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Investigation into the bacterial pollution in three Western Cape rivers, South Africa and the application of bioremediation strategies as clean-up technology

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INVESTIGATION INTO THE BACTERIAL POLLUTION IN THREE WESTERN CAPE RIVERS, SOUTH AFRICA AND THE APPLICATION OF BIOREMEDIATION STRATEGIES AS CLEAN-UP TECHNOLOGY

by

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Co-supervisor: AProf. Sehaam Khan

Bellville

DECLARATION

I, Arnelia Natalie Paulse, 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

The quality of South Africa's water sources is fast deteriorating due to an influx of pollutants from industrial and agricultural areas. In addition, urbanisation has led to the establishment of informal settlements along river systems. This study focuses on the importance of maintaining water quality and the management of water resources in order to ensure its sustainability in South Africa. The primary aim of this study was to determine the extent of bacterial contamination in three rivers namely the Berg-, Plankenburg- and Diep Rivers in the Western Cape, South Africa and to investigate the application of a bioremediation system as a possible treatment technology. Several aspects contributing to the contamination were addressed and different approaches were studied and reviewed. In all three rivers, four sampling sites were identified, which were sampled over a period of 9 to 12 months.

Contamination levels for the three rivers were evaluated by applying various enumeration techniques, which could provide an accurate indication of the planktonic bacterial pollution load in the river systems. The Most Probable Number (MPN) technique was used to determine the level of faecal coliforms and *E. coli*. The highest MPN, faecal coliform and *E. coli* counts of 3.5×10^7 micro-organisms/100 mℓ, 3.5×10^7 micro-organisms/100 mℓ and 1.7×10^7 micro-organisms/100 mℓ, respectively, were recorded at Site B2 in week 37 in the Berg River. Results showed that in all the river water sampled and evaluated, the total MPN count mostly exceeded the maximum limit of 2000 micro-organisms/100 mℓ (SABS, 1984) stipulated for river water throughout the study period. The heterotrophic plate count (HPC) method was used to determine the number of culturable micro-organisms in planktonic samples, while the flow cytometry (FCM) and epifluorescence microscopy (EM) with different fluorochromes (Acridine orange and BacLight™ Live/Dead stain) were employed to evaluate total bacterial counts in planktonic (water) samples. The highest HPC at the various sites sampled was 1.04×10^6 micro-organisms/mℓ (Berg River, Site B2), 7.9×10^4 micro-organisms/mℓ (Plankenbrug River, Site A) and 1.7×10^5 micro-organisms/mℓ (Diep River, Site B). Total cell counts as high as 3.7×10^7 micro-organism/mℓ (Berg River, Site B2), 5.5×10^8 micro-organism/mℓ (Plankenburg River, Site D) and 2.5×10^9 micro-organisms/mℓ (Diep River, Site B) were obtained by the FCM technique, which were significantly ($p < 0.05$) higher than the total counts obtained by epifluorescence microscopy. The results thus show that

the FCM technique was the most reliable method for determining the total cell count in river water samples. This technique makes use of computer software whereas epifluorescence microscopy involves manual counting which may lead to human error. In addition, the impact of residential, agricultural and industrial areas situated along these rivers was also investigated. Even though exact point sources of pollution could not be determined, it was found that all the sources, such as the storm water drainage pipes, the industrial as well as the agricultural areas, could contribute to increased MPN, heterotrophic and total bacterial counts.

This study also aimed at investigating and comparing the microbial contamination levels at various sites in the Plankenburg and Diep Rivers in the Western Cape, South Africa. Sampling of sites along the Plankenburg River started in June 2004 and continued for a period of one year until June 2005. Sampling of the Diep River sites started in March 2005 and continued for a period of nine months until November 2005. Faecal coliform (FC) and *E. coli* (EC) counts were determined by means of the Most Probable Number technique, 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). The highest microbial counts for the Plankenburg River were observed at site B where the highest MPN, FC, *E. coli* and total FCM counts of 9.2×10^6 (week 14), 3.5×10^6 (week 39) and 3.5×10^6 micro-organisms/100 mℓ (week 39) and 2.1×10^8 micro-organisms/mℓ (weeks 1 and 39) respectively, were recorded. The highest HPC recorded for the Plankenburg River was 7.9×10^6 micro-organisms/100 mℓ (week 44, site A). Site B is situated close to an informal settlement where waste effluents from storm water drainage pipes enter the river system. In addition, other possible contamination sources included agricultural (site A) and industrial (site C) areas bordering the Plankenburg River. The highest total MPN, FC and *E. coli* counts in the Diep River were 5.4×10^6 (week 23) and 1.6×10^6 micro-organisms/100 mℓ [FC and *E. coli*, respectively (both in week 23)], recorded at site B. The highest HPC and total FCM counts of 1.7×10^7 micro-organisms/100 mℓ (week 14) and 2.5×10^9 micro-organisms/mℓ (week 23), respectively, were also recorded at site B. This site was identified as the most contaminated site along the Diep River and served as an accumulation point for waste effluents from the residential and industrial areas, which included paint and machine manufacturers. Other sources situated along the Diep River included storage and maintenance facilities for steel containers, a waste water treatment plant and an oil-refinery. Most of the bacterial counts obtained for the

Plankenburg and Diep Rivers exceeded the accepted maximum limit for river water for most of the sampling period.

Bacterial species from the Berg- and Plankenburg Rivers were isolated and identified. The presence of various *Enterobacteriaceae* species isolated at all the sites in both rivers confirmed faecal contamination of these water sources over the entire sampling period. Opportunistic pathogens such as *Klebsiella* sp., *Serratia* sp., *Enterobacter* sp., *Shewanella* sp., *Aeromonas* sp., *Pseudomonas* sp., *Acinetobacter* sp. and *Citrobacter freundii* as well as pathogens such as *Bacillus cereus* and *B. anthracis* were also identified in both river systems.

All the respective articles are presented in the required format of the journal in which the article has been published or submitted to.

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BIOGRAPHICAL SKETCH

Arnelia Natalie Paulse was born in Paarl, South Africa, on the 22nd February 1974. She attended Ebenezer Primary School and matriculated at Klein Nederburg Senior Secondary in 1992. She enrolled at the University of the Western Cape in 1993 and obtained a B.Sc. degree in Zoology and Microbiology in 1998. In 1999 she completed a B.Sc. (Hons.) degree in Microbiology at the same university. Arnelia enrolled at the University of Stellenbosch in 2000 and obtained her M.Sc. degree in Microbiology in 2004. She is presently a part-time employee of Cape Peninsula University of Technology in the capacity of lecturer.

DEDICATION

This thesis is dedicated to my Father, Mother and Grandmother

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GLOSSARY

Department of Water Affairs and Forestry –	DWAF
Free basic water –	FBW
World Health Organisation –	WHO
United Children's Fund –	UNICEF
Polymerase Chain Reaction –	PCR
Colony forming units –	CFU
Viable-but-non-culturable –	VBNC
Adenosine-Tri-Phosphate –	ATP
South African Bureau of Standards –	SABS
European Economic Council –	EEC
Canadian Council Of Ministers of the Environment –	CCME
Free Water Surface –	FWS
Subsurface Flow –	SSF
Flow Cytometric Analysis –	FCM
Heterotrophic Plate Count –	HPC
Most Probable Number –	MPN
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LITERATURE REVIEW

1.1 INTRODUCTION

Access to safe, clean water is a daily commodity taken for granted by most people in first world countries. In many third world countries however, millions of people face morbidity and even mortality due to problems associated with the availability and access to safe drinking water and water for everyday use (Gerba, 1996; Straub & Chandler, 2003). Increasing population growth, urbanisation and the pollution of water sources, amongst other factors, have however, contributed to the decreased availability of the essential element, water.

Surface and groundwater are the most important water sources sustaining all forms of life. Groundwater can be defined as subsurface (underground) water, which is either stored in the pores between alluvial materials (sand, gravel, silt or clay), cracks and crevices in the ground below the water table or as water flowing within aquifer-underground layers of porous rock that are saturated from above or from structures sloping towards it. The rock beneath the earth's surface is known as the bedrock layer, which consists of many types of rock, such as sandstone, granite, and limestone. These rocks have varying amounts of void spaces in them where the groundwater accumulates (United States Geological Survey, 2006). The spaces in between the soil and rock are filled with air and water forming a zone referred to as the zone of infiltration. Below this zone, water occupies every available opening (called the zone of saturation). The water table is at the top of the zone of saturation, and groundwater lies beneath the water table **(Figure 1.1)**.

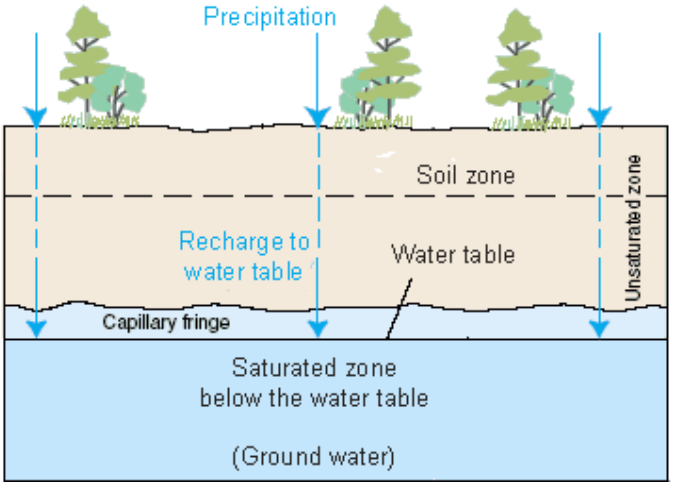


Figure 1.1 Groundwater accumulation beneath the soil surface indicating the zone of infiltration (unsaturated zone) and the saturation zone (United States Geological Survey, 2007).

Groundwater can either flow naturally to the earth's surface as a spring or it can form oases or swamps. It can also be withdrawn for agricultural, municipal and industrial

purposes using man-made wells (Hoyle, 2005). Surface water is found above the ground in streams, rivers, lakes, seas and the ocean (Hoyle, 2005). Primarily, it is used as a source of drinking water but is also used for recreational purposes, or by the thermoelectric power industry to cool electricity-generated equipment and finally for irrigation.

In first world countries, water plays an important role in the country's economy. In addition to satisfying society's basic needs for drinking water and sanitation as well as common household maintenance services, it is needed to produce steam and to cool turbines in thermal power stations. It is also necessary for the manufacturing industry where it is used to produce power (Flörke & Alcamo, 2004).

The volume of water used in the manufacturing sector varies tremendously between the different industries. The paper industry, for example, utilises more water in their processes than the textile industry. In addition, the food and chemical industries along with industries producing mineral products utilise more water in their processes than other industries. The volume of water used for irrigation however, depends on the water retention characteristics of the crops planted as well as the particular soil type. Local precipitation, temperature, wind and other climatic conditions, also play a role in the amount of water being utilised (Flörke & Alcamo, 2004).

In Europe, surface water contributes to approximately 75% of the total water abstracted for all general uses. Approximately 25% is obtained from groundwater sources, with only a small percentage obtained from desalinated seawater and the re-use of treated effluents **[Figure 1.2(a)]**. However, in certain European countries with sufficient aquifers, groundwater contributes to 75% of public water supplies. Of the total freshwater obtained from surface and groundwater sources, withdrawal is divided into water for urban use (14%), agriculture (30%), industry (10%), power generation and hydropower (32%) and undefined uses (14%) **[Figure 1.2(b)]** [European Environmental Agency (EEA), 1999]. It is important to note that the quality of water sources such as rivers, lakes and groundwater are however, being threatened by sewage and industrial waste contamination as well as excessive application of pesticides and fertilisers (Dickie, 2005).

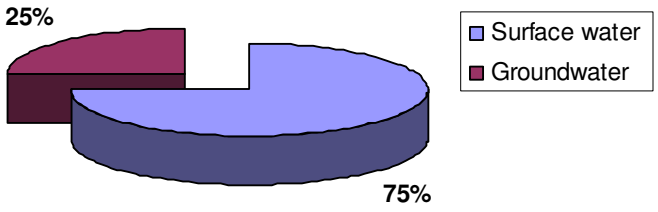


Figure 1.2 (a) Water resource availability in Europe (European Environmental Agency, 1999).

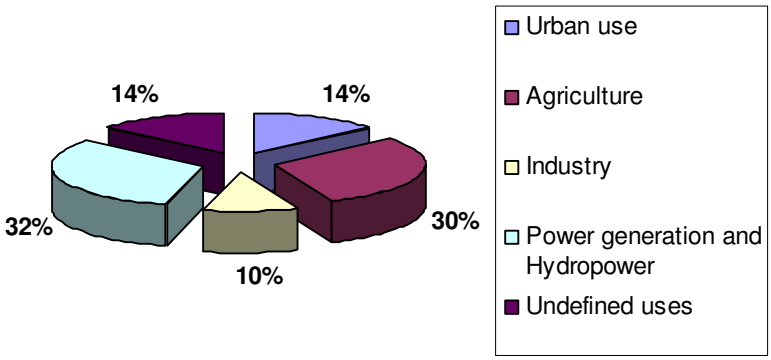


Figure 1.2 (b) Estimated water uses per sector in Europe in 1999 (European Environmental Agency, 1999)

In the United States approximately 50% of the population obtain all or part of their drinking water from a groundwater source, with 95% of the rural communities relying on groundwater for their drinking water supply (United States Geological Survey, 2000). Water statistics for the United States indicates that approximately 64% of total water withdrawals for general purposes are from fresh-surface water sources. Saline-surface water, primarily used for thermoelectric power generation, contributes to 15% of total water withdrawals, while 21% is drawn from a groundwater source (United States Environment Protection Agency, 2005) **[Figure 1.3(a)]**. Of the total amount of water available, the largest percentages are used for thermoelectric power and irrigation. **Figure 1.3(b)** indicates the estimated use of water per sector in the U.S.A from 2000 to 2004 (Hutson *et al*., 2004).

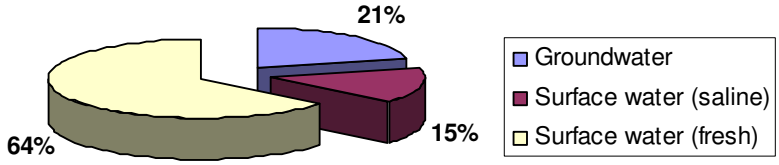


Figure 1.3 (a) The water availability per capita in the United States (United States Environmental Protection Agency, 2005)

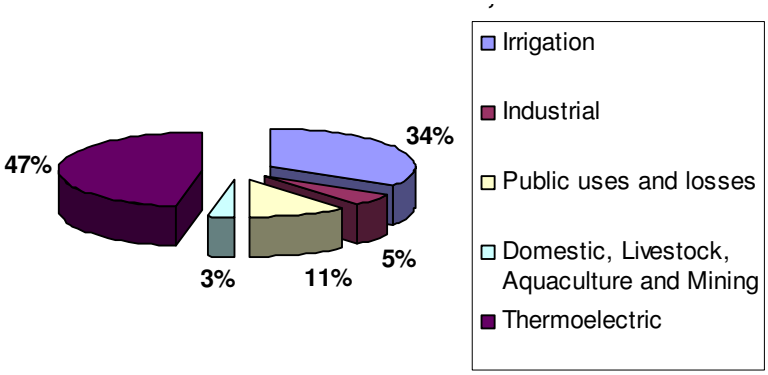


Figure 1.3 (b) Estimated water uses per sector in the United States from 2000 to 2004 (Hutson *et al*., 2004)

South Africa depends mainly on surface water resources (rivers and dams) for most of its urban, industrial and irrigation water. Groundwater (underground) resources are also extensively used, particularly in the rural and more arid areas [Department of Water Affairs and Forestry (DWAF), 2006/2007]. The country's existing water resource availability comprises 77% surface water, 9% groundwater and 14% re-use of return flows **(Figure 1.4)** (United Nations Educational, Scientific and Cultural Organisation, 2005).

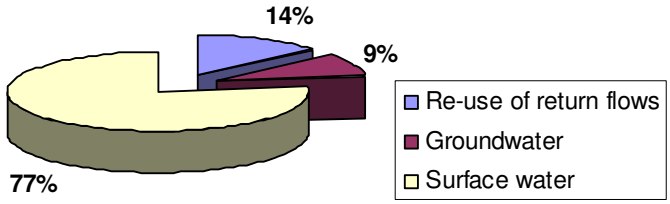


Figure 1.4 The water availability per capita in South Africa (United Nations Educational, Scientific and Cultural Organisation, 2005)

Even though groundwater represents only 9% of the total water resources, 74% of South Africa's rural communities use groundwater as their sole water source. In addition, the previous allocation of the groundwater uses per sector **(Figure 1.5)** included 4% for urban and 7% for rural usage, 5% for mining and quarries and 6% for stock watering.

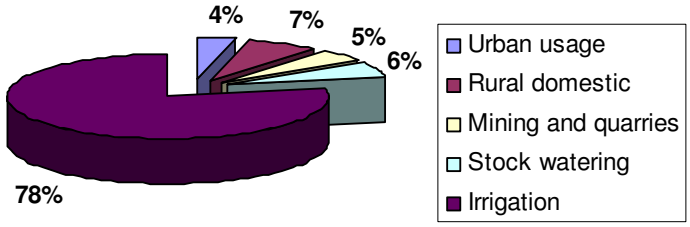


Figure 1.5 Groundwater utilisation per sector in South Africa (Department of Water Affairs and Forestry, 1986)

The total remaining 78% was used for irrigation purposes [Department of Water Affairs and Forestry (DWAF), 1986]. The latest statistics for surface water utilisation and water withdrawal from different sectors in South Africa are indicated in **Figures 1.6 (a)** and **(b)** with the country's total withdrawal from surface water resources amounting to approximately 10 200 million m^3 per year.

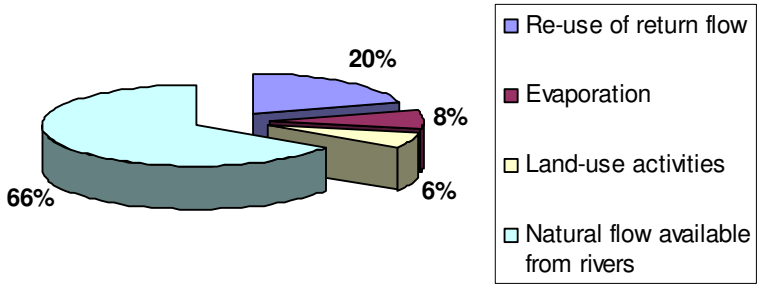


Figure 1.6 (a) Total withdrawals from surface water resources in South Africa (Department of Water Affairs and Forestry, 2006/2007)

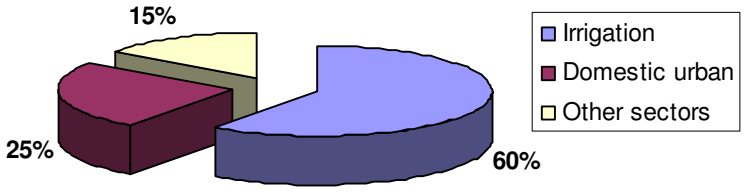


Figure 1.6 (b) Water withdrawal and allocation per sector in South Africa (Department of Water Affairs and Forestry, 2006/2007)

Human and economic well-being is directly and indirectly dependent on the services provided by rivers. These major services include possible sources of drinking water, subsistence fishing and direct irrigation, agricultural and industrial production and for recreational purposes such as boating or rafting. In many instances the value of the rivers and the services they provide are reduced when river systems are impaired or disturbed. The health state of rivers is therefore directly linked to the quality of the services they provide.

Due to the fact that South Africa is one of the 30 driest countries in the world, receiving approximately less than 500 mm rain per year, catchment storage dams are built across major rivers to block its flow. In times of excess flow, water is stored behind the dam wall in reservoirs (Water Research Commission, 2004). In 2005, 550 manmade government dams**,** with a total capacity of 37 000 million m^3 (Mm^3), were identified. These dams regulate the natural variable flow and facilitate the transfer of water between catchment areas and population service points. Approximately half of South Africa's annual rainfall is stored in dams (Collins, 2001). Communities also benefit from these dams, as they serve as a preventative measure against water scarcity during drought spells or flooding (McCartney *et al*., 2004).

In 2004, the 'Western Cape: State of the Environment Report' claimed that groundwater resources in the province was easily accessible compared to the rest of South Africa, which is generally water scarce. The report also stated that due to the mountainous areas of the South-western Cape, the province received some of the country's highest rainfall at > 2 500 mm/annum (Department of Environmental Affairs and Development Planning, 2004), which implied that the catchment dams were usually more than 80% full. The statistics however, have changed drastically in more recent years with dams being less than 50% full due to decreased rainfall.

The Orange River, the largest river in South Africa, and its tributaries run through approximately 48% of the total area of the country, including the mining and industrial heartland of the Gauteng area. In addition, it provides for 22% of South Africa's total water downflow. Apart from the Orange River, the Olifants River is the only other river that flows throughout the year (Swanevelder, 2004). South Africa's mining, power production and

agricultural activities are concentrated around this river catchment. It is estimated that approximately 10 000 operating boreholes are found in the catchment, with the total human consumption amounting to about 1 000 Mm^3. It has also been noted that of this total amount, surface water accounts for 900 Mm^3 while 100 Mm^3 is obtained from a groundwater source.

The largest percentage is used for irrigation (540 Mm^3 per year), primarily in the commercial farming sector (McCartney *et al*., 2004). The largest water transfer scheme in South Africa is the Lesotho Highlands Water Transfer Scheme, which provides for the industrial and business world of Gauteng, including Johannesburg and Pretoria. South Africa's landscape however, with its deep valleys and gorges, makes it difficult for the construction of suitable storage dams. The dams tend to be shallow, with a large surface area, which in conjunction to the country's hot, dry climate results in water evaporating from these dams. Generally, the volume of water stored in dams is also reduced due to a high silt (sludge) load. Rivers in the Western Cape are however, an exception as they carry relatively little sludge (Greater Good South Africa Trust/Report, 2005).

South Africa's drinking water is regarded as being the third best-treated water worldwide, making it completely safe for daily use and consumption. In the more affluent areas of South Africa, the average person utilises approximately 150 litres of water per day with a household of four utilising approximately 600 litres (Greater Good South Africa Trust/Report, 2005). The country however, is an urbanising nation where roughly 28 million people inhabit more than 3000 urban communities, including informal settlements. For this reason it is feared that the nation's water demand will greatly exceed availability of economically usable, fresh water resources (Greater Good South Africa Trust/Report, 2005). The total annual water consumption in 1999 was estimated to be in excess of 16 billion m^3. The latest statistics however, show estimates of 49.2 billion m^3 by the end of 2006 (DWAF 2006/2007). The Department of Water Affairs and Forestry (DWAF) predicts a growth of between four to six percent per year with the speculation that by 2010 the demand for water in South Africa will double.

1.1.1 Water provision

A municipal survey of water provision conducted in 1999, reported that many South Africans live more than 250 m from the nearest available water source. In addition, approximately 4.4 million people have access to a communal standpipe at a distance of less than 250 m (Goldblatt, 1999; Barnes, 2003). These huge numbers of inadequately serviced households clearly pose an enormous urban management challenge.

In 1994, the government committed itself to ensuring that all people will have access to at least 25 litres of clean water per capita per day (Department of Water Affairs and Forestry, 1994). This implies that this tranche of water provision, and the infrastructure

needed for individuals to obtain it, will be provided to those impoverished households, which are unable to pay for services, at no extra cost. This would then provide a major form of welfare assistance to many poor urban communities.

Free basic water (FBW) provision at 25 litres per capita per day however, is a substantial financial commitment. Water provision above this basic level would place unsustainable demands and pressure on national financial resources. Hence, inadequate revenue generation in the water sector means a decrease in the extension of services to new urban households, particularly those in new informal settlements (Goldblatt, 1999). This policy of FBW has however, caused much controversy as it was argued that 6 kilolitres of water per household per month would prove to be inadequate, as many low-income households use considerably more than the provided amount. This is mainly due to a higher average number of occupants per household, as well as old and out-dated infrastructures (McDonald, 2002; Earle *et al*., 2005). The 6-kilolitre provision was an extrapolation of the basic amount of 25 litres per person per day and was above the amount the World Health Organisation (WHO) considers to be 'basic access' (Howard & Bartram, 2003). Howard and Bartram (2003) also stated that the WHO considered access to a communal standpipe less than one kilometre from the home inadequate and a health risk. Even though the amount of FBW corresponded to the WHO's guideline of minimum water requirements for daily use, it has been increased to 6 000 litres per household within 200 metres of the dwelling. The need for water, however, is still higher, as the amount of water requirements for homegrown vegetables or for people with special needs, is not included in this estimate of water provided (Goldin, 2005). A comparative analysis of international water consumption trends, suggested that an average water consumption of between 100 and 200 litres per capita per day is sufficient to maintain a high standard of living (Goldin, 2005).

The South African Constitution states that everyone should have access to an environment that is not harmful to their health or well-being, which includes amongst others a constant supply of clean, safe water (Water Research Commission, 2007). Recently, new treatment advances, adopted from the Netherlands, have been introduced into wastewater treatment fields in South Africa and a full-scale, three-million-litre-a-day plant using this technology is currently being constructed in the Western Cape (Pringle, 2007). This type of technology may be used for the treatment of both domestic and industrial wastewaters. Thus far tests have also indicated that the final effluents conform to the minimum standards as set out by the Department of Water Affairs and Forestry (1996).

A previous study conducted in 2003 however, reported that the overall efficiency of drinking-water provision and water quality in the Western Cape, based on microbiological studies, was very poor. Approximately 62% of samples tested, failed the maximum acceptable limit for organisms, despite a higher standard of living and level of supply infrastructure in the Western Cape. The study also indicated that the quality of the

groundwater was lower than the surface water samples (Mackintosh & Colvin, 2003). The region is however, bearing the brunt of global climate change and it is predicted that reduced rainfall in the western parts of the country could result in a 10% reduction in run-off by the year 2015 (Yeld, 2004).

South Africa not only faces decreases in water availability but also increases in the contamination levels of those remaining water sources. Communities located in rural and semi-urban areas lack adequate domestic water supplies and wastewater treatment facilities and therefore, it is comprehensible that these communities would inhabit areas close to reliable water sources, i.e. rivers and dams (Gerba, 1996).

1.1.2 Informal settlements

Urbanisation and high population densities create challenges regarding the types, amount and quality of services, which rivers may offer. The fast-growing establishment of informal settlements along the riverbanks [**Figure 1.7(a) and (b)]** is also becoming a threat to the state of the rivers. One of the major impacts of these informal settlements is the contamination of the streams and waterways with raw sewage. Due to inadequate sanitation and wastewater removal facilities, faecal matter enters the river from the ground surface, or from pit latrines which seep below the ground into the streams. In the informal settlements it is often the case that only one faecal waste system is provided for more than one family [approximately 40 users per faecal waste system (Barnes, 2003)]. If not properly maintained, this could result in blockages and leakages of piping facilities causing sewers to discharge their waste load into the nearest river (South African River Health Programme, 2003).

Figure 1.7(a) An informal settlement in Brazil

Figure 1.7(b) An informal settlement in South Africa

The common indicator of faecal pollution in water is *Escherichia coli* (*E. coli*), an intestinal micro-organism. South African Guidelines, state that *E. coli* counts in excess of 400 cells per 100 ml results in major health risks. Water should thus be completely clear of this micro-organism in order to be regarded as safe for drinking water purposes. Counts of 1.08×10^6 micro-organisms/100 ml were however, recorded in the uMngeni River in the Pietermaritzburg area **(Table 1.1)**. The possible cause of increased microbial counts in this river was due to the presence of the informal community inhabiting the banks of the Palmiet River, which is a tributary of the uMngeni River. The commodities (i.e. fish, reeds etc.) and services (water for irrigation, etc.) provided by these rivers have clearly been compromised by the high microbial counts relating to faecal contamination, which in turn is an indication of poor management of human activities and infrastructure (South African River Health Programme, 2003).

Table 1.1 Number of *Escherichia coli* per 100 ml of river water and the conditions that led to current state of rivers in the Durban-Pietermaritzburg area (South African River Health Programme, 2003)

River affected	*E. coli* count / 100 ml water	Cause of contamination
Mayville Stream	290 000	Informal community - Inadequate sanitation
Bellair Stream	310 000	Informal community - Inadequate sanitation
Aller River	400 000	Informal community - Inadequate sanitation
Umhlangaan River	240 000	Broken sewers
iSiphingo River	10 000 000; exceeding 100 000, 60% of the time	Broken sewers
uMngeni River	1 080 000	Informal community on the Palmiet River
uMlazi River	720 000	Broken sewers
Slangspruit	610 000 – 670 000	Within city boundaries

The Buffalo River in the Eastern Cape used to be pristine at its source, but due to urban developments and dense peri-urban and rural settlements, immense pressure now impacts the middle and lower reaches of the river. The river also supports about 570 000 people by providing approximately 500 m^3 water per annum for daily use (River Health Programme, 2004). Research has shown that contamination of the Buffalo River basin was caused by overpopulation in a small catchment with inadequate water resources. Natural high salinity levels as well as dysfunctional and overloaded sewage works and treatment facilities contributed to the pollution load. Contamination of the Buffalo River basin extends beyond the estuary, affecting marine water quality, which ultimately could render the seawater and thus beaches unfit for recreational use (River Health Programme, 2004).

The Berg River (Western Cape), which rises in the Franschhoek and Drakenstein mountains, flows northwards past Paarl, Wellington, Hermon and Gouda, where it is joined by the Klein Berg and Vier-en-Twintig Rivers. Water quality and habitat integrity in the Berg River and lower reaches of these branches have also been reduced due to urban development **[Figure 1.8(a)]**.

Figure 1.8(a) Water pollution in the upper Berg River (Paarl), Western Cape region (Kruger, 2004)

Faecal run-off from informal areas has decreased the quality of river water in the Berg River area to such an extent, that agricultural exports could be jeopardized. Storm water drainage pipes flow directly into the river carrying with it large amounts of human and synthetic waste materials. Research has also shown that improper sewage infrastructures and the complete lack of proper sewage removal facilities **[Figure 1.8(b)]**, are the main reasons for contamination experienced in the areas surrounding the river (Kruger, 2004).

Figure 1.8(b) Waste accumulation in an informal settlement near Paarl, South Africa

1.1.3 Sewage and Waste Waterworks

Even though South Africa's potable water is primarily stored in dams, the country's rivers and groundwater sources are steadily becoming more contaminated. Poor management, the inability of sewage treatment plants to deal with the waste capacity as well as subsequent blockages of sewage systems, result in the discharge of partially treated and untreated effluent into rivers. Algal blooms and unacceptably high concentrations of faecal bacteria then flourish in these river systems (South African River Health Programme, 2003).

In the River Health Report (2003), the uMngeni catchment was divided into six resource units, with several corresponding to the drainage areas of the major dams in the area, while the uMlazi catchment forms a resource unit on its own. A resource unit is a sub-catchment of the larger study area which was chosen as a unit of reporting because it is a sensible geographic unit for river management.

The Pietermaritzburg Resource Unit, which encompasses most of the water sources in the Pietermaritzburg area, is bordered by the city centre, residential and industrial suburbs as well as informal housing developments. As a result of urbanisation and high population densities, many areas had little or no sanitation and waste removal services, which raised concerns about water quality. On occasion it was also reported that the extensive network of sewers discharged their contents into the river systems **[Figure 1.9 (a)]**. These sewer discharges in the uMngeni region complicate the pollution issue and may lead to diseases as the rivers in the area serve domestic purposes **[Figure 1.9 (b)]**. The quality of the water was further compromised when storm water from the city streets flowed into the river. Even though industrial effluents and illegal discharges into the rivers are few, they do occur. It has been found that water flowing through the Henley Dam (situated in the uMsunduze catchment area) is unnaturally low in nutrients and contains phytoplankton not normally

found in local rivers. The dam has subsequently been decommissioned as a supply dam due to its impact on water quality (South African River Health Programme, 2003).

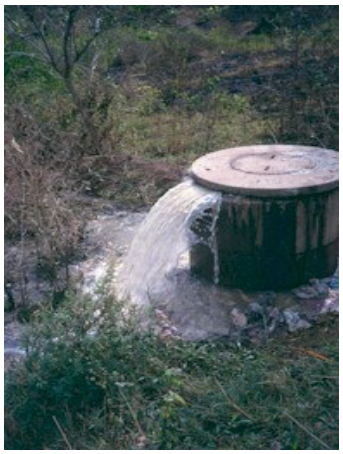
(a)   (b)

Figure 1.9 (a) An overflowing sewer system. (b) Inhabitants from the area using the rivers for domestic purposes (South African River Health Programme, 2003)

1.1.4 Industry and agriculture

Industrial waste which is either inadequately treated or not treated at all may also enter the river systems. This results in poor water quality, which poses a serious health risk for rural communities, since many households rely solely on untreated river water. Industrial factories located in the vicinity of the Pietermaritzburg Resource Unit also created major problems due to legal and illegal discharges into rivers **[Figure 1.10 (a and b)]**. Industries have however, proclaimed to incorporate strategies to reduce pollution from sewers, informal settlements, streets, illegal discharges, wastewater works and solid waste **[Figure 1.10 (c)]** (South African River Health Programme, 2003).

(a) (b) (c)

Figure 1.10 (a) Pollution from storm water drains in industrial suburbs, (b) Printing dye from nearby industrial facility is dumped into river, (c) Solid waste pollution along river (South African River Health Programme, 2003)

Municipal and winery effluents are the major causes of contamination in the Franschhoek valley [Western Cape, South Africa (SA)]. A lack of water flow from the Wemmershoek Dam, situated in the same area, results in severe and altered flow management downstream. The effects of agriculture also reduce river quality near the Tulbagh area (Western Cape, SA) where the water quality of the Berg River is poor. In addition, farming practices (riparian vegetation removal, bed modification, water abstraction) as well as alien vegetation (black wattle, river gum) impact the habitat reliability and flow of this river (State of the Rivers Report: Berg River, 2004).

1.2 WATERBORNE PATHOGENS

Several countries, such as those in the European Union and the United States have implemented treatment procedures, which adequately prevent waterborne disease outbreaks. Mechanisms such as advances in water treatment, source water protection and the real time monitoring of water quality are only some of the preventative measures that would or could be incorporated in areas where waterborne disease outbreaks occur (Allen *et al*., 2000). However, despite advances in preventing waterborne disease, severe outbreaks still occur. This not only happens in developing countries but also developing nations. Specific detection methods are therefore still needed to trace the origin of etiological agents, to identify "lapses" in water treatment and to identify new quality control processes and procedures.

Water serves as an inert carrier of infectious agents, such as the bacterial species *Salmonella*, *Campylobacter* and *Escherichia coli* (Gerba, 1996), as well as viruses, fungi, protozoa and helminths. The presence of these microbial agents in natural water sources presents a potentially significant human health risk. The young, elderly and immunocompromised are generally more susceptible as waterborne diseases may result in severe illness and even death (Theron & Cloete, 2002).

The World Health Organisation/United Nation Children's Fund's Global Water Supply and Sanitation Assessment 2000 Report, Water for People, estimates that approximately 6000 people, the majority of which are children, die every day due to water related diseases (WHO/UNICEF, 2008). In addition, diseases caused by waterborne pathogens, contribute to significant economical deficits, as the working community is affected by these diseases (Gerba, 1996).

The range of infectious waterborne diseases is continuously expanding, and pathogens once thought to be rare and insignificant to humans, have re-emerged as the cause of widespread disease. The incidence rate of cholera, for example, once thought to be a controlled disease, is rapidly increasing (Morse, 1995). This re-occurrence could possibly be ascribed to the lapse of public health measures. In addition, the re-emergence of diseases not only applies to developing countries, but also the industrialised world.

Waterborne outbreaks and the nature thereof have also changed drastically during the past 100 years. The change in the epidemiology and etiology of outbreaks are influenced by factors such as i) a decrease in individual cases and average number of cases; ii) an increase in the number of total outbreaks and of those in smaller community water systems; iii) a change in the effectiveness of predicting outbreaks by conventional testing methods and iv) a general change in the origin of diseases (Gradus, 1989).

1.2.1 Bacteria

1.2.1.1 *Helicobacter pylori*

Helicobacter pylori was referred to as *Campylobacter pylori* when, in 1982, it was cultured from the tissue of a stomach ulcer. An Australian physician described the organism as a Gram-negative, spiral-shaped bacterium, which shared biochemical and morphological characteristics with *Campylobacter* (Rusin *et al*., 2000). It was later revealed, through more detailed studies, that *C. pylori* should be classified in a new genus and in 1985, it was named *Helicobacter pylori*, which means spiral rod of the lower part of the stomach (Velásquez & Feirtag, 1999).

Helicobacter pylori has been implicated as the main cause of gastric disease, including peptic gastroduodenal ulcers, gastritis and even cancer (Ernst & Gold, 2000; Rusin *et al*., 2000). It produces large amounts of urease, the enzyme that converts urea to ammonia, which results in a high pH in the restricted region.

Humans have been indicated as the natural hosts of the organism, even though the reservoir for the organism has not been identified with certainty. Person-to-person transmission of *H. pylori* is probably the most common, but certainly not the only route of entry of the pathogen into the host (Rusin *et al*., 2000). Transmission may also involve other multiple pathways, such as zoonotic, iatrogenic, foodborne and water sources (Dunn *et al*., 1997; Van Duynhoven & de Jonge, 2001). The Polymerase Chain Reaction (PCR) has been used to detect *H. pylori* in faecal samples obtained from infected individuals with peptic ulcers, which suggests that the faecal-oral transmission is indeed possible (World Health Organisation, 2003a). This, therefore, supports the hypothesis that *H. pylori* is transmitted from person to person, as indicated by elevated prevalence rates of the pathogen among individuals living in institutions such as hostels and families with many members sharing the same sanitation facilities (Dunn *et al*., 1997; Goodman & Correa, 2000; Tindberg *et al*., 2001). It is recognised that the presence of *H. pylori* in the human stomach is especially prevalent among people living under poor hygienic conditions (Dubois, 1995; Bik *et al*., 2006). Furthermore, several studies have examined the possibility that *H. pylori* is waterborne, as *H. pylori*-specific DNA has previously been detected in water supplies

(Hegarty *et al*., 1999; Engstrand, 2001; Leclerc *et al*., 2002). A monoclonal antibody assay technique was used to successfully type the actively respiring bacteria in surface and groundwater samples in the USA. It was also discovered that its survival capacity in water might be related to the non-culturable coccoid form, which may persist for up to 20-30 days (Hegarty *et al*., 1999).

1.2.1.2 *Vibrio cholerae*

Vibrio cholerae is a motile, Gram-negative, curved rod that belongs to the family, *Vibrionaceae.* It is known to exist as the natural inhabitant of aquatic environments (Islam *et al*., 1994; Colwell, 1996) where it attaches to surfaces provided by plants, filamentous green algae, copepods (zooplankton), crustaceans and insects (Colwell, 1996). *Vibrio cholerae* is a facultative human pathogen and has been recognised as the causative agent of the well-known and well-studied human intestinal disease, cholera. The transmission of *V. cholerae* can be ascribed to poor sanitation practices in densely populated areas, where endemic toxigenic strains are the source of occasional outbreaks, due to the contamination of drinking water and/or improper food preparation. However, water contaminated with free-living *V. cholerae* cells are said to be the main origin of epidemics, followed to a lesser extent by contaminated food. Research has shown that there also seems to be a correlation between cholera outbreaks and the seasonal occurrence of algal blooms (Colwell, 1996). However, substantially more evidence is needed to assume that such occurrences lead to an enrichment of toxigenic *V. cholerae* strains, which are responsible for cholera outbreaks.

In the period December 2000 to February 2001, Swaziland's four regions reported a total of 819 cholera cases with 12 deaths recorded. The cause of the epidemic was suspected as being contaminated river water and unprotected springs which resulted from excessive rains. Even though no clear evidence could be found, it was suspected that a lack of economic resources might have influenced the cholera epidemic. When compared to previous outbreaks (in 1982 and 1992), it seemed unclear as to why the disease spread so rapidly. The situation therefore prompted questions as to whether emerging and re-emerging communicable diseases could influence the prevalence and extent of diarrhoeal disease in Swaziland's sub-region (World Health Organisation, 2001).

In South Africa (SA) cholera has been associated with the restricted access to good quality water and sanitation facilities. Even though the fatality rate was low during the 2000/2001-cholera epidemic in KwaZulu Natal (SA) (< 0.4%), 116 170 cases of infection were reported. During this period, tests showed that water supplied by municipalities was safe for daily use and consumption, while people relying on drinking water directly from the rivers were especially vulnerable (South African River Health Programme, 2003). In 2003 a

cholera outbreak was reported in the Mpumalanga region of South Africa, where 174 confirmed cases and three deaths were reported. The outbreak affected 27 areas bordering Swaziland and Mozambique. Mpumalanga was the third province, after the Eastern Cape and Kwa-Zulu Natal. The collective number of cholera outbreaks reported in South Africa from January to March 2003 was 2 362 (World Health Organisation, 2003a).

More recently, a cholera outbreak led to 1 298 deaths in Angola with tens of thousands infected. Contaminated drinking water, poor sanitation and dense urban living were regarded as the main cause of the outbreak, which began in February 2006 in Luanda. Communities in this rapidly growing capital live in desecrated, trash-filled slums with no reliable sources of clean water. Efforts to control the disease were hindered by the apparent slow response from the Angolan government and the World Health Organisation. Treatment centres and organisations such as Doctors without Borders, rather than government clinics or hospitals, treated more than 14 000 people affected by the cholera outbreak. In addition, little has since been done to improve conditions and supply safe drinking water to informal settlement dwellers (Timberg, 2006).

During November 2008, 6072 cases of cholera infections and 294 subsequent deaths were reported in nine provinces of Zimbabwe. In the South African province of Limpopo during the same time period, 187 cholera cases were treated and three deaths (one South African and two Zimbabweans) were reported. Cholera cases have also been reported in Zambia and Mozambique where people travelled through the Beitbridge area in Zimbabwe (Department of Health, 2008). An inadequate supply of clean drinking water and poor levels of hygiene have been implicated as the reasons for these outbreaks. According to the World Heath Organisation (WHO), 16 141 suspected cholera infections, with 775 reported deaths, have been documented in Zimbabwe since December 2008, with a fatality rate estimated at 4.8%. Serious challenges also exist with controlling the current state of the outbreak as 460 cholera cases and nine deaths have been reported in South Africa, mostly in the areas bordering Zimbabwe (WHO, 2008).

1.2.1.3 Salmonella

Salmonella are Gram-negative rods, which form part of the family *Enterobacteriaceae.* More than 2 000 serotypes have already been identified, all of which are known to be pathogenic to humans. Even though salmonellosis is primarily related to foodborne illnesses (infected beef and poultry), surface waters into which domestic waste or sewage, meat processing waste and stockyard wastes, are disposed, may lead to accidental water contamination and thus results in waterborne disease outbreaks. Symptoms associated with the disease include mild to severe gastroenteritis as well as in severe cases, death of infected individuals (Rusin *et al*., 2000).

Typhoid fever infections are encountered worldwide, but are primarily found in the developing countries where sanitary conditions are poor. It is prevalent on the Indian subcontinent, Southeast and Far East Asia, the Middle East, Africa, Central America, and South America and still affects approximately 12.5 million people each year (Corales, 2004).

Salmonella typhi is the causative agent of typhoid fever and remains endemic to many parts of South Africa, including Northern Gauteng, Kwa-Zulu Natal and the Transkei. It infects only humans and transmission occurs through consumption of contaminated food and beverages handled by persons shedding *S. typhi* in their stool. To a lesser extent, carriers may also shed the bacterium in their urine (Corales, 2004; Brusch *et al*., 2006). Sewage containing *S. typhi* can also contaminate water sources, while shellfish from polluted water may cause outbreaks. Canned meat produced, using contaminated water supplies, may also contribute to possible outbreaks (Corales, 2004; Brusch *et al*., 2006).

In 1996, an estimated amount of 600 cases of diarrhoea was reported in Gideon, Missouri. This outbreak was associated with an undisinfected groundwater supply and of the cases reported, 31 were caused by laboratory confirmed *S. typhimurium*. Fifteen hospitalisations and seven deaths in nursing homes, with four culture-confirmed cases for *S. typhimurium*, were also observed. Taste and odour complaints in the water distribution system led to the investigation and subsequent flushing of all hydrants. It was discovered that a sharp temperature drop in the system, caused stagnant and contaminated water levels in the storage tank to mix, which stirred up sediments contaminated with pathogens. Water inside the storage tanks was black and turbid and the presence of bird faeces was indicated as the probable cause of contamination (Clark *et al*., 1996). In another outbreak *Salmonella* serotype Saintpaul was indicated as the causative agent of 28 cases of gastroenteritis infections amongst over 200 workers at a large construction site in Central Queensland, Australia. The organism was isolated from a drinking water supply system and identified after the notification of similar cases by doctors from different towns during March 1999. The contaminated water system was identified by means of environmental sampling and confirmed by epidemiological testing. It was concluded that the original carriers of the organism were frogs and/or mice inhabiting the supply tanks (Taylor *et al*., 2000).

1.2.1.4 *Legionella*

Legionella, a Gram-negative bacillus, causes legionnares' disease and Pontiac fever. Symptoms associated with legionnares' disease include fever, coughing, abdominal pain and diarrhoea. Pneumonia is usually the result of the disease as respiratory failure may occur, with the disease also having a fatality rate of 15%. Pontiac fever is not associated with pneumonia or death and patients usually recover within two to five days after infection. The reservoir of the organism is primarily water, as it has been isolated from creeks, ponds and

the soils on riverbanks. The bacterium has also been known to survive in tap water and distilled water (Rusin *et al*., 2000). A study conducted by Zacheus and Martikainen (1994) indicated the presence of *L. pneumophila* in domestic potable water samples. It was found that 30% of hot water systems contained *Legionella*. The average number of *L. pneumophila* was 2.7 x 10^3 colony forming units/ml (or CFU/ml) within a range of 50 to 3.2 x 10^5 CFU/ml. *Legionella* was also isolated from hot water taps and showerheads. In the same study, *L. neumophila* was isolated from hot water systems which received chlorinated groundwater, unchlorinated groundwater and chlorinated surface water, respectively.

Amoebae and other protozoa have proved to be natural hosts and "amplifiers" of *Legionella* in the environment (Atlas, 1999; Swanson & Hammer, 2000) as its environmental survival capability and pathogenicity (virulence) is enhanced by growth within protozoa. Other factors such as their ability to enter a viable-but-non-culturable (VBNC) state and their occurrence within biofilms, also play a role in their survival and proliferation. Biofilms serve as ecological niches as well as the ultimate sources to which the disease can be traced. These factors, i.e. its VBNC state, association with protozoa as well as biofilms, complicate the detection and epidemiological investigation of *Legionella* (Atlas, 1999).

Worldwide, a variety of techniques are employed for culturing *Legionella* from environmental samples, a problem that has been addressed in various countries through standardised protocols (Australian Standard AS, 1991; Draft International Standard, 1996). In Southern Africa however, no such standards have been developed or are available. Local laboratories have therefore been using different detection methods, which result in a number of contradictory results regarding the quality of industrial water distribution systems. A study to provide Southern African laboratories and industries with appropriate guidelines for the development of detection methods for *Legionella* has however, been initiated (Bartie *et al*., 2003).

1.2.1.5 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a motile Gram-negative, bacillus that is either straight or slightly curved. The organism has been reported to be an important opportunistic human pathogen (it plays an important role in cystic fibrosis), which grows aerobically and does not ferment sugars. *Pseudomonas aeruginosa* is widely distributed in nature, particularly in water, soil and vegetation. It has simple nutritional requirements and tolerates a wide range of environmental conditions (Skerrett, 1999; Leclerc, 2003).

Pseudomonas aeruginosa is the most common source of infection in burn patients. It is also the cause of nosocomial pneumonia [pathogen responsible for ~16% of infections, (Wiblin, 1997)] as well as nosocomial urinary tract infections (pathogen responsible for 9.9% of infections) (Jain & Singh, 2007). *Pseudomonas aeruginosa* is also one of the leading

causes of surgical wound (Kluytmans, 1997; Todar, 2008) and bloodstream infections (Gordon *et al.,* 1998; Schutze *et al*., 2004). Water-related reservoirs that are commonly a source of contamination include swimming pools, whirl-pools, hot tubs and contact lens solutions. People with cystic fibrosis are at risk, but the cause of infection is as yet, unknown (Rusin *et al*., 2000).

Pseudomonas spp. were isolated from the Plankenburg River that runs through the town of Stellenbosch, in the Western Cape (South Africa) during a study conducted by Barnes (2003). These species were isolated at several sampling points along the river from water and biofilm samples obtained downstream from an informal settlement and where the river passes through an industrial area.

1.2.1.6 *Campylobacter*

Campylobacter is a Gram-negative curved rod that is relatively fragile and susceptible to environmental conditions. It was initially regarded as an animal pathogen, as it caused spontaneous abortion and enteritis in sheep and cattle. Since then, several *Campylobacter* species which cause disease in humans, have been identified. Common species include *C. jejuni, C. coli, C. lardis* and *C. fetus*. *Campylobacter jejuni* has however, been isolated in most reported cases of *Campylobacter* infections (Rusin *et al*., 2000).

Campylobacter jejuni is commonly found in the environment and can be isolated from the faeces of humans, livestock and wildlife. Surface water, stream sediment and sewage effluents have also been identified as reservoirs of *C. jejuni* (Altekruse *et al*., 1999). Primary routes of transmission appear to be ingestion of contaminated foods, or ingestion of untreated surface water (World Health Organisation, 2000). *Campylobacter jejuni* can survive for limited time periods in streams and ponds (Rusin *et al*., 2000) and a minimum of 500 to 800 organisms are sufficient to cause disease in humans (Black *et al*., 1988; Kothary & Babu, 2001). *Campylobacter jejuni* infections cause diarrhoea with fever, abdominal pain, nausea, headaches and muscle pain. Illness occurs two to five days after ingestion of contaminated food or water and usually lasts between seven and ten days (Rusin *et al*., 2000).

In South Africa statistics on the prevalence of *C. jejuni* outbreaks are not common mainly due to the fact that it is not a notifiable disease. Compared to other countries, where the organism and its infections are more frequently detected in the affluent communities, its prevalence in South Africa appears more in the lower socio-economic population groups (Department of Agriculture, 2000).

1.2.1.7 *Yersinia*

Yersinia enterolitica is a small rod-shaped Gram-negative bacterium which has previously been isolated from environmental (ponds and lakes) and food (meat, ice creams and milk) sources. Infective symptoms such as diarrhoea and vomiting which results from gastroenteritis, usually start 24 to 48 hours upon ingestion of contaminated food or water. Fever and abdominal pain can also be experienced and the infection may resemble the signs of appendicitis. *Yersinia* may also however, cause infection of other areas of the body such as wounds, joints and the urinary tract (Rusin *et al*., 2000).

This organism is widespread in water (i.e. streams and lakes) and thereby better able to adapt and survive in stream water (Terzieva & McFeters, 1991; Perdek *et al*., 2003). In the United States it is estimated that 3 000 to 20 000 humans are infected with *Yersinia* per year. In North America however, water is not considered as a major source of *Yersinia* infection. Due to the low number of reported human cases of yersiniosis this pathogen could be regarded as a waterborne disease of secondary importance. The importance of its pathogenicity however, lies in the fact that the organism could affect water sources, used in the food processing industry.

In a study conducted by Barnes (2003), *Yersinia* spp. was isolated from materials collected in the Plankenburg River, Stellenbosch (SA). The organism was one of a group of micro-organisms isolated from biofilms on stones obtained from an area downstream from the informal settlement of Kayamandi in Stellenbosch.

1.2.1.8 *Escherichia coli*

Escherichia coli is a Gram-negative rod found in the gastrointestinal tract of all warm-blooded animals where it forms part of the intestinal flora. Several strains however, are capable of causing gastrointestinal disease. Five classes of pathogenic *E. coli* are recognised and include enterotoxigenic, enteropathogenic, enteroinvasive, enterohaemorrhagic or verocytotoxic-producing and enteroaggregative *E. coli* (Kuntz & Kuntz, 1999; Rusin *et al*. 2000).

It has been reported that enterotoxigenic *E. coli* causes traveller's diarrhoea in persons from industrialised countries who visit developing countries. Furthermore, this particular strain of *E. coli* has been identified as the cause of diarrhoea in infants and children in under developed countries. The disease is caused by two toxins, namely the heat-labile toxin and the heat-stable toxin. Enterotoxigenic *E. coli* are species-specific which explains why humans are the carriers of the strains infecting other human hosts. Upon ingestion and subsequent incubation of 10 to 72 hours, symptoms such as cramps, vomiting, profuse diarrhoea and dehydration are experienced, which could last from three to five days. Only a

few outbreaks in the United States have been associated with enterotoxigenic *E. coli*, of which one resulted from the consumption of water contaminated with human sewage (Rusin *et al*., 2000).

Diarrhoeal disease caused by enteropathogenic *E. coli* is virtually confined to infants less than one year of age and it is the oldest recognised strain of *E. coli* to cause diarrhoea. Symptoms of infection include watery diarrhoea with mucus, fever and dehydration, which can be severe and result in a fatality rate of up to 50%. Even though the disease is under control in North America and Europe, it remains a major cause of infantile diarrhoea in South America, Africa and Asia (Smith & Cheasty, 1998; Rusin *et al*., 2000).

Enteroinvasive *E. coli* infections resemble that of *Shigella* infections. The illness begins with severe abdominal cramps, watery stools and fever. The disease targets all age groups and is self-limiting with no known complications. It is unclear which foods transmit enteroinvasive *E. coli*, but any food contaminated with human faeces from an infected individual could either directly or through contaminated water, cause disease. Upon ingestion, enteroinvasive *E. coli* may lead to intracellular bacterial multiplication, spread to adjacent cells, cause cell death and eventually inflammation and ulceration of the colonic mucosa (Menard *et al*., 1996; Rusin *et al*., 2000).

The enterohaemorrhagic or verocytotoxic strain of *E. coli* was first described in 1982. A multistate epidemic of haemorrhagic colitis occurred in the United States and was shown to be due to a specific serotype known as *E. coli* O157: H7. Two toxins, verotoxins I and II, are produced by *E. coli*, which closely resembles the toxin produced by *Shigella dysenteriae*. Symptoms of the illness include severe cramping and diarrhoea, which is initially watery and then becomes extremely bloody (Riley *et al*., 1983; Rusin *et al*., 2000). The disease could last up to eight days and is usually self-limiting. Younger victims of the disease could develop haemolytic-uremic syndrome, resulting in renal failure and haemolytic anaemia. Permanent kidney failure could also result. In older patients additional symptoms include fever and neurological effects, which could lead to thrombotic thrombocytopenic purpura. In the elderly, the illness can have a mortality rate of up to 50% (Smith & Cheasty, 1998).

Enteroaggregative *E. coli* has been implicated as the causative agent of diarrhoea in children in developing countries and could also result in major illness and death. Disease outbreaks have also indicated that this organism is responsible for foodborne illness in industrialised countries. This organism adheres to the intestinal mucosa and involves the release of enterotoxins and cytotoxins resulting in secretory diarrhoea and mucosal damage (Nataro *et al*., 1998).

The first case of enterohaemorrhagic *E. coli* in South Africa was diagnosed in 1990 in an elderly man who had undergone surgery for lower gastrointestinal bleeding (Browning *et al*., 1990). Effer *et al.* (1992) reported a large outbreak of *E. coli* O157:H7 infections in Swaziland (from October to November 1992) where a total of 40 912 patients

under the age of five experienced diarrhoeal complications. Consumption of beef and untreated water were identified as the sources of the illness. Muller *et al.* (2001) also investigated the occurrence of *E. coli* O157:H7 in selected water samples in South Africa. The 204 samples screened were selected from 15 different sites where water was used for human consumption. Even though none of the tested samples contained *E. coli* O157:H7, a few did however, exhibit enterohaemorrhagic *E. coli* virulence properties, such as Shiga toxin 1 and 2 and enterohaemolysin, which could cause serious health problems if ingested.

In 2005, medical researchers at the University of Stellenbosch, conducted intense research on water from rivers in the Western Cape region of South Africa. Rivers were extremely polluted by contaminants from human waste in nearby informal settlements. According to a study conducted in 2004, at least four different variants of *E. coli*, which causes diarrhoea, urinary tract infections and haemolytic uremic syndrome, were identified (Shaw, 2005).

1.2.1.9 Indicator organisms

The examination of environmental water samples for the presence of pathogens is generally considered to be time- and cost-consuming. It is therefore advisable to examine environmental water sources for indicator organisms whose presence is also indicative of certain pathogenic organisms. Certain non-pathogenic bacteria are used as indicators, as they can be easily isolated and enumerated and they occur in the faeces of all warm-blooded animals. The presence of these non-pathogenic bacteria in water therefore confirms the occurrence of faecal contamination, which could suggest that enteric pathogens may also exist in the water. The presence of coliform bacteria such as *Escherichia, Citrobacter, Enterobacter* and *Klebsiella* are usually found in numbers that are relative to the degree of faecal pollution. Coliform bacteria are more robust than disease-causing bacteria and their absence is usually indicative of bacteriologically safe water.

Coliform bacteria are also effective as indicators because they are easily detectable and are used as a standard for assessing the microbial numbers. The presence or absence of this group of organisms has been recognised by many countries as indicators of faecal contamination in drinking water, recreational bathing water, and wastewater discharges as well as various foods. The indicator organisms of faecal contamination should however, meet certain criteria as stipulated in **Table 1.2** (Gerba, 2000a).

It has however, been impossible for a single indicator organism to meet all the criteria as set out in **Table 1.2**. Various other microbial groups have therefore been suggested and adopted to fulfil the role of indicator organism (Gerba, 2000a). A fixed ratio of acceptable microbial numbers in 100 ml of drinking water ensures the safety of that water for human consumption (Gerba, 2000a).

Table 1.2 Criteria for the selection of an ideal indicator organism (Gerba, 2000a)

Criteria
The organism should be present in all types of water
The organism should be present whenever enteric pathogens are present
The organism should have a reasonably longer survival time than the hardiest enteric pathogen
The organism should not multiply in water sources
The testing method should be easy to perform
The density of the indicator organism should have some direct relationship to the degree of faecal pollution
The organism should be a member of the intestinal microflora of warm-blooded animals

Table 1.3 indicates the recommended maximum limits for indicator organisms in South African water.

Detection of pathogens however, is complex, expensive and time-consuming and it is advisable to monitor and control water quality on the basis of concentration of indicator organisms, rather than the pathogens themselves [DWAF, 1996c].

Table 1.3 Recommended guidelines for indicator organisms in South African waters [DWAF, 1996 (c)]

Organism	CFU per 100 ml
Total coliforms	≤5 (d)
Faecal coliforms	≤2000 (r); 10000 (i); 0 (d)
Enterococci (Faecal streptococci)	≤30 (full contact (r)]; ≤ 230 [interm. contact (r)]
Clostridium perfringens	No numerical guideline indicated
Staphylococcus (coagulase positive)	No numerical guideline indicated
Pseudomonas aeruginosa	No numerical guideline indicated
Acid-fast bacteria	No numerical guideline indicated
Coliphages	≤20 [full contact (r)]; ≤1 (d)
Escherichia coli	≤130 (r); 1 (i)
Bacteroides	No numerical guideline indicated

[d]Where water is used for domestic purposes, such as cooking, drinking etc.
[r]Where water is used for recreational purposes such as swimming, bathing etc.
[i]Where water is used for irrigational purposes

1.2.2 Viruses

Classified under the family *Reoviridae*, rotaviruses have a genome consisting of 11 double stranded RNA (dsRNA) segments surrounded by a distinctive two-layered protein capsid. Its particles are approximately 70 nm in diameter and three serological groups have been identified in humans and animals (Rusin *et al*., 2000). The route of transmission is by the faecal-oral route, either by environmental contact with contaminated surfaces or by ingestion of contaminated food or water. Rotaviruses have been known to be the most common cause of viral gastroenteritis in humans, especially acute infantile gastroenteritis (Kapikian & Chanock, 1996). In 1981, rotaviruses were linked to an outbreak of gastroenteritis in Colorado in the United States. Contaminated municipal water was indicated as the primary cause of viral infections. Group B rotaviruses have also been implicated as the causative agent of outbreaks involving millions of people in China in 1982 (Rusin *et al*., 2000).

Adenoviruses, on the other hand, belong to the Adenoviridae family. These viruses are dsDNA icosahedral viruses approximately 70 nm in diameter. Adenoviruses can replicate in the respiratory tract, the eye mucosa, intestinal tract, urinary bladder and liver (Rusin *et al*., 2000). They infect both humans and animals and are primarily spread through respiratory droplets, but can also be spread via faecal routes. Even though not many cases of waterborne adenovirus infections have been reported, the virus has previously been isolated from sewage, rivers, lakes, groundwater, drinking water and recreational bathing water (swimming pools are the major source) (Rosen, 2000).

Viral *Hepatitis* usually refers to infections caused by agents who attack the tissues of the liver. At least eight different *Hepatitis* viruses have been identified, including A, B, C, D and E strains. Even though the majority of infections are asymptomatic, common clinical features include symptoms such as anorexia, nausea, vomiting, right upper quadrant pain and elevated levels of the liver enzymes, aspartate aminotransferase and alanine aminotransferase. The hallmark of *Hepatitis* infections is jaundice, which tends to develop later (Hardie, 1999). *Hepatitis A* is a small, non-enveloped icosahedral particle, 27 nm in diameter containing a single-stranded RNA (ssRNA) genome (Hardie, 1999). The symptoms may range in severity from a mild illness lasting from one to two weeks, to a severely disabling disease lasting several months. The virus can be transmitted through direct person-to-person contact, exposure to contaminated water, ice or shellfish harvested from sewage-contaminated water (Hardie, 1999; Rusin *et al*., 2000). The *Hepatitis E* virus (HEV) has a diameter of 32 to 34 nm and consists of an ssRNA genome of approximately 7.5 kb (Krawczynski *et al*., 2000). It has been identified as the leading cause of acute viral hepatitis among young and middle-aged adults in the developing countries. Outbreaks have been linked to the consumption of sewage-polluted water and symptoms of infection characterised by jaundice, malaise, anorexia, abdominal pain and fever (Rusin *et al*., 2000). The first case

of *Hepatitis E* infection in Southern Africa was recorded in Maun, northern Botswana in 1985, where a waterborne outbreak led to 273 infected cases and at least four deaths (Byskov *et al*., 1989). An earlier *Hepatitis* outbreak, which occurred in Namibia in 1983 among people living in settlements lacking potable water and proper waste disposal facilities, could only be characterised and identified by molecular methods as being the epidemic *Hepatitis E* virus in the year 2000 (Isaacson *et al*., 2000).

The *Norwalk-like virus* belongs to the human enteric members of the family *Caliciviridae* and has also been identified as small round-structured viruses (Lodder *et al*., 1999). Small round-structured viruses have been implicated in and associated with food- and waterborne outbreaks of acute non-bacterial gastroenteritis (Kapikian & Chanock, 1996). Once positively identified as small round-structured viruses, these viruses could be linked to a number of outbreaks of gastroenteritis, which are recognized as the causative agents of diarrhoeal diseases worldwide (Dedman *et al*., 1998). Small round-structured viruses have been linked to municipal drinking water contaminated with sewage as well as recreational bathing water (Rosen, 2000).

In South Africa during a study conducted by Steele *et al.* (1988) at a Ga-Rankuwa Hospital, a large number of cases involving a diarrhoeal disease could not be associated with any identifiable infectious agent. Further investigation led to the implication of small round-structured viruses. The virus was found to be prevalent in the local population of that particular area and could be linked to cases of diarrhoea (Smit *et al*., 1997).

1.2.3 Microsporidia

Nägeli in 1857 first identified *Microsporidia* as pathogens in silkworms. In 1959, Matsubayashi *et al.* described the first human case of *Microsporidia*. Canals and other surface waters prove to be the primary environmental sources of this organism. Detection of human microsporidial species in environmental water samples has been reported in groundwater, indicating that the organism may be transmitted in the subsurface, where they can contaminate drinking wells (Dowd *et al*., 1998; Cotruvo *et al*., 2004).

In another study, samples of ground- and surface water were collected in France and examined by light microscopy and the Polymerase Chain Reaction (PCR). Even though no microsporidia spores were isolated from the groundwater samples, both techniques used identified the presence of microsporidia in a sample obtained from the river Seine and from three samples obtained from the river Loire. Deoxyribonucleic acid sequencing of the PCR product obtained from the sample from the river Seine, had a 98% homology to the microsporidium, *Encephalitozoon bieneusi*, thereby confirming its presence in the river Seine (Sparfel *et al*., 1997). This parasite is regarded as one of the most opportunistic pathogens in immunocompromised patients and is involved in various human diseases, which include

intestinal, ocular, sinus, pulmonary, and muscular and renal diseases in both immunocompetent and immunocompromised patients (Weber *et al*., 1994; Mathis *et al*., 2005).

1.2.4 Protozoa

The genus name *Cryptosporidium* was proposed by Tyzzer in 1907 for a protozoan parasite frequently found in mice. In 1912, a new species *Cryptosporidium parvum,* was also identified in the intestine of mice. In 1976 however, the first two cases of human cryptosporidiosis were reported in two separate groups (Meisel, 1976). Even though only eight cases of human infections occurred before 1982 (Adal *et al*., 1995) this protozoan parasite has attracted increasing interest due to it being the causative agent of several serious waterborne outbreaks (Fayer *et al*., 2000).

Factors such as sewage, muck or faeces from animals and faults in operation procedures during water preparation, have been identified as reasons for *Cryptosporidium* contamination of drinking water (Franzen & Müller, 1999). In addition, *Cryptosporidium* oocysts tend to be resistant to the common disinfectants used in drinking water treatments and have consequently become a major concern to public health and the drinking water industry (Xiao *et al*., 2000). In 1993, a massive *Cryptosporidium* outbreak in Milwaukee, in the United States, was caused by oocysts that passed through the filtration system of the city's water treatment plant. The source of the water was Lake Michigan and the two rivers discharging into the lake were swelled by heavy rains and snow runoffs thereby contaminating the rivers with agricultural wastes (Cicirello *et al*., 1997). In 1994, *Cryptosporidium* infected 78 people after exposure to contaminated tap water in Las Vegas, Nevada, in the Lake Mead watershed area. Sixty-one of the infected cases were immunocompromised individuals with HIV-infection. Many of the affected individuals died as a result of the infection (Goldstein *et al*., 1996). In 2000, a cryptosporidiosis outbreak was investigated by the Ohio Department of Health where more than 700 people were infected. After epidemiological and environmental studies were performed, recreational swimming pool water was indicated as the source of both human and bovine genotypes of *Cryptosporidium parvum* (Mathieu *et al*., 2004).

As with *Cryptosporidium*, *Giardia* has also emerged as a waterborne pathogen of concern for public health. This parasite is part of the pathogenic protozoa commonly found in the gastrointestinal tract and infects the small intestine in humans and other mammals, causing giardiasis (Cook, 1995; Nichols, 2000). Symptoms of giardiasis include diarrhoea, stomach cramps, fatigue and nausea. Surveys showed that *Giardia* cysts as well as *Cryptosporidium* oocysts were easily found in faecal specimens of feeding animals and in raw surface water samples (Hsu *et al*., 2000). The effective means of control of these

pathogenic waterborne parasites generally requires a multiple approach, which includes effective source protection, optimised treatment and an excellent drinking water distribution system. *Giardia* cysts can survive in the environment for extended periods. In 1989, DeRegnier *et al*. suspended *Giardia muris* cysts, a species that normally infects mice but which is often used as a model for *G. lamblia*, in a lake in Minneapolis and river water from the Mississippi River. It was found that at a depth of 375 cm and a temperature of 19.2±1.3ºC, cysts survived for 28 days in lake water. In the river water cysts remained viable for 56 days at a temperature of 6.6±0.4ºC and a depth of 750 cm.

1.2.5 Helminths

Helminths form part of the broader class of parasites, which include the roundworms, flatworms, tapeworms and flukes. They are complex, multicellular organisms that contain organs as well as tissues. These parasites usually develop in soil or to complete their lifecycle, develop within an intermediate host (Rusin *et al*., 2000). They can also be isolated from water, soil and contaminated foods. Helminth infections have immense impacts on the health and economy of developed as well as developing countries such as Africa, Asia, Central and South America. Jordan (1985) stated that schistosomiasis could be regarded as the most important water-based form of helminth infection and in 2006 Steinmann *et al*. confirmed its involvement in water-related illnesses. Transmission of schistosomiasis involves larval penetration of any parts of the skin exposed to water (Sturrock, 1993). In South Africa, schistosomiasis is restricted to the eastern and northern regions of the country, such as the provinces of Mpumalanga and KwaZulu-Natal. Infection is associated with an absence of piped water, good recreation and sanitary facilities, and therefore the foci of endemic areas are expected to be rural (Coopan *et al*., 1986).

Wolmarans (2007) evaluated the presence of the potential schistosome intermediate hosts, snails, in the Nwanedzi River catchment area. The study was performed to monitor or assess the prevalence of schistosome infections in humans in this endemic area of the Limpopo Province. Of the 35 habitats surveyed, *Bulinus globosus* was found in 23 habitats, while *Biomphalaria pfeifferi* was found in ten habitats. The prevalence of *Schistosome haematobium* and *Schistosome mansoni* infections were analysed by collecting stool and urine samples from 699 children from selected schools in the area. Both parasites were present in 62% of the male and 73% of the female participants. *Schistosome haematobium* alone was present in 8% males and 15% females, while 9% males and 19% females were infected with *S. mansoni*.

1.2.6 The Viable-but-non-culturable state (VBNC) of bacteria

Research has shown that certain environmental factors including temperature, pH, salinity and osmotic pressure, may lead to organisms entering a viable-but-non-culturable state (VBNC). It is important to note however, that after a period of starvation bacteria usually respond rapidly to nutrients when re-cultured, while cells in a VBNC state cannot grow on conventional culture plates. A VBNC state has been observed more often with the Gram-negative bacterial group, which represents the *Enterobacteriaceae*, *Vibrionaceae* (including *Aeromonas*) and genera of *Campylobacter*, *Helicobacter* and *Legionella*. This microbial state however, is still a phenomenon that is continually being researched especially where bacteria representative of aquatic habitats are concerned (World Health Organisation, 2003a).

It is widely recognised and accepted that conventional plate counts do not represent the total number of bacteria. In the late 1970's, non-culturable methods such as microscopy and ATP determination were introduced to observe cell viability. This demonstrated that many of these unculturable cells were indeed viable and capable of active metabolism and respiration. Fluorochromes such as Acridine Orange and the BacLight™ Viability probe, in conjunction with epifluorescence microscopy and flow cytometry respectively, are thus more commonly used, as they provide more accurate total cell counts in environmental samples (Ălvarez-Barrientos *et al*., 2000).

Another technique that has proven to be successful for the detection of micro-organisms in its VBNC state is adenosine tri-phosphate (ATP) bioluminescence, where cell viability assessment can be conducted by the detection of adenosine tri-phosphate (ATP) levels. As the ATP concentration is representative of the total biomass or viable population of the community (Van der Kooij & Veenendaal, 1993), ATP monitoring or ATP concentration determination can also be used in measuring bacterial population density ratios such as the biofilm formation potential and biofilm formation rate of water (Vrouwenvelder *et al*., 1997; Khan, 2004). In a study conducted by Khan (2004) ATP concentrations was used to determine biomass suspensions released from biofilm monitor rings, in a water treatment plant in Düsseldorf, Germany. It was found that whereas the heterotrophic plate count (HPC) only represents a fraction of the active micro-organisms present in the biomass suspension samples, cell numbers based on the ATP measurements represented a greater percentage of the total active micro-organisms present in these biomass samples.

Even though ATP bioluminescence has proved to be a complicated technique when used in complex systems obtained from food or water sources (ATP from different cell sources could be indistinguishable) (Schram, 1991), some researchers have used the technique as an indication of viability in *H. pylori* (Sörberg *et al*., 1996 and 1997; Velázquez & Feirtag, 1999). It was found that the determination of the ATP levels not only offer an alternative method to the Polymerase Chain Reaction technique (PCR) and deoxyribonucleic

acid (DNA) probing, it is also simpler, more economical and faster than current detection methods (Velázquez & Feirtag, 1999).

1.3 WATER QUALITY GUIDELINES

Organisational bodies such as the World Health Organisation (WHO) and The United Nations Children's Fund (UNICEF) have for many years been concerned with the health aspects associated with water source management. Research is constantly being conducted and as a result documents concerning the safety and state of the water environment and its importance to health are published regularly. According to the WHO, the most frequent adverse health deficits associated with exposure to faecally contaminated recreational water, are enteric diseases, such as gastroenteritis and diarrhoea, amongst others (World Health Organisation, 2001). A cause-effect relationship between faecal or bather-derived pollution and acute febrile respiratory illness, which is more severe than gastroenteritis, has also been identified (World Health Organisation, 2006).

For a recreational water environment to be classified as contaminated, a combination of evidential factors should be recognised or be present. The degree to which human excreta contaminates water entities should also be compared to counts of suitable faecal index bacteria standards, before the water source can be regarded as unsafe or contaminated. Sewage and river discharges as well as bather contamination are regarded as the three most important factors that influence the faecal contamination of recreational water environments. In microbial water quality assessments, the sampling programme should also be representative of a range of conditions, and water samples should be routinely collected during environmental and sanitary inspection. It is also important that a sufficient number of samples be collected in order to accurately estimate the possible microbial numbers to which recreational water users are exposed (World Health Organisation, 2003b).

River water is a multipurpose water source that serves domestic, recreational and agricultural uses in most areas of the world as well as in South Africa (DWAF, 1998). In South Africa two organisations, namely the South African Bureau of Standards (SABS) and Department of Water Affairs and Forestry (DWAF), are recognised for establishing and monitoring the quality of water sources. The SABS was established in terms of the Standards Act, 1945 (Act No. 24 of 1945). It contributes to the economic growth of not only South Africa but also Africa as a whole and ultimately aims to protect consumers and the environment by promoting uncompromised quality of products and services. The mission of DWAF is to ensure that the quality of water resources remains suitable for recognised water uses and that the viability of aquatic ecosystems is maintained and protected. The Department of Water Affairs and Forestry recognises four categories of water used in the

South African Water Act, which includes water for domestic, industrial, agricultural and recreational purposes.

It is a known fact that the quality of South Africa's available water resources, such as its rivers, are fast deteriorating. They do not meet the European and international minimum standards and the situation is not likely to improve in the near future. The SABS addresses only 27 parameters of water quality as opposed to the 70 presented by their European counterparts. **Table 1.4** compares the SABS's guidelines for metal and trace elements in water (SABS, 1984) to that of the European Economic Community (EEC, 1996) and Health Canada (Guidelines for Canadian Drinking Water Quality – CDWQ, 2007). The presence of these dangerous metals in concentrations higher than stipulated, raises concern about South Africa's ability to continue to produce water of a good quality and thus sustain life.

Microbial contamination of South African rivers, as in many other countries, may be strongly influenced by factors such as rainfall, leading to relatively short periods of elevated faecal pollution. Contamination of recreational water sources may lead to health problems due to the presence of infectious micro-organisms, which are derived from human sewage or animal sources (World Health Organisation, 2003a). The number of micro-organisms that may cause infection or disease depends upon the specific pathogen, the form in which it is encountered, the conditions of exposure and the host's susceptibility and immune status.

Table 1.4 SABS water specifications of metals or trace elements (SABS, 1984) compared to those of the European Economic Community (EEC) (1996) and Guidelines for Canadian Drinking Water Quality (CDWQ) (2007)

Metals and trace elements	SABS (1984) (mg/L)	EEC (1996) (mg/L)	CDWQ (2007) (mg/L)
Chlorides	600	25	≤250[AO]
Mercury	0.01	0.001	0.001
Cadmium	0.02	0.005	0.005
Arsenic	0.3	0.05	0.01
Manganese	1.0	0.05	≤0.05[AO]
Hexavalent Chr.	No limit	0.05	0.05
Cyanide	0.3	0.05	0.2
Phenols	0.01	0.0005	Archived
Sulphate	600	250	≤500[AO]
Aluminium	No limit	0.05	0.1/0.2[AO]
Colouration	20.0	1.0	≤15[TCU]

[AO] Maximum Accepted Concentrations (MAC), based on aesthetic considerations and listed as aesthetic objectives (AO) or based on operational considerations and listed as operational guidance values (OG).
[TCU]True Colour Unit.

For viral and parasitic protozoan illnesses to occur, the infectious dose of viable infectious units can be low (Haas *et al*., 1999; Okhuysen *et al*., 1999; Teunis & Havelaar, 1999). **Table 1.5** is an overview of the stipulated guidelines for the Canadian Council of Ministers of the Environment (CCME) (recreational), EEC, SABS, DWAF and the United States Environmental protection Agency (USEPA) (recreational) for recreational water quality. The guidelines presented by the Canadian government are constantly being revised or adjusted as new or more significant data becomes available.

Research has shown that the levels of *E. coli* and intestinal enterococci were steadily increasing in waters, which led to a new directive being adopted by the European Parliament and Council in 2006, which would progressively replace the previous directive set by the European Economic Community in 1975. The levels of *E. coli* in freshwater based on epidemiological studies (Kay *et al*., 2004), showed that the maximum admissible level for *E. coli* is now set at 900 micro-organisms/100ml in water. This new regulation was based on the fact that *E. coli* represents on average, 60 to 80% of faecal coliforms which is common in these water sources, making the new regulations stricter than the previous one (Servais *et al*., 2007).

1.3.1 Domestic water use

Domestic water as stipulated in the guidelines by DWAF (1996), refers to all the applications of water used in the domestic environment and includes water used for drinking, bathing, personal hygiene, washing (dishes), laundry, gardening, etc. Water supplies to domestic users can originate from impoundments such as dams, rivers and streams or from groundwater via boreholes.

In South Africa, consumers in cities generally receive a constant supply of conventionally treated domestic water of high quality. In rural areas, water receives only partial or minimal treatment, with a large percentage of water obtained from wells and boreholes. Isolated communities and villages without access to electricity or other services however, often use water directly from rivers or streams without treatment. In this case seasonal droughts or floods as well as contamination, may affect both the quantity and quality of the source water [DWAF, 1996a].

The Department of Water Affairs and Forestry stipulates that when surface water is used as a domestic water source, the heterotrophic bacterial counts should not exceed 100 micro-organisms/100ml, the total coliform count should not exceed five micro-organisms/100ml and no faecal coliforms should be present. If the counts as stipulated above exceed the limits, it could lead to a significant or increased risk of infectious disease transmission [DWAF, 1996a].

Table 1.5 Summaries of Water Quality Guidelines for Microbiological Indicators for various agricultural bodies

Micro-organisms	SABS (1984)	DWAF (1996)	EEC (2000)	CCME (2006)	USEPA (1992)
Escherichia coli	≤2000/100ml (recreation)	≤130/100ml (recreation); 1/100ml (irrigation)	0/100ml	0/100ml	≤400/100ml (recreation)
Faecal coliforms	0/100ml (domestic)	≤2000/100ml (recreation); 10000/100ml (irrigation); 0/100ml (domestic)	0/100ml; ≤10/100ml (in swimming baths)	200/100ml (recreation)	200-400/100ml (recreation)
Total coliform	0/100ml (domestic)	≤5/100ml (domestic)	No numerical guideline indicated	No numerical guideline indicated	No numerical guideline indicated
Heterotrophic Plate Count bacteria	100/ml (domestic)	≤100/ml (domestic)	No numerical guideline indicated	No numerical guideline required	No numerical guideline indicated
Enterococci	No numerical guideline indicated	≤30/100ml (full contact recreation); ≤ 230/100ml (intermediate contact)	100/100ml (bathing beach water)	≤20/100ml (recreation)	≤100/100ml (recreation)
Pseudomonas aeruginosa	No numerical guideline indicated	No numerical guideline indicated	0/100ml	≤2/100ml (recreation)	No numerical guideline indicated
Protozoa	No numerical guideline indicated	0/10L (domestic)	No numerical guideline indicated	No numerical guideline required	No numerical guideline indicated
Coliphages	No numerical guideline indicated	≤20/100ml (full contact recreation); ≤1/100ml (domestic)	No numerical guideline indicated	No numerical guideline indicated	No numerical guideline indicated
Enteric viruses	No numerical guideline indicated	0/100ml (full contact recreation); 0/10L (domestic)	No numerical guideline indicated	No numerical guideline required	No numerical guideline indicated

In 2006, the Western Cape's (South Africa) drinking water was described as "*a crisis beyond ominous*", when it was discovered that large quantities of raw sewage were contaminating rivers and streams that flow into the dams supplying the city of Cape Town with drinking water. Although tap water, serving households in the Cape Town area, was treated, it was suspected that power failures experienced throughout the province at that time, led to the overflow of raw sewage from faulty sewage systems, into rivers, streams and groundwater sources. The contaminated river or groundwater was then pumped through purification plants which were unable to cope with this high contamination load. According to DWAF an improved water pollution

management policy has been requested in order to minimise future recurrences (Steenkamp, 2006).

1.3.2 Recreational water use

In South Africa, recreational usage of water constitutes a wide variety of activities and as with domestic water can originate from impoundments such as dams, rivers or streams, or from groundwater via boreholes. Seasonal droughts and floods may however, also affect the quantity and quality of water and as a result, waterborne diseases, changes in water taste, odour and colour and increased cost of treatment, may result.

For recreational use, a faecal coliform and *E. coli* count of > 2000 micro-organisms/100 ml and > 130 micro-organisms/100 ml, respectively, may lead to serious disease infections [DWAF, 1996 (b)]. A previous study recorded a coliform bacterial count of up to 34 x 10^6 micro-organisms/100 ml in a Western Cape river in 2002 (Barnes, 2003). In 2004 Barnes conducted further studies on the same river and observed coliform counts of 56 x 10^7 micro-organisms/100 ml and 34 x 10^6 micro-organisms/100 ml (Gosling, 2004). These counts significantly exceed the stipulated guidelines and serve as a serious threat to human health and the economy, as the water downstream from the contaminated site was used as a source of irrigation.

1.3.3 Agricultural water use: Irrigation

Water used in the agricultural sector may originate from rivers, farm dams, large reservoirs, groundwater, municipal supplies and industrial effluent, with irrigated agriculture being the largest consumer of accessible water in South Africa. However, the agricultural sector not only has to contend with the deteriorating water quality but also with a diminishing supply of water for irrigation. Contaminated water used for irrigation could transfer human and animal pathogens to crops and vegetables as well as to humans, when these organisms are retained and survive on the surfaces of raw fruit and vegetables.

Bacterial pathogens require a high infective dose of 10 to 1000 organisms to cause infection. According to the WHO (2004) irrigation systems should have no more than 2000 organisms per 100 ml. The study conducted by Barnes (2003) also raised alarming concern to farmers downstream of the investigated river, as water from the river is used for irrigation (Health24, 2004).

1.4 BIOFILM FORMATION

In natural, industrial and medical environments most micro-organisms colonise surfaces including that of plants, rocks, and metals, where they exist and grow as organised biofilm

communities (Stickler, 1999). Biofilms can therefore be defined as layers of organisms, organic matter and inorganic material, which develop on biologically active or non-active surfaces. The micro-organisms are immobilised in a variety of polymeric compounds generally referred to as extracellular polymeric substances (EPS). The EPS contain various constituents such as polysaccharides, proteins, nucleic acids, lipids or humic substances (Mayer *et al*., 1999).

The formation of biofilms includes three stages of development before a mature biofilm can be observed. Initially, bacteria attach to an appropriate substrate after which bacterial growth and division lead to the colonisation of the surrounding area on the substrate. This leads to biofilm growth and maturation (**Figure 1.11)**. During this process new genes are expressed to synthesize slime (EPS). Cells communicate by exchanging signalling molecules, referred to as population dependent cell-to-cell communication or quorum sensing, a process proposed to aid in biofilm formation and maintenance (Lynch *et al*., 2002). The occurrences of biofilms that harbour various species of micro-organisms have been described extensively (LeChevallier *et al*., 1987). Numerous studies have shown that pathogenic and opportunistic pathogens such as *Pseudomonas, Mycobacter, Campylobacter, Klebsiella, Aeromonas, Legionella spp., Helicobacter pylori* and *Salmonella typhimurium* may associate and multiply in biofilm communities (Mackay *et al*., 1998).

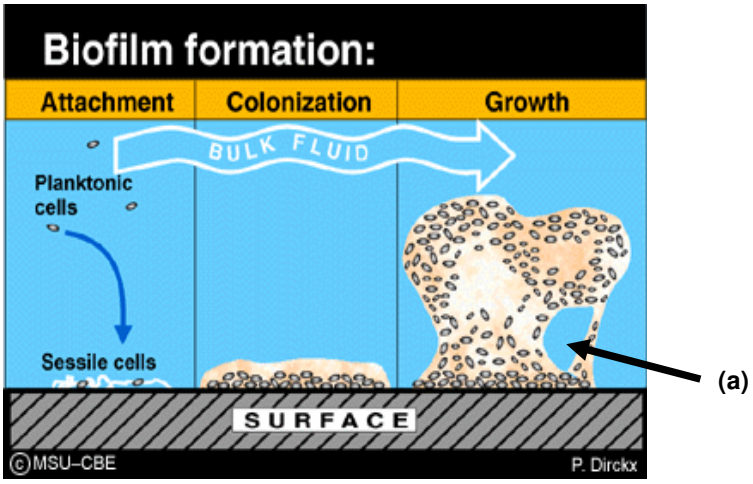


Figure 1.11 Systematic attachment, colonization and maturation of a biofilm. The arrow (a) indicates a channel through which water or fluid and nutrients are transported within the biofilm. Image adopted from P. Dirckx (1999)

Biofilms also have a major impact on biocorrosion and disinfection efficiency and may act as a reservoir for pathogenic and non-pathogenic micro-organisms. In a study conducted by Armon *et al*. (1997), two pathogenic bacteria *Legionella pneumophila* and *Salmonella typhimurium* WG-49 were introduced into a biofilm simulation flow system with sterile and non-sterile tap water. The survival of these biofilm communities on glass and

polyvinylchloride coupons was investigated. Experimental data showed that the survival of pathogenic micro-organisms in a biofilm is variable and depends on many factors. It was also found that the survival of *L. pneumophila* and *S. typhimurium* should raise important questions on their potential threat in water distribution systems.

According to Bisson and Johann (2004), temperatures at which water distribution systems are operated and the input of nutrients could allow for the growth of biofilms throughout these systems. Established biofilm communities in the water distribution systems could then in turn harbour *Legionellae* and protect them from adverse environmental conditions.

In the study conducted by Barnes (2003) it was found that farmers downstream of the Plankenburg River relied on biofilm formation on the surfaces of gabions (wire baskets filled with stones) to decrease the pollutant concentration in the river. This make-shift treatment system diverted the cleaner water to their farmland, thereby allowing them to use it for irrigation purposes (Barnes, 2003).

1.5 BIOREMEDIATION

The remediation of environmental sites contaminated by toxic pollutants is of major international concern. Bioremediation employs technology that uses micro-organisms to degrade these contaminants into less toxic or non-toxic entities or compounds. Pollutant-degradable micro-organisms, which have metabolic potential, can be manipulated or exploited and have been isolated for the bioremediation of contaminated environmental sites (Spain, 2000; Samanta, 2002; Parales & Haddock, 2004). Bioremediation also serves as an alternative to the traditional treatment techniques such as chlorination and ozonation etc., as it is a more cost- and time-effective approach (Kalogerakis & Psillakis, 2005). Several critical factors which include environmental conditions, presence of contaminants and nutrient availability, as well as the presence of degrading micro-organisms, should however, be considered when successfully employing bioremediation systems.

The two main types of bioremediation systems, which may be employed for the effective removal of contaminants from polluted aquatic environments, include wetland treatment systems and engineered bioreactors.

1.5.1 Wetland Treatment Systems

Wetland systems (natural or constructed) have gained increased interest due to their ability to reduce human pathogens in wastewater with varying but significant degrees of effectiveness (Karim *et al*., 2004). The diverse plant, animal, microbial and soil components, of which the wetland is composed, provides a high rate of biological activity. These components function together as a consortium to increase the efficiency of purification of the

influent wastewater and thereby also influence the overall quality of the effluent or outflow. In addition, wetland systems serve as cost-effective as well as environmentally sensitive treatment systems for the tertiary treatment of contaminated water or wastewater (Kadlec & Knight, 1996).

Two types of constructed wetland systems are generally used, viz. free water surface (FWS) and subsurface flow (SSF) systems.

1.5.1.1 Free Water Surface Systems

Free water surface wetland systems **(Figure 1.12 a and b)** are in principle similar to a natural marsh, as the water surface is exposed to the atmosphere. Submerged or floating plants such as duckweed, water hyacinths, bulrushes and cattails may be used in this wetland system. The wetland systems are usually flooded with water to a depth of 10 to 46 cm and the retention times of FWS's can vary considerably. These systems are usually lined with an impermeable plastic material to avoid total seepage and the soil that is used should have a permeability of 0.51 to 5.1 cm/hr, with the water more than 152.4 cm below the soil surface (Metcalf & Eddy, 1991). Micro-organisms attach to the various materials in the wetlands thereby effectively treating the incoming waste as it flows through the vegetation and soil.

(a) **(b)**

Figure 1.12 (a and b) Schematic diagrams of a free water surface (FWS) wetland system. Image (b) is adopted from S. Knight Merz (2000)

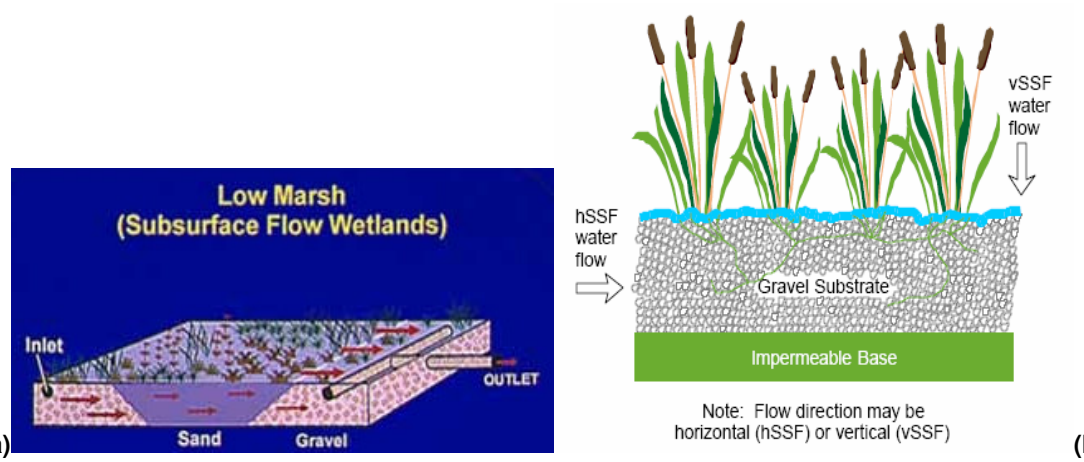
A study conducted by Reaves in 1995, aimed to determine the feasibility of using FWS's on dairy and swine wastewater in northern Indiana. It was found that these systems reduce the carbonaceous biochemical oxygen demand, faecal coliform bacteria, total suspended solids, phosphates, total phosphorus, ammonia-nitrogen as well as total nitrogen from both dairy and swine wastewater. In this type of constructed wetland system however, it was required that raw wastewater be pre-treated in lagoons, or similar separation systems, to remove solids from the waste stream. To ensure the success of the wetland systems, routine system maintenance and year-round management plans were incorporated (Reaves, 1995).

Constructed wetland systems have been applied as an important bioremediation strategy of animal wastewater treatment systems. In a previous study, four wetland cells were constructed based on a marsh-pond-marsh design in which the marsh sections were planted with cattails and bulrushes. Two cells within the wetland system were loaded with 16 kg nitrogen.ha^{-1}.day^{-1} with a detention of 21 days. The system effectively removed 51% of the added nitrogen. To the other two cells 32 kg nitrogen was added, with 37% of this nitrogen removed. The detention time was 10.5 days. It was found that removal efficiencies were more consistent during the warmer periods as compared to colder temperatures, with higher removal rates (more than 70%), for continuous marsh wetland systems. Phosphorus removal from the system ranged from 30 to 45% (Reddy *et al*., 2001).

In a study conducted by Stone *et al*. (2004), continuous marsh wetlands proved to be effective in treating swine lagoon effluent. The wetland constructed in 1995 at the North Carolina A&T University research farm, consisted of 10 m marsh followed by a 20 m pond and another 10 m marsh section planted with bulrushes and cattails. Even though the wetland system proved to be effective in the removal of nitrogen, with mean total nitrogen and ammonia-N concentration reductions of up to 30%, the system was not effective in the removal of phosphorus. The effective removal of phosphorus was limited to approximately eight percent. It was concluded that to accomplish more efficient removal of phosphorus in the wetland system, additional pre- and/or post-treatment would be required (Stone *et al*., 2004).

1.5.1.2 Sub-surface Flow Systems

In sub-surface flow (SSF) wetlands **(Figure 1.13 a and b)**, plants are not submerged in water but the water rather flows horizontally through a gravel-filled bed. In these types of wetlands, the gravel provides a surface area for microbial growth. Crites and Tchobanoglous (1998) suggested that the slope of these systems be less than 1% so that the transformation or removal of the chemical oxygen demand (COD), biological oxygen demand (BOD) and nitrogen and/or biological (bacteria) constituents from the wastewater, may be enhanced by the wetland systems.



(a) (b)

Figure 1.13 (a-b) Schematic diagrams of a sub-surface flow (SSF) wetland system. Image (b) is adopted from S. Knight Merz (2000)

According to Hammer (1989), multispecies micro-organisms in SSF wetlands may improve the removal of wastewater contaminants as these species may withstand fluctuating or changing loading rates more effectively. In a study of mixed (multi) species subsurface flow (SSF) wetlands with a retention time of approximately four days, *Cryptosporidium* was reduced by 53%, *Giardia* by 58%, enteric viruses by 98% and faecal coliform reduction averaged 98% (Karpiscak *et al*., 1996). A greater removal of the protozoan parasites *Giardia* (98%) and *Cryptosporidium* (87%) was also observed in a duckweed pond with a similar retention time. However, it was concluded that additional research was needed to obtain a greater understanding of the influence of wetland design and its potential for pathogen reduction (Maier *et al*., 2000).

1.5.2 Bioreactor Systems

In contrast to natural wetland systems, bioreactor systems are man-made bioremediation systems, which degrade various contaminants in water with micro-organisms through attached or suspended biological systems. Suspended growth systems may include activated sludge, fluidised beds or sequencing bed reactors. In these systems, contaminated groundwater is circulated in an aeration basin where a microbial population degrades organic matter and produces CO_2, H_2O and new cells. These cells form a sludge, which settles in a clarifier from which it is either recycled to an aeration basin or disposed of. Attached growth systems include upflow fixed film bioreactors, rotating biological contactors (RBC's) and trickling filters. Micro-organisms are attached to an immobile support matrix to aerobically degrade water contaminants (FRTR, 2000; Gerba, 2000b). Generally these treatment systems can be either anaerobic or aerobic and are based on the principle of removing organic material by a population of micro-organisms or biofilms attached to a medium such as polyvinylchloride or activated carbon, amongst others. Specific micro-organisms attach to an existing microbial population which have lost its biodegrading capabilities due to

contaminant toxicity. Through this process known as bioaugmentation, scientists are able to create 'superbugs' or micro-organisms that are able to degrade pollutants at significantly high rates. These organisms can be obtained by successive adaptations or genetic modifications under laboratory conditions. The introduction of these organisms however, has proved challenging, as often the micro-organism cannot establish a niche within the new environment and thus do not survive (Maier, 2000).

A plastic attachment medium containing polyvinylchloride (PVC) or polypropylene is commonly used in modern trickling filter systems and can be stacked approximately six to ten metres high due to their light weight. This filter system is referred to as a biotower. Rotating biological reactors (rotating biological contactors) and packed bed reactors are also common to a number of bioremediation technologies (Environmental Protection Agency, 2000).

In a trickling filter system **(Figure 1.14)** effluents, wastewater or contaminated water are pumped through a system onto the material, where the micro-organisms reside. These micro-organisms intercept the organic matter as it trickles past and decomposes or reduces the contamination load. Biofilms or zooleal films in these systems are primarily composed of bacteria, fungi, algae and protozoa. The increase in biofilm thickness, due to the constant passing of organic matter over the surface, leads to limited O_2 diffusion to the deeper layers of the biofilm, creating an anaerobic environment near the medium surface. Consequently, the biofilm becomes too heavy and the organisms eventually slough off from the surface, resulting in the formation of a new biofilm community (Bitton, 1994; Flint, 2001). Research has shown that the biological oxygen demand (BOD) removal for low-rate filter systems is approximately 85% (United States Environmental Protection Agency, 1977), while the removal of enteric pathogens by trickling filters is low and inconsistent. Filtration rates have also been shown to affect the removal of enteric viruses and other pathogenic micro-organisms as well (Moore *et al*., 1981).

Rotating biological reactors **(Figure 1.15)** or contactors are used in the secondary treatment of wastewater and involves contact between the wastewater and a biological medium in order to assist in contaminant removal. The simplest rotating biological contactor consists of a number of discs mounted on a shaft, which are allowed to rotate at specific angles to the flow of, for example, settled sewage.

The materials commonly used in manufacturing these discs are polythene, polyvinylchloride or expanded polystyrene. Discs are also usually contained in a furrow with approximately 40% of the discs submerged in the liquid medium. These types of bioreactors are usually fitted in a concrete tank with the surface of the wastewater passing through the tank, almost reaching the shaft. At a continuous rotation of one to two rotations per minute (rpm), a layer of biological growth, approximately two to four millimetres (mm) thick, could be established on the discs.

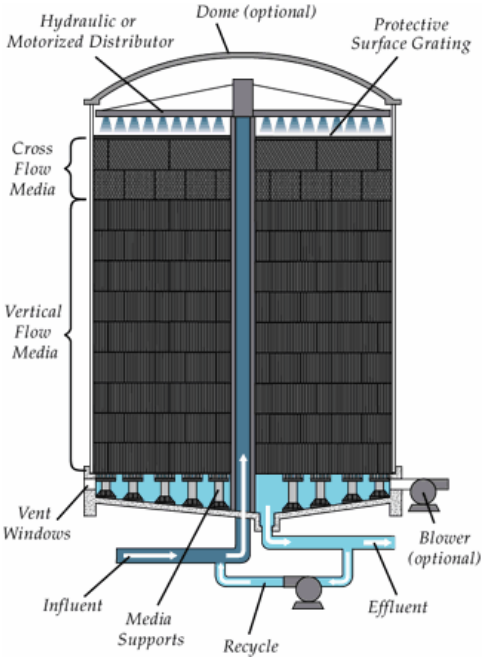


Figure 1.14 Schematic diagram of a trickling filter system. Images adopted from Brentwood Industries (2005)

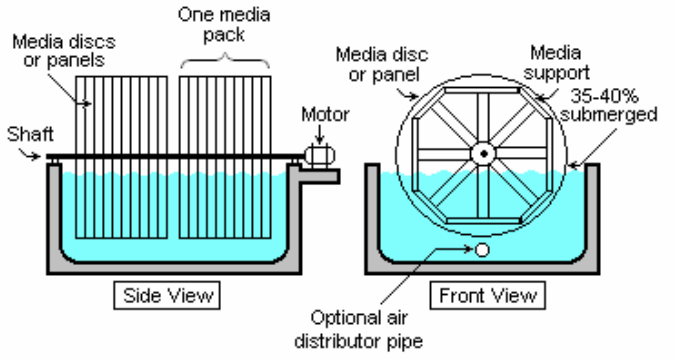


Figure 1.15 Schematic diagram of a rotating biological contactor (RBC). Image adopted from M. Beychok (2007)

The attached biological growth assimilates the organic material in the wastewater. This excess organic material is shaved off the discs into the tank where the solids are kept in suspension due to the discs' rotating action. The flow of the wastewater thereby pushes the solids out of the system into a clarifier, where it is separated and completely removed. As with every bioremediation system however, there are advantages and disadvantages. Advantages of this system, amongst others, include short contact periods because of the large active surface exposed to wastewater, short retention times, low power requirements and low sludge production and excellent process control. On the other hand, the complete coverage of the rotating biological contactor units is required in the northern climates, such

as Canada, in order to protect these systems from freezing. Furthermore, shaft bearings and mechanical drive units require frequent maintenance (Persaud, 1998).

In the packed bed reactor systems **(Figure 1.16 a - c),** materials flow through the reactor as a plug (Chaplin, 2004). These reactors, also referred to as plug flow reactors, are commonly used for immobilised enzymes and microbial cells. It is however, necessary to consider the pressure drop across the packed bed or column, as well as the column dimensions on the reaction rate. Packing offers the advantage of a lower pressure drop across the column (when compared to plates or trays), which is beneficial while operating under vacuum. In industrial applications, stacked columns are similar to packed beds which are used to perform separation processes. The column is a pressure container that consists of a closely stacked section, filled with dumped material or structured sections arranged into stacks (Seader & Henley, 2006).

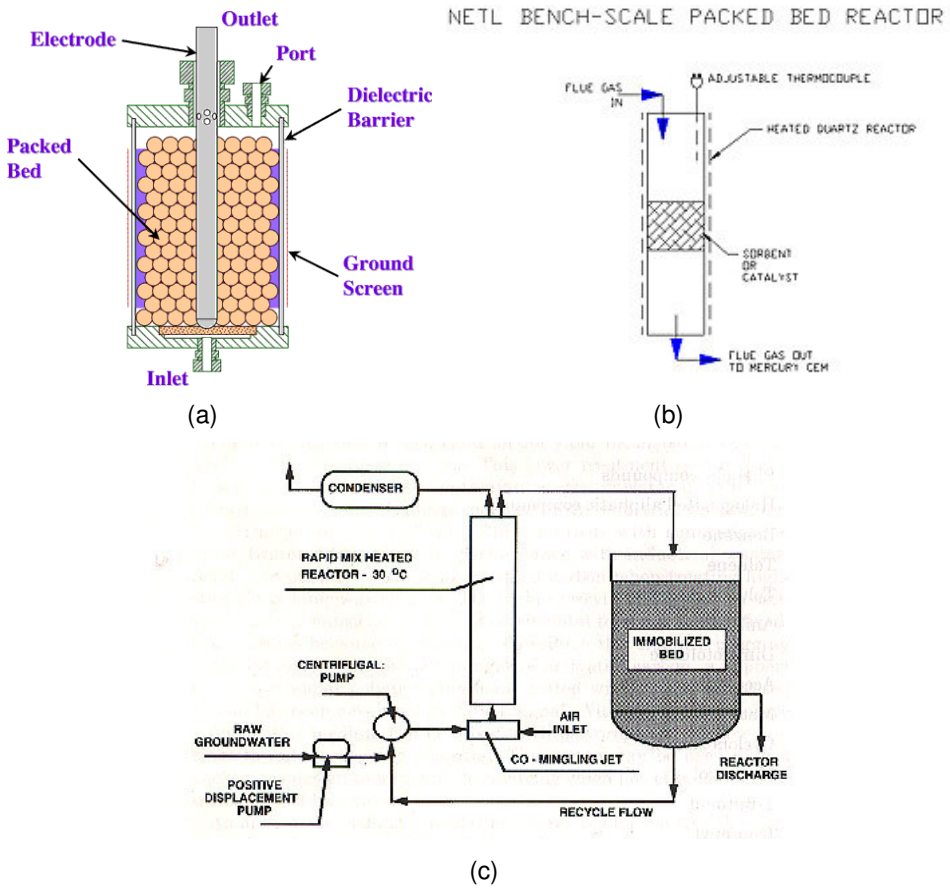


Figure 1.16 Examples of packed bed reactors. Image (a) adopted from Heath, W.: Gas Phase Corona Reactor, for Current Environmental Solutions. Image (b) adopted form the National Energy Technology Laboratory. Image (c) adopted from J.T. Cookson, Jr. (1995)

Once the surface of the used material is moistened by the liquid in the column, vapours pass across the wetted surface and cause mass transfer (the molecular and convective transfer of atoms and molecules within physical systems). The advantage of utilising packing

material in the system rather than trays, improves the separation of distillation columns. The use of materials in a bioreactor system in this manner, also allows for lower pressure drops across the column in the system, when compared to utilising trays or plates. The stacking material used may have different surface areas and void spaces which play an important role in the packing performance within the system (Seader & Henley, 2006). At present, packed bed- and fluidised bed reactors are more often used due to their high performance. Packed bed reactors are also the most popular reactors for immobolised biocatalysts because they allow for the use of the highest biocatalyst density (Kök *et al*., 2000).

In a previous study conducted by Sá and Boaventura (2001), a trickling bed biofilm reactor containing PORAVER particles, (a siliceous granular material similar to pumice stone) was used to evaluate phenol and total organic carbon removal efficiencies using the organism, *Pseudomonas putida* DSM 548. The researchers concluded that the calculated average biological yield during the pseudo steady-state operation was 0.8 g of biomass per gram of phenol removed. Total organic carbon removal efficiencies were also influenced by variations in temperature. In another study, a novel fibrous bioreactor was developed for treating odorous compounds in contaminated air. The study aimed at investigating the feasibility of such a reactor for the removal of aromatic volatile fatty acids, a common contaminant generated from anaerobic degradation of organic compounds. The bioreactor was effective in removing volatile fatty acids up to 32 $g/m^3/h$, beyond which the volatile fatty acids started accumulating in the recirculation liquid in the bioreactor system. These results indicated that the biofilm was unable to degrade all the volatile fatty acids introduced into the system (Tsang *et al*., 2008).

The behaviour of a microbial community, in terms of changes in the numbers of total bacterial population, autofluorescent methanogens, non-methanogens and morphology of the autofluorescent methanogens, was investigated using an anaerobic reactor, coupled with a conventional gravity settling tank and a continuous recycling system. The system was operated at an hydraulic retention time range of between 24 and 12 hours, using dairy wastewater as the substrate. A reduction in the number of the total bacterial population and autofluorescent methanogens was observed. The activity of the methane-forming bacteria decreased considerably at an hydraulic retention time of 16 and 12 hours. It was found however, that the conventional settling tank was less successful and that a membrane module would have been a more effective technique, where recycling would occur in the anaerobic treatment system (Demirel & Yenigün, 2005).

1.6 DETECTION, ISOLATION, ENUMERATION AND IDENTIFICATION OF WATERBORNE ORGANISMS

In order for authorities such as DWAF and the SABS to monitor the stipulated parameters for water sources, rapid and accurate water quality data needs to be obtained. Many water-

testing laboratories routinely use the heterotrophic plate count (HPC) technique to evaluate water quality (Australian Drinking Water Guidelines, 1996). In addition, several other methods, including direct microscopy such as epifluorescence microscopy, have been proposed as an alternative to plate counts for the enumeration of viable bacteria in water (Boulos *et al*., 1999). Flow cytometry (FCM) in conjunction with fluorescent dye technology, has also gained increased popularity as an enumeration technique employed for the detection of microbial numbers in water samples (Vives-Rego *et al*., 2000). It is essential however, that the techniques used to monitor the bacteriological quality of water need to be time- and cost-effective as well as reliable.

1.6.1 Most Probable Number Technique (MPN)

The most probable number (MPN) technique allows for the detection and the enumeration of gas-producing organisms in milk, water, soil and agricultural products. It should be noted however, that only viable organisms are enumerated by this technique and it is also important that the sample be prepared in a manner that randomly distributes the bacterium. The growth medium and incubation conditions employed should thus ensure that an inoculum that contains even one viable organism, produces detectable growth. The number of inocula producing growth at each dilution then provides an estimate of the original, undiluted concentration of bacteria in the sample. In order to obtain estimates over a broad range of possible concentrations, researchers use serial dilutions, with several tubes incubated at each dilution (Food and Drug Administration, 2001).

The effectiveness of the MPN technique has been observed as early as 1975 when a study conducted by Highsmith and Abshire (1975), incorporated the MPN method for detecting and enumerating *Pseudomonas aeruginosa* in water and wastewater. Media used in the presumptive and confirmatory tests, as stipulated in the 13th edition of Standard Methods from the Examination of Water and Wastewater, were compared to a modified preparation of the same media and was evaluated. Various water samples, including chlorinated tap water, creek water and the influent to a wastewater treatment facility, were tested. It was found that the modified media repeatedly yielded higher estimated MPN values of *P. aeruginosa* than the media listed in Standard Methods. *Pseudomonas aeruginosa* was present in all water samples except the tap water. The organism was present in numbers similar to that of the faecal coliforms and in even greater numbers than the faecal streptococci (Highsmith & Abshire, 1975).

Barnes (2003) used the multiple tube fermentation method in conjunction with the MPN technique to determine *E. coli* counts in the Plankenburg River. The *E. coli* levels observed from the various sampling points was compared to the maximum stipulated value of 2000 micro-organisms/100ml (SABS, 1986). These counts ranged from 0.13 x 10^2 micro-

organisms/100ml to 12.99 x 10^6 micro-organisms/100ml. Since the river flows through an area of informal housing schemes, the enumerative data obtained was valuable in studying the effects of failing sanitation facilities on the environment.

The MPN technique coupled with PCR (MPN-PCR) was also employed in a study conducted by Carey *et al*. (2006) to quantify the number of infectious *Cryptosporidium* oocysts recovered from raw water concentrates. The frequency of positive MPN-PCR results decreased as the oocysts numbers decreased. Similar results were also obtained when MPN was coupled to the foci detection method. It was however, found that the MPN-PCR method was a more effective method in the detection of infectious *Cryptosporidium* oocysts.

1.6.2 Heterotrophic Plate Count (HPC)

Heterotrophic organisms are broadly defined as micro-organisms that require an organic carbon source for growth. A variety of simple culture-based tests have been implemented to estimate the number of live heterotrophic organisms in water and collectively these tests are referred to as the heterotrophic plate count (HPC). However, no universal measurement for HPC exists and even though standardised methods have been formalised, test methods involve conditions that lead to a wide range of qualitative and quantitative results. Factors considered essential for the culturing of HPC's include conditions such as incubation temperature, incubation time periods and nutrient conditions. Depending on the media utilised, the incubation temperatures employed may range from 20ºC to 40ºC while incubation times may range from a few hours to a few days or even weeks. The concentration of available nutrients also influences HPC measurement. Heterotrophic plate counts however, do not specify the species of micro-organisms that might be detected (World Health Organisation, 2003a). It is also important to note that only a small proportion of metabolically active micro-organisms present in a water sample may grow and be detected under a given set of HPC test conditions. The microbial population which is detected may also differ significantly according to the test method that has been employed, for example the pour or spread plate technique or membrane filtration. In addition, the actual organisms recovered by the HPC test method may vary significantly between sampling sites, sampling seasons and consecutive samples at a single sampling site.

Micro-organisms detected and isolated through HPC usually form part of the natural microbiota of water and in some instances may also be derived from diverse contamination sources (World Health Organisation, 2003a). It is important to note that this technique only reveals the presence of culturable heterotrophic bacteria and does not account for the micro-organisms in the environment that become dormant (viable-but-non-culturable) due to stressful conditions. Careful consideration should therefore be taken when employing this

type of testing and it is suggested that results obtained be collaborated by one or more test methods which are capable of detecting more diverse groups of micro-organisms.

A study performed by LeChevallier *et al*. (1980), evaluated the possible effect of large numbers of heterotrophic plate count (HPC) bacteria on human health. Results for HPC bacteria ranged from one to 10^4 CFU/ml in drinking water. Even though these counts had no significant impact on health, there was great concern regarding the growth of HPC bacteria in bottled water and charcoal filters in household taps. It is recognised however, that even though the HPC is not a direct indication of the faecal content in water samples, it is indicative of the water quality. It has also been noted that these bacteria could interfere with coliform and faecal coliform detection when present in high numbers. Based on their results, LeChavellier *et al*. (1980) recommended that the HPC bacteria should not exceed 500 micro-organisms/ml in tap water.

The heterotrophic plate count technique was also applied in conjunction with a PCR-denaturing gradient gel electrophoresis fingerprinting technique to enumerate bacterial populations in ground- and bottled water. The two techniques were evaluated by comparing four kinds of mineral waters to one groundwater sample. Bacterial populations cultured on R2A plates were also subjected to the PCR- denaturing gradient gel electrophoresis technique and it was found that the HPC technique on its own, does not account for micro-organisms which enter a viable-but-non-culturable state (Dewettinck *et al*., 2001).

1.6.3 Microscopy and Fluorescent dyes

Various methods using direct microscopy have been employed as alternatives to the plate count method for the enumeration of viable micro-organisms such as heterotrophic bacteria in water samples or pure strains of *E. coli* in laboratory assays (Boulos *et al*., 1999). Microscopy refers to any technique used for producing visible images of structures or details too small to be seen by the human eye. Types of microscopy include optical and electron microscopy which involves the diffraction, reflection and refraction of radiation upon the studied subject in order to generate an image. This process may be carried out by wide field irradiation of the sample e.g. standard light or transmission electron microscopy or by scanning of a fine beam over the sample e.g. as in confocal and scanning electron microscopy (Roane & Pepper, 2002; United States Department of Energy, 2005). In conjunction with specific fluorochromes, such as Acridine Orange, 4',6-diamidino-2-phenylindole and BacLight™, Epifluorescence- and Confocal Laser Scanning Microscopy have become standard techniques currently used to estimate the abundance, biomass, biovolume, size and physiological activity of bacteria obtained from aquatic bodies (Lisle *et al*., 1999; McFeters *et al*., 1999). In addition, it can also be used to distinguish between live (viable), permeabilised (injured) and dead cells (Hiraoka & Kimbara, 2002). Even though

these enumeration techniques are fairly simple and easy to perform, care should be taken, as certain factors such as the presence of nonbacterial biomass or debris, may influence cell counts and hence, affect the reliability of resulting data. A study conducted by Zweifel and Hagström (1995) showed that large percentages of cells counted as bacteria in 4',6-diamidino-2-phenylindole-stained samples, were in fact particles without a genome, i.e. dead or ghost cells. In addition, it was found through transmission electron microscopic observations, that a large percentage of bacteria in marine samples had damaged intercellular integrity. The transmission electron microscope was suitable for estimating the percentage of intact bacterial cells versus damaged or empty cells by examining the internal structures and their morphology (Heissenberger *et al*., 1996; Manini & Danovaro, 2006). In other research studies, which questioned the validity of the transmission electron microscopy technique, it was concluded that overestimation of bacterial loads was due to the inclusion of nonbacterial or dead cell particles within microbial samples (Gasol *et al*., 1999).

1.6.4 Flow Cytometric Analysis (FCM)

Techniques employed in routine water quality analysis usually only assess the number of micro-organisms which are able to form visible colonies on solid medium, i.e. culturable heterotrophic bacteria. Consequently, the micro-organisms in the environment which are capable of maintaining metabolic activity while developing a recalcitrance or insurgence to nature, referred to as VBNC (viable-but-non-culturable) organisms (McDougald *et al*., 1998) are not enumerated. According to Kell *et al*. (1998) these micro-organisms exhibit measurable traits of physiological activity but fail to grow to a detectable level and therefore are referred to as ABNC or 'active but non-culturable'. These factors make it difficult to obtain a true indication of the bacteriological numbers in water by means of the heterotrophic plate count and thereby support the employment of flow cytometric analysis of environmental waters. Flow cytometry (FCM) has thus become an important and valuable tool for the detection of micro-organisms in aquatic environments and the assessment of water quality (Porter *et al*., 1996; Vives-Rego *et al*., 2000). This technique measures single bacterial cells at a rate of 1000 cells.s^{-1} (Vives-Rego *et al*., 2000) and bacterial data can therefore be acquired and processed rapidly and if necessary, allows researchers to respond quickly if corrective action needs to be implemented.

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 (Walberg *et al*., 1998; Current Protocols in Cytometry, 2005). The flow cytometer contains a doublet discrimination module, which uses pulse width and area to eliminate cell clumping (doublets and triplets). In conjunction with fluorescent dye technology such as the LIVE/DEAD® *Bac*Light™ bacterial viability stain, the technique allows for the

differentiation between bacterial cells and debris, as well as the enumeration of physiologically active bacteria (Boulos *et al*., 1999; Ramalho *et al*., 2001). The addition of fluorescent beads also allows for the calculation of absolute or total cell counts in samples. This 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.

In a previous study the FCM technique was applied for the detection and quantification of mycobacteria in metalworking fluids (Chang *et al*., 2004). Various conventional detection methods were available for the monitoring of microbial contamination in metalworking fluids. However, none were developed for the specific detection of mycobacteria in such media. Flow cytometric analysis, in conjunction with non-specific nucleic acid dyes, showed a good correlation with counts obtained by direct analysis using epifluorescent microscopy. It was found however, that the time required to complete FCM was 10 s/sample whereas the epifluorescent microscopy enumeration was 300 s/sample. The researchers thus supported the application of the FCM technique for microbial detection in complex fluids such as metalworking fluids.

Flow cytometric signatures using LIVE/DEAD® *Bac*Light™, were obtained for the active but non-culturable (ABNC) cells of *E. coli* and a coliform isolate *H03N1*, in a seawater microcosm. The population of ABNC cells could not be cultured using m-FC media (media for Faecal Coliforms), which is commonly used for the enumeration of faecal coliforms. The cells were resuscitated in phosphate buffer saline and then cultured in Luria broth after which they were subjected to the FCM technique. This technique was able to detect and differentiate between the ABNC cells in a mixed population of culturable cells, transition populations and dead cells. In addition, the FCM technique detected the presence of coliform bacteria, which were not detected by the membrane filtration technique (Sachidanandham *et al*., 2004).

1.6.5 Molecular Identification Techniques

Previously, identification of bacterial pathogens, or micro-organisms, resulted from techniques involving microscopy, serology and culturing. Culture-based techniques may however, exclude the identification of unculturable micro-organisms. Researchers thus emphasise the critical role of molecular techniques, such as the Polymerase Chain Reaction (PCR), due to its sensitivity in detecting micro-organisms (Fredericks & Relman, 1996). The use of molecular identification techniques has thus led to diverse microbial species being successfully identified or detected in environmental samples (Houpikian & Raoult, 2002).

Due to its high sensitivity, the polymerase chain reaction (PCR) allows for the successful identification of organisms present in low numbers and those that are difficult to culture (Buller *et al*., 1999). In addition, it is also a useful tool for taxonomic studies and the

identification of diverse species (Houpikian & Raoult, 2002). Contamination of PCR products as well as false-positive reactions can also occur. Non-sterile samples, such as faeces or sputum, the use of family- or species-restricted primers and in-situ hybridisation with specific nucleic probes could also pose as limitations of broad-range PCR applications (Fredericks & Relman, 1996; Relman, 1999).

In general, molecular identification techniques primarily begin with genomic DNA extraction procedures. This is followed by the PCR technique which is the 10^6-fold amplification of a specific amount of target DNA. During this enzymatic reaction a DNA polymerase enzyme is used to repeatedly copy a target DNA sequence during a series of 25 to 30 cycles. One such cycle has three steps, which firstly involves the denaturation of the double-stranded DNA (dsDNA) into two single strands of target or template DNA (ssDNA). Two dissimilar short pieces of single-stranded DNA called primers are added to the mixture. These primers are carefully chosen and commercially synthesised and are defined as oligonucleotides which have complementary sequences to the target ssDNA template. The second step in the PCR cycle is primer annealing, which involves the primers hybridising to the target DNA sequence, while the third and final step is extension of the target sequence. The result at the end of a cycle is two dsDNA molecules identical to the original double-stranded DNA molecule. This repetition of the PCR process, results in the amplification of the DNA and an exponential increase in the number of copies of the original DNA (Marlowe *et al*., 2000). Agarose gel electrophoresis and DNA sequencing is then used to conclude the identification process. Species-specific sequences can also be identified with the use of universal primers, which recognise regions that contain the 16S rDNA encoding gene, (Fredericks & Relman, 1996; Relman, 1999).

The PCR technique has also enabled laboratories to characterise organisms that have never been cultured. For example, PCR has in previous studies enabled the association of two diseases with novel etiological agents, i.e. *Bacillus henselae* in bacillary angiomatosis and *T. whipplei* in patients with Wipple disease (Relman *et al*., 1992).

In a study conducted by Grahn *et al.* (2003), the amplification of bacterial 16S rDNA fragments was used in conjunction with a pyrosequencing technique, for the detection, identification and typing of bacterial contaminants in PCR reagents. Waterborne bacteria such as *Pseudomonas*, *Stenotrophomonas*, *Xanthomonas*, *Ralstonia* and *Bacillus* were identified. Industrial water systems used for the manufacture of the reagents were identified as the possible source of contamination.

The 16S rRNA PCR technique has also been a successful tool in identifying mycobacterial species in raw and treated surface and groundwater sources as well as in the identification of biofilms in piping systems (Stinear *et al*., 2000; Falkinham *et al*., 2001; Le Dantec *et al*., 2002). Mycobacteria were also identified from cold and hot taps, ice, showers and bottled water with the application of this technique (Covert *et al*., 1999). In 1993,

Abbaszadegan *et al*. used reverse transcriptase-PCR to successfully amplify the conserved *VP1* gene in order to detect enteric viruses in groundwater. Reverse transcriptase-PCR has also been used to detect viruses, such as the *Sin Nombre virus* (SNV) or the *Hepatitis C virus* (HCV) (Mercer *et al*., 2001). The technique was also used by Chen *et al.* (2008) to conserve the 5'-nontranslated regions (5'-NTR) in enterovirus RNA in various samples obtained from environmental water collected in Taiwan during 2004–2005.

1.7 AIMS OF STUDY

The quality of water sources in South Africa is fast deteriorating and the increased demand for water by the country's growing population has in turn led to a rapid decrease in the availability of water sources. This study focuses on identifying agents responsible for the contamination and subsequent deterioration of important water sources such as the rivers in the Western Cape, South Africa. The primary aim of this study was to determine the extent of bacterial contamination in rivers in the Western Cape and to investigate the application of a bioremediation system as a possible treatment technology. Several aspects contributing to the contamination were addressed and different approaches were studied and reviewed. The primary aim was achieved as follows:

1.7.1.1 To identify and sample at various points along the Plankenburg- (Stellenbosch), Berg- (Paarl) and Diep Rivers every month for a period of one year.

1.7.1.2 To determine the level of bacterial contamination in the rivers by means of the Most Probable Number (MPN) and heterotrophic plate count (HPC) techniques.

1.7.1.3 To determine the total bacterial counts in the rivers by means of Flow Cytometric Analysis (FCM) using liquid counting beads and the LIVE/DEAD® *Bac*Light™ stain and Electron Microscopy (EM) using the LIVE/DEAD® *Bac*Light™ stain.

1.7.1.4 To isolate and identify planktonic bacterial pathogens present at the various sampling points along these rivers using molecular typing.

1.7.1.5 To isolate and identify pathogenic bacteria present in the biofilms (sessile) attached to the rocks and other materials in the rivers using molecular typing.

1.7.1.6 To observe the effective removal of these pathogens by developing and optimising an *in situ* assessment method, i.e. a laboratory-scale bioreactor system.

1.7.1.7 To develop an on-site bioreactor system for the reduction of total cell counts from the river water as a means of clean-up technology.

Comparison of enumeration techniques for the investigation of bacterial pollution in the Berg River, Western Cape, South Africa

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Abstract

The study was aimed at assessing techniques, which would provide an accurate indication of the planktonic bacterial pollution load in the Berg River, Western Cape, South Africa. Sampling of sites started in June 2004 and continued for a period of one year until June 2005. The Most Probable Number technique was used to determine the level of faecal coliforms and *Escherichia coli* (*E. coli*) while the heterotrophic plate count method was used to determine the amount of culturable micro-organisms in planktonic samples. The flow cytometry (FCM) and direct acridine orange count (DAOC) (epifluorescence microscopy) techniques were employed to evaluate total bacterial counts in planktonic (water) samples. The highest MPN and heterotrophic plate counts were recorded in week 37 at site B2 at 1.7 x 10^7 micro-organisms/100 mℓ and 1.04 x 10^6 micro-organisms/mℓ, respectively. In comparison, the viable FCM counts, were significantly ($p < 0.05$) higher for that period at 1.7 x 10^7 micro-organisms/mℓ. The highest total FCM count of 3.7 x 10^7 micro-organisms/mℓ was recorded in week 41 at site B2. In comparison the highest DAOC of 8.3 x 10^6 micro-organisms/mℓ was obtained in week 29 at site B2. Results showed that on average the heterotrophic plate count represented a fraction (< 3.65 %) of the total FCM counts. The total DAOC count also represented a fraction (< 43.08 %) of the total FCM count for most of the sampling period. Results therefore showed that the flow cytometry method proved to be more effective in evaluating microbial pollution in water samples.

Keywords: bacterial pollution; direct acridine orange count; flow cytometry; heterotrophic cell counts; planktonic organisms; river water

1. Introduction

Water scarcity is becoming a major problem in South Africa, as dams serving communities with drinking water and water for daily household use, have in recent years been less than 30% full (Department of Water Affairs and Forestry (DWAF), 2005). River water, in combination with groundwater, is considered a suitable alternative as an utilisable and potable water source. However, in South Africa, rivers are steadily becoming more contaminated and in some cases even toxic, due in large part, to urbanisation. Communities located in rural and some semi-urban areas, lack adequate domestic water supplies and wastewater treatment facilities. In many instances sullage and excreta from these informal settlements are discharged into storm water drainage pipes, which directly flow into nearby rivers. The low rainfall and increasingly high temperatures then leads to the proliferation of micro-organisms which in turn leads to a significant increase in waterborne diseases (Gerba, 1996; WHO-UNICEF, 1999). The contaminated river water also affects the farms downstream from the pollution source that utilises the water as a means of irrigation.

The Plankenburg- (Stellenbosch) and Berg Rivers (Paarl) are two rivers in the Cape Metropolitan-Boland area that are regarded as highly polluted. Informal settlements inhabit their banks, and storm water drainage pipes from the settlements flow directly into these rivers (Barnes, 2003). Previous studies have recorded an *E. coli* count as high as 2.44 x 10^9 CFU per 100 mℓ water in the Berg River at a Mbekweni storm water drainage pipe during the 2003 summer season (Barnes, 2003). Raw sewage spills from sewer pump stations in Wellington (near Paarl), overstressed sewer mains in the Paarl area as well as storm water effluent from informal settlements in Paarl

and Wellington areas were identified as possible sources of the pollution. These microbial counts significantly ($p < 0.05$) exceed the stipulated water quality guidelines indicating that these rivers, which serve as recreational and irrigational water sources, need to be monitored on a regular basis.

The Most Probable Number (MPN) technique is routinely used to determine levels of all gas-producing contaminants in river water, which include the faecal coliforms and *E. coli*. However, this technique does not determine or indicate the level of other culturable micro-organisms that might be present in the water. For this purpose a conventional heterotrophic plate-count technique is performed. There are however, two fundamental problems which inhibits the effectiveness of this quantitative culturing technique, i.e. the culturability of the samples on which the technique relies, as well as the selectivity of the medium on which bacteria are cultivated (Ward et al., 1992; Amann et al., 1995). Therefore, to base viability solely on the plate count method would not be sufficient proof of the total cell count. Furthermore, certain organisms such as *Vibrio cholerae* and *E. coli* have the ability to enter a non-culturable state in response to adverse environmental conditions (Xu et al., 1982; Colwell et al., 1985).

Flow cytometry and epifluorescence microscopy are techniques widely used not only to determine total cell counts, but also the ratio's of live (viable) cells to permeabilised (injured) and dead cells (Hiraoka and Kimbara, 2002). Epifluorescence microscopy, in conjunction with specific fluorochromes, has become one of the standard techniques that are currently used to estimate the abundance, biomass, biovolume, size and physiological activity of bacteria obtained from aquatic bodies (Hobbie et al., 1977; Kepner and Pratt, 1994; Lisle et al., 1999; McFeters et al., 1999). Even though this enumeration technique is fairly simple and easy to perform, care should be taken as

certain factors such as the presence of nonbacterial biomass or debris, if not eliminated or minimized, may influence cell counts and hence, affect the reliability of resulting data.

Flow cytometry could be regarded as a more reliable approach for the enumeration of micro-organisms. LIVE/DEAD BacLight™ Bacterial Kits (Molecular Probes, 1995) are used in flow cytometric analysis to investigate the microbiological effects of the two fluorochromes, SYTO 9 and Propidium iodide. These two fluorochromes are nucleic acid-binding stains and can be applied to estimate both viable and total bacterial counts in water samples (Boulos et al., 1999). An appropriate mixture of the two fluorochromes will stain bacterial cells with intact membranes green (SYTO 9) and those with damaged membranes a fluorescent red (Propidium iodide) (Braga et al., 2003).

The aim of this study was to assess enumeration techniques, which would provide an accurate indication or estimation of the planktonic bacterial pollution load in the Berg River, Western Cape, South Africa. The MPN technique was used to determine the level of faecal contamination in river water. The conventional plate count technique was used to determine heterotrophic counts in the water samples. In addition, all water samples were subjected to flow cytometric analysis and the direct acridine orange count technique using epifluorescence microscopy in order to obtain total cell counts, i.e. the culturable and non-culturable population.

2. Materials and Methods

2.1 Sampling Sites

Sampling sites at the Berg River location are indicated in Figure 2.1 and included Site A (agricultural farming area); Site B (informal settlement of Mbekweni - sites B1 and B2) and, Site C (Newton pumping station). Site B2 is known as Plot 8000 and is the site where storm water drainage pipes from the informal settlement flow directly into the river. The Newton pumping station services the residential area of Newton as well as certain sections of Mbekweni. Sampling of sites started in June 2004 and continued for a period of 1 year until June 2005. The temperature and pH of the river water was measured using a hand-held mercury thermometer and portable pH meter (Hanna Instruments), respectively. Water samples were collected in 1ℓ sterile Nalgene-polypropylene bottles and stored on ice to maintain a low temperature.

2.2 Most Probable Number & Heterotrophic Plate Counts

The MPN technique was modified by Barnes (2003) and involved the inoculation of sample water into lauryl tryptose tubes containing Durham tubes, followed by incubation for 48 hours at 37ºC (indicating all gas-producing organisms). All tubes indicating gas-formation were regarded as a positive presumptive test and the presumptive coliform count was read off De Mans tables (American Public Health Association, 1992, 1995). These positive tubes were re-inoculated into brilliant green bile broth and tryptone water tubes respectively, according to the guidelines set out by the South African Bureau of

Standards (SABS, 1984). These guidelines also incorporate the standard methods set out by the American Public Health Association, American Water Works Association and the Water Environment Federation (American Society for Microbiology, 1997). Positive tubes were incubated in a 44.5ºC waterbath for 24 hours. Gas-production in the brilliant green tubes (indicating faecal coliforms) was compared to growth in the tryptone water tubes (indicating *E. coli*). The presence of *E. coli* was confirmed with a colour change from clear to pink or red after the addition of Ehrlich's reagent into the tryptone water tubes.

Total heterotrophic counts were done in triplicate on Nutrient agar (NA) (Merck, Biolab Diagnostics) plates after serial dilutions (10^{-1} to 10^{-7}) of sample water were performed. Plates were incubated for 3-4 days at 37ºC. Thereafter, the number of visible cells [colony forming units (CFU)] were counted and recorded.

2.3 Flow cytometry (FCM)

For the flow cytometry-based assay, 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 therefore, 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 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 before analysis. The staining procedure was performed by combining equal volumes of PI (propidium iodide) (4 μℓ) and SYTO 9 (4 μℓ) in BacLight™, 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 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 beads allows 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.

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

Equation 1.

[Bead concentration recorded at 988/µℓ (value found on the vial of BD Liquid Counting Beads obtained from BD™)]

2.4 Direct Acridine Orange Count (DAOC)

The total number of micro-organisms in the water samples were measured by means of epifluorescence microscopy, with acridine orange (Sigma) as the fluorochrome. Samples (2 mℓ) were filtered through Millipore membrane filters with a pore size of 0.22 µm. Cells captured on the filter were stained with 2 mℓ acridine orange (160 mg/ℓ) for 5 minutes. Total cell counts were obtained using a Zeiss Epifluorescent microscope (100X magnification). A minimum of 5 different fields was enumerated for all the water samples, for each respective sampling time.

2.5 Statistical analysis

Repeated Measures Anovas (RMA) was performed on all data obtained as outlined in Dunn and 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.

Note: Unit clarification

The MPN results are expressed in micro-organisms per 100 mℓ, whereas the CFU, FCM and DAOC results are expressed in micro-organisms per mℓ.

3. Results and Discussion

3.1 Most Probable Number (MPN)

The levels of planktonic organisms associated with faecal pollution were evaluated by performing the MPN method (Figure 2.2). The MPN method distinguishes between the total number of possible gas-producing organisms, which includes faecal coliforms [(FC) indicates all indicator organisms] and *E. coli*-organisms within water samples (Oblinger and Koburger, 1975). On average the total MPN counts ranged from 1.6 x 10^3 micro-organisms/100 mℓ recorded at site A in the first week of sampling, to 3.5 x 10^7 micro-organisms/100 mℓ observed at site B2 in week 37 of the sampling period. In comparison, the lowest faecal coliform count of 1.7 x 10^2 micro-organisms/100 mℓ was recorded in week 17 at site A, whereas the highest FC count of 3.5 x 10^7 micro-organisms/100 mℓ water was observed at site B2 in week 37. Corresponding *E. coli* counts ranged from 0.36 x 10^2 micro-organisms/100 mℓ in week 1, to 1.7 x 10^7 micro-organisms/100 mℓ in week 37, both recorded at site B2. These results are significantly ($p < 0.05$) higher than the maximum limit of 2000 organisms/100 mℓ set for planktonic organisms in river water by the SABS Guidelines (1984). Furthermore, during the one-year sampling period, the *E. coli* counts fell within the accepted range less than 13 times for all samples (i.e. A, B1, B2 and C) analysed. It should also be noted that in two

distinct cases during the sampling period, namely week 1 at sites A and B1, no *E. coli* counts were detected. Results clearly show that increases in microbial activity was experienced at site B2 for most of the sampling period, with the highest MPN, FC and *E. coli* counts measured at this site.

3.2 Total heterotrophic plate counts

Results obtained for the average heterotrophic plate counts for the planktonic samples analysed at the respective sites in the Berg River are depicted in Figure 2.3. On average the heterotrophic plate counts ranged from 3×10^3 micro-organisms/mℓ recorded at various sites throughout the sampling period to 1.04×10^6 micro-organisms/mℓ recorded at site B2 in week 37. Even though the plate counts remained constantly low at all the sites for most of the sampling period, significant ($p < 0.05$) increases were observed in weeks 37 and 45 at site B2 where counts of 1.04×10^6 micro-organisms/mℓ and 3.6×10^5 micro-organisms/mℓ were recorded, respectively. High MPN counts (Figure 2.2) were also observed at site B2 for weeks 37 and 45. The high microbial input at site B2 could be ascribed to the fact that storm water drainage pipes from the informal settlement flow directly into the river at this site.

3.3 Flow cytometric analyses

Live/Dead ratios of planktonic populations were obtained using flow cytometer analyses, in conjunction with the Live/Dead BacLight™ probe and liquid beads (BD™). Two distinct populations of live and dead cells (R5 and R6, respectively) were observed by

distinguishing between their fluorescence intensities (Figure 2.4), i.e. either red or green fluorescence.

The total cell counts obtained are presented in Figure 2.5. The lowest planktonic counts of 1.5 x 10^6 and 1.6 x 10^6 micro-organisms/mℓ were recorded at sites B1 and B2 in weeks 5 and 49, respectively. In comparison, the highest total cell counts of 3.1 x 10^7, 3.7 x 10^7 and 3.5 x 10^7 micro-organisms/mℓ were observed in weeks 1, 41 and 45 respectively, all at site B2.

Comparisons of heterotrophic plate counts versus the viable cell counts obtained by flow cytometry are shown in Figure 2.6. It was observed that when compared to the CFU counts obtained by the heterotrophic plate count technique, flow cytometric (FCM) analysis yielded significantly ($p < 0.05$) higher viable counts in the planktonic samples.

The highest CFU count of 1.04 x 10^6 micro-organisms/mℓ (Figure 2.3) was recorded in week 37 at site B2. A corresponding FCM viable count of 1.72 x 10^7 micro-organisms/mℓ was recorded for the same sampling time. In addition, for weeks 1 and 41, FCM counts of 1.4 x 10^7 and 1.9 x 10^7 micro-organisms/mℓ, for viable micro-organisms, respectively were recorded at site B2, compared to the CFU counts of 3 x 10^4 and 3 x 10^3 micro-organisms/mℓ, recorded for the same week. As shown in Figures 2.2 and 2.3 significant increases in MPN and CFU counts were observed in weeks 37 and 45. The FCM results showed a significant increase in total cell counts in both these weeks, as well as in week 41. The temperatures recorded in weeks 37, 41 and 45 ranged from 19 to 25°C. However, irrespective of the high water temperature of 23.4°C measured in week 41, a low heterotrophic plate count was recorded at this site. Correspondingly, low MPN, FC and *E. coli* counts were also observed at this site for the same sampling time.

The relative values (by means of percentage ratios) of the heterotrophic plate count to the total FCM count, viable FCM count to total FCM count and heterotrophic plate count to viable FCM count are presented in Tables 2.1 – 2.3, respectively. Results showed that on average the heterotrophic plate count represented only a fraction [3.65% (week 37, site B2)] of the total biomass obtained by FCM analysis. In comparison, the viable FCM count accounted for 60.18% of the total FCM count for site B2 in the same week of sampling (Table 2.2). In addition, the heterotrophic plate count represented only a fraction (6.06%) of the viable FCM count for the same sampling period at site B2. The highest heterotrophic plate count represented 6.96% of the viable FCM count (week 45 at site B2) and only a fraction of 1.02% of the total FCM count (Table 2.1) for the same sampling site and period. Results clearly show that the FCM technique is the more reliable enumeration technique for microbial populations obtained from environmental samples. The higher FCM results could also be ascribed to the fact that this technique is able to detect certain populations in the environment, which enter a viable but non-culturable state when exposed to stressful conditions. It is thus evident that the traditional plate-count method only provides an indication of the viable-culturable cells present in the river water, and thereby only accounts for a fraction of the total viable population within samples, which could also explain the low MPN and CFU counts observed in week 41. In order to determine the exact level of pollution in the river water, it is thus essential that accurate methods of measuring total microbial activity for planktonic micro-organisms be employed.

Even though the flow cytometry technique provides accurate relative quantification (using fluorescent liquid beads to determine the percentage abundance) of cells in various sample types, technical limitations do exist. The essential reagents or components required for the FCM technique are costly and the limited applicability as a

result of the type of flow cytometer, adds to the restrictions of applying this technique for routine analysis (Montes et al., 2006).

3.4 Direct Acridine Orange Count (DAOC)

The average total cell counts of planktonic samples as obtained by DAOC analysis are represented in Figure 2.7. The total cell counts obtained from the DAOC method were lower in all the water samples, when compared to total counts obtained by flow cytometer analyses (Figure 2.8). The highest DAOC count of 8.3×10^6 micro-organisms/mℓ for planktonic samples was recorded in week 29 at site B2. The FCM analysis for week 29 yielded comparable total counts of 8.62×10^6 micro-organisms/mℓ. The lowest planktonic DAOC count of 4×10^4 micro-organisms/mℓ was recorded at site B1 in week 49, with a corresponding FCM count of 4.7×10^6 micro-organisms/mℓ. Even though the FCM counts fluctuated throughout the sampling period, results clearly show that the FCM method yields more accurate data for total cell counts than the DAOC method.

The DAOC also displayed questionable results when compared to the heterotrophic plate count data (Figure 2.3). The highest CFU counts of 1.04×10^6 micro-organisms/mℓ and 3.6×10^5 micro-organisms/mℓ were recorded in weeks 37 and 45, respectively at site B2. In comparison, the DAOC counts measured for the same sampling time were lower than the CFU counts, at 2.1×10^5 micro-organisms/mℓ and 1.3×10^5 micro-organisms/mℓ, respectively. Corresponding FCM viable counts of 1.72×10^7 and 5.2×10^6 micro-organisms/mℓ were measured at this site for the two weeks, respectively.

Percentage ratios of the heterotrophic plate count to total DAOC count as well as the total DAOC to total FCM counts are represented in Tables 2.4 and 2.5, respectively. Results showed that on average the heterotrophic plate count represented < 49.25% of the total DAOC count (site B1 in week 49). In comparison, the percentage ratio of the heterotrophic plate count to the total FCM count (Table 2.1) was recorded at 0.43% at site B1 for the same sampling period with the total DAOC count representing only 0.87% of the total FCM count. Discrepancies in the percentage ratio of the heterotrophic plate count and total DAOC counts were observed in weeks 37 (490.06 %) and 45 (286.26%) (Table 2.4), respectively. Generally, the total DAOC count should represent all culturable and non-culturable micro-organisms, while the heterotrophic plate count (culturable organisms) should thus only account for a fraction of the total DAOC count. In general, the total DAOC count on average represented < 43.08% of the total FCM count (Table 2.5) for most of the sampling period. However, in week 29 at site B2 the total DAOC count could be compared to the total FCM count as the DAOC count represented 96.35% of the total FCM count.

A significant limitation to be considered when using membrane filtration in retaining and concentrating bacteria is the lack of a sufficient number of cells to be counted on the filter's surface. An increased microbial population usually provides a level of acceptable reliability to the resulting data (Fry, 1990). Care should also be taken where increased volumes of water are filtered through a single membrane, as nonbacterial biomass and debris tend to clog filters routinely used in total-direct-count methods (Lisle et al., 2004). A factor, which could also influence results, is that the DAOC technique involves physical counting of the micro-organisms in conjunction with epifluorescence microscopy, whereas the FCM method analyses total cell counts by means of computer software.

4. Conclusions

The major conclusions of the study are as follows:

1. On average, the MPN counts notably exceeded the maximum limit of 2000 micro-organisms/100 mℓ (South African Bureau of Standards, 1984) for river water.
2. Only 2% of the total MPN, 23% of faecal coliforms and 30% of *E. coli* counts fell into the accepted maximum limit range.
3. The highest overall counts for MPN (faecal coliforms, *E. coli*), heterotrophic counts, DAOC and FCM were observed at site B2 which is the site where storm water drainage pipes from the informal settlement flows into the river.
4. Overall higher viable cell counts were obtained from FCM analysis when compared to cell counts obtained by means of the heterotrophic plate count technique, which could be ascribed to the fact that the heterotrophic plate count technique only accounts for viable culturable micro-organisms whereas FCM analysis detects viable-culturable micro-organisms as well as those in a viable-but-non-culturable state.
5. The heterotrophic plate count thereby represented only a fraction < 3.65% of the total FCM count and < 6.06% of the viable FCM count (site B2 in week 37) of the sampling period.
6. The heterotrophic plate counts represented < 49.25% of the total DAOC count with exceptions in weeks 37 and 45 where higher heterotrophic plate counts with percentage ratios of 490.06% and 286.26% respectively, for heterotrophic plate counts versus DAOC counts were recorded. This indicates that inconsistencies could be experienced with the DAOC technique based on the fact that this technique

involves physical counting of the micro-organisms in conjunction with epifluorescence microscopy, whereas FCM analysis employs computer software which is more reliable.

7. In addition, the FCM technique indicated significantly ($p < 0.05$) higher total counts than those observed by the DAOC technique. The only comparable DAOC to FCM count was observed in week 29 at site B2 where 96.35% percentage ratio was recorded. The FCM technique therefore proves to be a more effective technique to routinely compare and evaluate the presence of most if not all, populations in the river water samples.

5. Acknowledgements

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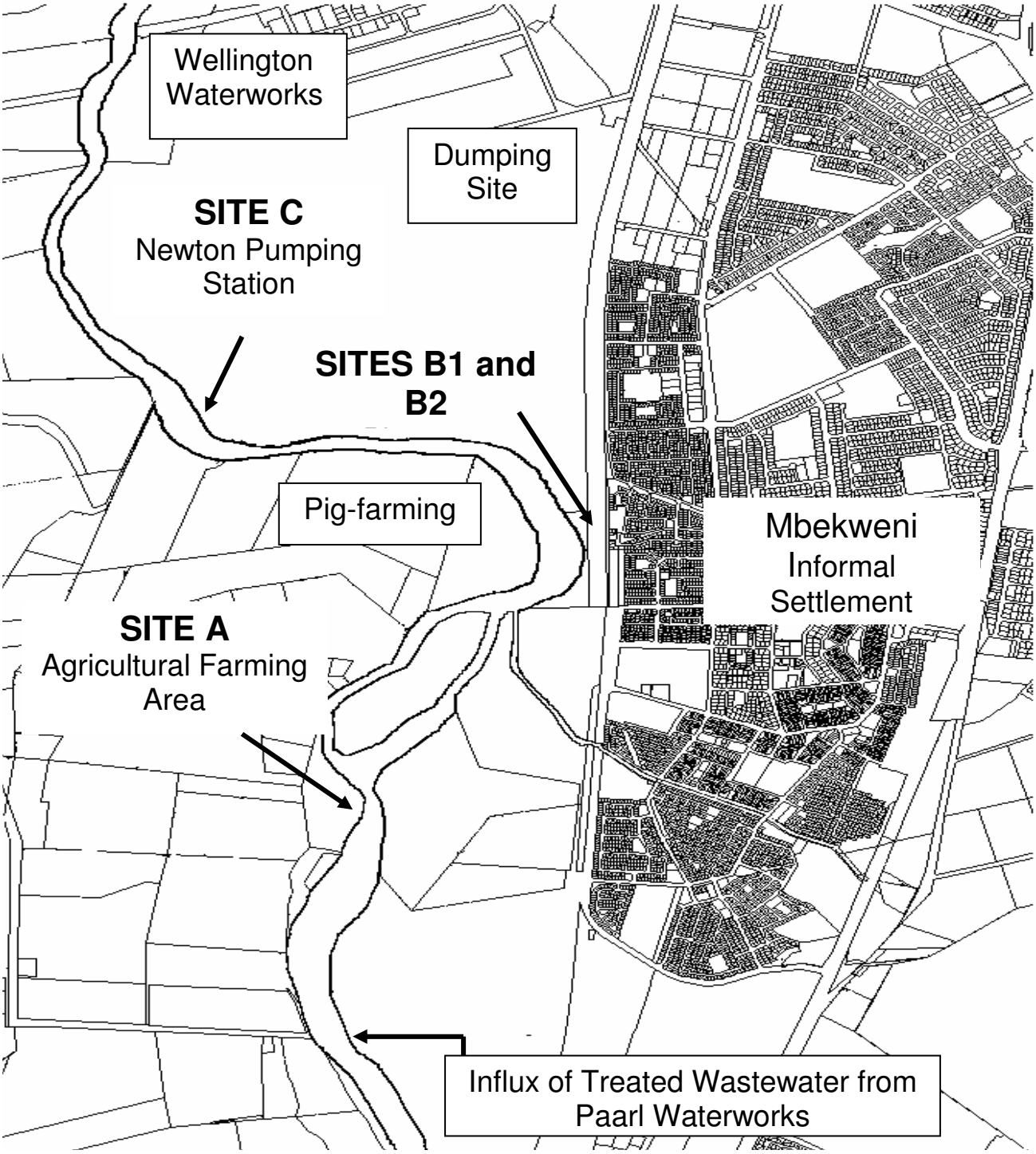

Figure 2.1

Map of the Berg River indicating the different sampling points: Site A - agricultural farming area; Sites B1 and B2 (Plot 8000) - close to the informal settlement of Mbekweni and Site C - the Newton pumping station.

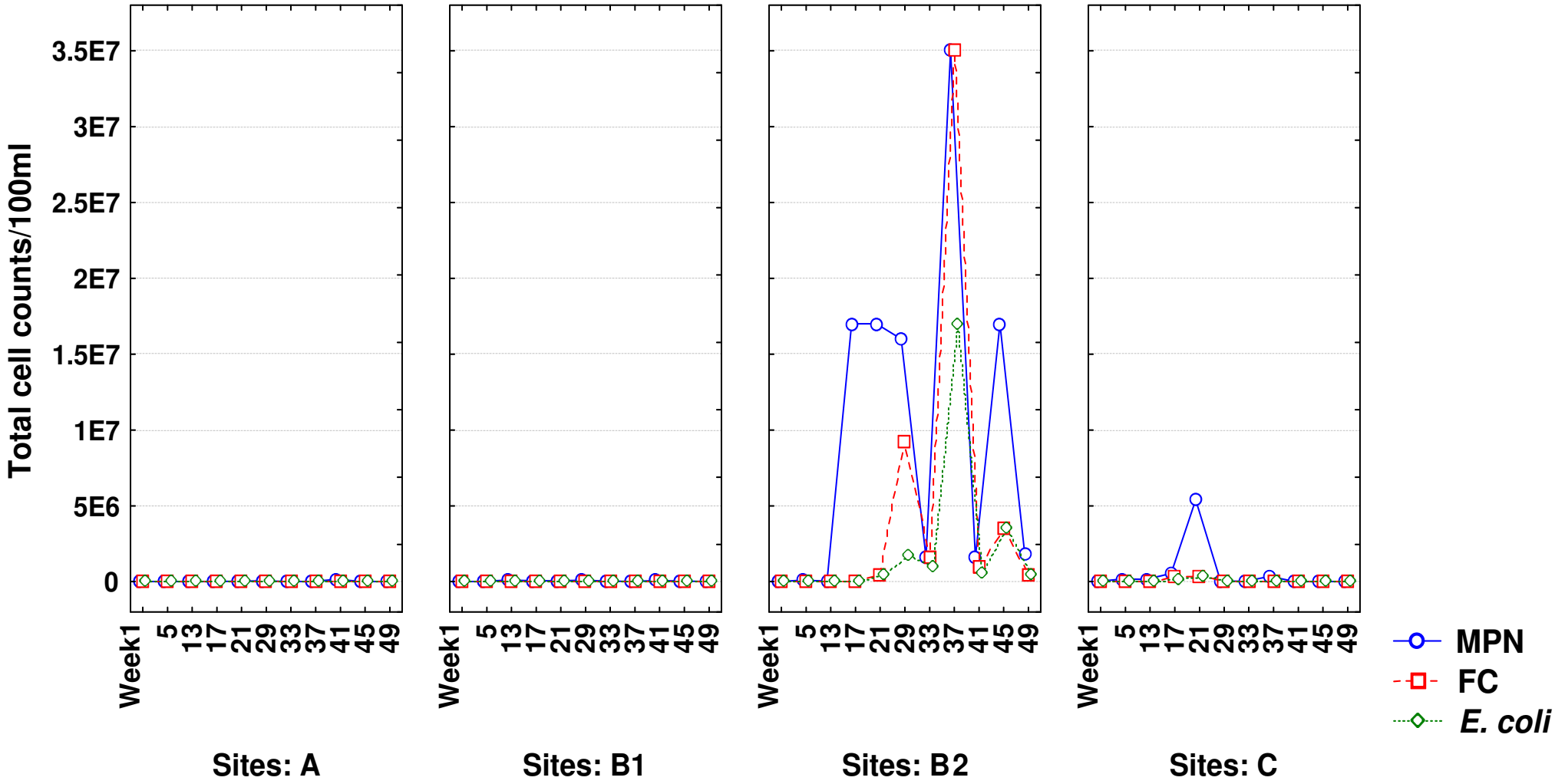

Figure 2.2

Comparison of Most Probable Number [(MPN), representing all possible gas-producing organisms], faecal coliforms (FC) and E. coli counts per 100mℓ of river water sample over the sampling period.

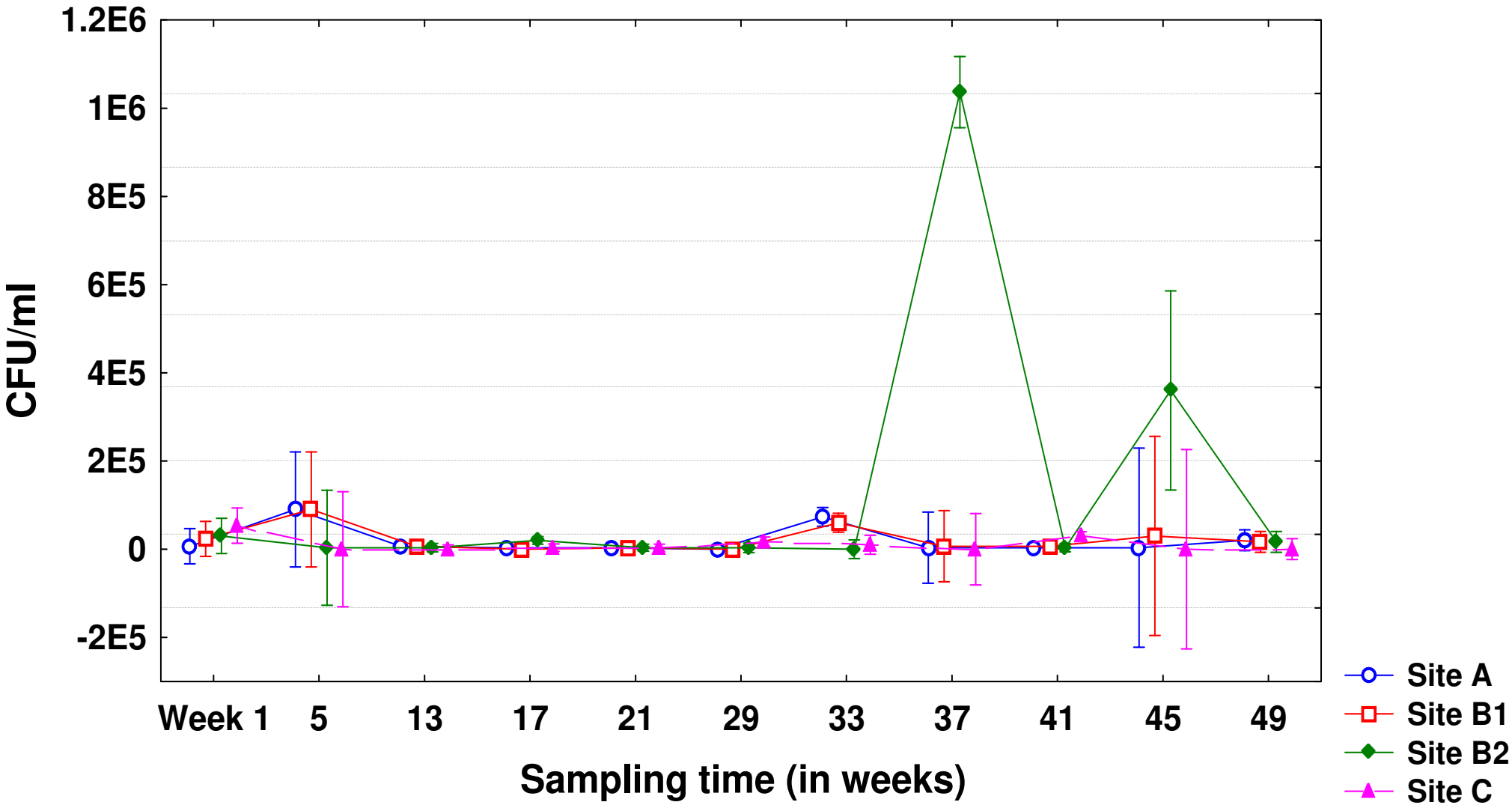

Figure 2.3

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

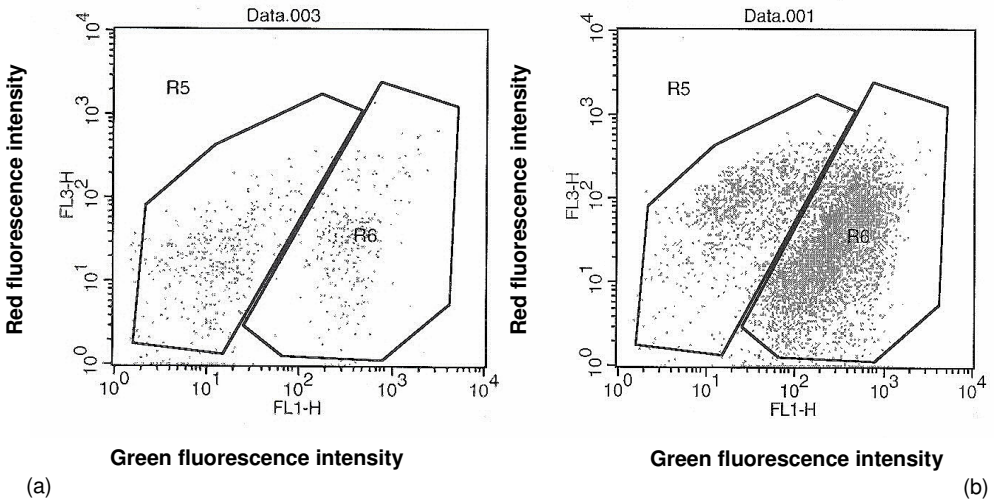

Figure 2.4

Flow cytometric analyses of river water samples: (a) total cell counts from a planktonic sample; (b) E.coli used as control, after staining with the BacLight™ probe. R5 and R6 indicate the dead and live populations, respectively.

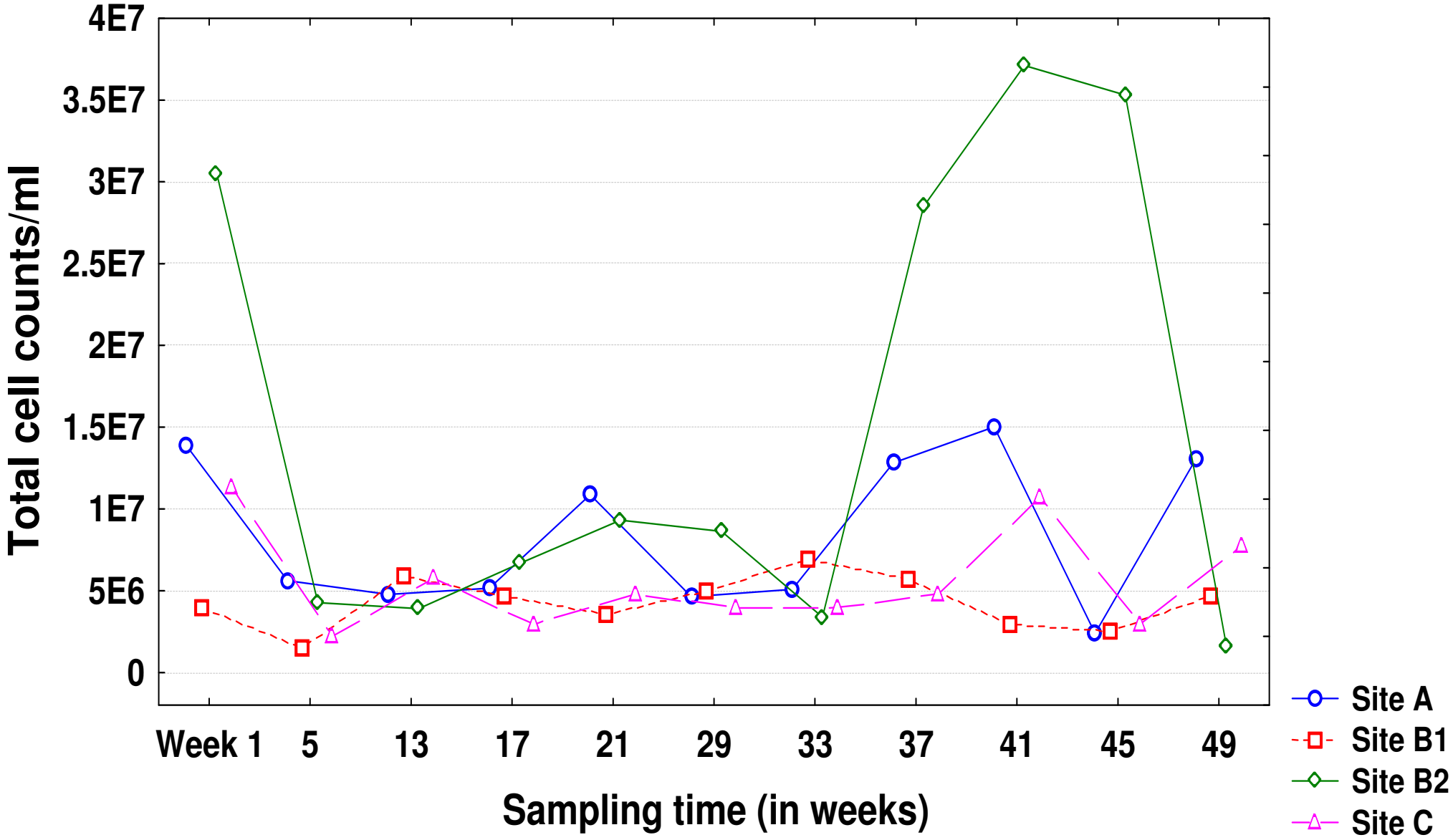

Figure 2.5

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

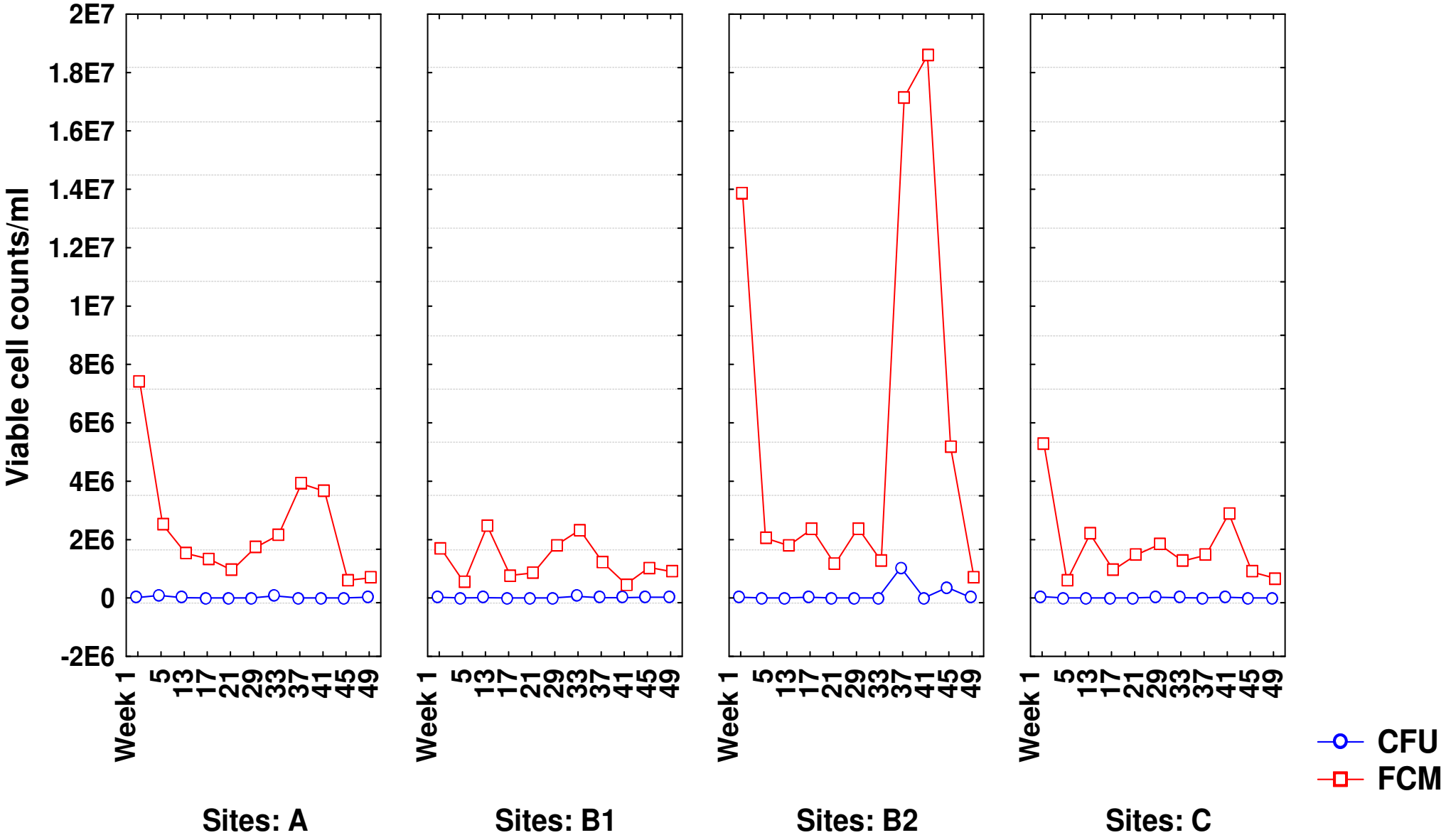

Figure 2.6

Comparison of colony forming units by heterotrophic plate counts to viable cell counts by means of flow cytometric analysis.

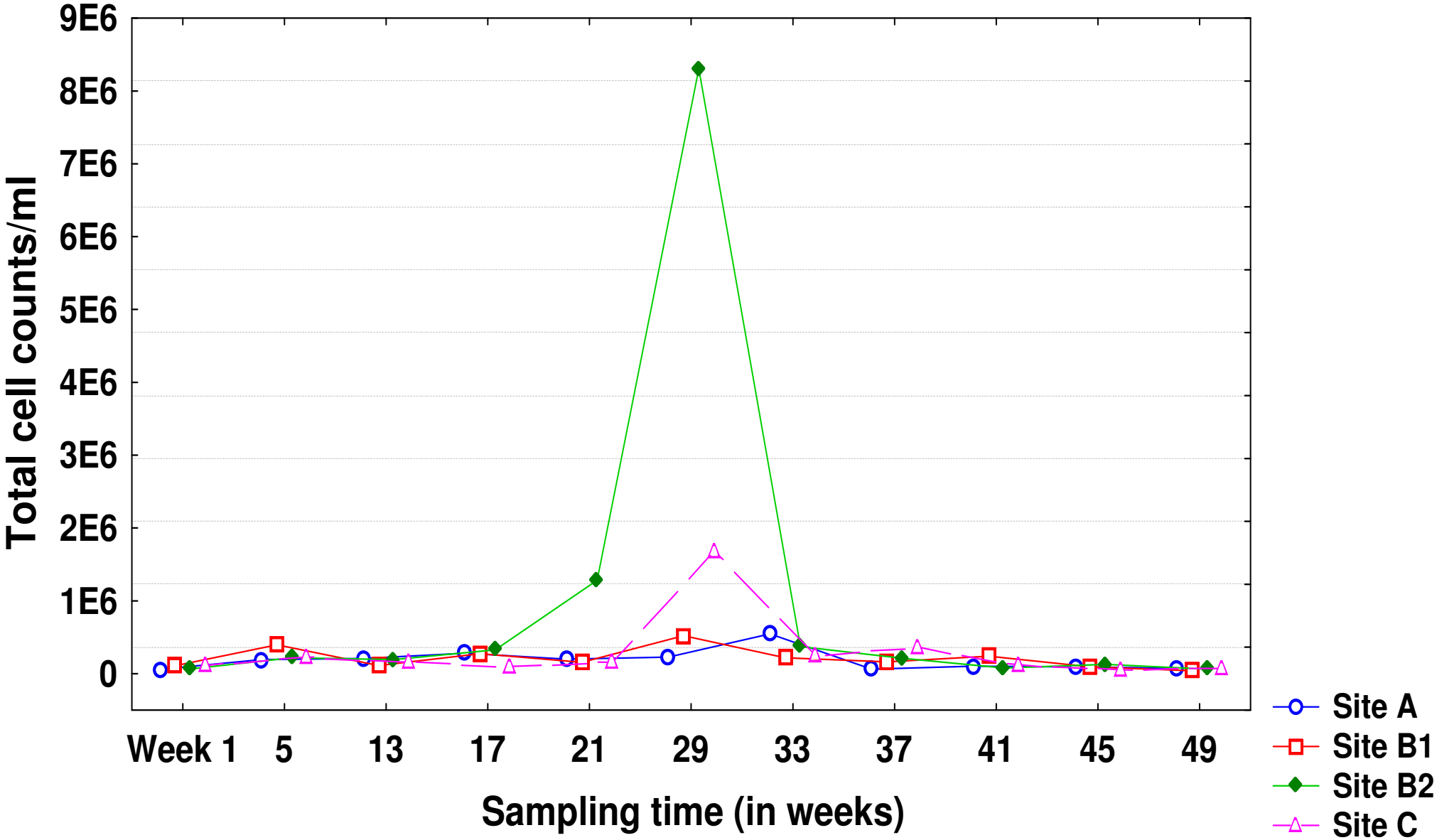

Figure 2.7

Enumeration of total bacteria for all sites analysed by means of the direct acridine orange count (DAOC).

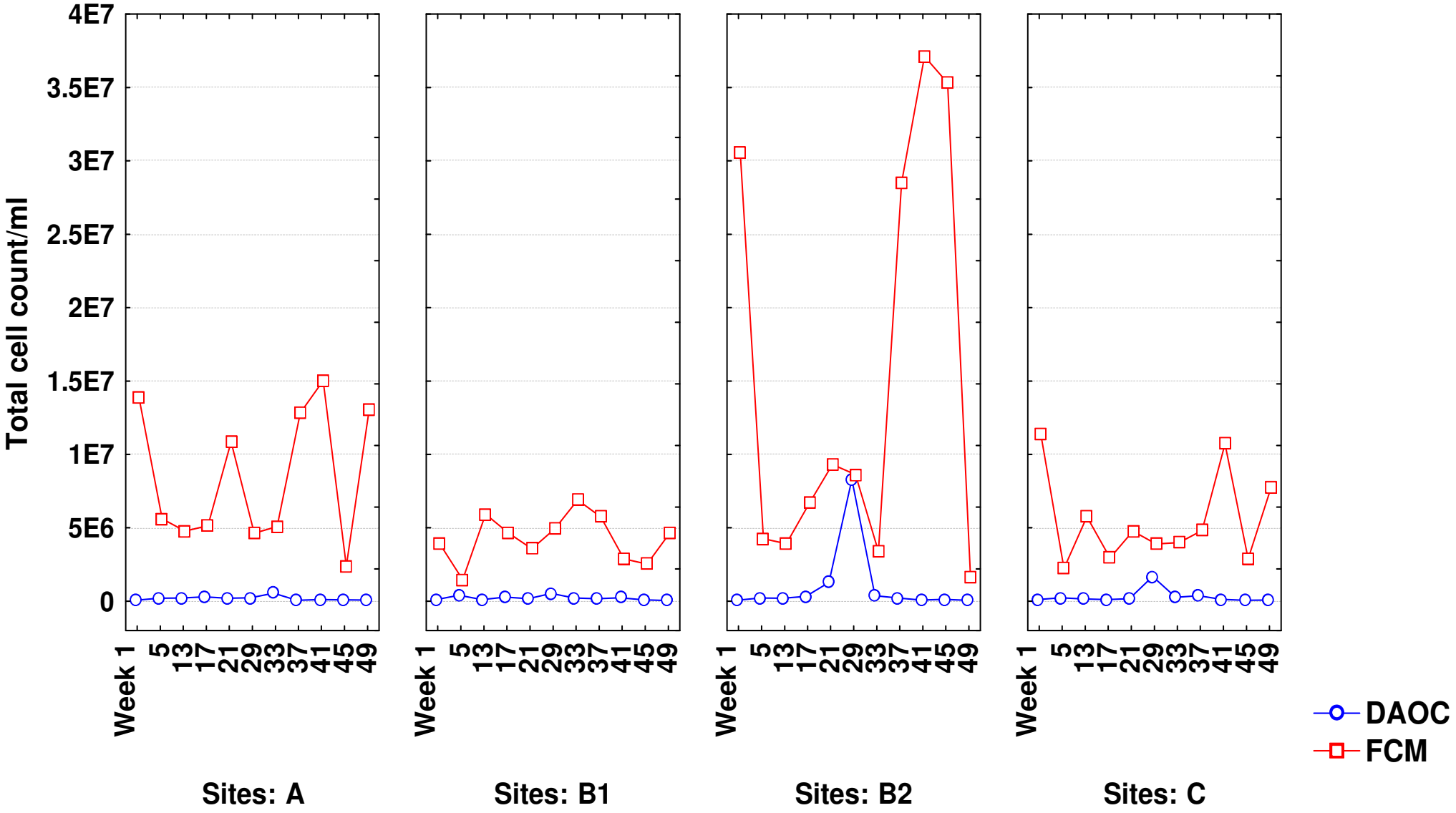

Figure 2.8

Comparison of total counts obtained by means of flow cytometric analysis to direct acridine orange count using epifluorescence microscopy.

Table 2.1 The average percentage ratio of heterotrophic plate counts to total cell numbers based on flow cytometric analysis for all samples analysed over the sampling period.

Parameter	1	5	13	17	21	29	33	37	41	45	49
A	0.07	1.61	0.21	0.06	0.03	0.00	1.38	0.02	0.02	0.13	0.15
B1	0.51	0.20	0.17	0.00	0.08	0.00	0.87	0.17	0.35	1.19	0.43
B2	0.10	0.00	0.08	0.30	0.03	0.03	0.00	3.65	0.01	1.02	1.23
C	0.44	0.00	0.00	0.10	0.06	0.51	0.25	0.00	0.28	0.00	0.00

Table 2.2 The average percentage ratio of viable cell numbers to total cell counts based on flow cytometric analysis for all samples analysed over the sampling period.

Parameter	1	5	13	17	21	29	33	37	41	45	49
A	53.48	44.87	32.11	25.87	8.77	37.31	42.46	30.54	24.37	25.66	5.25
B1	43.15	36.10	41.68	16.68	24.11	36.94	33.80	21.28	16.29	41.48	19.31
B2	45.51	48.15	45.94	35.39	12.44	27.78	37.38	60.18	50.09	14.64	44.20
C	46.48	26.64	38.02	32.56	31.02	47.04	31.70	30.52	26.99	30.84	8.64

Table 2.3 The average percentage ratio of heterotrophic plate counts to viable cell numbers based on flow cytometric analysis for all samples analysed over the sampling period.

Parameter	1	5	13	17	21	29	33	37	41	45	49
A	0.13	3.58	0.65	0.23	0.31	0.00	3.25	0.08	0.08	0.49	2.91
B1	1.18	0.57	0.40	0.00	0.35	0.00	2.58	0.82	2.13	2.88	2.22
B2	0.22	0.00	0.17	0.85	0.26	0.13	0.00	6.06	0.02	6.96	2.79
C	0.95	0.00	0.00	0.31	0.20	1.09	0.79	0.00	1.04	0.00	0.00

Table 2.4 The average percentage ratio of heterotrophic plate counts to total cell numbers based on direct acridine orange count analysis for all samples analysed over the sampling period.

Parameter	1	5	13	17	21	29	33	37	41	45	49
A	16.96	46.74	4.77	1.07	1.50	0.00	12.78	4.87	2.97	3.23	26.78
B1	18.39	0.76	8.88	0.00	1.91	0.00	27.43	6.21	4.17	32.25	49.25
B2	46.74	0.00	1.56	6.11	0.23	0.04	0.00	490.06	3.88	286.26	31.81
C	44.38	0.00	0.00	3.05	1.91	1.19	3.87	0.00	24.11	0.00	0.00

Table 2.5 The average percentage ratio of total cell numbers based on direct acridine orange count compared to total cell numbers based on flow cytometric analysis for all samples analysed over the sampling period.

Parameter	1	5	13	17	21	29	33	37	41	45	49
A	0.43	3.44	4.40	5.47	1.85	4.92	10.79	0.48	0.67	3.90	0.57
B1	2.76	27.08	1.90	5.94	4.42	10.56	3.18	2.81	8.32	3.70	0.87
B2	0.21	5.13	4.93	4.91	13.78	96.35	10.99	0.74	0.21	0.36	3.87
C	0.99	10.20	2.66	3.35	3.29	43.08	6.47	7.65	1.16	1.86	0.93

Comparison of microbial contamination at various sites along the Plankenburg- and Diep Rivers, Western Cape, South Africa

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Abstract

This study was aimed at investigating and comparing the microbial contamination levels at various sites in the Plankenburg and Diep Rivers in the Western Cape, South Africa. Sampling of sites along the Plankenburg River started in June 2004 and continued for a period of one year until June 2005. Sampling of the Diep River sites started in March 2005 and continued for a period of nine months until November 2005. Faecal coliform (FC) and *E. coli* (EC) counts were determined by means of the Most Probable Number technique, 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). The highest microbial counts for the Plankenburg River were observed at site B where the highest MPN, FC, *E. coli* and total FCM counts of 9.2×10^6 (week 14), 3.5×10^6 (week 39) and 3.5×10^6 micro-organisms/100 mℓ (week 39) and 2.1×10^8 micro-organisms/mℓ (weeks 1 and 39) respectively, were recorded. The highest HPC recorded for the Plankenburg River was 7.9×10^6 micro-organisms/100 mℓ (week 44, site A). Site B is situated close to an informal settlement where waste effluents from storm water drainage pipes enter the river system. In addition, other possible contamination sources included agricultural (site A) and industrial (site C) areas bordering the Plankenburg River. The highest total MPN, FC and *E. coli* counts in the Diep River were 5.4×10^6 (week 23) and 1.6×10^6 micro-organisms/100 mℓ [FC and *E. coli*, respectively (both in week 23)], recorded at site B. The highest HPC and total FCM counts of 1.7×10^7 micro-organisms/100 mℓ (week 14) and 2.5×10^9 micro-organisms/mℓ (week 23), respectively, were also recorded at site B. This site was identified as the most contaminated site along the Diep River and served as an accumulation point for waste effluents from the residential and industrial areas, which included paint and machine manufacturers. Other sources situated along the Diep River included storage and maintenance facilities for steel containers, a waste water treatment plant and an oil-refinery. Most of the bacterial counts obtained for the Plankenburg and Diep Rivers exceeded the accepted maximum limit for river water for most of the sampling period.

Keywords: river water; micro-organisms; most probable number; heterotrophic plate count; flow cytometry, industrial area, residential area, informal settlement.

1. Introduction

South Africa's major water sources are stored in dams and water abstraction schemes, which allow for the adequate and effective functioning of industry (e.g. power generation, food production, etc.), agriculture and domestic uses. Drought spells and point- and non-point source pollution has however, threatened the water availability, one of the country's most fundamental and indispensable national resources (Thukela Water Project Report, 2004).

Microbial agents, associated with waterborne outbreaks, include the bacterial organisms *Salmonella*, *Campylobacter* and *Escherichia coli (E. coli)* amongst others (Gerba, 1996), as well as viruses, protozoa, helminths and fungi. These micro-organisms may lead to significant health risks in humans, especially infants, the elderly and immunocompromised. In severe infections, waterborne diseases may result in chronic illness and even death (Theron & Cloete, 2002). Muller et al. (2001) investigated the occurrence of *E. coli* O157:H7 in selected river water samples in South Africa. The 204 samples screened were selected from 15 different sites where water was used for direct and indirect human consumption and domestic use. Even though none of the tested samples contained *E. coli* O157:H7, certain strains did however exhibit enterohaemorrhagic *E. coli* virulence properties, such as Shiga toxin 1 and 2 and enterohaemolysin, which could cause chronic health problems if ingested. In 2006 a cholera outbreak led to 1 298 deaths in Angola with tens of thousands of people infected. A survey showed that contaminated drinking water, poor sanitation and dense urban living were regarded as the main cause of the outbreak, which began in Luanda. Communities in this rapidly growing capital live in desecrated, garbage-filled slums with no reliable sources of clean water (Timberg, 2006). During November 2008, 6072 cases

of cholera infections and 294 subsequent deaths were reported in nine provinces of Zimbabwe. In the South African province of Limpopo during the same time period, 187 cholera cases were treated and three deaths (one South African and two Zimbabweans) were reported. Cholera cases have also been reported in Zambia and Mozambique where people travelled through the Beitbridge area in Zimbabwe (Department of Health, 2008). An inadequate supply of clean drinking water and poor levels of hygiene have been implicated as the reasons for these outbreaks. According to the World Heath Organisation (WHO), 16 141 suspected cholera infections, with 775 reported deaths, have been documented in Zimbabwe since December 2008, with a fatality rate estimated at 4.8%. Serious challenges also exist with controlling the current state of the outbreak as 460 cholera cases and nine deaths have been reported in South Africa, mostly in the areas bordering Zimbabwe (WHO, 2008).

The Plankenburg River is approximately ten kilometres long and services various activities in the residential, industrial and agricultural sectors. The river system runs through the town of Stellenbosch, which is well known for its many wine estates. Adjacent to the town is the informal housing scheme of Kayamandi, which comprises a population of more than 22 000 people. As documented by the Department of Water Affairs and Forestry (DWAF) (2001), 20% of Kayamandi's inhabitants live in brick houses with in-house water connections and flush toilets, while the remaining 80% occupy backyard shacks and informal dwellings. The river also serves as a source of irrigation to farmers in the agricultural areas both up- and downstream from the settlement. In addition, the river flows through Stellenbosch's industrial area, which includes amongst others a clothing factory, a well-known cheese factory, spray painting and mechanical workshops. Further downstream the river courses through an area of wineries and yoghurt and dairy producing plants (Barnes, 2003).

The Diep River catchment which is approximately 65 km in length is located in the South Western Cape Region and is bordered by industrial and residential areas. The estuary is about 900 hectares in area and consists of the Milnerton lagoon and the Rietvlei Nature Reserve and Boating Club (Lochner et al., 1994). The Diep River-Rietvlei system has silted up significantly over the past few years which has resulted in extensive erosion (Grindley & Dudley, 1988) and can therefore be regarded as a storage area for sediment-rich water during floods. The sedimentation rate is enhanced by vegetation in the vlei, especially where treated sewage water is being released. The river is bordered by various industrial establishments ranging from spray painting to chemical manufacturers as well as a wastewater treatment plant and an oil refinery, which could all have a significant impact on the water source and the surrounding environment.

The aim of this study was to investigate and compare the microbial contamination at various sites along the Plankenbrug and Diep Rivers in the Western Cape, South Africa. The level of faecal contamination in the river water samples was determined by the most probable number (MPN) technique, while the conventional plate count technique was used to determine heterotrophic counts (HPC). Flow cytometric analysis was used to obtain total cell counts (the culturable and non-culturable populations) in the collected water samples.

2. Materials and Methods

2.1 Sampling Sites

Three sampling sites were identified along the Plankenburg River (Stellenbosch) location: site A (Agricultural Farming and Residential Areas); site B (Informal Settlement of Kayamandi) and site C (Substation in Industrial Area) (Figure 3.1). Sampling of sites along the Plankenburg River started in June 2004 and continued for a period of one year until June 2005.

Sites for the Diep River (Milnerton) location: site A (Zoarvlei Nature Reserve - Industrial as well as Residential Areas); site B (Theo Marais Sportsclub - Industrial and Residential Area) and site C (Potsdam Wastewater Treatment Works) (Figure 3.2). Sampling of these sites started in March 2005 and continued for a period of nine months until November 2005 (a new site had to be selected after the initial site C dried up).

2.2 Sampling for Microbial contamination

The temperature and pH of the river water (Table 3.1) at the sampling locations were measured using a hand-held mercury thermometer and portable pH meter (Hanna Instruments). For microbial analysis water samples were collected in 1ℓ sterile Nalgene-polypropylene bottles and stored on ice to maintain a low temperature.

2.3 Most Probable Number (MPN)

The MPN technique was modified by Barnes (2003) and involved the inoculation of sample water into lauryl tryptose tubes containing Durham tubes, followed by incubation for 48 hours at 37ºC (indicating all gas-producing organisms). This differentiates between faecal coliforms [(FC) identifies all indicator organisms] and *E. coli* organisms within water samples (Oblinger & Koburger, 1975). Both these groups fall into the category of gas-producing micro-organisms (total MPN). All tubes indicating gas-formation were regarded as a positive presumptive test and the presumptive coliform count was read off De Mans tables (American Public Health Association, 1992, 1995). The positive tubes were re-inoculated into brilliant green bile broth and tryptone water tubes respectively, according to the guidelines set out by the South African Bureau of Standards (SABS, 1984). These guidelines also incorporate the standard methods set out by the American Public Health Association, American Water Works Association and the Water Environment Federation (American Society for Microbiology, 1997). Positive tubes were incubated in a 44.5ºC waterbath for 24 hours [44.5ºC promotes growth of faecal coliforms (Massa et al., 1988)]. Gas-production in the brilliant green tubes [indicating faecal coliforms (FC)] was compared to growth in the tryptone water tubes (indicating *E. coli*). The presence of *E. coli* was confirmed with a colour change from clear to pink or red after the addition of Ehrlich's reagent into the tryptone water tubes.

2.4 Heterotrophic plate counts (HPC)

Total heterotrophic counts were done in triplicate on Nutrient agar plates (NA) (Merck, Biolab Diagnostics) after serial dilutions (10^{-1} to 10^{-7}) of water samples were performed.

Plates were incubated for 3-4 days at 37ºC (pathogens infecting humans grow at this optimum temperature). Thereafter, the number of visible cells [colony forming units (CFU)] were counted and recorded.

2.5 Flow cytometry (FCM)

For the flow cytometry-based assay, individual samples were subjected to a Becton Dickinson FACSCalibur flow cytometer for analysis as outlined in Paulse et al. (2007). 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 therefore, 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 addition of fluorescent beads enables the calculation of absolute or total cell counts in samples. The absolute number (cells/$\mu\ell$) 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 before analysis. The staining procedure was performed by combining equal volumes of PI (propidium iodide) (4 μℓ) and SYTO 9 (4 μℓ) in BacLight™, 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 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 beads allows 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 or reference population to which unknown bacterial populations in planktonic samples could be compared during quantitative analyses (i.e. total cell count). Bacterial populations are reflected in a specified gating area on a flow cytometric scattergram or dotplot.

Equation 1. $$\frac{\text{Number of events in cell region}}{\text{Number of events in bead region}} \times \frac{\text{Number of beads / test}}{\text{test volume}} \times \text{dilution factor}$$

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

2.6 Statistical analysis

Repeated measures ANOVA (RMA) was 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 significant level of 5% was used as standards.

3. Results and Discussion

Temperature and pH were monitored at all sites along the Plankenburg and Diep Rivers and are presented in Tables 3.1 and 3.2, respectively. However, the results for microbial parameters investigated fluctuated throughout the sampling period and no distinct correlation between water temperature, pH and differences in microbial numbers could be drawn. It should also be noted that the MPN, FC, *E. coli* and HPC counts were calculated per 100 mℓ, while total, viable and dead FCM counts were calculated per mℓ.

3.1 Plankenburg River

3.1.1 Most Probable Number (MPN), Heterotrophic Plate Count (HPC) and Flow cytometric analysis (FCM).

The results obtained for MPN, FC, *E. coli* and HPC at site A along the Plankenburg River are represented in Figure 3.3, while the total FCM, as well as viable and dead FCM counts are represented in Figure 3.4. The MPN, FC, *E. coli* and HPC results

recorded at site B are represented in Figure 3.5 and the total FCM, viable and dead FCM are illustrated in Figure 3.6. Results obtained for site C along the Plankenburg River included total MPN, FC, *E. coli* and HPC counts represented in Figure 3.7 and total, viable and dead FCM counts are represented in Figure 3.8.

The rivers investigated serve multi-purpose functions and it is important to note that the South African Bureau of Standards (SABS, 1984) (river water) and Department of Water Affairs and Forestry (DWAF, 1996a-c) (i.e. for domestic, recreational and agricultural purposes) have stipulated guidelines for faecal coliform counts to safeguard these natural water sources. The maximum acceptable levels of *E. coli* in natural water sources, such as rivers, have been stipulated in the SABS guidelines (1984) and DWAF (1996a) as > 2000 micro-organisms/100 mℓ and > 130 micro-organisms/100 mℓ, respectively as counts above these levels may lead to waterborne infections. According to DWAF, where river water is used for recreational purposes, the FC counts should not exceed 2000 micro-organisms/100 mℓ (DWAF, 1996a), while an *E. coli* count of > 130 micro-organisms/100mℓ, could lead to serious disease infections.

Most of the results for the MPN and FC counts obtained from the Plankenburg River, on average exceeded the acceptable limit (SABS, 1984; DWAF, 1996a). During the sampling period at various sites along the Plankenburg River, no *E. coli* were detected. The highest total MPN counts observed at site A along the Plankenburg River (Figure 3.3) were 1.1×10^5 and 9.2×10^4 micro-organisms/100 mℓ recorded in weeks 23 and 44, respectively. The highest FC and *E. coli* counts recorded at this site were 5.4×10^3 (week 18) and 9.2×10^3 micro-organisms/100 mℓ (week 44), respectively. Total culturable microbial counts (HPC) obtained at site A are also depicted in Figure 3.3 with the highest HPC recorded at 7.9×10^6 micro-organisms/100 mℓ in week 44. The

total FCM counts recorded at site A ranged from 5.3 x 10^6 micro-organisms/mℓ (week 23) to 8.5 x 10^7 micro-organism/mℓ (week 5). A high FCM count was also observed in week 1 where 8.4 x 10^7 micro-organisms/mℓ were recorded.

Compared to the high HPC recorded at site A in week 44, a viable FCM count (Figure 3.4) of 2.1 x 10^7 micro-organisms/mℓ was observed. High viable FCM counts were also observed in weeks 5, 32 and 48 where counts of 7.4 x 10^7, 2.5 x 10^7 and 2.3 x 10^7 micro-organisms/mℓ were recorded. Corresponding HPC results recorded during weeks 5, 32 and 48 were 4 x 10^5, 1 x 10^7 and 3 x 10^4 micro-organisms/100 mℓ. As previously mentioned, the HPC results were calculated per 100 mℓ, while the viable FCM counts were calculated per mℓ. The HPC were thus significantly ($p < 0.05$) lower than the viable FCM count throughout the sampling period. The FCM technique distinguishes between ratios of live (viable), damaged (permeabilised) and dead (non-viable) cells, in conjunction with the LIVE/DEAD BacLight™ probe (Kell et al., 1998; Boulos et al., 1999). The results clearly show that micro-organisms that are generally undetected by conventional plate count techniques such as the HPC method can thus be detected by means of the FCM technique. In 2005, Pianetti et al. investigated the viability of *Aeromonas hydrophila* in various water samples by means of flow cytometry and compared the results to the conventional plate counting technique and spectrophotometric analysis. Results indicated that even though the optical density was low a greater *A. hydrophila* growth was observed with flow cytometric analysis while no plate count was obtained with the conventional plate count technique.

By comparing the total MPN to the total FCM counts in the current study, results clearly show that the MPN, which represents the total gas-producing micro-organisms, did not have any significant influence on the total FCM count recorded at site A. The

peaks indicating the high total FCM counts in Figure 3.4 do not correspond to the high MPN count observed in weeks 1 and 5, respectively. The results clearly show that bacterial pollutants other than the possible gas-producing micro-organisms (total MPN) could be responsible for the increased total FCM count. As mentioned, site A is bordered by agricultural farming and residential areas from which waste effluent flows into the river system, thereby contributing to possible contamination. Even though faecal contamination at this site does not contribute to increased health concerns, the faecal pollution observed at this site could be ascribed to the fact that this site serves as a cross-over point (humans and animals) between the residential area and one of the major roads leading into the town of Stellenbosch. A study conducted by Paulse et al. (2009) indicated that bacterial species such as *Bacillus* spp., *Comamonas* spp., *Brevundimonas* spp., and various members of the *Enterobacteriaceae* were present at this site. Bacterial species such as *Klebsiella oxytoca*, *Alcaligenes faecalis*, *Aeromonas* sp., *Lysobacter taiwanensis*, *Thermomonas fusca* and *Acinetobacter* sp., indicator organisms of faecal contamination, were also identified at this site. Waste materials such as rubber and wooden products, glass and plastic, foam as well as paper, impede water flow at this site, allowing for possible microbial growth and biofilm formation. In a previous study conducted by Paulse et al. (2007), increased microbial counts were obtained from materials to which biofilm communities were attached (results not shown). Research has also shown that pathogens survive longer in water and soil where organic matter, which provides attachment substrates and nutrients to micro-organisms, is readily available (Perri & Fallon, 1998; Fischer et al., 2003).

Compared to the MPN, FC and *E. coli* counts obtained at site A, a considerably higher influx of faecal contaminants could be observed at site B (Figure 3.5) along the Plankenburg River. The highest MPN counts recorded at this site were 4.6×10^6 (week

1), 9.2 x 10^6 (week 14), 3.5 x 10^6 (week 39) and 3.5 x 10^6 micro-organisms/100 mℓ (week 44). In addition, the highest FC and *E. coli* counts were 1.7 x 10^6 (week 14) and 3.5 x 10^6 (week 39) micro-organisms/100 mℓ, respectively. The highest HPC results were also recorded in weeks 14 and 39 where counts of 2.2 x 10^6 and 3.4 x 10^6 micro-organisms/100 mℓ were observed. The total FCM counts (Figure 3.6) at this site were also higher for most of the sampling period with exceptions in weeks 5, 32, 35 and 54. High FCM counts of 2.1 x 10^8 (weeks 1 and 39) and 1.5 x 10^8 (weeks 14 and 48) micro-organisms/mℓ were observed at this site. Results clearly show that increased activity from total gas-producing micro-organisms (total MPN) had a significant ($p < 0.05$) effect on the total FCM count recorded during the sampling period at site B as shown in weeks 1, 14 and 39 (not week 48 where a decreased total MPN was observed). The temperature and pH recorded at this site in weeks 1, 14 and 39 were 15°C (pH = 7.0), 11°C (pH = 7.1) and 25°C (pH = 6.5), respectively (Table 3.1). Even though an amplified microbial activity was recorded in week 39, where the highest water temperature of 25°C was measured, the microbial counts fluctuated with changes in water temperature and pH values throughout the sampling period.

High viable FCM counts (Figure 3.6) of 1.1 x 10^8 and 7.9 x 10^7 micro-organisms/mℓ were obtained with flow cytometric analysis and the HPC results obtained for the same samples were considerably lower (2.2 x 10^4 and 3.4 x 10^4 micro-organisms/mℓ, respectively).

It is evident from the results obtained that most of the general microbial activity was observed at site B where the highest MPN, FC, *E. coli* and FCM counts were recorded. This site is situated in close proximity to the informal settlement of Kayamandi where the sanitation systems are inadequate to satisfy the needs of the inhabitants.

The surrounding environment, storm water drainage systems and waste containers are used as a means of disposal of faecal matter. The high MPN, FC and *E. coli* counts could thus be indicative of the possible impact of increased faecal activity on a river system (DWAF, 2001). It has also been noted during the study period that faecal waste from surrounding public sanitary facilities is flushed into the river at this site, increasing faecal matter and thus possible faecal coliform growth.

A study conducted by Paulse et al. (2007) also revealed MPN, FC and *E. coli* counts exceeding the maximum allowable limit for gas-producing micro-organisms in the Berg River, Western Cape. The highest MPN and FC counts of 3.5 x 10^7 micro-organisms/100 mℓ respectively were observed at the site situated close to the informal settlement of Mbekweni. These high counts were ascribed to the possible influx of human and household waste from the informal settlement into the river system. In the study conducted by Paulse et al. (2009), bacterial species introduced into the river at site B (Plankenburg River) included various members of the *Enterobacteriaceae* group such as *Serratia* sp., *Citrobacter* sp., *Yersinia ruckeri* and *Enterobacter* sp. thereby confirming faecal contamination at this site. The genus, *Yersinia* is considered one of the major human pathogens and may be the causative agent of plague in susceptible individuals, thereby raising health concerns in this area. Site B is also surrounded by dense vegetation which slows the water flow contributing to an increase in possible faecal contaminants (Fischer et al., 2003). The increased *E. coli* counts at this site thus raise alarming concerns as the community inhabiting these riverbanks is exposed to possible pathogens on a daily basis. In addition, various waste materials such as glass, plastic containers, rubber (vehicle tyres), steel (shopping carts) and electrical wiring, apart from organic waste material from surrounding plants and trees contaminate the river at this site. These and other materials such as sediment found within a river

system may provide increased attachment surfaces for the colonisation of bacteria (Armon et al., 1997; Fischer et al., 2003).

The highest MPN counts recorded during the sampling period at site C (Figure 3.7) were 1.7 x 10^6 (week 35), 2.8 x 10^6 (week 39) and 2.3 x 10^6 micro-organisms/100 mℓ (week 48). The corresponding FC counts were 9.2 x 10^5, 1.8 x 10^6 and 7.8 x 10^5 micro-organisms/100 mℓ during the same sampling weeks. Similarly, high *E. coli* counts were also recorded in the same weeks with the highest count of 1.4 x 10^6 micro-organisms/100 mℓ, recorded in week 39. High HPC results (6.7 x 10^6 micro-organisms/100 mℓ) were also observed during this week at site C. During the present study, various materials sources observed at sites A and B, were also observed at site C. As mentioned, these materials provide ideal attachment surfaces to which micro-organisms adhere to and multiply in order to form biofilms (Armon et al., 1997).

The total FCM counts recorded at site C (Figure 3.8) ranged from 1.2 x 10^7 (week 32) to 1.2 x 10^8 micro-organisms/mℓ (weeks 48). Site C is situated directly after the informal settlement of Kayamandi and close to a substation in an industrial area. The dense vegetation on the riverbanks at this site could possibly also contribute to slower water flow, thereby promoting microbial growth and increased numbers (Fischer et al., 2003). Compared to HPC analysis, the viable cells obtained by FCM analysis, were significantly higher ($p < 0.05$) than counts obtained by the heterotrophic plate count technique (Figure 3.7). The highest viable FCM counts were observed in weeks 35 and 48 where counts of 6.8 x 10^7 and 8.3 x 10^7 micro-organisms/mℓ, respectively, were recorded. Corresponding HPC's recorded during weeks 35 and 48 were 1.3 x 10^4 and 3.0 x 10^3 micro-organisms/mℓ, respectively, once again indicating the reliability of the FCM technique. Monis et al. (2003) conducted a study on untreated and potable water

samples in South Australia. Water borne bacteria were enumerated by means of viable assays and flow cytometric analysis and the microbial numbers obtained, were compared to culture-based techniques. Counts obtained by the FCM technique yielded 5.56 x 10^2 and 3.94 x 10^4 active bacteria/mℓ compared to the culture based techniques, which were two to four log cycles less. The results thus suggested that flow cytometry be used to detect physiologically active bacteria from the various water sources.

In addition, downstream from this site the river services an agricultural area which greatly contributes to the international wine industry and thus the economy of the country. According to DWAF, river water used for irrigation, such as the Plankenburg River, should not exceed 1000 micro-organisms/100mℓ (*E. coli*) in the water source. Increased counts as high as those observed at site C, therefore could not only cause serious disease in humans (DWAF, 1996c), but also have major effects on the economy of the country.

Comparison of microbial counts recorded in the water samples obtained from the sites along the Plankenburg River indicate that site B was the most contaminated point. The results show that the lack of proper sanitary facilities and poor service delivery in the informal settlement located at site B could have contributed to the contamination of the river. Results clearly show that contamination at site B could have been influenced by human waste from public toilet systems situated on the river banks which are flushed into storm water drainage pipes which leads directly into the river at this site. The high microbial counts could lead to major health concerns as results for the MPN, FC, *E. coli*, HPC and FCM counts at all the sites investigated along the Plankenburg River exceeded the stipulated maximum limit for a water source used for recreational and irrigational purposes (DWAF, 1996a-c). Unless adequate waste removal and sanitary

facilities are incorporated, maintained and monitored, continued contamination of the river will not only lead to increased and major health concerns within these communities but will also have a negative impact on the environment.

3.2 Diep River

3.2.1 Most Probable Number (MPN), Heterotrophic Plate Count (HPC) and Flow cytometric analysis (FCM).

In the Diep River, the results obtained for MPN, FC, *E. coli* and HPC at site A are presented in Figure 3.9, while the total, viable and dead FCM counts are represented in Figure 3.10. The results recorded at site B are represented in Figure 3.11 and the total FCM, viable and dead FCM cells are illustrated in Figure 3.12. Counts obtained from site C included total MPN, FC, *E. coli* and HPC represented in Figure 3.13 and total, viable and dead FCM counts in Figure 3.14. The total MPN counts for the Diep River samples (Figure 3.9) recorded at site A ranged from 6.8 x 10^3 micro-organisms/100 mℓ (week 9) to 3.5 x 10^5 micro-organisms/100 mℓ (week 14). A high MPN count was also observed in week 32 where counts of 1.7 x 10^5 micro-organisms/100 mℓ were recorded. The highest FC and *E. coli* counts recorded at this site were 1.1 x 10^4 micro-organisms/100 mℓ, respectively, both recorded in week 5. The highest HPC counts of 1.7 x 10^6 micro-organisms/100 mℓ and 2.1 x 10^6 micro-organisms/100 mℓ were recorded in weeks 1 and 23, respectively. The highest total FCM counts (Figure 3.10) were recorded in weeks 14, 23 and 32 where counts of 2.6 x 10^8, 2.5 x 10^8 and 1.3 x 10^8 micro-organisms/mℓ, respectively, were observed. Significantly higher ($p < 0.05$) counts

were obtained for the viable FCM count in comparison to the HPC technique. The highest HPC of 2.1 x 10^4 micro-organisms/mℓ was recorded in week 23 with a corresponding viable FCM count of 2.4 x 10^8 micro-organisms/mℓ observed during the same sampling period. High viable FCM counts were also observed in weeks 14 and 32 where counts of 2.5 x 10^8 and 1.2 x 10^8 micro-organisms/mℓ were recorded. This site along the Diep River borders residential and industrial areas and a natural wetland system is also situated at site A (Friends of Rietvlei Group, 2006-2008). Site A is situated before the wetland system, while site B is located after the wetland system. Industries in the area include a steel container storage depot as well as various machine and tool manufacturers.

The highest MPN count recorded in the river at site B (Figure 3.11) (Theo Marais Sportsclub) was 5.4 x 10^6 micro-organisms/100 mℓ with corresponding FC and *E. coli* counts of 1.6 x 10^6 micro-organisms/100 mℓ, respectively, observed in week 23. A higher HPC range was also observed at this site with the lowest count of 4 x 10^5 micro-organism/100 mℓ. The highest HPC results recorded were 1.7 x 10^7 micro-organism/100 mℓ (week 14) and 9.4 x 10^6 micro-organism/100 mℓ (week 23). In comparison to the highest total FCM counts (Figure 3.12) recorded at site A (weeks 14, 23 and 32), higher counts were also recorded during weeks 23 and 32 (2.5 x 10^9 and 2.1 x 10^8 micro-organism/mℓ, respectively). Comparison of the viable FCM to the HPC results, showed that significantly higher ($p < 0.05$) counts were obtained with the FCM technique as observed in weeks 23, 32 and 36 where viable FCM counts of 2.3 x 10^9, 1.1 x 10^8 and 3 x 10^7 micro-organism/mℓ, respectively, were recorded. Correspondingly, HPC results were 9 x 10^6, 6 x 10^5 and 4 x 10^6 micro-organism/mℓ, respectively, during the same sampling weeks. This corresponds to the results obtained in a previous study

where significantly higher ($p < 0.05$) viable FCM counts compared to the HPC results were recorded in the Berg River (Paulse et al., 2007). In the study by Paulse et al. (2007) the highest HPC count of 1.04×10^6 micro-organisms/mℓ was recorded in week 37 (site B2) with a corresponding viable FCM count of 1.72×10^7 micro-organisms/mℓ recorded for the same sampling time. At site B2 high viable FCM counts were also recorded in weeks 1 and 41, where counts of 1.4×10^7 and 1.9×10^7 micro-organisms/mℓ, respectively were observed. The HPC results recorded during the same sampling period were 3×10^4 (week 1) and 3×10^3 (week 41) micro-organisms/mℓ. It was thus concluded that the FCM technique yielded a higher microbial count and is therefore a more reliable enumeration technique.

Compared to site A the results recorded at site B displayed evidence of significantly high gas-producing microbial growth and activity. This site is situated in an industrial area with industries ranging from spray painting, paint and chemical manufacturers to car wash facilities and pharmaceutical companies. A residential area is also situated in close proximity to site B. In addition, the site is also in an open area with minimum foliage for shade and where increased temperature could thus lead to higher water temperatures and thus increased microbial activity. Microbial results fluctuated at site B and no correlation could be drawn between the overall high counts experience in week 23 and the water temperature and pH (Table 3.2) recorded during the same sampling period.

Site C is situated at the Potsdam Wastewater Treatment Works. Results obtained at this site along the Diep River are represented in Figures 3.13 and 3.14. The highest MPN, FC and *E. coli* counts (Figure 3.13) recorded at this site were 5.4×10^5 (weeks 9 and 32), 3.5×10^5 (week 9) and 1.6×10^5 micro-organisms/100 mℓ (week 9),

respectively. Compared to site B, increased FC and *E. coli* counts (for most of the sampling period), were observed at site C which could indicate increased faecal contamination in this area. The Potsdam Water Treatment Works receives wastewater directly from the surrounding residential areas as well as an oil refinery. The highest HPC's recorded at site C were 1.9×10^6 micro-organisms/100 mℓ in week 14. The highest total FCM counts were observed in weeks 18, 23 and 36 where counts of 1.3×10^8, 2.8×10^8 and 1.3×10^8 micro-organisms/mℓ were recorded.

Overall an initial increase in the HPC results from site A to site B was followed by a decrease in the total HPC recorded at site C (with exception of week 32). Even though site C is an enclosed facility and the possibility of river water being used for domestic use is low, the microbial counts observed at this site may still raise major health concerns as the Diep River runs into the Rietvlei Nature Reserve, which is used for recreational purposes. As mentioned DWAF stipulates that where river water is used as a means of recreation, a faecal coliform and *E. coli* count of > 2000 micro-organisms/100 mℓ and > 130 micro-organisms/100 mℓ, respectively, may lead to serious disease infections (DWAF, 1996b). The overall results obtained from the three sites along the Diep River were thus significantly ($p < 0.05$) higher than the stipulated guidelines for most of the sampling period.

Site B along the Diep River was also identified as the most contaminated site. Waste effluent from residential and industrial areas accumulates at this site and could serve as sources of contamination. Results have also shown that the high microbial counts for the MPN, FC, *E. coli* (SABS, 1984; DWAF, 1996a-c), HPC and FCM exceeded the stipulated limit for river water for most of the sampling period. It should also be noted that the results show that the FCM technique is a more reliable

enumeration technique than the conventional plate count technique and it should be incorporated into the routine monitoring of environmental samples.

4. Conclusions

The major conclusions of the study thus include the following:

1. On average, the MPN, FC and *E. coli* levels within the Plankenburg River notably exceeded the maximum limit of 2000 micro-organisms/100 mℓ for river water throughout the study period (SABS, 1984; DWAF, 1996a-c).
2. Contamination of the river was also confirmed with the significantly high ($p < 0.05$) total FCM counts observed at all the sites along the Plankenburg River.
3. The MPN, FC, *E. coli*, HPC and FCM results obtained from the different sites along the Plankenburg River indicate that site B was the most contaminated site with higher counts than sites A and C.
4. Site B is situated close to the informal settlement where sanitation systems are inadequate to satisfy the needs of the inhabitants and where storm water drainage systems and waste containers are used as a means of disposal of faecal matter. In addition, sanitation systems located in the settlement are connected to storm water drainage pipes which enter the river system at this site.
5. Even though significantly lower microbial counts were observed at sites A and C, these sites are bordered by residential and industrial areas, respectively, which might have contributed to the increased microbial contaminants at these sites.
6. Agricultural run-off from farms adjacent to the river (Plankenburg River) could also have contributed to increased contaminant levels within the river at site A.

7. The MPN, FC, *E. coli*, HPC and FCM counts within the Diep River also exceeded the maximum limit of 2000 micro-organisms/100 mℓ for river water throughout the study period (SABS, 1984; DWAF, 1996a-c).
8. Overall, initial increases in the bacterial counts from site A to site B were followed by decreases in the bacterial counts recorded at site C. Results thus show that site B was the most contaminated site along the Diep River. Site B is located at the Theo Marais Sportsclub, which is surrounded by major industrial and residential areas. Waste effluent from these areas accumulates at this site contributing to possible contamination.
9. Site A is also bordered by industrial and residential areas, while site C receives waste water from a residential area as well as an oil-refinery. Even though no point- or non-point sources of contamination could be identified, the waste effluent present at the residential and industrial sites could influence water pollution at the respective sampling sites.
10. Comparisons of the HPC results to the viable FCM counts, which were significantly higher ($p < 0.05$) for the Plankenburg and Diep Rivers throughout the sampling period, proved that the FCM technique is a more reliable enumeration method to accurately determine the viable bacterial counts in environmental samples.
11. Overall, the significantly high microbial counts obtained for both river systems raises major human and environmental health concerns. The effectiveness of current monitoring and clean-up strategies by respective authorities and district municipalities should be revised to include techniques which accurately reflect the contamination levels of the river systems.

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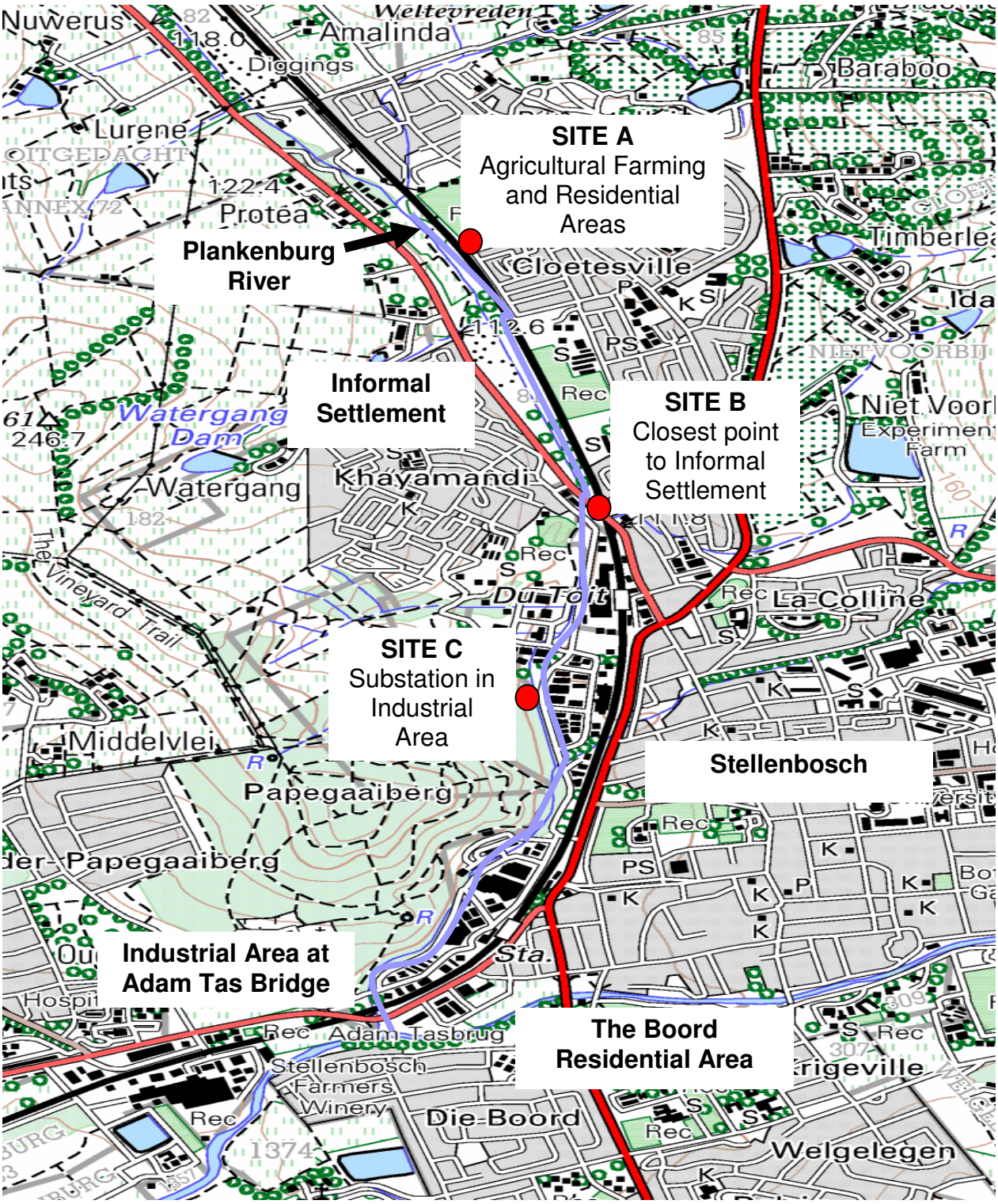

Figure 3.1

Map of the Plankenburg River indicating the different sampling points: Site A - agricultural farming as well as residential areas; Site B - close to an informal settlement and Site C - Substation in the industrial area.

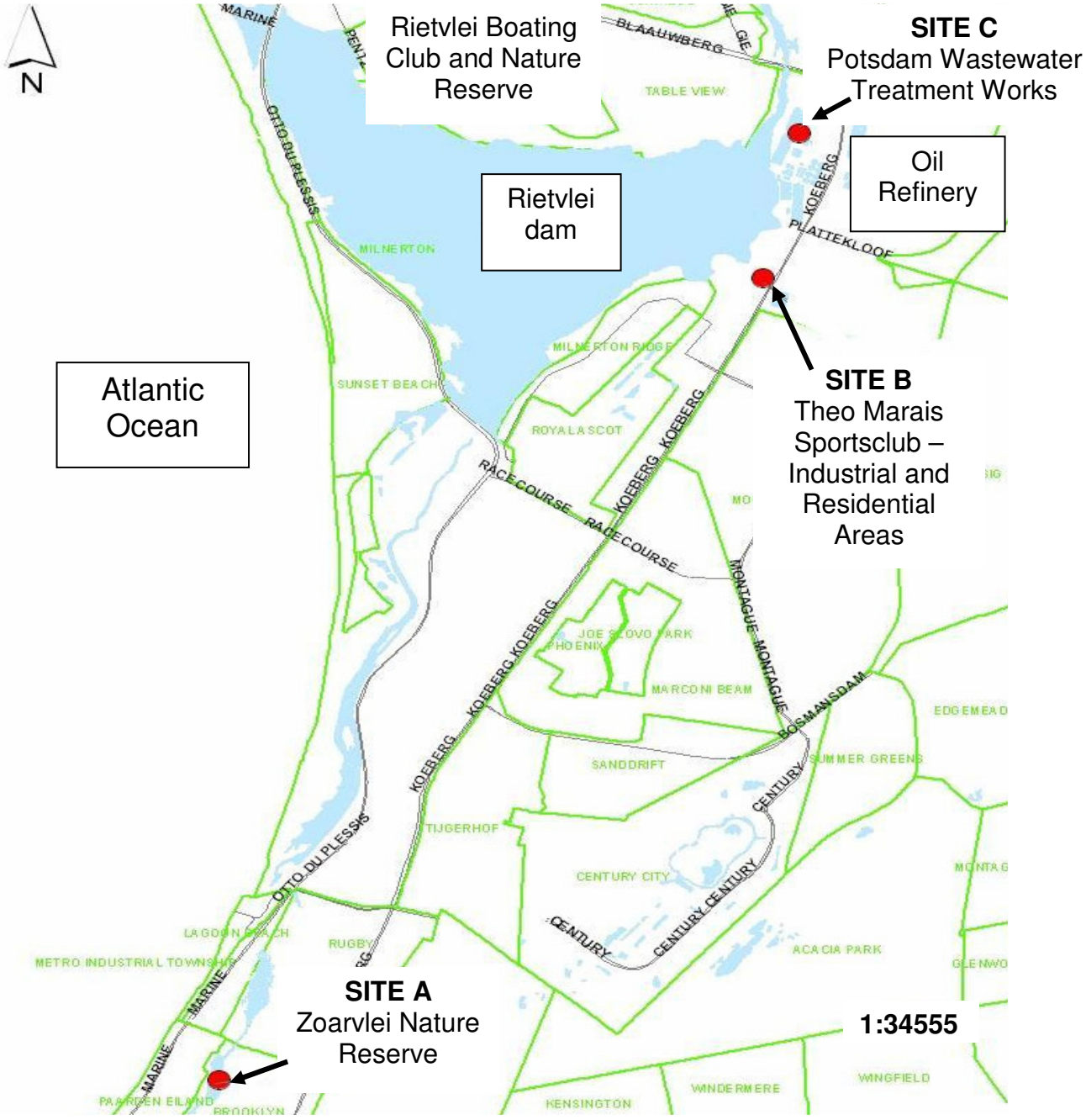

Figure 3.2

Map of the Diep River indicating the different sampling points: Site A - Zoarvlei Nature Reserve (industrial as well as residential areas); Site B - Theo Marais Sportsclub (Industrial and residential area); Site C - Milnerton Waterworks (close to the Oil Refinery and residential areas of Milnerton).

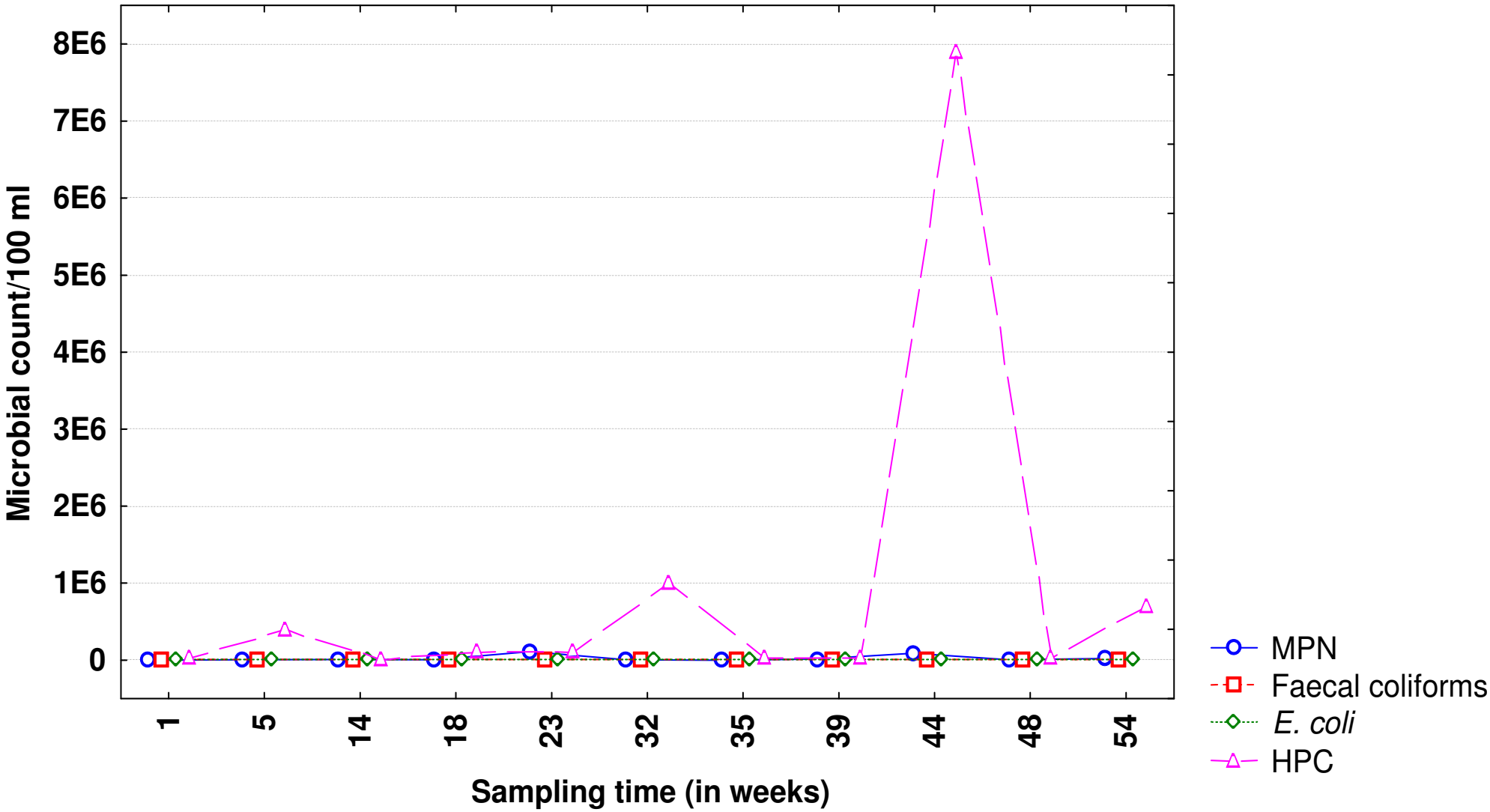

Figure 3.3

Comparison of Most Probable Number [(MPN), representing all possible gas-producing organisms], faecal coliforms (FC), E. coli and heterotrophic plate counts (HPC) per 100 mℓ of river water sample at site A (Plankenburg River) over the sampling period.

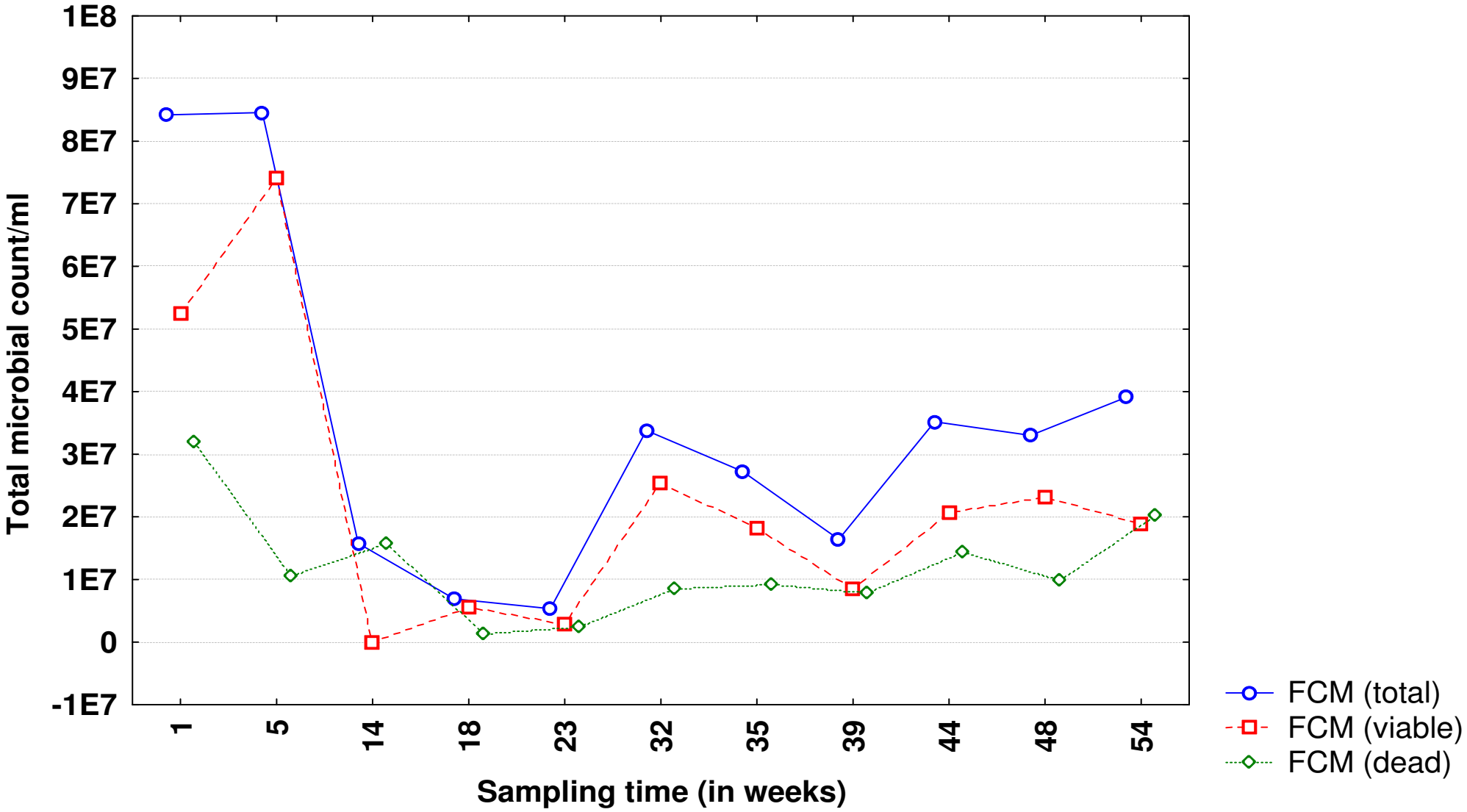

Figure 3.4

Enumeration of total, viable and dead bacterial cells within river water sample at site A (Plankenburg River) by means of flow cytometric analysis over the sampling period.

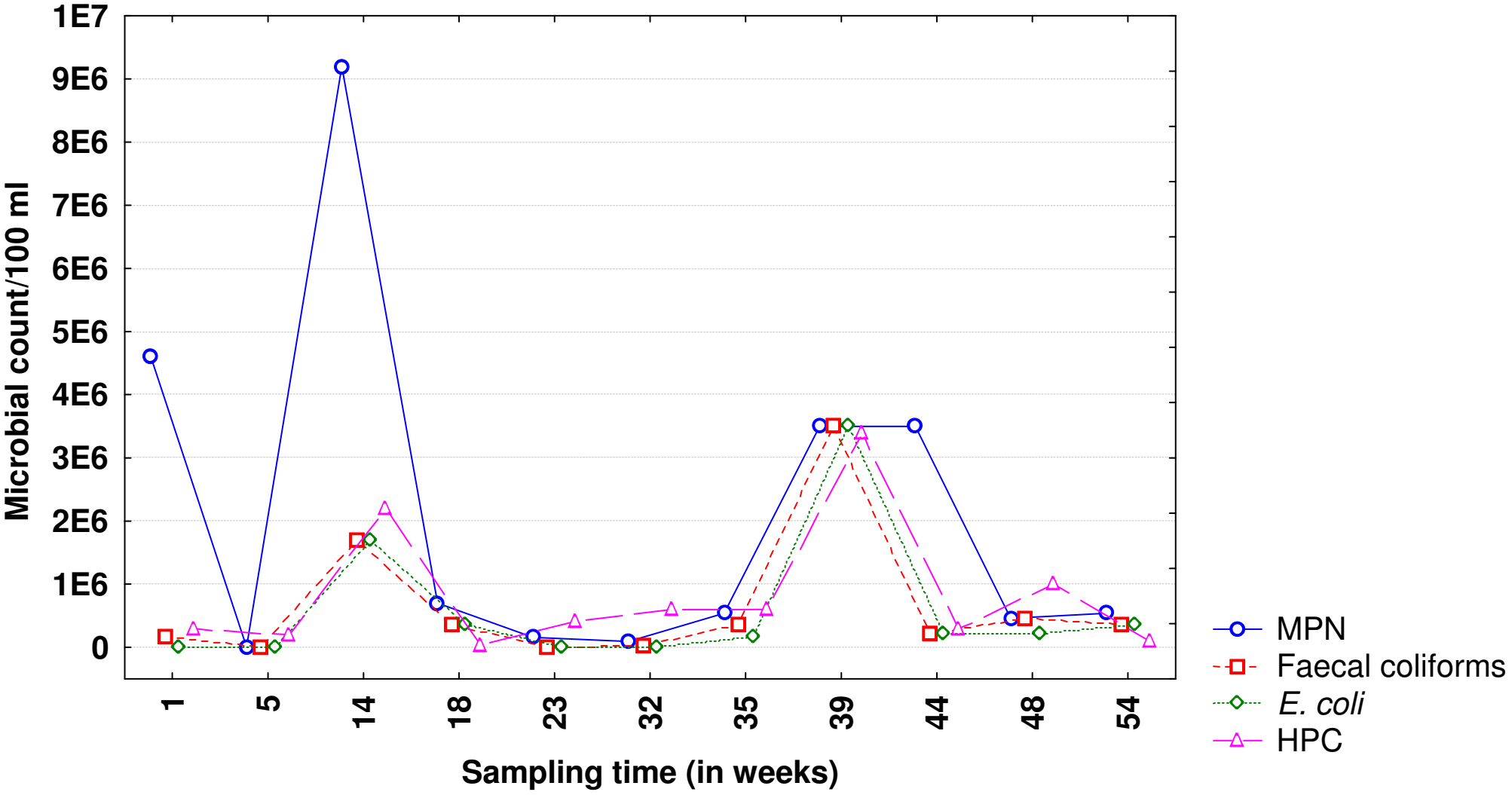

Figure 3.5

Comparison of Most Probable Number [(MPN), representing all possible gas-producing organisms], faecal coliforms (FC), E. coli and heterotrophic plate counts (HPC) per 100 mℓ of river water sample at site B (Plankenburg River) over the sampling period.

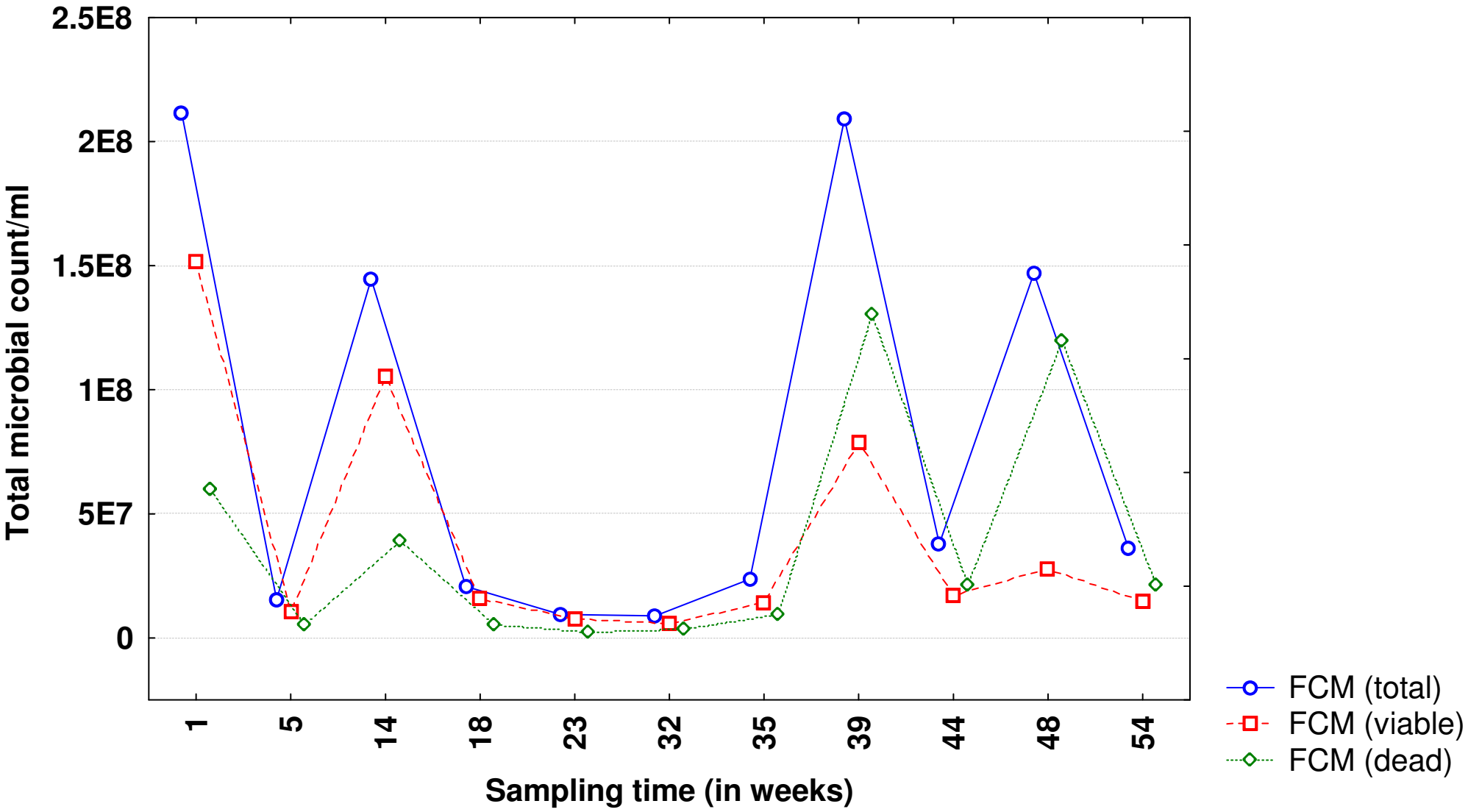

Figure 3.6

Enumeration of total, viable and dead bacterial cells within river water sample at site B (Plankenburg River) by means of flow cytometric analysis over the sampling period.

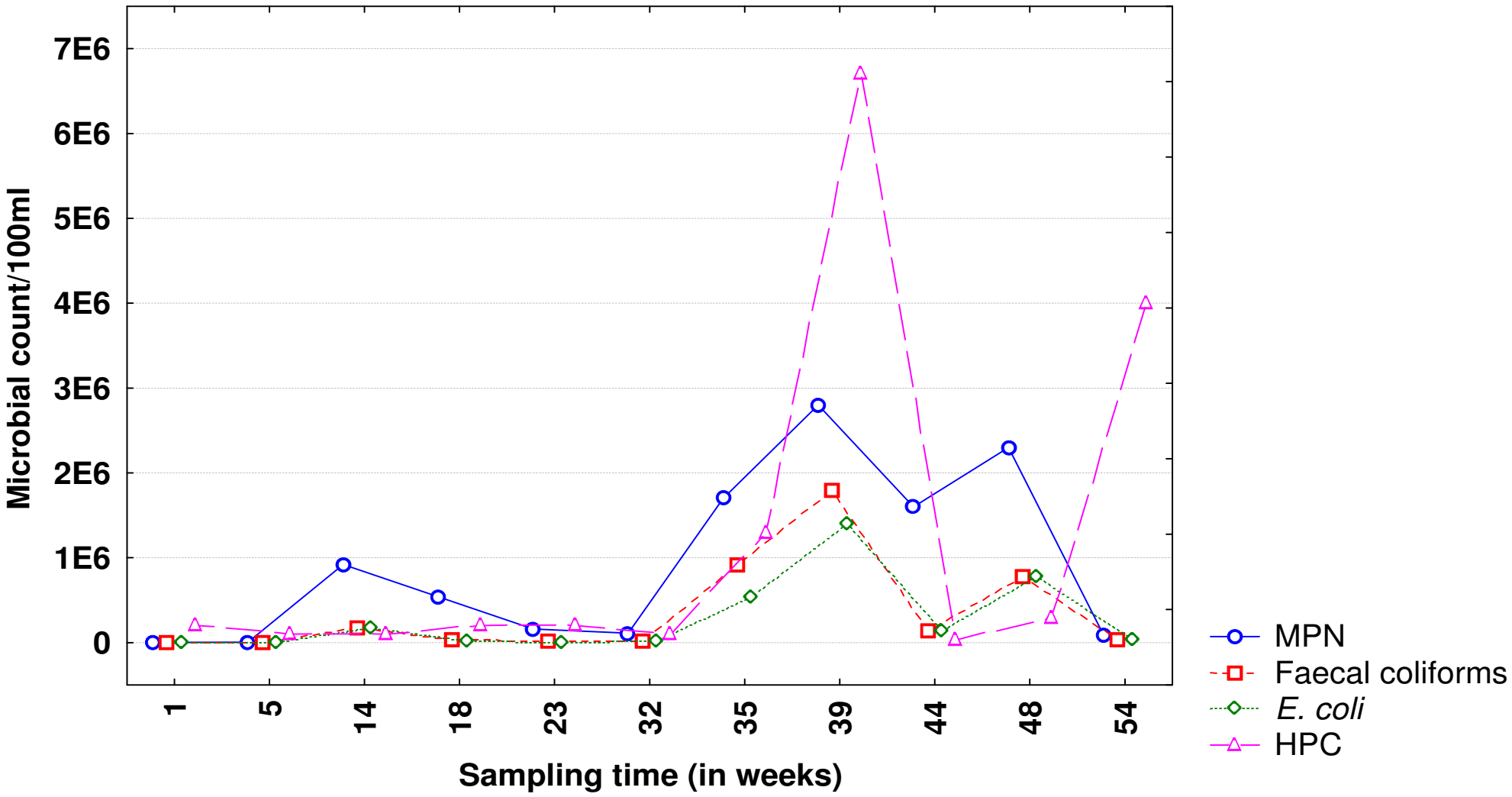

Figure 3.7

Comparison of Most Probable Number [(MPN), representing all possible gas-producing organisms], faecal coliforms (FC), E. coli and heterotrophic plate counts (HPC) per 100 mℓ of river water sample at site C (Plankenburg River) over the sampling period.

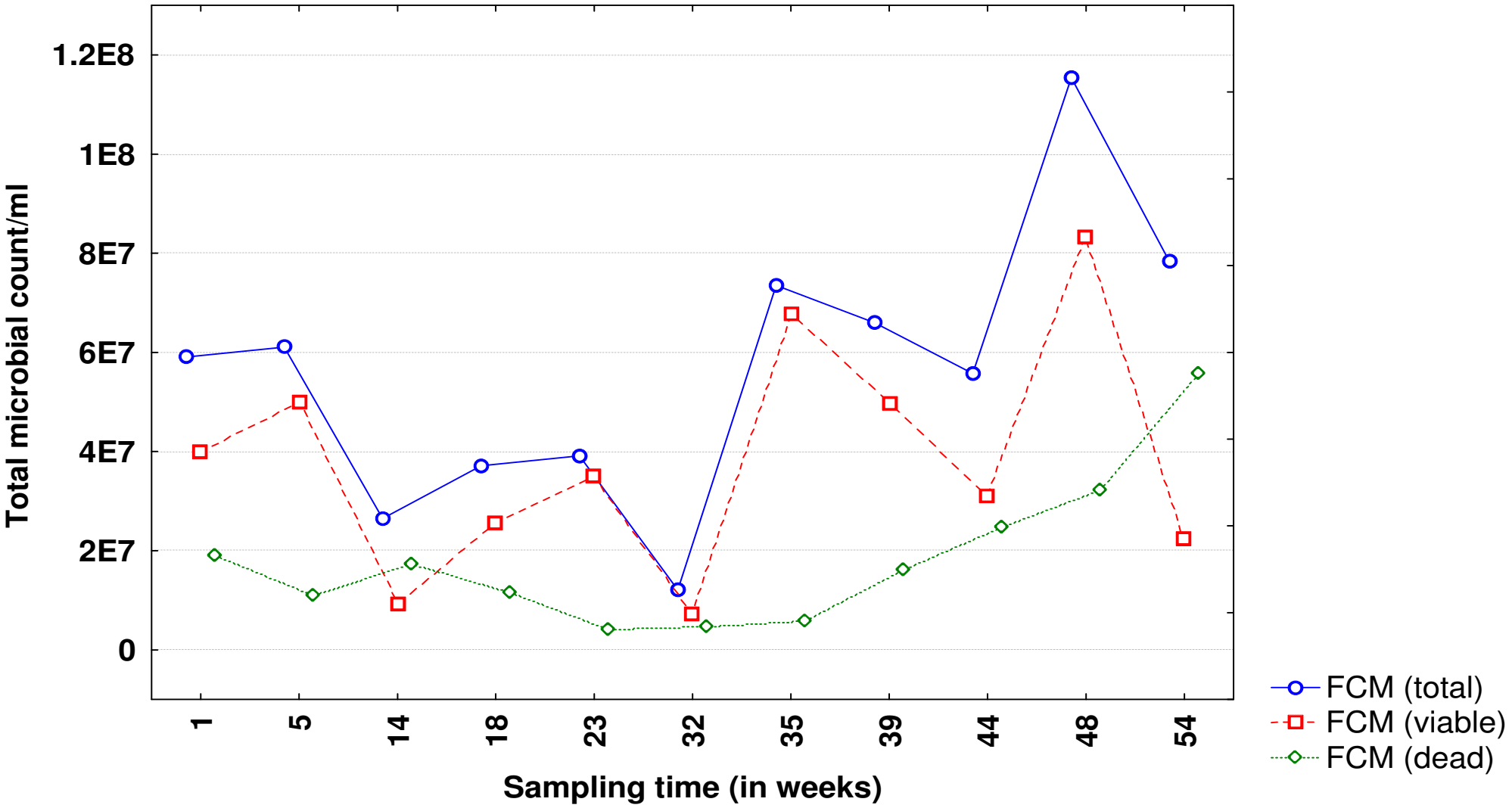

Figure 3.8

Enumeration of total, viable and dead bacterial cells within river water sample at site C (Plankenburg River) by means of flow cytometric analysis over the sampling period.

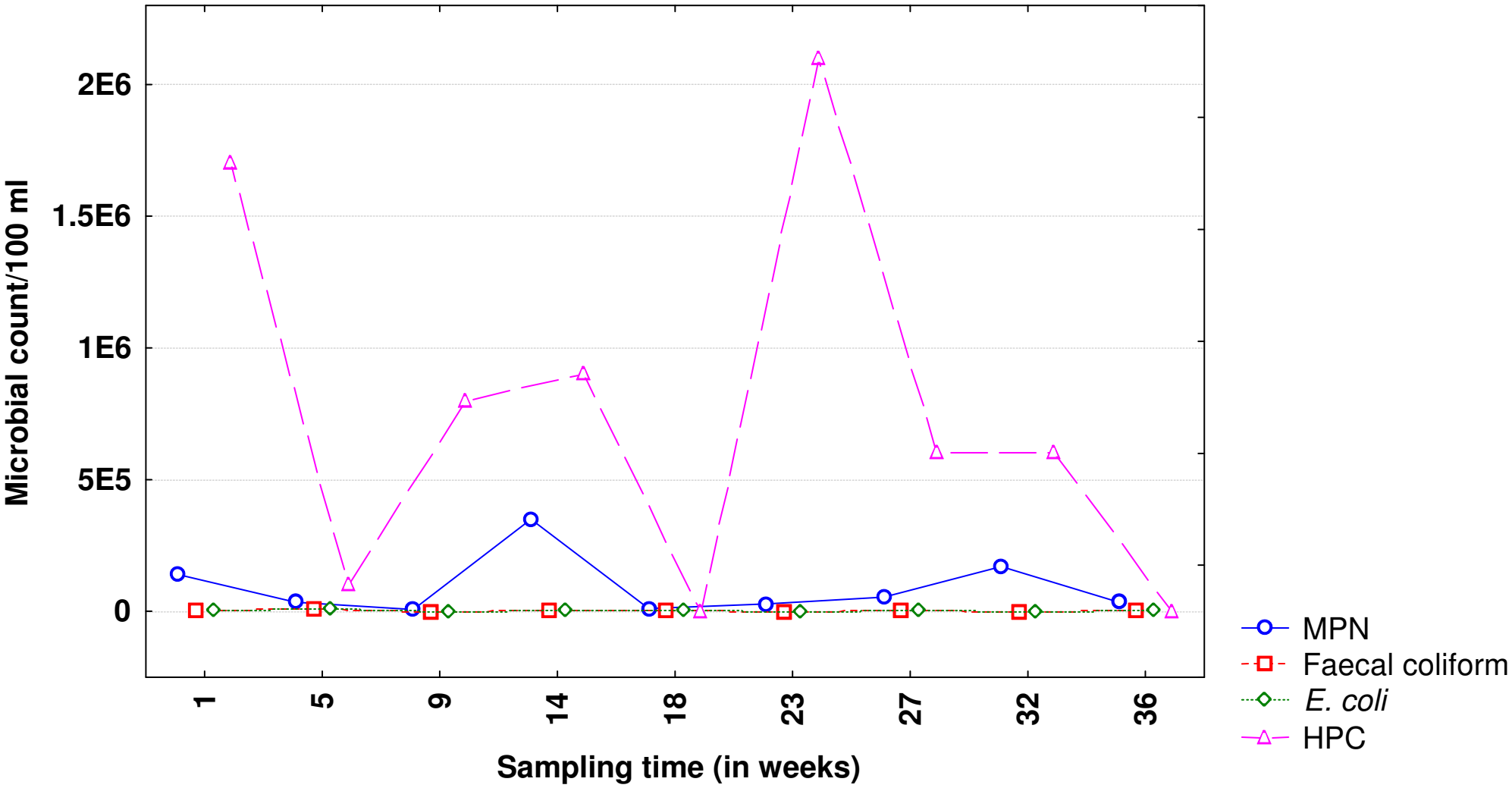

Figure 3.9

Comparison of Most Probable Number [(MPN), representing all possible gas-producing organisms], faecal coliforms (FC), E. coli and heterotrophic plate counts (HPC) per 100 mℓ of river water sample at site A (Diep River) over the sampling period.

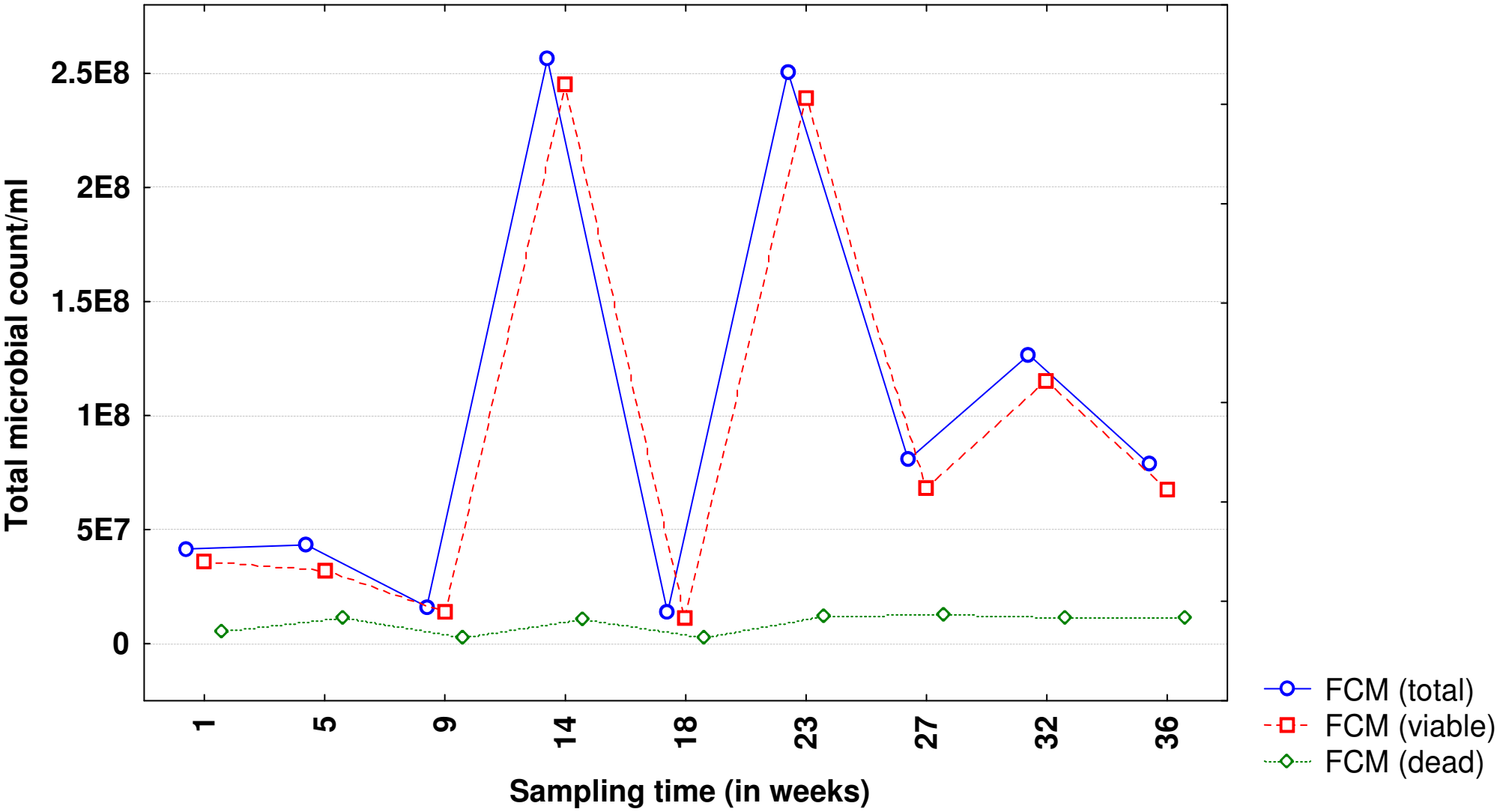

Figure 3.10

Enumeration of total, viable and dead bacterial cells within river water sample at site A (Diep River) by means of flow cytometric analysis over the sampling period.

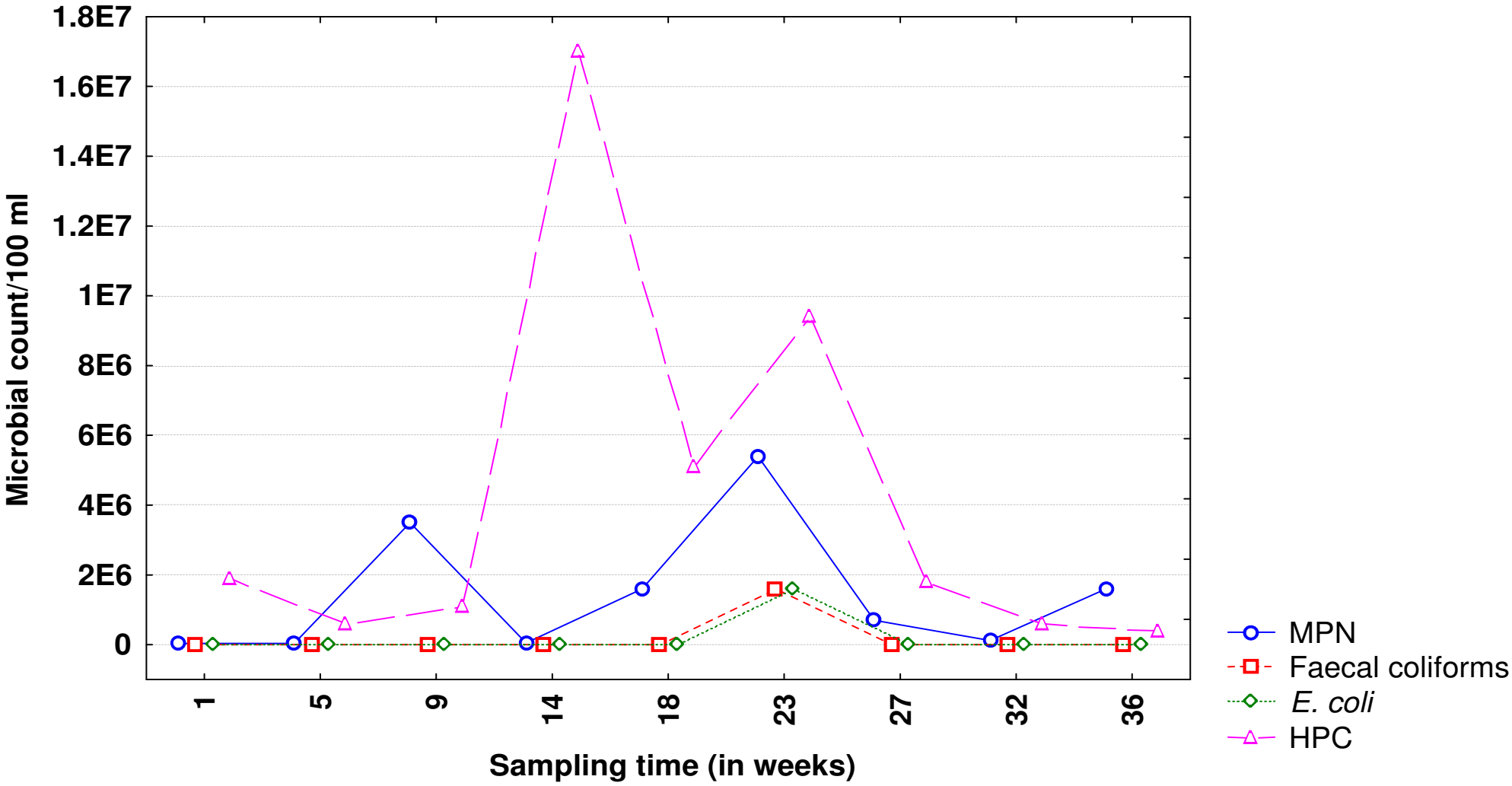

Figure 3.11

Comparison of Most Probable Number [(MPN), representing all possible gas-producing organisms], faecal coliforms (FC), E. coli and heterotrophic plate counts (HPC) per 100 mℓ of river water sample at site B (Diep River) over the sampling period.

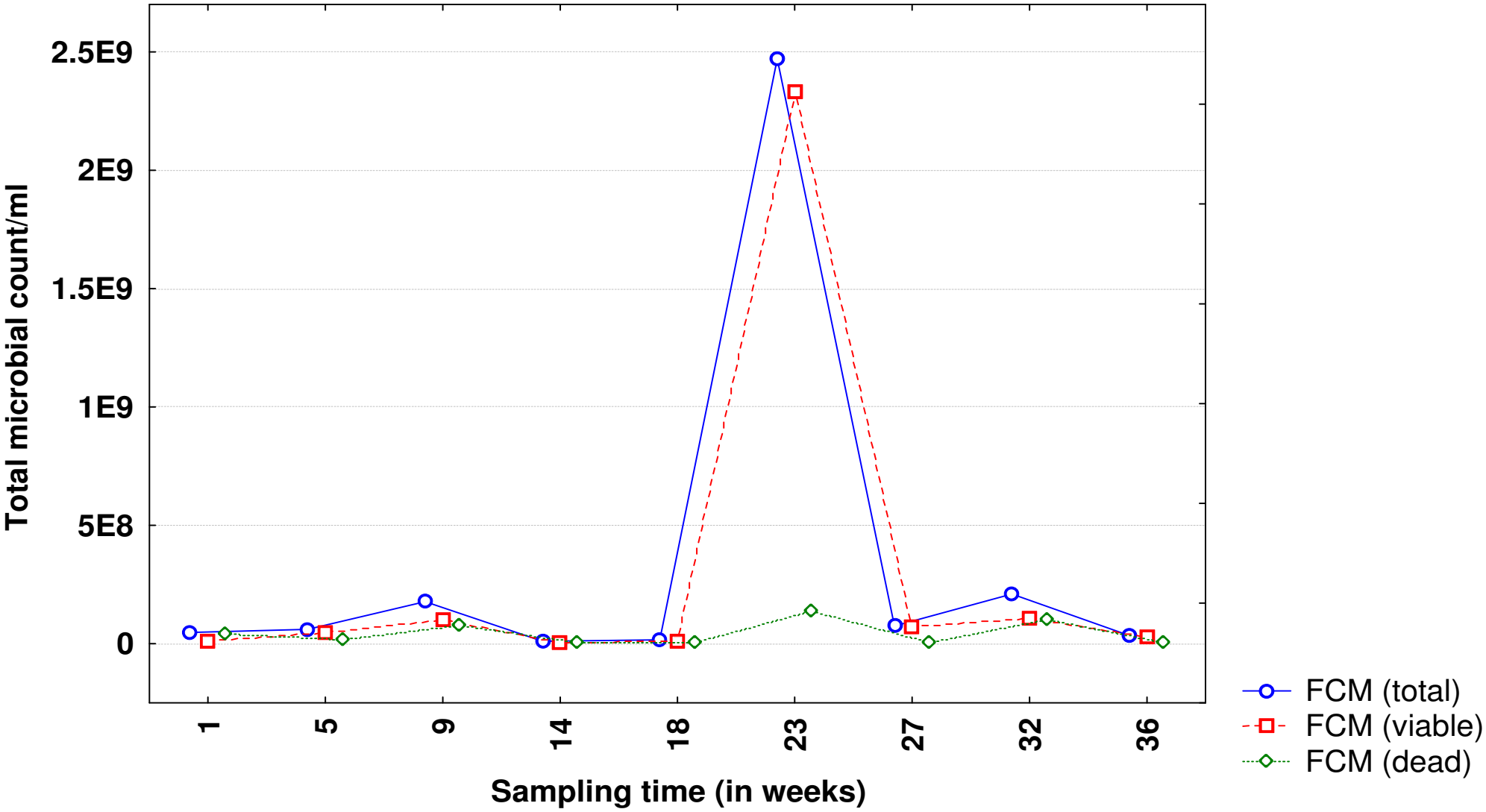

Figure 3.12

Enumeration of total, viable and dead bacterial cells within river water sample at site B (Diep River) by means of flow cytometric analysis over the sampling period.

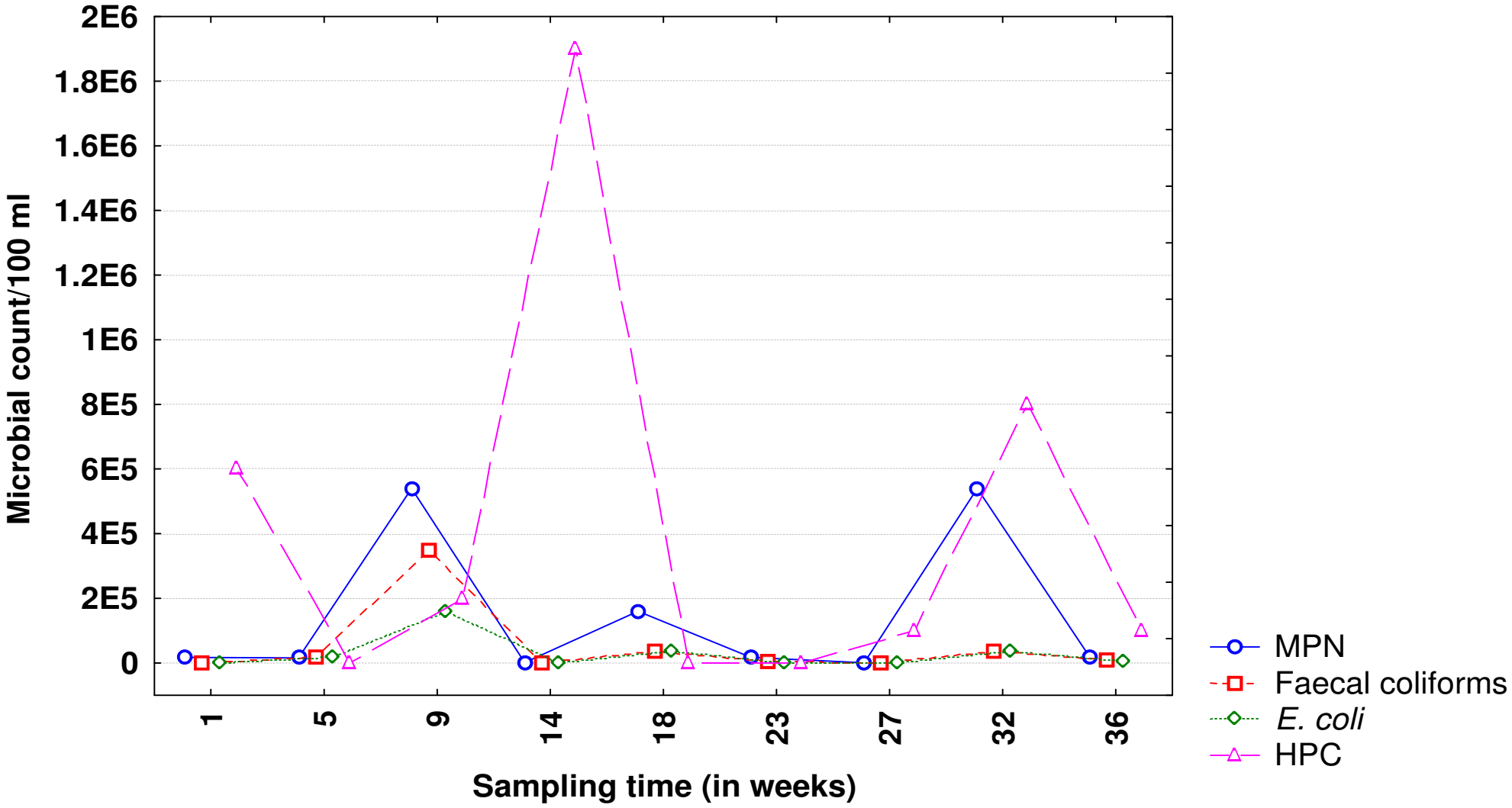

Figure 3.13

Comparison of Most Probable Number [(MPN), representing all possible gas-producing organisms], faecal coliforms (FC), E. coli and heterotrophic plate counts (HPC) per 100 m[of river water sample at site C (Diep River) over the sampling period.

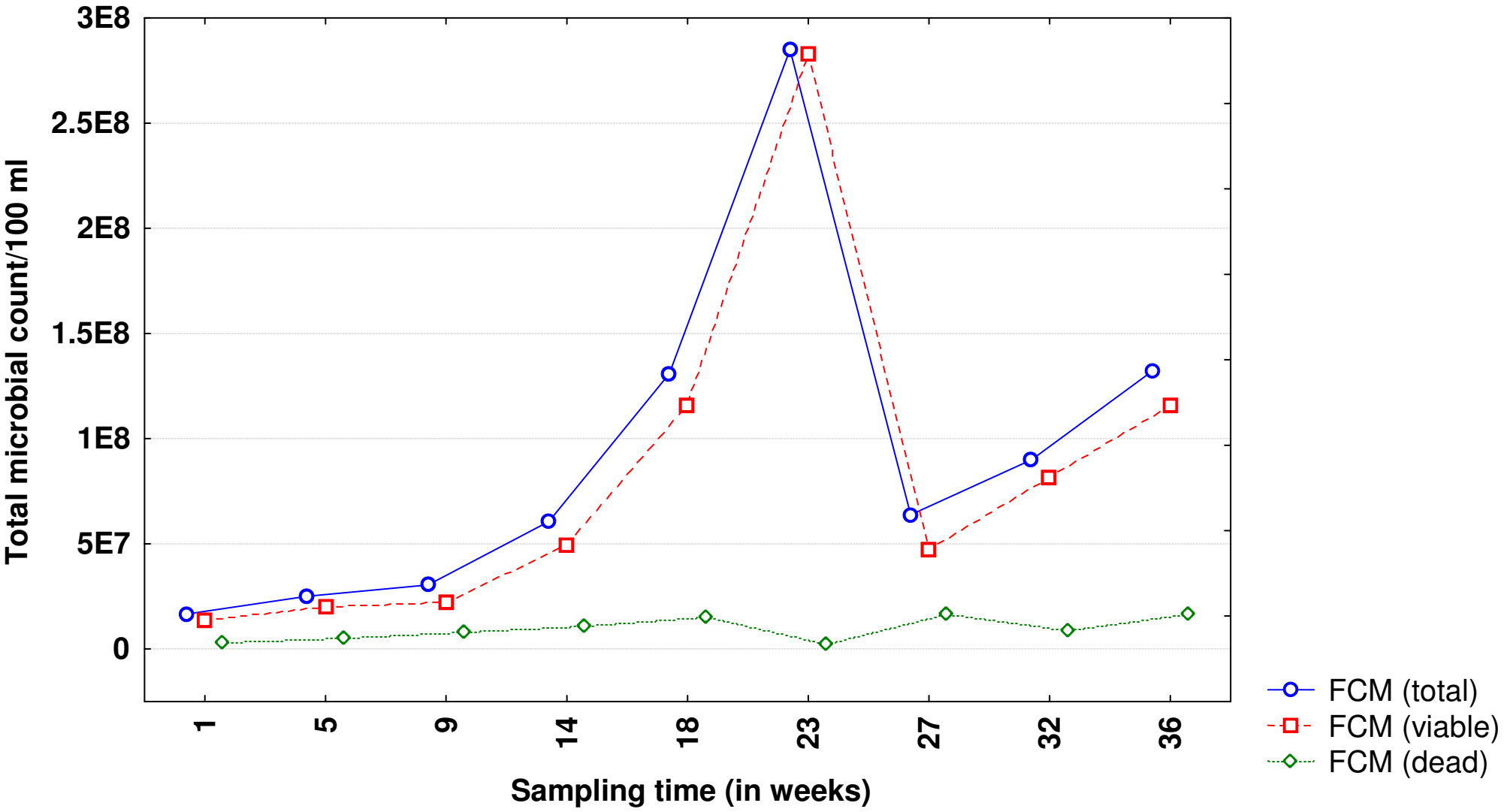

Figure 3.14

Enumeration of total, viable and dead bacterial cells within river water sample at site C (Diep River) by means of flow cytometric analysis over the sampling period.

Table 3.1 Water temperature and pH of the Plankenburg River from June 2004 to June 2005

Sampling date	Before point source of pollution (site A)		At point source of pollution (at Kayamandi-site B)		After point source of pollution (Substation-site C)	
	Water temp. (°C)	pH	Water temp. (°C)	pH	Water temp. (°C)	pH
Week 1	15	7.2	15	7.0	15.2	7.1
Week 5	13	7.0	13.1	7.1	14	7.0
Week 14	10.1	7.2	11.0	7.1	10.1	7.1
Week 18	18	7.0	18	6.8	17	7.2
Week 23	22	7.1	21	7.0	20	7.0
Week 32	21	6.7	22.4	6.8	22.5	6.8
Week 35	21	6.5	23	6.5	23.7	6.5
Week 39	26	6.9	25	6.5	24	6.7
Week 44	15	7.3	14	7.6	13.5	7.5
Week 48	14.5	7.3	14	7.1	14	7.2
Week 54	13	7.7	13.5	7.6	13	7.7

Table 3.2 Water temperature and pH of the Diep River from March 2005 to Nov 2005

Sampling date	Before point source of pollution		At point source of pollution (at Theo Marais)		After point source of pollution (at Potsdam Wastewater Treatment Works)	
	Water temp. (°C)	pH	Water temp. (°C)	pH	Water temp. (°C)	pH
Week 1	17	7.5	19	7.5	18	7.5
Week 5	13	7.3	16	7.7	18	7.7
Week 9	16.9	7.6	18	7.5	20	7.4
Week 14	12.3	7.3	15.5	8	17.4	7.8
Week 18	14.9	6	17	7.0	18	7.0
Week 23	14	6.5	17.8	7.0	17.9	7.0
Week 27	17	7	18.5	7.3	18.5	7.3
Week 32	19	7	18.8	7.5	22	7.3
Week 36	21	7.3	21.8	7.5	23.1	7.7

Isolation and Identification of Bacterial Pollutants from the Berg- and Plankenburg Rivers in the Western Cape, South Africa

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ABSTRACT

Bacterial species present in the Berg and Plankenburg Rivers (Western Cape, South Africa), were isolated from water and biofilm samples and identified. Sampling sites along the Berg River included Site A (agricultural farming area) and Site B (informal settlement). Sampling points along the Plankenburg River included Site A (agricultural farming and residential areas) and Site B (informal settlement). Deoxyribonucleic acid (DNA) extraction of representative isolates was performed and amplified using two different primer sets. Phylogenetic analysis was performed by aligning grouped DNA sequences with Clustal X (1.81) using default parameters and the Blosum matrix. Unrooted trees were constructed using the neighbour-joining program of *MEGA* version 4.1 (Molecular Evolutionary Genetics Analysis 4.1), while branching patterns were evaluated by pairing 1000 replicates. Various *Enterobacteriaceae* species were present at all the sites confirming faecal contamination. *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *Stenotrophomonas* sp. and *Bacillus cereus* were also isolated from the Berg River. In the Plankenburg River, *Bacillus anthracis* and *B. cereus* were identified at Site A while *Aeromonas* sp., *Acinetobacter* sp. and *Yersinia ruckeri* were isolated from Site B. This raises major concerns as population densities at Site B along both rivers are high and thus increases human exposure to the organisms.

Keywords: Enterobacteriaceae; faecal contamination; informal settlement; *Pseudomonas aeruginosa*; waterborne illnesses

1. Introduction

Most of the earth's surface is covered by water (70%), with only approximately one percent available as fresh water. Industrial and agricultural waste, sewage, human activities, human and animal excreta, in addition to organic and inorganic waste, are the main sources responsible for the deterioration of the valuable water bodies (Last, 2002). The organic waste products usually contain a wide variety of micro-organisms which include viruses, bacteria and protozoa, amongst others. Even though various micro-organisms occur naturally in water sources, environmental changes such as the influx of pollutants, may also increase the growth and proliferation of these micro-organisms (Colwell and Patz, 1998).

South Africa's water resources service various industrial, agricultural and domestic sectors, which may contribute to the point or non-point source contamination of these valuable water sources. In many areas of South Africa, and worldwide, informal housing schemes are often established along the banks of river systems. Due to a lack of adequate sanitary and waste removal facilities in the informal settlements, as well as poor management and disposal of sewage, the storm water drainage pipes leading directly to the rivers are often used as a means of disposal of human and animal waste (SARDC, 2002).

The most common bacterial pollutants isolated from water sources include *Escherichia coli*, *Vibrio cholerae*, *Campylobacter*, *Salmonella*, *Shigella* and *Aeromonas hydrophila* (Colwell and Patz, 1998). In addition, the occurrence of biofilms or encrustations in water sources, that harbour various types of micro-organisms (LeChavellier et al. 1987) allows for the multiplication of pathogens such as

Pseudomonas, *Mycobacter*, *Campylobacter*, *Klebsiella*, *Aeromonas*, *Legionella spp.*, *Helicobacter pylori* and *Salmonella typhimurium* (Mackay et al., 1998).

In South Africa, the presence of *E. coli* pathogens in sewage-contaminated river water and *E. coli* O157:H7 in sewage (Müller, 2000), was confirmed in an assessment report conducted in 2003. A study conducted by Diergaardt et al., (2004) confirmed the presence of *Campylobacter spp.*, such as *C. jejuni* and *C. coli* in environmental waters, implicating these water sources as potential reservoirs of these bacteria. Pathogens such as *Salmonella*, *Shigella*, *Vibrio cholera* and coliphages have also been isolated from the final effluent of wastewater treatment facilities in the Eastern Cape, South Africa. These treatment facilities were therefore regarded as point sources of pollution as they discharged their final effluent into respective water bodies, which formed part of the Indian Ocean (Momba et al., 2006).

Due to its high sensitivity, the polymerase chain reaction (PCR) allows for the successful identification of organisms present in low numbers and those that are difficult to culture (Buller et al., 1999). The 16S rRNA PCR technique has been a successful tool in identifying mycobacterial species in raw and treated surface and groundwater sources as well as in biofilms in piping systems. These micro-organisms were identified using a multiplex PCR technique (Le Dantec et al., 2002).

The aim of this study was to identify predominant bacterial species, isolated from planktonic and sessile samples, present in the Berg- and Plankenburg Rivers in the Western Cape, South Africa using PCR and DNA sequencing.

2. Materials and Methods

2.1 *Sampling Sites*

A previous study identified four sampling sites along the Berg River (**Fig. 4.1**) (Paulse et al., 2007). Sampling sites identified included Site A (agricultural farming area); and Site B (informal settlement - sites B1 and B2) and Site C (Newton pumping station). Site B2 (also known as Plot 8000) is the site where storm water drainage pipes from the informal settlement flow directly into the river. In certain areas human waste from a residential area as well as certain sections of the informal settlement, are directed to the Newton pumping station where solid and liquid waste is separated before the watery waste is dumped into the river. For this study, only planktonic and sessile samples collected from Sites A and B2 (**Fig. 4.1**) were analysed.

In another study (Paulse et al., 2008), four sampling sites, indicated in **Fig. 4.2**, were identified at the Plankenburg River location. Sampling sites along the Plankenburg River included Site A (agricultural farming and a residential area from Stellenbosch); Site B (informal settlement); Site C (industrial area) and, Site D (agricultural and industrial area). For this study, only planktonic and sessile samples collected from Sites A and B (**Fig. 4.2**) were analysed.

Sampling of sites for both the Plankenburg and Berg Rivers started in June 2004 and continued for a period of 1 year until June 2005. The temperature and pH of the river water at both sampling locations were measured using a hand-held mercury thermometer and portable pH meter (Hanna Instruments), respectively.

2.2 *Sampling*

Water samples were collected in 1L sterile Nalgene-polypropylene bottles (Cole-Palmer Instrument Company) and stored on ice to maintain the lowest possible temperature. Materials such as stones, glass, leaves, etc. were also collected from each sampling site and stored in sterile whirlpack bags for subsequent biofilm isolation.

2.3 *Sonication of Biofilm samples*

Glass, stones and leaves collected from the river were sonicated for 10 minutes in 60ml sterile dH_2O using a UMC 5 ultrasonication bath (Instrulab Inc.). The sonication step was repeated at least thrice depending on the amount of material collected from each sampling site. The bacterial suspension obtained was used for further microbiological analysis.

2.4 *DNA extraction and Agarose Gel Electrophoresis*

Cultures from planktonic and sessile samples (sonicated bacterial suspension) obtained from the sampling sites were spread-plated onto Nutrient Agar (NA) (Merck, Biolab Diagnostics) after serial dilutions (10^{-1} to 10^{-7}) 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). 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 1 hour at 90 volts to confirm the presence of genomic DNA.

2.5 *Polymerase Chain Reaction (PCR)*

The extracted DNA from individual samples was amplified using two primer sets, 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 1 hour to confirm successful amplification of the PCR product. A MassRuler™ DNA Ladder Mix, #SM0403 (Fermentas) was used to compare amplicon size.

2.6 *16S ribosomal RNA sequencing*

Successfully amplified PCR products (~600 kb) were purified using a High Pure PCR Product Purification Kit as per 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 Basic Local Alignment Search Tool (Blastn) (Altschul et al., 1997) obtained from the National Centre for Biotechnology Information website.

2.7 Phylogenetic analysis

All the DNA sequences obtained for the Berg- and Plankenburg Rivers (Sites A and B, respectively) were grouped and aligned with Clustal X (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.

3. Results and Discussion

Figure 4.3 (a and b) illustrates the amplified PCR products analysed on a 1.2% agarose gel against a MassRuler™ DNA Ladder Mix, #SM0403 (Fermentas) resembling the approximate size of 600 kb. The gel electrophoresis pictures indicating the representative samples (138 to 155) isolated at Site B in the Berg River, were amplified using both primer set 1 and 2 (Greisen et al., 1994). Whereas most samples represented in **Fig. 3a** were successfully amplified with primer set 1, samples 143, 147, 152 and 154 were not observed (**Fig. 3b**) when amplified with primer set 2. Samples sent for subsequent sequencing were therefore selected based on successful amplification with either primer sets.

The phylogenetic trees illustrated in **Figs. 4.4** to 4.**7** include various species of both the Gram-positive and Gram-negative heterotrophic bacteria. According to Nikaido (1996), Gram-negative bacteria tend to be more resistant to various lipophilic and amphiphilic inhibitors than Gram-positive bacteria. This indicates that these organisms possess survival mechanisms which gram-positive organisms lack and could thus explain why these organisms are surviving adverse environmental conditions. In all the trees the Gram-negative group make up the largest number of organisms isolated and are further subdivided into the *Enterobacteriaceae*, *Proteobacteria* and the high G+C Gram-negatives.

3.1 Berg River

Bacterial species isolated from the planktonic and sessile samples obtained from Site A (site situated upstream from the suspected point source of pollution), are presented in **Fig. 4.4**. Phylogenetic analysis based on the 16S rRNA of the bacterial strains isolated in 2004 and 2005 displayed acceptable bootstrap values. The tree shown in **Fig. 4.4** reflects the phylogenetic relationships of the isolated bacterial species to previously identified blast results. In addition to the *Enterobacteriaceae*, *Proteobacteria* and the high G+C Gram-negatives isolated from this site and the isolates from the low G+C Gram-positives were less diverse and consisted of *Staphylococcus epidermidis*, various *Bacillus* spp. and the *Firmicutes* bacterium.

The *Enterobacteriaceae* which is common and widespread in the environment, include the coliform bacteria, which are used as indicator organisms in evaluating faecal pollution in various water bodies (Prescott et al., 1990). The fact that *Enterobacter* sp., *Klebsiella* sp., *Serratia* sp., *Shewanella* sp. and *Aeromonas* sp., amongst others, were detected at Site A is a clear indication of faecal contamination of the river in this area. These isolates belong to the indicator coliform group of organisms of which most are harmless. However, their presence in a water system could lead to major health concerns as their presence indicate the presence of potential disease-causing bacterial strains i.e. pathogens (Centre for Disease Control, 1998).

In addition, the presence of *Pseudomonas* sp. and particularly, *Pseudomonas aeruginosa* constitute potential health threats to children, the elderly and immunocompromised patients. This organism is an opportunistic pathogen, which affects burn victims and may also cause urinary tract infections (Prescott et al., 1990).

According to a study by Miteva et al. (2004) isolates exhibiting multiple resistances, among the *Proteobacteria*, belong to the *Pseudomonas* sp.

The final group in this tree, the low G+C Gram-positives, consisted of *Staphylococcus epidermidis*, *Firmicutes* sp. and various *Bacillus* spp. In this subcluster *Staphylococcus epidermidis* branches off the *Bacillus* sp., *B. drentensis*, which exhibited a distinct difference from the rest of this genus. The clade consisting of the various *Bacillus* spp. contains one of the more important species of this genus, *B. cereus*, which may be responsible for food poisoning.

Another organism of interest is the free-living, non-glucose-fermenting, Gram-negative bacillus, *Stenotrophomonas maltophilia*. This organism has long been regarded as harmless but has over the years become increasingly important in infections involving humans (Gilligan & Whittier, 1999). This isolate has been linked to nosocomial infections in immunocompromised and cancer patients as well as transplant recipients and patients undergoing peritoneal dialysis (Taylor et al., 1999). Its presence in an area of the river utilised for irrigation could raise major health concerns as the organism is resistant to many broad-spectrum antibiotics including penicillins (Gilligan & Whittier, 1999). In addition to the above-mentioned micro-organisms isolated from Site A, various other water-borne bacterial species were also observed at this site. **Table 4.1** contains the names and accession numbers of all the isolates, pathogenic and non-pathogenic, identified at Site A in the Berg River.

It should also be noted that various species isolated at this site are non-pathogenic such as *Arthrobacter* sp. (Funke et al., 1996) and *Shewanella* sp. or not directly pathogenic to humans but form part of the normal flora of the human skin such as *Brevibacterium* sp. (Gruner et al., 1994) and *Staphylococcus epidermidis* (O'Gara & Humphreys, 2001). *Staphylococcus epidermidis* affects people with altered immune

systems and might be responsible for endocarditis and infection in these patients (Prescott et al., 1990).

To add to the major health concern of the inhabitants living in close proximity of this river, some of the bacterial clusters observed at Site A (i.e. agricultural area) were conserved when compared to micro-organisms identified at Site B. Hundreds of informal home dwellers as well as farm workers (agricultural area), live in close proximity to this site and many use the riverbanks as 'toilet schemes' as the sanitary facilities in the settlement are either in a poor state or completely non-existent in the vicinity. Even though *Escherichia coli* specifically, was not detected (Bussen & Standridge, 2001), this site was still a highly faecal contaminated site as the presence of the uncultured *Klebsiella* strain, Hg5-13 indicates (**Fig. 4.4**). This enteric organism detected at both Sites A and B, is an important human pathogen and may be a causative agent of pneumonia infections (Prescott et al., 1990).

The phylogenetic tree illustrated in **Fig. 4.5** (Site B), also displayed a high confidence percentage as most of the bootstrap values exhibited phylogenetic accuracies higher than 70%. As observed at Site A, the Gram-negative bacteria constituted the larger group of organisms isolated from this site. In addition to the *Klebsiella* sp. identified at this site and previously at Site A in the Berg River, bacterial species introduced into the river at Site B, included amongst others, *Citrobacter* sp., *Micrococcus* sp., *Corynebacterium* sp. and *Rhodococcus* sp. Even though most of these species are not well-known human pathogens, species such as *Citrobacter freundii* are opportunistic pathogens, which normally inhabit the intestine, and have been linked to alimentary infections and infections of the urinary tract, middle ear, gallbladder and meninges as well as bloodstream infections, brain abscess, neonatal sepsis, intra-abdominal sepsis and pneumonia (Pepperell et al., 2002). This organism has also been

associated with high mortality rates amongst patients with prolonged hospital stays, severe underlying illnesses, immunosuppression and foreign device insertions (Chen et al., 2002) and is familiar for its resistance to various antimicrobial agents (Wang et al., 2000). In **Fig. 4.5**, *Citrobacter freundii* belongs to the first subcluster of the phylogenetic tree, which included various species of the *Enterobacteriaceae*. This organism is not only closely related to the Hg5-13 strain of the *Klebsiella* sp. but also shares phylogenetic accuracies of 100%. The clade starting with *Citrobacter* through to *Klebsiella* branches off the clade which start with *Aeromonas* through to *Pseudomonas*, which in itself are pathogenic micro-organisms responsible for causing gastroenteritis, soft-tissue infections and bacteraemia [*Aeromonas* (Janda & Abbott, 1998)] and as previously mentioned, urinary tract infections [*Pseudomonas* (Prescott et al., 1990]. The fifth bacterial species in this subcluster, *Acinetobacter* sp., is a Gram-negative genus of bacteria belonging to the phylum *Proteobacteria* and are generally considered non-pathogenic to healthy individuals. The organism may however, be responsible for life-threatening infections in immunocompromised patients (Gerischer, 2008).

Even though various species isolated from Site B are not indicators of faecal contamination, their mode of transmission is either via contaminated soil, water or faeces. Also, although human or animal origin of individual isolates could not be specified, various species can be regarded as either pathogens or opportunistic pathogens to humans. Species such as *Burkholderia*, *Pseudomonas* and *Stenotrophomonas* have been isolated from Site B in the Berg River. Previous studies have indicated that *Burkholderia* sp. (Dance, 2000) and *Pseudomonas* sp. (Ruimy et al., 2001) were isolated from contaminated environmental samples as well as faecal samples. Research has shown that *Stenotrophomonas* sp. are affecting more humans (Gilligan & Whittier, 1999) and Site B is situated directly next to the informal settlement,

which raises concern as exposure to the organism could result in increased infection rates. Other studies have indicated the presence of *Stenotrophomonas* sp. in faeces and its carriage rates in humans (Kerr & Denton, 1998). During these studies, the carriage rates of the organism were found to be significantly high and up to 33% in people with haematological malignancy, as compared to the control group exhibiting a faecal carriage of 2.9%.

Also, various organisms isolated at Site A remained dominant at Site B and included species such as *Klebsiella*, *Aeromonas*, *Comamonas* and *Pseudomonas*. In addition, *Stenotrophomonas*, *Brevundimonas*, *Microbacterium*, and various *Bacillus* spp., including *Bacillus cereus* persisted at both Sites A and B. **Table 4.2** indicates the different bacterial species isolated from Site B and their GenBank accession numbers.

3.2 Plankenburg River

From the phylogenetic tree illustrated in **Fig. 4.6** (Site A), two distinct clusters could be identified with the Gram-negative bacteria comprising the largest. This cluster was divided into five subclusters and several clades. One subcluster included once again, members of the *Enterobacteriaceae* thereby confirming faecal contamination in the Plankenburg River. One of the isolated bacterial species resembled the organism, *Klebsiella oxytoca* strain 20 which formed a clade with an *Alcaligenes faecalis* strain GP16. It was however evident that even though these two organisms fell into the same Gram-negative group of organisms they can be regarded as two distinctly different species (i.e. representative of long nodes). Irrespective of their dissimilarities, both species along with the *Aeromonas* sp., *Lysobacter taiwanensis*, *Thermomonas fusca* and *Acinetobacter* sp. are all indicator organisms of faecal contamination. The presence

of these faecal indicators also relate to a previous study (Paulse et al., 2008) where significantly ($p < 0.05$) high faecal coliform and *E. coli* counts were recorded by means of the most probable number technique (MPN). During this study, faecal coliform and *E. coli* counts of 3.5×10^6 micro-organisms/100 ml respectively, were observed at Site B in the Plankenburg River.

The second cluster consisted of various species of the Gram-positive *Bacillus* genus in which two subclusters could be identified. Subcluster one consisted of four *Bacillus* spp. and one uncultured bacterium specie. The Bacillus species of interest is the organism, *Bacillus anthracis*, which is the causative agent of the disease, anthrax. Other *Bacillus* species observed in this subcluster included *B. drentensis*, *B. niacini*, *B. firmus*. The second subcluster consisted of *B. cereus*, which can cause food poisoning in humans as well as *B. aquimaris* and *B. pumilus* and various other *Bacillus* strains. Several other potentially pathogenic bacteria were detected at this site. *Aeromonas* sp. and particularly, *A. hydrophila* is associated with gastroenteritis, cellulitis and other diseases in humans. All the names and GenBank accession numbers of the water-borne bacteria isolated from Site A in the Plankenburg River are indicated in **Table 4.3**.

It is obvious that several of the bacterial species detected at Site A along the Plankenburg River were also observed at Site B (**Fig. 4.7**). As mentioned previously, the Gram-negative bacterial groups were more dominant than the Gram-positive micro-organisms. This could also be observed from the phylogenetic tree indicating the organisms isolated at Site B in the Plankenburg River.

Apart from the fact that two distinct clusters could be observed from the phylogenetic tree several smaller subclusters could be identified within each cluster. The subcluster containing various *Bacillus* species observed at Site A raised major

health concerns as pathogens such as *B. anthracis* and *B. cereus* were conserved between these two sites. In addition, *B. megaterium* was introduced at Site B, while species such as *B. drentensis*, *B. niacini*, and *B. firmus* detected at Site A were not isolated from Site B.

Other important bacterial species introduced at Site B included various members of the *Enterobacteriaceae* group such as *Serratia* sp., *Citrobacter* sp., *Yersinia ruckeri* and *Enterobacter* sp. thereby again confirming faecal contamination at this site. As previously mentioned this is the point of the river that is closest to the informal settlement. It is also the site where faecal wastewater from nearby sanitary facilities is flushed into the river. Bacterial species from the genus, *Yersinia* are considered major human pathogens and may be the causative agent of plague in susceptible individuals. As observed from all the previous phylogenetic trees discussed, the *Aeromonas* sp. always formed a clear link with the phylum, *Enterobacteriaceae* mainly due to the *Enterobacteriaceae-Vibrionaceae* lineage. This species belongs to the *Vibrionaceae* which consist of Gram-negative, straight or curved rods with polar flagella. This family also include the important *Vibrio* sp. of which *V. cholerae* and *V. parahaemolyticus* are the most well-known human pathogens. *Vibrio cholerae* is responsible for the disease cholera, while *V. parahaemolyticus* infections result in gastroenteritis in humans after the ingestion of contaminated seafood (Prescott et al., 1990). Even though studies of rRNA homology suggest that *Vibrio* sp. and *Aeromonas* sp. differ more widely, previous studies have reported the difficulty in identifying *Aeromoas* to the genospecies level (Abbott et al., 1992). It has also been found that commercial identification techniques failed to identify *Aeromonas* strains to the species level and sometimes also failed to distinguish these bacterial strains from phenotypically similar *Vibrio* spp. (Janda, 1991). Overall, the species diversity of micro-organisms isolated from Site B, were less

compared to the micro-organisms isolated from Site A. **Table 4.4** indicates the different bacterial species isolated from Site B and their GenBank accession numbers.

4. Acknowledgements

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Fig. 4.1 Map of the Berg River indicating the different sampling points: Site A – agricultural farming area; Sites B1 and B2 (Plot 8000) – situated close to the informal settlement and Site C - the Newton pumping station.

Fig. 4.2 Map of the Plankenburg River indicating the different sampling points: Site A - agricultural farming as well as residential areas; Site B - close to the informal settlement of Kayamandi; Site C - Substation in the industrial area and Site D - industrial area at Adam Tas Bridge.

Fig. 4.3a Polymerase Chain Reaction analysis of extracted DNA samples (BB1-138 to 155) [with primer set 1: forward (RW01) primer; reverse (DG74) primer] isolated from Site B in the Berg River. Lanes 1 –18: samples 138 to 155 (BB1); Lane A: Marker [MassRuler™ DNA Ladder Mix, #SM0403 (Fermentas)]; Lane B: Negative control.

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Fig. 4.4 Unrooted phylogenetic tree inferred from 16S rRNA sequence data, isolated from planktonic and sessile samples obtained from Site A in the Berg River in 2004 and 2005.

Fig. 4.5 Unrooted phylogenetic tree inferred from 16S rRNA sequence data, isolated from planktonic and sessile samples obtained from Site B in the Berg River in 2004 and 2005.

Fig. 4.6 Unrooted phylogenetic tree inferred from 16S rRNA sequence data, isolated from planktonic and sessile samples obtained from Site A in the Plankenburg River in 2004 and 2005.

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Table 4.4 Table of 43 isolates identified at Site B in the Plankenburg River, their codes and accession numbers

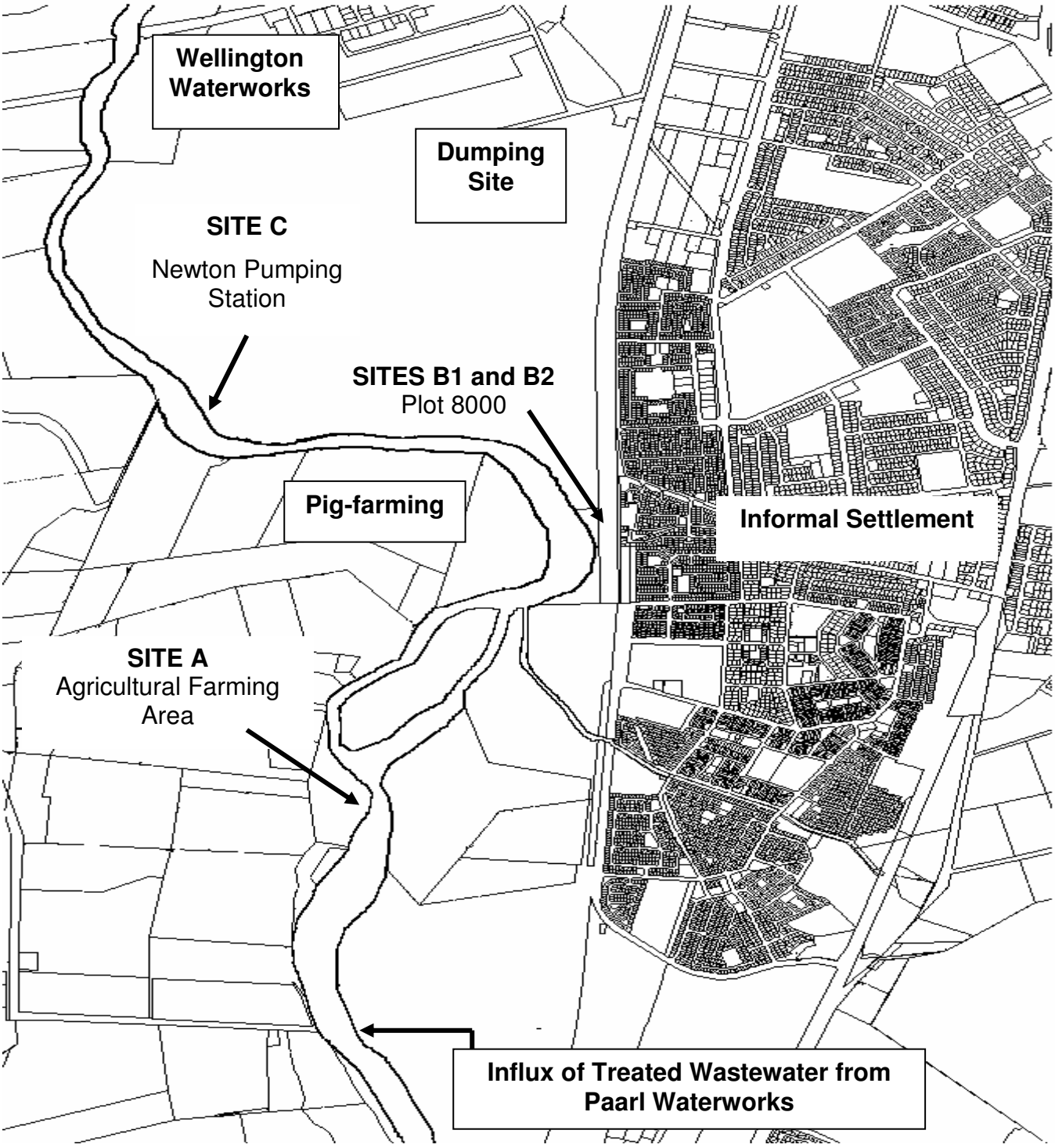

Fig. 4.1 Map of the Berg River indicating the different sampling points: Site A – agricultural farming area; Sites B1 and B2 (Plot 8000) – situated close to the informal settlement and Site C - the Newton pumping station.

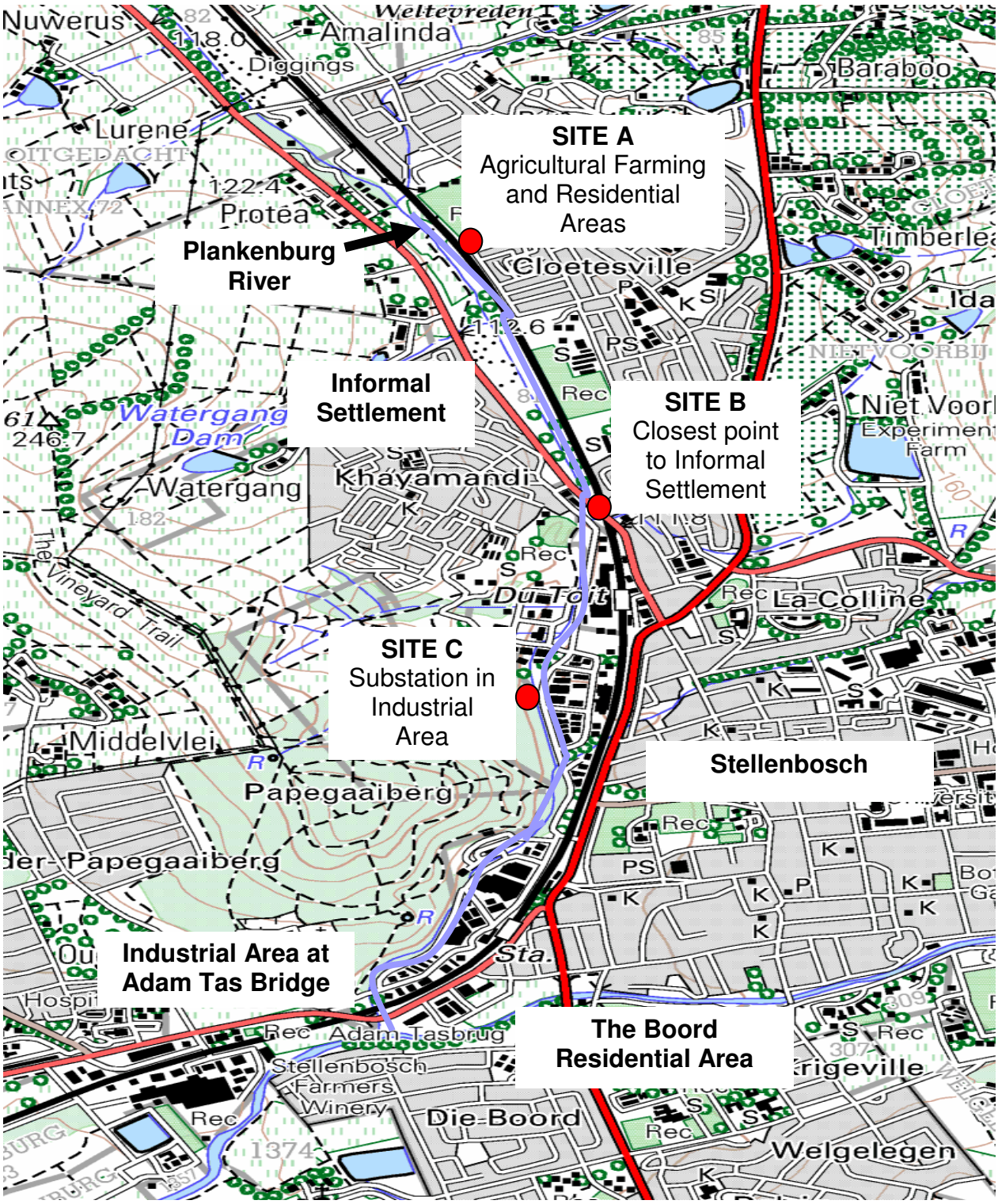

Fig. 4.2 Map of the Plankenburg River indicating the different sampling points: Site A - agricultural farming as well as residential areas; Site B - close to the informal settlement of Kayamandi; Site C - Substation in the industrial area and Site D - industrial area at Adam Tas Bridge.

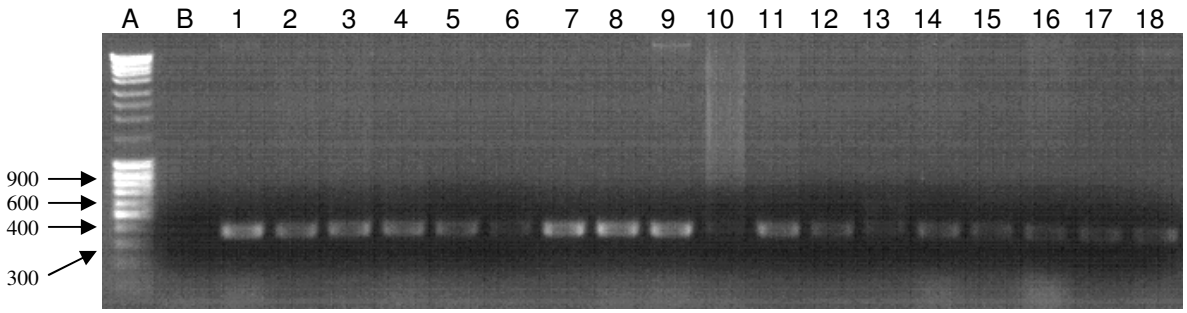

Fig. 4.3a Polymerase Chain Reaction analysis of extracted DNA samples (BB1-138 to 155) [with primer set 1: forward (RW01) primer; reverse (DG74) primer] isolated from Site B in the Berg River. Lanes 1 –18: samples 138 to 155 (BB1); Lane A: Marker [MassRuler™ DNA Ladder Mix, #SM0403 (Fermentas)]; Lane B: Negative control.

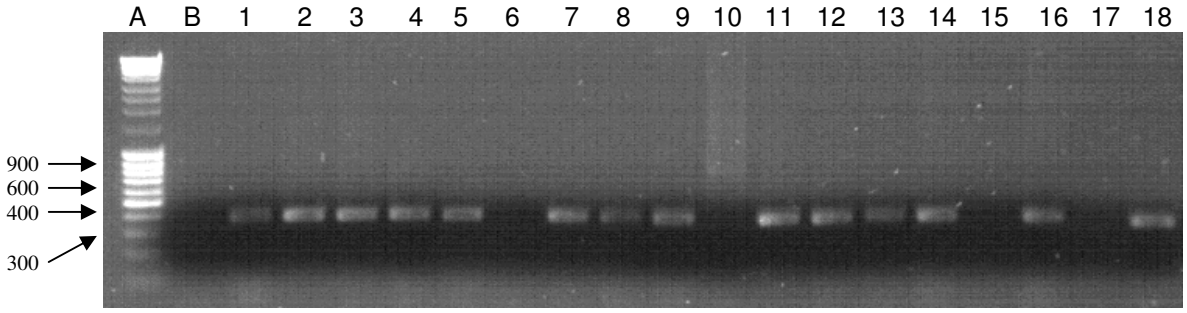

Fig. 4.3b Polymerase Chain Reaction analysis of extracted DNA samples (BB2-138 to 155) [with primer set 2: forward (RDR080) primer; reverse (DG74) primer] isolated from Site B in the Berg River. Lanes 1 – 18: samples 138 to 155 (BB2); Lane A: Marker [MassRuler™ DNA Ladder Mix, #SM0403 (Fermentas)]; Lane B: Negative control.

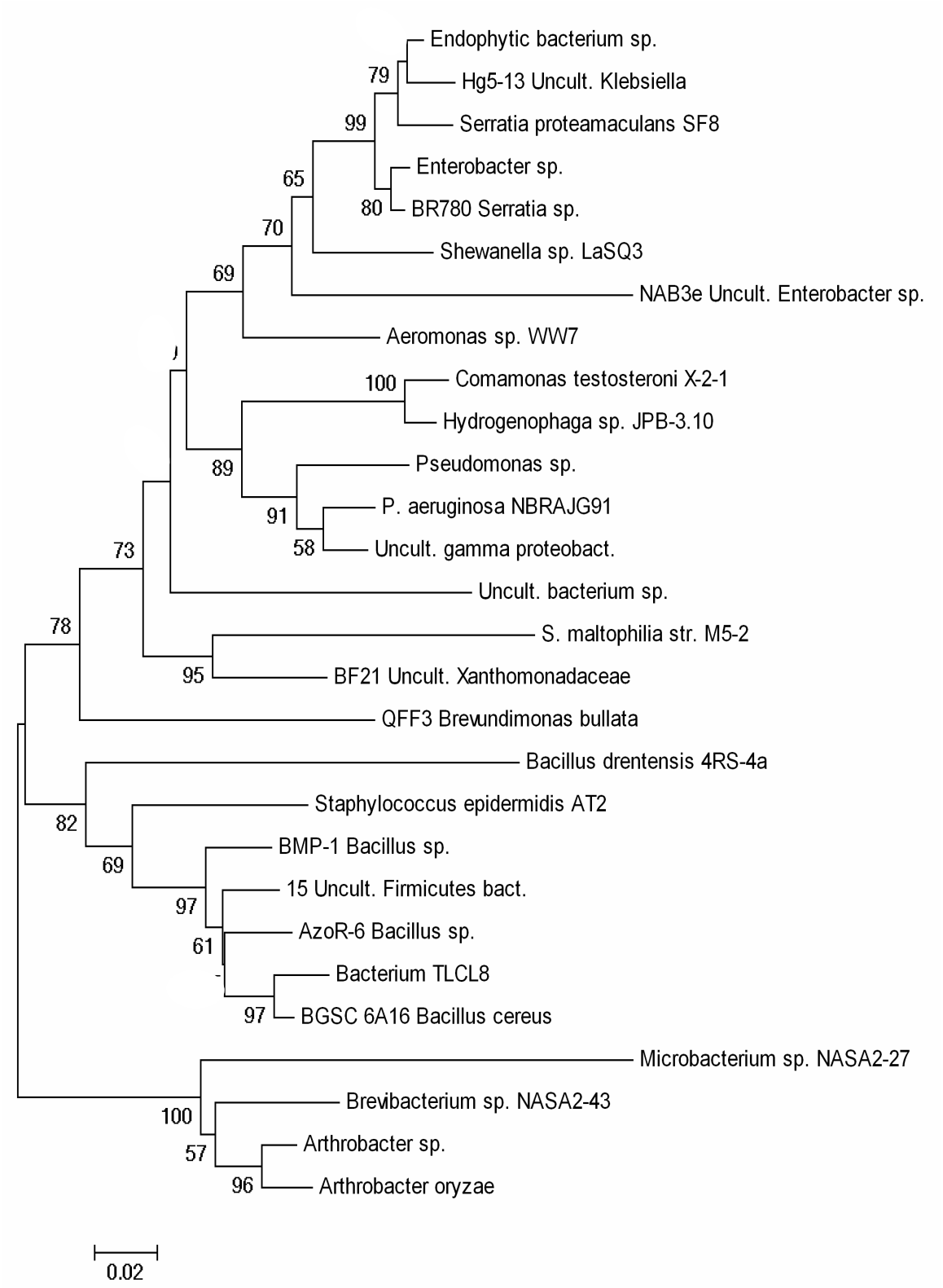

Fig. 4.4 Unrooted phylogenetic tree inferred from 16S rRNA sequence data, isolated from planktonic and sessile samples obtained from Site A in the Berg River in 2004 and 2005. Bootstrap values shown at nodes.

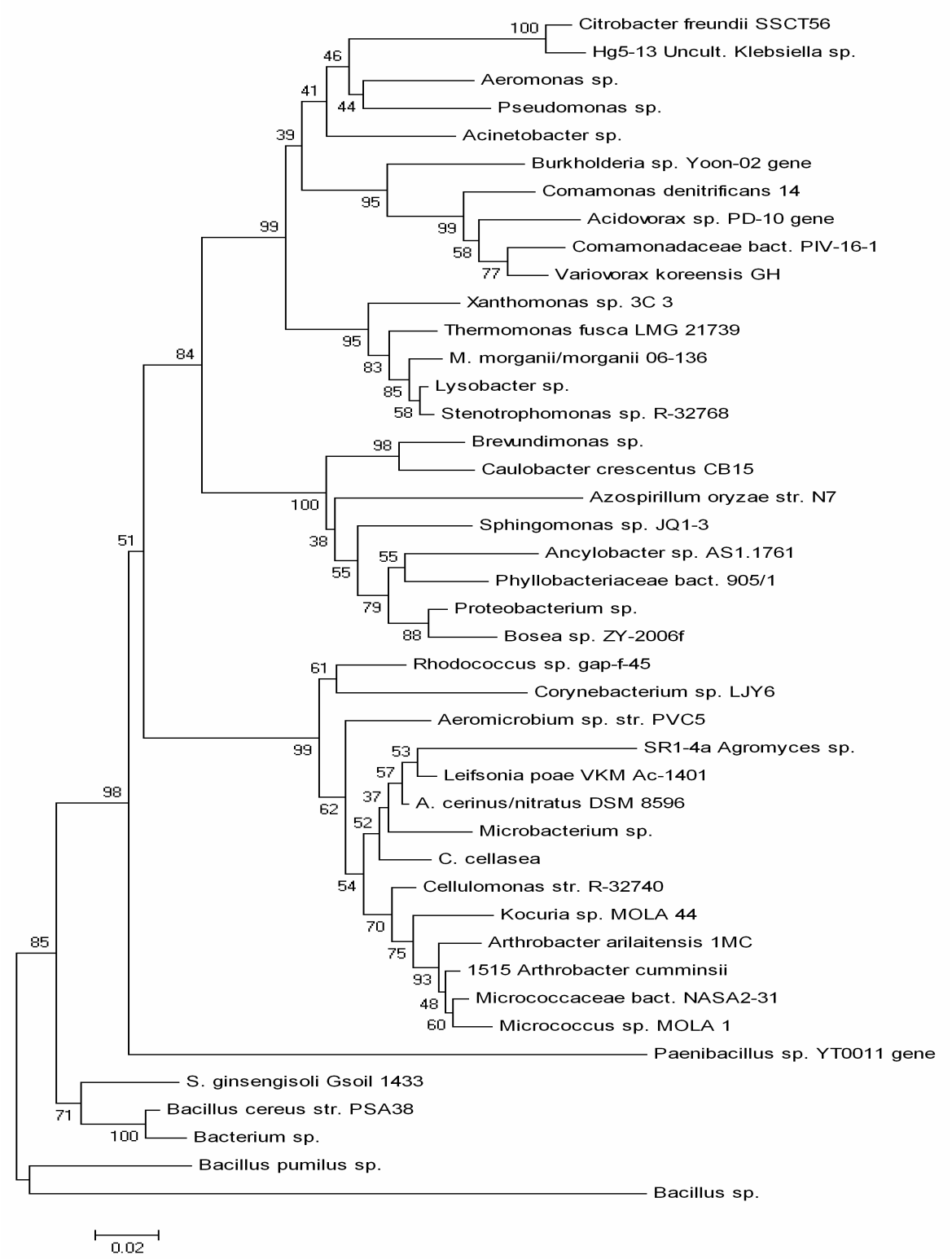

Fig. 4.5 Unrooted phylogenetic tree inferred from 16S rRNA sequence data, isolated from planktonic and sessile samples obtained from Site B in the Berg River in 2004 and 2005. Bootstrap values shown at nodes.

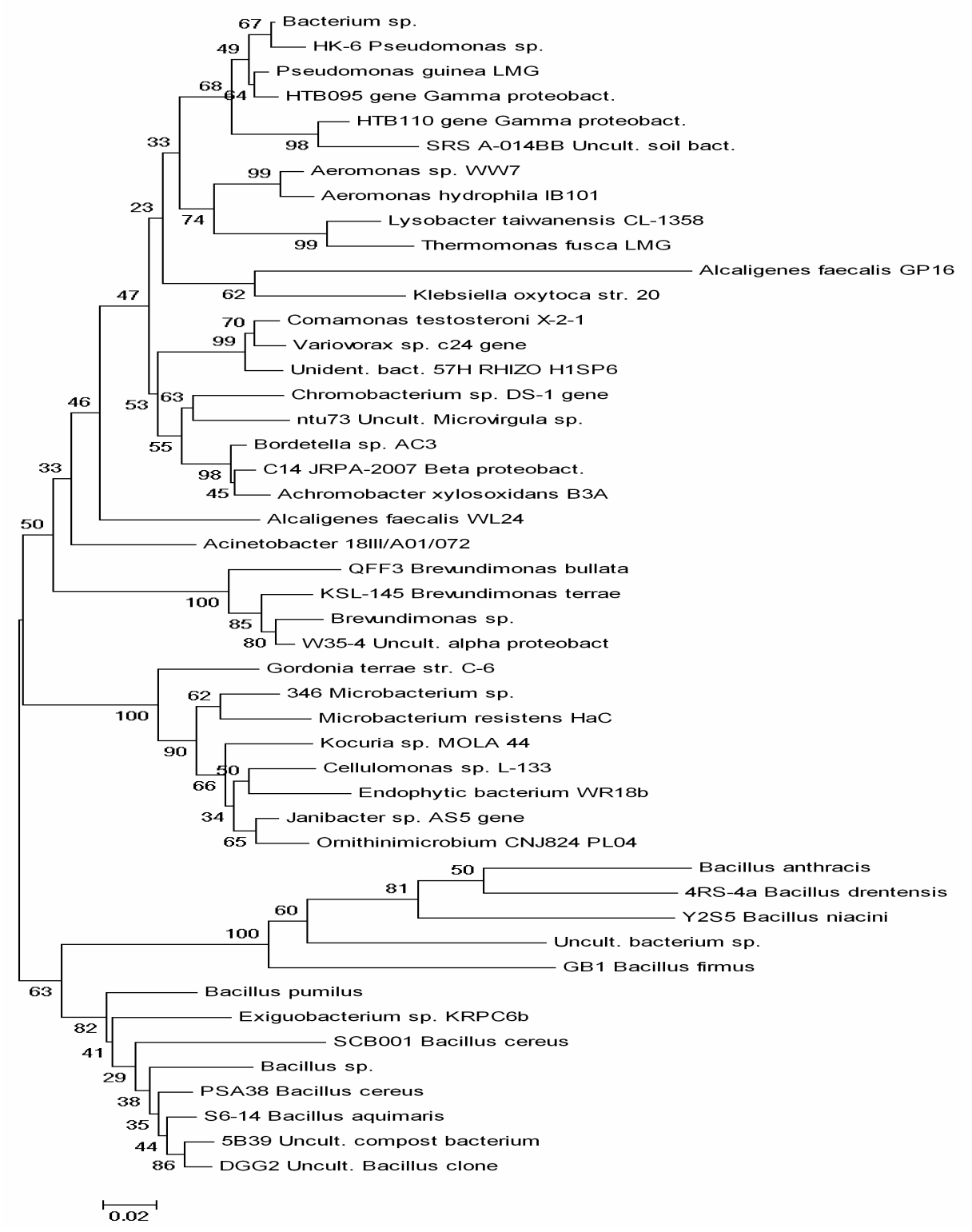

Fig. 4.6 Unrooted phylogenetic tree inferred from 16S rRNA sequence data, isolated from planktonic and sessile samples obtained from Site A in the Plankenburg River in 2004 and 2005. Bootstrap values shown at nodes.

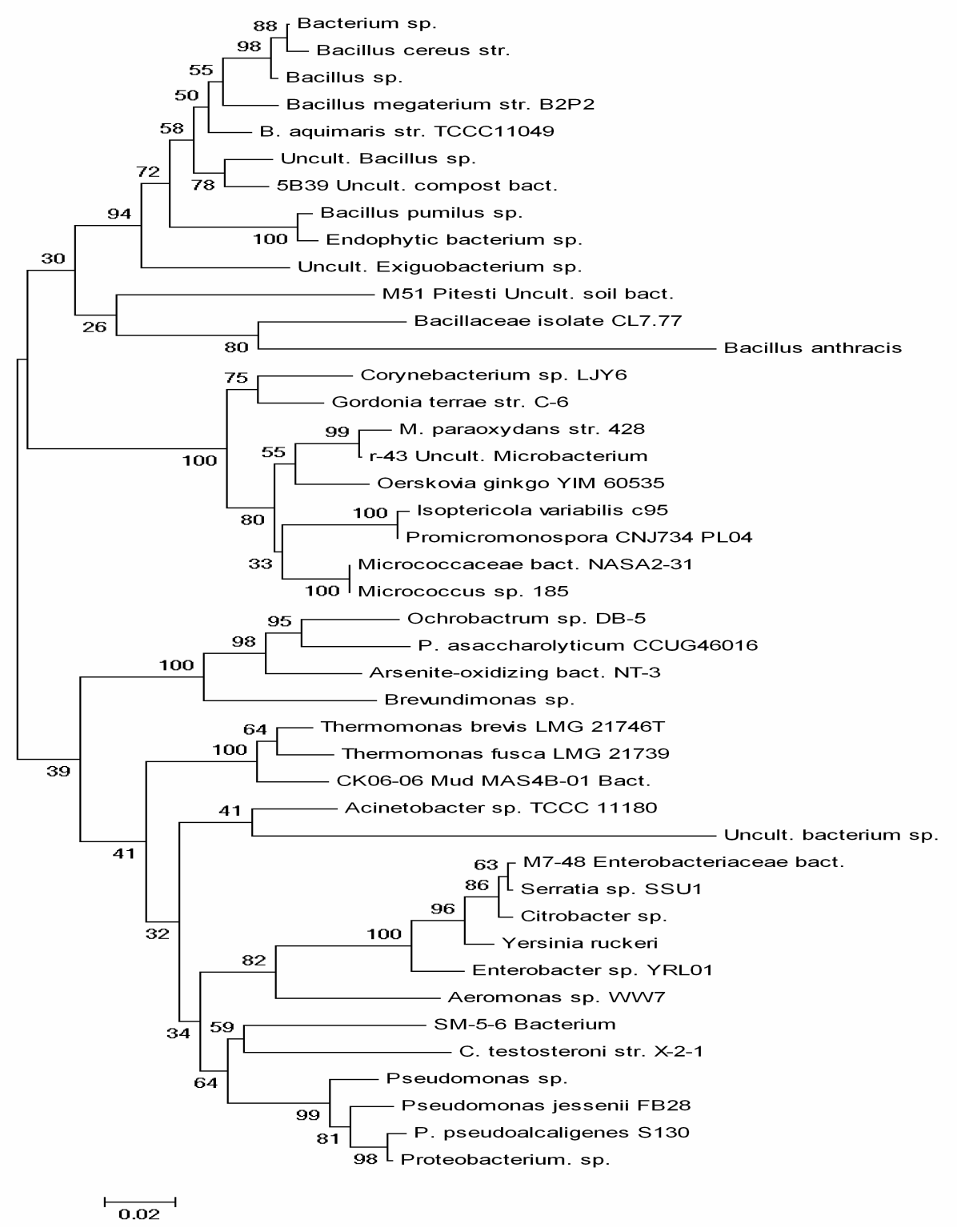

Fig. 4.7 Unrooted phylogenetic tree inferred from 16S rRNA sequence data, isolated from planktonic and sessile samples obtained from Site B in the Plankenburg River in 2004 and 2005. Bootstrap values shown at nodes.

Table 4.1 Table of 28 isolates identified at Site A in the Berg River, their codes and accession numbers

Name presented on tree	Organism	Accession number
Endophytic bacterium sp.	Endophytic bacterium sp.	EU088087.1
Hg5-13 Uncult. *Klebsiella*	Uncultured *Klebsiella* sp. strain Hg5-13	EU344923.1
Serratia proteomaculans SF8	*Serratia proteomaculans* strain SF8	EU760455.1
Enterobacter sp.	*Enterobacter* sp.	EU816586.1
BR780 *Serratia* sp.	*Serratia* sp. strain BR780	EF672647.1
Shewanella sp. LaSQ3	*Shewanella* sp. strain LaSQ3	EU075116.1
BAB3e Uncult. *Enterobacter* sp.	Uncultured *Enterobacter* sp. strain BAB3e	AY395025.1
Aeromonas sp. WW7	*Aeromonas* sp. strain WW7	EF433549.1
Uncult. Bacterium sp.	Uncultured Bacterium sp.	AY548935.1
Comamonas testosteroni X-2-1	*Comamonas testosteroni* strain X-2-1	EU668001.1
Hydrogenophaga sp. JPB-3.10	*Hydrogenophaga* sp. strain JPB-3.10	EU652485.1
Pseudomonas sp.	*Pseudomonas* sp.	EF426444.1
P. aeruginosa NBRAJG91	*Pseudomonas aeruginosa* strain NBRAJG91	EU661707.1
Uncult. gamma *proteobact.*	Uncultured gamma *Proteobacterium*	AF529346.1
S. maltophilia str. M5-2	*Stenotrophomonas maltophilia* strain M5-2	AY880273.1
BF21 Uncult. *Xanthomonadaceae*	Uncultured *Xanthomonadaceae* strain BF21	AM691113.1
QFF3 *Brevundimonas bullata*	*Brevundimonas bullata* strain QFF3	EU665637.1
Arthrobacter sp.	*Arthrobacter* sp.	AM933512.1
Arthrobacter oryzae	*Arthrobacter oryzae*	AB279889.1
Brevibacterium sp. NASA2-43	*Brevibacterium* sp. strain NASA2-43	EU029632.1
Microbacterium sp. NASA2-27	*Microbacterium* sp. strain NASA2-27	EU029616.1
Stahylococcus epidermidis AT2	*Stahylococcus epidermidis* strain AT2	EU021221.2
Bacillus drentensis 4RS-4a	*Bacillus drentensis* strain 4RS-4a	EU379279.1
AzoR-6 *Bacillus* sp.	*Bacillus* sp. strain AzoR-6	DQ279753.1
15 Uncult. *Firmicutes* bact.	Uncultured *Firmicutes* bacterium 15	EU647532.1
BMP-1 *Bacillus* sp.	*Bacillus* sp. strain BMP-1	DQ371431.1
Bacterium TLCL8	Bacterium TLCL8	EU086575.1
BGSC 6A16 *Bacillus cereus*	*Bacillus cereus* strain BGSC 6A16	AY310302.1

Table 4.2 Table of 43 isolates identified at Site B in the Berg River, their codes and accession numbers

Name presented on tree	Organism	Accession number
Citrobacter freundii SSCT56	*Citrobacter freundii* strain SSCT56	AB210978.1
Hg5-13 Uncult. *Klebsiella* sp.	Uncultered *Klebsiella* sp. strain Hg5-13	EU344923.1
Aeromonas sp.	*Aeromonas* sp.	EF433549.1
Pseudomonas sp.	*Pseudomonas* sp.	AJ968715.1
Acinetobacter sp.	Acinetobacter sp.	AY576723.1
Burkholderia sp. Yoon-02 gene	*Burkholderia* sp. strain Yoon-02 gene	AB259961.1
Comamonas denitrificans 14	*Comamonas denitrificans* strain 14	DQ836252.1
Acidovorax sp. PD-10 gene	*Acidovorax* sp. PD-10 gene	AB195159.1
Comamonadaceae bact. PIV-16-1	*Comamonadaceae* bacterium strain PIV-16-1	AJ505860.1
Variovorax koreensis GH	*Variovorax koreensis* strain GH	DQ432053.1
Xanthomonas sp. 3C 3	*Xanthomonas* sp. strain 3C 3	AY689031.1
Thermomonas fusca LMG 21739	*Thermomonas fusca* strain LMG 21739	AJ519988.1
Pseudoxanthomonas sp. R-24339	*Pseudoxanthomonas sp.* R-24339	AM231052.1
Lysobacter sp.	*Lysobacter* sp.	EU273938.1
Stenotrophomonas sp. R-32768	*Stenotrophomonas* sp. strain R-32768	AM403589.2
Brevundimonas sp.	*Brevundimonas* sp.	EU593764.1
Caulobacter crescentus CB15	*Caulobacter crescentus* strain CB15	AE005673.1
Azospirillum oryzae str. N7	*Azospirillum oryzae* str. strain N7	DQ682470.1
Sphingomonas sp. JQ1-3	*Sphingomonas* sp. strain JQ1-3	DQ118953.1
Ancylobacter sp. AS1.1761	*Ancylobacter* sp. strain AS1.1761	AY056830.1
Phyllobacteriaceae bact. 905/1	*Phyllobacteriaceae* bacterium strain 905/1	AM884149.1
Proteobacterium sp.	*Proteobacterium* sp.	AY040361.1
Bosea sp. ZY-2006f	*Bosea* sp. strain ZY-2006f	DQ987617.1
Rhodococcus sp. gap-f-45	*Rhodococcus* sp. strain gap-f-45	DQ530468.1
Corynebacterium sp. LJY6	*Corynebacterium* sp. strain LJY6	EU379022.1
Aeromicrobium sp. str. PVC5	*Aeromicrobium* sp. strain PVC5	AM421783.1
SR1-4a *Agromyces* sp.	*Agromyces* sp. strain SR1-4a	DQ102719.1
Leifsonia poae VKM Ac-1401	*Leifsonia poae* strain VKM Ac-1401	DQ232613.2
A. cerinus/nitratus DSM 8596	*Agromyces cerinus subsp. nitratus* strain DSM 8596	AM410681.1
Microbacterium sp.	*Microbacterium* sp.	EU714378.1
C. cellasea	*Cellulomonas cellasea*	X83804.1
Cellulomonas str. R-32740	*Cellulomonas* strain R-32740	AM403591.1
Kocuria sp. MOLA 44	*Kocuria* sp. strain MOLA 44	AM990819.1
Arthrobacter arilaitensis 1MC	*Arthrobacter arilaitensis* strain 1MC	DQ361012.1
1515 *Arthrobacter cumminsii*	*Arthrobacter cumminsii* strain 1515	EU086790.1
Micrococcaceae bact. NASA2-31	*Micrococcaceae* bacterium strain NASA2-31	EU029620.1
Micrococcus sp. MOLA 1	*Micrococcus* sp. strain MOLA 1	AM990777.1
Paenibacillus sp. YT0011	*Paenibacillus* sp. strain YT0011	AB362822.1
S. ginsengisoli Gsoil 1433	*Streptomyces ginsengisoli* Gsoil strain 1433	AB245381.1
Bacillus cereus str. PSA38	*Bacillus cereus* strain PSA38	EU346663.1
Bacterium sp.	Bacterium sp.	EU086575.1
Bacillus pumilus sp.	*Bacillus pumilus* sp.	EU660365.1
Bacillus sp.	*Bacillus* sp.	AM934695.1

Table 4.3 Table of 47 isolates identified at Site A in the Plankenburg River, their codes and accession numbers

Name presented on tree	Organism	Accession number
C14 JRPA-2007 Beta *proteobact.*	Beta *Proteobacterium* strain C14 JRPA-2007	EF599312.1
Bordetella sp. AC3	*Bordetella* sp. strain AC3	EU043370.1
Achromobacter xylosoxidans B3A	*Achromobacter xylosoxidans* strain B3A	EU360470.1
Chromobacterium sp. DS-1 gene	*Chromobacterium* sp. strain DS-1 gene	AB426118.1
ntu73 Uncult. *Microvirgula* sp.	Uncultured *Microvirgula* sp. strain ntu73	EU159477.1
Unident. bact. 57H RHIZO H1SP6	Unidentified bacterium strain 57H RHIZO H1SP6	EF606302.1
Comamonas testosteroni X-2-1	*Comamonas testosteroni* strain X-2-1	EU668001.1
Variovorax sp. c24 gene	*Variovorax* sp. strain c24 gene	AB167202.1
HK-6 *Pseudomonas* sp.	*Pseudomonas* sp. strain HK-6	DQ163021.1
Bacterium sp.	Bacterium sp.	EU086570.1
Pseudomonas guinea LMG	*Pseudomonas guinea* strain LMG	AM491811.1
HTB095 gene Gamma *proteobact.*	Gamma *Proteobacterium* strain HTB095 gene	AB010853.1
HTB110 gene Gamma *proteobact.*	Gamma *Proteobacterium* strain HTB110 gene	AB010851.1
SRS A-014BB Uncult. soil bact.	Uncultured soil bacterium strain SRS A-014BB	EF157964.1
Aeromonas sp. WW7	*Aeromonas* sp. strain WW7	EF433549.1
Aeromonas hydrophila IB101	*Aeromonas hydrophila* strain IB101	EU770274.1
Alcaligenes faecalis GP16	*Alcaligenes faecalis* strain GP16	DQ377464.1
Klebsiella oxytoca str. 20	*Klebsiella oxytoca* strain 20	DQ835530.1
Lysobacter taiwanensis CL-1358	*Lysobacter taiwanensis* strain CL-1358	DQ314555.1
Thermomonas fusca LMG	*Thermomonas fusca* strain LMG	AJ519988.1
Alcaligenes faecalis WL24	*Alcaligenes faecalis* strain WL24	EU727316.1
Acinetobacter 18III/A01/072	*Acinetobacter* strain 18III/A01/072	AY576723.1
QFF *Brevundimonas bullata*	*Brevundimonas bullata* strain QFF	EU665637.1
KSL-145 *Brevundimonas terrae*	*Brevundimonas terrae* strain KSL-145	DQ335215.1
Brevundimonas sp.	*Brevundimonas* sp.	DQ177489.1
W35-4 Uncult. alpha *proteobact.*	Uncultured alpha *Proteobacterium* strain W35-4	EU816932.1
Gordonia terrae str. C-6	*Gordonia terrae* strain C-6	EU590659.1
346 *Microbacterium* sp.	*Microbacterium* sp. strain 346	EU714366.1
Microbacterium resistens HaC	*Microbacterium resistens* strain HaC	EU675925.1
Kocuria sp. MOLA 44	*Kocuria* sp. strain MOLA 44	AM990819.1
Cellulomonas sp. L-133	*Cellulomonas* sp. strain L-133	EU420065.1
Endophytic bacterium WR18b	Endophytic bacterium strain WR18b	EU088020.1
Janibacter sp. AS5 gene	*Janibacter* sp. strain AS5 gene	AB299190.1
Ornithinimicrobium CNJ824 PL04	*Ornithinimicrobium* strain CNJ824 PL04	DQ448703.1
Bacillus anthracis	*Bacillus anthracis*	DQ105975.1
4RS-4a *Bacillus drentensis*	*Bacillus drentensis* strain 4RS-4a	EU379279.1
Y2S5 *Bacillus niacini*	*Bacillus niacini* strain Y2S5	EU221375.1
Uncult. bacterium sp.	Uncultured bacterium sp.	DQ816083.1
GB1 *Bacillus firmus*	*Bacillus firmus* strain GB1	EF101730.1
Bacillus pumilus	*Bacillus pumilus*	EU620415.1
Exiguobacterium sp. KRPC6b	*Exiguobacterium* sp. strain KRPC6b	DQ375558.1
SCB001 *Bacillus cereus*	*Bacillus cereus* strain SCB001	DQ466089.1
PSA38 *Bacillus cereus*	*Bacillus cereus* strain PSA38	EU346663.1
Bacillus sp.	*Bacillus* sp.	EU177796.1
S6-14 *Bacillus aquimaris*	*Bacillus aquimaris* strain S6-14	EU624438.1
5B39 Uncult. compost bacterium	Uncultured compost bacterium strain 5B39	DQ346644.1
DGG2 Uncult. *Bacillus* clone	Uncultured *Bacillus* clone DGG2	AY082367.1

Table 4.4 Table of 43 isolates identified at Site B in the Plankenburg River, their codes and accession numbers

Name presented on tree	Organism	Accession number
M7-48 *Enterobacteriaceae* bact.	*Enterobacteriaceae* bacterium strain M7-48	EU530471.1
Serratia sp. SSU1	*Serratia* sp. strain SSU1	EF032328.1
Citrobacter sp.	*Citrobacter* sp.	EF491831.1
Yersinia ruckeri	*Yersinia ruckeri*	EU401667.1
Enterobacter sp. YRL01	*Enterobacter* sp. strain YRL01	EU373405.1
Aeromonas sp. WW7	*Aeromonas* sp. strain WW7	EF433549.1
Acinetobacter sp. TCCC 11180	*Acinetobacter* sp. strain TCCC 11180	EU567051.1
SM-5-6 Bacterium	Bacterium strain SM-5-6	AY773137.1
C. testosteroni str. X-2-1	*Comamonas testosteroni* strain X-2-1	EU668001.1
Pseudomonas sp.	*Pseudomonas* sp.	DQ163021.1
Pseudomonas jessenii FB28	*Pseudomonas jessenii* strain FB28	AM933519.1
P. pseudoalcaligenes S130	*Pseudomonas pseudoalcaligenes* strain S130	EF095716.1
Proteobacterium sp.	*Proteobacterium* sp.	AB010851.1
CK06-06 Mud MAS4B-01 Bact.	Bacterium strain CK06-06 Mud MAS4B-01	AB371716.1
Thermomonas brevis LMG 21746T	*Thermomonas brevis* strain LMG 21746T	AJ519989.1
Thermomonas fusca LMG 21739	*Thermomonas fusca* strain LMG 21739	AJ519988.1
Brevundimonas sp.	*Brevundimonas* sp.	AJ244650.1
Arsenite-oxidising bact. NT-3	Arsenite-oxidising bacterium strain NT-3	AY027502.1
Ochrobactrum sp. DB-5	*Ochrobactrum* sp. strain DB-5	EU439404.1
P. assaccharolyticus CCUG46016	*Peptostreptococcus assaccharolyticus* strain CCUG46016	AM180485.1
M51 Pitesti Uncult. soil bact.	M51 Pitesti Uncultured soil bacterium	DQ378267.1
Isoptericola variabilis c95	*Isoptericola variabilis* strain c95	AB167235.1
Promicromonospora CNJ734 PL04	*Promicromonospora* strain CNJ734 PL04	DQ448724.1
Micrococcaceae bact. NASA2-31	*Micrococcaceae* bacterium strain NASA2-31	EU029620.1
Micrococcus sp. 185	*Micrococcus* sp. strain 185	EU714334.1
Oerskovia ginkgo YIM 60535	*Oerskovia ginkgo* strain YIM 60535	EU200684.1
M. paraoxydans str. 428	*Microbacterium paraoxydans* strain 428	EU714370.1
r-43 Uncult. *Microbacterium*	Uncultured *Microbacterium* strain r-43	EU816944.1
Corynebacterium sp. LJY6	*Corynebacterium* sp. strain LJY6	EU379022.1
Gordonia terrae str. C-6	*Gordonia terrae* strain C-6	EU590659.1
Uncult. *Exiguobacterium* sp.	Uncultured *Exiguobacterium* sp.	EU341181.1
Bacillus pumilus sp.	*Bacillus pumilus* sp.	EU379284.1
Endophytic bacterium sp.	Endophytic bacterium sp.	EU088021.1
Uncult. *Bacillus* sp.	Uncultured *Bacillus* sp.	EU817574.1
5B39 Uncult. compost bact.	Uncultured compost bacterium strain 5B39	DQ346644.1
B. aquimaris str. TCCC11049	*Bacillus aquimaris* strain TCCC11049	EU231632.1
Bacillus megaterium str. B2P2	*Bacillus megaterium* strain B2P2	EU221370.1
Bacillus sp.	*Bacillus* sp.	DQ084545.1
Bacterium sp.	Bacterium sp.	EU086570.1
Bacillus cereus str.	*Bacillus cereus* strain	AY310302.1
Uncult. bacterium sp.	Uncultured bacterium sp.	gb|AF143844.1|
Bacillaceae isolate CL7.77	*Bacillaceae* isolate strain CL7.77	FM174171.1
Bacillus anthracis	*Bacillus anthracis*	DQ105975.1

GENERAL CONCLUSIONS

5.1 COMPARISON OF ENUMERATION TECHNIQUES FOR THE INVESTIGATION OF BACTERIAL POLLUTION IN THE BERG RIVER, WESTERN CAPE, SOUTH AFRICA

The water quality of the rivers in South Africa and more specifically, the Western Cape, has deteriorated in recent years, mainly due to the influence of human activity along riverbanks. Several articles have highlighted health concerns sparked by faecal contamination in Western Cape Rivers. Two rivers in the Cape Metropolitan-Boland area that have been highly affected by faecal pollutants are the Plankenbrug River in Stellenbosch and Berg River in Paarl. The current study was aimed at assessing techniques, which would provide an accurate indication of the planktonic bacterial pollution load in the Berg River, Western Cape, South Africa. Various enumeration techniques were employed to determine different types of micro-organisms in planktonic (water) samples. All figures and tables in this section refer to Article one as discussed in Chapter two.

Sampling at the Berg River sites **(Figure 2.1)** started in June 2004 and continued for a period of 1 year until June 2005. Four sampling sites were identified and included Site A (agricultural farming area); Site B (informal settlement of Mbekweni - sites B1 and B2) and Site C (Newton pumping station). The Newton pumping station services the residential area of Newton as well as certain sections of Mbekweni. Enumeration techniques such as the Most Probable Number (MPN) technique were used to determine levels of all gas-producing micro-organism, including faecal coliforms and *E. coli*. The heterotrophic plate count (HPC) was also employed to determine the number of culturable micro-organisms in planktonic samples. This technique determines the amount of culturable micro-organisms but does not account for micro-organisms in a viable-but-non-culturable state. Flow cytometric analysis (FCM) and the direct acridine orange count (DAOC) (epifluorescence microscopy) technique were employed to evaluate total bacterial counts in planktonic (water) samples.

On average the total MPN counts **(Figure 2.2)** ranged from 1.6 x 10^3 micro-organisms/100 mℓ recorded at site A in the first week of sampling, to 3.5 x 10^7 micro-

organisms/100 mℓ observed at site B2 in week 37 of the sampling period. In comparison, the lowest faecal coliform count of 1.7 x 10^2 micro-organisms/100 mℓ was recorded in week 17 at site A, whereas the highest FC count of 3.5 x 10^7 micro-organisms/100 mℓ water was observed at site B2 in week 37. Corresponding *E. coli* counts ranged from 0.36 x 10^2 micro-organisms/100 mℓ in week 1, to 1.7 x 10^7 micro-organisms/100 mℓ in week 37, both recorded at site B2. These results are significantly ($p < 0.05$) higher than the maximum limit of 2000 organisms/100 mℓ stipulated for planktonic organisms in river water by the SABS Guidelines (1984). Site B2 (Plot 8000) is the site where storm water drainage pipes from the informal settlement flow directly into the river.

Results obtained for the average heterotrophic plate counts for the planktonic samples analysed at the respective sites in the Berg River are depicted in **Figure 2.3**. On average the heterotrophic plate counts ranged from 3 x 10^3 micro-organisms/mℓ recorded at various sites throughout the sampling period to 1.04 x 10^6 micro-organisms/mℓ recorded at site B2 in week 37.

In comparison, the total cell counts obtained by the FCM technique **(Figures 2.4 and 2.5)** ranged from 1.5 x 10^6 (site B1, week 5) and 1.6 x 10^6 micro-organisms/mℓ (site B2, week 49) to 3.7 x 10^7 micro-organisms/mℓ in week 41 (site B2). Comparison of the CFU counts obtained by the heterotrophic plate count technique, to the viable flow cytometric (FCM) analysis counts yielded significantly ($p < 0.05$) higher viable counts **(Figure 2.6)** in the planktonic samples. The highest CFU count of 1.04 x 10^6 micro-organisms/mℓ (**Figure 2.3**) was recorded in week 37 at site B2. A corresponding FCM viable count of 1.72 x 10^7 micro-organisms/mℓ was recorded for the same sampling time. The relative values (by means of percentage ratios) of the heterotrophic plate count to the total FCM count, viable FCM count to total FCM count and heterotrophic plate count to viable FCM count are presented in **Tables 2.1 – 2.3**, respectively. Results showed that on average the heterotrophic plate count represented only a fraction [3.65% (week 37, site B2)] of the total biomass obtained by FCM analysis. The viable FCM count accounted for 60.18% of the total FCM count for site B2 in the same week of sampling **(Table 2.2)**. In addition, the heterotrophic plate count represented only a fraction (6.06%) of the viable FCM count for the same sampling period at site B2. The highest heterotrophic plate count represented 6.96% of the viable FCM count (week 45

at site B2) and only a fraction of 1.02% of the total FCM count **(Table 2.1)** for the same sampling site and period. Results clearly show that the FCM technique is the more reliable enumeration technique for microbial populations obtained from environmental samples. The higher FCM results could also be ascribed to the fact that this technique is able to detect certain populations in the environment, which enter a viable but non-culturable state when exposed to stressful conditions.

The total cell count obtained by the FCM technique was compared to total cell counts obtained by the DAOC **(Figure 2.7)** method. The total cell counts obtained from the DAOC method were lower in all the water samples, when compared to total counts obtained by flow cytometer analyses **(Figure 2.8)**. The highest DAOC count of 8.3×10^6 micro-organisms/mℓ for planktonic samples was recorded in week 29 at site B2. The FCM analysis for week 29 yielded comparable total counts of 8.62×10^6 micro-organisms/mℓ. The lowest planktonic DAOC count of 4×10^4 micro-organisms/mℓ was recorded at site B1 in week 49, with a corresponding FCM count of 4.7×10^6 micro-organisms/mℓ. Even though the FCM counts fluctuated throughout the sampling period, results clearly show that the FCM method yields more accurate data for total cell counts than the DAOC method. The total DAOC count also represented a fraction (< 43.08%) of the total FCM count **(Table 2.5)** for most of the sampling period. In comparison, the percentage ratio of the heterotrophic plate count to the total FCM count **(Table 2.1)** was recorded at 0.43% at site B1 for the same sampling period with the total DAOC count representing only 0.87% of the total FCM count. Discrepancies in the percentage ratio of the heterotrophic plate count and total DAOC counts were observed in weeks 37 (490.06 %) and 45 (286.26%) **(Table 2.4)**, respectively. These results indicate the inconsistencies which could be experienced with the DAOC technique as this technique involves physical counting of the micro-organisms in conjunction with epifluorescence microscopy. The FCM method analyses total cell counts by means of computer software. Results therefore showed that the flow cytometry method proved to be more effective in evaluating microbial pollution in water samples. It is also recommended that the levels of bacterial contaminants, amongst others, in water courses such as the Berg River and other rivers be more routinely evaluated or monitored.

5.2 COMPARISON OF MICROBIAL CONTAMINATION AT VARIOUS SITES ALONG THE PLANKENBURG- AND DIEP RIVERS, WESTERN CAPE, SOUTH AFRICA

Irregular rainfall patterns, drought spells as well as point- and non-point source pollution has increased in recent years in South Africa. Water availability and quality are thus threatened to such an extent that the need for alternative water resources exists (Thukela Water Project Report, 2004). Nationally, river water has become highly contaminated due to the growing number of communities inhabiting the banks of major water courses. The lack of adequate and functional sanitary facilities have led to faecal and solid waste being dumped into rivers and along riverbanks, which seep into rivers thereby causing potential pathogens to accumulate and proliferate in these water sources.

The aim of the present study was to investigate and compare microbial contamination at various sites along the Plankenburg and Diep Rivers in the Western Cape, South Africa. All the figures mentioned in this section refer to Article two as presented in Chapter three. Sampling of the Plankenburg River sites started in June 2004 and continued on a monthly basis until June 2005. Three sampling sites were identified for the Plankenburg River (Stellenbosch) **(Figure 3.1)** location and included Site A (Agricultural Farming and Residential Areas); Site B (Informal Settlement of Kayamandi) and Site C (Substation in Industrial Area). Sampling in the Diep River **(Figure 3.2)** started in March 2005 and continued for a period of nine months until November 2005. Sites for the Diep River (Milnerton) location included Site A (Zoarvlei Nature Reserve - Industrial as well as Residential Areas); Site B (Theo Marais Sportsclub - Industrial and Residential Area) and Site C (Potsdam Wastewater Treatment Works). Faecal coliform (FC) and *E. coli* (EC) counts were determined by means of the Most Probable Number technique, while the number of culturable cells were determined using the heterotrophic plate count (HPC) technique. Total microbial counts were determined by Flow cytometric analysis (FCM). Temperature and pH were monitored at all sites along the Plankenburg and Diep Rivers and are presented in Tables **3.1** and **3.2**, respectively.

Overall, the highest microbial counts (**Figures 3.5** and **3.6**) were observed at site B (Plankenburg River) where the highest MPN, FC and *E. coli* counts of 9.2×10^6 (week

14), 3.5 x 10^6 (week 39) and 3.5 x 10^6 micro-organisms/100 mℓ (week 39), respectively, were recorded. The highest HPC recorded along the river was 7.9 x 10^6 micro-organisms/100 mℓ (week 44, site A) (**Figure 3.3**), while significantly high total cell counts (total FCM) (**Figure 3.6**) of 2.1 x 10^8 (weeks 1 and 39, respectively) and 1.5 x 10^8 micro-organisms/mℓ (weeks 14 and 48, respectively) were recorded at site B. Overall, the results show that site B was the most contaminated site along the Plankenburg River during the sampling period. As mentioned, site B is situated close to the informal settlement where sanitation systems are inadequate to satisfy the needs of the inhabitants and where storm water drainage systems and waste containers are used as a means of disposal of faecal matter. In addition, sanitation systems located in the settlement are connected to storm water drainage pipes which enter the river system at this site. The dense vegetation on the riverbanks at this site could possibly also contribute to slower water flow, thereby promoting microbial growth and increased numbers. In addition, other possible contamination sources included agricultural (farming practices) and industrial (spray-painting facilities, machine manufacturers and a cheese factory) areas bordering the Plankenburg River.

Compared to sites A and C, the highest microbial counts were observed at site B along the Diep River. The highest total MPN, FC and *E. coli* counts recorded in the Diep River were 5.4 x 10^6 (week 23) and 1.6 x 10^6 micro-organisms/100 mℓ [FC and *E. coli*, respectively (both in week 23)], recorded at site B (the Theo Marais Sportsclub). The highest HPC and total FCM counts were also recorded at site B and indicated significantly high ($p < 0.05$) counts of 1.7 x 10^7 micro-organisms/100 mℓ (week 14) and 2.5 x 10^9 micro-organisms/mℓ (week 23), respectively, thereby implicating the site as the most contaminated along the river. This site is an accumulation point for waste effluent flowing from the residential and industrial areas of Milnerton and Paarden Island, which include paint and machine manufacturers. Other sources situated along the Diep River included storage and maintenance facilities for steel containers, a waste water treatment plant and an oil-refinery, which could also have contributed to the elevated bacterial pollutants along the Diep River. The significantly high ($p < 0.05$) counts observed in the Diep River exceeded the accepted maximum limit for river water and may therefore lead to major health concerns as the river runs into the Rietvlei Nature Reserve, which is used for recreational purposes.

Most of the counts exceeded the accepted maximum limit for river water for the Plankenburg and Diep Rivers for most of the sampling period. Comparison of the HPC results to the viable FCM counts clearly show that the FCM technique yielded significantly ($p < 0.05$) higher counts and indicated that this technique is a more reliable enumeration method to determine more accurately, the viable microbial counts in environmental samples.

5.3 ISOLATION AND IDENTIFICATION OF BACTERIAL POLLUTANTS FROM THE BERG- AND PLANKENBURG RIVERS IN THE WESTERN CAPE, SOUTH AFRICA

Organic and inorganic waste, as well as industrial and agricultural waste, sewage, human activities, human and animal excreta, are the main sources responsible for the deterioration of the valuable water bodies (Last, 2002). In South Africa, water resources such as rivers, service various industrial, agricultural and domestic sectors, which may also contribute to the point or non-point source contamination of these valuable water sources. Waterborne outbreaks have been associated with microbial agents, such as *Salmonella* sp., *Campylobacter* sp. and *Escherichia coli* amongst others (Gerba, 1996), as well as viruses, protozoa, helminths and fungi. These micro-organisms have led to significant health risks in humans with lowered resistance levels such as infants, the elderly and immunocompromised individuals (Theron & Cloete, 2002).

The aim of this study was to identify the predominant bacterial species, isolated from planktonic and sessile samples, present in the Berg- and Plankenburg Rivers in the Western Cape, South Africa using 16S rRNA PCR and DNA sequencing. For this study only the sites upstream from and at the informal settlements were selected for analysis. Micro-organisms isolated from water and biofilm samples obtained from the Berg- and Plankenburg Rivers were analysed. All figures mentioned in this section refer to Article three as discussed in Chapter four.

Sampling at the Berg- and Plankenburg River-sites started in June 2004 and continued for a period of 1 year until June 2005. Four sampling sites were identified along the Berg River **(Figure 4.1)** and included Site A (agricultural farming area); Site B (informal settlement - sites B1 and B2) and Site C (Newton pumping station). Site B2 (Plot 8000) was identified as the site where storm water drainage pipes from the informal

settlement flow directly into the river. The Newton pumping station services the residential area of Newton as well as certain sections of Mbekweni. Sampling sites along the Plankenburg River **(Figure 4.2)** included Site A (agricultural farming and a residential area from Stellenbosch); Site B (informal settlement); Site C (industrial area) and, Site D (agricultural and industrial area).

Deoxyribonucleic acid (DNA) extractions of isolated micro-organisms were performed using the High Pure PCR Template Preparation Kit as per manufacturer's instructions (Roche Diagnostics) followed by subsequent 16S rRNA sequence amplification by means of the Polymerase Chain Reaction, sequenced and identified using the Basic Local Alignment Search Tool (Blastn) (Altschul et al., 1997) obtained from the National Centre for Biotechnology Information website. A MassRuler™ DNA Ladder Mix, #SM0403 (Fermentas) was used to compare amplicon size. Subsequent DNA concentrations of PCR products were determined by means of the Qubit™ fluorometer (Invitrogen) using the Quant-iT™ dsDNA BR (Broad-range) Assay kit 2 – 1000 ng as per manufacturer's instructions (Molecular probes and Invitrogen). Phylogenetic analysis was performed by aligning grouped DNA sequences with Clustal X (1.81) using default parameters and the Blosum matrix. Unrooted trees were constructed using the neighbour-joining program of *MEGA* version 4.1 (Molecular Evolutionary Genetics Analysis 4.1), while branching patterns were evaluated by pairing 1000 replicates.

Figure 4.3a indicates representative samples isolated at Site B in the Berg River, which were amplified using primer set 1 (Greisen et al., 1994), while the amplified PCR samples represented in **Figure 4.3b** were obtained using primer set 2 (Greisen et al., 1994). The unrooted phylogenetic trees illustrated in **Figures 4.4** to **4.7** include various species of both the Gram-positive and Gram-negative heterotrophic bacteria. Bacterial species isolated from the planktonic and sessile samples obtained from Site A in the Berg River (**Figure 4.4**) displayed a wide diversity of micro-organisms. The presence of various members of the *Enterobacteriaceae*, such as *Klebsiella* sp., *Serratia* sp., *Enterobacter* sp., *Shewanella* sp. and *Aeromonas* sp., confirm faecal contamination in the river. In addition, pathogenic micro-organisms including *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia* and *Bacillus cereus* were also isolated at this site. Research has shown that *Stenotrophomonas* sp. are affecting more humans (Gilligan and Whittier, 1999) and the fact that Site B is situated directly next to the informal

settlement, raises concern as exposure to the organism could result in increased infection rates. *Staphylococcus epidermidis* which form part of the normal flora of the human skin (O'Gara & Humphreys, 2001) was also identified at this site. This organism however, may affect people with altered immune systems and might be responsible for endocarditis and infection in these patients (Prescott et al., 1990). *Enterobacteriaceae* species isolated at Site B along the Berg River (**Figure 4.5**) also included *Citrobacter freundii* and *Pseudomonas* sp. Compared to Site A, a greater species diversity was observed at Site B. Various bacterial species were introduced at this Site B and included species such as *Burkholderia* sp., *Acidovorax* sp., *Variovorax koreensis*, *Thermomonas* sp., *Lysobacter* sp., and *Kocuria* sp., amongst others. Similarly many species were conserved between the sites for example pathogenic micro-organisms such as *Klebsiella* sp., *Aeromonas* sp., *Pseudomonas* sp., *Stenotrophomonas* sp. and *Bacillus cereus*.

From the phylogenetic tree illustrated in **Figure 4.6** (Plankenbrug River, Site A), two distinct clusters could be identified with the Gram-negative bacteria comprising the largest. This cluster was divided into five subclusters and several clades. One subcluster included once again, members of the *Enterobacteriaceae* thereby confirming faecal contamination in the Plankenburg River. Species such as *Aeromonas hydrophila*, *Alcaligenes faecalis*, *Klebsiella oxytoca* and various other species were also identified at this site. *Aeromonas* sp. and particularly, *A. hydrophila* is associated with gastroenteritis, cellulitis and other diseases in humans. Bacterial species such as *Lysobacter taiwanensis*, *Thermomonas fusca* and *Acinetobacter* sp. were also identified at this site and are all indicator organisms of faecal contamination. The presence of these faecal indicators also relate to a previous study (Paulse et al., 2008) where significantly ($p < 0.05$) high faecal coliform and *E. coli* counts were recorded by means of the most probable number technique (MPN). During this study, faecal coliform and *E. coli* counts of 3.5×10^6 micro-organisms/100 ml respectively, were observed at Site B in the Plankenburg River. In addition, pathogens such as *Bacillus cereus* and *B. anthracis* were also identified at this site. The presence of these two organisms is responsible for major health illness such as food poisoning (*B. cereus*) and the disease, anthrax (*B. anthracis*). These organisms also raise concerns as both organisms were conserved between Site A and Site B (**Figure 4.7**), where increased population numbers are observed, thus resulting in increased exposure to the pathogens and thereby

increased possible infections. Other important bacterial species introduced at Site B included various members of the *Enterobacteriaceae* group such as *Serratia* sp., *Citrobacter* sp., *Yersinia ruckeri* and *Enterobacter* sp. thereby again confirming faecal contamination at this site. As previously mentioned this is the point of the river that is closest to the informal settlement. It is also the site where faecal wastewater from nearby sanitary facilities is flushed into the river. Bacterial species from the genus, *Yersinia* are considered major human pathogens and may be the causative agent of plague in susceptible individuals.

5.4 MAJOR FINDINGS OF THE STUDY

5.4.1.1 On average, the MPN counts notably exceeded the maximum limit of 2000 micro-organisms/100 mℓ (South African Bureau of Standards, 1984) for river water.

5.4.1.2 Only 2% of the total MPN, 23% of faecal coliforms and 30% of *E. coli* counts fell within the accepted maximum limit range.

5.4.1.3 The highest overall counts for MPN (faecal coliforms, *E. coli*), heterotrophic counts, DAOC and FCM were observed at site B2 which is the site where storm water drainage pipes from the informal settlement flows into the river.

5.4.1.4 Overall higher viable cell counts were obtained from FCM analysis when compared to cell counts obtained by means of the heterotrophic plate count technique, which could be ascribed to the fact that the heterotrophic plate count technique only accounts for viable culturable micro-organisms whereas FCM analysis detects viable-culturable micro-organisms as well as those in a viable-but-non-culturable state.

5.4.1.5 The heterotrophic plate count thereby represented only a fraction < 3.65% of the total FCM count and < 6.06% of the viable FCM count (site B2 in week 37) of the sampling period.

5.4.1.6 The heterotrophic plate counts represented < 49.25% of the total DAOC count with exceptions in weeks 37 and 45 where higher heterotrophic plate counts with percentage ratios of 490.06% and 286.26% respectively, for heterotrophic

plate counts versus DAOC counts were recorded. This indicates that inconsistencies could be experienced with the DAOC technique.

5.4.1.7 In addition, the FCM technique indicated significantly ($p < 0.05$) higher total counts than those observed by the DAOC technique. The only comparable DAOC to FCM count was observed in week 29 at site B2 where 96.35% percentage ratio was recorded. The FCM technique therefore proves to be a more effective technique to routinely compare and evaluate the presence of most if not all, populations in the river water samples.

5.4.2.1 On average, the MPN, FC and *E. coli* levels within the Plankenburg River notably exceeded the maximum limit of 2000 micro-organisms/100 mℓ for river water throughout the study period (SABS, 1984; DWAF, 1996a-c).

5.4.2.2 Contamination of the river was also confirmed with the significantly high ($p < 0.05$) total FCM counts observed at all the sites along the Plankenburg River.

5.4.2.3 The MPN, FC, *E. coli*, HPC and FCM results obtained from the different sites along the Plankenburg River indicate that site B was the most contaminated site with higher counts than sites A and C.

5.4.2.4 Site B is situated close to the informal settlement where sanitation systems are inadequate to satisfy the needs of the inhabitants and where storm water drainage systems and waste containers are used as a means of disposal of faecal matter. In addition, sanitation systems located in the settlement are connected to storm water drainage pipes which enter the river system at this site.

5.4.2.5 Even though significantly lower microbial counts were observed at sites A and C, these sites are bordered by residential and industrial areas, respectively, which might have contributed to the increased microbial contaminants at these sites.

5.4.2.6 Agricultural run-off from farms adjacent to the river (Plankenburg River) could also have contributed to increased contaminant levels within the river at site A.

5.4.2.7 The MPN, FC, *E. coli*, HPC and FCM counts within the Diep River also exceeded the maximum limit of 2000 micro-organisms/100 mℓ for river water throughout the study period (SABS, 1984; DWAF, 1996a-c).

5.4.2.8 Overall, initial increases in the bacterial counts from site A to site B were followed by decreases in the bacterial counts recorded at site C. Results thus show that site B was the most contaminated site along the Diep River. Site B is located at the Theo Marais Sportsclub, which is surrounded by major industrial and residential areas. Waste effluent from these areas accumulates at this site contributing to possible contamination.

5.4.2.9 Site A is also bordered by industrial and residential areas, while site C receives waste water from a residential area as well as an oil-refinery. Even though no point- or non-point sources of contamination could be identified, the waste effluent present at the residential and industrial sites could influence water pollution at the respective sampling sites.

5.4.2.10 Comparisons of the HPC results to the viable FCM counts, which were significantly higher ($p < 0.05$) for the Plankenburg and Diep Rivers throughout the sampling period, proved that the FCM technique is a more reliable enumeration method to accurately determine the viable bacterial counts in environmental samples.

5.4.2.11 Overall, the significantly high microbial counts obtained for both river systems raises major human and environmental health concerns. The effectiveness of current monitoring and clean-up strategies by respective authorities and district municipalities should be revised to include techniques which accurately reflect the contamination levels of the river systems.

5.4.3.1 The presence of *Klebsiella*, *Serratia*, *Enterobacter*, *Shewanella* and *Aeromonas* species shows that faecal contamination could be observed in both the Berg and Plankenburg Rivers over the one year sampling period, i.e. from 2004 to 2005, and at all the sites sampled.

5.4.3.2 Members of the family, *Enterobacteriaceae*, isolated at Sites A in the Berg River included *Klebsiella* sp., *Serratia* sp., *Enterobacter* sp., *Shewanella* sp. and *Aeromonas* sp.

5.4.3.3 Pathogenic and opportunistic pathogens such as *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *Staphylococcus epidermidis*, and *Bacillus cereus* have also been identified at Site A in the Berg River.

5.4.3.4 Faecal indicators isolated from Site B in the Berg River included *Citrobacter freundii*, *Klebsiella* sp., *Aeromonas* sp., *Pseudomonas* sp. and *Acinetobacter* sp.

5.4.3.5 Pathogenic and opportunistic pathogens such as *Stenotrophomonas* sp. and *Bacillus cereus* have also been identified at Site B (Berg River).

5.4.3.6 The *Enterobacteriaceae* isolated from Site A in the Plankenburg River included *Aeromonas* sp., *Alcaligenes faecalis*, *Klebsiella* sp. and *Acinetobacter* sp.

5.4.3.7 In addition to abovementioned water-borne isolates, pathogenic species such as *Bacillus anthracis* and *B. cereus* were also identified at Site A in the Plankenburg River.

5.4.3.8 Bacterial species such as *Serratia*, *Citrobacter*, *Enterobacter*, *Aeromonas*, *Acinetobacter* and *Yersinia ruckeri* were isolated from Site B in the Plankenburg River.

5.4.3.9 Various pathogenic species has been conserved between Site A and Site B (Plankenburg River), such as *Bacillus anthracis* and *B. cereus*. In addition, other members of this species have also been conserved between sites even though some has been lost while others have added.

5.4.3.10 Most of the bacterial species isolated are either pathogenic or opportunistic pathogens, which may lead to severe illnesses to inhabitants of these river areas.

5.5 RECOMMENDATIONS

By the enumeration techniques employed throughout the study, it is clear that the FCM technique was the most effective in determining more accurate bacterial counts in environmental and more specifically, water samples. It is therefore recommended that this technique be incorporated as the major means of determining whether or not bacterial counts fall within the accepted maximum limit of a specific group of organisms in a specified body of water. Also, due to the fact that urbanisation in a developing country, such as South Africa, would lead to increasing numbers of people inhabiting riverbanks and areas close to water sources, it be advised that these areas be made fit and safer for human living. If not possible, these communities should be relocated to

areas with adequate and efficient sanitary facilities and sufficient water facilities for daily use.

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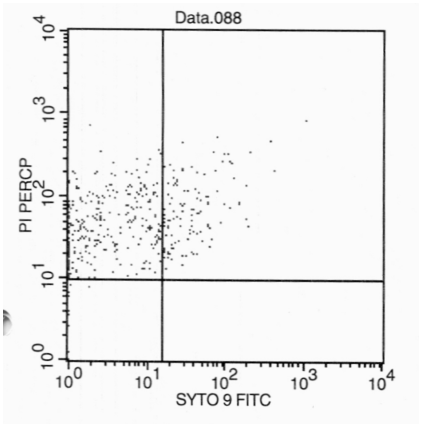
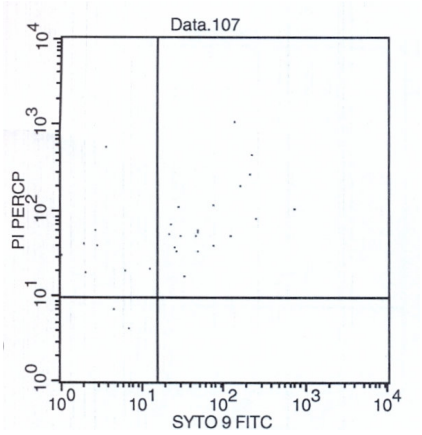
APPENDIX A

Laboratory-scale bioreactor (i)

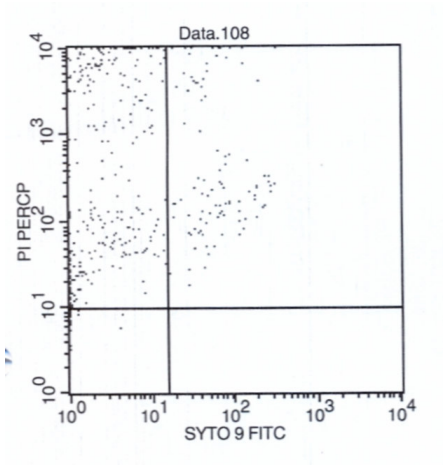


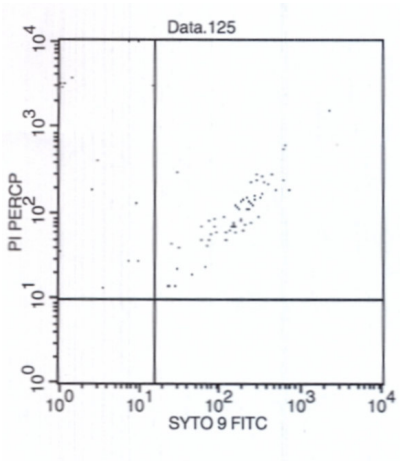


Initial sample (day one) **Final sample (day 20)**

Dotplots indicating the viable and non-viable bacterial populations, from laboratory-scale bioreactor system (i), obtained by FCM analysis. The two left quadrants indicate the dead bacterial cells and the right quadrants the live bacterial cells.

Laboratory-scale bioreactor (ii)





Initial sample (day one) **Final sample (day 18)**

Dotplots indicating the viable and non-viable bacterial populations, from laboratory-scale bioreactor system (ii), obtained by FCM analysis. The two left quadrants indicate the dead bacterial cells and the right quadrants the live bacterial cells.

APPENDIX B

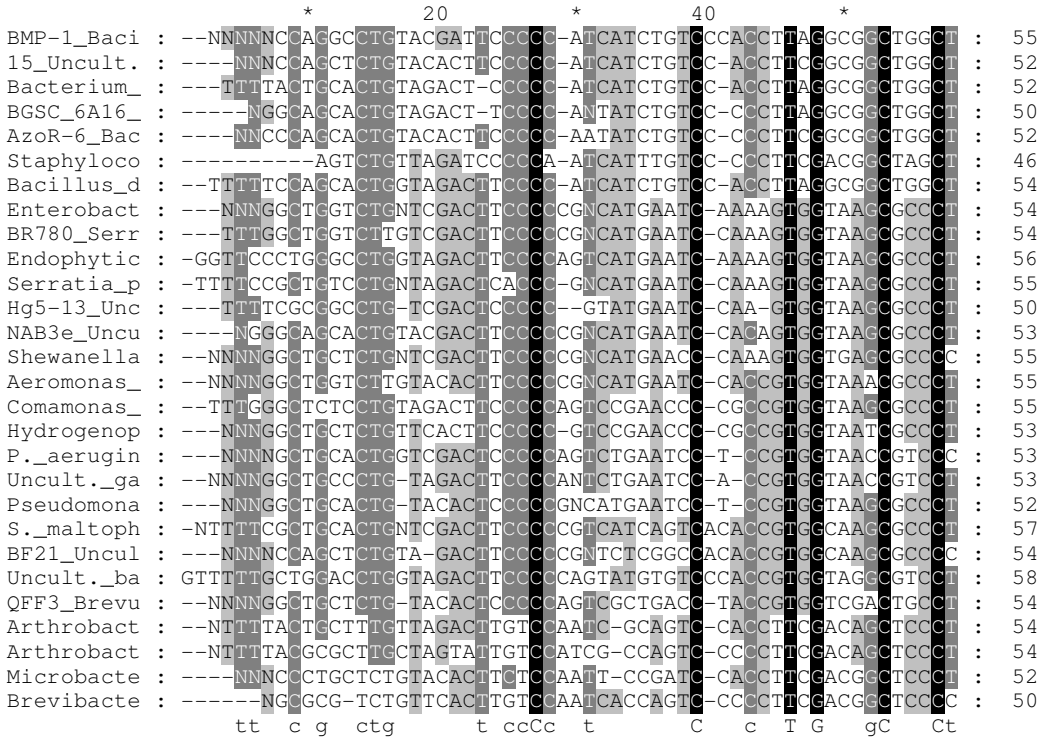


Figure 1 Alignment of the amino acid sequences of 28 waterborne bacterial species isolated from Site A in the Berg River. The alignment was carried out by the multiple alignment of ClustalX (1.81). Genedoc software was used for homology shading. The abbreviations of the isolates are given in the text. Gaps introduced into the alignment are indicated with dashes. Four shading levels were set: black for 100% identity and grey for 70% identity.

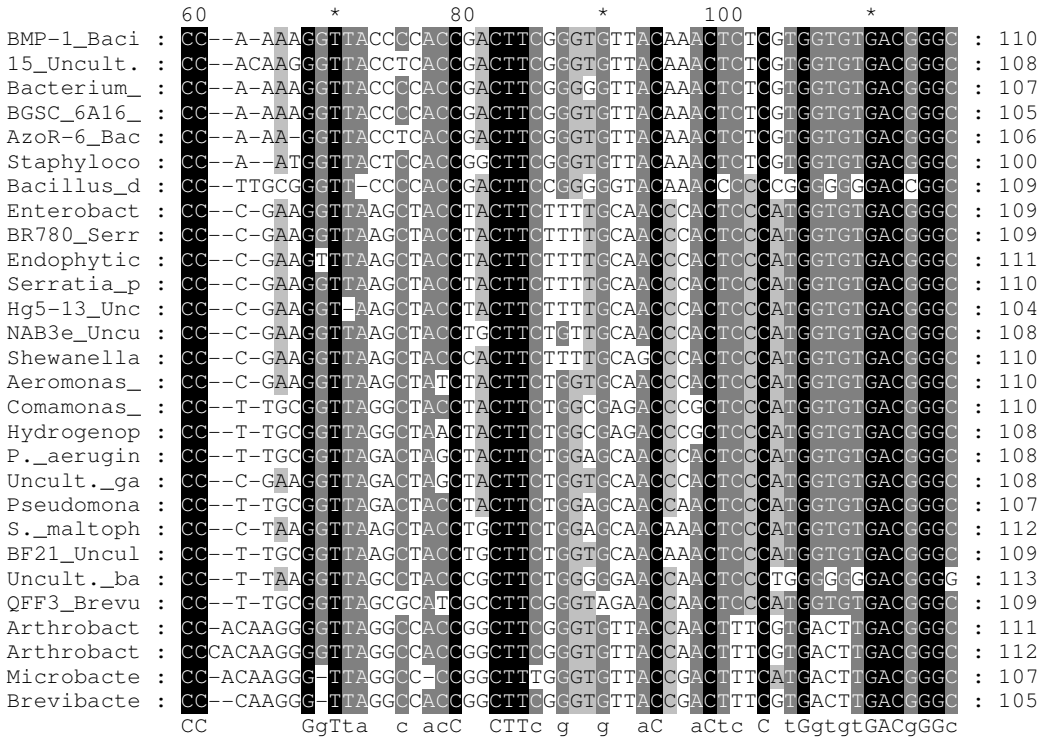


Figure 1-*Continued*

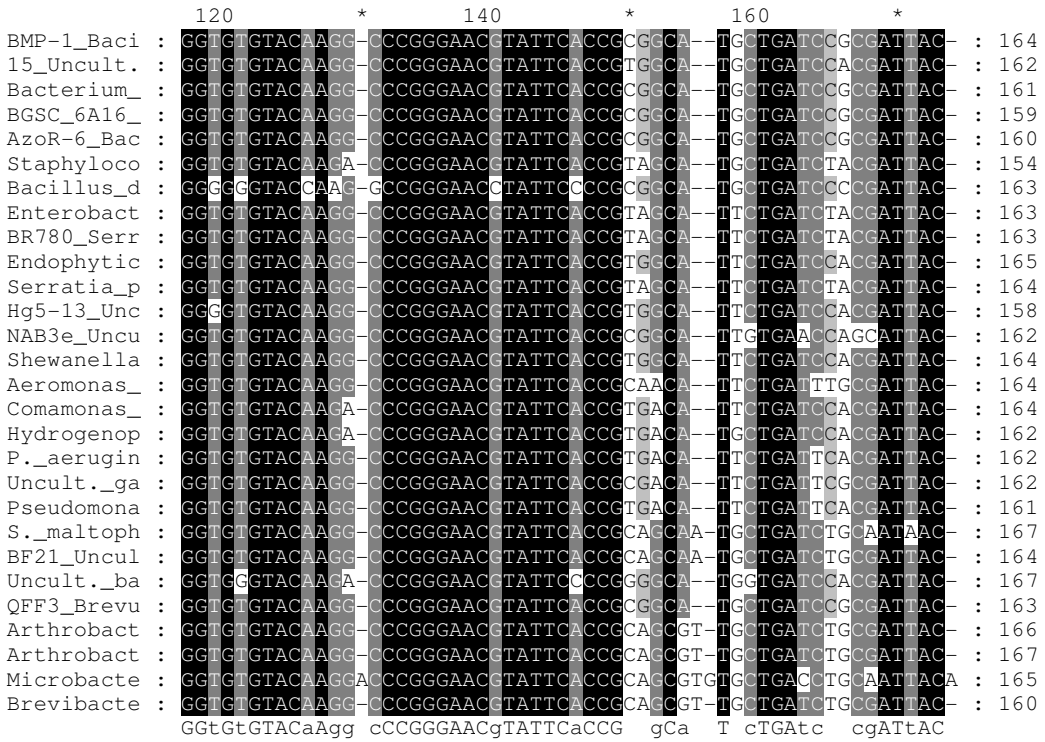


Figure 1-*Continued*

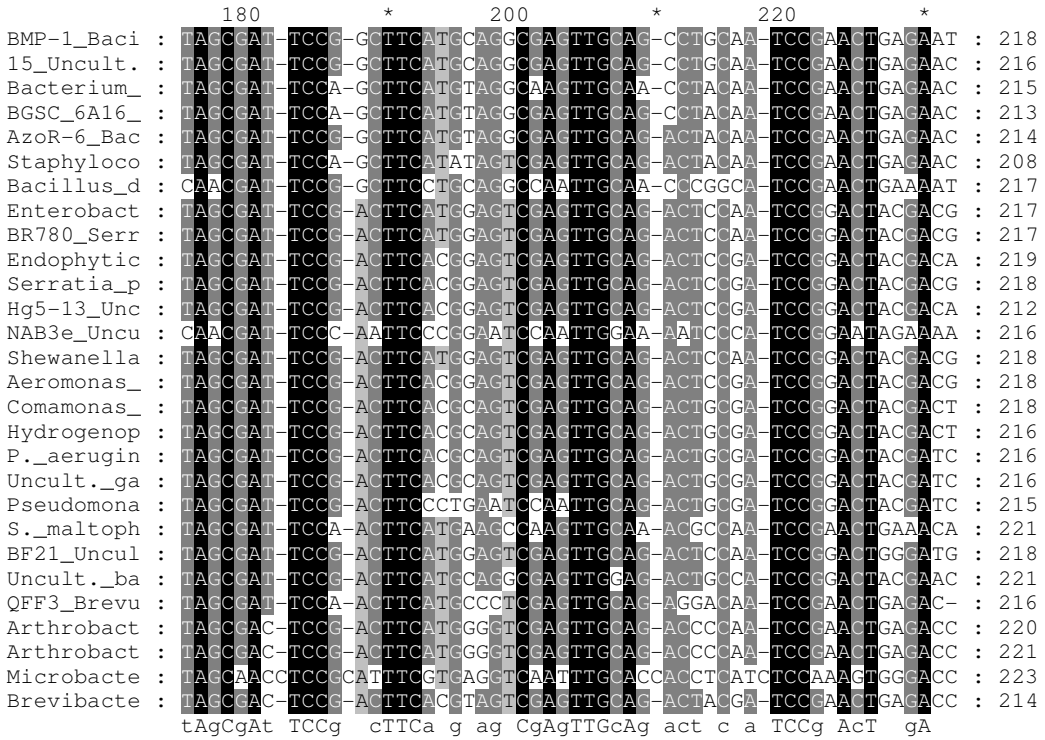


Figure 1-*Continued*

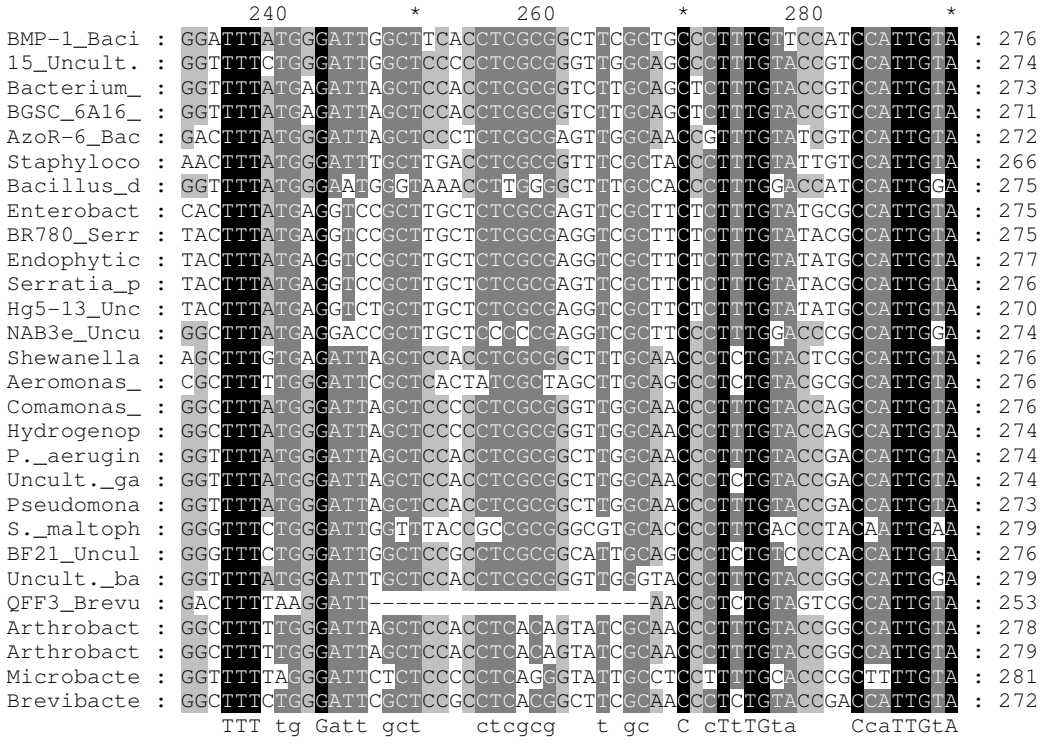


Figure 1-*Continued*

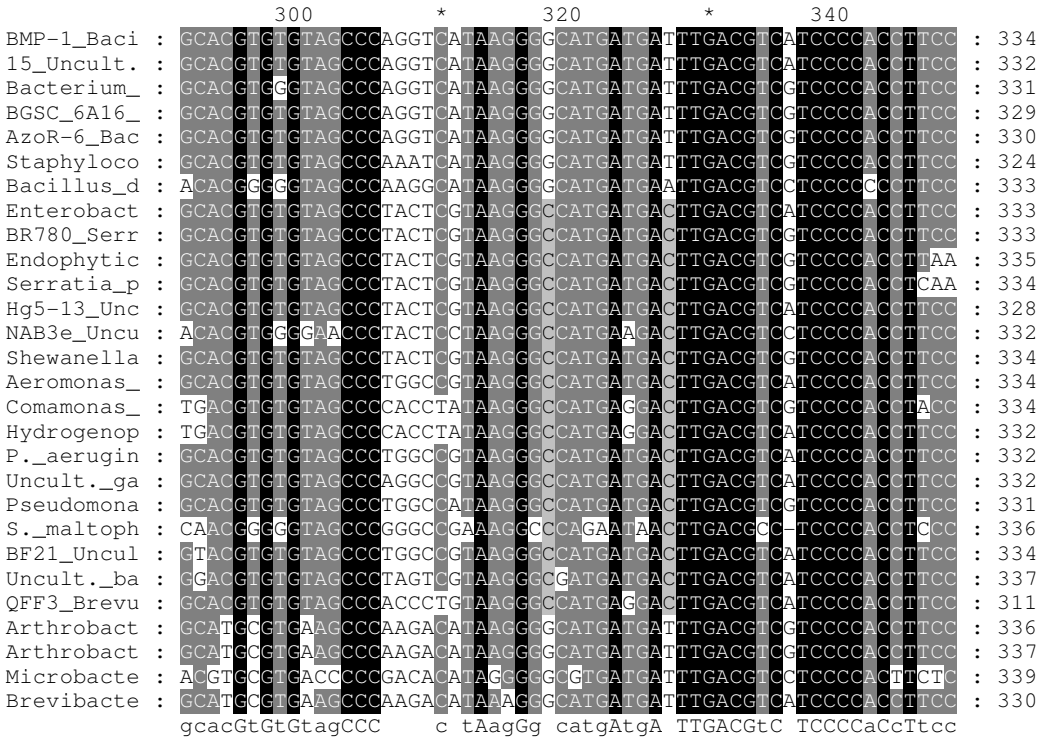


Figure 1-*Continued*

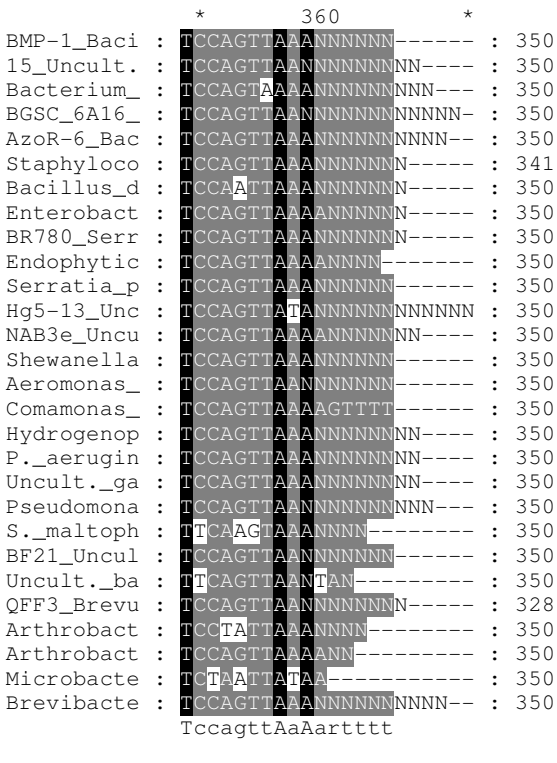


Figure 1-*Continued*

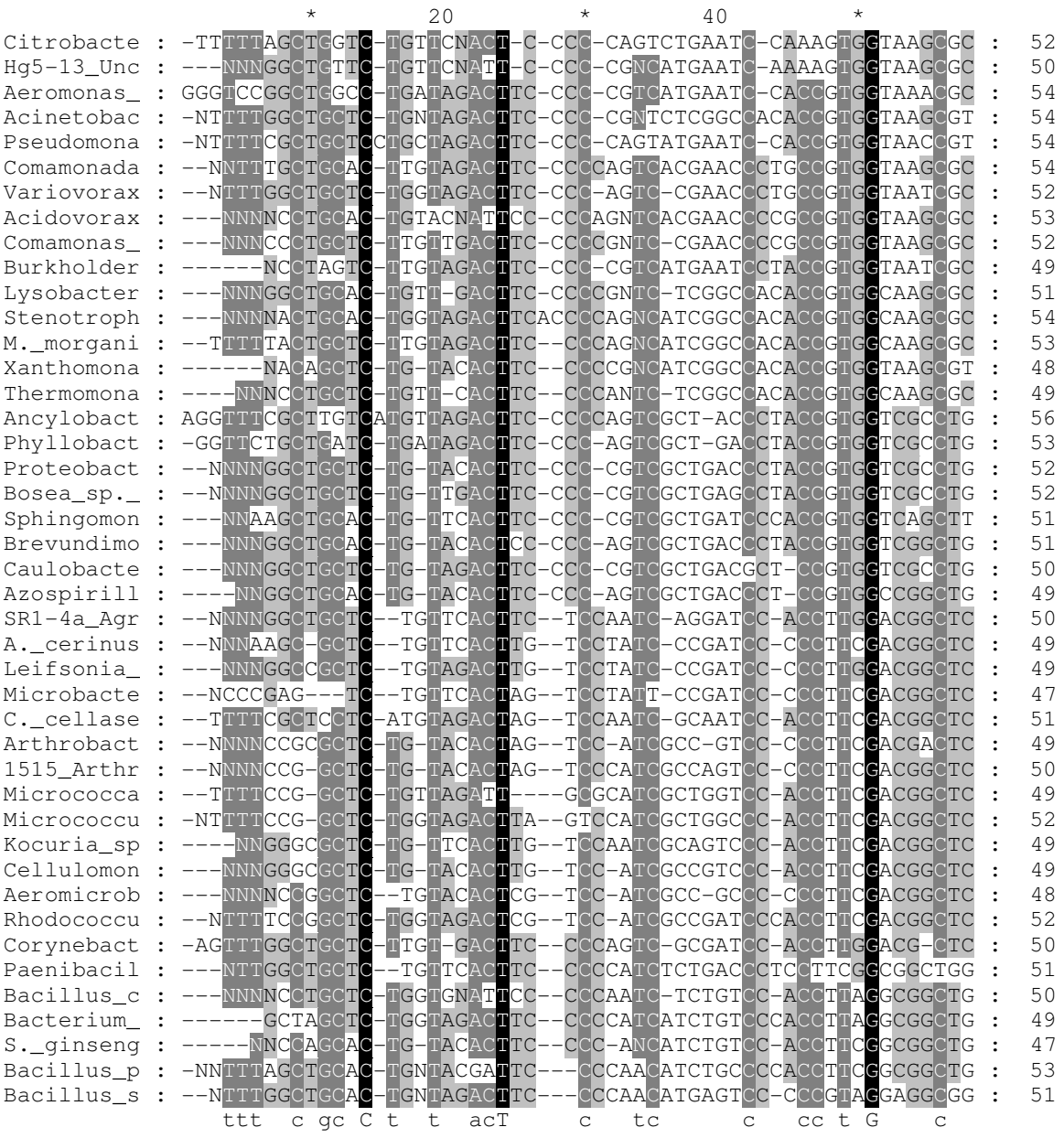


Figure 2 Alignment of the amino acid sequences of 43 waterborne bacterial species isolated from Site B in the Berg River. The alignment was carried out by the multiple alignment of ClustalX (1.81). Genedoc software was used for homology shading. The abbreviations of the isolates are given in the text. Gaps introduced into the alignment are indicated with dashes. Four shading levels were set: black for 100% identity and grey for 70% identity.

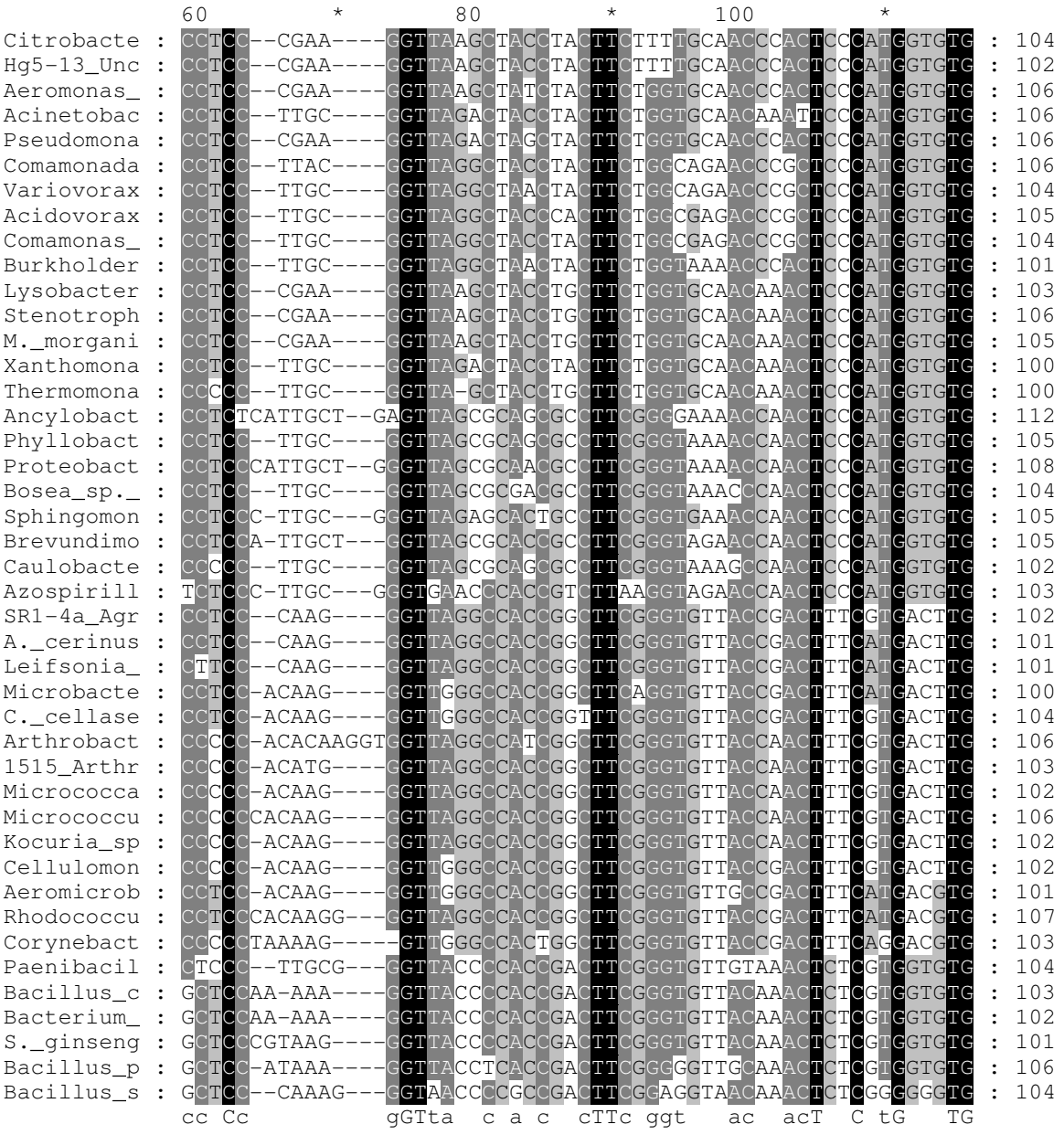


Figure 2-*Continued*

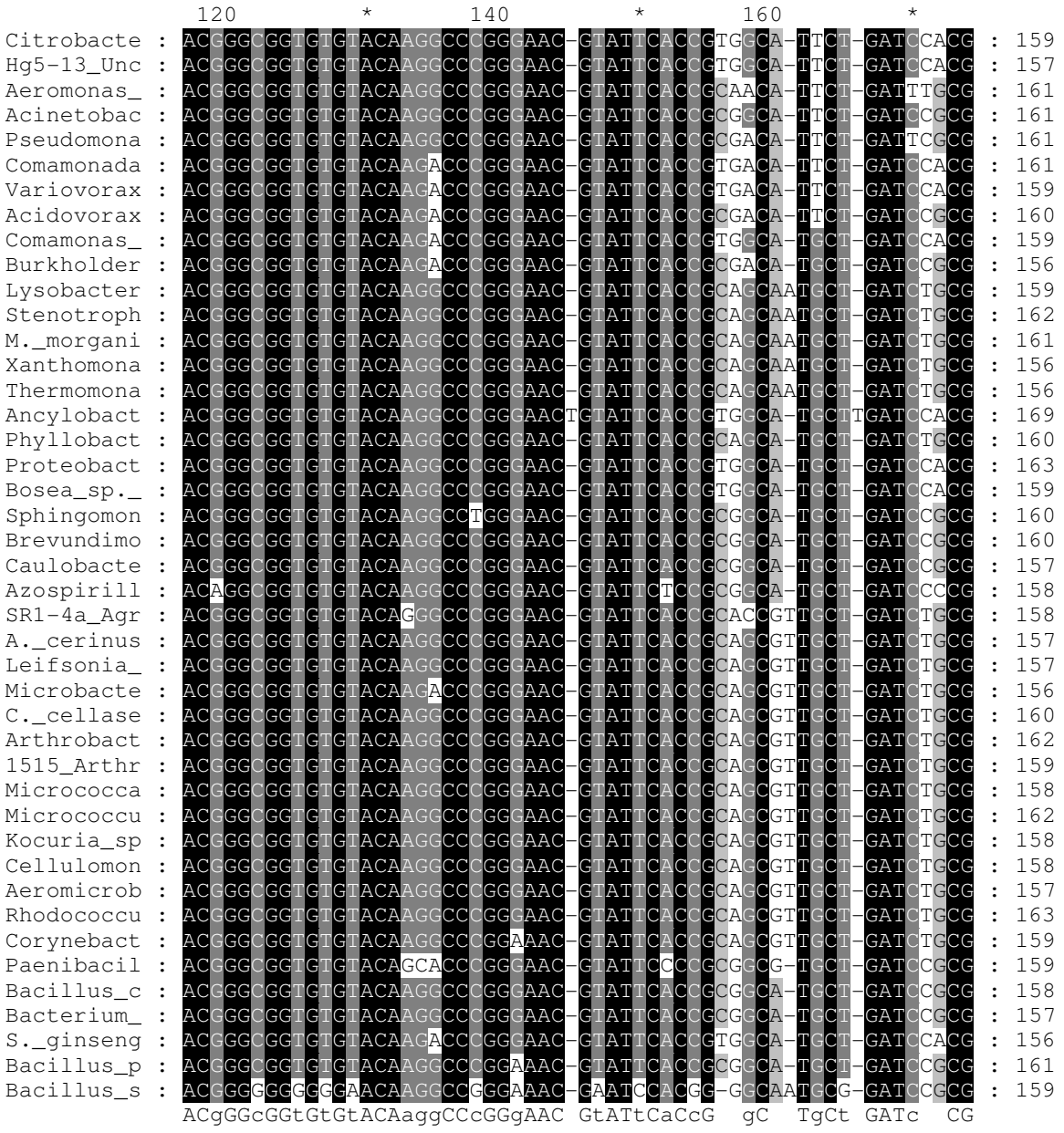


Figure 2-*Continued*

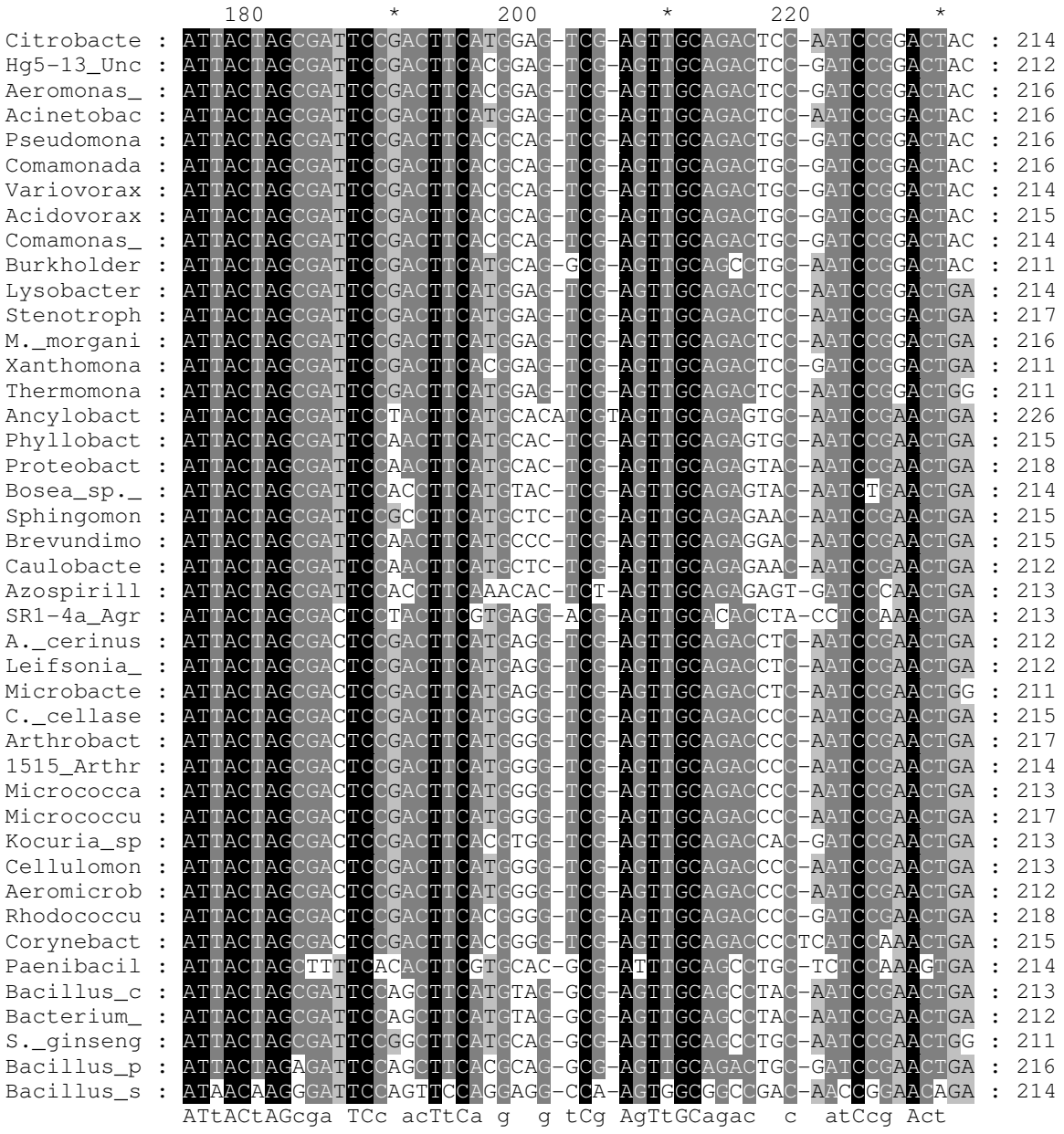


Figure 2-*Continued*

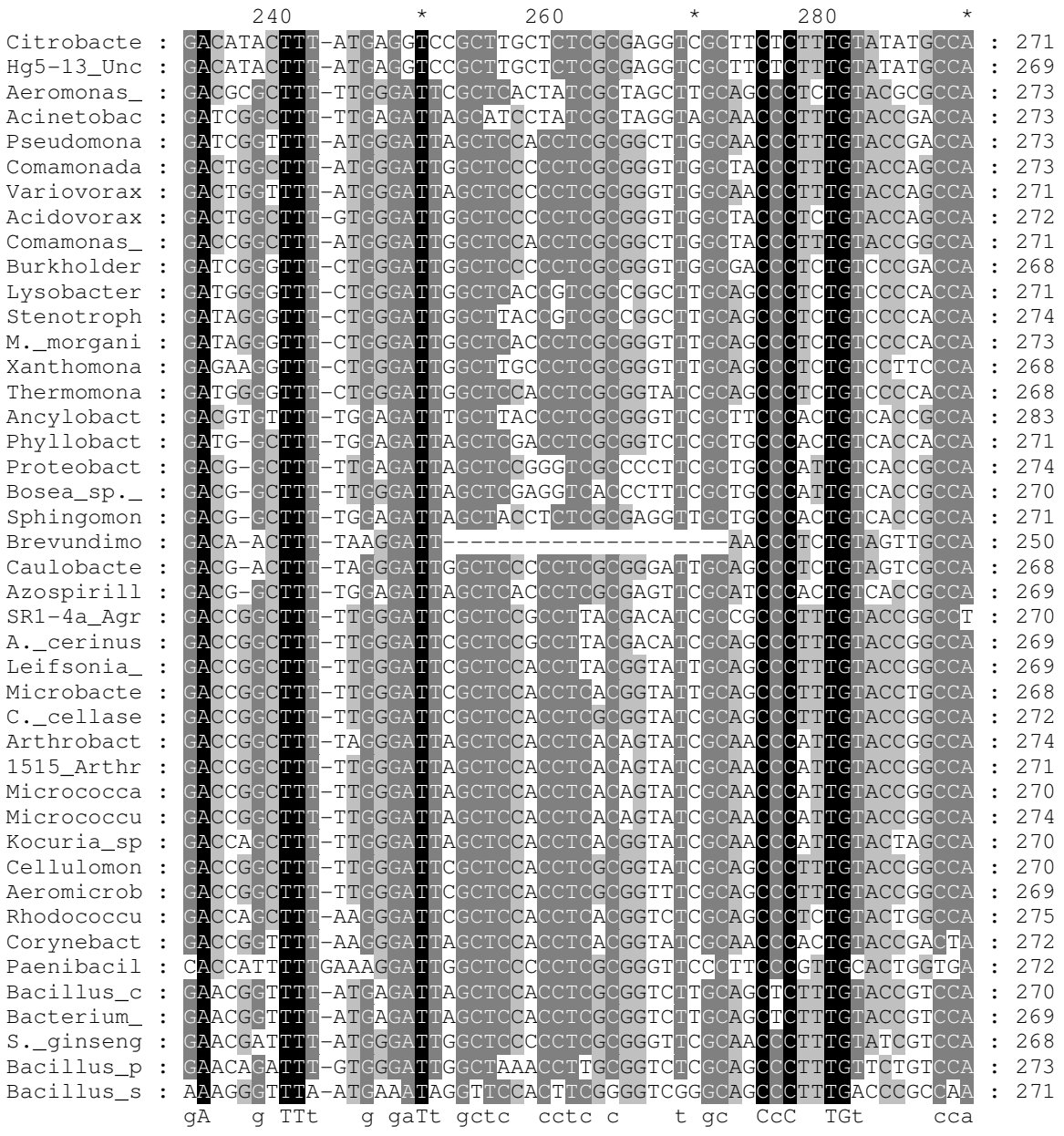


Figure 2-*Continued*

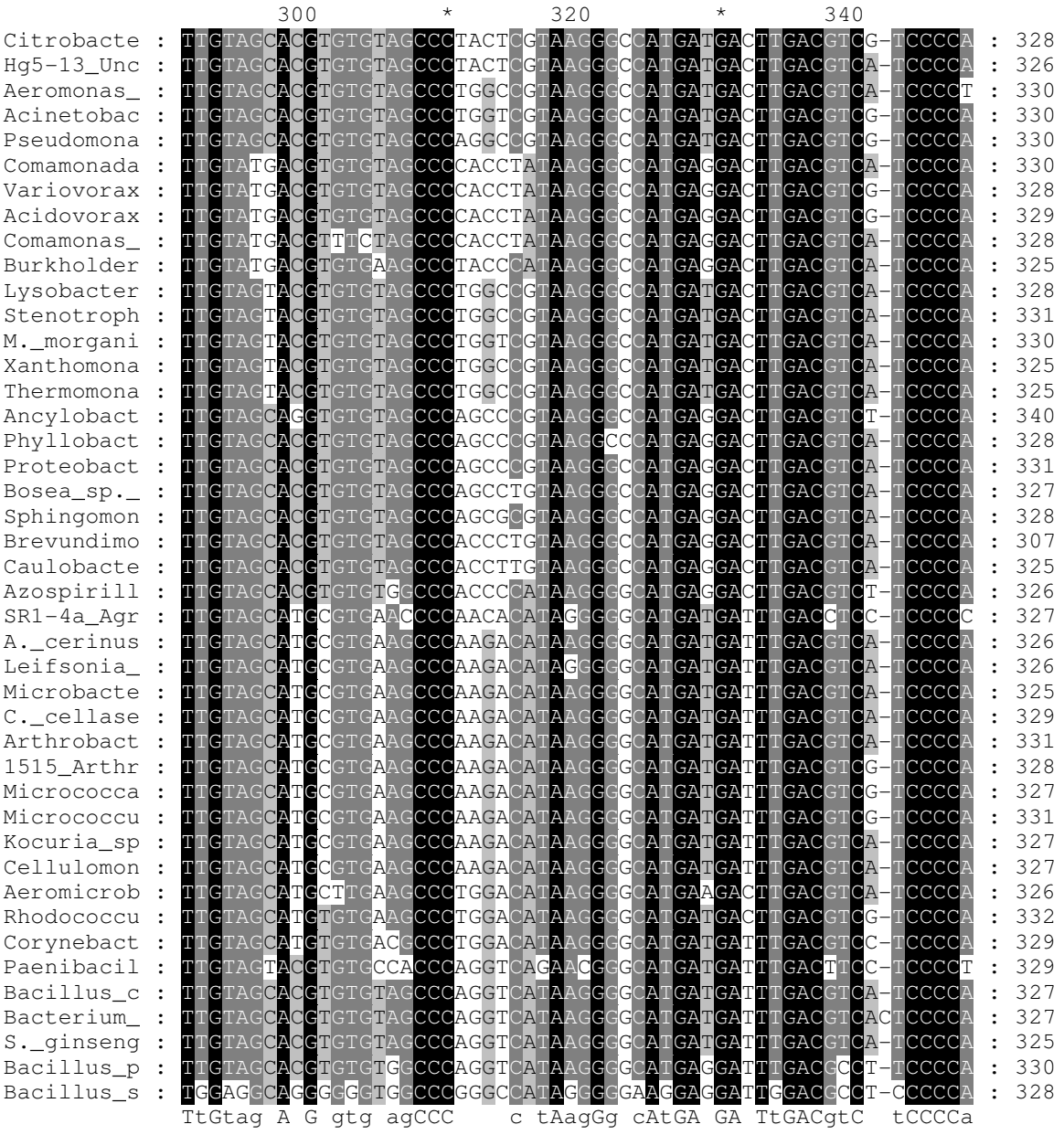


Figure 2-*Continued*

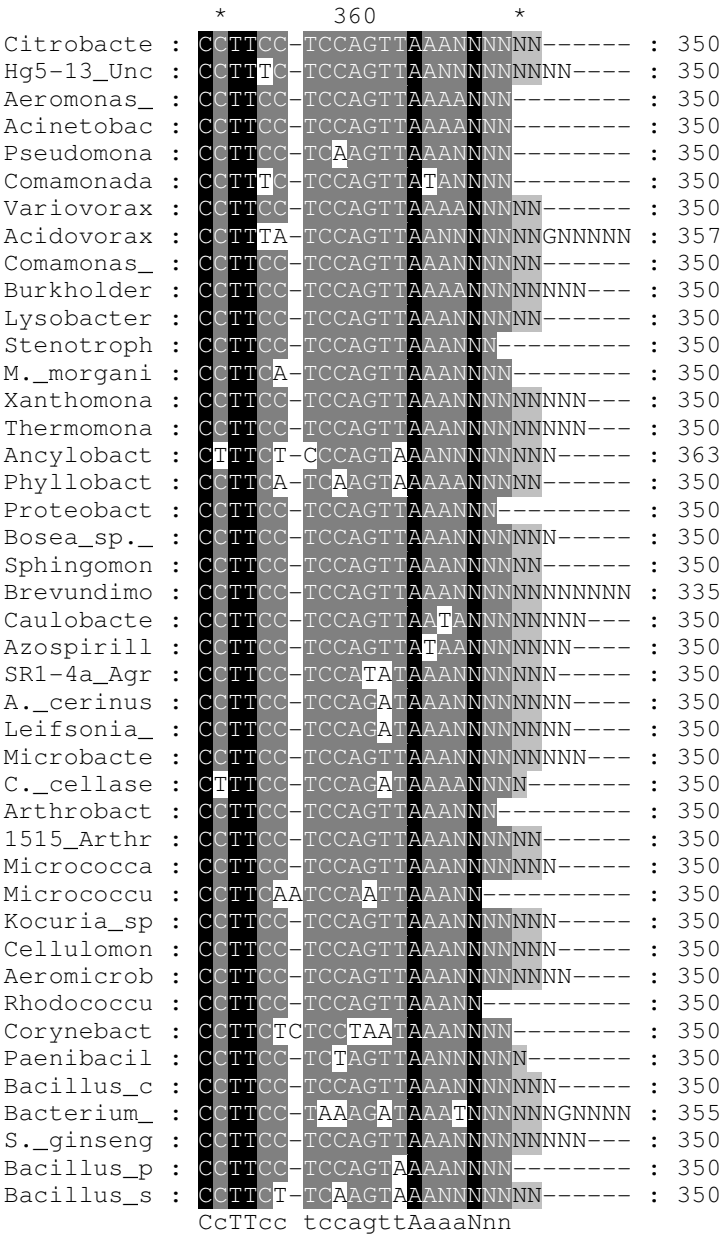


Figure 2-*Continued*

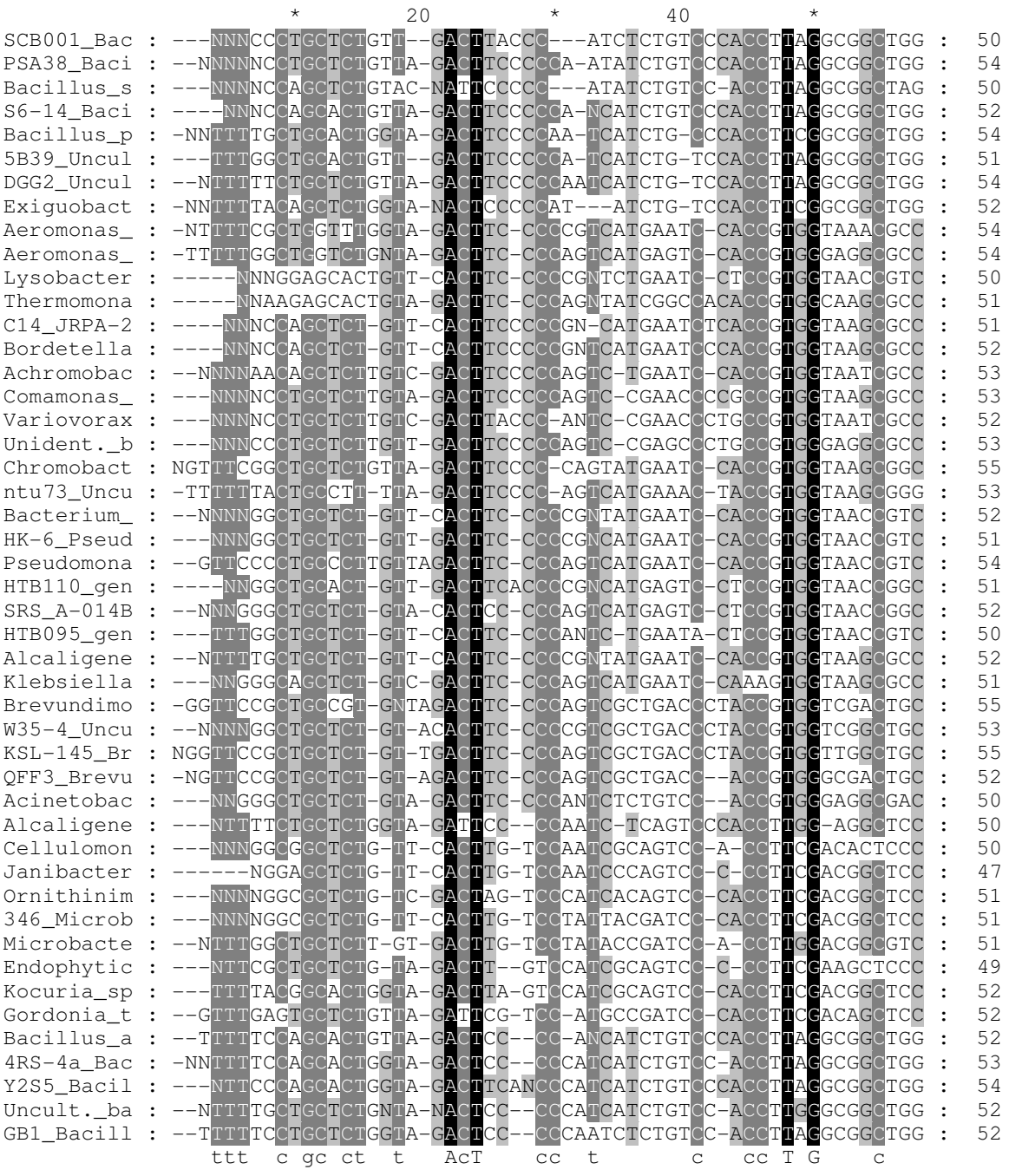


Figure 3 Alignment of the amino acid sequences of 47 waterborne bacterial species isolated from Site A in the Plankenburg River. The alignment was carried out by the multiple alignment of ClustalX (1.81). Genedoc software was used for homology shading. The abbreviations of the isolates are given in the text. Gaps introduced into the alignment are indicated with dashes. Four shading levels were set: black for 100% identity and grey for 70% identity.

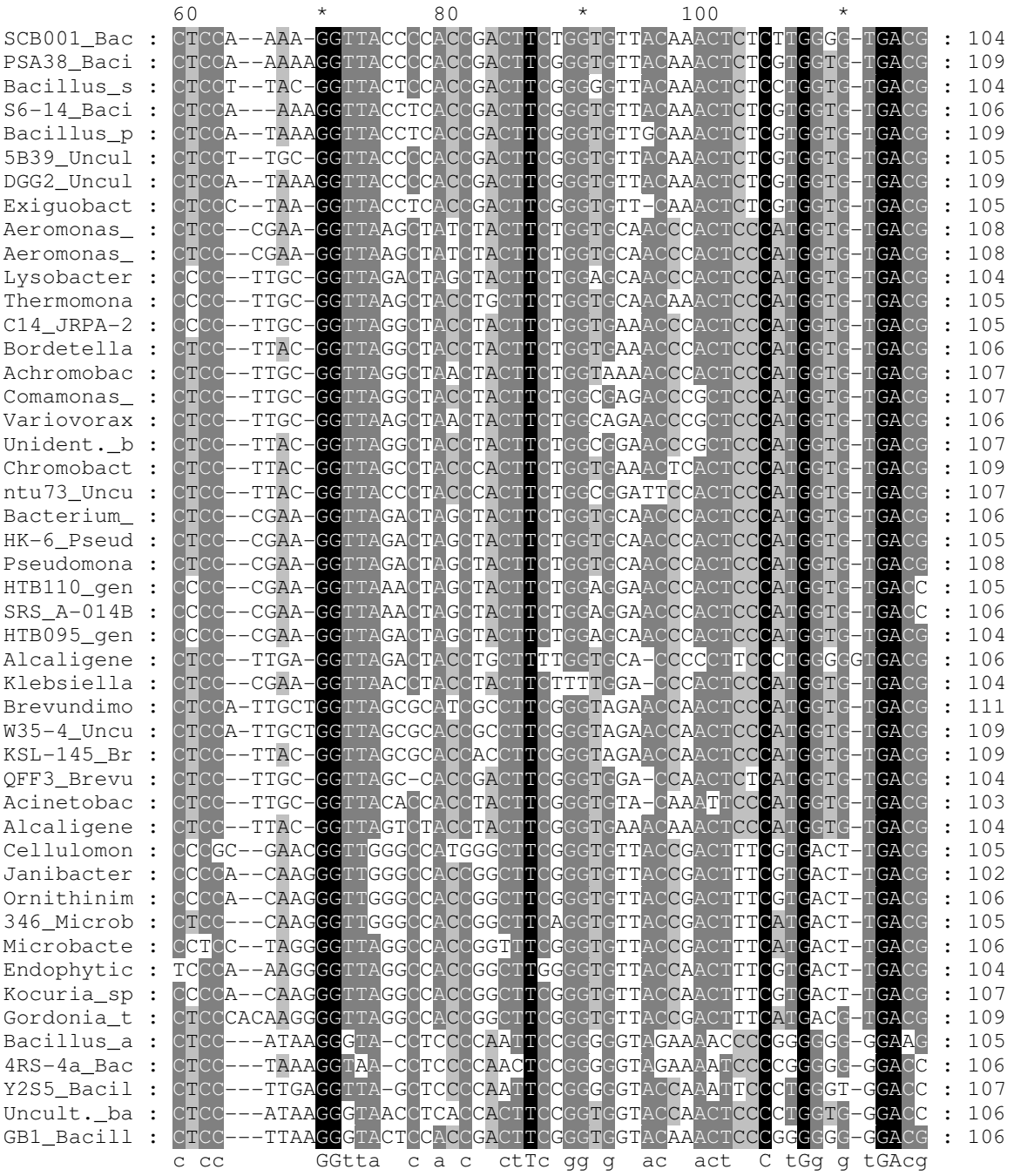
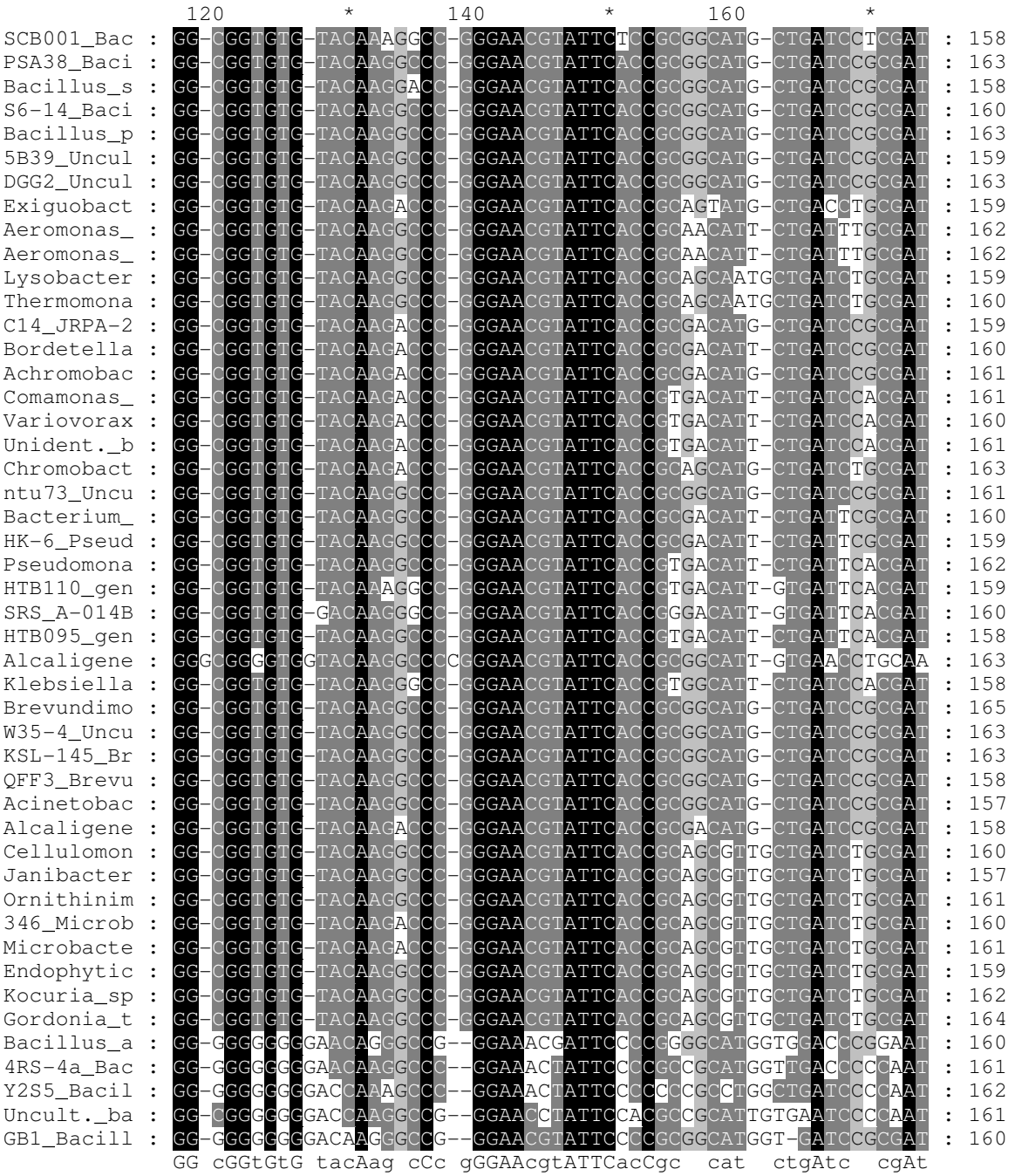


Figure 3-*Continued*



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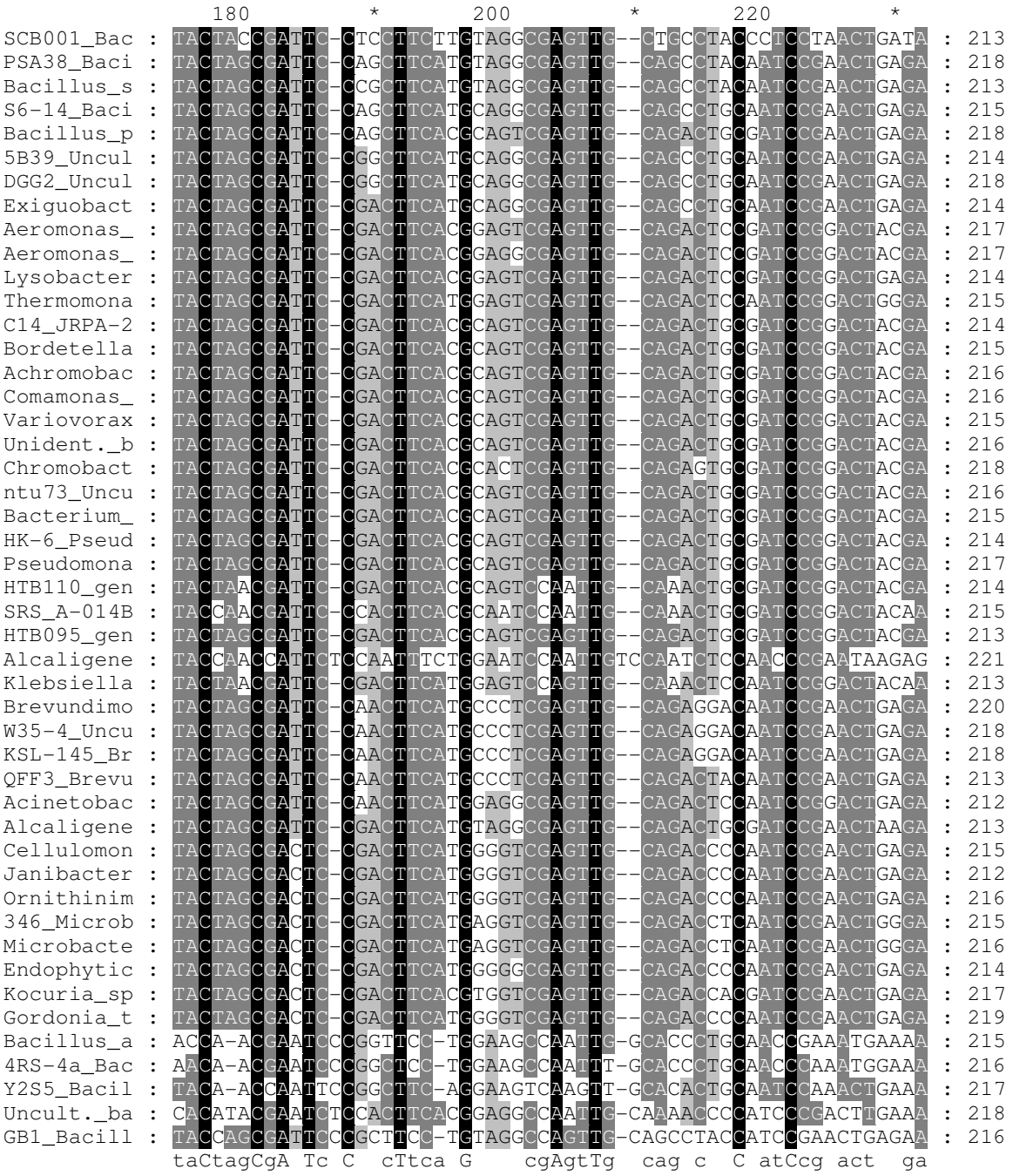

Figure 3-*Continued*

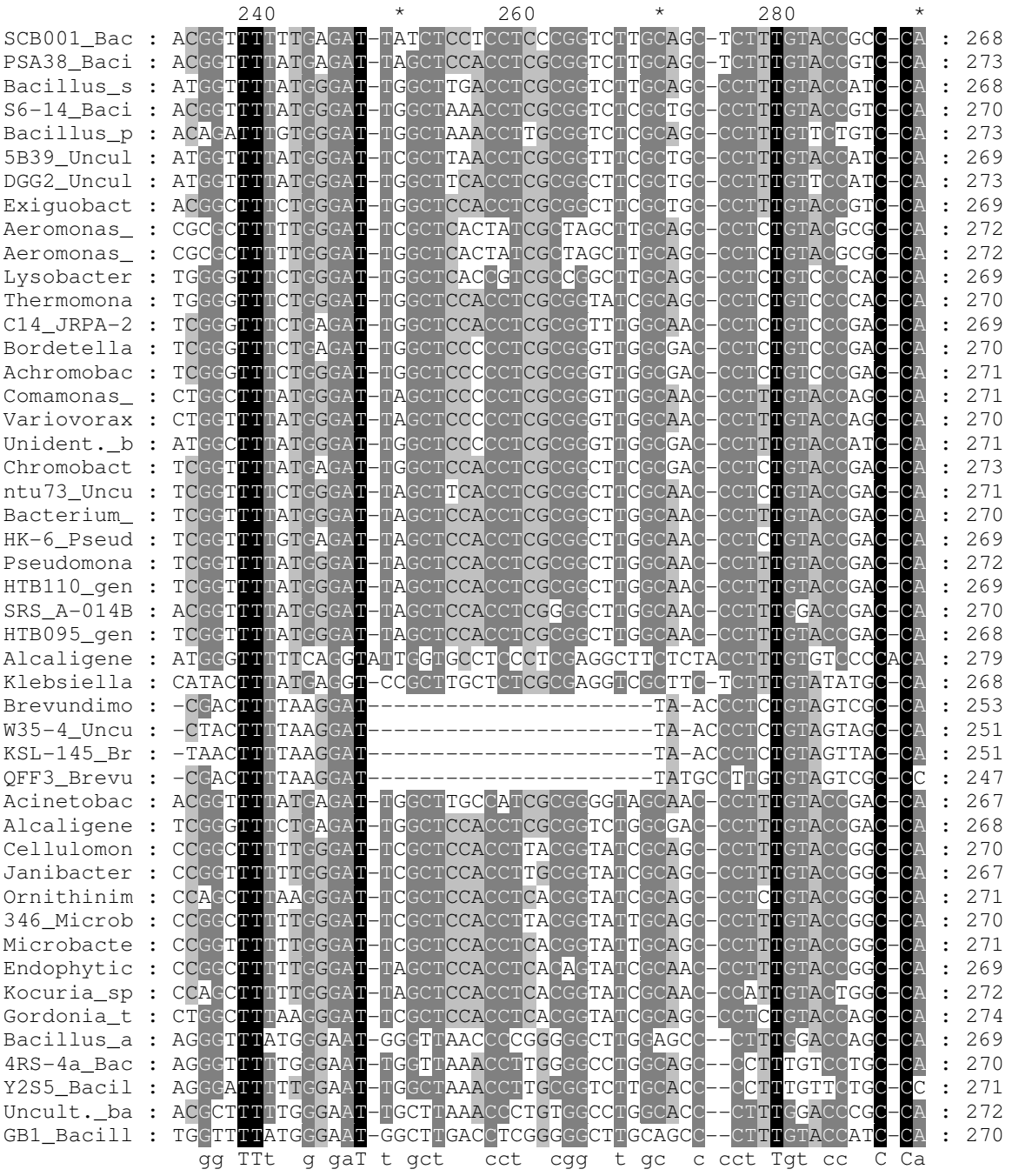

Figure 3-*Continued*

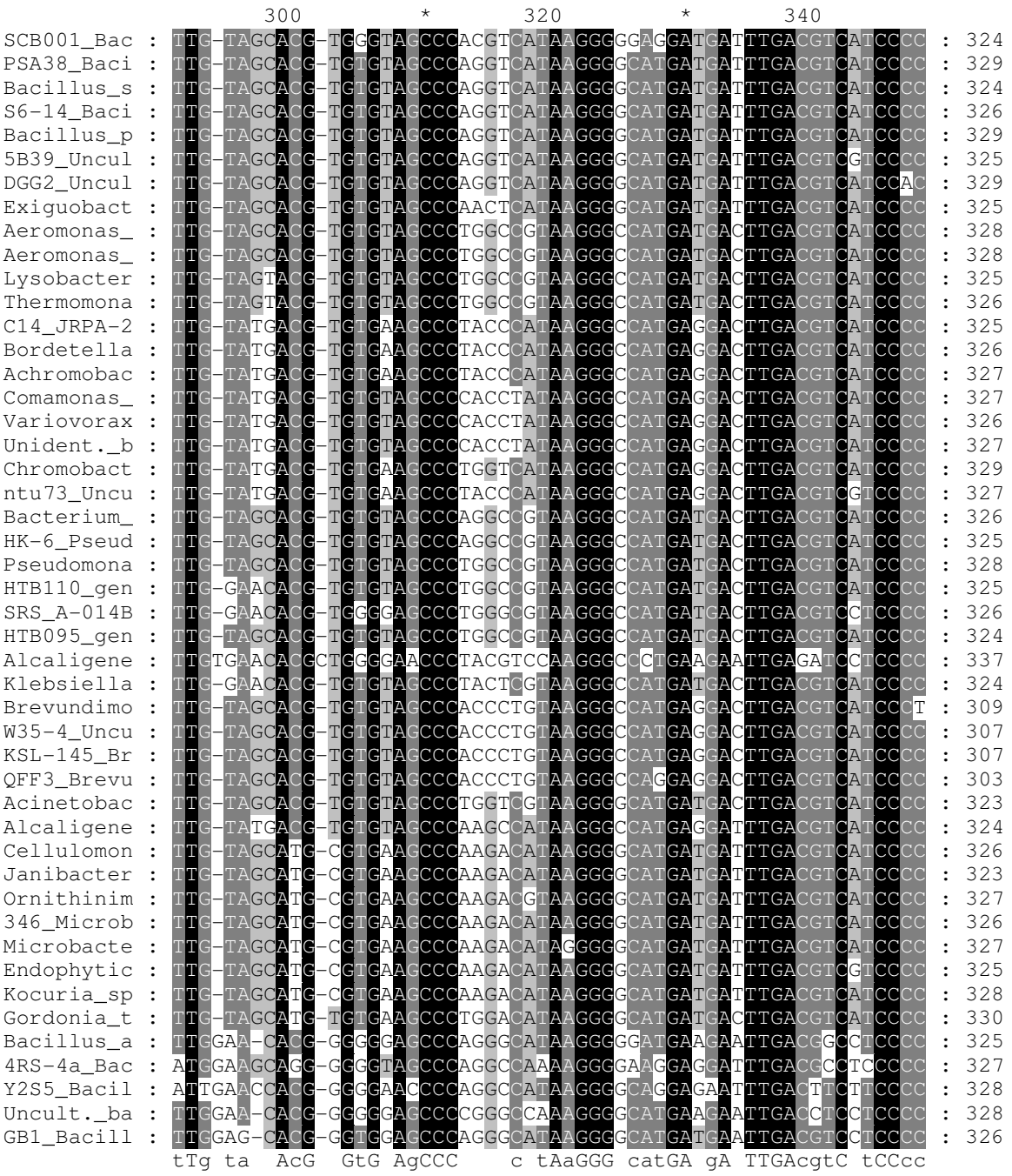

Figure 3-*Continued*

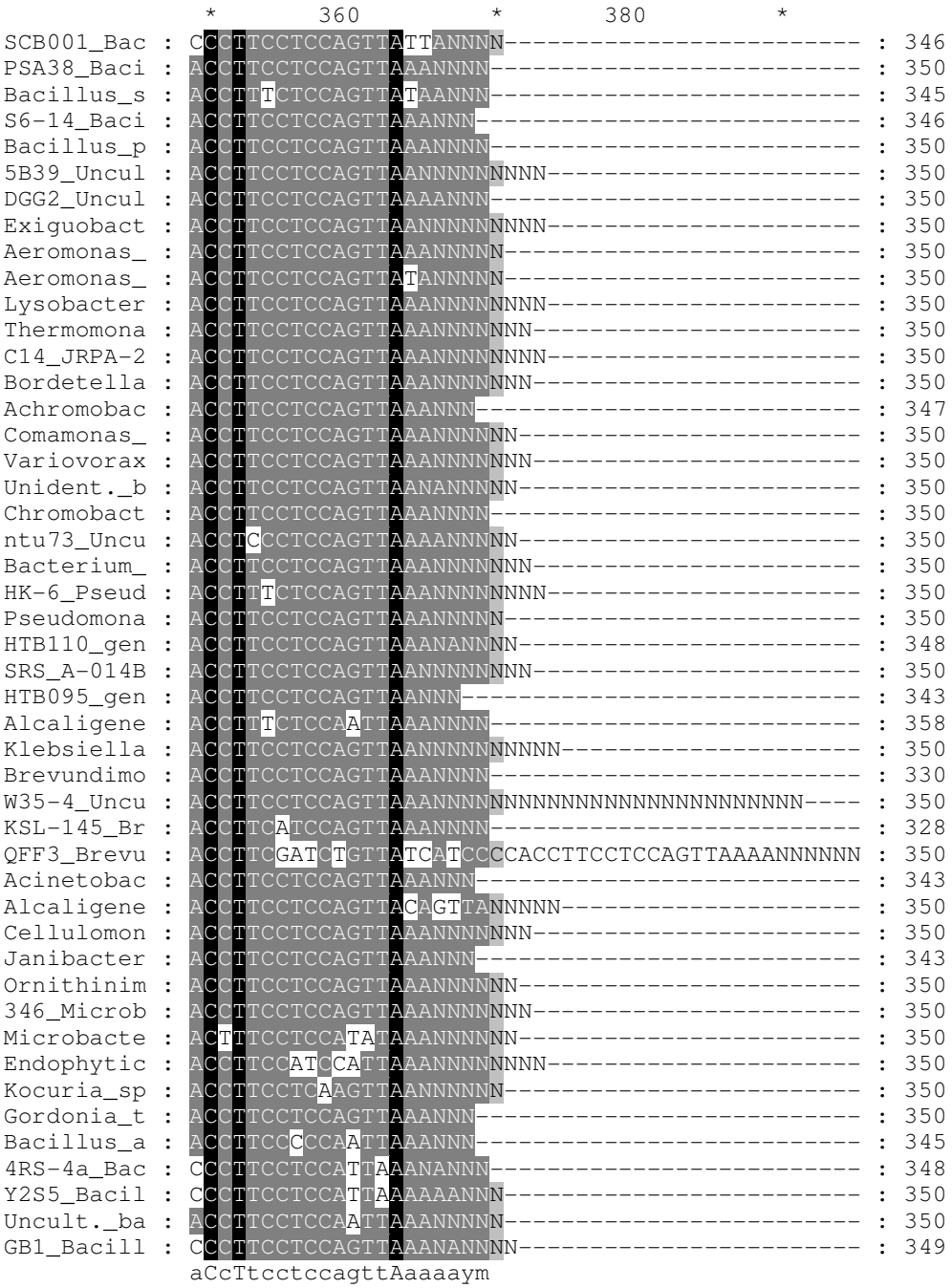

Figure 3-*Continued*

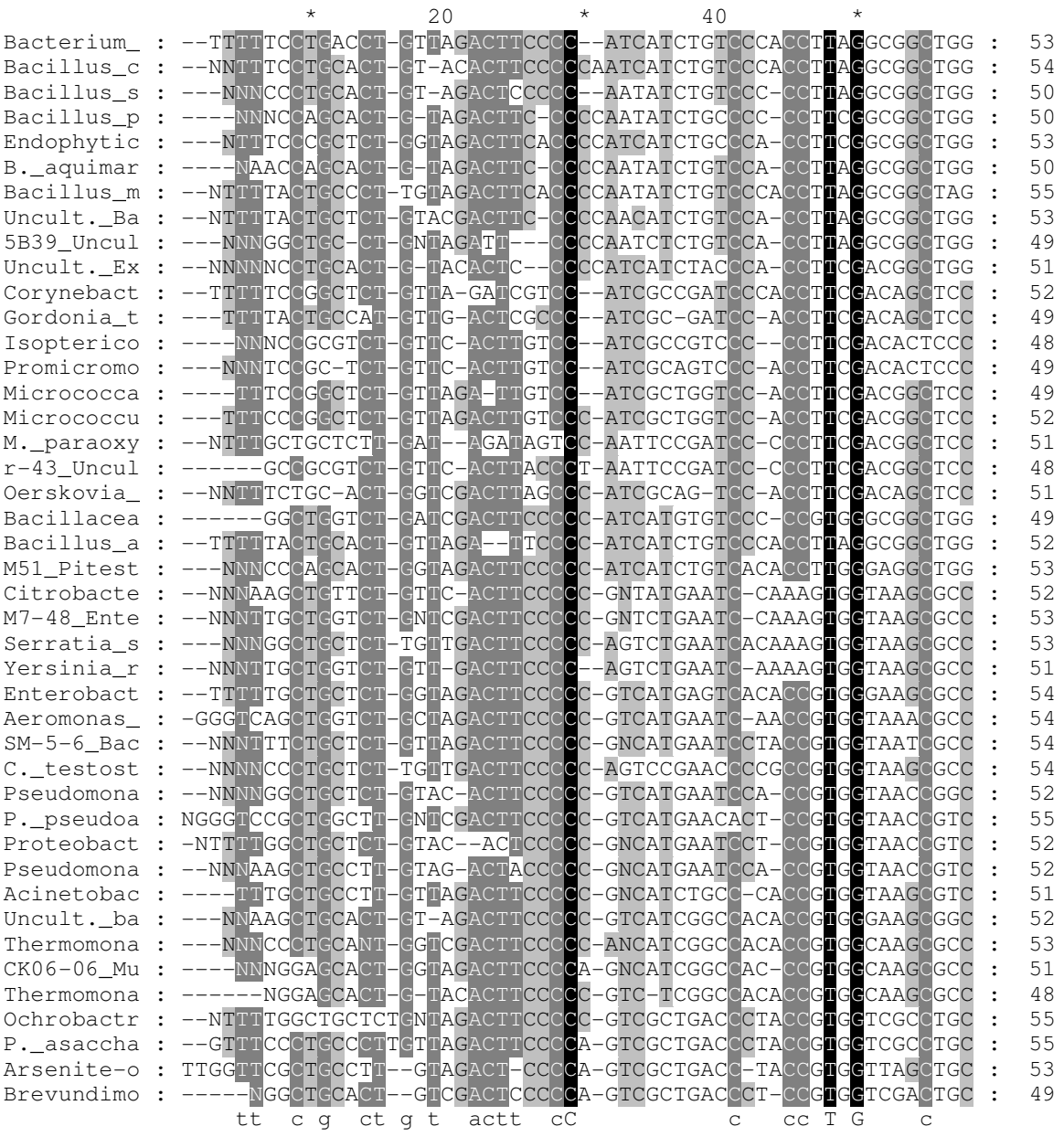

Figure 4 Alignment of the amino acid sequences of 43 waterborne bacterial species isolated from Site B in the Plankenburg River. The alignment was carried out by the multiple alignment of ClustalX (1.81). Genedoc software was used for homology shading. The abbreviations of the isolates are given in the text. Gaps introduced into the alignment are indicated with dashes. Four shading levels were set: black for 100% identity and grey for 70% identity.

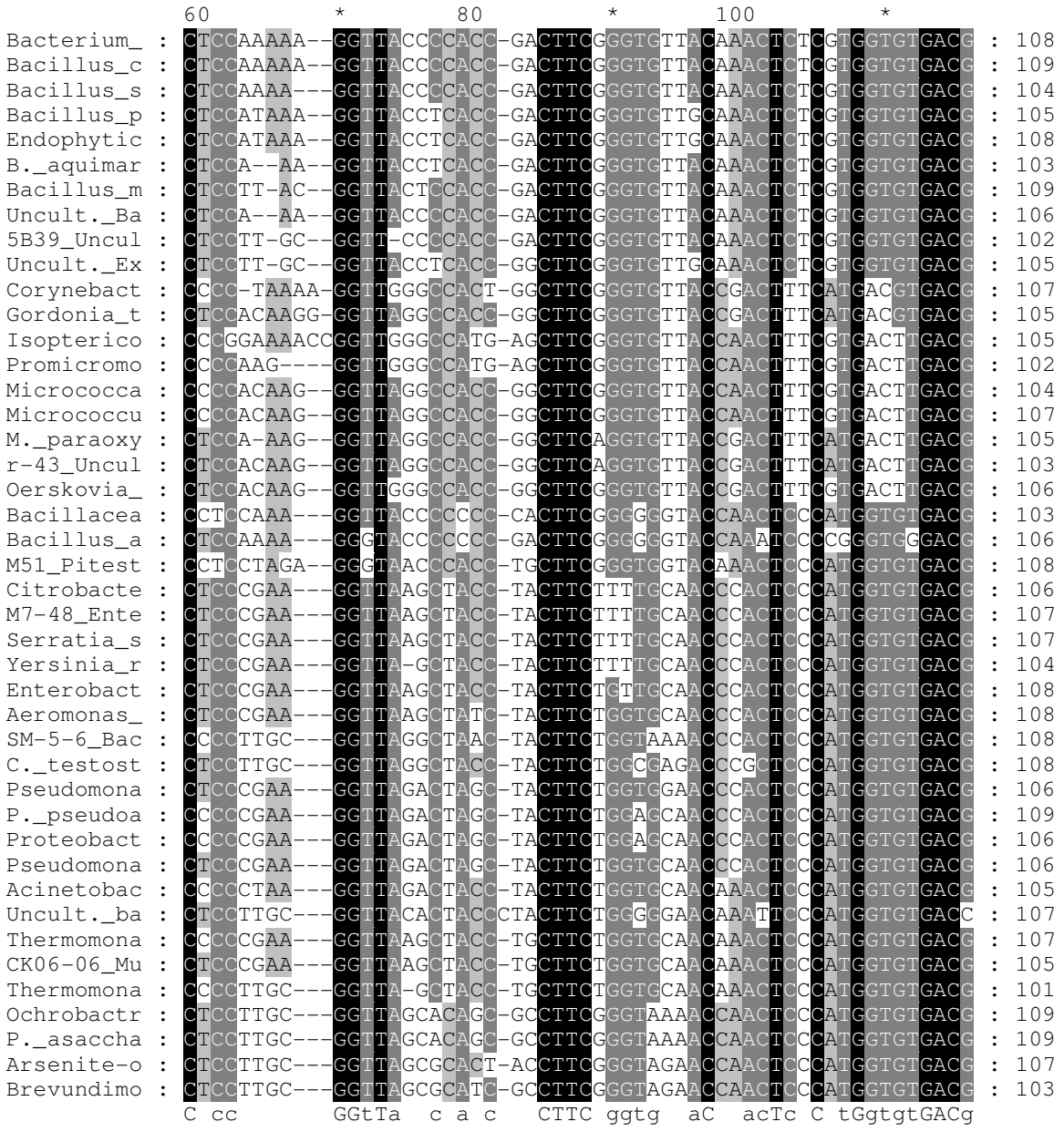

Figure 4-*Continued*

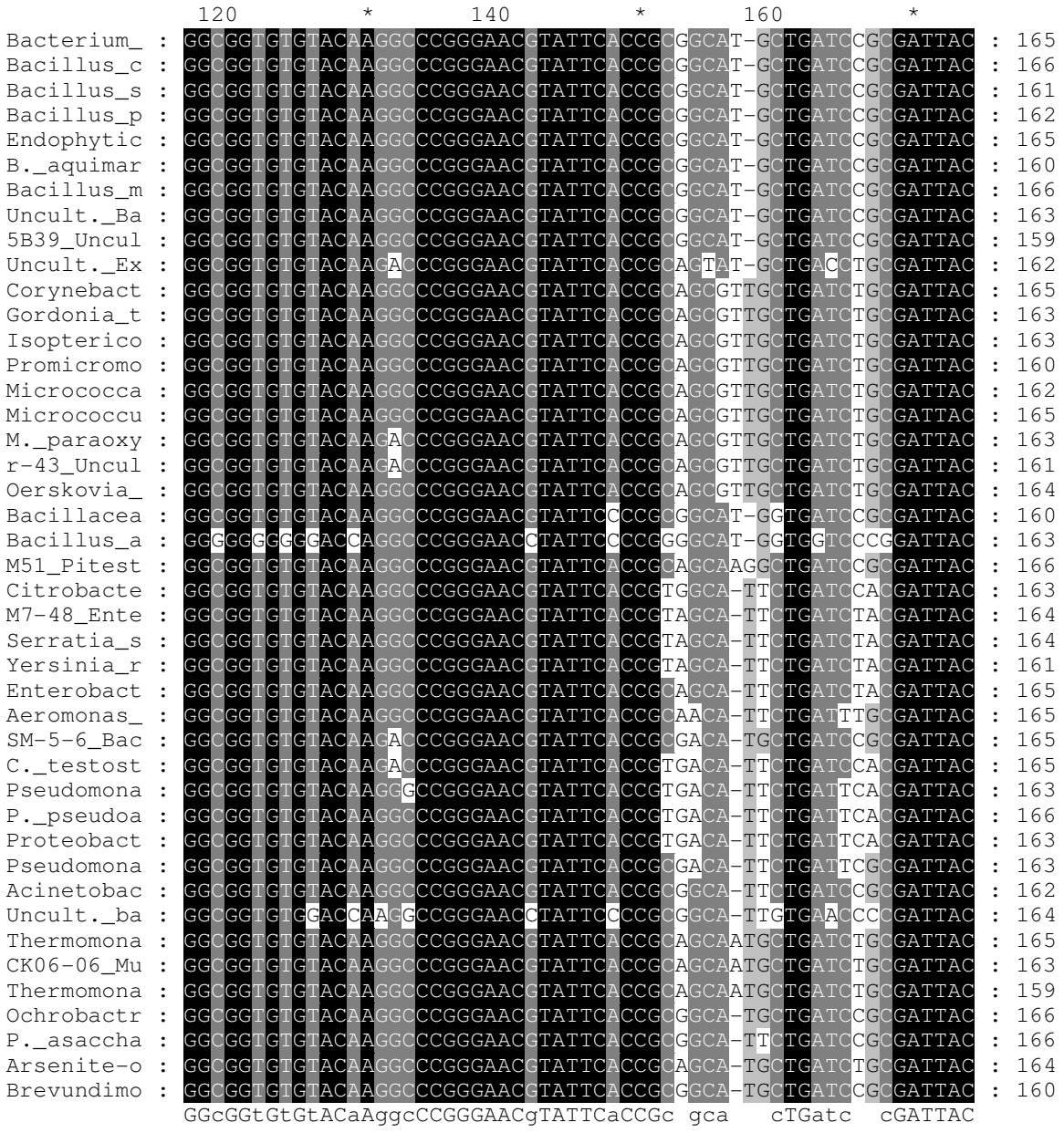

Figure 4-*Continued*

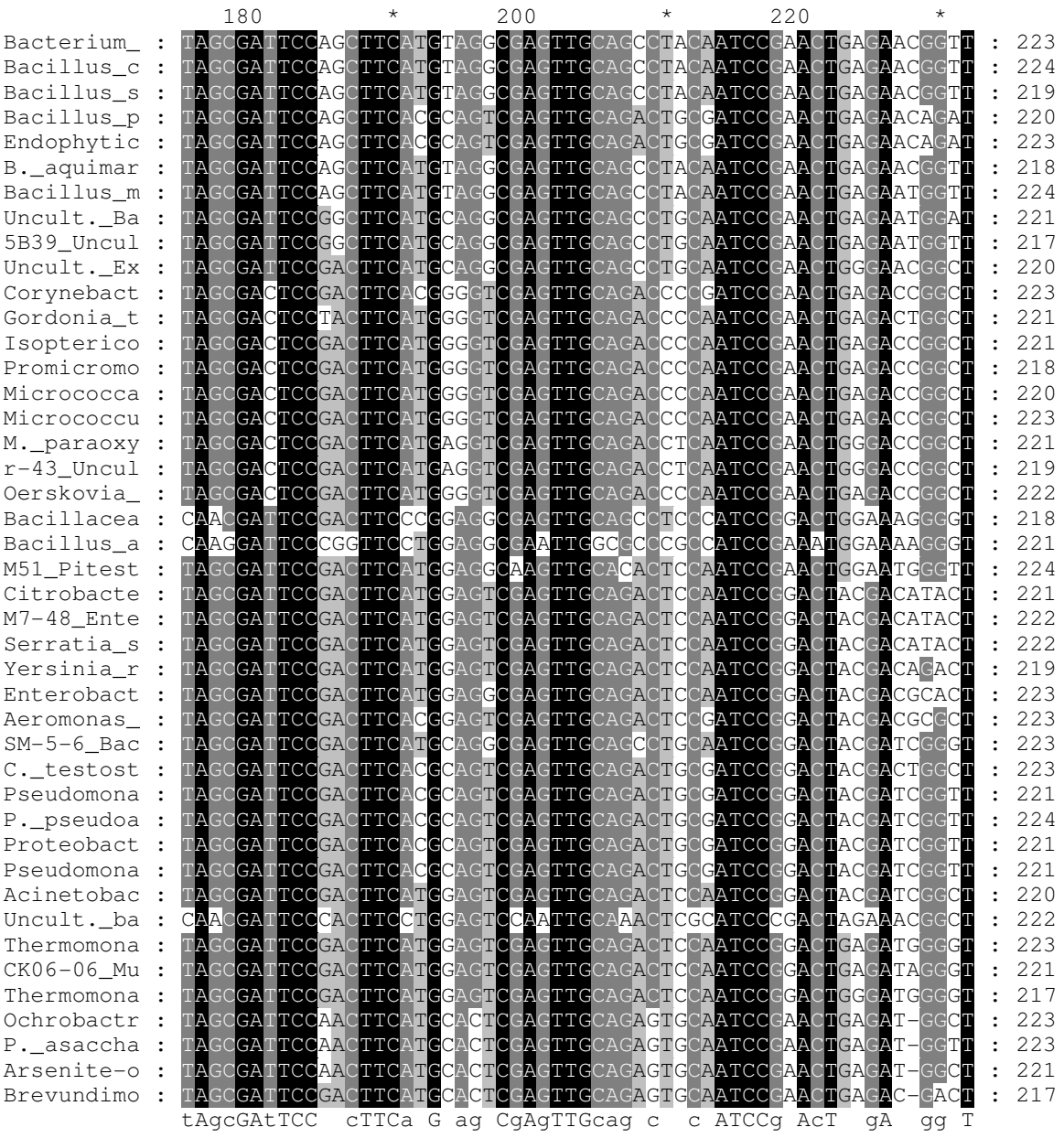

Figure 4-*Continued*

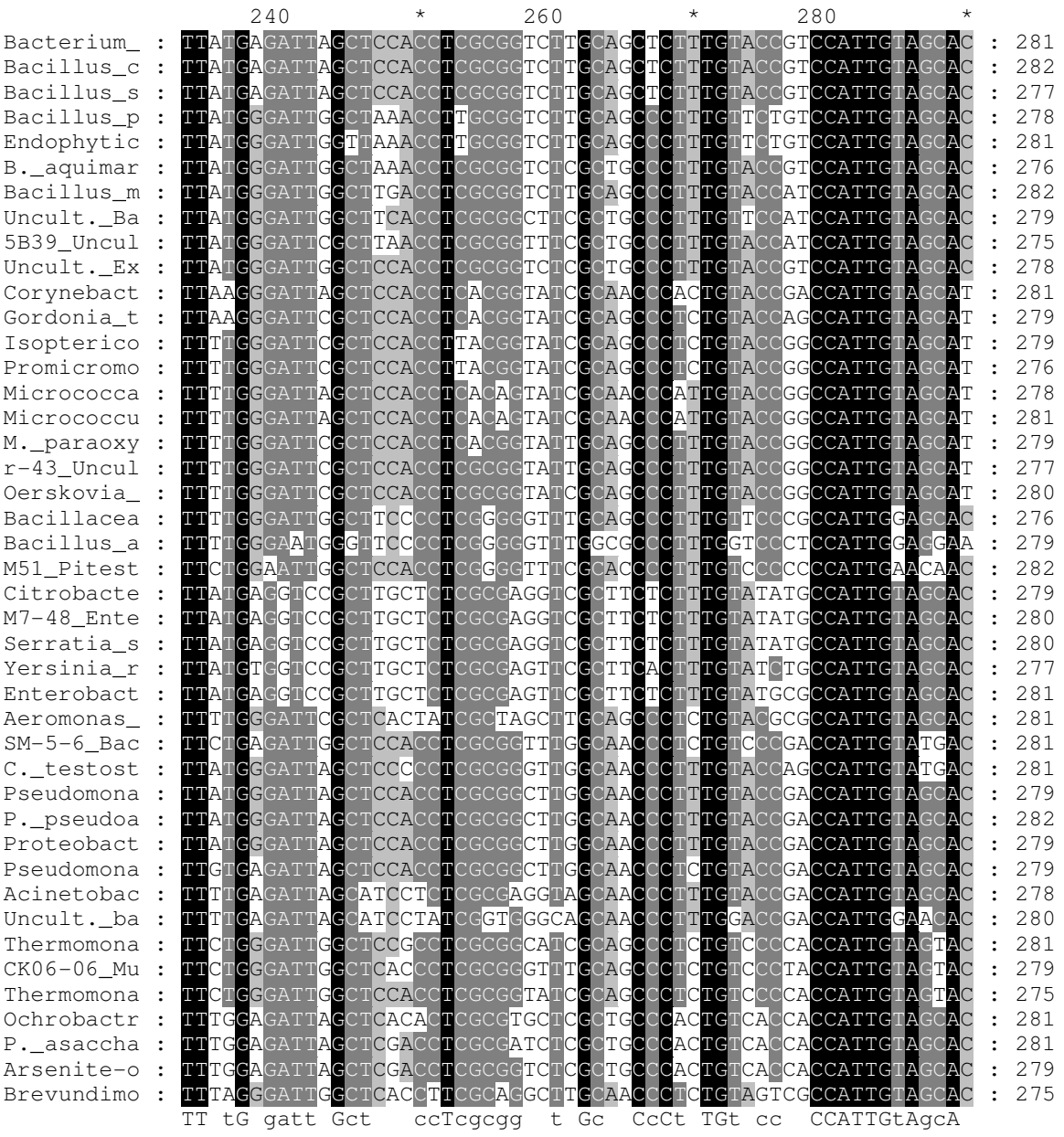

Figure 4-*Continued*

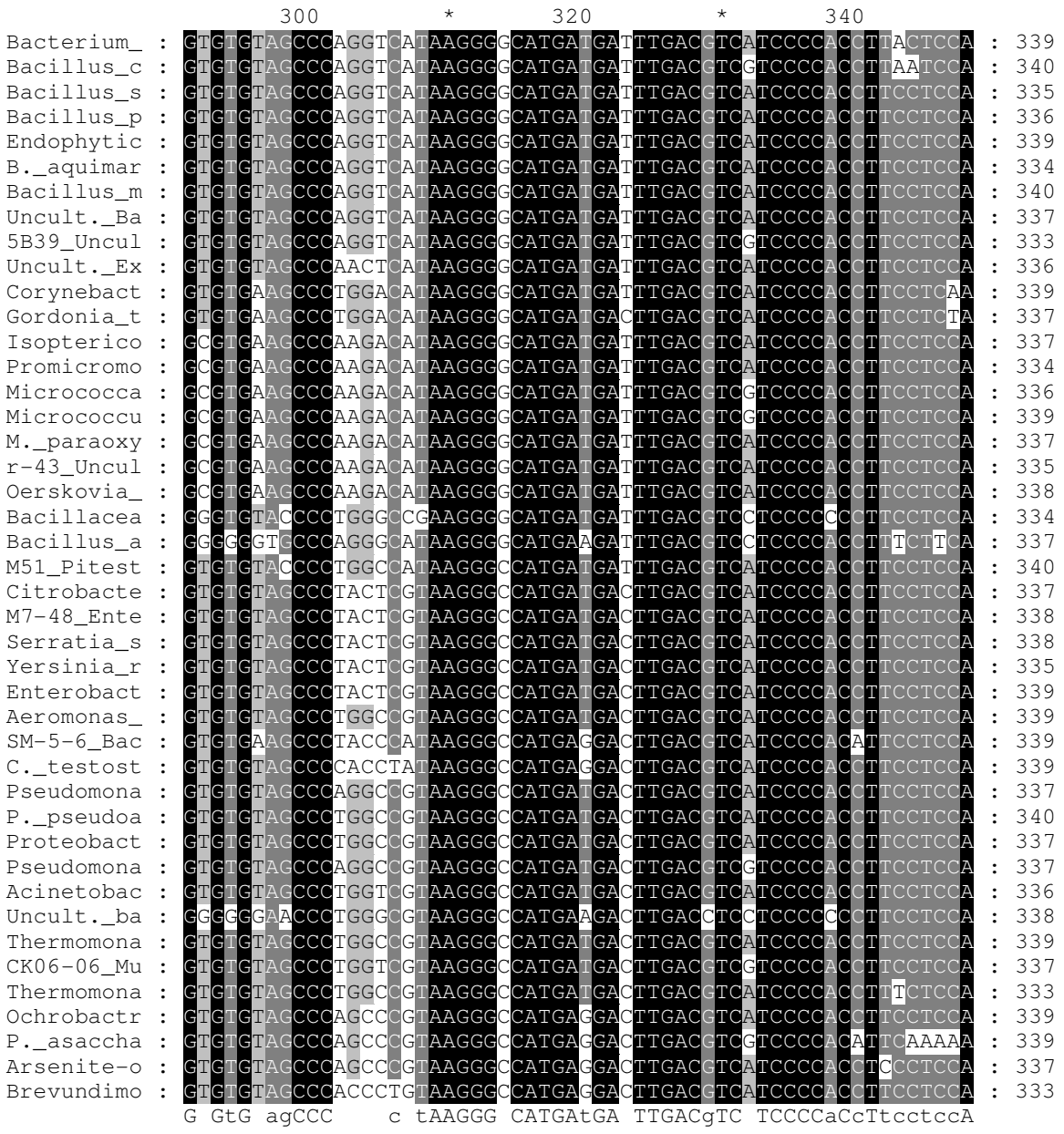

Figure 4-*Continued*

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                *       360
Bacterium_ : GTTAAANNNNN------ : 350
Bacillus_c : GTTAAANNNN------- : 350
Bacillus_s : GTTAAANNNNNNNN-- : 350
Bacillus_p : GTTAAANNNNNNN--- : 350
Endophytic : GTTAANNNNNN------ : 350
B._aquimar : GTTAANNNNNNNNNN- : 350
Bacillus_m : GTTAAAANNN------- : 350
Uncult._Ba : GTTAAANNNNNN---- : 350
5B39_Uncul : GTTAANNNNNNNNNNN : 350
Uncult._Ex : GTTAAANNNNNNN--- : 350
Corynebact : GTTAAANNNNN------ : 350
Gordonia_t : GTTAAANNNNNN---- : 350
Isopterico : GTTAANNNNNNN---- : 350
Promicromo : GTTAANNNNNNNNN- : 350
Micrococca : GTTAAANNNNNNN--- : 350
Micrococcu : GTTAAANNNNN------ : 350
M._paraoxy : GTTAANNNNNNN---- : 350
r-43_Uncul : GTTAAANNNNNNNN-- : 350
Oerskovia_ : TATATANNNNN----- : 350
Bacillacea : ATTAAGNGGGNGGNNN- : 350
Bacillus_a : GTTAAANNNNNN---- : 350
M51_Pitest : GTAAANNNNN------- : 350
Citrobacte : GTTAAANNNNNN---- : 350
M7-48_Ente : GTTAAANNNNN----- : 350
Serratia_s : GTTAAANNNNN----- : 350
Yersinia_r : GTTAAANNNNNNNN-- : 350
Enterobact : GTTAAANNNNN------ : 350
Aeromonas_ : GTTAAAANNN------ : 350
SM-5-6_Bac : GTTAAANNNNN------ : 350
C._testost : GTTAAANNNNN------ : 350
Pseudomona : GTTATAAANNNNN---- : 350
P._pseudoa : GTTAAAANNN------- : 350
Proteobact : GTTAANNNNNNNN---- : 350
Pseudomona : GTTAANNANNNNN---- : 350
Acinetobac : GTTAAANNNNNNN--- : 350
Uncult._ba : ATTAANNNNNN----- : 350
Thermomona : GTTAAANNNNN------ : 350
CK06-06_Mu : GTTAAANNNNNN---- : 350
Thermomona : GTTAANNNNNNNNNNN : 350
Ochrobactr : GTTAAAANNNN------ : 350
P._asaccha : GTTAAANNNNN------ : 350
Arsenite-o : GTTAAAANNNNN---- : 350
Brevundimo : GTTAAANNNNNNNNNN : 350
             gttAaaAaGGn
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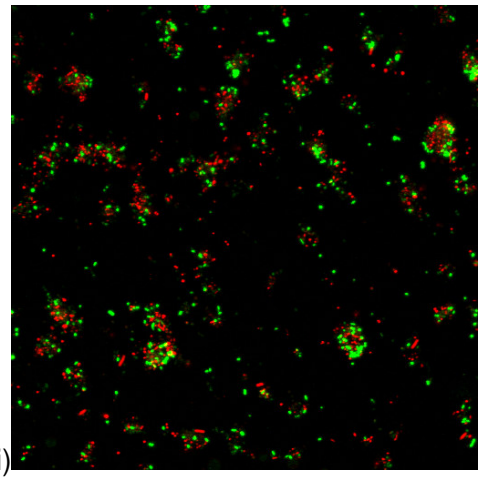
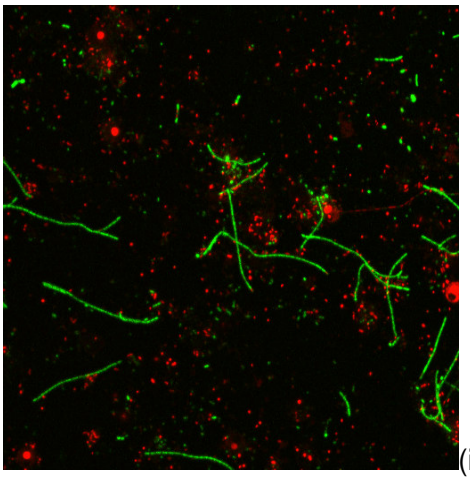
Figure 4-*Continued*

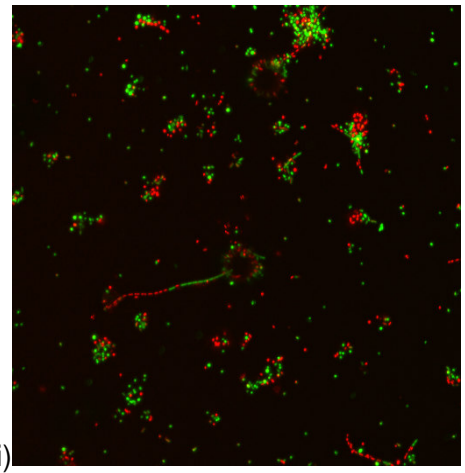
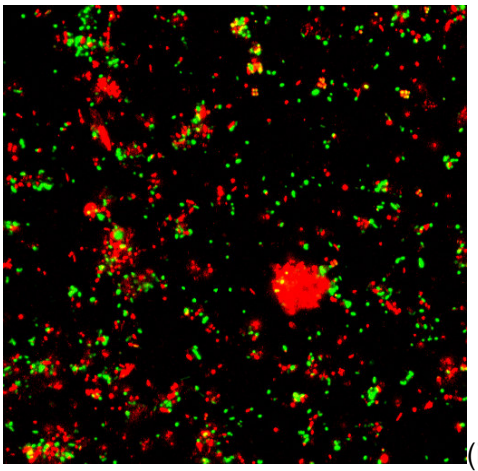
APPENDIX C

Confocal Laser Scanning Microscopy and Scanning Electron Microscopy images of bacterial communities (biofilms) grown on Pederson's devices. Water samples were obtained over the one year sampling period of the Berg River (2004 to 2005). The biofilms were stained with the LIVE/DEAD™ BacLight stain: green represents live cells and red represent dead cells within the bacterial community.

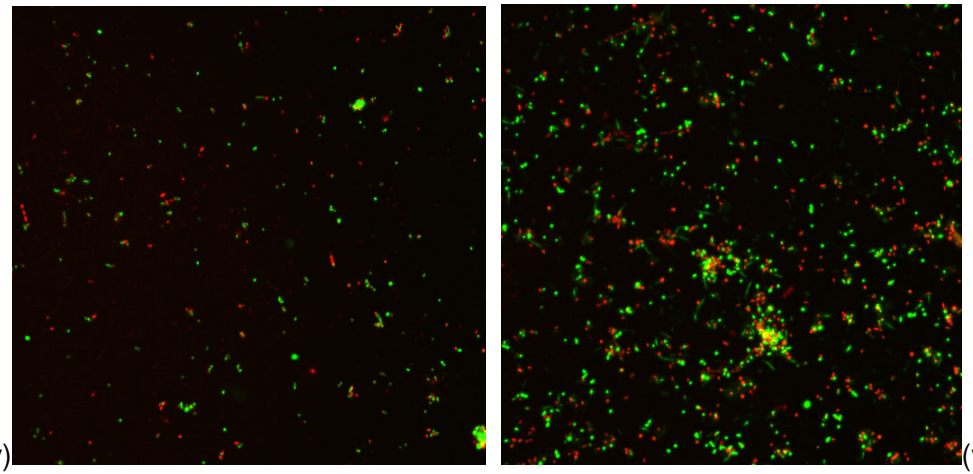
Biofilm communities grown on Pederson's devices: water samples collected during the first two months of the sampling period [June and July 2004 (i)]) and [September 2004 (ii)]:

(i)   (ii)

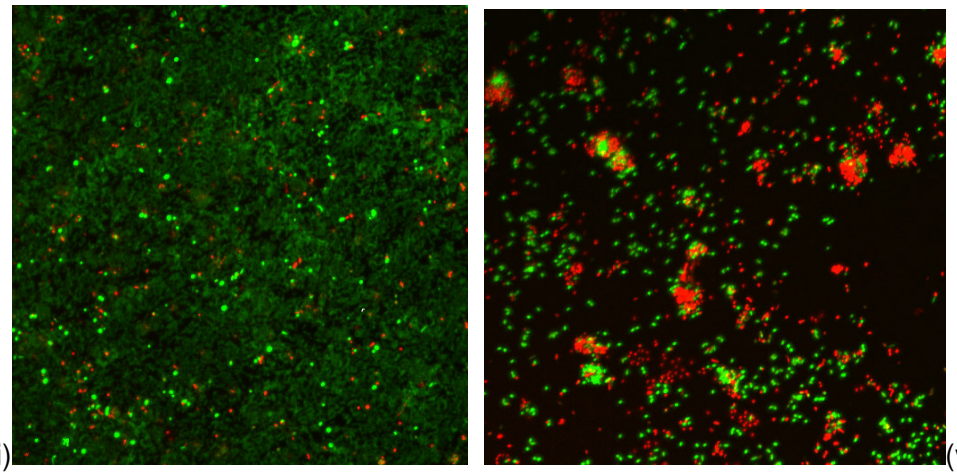
Biofilm communities grown on Pederson's devices: water samples collected during October 2004 (iii) and November 2004 (iv):

(iii)   (iv)

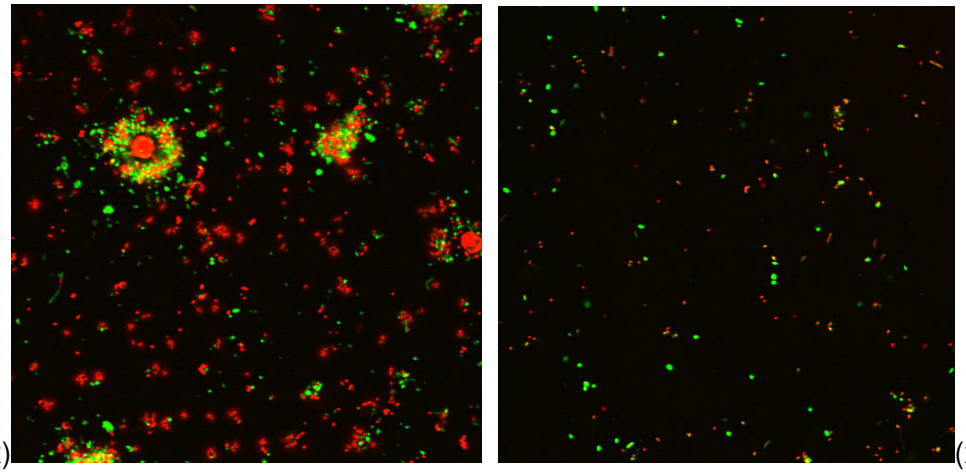
Biofilm communities grown on Pederson's devices: water samples collected during January 2005 (v) and February 2005 (vi):

(v)  (vi)

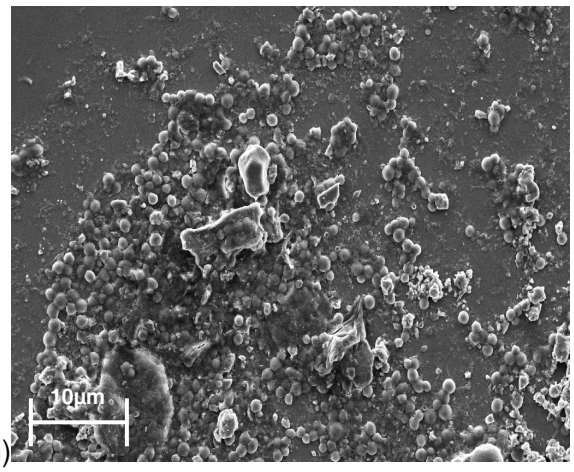
Biofilm communities grown on Pederson's devices: water samples collected during March 2005 (vii) and April 2005 (viii):

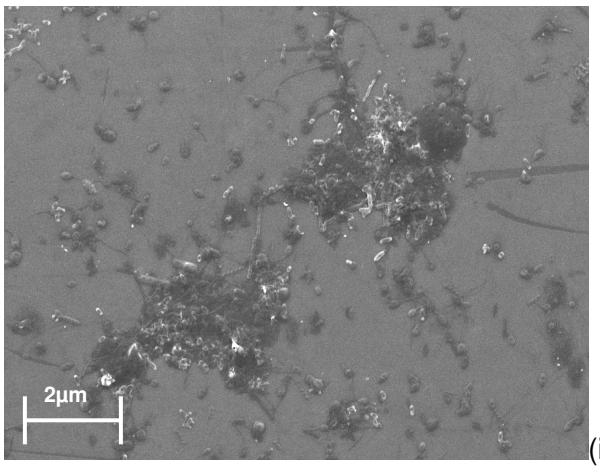
(vii)  (viii)

Biofilm communities grown on Pederson's devices: water samples collected during May 2005 (ix) and June 2005 (x):

(ix)  (x)

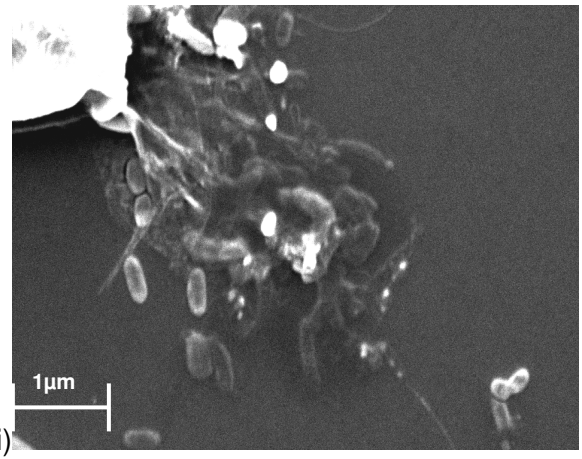
Biofilm communities grown on Pederson's devices: water samples collected during the first two months of the sampling period [June and July 2004 (i)]) and [September 2004 (ii)]:

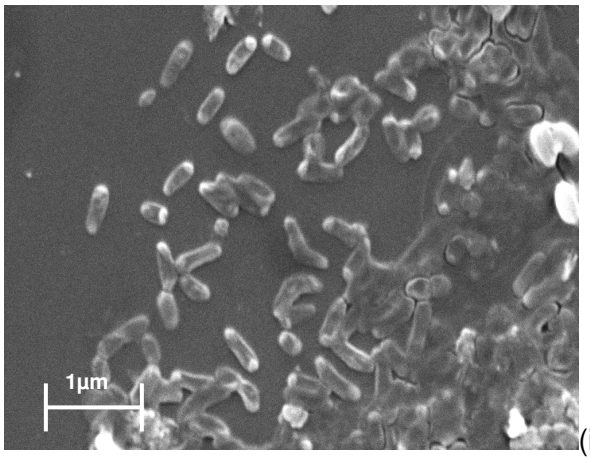
(i) 



(ii)

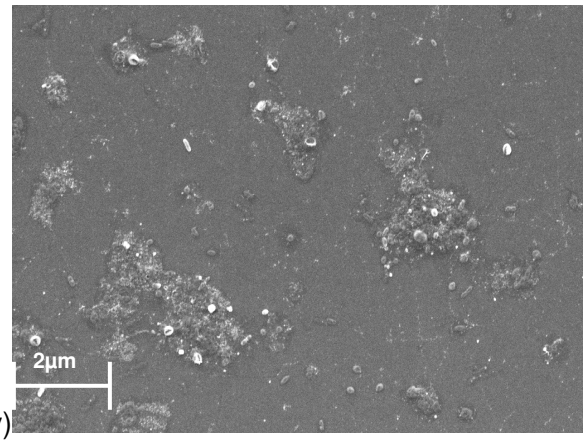
Biofilm communities grown on Pederson's devices: water samples collected during October 2004 (iii) and November 2004 (iv):

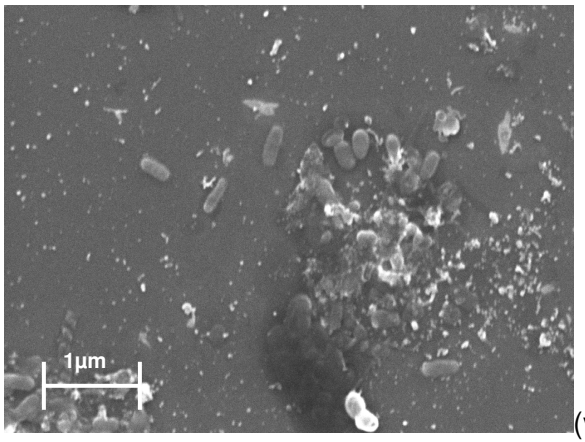
(iii) 



(iv)

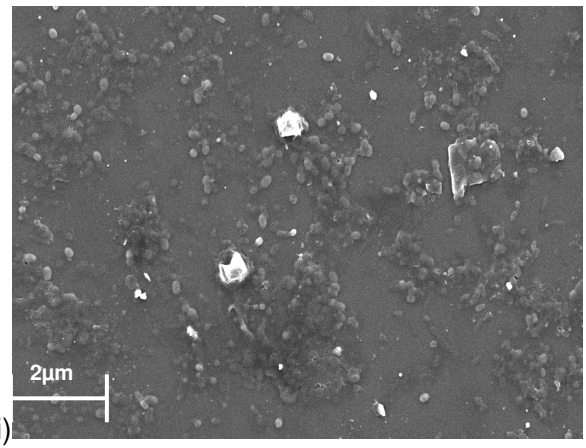
Biofilm communities grown on Pederson's devices: water samples collected during January 2005 (v) and February 2005 (vi):

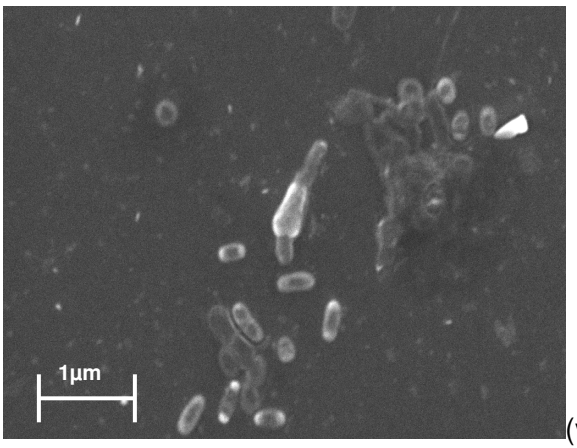
(v) 



(vi)

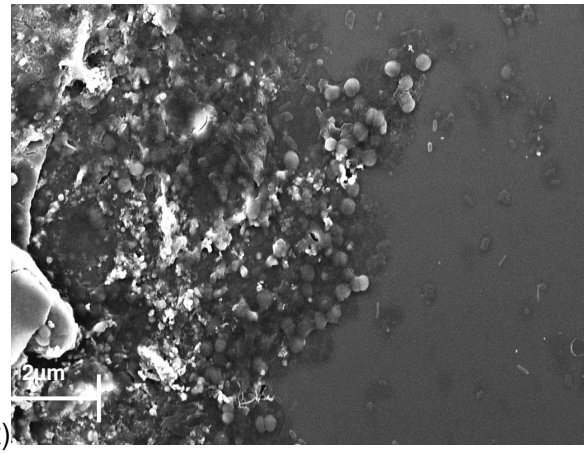
Biofilm communities grown on Pederson's devices: water samples collected during March 2005 (vii) and April 2005 (viii):

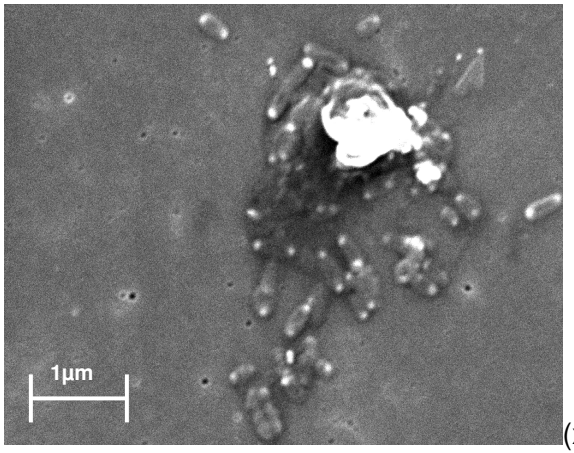
(vii) 



(viii)

Biofilm communities grown on Pederson's devices: water samples collected during May 2005 (ix) and June 2005 (x):

(ix) 

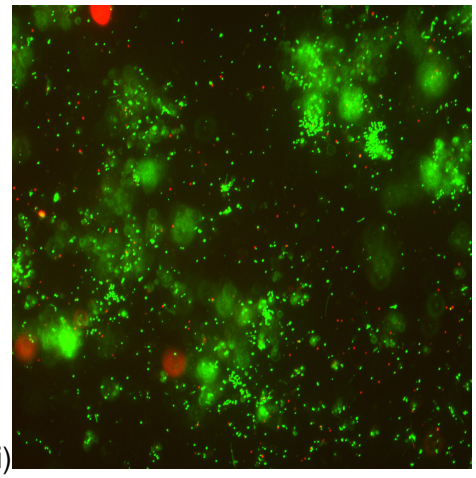
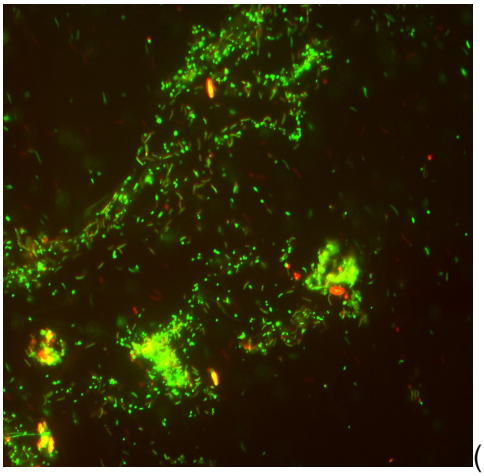


(x)

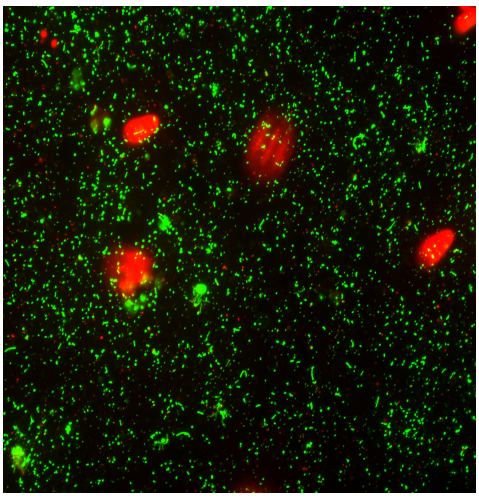
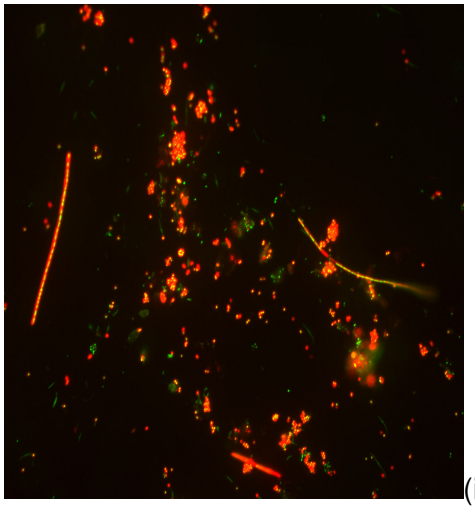
APPENDIX D

Epifluorescence and Scanning Electron Microscopy images of bacterial communities (biofilms) grown on Pederson's devices. Water samples were obtained over the one year sampling period of the Plankenburg River (2004 to 2005). The biofilms were stained with the LIVE/DEAD™ BacLight stain: green represents live cells and red represent dead cells within the bacterial community.

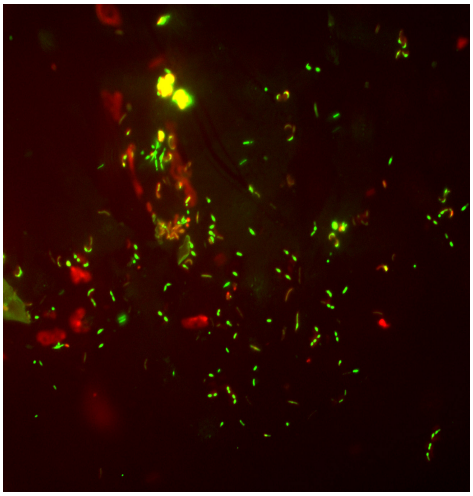
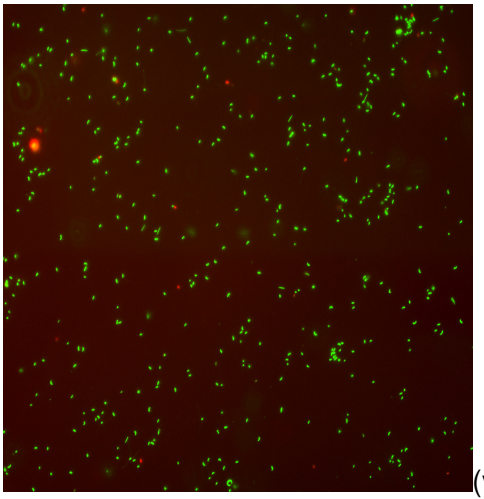
(A) Biofilm communities grown on Pederson's devices: water samples collected during the first two months of the sampling period [June 2004 (i) and July (ii)]:

(i)   (ii)

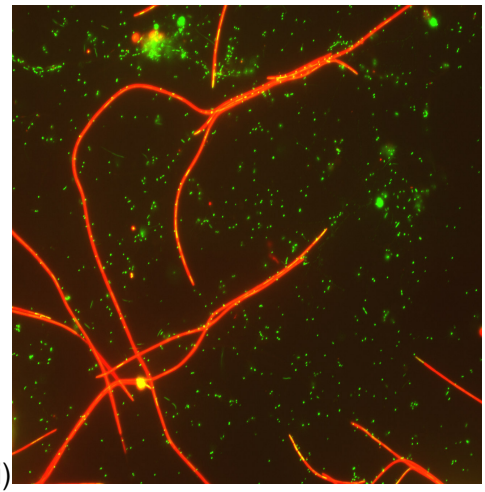
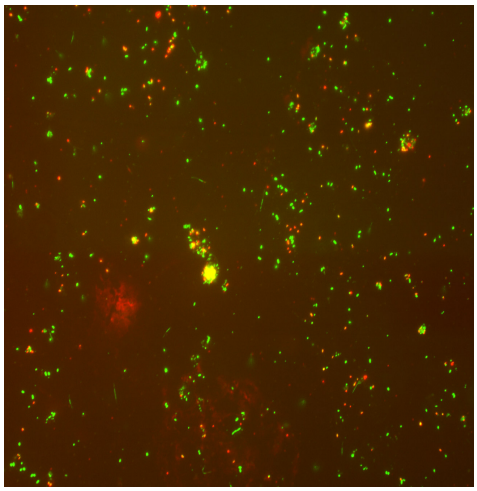
Biofilm communities grown on Pederson's devices: water samples collected during September 2004 (iii) and October 2004 (iv):

(iii)   (iv)

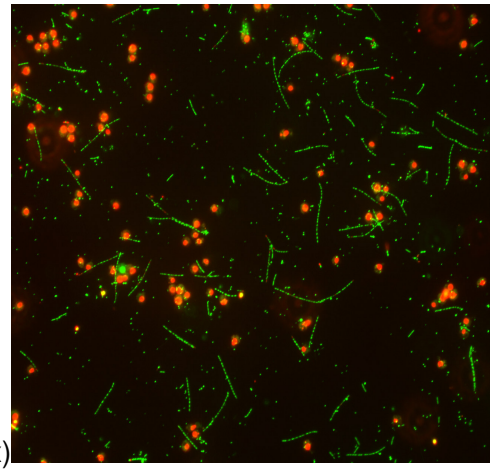
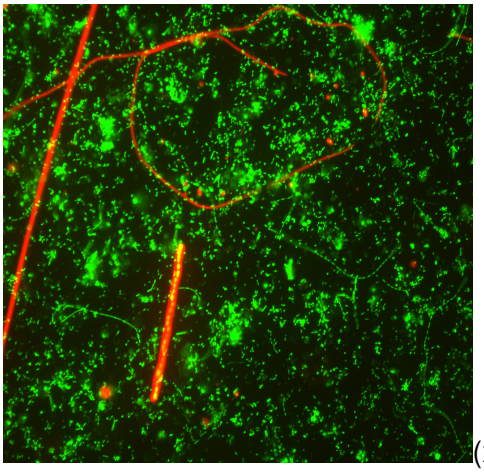
Biofilm communities grown on Pederson's devices: water samples collected during November 2004 (v) and January 2005 (vi):

(v)   (vi)

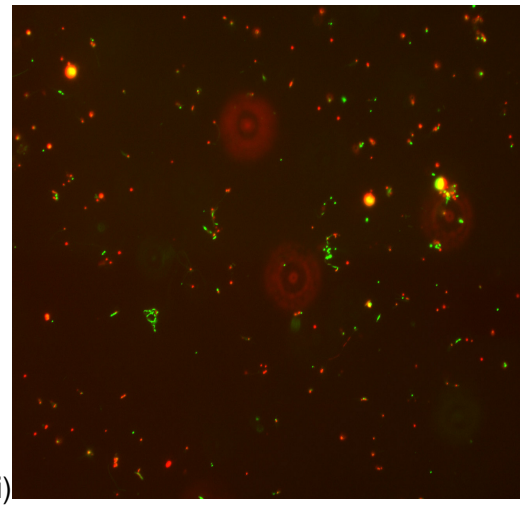
Biofilm communities grown on Pederson's devices: water samples collected during February 2005 (vii) and March 2005 (viii):

(vii)   (viii)

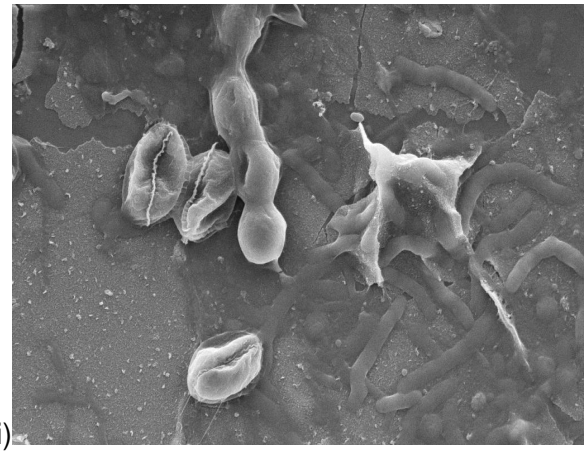
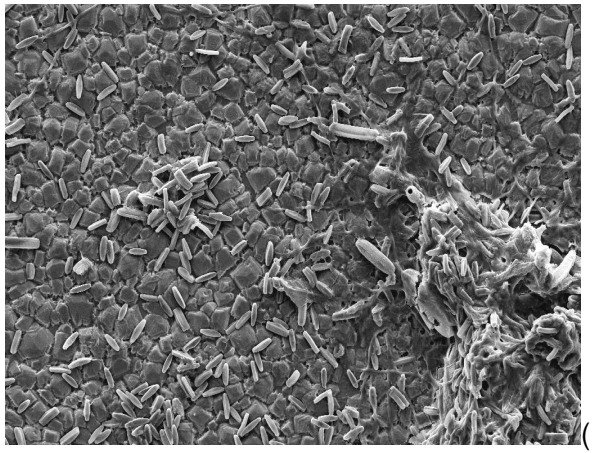
Biofilm communities grown on Pederson's devices: water samples collected during April 2005 (ix) and May 2005 (x):

(ix)   (x)

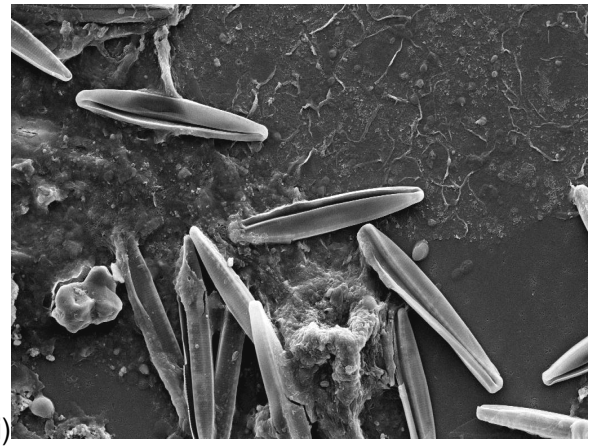
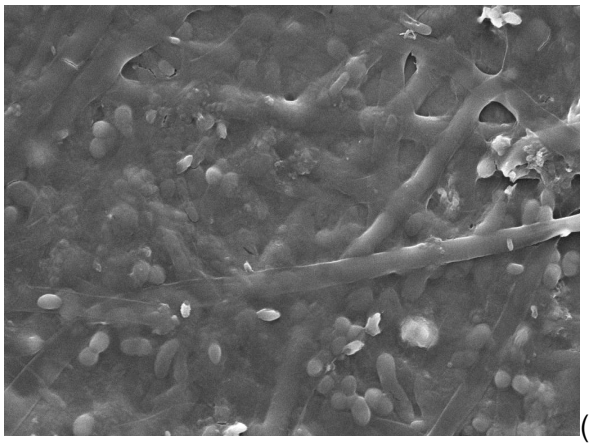
Biofilm communities grown on Pederson's devices: water samples collected during June 2005 (xi)

(xi) 

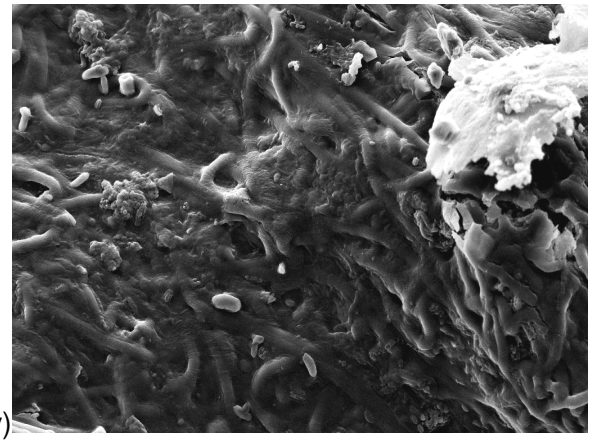
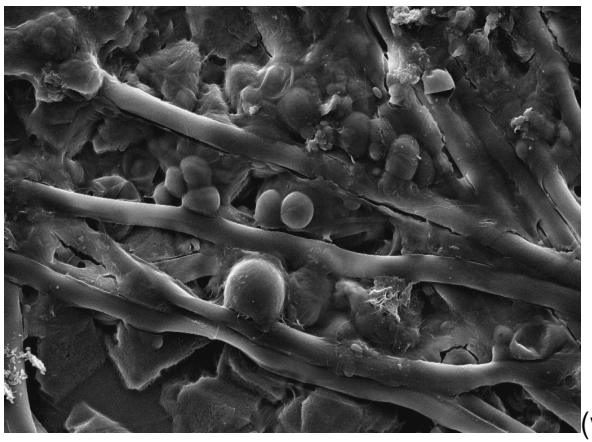
(A) Biofilm communities grown on Pederson's devices: water samples collected during the first two months of the sampling period [June 2004 (i) and July (ii)]:

(i)   (ii)

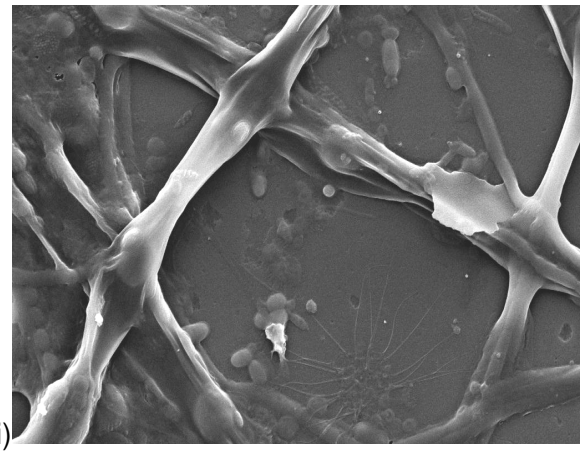
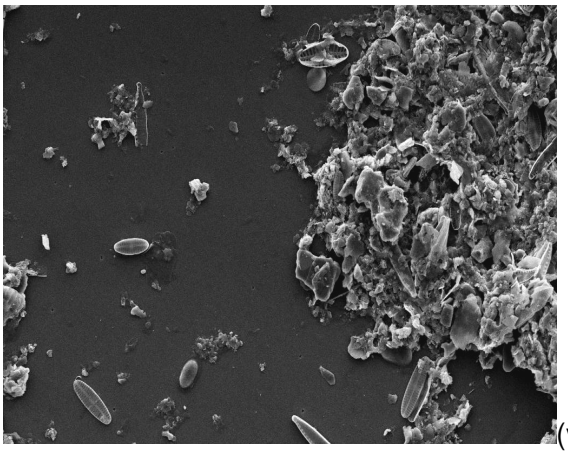
Biofilm communities grown on Pederson's devices: water samples collected during September 2004 (iii) and October 2004 (iv):

(iii)   (iv)

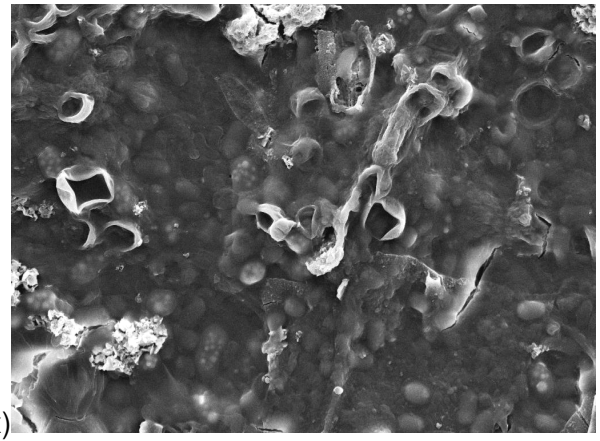
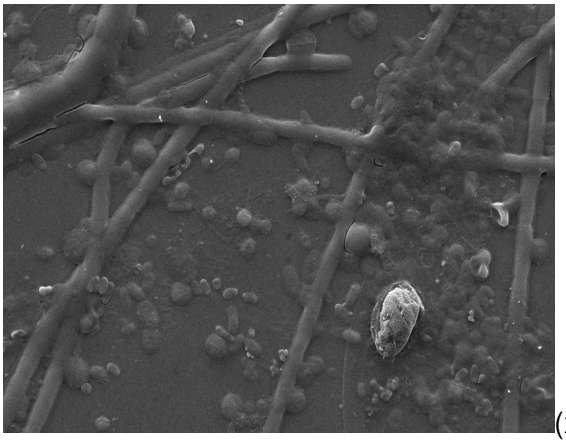
Biofilm communities grown on Pederson's devices: water samples collected during November 2004 (v) and January 2005 (vi):

(v)   (vi)

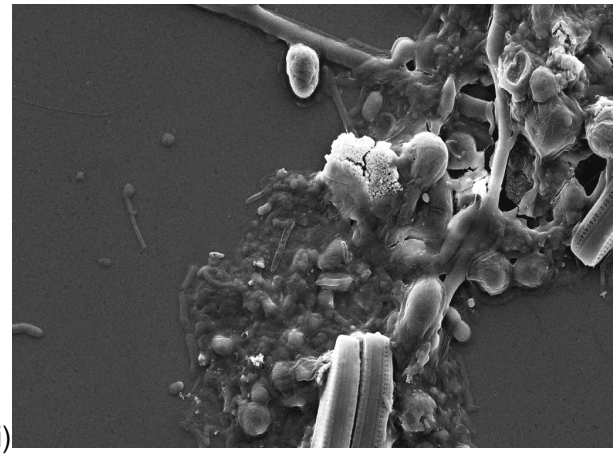
Biofilm communities grown on Pederson's devices: water samples collected during February 2005 (vii) and March 2005 (viii):

(vii)   (viii)

Biofilm communities grown on Pederson's devices: water samples collected during April 2005 (ix) and May 2005 (x):

(ix)   (x)

Biofilm communities grown on Pederson's devices: water samples collected during June 2005 (xi)

(xi) 

APPENDIX E

Bioremediation of Bacterial Pollutants in the Plankenburg River in the Western Cape, South Africa

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ABSTRACT. The aim of this study was to develop and optimise two laboratory-scale and one large-scale bioreactor systems to reduce the level of bacterial river water contaminants. The large-scale bioreactor system was established on-site along the Plankenburg River, Stellenbosch, South Africa. Total cell counts were analysed by means of flow cytometric analysis (FCM) and epifluorescence microscopy (EM) (laboratory-scale bioreactor systems only). In bioreactor (i), the initial total cell count of 2.8×10^7 micro-organisms/mL was reduced to 5.9×10^6 micro-organisms/mL. The reduction in bacterial pollutants was thus 79% (FCM) and 86% (EM). In bioreactor (ii) total cell counts were decreased by 81% (FCM) and 42% (EM). Even though the EM technique indicated a higher bacterial pollutant reduction, higher and more accurate cell counts were obtained with the FCM technique. The initial cell count obtained for the on-site bioreactor system (day one) was 5.74×10^7 micro-organisms/mL and 3.9×10^7 micro-organisms/mL (final day) indicating a 32% reduction in bacterial pollutants. Currently, the on-site large-scale bioreactor system is being optimised to evaluate a higher pollutant reduction potential.

KEYWORDS. Bacterial reduction; biofilm; bioreactor; bioremediation; epifluorescence microscopy, flow cytometry.

BRIEF. This study involved evaluating bacterial pollutants in river water sources and the application of bioremediative techniques as a means of clean-up strategy.

Introduction

In recent years the quality of South Africa's rivers has fast been deteriorating. They do not meet the European and international minimum standards and the situation is unlikely to improve in the near future. The World Wide Fund for Nature (WWF) has also warned that South Africa could become one of the driest places on earth due to climate change and decreased rainfall percentages (1). Irregular rainfall patterns then result in short periods of elevated faecal pollution, as run-off from informal settlements and the agricultural and industrial sectors, contribute to the microbial contamination of these rivers.

River water in South Africa serves the domestic, recreational and agricultural sectors (2). Contamination of recreational water by infectious micro-organisms, derived from human sewage or animal sources, may lead to an increased incidence of waterborne diseases. The number of micro-organisms that cause infection or disease depends upon the specific pathogen, the form in which it is encountered, the conditions of exposure and the host's susceptibility and immune status (3).

Most of South Africa's river systems situated around major cities have unacceptably high levels of pollution and an estimated 25% of the country's freshwater resources contain agents which promote growth of pathogens and algae (4). In addition, artificial or man-made waste products such as glass- and paper products significantly contribute to contamination in South African rivers. In these natural environments artificial waste products provide increased surfaces for microbial colonisation where they exist and grow as organised biofilm communities (5). Previous studies have also shown that pathogenic and opportunistic pathogens such as *Pseudomonas, Mycobacter, Campylobacter, Klebsiella, Aeromonas, Legionella spp.,*

Helicobacter pylori and *Salmonella typhimurium* may be associated with and multiply in biofilm communities (6). In a study conducted on the Plankenburg River, South Africa (7) contamination in the river was so severe that farmers downstream constructed structures such as gabions (wire baskets filled with stones) to decrease the pollutant concentration in the river water. This make-shift treatment system diverted the cleaner water to their farmland, thereby allowing the farmers to use the water for irrigation purposes.

In a previous study (8) various enumeration techniques were employed to determine the number of bacterial contaminants in the Berg River. The most probable number technique (MPN) (technique estimating gas-producing bacteria) counts were significantly high and exceeded the maximum limit of 2000 micro-organisms/100 mL (5) for river water. In addition, total bacterial counts at the site investigated along the Berg River were recorded at 3.7×10^7 micro-organisms/mL (FCM) and 8.3×10^6 micro-organisms/mL (EM). In South Africa awareness has been raised regarding the state of the rivers and the damage done to existing clean-up systems such as wetlands. Alternative strategies should, however, be established to alleviate the contamination problem in South Africa's surface water sources.

Bioremediation has become a developing technology that uses biological components, such as micro-organisms, to alter contaminants into less toxic or non-toxic compounds or substances (9). The primary objective of bioremediative technology is to manipulate or exploit naturally occurring biodegradative processes to clean up contaminated sites, such as water or soil environments (10). Engineered processes take advantage of the reliability of micro-organisms and thereby suggest that organic compounds which are produced biologically, be destroyed biologically (11). The use of biological processes, such as bioremediation, has thus become a favourable treatment of organic compounds in the environment due to its cost effectiveness and environmental acceptability (12).

The current study investigated the reduction in bacterial river water contaminants by developing and optimising an in situ laboratory-scale bioremediation system. A large-scale bioreactor system was then established on-site at the Plankenburg River, Stellenbosch, South Africa to evaluate the reduction of total microbial cell counts in the river. The total cell counts were analysed by means of flow cytometric analysis and epifluorescence microscopy (laboratory-scale bioreactor systems only).

Materials and Methods

Sampling sites. Based on results obtained from a previous study (8) the water used for the laboratory-scale bioreactor systems were collected from a contaminated site along the Berg River [8 (Site B2)]. This site was one of four sites investigated, i.e. Site A (agricultural farming area); Site B (informal settlement - sites B1 and B2) and Site C (Newton pumping station), along the Berg River. Bacterial analyses by means of the Most Probable Number (MPN) and flow cytometry (FCM) techniques indicated significantly ($p < 0.05$) high gas-producing and total bacterial counts, respectively at this site. Site B2 is situated in an area where storm water drainage pipes directly flow into the river, carrying human and household waste from the informal settlement.

Material used as attachment surfaces – Bioballs™. In the first laboratory-scale bioreactor system white Bioballs™, as indicated in Figure 1 (a), were used as attachment surfaces. These were however, replaced in the second laboratory-scale bioreactor and on-site bioreactor systems with the blue Bioballs™ [Figure 1 (b)], because of a greater attachment surface area. The blue Bioballs™ are produced from a combination of acrylonitrile, butadiene and styrene (ABS). They have a surface area of 20 cm^3 and can be stacked close to each other.

This not only increases the attachment surface area for biofilm growth, but also the retention time within the system.

Laboratory-scale bioreactors. Two laboratory-scale bioreactor systems (Figure 1) were evaluated over a period of two and three weeks, respectively. The first system (i), containing the white Bioballs™, was constructed in an enclosed building minimising the onslaught of environmental factors, while the second system (ii) (filled with the blue Bioballs™) was constructed outside to monitor the effect of natural environmental conditions. On both occasions, 200 L of water was collected from Site B2 along the Berg River and pumped through the bioremediation systems filled with the different Bioballs™. The horizontal bioreactor systems (35 cm x 30 cm x 100 cm) were operated at a flow rate of 1000L/hr (Ecopool 6 pump) and a retention time of three minutes. The bioreactor systems were sampled daily for a period of 20 and 18 days, respectively. Both laboratory-scale reactors were batch or closed systems.

On-site bioreactor. Initially, the on-site bioremediation system was constructed next to a storm water drainage pipe running from the informal settlement to the Berg River. This site was later however, replaced with Site C along the Plankenburg River due to human interferences, vandalism and power failures experienced at the Berg River site. The new site was identified in a previous study (13) and significantly high MPN and total cell counts were also recorded at this site, which is situated downstream from an informal settlement in Stellenbosch. The bioreactor system (Figure 2) consisted of six 500 L containers filled with blue Bioballs™, interconnected to each other with polyvinylchloride piping and connected to a well-point pump drawing sample water from the river. The system was operated at a flow rate of 1000L/hr and at a retention time of 120 minutes. The system was sampled twice a week for a minimum of 10 weeks.

Flow cytometry (FCM). Flow cytometric analysis as outlined by (8) was employed in the present study.

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

Equation 1.

[Bead concentration, used for lab-scale reactors, recorded at 988/μL for BD Liquid Counting Beads and at 49827 beads per Trucount™ tube. Bead concentration, used for on-site reactor system, recorded at 986/μL for BD Liquid Counting Beads, all products obtained from BD™].

Epifluorescence microscopy (EM). The total number of micro-organisms in the laboratory-scale bioreactor samples was enumerated by means of epifluorescence microscopy, with the BacLight LIVE/DEAD™ bacterial stain (The Scientific Group) as the fluorochrome. Samples (2 mℓ) were filtered through black nitrocellulose membrane filters with a pore size of 0.22 μm. Cells captured on the filter were stained with 2 mL BacLight LIVE/DEAD™ bacterial stain for 5 minutes. Total cell counts were obtained using a Zeiss Epifluorescent microscope (100X magnification). A minimum of 5 different fields were enumerated for all the water samples, for each respective sampling time.

Statistical analysis. Statistical analysis was determined as outlined by (8).

Results and Discussion

Total bacterial counts for bioreactors (i) and (ii) are presented in Figures 3 and 5.

Laboratory-scale bioreactor (i). Although counts observed fluctuated over time, a significant ($p < 0.05$) decrease in the total cell count (FCM) could be observed between the initial and final counts in the water samples collected. The total bacterial FCM count (Figure 3) recorded on the first day of sampling was 2.8×10^7 micro-organisms/mL in bioreactor (i), while

the final total count observed on day 20 was 5.9 x 10^6 micro-organisms/mL. In comparison, total counts obtained by epifluorescence microscopy yielded a highest initial count of 1.56 x 10^6 micro-organisms/mL and a final total count of 2.2 x 10^5 micro-organisms/mL. The reduction in bacterial pollutants for the first bioreactor was thus calculated at 79% for the FCM technique. Bacterial removal as indicated by the initial and final results obtained from the EM technique was 86%. The comparison of total cell count results however, showed that the EM results do not represent the actual total cell count recorded. Figure 5 indicates a higher total cell count obtained by the FCM technique compared to the total cell count obtained by the EM technique. The EM technique involves the physical counting of micro-organisms, which could lead to human error and thus inaccurate bacterial numbers. In comparison, the FCM technique makes use of computer software, which is more reliable in counting bacterial cells (8). The fluctuating counts observed during the analysis of the bioreactor system could also possibly be ascribed to the fact that the bacterial biofilm communities accumulating on the surfaces of the Bioballs™ within the bioreactor system sloughed off from the attachment surfaces. These bacterial cells are thus suspended in the planktonic or aqueous medium within the system. Similarly, the thickness of the biofilm creates anaerobic conditions for the bacterial cells which accumulate on the bottom of the biofilm layer, subsequently leading to these cells dying off (14). Figure 4 illustrates the number of viable bacterial cells versus dead cells (FCM analysis) within the bioreactor samples. The higher dead cell count (5.84 x 10^7 micro-organisms/mL) observed on the first day of sampling could be ascribed to the fact that certain micro-organisms could not initially adapt to the changed environmental conditions when moved from their natural habitat, i.e. the river, to the bioreactor system (15). The viable cell count recorded on the first day was 2.23 x 10^7 micro-organisms/mL. By day 6, however, the bacterial cells were able to adapt as the viable cell count steadily increased and followed this trend for most of the sampling period.

Laboratory-scale bioreactor (ii). In the second bioreactor system an initial total count of 7.8×10^7 micro-organisms/mL (day 1) and a final count of 1.5×10^7 micro-organisms/mL (day 18) (Figure 5) for FCM analysis, was recorded. The total cell counts in bioreactor (ii) was decreased by 81%. The decrease in total count as observed by epifluorescence microscopy analysis was 42% with the initial and final counts recorded at 3.54×10^6 micro-organisms/mL and 2.04×10^6 micro-organisms/mL, respectively. As with bioreactor (i), the total cell counts as obtained by FCM analysis, fluctuated over the sampling period. In addition, the dead bacterial cell count obtained by FCM analysis was also initially higher [as observed in bioreactor (i)], in comparison to the viable bacterial cells obtained from the bioreactor (ii) (Figure 6).

As previous studies indicated (8), the FCM technique overall generally yields higher total counts in comparison to the EM technique. In addition to the physical counting proving to be disadvantageous in obtaining accurate bacterial counts, the possibility of nonbacterial biomass and debris captured on the membrane filter used for EM analysis, could also influence the bacterial enumeration (6). Results also clearly show that a higher bacterial pollutant removal was obtained with the blue Bioballs™ [bioreactor (ii)] in comparison to the white Bioballs™ employed in bioreactor (i). The blue Bioballs™ provide a larger attachment surface and thus proved more effective in the reduction of bacterial numbers in the river water samples. Based on results obtained, the total cell counts for the on-site bioreactor samples were analysed by the FCM technique only and the blue Bioballs™ were used as attachment surfaces in the on-site large-scale bioreactor.

On-site bioreactor system. The results obtained for the on-site bioreactor system are presented in Figures 7 and 8. Scanning electron microscopy (SEM) images also indicate biofilm formation on the surface of the Bioballs™ (Figure 9). It is evident from the SEM images that the biofilms (i.e. bacterial communities) formed on the surfaces of the Bioballs™ produce a substance (extracellular polysaccharides or EPS) which serves as entrapment material for debris

and pollutants. According to previous studies, biofilms and the extracellular polymeric substances produced by these biofilms, may aid in various pollutant removal strategies, depending on its ability to adhere to particular support surfaces (16; 17). The initial total FCM bacterial cell count recorded (day 1) in the large-scale bioreactor was 5.74×10^7 micro-organisms/mL. Cell counts continued to fluctuate during the two-month sampling period, which could possibly be ascribed to cells sloughing off the attachment surfaces, the influence of environmental factors and the effect of constant power failures in the area. The final total count recorded on the last day of sampling was 3.9×10^7 micro-organisms/mL (day 19) (Figures 7 and 8) indicating a 32% decrease in bacterial pollutants. Currently, the large-scale on-site bioreactor is being optimised to evaluate the maximum pollutant removal potential of the system. In addition, various mechanisms such as circulation or aeration systems could be introduced into the bioreactor system to ensure more effective removal of possible contaminants. Another possible means of improving such a bioremediation system is to extend the retention times at which the system operates. As power failures had a major effect on the effective running of the bioreactor system in the abovementioned study, alternative power or electrical back-up systems should be considered to ensure smooth operation of the system.

ACKNOWLEDGMENTS. The National Research Foundation (NRF) and Cape Peninsula University of Technology (CPUT) for financial support. The Paarl and Stellenbosch Municipalities are thanked for their assistance.

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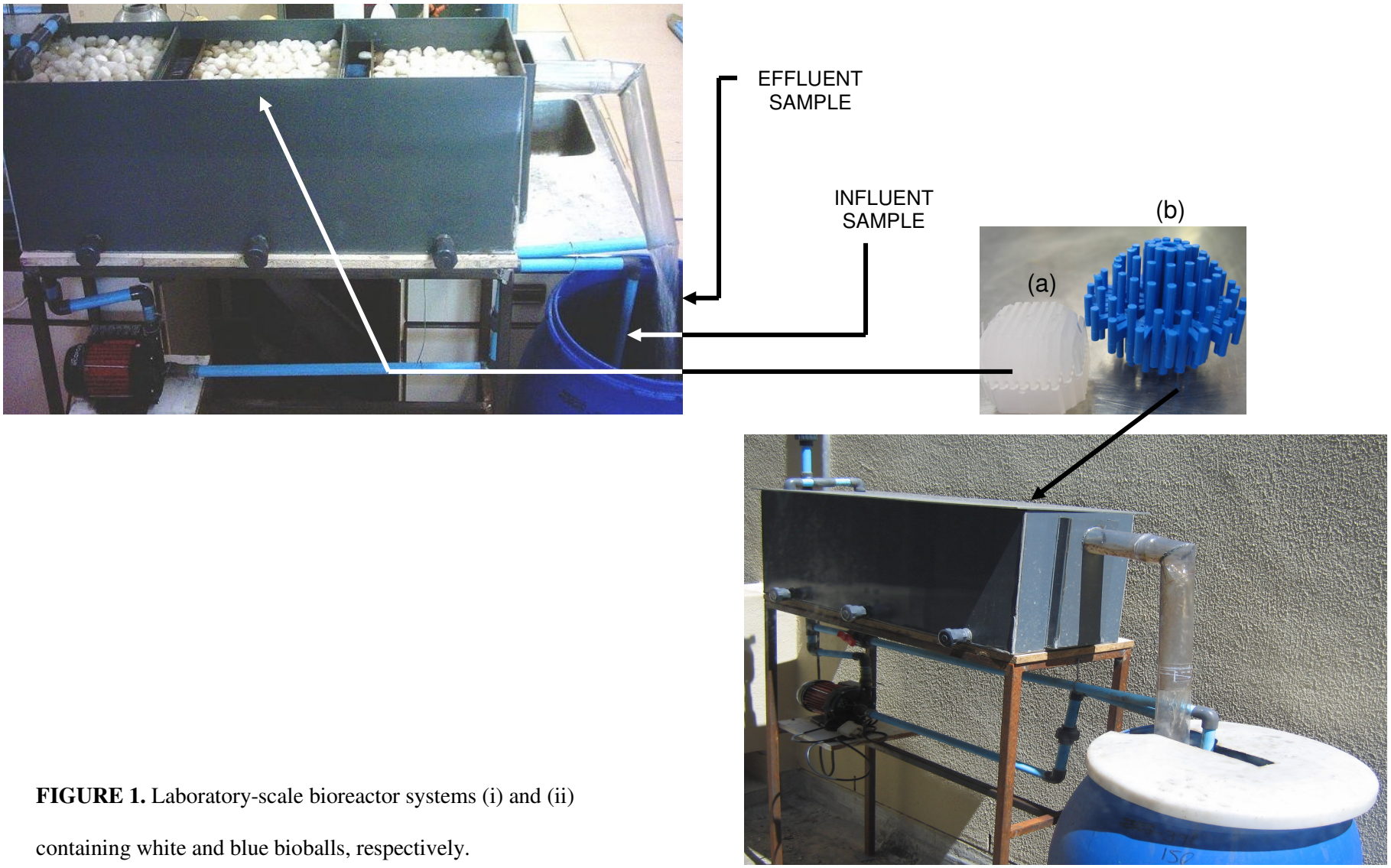

FIGURE 1. Laboratory-scale bioreactor systems (i) and (ii) containing white and blue bioballs, respectively.

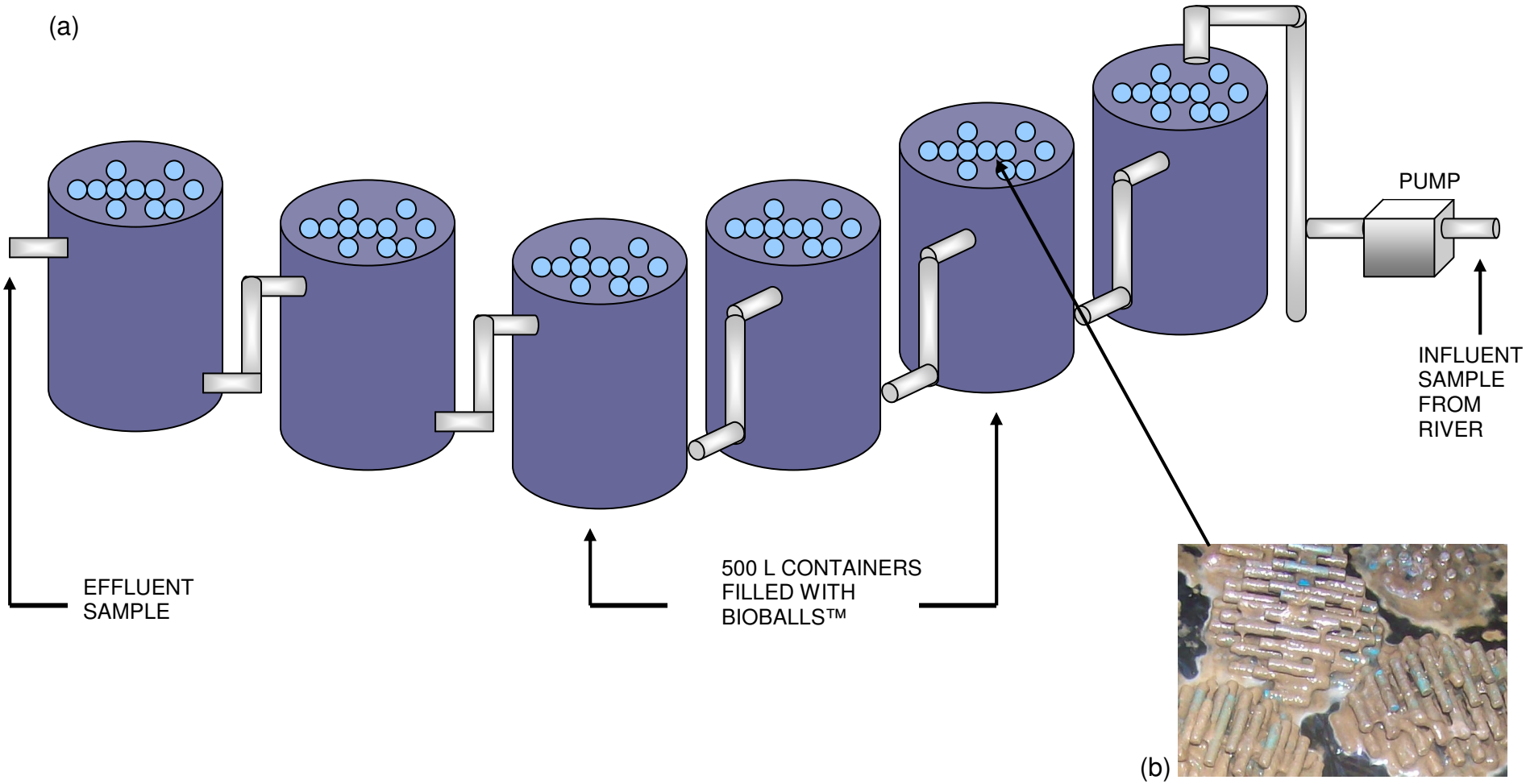

FIGURE 2. (a) Schematic diagram of on-site bioreactor system containing blue Bioballs™. (b) Bioballs™ serving as attachment surface.

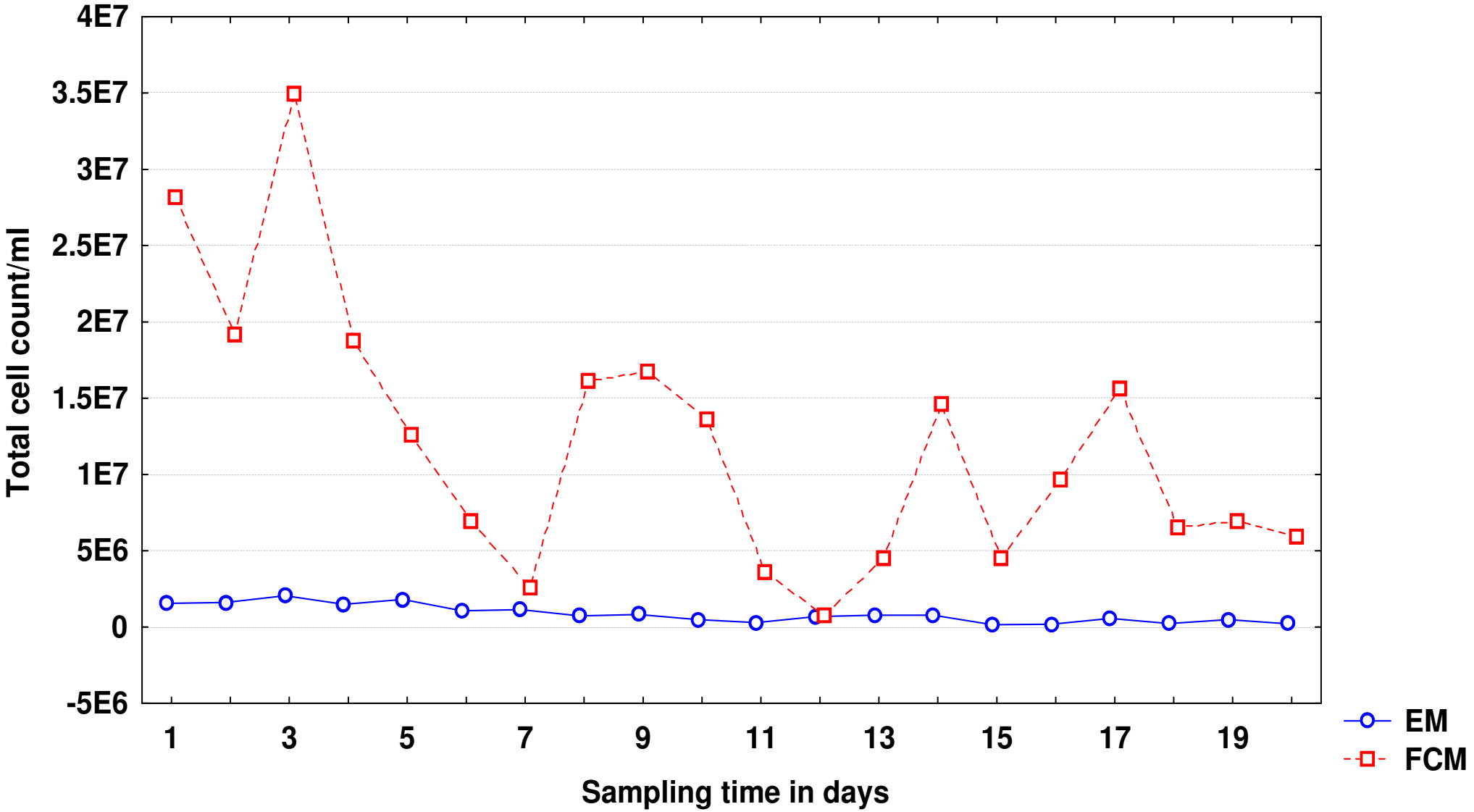

FIGURE 3. Total bacterial cell count obtained from bioreactor system (i) based on epifluorescence microscopy (EM) and flow cytometric analysis (FCM, using LIVE/DEAD BacLight™ stain).

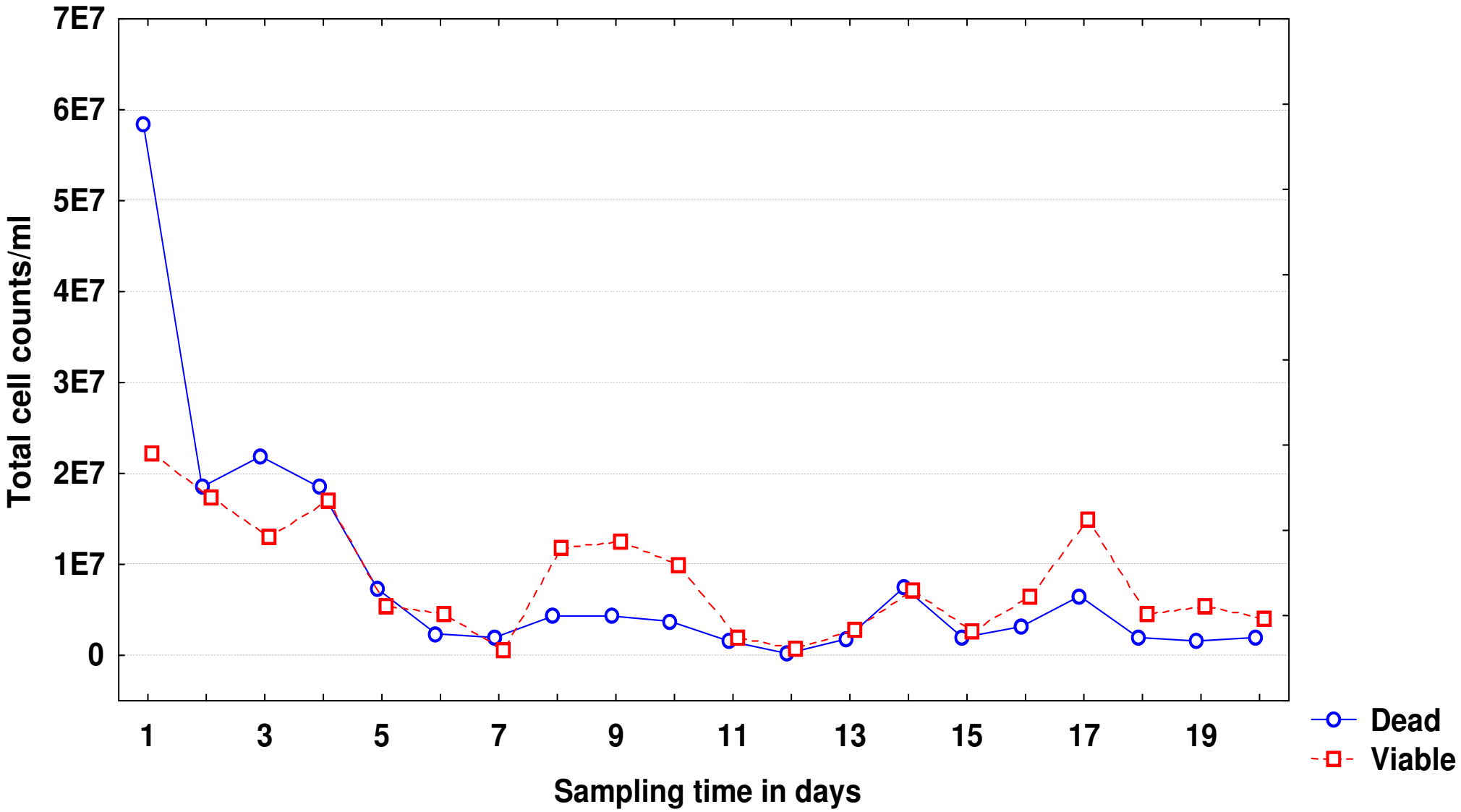

FIGURE 4. Total viable bacterial cell count obtained from bioreactor system (i) versus the dead bacterial cells based on flow cytometric analysis (FCM, using LIVE/DEAD BacLight™ stain).

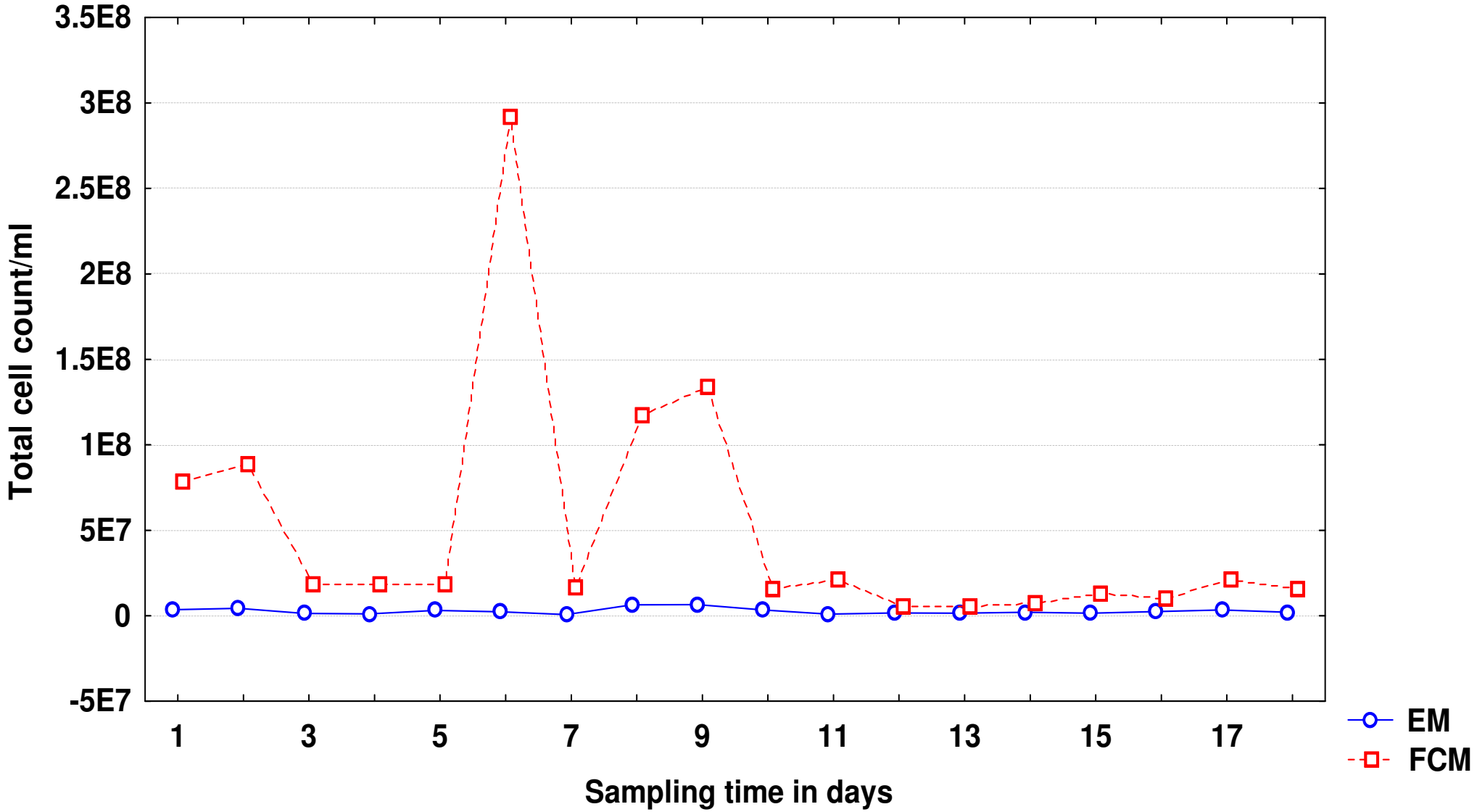

FIGURE 5. Total bacterial cell count obtained from bioreactor system (ii) based on epifluorescence microscopy (EM) and flow cytometric analysis (FCM, using LIVE/DEAD BacLight™ stain).

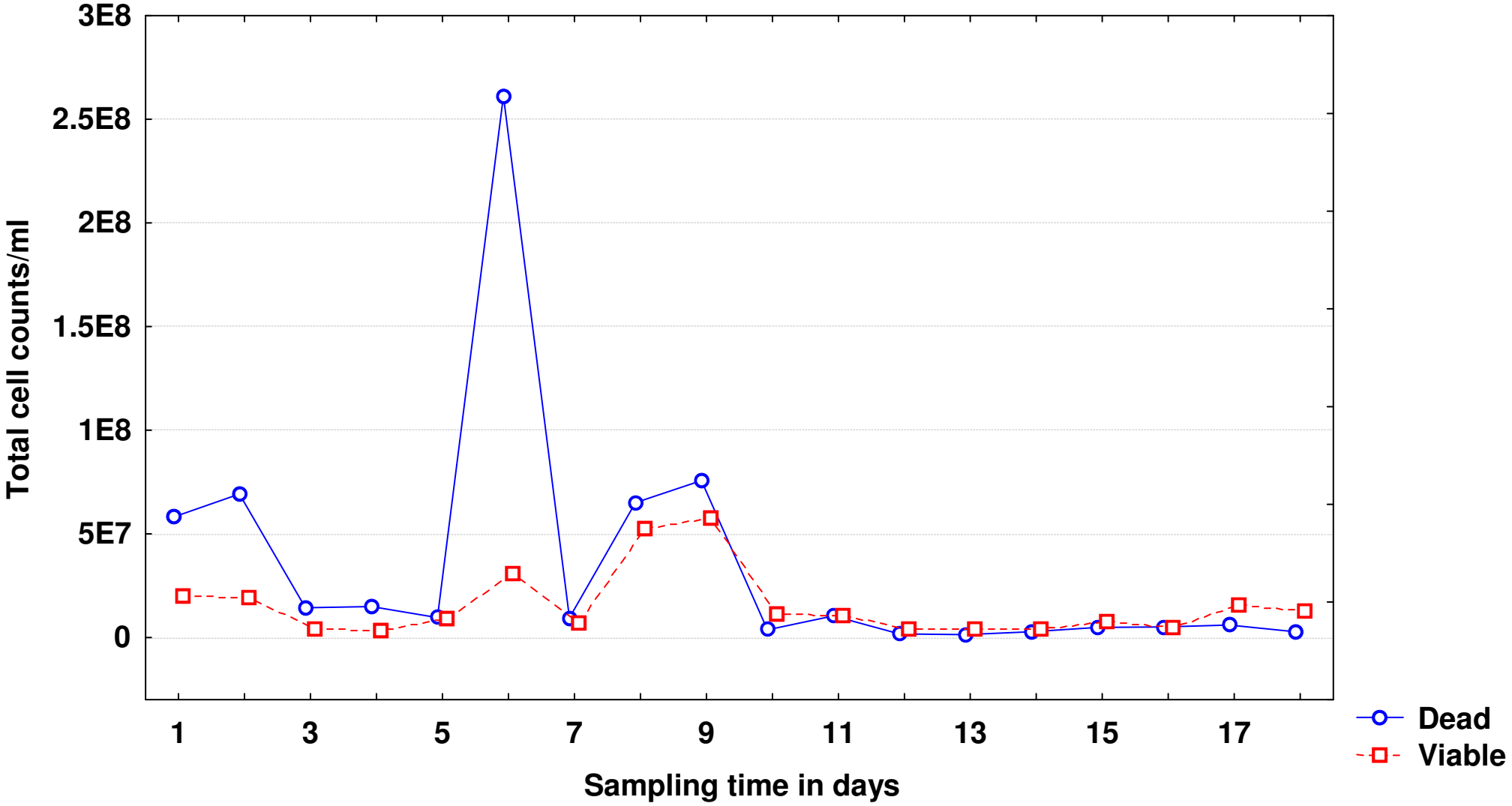

FIGURE 6. Total viable bacterial cell count obtained from bioreactor system (ii) versus the dead bacterial cells based on flow cytometric analysis (FCM, using LIVE/DEAD BacLight™ stain).

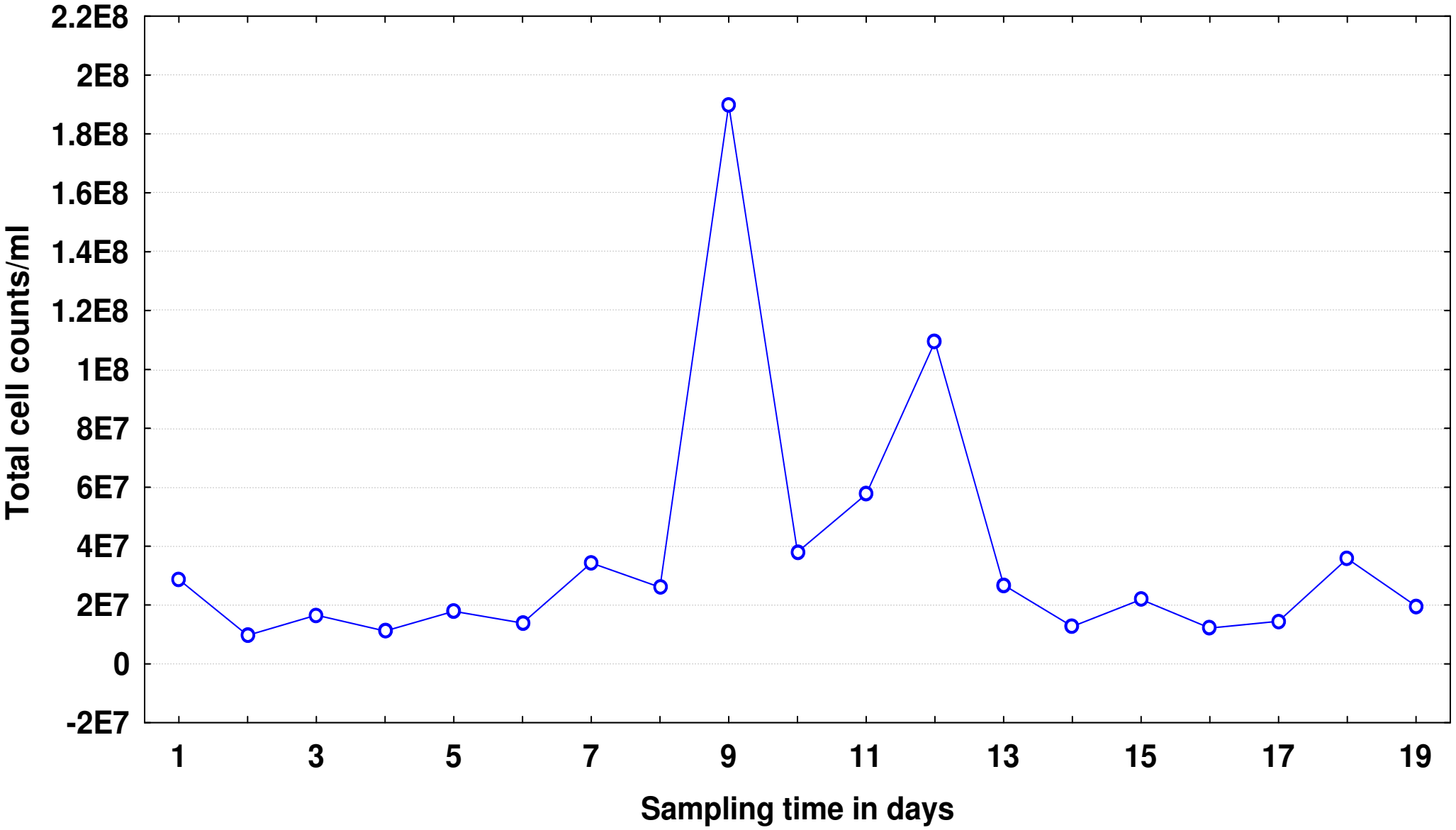

FIGURE 7. Total bacterial cell count obtained from on-site bioreactor system based on flow cytometric analysis (FCM, using LIVE/DEAD BacLight™ stain).

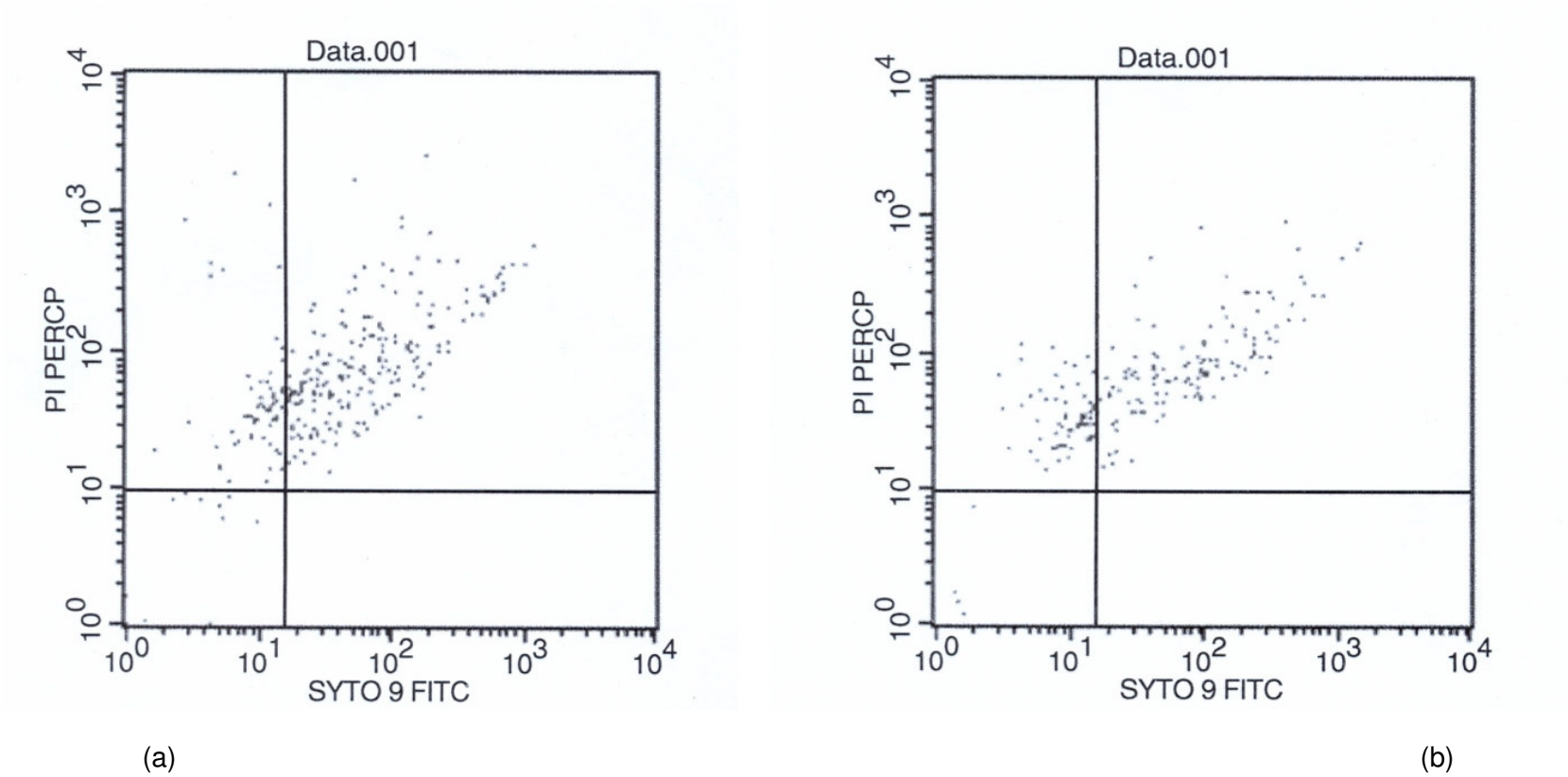

FIGURE 8. Dotplots of initial (a) and final (b) total FCM counts, respectively obtained from the on-site bioreactor system by means of flow cytometric analysis.

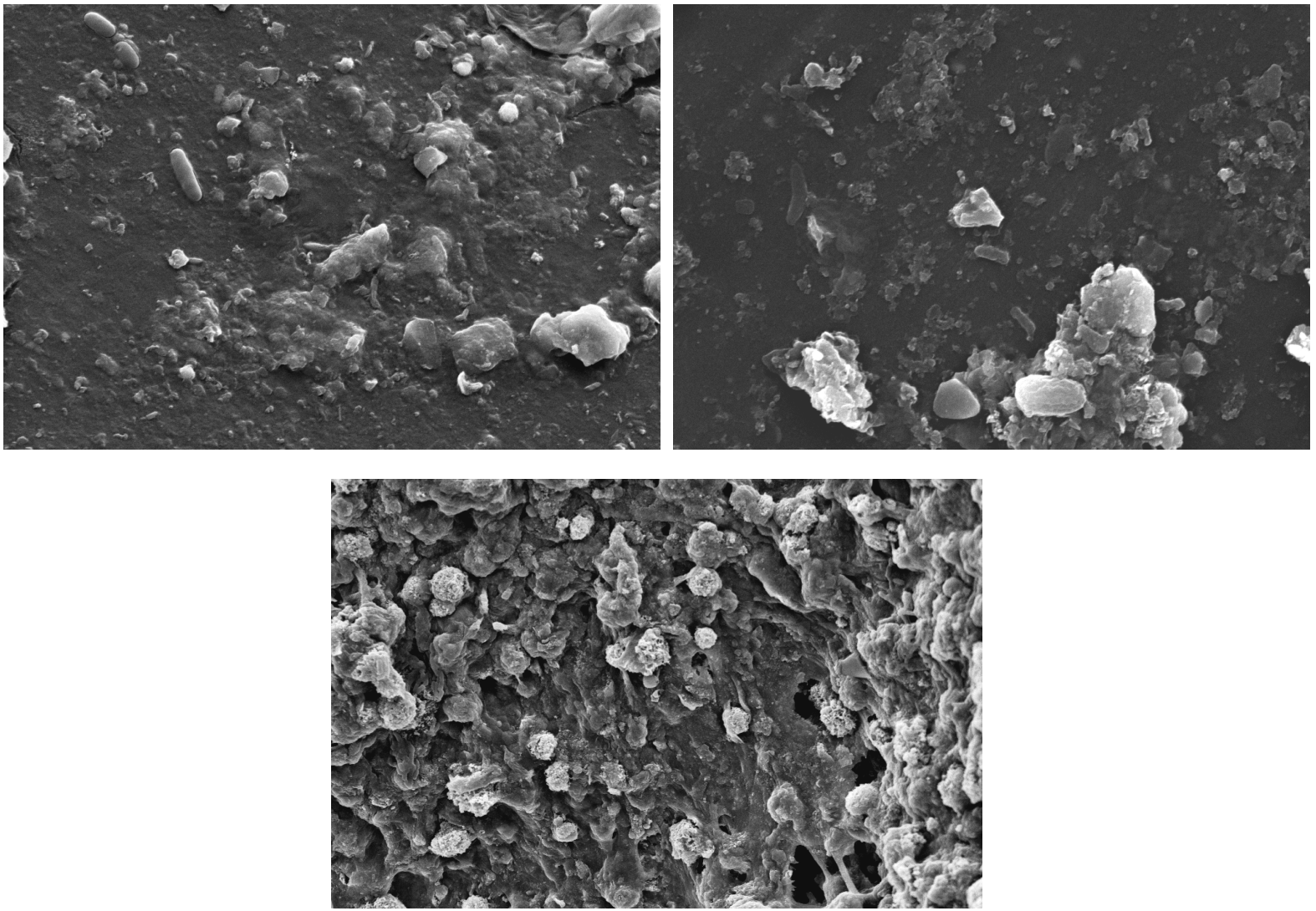

FIGURE 9. Scanning electron microscopy images of biofilm formation on Bioballs™ within the on-site bioreactor system.