Chapter 3

MATERIALS AND METHODS

3.1 INFORMED AND ETHICAL CONSENT

Ethical clearance for this study was obtained from the Provincial DOH in Polokwane, the capital city of the Limpopo Province, South Africa in which the study area (Vhembe regiont) was situated. Ethical permission was also obtained from the University of Venda, Thohoyandou, South Africa from which the study was carried out and the University of Pretoria, Gauteng Province, South Africa where the study was registered. The project was also registered at the University of Venda's Research Department. In each of the two study villages, the study layout was explained to the headman or chief whom granted permission to conduct this study. In addition, the head of each study household in both rural villages gave written consent to take part in the study (Appendix A).

3.2 SCHEMATIC OUTLINE OF STUDY DESIGN

This study contained three specific objectives as indicated in Chapter 1. Objectives one and two was carried out using field based studies, while objective three was primarily a laboratory based study. Sections 3.3, 3.4 and 3.5 described the methodology used to prove each of these objectives.

3.3 OBJECTIVE ONE: TO ASSESS AN INTERVENTION STRATEGY TO IMPROVE THE DRINKING WATER QUALITY IN RURAL HOUSEHOLDS

This section described the methodology used to assess the implementation of an intervention strategy to improve the microbiological quality of drinking water in households from two typical rural communities in the Vhembe region, Limpopo Province of South Africa with little of no water and sanitation infrastructure (Fig 3.1).

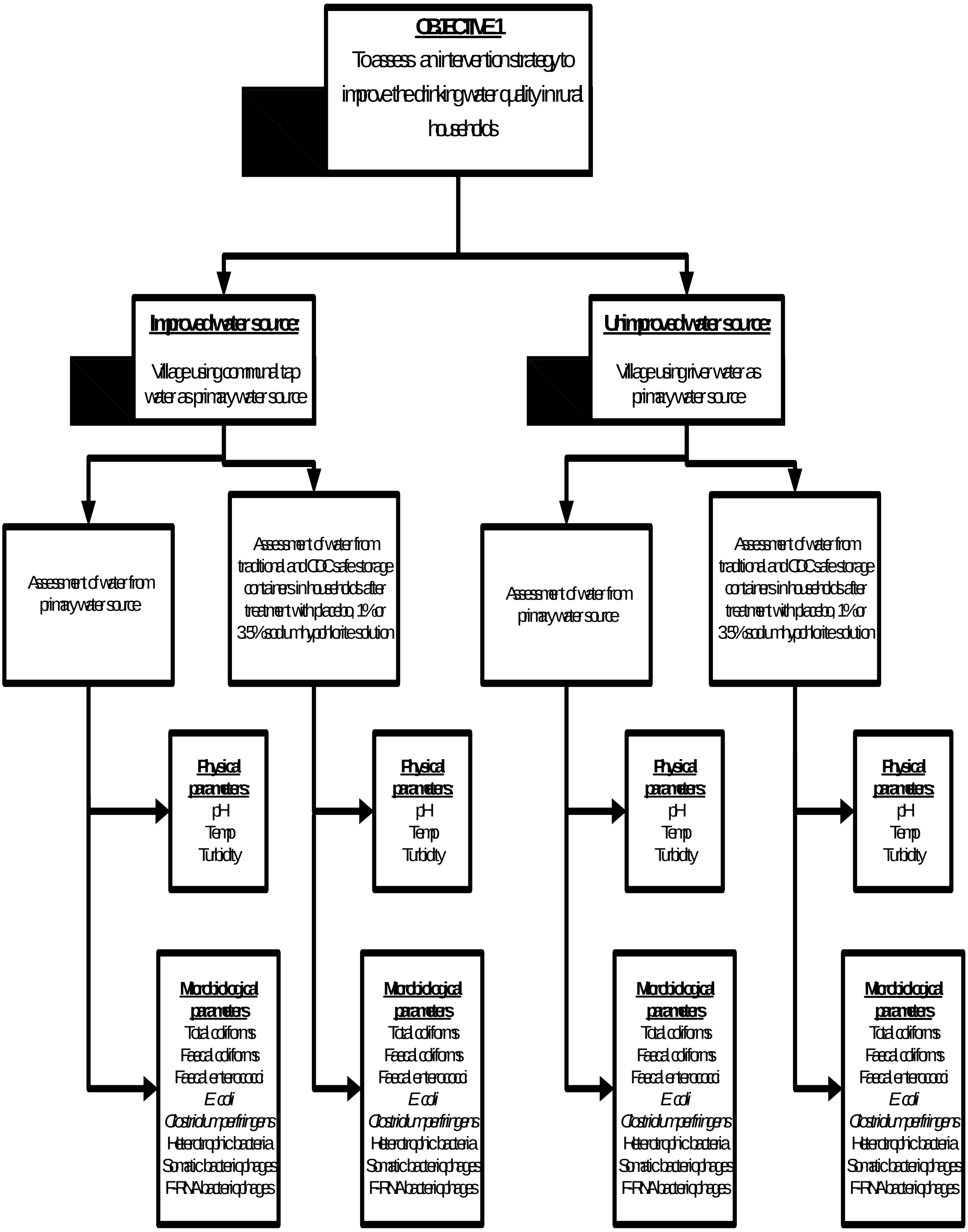

Figure 3.1: **Schematic outlay of the study design of objective one to assess an intervention strategy to improve the drinking water quality at the point-of-use in rural households of South Africa**

3.3.1 Study site and household selection

Two rural villages in the Vhembe region were selected for this study. One village used an improved water source while the other village used an unimproved water source. In village 1 the primary water source included communal taps with untreated water (Fig 3.2). The water was pump directly from a aquifer into a large open reservoir from where it was pumped to the communal taps used by the study households. In village 2 the primary water source included the Sambandou River (Fig 3.3), which was also used for livestock watering, washing of clothes and recreational activities by the community.

Figure 3.2: Typical communal taps used by rural households in village 1 in the Vhembe region of the Limpopo Province, South Africa

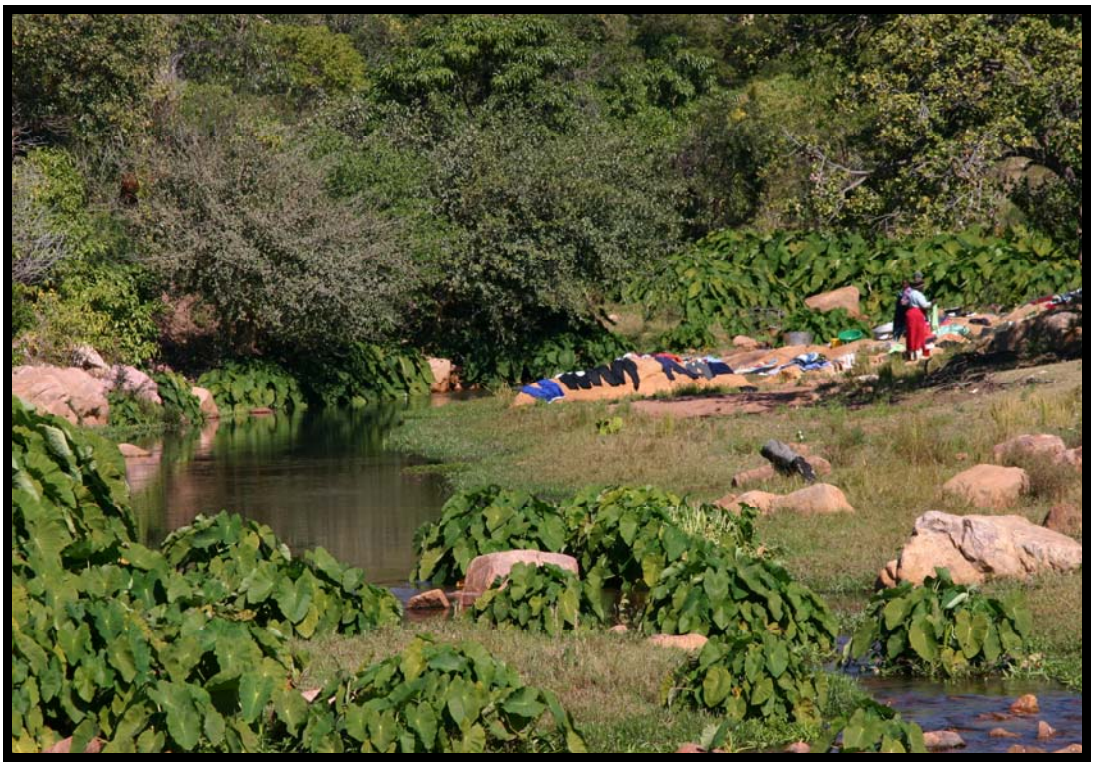

Figure 3.3: The Sambandou River used by rural households in village 2 in the Vhembe region of the Limpopo Province, South Africa

In each village 60 households were randomly recruited and assigned into one control and five intervention groups. The format of the intervention trial is presented in Table 3.1. A group meeting was held with all the selected study households in each village to explain the purpose of the study before the study commenced. Care was taken to make sure that all the study households were blinded to the concentration of the sodium hypochlorite solution. At the household group meetings, the people were only informed that different concentrations of sodium hypochlorite were going to be evaluated during the study period and that the different sodium hypochlorite concentrations will have different smells (eg. strong to weak).

The intervention study was carried out over a period of 4 months (section 3.3.2) in which the water quality of the traditional 20 litre water storage container (called a "tshigubu") was compared to water quality of the 20 litre CDC safe storage container, with the addition of either a placebo (which consisted of distilled water), 1% or 3.5% sodium hypochlorite solution. The control group of households used their traditional storage container and received the placebo solution (Table 3.1).

The households in intervention groups I and II used their traditional water storage container (Table 3.1). However, households in intervention group I received the 1% sodium hypochlorite solution, while the households in intervention group II received the 3.5% sodium hypochlorite solution (Table 3.1).

Each of the households in intervention groups III, IV and V received two CDC safe storage containers to replace their traditional household storage containers. The households in intervention group III received the placebo solution, while the households in intervention groups IV and V received the 1% and 3.5% sodium hypochlorite solutions respectively (Table 3.1). The intervention households in groups III, IV and V were visited individually and given clear instructions and education by a trained field worker (speaking the local language of the household) concerning the proper use and cleaning of the CDC safe storage container (Table 3.1).

Table 3.1: Summary of the intervention trial carried out in each of two rural villages in the Vhembe region of the Limpopo Province, South Africa

Study group	Description of each group	Number of households per group
Control	Traditional household container plus the addition of 5 ml placebo solution	10 households
Intervention I	Traditional household container plus the addition of 5 ml of a stabilized 1.0% sodium hypochlorite solution	10 households
Intervention II	Traditional household container plus the addition of a predetermined volume of a stabilized 3.5% sodium hypochlorite solution	10 households
Intervention III	CDC safe storage container plus the addition of 5 ml placebo solution	10 households
Intervention IV	CDC safe storage container plus the addition of 5 ml of a stabilized 1.0% sodium hypochlorite solution	10 households
Intervention V	CDC safe storage container plus the addition of a predetermined volume of a stabilized 3.5% sodium hypochlorite solution	10 households

In addition, all the study households in both the villages was educated on the proper storage conditions and the correct procedure of adding the placebo and sodium hypochlorite solutions to the water in the storage containers. Every third week, each

study household were given a freshly prepared bottle of placebo, 1% or 3.5% sodium hypochlorite solution. All old solution bottles were removed and replaced with fresh solutions. To be consistent, the placebo, 1% and 3.5% sodium hypochlorite solutions were distributed in similar bottles and given to the households at the same time. Using a teaspoon as measuring device, all study households in both villages were trained to add 5 ml of their given solution to the water storage container each time water from the source was collected. The volume of 5 ml was chosen as a standard for all households because:

- It is the recommended dosage stipulated by the DOH in South Africa for using the 3.5% sodium hypochlorite solution. The results of ten repeated experiments have indicated that 5 ml of the 3.5% sodium hypochlorite solution gave a free chlorine concentration of 3.8 $mg.l^{-1}$ after 60 min and 0.8 $mg.l^{-1}$ after 24 h (Appendix B).
- Laboratory studies were performed to determine the chlorine demand curve (Section 3.3.1.1) for the 1% sodium hypochlorite solution with water from both water sources. The results of ten repeated experiments have showed that 5 ml of the 1% sodium hypochlorite solution gave a free chlorine concentration of 1.5 $mg.l^{-1}$ after 60 min as stipulated by the CDC.
- To be consistent, households who received the placebo solution were advised to use a 5 ml volume of the solution provided.

3.3.1.1 Determination of the chlorine demand curve for containers receiving the 1% sodium hypochlorite solution

In South Africa, the 1% sodium hypochlorite solution as described by the CDC is not commercially available. It was therefore specially prepared for this study by a manufacturer, *TS Marketing*, situated in Polokwane, South Africa.

The volume of 1% sodium hypochlorite solution needed for each water source type had to be determined because parameters such as turbidity, pH and temperature of the water source could influence the volume of sodium hypochlorite solution needed to obtain a free chlorine residual concentration of 1.5 $mg.l^{-1}$ (Dr R Quick, CDC, Atlanta, USA, personal communication). To determine the correct dosage of the 1% sodium hypchlorite solution to the storage containers, a chlorine demand curve for each water

source was determined using the N, N-diethyl-phenylenediamine (DPD) colorimetric method according to the manufacturer's specification (Merck, Darmstadt, Germany). Briefly; different volumes (5 ml, 10 ml, 15 ml and 20 ml) of the 1% stabilized sodium hypochlorite solution was added to separate traditional and CDC safe storage containers which each contained 20 litres of the specific water source. The free chlorine residual was measured at 30 min time intervals in each container for 5 hours after thoroughly shaking the container and taking a 10 ml representative sample. The results of ten repeated experiments indicated that a volume of 5 ml of a 1% sodium hypochlorite solution corresponded to a free chlorine residual of 1.5 $mg.l^{-1}$ after 60 min for the surface and ground water sources used in this study.

3.3.1.2 Questionnaire administration at each study household

A comprehensive questionnaire was used in this study to obtain baseline characteristics of the study households in the two rural villages (Appendix C). The original questionnaire was formulated in English and the interview was conducted in either Tshivenda or Xitsonga with the female head of each household. Two postgraduate students from the University of Venda were trained as field workers to conduct the survey. Both students were fluent in English, Tshivenda and Xitsonga. Data on household demographics, water source, water collection practices, water transportation practices, water storage practices, sanitation, prevalence of diarrhoea during the past six months prior to the interview and general observations made by the interviewer during the interview, were recorded.

3.3.2 Assessment of the effectiveness, compliance and sustainability of a household intervention using an improved storage container and a sodium hypochlorite solution

The principle objective of this study was to evaluate the effectiveness, compliance and sustainability of an intervention consisting of the CDC household storage container with the addition of a 1% or 3.5% sodium hypochlorite solution in rural communities of South Africa. In order to determine the effectiveness and level of compliance of the intervention, all the visits during the intervention study were unannounced and the households were blinded with regards to the concentration of the sodium hypochlorite

solutions. This was necessary to determine if the households were using the intervention on a particular day.

Water samples were collected once a month for 4 months from the primary water source in each village and each of the study household water storage containers (traditional or CDC safe storage containers). Aseptic techniques were used to collect 2 000 ml water samples in sterile Nalgene (Merck, Darmstadt, Germany) collection bottles for microbiological analyses. During collection of communal tap water samples in village 1, the water from the tap was allowed to run for 1 min before a sample was taken. During collection of river water samples in village 2, care was taken to collect samples at the exact sites used by the study households as their water collection points in the river. All samples were transported on ice to the laboratory and processed within 8 h.

Source water samples as well as water samples collected from intervention and non-intervention households (Table 3.1) were tested for physical (section 3.3.2.1) and microbiological parameters (sections 3.3.2.2 and 3.3.2.3). In measuring and comparing the concentration of physical parameters and counts of microbiological parameters in drinking water samples in each household group (Table 3.1), the effectiveness of the intervention was assessed.

3.3.2.1 Physico-chemical analyses of water samples

Temperature and pH measurements were determined in 100 ml volumes of water samples using a Basic-20 pH meter (Crison Instruments, South Africa) and a Silberbrand laboratory thermometer (Merck, Darmstadt, Germany) respectively. The turbidity of each water sample was determined in 10 ml volumes of water samples using a portable HI93703 Microprocessor turbidity meter (HANNA Instruments, Germany). The pH and turbidity meters were calibrated according to the manufacturer's instructions. Free chlorine residuals of water samples were determined in 10 ml of each of the water samples using the N, N-diethyl-phenylenediamine (DPD) colorimetric method according to the manufacturer's specifications (Merck, Darmstadt, Germany).

3.3.2.2 Enumeration of indicator bacteria in the water samples

Indicator bacteria used to assess the microbiological quality of the water samples included heterotropic bacteria, total coliforms, faecal coliforms, faecal enterococci, *E. coli* and *C. perfringens*. These indicator microorganisms were determined in the primary water sources as well as the household water storage container samples.

Selective media were used and prepared in 90 mm Petri plates (Merck, Darmstadt, Germany) according to the manufacturer's instructions. Plate Count Agar (Difco Laboratories, Detroit, MI, USA) was used for the enumeration of heterotrophic microorganisms. Total coliform bacteria were enumerated on mEndo agar (Difco Laboratories, Detroit, MI, USA). Faecal coliform bacteria were enumerated on mFc agar (Difco Laboratories, Detroit, MI, USA). Faecal enterococci bacteria were enumerated on mEnterococcus agar (Difco Laboratories, Detroit, MI, USA). *Clostridium perfringens* OPSP agar with supplements A and B (Oxoid Ltd., Basingstoke, Hampshire, England) was used for the enumeration of *C. perfringens* vegetative cells and spores.

Water samples were assessed in duplicate for the presence of total coliforms, faecal coliforms, faecal enterococci and *C. perfringens* using the membrane filtration technique (Standard Methods, 1995). Sterile filtration membranes (0.45 µm pore size, 47 mm diameter) (Millipore, Johannesburg, South Africa) were prepared by passing 10 ml volumes of each water sample through the membranes using a vacuum pump (Model CP5PM75544; Millipore, Johannesburg, South Africa). The membranes were placed right side up on the respective agar plates. Total coliform and faecal enterococci plates were inverted and incubated (Labotec series 2000 digital incubator; Labotec, Johannesburg, South Africa) aerobically at 37°C for 24 h and 48 h respectively. Metallic green colonies were counted as positive colonies for total coliform bacteria, while pink colonies were counted as positive for faecal enterococci bacteria.

Faecal coliform plates were inverted and incubated (Labotec series 2000 digital incubator; Labotec, Johannesburg, South Africa) aerobically at 44.5ºC for 24 h and dark blue or violet colonies were considered positive colonies. *Clostridium perfringens* plates were inverted and incubated (Labotec series 2000 digital incubator; Labotec,

Johannesburg, South Africa) in micro-aerophillic conditions at 37°C for 24 h using Anaerogen sachets (Oxoid Ltd., Basingstoke, Hampshire, England). Dark brown to black colonies (both vegetative cells and spores) were counted. After incubation, all representative colonies on each plate were counted and multiplied by a factor 10 in order to report the counts as colony forming units per 100 ml (cfu.100 ml^{-1}).

Escherichia coli bacteria was enumerated as follows: membranes from the mFc agar plates containing faecal coliform bacteria were removed and placed directly onto Nutrient-MUG agar (Merck, Darmstadt, Germany) plates and incubated aerobically (Labotec series 2000 digital incubator; Labotec, Johannesburg, South Africa) at 37°C for 24 h. Plates were removed from the incubator and observed under a 366 nm ultraviolet light source (Merck, Darmstadt, Germany). Fluorescent colonies were counted as presumptive *E. coli* bacteria and the counts expressed as cfu.100 ml^{-1} (Difco Manual; Difco Laboratories, Detroit, MI, USA). Each presumptive *E. coli* colony was confirmed using Gram-staining and indole tests with Kovac's reagent (Merck, Darmstadt, Germany) according to the techniques described by Mac Faddin (1980). All Gram negative, indole positive colonies were recorded as *E. coli* (Mac Faddin, 1980).

The number of heterotrophic counts was determined as colony forming units per millilitre (cfu.ml^{-1}) using the pour plate method (Standard Methods, 1995). Briefly; ten fold serial dilutions of each water sample were prepared in sterile distilled water. One ml of each dilution was added to 9 ml Plate Count Agar that was kept in sterile 16 mm test tubes (Adcock Ingram Pty Ltd., Johannesburg, South Africa) at 55ºC in a water bath (Labotec, Johannesburg, South Africa). The test tubes were vortexed to mix the water sample and the agar and poured into sterile 90 mm Petri dishes (Merck, Darmstadt, Germany). After solidification of the agar, the plates were inverted and incubated (Labotec series 2000 incubator; Labotec, Johannesburg, South Africa) under aerobic conditions at 37°C for 48 h.

3.3.2.3 Enumeration of somatic and male specific F-RNA bacteriophages in the water samples

Standard ISO methods were used to determine the presence of somatic bacteriophages (ISO, 2000) and male specific F-RNA bacteriophages (ISO, 1995) in the water samples. The following reagents were used in the preparation of bacterial hosts and agar plates:

Calcium-Glucose solution:

The Calcium-Glucose solution contained 3 g Calsium-Chloride ($CaCl_2.2H_2O$) (Merck, Darmstadt, Germany) and 10 g Glucose (Merck, Darmstadt, Germany) dissolved in 100 ml distilled water. The solution was decontaminated by membrane filtration using 0.22 µm syringe membranes (Merck, Darmstadt, Germany) (ISO, 1995).

Nalidixic Acid solution:

Nalidixic Acid (2.5 g) (Sigma Chemicals Co., St Louis, MO, USA) was dissolved in 2 ml Sodium Hydroxide solution (1 $mol.l^{-1}$ NaOH) (Merck, Darmstadt, Germany) and 98 ml distilled water. The solution was decontaminated by using 0.22 µm syringe membrane filtration (Merck, Darmstadt, Germany) (ISO, 1995).

3.3.2.3.1 Preparation of bacterial hosts for the detection of bacteriophages

Escherichia coli strain WG5 (ISO, 2000) was used as bacterial host to isolate somatic bacteriophages. The bacterial host was grown overnight at 37ºC in a Labotec 2000 digital incubator (Labotec, Johannesburg, South Africa) in Nutrient Broth prepared according to the manufacturer's specifications (Merck, Darmstadt, Germany).

The *S. typhimurium* nalidixic acid and kanamycin resistant WG49 strain (NCTC 12484) containing an *E. coli* plasmid which codes for sex pili production was used as the bacterial host for the detection of male specific F-RNA bacteriophages (Havelaar and Hogeboom, 1984; ISO, 1995). The host was grown in Tryptone Yeast Extract which was prepared as follows: 10 g Trypticase Peptone (Difco Laboratories, Detroit, MI, USA), 1 g Yeast Extract (Difco Laboratories, Detroit, MI, USA) and 8 g Sodium

Chloride (Merck, Darmstadt, Germany) was dissolved in 1 000 ml distilled water and autoclaved at 121°C for 15 min (ISO, 1995). The agar was allowed to cool to 50ºC, the pH was aseptically adjusted to 7.2 using a Basic-20 pH meter (Crison Instruments, South Africa) and 10 ml Calcium-Glucose solution (section 3.3.2.3) was aseptically added. One vial of a host stock culture was added to 50 ml Tryptone Yeast Extract broth and incubated at 100 rpm on a Labcon Platform shaking incubator (Labotec; Johannesburg, South Africa) at 37°C until the F-pili developed onto which the bacteriophages attached to infect the bacteria cell (ISO, 1995).

The absorbance of the growth suspension was measured at 30 min intervals from time 0 min against a blank reference at 560 nm using a Spectro 22 Digital Spectrophotometer (Labomed Inc., USA) until an absorbance of 0.75 was obtained at which the sex pili were produced (Grabow, 2001). The host suspension was removed from the incubator, placed on ice and used within 2 h (ISO, 1995).

3.3.2.3.2 Preparation of bottom agar plates for the detection of somatic bacteriophages

Somatic bacteriophage bottom agar plates contained 14 g Bacto agar (Difco Laboratories, Detroit, MI, USA), 13 g Tryptone (Difco Laboratories, Detroit, MI, USA), 8 g Sodium Chloride (Merck, Darmstadt, Germany) and 1.5 g Glucose (Merck, Darmstadt, Germany) which were dissolved in 1 000 ml distilled water and autoclaved at 121°C for 15 min. The agar was allowed to cool to 50ºC and 1 ml Nalidixic Acid solution (section 3.3.2.3) were added using aseptic techniques (ISO, 2000). Twenty millilitre volumes of the prepared solution was poured into 90 mm Petri dishes (Merck, Darmstadt, Germany) and allowed to solidify (ISO, 2000).

3.3.2.3.3 Preparation of bottom agar plates for the detection of male specific F-RNA bacteriophages

Male specific F-RNA bacteriophage bottom agar plates contained 10 g Trypticase peptone (Difco Laboratories, Detroit, MI, USA), 1 g Yeast Extract (Difco Laboratories, Detroit, MI, USA), 8 g Sodium Chloride (Merck, Darmstadt, Germany), 12 g Bacto agar (Difco Laboratories, Detroit, MI, USA) which was dissolved in 1 000 ml distilled

water, autoclaved at 121°C for 15 min and adjusted to a pH of 7.2 using aseptic techniques. A volume of 10 ml of a Calcium-Glucose solution (section 3.3.2.3) was added aseptically to the medium and 20 ml volumes of the prepared agar solution was poured into 90 mm Petri dishes (Merck, Darmstadt, Germany) and allowed to solidify (ISO, 1995).

3.3.2.3.4 Preparation of top agar plates for the detection of somatic bacteriophages

The somatic bacteriophage top agar contained 8 g Bacto agar (Difco Laboratories, Detroit, MI, USA), 10 g Tryptone (Difco Laboratories, Detroit, MI, USA), 8 g Sodium Chloride (Merck, Darmstadt, Germany), 3 g Glucose (Merck, Darmstadt, Germany), 5 ml of a 1 M Sodium Carbonate (Merck, Darmstadt, Germany) solution and 1 ml of a 1 M Magnesium Chloride (Merck, Darmstadt, Germany) solution. One ml Nalidixic Acid solution (section 3.3.2.3) was added to the top agar using aseptic techniques (ISO, 2000).

3.3.2.3.5 Preparation of top agar plates for the detection of male specific F-RNA bacteriophages

The male specific F-RNA bacteriophage top agar contained 10 g Trypticase Peptone (Difco Laboratories, Detroit, MI, USA), 1 g Yeast Extract (Difco Laboratories, Detroit, MI, USA), 8 g Sodium Chloride (Merck, Darmstadt, Germany) and 6.5 g Bacto agar (Difco Laboratories, Detroit, MI, USA) which were dissolved in 1 000 ml distilled water and autoclaved at 121°C for 15 min. The pH of the agar solution was aseptically adjusted to 7.2 after autoclaving using a Basic-20 pH meter (Crison Instruments, South Africa) and 10 ml Calcium-Glucose solution (section 3.3.2.3) together with 4 ml Nalidixic Acid solution (section 3.3.2.3) added (ISO, 1995).

3.3.2.3.6 Double agar layer plate assay for the detection of somatic and male specific F-RNA bacteriophages in a water sample

Ten fold serial dilutions were made of each water sample using distilled water. The top agar was melted in a 56°C waterbath (Model GFL 1083; Labotec, Johannesburg, South Africa) and prepared as follows: three ml volumes of the top agar were aliquoted into 10

ml conical test tubes (Adcock Ingram Scientific Pty Ltd., Johannesburg, South Africa) and kept liquefied in a 56^{o}C waterbath (Model GFL 1083; Labotec, Johannesburg, South Africa). To each test tube, 1 ml of the prepared host for each bacteriophages type (section 3.3.2.3.1) and 1 ml of a tenfold dilution of the water sample was added. The test tubes were mixed by hand before pouring the solution onto a pre-marked bottom agar plate (section 3.3.2.3.2 and section 3.3.2.3.3). The plates were allowed to solidify and incubated inverted at 37ºC for 24 h in a Labotec 2000 digital incubator (Labotec, Johannesburg, South Africa).

3.3.2.3.7 Presence-Absence spot test for determination of somatic and male specific F-RNA bacteriophages in the water samples

The Presence-Absence test was used to detect somatic and male specific F-RNA bacteriophages following a procedure described by Uys (1999). The Presence-Absence test instead of the double agar layer test was used to analyse 500 ml instead of 1 ml of the water sample. The *Escherichia coli* strain WG5 (ISO, 2000) and *Salmonella typhimurium* WG49 (ISO, 1995) were used respectively as bacterial hosts to isolate somatic and male specific F-RNA bacteriophages from the water samples.

Each water sample was mixed to have a homogenous suspension and 500 ml was poured into a sterile plastic 1 000 ml water collection bottle to which 5 g Trypticase Peptone (Difco Laboratories, Detroit, MI, USA), 0.5 g Yeast Extract (Difco Laboratories, Detroit, MI, USA), 4 g Sodium Chloride (Merck, Darmstadt, Germany) and 5 ml of a Calcium-Glucose solution (section 3.3.2.3) were added. Host cultures were prepared according to ISO procedures (section 3.3.2.3.1). One millilitre of the specific host culture was added to each of the water samples and incubated (Labotec series 2000 digital incubator; Labotec, Johannesburg, South Africa) at 37°C for 24 h. The presence of either somatic or F-RNA bacteriophages were determined by spotting 5 µl from each Presence-Absence water sample onto a pre-prepared lawn of host bacteria in 90 mm Petri dishes (Merck, Darmstadt, Germany) (Uys, 1999). The plates were incubated at 37ºC for 24 h and zones of cell lysis (plaques) were considered positive and reported as Present for each water sample (Uys, 1999).

3.3.2.4 Compliance of households in two villages with the intervention using an improved storage container and a sodium hypochlorite solution

During each of the unannounced water collection visits (section 3.3.2), the free chlorine residual in household storage containers was determined as described in section 3.3.2.1. Comparisons of the free chlorine concentrations detected in the household storage containers were used to determine if the point-of-use water treatment intervention resulted in any improvement in the drinking water quality. In addition, a qualitative survey was administered at the end of the intervention study to determine the degree of satisfaction of the consumers with the point-of-use water treatment intervention. The questionnaire was used to solicit information on problems regarding the taste and smell of the water after treatment, problems in the use of the sodium hypochlorite solution or problems with the CDC safe storage containers.

3.3.2.5 Sustainability of the intervention study in two rural villages

The sustainability of the intervention introduced to the study households in each of the two rural villages was assessed twice after the 4 months intervention study was completed. No new bottles of the placebo or 1% and 3.5% sodium hypochlorite solutions were given to any of the households after the end of the formal intervention trial. The first assessment was carried out 6 months after the completion of the formal intervention trial while the second assessment was carried out 12 months after completion of the formal intervention trial. The same procedures discussed in section 3.3.2.1 to section 3.3.2.3.7 were used.

3.3.3 Statistical analyses of intervention study data

The Stata Release 8.0 (Stata Corporation, College Station, Texas, USA) statistical software package was used throughout this study for all analysis. All the raw data is kept electronically by Prof PJ Becker in the Biostatistics Unit at the Medical Research Council, in Pretoria, South Africa and could be made available on request by mutual agreement.

All parameters in the household questionnaires used on the baseline characteristics were of a categorical nature describing certain water, hygiene and sanitation practices at the household level. The Stata Release 8.0 statistical software package was used in the process of cleaning and editing the data and to do comparative analyses. Data was summarized making use of frequencies, percentages and cross-tabulations.

The results for the water samples collected from the primary water sources and water samples collected from the household storage containers were summarised for each water source and household group as outlined in Table 3.1. According to Standard Methods (1995) the best estimate of central tendency of log normal data is the geometric mean which was used in this section. In all comparison analysis differences were considered statistical significant if $P < 0.05$. In addition, the association or link between household demographic and hygiene practices and water quality, measured in terms of *E. coli* counts, was determined using Poisson regression which adequately deals with counts and zeros.

Analyses of variance (ANOVA) is a parametric test and assumes that the data analysed are normal distributed around the mean with similar variance (Helsel and Hirsch, 1995). If the assumption of equal variance was violated, the Welsch approach was used in parametric testing. In this study, the instances where data did not pass normality were considerably more than instances where data did not comply with these assumptions, non-parametric tests were employed.

Non-parametric testing has considerable power in comparing non-normal as well as normal data. The following tests were included in this study (Helsel and Hirsch, 1997):

- The *Rank Sum Test*, also referred to as the *Mann-Whitney Rank U-Test*, a non-parametric procedure was used to test for a difference in medians between two groups.
- The *Wilcoxon Matched Pairs Signed Ranks Test*, a non-parametric procedure was used on paired data sets.
- The *ANOVA on Ranks* and the *Kruskal-Wallis* tests were used to test for differences between three and more study groups. *Multiple Comparison* tests (MCT's) were used to ascertain where group differences were.

According to Helsel and Hirsch (1997), Box plots visually displayed microorganism counts in the improved and the traditional household storage container. Figure 3.4 gives a visual presentation of a Box plot.

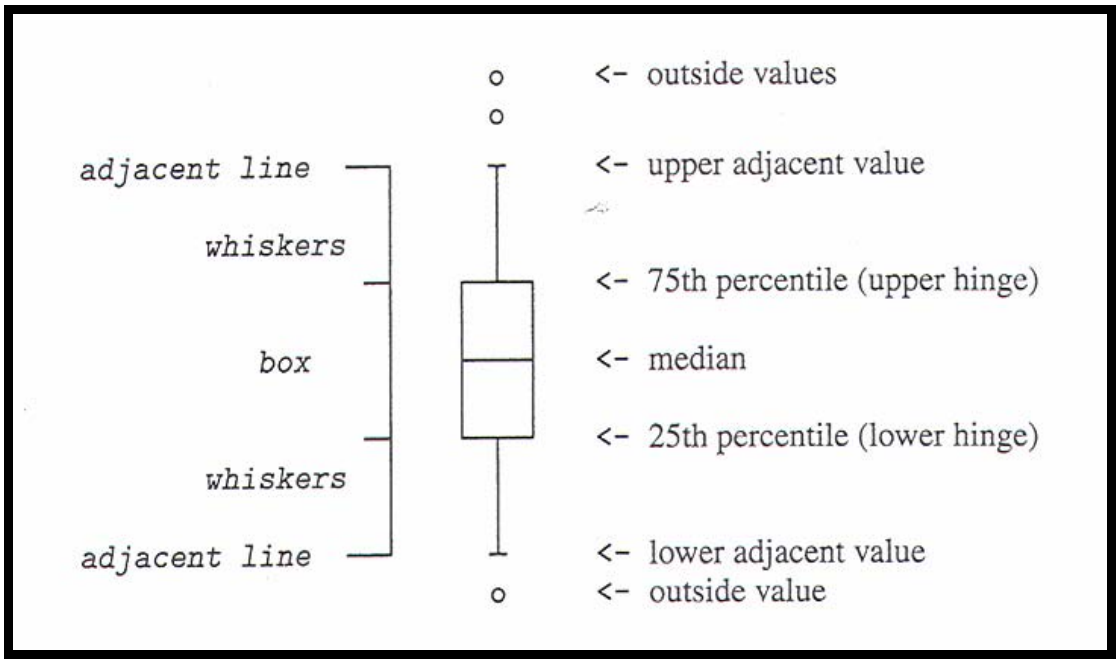

Figure 3.4: Visual presentation of a Box plot used in this study to compare the microbiological counts between the traditional and CDC safe storage containers in the study households from two rural villages in the Vhembe region of the Limpopo Province, South Africa (Helsel and Hirsch, 1995)

- The centre line in the Box plot gives the median, often the preferred measure of central tendency as it is resistant to the effects of outliers (Helsel and Hirsch, 1997).
- The inter-quartile range (variation or spread of the data) is the upper and lower boundaries forming the Box height and indicates the spread of data between the 25^{th} and the 75^{th} percentile. The closer the data are clustered to the median within the inter-quartile range, the less variation (more stable) the data set is (Helsel and Hirsch, 1997).
- The skewness (also referred to as the quartile skew) is represented by the relative size of the Box halves. The further the median line is from the middle of the box, the more skewed the data is distributed around the mean. This implies the use of non-parametric methods of analyses (Helsel and Hirsch, 1997).

- The caps whiskers on the lines protruding above and below the 75th and the 25th percentiles represent the distance 1.5 x inter-quartile range above and below the latter. The circle symbols beyond the caps and whiskers indicate outliers (Helsel and Hirsch, 1997).

3.4 OBJECTIVE TWO: TO DISTINGUISH BETWEEN FAECAL POLLUTION OF ANIMAL OR HUMAN ORIGIN USING MOLECULAR TYPING OF MALE SPECIFIC F-RNA BACTERIOPHAGE SUBGROUPS

This section described the methodology used to assess the origin of faecal contamination in the household water storage containers. Molecular genotyping of male specific F-RNA bacteriophages was used to distinguish between the four different male specific F-RNA bacteriophage subgroups.

3.4.1 Water sample collection

An additional forty households (not the same households used in section 3.3) were randomly selected in each rural village described in section 3.3.1 to participate. Two rounds of water collection from the households and the water sources were carried out over a period of 5 months. Water samples (2 000 ml) were collected aseptically in sterile Nalgene water collection bottles (Merck, Darmstadt, Germany) from 7 communal taps in village 1 (Fig 3.2) and from 4 points on the Sambandou River in village 2 (Fig 3.3).

Two water storage containers in each household were randomly selected and vigorously shaken before water samples were collected. Water samples (1 000 ml each) were collected aseptically in sterile Nalgene water collection bottles (Merck, Darmstadt, Germany) from each of the two selected storage containers in each household on both rounds of water collection. The two water samples from each household were pooled into one sample (2 000 ml) representative of the household container water, placed on ice and transported to the laboratory for further analyses within 8 h. After the first round of water sampling, 20 households in each village were randomly selected and provided with two CDC safe storage containers each. These households were requested to use the CDC safe storage containers instead of the traditional household storage containers. The water samples obtained from the households using the CDC safe storage container were compared with water samples obtained from the households in the same village using traditional storage containers in order to determine the impact of an improved storage container on the origin of faecal pollution (Fig 3.5).

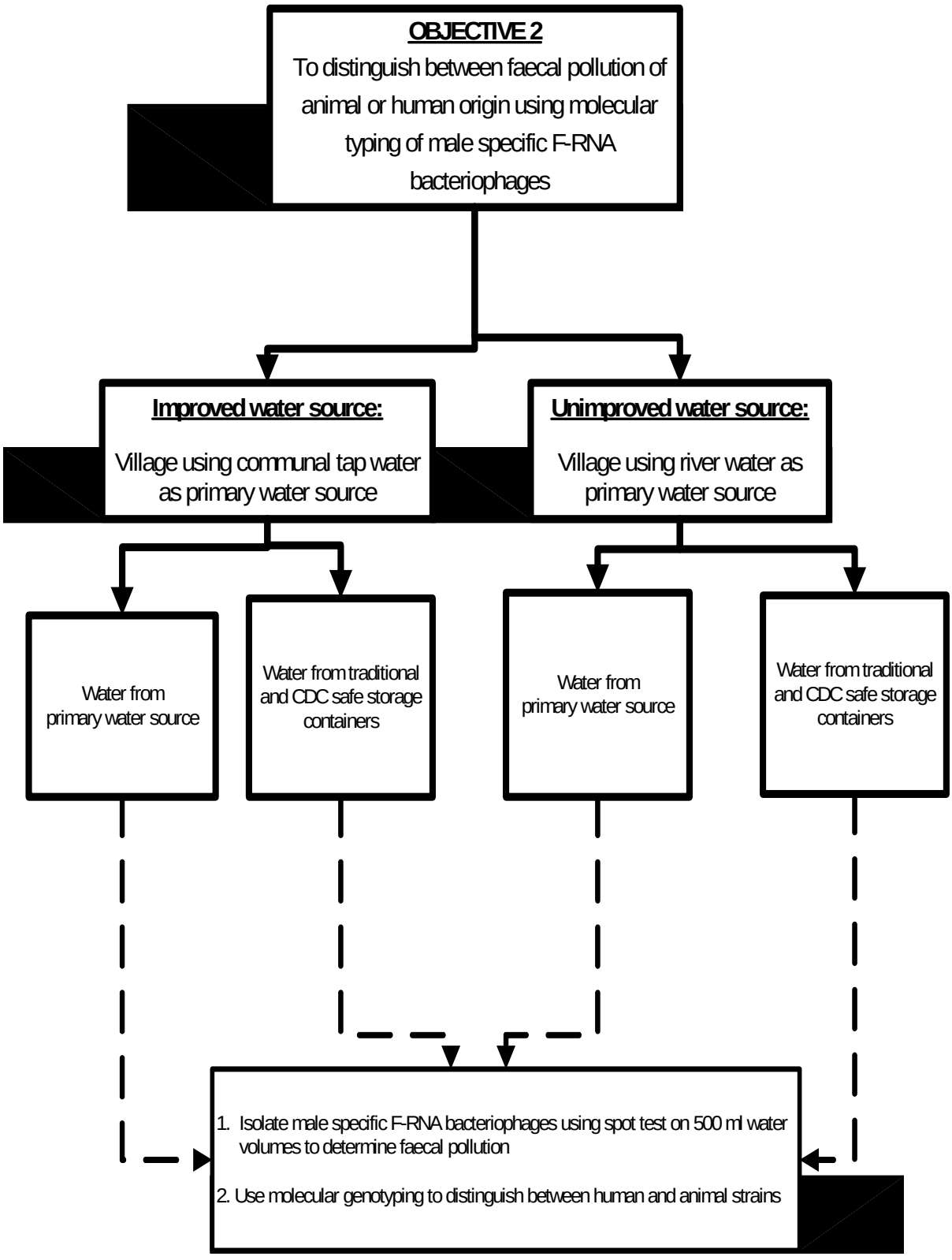

Figure 3.5: **Schematic outlay of the study design of objective two to distinguish between faecal pollution of human or animal origin in the primary water sources as well as the traditional and CDC safe storage containers**

3.4.2 Isolation and identification of male specific F-RNA bacteriophages

Preparation of the bacterial host for the detection of male specific F-RNA bacteriophages in the water samples was done as described in section 3.3.2.3. The preparation of bottom agar plates and top agar plates for direct plaque assays for the isolation of male specific F-RNA bacteriophages was carried out to the procedures described in sections 3.3.2.3.3 and 3.3.2.3.5 respectively (ISO, 1995). The Calcium-Glucose and Nalidixic Acid solutions were prepared as described in section 3.3.2.3 (ISO, 1995). The double agar plate assay procedure as described in section 3.3.2.3.6 was used for direct plaque assays for the isolation of male specific F-RNA bacteriophages.

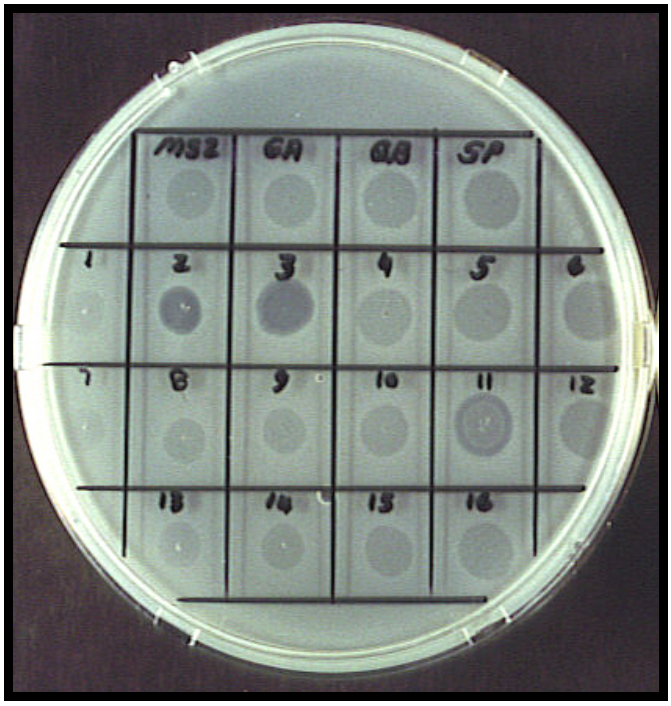

Figure 3.6: A Petri plate indicating spots of positive male specific F-RNA bacteriophage controls and water samples (Uys, 1999)

The Presence-Absence test to determine the presence of male specific F-RNA bacteriophages in the water samples was carried out as described in section 3.3.2.3.7. A total of 16 water samples were spotted on one plate (Fig 3.6). Representative strains of male specific F-RNA bacteriophage consisting of MS2 (subgroup I), GA (subgroup II), QB (subgroup III) and F1 (subgroup IV) [donated by Prof MD Sobsey, University of North Carolina, Chapel Hill, USA] were used as positive controls on each plate (Fig 3.6).

3.4.3 Preparation of phage plates for hybridisation, phage transfer and membrane fixation

The method described by Schaper and co-workers (2002a) were used. The phage spot plates (section 3.4.2) were removed from the 37ºC incubator (Labotec 2000 digital incubator; Labotec, Johannesburg, South Africa) and placed in a fridge at 4ºC for 30 min to solidify and dampen the agar to facilitate phage transfer. The plates were removed from the fridge and covered with a Nylon membrane (Roche Diagnostics, GmbH, Mannheim, Germany) for 1 min for the fixation of the bacteriophages onto the membranes. Following fixation, the bacteriophage RNA would have to be released and denatured to expose the bases to the complementary oligonucleotides. Therefore, the membranes were removed from the plates, placed in plastic containers and submerged in 40 ml of a 0.1 M Sodium Hydroxide (NaOH) (Merck, Darmstadt, Germany) solution for 5 min. This was followed by a neutralisation step where the membranes were placed into clean plastic containers and submerged in 40 ml of 0.1 M Sodium Acetate ($CH_3COOHNa$; pH 6) (Merck, Darmstadt, Germany) solution for 1 min. The nucleic acids were cross linked to the membranes by a 5 min exposure of both sides of the membranes to an ultra violet transilluminator (Model TL-302; Spectroline, Germany). The fixed membranes were used immediately for hybridisation.

3.4.4 Hybridisation of fixed male specific F-RNA bacteriophages

The hybridisation method of Beekwilder and co-workers (1996) as modified by Schaper and co-workers (2002a) was used. Each fixed membrane (section 3.4.3) was placed into a hybridisation bag (Roche Diagnostics, GmbH, Mannheim, Germany) with 5 ml of prehybridisation solution. The prehybridisation solution contained 6 X Saline Sodium Citrate (SSC) (Amfresco, Solo, Ohio, USA); 0.1% Sodium Dodecyl Sulphate (SDS) (Merck, Darmstadt, Germany); 1 x Denhardt solution (Invitrogen Ltd., Paisley, Scotland) and 0.1 $mg.ml^{-1}$ Salmon Sperm DNA (Invitrogen Ltd., Paisley, Scotland) (Sambrook *et al.*, 1989). The Salmon Sperm DNA was denatured at 99ºC for 10 min in a PCR thermocycler (Pharmacia LKB Gene ATAQ, Upsalla, Sweden) and kept at 4ºC until used.

The hybridisation bags were sealed and placed in a shaking incubator (Hub O'Matic, K Huber Engineering, South Africa) at 25ºC for 10 min. Hybridisation was carried out in the same hybridisation bags by adding 2.5 pmol.ml^{-1} of the digoxy-labelled probes (Sigma-Genosys; Sigma Chemicals Co., St Louis, MO, USA) described by Hsu and co-workers (1995) (Table 3.2).

Table 3.2: Nucleotide sequences of male specific F-RNA bacteriophage probes used in this study (Hsu *et al*., 1995)

Phage subgroup	Probe sequence	Basepairs
I	5`-CTAAGGTATGGACCATCGAGAAAGGA-3`	26
II	5`-CCATGTTATCCCCCAAGTTGCTGGCTAT-3`	27
III	5`-ATACTCAGTGAARTACTGCTGTGT-3`	24
IV	5`-GGCATAGATTCTCCTCTGTAGTGCG-3`	25

The bags were resealed and placed in a waterbath (Labotec, Johannesburg, South Africa) at 37ºC for 60 min. After hybridisation, the membranes were removed from the hybridisation bags and placed into clean plastic containers and washed twice using large volumes of a buffer containing 0.3 x SSC (Amfresco, Solo, Ohio, USA) and 0.1% SDS (Merck, Darmstadt, Germany). These washing steps were carried out in a waterbath (Labotec, Johannesburg, South Africa) at 37ºC for 10 min.

3.4.5 Chemiluminescent detection of hybridised male specific F-RNA bacteriophage plaques

A digoxigenin (DIG) Wash and Block Buffer Set (Roche Diagnostics, GmbH, Mannheim, Germany) containing washing, blocking and detection solutions was used. The membranes were washed with the washing buffer (Roche Diagnostics, GmbH, Mannheim, Germany) at 37ºC for 2 min in a waterbath (Labotec, Johannesburg, South Africa) and blocked at 37ºC for 15 min (Labotec waterbath) using 80 ml blocking solution (Roche Diagnostics, GmbH, Mannheim, Germany) per membrane.

Each membrane was incubated at 25ºC for 30 min in a shaking incubator (Hub O'Matic, K Huber Engineering, South Africa) at 100 rpm in 20 ml blocking solution. The blocking solution contained 1 µl Anti-digoxigenic-AP Fab fragments (Roche

Diagnostics, GmbH, Mannheim, Germany). The membranes were washed twice with washing solution (Roche Diagnostics, GmbH, Mannheim, Germany) at 25ºC for 15 min at 100 rpm in a shaking incubator (Hub O'Matic, K Huber Engineering, South Africa). The membranes were treated with 20 ml detection solution (Roche Diagnostics, GmbH, Mannheim, Germany) at 25ºC for 5 min at 100 rpm in a shaking incubator (Hub O'Matic, K Huber Engineering, South Africa).

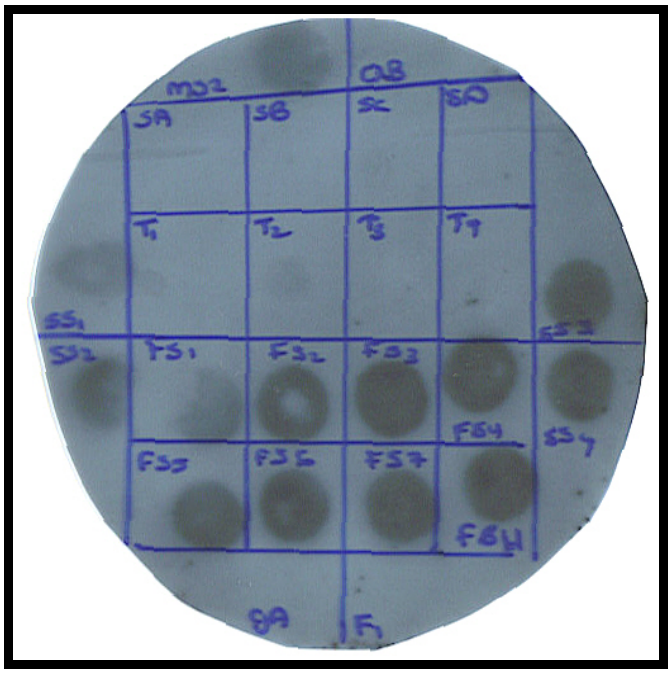

Figure 3.7: An X-Ray film showing MS2 probes hybridised to male specific F-RNA bacteriophage nucleic acid in river and tap water samples

A 1:100 dilution of a CDP (disodium 2-chloro-5-4(methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1.3,7]decan}-4-yl)-1-phenyl phosphate) detection substrate solution (Roche Diagnostics, GmbH, Mannheim, Germany) was added to the detection solution (Roche Diagnostics, GmbH, Mannheim, Germany) and incubated with the membranes at 25ºC for 2.5 min. The membranes were sealed in new hybridisation bags (Roche Diagnostics, GmbH, Mannheim, Germany) and exposed for 5 to 8 min to X-Ray Lumi film (Roche Diagnostics, GmbH, Mannheim, Germany) in a developing cassette. The film was developed using developing, stopping and fixing solutions as described by the manufacturer (AXIM, Midrand, South Africa). Probes hybridised to F-RNA phage nucleic acid yielded black circular spots on the X-Ray film (Fig 3.7).

3.5 OBJECTIVE THREE: TO DETERMINE THE SURVIVAL OF INDICATOR AND WATERBORNE PATHOGENS IN THE IMPROVED CDC SAFE STORAGE CONTAINER

Laboratory based seeding experiments were carried out after the rural household intervention study (section 3.3) in the field. This was done to determine how long indicator and pathogenic microorganisms would survive in surface and groundwater sources used by rural communities for domestic purposes inside the 20 litre CDC safe water storage container with or without the addition of a sodium hypochlorite solution.

3.5.1 Water samples

Surface water was obtained from the Levuvhu River in the Dididi region of the Limpopo Province and groundwater was obtained from a community borehole in the Sambandou region of the Limpopo Province, South Africa. The CDC safe storage containers were filled with 20 litres of either river or borehole water directly from the respective sources and transported to the laboratory where the containers were used as outlined in section 3.5.2 and Fig 3.8.

3.5.2 Laboratory based survival study outline

The survival study was set up to contain three groups of 3 CDC safe storage containers for each water source as outlined in Figure 3.8. In each group of CDC safe storage containers, the first container was used to determine the numbers of natural occurring heterotrophic bacteria, total coliforms, faecal coliforms, *E. coli*, faecal enterococci, *C. perfringens*, somatic bacteriophages, male specific F-RNA bacteriophages, *S. typhimurium* and Enteroviruses in the respective water sources. The second container was seeded with 10^6 plaque forming units per millilitre (pfu.ml^{-1}) Coxsackie B1 virus (National Institute of Virology, Johannesburg, South Africa), 10^9 cfu.ml^{-1} male specific F-RNA subgroup II (MS2) bacteriophages [donated by Prof MD Sobsey, University of North Carolina, Chapel Hill, USA] and 10^7 cfu.ml^{-1} *E. coli* (ATCC 13706). The third container was seeded with 10^9 colony forming units per millilitre (cfu.ml^{-1}) somatic bacteriophages (ATCC 73378) and 10^6 cfu.ml^{-1} *S. typhimurium* (NCTC 12484).

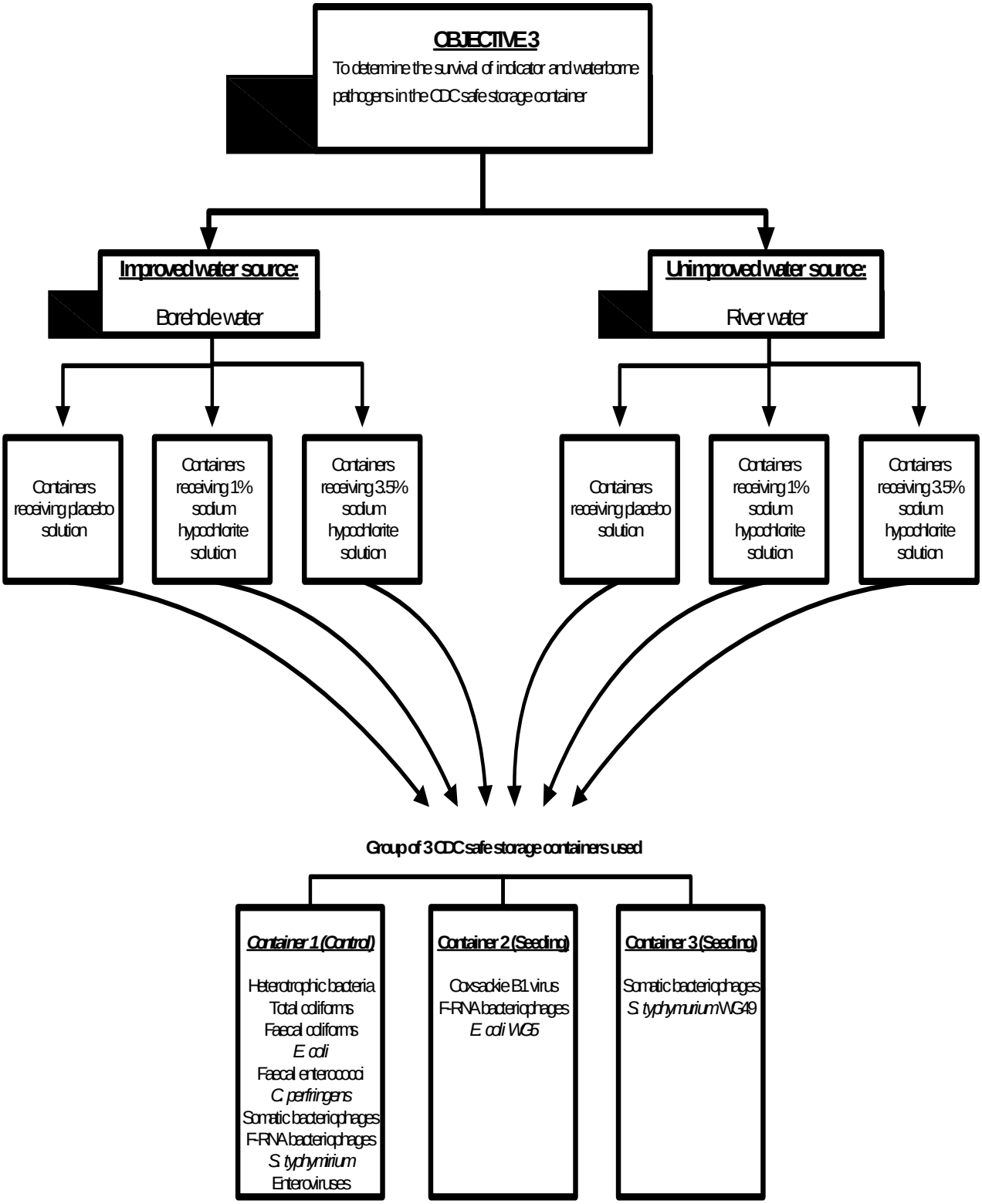
OBJECTIVE 3
To determine the survival of indicator and waterborne pathogens in the CDC safe storage container
Improved water source:
Borehole water
Unimproved water source:
River water
Containers receiving placebo solution
Containers receiving 1% sodium hypochlorite solution
Containers receiving 3.5% sodium hypochlorite solution
Containers receiving placebo solution
Containers receiving 1% sodium hypochlorite solution
Containers receiving 3.5% sodium hypochlorite solution
Group of 3 CDC safe storage containers used
Container 1 (Control)
Heterotrophic bacteria
Total coliforms
Faecal coliforms
E. coli
Faecal enterococci
C. perfringens
Somatic bacteriophages
F-RNA bacteriophages
S. typhymirium
Enteroviruses
Container 2 (Seeding)
Coxsackie B1 virus
F-RNA bacteriophages
E. coli WG5
Container 3 (Seeding)
Somatic bacteriophages
S. typhymurium WG49

Figure 3.8: Schematic outlay of the laboratory study design of objective three to determine the survival of indicator microorganisms and waterborne pathogens in the CDC safe storage container

The first group of CDC safe storage containers was referred to as the control group and 5 ml of a placebo solution was added to each of the 3 containers (section 3.3.1). The second group of CDC safe storage containers each received 5 ml of a 1% sodium hypochlorite solution (sections 3.3.1 and 3.3.1.1). The third group of CDC safe storage containers each received 5 ml of a 3.5 % sodium hypochlorite solution (section 3.3.1).

The experiment was repeated two times in triplicate using representative 1 000 ml water samples. Care was taken to collect water after thoroughly shaking each container in the same way and at the same time each day for the duration of each experiment. The numbers of different microorganisms (naturally occurring and seeded microorganisms) in the containers were determined at time zero prior to the addition of the placebo and sodium hypochlorite solutions and again 60 min after the addition of the respective placebo and sodium hypochlorite solutions. Thereafter water samples were taken at the same time from all containers after 24 h, 48 h and after five days. Chlorine was neutralized with the addition of 2 ml of a 1 M Sodium Thiosulphate (Merck, Darmstadt, Germany) solution to the collected water samples.

3.5.3 Physico-chemical analyses of water samples

Measurements of temperature, pH and turbidity of water samples were carried out as described in section 3.3.2.1. Free chlorine residuals of water samples were determined using the N, N-diethyl-phenylenediamine (DPD) colorimetric method as described in section 3.3.2.1.

3.5.4 Enumeration of naturally occurring indicator bacteria and bacteriophages in the water samples (Container 1)

Standard methods (1995) as described in sections 3.3.2.2 and 3.3.2.3 were employed in the detection of heterotrophic bacteria, total coliforms, faecal coliforms, faecal enterococci, *C. perfringens* bacteria, somatic and male specific F-RNA bacteriophages. *Escherichia coli* bacteria were enumerated on Eosin Methylene Blue (EMB) agar

(Merck, Darmstadt, Germany) prepared according to the manufacturer's specifications in 90 mm Petri plates (Merck, Darmstadt, Germany). *Salmonella typhimurium* bacteria were enumerated on MacConkey agar (Merck, Darmstadt, Germany), which were prepared according to the manufacturer's specifications in 90 mm Petri plates (Merck, Darmstadt, Germany).

Ten fold serial dilutions were made for each water sample in distilled water. The spread plate method was used and 0.1 ml of each water dilution was spread onto the individually pre-marked 90 mm Petri plates (Merck, Darmstadt, Germany) that were inverted and incubated in a Labotec series 2000 digital incubator (Labotec, Johannesburg, South Africa) at 37°C for 24 h under aerobic conditions. Typical colony growth, which included pink colonies for *S. typhimurium* and purple colonies for *E. coli* were counted and expressed as colony forming units per millilitre (cfu.ml^{-1}).

3.5.5 Enumeration of naturally occurring Enteroviruses in the water samples (Container 1)

Buffalo Green Monkey (BGM) kidney cell cultures were used to determine the prevalence of any naturally occurring Enteroviruses in the original river and borehole water samples (Grabow *et al*., 1990; Potgieter, 1997). The cells were grown in 25 cm^2 tissue culture flask (Corning, USA) to confluent monolayers, washed with 5 ml sterile Phosphate Buffered Saline (PBS; pH 7) (Sigma Chemicals Co, St Louis, MO, USA) and starved for 60 min in 1 ml serum free Eagle's Minimum Essential Media (EMEM) (Highveld Biological, Pty. Ltd, Kelvin, South Africa). After withdrawal of the starvation media, 1 ml of the water sample was inoculated onto the cells and left at 37ºC for 60 min with gentle hand rotation every 15 min.

The inoculum was removed from the cells and 5 ml EMEM (Highveld Biological, Pty. Ltd, Kelvin, South Africa) containing 1% inactivated bovine serum (Delta Bioproducts, Johannesburg, South Africa) and 1% of an antibiotic solution prepared to contain 10 000 Units.ml^{-1} Penicillin (BioWhittaker, Walkersville, MD, USA), 5 000 Units.ml^{-1} Streptomycin (BioWhittaker, Walkersville, MD, USA), and 100 Units.ml^{-1} Nystatin (Sigma Chemicals Co., St Louis, MO, USA), were added. The infected cell flasks were incubated (Galaxy CO_2 incubator, Biotech Northants, England) at 37ºC in the presence

of 5% CO_2 for 21 days and blind passages performed every three days (Grabow *et al*., 1990; Pinto *et al*., 1994; Potgieter, 1997).

A commercial viral RNA extraction kit (Qiagen, Hilden, Germany) was used to extract viral RNA from 2 ml infected BGM tissue culture fluid. Reagents used in the RT-PCR and nested PCR reactions were obtained from Promega (Promega Corp., Madison WI, USA) and Boehringer (Boehringer Mannheim GmbH, Germany) and all primers were obtained from Sigma (Sigma Genosys Ltd., Pampisford, Cambridgeshire, United Kingdom). A positive control (cell cultured coxsackie B1 virus) and a negative control (nuclease free water; Promega Corp., Madison WI, USA) were included in both the reverse transcriptase polymerase chain reaction (RT-PCR) and nested PCR reactions. A Techne Genius thermocycler (Techne, Cambridge, United Kingdom) was used for the RT-PCR and nested PCR reactions.

The published primer set (Gow *et al*., 1991, Egger *et al*., 1995) used in the RT-PCR reaction included primer EP1 (5'-$_{64}$CGGTACCTTTGTGCGCCTGT$_{83}$-3') and primer EP4 (5'-$_{459}$TTAGGATTAGCCGCATTCAG$_{478}$-3') which gave a 414 base pair (bp) product. The RT-PCR reaction was carried out in a 50 µl volume containing 50 pmol of each of the EP1 and EP4 primers, 1 µl of 5 U *Tfl* DNA polymerase; 15 µl extracted RNA, 1 µl of 5 U avian myeloblastosis virus reverse transcriptase (AMV-RT); 1x AMV/*Tfl* reaction buffer, 1.5 mM Magnesium sulphate ($MgSO_4$) and a dideoxy nucleotide triphosphates (dNTP - final concentration of 0.2 mM) mix (Gow *et al*., 1991; Kuan, 1997). A reverse transcriptase step at 48ºC for 45 min was followed by 30 cycles of DNA denaturation at 94ºC for 1 min, annealing at 56ºC for 1 min and an extension at 72ºC for 1 min. The RT-PCR reaction was ended with a final extension step at 72ºC for 10 min (Gow *et al*., 1991).

The nested PCR reaction was carried out with published primer set E1 (5'-$_{166}$AAGCACTTCTGTTTCCC$_{182}$-3') and E2 (5'-$_{447}$ATTCAGGGGCCGGAGGA$_{463}$-3') to give a final product of 297 bp (Gow *et al*., 1991; Kuan, 1997). The 50 µl nested PCR mixture contained 1 µl RT-PCR product, 50 pmol of each primer; 10 mM Tris-HCl (pH 9), 50 mM Potassium Chloride (KCl), 0.1% Triton X-100, 1.5 mM Magnesium Chloride ($MgCl_2$), dNTP mix (0.2 mM final concentration) and 1.5 U *Taq* DNA polymerase (Kuan, 1997). The nested PCR reaction started with a DNA denaturation step at 94ºC for 3 min, which was followed by 30 cycles of 94ºC for 1 min, annealing at

45ºC for 1 min and an extension at 72ºC for 1 min and ended with a final extension at 72ºC for 10 min (Kuan, 1997).

The amplified products from the RT-PCR and the nested PCR reactions were separated on a 2% agarose gel (Seakem LE agarose, Bioproducts, USA) using a Medicell Primo gel apparatus (Holbrook, NY). The size of the products was determined using a 100 bp molecular weight marker (Promega Corp., Madison WI, USA) (Gow *et al*., 1991; Kuan, 1997).

3.5.6 Enumeration of selected seeded pathogenic bacteria and bacteriophages in the water samples (Container 2 or 3)

Methods used to determine the survival of *E. coli* and *S. typhimurium* bacteria were described in section 3.5.4. Methods used to determine the survival of somatic and male specific F-RNA bacteriophages were described in section 3.3.2.3.

3.5.7 Enumeration of seeded Enteroviruses in the water samples (Container 3)

The survival of Coxsackie B1 virus in the water samples prior to and after the addition of the sodium hypochlorite solutions was determined using BGM cells (Potgieter, 1997). The BGM cells were grown in 75 cm^2 tissue culture flasks (Corning, USA) to confluent monolayers using EMEM (Highveld Biological, Pty. Ltd, Kelvin, South Africa). The EMEM contained 10% inactivated bovine serum (Delta Bioproducts, Johannesburg, South Africa) and 1% of an antibiotic solution (section 3.5.5). The flasks, containing BGM cell monolayers were trypsinised, by removing the growth medium and adding 5 ml activated Trypsine Versene solution (Highveld Biological, Pty. Ltd, Kelvin, South Africa) for 1 min. The Trypsine Versene solution was removed and the cells resuspended in fresh 10% EMEM-bovine serum-antibiotic medium (Potgieter, 1997). Cells were counted using a haemocytometer (Merck, Darmstadt, Germany) and seeded into 96 well microtitre cell culture plates (Corning, USA) in 200 µl volumes per well. The plates were incubated (Galaxy CO_2 incubator, Biotech Northants, England) overnight at 37ºC in the presence of 5% CO_2. This procedure yielded confluent cell monolayers in each well within 24 h.

End point titrations were carried out as follows: ten fold serial dilutions of each water sample were made in EMEM without serum or antibiotics. The cells in the microtitre plates were rinsed twice with sterile pH 7.2 Phosphate Buffered Saline (PBS) solution (Sigma Chemicals Co., St Louis, MO, USA). Six wells on the microtitre plate were used for each dilution, with six wells respectively for the positive and negative controls. The plates were inoculated with 20 µl of the ten fold dilutions per well. The positive control was a direct inoculation of the Coxsackie B1 virus stock. The negative control consisted of EMEM containing 2% inactivated bovine serum (Delta Bioproducts, Johannesburg, South Africa) and 1% of an antibiotic solution (section 3.5.5). The plates were incubated at 37ºC for 60 min (Galaxy CO_2 incubator, Biotech Northants, England) in the presence of 5% CO_2 with gentle hand rotation every 15 min (Potgieter, 1997).

After incubation, each well received 180 µl of a 2% EMEM-bovine serum-antibiotic solution. The plates were incubated (Galaxy CO_2 incubator, Biotech Northants, England) at 37ºC in the presence of 5% CO_2. The plates were examined daily for a period of 10 days for the presence of cytopathogenic effect (CPE). The 50% endpoints for each water sample were determined by the $TCID_{50}$ Kärber formula described by Grist and co-workers (1979).

3.5.8 Statistical analysis of the laboratory based survival study

The triplicate counts of each of the two experiments were averaged by calculating the geometric means. In cases where microbial counts were not detected, the counts were treated as 0.1 in order to calculate the geometric mean. The means were then log 10 transformed and log 10 reduction values calculated for each microorganism. A zero observation, i.e. no growth detected, was denoted by “n.d” (not detected) since log of zero is not defined.