not ten minutes (Kurtz and Lee, 1987). HAstV is morphologically stable at ultralow temperatures (-70°C to -85°C) for six to ten years, but disruption may occur with repeated freezing and thawing (Williams, 1989).

In water, HAstV appears to be more resistant at low temperatures and may survive in seawater for extended periods of time, but is more rapidly inactivated in marine than fresh water. HAstV shows log reductions of up to 3 units in the presence of 1 mg of free chlorine/litre of water after 120 min (Abad *et al*., 1997)

8.3.2 *Clinical Manifestations*

An incubation period of three to four days is most common (Kurtz, 1994), although longer and shorter incubation periods have been documented (Midthun *et al.*, 1993). Clinical symptoms include a watery diarrhoea, generally lasting two to three days (Greenberg and Matsui., 1992), but prolonged diarrhoea lasting one to two weeks with virus shedding has also been reported (Kurtz, 1994). Diarrhoea is accompanied by fever, malaise, nausea, vomiting and abdominal discomfort (Kurtz and Lee., 1987; Blacklow and Greenberg, 1991; Greenberg and Matsui., 1992). Persistent HAstV infections have recently been recognised in immunocompromised patients, and in some studies HAstV has been found to be the most common virus associated with gastroenteritis in patients with HIV (Grohmann *et al.*, 1993) and bone marrow transplant recipients (Cox *et al.*, 1994).

8.3.3 *Epidemiology*

The predominant route of transmission of HAstV is the faecal-oral route (Midthun *et al.*, 1993; Kurtz, 1994), with direct spread in families or institutions such as hospitals, schools, day care centres and homes for the elderly (Nazer *et al.*, 1982; Herrmann *et al.*, 1991; Kotloff *et al.*, 1992). The most susceptible populations to infection are paediatric (Ford-Jones *et al.*, 1990), geriatric and immunocompromised patients (Grohmann *et al.*, 1993; Cox *et al.*, 1994). Infection can be community (Kotloff *et al.*, 1992; Utagawa *et al.*, 1994) or nosocomially acquired (Esahli *et al.*, 1991) and contaminated food and water (Kurtz and Lee, 1987; Oishi *et al.*, 1994) are being implicated as vehicles of infection more frequently. HAstV appear to occur worldwide and have been detected in China, Japan, Thailand, Australia, SA (Marx *et al*., 1998a), Malawi, Italy Sweden, United Kingdom and the United States (Moe *et al*., 1991).

The prevalence of HAstV in SA appears to be similar to that reported in other developing countries, as indicated by a comparative epidemiological study of HAstV and rotavirus gastroenteritis in young children in SA (Marx *et al.*, 1998a; Steele *et al.*, 1998). The true incidence of HAstV-associated gastroenteritis is, however, difficult to assess as the diarrhoeal episode is usually mild and does not usually require medical attention (Greenberg and Matsui, 1996). Although the determinants of immunity to HAstV are not well understood, it is generally assumed that antibody acquired to HAstV early in life renders long-lasting immunity (Kurtz *et al*., 1978; Greenberg and Matsui., 1992).

8.3.4 *Prevention*

Prevention of HAstV transmission is an essential element of control. Strict enforcement of hygiene procedures, personal hygiene and special disposal and disinfection of contaminated material may decrease transmission within a family or institution. It is uncertain what the value of a HAstV vaccine would be. Epidemiological studies, however, suggest that the development of a vaccine would be a worthwhile objective (Herrmann *et al.*, 1990; Cruz *et al.*, 1992; Greenberg and Matsui, 1996). Furthermore, the imminent licensing of a rotavirus vaccine may lead to AstV becoming the primary cause of gastroenteritis among children in the near future (Keddy, 1998).

8.4 The role of food and water in the transmission of HAV, HuCV and HAstV infection

8.4.1 *Introduction*

The provision of food and water supplies has become increasingly complicated as society becomes more urbanized and the global economy more complex (Conlon, 1999). Often, there are large, centralized facilities for food production such as school cafeterias (Oishi *et al*., 1994) and restaurants (Kaul et al., 2000), with the risk that contamination early in the production process may lead to widespread infection. Water becomes a vehicle for a

variety of infections when contaminated by human or animal faeces (Conlon, 1999). Studies conducted in the past decade have shown that HAstV (Ferrari and Torres, 1998), and more specifically HAV, are being incriminated on a more frequent basis in common source disease outbreaks involving food and water (Weltman *et al.,* 1996; Petterson and Ashbolt, 2001; Taylor *et al.*, 2001). This situation is aggravated by the fact that these viruses are relatively resistant to inactivation by heat and chemical disinfectants and can retain their infectivity for several days in water and on fruits and vegetables that are often consumed raw. Three major types of foods are responsible for outbreaks of viral foodborne disease. These foods are:

i) shellfish harvested from sewage polluted waters (Bosch *et al*., 2001)
ii) food products contaminated by sewage-polluted irrigation water (Petterson and Ashbolt, 2001)
iii) delicatessen and other foods (Weltman *et al*., 1996; Daniels *et al.*, 2000).

8.4.1.1 *Minimally processed foods*

Minimally processed foods (MPFs) include salads, vegetables, fruits and other fresh produce that require only minimal processing before consumption. Fruits and vegetables are usually contaminated through human contact during harvesting or processing. Contamination via wastewater and sludge, used for crop irrigation and fertilization, have also been documented (Petterson and Ashbolt, 2001).

The threat of foodborne viral diseases has triggered worldwide interest in the fate of viruses on fresh produce irrigated with sludge or sewage effluents (Petterson and Ashbolt, 2001). A serious threat probably stems from vegetables and fruits that have a relatively short growth period (eg, radishes) and are eaten raw. Prior to harvesting, MPFs may also be contaminated by wastewater effluents used for irrigation purposes. This practice has been responsible for many disease outbreaks caused by bacteria, protozoa, parasitic helminthes and viruses (Bitton, 1980). MPFs are subject to contamination by food handlers after harvesting. An outbreak of HA in Scotland was linked to the consumption of raspberry mousse prepared from frozen raspberries (Reid and Robertson, 1987). The raspberries were obtained from several farms, and the three producers were implicated indirectly in a previous outbreak. Contamination of raspberries apparently occurred at the time of picking or packing, probably by a HAV excreter (Reid and Robertson, 1987). A multistate outbreak of HAV was traced to frozen strawberries processed in a single plant in California in 1990 (Niu *et al*., 1992). Nine hundred students, teachers and staff in Georgia and Montana developed HA from eating strawberry desserts. Epidemiological data indicated that contamination did not occur from an infected worker within the plant but most likely from an infected picker. HA has been linked to the consumption of commercially distributed lettuce (Rosenblum *et al.*, 1990). In 1988, 202 cases of infection in Kentucky occurred during a seven-week period. Case-control studies indicated that 71% of infected persons had eaten at one or more of three restaurants. HA was associated with consumption of uncooked diced tomatoes in Arkansas (Williams *et al.*, 1995). Twenty-five cases were

reported and evidence suggests that ill persons had eaten in one suspect restaurant. A common-source outbreak of HA occurred among 43 people who ate contaminated green onions at a restaurant in Ohio in 1998 (Dentinger *et al.*, 2001). The source of contamination was believed to be workers on one of two farms in Mexico, which had supplied the onions.

8.4.1.2 *Shellfish*

The most well-researched food type associated with foodborne viruses are molluscan shellfish such as oysters, mussels and cockles, which are usually found in shallow coastal or estuarine waters, commonly near sewage outlets. These shellfish are filter feeders that concentrate virus particles from surrounding water (Abad *et al.*, 1997; Bosch *et al*., 2001).

Human disease due to the consumption of shellfish contaminated by viruses was first recognized in 1956 with the report of a large outbreak of clam-associated HAV (Gerba, 1988; Jaykus, 1997). Since then, epidemiological evidence linking the transmission of enteric viral disease, mainly hepatitis and gastroenteritis, to shellfish has continued to increase (Gerba, 1988; Jaykus, 1997). In 1995, A mussel-associated HA epidemic occurred in Germany. A total of 42 people showed clinical symptoms after consuming pizza with contaminated mussels. Three of these patients infected a further 32 people after preparing food in their family restaurant (Kaul *et al.*, 2000). An outbreak of HA, affecting 183 people, recently occurred in Spain. Epidemiological evidence pointed to an association of the outbreak with consumption of Coquina clams, imported from Peru (Bosch *et al.*, 2001). Extreme cases include the outbreak of 300 000 cases of HA and 25 000 cases of viral gastroenteritis in Shangai in 1988 by shellfish harvested from a sewage-polluted estuary (Halliday *et al.*, 1991). HAV, Norwalk-like viruses and AstVs are recognized as the predominant enteric viruses linked epidemiologically to shellfish-associated viral diseases (Richards, 1985; Le Guyader *et al.*, 2000; Schwab *et al.*, 2001).

8.4.1.3 *Delicatessen and other food sources*

A variety of foods that are uncooked, undercooked or contaminated after cooking are all possible vehicles for HAV and HAstV. As early as 1968, a community outbreak of HA occurred in West Branch, Michigan where the source of contamination was traced to glazed doughnuts from a bakery. A similar common-source outbreak occurred 26 years later in Rochester, in the New York area (Weltman *et al.*, 1996). In March 1988, an outbreak of acute gastroenteritis occurred among students at a Texas university where 125 students fell ill. Case-control studies revealed that illness was significantly associated with eating ham from the university's main cafeteria deli bar. The suspected source of contamination was a food handler (Daniels *et al.*, 2000). An outbreak of HAstV occurred amongst students and teachers in Japan when contaminated food was consumed at the school cafeteria (Oishi *et al.*, 1994). The source of contamination was believed to be a food handler.

8.4.1.4 *Water*

Current water treatment practices are unable to provide virus-free wastewater effluents; consequently, human pathogenic viruses are routinely introduced into marine, dam and estuarine waters through the discharge of treated and untreated sewage (Abad *et al.*, 1997). Although faecally-polluted water has been implicated as a possible source of HA and gastroenteritis in South African canoeists (Taylor *et al.*, 1995), there is little data on the presence of these viruses in water sources used for domestic, agricultural and recreational purposes in SA (Taylor *et al.*, 2001). This is probably due to the lack of an infrastructure for the detection and recording of such infections (Grabow, 1996). There is no reason to believe that risks of waterborne disease in SA are any different from those in the rest of the world. Escalating demands and pollution of already limited water sources, particularly in rural and developing communities, may even elevate the risks (DAWN, 2001).

Common-source outbreaks of HAV, HuCV and HAstV have been reported in various water systems (Bosch et al., 1991). Amongst these recreational waters (Gammie and Wyn-Jones, 1997; Taylor *et al.*, 2001), surface water systems, domestic water and irrigation water (Devaux, *et al.*, 2001; Petterson and Ashbolt, 2001) feature most prominently. Subsoil water, which is acquired through boring, is also a potential source for viral contamination (DAWN, 2001). In regions where the water table is very low, people use water that is acquired in the initial stage of boring. This is rainwater that has potentially been contaminated with sewage. Contamination of recirculated water used for hydrocooling or of ice that is layered on top of fruits and vegetables before shipping is also a potential source of contamination (Dentinger *et al.*, 2001).

8.4.2 *Viral recovery and detection from food and water sources*

A variety of methods have been investigated for processing shellfish samples, all including several steps towards separation of virus particles from shellfish tissues before viral RNA purification and amplification. Two general schemes, designated extraction-concentration and adsorption-elution-concentration, are used. Such methods generally imply a combination of different techniques and are preceded in all cases by mechanical homogenization of the shellfish. More recently, a simplified procedure based on the enzymatic liquefaction of shellfish digestive tissues, followed by clarification of the lysate using dichloromethane extraction has been developed (Legeay *et al.*, 2000). There is no information on the penetration of plant tissue by HAV and HAstV. Therefore, until raw fruits and vegetables are processed or included in any other food items, virus is present only on their outer surfaces, thus making recovery and detection of viruses from MPFs very specific. In contrast to grapes and tomatoes, which have smooth surfaces, lettuce and strawberries have rugged surfaces which are difficult to clean properly without damaging the surface, and which allow the prevalence of moist conditions, thus prolonging virus survival (Bitton, 1980).

In the past, viruses have generally been extracted from MPFs by using an elution-precipitation approach with some variations. Homogenization is avoided, and viruses are

recovered from surfaces more efficiently if the entire food substance is not incorporated into a test suspension. Other variations from the elution-precipitation include the use of an unusually alkaline buffer, to recover virus from frozen strawberries (Schwab *et al.*, 2000). Methods used involved washing of food samples with a buffer such as a guanidinium-phenol-based reagent for immediate extraction (Schwab *et al.*, 2000), phosphate buffered saline (PBS), or any pH specific buffer, depending on the surface pH of the food being processed. Chloroform-isopropanol or polyethanol-glycol precipitation of viral RNA after washing is followed by RNA extraction with TRIzol® reagent or any one of the commercial RNA extraction kits (Schwab *et al.*, 2000).

Traditional methods of detecting human enteric viruses in shellfish have relied on infectivity assays in cell cultures (Casas and Suňén, 2000). Cell culture, although being the only method that reveals infectiousness, is limited mainly by lack of sensitivity, lengthy analysis time and a lack of susceptible cell lines (Casas and Suňén, 2000).

Methods for the recovery and detection of HAV and HAstV, as well as other enteric viruses such as rotavirus, adenovirus and NLVs, are rapidly advancing with the advent of molecular techniques (Schwab *et al.*, 2000). Since the nature of a foodborne outbreak requires immediate results for accurate assessment and prevention, an essential factor is time. Recovery procedures described previously are followed by viral RNA amplification by RT-PCR with virus-specific primers.

8.5 Significance of food- and waterborne viral infections

Changes in human demographics, food preferences, food production and distribution systems, and lack of support for public health resources and infrastructure have led to the emergence of novel as well as traditional food- and waterborne diseases (Grabow, 1996). With increasing travel and trade opportunities, the risk of contracting and spreading food- and waterborne illnesses now exist locally, regionally, and even globally (Ford and Colwell, 1996).

Governments across the globe are increasingly finding themselves urgently in need of upgrading their domestic food safety systems. However, in many developing countries, such as SA, there is no comprehensive food safety system in place to upgrade.

There is very little data on the presence of HAV, HuCV and HAstV in food sources and domestic, agricultural and recreational water sources in SA. This is partly due to the lack of infrastructure for the detection and recording of such infections (Grabow, 1996). There is no reason to believe that risks of food- and waterborne disease in SA are any different from those in the rest of the world. Escalating demands and pollution of already limited water sources, particularly in rural and developing communities, may even elevate risks (DAWN, 2001). Common-source viral foodborne outbreaks, such as SRSV-associated

gastroenteritis, have been described in SA (Taylor *et al.,* 1993), but to date, no food- and waterborne outbreaks of HA or HAstV have been documented due to the relative underreporting of these diseases in SA. HAV and HAstV have been detected in raw and treated water sources (Taylor *et al.*, 2001a, Marx *et al.*, 1997; Grabow *et al.*, 2001) confirming the risk of water-borne transmission of these viruses.

CHAPTER 9

9. DEVELOPMENT AND ASSESSMENT OF VIROLOGICAL TECHNIQUES FOR THE RECOVERY AND DETECTION OF VIRUSES FROM MINIMALLY PROCESSED FOODS

9.1 Materials and methods

9.1.1 *Recovery of viruses from minimally processed foods*

The optimisation of methods for the recovery of viruses from MPFs were based on the findings of Bidawid *et al.* (2000) and Schwab *et al*. (2001). As MPFs are contaminated by the handler and/or the irrigation or processing water, the experimental procedure addressed the choice of recovery buffer and optimal RNA extraction procedure for detection of viruses on the surface of the MPFs. For the optimisation experiments, strawberries were chosen as an example of rough MPFs and grapes as an example of smooth MPFs. Details of the experimental procedure are summarised in Table 9.1.

Table 9.1: Summary of procedures for the recovery and detection of viruses from MPFs

BUFFER	TEMPERATURE	TIME	MPF	EXTRACTION
PBS	25ºC	24 h	Strawberries	QIAamp kit TRIzol
			Grapes	
	4ºC	24 h	Strawberries	QIAamp kit TRIzol
			Grapes	
MEM	25ºC	24 h	Strawberries	QIAamp kit TRIzol
			Grapes	
Tris-HCl	25ºC	24 h	Strawberries	QIAamp kit TRIzol
			Grapes	

9.1.1.1 *Seeding of the MPFs*

Prior to seeding the MPFs with HAV and/or HAstV, all possible contaminants on the surface of the MPFs were removed by first rinsing the MPF in a dilute solution of Extran® MA 01 (Merck) followed by rinsing in running tap water and then twice in distilled water. The MPFs were then air-dried and disinfected by exposure, on all sides, to UV light for 1 min.

MPFs (approximately 50g) were seeded with 500 µl of a 1:10 dilution ($10^{5.5}$ $TCID_{50}$) of the virus by gently swirling the fruit in the virus suspension for five minutes. After being left to dry for 1 h in a Class II biological safety cabinet (Labaire), the MPFs were placed in a sterile container, covered with parafilm (Parafilm™ "M", American National Can, Chicago, IL.) and held overnight at the desired temperature.

9.1.1.2 *Comparison of recovery buffers*

The recovery efficacies of three buffers were tested, i.e. PBS pH 7.4 (Sigma), Tris-HCl pH 8.3 and MEM pH 7.2 (NIV). The seeded MPFs were gently swirled in 25 ml of the respective buffer for 5 min to recover the virus from their surfaces after which they were removed from the buffer and discarded. As an alternative, the seeded MPFs were also left in 25 ml of buffer overnight at 4ºC and processed as described above. Viruses were reconcentrated from the buffer, to a final volume of 2 ml, using the PEG/NaCl technique (Minor, 1991)

9.1.1.3 *Comparison of RNA extraction procedures*

Two methods, namely TRIzol® reagent, as per manufacturer's instructions, and the QIAamp viral RNA kit, were compared for the extraction of total RNA from the recovered viral suspensions. Of the 2 ml viral suspension 120 µl was used for the TRIzol® extraction and 140 µl for the QIAamp extraction, as per manufacturer's instructions.

9.1.2 *Recovery of viruses from irrigation water*

For optimisation of viral recovery and extraction from irrigation water, 1 litre of tap water was treated with 1 ml of Na_2SO_3 to neutralise free chlorine. The water was then seeded with 100 µl of a HAV/HAstV viral suspension. The PEG/NaCl as described by Minor (1991) or SiO_2 method as described by Baggi and Peduzzi (2000) were compared for the recovery of the viruses from 1 L of the seeded tap water. Viral RNA was extracted from the viral concentrates using the TRIzol® reagent method and viruses were subsequently detected by RT-PCR.

9.1.3 *Molecular detection of viruses*

9.1.3.1 *RT-PCR for HAV*

The RT-PCR oligonucleotide probe assay (Taylor *et al*., 2001) was used for the detection of HAV.

9.1.3.2 *RT-PCR for HAstV*

The RT-PCR oligonucleotide probe assay as described by Marx *et al*. (1998b) and modified by Taylor *et al*. (2001a) was used for the detection of HAstVs.

9.1.3.3 *RT-PCR for HuCV*

A sensitive and specific RT-PCR was developed and optimised for the detection of HuCVs using the primer pair described by Jiang *et al*. (1999) for the detection of both noro- and sapoviruses .

9.1.3.4 *Multiplex RT-PCR for the simultaneous detection of HAV and HuCV*

A multiplex RT-PCR, described by Schwab *et al*. (2000), was assessed for the detection of HAV and HuCVs.

9.2 Results and Discussion

9.2.1 *Recovery of viruses from minimally processed foods*

The viral titre of the HAV stock culture used to seed the MPFs was $10^{5.5}$ ml^{-1} . The titre of virus recovered from the different MPFs, in the selected buffer systems are summarised in table 9.2. Titres ($TCID_{50}$) were calculated using the Kärber formula.

Table 9.2: Comparison of titres of HAV recovered from MPFs using different buffers.

Buffer	Grape $TCID_{50}.ml^{-1}$	Strawberry $TCID_{50}.ml^{-1}$
PBS	$10^{2.3}$	$10^{2.2}$
PBS control	$10^{3.7}$	$10^{2.8}$
MEM	$10^{2.7}$	$10^{1.8}$
MEM control	$10^{2.0}$	$10^{2.2}$
TRIS-HCl	$10^{2.2}$	$10^{1.7}$
TRIS-HCl control	$10^{2.7}$	$10^{2.2}$

It is evident from the above comparison that viral recovery from grapes was more efficient than viral recovery from strawberries, i.e. a 2.7-log reduction in viral titre was observed with the strawberries, while only a 1.8-log reduction was noted for grapes with PBS. However the titre of virus in the control buffer was also lower than the stock titre suggesting that viral infectivity decreased during the experimental period, possibly due to factors such as temperature and time.

9.2.2 *Comparison of viral recovery buffer and viral RNA extraction methods from minimally processed foods*

The HAV RT-PCR results for the experimental procedures comparing and optimising viral recovery buffers and RNA extraction techniques for the recovery and detection of viruses, by RT-PCR, from the surfaces of MPFs are presented in Figure 1a. The confirmatory oligonucleotide hybridisation results are presented in Figure 1b. From these results it is evident that viruses were recovered and detected more efficiently from smooth MPFs, i.e. the grapes (positive RT-PCR signals in lanes 2-4, 6 and 7), than rough juicier MPFs, i.e.

strawberries (positive RT-PCR signal in lane 12). With regard to viral recovery from grapes, more prominent signals were obtained with the PBS/TRIzol buffer-extraction combination (positive RT-PCR signal in lane 2). Weaker signals were obtained with PBS/QIAamp recovery (lane 3), MEM/TRIzol recovery (lane 4) and MEM/QIAamp recovery (lane 5). The Tris-HCl/TRIzol recovery combination (lane 6) showed a signal weaker than that of lane 2, but stronger than lanes 3, 4 and 5. Viral recovery combinations of buffer and extraction method from strawberries showed only a positive signal with Tris-HCl/TRIzol recovery (positive RT-PCR signal in lane 12).

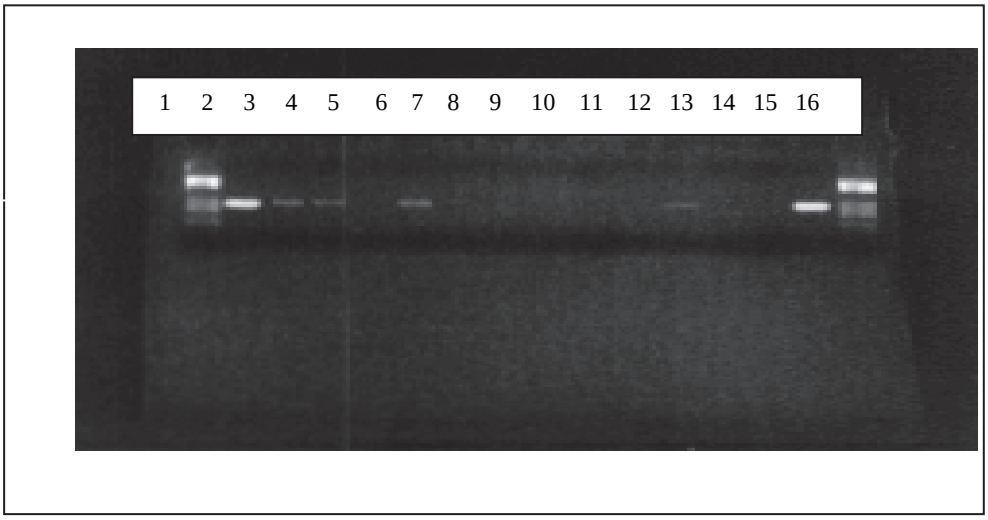

Figure 9.1a: A 2 % agarose gel of a HAV RT-PCR assay showing comparison of virus recovery buffers and RNA extraction techniques for minimally processed foods. Lane 1: MW Marker V (Roche); Lanes 2 – 7 show combinations of buffer and RNA extraction method used on grapes (Lane 2: PBS and TRIzol; Lane 3: PBS and QIAamp; Lane 4: MEM and TRIzol; Lane 5: MEM and QIAamp; Lane 6: Tris-HCl and TRIzol; Lane 7: Tris-HCl and QIAamp). Lanes 8 – 13 show combinations of buffer and RNA extraction method used on strawberries. (Lane 8: PBS and TRIzol; Lane 9: PBS and QIAamp; Lane 10: MEM and TRIzol; Lane: 11 MEM and QIAamp; Lane 12: Tris-HCl and TRIzol; Lane 13: Tris-HCl and QIAamp). Lane 14: Negative RT-PCR control; Lane 15: Positive RT-PCR control (cell-cultured HAV strain pHM-175); Lane 16: MW Marker V (Roche).

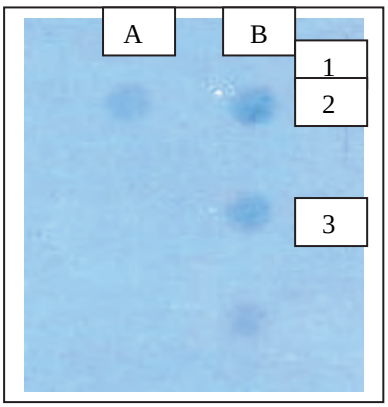

Figure 9.1b: A representative portion of the HAV hybridisation results showing the different buffers optimised.

A1: PBS, B1: PBS control, A2: MEM, B2: MEM control, A3:Tris-HCl, B3: Tris-HCl control.

The best recovery and detection results of HAV were however obtained when the MPF was washed overnight in PBS at 4°C followed by TRIZOL® viral RNA extraction. This optimised combination of buffer, washing time and

temperature and RNA extraction procedure was subsequently applied to all field samples received throughout the study.

Virus was more readily recovered and detected from the seeded grapes than the strawberries. This could possibly be due to the nature of the fruit, i.e. the strawberries are juicier and fleshier and more prone to damage during processing than the grapes. The rough surface may retain the virus more readily than the smooth surface of the grape. Seepage during processing could result in the presence of RT-PCR inhibitors. In addition strawberries have been shown to have anti-viral properties *in vitro* (Dr J Barnes, Dept of Community Health, University of Stellenbosch, personal communication).

CHAPTER 10

10. MICROBIOLOGICAL ANALYSIS OF IRRIGATION WATER AND ASSOCIATED MINIMALLY PROCESSED FOODS

10.1 Materials and methods

10.1.1 *Water and food samples*

10.1.1.1 *Commercially available minimally processed foods*

Samples (8) of commercially available MPFs were purchased from retail outlets. The number and source of MPF samples are summarised in Table 3.

Table 10.1: Source of commercially available MPFs

SOURCE	SAMPLE	NUMBER OF SAMPLES
Glenwood farms	Strawberries	6
Naboomspruit	*Cherry tomatoes	2

*Cherry tomatoes were used as grape alternatives due to the seasonal unavailability of grapes as the study progressed**.**

10.1.1.2 *Irrigation water and associated minimally processed foods from a rural area*

A total of nine sets of samples, i.e. irrigation and one or more associated MPFs, were received from Venda, five in 2001 and four in 2002. Water samples included borehole and river water and MPFs included tomatoes, onions and cabbages (Table 10.2; APPENDIX B)

10.1.2 *Bacteriological analysis of water and food samples*

10.1.2.1 *Water samples*

The water samples were analysed for total coliforms, faecal coliforms, enterococci and *Clostridium perfringens* using the membrane filtration technique. A sample volume of 10 ml was used for each assay and each assay was done in duplicate. The heterotrophic plate count was performed using the pour-plate method.

10.1.2.2 *MPF samples*

The water samples were analysed for total coliforms, faecal coliforms, enterococci and *Clostridium perfringens* using the spread plate technique and a sample volume of 0,1 ml.

Table 10.2: Source and type of irrigation water and MPFs from a rural area

SOURCE	DATE	SAMPLE
Tshituni Borehole (GP farmers)	2002-06-20	Borehole water Tomatoes Cabbage

	2002-06-09 2002-07-16 2002-08-20	Borehole water Tomatoes Cabbage Borehole water Tomatoes Cabbage Borehole water Cabbage
Tshikuwi River	2002-06-20 2002-06-09 2002-07-16 2002-08-20	River water Tomatoes Onions River water Onions River water Tomatoes Onions River water Tomatoes
Mawoni Borehole	2002-06-20 2002-06-09 2002-07-16 2002-08-20	Borehole water Tomatoes Cabbage Borehole water Tomatoes Cabbage Borehole water Tomatoes Cabbage Borehole water Tomatoes Cabbage
Phiphidi Borehole	2002-06-20 2002-06-09 2002-07-16 2002-08-20	Borehole water Onions Cabbage Borehole water Cabbage Borehole water Tomatoes Cabbage Borehole water

10.1.2.3 *Pathogens*

Water and MPFs were analysed for *Shigella* using SS agar, *Salmonella* using XLD agar and *E. coli* using EMB agar

10.1.2.4 *Identification of isolates*

Bacterial isolates were identified using standard biochemical assays.

10.1.3 *Recovery of viruses from water and food samples*

10.1.3.1 *Recovery from MPFs*

The MPF samples from Venda were washed on site, where possible, in 25 ml of PBS/50g MPF sample. The MPF-PBS wash was kept at 4°C and forwarded to the laboratory in Pretoria for viral analysis. Virus was recovered from the PBS using the PEG/NaCl viral recovery method. RNA was extracted from the viral concentrate using TRIzol® reagent.

10.1.3.2 *Viral recovery from the irrigation water*

Two litre samples of irrigation water were taken at the relevant borehole and river sites and sent to the laboratory in Pretoria. Viruses were recovered from the sample using the PEG/NaCl viral recovery method. RNA was extracted from the viral concentrate, for viral detection, using the TRIzol® reagent.

10.1.3.3 *Isolation and propagation of viruses in cell culture*

Cell culture flasks ($25cm^2$) Were seeded with the appropriate volume of 10^5 cells/ml and incubated for 24-48 h at 37ºC in a humidified 5% CO_2 incubator to provide a semi-confluent (70-80%) monolayer. Prior to infection with viruses recovered from water or MPF samples, the growth medium was withdrawn from and the cells were starved for 1 h at 37ºC in prewarmed serum-free MEM. After withdrawal of the starvation medium, the appropriate volume of virus or treated sample was left to adsorb to the cells for 1 h at 37ºC, with gentle swirling every 15 min.

For the isolation and propagation of HAV the appropriate volume of maintenance media was then added to the FRHk-4R cells followed by incubation at 37ºC for 21 days, with the replacement of the maintenance medium 10 days prior to harvesting. For HAstV and HRV, the appropriate volume of serum-free medium containing 10µg ml^{-1} trypsin (Trypsin 250; Difco, Detroit, MI) was added to each flask of PLC/PRF/5 and Caco-2 cell cultures and the infected cells incubated at 37ºC for 7 days, after which they were blind-passaged and the cells harvested after a further 7 days incubation at 37ºC.

10.1.4 *Molecular detection of viruses*

Cell cultures were examine for cytopathic effect prior to being harvested and analysed for HAV, HAstV and HuCVs as described previously (see 9.1.3)

10.2 Results and Discussion

10.2.1 *Commercially available minimally processed foods*

No viruses were detected in the limited number of samples assayed.

10.2.2 *Irrigation water and associated minimally processed foods from rural areas*

10.2.2.1 *Virological analysis*

HAV and HAstV were not detected in any of the irrigation waters and MPF samples tested to date. .In all instances the oligonucleotide hybridisation assay results correlated with, and therefore confirmed the RT-PCR results obtained. HuCVs were also not detected in any of the irrigation waters and MPF samples analysed. HRVs were detected in one tomato sample, collected on 2002-06-26 and irrigated with borehole water from the Tshituni Borehole. No viruses were detected in the corresponding borehole water. HRVs were also detected in the Tshikuwi River water drawn on 2002-07-09 while no viruses were detected in the associated MPFs. The presence of HRVs in the water samples could possibly be ascribed to the season as HRV infection peaks during the winter months.

The overall low occurrence of viruses could be ascribed to a number of factors. Borehole water is generally considered clean unless viral contamination by an external source has occurred no viruses were expected to be present in the borehole water samples. The small sample volume may have also influenced the results. River water samples were all collected at the same time of day from the same locations, but varying weather conditions at the time of sampling, i.e. heavy rains, could effect the outcome of the irrigation water field sample results.

10.2.2.2 *Bacteriological analysis*

Results from the analyses for indicator organisms and pathogenic bacteria are summarised in Appendix C. These data clearly indicate that at certain times there is evidence of human faecal contamination at levels, i.e. > 1000 cfu·100ml^{-1},which are unacceptable for irrigation water used for human produce. The detection of bacterial pathogens in some of the irrigation water samples as well as the MPFs suggests that the pathogenic bacteria may pose a greater health risk than the enteric viruses. This however needs further investigation to quantify the actual health risk posed to persons consuming the MPFs.

CHAPTER 11

11. GENERAL DISCUSSION AND CONCLUSIONS

The absence of viruses on commercially available MPFs is encouraging. However it must be borne in mind that only a limited number of samples were analysed. Analysis of more and/or different MPFs from different regions could present a totally different picture.

It is evident from the results that in the Venda region selected sources of irrigation water are faecally polluted and could possibly serve as a source of contamination to the associated MPFs or source of infection to persons using these water resources for domestic purposes. The detection of pathogenic bacteria in certain irrigation water and on selected MPFs could pose a health risk to consumers. Whether the bacteria on the MPFs originated from the irrigation water or from subsequent handling of the MPFs is not known and further research is required to quantify the health risk.

CHAPTER 12

12. REFERENCES

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APPENDIX C

Part B: Microbiological quality of irrigation water samples from Venda 2001

Sample identification	Date	Total coliforms (counts/100ml)	Faecal coliforms (counts/100ml)	Enterococci (counts/100ml)	Heterotrophic count (counts/100ml)	*C. perfringnes* (counts/100ml)
Mawoni Borehole	2001-10-03	950	240	0	2.0×10^4	480
	2001-10-10	110	20	190	1.0×10^3	70
	2001-10-17	410	320	0	9.0×10^2	30
	2001-11-07	80	0	20	9.0×10^2	0
	2001-11-21	3850	2740	610	2.0×10^3	470
Mawoni River	2001-10-03	4160	3780	480	2.1×10^8	2470
	2001-10-10	3600	3090	2270	3.7×10^6	1360
	2001-10-17	260	0	840	5.0×10^5	400
	2001-11-07	4800	280	2520	1.5×10^8	1340
	2001-11-21	4890	3260	2080	4.9×10^8	1950
GP Farmers (Tshituni) Borehole	2001-10-03	1660	1320	0	5.0×10^3	820
	2001-10-10	650	580	180	5.0×10^4	70
	2001-10-17	90	0	0	6.0×10^4	0
	2001-11-07	0	0	30	2.0×10^2	0
	2001-11-21	1200	1150	490	9.0×10^3	20
Smokey River	2001-10-03	3380	2720	390	1.5×10^7	700
	2001-10-10	2340	270	1580	1.0×10^8	1000
	2001-10-17	560	0	970	1.4×10^5	700
	2001-11-07	3440	450	2310	4.6×10^8	990
	2001-11-21	2530	950	3480	1.2×10^6	1270

Part B: Microbiological quality of the irrigated minimally processed food samples from Venda 2001

Sample identification	Date	Total coliforms (counts/100ml)	Faecal coliforms (counts/100ml)	Enterococci (counts/100ml)	Heterotrophic count (counts/100ml)	*C. perfringens* (counts/100ml)
Mawoni cabbage	2001-10-03	390	560	2800	3.0×10^{8}	520
	2001-10-10	520	410	3130	2.3×10^{8}	430
	2001-10-17	410	350	4620	8.0×10^{7}	530
	2001-11-07	360	410	3790	1.2×10^{7}	270
	2001-11-21	380	280	1100	2.8×10^{8}	360
Mawoni onions	2001-10-03	390	260	1420	8.3×10^{7}	350
	2001-10-10	340	270	1400	8.0×10^{6}	310
	2001-10-17	300	220	1220	8.0×10^{5}	300
	2001-11-07	250	210	0	1.0×10^{6}	0
	2001-11-21	130	60	180	8.0×10^{5}	280
Mawoni tomatoes	2001-10-03	420	310	590	5.0×10^{7}	190
	2001-10-10	410	310	550	5.0×10^{6}	230
	2001-10-17	270	140	300	7.0×10^{6}	130
	2001-11-07	270	150	100	1.7×10^{7}	70
	2001-11-21	710	320	330	2.8×10^{8}	1350
GP farmers (Tshituni) tomatoes	2001-10-03	120	0	0	1.2×10^{5}	270
	2001-10-10	60	0	0	7.0×10^{4}	250
	2001-10-17	0	0	0	9.0×10^{2}	150
	2001-11-07	0	0	0	3.0×10^{5}	0
	2001-11-21	580	340	230	6.0×10^{6}	0
Smokey tomatoes	2001-10-03	240	220	0	4.0×10^{7}	480
	2001-10-10	250	220	0	1.4×10^{7}	290
	2001-10-17	270	60	0	4.0×10^{5}	330
	2001-11-07	280	220	0	4.0×10^{6}	460
	2001-11-21	740	340	200	2.1×10^{8}	290
Smokey onions	2001-10-03	370	270	1290	7.3×10^{7}	0
	2001-10-10	280	250	1470	4.3×10^{6}	0
	2001-10-17	340	210	2270	9.0×10^{3}	0
	2001-11-07	410	160	0	3.0×10^{6}	0
	2001-11-21	390	260	1860	1.9×10^{7}	240

Part B: Microbiological quality of irrigation water samples from Venda 2002

Sample identification	Date	Total coliforms (counts/100ml)	Faecal coliforms (counts/100ml)	Enterococci (counts/100ml)	Heterotrophic count (counts/100ml)	*C .perfringens* (counts/100ml)	Somatic coliphages (counts/1ml)	F-RNA coliphages (counts/1ml)
Mawoni Borehole	2002-06-05	80	0	0	4.3×10^2	10	0	0
	2002-07-10	0	0	0	1.0×10^5	0	0	0
	2002-07-17	0	0	0	1.0×10^2	0	0	0
	2002-08-22	0	0	0	6.0×10^6	0	0	0
Phiphidi Borehole	2002-06-05	350	60	0	1.0×10^6	30	0	0
	2002-07-10	10	0	0	1.0×10^3	150	0	0
	2002-07-17	100	10	20	1.0×10^6	0	0	0
	2002-08-22	40	10	0	1.4×10^7	0	0	0
GP Farmers (Tshituni) Borehole	2002-06-05	$>10^4$	$>10^4$	40	7.0×10^6	10	0	0
	2002-07-10	600	80	180	1.8×10^4	10	0	0
	2002-07-17	450	40	20	1.0×10^4	0	0	0
	2002-08-22	300	120	30	6.0×10^6	0	0	0
Tshikuwi River	2002-06-05	0	0	$>10^4$	1.8×10^6	110	24	0
	2002-07-10	1310	1090	260	5.0×10^6	40	21	0
	2002-07-17	$>10^4$	$>10^4$	390	4.0×10^6	180	19	0
	2002-08-22	220	140	80	2.1×10^7	0	12	0

Microbiological quality of the irrigated minimally processed food samples from Venda 2002

Sample identification	Date	Total coliforms (counts/100ml)	Faecal coliforms (counts/100ml)	Enterococci (counts/100ml)	Heterotrophic count (counts/100ml)	*C.perfringens* (counts/100ml)
Mawoni cabbage	2002-06-05	10	0	0	1.0×10^{1}	0
	2002-07-10	3	0	0	1.0×10^{2}	0
	2002-07-17	0	0	0	1.0×10^{1}	0
	2002-08-22	40	10	0	1.0×10^{1}	0
Mawoni tomatoes	2002-06-05	90	20	20	1.3×10^{1}	0
	2002-07-10	40	10	140	2.0×10^{1}	10
	2002-07-17	0	0	20	1.0×10^{2}	0
	2002-08-22	50	10	0	1.0×10^{2}	0
GP farmers cabbage (Tshituni)	2002-06-05	480	410	900	8.0×10^{7}	90
	2002-07-10	680	510	50	4.0×10^{7}	30
	2002-07-17	970	940	950	1.0×10^{6}	100
	2002-08-22	470	250	880	5.7×10^{8}	270
GP farmers tomatoes (Tshituni)	2002-06-05	120	80	0	8.2×10^{6}	20
	2002-07-10	440	110	30	1.0×10^{6}	20
	2002-07-17	560	360	120	3.0×10^{5}	50
	2002-08-22	90	40	80	2.0×10^{5}	0
Tshikuwi onion	2002-06-05	10	0	10	1.2×10^{6}	10
	2002-07-10	450	220	550	8.0×10^{7}	0
	2002-07-17	320	300	80	4.8×10^{6}	50
	2002-08-22	90	70	0	7.0×10^{7}	120
Tshikuwi tomatoes	2002-06-05	80	60	0	6.0×10^{7}	0
	2002-07-10	950	850	300	8.0×10^{7}	10
	2002-07-17	120	90	50	4.0×10^{6}	50
	2002-08-22	320	250	120	1.8×10^{7}	110
Phiphidi cabbage	2002-06-05	40	20	0	8.0×10^{6}	80
	2002-07-10	280	220	10	1.4×10^{7}	20
	2002-07-17	70	60	0	2.0×10^{5}	30
	2002-08-22	80	60	10	4.0×10^{7}	40
Phiphidi onions	2002-06-05	70	20	90	8.0×10^{5}	0
	2002-07-10	80	50	40	4.0×10^{7}	0
	2002-07-17	30	10	20	1.0×10^{5}	0
	2002-08-22	90	60	160	1.0×10^{5}	40

Part B: Pathogenic bacteria isolated from irrigated water and food samples 2001

Sample	*Salmonella*	*Shigella*	*E. coli*
Mawoni cabbage	4	2	1
Mawoni onions	1	2	2
Mawoni tomatoes	4	2	3
GP farmers tomatoes	0	0	2
Smokey tomatoes	0	2	2
Smokey onions	2	0	2
Mawoni Borehole	2	0	1
Mawoni River	2	2	1
GP Farmers Borehole	0	0	1
Smokey River	2	1	1

Part B: Pathogenic bacteria isolated from irrigated water and food samples 2002

Sample	*Salmonella*	*Shigella*	*E. coli*
Mawoni cabbage	0	2	2
Mawoni tomatoes	1	2	2
GP farmers cabbage	3	3	2
GP farmers tomatoes	0	1	1
Tshikuwi onion	1	1	1
Tshikuwi tomatoes	0	1	1
Phiphidi cabbage	0	0	0
Phiphidi onions	0	0	1
Mawoni Borehole	0	0	3
Phiphidi Borehole	0	0	1
GP Farmers Borehole (Tshituni)	0	1	2
Tshikuwi River	1	1	1

APPENDIX A

Part A: Sampling points on the Plankenbrug river

APPENDIX B

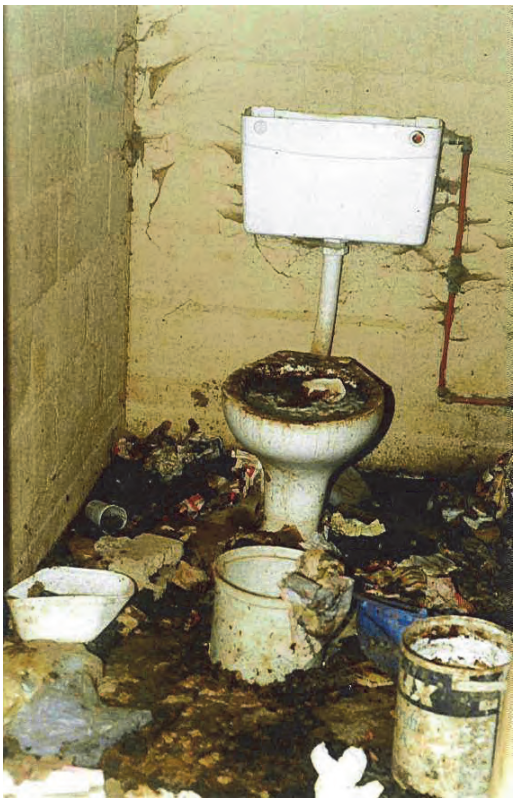
Part A: The Plankenbrug River during low flow conditions (summer) as it flows past Kayamandi on the outskirts of Stellenbosch. At this point the river has already received the discharge from several storm-water drains laden with faecal and other pollution.

Part A: The Plankenbrug River below Kayamandi is overgrown, polluted with solid waste and the riparian housekeeping such as removal of alien invader plants is poor.

Part A: Dense foam that does not abate for a considerable distance downstream points to chemical pollution (possibly with soaps and detergents) or material rich in certain kinds of proteins.

Part A: About 80% of the inhabitants of Kayamandi only have access to communal toilets which are not in a good state of repair. Vandalism contributes to this state of affairs, but a low level of maintenance aggravates the situation. At some of the communal blocks there are reported to be up to 40 users per toilet.

Part A: The communal toilet blocks have taps and basins along the back wall. A similar state of disrepair is evident here. There are very few standing taps along the streets and service points such as this one is also the only source of water for most shack dwellers.



Part A: Many of the toilets were in such a non-functional state that major renovations were necessary during the clean up and education campaigns described in this report.

Part A: Due to the state of disrepair of the facilities, the environment is being polluted with sewage and this is spread over the ground surface by run-off, blowing of contaminated dust and rubbish and wandering animals.

Part A: A workshop of sidewalk vendors and other small business entrepreneurs to find solutions for the large amount of solid waste and animal blood and offal reaching the river via the storm-water drains. The meeting was held in the newly renovated Community Hall, which was used as a practice project for the artisans trained by the DANCED project.

Part A: One of the members of the Kayamandi community who received training in plumbing during the project. The plumbers who were trained were meant to help with the upkeep of the communal toilet facilities.

Part A: Community members being trained in the unblocking and repair of drains around the communal toilet blocks. All these trainees received their basic certificates in plumbing from the training team of the Department of Labour.

Part B: Tomatoes from the irrigated fields in the Venda region

Part B: Cabbages in irrigated fields in the Venda region

Part B: Sampling of borehole water for microbiological analysis

Part B: Sampling of minimally processed foods (cabbages) for microbiological analysis

APPENDIX C

Part B: Microbiological quality of irrigation water samples from Venda 2001

Sample identification	Date	Total coliforms (counts/100ml)	Faecal coliforms (counts/100ml)	Enterococci (counts/100ml)	Heterotrophiccount (counts/100ml)	*C. perfringnes* (counts/100ml)
Mawoni Borehole	2001-10-03	950	240	0	2.0×10^4	480
	2001-10-10	110	20	190	1.0×10^3	70
	2001-10-17	410	320	0	9.0×10^2	30
	2001-11-07	80	0	20	9.0×10^2	0
	2001-11-21	3850	2740	610	2.0×10^3	470
Mawoni River	2001-10-03	4160	3780	480	2.1×10^8	2470
	2001-10-10	3600	3090	2270	3.7×10^6	1360
	2001-10-17	260	0	840	5.0×10^5	400
	2001-11-07	4800	280	2520	1.5×10^8	1340
	2001-11-21	4890	3260	2080	4.9×10^8	1950
GP Farmers (Tshituni) Borehole	2001-10-03	1660	1320	0	5.0×10^3	820
	2001-10-10	650	580	180	5.0×10^4	70
	2001-10-17	90	0	0	6.0×10^4	0
	2001-11-07	0	0	30	2.0×10^2	0
	2001-11-21	1200	1150	490	9.0×10^3	20
Smokey River	2001-10-03	3380	2720	390	1.5×10^7	700
	2001-10-10	2340	270	1580	1.0×10^8	1000
	2001-10-17	560	0	970	1.4×10^5	700
	2001-11-07	3440	450	2310	4.6×10^8	990
	2001-11-21	2530	950	3480	1.2×10^6	1270

Part B: Microbiological quality of the irrigated minimally processed food samples from Venda 2001

Sample identification	Date	Total coliforms (counts/100ml)	Faecal coliforms (counts/100ml)	Enterococci (counts/100ml)	Heterotrophic ount (counts/100ml)	*C. perfringens* (counts/100ml)
Mawoni cabbage	2001-10-03	390	560	2800	3.0×10^8	520
	2001-10-10	520	410	3130	2.3×10^8	430
	2001-10-17	410	350	4620	8.0×10^7	530
	2001-11-07	360	410	3790	1.2×10^7	270
	2001-11-21	380	280	1100	2.8×10^8	360
Mawoni onions	2001-10-03	390	260	1420	8.3×10^7	350
	2001-10-10	340	270	1400	8.0×10^6	310
	2001-10-17	300	220	1220	8.0×10^5	300
	2001-11-07	250	210	0	1.0×10^6	0
	2001-11-21	130	60	180	8.0×10^5	280
Mawoni tomatoes	2001-10-03	420	310	590	5.0×10^7	190
	2001-10-10	410	310	550	5.0×10^6	230
	2001-10-17	270	140	300	7.0×10^6	130
	2001-11-07	270	150	100	1.7×10^7	70
	2001-11-21	710	320	330	2.8×10^8	1350
GP farmers (Tshituni) tomatoes	2001-10-03	120	0	0	1.2×10^5	270
	2001-10-10	60	0	0	7.0×10^4	250
	2001-10-17	0	0	0	9.0×10^2	150
	2001-11-07	0	0	0	3.0×10^5	0
	2001-11-21	580	340	230	6.0×10^6	0
Smokey tomatoes	2001-10-03	240	220	0	4.0×10^7	480
	2001-10-10	250	220	0	1.4×10^7	290
	2001-10-17	270	60	0	4.0×10^5	330
	2001-11-07	280	220	0	4.0×10^6	460
	2001-11-21	740	340	200	2.1×10^8	290
Smokey onions	2001-10-03	370	270	1290	7.3×10^7	0
	2001-10-10	280	250	1470	4.3×10^6	0
	2001-10-17	340	210	2270	9.0×10^3	0
	2001-11-07	410	160	0	3.0×10^6	0
	2001-11-21	390	260	1860	1.9×10^7	240

Part B: Microbiological quality of irrigation water samples from Venda 2002

Sample identification	Date	Total coliforms (counts/100ml)	Faecal coliforms (counts/100ml)	Enterococci (counts/100ml)	Heterotrophic count (counts/100ml)	C .perfringens (counts/100ml)	*Somatic coliphages* (counts/1ml)	*F-RNA coliphages* (counts/1ml)
Mawoni Borehole	2002-06-05	80	0	0	4.3×10^2	10	0	0
	2002-07-10	0	0	0	1.0×10^5	0	0	0
	2002-07-17	0	0	0	1.0×10^2	0	0	0
	2002-08-22	0	0	0	6.0×10^6	0	0	0
Phiphidi Borehole	2002-06-05	350	60	0	1.0×10^6	30	0	0
	2002-07-10	10	0	0	1.0×10^3	150	0	0
	2002-07-17	100	10	20	1.0×10^6	0	0	0
	2002-08-22	40	10	0	1.4×10^7	0	0	0
GP Farmers (Tshituni) Borehole	2002-06-05	$>10^4$	$>10^4$	40	7.0×10^6	10	0	0
	2002-07-10	600	80	180	1.8×10^4	10	0	0
	2002-07-17	450	40	20	1.0×10^4	0	0	0
	2002-08-22	300	120	30	6.0×10^6	0	0	0
Tshikuwi River	2002-06-05	0	0	$>10^4$	1.8×10^6	110	24	0
	2002-07-10	1310	1090	260	5.0×10^6	40	21	0
	2002-07-17	$>10^4$	$>10^4$	390	4.0×10^6	180	19	0
	2002-08-22	220	140	80	2.1×10^7	0	12	0

Microbiological quality of the irrigated minimally processed food samples from Venda 2002

Sample identification	Date	Total coliforms (counts/100ml)	Faecal coliforms (counts/100ml)	Enterococci (counts/100ml)	Heterotrophic count (counts/100ml)	C.perfringens (counts/100ml)
Mawoni cabbage	2002-06-05	10	0	0	1.0×10^{1}	0
	2002-07-10	3	0	0	1.0×10^{2}	0
	2002-07-17	0	0	0	1.0×10^{1}	0
	2002-08-22	40	10	0	1.0×10^{1}	0
Mawoni tomatoes	2002-06-05	90	20	20	1.3×10^{1}	0
	2002-07-10	40	10	140	2.0×10^{1}	10
	2002-07-17	0	0	20	1.0×10^{2}	0
	2002-08-22	50	10	0	1.0×10^{2}	0
GP farmers cabbage (Tshituni)	2002-06-05	480	410	900	8.0×10^{7}	90
	2002-07-10	680	510	50	4.0×10^{7}	30
	2002-07-17	970	940	950	1.0×10^{6}	100
	2002-08-22	470	250	880	5.7×10^{8}	270
GP farmers tomatoes (Tshituni)	2002-06-05	120	80	0	8.2×10^{6}	20
	2002-07-10	440	110	30	1.0×10^{6}	20
	2002-07-17	560	360	120	3.0×10^{5}	50
	2002-08-22	90	40	80	2.0×10^{5}	0
Tshikuwi onion	2002-06-05	10	0	10	1.2×10^{6}	10
	2002-07-10	450	220	550	8.0×10^{7}	0
	2002-07-17	320	300	80	4.8×10^{6}	50
	2002-08-22	90	70	0	7.0×10^{7}	120
Tshikuwi tomatoes	2002-06-05	80	60	0	6.0×10^{7}	0
	2002-07-10	950	850	300	8.0×10^{7}	10
	2002-07-17	120	90	50	4.0×10^{6}	50
	2002-08-22	320	250	120	1.8×10^{7}	110
Phiphidi cabbage	2002-06-05	40	20	0	8.0×10^{6}	80
	2002-07-10	280	220	10	1.4×10^{7}	20
	2002-07-17	70	60	0	2.0×10^{5}	30
	2002-08-22	80	60	10	4.0×10^{7}	40
Phiphidi onions	2002-06-05	70	20	90	8.0×10^{5}	0
	2002-07-10	80	50	40	4.0×10^{7}	0
	2002-07-17	30	10	20	1.0×10^{5}	0
	2002-08-22	90	60	160	1.0×10^{5}	40

Part B: Pathogenic bacteria isolated from irrigated water and food samples 2001

Sample	*Salmonella*	*Shigella*	*E. coli*
Mawoni cabbage	4	2	1
Mawoni onions	1	2	2
Mawoni tomatoes	4	2	3
GP farmers tomatoes	0	0	2
Smokey tomatoes	0	2	2
Smokey onions	2	0	2
Mawoni Borehole	2	0	1
Mawoni River	2	2	1
GP Farmers Borehole	0	0	1
Smokey River	2	1	1

Part B: Pathogenic bacteria isolated from irrigated water and food samples 2002

Sample	*Salmonella*	*Shigella*	*E. coli*
Mawoni cabbage	0	2	2
Mawoni tomatoes	1	2	2
GP farmers cabbage	3	3	2
GP farmers tomatoes	0	1	1
Tshikuwi onion	1	1	1
Tshikuwi tomatoes	0	1	1
Phiphidi cabbage	0	0	0
Phiphidi onions	0	0	1
Mawoni Borehole	0	0	3
Phiphidi Borehole	0	0	1
GP Farmers Borehole (Tshituni)	0	1	2
Tshikuwi River	1	1	1