1-1-2008

Investigation into the bacterial contamination in a spring water distribution system and the application of bioremediation as treatment technology

Latiefa Behardien
Cape Peninsula University of Technology

Cape Peninsula
University of Technology

INVESTIGATION INTO THE BACTERIAL CONTAMINATION IN A SPRING WATER DISTRIBUTION SYSTEM AND THE APPLICATION OF BIOREMEDIATION AS TREATMENT TECHNOLOGY

by

LATIEFA BEHARDIEN

Thesis submitted in fulfilment of the requirements for the degree

Master of Technology: Food Technology

in the Faculty of Applied Science

at the Cape Peninsula University of Technology

Supervisor: AProf. Wesaal Khan
Co-supervisor: AProf. Sehaam Khan

Cape Town

DECLARATION

I, Latiefa Behardien, declare that the contents of this thesis represent my own unaided work, and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

Signed **Date**

ABSTRACT

Spring water bottled and sold for human consumption can only be subjected to certain treatment processes such as separation from unstable constituents by decantation, filtration and aeration, ultraviolet irradiation and ozonation. A spring water distribution system in the Western Cape, South Africa was experiencing microbiological problems. The aim of the study was to investigate bacterial contamination in the spring water distribution system and the application of bioremediation as treatment technology. Sampling at various points in the spring water distribution bottling system started in February 2004 and continued until November 2004.

The acceptable microbiological limits for bottled spring water clearly states that the total viable colony count should be < 100 organisms per ml of water. Analysis of samples by the heterotrophic plate count (HPC) technique indicated significantly ($p < 0.05$) high counts which did not conform to the microbiological limit. The heterotrophic plate counts recorded for weeks one, four, eight & 46 in the final bottled water (Site J) were 3.66×10^7 cfu/ml, 9.0×10^6cfu/ml, 2.35×10^7 cfu/ml and 5.00×10^4 cfu/ml, respectively. The total cell counts [Flow cytometry analyses (FCM)] recorded for week one, four, eight & 46 in the final bottled water (Site J) were 5.44×10^7 microorganisms/ml, 8.36×10^7 microorganisms/ml, 9.09×10^7 microorganisms/ml and 5.70×10^7 microorganisms/ml, respectively. The higher viable total cell counts (FCM) indicate that flow cytometry was able to detect cells in the water sample that enter a viable but not culturable state and that the heterotrophic plate count technique only allowed for the growth of the viable and culturable cells present in the water samples. This indicated that the HPC is not a clear indication of the actual microbial population in the water samples. It could be concluded that FCM technique was a more reliable technique for the enumeration of microbial populations in bottled water samples. Various organisms were identified by means of the Polymerase Chain Reaction (PCR) using 16S rRNA specific primers. Purified PCR amplicons were sequenced and Phylogenetic trees were constructed. Neighbour-joining phylogenetic tree analysis of the bacterial species present in the water samples was performed. The dominant bacterial isolates that were sequenced from the various water samples throughout weeks one, four, eight and 46 were *Bacillus sp*. and *Enterobacteriaceae.* The pathogenic species isolated throughout the sampling

period included *Escherichia sp., Pseudomonas sp., Shigella boydii, Bacillus and Staphylococcus sp.*

A laboratory-scale bioreactor was constructed and water samples were analysed over a period of two weeks. Water samples were analysed using FCM and Direct Acridine Orange Count (DAOC) in conjunction with epiflourescence microscopy (EM). The FCM counts ranged from 1.53 x 10^7 microorganisms/ml in the initial sample (Day 0) to 1.16 x 10^7 microorganisms/mℓ in the final sample (Day 13). The results indicated a 24% decrease in the microbial numbers however, it was still above the limit of < 100 organisms/ml as set out by the South African Standards of Bottled Water, (2003). The total cell counts obtained by the DAOC method ranged from 1.43 x 10^6 microorganisms/ml to 9.54 x 10^5 microorganism/ml on day 13 (final). The results indicated a 33% decrease in microbial numbers. The total cell counts analysed by flow cytometry fluctuated throughout the sampling period. The total cell counts obtained from the DAOC method were lower in all the water samples when compared to the total counts obtained by flow cytometric analyses. Even though the FCM counts fluctuated throughout the sampling period, results clearly show that the FCM method yielded more accurate data for total cell counts than the DAOC method. Due to external environmental conditions such as changes in the weather conditions the results fluctuated and the final results clearly indicated that further studies are required to optimise the bioreactor system for its application in the spring water industry.

ACKNOWLEDGEMENTS

I wish to thank:

THE ALMIGHTY for my health, strength and guidance in completing this thesis.

PROF. WESAAL KHAN, Department of Agricultural and Food Sciences, Faculty of Applied Science, Cape Peninsula University of Technology, Cape Town who acted as my promoter for her patience, guidance and motivation throughout the project.

PROF. SEHAAM KHAN, Department of Biomedical Sciences, Faculty of Health and Wellness Sciences, Cape Peninsula University of Technology, Cape Town who acted as my co-promoter for her guidance throughout the project.

MY EMPLOYER who stands for quality, excellence, value, style and so much more and who has taught me through experience that constant innovation and excellence is a continuous evolvement of not only your job but yourself.

THE CAPE TOWN WEATHER BUREAU for assisting with climatological information.

THE MINERAL WATER SUPPLIER in the Western Cape for allowing us to sample in the distribution system.

MY PARENTS Mariam Behardien, Faried Behardien (late), Rashieda Lester and Ebrahim Lester for their support, guidance and unconditional love, for teaching me good values and for always keeping me in their prayers.

ALWIE LESTER, my beloved husband for loving and supporting me no matter what.

MY SISTERS AND BROTHERS for caring and supporting me through the toughest times in my life and for always being there.

MY FRIENDS AND COLLEAGUES especially Arnelia Paulse and Vanessa Jackson for their assistance and continuous support throughout this time and Michael Tobin for lending a helping hand.

MEDHANI ABRAHAMS for his assistance wherever he may be.

THE NATIONAL RESEARCH FOUNDATION and **CAPE PENINSULA UNIVERSITY OF TECHNOLOGY** for their financial support towards this research. Opinions expressed in this thesis and the conclusions arrived at, are those of the author, and are not necessarily to be attributed to the National Research Foundation or CPUT.

DEDICATION

I dedicate this thesis to my family and my hubby for always being there and for being a part of who I am.

BIOGRAPHICAL SKETCH

LATIEFA BEHARDIEN was born in Cape Town on 15 August 1974. She matriculated from Alexander Sinton High School in Crawford, Cape Town, in 1992. In 1995, she obtained her National Diploma in Food Technology at the then Cape Technikon (now Cape Peninsula University of Technology). She started working as an assistant microbiologist for one of the big retailers in South Africa and continued her studies on a part time basis at the Technikon, completing her BTech degree in Food Technology in 1998. Her job function changed to Food Technologist looking after various product profiles still within the same company. She furthered her studies by completing a Post Graduate Diploma in Business Management at the Graduate School of Business in Cape Town in 2006. She is still currently employed at the same company as a Food Technologist where she has 13 years service.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

GLOSSARY

South African National Bottled Water Association	SANBWA
Parts per Million	ppm
Ultraviolet	UV
Ozone	O_3
United States	US
South African National Standards	SANS
Colony Forming Units	CFU
Heterotrophic Plate Counts	HPC
Viable-but-non-culturable	VBNC
Polymerase Chain Reaction	PCR
Epiflourescent Microscopy	EM
Flow Cytometric Analysis	FCM
Confocal Laser Scanning Microscopy	CLSM
DNA	Deoxyribonucleic acid
DAOC	Direct acridine orange count

LITERATURE REVIEW

1.1 INTRODUCTION

1.1.1 Spring water

South Africa bottles approximately 190 million litres of spring water per year, generating over a billion Rand in turnover [South African Natural Bottled Water Association (SANBWA), 2006]. This spring water is derived from a subterranean source where the water flows naturally in aquifers below the surface of the earth. Over time the pressure below the surface of the earth becomes greater than the atmospheric pressure, thereby naturally expelling the water through various soil and rock layers, to the surface as a spring **(Figure 1.1)** (LaMoreaux & Tanner, 2002). As the water filters through the underground rock layers it absorbs minerals, which differ from spring to spring depending on the rock layers it filters through.

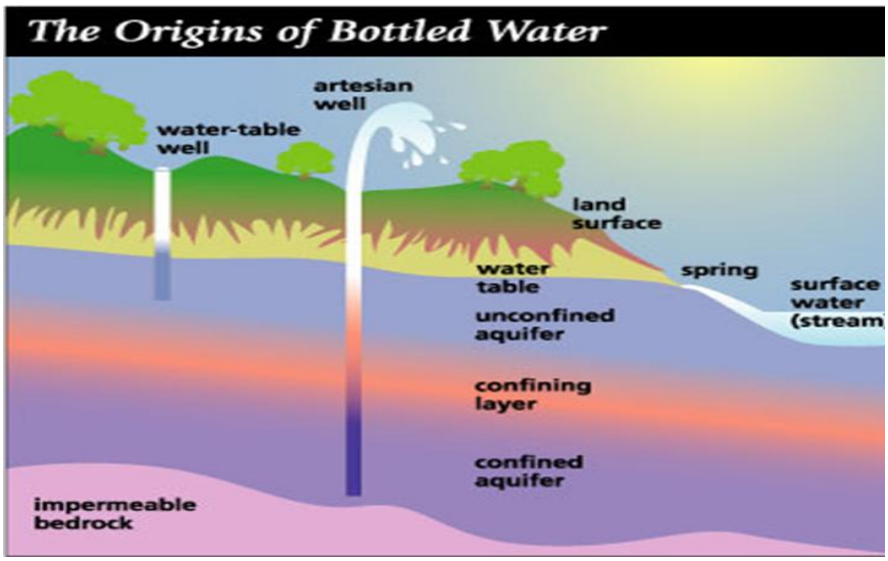


Figure 1.1 Spring formation (Ghettoplanet, 2003)

Springs are classified according to the rock type at the site where the spring occurs, the way in which the spring was formed, the temperature of the water and the volume of the water discharged. Artesian springs are formed when groundwater is forced to the surface of the earth due to underground pressure. Perennial springs flow continuously throughout the year and drain a large surface area, whereas intermittent springs only flow at certain times of the year (Hoyle, 2005). Spring formation however, occurs not only on the surface of the earth, but can also form on the surface of the ocean floor. The thermal springs found beneath the ocean are

called hydrothermal vents and can expel water with a temperature of up to 400°C. These hydrothermal vents are commonly found near volcanically active areas where the water expelled is rich in dissolved chemicals and complex microbial communities (Hydrothermal Vent Biology, 2006).

1.1.2. Bottled water

Groundwater is found below the earth's surface in natural rock formations called aquifers and can be brought to the surface either naturally by lakes or streams, or artificially by drilling a borehole into the aquifer. The water can also be forced to the surface of the earth by the build up of natural pressure below the ground as a spring (LaMoreaux & Tanner, 2002). In nature, surface water includes water found in rivers and oceans, and waters stored as ice and snow. This water may contain minerals, organic matter, and microorganisms which may affect the organoleptic properties and quality of the water. This implies that surface water, which is often used as a potable water source, may require treatment before use. In contrast, groundwater which possesses a lower turbidity and microbial count than surface water, is not directly exposed to environmental factors, and may thus require little or no treatment.

Natural spring water is obtained directly from underground water sources and is collected under conditions that maintain its natural chemical composition and microbiological purity. According to the South African National Standards for Bottled Water (2003), the source of the spring must not be situated at, or close to any potential sources of pollution, such as sewerage, farming operations, waste disposal, industrial activities or any combination of the above pollutants. The untreated water at the spring source must also comply with the microbiological criteria as stipulated in **Table 1.1**

Table 1.1 Microbial specifications for untreated spring water sources (South African National Standards, Bottled Natural Water, 2003)

Microorganism	*Limit
Coliform	Absent per 100ml
Faecal coliform	Absent per 100ml
Total viable colony count/ Heterotrophic total plate count	<100 organisms per 1ml
Clostridium spores	Absent per 100ml

*The above criteria are subject to analysis within 24 hours of the sample being collected.

Bottled water can fall into various categories and according to the South African Natural Bottled Water Association (2006) there are three general classes of

bottled water. Class one is natural water, which is derived from an underground source and where the physical and chemical properties of the water are maintained using a minimum level of filtration. Class two, is water defined by its origin such as for example a spring, and which undergoes a limited amount of acceptable treatments. The last class of bottled water is prepared water which is classified as water from an acceptable source such as a municipal water supply, and which can be subjected to any treatment that will allow it to be safe and healthy for the consumer. In developed countries, the demand for bottled water is determined by convenience, the perception that bottled water may be safer than the local municipal water and taste preferences. In many areas of developing countries however, factors such as the lack of potable, reliable or safe water as well as potentially contaminated water, influences the demand for bottled water. This spring water, which is bottled and sold for human consumption, differs from normal drinking water in that it contains certain mineral salts and trace elements in relative proportions **(Table 1.2)**. It should also be bottled close to or at the source and can only be subjected to treatments permissible under the Foodstuffs, Cosmetics and Disinfectants Act (South Africa, Department of Health, 2004).

Table 1.2 Permissible limits of trace elements found in natural mineral water (South Africa, Department of Health, 2004).

Substances	**Maximum Limit (mg/l)**
Antimony	0.005
Arsenic	0.01 (as total arsenic)
Barium	0.7
Borate	0.5 (as total Boron)
Cadmium	0.003
Chromium	0.05
Copper	1
Cyanide	0.07
Fluoride	1.5
Lead	0.01
Manganese	0.5
Mercury	0.001
Molybdenum	0.07
Nickel	0.02
Nitrate	50, calculated as nitrate
Nitrite	0.2, calculated as nitrite
Organophosphate pesticides	Below limit of quantification
Organochlorine pesticides and polychlorinated biphenyls	Below limit of quantification
Selenium	0.01
Surface active agents	Below limit of quantification
Uranium	0.002

The general classes of bottled water are further subdivided into various categories, some of which have functional elements and minerals added to the water for health benefits. Mineral water is derived from a tapped source of a borehole or spring that is protected and contains more than 250 parts per million (ppm) of total dissolved solids. The minerals should be naturally occurring and may not be added to the water. Bottled water defined by its origin, originates from an underground source or surface water system, e.g. spring water, artesian water and glacial water. Prepared water is bottled water which does not comply with any of the provisions stipulated for water, is defined by its origin and can originate from any type of water supply. Spring bottled water is water sourced from a confined aquifer from which the water flows naturally to the earth's surface. The water is collected from the spring or a borehole tapping the underground formation. This water when bottled can either be classified as still spring water, or it can be carbonated and labelled as sparkling spring water (South Africa, Department of Health, 2004). In addition, fluoridated water is water that contains added fluoride within set limitations. The fluoride is added as a functional additive to aid in the building up of strong teeth by preventing tooth decay. Sterile water should comply with the requirements of "sterility testing" while well water is water derived from a well drilled into an aquifer (Morelli, 1994).

1.1.3 Spring water contamination and pollution

Pollutants that contaminate surface water may be the same pollutants that contaminate groundwater, as compounds from the surface can move through the soil into aquifers. Advances in modern technology have led to the development of various treatment processes to try and control and eliminate all forms of contamination from potable water, as time- and cost-effectively as possible. However, depending on the pollution load, the treatment process may prove inadequate in eliminating all forms of contamination from the water. Furthermore, the water may become re-contaminated after treatment. Humans may also contract diseases such as dysentery, typhoid fever, minor skin irritations and respiratory disorders, from microbial pathogens contaminating the water sources. Pathogens associated with water and which could cause disease, include microorganisms such as viruses, bacteria, fungi, helminths and protozoa (Krantz & Kifferstein, 2003). In addition, microorganisms in aqueous environments, encounter a large number of solid surfaces. These organisms exhibit a tendency to attach and accumulate on the surfaces in cell aggregates. A biofilm is formed when these adherent microbial

communities, surrounded by a matrix composed of microbial polymer, grow and actively multiply, entrapping nutrients and other microorganisms (Characklis & Marshall, 1990).

The major factors that influence the type of microorganisms found in finished bottled water can include the type and quality of the source water, the efficacy of treatment and disinfection, temperature as well as the design and construction of the bottling system (LeChevallier *et al.,* 1991). Hydrogeological data should be collected at the spring and in the surrounding area so that possible points of contamination can be eliminated and the critical areas protected. Precautions should also be taken to avoid pollution by external influences. The surrounding area should be checked for possible dumping sites of radioactive substances, fertilisers and pesticides and should not be in the path of potential sources of underground contamination, such as septic tanks, sewers and industrial waste ponds (Codex Alimentarius Commission, 1985).

Microbial contaminants are however, not the only contaminants found in spring water, as inorganic and organic contaminants may also be present **(Table 1.3)** (Tchobanoglous & Schroeder, 1985). Typical inorganic compounds include; dissolved gases such as oxygen, nitrogen, carbon dioxide; metals and cations such as aliminium, arsenic, lead, mercury and calcium; and anions such as fluoride, chloride, nitrate, nitrite, phosphate, carbonate and cyanide (LaMoreaux & Tanner 2002). While some of these compounds are required in trace amounts by the human body, prolonged exposure may have adverse effects on the health of humans and the environment.

Table 1.3 Typical groundwater contaminants * (Tchobanoglous & Schroeder, 1985)

Class	Typical contaminants found in groundwater.
Floating and suspended materials	None
Colloidal materials	Trace organic and inorganic constituents, microorganisms
Dissolved material	Inorganic salts, trace organic compounds, Iron and manganese, hardness ions
Dissolved gases	Carbon dioxide, Hydrogen sulphide
Immiscible liquids	Unusual in ground water aquifers

*Specific water quality objectives may be related to drinking water standards.

1.2 SPRING WATER PURIFICATION PROCESSES

The production of good quality spring water, which is microbiologically and chemically safe for human consumption, implies that certain water sources may need to undergo

specific treatment processes. The specific treatment process is dependent on the initial quality of the water and can include procedures such as screening, filtration, sedimentation, coagulation and disinfection. As groundwater is the main water source of bottled mineral water, the typical contaminants found in this water source **(Table 1.3)** may have to be removed to comply with specific water quality criteria (Tchobanoglous & Schroeder, 1985). However, natural bottled and mineral water can only be subjected to certain treatment processes such as separation from unstable constituents by decantation, filtration, aeration, or by any process that will ensure that the natural mineral content of the water is not modified or altered in any way, such as ultraviolet irradiation and ozonation (South Africa, Department of Health, 2004).

1.2.1 Sedimentation and flotation

The sedimentation process generally involves the removal of suspended solids from water. As the density of the particles is usually higher than that of the surrounding water, the particles migrate to the bottom of the settling basin, reducing the concentration of suspended particles removed by the filters. Factors that influence the degree of sedimentation include; the size, shape and weight of the particles, the temperature and viscosity of the water, the retention time, the number, areas and depth of the basins, surface overflow rate, and the inlet and outlet design (Senior & Dege, 2005). Sedimentation differs from flotation in that flotation involves the separation of solid and liquid particles of a much lower density from the water (Matilainen *et al.,* 2002; Vinneras & Johnson, 2002). Sedimentation and flotation cannot however, be used to remove microbial contaminants or very fine particles in suspension, as they have a low capacity for the removal of extremely small particles (Senior & Dege, 2005).

1.2.2 Filtration

Filtration can be defined as a process whereby water is forced through a medium (usually a membrane or filter), in order to remove different types of particles (Morelli, 1994). Generally, sedimentation and flotation do not remove all suspended matter from water and filtration is then required as a secondary treatment process. Filter mediums can consist of, amongst others, sand, activated carbon, stainless steel mesh, glass fibre and cellulose filters (Pall Filters™, 2004). Factors important in selecting a proper filter for a particular application include, the size, shape and

hardness of the particles to be removed, the quantity of the particles, the nature and volume of the fluid to be filtered, the flow rate of the fluid, pressure, fluid temperature, properties of the fluid, and whether there is space available for particle collection (Senior & Dege, 2005).

Filter types can further be classified into non-fixed pore filter media and fixed pore filter media. Non-fixed pore filter media i.e. felts, woven yarns, asbestos pads and loosely packed fibreglass, are constructed of a thick non-fixed medium capable of trapping particles of a specific size. The only disadvantage of this type of filter medium is that a release of collected particles can occur when an increase in pressure causes the pore size of the filter medium to enlarge. When using non-fixed pore filter media the particle retention force must always be greater than the dislodging force of the fluid. Fixed pore filter media i.e. woven wire mesh filters and woven cloth filters, are constructed of a specific material, which does not allow for the distortion of the pore size at high pressure. Therefore, fixed pore filters are superior for most applications and have a higher waste retention capacity per unit area (SANBWA, 2004).

Sand filters are driven by gravity, and can be divided into two categories namely, slow rate sand filters and high rate sand filters. Slow rate sand filters consist of a 0.5 - 1.5 m layer of silica sand with an effective particle size of 0.01 - 0.6 mm, while high rate sand filters are 0.4 - 1.2 m deep, with a coarser sand of an effective particle size of 0.5 - 1.0 mm (Tebbut, 1992). Slow rate sand filters produce good quality water whereas after high rate sand filtration, terminal disinfection is usually required. Research has however, shown that microbial communities are known to attach to slow rate sand filters, but not to the high rate sand filters increasing the possibility of pollution (Brennan *et al.,* 1990).

Activated carbon filters are also widely used in the potable water industry and consist of fine or coarse-grained carbons. Charcoal is a type of carbon which has a high surface area and is commonly used in household water filters. Carbon filter sizes usually range from 0.5 to 50 µm and its efficacy is dependent on the flow rate of the water. Carbon filters are most effective in the removal of chlorine, sediment and volatile organic compounds, but as it does not bind well to certain chemicals, strong acids and most inorganic compounds such as sodium, lithium and iron, its removal efficacy is limited (Matilainen *et al*., 2002). However, it has been reported that carbon filters in the spring water industry can impart taints to the water and therefore are not always used in spring water distribution systems.

Filter sizes used in the bottled water industry are usually 0.5 µm absolute which means that they have a high degree of accuracy. In the spring water bottling process it is also important to consider the following factors when selecting the desired filtration process; the source of the water to be filtered, the flow rate, surface area, temperature, pressure and the degree of filtration. In addition, the prefiltration process should also be considered when selecting the filtration system for a spring water bottling plant. One of the major disadvantages of filtration is that the effectiveness of a filter cannot be detected visually and can only be analysed through microbiological analysis techniques. Filtration system defects can also be detected by pressure systems where a drop in pressure usually signifies an ineffective filtration process. This pressure drop must be continually monitored in order to detect early signs of malfunctions (Pall Filters™, 2004).

1.2.3 Ultraviolet Irradiation

In the spring water industry, ultraviolet irradiation is commonly employed as a non-chemical disinfectant treatment of the water source. The process involves water passing through a chamber where it is exposed to ultraviolet light at a wavelength of 254 nm (Margolin, 1997). When microorganisms are exposed to UV energy, the DNA in their cells is disrupted, effectively inactivating them and preventing reproduction. The resistance of microorganisms to the UV energy will differ, however the vast majority of bacteria, protozoa, viruses, fungi, moulds, yeast and algae are inactivated when exposed to a sufficient dose (Morelli, 1994). The efficiency of UV irradiation is also increased when used in conjunction with other treatment processes such as sedimentation and filtration. However, as is the case with most disinfectant processes, the efficiency of ultraviolet irradiation is dependent on the quality of the incoming water. For a UV light to function optimally the light requires a few minutes to heat up before water passes over the lamp. Regular cleaning of the quartz is also important for full transmissivity and efficacy is reduced if the turbidity of the water passing over the lamp is high (Senior & Dege, 2005).

1.2.4 Ozonation

Ozone (O_3) is a tri-atomic form of oxygen created by passing dry oxygen or air through a high voltage corona discharge in a controlled oxygen atmosphere (Morelli, 1994). It is slightly soluble in water and due to its instability, leaves no residue. Ozonation is frequently used in the spring water disinfection process as it can

disinfect the rinsing water, the bottling equipment, the bottles (sealed cap) as well as the air above the water. The application of ozone treatment to the water allows for an effective treatment against microbial contamination which thus also protects the consumer (Swancara, 2007). The ozone is added just prior to bottling of the water and once it is dissolved in the water, it undergoes three simultaneous reactions, which include disinfection, chemical oxidation and decomposition. The disinfection reaction in the water is effective against bacteria, viruses and parasites such as *Giardia* and *Cryptosporidium*. The presence of organic and inorganic materials such as iron, manganese and sulphur in the water can result in problems associated with odour and taste. The chemical oxidation reaction thus also effectively removes these odours and tastes. The instability of ozone however, causes it to decompose rapidly to oxygen (National Drinking Water Clearinghouse, 1996). Ozone can also react with natural organic substances to form low molecular weight by-products. These substances could promote biological growth in distribution systems thereby decreasing the efficacy of ozone. Ozone should then be used in combination with other disinfecting systems capable of maintaining an active residual for longer time periods (Glaze, 1987).

1.3 SPRING WATER DISTRIBUTION SYSTEMS

A spring water distribution system is a system of pipes that connects the spring water source to the final point of bottling and allows the water to flow through a series of filters as a result of gravity and pumps **(Figure 1.2)**

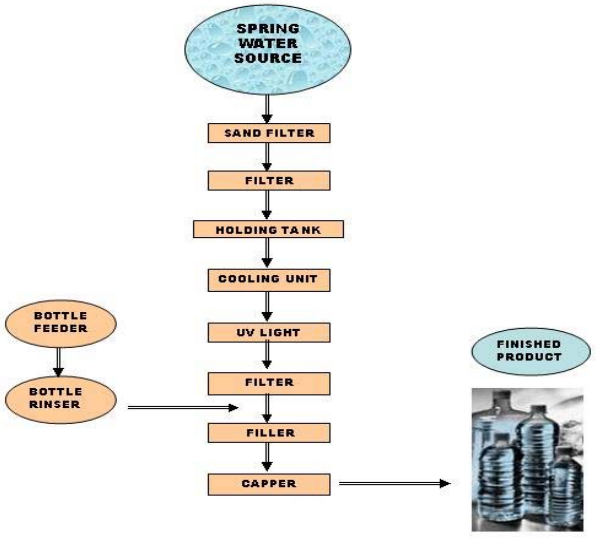


Figure 1.2 Flow diagram representing a typical spring water bottling process.

The source of the spring is usually covered to protect against possible contamination by soil or external pollutants (British Soft Drinks Association, 1995). The piping used in the spring water distribution system should also comply with the stipulated requirements. Inert materials such as ceramic or stainless steel are widely used as they prevent deterioration by water, handling, servicing or disinfection. In addition, the piping system should be easily accessible to allow for good cleaning and the contact piping surface should be smooth for easy water flow. The distribution system should also be designed with no dead ends and it should be self draining, so as to prevent any stagnant water accumulating, which could serve as a possible source of contamination (Senior & Dege, 2005). Treatment systems such as filtration, UV irradiation and ozonation are also incorporated into the spring water distribution system to ensure a good quality bottled water. Bottling of the water needs to occur very close to or at the actual source to ensure that the water is free from microbial contamination and is fit for human consumption. The quality of the packaged bottled water is further ensured by law, with the maximum levels of essential elements stipulated in **Table 1.2** (South Africa, Department of Health, 2004).

1.3.1 Cleaning and disinfection of spring water distribution systems

Detergents and sanitisers used in a spring water distribution system should be suitable for the cleaning of the piping system and must be approved for cleaning of food contact surfaces. It is also essential that all residues be thoroughly removed by rinsing the system prior to the bottling of the water. In addition, sanitisation of all equipment is crucial to the bottling process as the water could possibly be contaminated with microorganisms attached to the piping in the distribution system. In the U.S., sanitisers must reduce the microbial activity of *Escherichia coli* and *Staphylococcus aureus* by as much as 99.99% or five logs in 30 sec at 25°C. The following factors should also be taken into consideration for the effective sanitisation of the distribution line; contact time, concentration and temperature relationships, pH, intimate contact with cell walls of all microorganisms and the type of microorganisms associated with the system (American Water Works Association, 1999; Senior & Dege, 2005). Sanitisers commonly used for the disinfection of the spring water distribution system include chlorine and oxonia.

1.3.1.1 Chlorine

Chlorine (Cl_2) is one of the most effective and inexpensive organic and inorganic biocides (Mittelman, 1986). It is also one of the most common disinfectants used by water treatment plants to disinfect the piping system as it is effective against almost all microorganisms. The chlorination process involves the addition of chlorine to the cleaning water in the form of a gas (Cl_2 gas), solid [chlorine dioxide (ClO_2)], or liquid [sodium hypochlorite (NaoCl), and calcium hypochlorite $Ca(OCl)_2$] (Morelli, 1994; Tchobanoglous & Schroeder, 1985; National Drinking Water Clearinghouse, 1996). The concentration of chlorine required for disinfection of the piping system is dependent on the amount of microbial organisms present. The quantity of chlorine required to eliminate all living organisms from the water and to react with other organic substances is then called the chlorine demand (Morelli, 1994). Factors which influence the disinfection efficiency of chlorine include the initial contact time, the concentration and form of disinfectant, microbial load, pH and temperature (National Drinking Water Clearinghouse, 1996). However, chlorination may lead to the formation of chloroform and other trihalomethanes. The initial dose of chlorine therefore depends on the effect it will have on the organoleptic quality of the water as well as the formation of the trihalomethanes (Symons, 1981).

The advantages of chlorine as a sanitiser include its cost effectiveness, low temperature efficacy, non-film forming, hard water tolerance and broad spectrum of activity (National Drinking Water Clearinghouse, 1996). However, chlorine could corrode the piping material, is unstable and has the potential to form by-products.

1.3.1.2 Oxonia

Oxonia Active™ is a clear, colourless liquid that is generally applied as a broad-spectrum peroxyacetic acid, antimicrobial agent in sanitation. It is however, also widely used for sanitising the piping system of the spring water distribution system and is effective against a wide range of microorganisms such as *Staphylococcus aureus, Escherichia coli, Listeria monocytogenes, Salmonella typhimurium, Pseudomonas aeruginosa, Vibrio cholerae* and *Saccharomyces cerevisiae*. When used at the recommended dosage, Oxonia does not corrode materials, such as stainless steel (types 304 and 316) or aluminium, all of which are generally used within the spring water distribution system. Furthermore, Oxonia is environmentally friendly and rapidly breaks down into water, oxygen and acetic acid (Ecolab™, 2004). Additional advantages of Oxonia include, no residue formation, it has a broad

spectrum of bactericidal activity, and has activity over a broad pH range of up to 7.5. Oxonia however, has limited activity against fungi, can be corrosive to soft metals and exhibits metal ion sensitivity (Senior & Dege, 2005).

1.4 MICROORGANISMS ASSOCIATED WITH BOTTLED WATER

The South African National Standards for Bottled Water, (2003) states that natural mineral water is not allowed to undergo any disinfection process. This implies that the source water must be of good microbial quality and the piping system should be effectively sanitised to prevent contamination of the treated spring water. In addition to the selection of the groundwater source, the integrity of the source water, system disinfection, bottling process and packaging material, are crucial factors in ensuring the delivery of a safe healthy product to the consumer. To maintain and manage the microbial risk of the water source, faecal contaminant indicator organisms must be absent and the risk of actual contamination must be minimal. Spring-, bottled-, well- and borehole water could however, be susceptible to contamination by enteric pathogens such as parasites, bacteria and viruses (Manaia *et al.,* 1990). Waterborne infections are also usually associated with warm, moist environments, where the bacterial counts are high and disinfection is generally deficient (Le Chevallier *et al.,* 1988).

According to the World Health Organisation (2001) Microbial Methods, the Heterotrophic Plate Count (HPC) is generally used to assess the microbial quality of bottled water. In a random survey into the quality of bottled water conducted in South Africa by Ehlers *et al.* (2004), 10 brands of bottled water were analysed over a period of three months. The heterotrophic plate counts analysed ranged from 1.0^{1} cfu.ml^{-1} to 8.89 x 10^{3} cfu.ml^{-1} in the bottled water. Two of the ten brands had high HPC counts which exceeded the SANS limit of < 100 organisms/ml. The presence of these high numbers in the source water could have been due to the natural microbial flora present in the water multiplying after bottling or microbial contamination could have occurred due to the inadequate sanitisation of bottles or equipment. Heterotrophic plate count measurement of bottled water is used not only to indicate the level of disinfection of the distribution and bottling system but also assists in indicating if any changes in the water occurred from the borehole to the final bottled product (Leclerc & Moreau, 2002). During a surveillance study for waterborne disease outbreaks in the United States between 1993 and 1994, 30 outbreaks associated with drinking water were reported. These outbreaks resulted in illness in

an estimated 405 366 people. Etiologic agents were identified in 25 of the 30 outbreaks with the following pathogens responsible for isolated outbreaks *Campylobacter jejuni*, *Shigella sonnei*, *Shigella flexneri* and non *O1 Vibrio cholerae* (Kramer *et al.,* 1996).

The microbial agents indicated in **Table 1.4** present a serious risk of disease whenever they are present in concentrations exceeding the stipulated limits in water supplies and their elimination from it should be given high priority.

Table 1.4 Waterborne pathogens and their significance in water supplies (World Health Organisation, 1996)

Pathogen	Health Significance	Main Route of Exposure[a]	Persistence in Water Supplies[b]	Relative Infective dose[c]
Bacteria				
Campylobacter jejuni, C. coli	High	O	Moderate	Moderate
Pathogenic *Escherichia coli*	High	O	Moderate	High
Salmonella typhi	High	O	Moderate	High
Other Salmonellae	High	O	Long	High
Shigella spp.	High	O	Short	Moderate
Vibrio Cholera	High	O	Short	High
Yersinia enterocolitica	High	O	Long	High (?)
Legionella	Moderate	I	May multiply	High
Pseudomonas aeruginosa	Moderate	C, IN	May multiply	High (?)
Aeromonas spp.	Moderate	O, C	May multiply	High (?)
Mycobacterium, atypical	Moderate	I, C	May multiply	?
Viruses				
Adenoviruses	High	O, I, C	?	Low
Enteroviruses	High	O	Long	Low
Hepatitis A	High	O	Long	Low
Hepatitis E	High	O	?	Low
Norwalk virus	High	O	?	Low
Rotavirus	High	O	?	Moderate
Small round viruses	Moderate	O	?	Low (?)
Protozoa				
Entamoeba histolytica	High	O	Moderate	Low
Giardia intestinalis	High	O	Moderate	Low
Cryptosporidium parvum	High	O	Long	Low
Acanthamoeba spp.	Moderate	C, I	May multiply	?
Naelgleria fowlerei	Moderate	C	May multiply	Low
Balantidium coli	Moderate	O	?	Low
Helminths				
Dracunculus medinensis	High	O	Moderate	Low
Schistosoma spp.	Moderate	C	Short	Low

?- Not known or uncertain

a- O = oral (ingestion); I = inhalation in aerosol; C = contact with skin; IN = ingestion in immunosupressed patients

b- Detection period for infective stage in water at 20ºC: short = up to 1 week; moderate = 1 week to 1 month; long = over 1 month

c- Dose required to cause infection in 50 % of healthy adult volunteers

According to the South African National Standards for Bottled Water (2003) the acceptable microbiological limits for bottled spring water clearly states that coliform bacteria and faecal coliform bacteria must be absent per 100ml and the total

viable colony count should be < 100 organisms per 1ml of water **(Table 1.5).** It is also essential that the water be analysed within 24 hours of bottling.

Table 1.5 Microbiological criteria as set out by the South Africa National Standards for Bottled Natural Water, 2003.

Microorganisms	n	c	m	M	Method
Coliforms	5x250ml	1	0	1	ISO/DIS 9308/1
Faecal streptococci	5x250ml	1	0	1	(ISO 7899/2)
Spore-forming sulfite-reducing anaerobes	5x250ml	1	0	1	(ISO 6461/2)
Pseudomonas aeruginosa	5x250ml	0	0	-	(ISO 8360/2)

n = No of units making up the sample

c = the no of units in the samples that can fall between m and M

m = threshold below which all results are considered satisfactory.

M = acceptability threshold, the results are considered unsatisfactory if one or more units yields values = or > M.

Leclerc and Moreau (2002) investigated the microbiological safety of natural mineral water at source and after bottling. As the nutrient load in the source water is usually low the bacterial populations were generally heterotrophic and therefore may enter a viable but non cultural state. The number of viable counts however, increased rapidly after bottling, reaching $10^4 – 10^5$ colony forming units ml $^{-1}$ within three to seven days. The major bacteria isolated from the natural mineral water were identified as *Pseudomonas* fluorescent spp., *Pseudomonas* non-fluorescent species, *Acinetobacter, Alcaligenes*, *Comamonas* spp., *Cytophages*, *Flavobacterium, Arthrobacter* and *Corynebacterium*.

The flora of natural mineral water has been studied in great detail and the major groups of bacteria isolated from mineral water include amongst others *Escherichia coli*, *Salmonella typhi*, *Pseudomonas* fluorescent and non-flourescent spp, *Alcaligenes*, Proteo bacteria, *Cytophaga* spp. (Leclerc & Moreau, 2002), and *Vibrio cholerae* (Kramer *et al.,* 1996). Spring bottled water cannot be exposed to any disinfection treatment processes which can modify the biological content of the water and therefore the microbiological integrity of the water at the spring source is very important. The microorganisms associated with spring bottled water can also generally be related to the type of microbial pollutants in the soil and the surrounding environment, and it is thus important to assess the microbial risk posed to the bottled water and therefore to the consumer (Leclerc & Moreau, 2002).

1.4.1 Bacteria

1.4.1.1 *Salmonella*

Salmonella is a gram-negative, rod-shaped bacterium, and includes more than 1000 different strains and serotypes. The most commonly found strains include *Salmonella typhi, S. enteritidas, S. paratyphi and S. typhamurium*, with *S. typhi* being the most pathogenic strain causing typhoid fever. Infection with *Salmonella* causes salmonellosis, with the symptoms including the onset of fever accompanied by severe abdominal pain, nausea, vomiting and diarrhoea (Klein, 2002). The severity of salmonellosis is dependant on the serotype of the organism, the dosage of bacteria ingested and certain host factors, such as age and concurrent illness, amongst others (Lloyd, 1983).

Waterborne outbreaks have mostly been associated with *Salmonella typhi* and much less frequently with *S. paratyphi* or other *Salmonella* serotypes (Cohn *et al.,* 1999). Most salmonellae are primarily pathogens of animals, and can be transmitted when faecal contamination of a groundwater or surface water source occurs. Inadequately treated and disinfected drinking water, are the main causes of epidemic waterborne outbreaks caused by *Salmonella* spp (Ryan, 2004). Salmonellae can be found in open wells as a result of the drainage or flooding of contaminated surface water into unprotected well shafts. It is uncommon for salmonellae to be isolated from piped water supplies, and the presence of *Salmonella* in potable water or even bottled mineral water may be indicative of a serious fault in the design or management of the water distribution- or bottled water plants (Lloyd, 1983; Cohn *et al.,* 1999). Kramer *et al.* (1996) investigated the waterborne disease outbreaks in the United States for the period 1993 to 1994. Over the two year period 17 states were monitored and a total of 30 outbreaks were associated with drinking- and commercially bottled water. Based on their results *Salmonella* serotype *typhimurium* was responsible for one of the outbreaks resulting in several deaths.

1.4.1.2 *Escherichia coli*

Escherichia coli (*E. coli*) belongs to the family of *Enterobacteriaceae* and is a normal inhabitant of the intestine. It is a specific indicator of faecal contamination of water sources (Leclerc, 2002). The strains of *E. coli* can be divided into five main groups namely, enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), enteroheamorrhagic (EHEC), and facultatively eneteropathogenic (FEEC) (Jay, 1992). The enteroinvasive strains of *E. coli* attack the colon resulting in the

production of symptoms similar to that of shigellosis, while infection with the enterotoxigenic class results in cholera like symptoms (Tartakow & Vorperian, 1980). Epidemiological evidence suggests that the enterotoxigenic strains are the major cause of *E. coli* diarrhoea in developing countries (World Health Organisation, 1996). Vero cytotoxin-producing *E.coli* (VTEC) produces harmful toxins which can cause severe disease in humans. The most harmful VTEC strain is O157, which is termed 0157:H7 VTEC. The organisms belonging to this group cause disease ranging from mild diarrhoea to haemorrhagic colitis, resulting in blood stained diarrhoea accompanied by severe abdominal pain (Ryan, 2004). According to the World Health Organisation (1996) it also causes haemolytic uraemic syndrome found commonly in infants and young children, causing acute renal failure and haemolytic anaemia.

1.4.1.3 *Pseudomonas aeruginosa*

Pseudomonas is a gram-negative anaerobic rod and is commonly found in faeces, soil, water, marine environments and plants. The strain *Pseudomonas aeruginosa (P. aeruginosa)* is an opportunistic pathogen and its presence in water usually indicates a general lack of cleanliness and disinfection in a distribution system. The incidence of illnesses caused by *P. aeruginosa* not only results from contaminated water, but may also occur as a result of contaminated food and equipment in contact with the water (Jay, 1992).

The presence of *Pseudomonas* spp. in potable water causes a serious deterioration in the bacteriological quality and can affect the organoleptic (taste, odour and turbidity) properties of the water. Pseudomonads are one of the most frequently isolated organisms from natural mineral water as they are easily adaptable to colonisation in groundwater due to the organic carbon source present in the soil (Senior & Dege, 2005). Mavridou (1992) investigated the bacterial flora of non-carbonated natural mineral water in the United Kingdom. The microbiological analysis of the water indicated that *Pseudomonas* was one of the most dominant species present in this water type.

1.4.1.4 *Vibrio cholerae*

Vibrio cholerae (*V. cholerae*) is a gram negative, curved, rod-shaped bacterium that belongs to the genus *Vibrio*. There are two major strains of *V. cholerae* called classic and El Tor and various other serotypes also exist (Ryan, 2004). Cholera is a waterborne disease caused by the organism *V. cholerae* which belongs to serovar

0 group 1. The isolation of *V. cholera* 01 from water sources used for drinking is usually an indication of faecal contamination and the symptoms associated with cholera include diarrhoea, abdominal pain, cramps, nausea, vomiting and dizziness (Jay, 1992). In the 19th century the first waterborne pathogens recognised were *V. cholerae* and *S. enterica*. In Spain and Portugal bottled water was associated with outbreaks of cholera and typhoid fever (Mavridou, 1992; Warburton *et al.,* 1992; Warburton, 2000). A study by Blake *et al.* (1977) investigating a cholera outbreak in Portugal, also identified bottled water as one of the possible sources of contamination along with shellfish and undercooked chicken. In 2000, the first known outbreak of *Vibrio cholerae* O1 infection occurred on Ebeye Island, Republic of the Marshall Islands, southwest of Hawaii, due to inadequately chlorinated water. During the period December 2000 to 8th January 2001, 278 individuals on Ebeye Island were diagnosed at the local hospital with diarrhoea, but only 103 of these cases were attributed to cholera, resulting in six fatalities (World Health Organisation, 2000).

1.4.2 Viruses

A virus is a tiny particle of genetic material comprising of either deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) encased in a protein shell called a viral coat or capsid. Viruses cannot reproduce by themselves and have to enter a host cell to enable it to replicate. When a virus enters a cell and starts reproducing itself, it is called a viral infection. If a virus contains DNA it injects the DNA into the host cell's DNA, but if the virus contains RNA it must first convert its RNA to DNA by utilising the host cell. DNA containing viruses include Adenoviruses, Papillomaviruses, Herpes viruses and Hepatitis B viruses. Some RNA containing viruses include Influenza and Enterovirus (Biological Virus, 2008).

The enteric virus has only recently been recognised as a waterborne pathogen since being detected in many drinking water supplies across the world. The viruses of greatest significance in the waterborne transmission of infectious diseases are essentially those that multiply in the intestine of humans and are excreted in large numbers in the faeces of the infected individuals. Infections caused by enteric viruses found in contaminated water can possibly cause gastro-enteritis and hepatitis (Zaoutis, 1998). Beuret *et al.* (2002) monitored 159 samples of various mineral water brands in Europe over a one year period to investigate the microbial flora. Norovirus sequences were isolated from three leading European mineral water brands and in

53 of the 159 mineral water samples tested. Waterborne outbreaks caused by viruses have also been evident in developing countries (Murphy *et al.,* 1983).

Ehlers *et al.* (2005) investigated the detection of enteroviruses in untreated and treated drinking water supplies in South Africa. During the period July 2000 to June 2002 drinking water from boreholes, rivers, dams and springs were tested for enteroviruses. Enteroviruses were detected in 26.7% dam/spring water, 25.3% borehole water, 18.7% drinking water, 28.5% river water and in 42.5% sewage water.

Viruses and protozoan cysts are persistent organisms in the environment and are found to be more resistant to water treatment processes. In certain studies, viruses were isolated from water meeting coliform standards, indicating that the absence of coliforms is not a good indicator of virus free water (Sobsey *et al.,* 1995).

1.4.3 Helminths

Helminths, generally describe parasitic worms that vary in size from 1mm to an excess of 1m. They are classified according to the external and internal morphology of their various life stages and they can be hermaphroditic and bisexual. General classes of helminths are Flukes (Trematodes), tapeworms (Cestodes) and Roundworms (Nematodes) (Wakelin, 2007). Helminths (nematodes and tape worms) are also common intestinal parasites that have often been isolated from wastewaters (Toze, 1999). Guinea worms occur in rural areas where piped water supplies are not always available. They can only be controlled by ensuring the safety of boreholes and wells and preventative measurements include filtering prior to drinking, and in some instances chemical treatments of ponds and open wells.

Schistomasomiasis is a disease caused by the infected larvae of *Schistosoma* spp. which is capable of penetrating the human skin and mucous membrane. These infections are caused by contact with the infected water during bathing, washing or while working in the contaminated water. According to the World Health Organisation (1993) Schistosomal infections are a hazard of irrigational and recreational water use, rather than drinking water.

1.4.4 Fungi

Fungi are heterotrophic in nature and therefore depend on the presence of organic material as a source of food. They can either be saprophytic that is (they utilise dead organic material as a food source) or parasitic (they utilise living cells for growth) (Jay, 1992). Generally, fungal species flourish more during summer when

temperatures of the source waters are high (Niemi *et al.,* 1982). Research conducted by Cooke (1986) also demonstrated that although fungi occur in low densities in source water, filtration and chemical coagulation prior to filtration, will increase their removal efficiency, but provide no barrier against these organisms. Gottlich *et al*. (2002) conducted a survey on drinking water derived from groundwater in Germany over a 12 month period to assess the fungal flora present. The fungal flora consisted predominantly of *Acremonium*, *Penicillium*, *Exophilia* and *Phialophora* species. To date no waterborne disease outbreaks have been reported (Bennet, 1994), although a few fungal species are pathogenic. Inhalation of these fungal spores by immunocompromised individuals may also cause respiratory problems, including pneumonia and fever, but symptoms are usually very mild. Fungal contamination of water distribution systems is however, more often associated with taste and odour problems, rather than disease. A number of fungi such as *Aspergillas, Fusarium, Penicillium, Chaetomium* and *Stachybotrys* under special conditions of moisture and temperature also produce toxins called mycotoxins (Pohland, 1993). Mycotoxins, depending on the type of toxin and the concentration, can cause illness in humans and animals.

1.4.5 *Cryptosporidium*

Cryptosporidium is an enteric protozoan pathogen of the phylum, *Apicomplexa,* and is most commonly associated with waterborne disease. A number of species of *Cryptosporidium* infect mammals and in humans the main cause of disease are *Cryptosporidium parvum* and *Cryptosporidium hominis.* The parasite is transmitted by oocysts, which once ingested excysts in the small intestine and results in infection of the intestinal epithelial tissue. Even though these oocysts are highly resistant to disinfection they can be removed from the source water by filtration or boiling (Senior & Dege, 2005).

Cryptosporidiosis is a gastrointestinal disease caused by this parasite commonly found in contaminated, untreated or unfiltered water (Lenntech Water Treatment, 2005). It is typically an acute short-term infection but can become severe in children and immunocompromised individuals. Goldstein *et al.* (1996) investigated the magnitude and source of the Cryptosporidiosis outbreak amongst individuals with Human Immunodeficiency Virus (HIV) in Nevada, Texas. Three cases of cryptosporidiosis were identified in 1992, 23 cases in 1993 and 78 laboratory cases in the first quarter of 1994. Of the 78 confirmed cases in 1994, 61 adults were HIV

positive of which 32 died by June of that year. Twenty of the HIV fatalities had cryptosporidiosis listed as cause of death on their death certificates. The outbreak was associated with municipal drinking water and highlighted the need to prevent cryptosporidiosis infections especially in immunocompromised individuals.

1.4.6 Microbial Indicators of Bottled Water Quality

The monitoring, isolation and identification of pathogens in water is a time-consuming task as, in most cases, the pathogens are present in low concentrations and large volumes of water samples are usually required. The routine analysis of drinking water thus involves testing for microorganisms such as coliforms, faecal streptococci, *P. aeruginosa* and sulphite reducing anaerobes as indicators of treatment efficiency, and water quality in the distribution system (Leclerc *et al.,* 2001). An ideal indicator organism should meet all of the following criteria; be present in faecal material in large numbers, should be stable, non- pathogenic and suitable for all drinking water varieties, be present when the pathogen of concern is present and absent in clean uncontaminated water, behave similarly to the pathogen of concern when exposed to certain treatment processes, be easily detectable by simple and inexpensive methods and have a high indicator pathogen ratio (Jay, 1992).

Coliforms are present in large quantities in soil, and their presence in water usually indicates a health risk. The water source should then be routinely monitored for faecal contamination. Coliform bacteria, when detected in treated water supplies, could also be indicative of inadequate treatment, or post treatments and thus the coliform group of organisms are primarily used as indicators in bottled water analysis (World Health Organisation, 1996). Their survival in distribution systems have been attributed to the possible formation of biofilms on pipe surfaces, but despite this finding, coliform isolation from biofilms present in water distribution systems has rarely been reported (Camper *et al.,* 1991; Le Chevallier *et al.,* 1996).

An acceptable pathogen indicator such as *E. coli,* which is also routinely tested for, must be present when the pathogen is present and must be easily detected and quantified (Gleeson & Gray, 1997). The presence of heterotrophic plate count organisms in bottled mineral water is also used as an indicator of water quality and the microbial status from the spring to the finished bottled water (Moreau, 2001).

The emergence of new pathogens such as *Cryptosporidium* implies that extensive analysis should be done on water samples even if the common indicator

organisms are absent. It is therefore important to not only use the indicator organisms as a marker for microbiologically safe water but to also incorporate testing procedures for susceptible pathogenic organisms, such as *Cryptosporidium* (Senior & Dege, 2005).

1.5 BIOFILM FORMATION IN SPRING WATER DISTRIBUTION SYSTEMS

Biofilms can be defined as a complex community of microorganisms, microbial products and detritus deposited on an organic or inorganic surface (Characklis, 1981; Characklis & Marshall, 1990). In some instances the attached community may form in patches on the surface or may cover the entire surface. In both cases the biofilm offers microorganisms greater access to nutrients, protection from antimicrobial agents and acts as a buffer if conditions change in the environment (Geesey *et al.,* 1992). Generally biofilms consist largely of bacteria although other groups of microorganisms, such as fungi or algae, can also join the microbial community. The biofilm formation process involves three phases that include the attachment of microorganisms, colonisation, and growth of microorganisms as indicated in **Figure 1.3** (Forsythe, 2000; Trachoo, 2003).

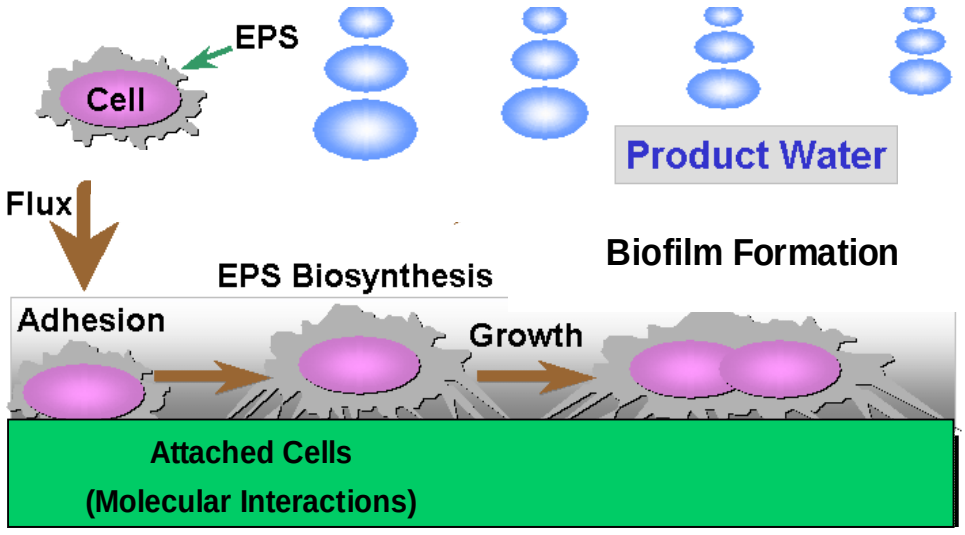


Figure 1.3 Diagrammatic representation of biofilm formation (Adapted from MicroMem Analytical, 2003)

The first step in biofilm formation involves the conditioning of the surface by the adsorption of organic and inorganic molecules Bacterial attachment to the conditioned surface can then occur by several mechanisms such as (i) sedimentation

and Brownian motion of cells, (ii) convection currents within the bulk liquid, (iii) active movement by motile bacteria or (iv) electrostatic and physical interactions between the bacterial cell and the surface (Costerton *et al.,* 1994, Costerton *et al.,* 1999 and Trachoo, 2003).

The microorganisms attach themselves to the surface by means of 'sticky' organic polymers called extracellular polymeric substances (EPS). This EPS acts as a glue to attach the microorganisms to the surface, which implies that they cannot easily be dislodged and the cells are protected from desiccation (Lindsay, 2002). The attachment of bacteria to the surface is also affected by factors such as, nutrient concentration, nutrient availability, temperature, pH, flow of water and electrolyte concentration (Lappin–Scott *et al.,* 1992). This step is followed by the adhesion of the microorganisms to the surface in a two-phase process, where the first phase is reversible and attachment occurs by weak forces and the bacteria still exhibit Brownian motion, implying that the bacteria can be easily removed by washing. The second phase is irreversible and the adhesion of the bacteria is firm and no Brownian motion can be exhibited by the organisms (Blenkinsopp & Costerton, 1991). Biofilms can however, detach from the surface and attach to a new surface thus effectively forming a new biofilm (Lappin–Scott *et al*., 1992). The detachment of sections of the biofilm from the surface is considered a community survival mechanism as it allows the microorganisms to attach to a new surface and establish growth (Geldreich & Rice, 1987). Factors which affect biofilm detachment include biofilm thickness, shear stress of the water, nutrient availability and the velocity of the water (Lappin–Scott *et al.,* 1992).

A biofilm community can survive disinfection and can thus attach to the surface of the distribution system at the time of installation or during repairs of the line or line breaks during maintenance (Geldreich, 1990). Heterotrophic bacteria present in these biofilms may cause aesthetic and organoleptic problems in the water quality, such as odours and taints. In addition, biofilms can also contribute to the bio-deterioration of medical and industrial processing systems for example; an increase in the resistance of heat transfer occurs when they accumulate on the surface of heat transfer equipment; corrosion may occur when biofilms accumulate on distribution piping, and in the medical field biofilms are responsible for various health problems such as urinary tract infections and infections related to implants, (Characklis & Marshall, 1990; Lindsay, 2002). A study investigating the bacterial, chemical and mineralogical characteristics of tubercles in distribution pipelines indicated that no

bacterial count could be detected in the final drinking water, but upon investigation of the piping system, analysis indicated multiple microorganisms attached to the surface of the piping material. Scanning Electron Microscopic (SEM) analysis of the piping material displayed complex communities of microorganisms (Tuovinen *et al.,* 1980).

1.5.1 Factors that affect biofilm growth

Microorganisms are ubiquitous in nature and flourish in conditions optimum to their growth and proliferation. As mentioned previously, factors that affect the attachment of microorganisms to the surface of the water distribution system include; temperature, pH, flow of water and electrolyte concentration (Lappin–Scott *et al.,* 1992). Nutrient availability is also important for the growth of the microorganisms in the biofilm as the principal nutrient sources such as organic carbon, phosphorous, and nitrogen are important for the growth and proliferation of coliforms and heterotrophic bacteria. Temperature is one of the most important factors controlling the rate of biofilm growth, with most microbial growth observed at temperatures of 15°C or higher. Rainfall is another factor which influences the microbial quality of drinking water as it acts as a catalyst for coliform growth (Le Chevallier*,* 1990). In addition Le Chevallier *et al.* (1988) concluded that environmental factors, the presence and effectiveness of disinfectant residuals, internal corrosion and hydraulic properties, all affect biofilm growth.

Geldreich (1988) observed that an increase in velocity of source water caused a greater influx of nutrients to the pipe surface and greater transport of disinfectants and greater shearing of biofilms from the pipe surface. During peak bottling seasons, such as summer, the rate of consumption of bottled water increases, which implies that the rate of flow velocity increases with the output of bottled water. An increase in temperature due to the warmer seasons also increases the risk of biofilm formation. Sanitisation of the system and the employment of permissible treatments are thus essential in ensuring the quality of the bottled water.

1.6 METHODS AND TECHNIQUES

1.6.1 Cultivation dependent techniques

Cultivation techniques are age old methods that employ knowledge of the nutritional requirements of the microorganisms being cultivated. The heterotrophic plate count technique, for example, uses R2A agar, nutrient agar, or plate count agar as a

nutrient source for the enumeration of aerobic bacteria. The Heterotrophic Plate Count techniques include amongst others, the pour plate, spread plate and the membrane filter technique (Resoner, 1990). One of the disadvantages of this type of technique however, is that it only detects the microorganism selected for growth under specific conditions (Hurst *et al.,* 2002). Another limitation of this technique is that some viable organisms are not able to grow on the plates as they are not culturable. These organisms are referred to as viable but non culturable (VBNC) or active but not culturable (ABNC). Certain microorganisms enter the viable but non culturable condition when they are exposed to a stressful environment (Hurst *et al*., 2002).

In a survey of the microbiological quality of bottled water sold in the UK, eight brands of domestic and imported bottled water were microbiologically analysed within three hours of purchase. The purpose of the microbiological study was to qualitatively and quantitatively assess the status of the bottled water and to assess the changes during storage of the water. The surface plate count technique was used, with Plate Count agar as the nutrient source to assess the microbial numbers. Initial counts of 10^4 cfu were obtained with *Pseudomonas spp* being the most dominant species (Benito & Sutherland, 1999).

The Most Probable Number (MPN) technique is also routinely used to determine levels of contamination in river water as it is specific for determining the total number of gas-producing organisms, which includes faecal coliforms and *E. coli*, within the water samples (Oblinger & Koburger, 1975). However, this technique does not determine or indicate the level of other culturable microorganisms which might be present in the water. The membrane filter method (MF) is specific to the determination of a vast number of microorganisms in bottled water because of its simplicity, speed and precision and results are usually expressed per 100 ml or 250 ml of test sample. A known volume of sample is passed through a membrane (usually 0.45 μm), after which the membrane is placed on agar media and incubated (Jay, 1992). The MF method can only be used for low turbidity waters (Le Chevallier *et al.,* 1982), however the overall efficiency of the MF method has been increased by the introduction of fluorescent dyes (Jay, 1992).

Swabbing, sonicating, scraping and agar contact methods are all examples of conventional cultivating methods used for biofilm sampling and enumeration (Frank and Kofti, 1990). However, these techniques may not always detect the presence of biofilms as in many cases the cells are compactly attached to the surface. A

limitation of the above cultivation methods for bacteriophage detection is that bacteriophages are usually present in very low numbers in the initial biofilm sample size and not easily detectable by certain cultivation techniques. The absence presence method was used as a qualitative method for the detection of bacteriophage indicators. This technique is similar to the MPN method. The MPN technique involves the inoculations of the sample water in specific media containing Durham tubes, followed by incubation at a specific temperature for a specific time. For the qualitative detection of bacteriophages a larger volume of water (500 ml) is enriched with the growth medium and the host bacteria and then incubated (Grabow, 2001).

1.6.2 Microscopy

Microscopy is one of the earliest techniques employed by microbiologists in the study of the morphology of bacteria. One of the most important functions of the microscopic technique is to achieve the least amount of disturbance within the system being observed. The microscope itself has not been sufficient to assess the microenvironment, taxonomic relationship and state of certain organisms, but with the use of fluorescent reporter molecules, enhanced image devices like confocal laser scanning devices, cameras and specific computer software, detailed analyses of samples is possible (Hurst *et al.,* 2002).

Microscopy is the only technique whereby bacterial biofilms can be studied at the single cell level *in situ.* Total cell numbers in environmental samples can also successfully be determined using microscopic techniques. This method has been used considerably as it has been successfully employed for the detection of the characteristics, physiology of microorganisms and community interactions of biofilms (Kumar & Anand, 1998). Confocal Laser Scanning Microscopy (CLSM), Epifluorescent- and Phase Contrast microscopy are all widely used microscopic methods. Phase contrast microscopy involves the use of high resolving power and magnification but is moderately expensive in relation to other microscopic techniques (Hurst *et al.,* 2002). It is however, the most convenient microscopic technique and it is readily used in continuous observation systems as it does not need staining or drying of cells (Lawrence *et al.,* 1997).

Confocal Laser Scanning Microscopy was also used in a study investigating the dynamics of *Pseudomonas sp.* biofilms grown in flow chambers as motility plays a role in the formation of the structures in biofilms (Tolker-Nielsen *et al*., 2000).

Protozoa can usually be detected and quantified by the use of microscopic techniques such as epifluorescence, bright field, phase contrast and differential interference contrast (DIC) microscopy. Electron microscopy is also one of the frequent methods used to assess biofilm structure and viruses in water (Hurst *et al.,* 2002). Scanning Electron Microscopy (SEM) is used to determine the number and distribution of microorganisms adhering to the surface, but lacks the resolution to give sufficient information on the adhesive structures of biofilms (Marshall, 1997). Although SEM shows the basic structure of biofilms it is disadvantaged by the fact that the bacterial cells are fixed and killed during staining.

Mueller *et al.* (2001) used CLSM to analyse biofilms on glass windows to determine their composition and thickness. In the study fluorescent probes were used and CLSM together with fluorescent *in situ* hybridisation proved to be successful in the characterisation of the biofilms. In a study conducted by Lawrence *et al.,* (1994) CLSM was used in conjunction with fluorescein and size-fractionated fluor conjugated dextrans to directly monitor and determine the diffusion coefficients within biofilms. It was also concluded in this study that fluorescent probes could be monitored in the biofilm matrix using CLSM. In a study conducted by Garabetian *et al*. (1999) epifluorescent microscopy was used to assess whether storage of freshwater samples affected the bacterial counts in the water. The water samples were stored at 4 °C and -18 °C for short (7 & 14 days) and long term (160 & 240 days) by using formalin – fixing. The study was carried out in the field and although bacterial reductions were observed, the technique was unsuitable due to the time it took to immediately filter, stain and microscopically examine the slides under the microscope.

1.6.3 Fluorescent staining

One of the most significant methods in determining the total bacterial population in water is the direct counting method, which can be achieved by using various fluorescent dyes such as acridine orange [3.6-bis (dimethylamino acridium chloride] and 4', 6-diamidino-2- phenylindole (DAPI) (Hurst *et al.,* 2002). 6-Diamidino-2-phenylindole has replaced acridine orange because of its greater fluorescent stability (Porter & Greig, 1980). Confocal Laser Scanning Microscopy (CLSM) also allows for immediate estimation of viable bacteria using fluorescent dyes. The fluorescent dyes change colour depending on the state of the cells i.e. dead, alive or injured.

The Live/Dead Baclight™ bacterial viability assay utilises mixtures of SYTO9 green fluorescent nucleic acid stain and the red fluorescent nucleic acid stain, propidium iodide. These stains differ both in their spectral characteristics and in their ability to penetrate healthy bacterial cells. When mixed in the recommended proportions, SYTO9 stain produces green fluorescent staining of bacteria with intact cell membranes, while propidium iodide produces red fluorescent staining of bacteria with damaged membranes (Molecular Probes, 2002). BacLight™ has been employed as a rapid epifluorescence staining method used to assess the viable and non–viable total counts of bacteria in drinking water. Boulos *et al.* (1999) investigated the application of this rapid staining technique to enumerate the viable and total bacteria in drinking water. The study was aimed at comparing the viable results and total counts from the BacLight™ Viability Kit to 5-cyano-2.3ditolyl tetrazolium (CTC) counts and acridine orange, respectively. The viable BacLight™ counts were comparable to the CTC counts in the absence of stress and the BacLight™ total counts were also comparable to the acridine orange counts differing by < 0.1 log/ml.

1.6.4 Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) is a method that allows for the multiple production of target DNA by utilising a thermostable polymerase enzyme. The identification of organisms or the detection of microbial pathogens is achieved by using short sections of synthetic single stranded DNA called oligonucleotide primers. Primers can be designed for specific organisms or groups of organisms (Toze, 1999). The most frequently used PCR method employs a temperature of < 90 °C to separate the DNA into two single strands. A lower temperature is then applied which allows for the primers to attach to a target section of DNA. After the primer attachment a copy of the target DNA is produced by the polymerase enzyme. This DNA replication takes place at an intermediate temperature which falls between the previous two temperature cycles. After about 25-30 cycles more than 10^9 copies of the target DNA is produced (Glick & Pasternak, 1994). A standard PCR method can be employed for DNA replication or it can be modified to semi-nested PCR utilising a second PCR reaction with additional primers or nested PCR which uses a new set of primers. The modified PCR technique improves the detection efficiency because of the additional amplification of the already amplified DNA (Gajardo *et al.,* 1995).

The advantages of PCR in relation to other culture techniques or standard methods used for the detection of microbial pathogens in water, is that it is specific, sensitive, rapid, accurate and can detect small amounts of nucleic acids in a sample. This reaction involves the amplification and detection of DNA sequences and viable and dead microbial cells can also be amplified (Amman *et al.,* 1995). Polymerase Chain Reaction as a method potentially decreases the detection time and the cost associated with microbial detection in water (Atmar *et al.,* 1995). However, limitations of the PCR technique include the detection of false positives by detecting non–viable microorganisms, naked nucleic acids or by possible contamination during testing (Toranzos & Bej, 1997). Vivier *et al.* (2004) routinely monitored drinking water over a one year period for the presence of Enteroviruses. A nested PCR approach followed by enzyme restriction was employed for both detecting and typing of any Enteroviruses as it is simple, rapid and able to detect viruses in large volumes of water samples. In a random survey of the microbiological quality of bottled water in South Africa the molecular detection of calici-entero and rotoviruses was done using the PCR technique. Ten different bottled water samples were analysed and no enteric viruses, bacteriophages, faecal coliform bacteria, Enterrococci and *C. perfringens* were detected using the reverse transcriptase-PCR technique (Ehlers *et al.,* 2004). A study conducted by Girones *et al.* (1995) on the detection of Adenovirus and Enterovirus using the PCR amplification technique demonstrated that DNA viruses and RNA viruses were detected in the same sample of polluted water analysed. This technique therefore reduced the time required for virus detection in water samples. Amman *et al.* (1995) investigated the phylogenetic identification and *in situ* detection of individual microbial cells without cultivation from marine and soil environments. The opportunistic pathogen *Vibrio vulnificus* was isolated from water by concentrating the cells on membrane filters and using fluorescently-labelled oligonucleotide probes for tRNA sequences. A reverse transcriptase PCR system was used for the reverse transcription and PCR amplification. Enteroviruses were detected in 11% and 16% of the drinking water samples collected from two different treatment plants. Tsen *et al.* (1998) utilised PCR and selected regions of the *E. coli* 16S rRNA gene to detect *E. coli* cells in water, and by the addition of an enrichment step a detection limit of as low as one *E. coli* cell /100ml was determined. A study conducted by Kong *et al*. (1995) investigated the feasibility of detecting waterborne pathogens in sea water samples from the Hong Kong Island and the Ngau Tau Kok Island in Kowloon using multiplex PCR. Multiplex PCR utilises a combination of

primer sequences, which have been tested for self-complementarity and inter-primer annealing. Combination primers were used to simultaneously detect target DNA of *Salmonella typhimurium, Vibrio cholerae* and *E. coli*.

1.6.5 Flow Cytometry

Flow cytometry (FCM) is considered an alternative method to the microscopic enumeration of total cells (Hurst *et al.,* 2002). This method employs the principles of light scattering, light excitation and the emission of fluorochrome molecules to generate data from particles or cells ranging in cell sizes of 0.5 µm to 40 µm in diameter (Current Protocols in Flow Cytometry, 2005). A typical flow cytometer consists of a light source, collection optics, electronics and a computer to translate the signals to data. Flow cytometry can also be referred to as fluorescent activated cell sorting (FACS) and is used to sort and measure different types of cells by fluorescent labelling of markers on the surface of the cell. Fluorescent beads can also be added to aid in the calculation of the total or absolute cell count. Flow cytometry is a rapid method that allows for a large amount of data to be available in a very short time period (Javois, 1999). Although FCM is a rapid technique it also has certain technical limitations, as the reagents required for analysis are costly and there could be disadvantages depending on the type of flow cytometer used (Montes *et al*., 2006).

Monis *et al.* (2003) investigated the enumeration of waterborne bacteria using viability assays and flow cytometry and compared it to culture-based techniques. Untreated water as well as potable water samples were taken at various locations around South Australia. Due to the short testing time, flow cytometry was used along with the BacLight™ bacterial viability kit and carboxyfluorescein diacetate (CFDA) to detect physiologically active bacteria in the water samples. The FCM technique yielded 5.56×10^2 and 3.94×10^4 active bacteria.ml^{-1} in comparison to the culture based techniques which were 2-4 log cycles less. A range of fluorescent dyes were utilised along with flow cytometry to rapidly enumerate the viable bacterial planktonic communities in freshwater environments in Cumbria in the United Kingdom (Porter *et al.,* 1995). It was possible to enumerate viable cell counts within two hours after sampling at the site using carboxyfluorescein diacetate or chemchrome B with a detergent-mediated permeabilisation step to aid in the prevention of overlapping between background fluorescence and labelled cells.

Pianetti *et al.* (2005) investigated the viability of *Aeromonas hydrophila* in different types of water by means of flow cytometry in comparison with the plate counting technique and spectrophotometric analysis. *Aeromonas spp.* in water can pose a risk to human health and can also cause gastroenteritis, haemolytic uremic syndrome and septicaemia. *Aeromonas hydrophilia* was inoculated into samples of river water, brackish water, spring water, mineral water and drinking water. The bacterial count was then determined on a daily basis over a 30 day period. Cell membrane integrity was assessed by staining the cell with SYBR Green I (Molecular Probes) and propidium iodide (PI) (Molecular Probes) fluorescent probes which allowed for the distinction between intact membrane live cells (green), damaged membrane cells (green plus orange red) and cells with compromised membranes (orange red – dead cells). The tests indicated that flow cytometric analysis yielded *A. hydrophila* growth even when there was no plate count and the optical density was low. Paulse *et al.* (2007) assessed various enumeration techniques to investigate the planktonic bacterial population in the Berg River, Western Cape, South Africa. The heterotrophic plate count technique was used to determine the number of culturable microorganisms in the water samples and flow cytometry was used to evaluate the total bacterial counts. The study indicated that the average heterotrophic plate count represented only a fraction of the total FCM counts and < 6.06% of the viable FCM count.

1.7 BIOREMEDIATION

Bioremediation is the process whereby microorganisms utilise their metabolic potential to clean up contaminated environments under controlled conditions (Watanabe & Baker, 2000). It utilises these living organisms and their metabolic components to reduce contaminants in the environment into less toxic forms (Mueller *et al.,*1996). This biological process utilises either naturally occurring organisms, or organisms introduced to the site, to degrade the toxic compounds. The process of introducing microorganisms to the site to speed up the biodegradation process is known as bioaugmentation. The reduction in the level of contaminants is often the result of various diverse groups of microorganisms interacting together and functioning in the contaminated environments under various specific conditions. A limitation of bioremediation is that it can be time consuming and can only be applied to biodegradable compounds. Bioreactors are thus a long term technology and may take up to several years for degradation of the desired compound (Vidali, 2001).

Bioremediation has been applied to clean up contaminated water sludges and waste streams as the process is relatively cost effective and usually entails the employment of simple technology (Boopathy, 2000). It is important to assess the suitability of the environment for this biological process in order to achieve the desired results, as bioremediation cannot be used to degrade all types of contaminants, for example chlorinated hydrocarbons, which are often resistant to microbial degradation (Coldberg & Young, 1995; Vidali, 2001).

The presence of oxygen, pH, nutrient availability and the ability of the microbial population to degrade the pollutants are all important factors in the optimisation of the bioremediation process. A bioreactor utilises microorganisms to degrade contaminants in water through suspended biological systems. Organic matter in contaminated groundwater is aerobically degraded by microorganisms while being circulated through an aerated basin to carbon dioxide, water and new cells (Vidali, 2001).

The organisms primarily employed in bioremediation can be subdivided into aerobic or anaerobic organisms, lignolytic fungi and methylotrophic bacteria. *Methylotrophs* are aerobic bacteria that utilize methane as an energy and carbon source. Aerobic bacteria require oxygen to grow and include *Pseudomonas, Alcaligenes, Mycobacterium* and *Rhodococcus.* Anaerobic bacteria flourish in the absence of oxygen, but they are not as widely used as aerobic bacteria for the reduction of contaminant levels. *Lignolytic fungi* have the ability to degrade environmental pollutants such as saw dust, corn cobs and straw (Allard & Neilson, 1997).

As bioremediation is a natural process, it is widely accepted by the public and allows for the complete destruction of a wide variety of contaminants in a cost effective manner. Various bioremediation techniques can also be employed depending on the aeration of the area and the degree of saturation. *In situ* can be defined as a bioremediation technique that can be applied to soil and water with minimalistic disturbances to the environment. *Ex situ* is the process whereby the site has been moved from its original location either by pumping of the water or by excavating of the soil.

There are three basic bioreactor configurations namely, slurry bioreactors, solid state fixed bed bioreactors and rotating drum dry solid bioreactors. Slurry bioreactors are usually used for the degradation of toxic compounds in contaminated soil. A slurry bioreactor consists of a containment vessel and a stirring apparatus

used to ensure mixing of the solid, liquid and gas phase in the contaminated soil to speed up the bioremediation process. One of the limitations of the slurry bioreactor is that the soil must be pre-treated by possible excavation before being transferred to the bioreactor (Vidali, 2001). A trickling filtration system is a solid state fixed bed bioreactor that consists of a bed of highly permeable media, a water distributor and an underdrain system. Wastewater is trickled over the filter bed and the microorganisms attached to the filter bed to degrade the organic contaminants. The filter media can either be plastic, wood or rock (Remediation Technologies, 2007). Rotating drum bioreactors consists of disks partially immersed into the water to be treated. Rotation of the disks allows the attached biomass to interact alternatively with the treated water and the air. This type of bioremediation is simple and inexpensive and utilises low energy consumption, but one of the major disadvantages is that the quality of the effluent produced is not that high (Langwaldt & Puhakka, 2000).

Phenol is a water soluble toxic compound, highly mobile and therefore easily absorbed into water systems, imparting severe odour and taste problems. A study conducted by Boaventura (2001) to assess the biodegradation of phenol by *Pseudomonas putida* in a trickling bed reactor, yielded an average biological yield during the pseudo steady state operation of 0.8 g of biomass produced per gram of phenol removed. In Germany, the *in situ* bioremediation of chlorobenzene-contaminated ground water was tested under anoxic conditions in the presence of nitrate and under mixed electron acceptor conditions (oxygen & nitrate), by utilising hydrogen peroxide as the oxygen releasing compound in a flow through fluidized bed bioreactor. A few aerobic bacterial species responsible for the degradation of chlorobenzene include *Pseudomonas* and *Rhodococcus*. The addition of the hydrogen peroxide indicated an enhanced level of chlorobenzene degradation in relation to the nitrate presence only (Vogt *et. al*., 2004).

1.8 OVERALL AIM OF STUDY

The primary aim of the research was to investigate the bacterial contamination at various points in a spring water bottling system in the Western Cape, South Africa. To achieve this aim, a hypothesis stating that the good quality of groundwater does not support notable bacterial growth, was investigated. An alternative to this hypothesis would be that the lack of disinfection procedures within the spring water industry, leads to an increased risk of microbial contamination and biofilm formation.

The influence and control of various parameters on bacterial growth within the bottled water industry was thus investigated as follows:

1.8.1 Microbiological analysis of water samples to investigate the level of bacterial pollution in the spring water bottling system.

- Heterotrophic plate counts on R2A agar and Nutrient agar.
- Flow cytometry in conjunction with Liquid counting beads and the Live/Dead BacLight™ stain to determine the total cell counts (dead & viable).
- Isolation of predominant organisms and molecular typing using the polymerase chain reaction, followed by 16S ribosomal RNA sequencing.

1.8.2 The application of bioremediation for the possible treatment and reduction of microbial contamination in spring water by the optimisation of a laboratory - scale bioreactor system.

- Flow Cytometry in conjunction with Liquid counting beads and the Live/Dead BacLight™ stain to determine the total cell counts (dead & viable).
- Direct Acridine Orange Counts in conjunction with epifluorescent microscopy to determine the total cell counts (dead & viable).

Investigation into the Microbial Contamination in a Spring Water Distribution System, Western Cape, South Africa

L Behardien[1], AN Paulse[2], VA Jackson[2], S Khan[2] & * W Khan[3]

[1] Department of Food Technology, Faculty of Applied Science, Cape Peninsula University of Technology, Cape Town, 8000, South Africa.

[2] Department of Biomedical Sciences, Faculty of Health and Wellness Sciences, Cape Peninsula University of Technology, Bellville, 7535, South Africa.

[3] Department of Agriculture and Food Science, Faculty of Applied Science, Cape Peninsula University of Technology, Cape Town, 8000, South Africa.

* To whom all correspondence should be addressed.

Tel: +27 21 460 3175, Fax: +27 21 460 3193; e-mail: khanw@cput.ac.za

Abstract

The aim of the study was to investigate the microbial contamination in a spring water distribution system in the Western Cape, South Africa. Sampling at various points from the spring and throughout the bottling system started in February 2004 and continued until November 2004. The number of culturable cells were determined using the heterotrophic plate count (HPC) technique and total microbial counts were evaluated by Flow Cytometric analysis (FCM). Heterotrophic plate counts in the final bottled water ranged from 1.34 x 10^8 cfu/ml (week 1) to 5.00 x 10^4 cfu/ml (week 46). The total cell counts ranged from 2.09 x 10^8 microorganisms/ml (week 1) to 5.70 x 10^7 microorganisms/ml (week 46, site J). The current water legislation states that the heterotrophic plate count of the final bottled water must be < 100 organisms/ml within 24 hours of bottling. In week 46 differences in the heterotrophic plate counts and the viable cell counts could be observed at all the sampling sites. The higher FCM counts indicated that the flow cytometry technique was able to detect viable but not culturable organisms in the water and that the heterotrophic plate count technique only allowed for growth of the viable and culturable cells present in the water samples. It can be concluded that the FCM technique was a more reliable technique for the quantitative enumeration of microbial populations in water samples. 16S ribosomal RNA of the bacterial species isolated from the water samples was amplified with PCR and phylogenetic trees were constructed using the neighbour-joining algorithm. The sequenced isolates from the various water samples belonged to the major groups *Bacillus sp*, and *Enterobacteriaceae* such as *Shigella boydii*, *Serratia sp., Enterobacter asburiae* and *Pseudomonas sp.*

Keywords: bacterial contamination, flow cytometry, heterotrophic plate count, molecular typing, spring water distribution system.

1. Introduction

Over time the pressure below the surface of the earth becomes greater than the atmospheric pressure, thereby naturally expelling water through various soil and rock layers to the surface as a spring (U.S Department of the Interior Geology Survey, 2006). Spring water is generally derived from a subterranean source where the water flows naturally in aquifers below the surface of the earth [South African National Standards (SANS, 2003)]. As the water filters through the underground rock layers it absorbs various minerals, which differ depending on the rock layers it filters through. Mineral water therefore differs from drinking water in its chemical composition and mineral content.

Natural spring water is obtained directly from these underground water sources, and is collected under conditions to maintain its natural chemical composition and microbiological purity. According to the South African National Standards for Bottled Water (2003) the source of the spring must not be situated at or close to any danger of pollution by sewerage, farming operations, waste disposal or industrial activities or any combination of the above pollutant sources. Natural bottled water can also only be subjected to certain treatment processes such as the separation from unstable constituents by decantation and or filtration, aeration, and by any process that will ensure that the natural mineral content is not modified, such as ultraviolet irradiation and ozonation (South Africa, Department of Health, 2004).

Surface water sources are known vehicles of enteric pathogens such as viruses, parasites and bacteria (Manaia *et al*., 1990). The microorganisms associated with spring water, which is derived from a ground water source, can also generally be related

to the type of microbial pollutants in the soil and the surrounding environment. It is thus essential to assess the microbial contamination risks or the level of pollution at the location of the spring (Leclerc & Moreau, 2002). Coliform bacteria, when detected in treated water supplies, could also be indicative of inadequate treatment, or post treatments of the water system (World Health Organisation, 1996). Coliforms are present in large quantities in soil, and if found in water they usually present a significant health risk. The water source should then be routinely tested for faecal contamination and coliforms are therefore used as indicator organisms in bottled water analysis (Ryan, 2004).

Blake *et al*. (1977) investigated a cholera outbreak in Portugal and identified bottled water as one of the possible sources of contamination along with shell fish and uncooked chicken. A total of 2467 confirmed hospitalised cases associated with this outbreak were reported. Beuret *et al*. (2002) monitored three brands of natural mineral waters in Europe over a one year period to investigate and identify the microbial flora present. Norovirus sequences were isolated from three leading European brands of still mineral water, during the course of this investigation. Research by Leclerc (2002) on the microbiological safety of bottled water, identified the major species of bacteria associated with natural mineral water as *Pseudomonas* fluorescent species, *Pseudomonas* non-fluorescent species, *Acinetobacter*, *Alcaligenes*, *Comamonas spp*., *Cyto-phaga Flavobacterium*, *Arthrobacter* and *Corynebacterium*.

Biofilms can be defined as a complex community of microorganisms, microbial products and detritus deposited on an organic or inorganic surface (Characklis and Marshall, 1990). The biofilm community can survive disinfection and can also attach to the surface of the distribution system at the time of installation or during repairs of the line or line breaks (Geldreich, 1990). Heterotrophic bacteria present in these biofilms

may cause aesthetic and organoleptic problems in the water quality such as odours and taints. In addition, biofilms can also contribute to the bio-deterioration of medical and industrial processing systems (Lindsay, 2002). Biofilm accumulation on heat transfer equipment and distribution piping, may respectively lead to an increase in the resistance of heat transfer and corrosion. In the medical field, biofilms are responsible for various health problems, such as urinary tract infections and infections related to implants (Characklis and Marshall, 1990).

Geldreich and Rice (1987) observed that an increase in velocity caused a greater influx of nutrients to the pipe surface, greater transport of disinfectants and greater shearing of biofilms from the pipe surface. During peak bottling seasons, especially summer, an increase in the rate of consumption of bottled water occurs. This demand in water output within the distribution system causes an increase in flow velocity. Sanitisation of the system and implementation of permissible treatments are thus essential in ensuring the quality of the bottled water.

Heterotrophic plate count bacteria are generally used to assess the microbial quality of bottled water (SANS, 2003). The heterotrophic plate count technique (HPC) enumerates aerobic and facultative anaerobic bacteria found in the water, as these organisms are capable of growing on organic compounds found in the culture medium. In a random survey of bottled water conducted in South Africa by Ehlers *et al*. (2004), heterotrophic plate counts ranging from 1.1×10^2 cfu. mℓ to 5.4×10^2 cfu. mℓ were recorded. It was concluded that the presence of these high numbers were due to the natural microbial flora present in the source water and could thus be used to indicate the level of disinfection of the distribution and bottling system required (Leclerc and Moreau, 2002). One of the disadvantages of the heterotrophic plate count technique is that it only detects the microorganism selected for growth under specific growth conditions.

Another limitation of this technique is that some viable organisms are not able to grow on the plates as they are not culturable and enter a viable but non culturable state (VBNC) (Hurst *et al*., 2002). Certain microorganisms enter this VNBC state when they are exposed to stressful environments (Roszak and Coldwell, 1987).

Flow cytometry can be referred to as fluorescent activated cell sorting (FACS) and is used to sort and measure different types of cells by the fluorescent labelling of markers on the surface of the cell (Javois, 1999). The addition of fluorescent beads in conjunction with the Live/Dead BacLight™ viability probe allows for the enumeration of total bacteria in the water samples. Monis *et al*. (2003) investigated the enumeration of water-borne bacteria using viability assays and flow cytometry and compared it to culture-based techniques. Untreated water as well as potable water samples were collected at various locations around South Australia. Flow cytometric evaluation was employed in conjunction with the BacLight™ bacterial viability probe and carboxyfluorescein diacetate (CFDA) to detect physiologically active microorganisms or bacteria in the water samples. The FCM technique yielded 5.56 x 10^2 and 3.94 x 10^4 active bacteria per mℓ in comparison to the culture based techniques which were two to four log cycles less. Due to the short testing time, it was recommended that flow cytometry be used to detect physiologically active bacteria from potable and raw waters in Australia. Paulse *et al.* (2007) assessed various enumeration techniques to investigate the planktonic bacterial population in the Berg River, Western Cape, South Africa. The heterotrophic plate count technique was used to determine the number of culturable microorganisms in the water samples and flow cytometry was used to evaluate the total bacterial counts. The study indicated that the average heterotrophic plate count represented only a fraction (< 3.65%) of the total FCM counts and < 6.06% of the viable FCM count.

The advantages of the Polymerase Chain Reaction (PCR) in relation to other culture techniques or standard methods used for the detection of microbial pathogens in water, is that it is specific, sensitive, rapid, accurate and can detect small amounts of nucleic acids in a single sample. This reaction involves the amplification and detection of DNA sequences (Amman *et al.*, 1995). A study conducted by Kong *et al.* (1995) investigated the feasibility of multiplex PCR to detect water borne pathogens in sea water samples from the Hong Kong Island and the Ngau Tau Kok Island in Kowloon. Multiplex PCR utilises a combination of primer sequences which have been tested for self-complementarity and inter-primer annealing. A combination of primers was used to simultaneously detect target DNA of *Salmonella typhimurium*, *Vibrio cholera* and *Escherichia coli*. Tsen *et al*. (1998) utilised PCR to select regions of the *E. coli* 16S rRNA gene to detect these cells in water. The addition of an enrichment step allowed for a detection limit of as low as one *E. coli* cell /100 mℓ.

A spring water distribution system in the Western Cape, South Africa experienced quality problems associated with bacterial contamination. The aim of this study was to investigate the bacterial contamination in this spring water bottling system. The level of heterotrophic plate counts (HPC) in the water samples at various sites throughout the system were determined by the conventional plate count technique. In addition, flow cytometric analysis was used to obtain total cell counts (the culturable and non-culturable populations) in the collected water samples at the various sites. Identification of microorganisms in the water samples was performed by means of molecular typing.

2. Materials and Methods

2.1 Sampling Sites

Sampling sites at the spring water distribution system in the Western Cape, South Africa are indicated in Figure 1. The sites include; Site A (borehole one); Site B (borehole two); Site C (Dositron – Flushing point); Site D (between 0.3 µm and UV steriliser – outside factory), Site E (After UV); the bottling line then splits into two lines and either one of the lines can be used for bottling. Site F (Line one after 0.35µm filter); Site G (Line one after 0.2 µm filter); Site H (Line two after 0.35 µm filter); Site I (Line two after 0.2 µm filter) and Site J (at filler- final bottling point). Sampling of these sites started in March 2004 (week one and week four) and continued in April (week eight) until November 2004 (week 46). Water samples were collected in 1L sterile Nalgene-polypropylene bottles and stored on ice to maintain a low temperature.

2.2 Heterotrophic Plate count technique and Pure culture isolation

Total heterotrophic plate counts were performed in duplicate on R2A Agar (Merck, Biolab Diagnostics) after serial dilutions 10^{-1} to 10^{-7} of sample water was performed. According to Geldreich (1996) R2A has gained increased popularity as it yields significantly higher bacterial counts than plate count agar, which does not permit growth of certain bacteria that may also be present in the water samples. Plates were incubated for 24 to 48 hrs at 37°C. Thereafter the number of visible cells, or colony forming units (CFU's) were counted and recorded. Distinct visible cells CFU's were

identified and subcultured onto Nutrient Agar (NA) (Merck, Biolab Diagnostics) plates for further purification of cultures.

2.3 Flow cytometry (FCM)

The flow cytometer analysis outlined by Paulse *et al*. (2007) was employed in the present study. Individual samples were subjected to a Becton Dickinson FACSCalibur flow cytometer for analysis. The Becton Dickinson FACSCalibur flow cytometer has a 15 mW, 488 nm argon-ion laser. A Doublet Discrimination Module, which uses pulse width and area to eliminate cell clumping (doublets and triplets), in conjunction with a LIVE/DEAD™ bacterial stain, allows for the differentiation between bacterial cells and debris. Flow cytometry employs the principles of light scattering, light excitation and the emission of fluorochrome molecules to generate data from particles or cells in the size range of 0.5 µm to 40 µm in diameter (Current Protocols in Cytometry, 2005). The addition of fluorescent beads enables the calculation of absolute or total cell counts in samples. The absolute number (cells/µℓ) of positive cells in a sample can be determined by comparing cellular events to the bead events measured by the flow cytometer. For this study, the bacterial population was identified and gated on a forward scatter (FSC) versus a side scatter (SSC) dotplot and a SSC versus fluorescence channel 2 (FL-2) at 585/42 nm dotplot. The bead count was identified and gated on a SSC versus fluorescence channel (FL-1) dotplot. All parameters were measured using a logarithmic amplification scale. A threshold of 52 FSC channels was set to remove sample debris. Only bacterial cells satisfying both gates were collected for subsequent analysis. Depending on the amount of debris present, certain samples were filtered through a 0.45 µm filter prior to analysis, The pore size of the filter was such that it allowed the

bacteria to pass through and yet retaining the debris. The stain was made up by combining equal volumes of PI (propidium iodide) (4 µℓ) and SYTO 9 (4 µℓ) in 1 mℓ sterile distilled H_2O. The stained samples (1 mℓ sample stained with 200 µℓ BacLight™ viability probe) were stored in the dark for 15 minutes, after which 50 µℓ liquid counting beads (BD™ Cell Viability Kit, BD™ Liquid Counting Beads) were added. The samples were then subjected to the flow cytometer for analysis and the concentrations of total cell populations were determined (Equation 1). In order to avoid excessive compensation of fluorescence overlap, SYTO 9 green emittance fluorescence was measured in fluorescence channel 1 (FL-1) at 530/30 nm and the PI was measured in fluorescence channel 3 (FL-3) at 670/LP nm. As previously mentioned, the addition of the beads allowed for the calculation of total cell counts (i.e. viable plus dead cells) in samples. After optimisation, each water sample was subjected to the flow cytometer until a total of 250 counting bead events were detected. An *E. coli* laboratory strain was used as control.

Equation 1

$$\frac{\text{Number of events in cell region}}{\text{Number of events in bead region}} \times \frac{\text{Number of beads / test}}{\text{test volume}} \times \text{dilution factor}$$

[Bead concentration recorded at 988/µℓ for BD Liquid Counting Beads and at 49827 beads per Trucount™ tube, both obtained from BD™]

2.4 DNA extraction and Agarose Gel Electrophoresis

Cultures from planktonic samples obtained from the sampling sites were spread-plated onto Nutrient Agar (NA) (Merck, Biolab Diagnostics) after serial dilutions (10^{-1} to 10^{-7}) of

sample water were performed. Plates were incubated for 3-4 days at 37ºC. Thereafter, distinct visible cells [colony forming units (CFU)] were identified based on morphological differences and re-streaked onto clean NA plates for isolation of pure cultures. Deoxyribonucleic acid (DNA) extraction was performed using the High Pure PCR Template Preparation Kit as per manufacturer's instructions (Roche Diagnostics, Germany). Extracted DNA samples (10 µℓ) were electrophoretically analysed on a 0.8% molecular grade agarose gel containing 12 µℓ of 0.5 µg/mℓ ethidium bromide, using 1 x Tris-acetate- ethylenediamine tetraacetic acid (TAE) electrophoresis buffer and run for one hour at 90 volts to confirm the presence and quality of genomic DNA.

2.5 Polymerase Chain Reaction (PCR)

The extracted DNA from individual samples was amplified using two primer sets specified for most pathogenic bacteria, respectively. Amplification of target DNA samples (5 µℓ) by PCR was performed in a total reaction volume of 50 µl containing a 10mM dNTP mix (1 µℓ), 25 mM $MgCl_2$ (4 µℓ), 5 x PCR Buffer with $(NH_4)_2SO_4$ (10 µℓ), 10 µM forward (RW01) primer [AAC TGG AGG AAG GTG GGG AT] (2.5 µℓ), 10 µM reverse (DG74) primer [AGG AGG TGA TCC AAC CGC A] (2.5 µl) (Greisen *et al*., 1994), GoTaq DNA polymerase (0.25 µℓ) and sterile distilled H_2O (24.75 µℓ). For the second primer set all the reagents mentioned above were added proportionally, together with 10 µM forward (RDR080) primer [AAC TGG AGG AAG GTG GGG AC] (2.5 µℓ) and 10µM reverse (DG74) primer [AGG AGG TGA TCC AAC CGC A] (2.5 µℓ) (Greisen *et al*., 1994) to obtain a total volume of 50 µℓ for subsequent amplification. The PCR procedure included an initial denaturation step of 5 minutes at 95°C, followed by 30 cycles of amplification (25 seconds at 95°C, 25 seconds 55°C and 1 minute at 72°C). The final

extension step was performed at 72°C for 10 minutes. Ten microliters of the amplified DNA fragments of the PCR reactions were analysed on a 1.2% agarose gel containing 12 µℓ of 0.5 µg/mℓ ethidium bromide, using 1 x Tris-acetate- ethylenediamine tetraacetic acid (TAE) electrophoresis buffer and run at 90 volts for one hour to confirm successful amplification of the PCR product.

2.6 16S ribosomal RNA sequencing

Successfully amplified PCR products (~400 kb) were purified using a High Pure PCR Product Purification Kit as per the manufacturer's instructions (Roche Diagnostics). The DNA concentrations were determined using the Qubit™ fluorometer (Invitrogen) and the Quant-iT™ dsDNA BR (Broad-range) Assay kit 2–1000 ng as per manufacturer's instructions (Molecular probes and Invitrogen). Samples were loaded onto 96-well plates (15 µℓ per sample), dried in a speed vac with medium heat for 30 to 60 minutes (depending on the volumes) and sent for subsequent sequencing where the Applied Biosystems Big Dye Terminator v3.1 Cycle sequencing Kit was used for the sequencing reactions, as per manufacturers' protocols. Sequences were identified using the (Blastn) or Local Alignment Search Tool Basic (Altschul *et al*., 1997) obtained from the National Centre for Biotechnology Information website.

2.7 Phylogenetic analysis

All the DNA sequences obtained from water at the various sites over the four sampling weeks were grouped and aligned with ClustalX (1.81) using default parameters and the

Blosum matrix. An unrooted tree was constructed using the neighbour-joining (Saitou and Nei, 1987) program of *MEGA* version 4.1 (Molecular Evolutionary Genetics Analysis 4.1) (Tamura *et al*., 2007). Branching patterns were evaluated by pairing 1000 replicates.

2.8 Statistical analysis

Repeated measures ANOVA (RMA) were performed on all HPC and FCM data obtained as outlined in Dunn and Clark (1987) using Statistica^{TM}. In each RMA, the residuals were analysed to determine if they were normally distributed. In all hypothesis tests, a significant level of 5% was used as standards.

3. Results and Discussion

3.1 Heterotrophic Plate Counts (HPC)

Total culturable microbial counts obtained by the HPC technique for all the sampling sites throughout the study period are presented in Figure 2. The HPC recorded in week one ranged from 1.34 x 10^8 cfu/mℓ at the borehole (Site A) to 3.66 x 10^7 cfu/mℓ in the final bottled water (Site J). The initial count at the borehole was significantly high and as this sample was collected in February [average temperature 25.2 °C] (Cape Town Weather Bureau, 2008), the high counts could be ascribed to the fact that increased temperatures favour microbial growth. The borehole water was untreated and according

to the South African National Standards, (2003) the HPC specification for untreated spring water is < 100 organisms/mℓ. The recorded counts thus significantly ($p < 0.05$) exceeded the standard specification.

The highest count of 2.02×10^8 cfu/mℓ in week one was observed after the 0.35 µm filter in line two (Site H) while the lowest count of 3.0×10^7 cfu/mℓ was observed after ultraviolet irradiation (Site E). The spring water system sampled was experiencing problems with bacterial contamination and the high counts could be ascribed to the fact that the 0.35 µm filter of line two was contaminated, clogged or faulty or that a biofilm was present in the distribution system which periodically sloughed off and served as a continuous source of contamination. In spring water distribution systems however, filters must be backwashed on a regular basis to maintain the integrity of the filter system. It is essential to monitor the integrity of the filters, as non-fixed pore filters enlarge in pore size after high water volumes have passed through them, thus resulting in the release of trapped contaminants into the filtered water (Pall Filters, 2004). The lowest count observed after ultraviolet irradiation (UV) indicated that the UV treatment was effective in reducing the number of microorganisms, however, the initial number of microorganisms in the water was significantly high, thus the UV irradiation only reduced the microorganisms by one log cycle. Senior and Dege (2005) confirmed that the efficiency of UV irradiation as with any other disinfection process is dependant on the quality of the incoming source water. During peak bottling seasons, such as summer, the rate of consumption of bottling water also increases which implies that the rate of flow velocity increases with the output of bottled water. Geldreich (1990) observed that an increase in flow velocity of water caused a greater influx of nutrients to the pipe surface, greater transport of disinfectants and greater shearing of biofilms from the pipe surface which could account for the fluctuations in HPC counts observed.

The HPC recorded in week four ranged from 4.50 x 10^6 cfu/mℓ at the borehole (Site A) to 9.00 x 10^6 cfu/mℓ in the final bottled water (Site J). Increases in cfu/ml were recorded after the 0.3 µm filter (Site D) at 3.10 x 10^8 cfu/mℓ and again after the 0.35 µm filter in line two (Site H) at 2.39 x 10^8 cfu/mℓ. Blocked or contaminated filters could have influenced the significant increase in HPC counts recorded after these filters (Site D and H). A significant decrease is however, noted after the water passes through the UV system (Site E), line one at both the 0.35 µm and 0.2 µm filters and the 0.2 µm filter in line two (Site I). This indicates that these filters were still functioning at their maximum efficiency. An increase in the microbial count was recorded in the final bottled water (Site J), which indicated that the filler ports were either contaminated or had not been sanitised correctly. The final microbial count of 9.0 x 10^6 cfu/mℓ (Site J) significantly exceeded the South African National Standard for HPC for bottled water of < 100 organisms/mℓ.

The HPC recorded in week eight ranged from 2.70 x 10^8 cfu/mℓ at source (Site A) to 2.35 x 10^7 cfu/mℓ in the final bottled water (Site J). The highest count was recorded at the borehole (Site A) indicating that the source water still contained significantly high numbers of microorganisms, which would influence the filtration process and the HPC count in the final bottled water. The lowest microbial count in the system of 3.0 x 10^6 cfu/mℓ was observed at the small borehole (Site B). The high microbial count at the larger borehole can be due to the large surface area in comparison to the smaller borehole. The HPC count in the final bottled water (Site J) in week eight was 2.35 x 10^7 cfu/mℓ in comparison to weeks one and four where counts of 3.66 x 10^7 cfu/mℓ and 9.00 x 10^6 cfu/mℓ were recorded, respectively. The distribution system was undergoing chlorine and oxonia disinfection treatment processes to reduce microbial counts, but the disinfection process was still not adequate to reduce the

number of microorganisms to the acceptable level of < 100 microorganisms/mℓ in the final bottled water. This implied that based on the results for weeks one, four and eight the spring water distribution system investigated, required additional chlorine disinfections washes and the filters had to be sanitised, backwashed or replaced to achieve the legal requirement.

The HPC recorded in week 46 ranged from 4.5×10^5 cfu/mℓ at source (Site A) to 5.0×10^4 cfu/mℓ in the final bottled water (Site J). The graph in figure 2 does not accurately reflect the values recorded in week 46 due to scaling constraints. The lowest HPC count was recorded in the final bottled water while the highest microbial count of 7.5×10^5 cfu/mℓ was observed after UV irradiation (Site E). In week 46 a significant reduction in HPC in comparison to week one, four and eight was recorded for all sites as the system had been routinely disinfected with chlorine soaks and oxonia. Contact times and dosage for disinfecting the system were increased and the filters were disinfected and backwashed. However, the HPC count in the final bottled water still significantly ($p < 0.05$) exceeded the acceptable limit of < 100 organisms/mℓ.

3.2 Flow Cytometric Analysis (FCM)

The live and dead ratios of planktonic populations were obtained using flow cytometer analysis, in conjunction with the Live/Dead BacLight™ viability probe and liquid counting beads (BD™). Two distinct populations of live and dead cells were observed by distinguishing between their fluorescence intensities, i.e. either red or green. Results obtained for the average live/dead and total ratios of the planktonic populations were analysed at the respective sites over the specific time period. The total cell counts obtained by flow cytometric analysis are presented in Figures 3 and 4 (a & b).

The total cell counts obtained in week one ranged from 2.09 x 10^8 microorganisms/mℓ at the large borehole (Site A) to 5.44 x 10^7 microorganisms/mℓ in the final bottled product (Site J). In addition, significantly high total cell counts were observed at the small borehole (Site B) at 2.03 x 10^8 microorganisms/mℓ, Dositron/flushing point (Site C) at 1.88 x 10^8 microorganisms/mℓ, and after the 0.35 µm filter in line one (Site F) at 1.56 x 10^8 microorganisms/mℓ**.** The high total counts recorded in the source water at the two boreholes (Site A and B) could have been due to high temperatures experienced, [average temperature 25.2 °C (Cape Town Weather Bureau, 2008)} which favoured microbial growth and proliferation. Alternatively as the distribution system was experiencing problems, an external contamination source could have contributed to the elevated microbial numbers as the two borehole sites may not have been tapped or sealed securely. The increased total cell count observed in the final bottled water indicated that the filler or filling nozzle was either contaminated, or that a biofilm was present in the filling system which sloughed off and contaminated the water. Based on the results obtained for the total cell counts in the final bottled water and throughout the system it was evident that the distribution system needed to be disinfected on a regular basis.

The marked increase in the total cell count observed after the 0.35 µm filter in line one (Site F) indicated that the filter was blocked and required sanitisation, backwashing or replacement. A decrease in the total cell counts at Site D at 6.13 x 10^7 microorganisms/mℓ, Site G at 2.58 x 10^7 microorganisms/mℓ, and Site I at 2.11 x 10^7 microorganisms/mℓ, can be observed which indicated the efficacy of these filters in the filtration process. These results indicate that the filter system at these sites successfully retained some of the bacterial load and supported the implementation of multiple filter systems in a spring water distribution system. A further reduction in the

total cell count was observed after the ultraviolet irradiation treatment (Site E) at 3.6×10^7 microorganisms/mℓ, which indicated that the UV irradiation treatment was effective in reducing the microbial numbers.

The total cell counts in week four ranged from 3.90×10^7 microorganisms/mℓ at the large borehole (Site A) to 8.36×10^7 microorganisms/mℓ in the final bottled product (Site J), with the lowest total cell count observed at the Dositron unit (Site C) at 2.13×10^7 microorganisms/mℓ. The total cell count for Site F in week four could not be measured as the sample vial broke. The high total counts in the final bottled water in week four indicated that although the bottling system was sanitised with chlorine and oxonia, an increase in the cell count was recorded from source to final product, which implied that there was definitely a source of contamination in the distribution system. Tchobanoglous and Schroeder (1985) indicated that the factors which influence the disinfection efficiency of chlorine include the initial contact time, concentration and form, microbial load, pH and temperature. The bottling system of the site investigated was dosed with concentrated chlorine and left to stand for two days to increase its contact time. The initial counts in the source water in week four of 3.90×10^7 microorganisms/mℓ and 3.88×10^7 microorganisms/mℓ at Sites A and B, respectively were however, lower than the microbial counts recorded at source in week one at 2.09×10^8 microorganisms/mℓ (Site A) and 2.03×10^8 microorganisms/mℓ (Site B). The results indicate that the precautions implemented to secure the borehole sites such as the tapping of the boreholes, improved the source water quality. In comparison to the other sampling weeks no significant increase in the total cell counts were recorded from sites A to I. A significant increase ($p > 0.05$) in total cell counts were observed in the final bottled water at site J, but limits for total cell counts in the final product are not stipulated by the South African National Standards, (2003).

The total cell counts in week eight ranged from 6.25 x 10^7 microorganisms/mℓ (Site A) to 9.09 x 10^7 microorganisms/mℓ (Site J). The highest total cell count of 2.02 x 10^8 microorganisms/mℓ was recorded after UV irradiation (Site E). For a UV light to function optimally the quartz must be cleaned regularly to ensure full transmissivity and efficacy (Senior and Dege, 2005). Sommer and Cabaj (1993) evaluated the efficiency of a UV plant for the disinfection of drinking water and concluded that biodosimetric conditions should be used to monitor disinfection efficiency. This process involves the addition of organisms of interest to the water and the determination of the colony counts before and after treatment with UV. Dose-response curves therefore assist in the evaluation of the UV treatment of the water. High counts of 1.80 x 10^8 microorganisms/mℓ at the Dositron (Site C) and 1.69 x 10^8 microorganisms/mℓ after the 0.2 µm filter line two (Site I) were also observed, which indicated that the dositron (point where sanitiser is added to the system) was perhaps not sealed or was exposed to an external source of contamination. Membrane fouling which is caused by the accumulation of chemicals, particles and growth of microorganisms on the membrane surface could also have contributed to the increase in microbial counts (Guidelines for Canadian Water Quality, 2008).

Due to the consistent contamination experienced, the production at the supplier was stopped and the problem was investigated. Sampling was resumed after three months to measure the efficiency of the treatment procedures implemented. The total cell counts recorded in week 46 ranged from 2.69 x 10^8 microorganisms/mℓ at the large borehole (Site A) to 5.70 x 10^7 microorganisms/mℓ in the final bottled product (Site J). Results in week 46 fluctuated with the highest total cell counts of 5.00 x 10^8 microorganisms/mℓ and 5.08 x 10^8 microorganisms/mℓ observed after UV irradiation treatment (Site E) and the 0.2 µm filter in line one (Site G), respectively. The

high total cell count recorded after the UV irradiation treatment in week 46, compared to week one, four and eight indicated the reduction in the efficiency of the quartz and clearly showed that the lamp needed replacement. The total cell count of 2.69 x 10^8 microorganisms/mℓ observed in the large borehole (Site A) in week 46 was also higher than the counts recorded in weeks one, four and eight. This could be ascribed to the fact that microbial contamination was still taking place in the source water. The high count recorded after the 0.2 µm filter (Site G) indicated that the filter was still blocked and that the disinfection process employed was not adequate in addressing the contamination of the filters.

In comparison to the other sampling weeks, low HPC counts were observed in week 46, which indicated that even though the counts still exceeded the stipulated HPC limit, the treatment procedures implemented were effective in reducing the CFU counts. However, the flow cytometry results showed that the total cell counts of week 46 were higher than all the other weeks sampled, which clearly indicates that the HPC count was not a true reflection of the microbial numbers in the spring water distribution system.

The heterotrophic plate counts were compared to the viable cell counts as obtained by flow cytometry. The results for week 46 only (Figure 5) are discussed as a representation of results as significant differences were recorded in the HPC and FCM counts for this week. The graph in figure 5 does not accurately reflect the heterotrophic values recorded in week 46 due to scaling constraints. These results showed that the flow cytometric (FCM) analysis yielded higher viable counts in the water sampled at the various sites. The highest CFU count of 7.50 x 10^5 microorganisms/mℓ was recorded after the UV irradiation process (Site E). A corresponding FCM viable count of 2.17 x 10^6 microorganisms/mℓ was recorded for the same sampling site. The highest viable FCM count for week 46 was observed at the Dositron (Site C) at 4.40 x 10^7

microorganisms/mℓ. A corresponding CFU count of 3.00 x 10^5 microorganisms/mℓ was recorded for the same sampling site. Differences in the heterotrophic plate counts and the FCM viable cell counts could also be observed at all the sampling sites in the distribution system with the HPC result in the bottled water (Site J) at 5.0 x 10^4 microorganisms/mℓ also lower than the viable FCM count of 9.86 x 10^5 microorganisms/mℓ. Similarly the HPC count at Site G at 2.00 x 10^5 microorganisms/mℓ in comparison to the corresponding FCM count of 3.94 x 10^5 microorganisms/mℓ was also lower. The higher FCM counts indicated that this technique was able to detect cells in the water sample that enter a viable but non culturable state (Paulse *et al*., 2007). The current water legislation however, states that the heterotrophic plate count of the final bottled water must be < 100 organisms/mℓ within 24 hours of bottling however, no stipulation regulation for FCM in bottled water could be found. Results clearly showed that in comparison to the FCM technique, the heterotrophic plate count technique, only allows for growth of the viable and culturable cells present in the water samples and that it is not an accurate method to assess the actual viable microbial population in the bottled water samples.

3.3 Phylogenetic analysis

Figure 6 (a) and (b) represents the purified PCR agarose gel electrophoresis photos of week 4 using both primer sets 1 and 2. Lane one contains the DNA ladder # SM0402 and lane two contains the negative control. Phylogeny of 180 sequences were analysed and there were many similar species that were repeatedly isolated from the various sampling points over the sampling periods. Their duplicate species were excluded as shown in Figures 7 to 10. Species that were similar and belonged to the same family

grouped together to form clades. Bootstrap values for all scores were on average above 90. The microbial flora isolated over the sampling periods was mainly *Pseudomonas sp*., *Bacillus sp*., *Staphylococcus sp.* and *Stenotrophomonas sp.* Pathogens isolated over the sampling period include *Pseudomonas sp., Shigella, and Staphyloccocus sp. Pseudomonas* is an opportunistic pathogen that if present in the water, indicates a lack of disinfection and cleanliness in the distribution system and is also one of the most dominant species isolated from non carbonated natural mineral water (Mavridou, 1992). *Shigella* is an enteric pathogen and-one of the major foodborne bacteria causing illness (Jay 1992). *Bacillus sp.* only contains two species that are pathogenic which is *B. anthracis* (cause of anthrax) and *B. cereus.* Most non pathogenic *Bacillus sp.* can cause foodborne gastroenteritis.

In week one, 16 diverse species were isolated and a phylogenetic tree was constructed (Figure 7). The HPC counts in this time period were significantly high ($p < 0.05$) and exceeded the limit specified by the South African National Standards (2003) of < 100 organisms/mℓ. High microbial counts were isolated at the borehole and after the 0.35µm filter line 2 which indicated the points of contamination within the system. These high counts indicated that the current sanitisation system was not adequate as the counts were not within the legal limit of < 100 organisms/mℓ (South African National Standards, 2003).

As shown in (Figure 7), amongst the organisms isolated were *Shigella boydii, Serratia sp. SB, Enterobacter asburiae.* All or which *belong* to the family *Enterobacteriaceae. Serratia* belongs to the coliform group and occurs in the environment, where their presence usually indicate faecal contamination. In developing countries contaminated drinking water is still also a major cause of shigellosis (Ray, 2004). *Stenotrophomonas* sp. are ubiquitous in the environment and in

immunocompromised individuals *S. matophilia* can lead to nosocomial infections (Jay, 1992). *Pseudomonas* and *Stenotrophomonas* sp. have been frequently isolated from mineral water (Leclerc, 2002).

In week four, 17 diverse species were isolated and a phylogenetic tree was constructed (Figure 8). *Serratia sp.* SB and *Shigella boydii* was not isolated in week four which could indicate that the current sanitisation process of the distribution system was effective against these bacteria. All the species in this sampling week with the exception of *Pseudomonas sp.* and *Bacillus* sp. were introduced in this sampling period. In week four the highest HPC counts were recorded at the filters (Figure 2) and it could be at these contamination points where these species were introduced. The presence of the *Pseudomonas sp*. and *Bacillus* also indicated that the sanitisation process, although adjusted was still not effective in the elimination of these organisms from the water. Factors that influence the efficiency of chlorine disinfection include contact time, concentration, microbial specie, microbial load, pH and temperature (Tchobanoglous and Schroeder, 1985).

In week eight, 41 diverse species were isolated and a phylogenetic tree was constructed (Figure 9). The highest number of species diversity isolated was in this week. The highest HPC count for all the sampling periods was recorded in week eight at the large borehole (Figure 2) and clearly indicated the most pronounced point of contamination and species introduction. The borehole must be tapped securely to prevent microorganisms from entering the system. The species introduced in this sampling period included *Commamonas aquatica, Proteus sp. K10, 7 Proteus mirabilis, Hafnia alvei CCUG 429, Enterobacter sp. NJ-64, Enterobacter sp. MB-1-6-6, Amorphomonas oryzae B46, Uncultured soil bacterium clone TG8, Rhizobium soli, Arthrobacter sp. W17, Staphylococcus pasteuri strain SS-08, Staphylococcus*

epidermidis strain AT2, Staphylococcus sp. SA6, Uncultured soil bacterium clone TG8, Endophytic bacterium WS7b strain DS-42 and Brevibacterium sp. NASA2-43. The presence of *Pseudomonas sp. and Bacillus sp.* again indicate that these organisms were not eliminated during the sanitisation process. The sanitisation process and conditions were either insufficient to eliminate these organisms or they could have formed a biofilm within the distribution system. In a routine monitoring study of rural drinking water *S. aureus* was also isolated in 6% of the water samples (Le Chavellier and Seidler, 1980). *Enterobacter sp.* as well as certain other species belong to the family Enterobacteriaceae are usually used as indicator organisms. *Stenotrophomonas sp. w*as eliminated in week four, but reintroduced in week eight which indicated that it could be part of the biofilm within the distribution system. Production at the plant was stopped as the HPC counts were consistently higher than the accepted limit of < 100 organisms/mℓ (South African National Standard, 2003).

In week 46, only 13 different species were isolated and a phylogenetic tree was constructed (Figure 10). The low species diversity correlates with the HPC count observed in week 46, which was the lowest count for all the sampling periods (Figure 2), but in comparison to the FCM count, which was the highest (Figure 3), it indicated an increase in the number or organisms. Although most of the species isolated in the previous sampling period were eliminated through the chlorine soaks, increased concentration and contact times of the sanitiser, the number of viable organisms increased. The presence of *Pseudomonas sp. and Bacillus sp.* again indicate that these species were not eliminated during the sanitisation process and their persistence definitely indicate a biofilm in the system. A study conducted by Percival *et al.* (1998) on the development of biofilms on stainless steel pipes in a mains water system indicated that the dominant specie isolated were *Pseudomonas spp.* and *Alcaligenes sp*. In week

46, the following species *Escherichia sp*. *Aeromonas, Endophytic* bacteria and *Brevundimonas sp.* were introduced. *Escherichia sp.* B4 belong to the family *Enterobacteriaceae* and certain strains cause foodborne gastroenteritis. The presence of *Escherichia coli* also usually indicates faecal contamination (Ray, 2004). *Aeromonas sp.* are widely distributed in nature and can be found in freshwater, saltwater and soil and are considered opportunistic pathogens. LeChavellier *et al.* (1982) investigated the presence and health significance of *Aeromonas* sp. in chlorinated drinking water. *Aeromonas sp.* were isolated from 27% of the 183 chlorinated drinking water samples collected over an 18 month period. The presence of *Aeromonas sp*. could not be correlated with the presence of coliforms, but certain strains can cause diarrhoea and have the potential of possibly causing water associated gastroenteritis in humans.

In week 46, the HPC and specie diversity was significantly reduced, but the HPC was still not within the limit of < 100 organisms/mℓ (South African National Standards, 2003). It is important to understand quantitatively the bacterial diversity in the bottling water distribution system and the correct number in order to apply and optimise the correct sanitisation procedure. As bottled water cannot be subjected to any chemical treatments during and after bottling it is important to apply effective sanitisation, manage the filter integrity, UV irradiation and secure the spring source in order to supply safe water free of any contamination.

4. Conclusions

The major conclusions of the study were as follows:

1. The lowest HPC count was recorded in week 46 but still notably exceeded the maximum limit of < 100 microorganisms/mℓ (South African National Standards for Bottled Water, 2003).
2. The total cell counts obtained by the FCM method were higher in week 46 at all the sites throughout the sampling period, when compared to the heterotrophic plate counts.
3. The higher FCM counts indicated that the flow cytometry technique was able to detect certain cells in the sample that enter a viable but not culturable state and that the heterotrophic plate count technique only allowed for growth of the viable and culturable cells present in the water samples.
4. Flow cytometry proved to be a rapid and more reliable technique for the assessment of total bacterial count in water samples. New updated methods for the analysis of drinking water, which could include the FCM technique, should be included in the proposed techniques for determining the maximum and minimum levels as set out by the South African National Standards for Bottled Water.
5. The dominant species *Pseudomonas sp*. and *Bacillus sp*. were isolated throughout the sampling period from week one to week 46. The pathogenic organisms isolated from all the sampling periods included *Escherichia sp*., *Pseudomonas sp., Shigella boydii, Bacillus sp.* and *Staphyloccocus sp.*
6. The highest species diversity in comparison to all the other sampling periods was recorded in week eight, which correlated with the highest HPC count recorded at the borehole. These counts could have been influenced by the fact that the borehole was not secured tightly which allowed the microorganisms to enter the system.

7. It is important to understand quantitatively the viable bacterial load and the species diversity in the bottling water distribution system in order to apply and optimise the most efficient sanitisation procedure. As bottled water cannot be subjected to any chemical treatments during and after bottling it is also important to understand the survival capacity of the pathogenic and indicator organisms.
8. Production at the supplier was stopped and although the HPC counts were reduced there was still quality problems detected in the final bottled water.

5. Acknowledgements

The National Research Foundation (NRF) and Cape Peninsula University of Technology (CPUT) for financial support.

6. References

ALTSCHUL SF, MADDEN TL, SCHÄFFER AA, ZHANG J, ZHANG Z, MILLER W and LIPMAN DJ (1997) "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs". *Nucleic Acids Research* **25** 3389-3402.

AMMAN RL, LUDWIG W and SCHLEIFER KH (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiology Review* **59** 143-169.

BEURET C, KOHLER D and BAUMGARTNER A (2002) Norwalk-likevirus sequences in mineral waters: One year monitoring of three brands. *American Society for Microbiology* **68** 1925-1931.

BLAKE PA, ROSENDBERG ML FLORENCIA J, COSTA JB, QUINTINO LDP and GANGAROSA EJ (1977) Cholera in Portugal, 1974. II Transmission by bottled water mineral water. *American Journal Epidemiology* **105** 344 – 348.

CAPE TOWN WEATHER BUREAU. (2008) Cape Town.

CHARACKLIS WG and MARSHALL KC (1990) Biofilms: a basis for an interdisciplinary approach. Wiley: New York.

CURRENT PROTOCOLS IN FLOW CYTOMETRY, (2005) Introduction. John Wiley & Sons (via Wiley InterScience). Cited online at > URL http://www3.interscience.wiley.com/cgi-bin/mrwhome/104554804/HOME. [Accessed on 2006/11/22].

DUNN OJ and CLARK VA (1987) *Applied Statistics: Analysis of variance and regression* (2nd Ed.) John Wiley & Sons. London, UK.

EHLERS MM, VAN ZYL WB, PAVLOV DN and MULLER EE (2004) Random survey of the microbial quality of bottled water in South Africa. *Water SA* **30**(2) 203 – 210.

GELDREICH EE and RICE EW (1987) Occurrence, Significance, and Detection of *Klebsiella* in Water Systems. *Journal of American Water Works Association* **79**(5) 74.

GELDREICH EE (1990) "*Microbial Quality Control in Distribution Systems." Water quality and treatment,* (4th Ed). In Pontius, F.W. (ed). American Water Works Association. McGraw-Hill, Inc. New York.

GELDREICH EE (1996) *Microbial quality of water supply in distribution systems.* CRC Press Inc: Florida, 374.

GREISEN K, LOEFFELHOLZ M, PUROHIT A and LEONG D (1994) PCR primers and probes for the 16S rRNA gene of most species of pathogenic bacteria, including bacteria found in cerebrospinal fluid. *Journal of Clinical Microbiology* **32** 335-351.

GUIDELINES FOR CANADIAN DRINKING WATER QUALITY, (2008). Treatment Technology. mhtml:file:E:\articles\Page9 [30 April 2004].

HURST JC, CRAWFORD RL, KNUDSEN GR, MCINERNEY MJ and STETZENBACH LD (2002) *Manual of Environmental Microbiology.* 2nd ed. American Society for Microbiology. Washington DC.

JAVOIS LC (1999) Immunocytochemical *Methods and Protocols.* Totawa, NJ: Human Press. http://www.answers.com/topic/flow-cytometry [27 August 2007].

JAY MJ (1992) *Modern Food Microbiology*. 4th ed. Chapman and Hall:New York.

KONG RYC, DUNG WF, VRIJMOED LLP and WU RSS (1995) Co-detection of three species of water borne bacteria by multiplex PCR. *Marine Pollution Bulletin,* **31**(4-12) 317–324.

LECHEVALLIER MW and SEIDLER RJ (1980) *Staphylococcus aureus* in rural drinking water. *Applied and Environmental Microbiology* **30**(4) 739-742.

LECEHAVALLIER MW, EVANS TM, SEIDLER RJ, DAILY OP, MERREL BR, ROLLINS DM and JOSEPH SW (1982) *Aeromonas sobria* in chlorinated drinking water supplies. *Microbial Ecology* **8** 325-333.

LECLERC H (2002) Microbiology of Natural Mineral Waters. http://www.centre-evian.com/fondDoc/dos-science/11858.html [18 September 2003].

LECLERC H and MOREAU A (2002) Microbiological safety of natural mineral water. FEMS *Microbiology reviews* **26**(2), 207-222.

LINDSAY D (2002) Biofilms a hidden challenge in food processing. *Food Review* 25–27.

MANAIA CM, NUNES OC, MORAIS PV and DA COSTA MS (1990) Heterotrophic plate counts and the isolation of bacteria from mineral waters on selective and enrichment media. *Journal of Applied Bacteriology* **69** 871 – 876.

MAVRIDOU A (1992) A study of the bacterial flora of non-carbonated natural mineral water. *Journal of Applied Bacteriology* **73** 355 – 361.

MONIS PT, SAINT PCP, HOEFEL D, GROOBY WL and ANDREWS S (2003) Enumeration of water borne bacteria using viability assays and flow cytometry: a comparison to culture-based techniques. *Journal of Microbiology Methods* **55**(3) 585-597.

PALL FILTERS™. (2004) *Focus on filtration.*

PAULSE AN, JACKSON VA and KHAN W (2007) Comparison of enumeration techniques for the investigation of bacterial pollution in the Berg River, Western Cape, South Africa. *Water SA* **33** 165-173.

PERCIVAL SL, KNAPP JS, EDYVEAN GJ and WALES DS (1998) Biofilms, mains water and stainless steel. *Water Research* **32**(7) 2187-2201.

RAY B (2004) *Fundamental Food Microbiology*. 3rd ed. CRC Press: Florida, 374.

ROSZAK DB and COLDWELL RR (1987) Survival strategies of bacteria in the natural environment. *Microbiology Review* **51** 356-379.

RYAN KJ (2004) *Medical Microbiology*. 4th ed. Mc Graw Hill: New York:

SAITOU N and NEI M (1987) The neighbour-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4** 406-425.

SENIOR D and DEGE N (2005) *Technology of bottled water*. Blackwell Publishing Ltd, Oxford, UK.

SOMMER R and CABAJ A (1993) Evaluation of the efficiency of a UV plant for drinking water disinfection. *Water Science and Technology* **27** 357-362.

SOUTH AFRICA, DEPARTMENT OF HEALTH. (2004) Foodstuffs, Cosmetics and Disinfectants Act: *Act 54 of 1972.* Regulations governing bottled waters including natural mineral waters. Pretoria: Government Printer: No R 502.

SOUTH AFRICAN NATIONAL STANDARDS. (2003) Bottled Natural Water. SANS 1657:2003 Ed 1.3. Notice 1373 of 8 November 2002.

TAMURA K, DUDLEY J, NEI M and KUMAR S (2007) *MEGA4*: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.1. *Molecular Biology and Evolution* **24** 1596-1599.

TCHOBANGLOUS G and SCHROEDER ED (1985) *Water Quality: Characteristics; Modelling; Modification*. Addison-Wesley, United States of America.

TSEN HY, LIN CK and CHI WR (1998) Development and use of 16S rRNA gene targeted PCR primers for the identification of *Escherichia coli* cells in water. *Journal of Applied Microbiology* **85** 554-560.

UNITED STATES DEPARTMENT OF THE INTERIOR U.S. GEOLOGICAL SURVEY. http://ga.water.usga.gov/edu/watercyclesprings.html [14 August 2006].

WORLD HEALTH ORGANISATION (1996) Guidelines for drinking water quality 2nd (ed). Weiner Verlag, WHO, Austria:

7. List of Figures and Tables

Figure 1 Diagram of the borehole and bottling plant indicating sampling points.

Figure 2 Average heterotrophic plate counts for the sites analysed recorded over the sampling period.

Figure 3 Enumeration of total bacteria by means of flow cytometric analysis (FCM) recorder over the sampling period.

Figure 4a Dotplots of planktonic samples obtained at the borehole for week 46 of the distribution site in the Western Cape by means of Flow cytometric analyses (FCM).

Figure 4b Dotplots of planktonic samples obtained in the final bottled water for week 46 of the distribution site in the Western Cape by means of Flow cytometric analyses (FCM).

Figure 5 Comparison of heterotrophic plate count to viable FCM count for week 46 for the sites analysed.

Figure 6a Polymerase Chain Reaction analysis of extracted DNA samples (BB1-138 to 155) [with primer set 1: forward (RW01) primer; reverse (DG74) primer] for sampling week 4. Lanes 1 –18: samples 14 to 32; Lane A: Marker [MassRuler™ DNA Ladder Mix, #SM0403 (Fermentas)]; Lane B: Negative control.

Figure 6b Polymerase Chain Reaction analysis of extracted DNA samples (BB1-138 to 155) [with primer set 2: forward (RW080) primer; reverse (DG74) primer] for sampling week 4. Lanes 1 –18: samples 14 to 32; Lane A: Marker [MassRuler™ DNA Ladder Mix, #SM0403 (Fermentas)]; Lane B: Negative control.

Figure 7 Unrooted Phylogenetic tree inferred from 16S rRNA sequence data, isolated from bacterial samples obtained from water samples taken in week 1 from the distribution system in Western Cape, South Africa. Distance matrices were constructed from the aligned sequences and created for multiple base changes at single position by the BLOSUM algorithm. Bootstrap values are shown at nodes.

Figure 8 Unrooted Phylogenetic tree inferred from 16S rRNA sequence data, isolated from bacterial samples obtained from water samples taken in week 4 from the distribution system in Western Cape, South Africa. Distance matrices were constructed from the aligned sequences and created for multiple base changes at single position by the BLOSUM algorithm. Bootstrap values are shown at nodes.

Figure 9 Unrooted Phylogenetic tree inferred from 16S rRNA sequence data, isolated from bacterial samples obtained from water samples taken in week 8 from the distribution system in Western Cape, South Africa. Distance matrices were constructed from the aligned sequences and created for multiple base changes at single position by the BLOSUM algorithm. Bootstrap values are shown at nodes.

Figure 10 Unrooted Phylogenetic tree inferred from 16S rRNA sequence data, isolated from bacterial samples obtained from water samples taken in week 46 from the distribution system in Western Cape, South Africa. Distance matrices were constructed from the aligned sequences and created for multiple base changes at single position by the BLOSUM algorithm. Bootstrap values are shown at nodes.

Table 1 Table of 16 isolates, their codes and accession numbers for organisms isolated from week 1 sampling period.

Table 2 Table of 17 isolates, their codes and accession numbers for organisms isolated from week 4 sampling period.

Table 3 Table of 43 isolates, their codes and accession numbers for organisms isolated from week 8 sampling period.

Table 4 Table of 13 isolates, their codes and accession numbers for organisms isolated from week 46 sampling period.

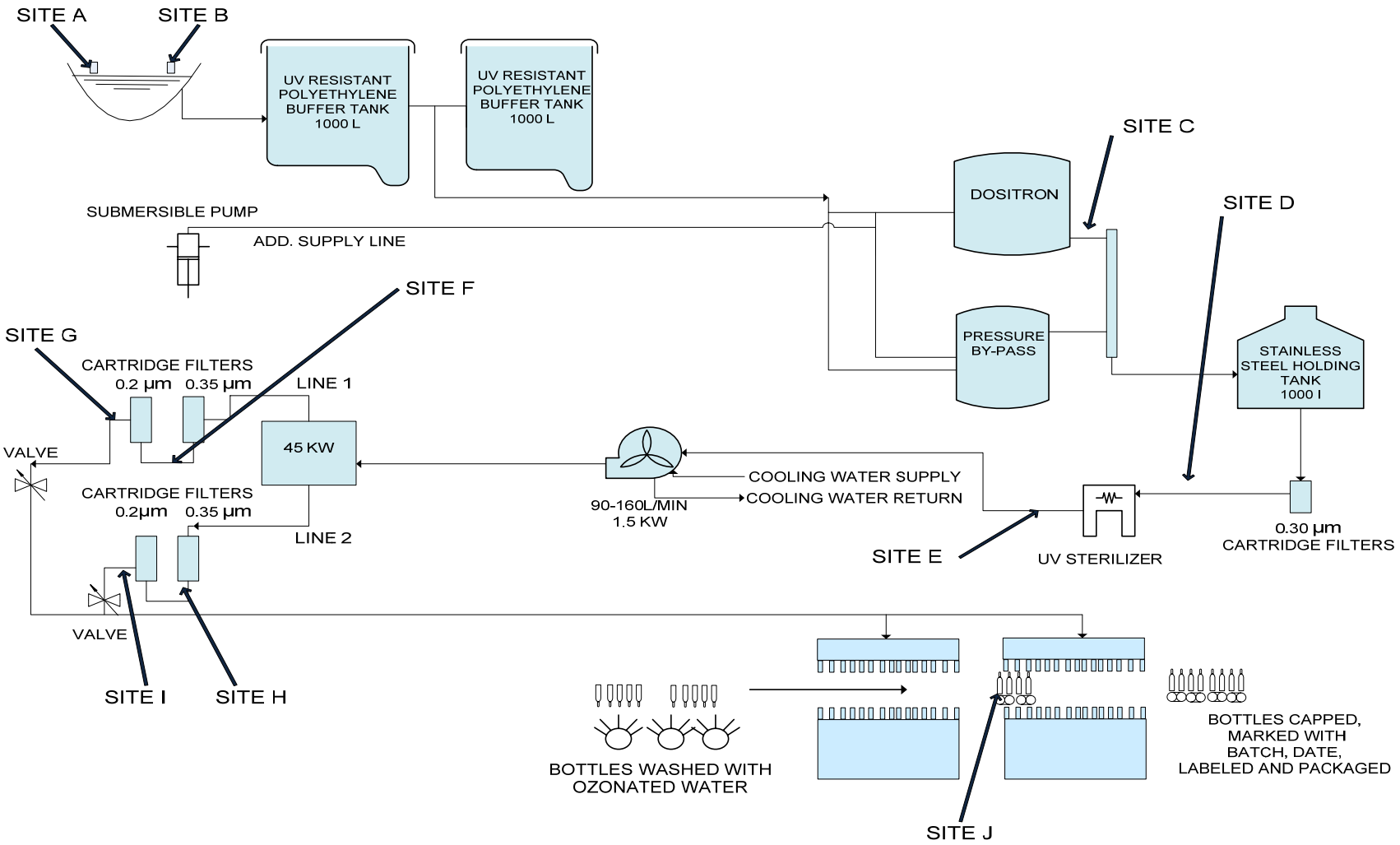


Figure 1 Diagram of the borehole and bottling plant indicating sampling points.

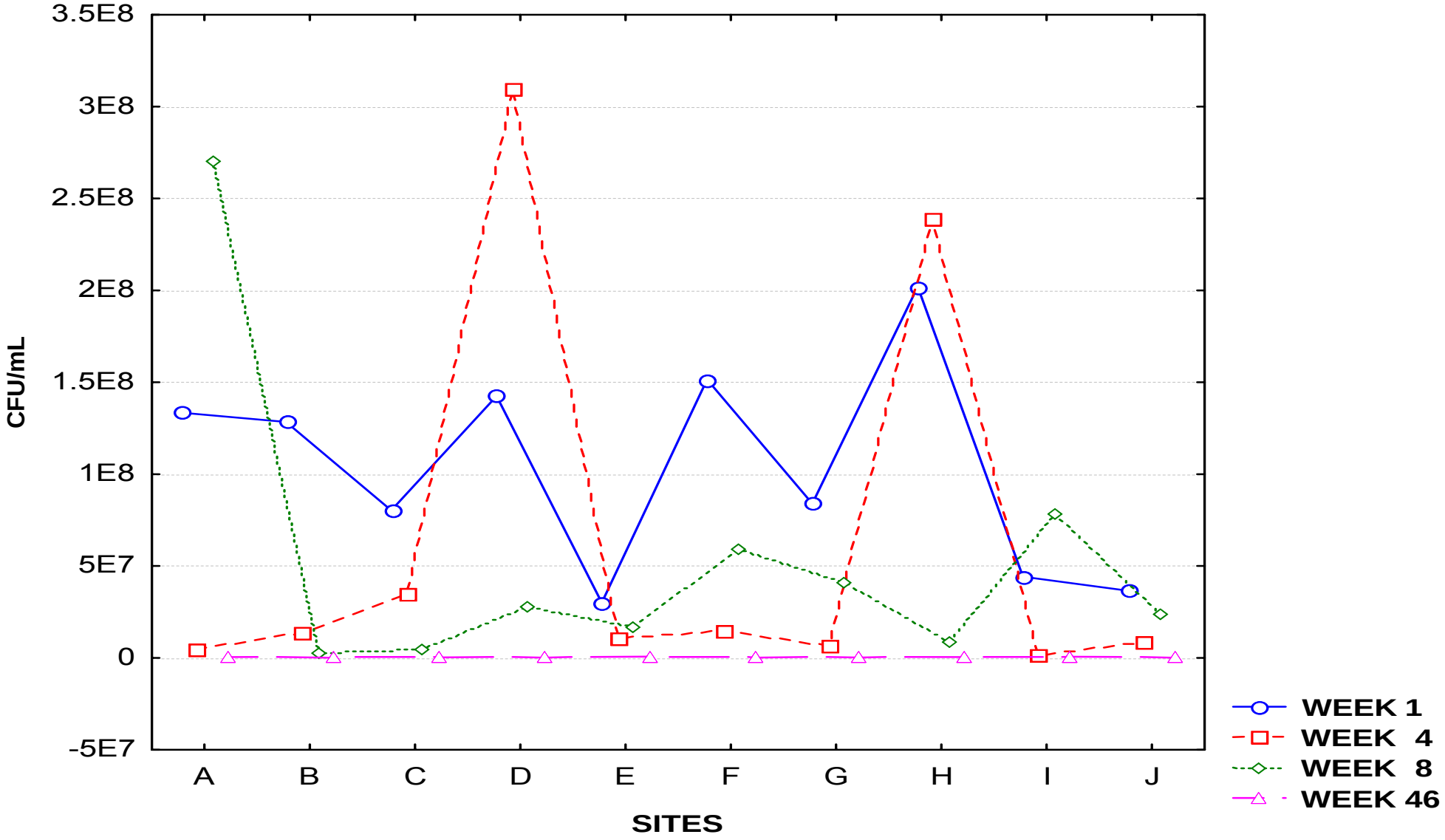


Figure 2 Average heterotrophic plate counts for the sites analysed recorded over the sampling period.

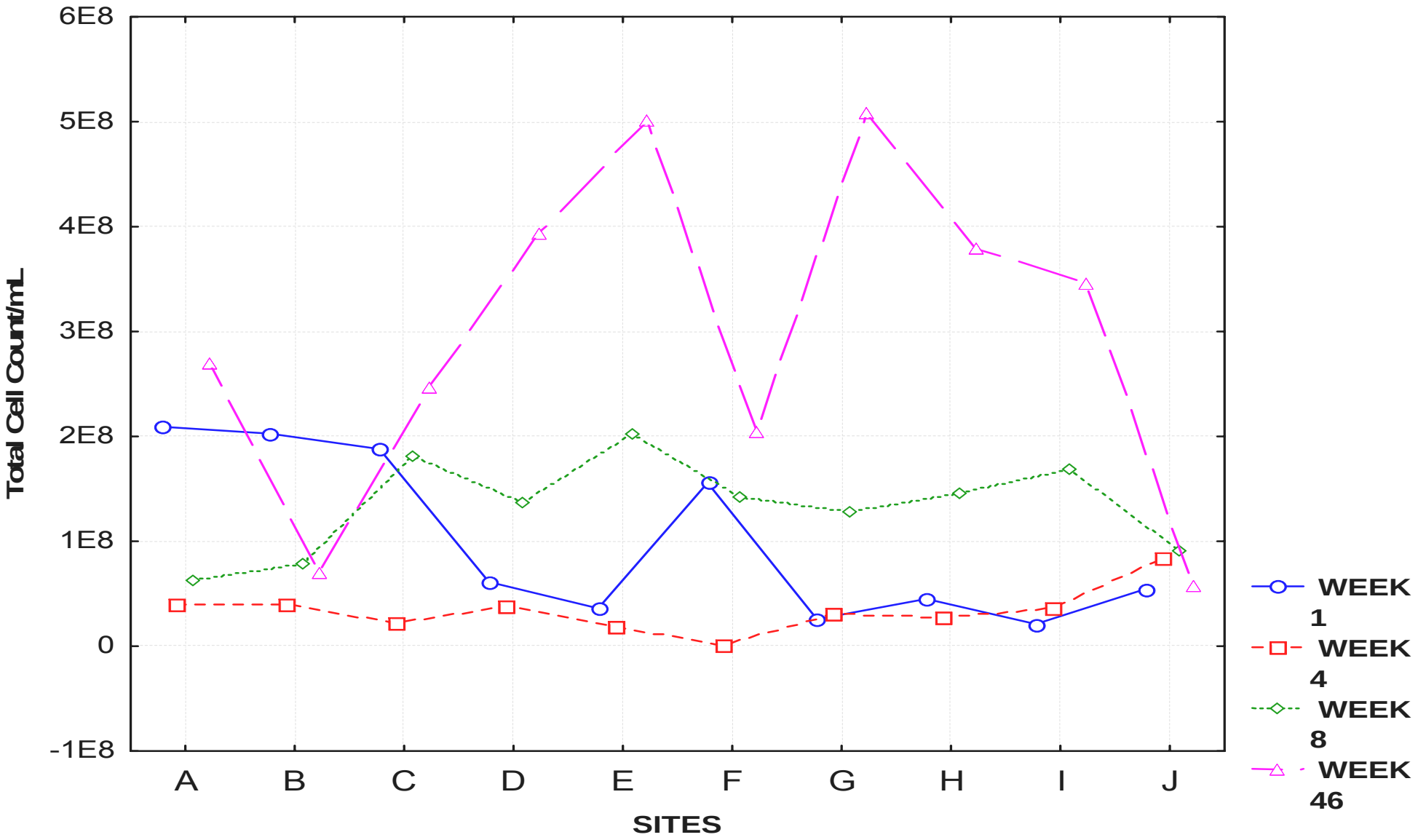


Figure 3 Enumeration of total bacteria by means of flow cytometric analysis (FCM) recorder over the sampling period.

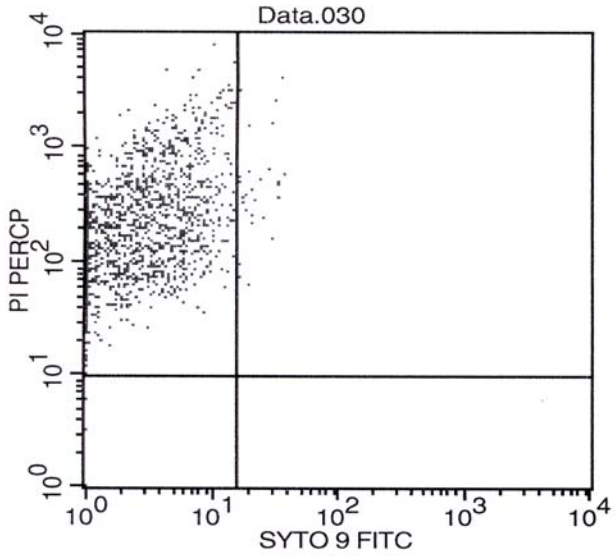


Figure 4a Dotplots of planktonic samples obtained at the borehole for week 46 of the distribution site in the Western Cape by means of Flow cytometric analyses (FCM).

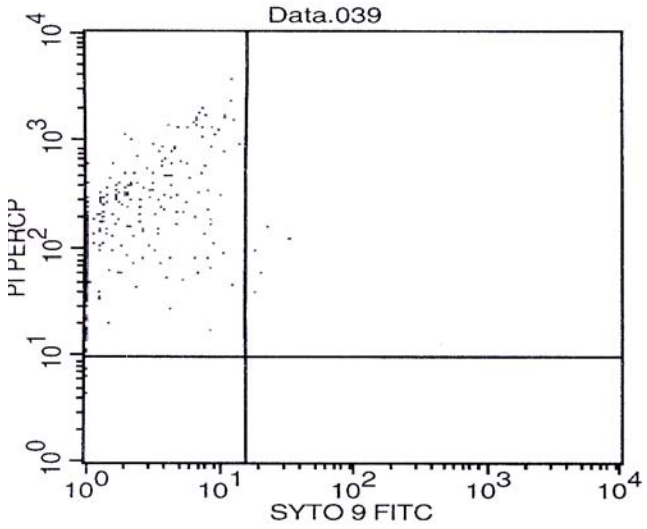


Figure 4b Dotplots of planktonic samples obtained in the final bottled water for week 46 of the distribution site in the Western Cape by means of Flow cytometric analyses (FCM).

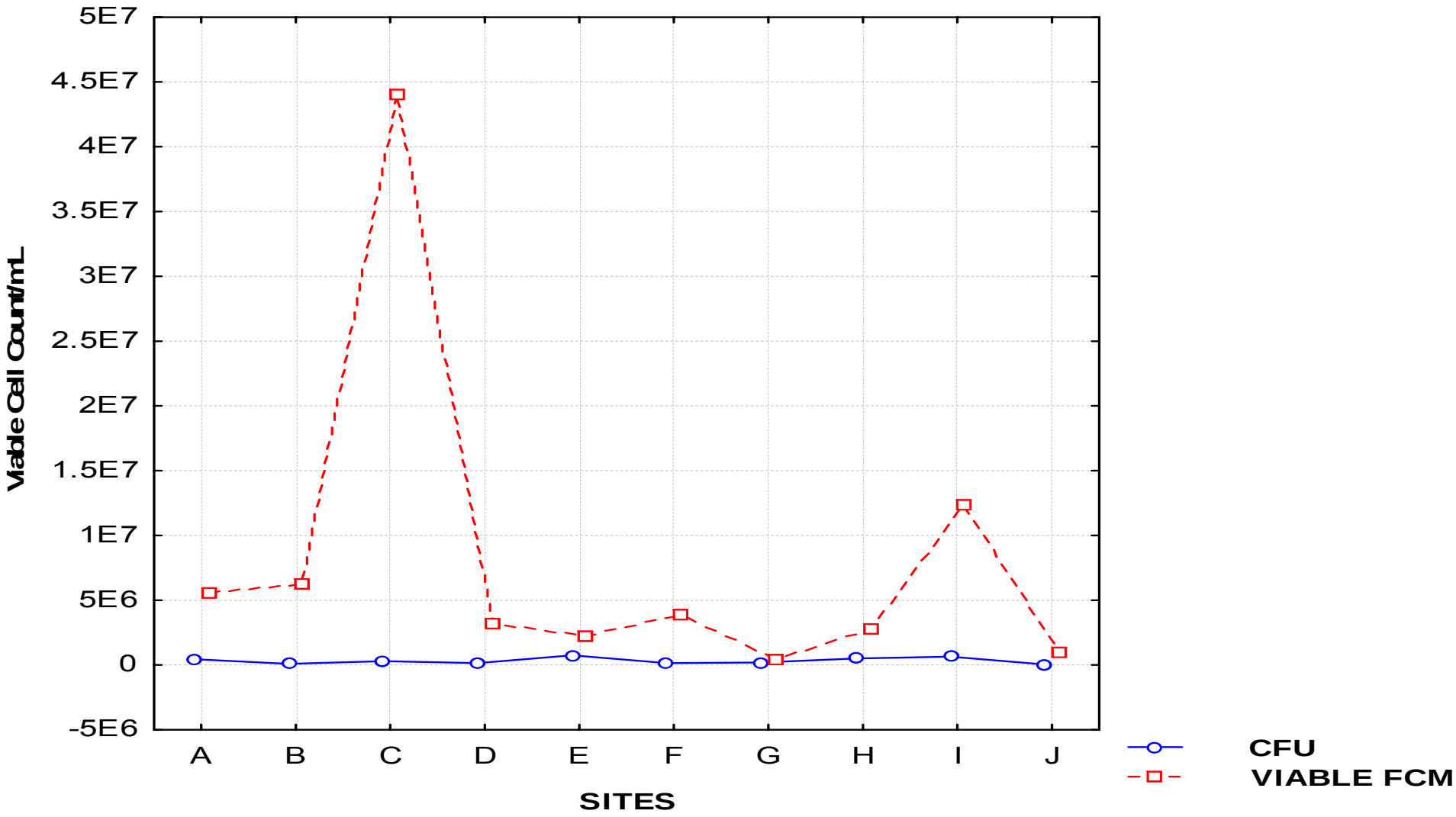


Figure 5 Comparison of heterotrophic plate count to viable FCM count for week 46 for the sites analysed.

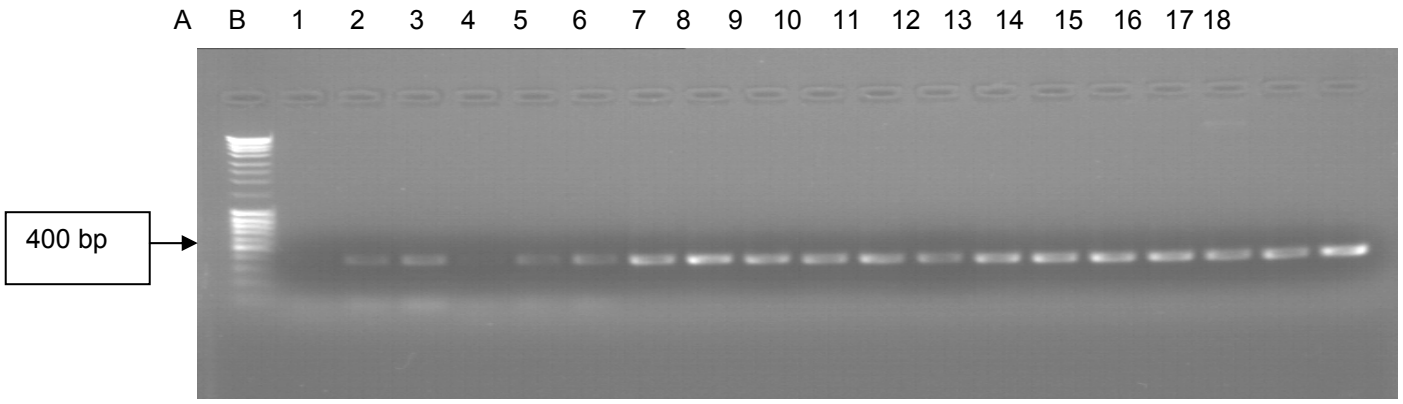


Figure 6a Polymerase Chain Reaction analysis of extracted DNA samples (BB1-138 to 155) [with primer set 1: forward (RW01) primer; reverse (DG74) primer] for sampling week 4. Lanes 1 –18: samples 14 to 32; Lane A: Marker [MassRuler™ DNA Ladder Mix, #SM0403 (Fermentas)]; Lane B: Negative control.

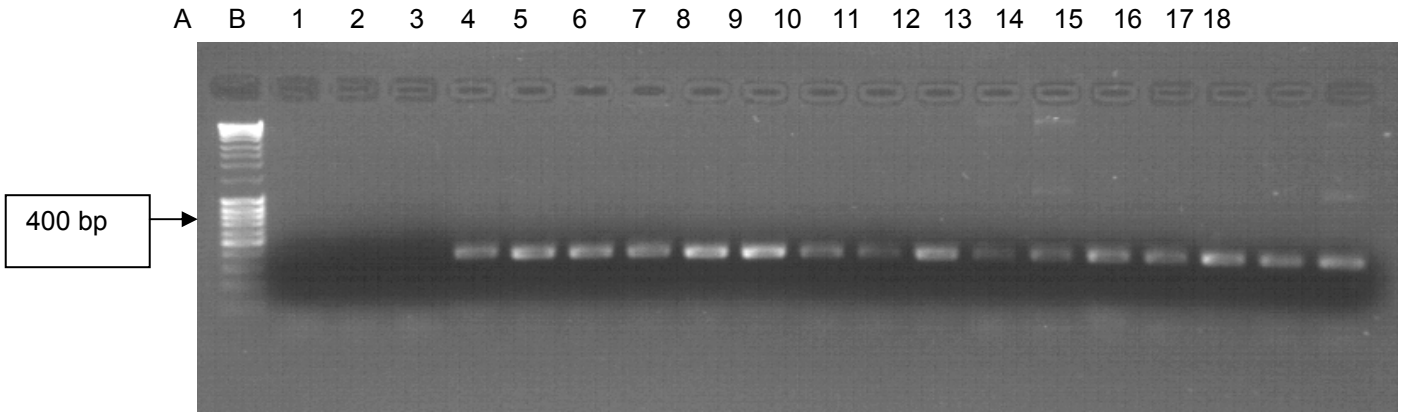


Figure 6b Polymerase Chain Reaction analysis of extracted DNA samples (BB1-138 to 155) [with primer set 2: forward (RW080) primer; reverse (DG74) primer] for sampling week 4. Lanes 1 –18: samples 14 to 32; Lane A: Marker [MassRuler™ DNA Ladder Mix, #SM0403 (Fermentas)]; Lane B: Negative control.

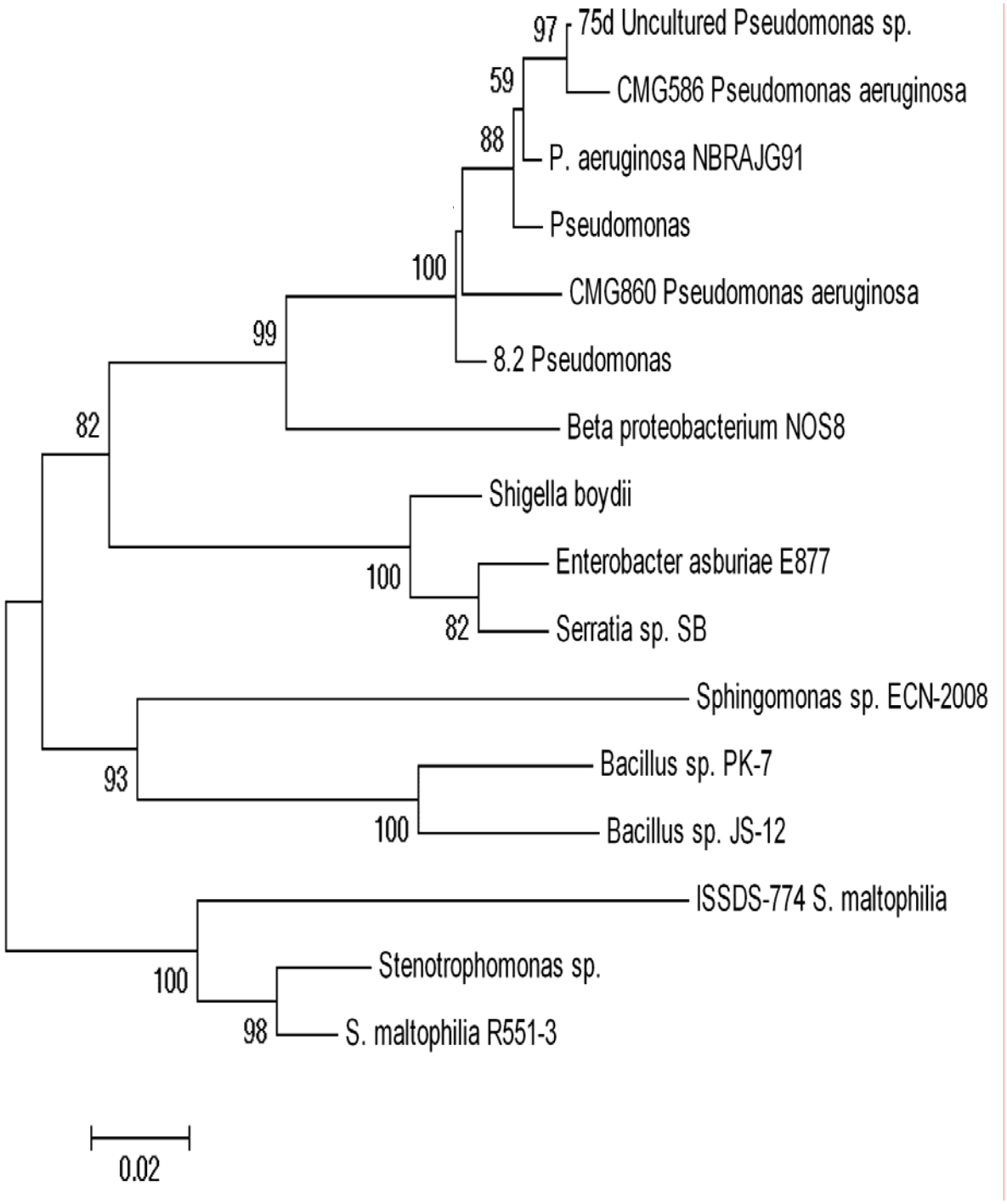


Figure 7 Unrooted phylogenetic tree inferred from 16S rRNA sequence data, isolated from bacterial samples obtained from water samples taken in week 1 from the distribution system in Western Cape, South Africa. Distance matrices were constructed from the aligned sequences and created for multiple base changes at single position by the BLOSUM algorithm. Bootstrap values are shown at nodes.

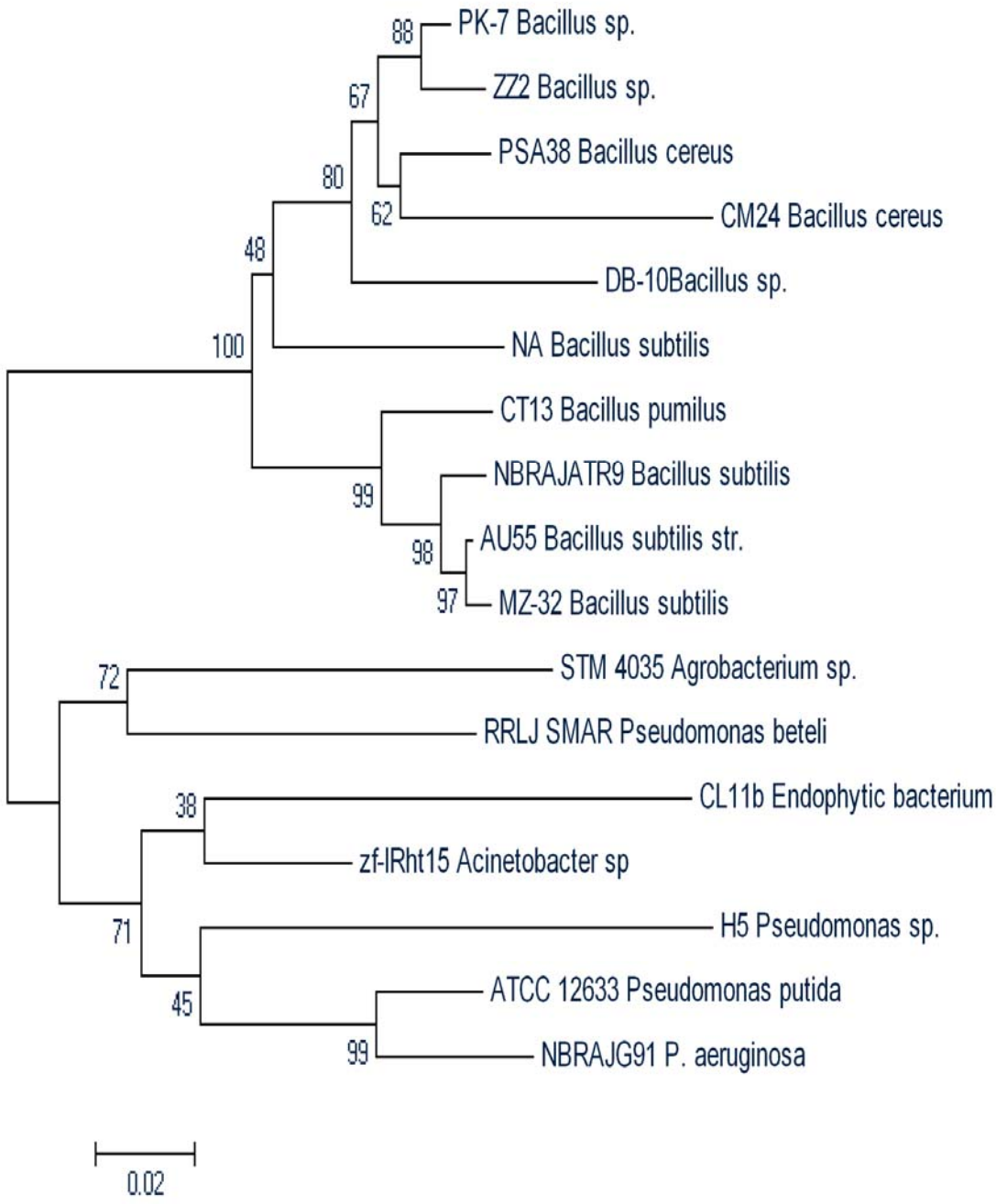


Figure 8 Unrooted phylogenetic tree inferred from 16S rRNA sequence data, isolated from bacterial samples obtained from water samples taken in week 4 from the distribution system in Western Cape, South Africa. Distance matrices were constructed from the aligned sequences and created for multiple base changes at single position by the BLOSUM algorithm. Bootstrap values are shown at nodes.

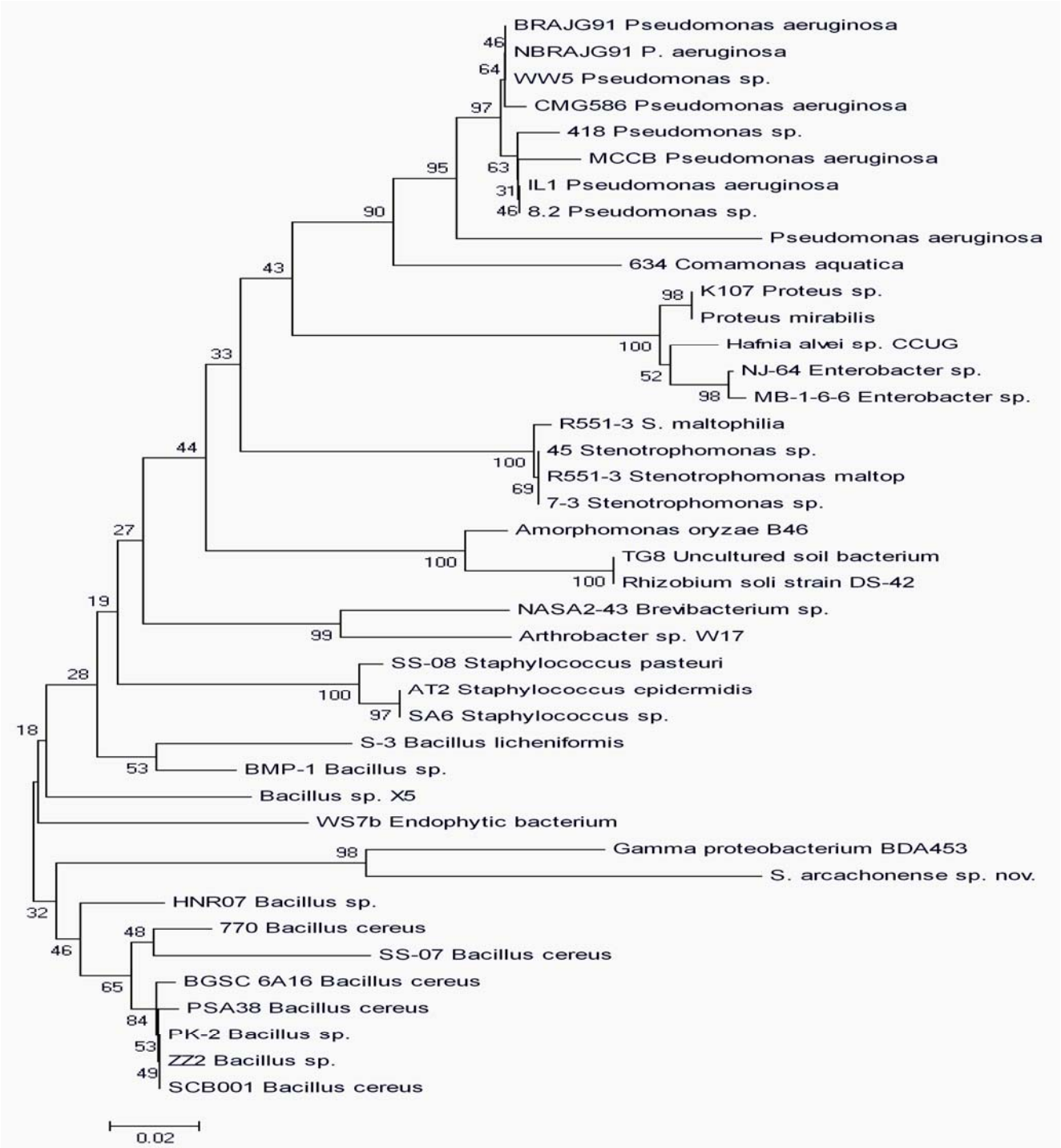


Figure 9 Unrooted phylogenetic tree inferred from 16S rRNA sequence data, isolated from bacterial samples obtained from water samples taken in week 8 from the distribution system in Western Cape, South Africa. Distance matrices were constructed from the aligned sequences and created for multiple base changes at single position by the BLOSUM algorithm. Bootstrap values are shown at nodes.

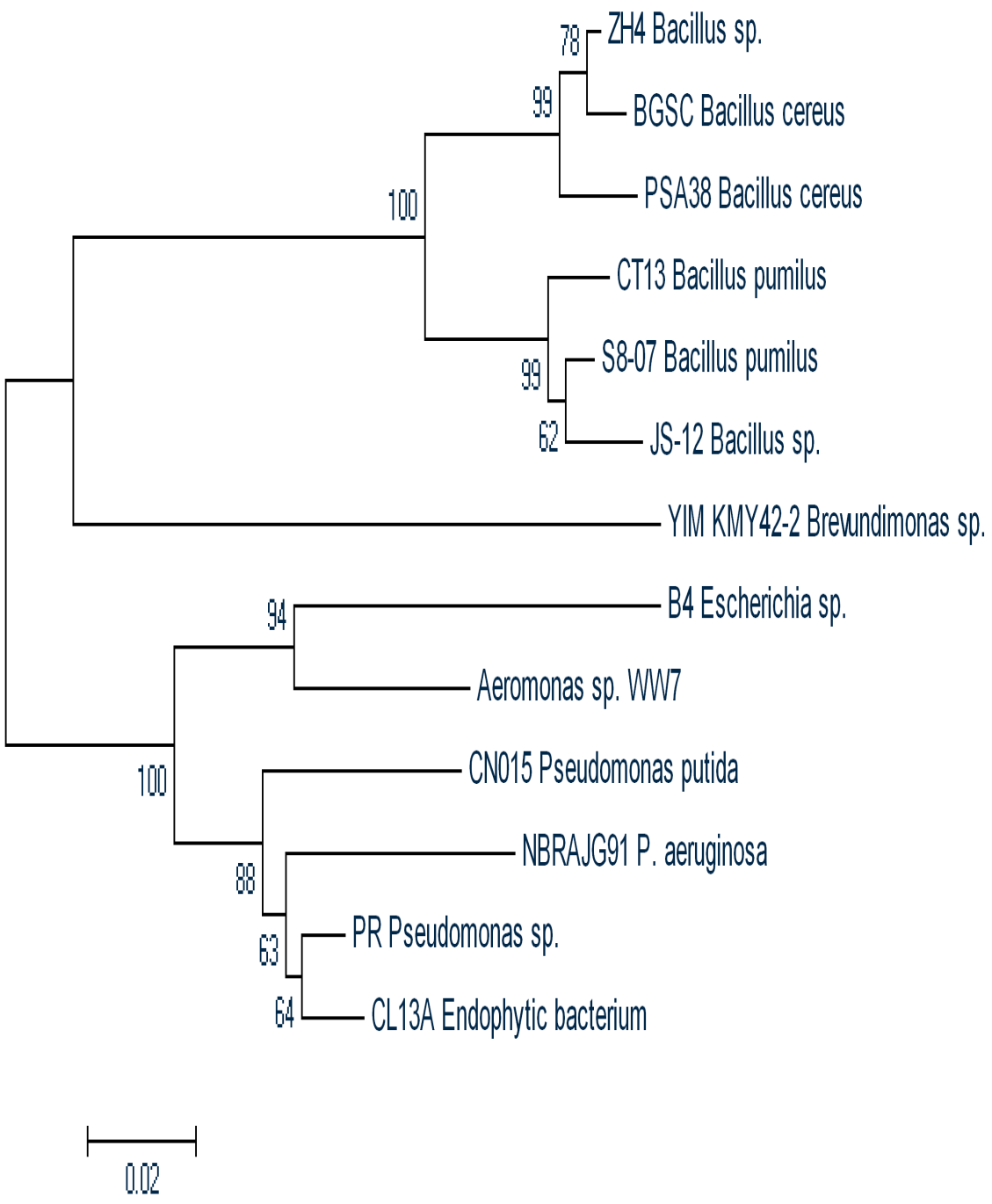


Figure 10 Unrooted phylogenetic tree inferred from 16S rRNA sequence data, isolated from bacterial samples obtained from water samples taken in week 46 from the distribution system in Western Cape, South Africa. Distance matrices were constructed from the aligned sequences and created for multiple base changes at single position by the BLOSUM algorithm.

Table 1 Table of 16 isolates, their codes and accession numbers for organisms isolated from week 1 sampling period.

Name presented on tree	Organism	Accession number
75d Uncultured *Pseudomonas sp.*	Clone_75d_Uncultured_*Pseudomonas sp.*	EF593077.1
CMG586 Pseudomonas aeruginosa	Pseudomonas aeruginosa strain CMG586	EU194236.1
NBRAJG91 *P. aeruginosa*	*Pseudomonas aeruginosa strain* NBRAJG91	*EU661707.1*
WW5 *Pseudomonas sp*	*Pseudomonas sp.* WW5	*EF433547.1*
CMG860 *Pseudomonas aeruginosa*	*Pseudomonas aeruginosa strain* CMG860	*EF511771.1*
Pseudomonas sp.	*Pseudomonas sp.*	*EF426444.1*
Beta proteobacterium NOS8	*Beta proteobacterium* NOS8	*AB076846.1*
Shigella boydii	*Shigella boydii*	*AB273731.1*
Serratia sp. SB	*Serratia sp. SB*	*EU816383.1*
E877 *Enterobacter asburiae*	*Enterobacter asburiae strain* E877	*EF059885.1*
Sphingomonas sp. ECN-2008	*Sphingomonas sp.* ECN-2008	*AM940945.1*
Bacillus sp. PK-7	*Bacillus sp.* PK-7	*EU685824.1*
JS-12 *Bacillus sp*	*Bacillus sp.* JS-12	*EF040535.1*
ISSDS-774 *S. maltophilia*	*Stenotrophomonas maltophilia strain* ISSDS-774	*EF620464.1*
Stenotrophomonas sp.	*Stenotrophomonas sp.*	*AJ884482.1*
R551-3 *S. maltophilia*	*Stenotrophomonas maltophilia* R551-3	*CP001111.1*

Table 2 Table of 17 isolates, their codes and accession numbers for organisms isolated from week 4 sampling period.

Name presented on tree	Organism	Accession number
PK-7 *Bacillus sp*	*Bacillus sp.* PK-7	EU685824.1
ZZ2 *Bacillus sp*	*Bacillus sp.* ZZ2	DQ113449.1
PSA38 *Bacillus cereus*	*Bacillus cereus strain* PSA38	EU346663.1
CM24 *Bacillus cereus*	*Bacillus cereus strain* CM24	EU660318.
DB-10*Bacillus sp*	*Bacillus sp.* DB-10	EU439408.1
NA *Bacillus subtilis*	*Bacillus subtilis strain* NA	EF064205.1
CT13 *Bacillus pumilus*	*Bacillus pumilus strain* CT13	EU660365.1
NBRAJATR9 *Bacillus subtilis*	*Bacillus subtilis strain* NBRAJATR9	EU661710.1
AU55 *Bacillus subtilis str.*	*Bacillus subtilis strain* AU55	EF032684.1
MZ-32 *Bacillus subtilis*	*Bacillus subtilis subsp. subtilis* MZ-32	EF422864.1
STM 4035 *Agrobacterium s.p*	*Agrobacterium sp.* STM 4035	EF152474.1
RRLJ SMAR *Pseudomonas beteli*	*Pseudomonas beteli strain* RRLJ SMAR	DQ299947.1
CL11b *Endophytic bacterium*	*Endophytic bacterium* CL11b	EU088087.1
zf-IRht15 *Acinetobacter sp*	*Acinetobacter sp.* zf-IRht15	DQ223660.1
H5 *Pseudomonas sp*	*Pseudomonas sp.* H5	DQ268826.1
ATCC 12633 *Pseudomonas putida*	*Pseudomonas putida strain* ATCC 12633	AF094736.1
NBRAJG91 *P. aeruginosa strain*	*Pseudomonas aeruginosa strain* NBRAJG91	EU661707.1

Table 3 Table of 43 isolates, their codes and accession numbers for organisms isolated from week 8 sampling period.

Name presented on tree	Organism	Accession number
NBRAJG91 P. aeruginosa	*Pseudomonas aeruginosa strain* NBRAJG91	EU661707.1
NBRAJG91 P. aeruginosa	*Pseudomonas aeruginosa strain* NBRAJG91	EU661707.1
WW5 Pseudomonas sp.	*Pseudomonas sp.* WW5	EF433547.1
CMG586 Pseudomonas aeruginosa	*Pseudomonas strain* CMG586	EU194236.1
418 Pseudomonas sp	*Pseudomonas sp.* 418	EU841539.1
MCCB Pseudomonas aeruginosa	*Pseudomonas aeruginosa isolate* MCCB	EF053508.2
IL1 Pseudomonas aeruginosa	*Pseudomonas aeruginosa isolate* IL1	DQ989211.2
8.2 Pseudomonas sp	*Pseudomonas sp.* 8.2	EF426444.1
Pseudomonas aeruginosa	*Pseudomonas aeruginosa*	EU327890.1
634 Comamonas aquatica	*Comamonas aquatica strain* 634	EU841530.1
K107 Proteus sp	*Proteus sp.* K107	EU710747.1
Proteus mirabilis	*Proteus mirabilis*	DQ777867.1
Hafnia alvei CCUG 429	*Hafnia alvei CCUG* 429	FM179944.1
NJ-64 Enterobacter sp	*Enterobacter sp.* NJ-64	AM421983.1
MB-1-6-6 Enterobacter sp.	*Enterobacter sp.* MB-1-6-6	EU816586.1
45 Stenotrophomonas sp	*Stenotrophomonas sp.* 45	AY856845.1
R551-3 Stenotrophomonas maltop.	*Stenotrophomonas maltophilia* R551-3	CP001111.1
7-3 Stenotrophomonas sp.	*Stenotrophomonas sp.* 7-3	EU054384.1
Amorphomonas oryzae B46	*Amorphomonas oryzae* B46	AB233493.1
TG8 Uncultured soil bacterium	*Uncultured soil bacterium clone* TG8	DQ297948.2
Rhizobium soli strain DS-42	*Rhizobium soli strain* DS-42	EF363715.1
NASA2-43 Brevibacterium sp.	*Brevibacterium sp.* NASA2-43	EU029632.1
Arthrobacter sp. W17	*Arthrobacter sp.* W17	EU596424.1
SS-08 Staphylococcus pasteuri	*Staphylococcus pasteuri strain* SS-08	EU624447.1
AT2 Staphylococcus epidermidis	*Staphylococcus epidermidis strain* AT2	EU021221.2
SA6 Staphylococcus sp	*Staphylococcus sp.* SA6	AY864655.1
TG8 Uncultured soil bacterium	Uncultured soil bacterium clone TG8	DQ297948.2
BMP-1 Bacillus sp.	*Bacillus sp.* BMP-1	DQ371431.1
Bacillus sp. X5	*Bacillus sp. X5*	EU236728.1
WS7b Endophytic bacterium	*Endophytic bacterium* WS7b	EU088038.
Gamma proteobacterium BDA453	*Gamma proteobacterium* BDA453	AB304258.1
S.arcachonense sp. nov.	*S.arcachonense sp. nov.*	Y11561.1
HNR07 Bacillus sp.	*Bacillus sp.* HNR07	EU373351.1
770 Bacillus cereus	*Bacillus cereus strain* 770	EU430093.1
SS-07 Bacillus cereus	*Bacillus cereus strain* SS-07	EU624445.1
BGSC 6A16 Bacillus cereus	*Bacillus cereus strain* BGSC 6A16	AY310302.1
PSA38 Bacillus cereus	*Bacillus cereus strain* PSA38	EU346663.1
PK-2 Bacillus sp	*Bacillus sp.* PK-2	EU685821.1
ZZ2 Bacillus sp.	*Bacillus sp.* ZZ2	DQ113449.1
SCB001 Bacillus cereus	*Bacillus cereus strain* SCB001	DQ466089.1

Table 4 Table of 13 isolates, their codes and accession numbers for organisms isolated from week 46 sampling period.

Name presented on tree	Organism	Accession number
ZH4 *Bacillus sp*	*Bacillus sp.* ZH4	*EU236750.1*
BGSC *Bacillus cereus strain*	*Bacillus cereus strain* BGSC	*AY310302.1*
PSA38 *Bacillus cereus*	*Bacillus cereus strain* PSA38	*EU346663.1*
CT13 *Bacillus pumilus*	*Bacillus pumilus strain* CT13	*EU660365.1*
S8-07 *Bacillus pumilus*	*Bacillus pumilus strain* S8-07	*EU620415.1*
JS-12 *Bacillus sp*	*Bacillus sp.* JS-12	*EF040535.1*
YIM KMY42-2 *Brevundimonas sp*	*Brevundimonas sp.* YIM KMY42-2	*DQ358649.1*
B4 *Escherichia sp*	*Escherichia sp.* B4	*EU722735.1*
WW7 *Aeromonas sp*	*Aeromonas sp.* WW7	*EF433549.1*
CN015 *Pseudomonas putida*	*Pseudomonas putida strain* CN015	*EU364531.1*
NBRAJG91 *P. aeruginosa*	*Pseudomonas aeruginosa strain* NBRAJG91	*EU661707.1*
PR *Pseudomonas sp*	*Pseudomonas sp.* PR	*EU816382.1*
CL13A *Endophytic bacterium*	*Endophytic bacterium* CL13A	*EU088094.1*

Application of Bioremediation as Treatment Technology in a Spring Water Distribution System.

L. Behardien[1], A. Bester[2], J.H. Neethling[3] & W.Khan[4]

[1] *Department of Food Technology, Faculty of Applied Science, Cape Peninsula University of Technology, Cape Town, 8000, South Africa.*

[2] *Department of Chemical Engineering, Faculty of Engineering, Cape Peninsula University of Technology, Cape Town, 8000, South Africa.*

[3] *Department of Biomedical Sciences, Faculty of Health and Wellness Sciences, Cape Peninsula University of Technology, Bellville, 7535, South Africa.*

[4] *Department of Agricultural and Food Science, Faculty of Applied Science, Cape Peninsula University of Technology, Cape Town, 8000, South Africa.*

* To whom all correspondence should be addressed.

Tel: +27 21 460 3175, Fax: +27 21 460 3193; e-mail: khanw@cput.ac.za

Abstract

The aim of this study was to investigate the application of bioremediation as treatment in a spring water bottling system in the Western Cape, South Africa. The spring water bottling system was experiencing microbial quality problems. A laboratory scale bioreactor was constructed and water samples were analysed over a two week period. Flow cytometry (FCM) and the Direct Acridine Orange Count (DAOC) with Epiflourescent Microscopy (EM) were used for the enumeration of microorganisms in the samples. The total cell counts analysed by flow cytometry ranged from 1.53 x 10^7 microorganisms/ml in the initial sample to 1.16 x 10^7 microorganisms/ml on day 13 (final). The results indicated a 24% decrease in the microbial numbers. The total cell counts obtained by the DAOC method ranged from 1.43 x 10^6 microorganisms/ml in the initial sample to 9.54 x 10^5 microorganisms/ml on day 13 (final). The results indicate a 33% decrease in microbial numbers. The total cell counts obtained by the DAOC method were lower in all the water samples when compared to the total counts obtained by flow cytometric analyses. Although the FCM counts fluctuated throughout the entire sampling period, results clearly showed that the FCM method was more effective for total cell count determination than the DAOC method. Even though a decrease in the total cell counts (24%, FCM; 33%, DAOC) were observed further studies are required to optimise the bioreactor system for its application in the spring water distribution system.

Keywords: bacterial contamination, bioremediation, direct acridine orange count (DAOC), epifluorescence microscopy, flow cytometry, spring water bottling system

Introduction

Spring water is formed when water that filters through the earth's layers is forced through natural underground pressure to the earth's surface in the form of a spring (LaMoreaux & Tanner, 2002). If the spring water is bottled and sold for human consumption the source of the spring must not be situated at or close to any danger of pollution by sewerage, farming operations, waste disposal or industrial activities or any combination of the above pollutant sources (South African National Standard, 2003). This natural bottled water can only be subjected to certain treatment processes such as separation from unstable constituents by decantation, filtration, aeration, and by any process that will ensure that the natural mineral content is not modified, such as ultraviolet irradiation and ozonation (South African Department of Health, 2004). According to the South African National Standards for Bottled Water (2003) coliform bacteria and faecal coliform bacteria must be absent per 100 ml of water and the total viable colony count should be < 100 organisms per 1 ml of water.

Bioremediation is the process by which living organisms utilise their metabolic potential to degrade or transform hazardous organic compounds in order to clean up contaminated environments under controlled conditions (Watanabe & Baker, 2000; Spain, 2000; Samanta, 2002 and Parales & Haddock, 2004). This biological process utilises either naturally occurring organisms, or organisms introduced to the site, to degrade toxic compounds. Microbial bioremediation is the process whereby microorganisms convert harmful chemicals into harmless compounds or utilisable matter through enzymatic reactions. The reduction in the level of contaminants is often the result of various diverse groups of microorganisms interacting with one another and functioning in the contaminated environments under various specific conditions (Vidali, 2001).

A bioreactor utilises microorganisms to degrade contaminants in water through suspended biological systems. Organic matter in contaminated ground water is aerobically degraded by microorganisms while being circulated through an aerated basin to carbon dioxide, water and new cells. Contaminated water sludges and waste streams have also successfully been treated utilising bioremediation technology, which is simple and cost effective (Boopathy, 2000). It is however, important to assess the suitability of the environment for this biological process, as bioremediation cannot be used to degrade all types of contaminants, such as chlorinated hydrocarbons, as they are often resistant to microbial degradation (Coldberg & Young, 1995; Vidali, 2001). Factors such as oxygen, pH, nutrient availability and the ability of the microbial population to degrade pollutants are all important for the optimisation of the bioremediation process (Vidali, 2001).

Microscopy is one of the only techniques where bacterial cells and in particular biofilms can be studied at the single cell level *in situ.* This method has gained considerable attention as it has been successfully employed for the detection of the characteristics, physiology and interactions of microbial cells, (Kumar & Anand, 1998). Confocal Laser Scanning Microscopy (CLSM), Epifluorescent-, Electron- and Phase Contrast microscopy are all widely used methods in the determination of the biofilm structure (Hurst *et al*., 2002). In a study conducted by Garabetian *et al*. (1999) epiflourescent microscopy was used to assess whether the storage of freshwater samples affected the total bacterial counts in water. The water samples were stored at 4 ºC and -18 ºC for short (7 and 14 days) and long (160 and 240 days) periods by using formalin–fixing. The study was performed in the field and although bacterial reductions were observed, filtering, staining and microscopic examination of the slides under the microscope was time-consuming.

Flow cytometry (FCM) is a rapid and reliable technique to assess single or multiple microorganisms by means of fluorochromes (Current Protocols in Flow Cytometry, 2005). The addition of fluorescent beads in conjunction with the Live/Dead BacLight™ viability stain allows for the enumeration of total bacteria in the water samples (Monis *et al*., 2003). According to Hiraoka & Kimbara (2002) both flow cytometry and epifluorescence microscopy are techniques widely used to determine total cell counts and also the ratios of live (viable) cells to permeabilised (injured) and dead cells. Paulse *et al*. (2007) also used flow cytometry and epifluorescence microscopy to determine total cell counts in the Berg River, Western Cape, South Africa. Results showed that the flow cytometry method was effective in routinely comparing and evaluating the presence of total cell counts in the river water samples.

The aim of this study was to investigate the application of bioremediation as treatment technology in a spring water bottling system in the Western Cape, South Africa. Flow cytometric analysis and direct acridine orange count (DAOC) using epifluorescence microscopy was used to obtain total cell counts (the culturable and non-culturable populations).

Materials and Methods

Water collection

The water used for the laboratory-scale bioreactor systems was collected from a spring water bottling plant in the Western Cape, South Africa. This bottling plant was selected as bacterial contamination was experienced throughout the water distribution system. Significantly high total cell counts were also obtained by the FCM technique at various sampling sites in the distribution system (Behardien *et al*., 2008).

Laboratory-scale bioreactor

A laboratory-scale bioremediation system **(Figure 1)** was evaluated over a period of two weeks. Two hundred litres of water collected from the spring water distribution system was pumped through the batch laboratory-scale horizontal bioreactor (35 cm x 30 cm x 100 cm) at a flow rate of 1000 L/h (Ecopool 6 pump) and a retention time of three minutes.

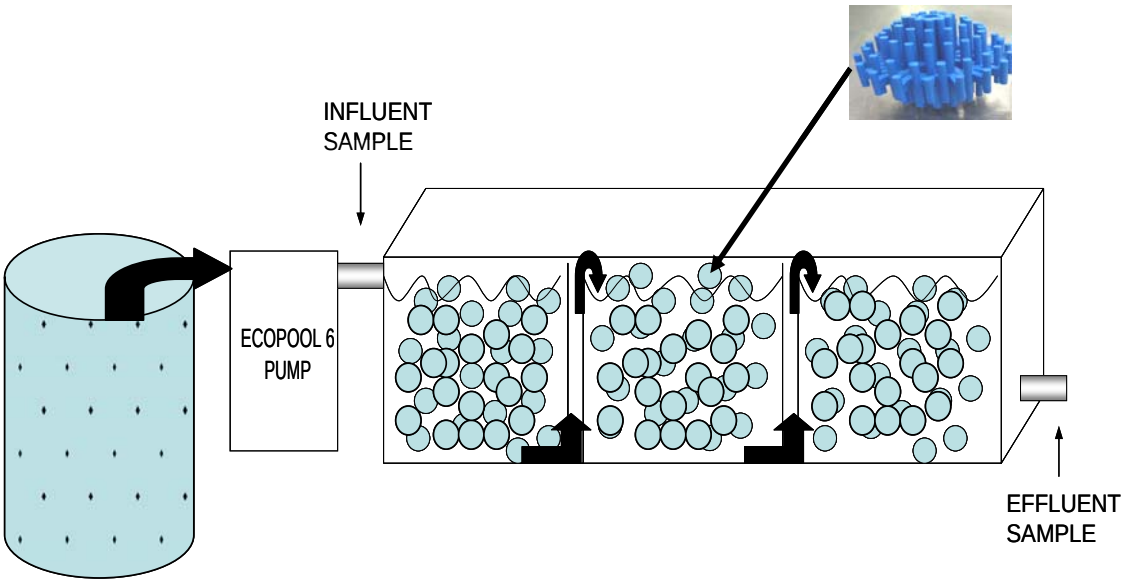


Figure 1
Schematic diagram of the laboratory-scale bioreactor system with Bioballs™ as the attachment surface.

Material used as attachment surfaces – Bioballs™

Each of the three compartments of the bioreactor system were filled with Bioballs™ **(Figure 1)**, which is produced from a combination of acrylonitrile, butadiene and styrene (ABS). The Bioballs™ have a surface area of 20 cm^3 and can be stacked close to each other in a system. This not only increases the attachment surface area for biofilm growth, but also the retention time within the system.

Direct Acridine Orange Count (DAOC)

The total number of microorganisms in the effluent water samples was determined by means of epifluorescence microscopy, with acridine orange (Sigma) as the fluorochrome. The water samples (2 ml) were filtered through Millipore membrane filters with a pore size of 0.22 µm. Cells were stained with 2 ml acridine orange (160 mg/L) for 5 minutes. Total cell counts were obtained using a Zeiss microscope (100X magnification). A minimum of five different fields was enumerated for each sampling time.

Flow cytometry (FCM)

The flow cytometer analysis as outlined by Paulse *et al*. (2007) was employed in the present study. For the flow cytometry-based assay of samples, individual samples were subjected to a Becton Dickinson FACSCalibur flow cytometer for analysis. The Becton Dickinson FACSCalibur flow cytometer has a 15 mW, 488 nm argon-ion laser. A Doublet Discrimination Module, which uses pulse width and area to eliminate cell clumping (doublets and triplets) is ideal for DNA analysis. Flow cytometry employs the principles of light scattering, light excitation and emission of fluorochrome molecules to generate data from particles or cells in the size range of 0.5 µm to 40 µm in diameter (Current Protocols in Cytometry, 2005). The bacterial population was identified and gated on a forward scatter (FSC) versus a side scatter (SSC) dotplot and a SSC versus fluorescence channel 2 (FL-2) at 585/42 nm dotplot. The bead count was identified and gated on a SSC versus fluorescence channel (FL-1) dotplot. All parameters were measured using a logarithmic amplification scale. A threshold of 52 FSC channels were set to remove sample debris. Only bacterial cells satisfying both gates were collected for subsequent analysis. Depending on the amount of debris present, certain samples were filtered through a 0.22 µm filter before analysis. The staining procedure was performed by combining equal volumes of PI (propidium iodide) (4 µl) and SYTO 9 (4 µl) in the BacLight™ viability probe and dissolved in 1 ml sterile distilled H_2O. The stained samples (1 ml sample stained with 200 µl BacLight™) were kept in the dark for 15 minutes, after which 50 µl liquid counting beads (BD™ Cell Viability Kit, BD™ Liquid Counting Beads) were added. The samples were then analysed with the flow cytometer and the concentrations of total cell populations were determined (Equation 1). In order to avoid excessive compensation of fluorescence overlap, SYTO 9 green emittance fluorescence was measured in fluorescence channel 1 (FL-1) at 530/30 nm and the PI was measured in fluorescence channel 3 (FL-3) at 670/LP nm. The samples were acquired until a total of 250 counting bead events were detected. An *Escherichia coli* laboratory strain was used as control.

Equation 1

$$\frac{\text{Number of events in cell region}}{\text{Number of events in bead region}} \times \frac{\text{Number of beads / test}}{\text{test volume}} \times \text{dilution factor}$$

[Bead concentration recorded at 988/µℓ for BD Liquid Counting Beads and at 49827 beads per Trucount™ tube, both obtained from BD™]

Statistical Analysis

Repeated Measures Anovas (RMAs) were performed on all data obtained as outlined in Dunn & Clark (1987) using Statistica™. In each RMA the residuals were analysed to determine if they were normally distributed. In all hypothesis tests a significance level of 5% was used as standard.

Results and Discussion

Flow Cytometric Analysis (FCM) and Direct Acridine Orange Count (DAOC)

The total cell counts obtained by FCM analysis and DAOC using epiflourescent microscopy are presented in **Figures 2 & 3**. The live and dead ratios of samples were obtained using flow

cytometer analysis, in conjunction with the Live/Dead BacLight™ availability probe and liquid counting beads (BD™). Two distinct populations of live and dead cells were observed by distinguishing between their fluorescence intensities, i.e. either red or green. The total cell counts for FCM analysis ranged from 1.53 x 10^7 microorganisms/ml in the initial sample to 1.16 x 10^7 microorganisms/ml on day 13. A steady increase in the microbial count was noted over the first four days, with a count of 3.35 x 10^7 microorganisms/ml recorded on day four. This increase in the microbial numbers could have been due to nutrient availability in the water and favourable growth conditions. After day four the results decreased rapidly from 3.35 x 10^7 microorganisms/ml (Day 4) to 1.08 x 10^7 microorganisms/ml on day five. The rapid decrease in the microbial numbers could be due to a decrease in temperature and depletion of nutrients. The growth of microorganisms is also dependant on their environmental conditions and a change in the conditions can either alter their growth rate or kill them. Certain microorganisms also enter a viable but non-culturable (VNBC) state when they are exposed to a stressful environment (Roszak & Coldwell, 1987). Results continued to fluctuate over the study period with a final count of 1.16 x 10^7 microorganisms/ml observed on day 13. These results indicated a 24% decrease in the microbial numbers. The presence of oxygen, pH, nutrient availability and the ability of the microbial population to degrade pollutants are all important factors in the optimisation of the bioremediation process (Vidali, 2001).

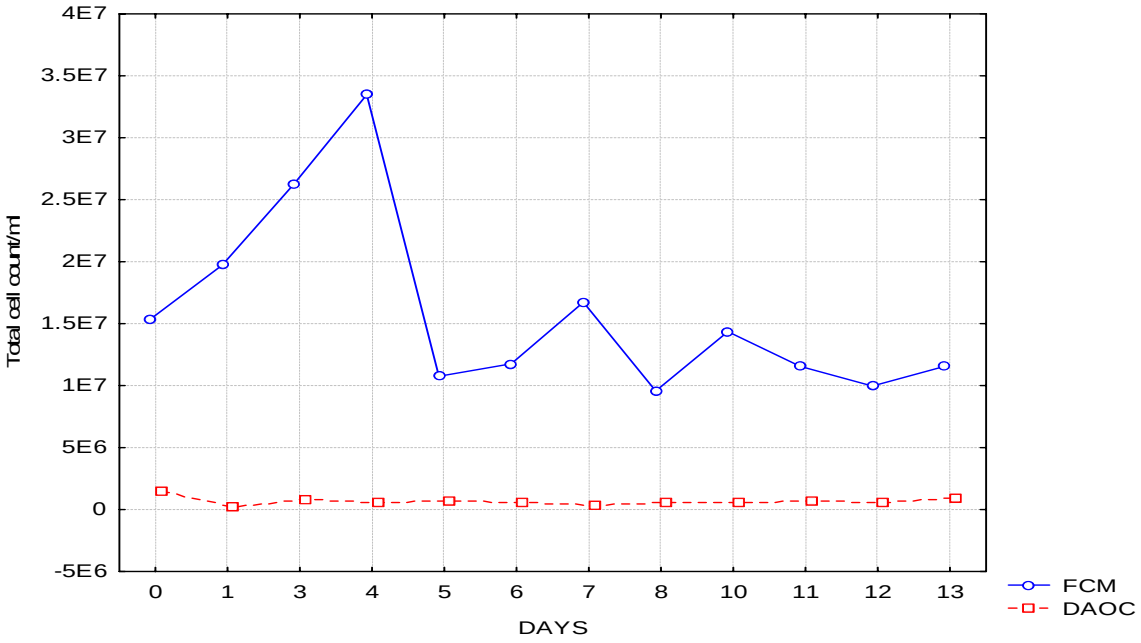


Figure 2
Comparison of total cell counts for bioreactor samples as obtained by flow cytometry (FCM) and epiflourescent microscopy (DAOC).

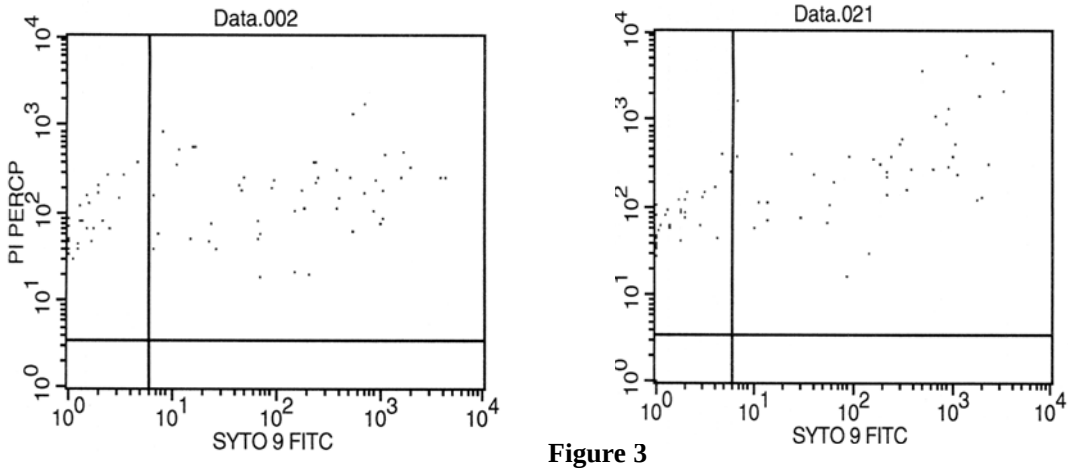


Figure 3

Dotplots of planktonic samples obtained at initial (day 0) and final day (day 13) of the bioreactor by Flow cytometric analyses (FCM).

The total cell counts obtained by the DAOC method were lower in all the water samples, when compared to total counts obtained by flow cytometry analyses. The highest DAOC count of 1.43 x 10^6 microorganisms/mℓ was recorded in the initial water sample with a corresponding FCM count of 1.53 x 10^7 microorganisms/ml. The lowest DAOC count of 1.76 x 10^5 microorganisms/mℓ was recorded on day two with a corresponding FCM count of 1.97 x 10^7 micro-organisms/mℓ. Even though the FCM counts fluctuated throughout the sampling period, results clearly showed that the FCM method yielded more accurate data for total cell counts. Paulse *et al.* (2007) assessed various enumeration techniques during an investigation into the planktonic bacterial population in the Berg River, Western Cape, South Africa. Higher FCM than DAOC total cell counts were detected indicating that this technique was able to detect cells in the water sample that enter a viable but non culturable state. The FCM technique indicated significantly ($p < 0.05$) higher total counts than those observed by the DAOC technique. The DAOC method involves the manual counting of the microorganisms in conjunction with epifluorescence microscopy, which implies that more scope for human error exists.

Results obtained for the average live/dead total cell count by FCM analysis for each sample over the specific time period are presented in **Figure 4**. The live vs. the dead cell counts fluctuated throughout the two week study period. The live cell count of 9.77 x 10^6 microorganisms/ml in the initial sample was significantly ($p < 0.05$) higher than the corresponding dead cell count of 5.58 x 10^6 microorganisms/ml. On day four a significant increase in the live cell count was noted which again could be attributed to favourable growth conditions. The final live cell count on day 13 was 4.58 x 10^6 microorganisms/ml in comparison to the dead cell count of 6.98 x 10^6 microorganisms/ml **(Figure 4)**. The current water legislation however, states that the heterotrophic plate count of the final bottled water must be < 100 organisms/ml within 24 hours of bottling however, no regulation for FCM in bottled water could be found.

Melin *et al*. (1998) expressed that the main limitation for groundwater bioremediation is thought to be temperature as it slows down the degradation rates. Due to the influence of external environmental conditions such as changes in weather conditions, the results fluctuated and even though a decrease in total cell counts is obtained (FCM & DAOC) further studies are required. A bioreactor system could however possibly be implemented as primary treatment directly after the borehole, as this will not alter the chemical composition of the

water. Once the primary treatment is implemented the water can continue throughout the distribution system which effectively reduces the clogging of filters.

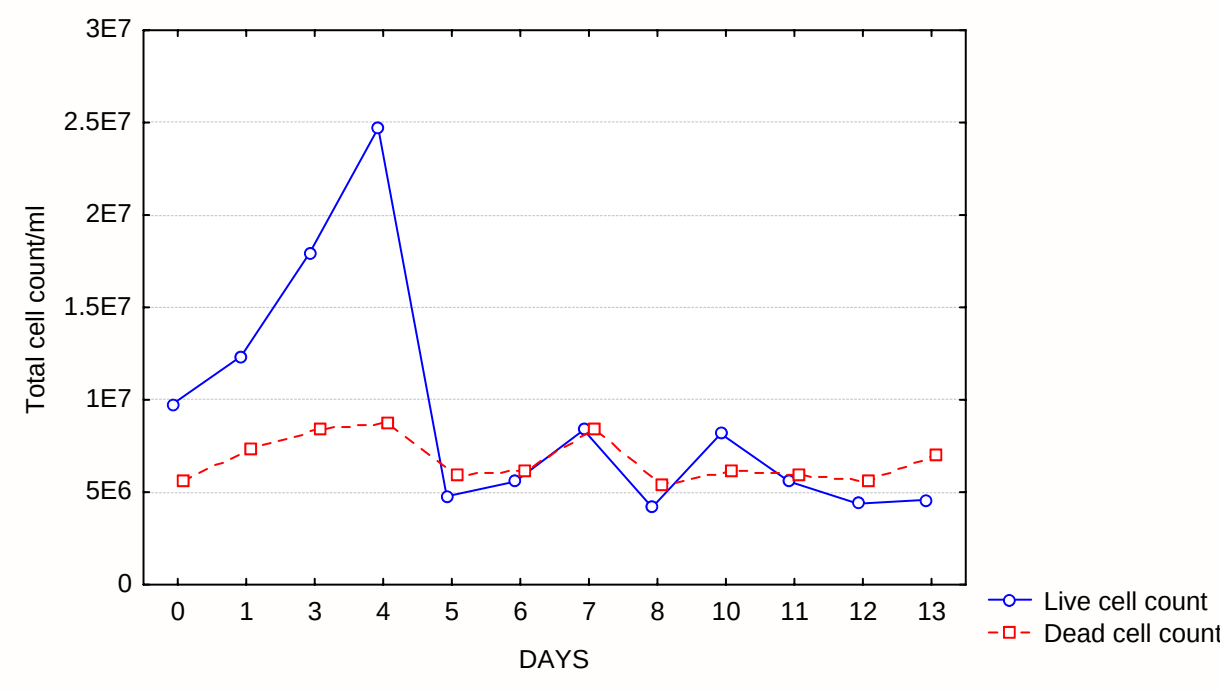


Figure 4
Enumeration of live and dead cell counts by means of flow cytometry (FCM) for the sampling period.

Conclusions

The major conclusions of the study include the following:

1. The FCM count ranged from 1.53 x 10^7 microorganisms/mℓ in the initial sample to 1.16 x 10^7 microorganisms/mℓ in the final sample. Direct acridine orange counts ranged from 1.43 x 10^6 microorganisms/mℓ in the initial sample to 9.54 x 10^5 microorganisms/mℓ in the final sample.

2. The total cell counts obtained by the DAOC method were lower in all the water samples, when compared to total counts obtained by flow cytometric analyses. Even though the FCM counts fluctuated throughout the sampling period, results clearly showed that the FCM method yielded more accurate data for total cell counts than the DAOC method.

3. Although there was a decrease in the viable cell count it was still significantly higher than the heterotrophic plate count limit as defined by the South African Standards for bottled water of < 100 organisms/ml. There is no FCM standard specified for bottled spring water.

4. Even though a decrease in the total cell counts (24%, FCM; 33%, DAOC) were observed further studies are required to optimise the bioreactor system for its application in the spring water distribution system.

References

Behardien, L., Paulse, A.N., Jackson, V.A., Khan, S. and Khan, W. (2008). Investigation into the microbial contamination in a spring water distribution system, Western Cape, South Africa. *Water SA,* (Submitted for publication).

Boopathy, R. (2000). Factors Limiting bioremediation technologies. *Bioresource Technology,* **74**(1), 63-67.

Coldberg, P.J.S. and Young, L.Y. (1995). Anaerobic degradation of nonhalogenated homocyclic aromatic compounds coupled with nitrate, iron, or sulfate reduction. In *microbial transformation and degradation of toxic organic chemicals*. Wisley-Liss, New York, 307-330.

Current Protocols in Flow Cytometry. (2005). *Introduction.* John Wiley & Sons (via Wiley Inter Science).
http://www3.interscience.wiley.com/cgi-bin/mrwhome/104554804/HOME
[18 August 2005].

Dunn, O.J. and Clark, V.A. (1987). Applied Statistics: Analysis of variance and regression (2nd Ed). John Wiley & Sons, London, UK:

Garabetian, F., Petit, M. and Lavandier, P. (1999). Does storage affect epifluorescence microscopic counts of total bacteria in freshwater samples. *Academy Science,* **322**, 779-784.

Hiraoka, Y. and Kimbara, K. (2002). Rapid assessment of the physiological status of the polychlorinated biphenyl degrader Comamonas testosteroni TK102 by flow cytometry. *Journal of Applied Environmental Microbiology,* **68**(4), 2031-2035.

Hurst, J.C., Crawford, R.L., Knudsen, G.R., McInerney, M.J. and Stetzenbach, L.D. (2002). *Manual of Environmental Microbiology*. 2nd ed. American Society for Microbiology. Washington DC.

Kumar, C.G. and Anand, S.K. (1998). Significance of microbial biofilms in food industry. *International Journal of Food Industry,* **42**, 9-27.

LaMoreaux, P.E. and Tanner, J.T. (2002*). Springs and bottled waters of the world: Ancient history, source, occurrences.* Springer Verlag, Netherlands.

Melin, E., Jarvinen, K. and Puhakka, J. (1998). Effects of temperature on chlorophenol biodegradation kinetics in fluidised-bed reactors with different biomass carriers. *Journal of Water Research,* **32**(1), 81-90.

Monis, P.T., Saint, PC.P., Hoefel, D., Grooby, W.L. and Andrews, S. (2003). Enumeration of water borne bacteria using viability assays and flow cytometry: a comparison to culture-based techniques. *Journal of Microbiology Methods,* **55**(3), 585-597.

Parales, R.E. and Haddcok, J.D. (2004). Biocatalytic degradation of pollutants. *Current Opinions Biotechnology,* **13**, 249-252.

Paulse, A.N., Jackson, VA. and Khan, W. (2007). Comparison of enumeration techniques for the investigation of bacterial pollution in the Berg River, Western Cape, South Africa. *South African Journal of Water Research,* **33**, 165-173.

Roszak, D.B. and Coldwell, R.R. (1987). Survival strategies of bacteria in the natural environment. *Microbiological Review,* **51**, 356-379.

Samanta, S.K. (2002). Polycyclic aromatic hydrocarbons: Environmental pollution and bioremediation. *Trends Biotechnology,* **20**, 243-248.

South African National Standards. (2003). Bottled Natural Water. SANS 1657:2003 Ed 1.3. Notice 1373 of 8 November 2002.

South Africa, Department of Health. (2004). Foodstuffs, Cosmetics and Disinfectants Act: Act 54 of 1972. Regulations governing bottled waters including natural mineral waters. No R 502.

Spain, J.C. (2000). *Biodegradation of nitroaromatic compounds*. Lewis Publishers, Washington, DC.

Vidali, M. (2001). Bioremediation. *Pure Application Chemistry,* **7**, 1163-1172.

Watanabe, K. and Baker, P.W. (2000). Environmentally relevant microorganisms. *Journal of Bioengineering,* **89**, 1-11.

GENERAL CONCLUSIONS

4.1 INVESTIGATION INTO THE MICROBIAL CONTAMINATION IN A SPRING WATER DISTRIBUTION SYSTEM, WESTERN CAPE, SOUTH AFRICA.

Spring bottled water is chemically untreated and must comply with stringent microbiological criteria as stipulated by the South African National Standards for Bottled Water (2003). In developed countries the demand for bottled water is determined not only by safety but by taste preference. In developing countries however, the lack of potable water has influenced the demand for bottled water. The perception that bottled water is safer than tap water has also led to an increase in the demand for bottled water. The water from the borehole goes through a series of filtration processes, and ultraviolet irradiation before bottling and can only be subjected to certain treatment processes that will ensure that the natural mineral content of the water is not altered in any way (South African Standards, 2003). Spring water cannot undergo any chemical disinfection treatments. The spring water bottling system in the Western Cape, South Africa investigated in this study was experiencing problems associated with bacterial contamination.

Sampling of the sites in the spring water bottling plant started in March 2004 (week one & week four) and continued in April (week eight) until November 2004 (week 46) to investigate the bacterial contamination in the spring water bottling system. Figures in this section refer to article 1 (chapter 2). Sampling sites at the spring water distribution system in the Western Cape, South Africa indicated in **(Figure 1)** included; Site A (borehole 1); Site B (borehole 2); Site C (Dositron – Flushing point); Site D (between 0.3 µm and UV steriliser –outside factory), Site E (After UV); the bottling line then splits into two lines and either one of the lines can be used for bottling. Site F (Line one after 0.35µm filter); Site G (Line one after 0.2 µm filter); Site H (Line two after 0.35 µm filter); Site I (Line two after 0.2 µm filter) and Site J (at filler- final bottling point).

The number of culturable cells were determined using the heterotrophic plate count (HPC) technique **(Figure 2)**. R2A and nutrient agar were used as the nutrient sources for the enumeration of aerobic bacteria. The heterotrophic plate count measurement in bottled water is used not only to indicate the level of disinfection of the distribution and bottling system but also assists in indicating if any changes in the water occurred from the borehole to the final bottled product (Leclerc &

Moreau, 2002). The heterotrophic plate counts recorded for weeks one, four, eight & 46 in the final bottled water (Site J) were 3.66 x 10^7 cfu/ml, 9.0 x 10^6cfu/ml, 2.35 x 10^7 cfu/ml and 5.00 x 10^4 cfu/ml, respectively. The current water legislation states that the heterotrophic plate count of the final bottled water must be < 100 organisms/ml within 24 hrs of bottling (South African National Standards, 2003), which implies that the counts recorded in the final bottled water obtained for all the sampling periods were significantly higher than the accepted limit. High HPC counts were recorded at the filters, the UV light and the borehole and although the sanitisation process was adjusted and the final count in week 46 was much lower than all the other sampling weeks, it was still not within the acceptable legal limit. One of the disadvantages of the HPC technique however, is that it only detects the microorganism selected for growth under specific conditions (Hurst *et al.,* 2002). Certain microorganisms also enter a viable but non-culturable (VNBC) state when they are exposed to a stressful environment (Roszak & Coldwell, 1987).

The total cell counts were evaluated by flow cytometric analysis (FCM) in conjunction with the Live/Dead BacLight™ viability probe and liquid counting beads (BD™) **(Figures 3 & 4)**. Flow cytometry can also be referred to as fluorescent activated cell sorting (FACS) and is used to sort and measure different types of cells by labelling the surface of the cell with fluorescent markers. Fluorescent beads can also be added to aid in the calculation of the total or absolute cell counts. Flow cytometry is a rapid method that allows for a large amount of data to be available in a very short time period (Javois, 1999). The total cell counts recorded for week one, four, eight & 46 in the final bottled water (Site J) were 5.44 x 10^7 microorganisms/ml, 8.36 x 10^7 microorganisms/ml, 9.09 x 10^7 microorganisms/ml and 5.70 x 10^7 microorganisms/ml, respectively. According to the South African National Standards, (2003) the HPC specification for untreated spring water is < 100 organisms/ml. The recorded counts significantly ($p < 0.05$) exceeded the standard specification.

Significant differences between the total cell counts obtained by HPC and the viable cell counts by the FCM technique were also noted in week 46 **(Figure 5).** The highest HPC count of 7.50 x 10^5 microorganisms/ml was recorded at Site E in comparison to the viable FCM count of 2.17 x 10^6 microorganisms/ml. The differences in the heterotrophic plate counts and the viable cell counts observed in the final bottled water in comparison to the viable FCM count indicated that the flow cytometry technique was able to detect viable but not culturable cells, whereas the

heterotrophic plate count technique only allowed for growth of the viable and culturable cells present in the water samples. Heterotrophic plate count results recorded were thus not a clear indication of the actual microbial numbers in the water samples. Monis *et al.* (2003) investigated the enumeration of waterborne bacteria using viability assays and flow cytometry and compared it to culture-based techniques. Untreated water as well as potable water samples were collected at various locations around South Australia. Due to the short testing time flow cytometry was used along with the LIVE/DEAD BacLight™ bacterial viability kit and carboxyfluorescein diacetate (CFDA) to detect physiologically active bacteria from the water samples. The FCM technique yielded 5.56 x 10^2 and 3.94 x 10^4 active bacteria per ml in comparison to the culture based techniques which were two to four log cycles less. It can thus be concluded that the FCM technique is a more reliable technique for the enumeration of microbial populations in water samples.

DNA was extracted from pure culture isolates from individual water samples and was amplified using two primer sets. The PCR product was sent for sequencing and subsequently blasted and aligned with ClustalX (1.81) using default parameters and the Blosum matrix. An unrooted tree was constructed using the neighbour-joining (Saitou & Nei, 1987) program of *MEGA* version 4.1 (Molecular Evolutionary Genetics Analysis 4.1) (Tamura *et al*., 2007). Branching patterns were evaluated by pairing 1000 replicates. Phylogenetic trees were constructed for week one, four, eight and 46 **(Figure 7-10)**. The phylogeny of the 180 isolates were analysed using the neighbour-joining algorithm of CLUSTAL X. There were many species that were repeatedly isolated from the various sampling points over the sampling periods and therefore duplicates were excluded. The number of diverse isolates for the sampling weeks one, four, eight & 46 were 17, 17, 41 and 13 respectively. The most dominant species present throughout the four week sampling period were *Pseudomonas sp. and Bacillus sp.* which indicated that these species were not eliminated during the sanitisation process. In a study conducted by Percival *et al.* (1998) on the development of biofilms on stainless steel pipes in a mains water system indicated that the dominant species isolated were *Pseudomonas spp.* and *Alcaligenes sp*. A biofilm community can survive disinfection and can thus attach to the surface of the distribution system (Geldreich, 1990). Other pathogenic bacteria isolated from the water samples in this study included *Bacillus sp, Staphylococcus sp*. and *Shigella boydii*.

In week eight the highest number of species was isolated in comparison to all the other sampling periods. The highest HPC counts were also isolated at the borehole in comparison to all the other sampling periods which clearly indicated that there was a new external source of contamination entering the distribution system via the borehole. The organisms isolated throughout the sampling period include amongst others *Bacillus pumilis, Shigella boydii, Serratia sp. SB, Enterobacter asburiae, Stenotrophomonas* sp., *Serratia sp., Stenotrophomonas maltophilia, Acinetobacter, Comamonas, Enterobacter sp., Staphylococcus sp., Aeromonas sp.* and *Escherichia sp*. The flora of natural mineral water has been studied in great detail and the major groups of bacteria isolated from mineral water include amongst others *Escherichia coli*, *Salmonella typhi*, *Pseudomonas fluorescent* and *non-flourescent* spp, *Alcaligenes*, Proteo bacteria, *Cytophaga* spp. (Leclerc & Moreau, 2002), and *Vibrio cholerae* (Kramer *et al.,* 1996).

In week 46 *Escherichia sp.* were isolated, which is indicative of faecal contamination. It is important to understand quantitatively the viable bacterial load and the microbial diversity in the bottling water distribution system in order to apply and optimise the correct sanitisation procedure. As bottled water cannot be subjected to any chemical treatments during and after bottling it is important to understand what the survival capacity of the pathogenic and indicator organisms are within the distribution system in order to apply an effective sanitisation procedure throughout the distribution system, especially the filter systems.

4.2 APPLICATION OF BIOREMEDIATION AS TREATMENT TECHNOLOGY IN A SPRING WATER DISTRIBUTION SYSTEM.

Bioremediation is the process by which living organisms utilise their metabolic potential to degrade or transform hazardous organic compounds to clean up or remediate contaminated environments under controlled conditions (Watanabe & Baker, 2000; Spain *et al*., 2000; Samantha, 2002 & Parales & Haddock, 2004). The biofilm allows microorganisms to attach to surfaces and not only provides access to nutrients but also protection against disinfection agents.

Bioremediation was assessed as a primary treatment technique in the spring water bottling system in the Western Cape, South Africa to reduce the number of microorganisms in the spring water. The figures in this section refer to article 2

(chapter 3). A laboratory scale bioreactor **(Figure 1)** was constructed and water samples were analysed over a two week period.

Flow cytometry (FCM) and Direct Acridine Orange Count (DAOC) along with epiflourescent microscopy (EM) are both rapid techniques for the enumeration of microorganisms from samples. Flow cytometry can also be referred to as fluorescent activated cell sorting (FACS) and is used to sort and measure different types of cells by fluorescent labelling of markers on the surface of the cell. Fluorescent beads can also be added to aid in the calculation of the total or absolute cell count. The total cell counts analysed by flow cytometry ranged from 1.53×10^7 microorganisms/ml in the initial sample to 1.16×10^7 microorganisms/ml on day 13 **(Figures 2 & 3).** The results indicated a 24% decrease in the microbial numbers. The FCM counts fluctuated throughout the sampling period. The average live/dead total cell counts were also analysed by FCM **(Figure 4)**. The live cell count of 9.77×10^6 microorganisms/ml in the initial sample was significantly ($p < 0.05$) higher than the corresponding dead cell count of 5.58×10^6 microorganisms/ml. The final live cell count on day 13 was 4.58×10^6 microorganisms/ml in comparison to the dead cell count of 6.98×10^6 microorganisms/ml.

The total number of microorganisms in the water samples was determined by means of epifluorescence microscopy, with acridine orange (Sigma) as the fluorochrome. The total cell counts obtained by the DAOC method ranged from 1.43×10^6 microorganisms/ml in the initial sample to 9.54×10^5 microorganisms/ml on day 13 (final). The results indicate a 33% decrease in microbial numbers. The total cell counts obtained from the DAOC method was lower in all the water samples in comparison to the FCM counts **(Figure 2)**. The lowest DAOC count of 1.76×10^5 microorganisms/ml was recorded on day two with a corresponding FCM count of 1.97×10^7 microorganisms/ml. Even though the FCM counts fluctuated throughout the sampling period, results clearly showed that the FCM method more accurately reflected the number of total cell counts in the collected samples. Paulse *et al.* (2007) assessed various enumeration techniques during an investigation into the planktonic bacterial population in the Berg River, Western Cape, South Africa. The FCM counts detected were higher than HPC counts indicating that this technique was able to detect cells in the water sample that enter a viable but non culturable state.

The DAOC method involves the physical counting of the microorganisms in conjunction with epifluorescence microscopy, whereas the FCM technique analysis total cell counts by means of computer software. Although a 24% reduction in the

microbial count was noted it was still above the limit of < 100 organisms/ml as set out by the South African Standards of Bottled Water, (2003). Due to external environmental conditions such as changes in the weather conditions the results fluctuated and the final results clearly indicated that further studies are required to optimise the bioreactor system for its application in the spring water industry.

4.3 Major Findings of the Study.

The significant results from this study were as follows:

1. Significantly high heterotrophic plate counts were recorded for weeks one, four, eight & 46 in the final bottled water (Site J) of 3.66 x 10^7 cfu/ml, 9.0 x 10^6cfu/ml, 2.35 x 10^7 cfu/ml and 5.00 x 10^4 cfu/ml, respectively. These counts were not within the specified limit of < 100 organisms/ml (South African National Standards, 2003) which clearly indicated bacterial contamination in the distribution system.

2. The total cell counts analysed by FCM recorded for week one, four, eight & 46 in the final bottled water (Site J) were 5.44 x 10^7 microorganisms/ml, 8.36 x 10^7 microorganisms/ml, 9.09 x 10^7 microorganisms/ml and 5.70 x 10^7 microorganisms/ml, respectively. New updated methods for the analysis of drinking water, which could include the FCM technique, should be included in the proposed techniques for determining the maximum and minimum levels as set out by the South African National Standards for Bottled Water. The heterotrophic plate count technique only allowed for growth of the viable and culturable cells present in the water samples, but as observed the FCM technique was able to detect those cells in the water that enter a viable-but-non-culturable state yielding higher FCM counts which was more accurate than the HPC counts.

3. The species that were dominant throughout the sampling period from week 1 to week 46 belonged to the *Pseudomonas sp*. and *Bacillus sp*. groups. The pathogens isolated throughout the sampling period included *Escherichia sp*., *Pseudomonas sp., Shigella boydii, Bacillus sp.* and *Staphylococcus sp.*

4. During the bioremediation study the (FCM & DAOC) results fluctuated, but an overall decrease in the total cell counts was observed. The total cell counts analysed by flow cytometry ranged from 1.53 x 10^7 microorganisms/ml in the initial sample to 1.16 x 10^7 microorganisms/ml on day 13 (final) indicating a 24% decrease in the microbial numbers. The total cell counts obtained by the DAOC method ranged from 1.43 x 10^6 microorganisms/ml in the initial sample to 9.54 x 10^5 microorganisms/ml on day 13 (final) indicating a 33% decrease in the microbial numbers. Further studies are required for the possible implementation of a bioreactor system as primary treatment directly after the borehole to try and reduce clogging of the filter system and as to possibly minimise the microbial contamination.

REFERENCES

Allard, A.S. & Neilson, A.H. 1997. Bioremediation of organic waste sites: A critical review of microbiological aspects. *International Journal of Biodeterioration and Biodegredation,* 39: 253-285.

Altschul, Stephen F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D.J. 1997. "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs". Nucleic Acids Research, 25: 3389-3402.

Amman, R.L., Ludwig, W., Schleifer, K.H. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiology Review,* 59: 143-169.

American Water Works Association, 1999. *Water quality and treatment*. 5th ed. United States of America: McGraw-Hill.

Atmar, R.L., Neill, F.H., Romalde, J.L., Guyader, F., Woodley, C.M., Metcalf, T.G. Estes, M.K. 1995. Detection of Norwalk virus and Hepatitis A virus in shellfish tissues with the PCR. *Applied Environmental Microbiology,* 61: 3014-3018.

Bennet, J.E. 1994. Fungal Infections. In Isselbacher, K. J., Braunwald, E., Wilson, J. D., Martin, J. B., Fauci, A.S & Kasper, D.L. (eds). *Harrison's Principles of International Medicine.* New York: McGraw-Hill: 854-865.

Behardien, L., Paulse, A.N., Jackson, V.A., Khan, S. & Khan, W. 2008. Investigation into the microbial contamination in a spring water distribution system, Western Cape, South Africa. *Water SA.* (Submitted for publication).

Benito, A. & Sutherland, J.P. 1999. A survey of the microbiological quality of bottled water sold in the UK and changes occurring during storage. *International Journal of Food Microbiology,* 48: 59-65.

Beuret, C., Kohler, D., Baumgartner,A., Luthi, T. 2002. Norwalk-like virus sequences in mineral waters: One year monitoring of three brands. *Applied and Environmental Microbiology*, 68(4):1925-1931.

Biological viruses <E:\viruses.htm [11 Feb 2008].

Bishop, R.F. 1994. Natural history of human rotavirus infections. In: Kapikian, A.Z (ed). *Viral infections of the gastrointestinal tract.* 2nd ed. New York: Marcell Dekker.

Blake, P.A., Rosenberg, M.L. Florencia, J. Costa, J.B. Quintino, L.D.P., Gangarosa, E.J. 1977. Cholera in Portugal, 1974. II Transmission by bottled water mineral water. *American Journal of Epidemiology,* 105: 344 – 348.

Blenkinsopp, S.A. & Costerton, J.W. 1991. Understanding Bacterial Biofilms. London, England: Elserivier Science.

Boaventura, R.A.R. 2001. Biodegradation of phenol by *Pseudomonas putida* DSM 548 in a trickling bed reactor. *Biochemical Engineering Journal,* 9: 211 -219.

Boopathy, R. 2000. Factors Limiting bioremediation technologies, *Bioresource Technology,* 74 (1): 63-67.

Brennan, J.G., Butters, J.R., Cowell, N.D. & Lilley, A.E.V. 1990. *Food Engineering Operations*. 3rd ed. London, England: Elserivier Applied Science.

Boulos, L. Presvost, M., Barbeau, B., Coallier, J., Desjardins, R. 1999. LIVE/DEAD Baclight™: application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water. *Journal of Microbiology Methods*, 37: 77-86.

British Soft Drinks Association, 1995. *The Guide to Good Bottled Water Standards. A useful Guide to technical aspects of designing and managing a bottled water source.* England: BSDA.

Camper, A.K., McFeters, G.A., Characklis, W.G. & Jones, W.L. 1991. Growth kinetics of coliform bacteria under conditions relevent to drinking water distribution systems. *Journal of Applied Environmental Microbiology,* 57: 2233-2239.

Cape Town Weather Bureau, 2008. Cape Town.

Characklis, W.G. 1981. Fouling biofilm development: a process analysis. *Journal of Biotechnology Bioengineering*, 23: 1923-1960.

Characklis, W.G. & Marshall, K.C. 1990. *Biofilms: A basis for an interdisciplinary approach*. New York: John Wiley & Son.

Codex Alimentarius Commission. 1985. Recommended International Code of Hygiene Practice for the Collecting, Processing and Marketing of Natural Mineral Water. Europe: CAC/RCP 33-1985.

Cohn, P.D., Cox, M. & Berger, P.S. 1999. Health and aesthetic aspects of water quality. In: *Water Quality and Treatment.* 5th ed. American Water Works Association (eds.). United States of America: McGraw-Hill.

Coldberg, P.J.S. & Young, L.Y. 1995. *Anaerobic degradation of nonhalogenated homocyclic aromatic compounds coupled with nitrate, iron, or sulfate reduction.* In *microbial transformation and degradation of toxic organic chemicals*. New York: Wisley-Liss: 307-330.

Cooke, W.B. 1986. *The fungi of our mouldy earth*. Berlin, West Germany: J.Cramer Publishers.

Costerton, J.W., Lewandowski, Z., De Beer, D., Caldwell, D., Korber, D & James, G. 1994. Biofilms, the customised microniche. *Journal of Bacteriology*, 176: 2137-2142.

Costerton, J.W., Stewart, P.S. & Greenberg, E.P. 1999. Bacterial biofilms: A common cause of persistent infections. *Science*, 284: 1318-1322.

Current Protocols in Flow Cytometry, 2005. *Introduction.* John Wiley & Sons (via Wiley Inter Science).
http://www3.interscience.wiley.com/cgi-bin/mrwhome/104554804/HOME [18 August 2005].

Dunn, O.J. & Clark, V.A. 1987. Applied Statistics: Analysis of variance and regression (2nd Ed). London, UK: John Wiley & Sons.

Ecolab™. 2004. Oxonia Active® (Pamphlet). Cape Town.

Ehlers, M.M., Van Zyl, W.B., Pavlov, D.N. & Muller, E.E. 2004. Random survey of the microbial quality of bottled water in South Africa. *Water SA,* 30(2): 203 – 210.

Ehlers, M.M., Grabow, W.O.K., Pavlov, D.N. 2005. Detection of enteroviruses in untreated and treated drinking water supplies in South Africa. *Water Research,* (39): 2253 – 2258.

Forsythe, S.J. 2000. *The microbiology of safe food*. London: Blackwell Science.

Frank, J.F. & Kofti, R.A. 1990. Surface adherent growth of *Listeria monocytogenes* is associated with increased resistance to surfactant sanitizers and heat. *Journal of Food Prot.,* 53: 550-554.

Gajardo, R., Pinto, R.M., Bosch, A. 1995. Polymerase chain reaction amplification and typing of rotavirus in environmental samples. *Water Science Technology*, 31: 371-374.

Garabetian, F., Petit, M., Lavandier, P. 1999. Does storage affect epifluorescence microscopic counts of total bacteria in freshwater samples. *Academy Science,* 322: 779-784.

Geesey, G.G., Stupy, M.V.O. & Bremer, P.J. 1992. Dynamics of biofilms. *International Journal of Biodeterioration and Biodegradation*, 30: 135 – 154.

Geldreich, E.E. & Rice, E.W. 1987. Occurrence, significance and detection of *Klebsiella* in water systems. *Journal of the American Water Works Association,* 79(5).

Geldreich, E.E. 1988. Coliform non-compliance nightmares in water supply distribution systems. In: *Water Quality: A Realistic Perspective*. College of England. University of Michigan: Ann Arbor: 55-74.

Geldreich, E.E. 1990. Microbial quality control in distribution systems." *Water quality and treatment*. 4th ed. F.W. Pontius, (ed). American Water Works Association. New York: McGraw-Hill.

Geldreich, E. E. 1996 Microbial quality of water supply in distribution systems. CRC Press Inc.

Ghettoplanet, 2003. Spring Formation. www.ghettoplanet.com/index.php?itemid=6 [13 January 2008].

Girones, R., Puig, M., Allard, A., Lucena, F., Wadell, G. & Jofre, J. 1995. Detection of adenovirus and enterovirus by PCR amplification in polluted water. *Journal of Water Science and Techology,* 31*:* 351-357.

Glaze, W.H. 1987. Drinking water treatment with ozone. *Journal of Environmental Science and Technology*, 21: 224.

Gleeson, C. & Gray, N. 1997. *The coliform index and waterborne disease problems of microbial drinking water assessment*. London: E & FN Spon.

Glick, R.B. & Pasternak, J.J. 1994. *Molecular biotechnology. principles and applications of recombinant DNA*. Washington DC: ASM Press.

Goldstein, S.T., Dennis, A., Juranek, D.D., Ravenholt, O., Hightower, A.W., Martin, D.G., Mesnik, J.L., Griffiths, S.D., Bryant, A.J., Reich, R.R., Herwaldt, B.L. 1996. Crytosporidiosis: An Outbreak Associated with Drinking water Despite State-of-the-Art Water Treatment. http://www/annals.org/cgi/content/full/124/5/459 [11 June 2007].

Gottlich, E., van der Lubbe, W., Lange, B., Fielder, S., Melchert, I., Michael, R., Flemming, H.C., de Hoog, S. 2002. Fungal flora in groundwater derived drinking water. *International Journal of Hygiene and Environmental Health*, 205(4): 269-279.

Grabow, W.O.K. 2001. Bacteriophages: Update on application as models for viruses in water. *Water SA*, 27: 251-268.

Greisen, K., Loeffelholz, M., Purohit, A. and Leong, D. 1994. PCR primers and probes for the 16S rRNA gene of most species of pathogenic bacteria, including bacteria found in cerebrospinal fluid. Journal of Clinical Microbiology 32, 335-351.

Guidelines for Canadian Drinking Water Quality, 2008. Treatment Technology. mhtml:file:E:\articles\Page9 [30 April 2004].

Hiraoka, Y. & Kimbara, K. 2002. Rapid assessment of the physiological status of the polychlorinated biphenyl degrader *Comamonas testosteroni* TK102 by flow cytometry. *Journal of Applied Environmental Microbiology*. 68(4): 2031-2035.

Hoyle, B. 2005. Groundwater. In Lerner, K., Lerner, B. & Baker, L. (eds). *Encyclopaedia of Water Science*, 3: 411-414.

Huang, X. & Madan, A. (1999) CAP3: A DNA sequence assembly program. Genome Research 9, 868-877.

Hurst, J.C., Crawford, R.L., Knudsen, G.R., McInerney, M.J., Stetzenbach, L.D. 2002. *Manual of Environmental Microbiology*. 2nd ed. Washington DC: American Society for Microbiology.

Hydrothermal Vent Biology. 2006. http://www.csa.com/discoveryguides/vent/review.pdf [24 August 2007].

Javois, L.C. 1999. Immunocytochemical Methods and Protocols. Totawa: NJ:Human http://www.answers.com/topic/flow-cytometry [27 August 2007].

Jay, M.J. 1992. *Modern Food Microbiology*. 4th ed. New York: Chapman and Hall.

Klein, P.H. 2002. *MICROBIOLOGY*. 5th ed. New York: MC Graw-Hill.

Kong, R.Y.C., Dung, W.F., Vrijmoed, L.L.P., Wu, R.S.S. 1995. Co-detection of three species of water borne bacteria by multiplex PCR. *Marine Pollution Bulletin,* 31(4): 317 – 324.

Kramer, M.H., Herwaldt, B.L., Craun, C.F., Calderon, R.L., Juranek, D.D. 1996. *Surveillance for Waterborne Disease Outbreaks* - United States. http://www.ncbi.nlm.gov/sites/entrez [26 September 2007].

Krantz, D. & Kifferstein, B. 2003. *Water Pollution*. http://www.umich.edu/~gs265/society/waterpollution.htm [04 November 2003].

Kumar, C.G. & Anand, S.K. 1998. Significance of microbial biofilms in food industry. *International Journal of Food Industry*, 42: 9-27.

LaMoreaux, P.E. & Tanner, J.T. 2002. *Springs and bottled waters of the world: Ancient history, source, occurrences*. Netherlands: Springer Verlag.

Langwaldt, J.A. & Puhakka, J.A. 2000. On-site biological remediation of contaminated groundwater. *Environmental Pollution*, 107: 187 – 197.

Lappin–Scott, H.M., Costerton, J.W., Marie, T.J. 1992. Biofilms and biofouling. *International Journal Lederberg (*ed*.),* 277 – 284. Encyclopaedia of Microbiology. San Diego: Academic Press

Lawrence, J.R., Wolfaardt, G.M. & Korber, D.R. 1994. Determination of diffusion coefficients in biofilms by confocal laser microscopy. *Journal of Applied Environmental Microbiology*, 60: 1166-1173.

Lawrence, J.R., Korber, D.R., Wolfaardt, G.M. & Caldwell, D.E. 1997. Analytical imaging and microscopy techniques. In Hurst, C.J., Knudsen, G.R., McInerney, M.J., Stetzenbach, L.D. & Walter, M.V. (eds). *Manual of Environmental Microbiology.* Washington DC: American Society for Microbiology.

LeChevallier, M.W. & Seidler, R.J. (1980). *Staphylococcus aureus* in rural drinking water. *Applied and Environmental Microbiology*, 30(4): 739-742.

LeChevallier, M.W., Evans, T.M., Seidler, R.J., Daily, O.P., Merrel, B.R., Rollins, D.M. and Joseph, S.W. 1982. *Aeromonas sobria* in chlorinated drinking water supplies. Microbial Ecology 8, 325-333.

LeChevallier, M.W., Cawthorn, C.D. & Lee, R.G. 1988. Factors promoting survival of bacteria in chlorinated water supplies. *Journal of Applied Environmental Microbiology,* 54: 649 –654.

LeChevallier, M.W. 1990. Coliform regrowth in drinking water. *Journal of American Water Works Association,* 82: 74-86.

LeChevallier, M.W., Lowry, C.D. & LEE, R.G. 1991. Disinfecting biofilms in a model distribution system. *Journal of American Water Works Association,* 82: 87-99.

LeChevallier, M.W., Welch, N.J. & Smith, D.B. 1996. Full-scale studies of factors related to coliform regrowth in drinking water. *Journal of Applied Environmental Microbiology,* 57: 857-862.

Leclerc, H., Mossel, D.A.A, Edberg, S.C. & Struijk, C.B. 2001. Advances in the bacteriology of the coliform group: their suitability as markers of microbial water safety. *Annual Review of Microbiology*, 55:134-201.

Leclerc, H. 2002. *Microbiology of natural mineral waters*. http://www.centre-evian.com/fondDoc/dos-science/11858.html [18 September 2003].

Leclerc, H. & Moreau, A. 2002. Microbiological safety of natural mineral water. *FEMS Microbiology reviews*, 26(2): 207-222.

Lenntech Water Treatment & Air Purification Holding B.V. 2005. *Necessity of drinking water disinfection.* http://www.lenntech.com/water-disinfection.necessity-drinking-water-disinfection.htm. [14 August 2007].

Lindsay D. 2002. Biofilms a hidden challenge in food processing. *Food Review:* 25 – 27. June 2002.

Lloyd, B. 1983. *Salmonella*, enteric fevers and salmonelloses. In: Feachem, R.G. (ed). *Sanitation and disease. Health aspects of excreta and wastewater management*. England: John Wiley & Sons.

Manaia, C.M., Nunes, O.C., Morais, P.V., Da Costa, M.S. 1990. Heterotrophic plate counts and the isolation of bacteria from mineral waters on selective and enrichment media. *Journal of Applied Bacteriology*, 69: 871 – 876.

Margolin, A.B. 1997. Control of microorganisms in source water and drinking water. In Hurst, D.J. Knudsen, G.R., McInerney, M.J., Stetzenbach, L.D. & Walter, M.V. 1997. *Manual of Environmental Microbiology.* Washington DC: American Society for Microbiology.

Marshall, K.C. 1997. Colonisation, adhesion and biofilms. In Hurst, D.J. Knudsen, G.R., McInerney, M.J., Stetzenbach, L.D. & Walter, M.V. 1997. *Manual of Environmental Microbiology.* Washington DC: American Society for Microbiology.

Matilainen, A., Lindqvist, N., Korhonen, S. & Tuhkanen, T. 2002. Removal of NOM in the different stages of the water treatments process. *Environmental International,* 28: 457-465.

Mavridou, A. 1992. A study of the bacterial flora of non-carbonated natural mineral water. *Journal of Applied Bacteriology*, 73: 355 – 361.

Melin, E., Jarvinen, K. & Puhakka, J. 1998. Effects of temperature on chlorophenol biodegradation kinetics in fluidised-bed reactors with different biomass carriers. *Journal of Water Research*. 32(1):81-90.

Micromem Analytical. 2003. *Micromem Analytical: A division of Orange County Water District.* http://www.micromemanalytical.com/ (03 August 2003).

Mittelman, M.W. 1986. Biological fouling of purified water systems: Part 3, Treatment. *Microcontamination,* 4(1): 30-40&70.

Moreau, A. 2001. Surveillance microbiologique des eaux minerals naturalles. *Bulletin de la Soceite Francaise de Microbiologie*, 16: 13-19.

Molecular probes. 2002. *Fluorescence Microplate Assays*. 7th ed. Molecular Probes Inc. USA. http://www.probes.com/

Monis, P.T., Saint, P.C.P., Hoefel, D., Grooby, W.L., Andrews, S. 2003. Enumeration of water borne bacteria using viability assays and flow cytometry: A comparison to culture-based techniques. *Journal of Microbiology Methods*, 55(3): 585-597.

Montes, M. Jaensson, E.A., Orozco, A.F. Lewis, D.E. & Corry, D.B. 2006. A general method for bead-enhanced quantitation by flow cytometry. *Journal of Immunological Methods.* http://www.caspases.org/showabstract.php?pmid=17067632 [13 January 2007].

Morelli, C.D. 1994. *Water Manual*. 3rd ed. New York: Keller International.

Mueller, J.G., Cerniglia, C.E., Pritchard, P.H. 1996. *Bioremediation of environments contaminated by polycyclic aromatic hydrocarbons*. In *Bioremediation: Principles and Applications* :125-194. Cambridge: Cambridge University Press.

Mueller, E., Drewello, U., Drewello, R., Weissmann, R. & Wuertz, S. 2001. In situ analysis of biofilms on historic window glass using confocal laser scanning microscopy. *Journal of Cultural Heritage,* 2: 31-42.

Murphy, A.M., Grohmann, G.S. & Sexton, M.F.H. 1983. Infectious gastroenteritis in Norfolk Island and recovery of viruses from drinking water. *Journal of Hygiene,* 91:139-146.

National Drinking Water Clearinghouse. 1996. Tech Brief one: *Disinfection Fact Sheet.* USA: NDWC.

Niemi, R.M., Knuth, S., Lundstrom, K. 1982. *Actinomycetes* and fungi in surface waters and in potable water. *Journal of Applied Environmental Microbiology,* 43:378-388.

Oblinger, J.L. & Koburger, J.A. 1975. Understanding and teaching the most probable number technique. *Journal of Milk Food Technology,* 38(9):540 – 545.

Pall Filters™. 2004. Focus on filtration. (pamphlet).

Parales, R.E. & Haddcok, J.D. 2004. Biocatalytic degradation of pollutants. *Current. Opinions Biotechnology*, 13: 249-252.

Paulse, A.N., Jackson, VA., Khan, W. 2007. Comparison of enumeration techniques for the investigation of bacterial pollution in the Berg River, Western Cape, South Africa. *Water SA*, 33: 165-173.

Pianetti, A., Falcioni, T., Bruscolini, F., Sabatini, L., Sisti, E., Papa, S. 2005. Determination of the viability of *Aeromonas hydrophilia* in different types of water by flow cytometry, and comparison with classical methods. *Journal of Applied Environmental Microbiology*, (12):7948-7954.

Percival, S.L, Knapp, J.S, Edyvean, G.J. and Wales, D.S. 1998. Biofilms, mains water and stainless steel. *Water Research,* 32(7): 2187-2201.

Pohland, A.E. 1993. Mycotoxins in review. *Food Addit. Contamination*, 10:17-28.

Porter, J., Diaper, J., Edwards, C., Pickup, R. 1995. Direct measurement of natural planktonic bacterial community viability by flow cytometry. *Journal of Applied Environmental Microbiology*, 61(7): 2783-2786.

Porter, K.G. & Greig, Y.S. 1980. The use of DAPI for identification and counting of aquatic Microflora dimnil*. Oceanography,* 25: 943-948.

Ray, B. 2004. Fundamental Food Microbiology. 3rd ed. Florida: CRC Press. 374.

Remediation Technologies Screening Matrix. 2007. *Bioreactors*. http://www.frtr.gov/matrix2/section4/4-42.html [24 August 2007].

Resoner, D.J. 1990. Monitoring heterotrophic bacteria in potable water. In *Drinking Water Microbiology: Progress and Recent Developments.* New York: Springer: *452-477.*

Roszak, D.B. & Coldwell, R.R. 1987. Survival strategies of bacteria in the natural environment. *Microbiology. Review*, 51: 356-379.

Ryan, K.J. 2004. *Medical Microbiology*. 4th ed. New York: Mc Graw Hill.

Samanta, S.K. 2002. Polycyclic aromatic hydrocarbons: Environmental pollution and bioremediation. *Trends Biotechnology,* 20: 243-248.

Saitou, N. & Nei, M. 1987. The neighbour-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biol. Evol*., 4: 406-425.

Senior, D. & Dege, N. 2005. *Technology of bottled water.* Oxford, UK: Blackwell Publishing.

Sobsey, M.D., Battigelli, D.A., Handzel, T.R. & Schwab, K.J. 1995. Male specific coliphages as indicators of viral contamination of drinking water. *American Waterworks Association Research Foundation,* Denver:150

Sommer, R. and Cabaj, A. 1993. Evaluation of the efficiency of a UV plant for drinking water disinfection. Water Science and Technology 27, 357-362.

South African National Standards. 2003. Bottled Natural Water. SANS 1657:2003 Ed 1.3. Notice 1373 of 8 November 2002.

South Africa, Department of Health. 2004. Foodstuffs, Cosmetics and Disinfectants Act: *Act 54 of 1972.* Regulations governing bottled waters including natural mineral waters. Pretoria: Government Printer: No R 502.

South African National Bottled Water Association (SANBWA), 2006. Making it Crystal Clear. *Food Review,* September 2006: 24 – 25.

Spain, J.C. 2000. *Biodegradation of nitroaromatic compounds*.: Washington, DC: Lewis Publishers.

Swancara, J. 2007. Ozone as a disinfectant. *Water Quality Products*, September 2007, 12(9).

Symons, J.M. 1981. Treatment techniques for controlling trihalomethanes in drinking water. EPA-60012-81-16,USEPA, Cincinnati. OH.

Tamura K, Dudley J, Nei, M. and Kumar, S. (2007) *MEGA4*: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.1. Molecular Biology and Evolution 24, 1596-1599.

Tartakow, I.J. & Vorperian, J.H. 1980. *Foodborne and waterborne diseases. Their epidemiological characteristics.* Wesport, CT,USA: AVI Publishing.

Tchobanoglous, G. & Schroeder, E.D. 1985. *Water Quality: Characteristics; Modelling; Modification*. United States of America: Addison-Wesley.

Tebbut, T.H.Y. 1992. *Principles of water quality control.* Great Britain: Pergamon Press.

Tolker-Nielsen, T., Brinch, U.C., Ragas, P.C., Anderson, J.B., Jacobson, C.S., Molin, S. 2000. Development and dynamics of *Pseudomonas sp*. biofilms. *Journal of Bacteriology*, 182(22):6482-6489.

Toranzos, G.A. & Bej, A.K. 1997. Environmental applications of nucleic acid amplification techniques. In Toranzod, G.A. Ed, 1997. *Environmental applications of nucleic acid amplification techniques.* Lancaster, PA: Technomic Publishing:1-35.

Toze, S. 1999. PCR and the detection of microbial pathogens in water and wastewater. *Water Research,* 33(17):3545-3556.

Trachoo, N. 2003. Biofilms and the food industry. *Songklanarin Journal of Science and Technology,* 25(6):807 – 815.

Tsen, H.Y., Lin, C.K. & Chi, W.R. 1998. Development and use of 16S rRNA gene targeted PCR primers for the identification of *Escherichia coli* cells in water. *Journal of Applied Microbiology,* 85:554-560.

Tuovinen, O.H., Button, K.S., Vuorinen, A., Carison, L. 1980. Bacterial, chemical and mineralogical characteristics of tubercles in distribution pipelines. *Journal of American Water Works Association*, 72(11): 626-635.

Unites States Department of the Interior U.S *Geological Survey.* 2006. http://ga.water.usga.gov/edu/watercyclesprings.html (14 August 2006).

Vidali, M. 2001. Bioremediation. *Pure Application Chemistry*, 7:1163-1172.

Vinneras, B. & Johnson, H. 2002. Faecal separation for nutrient management – evaluation of different separation techniques corresponding. *Urban Water,* 4: 321-329.

Vivier, J.C., Ehlers, M.M., Grabow, W.O.K. 2004. Detection of enteroviruses in treated drinking water. *International Journal of Water Research,* 38:2699-2705.

Vogt, C., Alfreider, A., Lorbeer, H., Hoffmann, D., Wuensche, L., Babel. W. 2004. Bioremediation of chlorobenzene-contaminated groundwater in an in situ reactor mediated by hydrogen peroxide. *Journal of Contaminant Hydrology,* 68: 121-141.

Wakelin, D. 2007. *Helminths: Pathogenesis and Defenses.* http://www.gsbs.utmb.edu/microbook/ch087.htm [26 September 2007].

Warburton, D.W., Dodds, K.L., Burke, R., Jonston, M.A. & Laffey, P.J. 1992. A review of the microbiological quality of bottled water sold in Canada between 1981 and 1989. *Canadian Journal of Microbiology*, 38: 12-19.

Warburton, D.W. 2000. The microbiological safety of bottled waters. In: Farber, J.M. & Ewen, E.D. (eds.) *Safe Handling of Foods*. New York: Marcel Dekker.

Watanabe, K. & Baker, P.W. 2000. Environmentally relevant microorganisms. *Journal of Bioengineering*, 89: 1-11.

World Health Organisation. 1993. *The control of schistosomiasis:second report of the WHO Expert Committee*. No. 830. Geneva.

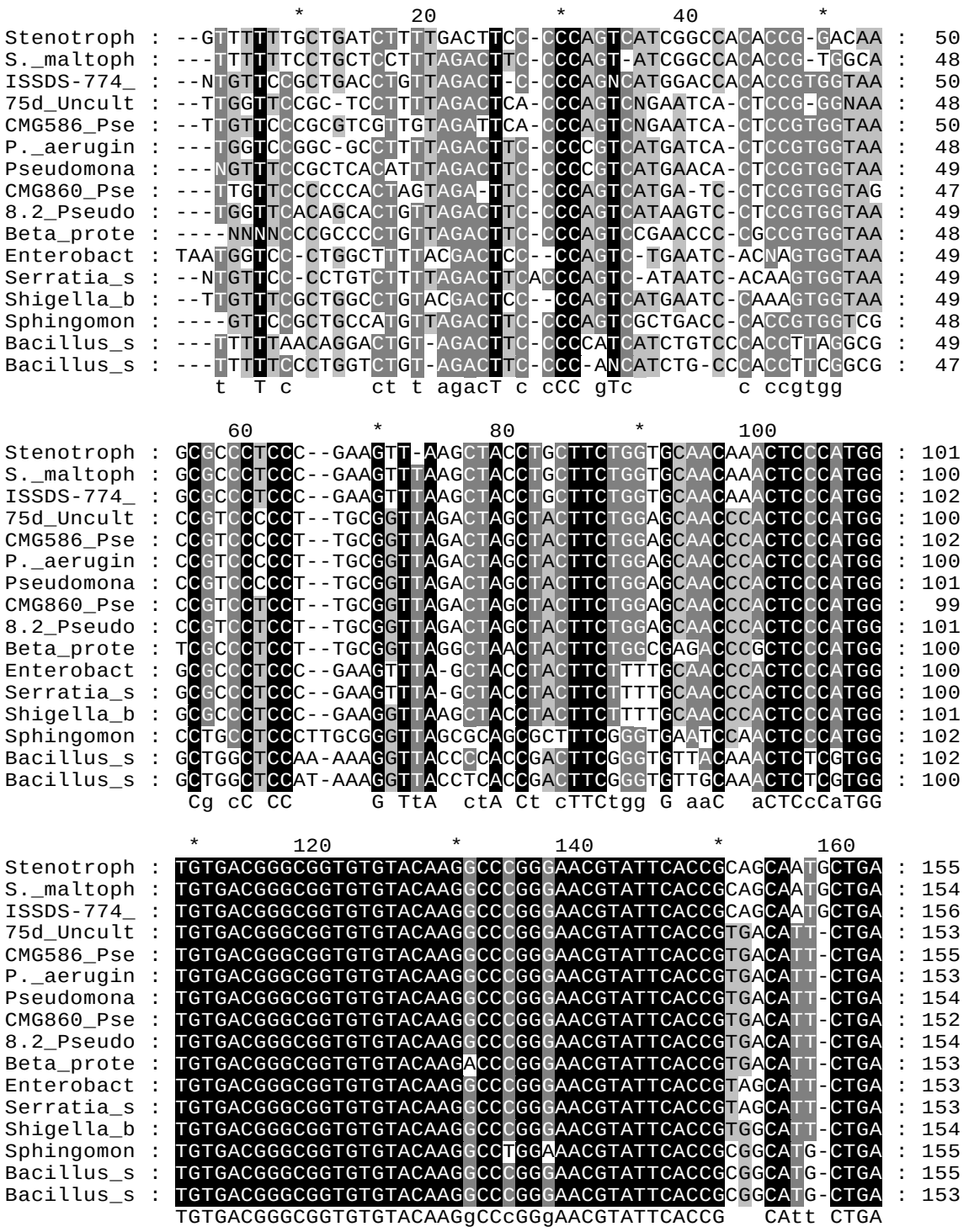
World Health Organisation. 1996. *Guidelines for drinking water quality* (2nd ed). Austria: Weiner Verlag.

World Health Organisation. 2000. Cholera. *Weekly Epidemiology Record* 2001; 76: 233-40.

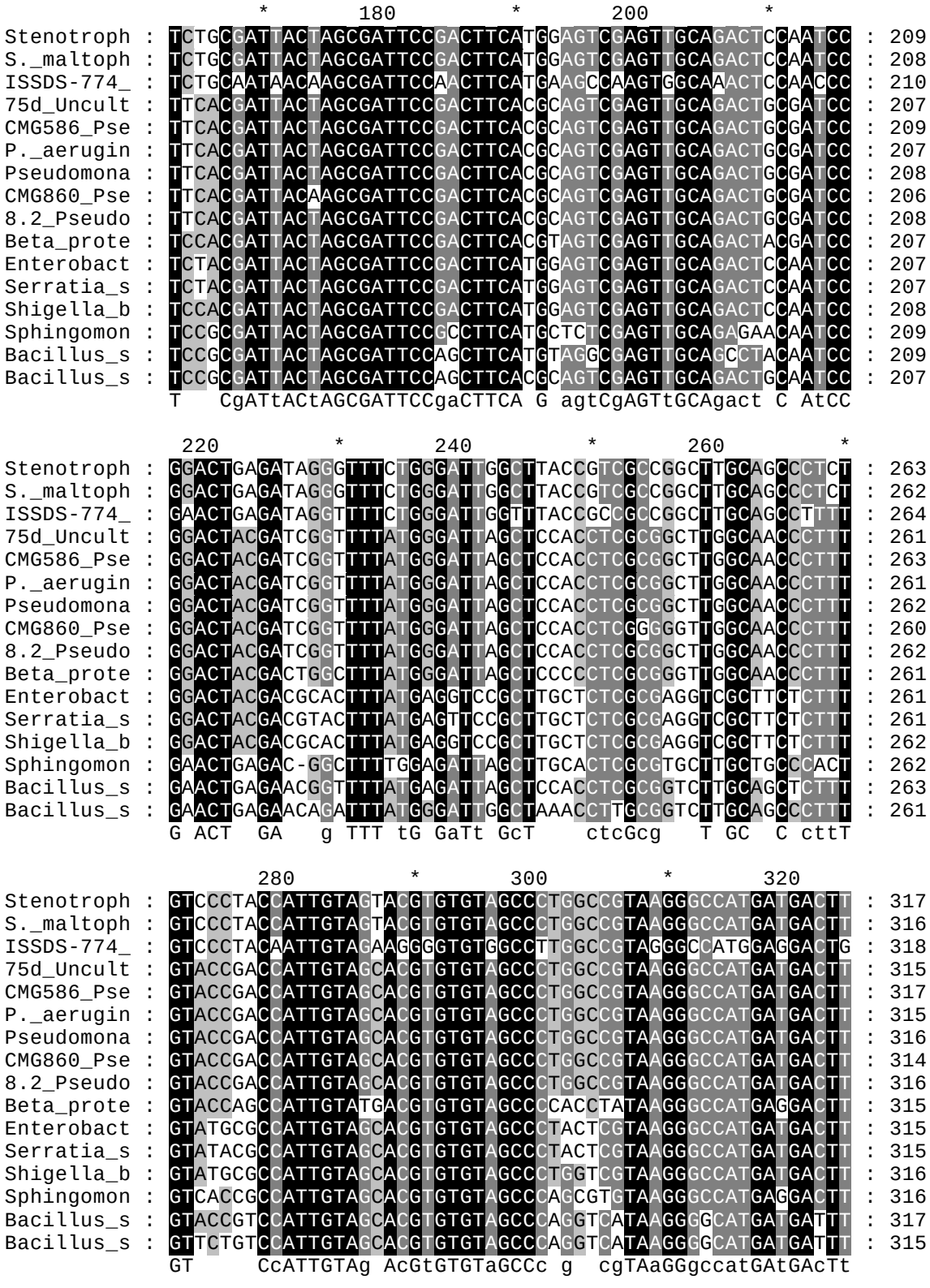
World Health Organisation. 2001. *Guidelines for Drinking Water Quality.* 2nd ed. Vol 1: Microbiological Methods. Geneva.

Zaoutis, T. 1998. Enterovirus infections. *Pediatr*. *Review*, 19: 183-191.

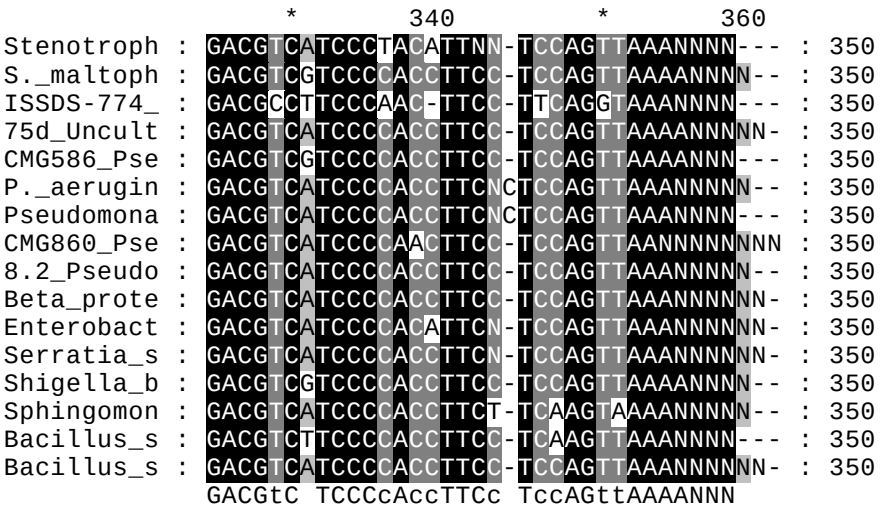
APPENDIX A



Addendum A: Alignment of the amino acid sequences of 16 isolates obtained from different sampling sites in the spring water distribution system in week 1. The alignment was carried out by the multiple alignment of Clustal X (1.81). Genedoc software was used for homology shading. Gaps introduced into the alignment are indicated with dashes. Four shading levels were set.

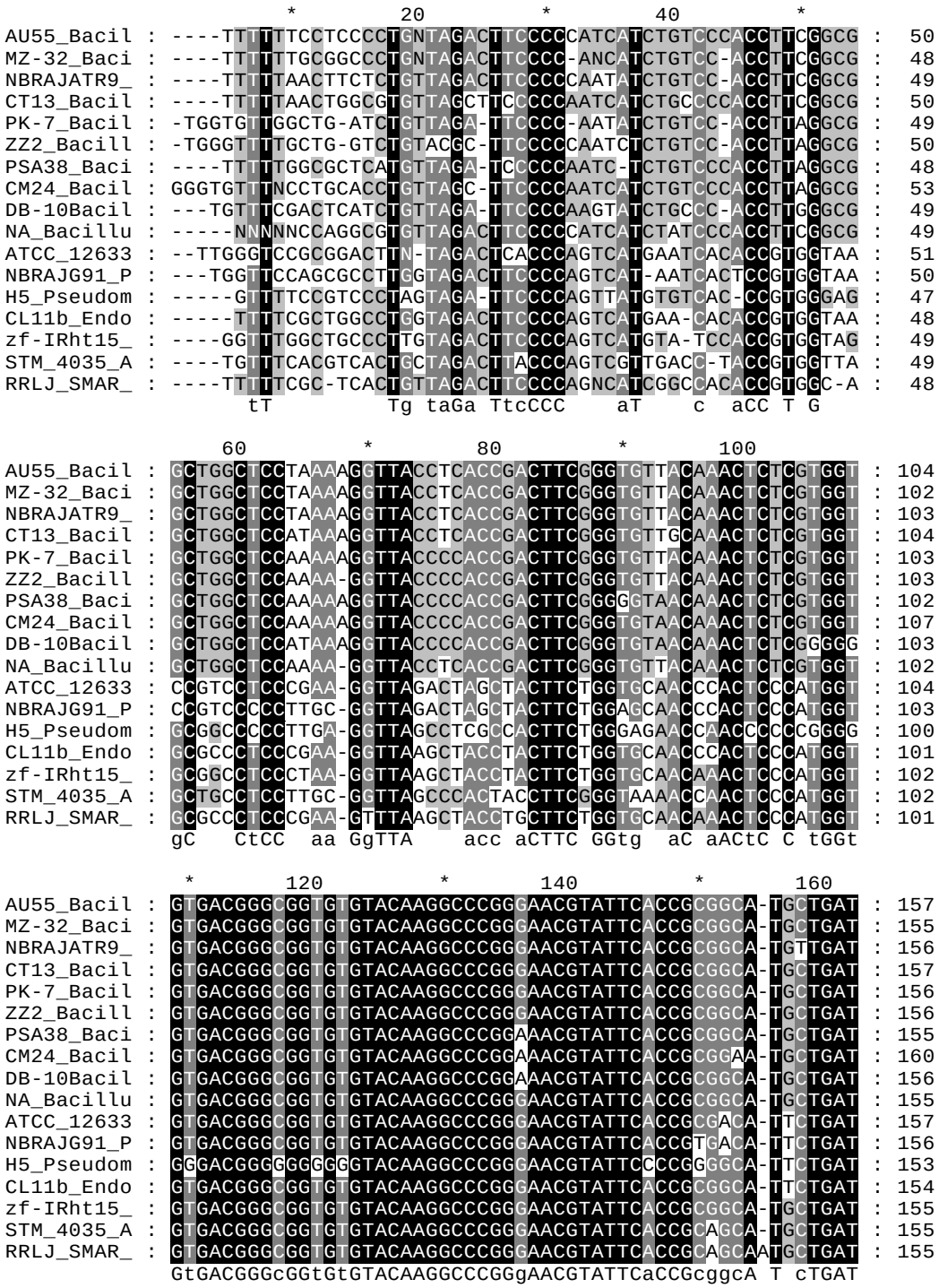


Alignment of the amino acid sequences of 16 isolates obtained from different sampling sites in the spring water distribution system in week 1 continue.

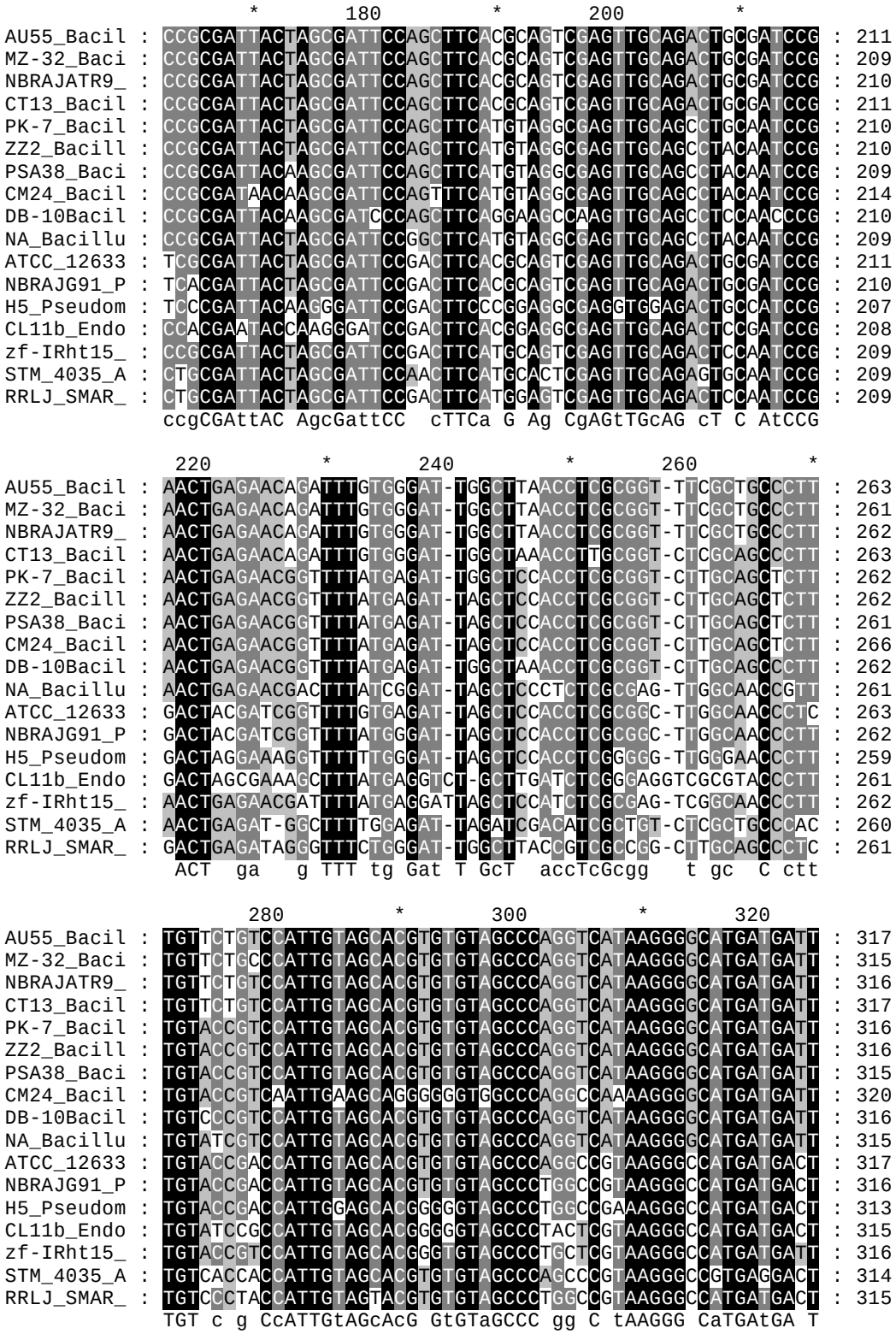


Alignment of the amino acid sequences of 16 isolates obtained from different sampling sites in the spring water distribution system in week 1 continue.

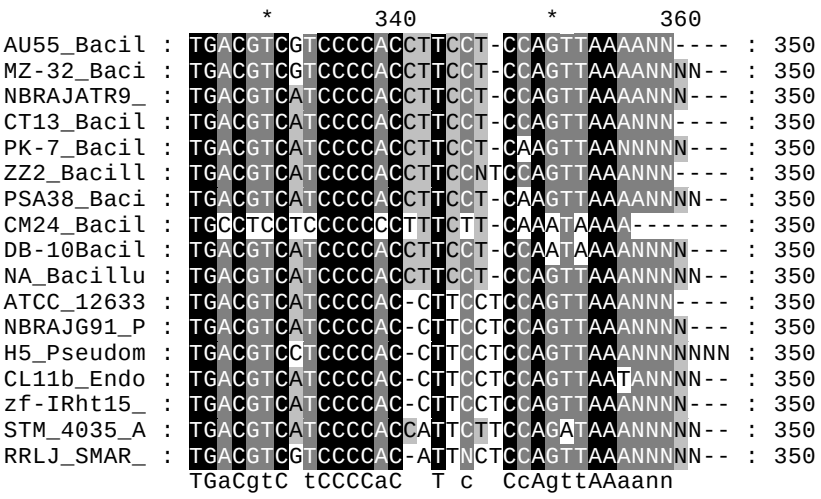
APPENDIX B



Addendum B: Alignment of the amino acid sequences of 17 isolates obtained from different sampling sites in the spring water distribution system in week 4. The alignment was carried out by the multiple alignment of Clustal X (1.81). Genedoc software was used for homology shading. Gaps introduced into the alignment are indicated with dashes. Four shading levels were set.

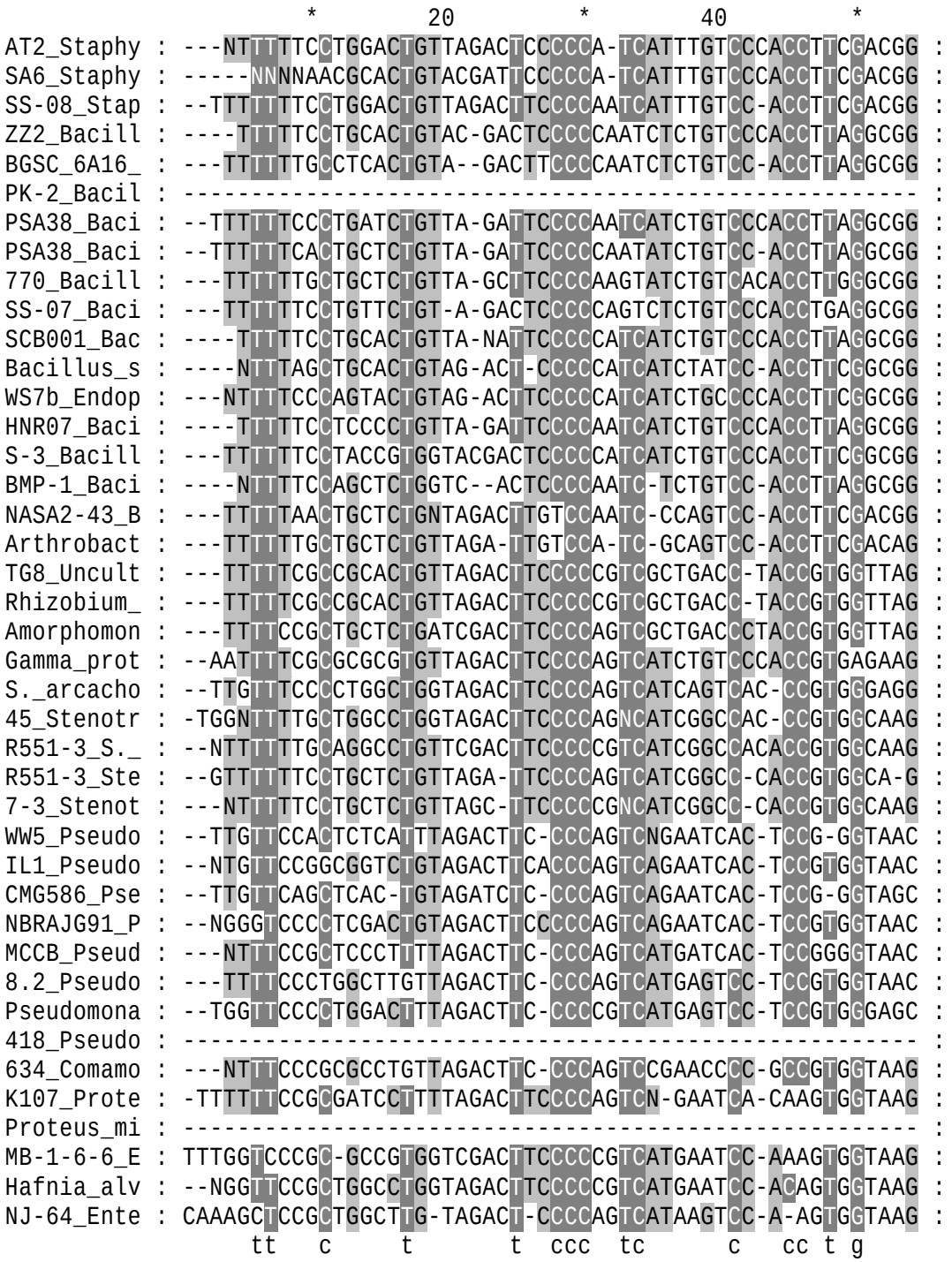


Alignment of the amino acid sequences of 17 isolates obtained from different sampling sites in the spring water distribution system in week 4 continue.

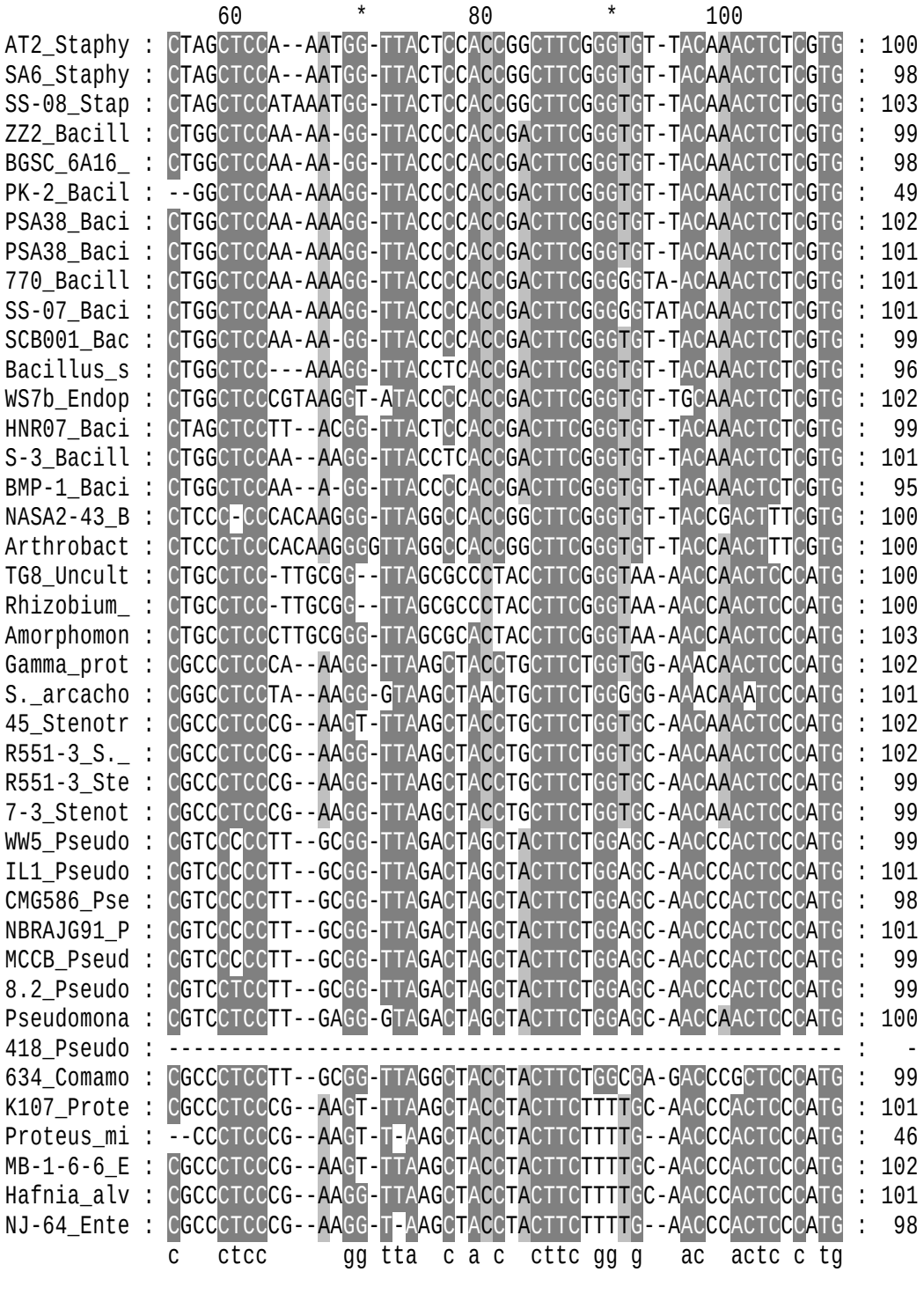


Alignment of the amino acid sequences of 17 isolates obtained from different sampling sites in the spring water distribution system in week 4 continue.

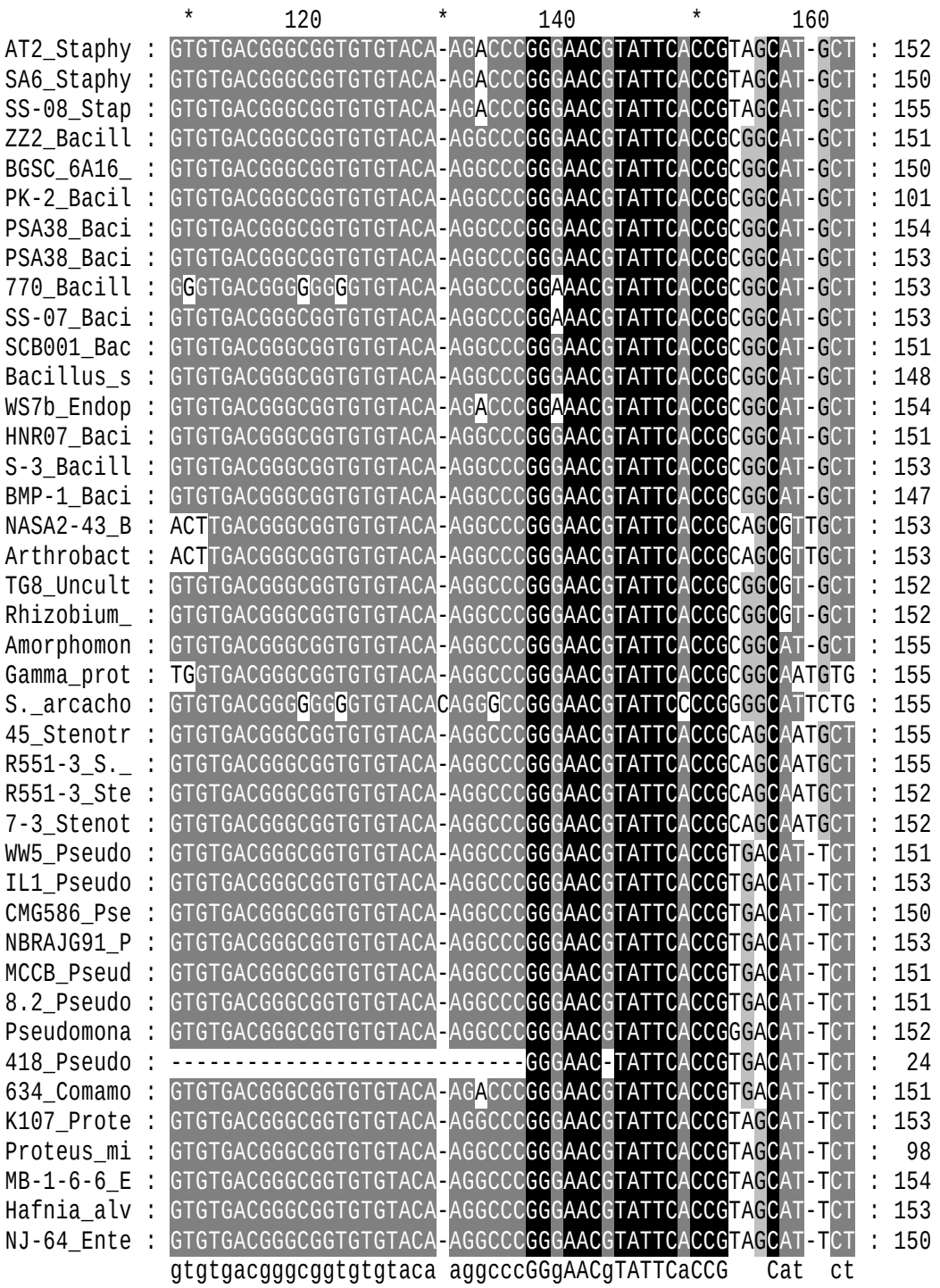
APPENDIX C



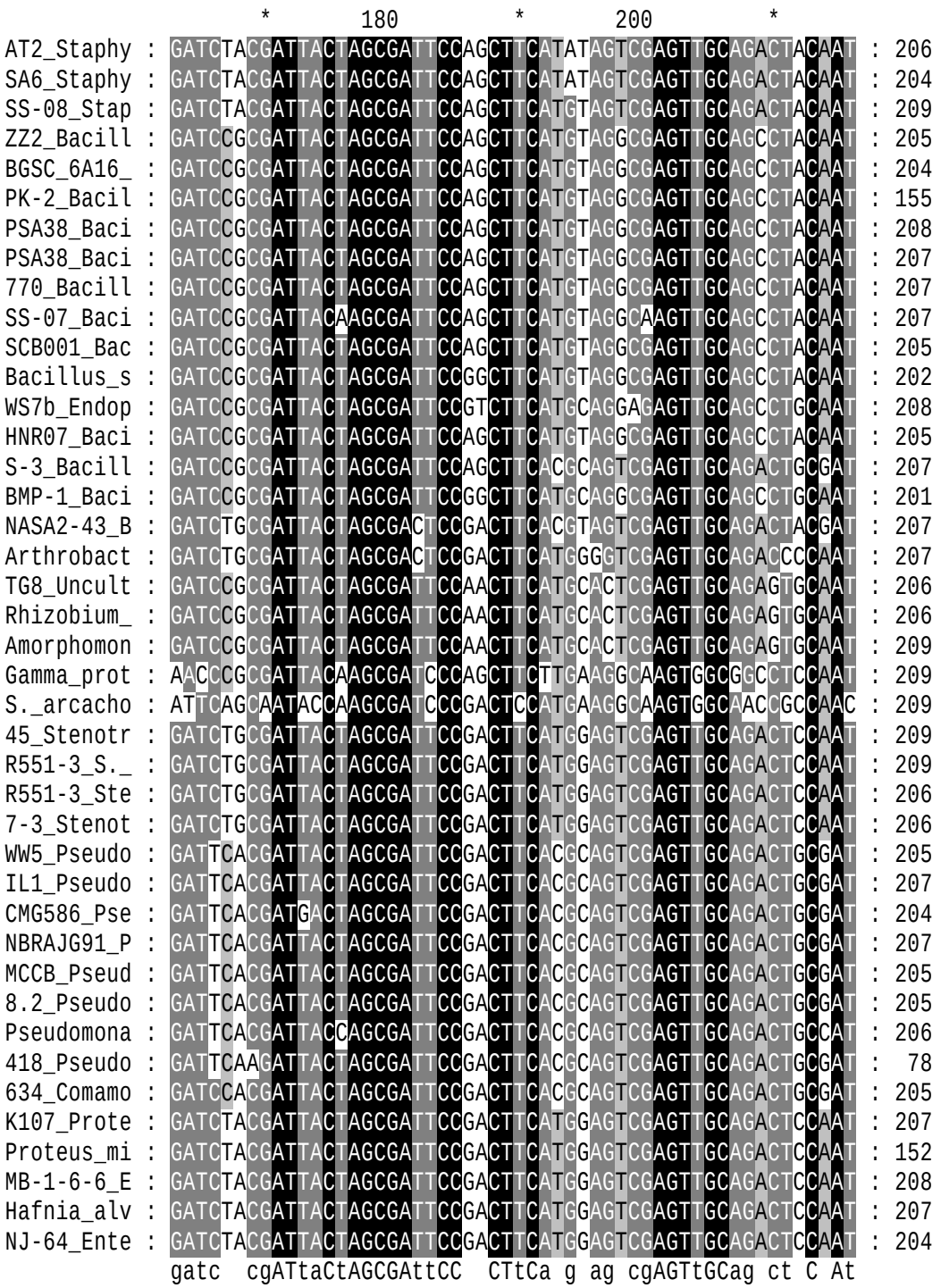
Addendum C: Alignment of the amino acid sequences of 41 isolates obtained from different sampling sites in the spring water distribution system in week 8. The alignment was carried out by the multiple alignment of Clustal X (1.81). Genedoc software was used for homology shading. Gaps introduced into the alignment are indicated with dashes. Four shading levels were set.



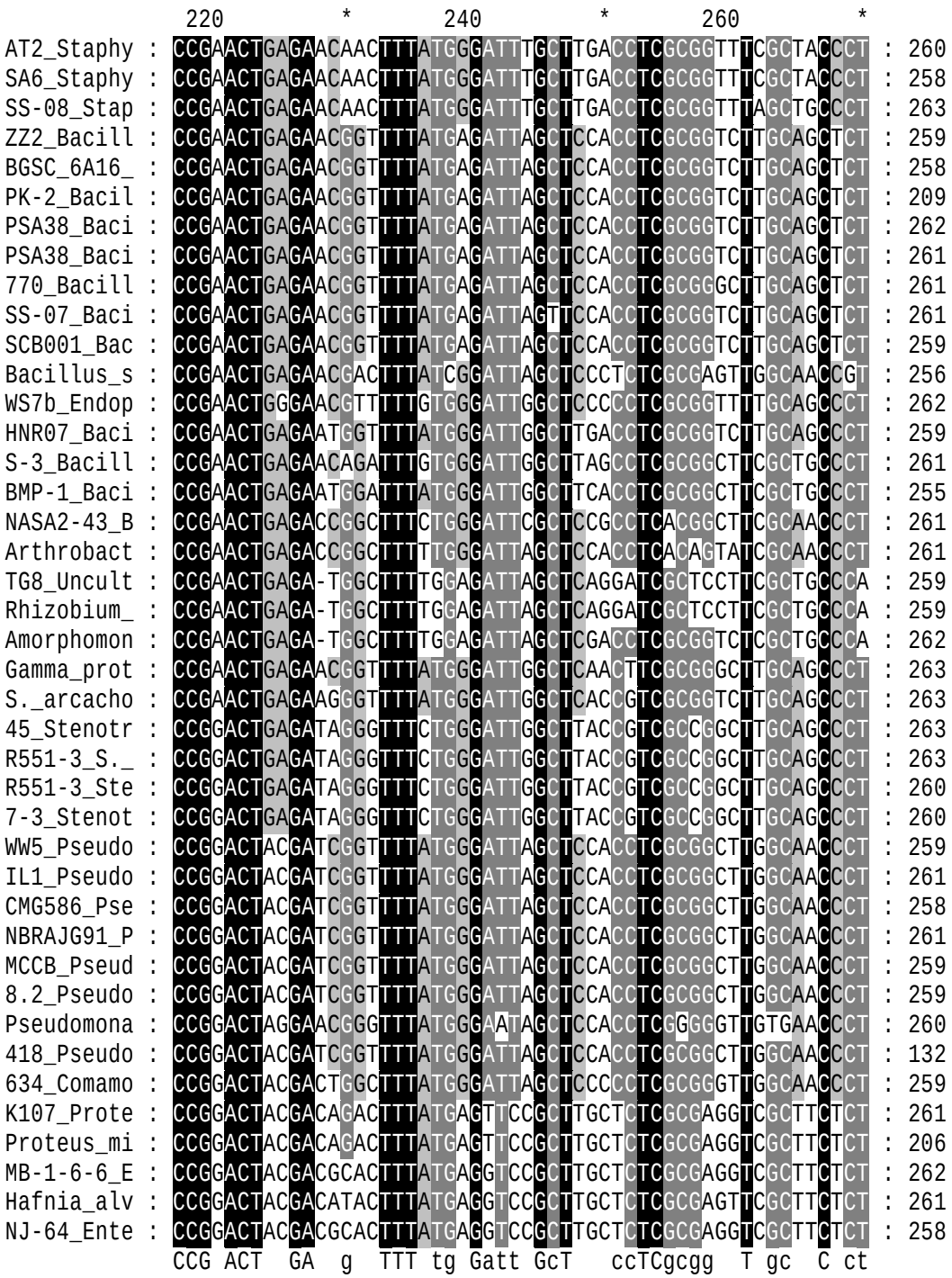
Alignment of the amino acid sequences of 41 isolates obtained from different sampling sites in the spring water distribution system in week 8 continue.



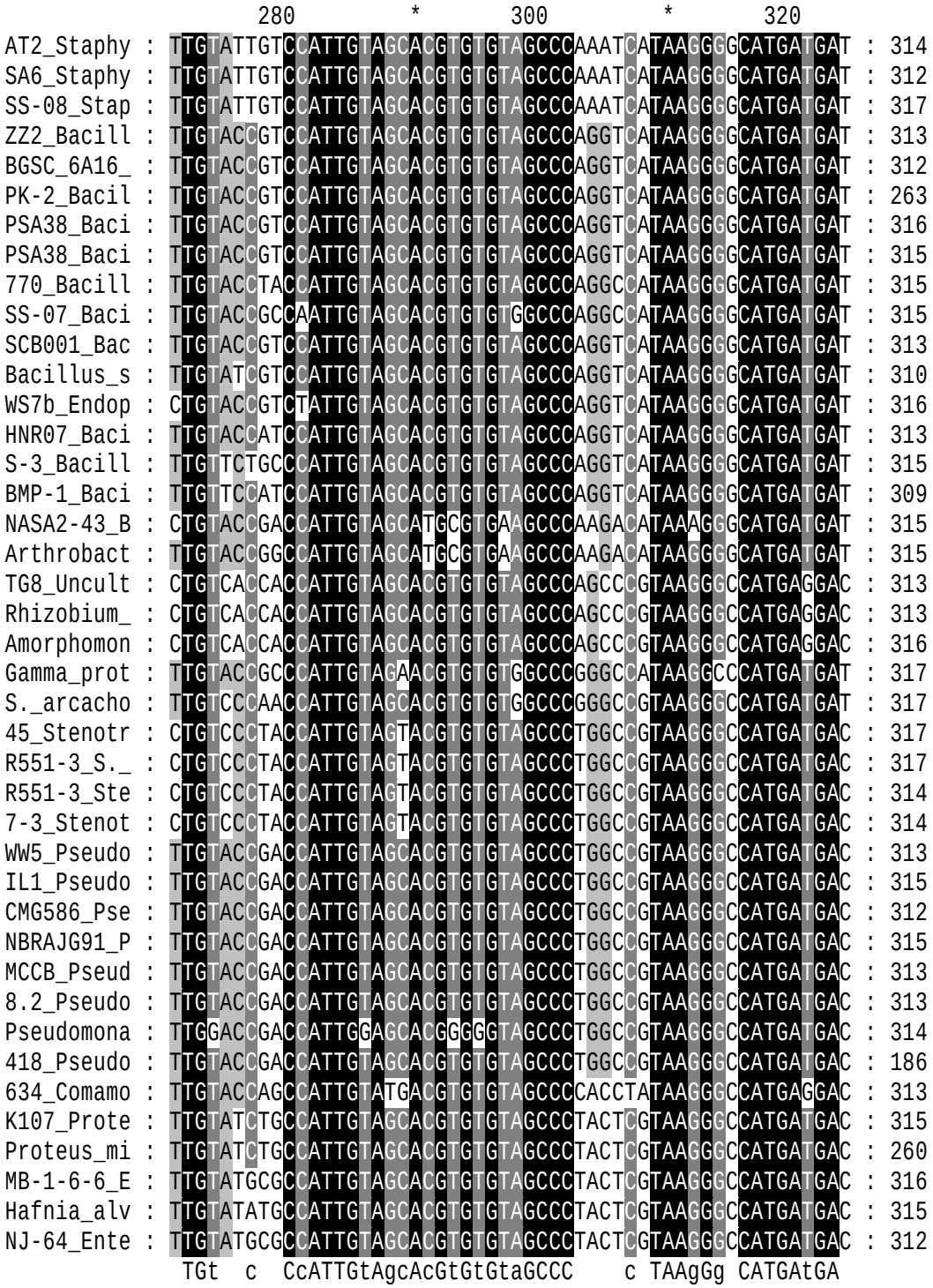
Alignment of the amino acid sequences of 41 isolates obtained from different sampling sites in the spring water distribution system in week 8 continue.



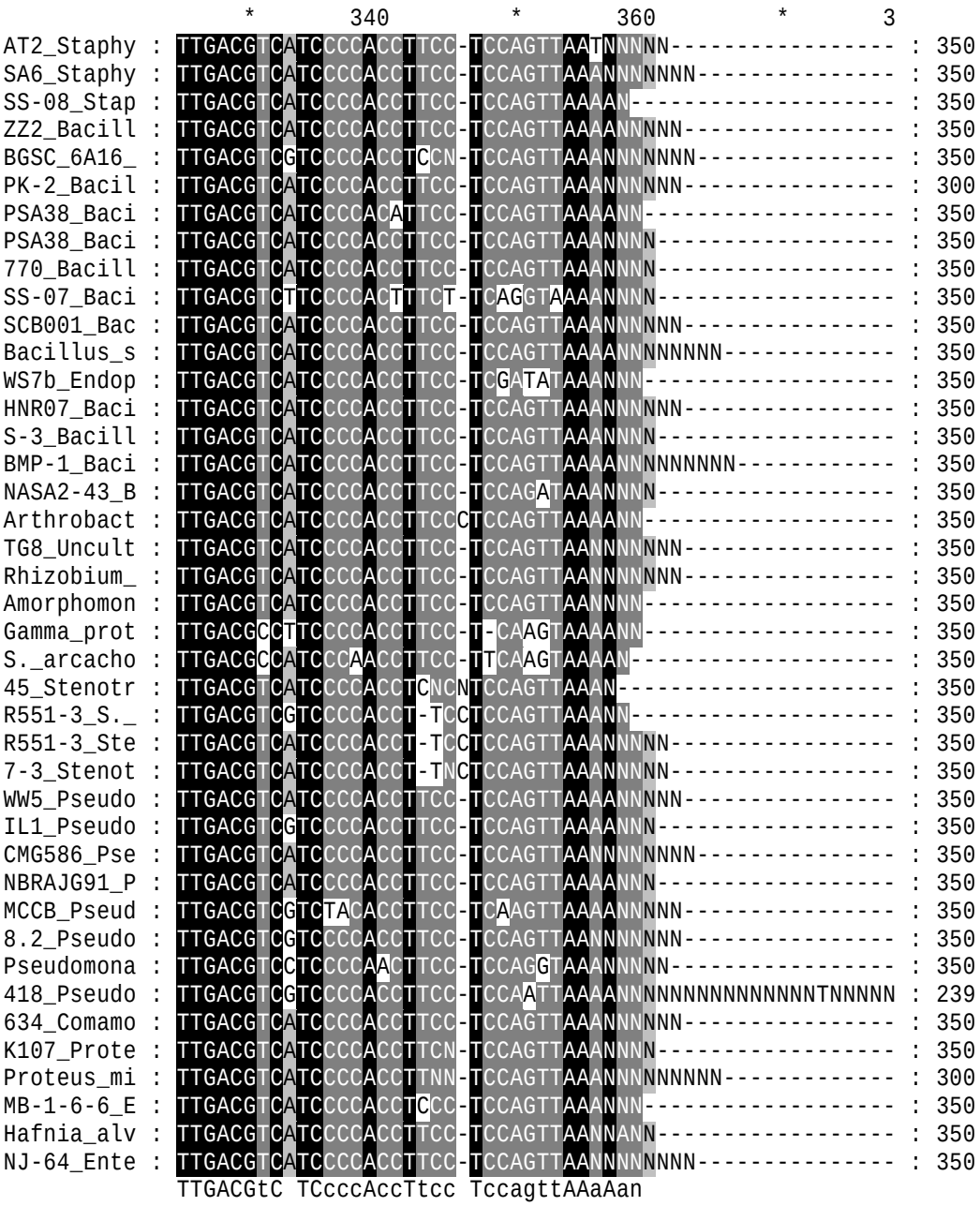
Alignment of the amino acid sequences of 41 isolates obtained from different sampling sites in the spring water distribution system in week 8 continue.



Alignment of the amino acid sequences of 41 isolates obtained from different sampling sites in the spring water distribution system in week 8 continue.

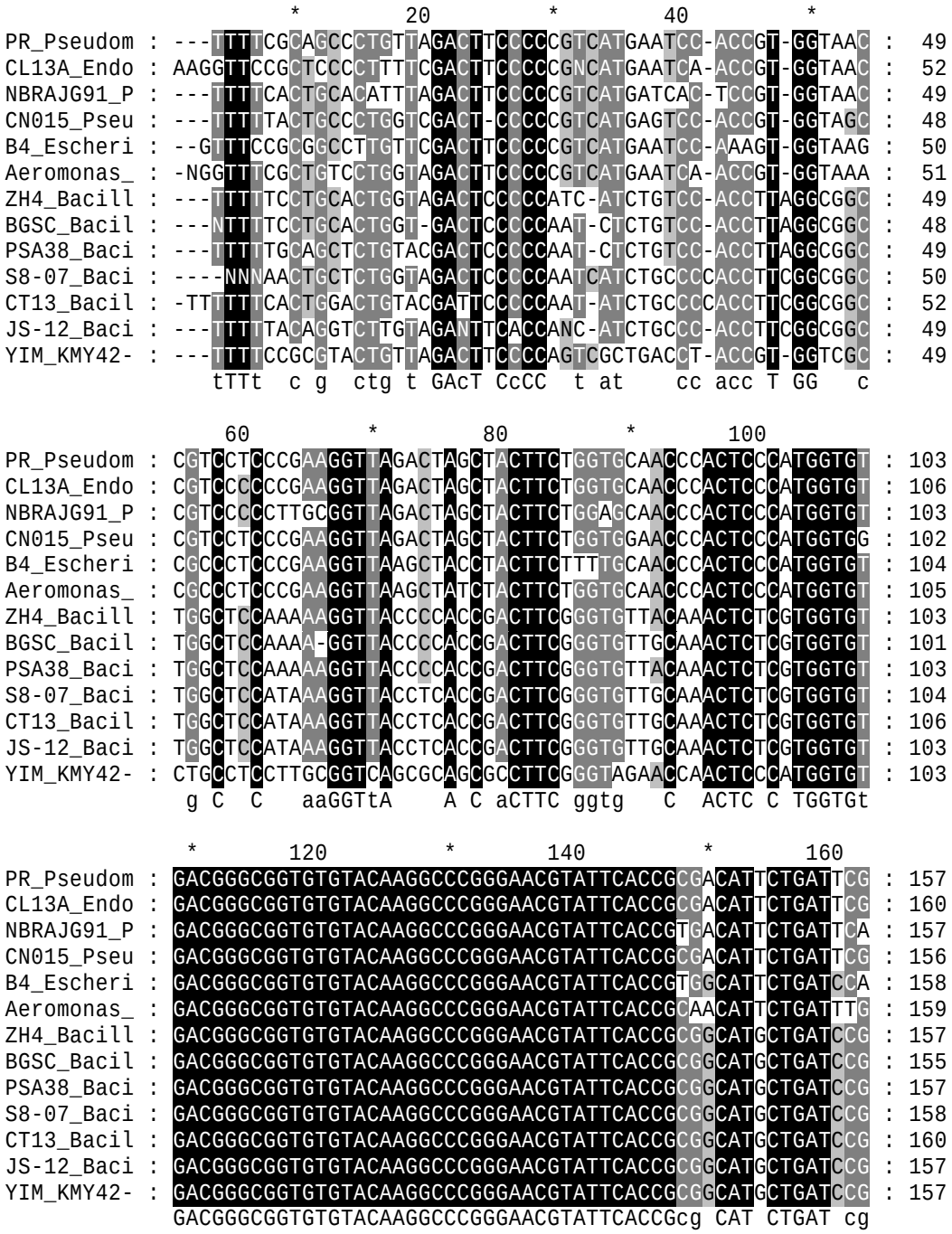


Alignment of the amino acid sequences of 41 isolates obtained from different sampling sites in the spring water distribution system in week 8 continue.

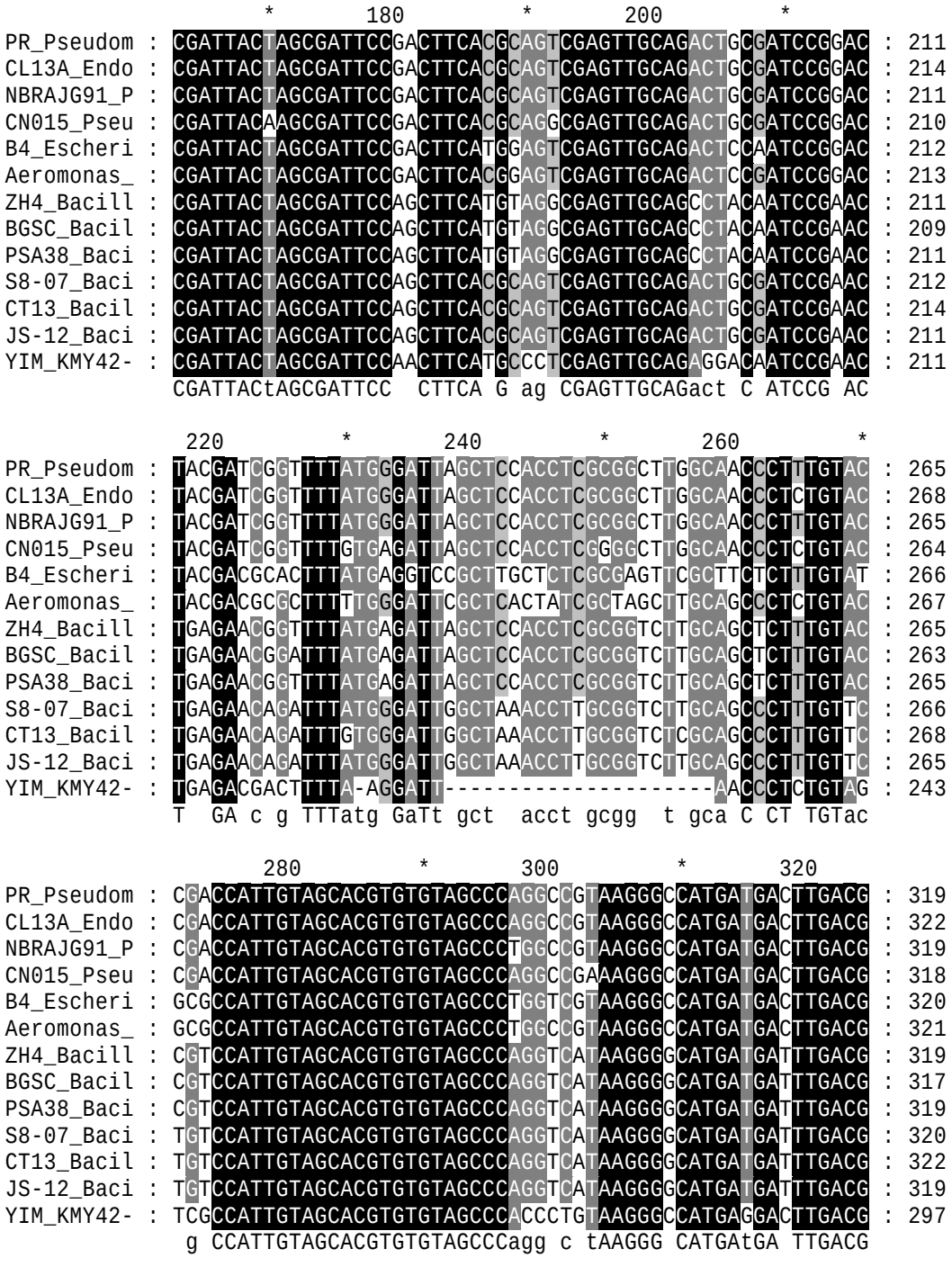


Alignment of the amino acid sequences of 41 isolates obtained from different sampling sites in the spring water distribution system in week 8 continue.

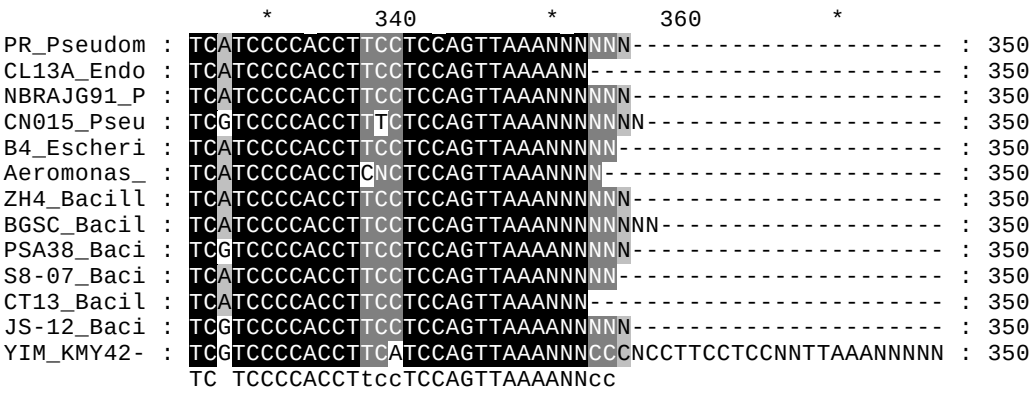
APPENDIX D



Addendum D: Alignment of the amino acid sequences of 13 isolates obtained from different sampling sites in the spring water distribution system in week 46. The alignment was carried out by the multiple alignment of Clustal X (1.81). Genedoc software was used for homology shading. Gaps introduced into the alignment are indicated with dashes. Four shading levels were set.



Alignment of the amino acid sequences of 13 isolates obtained from different sampling sites in the spring water distribution system in week 46 continue.



Alignment of the amino acid sequences of 13 isolates obtained from different sampling sites in the spring water distribution system in week 46 continue.

APPENDIX E

PRIMER SET i	RW01 (Forward) & DG74 (Reverse)
PRIMER SET ii	RD080 (Forward) & DG74 (Reverse)
MARKER	Mass Ruler ™DNA ladder, # SM0402

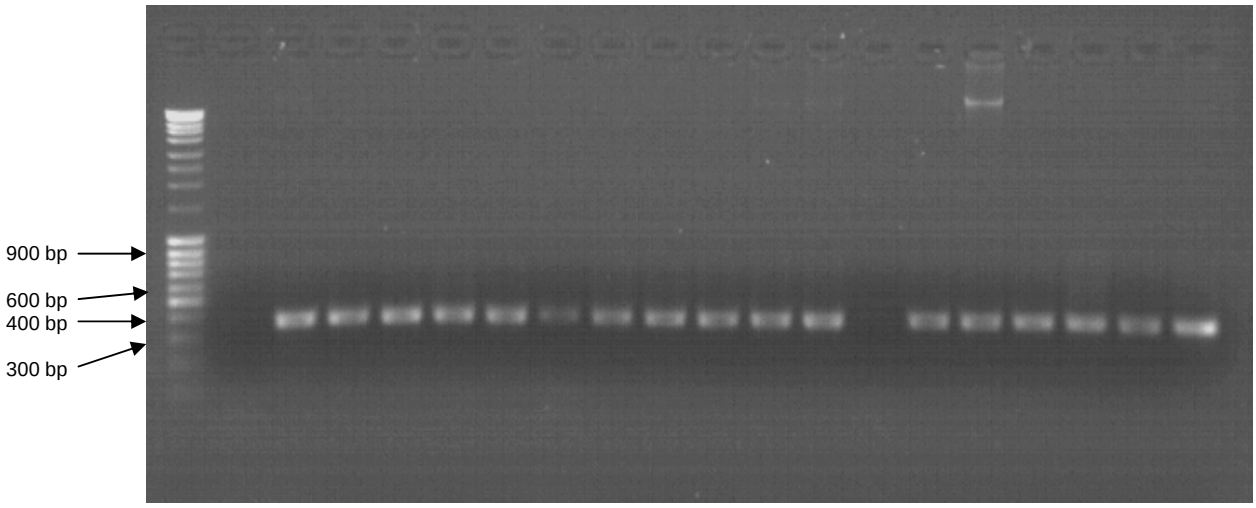
WEEK 1		**WEEK 4**		**WEEK 8**		**WEEK 46**	
A1i	A1ii	B1i	B1ii	C1i	C1ii	D1i	D1ii
A2i	A2ii	B2i	B2ii	C2i	C2ii	D2i	D2ii
A3i	A3ii	B3i	B3ii	C3i	C3ii	D3i	D3ii
A4i	A4ii	B4i	B4ii	C4i	C4ii	D4i	D4ii
A5i	A5ii	B5i	B5ii	C5i	C5ii	D5i	D5ii
A6i	A6ii	B6i	B6ii	C6i	C6ii	D6i	D6ii
A7i	A7ii	B7i	B7ii	C7i	C7ii	D7i	D7ii
A8i	A8ii	B8i	B8ii	C8i	C8ii	D8i	D8ii
A9i	A9ii	B9i	B9ii	C9i	C9ii	D9i	D9ii
A10i	A10ii	B10i	B10ii	C10i	C10ii	D10i	D10ii
A11i	A11ii	B11i	B11ii	C11i	C11ii	D11i	D11ii
A12i	A12ii	B12i	B12ii	C12i	C12ii	D12i	D12ii
A13i	A13ii	B13i	B13ii	C13i	C13ii	D13i	D13ii
A14i	A14ii	B14i	B14ii	C14i	C14ii	D14i	D14ii
A15i	A15ii	B15i	B15ii	C15i	C15ii	D15i	D15ii
A16i	A16ii	B16i	B16ii	C16i	C16ii	D16i	D16ii
A17i	A17ii	B17i	B17ii	C17i	C17ii	D17i	D17ii
A18i	A18ii	B18i	B18ii	C18i	C18ii	D18i	D18ii
A19i	A19ii	B19i	B19ii	C19i	C19ii	D19i	D19ii
A20i	A20ii	B20i	B20ii	C20i	C20ii	D20i	D20ii
A21i	A21ii	B21i	B21ii	C21i	C21ii		
A22i	A22ii	B22i	B22ii	C22i	C22ii		
A23i	A23ii	B23i	B23ii	C23i	C23ii		
A24i	A24ii	B24i	B24ii	C24i	C24ii		
A25i	A25ii	B25i	B25ii	C25i	C25ii		
A26i	A26ii	B26i	B26ii	C26i	C26ii		
A27i	A27ii	B27i	B27ii	C27i	C27ii		
A28i	A28ii	B28i	B28ii	C28i	C28ii		
A29i	A29ii	B29i	B29ii	C29i	C29ii		
A30i	A30ii	B30i	B30ii	C30i	C30ii		
A31i	A31ii	B31i	B31i	C31i	C31ii		
A32i	A32ii	B32i	B32ii	C32i	C32ii		
A33i	A33ii	B33i	B33ii	C33i	C33ii		
A34i	A34ii	B34i	B34ii	C34i	C34ii		
A35i	A35ii			C35i	C35ii		
A36i	A36ii			C36i	C36ii		
A37i	A37ii			C37i	C37ii		
A38i	A38ii			C38i	C38ii		
A39i	A39ii			C39i	C39ii		
A40i	A40ii			C40i	C40ii		
A41i	A41ii			C41i	C41ii		
				C42i	C42ii		
				C43i	C43ii		

				C44i	CA44ii		
				C45i	C45ii		
				C46i	C46ii		
				C47i	C47ii		
				C48i	C48ii		
				C49i	C49ii		
				C50i	C50ii		
				C51i	C51ii		
				C52i	C52ii		
				C53i	C53ii		
				C54i	C54ii		
				C55i	C55ii		
				C56i	C56ii		
				C57i	C57ii		
				C58i	C58ii		
				C59i	C59ii		

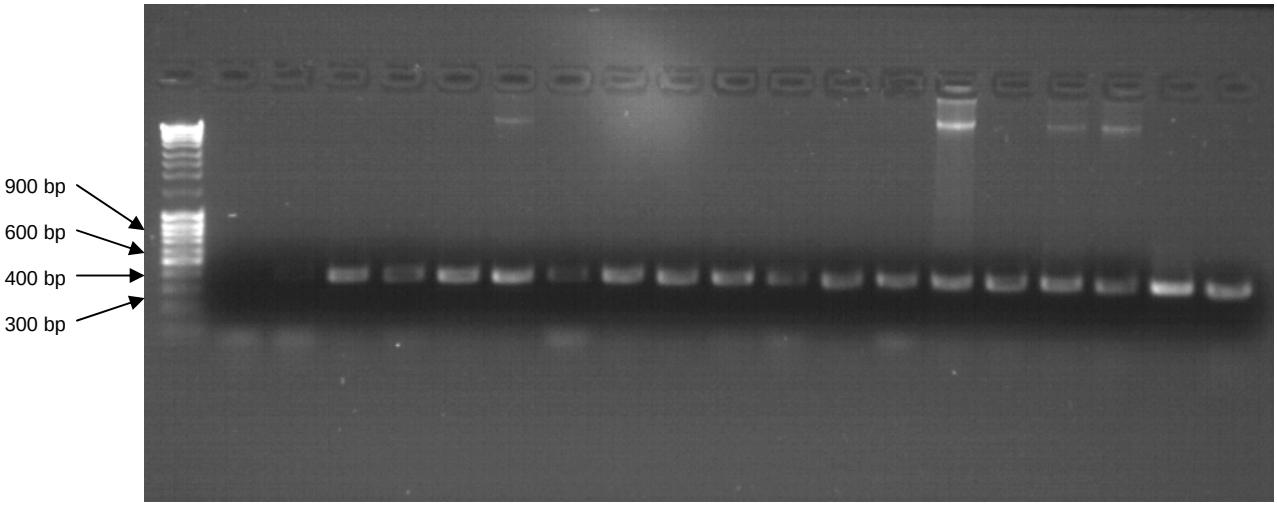
Table 5. Codes for agarose gel electrophoresis pictures of purified PCR samples for the sampling weeks using both primer sets.

APPENDIX F

GEL 1–WEEK 1–PRIMER 1

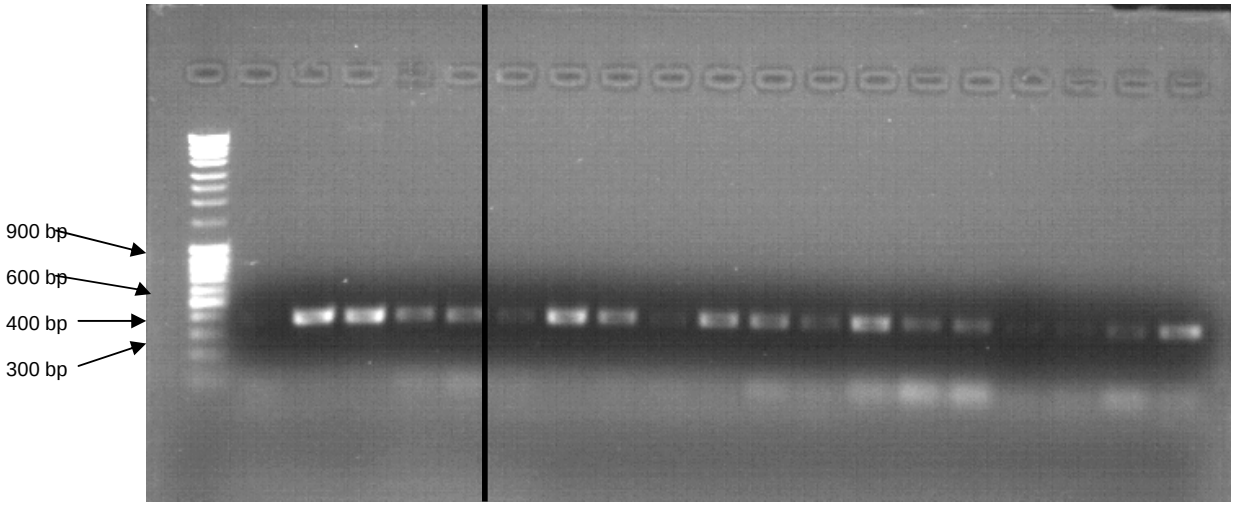


GEL 2–WEEK 1–PRIMER 1

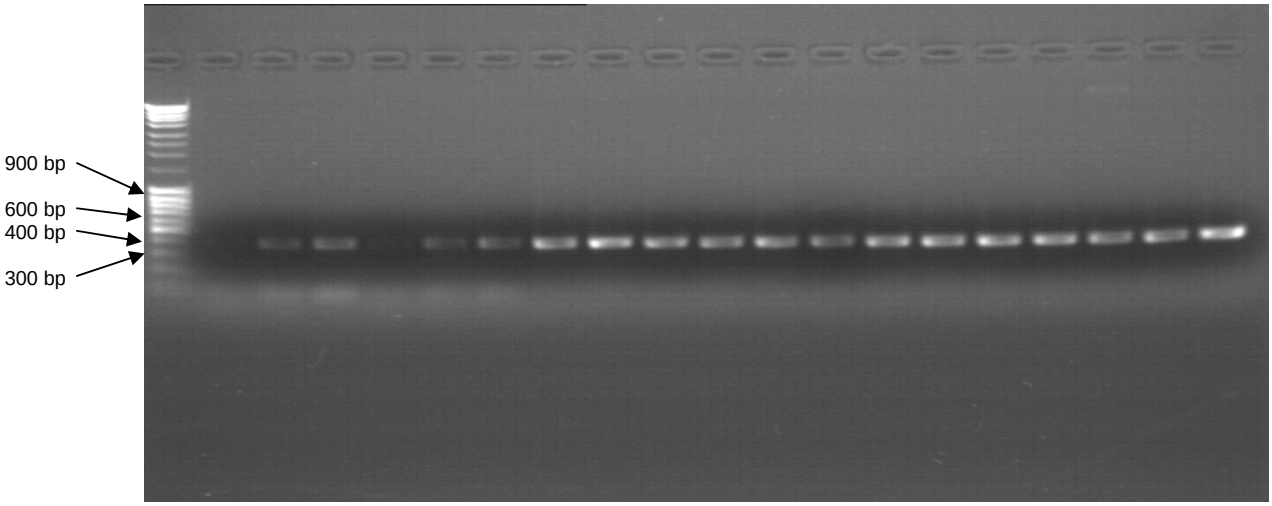


Agarose gel electrophoresis pictures of purified PCR samples for the sampling weeks using both primer sets.

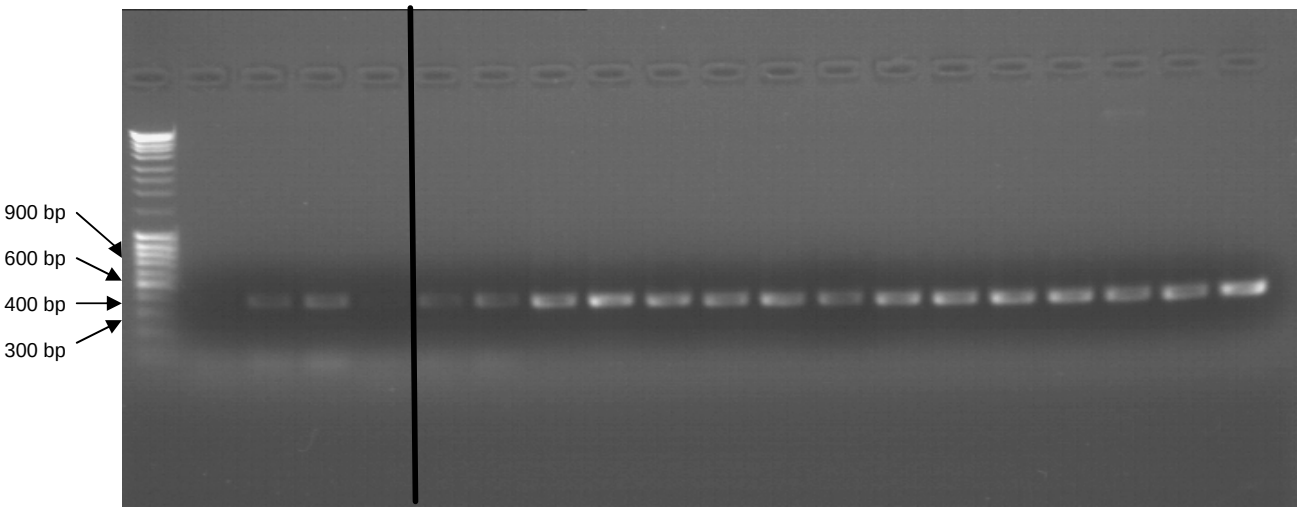
GEL 3-WEEK 1-PRIMER 1 WEEK 4-PRIMER 1



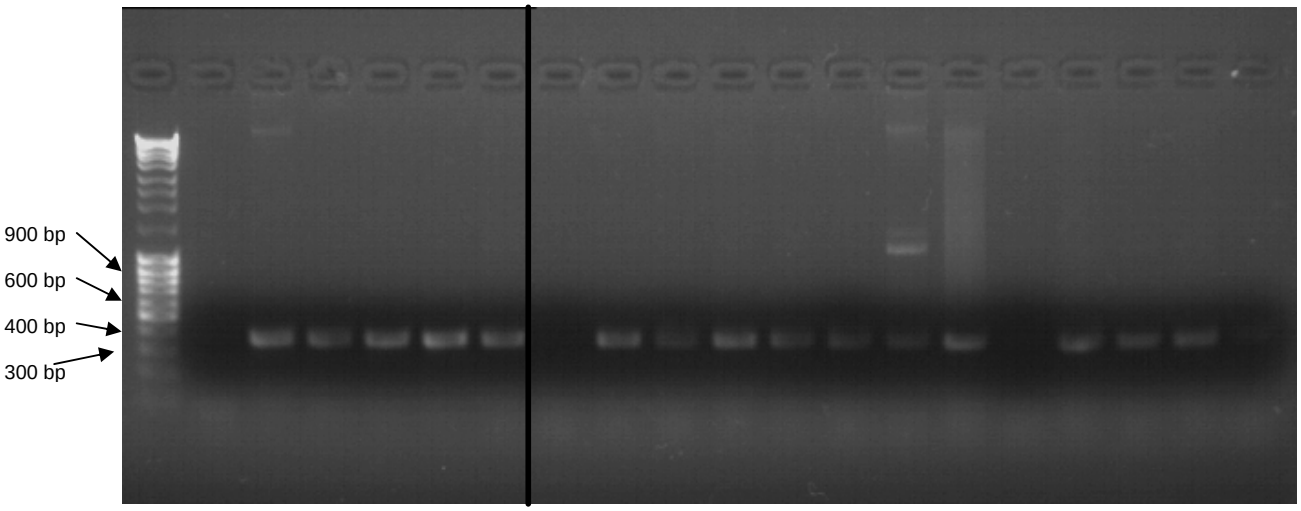
GEL 4-WEEK 4-PRIMER 1



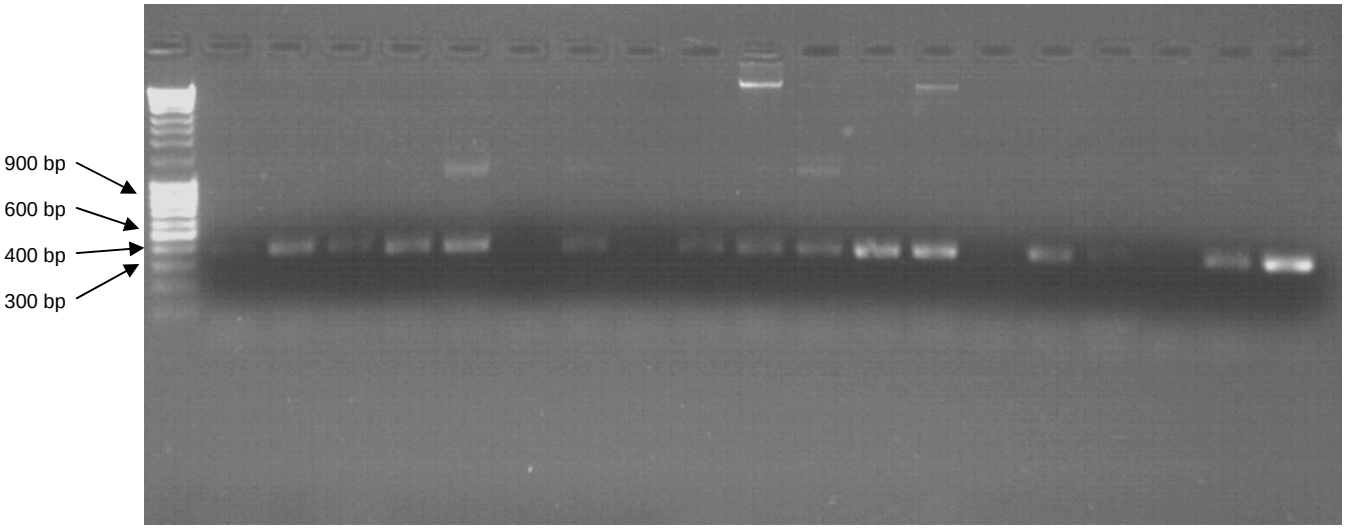
GEL 5-WEEK-1PRIMER 1 WEEK 4-PRIMER 1



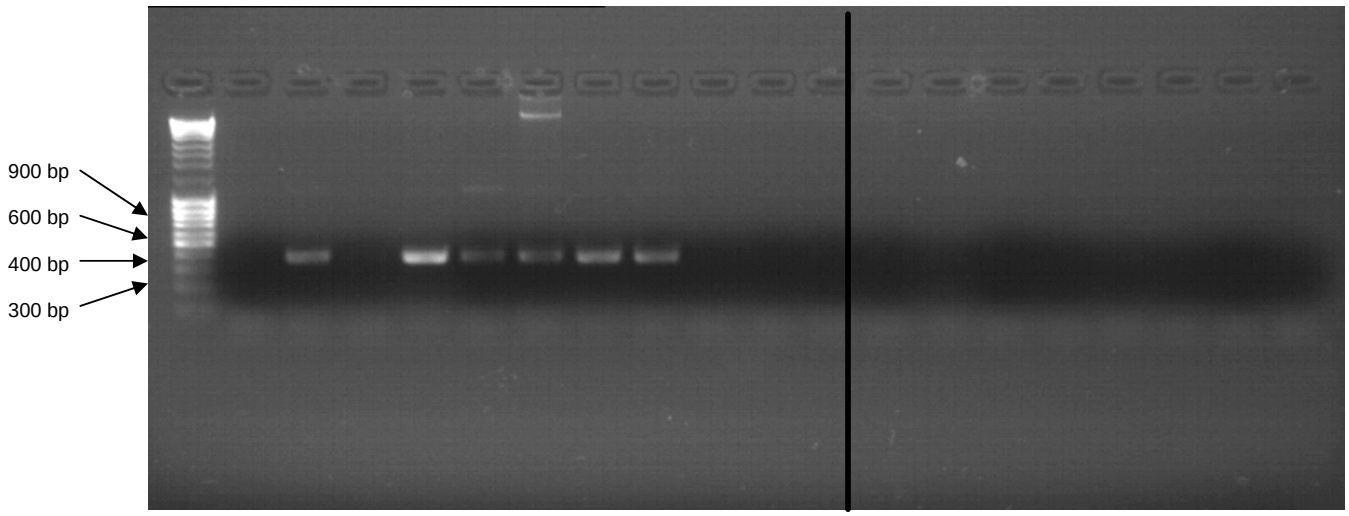
GEL 6-WEEK 8-PRIMER 2 WEEK 1-PRIMER 2



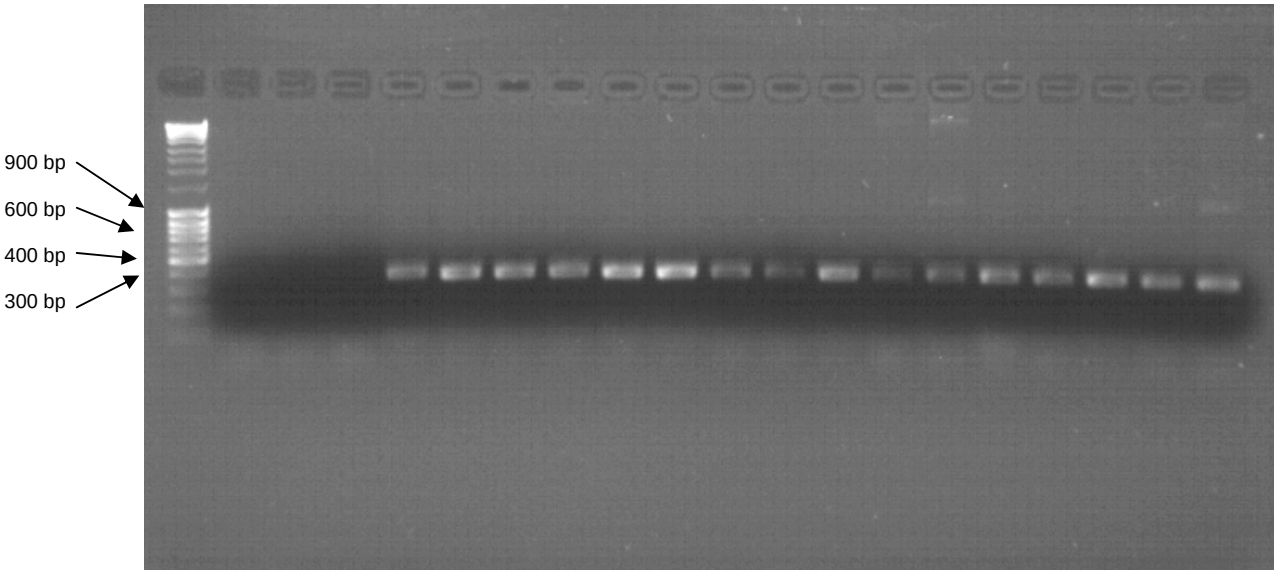
GEL 7-WEEK 1-PRIMER 2



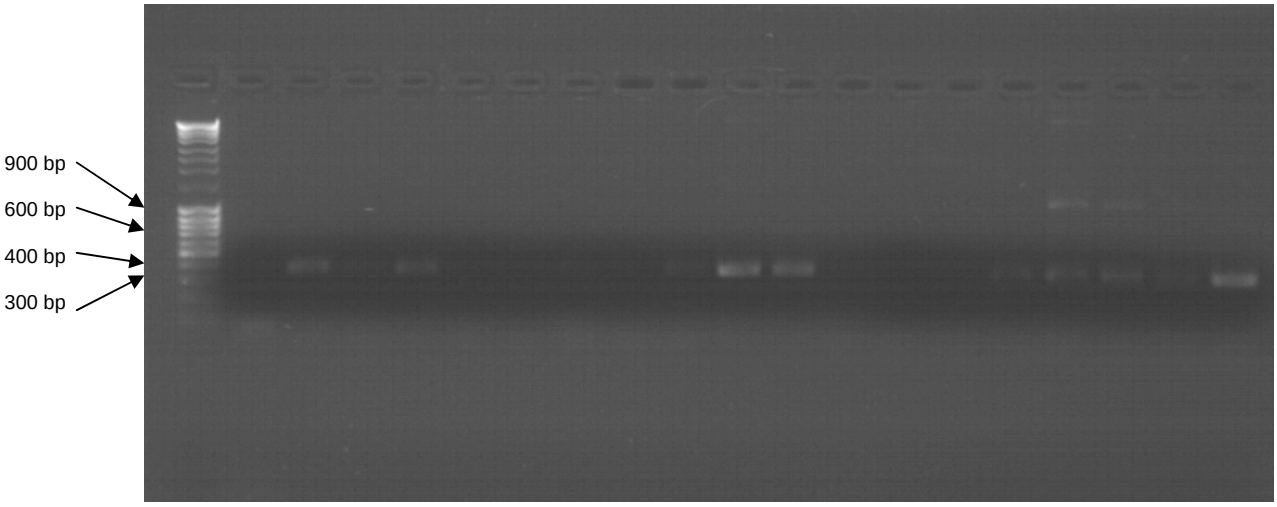
GEL 8-WEEK 1-PRIMER 2 WEEK 4-PRIMER 2



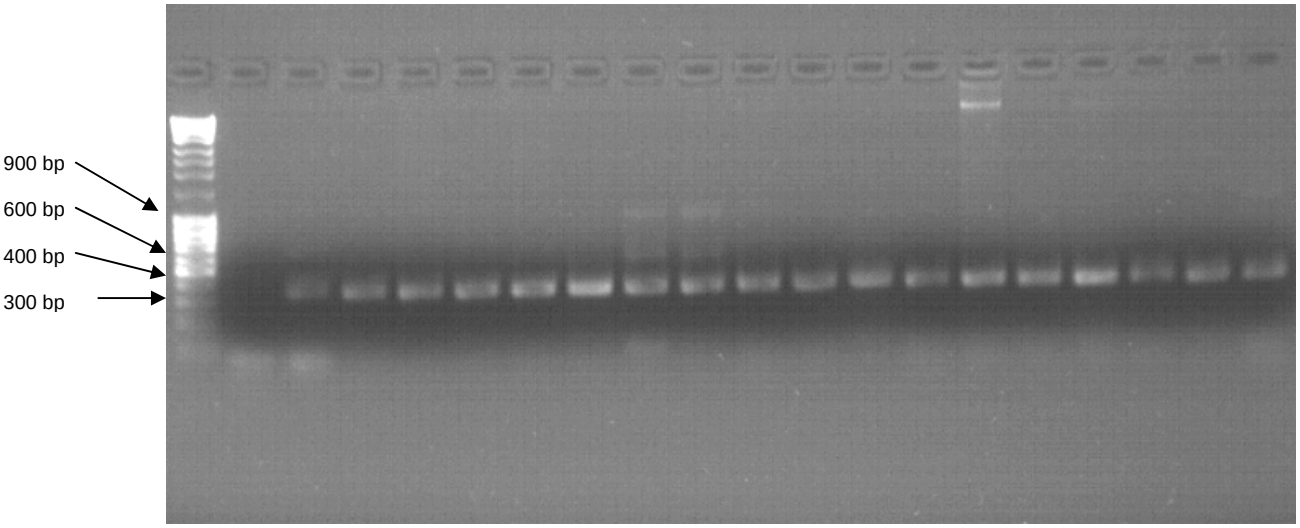
GEL 9-WEEK 4-PRIMER 2



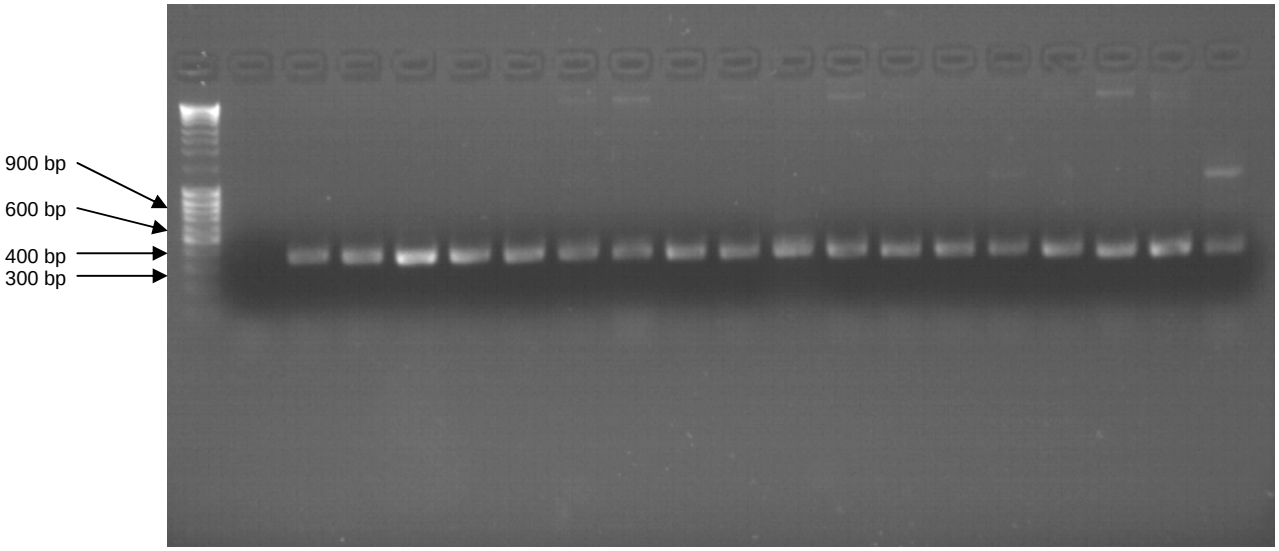
GEL 10-WEEK 8-PRIMER 2



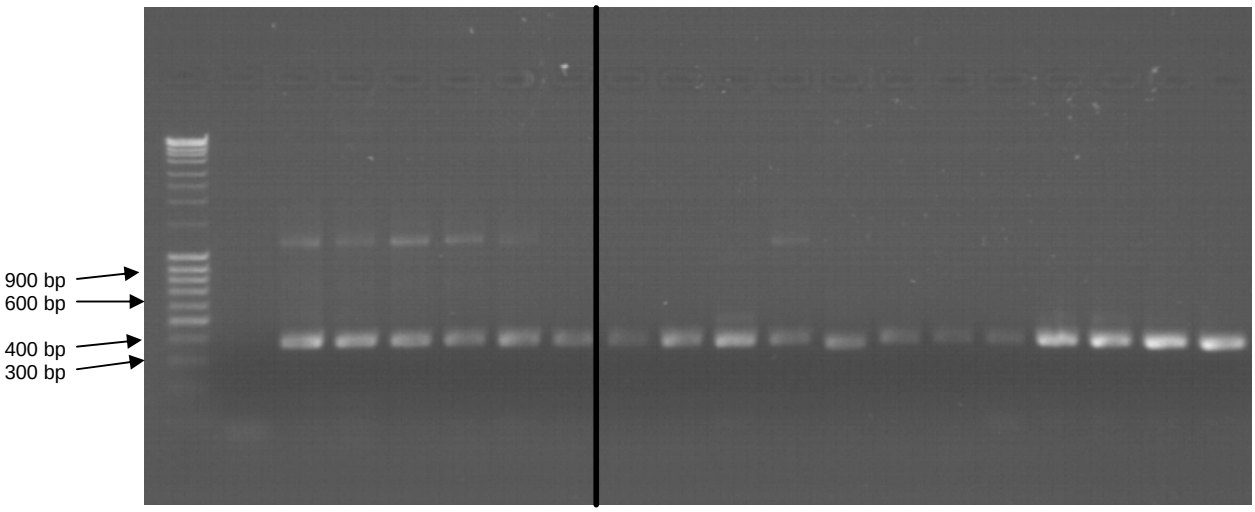
GEL 11-WEEK 8-PRIMER 2



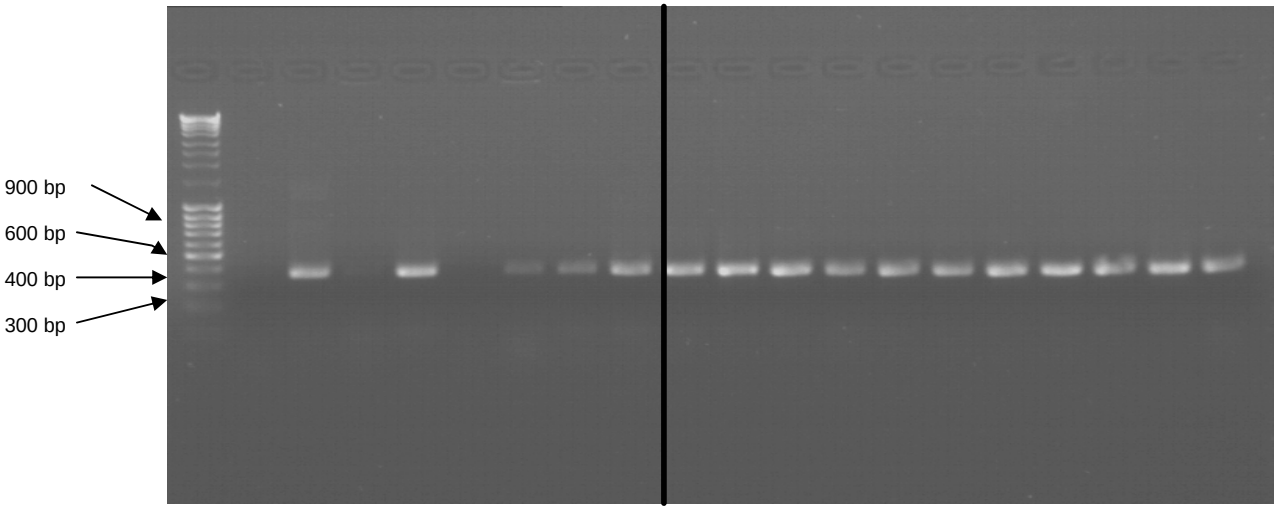
GEL 12-WEEK 8-PRIMER 2



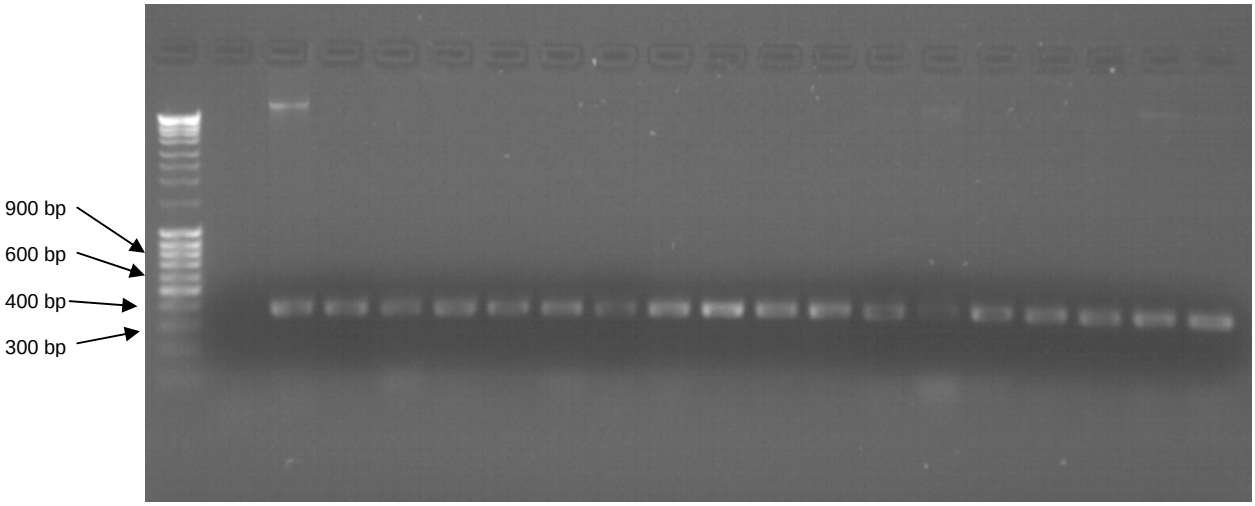
GEL 13-WEEK 46-PRIMER 2 WEEK 46-PRIMER 2



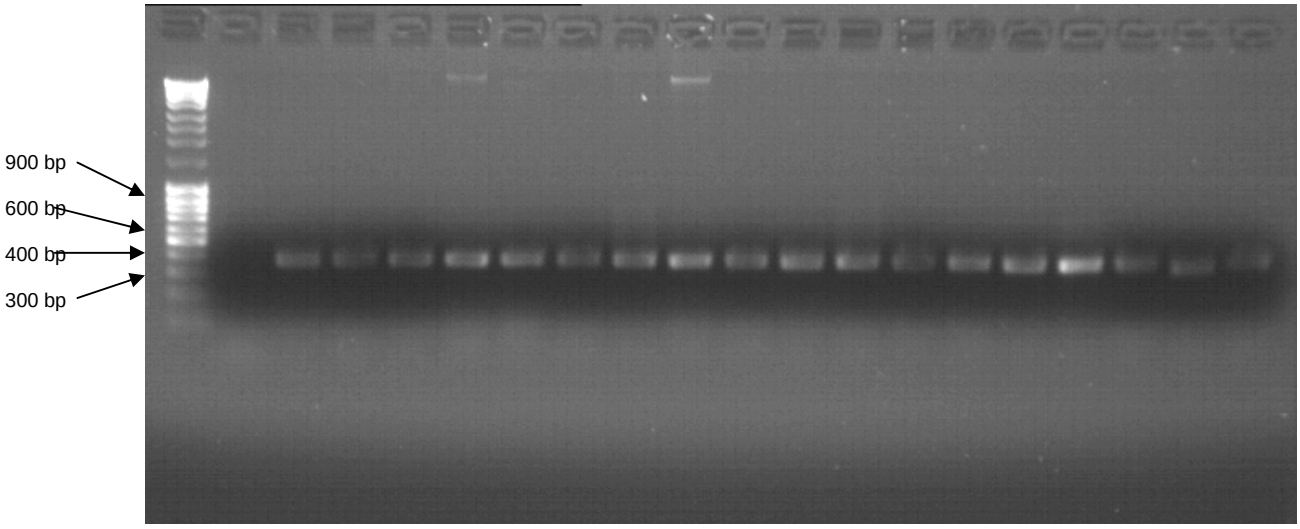
GEL 14-WEEK 46-PRIMER 2 WEEK 8-PRIMER 1



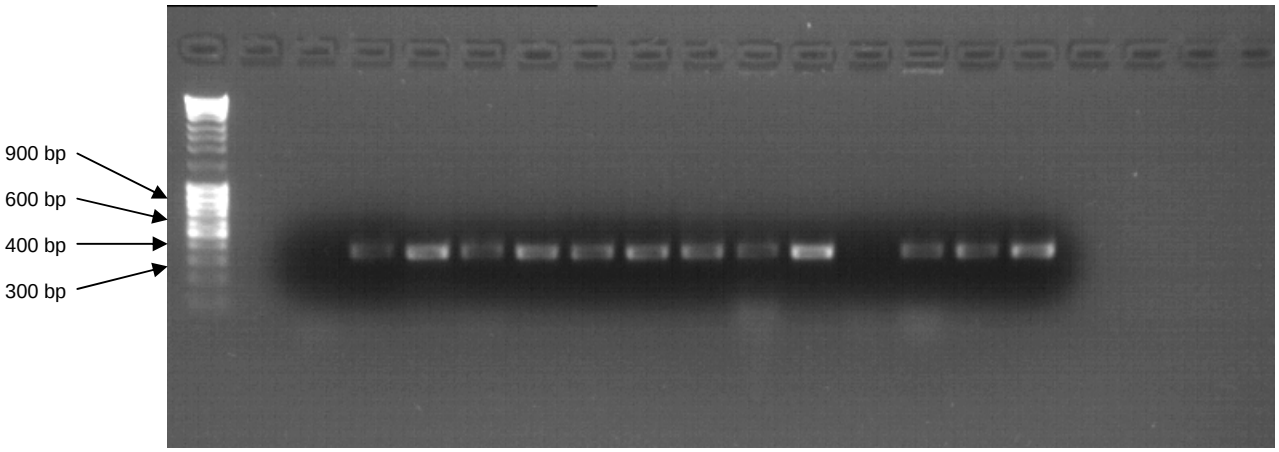
GEL 15-WEEK 8-PRIMER 1



GEL 16-WEEK 8-PRIMER 1

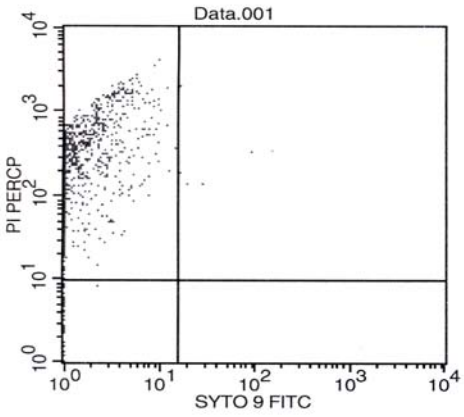


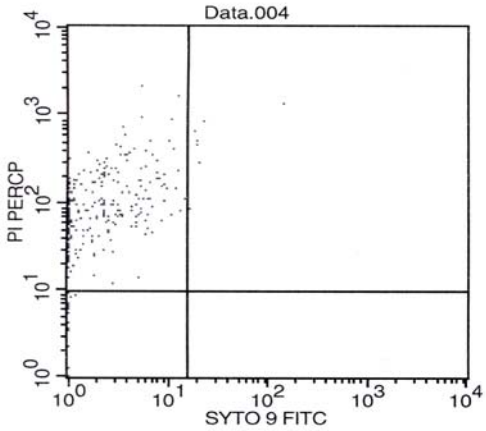
GEL 17-WEEK 46-PRIMER 1



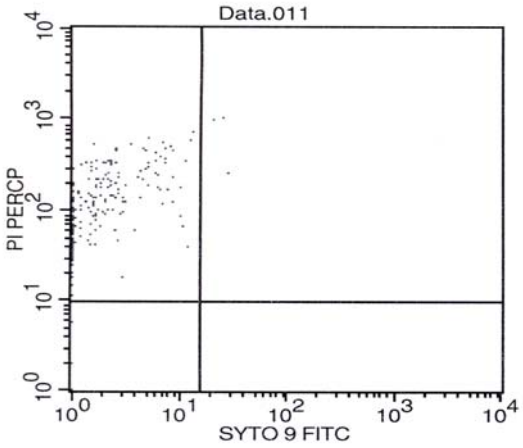
APPENDIX G

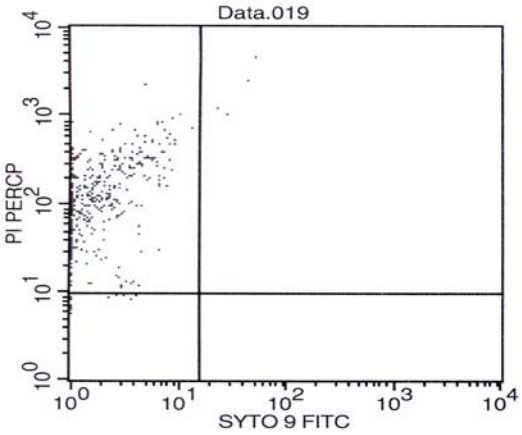
Appendix G: Dotplots of planktonic samples obtained at the borehole and final product for the distribution site in the Western Cape by means of Flow cytometric analyses (FCM).



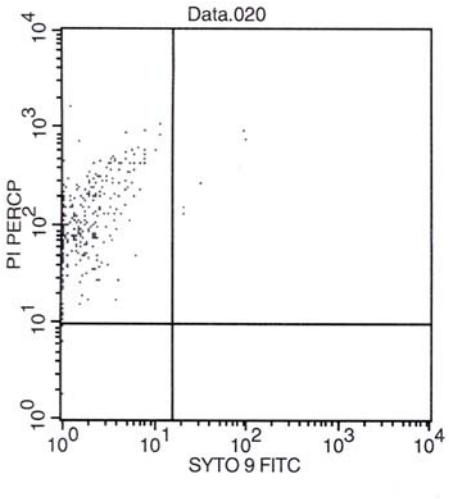


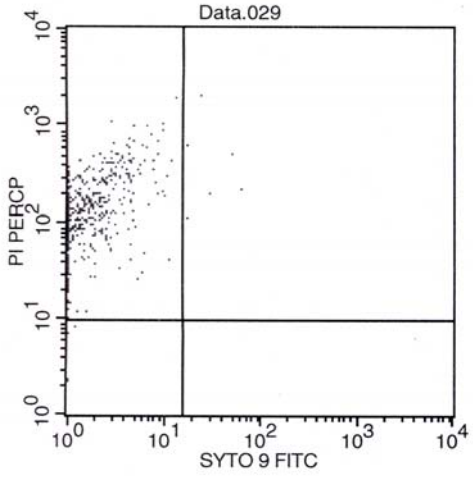
Dotplots of planktonic samples obtained at the borehole and final bottled water for week 1 of the distribution site in the Western Cape by means of Flow cytometric analyses (FCM).





Dotplots of planktonic samples obtained at the borehole and final bottled water for week 4 of the distribution site in the Western Cape by means of Flow cytometric analyses (FCM).





Dotplots of planktonic samples obtained at the borehole and final bottled water for week 8 of the distribution site in the Western Cape by means of Flow cytometric analyses (FCM).