MANAGEMENT OF THE MICROBIAL WATER QUALITY IN CATCHMENTS

Report to the
Water Research Commission

by

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EXECUTIVE SUMMARY

Management of the microbiological quality of water requires a good understanding of the behaviour of microorganisms at an ecosystem level. The behaviour, which is linked to the microbial population structure and diversity, consequently influences the effectiveness of catchment level mitigation strategies that contribute to sustainable management of our surface water resources. Managing microbial loads at catchment levels could provide both financial and health benefits for downstream users.

Accordingly two distinct, but somewhat related, topics were focussed on during the tenure of this project: Firstly, a literature survey was conducted to summarize the current mitigation approaches and practices applicable at catchment level, and secondly the applicability of novel molecular approaches for the determination of true microbial diversity in surface waters was investigated.

The objectives were to:

- Provide an overview of current mitigation approaches and practices applied at catchment level;
- Assess the effectiveness of each mitigation method in the production of a better quality water based on published studies;
- Evaluate the applicability of new molecular approaches for the true diversity of the microbial communities in surface waters; and
- Determine future research needs and priorities.

Mitigation strategies for the management of microbial water quality in catchments

The literature on vegetated buffer strips and wetlands as mitigation measures and their effectiveness for reducing faecal bacteria loads at catchment level were perused. To gain insight into the behaviour of bacteria, other than faecal bacteria, two molecular approaches were used to determine the true diversity of bacterial communities of surface water as opposed to measuring only faecal indicators.

The performance of vegetated buffer strips and wetlands in terms of reducing contaminating organisms has been determined using faecal indicators. The efficiency of vegetated buffer strips is dependent on local rainfall intensity, vegetation types, soil types, length and slope of the strips. Shallow and slow flow enhances their efficiency. At high flow *E. coli* recovery varied between 16 and 62% while at low flow recovery was as low as <1% to 5%.

Wetlands are intermediary wet areas between water and land. They occur naturally but can also be constructed. They are characterised by wet soils and plants adapted to the wet conditions. Inorganic chemicals are converted to organic chemicals by wetlands, which in turn provide nutrients for growth of the wetland vegetation. The effectiveness of wetlands is dependent on the rate of effluent flow and its quality that enters the wetland, die-off rate of organisms, rate of removal by filtration and sedimentation, rate of addition of transient or resident animal sources and the rate of predation. They have been shown to remove various bacteria and viruses at rates that range from 41% to 99%.

Pre-treatment processes, for example waste stabilisation ponds, soil infiltration areas and settling basins are well known methods for removal of solids, nutrients and pathogens, before discharging to a secondary technology. Water stabilisation ponds are similar to vegetation buffer strips and wetlands in respect of attenuation and retention of faecal bacteria. They effectively reduce pathogenic bacteria through ultra violet irradiation from the sun, high pH and predation in the ponds. Soil infiltration has also been shown as an effective method for improving contaminated water. Feedlot runoff treatment is often achieved by soil infiltration areas. The microbial quality of the water is improved by infiltration of the contaminated water into the soil. An additional pre-treatment process is settling of water in settling basins. The latter improves water quality by holding

or storing, undisturbed water without mixing for long enough periods to allow particles to settle out with gravity.

Land management engineering is another option to prevent the transfer of microbial contamination to surface waters. Flow connection modelling using GIS and field observations provides information for flow directions due to contours land management features and subsequently allow for landscape engineering to disconnect and redirect flows away from sensitive areas and water bodies.

Applicability of new molecular approaches to determine microbial diversity
Limited information exits on the actual composition of the microbes in the water and how these could perhaps affect the efficiency of the mitigation methods.

New developments in the molecular field provide alternative sequencing and microarray technologies that enable a more holistic approach when determining the microbial quality of water resources. Assessment of the microbial quality of surface waters can now be based upon the bacterial community composition rather than the presence of indicator organisms alone. Accurate information on the microbial diversity of surface waters could help in assessing and modelling the potential health risks to water users in the catchment.

Two methods, pyrosequencing and phylochip analysis, were applied to a limited number of samples to gain information on possible mitigation procedures for wetlands through the management of the microbiological quality of surface waters.

Findings showed that that both pyrosequencing and phylochip analysis reveal a much greater diversity of the bacterial population in the water samples when compared to Denaturing Gradient Gel Electrophoresis (DGGE) analysis. DGGE only revealed the most dominant phyla and is only useful for determining the most dominant members of a bacterial community. Pyrosequencing or phylochips were also able to reveal taxa that are present at low levels.

Primary phylochip data only provided an indication of presence/absence and not of abundance. However, using the fluorescence intensity data of the individual hybridizations, the phylochip data also provided an indication of abundance which could be correlated with the pyrosequencing results.

Although a detailed analysis of the bacterial species diversity and abundance could be obtained from these new molecular methods it was evident that species and genera representing water-borne pathogens were seldom detected and if detected, were present at very low levels. The impact and response of natural freshwater ecosystems to contamination with human wastes could thus be accurately analysed for bacterial species diversity and dynamics using these methods. The water-borne pathogens typically only represent a small fraction of the total bacterial community and are not easily detected when doing whole community diversity analyses. As a result health risk assessments cannot be performed using these methods.

For risk assessments, direct analysis of the water-borne pathogens or their indicators is preferable. Molecular approaches targeting water-borne pathogens directly such as q-PCR are at present still the methods of choice. This situation may change in future, but it does not seem that the current developments in sequencing technologies and microarrays will provide a viable alternative.

This study has shown that pyrosequencing and phylochips can provide insight into the bacterial community composition in contaminated waters. However, even in highly contaminated water sources the indicator organisms and pathogens are present as a low percentage of the total bacterial population. As the techniques have difficulty in detecting taxa present as a low percentage, the data generated by pyrosequencing and phylochips are not suitable for use in microbial risk assessment methods. For now the main application of these techniques will be to study the overall microbial diversity in aquatic ecosystems; providing pertinent information on the microbial composition and dynamics of these systems.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACRONYMS

bp	Base pairs
CCD	Charge Coupled Device
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
E. coli	*Escherichia coli*
EtOH	Ethanol
EU	European Union
FISH	Fluorescent in-situ hybridization
GIS	Geographic Information System
GPS	Global Positioning System
LB	Luria Bertani
MPN	Most Probable Number
NFW	Nuclease free water
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
T-RFLP	Terminal- restriction fragment length polymorphism
TAE	Tris-HCL, acetic Acid and EDTA (buffer)
TDS	Total Dissolved Solids
TKN	Total Kjeldahl Nitrogen
TOC	Total Organic Carbon
USA	United States of America
UV	Ultra Violet

CHAPTER 1: BACKGROUND

1.1 INTRODUCTION

Current methods used to declare water fit for use have a number of shortcomings. These methods are based on the detection of indicator organisms that give an indication of the presence of faecal contamination of the water, but no indication of the presence of any other bacterial communities or possible pathogens. With the rapid development of molecular methods a realization of the immense diversity within populations of bacteria, not previously taken into account when determining the quality of water, has emerged.

1.2 PROJECT AIMS

This study provided information on mitigation procedures for wetlands through the management of the microbiological quality of surface waters. This management will be based on the characterization of the behaviour of microorganisms at catchment and wetland level using molecular methods.

The overall aims included:

- Evaluation of methods for mitigation of microbial pollution of surface water at catchment level; and
- Application of new molecular approaches for determining the true microbial diversity of surface waters at catchment level.

The initial project objectives were:

1. Provide an overview of current mitigation approaches and practices applied at catchment level;
2. Assess the effectiveness of each mitigation method in the production of a better quality water based on published studies;
3. Evaluate the applicability of new molecular approaches to determine the true diversity of the microbial communities in surface waters; and
4. Determine future research needs and priorities.

All the project objectives were met.

1.2.1 Literature survey

Before embarking on any pilot mitigation study in South African catchments to improve the microbial water quality of surface waters, it is important to investigate international approaches and practices. The main aim of this literature survey was to scrutinize international literature (both journals and official reports) for descriptions of mitigation attempts. An initial screening of the literature has indicated that thus far little attention has been given to the mitigation of microbial loads in urban and rural catchments due to human contamination. Attention was therefore also given to studies dealing with pollution from livestock and other farming activities (Edwards, 2003, Collins *et al.*, 2007). In these studies vegetative filter or buffer strips, retention areas and wetlands have been evaluated.

Based on the findings of the scoping study a list of future research needs and priorities was compiled.

1.2.2 Molecular methods

The current knowledge of the microbial quality of surface waters is mainly based on the presence of indicator organisms and in some cases the cultivation of selected pathogens. It is, however, well

known that there are many problems associated with this approach. Not all microbes can be cultured and the bacteria that grow may not necessarily be the dominant species in the community. Some researchers estimate that less than 1% of the total microbial community can be cultured. Even when the targeted species can be cultured, specific strains may dominate and may mask the true diversity of the community and the health risks associated with the bacterial population.

The development of alternative sequencing and microarray technologies during the last five years has revolutionized microbiology (Kunin *et al*., 2008). These developments provide us for the first time with the opportunity to have a more holistic approach when determining the microbial quality of water resources. Using this approach the assessment of the microbial quality of surface waters will be based upon the bacterial community composition rather than the presence of indicator organisms alone. It is foreseen that accurate information on the microbial diversity of surface waters would help in assessing and modelling the potential health risks to water users in the catchment.

The applicability of some of these new techniques for surface waters (known for their complex bacterial communities) was evaluated. As this was merely a scoping study to evaluate applicability, only a limited number of samples were analyzed. The samples were taken from an area representative of conditions in many South African catchments and that are known to experience definite microbial water quality problems (Olifants River Catchment). Two techniques, pyrotags and the phylochip were evaluated.

CHAPTER 2: MITIGATION STRATEGIES FOR THE MANAGEMENT OF MICROBIAL WATER QUALITY IN CATCHMENTS

2.1 INTRODUCTION

Health protection by the control of faecal indicator organisms is a central aim of new catchment-scale water quality management required in the USA by the Clean Water Act and in the EU as the Water Framework Directive (Kay *et al*., 2008). Potential sources of microbial faecal indicator organisms at the catchment scale can include human sewage discharges, agricultural sources derived from livestock, wildlife sources and urban surfaces and drainage (Edwards and Merrilees, 2003). Best management practices when managing microbial loads or faecal indicator organisms include those that focus on containment of microbes at the source and practices that target the transfer and connectivity of microbial flows through the landscape (Edwards and Merrilees, 2003). Many best management practices or mitigation strategies designed to reduce catchment scale flux of faecal indicator organisms require interventions and have associated costs (Kay *et al*., 2007). In order to successfully prevent the microbial contamination of catchments, the range of mitigation tactics designed to protect these water sources must be investigated.

The introduction of best management practices at the source in an agricultural scenario such as slurry storage, stream fencing and water troughs can reduce concentrations of faecally-associated pathogens at a catchment level, but this is not enough to ensure compliance with water quality standards (Inamdar *et al*., 2002). Vegetated filter strips and wetland retention areas are widely recommended in literature as best management practices that are situated adjacent to potential receiving waters (Edwards and Merilees, 2003). Wetland and buffer strips rely on retention as a means for microbial attenuation and have been successful in specific catchments situations (Edwards and Merrilees, 2003). Vegetative buffer strips serve as an interception targeting overland flow or run-off events, whereas constructed wetlands are especially suited to treating regular supplies of effluent with subsurface transport routes or channelled surface flow (Edwards and Merrilees, 2003). It is important to note that faecal indicator organism transport is highly episodic and dependant on season. Therefore factors such as high and low flow, winter and summer as well as different land use types should be considered when employing mitigation strategies (Kay *et al*., 2007). Reduction of faecal indicator organism risk at the source through the control of polluted water is a more feasible management option, although this is not well researched at the catchment level (Edwards and Merrilees, 2003). The combination of different catchment mitigation strategies may offer improved protection (Oliver *et al*., 2007). Retention zones require long-term maintenance as they may rapidly change from being contaminant sinks to becoming contaminant sources which may be located adjacent to surface water channels (Edwards and Merrilees, 2003).

2.2 VEGETATIVE FILTER STRIPS OR BUFFER STRIPS

Vegetative filter strips or buffer strips are increasingly viewed as practical low-cost management options for improving the quality of surface runoff from pollutant sources and improving erosion protection (Woerner and Lorimer, 2003). Vegetative buffer strips are strips of vegetation that act as a filter for sediments and nutrients, trapping bacteria and pathogens, and providing a non-disturbance area for runoff-producing areas (Barling and Moore, 1994). Vegetative filter strips may also be a riparian zone situated along the banks of a river or stream. This influences bank and channel stability and has a primary role in determining the structure and function of the stream habitat (Barling and Moore, 1994). Vegetative filter strips can be specifically planted or pre-existing.

2.2.1 Effect of Vegetated Buffer Strips on Faecal Indicator Organisms

The potential for vegetated buffer strips to reduce sediment load and contaminants such as pesticides and nutrients has been widely reported. These methods are regularly recommended as best management practices in national programmes aimed at reducing diffuse pollution (Edwards

and Merrilees, 2003). The effects however, on faecal indicator organism attenuation have been difficult to quantify or predict because many factors affect microbial populations, such as climate, rainfall amount and intensity, and die-off. Supporting information regarding the actual design, best location, long-term management and effectiveness of vegetated filter strips shows little consensus (Edwards and Merrilees, 2003).

The reduction of runoff of microbial contaminants by buffer strips has been shown. Implementation of vegetated buffer strips of about 3-4 m in width at the edge of near-stream fields receiving animal manure may decrease or eliminate the transport of faecal coliform bacteria into the water system under well-maintained conditions (Roodsari *et al*., 2005). This study illustrated that the amount of faecal coliforms in the runoff was decreased from 68% to 1% in bare versus vegetated clay loam plots and from 23% to non-detectable levels in bare versus vegetative sandy loam plots. Fajardo *et al*. (2001) reported faecal coliform removal rates between 64% and 87% when using small scale simulated runoff events with stockpiled manure. Another study used vegetated buffers for reducing transport of faecal coliform bacteria from agricultural fields amended with dairy cow manure. It was found that the presence of vegetated buffers of any size from 1-25 m, generally reduced faecal coliform concentration by more than 99% (Sullivan *et al*., 2007). Young *et al*. (1980) carried out studies on feed lots where filter strips sown with barley, sorghum and other food crops reduced total coliforms and total *Streptococcus* counts by 69% and 70% respectively. Lim *et al*. (1998) showed that pasture runoff to vegetative filter strips exhibited complete removal of faecal coliforms within the first 6.1 m of the buffer. Standard methods such as membrane filtration and plate counts were used to determine bacterial counts in these studies.

In contrast, when swine wastewater was applied to riparian filter strips, faecal coliform numbers did not decline as water moved down slope, regardless of the vegetation type or season. However, total and faecal coliform numbers in soil water and shallow ground water declined approximately ten-fold every 7 days in the first 14 days, regardless of vegetation or season (Entry *et al*., 2000). Schellinger and Clausen (1992) reported no significant difference in nutrients or bacteria from commercial dairy farm runoff across a vegetative filter strip sown with grasses, although it was suggested that strip performance could be improved by design. When the effectiveness of vegetated buffer strips was investigated by applying cattle slurry, faecal bacterial levels (especially *Streptococcus*) remained high, even in runoff collected 8 m down slope after the third rainfall event. High levels were also detected in adjacent plots indicating lateral movement, which suggests significant risks associated with bacterial contamination (Núñez-Delgado *et al*., 2002). Standard methods such as plate counts were also used to determine bacterial counts in these studies. The application of livestock slurry should be avoided during periods when there is a high probability of major intensity rainfall events, since runoff of slurry contaminants is highly likely during such periods (Núñez-Delgado *et al*., 2002). It should be noted that buffer strips are of little use for treating contaminants that move along subsurface routes or as concentrated, channelled surface flow (Edwards and Merrilees, 2003).

2.2.2 Factors Affecting Operational Efficiency of Vegetated Filter Strips

The operational efficiency of vegetated filter strips will be dependent on the location, climatic factors such as rainfall intensity and duration, the type of vegetation and the soil properties, as texture affects the infiltration rate (Barling and Moore, 1994). The conflicting results obtained from studies observing the effectiveness of vegetated buffer strips show that factors controlling microbial transport are not sufficiently understood. This results in discrepancies in design recommendations for buffer strips. For this reason, effective buffer strips should be regularly maintained and should be used in conjunction with other on site management strategies (Barling and Moore, 1994).

2.2.2.1 Length and Slope

It has been illustrated that even relatively short filter strips can distinctly improve quality of runoff from grassed areas receiving cattle manure (Lim *et al*., 1998). Sullivan *et al*. (2007) determined

that although smaller vegetative buffers (1-3 m) removed faecal coliforms from runoff just as well as larger buffers (8-25 m), there may be other environmental benefits associated with larger buffers such as nutrient management, wildlife habitat and stream shading. It was shown that size of the vegetated buffers was not an important determinant for bacterial removal efficiency. This is in contradiction to the studies of Young *et al*. (1980) and Coyne *et al.* (1998). Young *et al*. (1980) found a reduction in total coliforms by 69% with vegetated buffer strips, however it was determined that a length of 36 m long would be required to reduce the total coliform count to recommended levels. In a study conducted by Coyne *et al*. (1998), it was determined that grass filters as short as 4.5 m were able to trap most of the bacteria and filter strips of longer than 45 m had only slightly greater trapping efficiency, however the faecal coliform concentrations greatly exceeded water quality standards when runoff occurred. These studies were however based on artificial irrigation with high rates to force soil saturation and overland flow, whereas in the study of Sullivan *et al*. (2007) natural rainfall produced overland flow and sampling was undertaken during high rainfall events. A wide variety of recommendations for optimum widths exits in literature. A range of buffer widths from 3 to 200 m were found to be effective, depending on site-specific conditions (Castelle *et al*., 1994). A width of from 5 to 15 m seems to be the most commonly used (Edwards and Merrilees, 2003). Generally smaller buffers are adequate when the buffer strip is in good condition, the adjacent land use intensity is low, and the wetland or stream has a low functional value (Castelle *et al*., 1994). The slope of the buffer strip also influences its effectiveness due to the impact on the infiltration of contaminants to the soil. The length of the filter strip should generally be increased with increasing slope to ensure that the infiltration capacity and contaminant retention are not reduced (Núñez-Delgado *et al*., 1995).

2.2.2.2 Flow Rate

Filter strips are most effective when flow is shallow and slow. Flow from hilly terrain rapidly concentrates, producing higher flow that submerges the vegetation and reduces the effectiveness of the filter strip. In such locations, grass waterways (a particular form of buffer strip) should be used more extensively as water conduits without causing concentrated flow erosion (Barling and Moore, 1994). Vegetated buffer strips become highly inefficient under channelled flow conditions (Edward and Merrilees, 2003). Field studies have been carried out to determine the effect of flow rate on the recovery of microbes from surface runoff applied to buffer strips (Collins *et al*., 2003). At high flow recovery varied between 16% and 62% for *E. coli* and between 13% and 89% for *Campylobacter*. In contrast, the recovery at slow flow ranged between <1% and 5% for both microbes indicating that the buffer strips provided for >95% attenuation. This raises important implications for buffer strip design, especially if large amounts of faecal contamination are only delivered by surface runoff during high rainfall events (Collins *et al*., 2003). The higher the rainfall precipitation, the greater the probability that the effluent applied to the site will not infiltrate into the soil, but will be rapidly removed as surface runoff with reductions in purification efficiency (Núñez-Delgado *et al*., 1995).

2.2.2.3 Vegetation

Buffer vegetation forms a physical barrier that slows surface flow rates and mechanically traps sediment and debris (Castelle *et al*., 1994). The preferred vegetation types for filter strips need to be determined based on the hydraulic characteristics and their resilience to low moisture availability while reducing corrosion and sediment transport and runoff (Barling and Moore, 1994). Native vegetation species should be chosen that are appropriate for the climate and soil. A buffer strip is considered in good condition when there is dense native vegetation and undisturbed soils (Castelle *et al*., 1994). Roodsari *et al*. (2005) showed that vegetation had a stronger influence than soil type, although both factors were important in minimising runoff of manure-borne faecal coliforms by affecting vertical transport and infiltration. A study was undertaken by Entry *et al*. (2000) to determine if maidencane (a species recommended for constructed wetlands) would be effective in reducing total and faecal coliform bacteria in swine wastewater compared with forest and grass vegetation. The total and faecal coliform numbers did not decline regardless of vegetation type or season.

2.2.2.4 Soil Properties

Buffer strips are more effective at removing sediment than nutrients from flow and are more effective at removing coarse sediment than fine sediment from the flow. This is due to the settling characteristics of the sediment as coarse sediment settles first (Barling and Moore, 1994). Edwards and Merrilees (2003) suggested that substantial faecal indicator organisms are associated with smaller clay or silt aggregates and lighter solid faecal material. This limits the efficacy of vegetated buffer strips in reducing faecal indicator organisms unless high infiltration capacities can be maintained. Continued sedimentation of coarser material leads to reductions in infiltration capacity and loss of filter strips efficiency (Edwards and Merrilees, 2003). Few studies have sought to understand how faecal indicator organisms separate with different sedimentary fractions and how particle size and mineralogy influence attachment, transport and survival in freshwater systems (Kay *et al*., 2007). Roodsari *et al*. (2005) and Sullivan *et al*. (2007) suggest that infiltration is the primary mechanism responsible for attenuation of faecal coliform surface transport. In terms of design, infiltration capacity should not be evaluated in isolation and the potential susceptibility of groundwater supplies to contamination should be kept in mind (Núñez-Delgado *et al*., 1995). In a study carried out by Collins *et al*. (2003), where field studies were used to determine the ability of buffer strips to trap *Campylobacter* and *E. coli* washed in by surface runoff, it was noted that the fate of the microbes carried within the non-recovered water was unknown and it was speculated that movement to deep groundwater and die-off within the soil water may occur.

2.3 WETLANDS

Wetlands are intermediary areas between water and land and are distinguished by wet soils, plants that are adapted to wet soils and a water table depth that maintains these characteristics (Kivaisi, 2001). Incoming nutrients support the growth of vegetation, which converts inorganic chemicals into organic materials, which forms the basis of the wetland food chain (Kivaisi, 2001). Wetlands have a wide diversity of wildlife and may protect against soil erosion along river banks. Wetland systems are effective in reducing organic and inorganic contaminants and pathogens and serve as ‘natural purifiers of water’ (Kivaisi, 2001). The effectiveness of wetlands depends on a number of mechanisms that influence bacterial removal efficiency such as the rate of effluent flow and quality, die-off rate, rate of removal by filtration (by plant roots) and sedimentation, rate of addition from transient (or resident) animal sources and the rate of predation (Perkins and Hunter, 1999).

Natural wetlands are able to provide high levels of wastewater treatment. There is however, concern regarding the long term degradation of natural wetlands due to additional nutrient and hydraulic loadings from wastewater and the potential harmful effects of pathogens and toxic materials (Kivaisi, 2001). For this reason constructed wetland technology is employed to imitate the conditions of natural wetlands. Constructed wetlands have been shown to be capable of removing a wide variety of contaminants, including bacterial pollution (Ottová *et al*., 1997). Constructed wetlands consist of two main types; surface flow and subsurface flow. Surface flow wetlands (Figure 1) are very similar to natural wetlands where water flows over the soil surface from an inlet to an outlet point (Sleytr *et al*., 2007). The water surface is exposed to the atmosphere and plants are rooted at the bottom of the wetland in some type of substrate (gravel or soils) (Gerba *et al*., 1999).

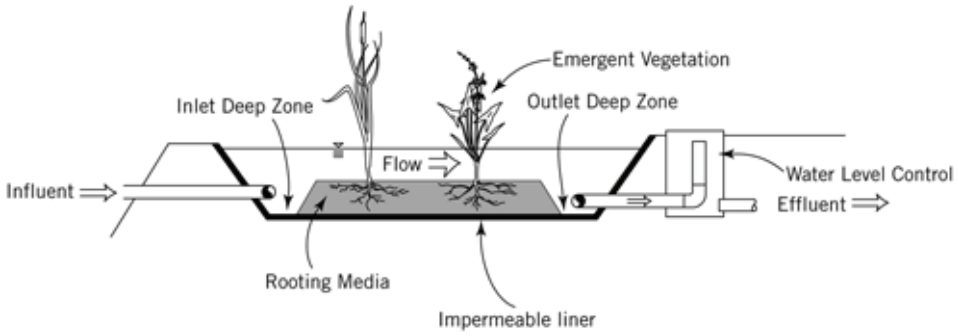

Figure 1: Small-scale surface flow constructed wetland (Wallace and Knight, 2006).

In subsurface wetlands (Figure 2), plants are not submerged in water, but rather the water typically flows horizontally through the gravel substrate, unexposed to the surface allowing a larger surface area for microbial activity (Gerba *et al*., 1999). The water travels around the roots of plants which play a role in facilitating the treatment process and are also commonly known as reed bed systems (Fyfe, 2004). Subsurface wetlands are considered more effective as greater reductions in pathogens occur (Hill and Sobsey, 2001) due to greater contact area of the water with bacteria and substrate (Sleytr *et al*., 2007).

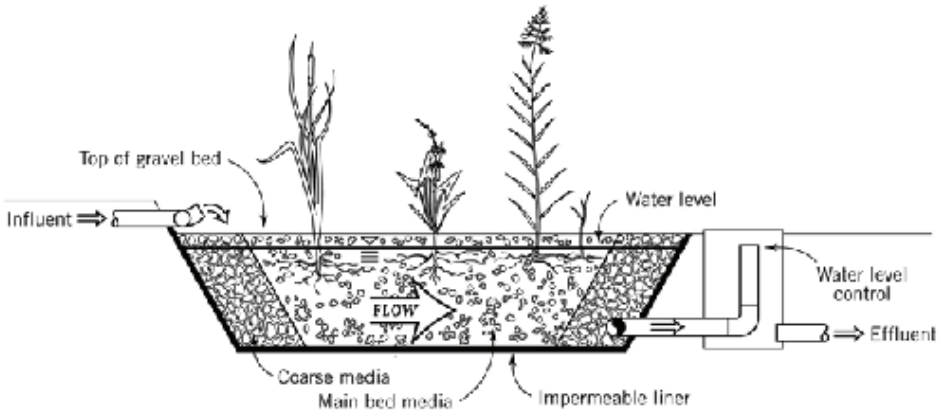

Figure 2: Small-scale subsurface flow constructed wetland (Wallace and Knight, 2006).

Wetlands are especially suited to the treatment of effluent from septic systems and have been successfully used on a variety of agricultural wastewaters (Edwards and Merrilees, 2003) and domestic wastewaters (Cooper *et al*., 1997; Schreijer *et al*., 1997). The focus of wetland research has been on the treatment of effluents rather than dealing with intermittent diffuse source pollution. A survey in the USA demonstrated the range of farm wastes treated by wetland systems, which included dairy manure, effluent/wash water, runoff from concentrated cattle feeding operations and pig and poultry manure (Knight *et al*., 2000). In order to maintain long-term health of the wetland system, the review indicated the potential need for pre-treatment stages (Knight *et al*., 2000). Caution should also be taken when retention areas are introduced such as wetlands as these represent a potential risk for children and livestock (Edwards and Merrilees, 2003)

2.3.1 Effect of Wetlands on Faecal Indicator Organisms

The effect of wetlands on the reduction of microbial contaminants has been shown for both agricultural and domestic runoff. Dairy farm effluent passing through constructed wetlands receiving a hydraulic loading rate of 0.013 m^3/m^2 per day in summer and 0.01 m^3/m^2 per day in winter reported 99.3% removal of faecal coliforms in the summer and 95.8% in the

winter (Kern *et al*., 2000). Perkins and Hunter (2000) examined the removal of enteric bacteria from partially treated sewage effluent in a surface flow constructed wetland. The mean removal efficiencies were 86-94% for faecal coliform bacteria and 83-90% for faecal *Streptococci* bacteria. Decamp and Warren (2000) compared *E. coli* removal from sewage in plot scale and laboratory microcosms with a subsurface (or horizontal) flow design. A removal rate of 41-72% was found in the lab scale studies and a much higher rate of attenuation of 97-99% in the larger plot scale studies, which may be attributed to less efficient communities developed in the microcosms. In surface flow constructed wetlands for swine wastewater, overall reductions of *Salmonella*, faecal coliforms and *E. coli* were 96, 98 and 99% respectively (Hill and Sobsey, 2001). Viral indicators were reduced by between 98 and 99%. When using a laboratory scale subsurface constructed wetland reactor, *Salmonella* and faecal coliform reductions were 99.8% and 99.9% at a loading of 19 $TKNha^{-1}d^{-1}$ and were 80 and 98% at a loading of 40 $TKNha^{-1}d^{-1}$ indicating that greater removals can be achieved using subsurface design and lower TKN (total Kjeldahl nitrogen) loading rates (Hill and Sobsey, 2001). In a pilot scale constructed wetland receiving raw wastewater, heterotrophic bacteria and coliform removal rates between the inflow and outflow were determined to be >99% (Vacca *et al*., 2005).

2.3.2 Factors Affecting Operational Efficiency of Wetlands

The main factors affecting water quality in wetlands are the hydrology of the region, vegetation and soil (Kivaisi, 2001). The processes that occur in wetlands in order for chemical and biological contaminants to be removed include sedimentation, filtration, adsorption and predation. The effectiveness of these removal processes relies on an adequate contact time with the substrate and vegetation (Hodgson *et al*., 2004) and adsorption onto soil particles (Kern *et al*., 2000). Factors such as sunlight inactivation and protozoan predation are also considered to be responsible for bacterial retention (Sinton *et al*., 2002). Microbial communities in wetland systems are strongly influenced by the conditions of operation, the filter material, and the plants (Vacca *et al*., 2005).

2.3.2.1 Effluent Flow

Effluent flow has a strong influence on bacterial removal. Hodgson *et al*., (2004) showed that bacterial removal is inversely proportional to the effluent flow. Constructed wetland beds were particularly inefficient at removing faecal *Streptococci* during high flow rates through the system. This finding is reiterated in a study on the removal of enteric bacteria in a surface flow constructed wetland for partially treated sewage (Perkins and Hunter, 2000). The key factor for retention of bacteria in wetlands is considered to be adsorption onto soil particles (Kern *et al*., 2000). Treatment systems conducive to the settlement of fine particles may effectively remove sediment bound coliphages and therefore possibly enteric viruses from storm water (Davies *et al*., 2003).

2.3.2.2 Vegetation

The presence of extensive vegetation in wetlands obstructs the flow of effluent which allows more effective sedimentation of fine particles (< 2 µm in size) that have slower settling velocities (Davies *et al*., 2003). In addition to the filtering role of macrophytes, they are also responsible for the uptake and storage of nutrients, stabilising the sediment surface to prevent soil erosion and releasing oxygen which increases degradation of organic matter (Brix, 1997). Some macrophytes have the ability to produce root exudates which are toxic to a range of bacteria, including *E. coli* (Gopal and Goel, 1993). The improved development of populations of bacteria such as *Pseudomonas* with antibiotic activity in the rhizosphere may also result in coliform die-off (Ottová *et al*., 1997). Macrophytes provide a large surface area for the development of attached microbial growth and this resulting biofilm is responsible for the majority of microbial processing that occurs in constructed wetlands (Brix, 1997). Plants typically used in constructed wetlands include rushes, cattails, curly dock, duckweed, knotgrass, smartweed, spiked bulrush, reeds, sedge and sulfuria (Woerner and Lorimor, 2003).

2.3.2.3 Planting Medium

The type of planting medium such as gravel or soil, may influence the effectiveness of subsurface wetlands in reducing coliform numbers. Decamp and Warren (2000) compared *E. coli* removal in plot scale and laboratory microcosms with a subsurface (or horizontal) flow design. A faster removal rate was observed in the planted gravel beds than the unplanted soil beds and most of the removal occurred within the first two thirds of the planted gravel pilot scale system. This is possibly due to the inflowing water being made to filter or percolate through the planted gravel bed rather than flow over the surface. Although no data on soil pore sizes were available from their study, Decamp and Warren (2000) suggested that soil substrates are likely to provide bacteria with better protection against predation than gravel substrates as it is generally found that soils have a lower porosity than gravel and therefore smaller pores.

2.3.3 Disadvantages of Constructed Wetlands

Although wetlands offer many advantages, there are also disadvantages associated with them. Wetlands are potential habitats for disease vectors such as mosquitoes, which carry malaria, filariasis, and encephalitis and snails which act as vectors for schistosomiasis (Kivaisi *et al*., 2001). Surface flow constructed wetlands provide a habitat for mosquitoes as shallow water and dense vegetation promote their activity (Russel, 1999). Mosquito management must be an integral part of wetland design and maintenance in order to reduce health hazards (Russel, 1999). Suggested steps for the prevention of mosquitoes include; the strategic removal of non-crucial floating vegetation, providing open areas for wind and easy access to mosquito larvae for predator fish, the introduction of nematodes parasitic to mosquitoes, the application of low organic loading to avoid anaerobic conditions in the water and the application of chemical agents that have no residual effect in the environment (Kivaisi, 2001). The provision of fish such as *Gambusia* species will harvest nuisance insects and maintain an acceptable insect population. As the wetland system develops there will also be a natural immigration of other predator species such as frogs and newts (although newts do not occur in South Africa) which will further contribute to insect control (Wood, 1995). The surface plant or litter mass also play a role in limiting the development of insects such as mosquitoes and gnats in water that has ponded on the surface by absorbing the wastewater into the litter mass and over-shading any open water (Wood, 1995).

All wetlands have their own characteristic odour which varies depending on the quantity of influent wastewater and dissolved oxygen (Kivaisi, 2001). Plants can play a role in limiting odours as the plants and the associated litter layer act as natural odour biofilters. This allows wetlands to be safely positioned relatively close to the community they serve (Wood, 1995).

2.3.4 Constructed Wetlands in an African Context

In most developing countries there are few wastewater treatment facilities. This is mainly due to the high costs of the treatment processes and lack of effective environmental pollution control law enforcement. Constructed wetland systems are not extensively used due to lack of awareness and local expertise in developing the technology (Kivaisi, 2001). A study was undertaken in South Africa to evaluate a pilot scale subsurface flow wetland for the treatment of primary settled sewage 10 years after commissioning (Batchelor and Loots, 1997). It was noted prior to this undertaking that research programmes on constructed wetlands initiated in South Africa were generally based on European and American experiences (Batchelor and Loots, 1997). When a nitrifying column was introduced after the subsurface flow followed by a surface flow wetland for polishing, it was found that effluent complied with South African general standards for nutrient removal efficiency. The effect on microbial attenuation was not examined in this study. Constructed wetlands are low cost, easily operated and maintained and have a strong potential for developing countries, particularly in small rural communities (Kivaisi, 2001). Most developing countries have warm tropical or subtropical climates with a rich diversity of biota that are conducive to higher biological activity and productivity resulting in better wetland system performance (Kivaisi, 2001). An example

of a successful wetland system in an African scenario is that of a 0.5 ha wetland designed in Nairobi, Kenya, for the treatment of restaurant and swimming pool resort wastewater. The combined pond and subsurface wetland functioned well in terms of wastewater treatment, with faecal coliform removal efficiencies of 99.4-99.9% (Nyakang'o and Van Bruggen, 1999). The wetland was designed to treat wastewater as well as to provide an aesthetically pleasing landscape. Constructed wetlands are potentially well-suited to smaller communities where municipal land surrounding schools, hospitals and hotels and rural areas is not in short supply (Kivaisi, 2001).

2.4 PRE-TREATMENT MICROBIAL MITIGATION MEASURES

Pre-treatment mechanisms are often required before discharging to a secondary treatment technology to remove nutrients, solids and pathogens. The more easily collectable materials are removed which allows for polishing of effluent in the secondary treatment.

2.4.1 Waste Stabilisation Ponds/Farm Ponds

Waste stabilisation ponds reduce organics, nutrients and pathogens in wastewater to produce an effluent suitable for reuse or disposal (Fyfe, 2004). They are commonly used in the agricultural sector for treating livestock wastes. They are similar mitigation measures to vegetated buffer strips and wetlands in that they rely on retention to attenuate faecal microbes. Waste stabilisation ponds are mostly used in combination with subsequent effluent treatment processes such as constructed wetlands to produce a less polluting effluent prior to disposal (Fyfe, 2004). The mechanisms responsible for treatment in waste stabilisation ponds include settling, bacterial decomposition (anaerobic and aerobic) and precipitation (Fyfe, 2004). Ponds used for livestock operation waste management can be aerobic, anaerobic or facultative. Specifically designed waste stabilisation ponds are considered to be effective in removing pathogenic bacteria (Von Sperling, 2005). Larger protozoan pathogens are removed from ponds due to sedimentation. Bacteria are eliminated by processes of ultraviolet (UV) radiation, high pH and predation within the pond environment (Oliver *et al*., 2007). The depth of the pond is an important factor as it influences the dissolved oxygen concentrations and has an impact on the penetration of UV radiation through the water (Von Sperling, 2005). Viruses are difficult to remove by facultative ponds and as the potential infective dose is very low, this poses a bigger threat (Kivasi *et al*., 2001). Untreated ponds are often viewed as hazardous contaminant sources within farm environments as they receive and harbour contaminated surface runoff, septic tank effluents and faecal material (Malaney *et al*., 1962). Farm ponds are a rich source of pathogens such as *Salmonella* and *Campylobacter* from livestock and may provide birds with pathogen-contaminated water for consumption and bathing, resulting in transfer of these pathogens elsewhere (Jones, 2005).

2.4.2 Soil Infiltration Areas

Soil infiltration areas are used as a pre-treatment mechanism to condition waste before it enters a vegetated filter strip or constructed wetland. A soil medium is loaded with runoff allowing the liquid to infiltrate the soil. Field drainage tile may be used to intercept the filtrate and carry it to a secondary form of treatment (Woerner and Lorimor, 2003). Research into pathogen transport and fate in porous media or soil-based wastewater treatment systems has been limited (van Cuyk *et al*., 2004), although the benefits for the removal of nutrients has been shown (Prantner *et al*., 2001). Research has been undertaken to quantify the removal of viruses and bacteria using microbial tracers in soil treatments systems. A soil infiltration site for wastewater application that had been in continuous operation for over 30 years was examined for the accumulation of a tracer virus, indigenous enteroviruses, and enteric indicator bacteria in the soils and underlying groundwater (Schaub and Sorber, 1977). Indigenous and tracer virus were sporadically detected in the groundwater at horizontal distances of 183 m from the application zone. Enteric indicator bacteria were readily concentrated on the soil surface mat, however comparison studies with

faecal *Streptococcus* revealed that bacteria were capable of penetrating the surface and were able to migrate to the groundwater where the enteric and tracer viruses were detected (Schaub and Sorber, 1977). Van Cuyk *et al*. (2004) observed a 2 to 3 log (99-99.9%) removal of virus and a near complete removal of faecal coliform bacteria during soil treatment of wastewater with an unsaturated flow through 60-90 cm of sandy medium. Episodic release of virus and bacteria was observed during the early period of operation. It was suggested that this may be attributable to an underdeveloped biomat at the infiltrative surface (van Cuyk *et al*., 2004). A biomat can also be referred to as a clogging zone which develops at the soil infiltrative surface. This leads to reduced permeability, more uniform infiltration and an unsaturated flow (van Cuyk *et al*., 2001). The clogging layer develops from precipitated and filtered solids and from biological processes within the biomat. The clogging layer is unfavourable as it lowers the hydraulic conductivity, however it has the advantage of creating an excellent biofilter that enhances the treatment efficiency of the system (Blazejewski and Murat-Blazejewska, 1997). In wastewater soil infiltration systems, clogging must therefore occur to a balanced extent in order to allow for the advanced purification that is required without becoming hydraulically too restrictive (van Cuyk *et al*., 2001). The same problem is encountered in the maintenance of constructed wetlands with subsurface flow (Blazejewski and Murat-Blazejewska, 1997). In a study assessing the removal of bacteriophages from wastewater in septic tank drain fields it was observed that significant increases in bacteriophage elution occurred with rainfall during the first 10 days of the study, indicating that soils are limited in their ability to properly assimilate sewage effluent (Nicosia *et al*., 2001).

2.4.3 Settling Basins

Solids removal with the use of settling basins is considered the first treatment of any open feedlot runoff treatment system before transferring to a secondary treatment technology, and is currently required by many USA state laws (Woerner and Lorimer, 2003). The settling basin works on the basis that the microbial quality of water can be improved by holding or storing it undisturbed without mixing for long enough to allow larger particles to sediment or settle out with gravity. The settled water can then be carefully removed and recovered by decanting without disturbing the sedimented particles (Sobsey, 2002). Storing water for a few hours will allow large, dense particles, such as inorganic sands and silts, and any microorganisms associated with larger, denser particles to sediment out. Clay particles and smaller microorganisms will, however, not settle under these conditions (Sobsey, 2002). Solids in manure from Australian feedlots were examined and two components were observed; large particles that settled rapidly within ten minutes and small particles that required extremely long settling times (Lott *et al*., 1994). It has been found that longer settling times of 1-2 days will remove larger microbes, including helminth ova and some protozoan parasites, certain algae, and larger clay particles. Reductions of helminth ova and some protozoans can exceed 90% at this storage time (Sobsey, 2002). Most viruses and bacteria and fine clay particles are too small to be settled out by simple gravity sedimentation. Therefore, sedimentation is not consistently effective for reducing microbial contamination. Overall reductions of viruses and bacteria by sedimentation rarely exceed 90% (Sobsey, 2002). Properly designed and managed solid settling basins should remove about 30% of nitrogen and phosphorous from the runoff of swine lots and approximately 80% from bovine lot runoff (Woerner and Lorimor, 2003). Settling improves the turbidity of the water which makes it more amenable to treatment by other methods such as increased UV penetration from sunlight and a decreased demand for oxidants (e.g. chlorine) (Sobsey, 2002).

2.5 LAND MANAGEMENT ENGINEERING

In order to prevent the transfer of microbial contamination to surface waters, there is an option to engineer the land to reduce, but not eliminate the delivery of faecal microbes (Oliver *et al*., 2007). The introduction of geographic information systems (GIS) has dramatically improved visual modelling approaches to land management (Oliver *et al*., 2007). Heathwaite *et al*. (2005) have used modelling to manage critical source areas using flow connectivity simulation by combining critical source areas and flow accumulation models. The model output emphasises the impact of

land management features such as tramlines, tracks and field drains in concentrating flow towards receiving waters and increasing the risk of delivery of pollutants derived from diffuse agricultural sources (Heathwaite *et al*., 2005). Flow connection modelling also allows for assumptions to be made for flow directions due to the contours derived from digital terrain models (Lane *et al*., 2004). If landscape engineering can be employed to disconnect flows then contaminants will also be disconnected from surface waters (Oliver *et al*., 2007). Field characterisation cannot be carried out by GIS modelling alone. It order to obtain a more realistic understanding a combination of field visits, farmer interviews and nationally available data sets at different spatial resolutions are required (Oliver *et al*., 2007).

2.6 CONCLUSIONS

It is of utmost importance to protect catchment areas from contamination arising from human activities and animal sources. The understanding of faecal indicator organism fate and transport at the catchment level is rudimentary and further understanding of the microbial dynamics in terrestrial ecosystems is required (Kay *et al*., 2008). Studies that have been documented on wetlands, for example, are usually limited to total and/or faecal coliform removal, measured in terms of colony forming units or most probable number techniques in samples from the inflow and outflow of wastewater. These standard techniques are sensitive and well-established but provide evidence only on the presence or absence of bacteria and not on the community structure (Vacca *et al*., 2005). Due to landscapes being complex, the likelihood of a single management strategy providing complete protection of receiving waters from microbial contamination is minimal. Comprehensive multi-stage strategies of mitigation are therefore essential for minimising risk (Oliver *et al*., 2007). More knowledge is required on how combinations of the available intervention strategies, such as vegetated buffer strips and wetlands, perform in reducing faecal indicator organism delivery at the catchment scale (Kay *et al*., 2008). Integrated mitigation strategies should successfully limit microbial contamination of water by removing or significantly reducing source, transfer or delivery components so that microbial contamination of surface runoff does not occur (Oliver *et al*., 2007).

2.7 RECOMMENDATIONS FOR FUTURE RESEARCH

- More knowledge is required on how combinations of the available intervention strategies perform in reducing faecal indicator organism delivery at the catchment scale, therefore comprehensive multi-stage strategies should be evaluated.

- As research programmes on constructed wetlands initiated in South Africa are generally based on European and American experiences, it is foreseen that pilot studies could be initiated where mitigation approaches can be thoroughly evaluated under local conditions.

- Where research programmes on constructed wetlands were initiated in South Africa, the effects on microbial attenuation were not examined; therefore there is a need to evaluate mitigation approaches with regards to microbial attenuation.

- Further investigation of microbial dynamics is required in order to obtain an understanding of microbial fate and transport at the catchment level.

CHAPTER 3: APPLICABILITY OF NEW MOLECULAR APPROACHES

3.1 INTRODUCTION

In South Africa many water users are impacted by the poor microbial quality of surface waters. A major contributor to the poor quality is a general lack of sanitation services or the poor maintenance of existing services in many areas. The production of treated drinking water and water for recreation and irrigation are some of the uses affected but informal or developing communities without access to basic water service are the most severely impacted. They have to rely on untreated surface water for most if not all of their domestic needs. In these communities water-borne diseases are quite widespread and often difficult to control (Lonnen *et al.,* 2005; Toze*,* 1999; Boyle *et al.,* 2008).

The microbial quality of water and its potential effect on human health are in most cases only assessed by the analysis of faecal indicator microorganisms, mainly faecal coliforms or *E. coli*. Heterotrophic plate counts could also be performed to obtain an estimate of the total bacterial load in the water. In exceptional cases the water may also be tested for the presence of specific pathogens but (in our experience) it is unlikely that these analyses would cover the full range of bacterial pathogens that could potentially be present.

Health impact assessments provide crucial information required for the development of catchment management strategies and water safety plans. Assessment of the potential human health impact of surface waters will be greatly improved if a better estimation of all the pathogens present in the water can be made. Accurate methods to estimate the diversity of parasites and viruses are not yet available but progress has been made in terms of estimating the bacterial diversity present in water. The current methods used such as 16S rRNA cloning, DGGE, T-RFLP and FISH is based on a variety of molecular or sequencing techniques. These methods have, however, been reported to give an incomplete overview of the microbial diversity when a sample containing a complex microbial community is analyzed, as they tend to favour the more abundant taxa. New sequencing technologies, however, seem to resolve this problem by by-passing cloning and culturing steps that may result in these biases (Armougom & Raoult, 2009; Claesson *et al.,* 2009).

Pyrosequencing is one of the recently developed high-throughput sequencing approaches and was released by 454 Life Science. This method allows the sequencing of a large number of mixed PCR products such as the partial 16S rRNA sequences in parallel (Claesson *et al*., 2009). One PicoTiterPlate™ can provide an estimated 200 000 useful reads that can be further analysed (Huse *et al*., 2007). The huge amount of reads (pyrotags) that are produced in one pyrosequencing reaction yields great sampling depth and provides a better picture of the size and diversity of a particular community.

Due to the vast amount of data and the relatively short read lengths that are produced by pyrosequencing, it is almost impossible to construct any form of a tree to determine the phylogenetic position and identity of the pyrotags. In response, some tree independent tools have been developed to assist in the assigning of the sequences to specific taxonomic groups. These tools include the Greengenes classifier and the RDP-II classifier. When using tree-based methods, the tree seems to vary depending on the gene used, whereas with the tree-independent tools, a stable and precise taxonomic assignment can be made although in most cases only to genus level (Armougom & Raoult, 2009).

In light of the large number of pyrotags that can be obtained in one sequencing run, samples are often pooled without losing any information on species diversity. Pooling of samples also helps to reduce the analysis costs. The largest number of independent samples that a picotiter plate can take is sixteen, as the plate can be divided into only sixteen regions. A bar-coding strategy has, however, been developed to accommodate more samples per plate. For this application the primer that is used in the original amplification step has various sections. It has a universal 454 adaptor,

followed by a barcode or multiplex identifier of between 2 to 12 nucleotides in length. Finally the primer contains the specific sequence required for the amplification of the targeted region (Armougom & Raoult, 2009; Claesson *et al*., 2009)

This approach has been used for a number of applications and types of samples. Huber and colleagues used pyrosequencing to sequence over 900 000 V6 amplicons of bacterial and archaeal origin from two different ocean sites (Huber *et al*., 2007). Humblot & Guyot (2009) used the same approach to determine the bacterial diversity in fermented foods by sequencing the V3 region. Most of the studies thus far using the pyrosequencing approach have focused on the bacterial diversity within the human gut microbiome (Claesson *et al*., 2009).

High-throughput methods for the analysis of bacterial microbial diversity do not always make use of a sequencing approach. DNA microarrays can detect many genes and this technique has been used extensively in gene expression profiling. It has since also proved successful in identifying bacteria as well as determining the diversity within a microbial community. A number of phylogenetic-based arrays that allow hybridization of target DNA to a single-stranded probe that correspond to either full or partial 16S sequences have been developed (Claesson *et al*., 2009). The Lawrence Berkeley National Laboratory developed an Affymetrix microarray product called the PhyloChip. The PhyloChip has been used to identify up to 8900 unique microorganisms from various environments in one experimental run. The PhyloChip contains hundreds of 16S rRNA based probes (15- to 25-mer) that are fixed on a glass slide. The DNA is fluorescently labelled during amplification of the target DNA in the sample and then hybridized to the probes which are attached to the solid surface. The slide is then viewed under a fluorescence microscope that is fitted with a cooled charge-coupled device (CCD) camera to detect all the hybridization reactions. These data are then processed and a list of all the known species present in a sample is compiled. Novel bacterial species can unfortunately not be detected with this approach (Andersson *et al*., 2008; Armougom & Raoult, 2009).

During this study the applicability of two new molecular approaches (pyrotags and phylochips) for determining the true bacterial diversity of surface waters at catchment level was determined. The results were also compared with the more widely used Denaturing Gradient Gel Electrophoresis (DGGE) analysis widely used in this type of study (Gianazza *et al*., 1998; Burtscher *et al*., 2009). As this was merely a scoping study to evaluate applicability, only a limited number of samples were analyzed. The samples for analysis were taken from an area representative of conditions in many South African catchments that experience definite microbial water quality problems (Olifants River catchment).

3.2 MATERIALS AND METHODS

3.2.1 Sampling and analyses

One of the sub-catchments in the larger Olifants River catchment was selected for sampling. Three water samples were collected at different points along the same river. Sample A was collected upstream from an informal settlement near Ogies. The sample was collected in an area that drained the coal mine, upstream of the settlement. Sample B was collected within the settlement close to the sewerage works. Signs of regular contamination of the river with raw sewage as well as normal drainage from the settlement were clearly evident. Sample C was collected downstream of the settlement. The three samples were representative of the following conditions: Sample A – no faecal pollution, Sample B – heavy faecal pollution, Sample C – heavy faecal pollution, with a degree of natural remediation. The GPS coordinates for the samples were recorded. All three samples were analyzed for electric conductivity, iron, nitrate and nitrite, pH, total dissolved solids, total organic carbon and the level of *E. coli* present in the sample was also determined.

3.2.2 DNA extraction and 16S rRNA amplification

For the molecular analyses the samples were concentrated by filtering and subsequently resuspended into 20 ml of PBS (20 mM). Ten ml of sample A and 1 ml of sample B and C were then centrifuged at 7500 rpm for 10 minutes. The resulting pellet was resuspended in 1 ml PBS (10 mM) as part of a washing step. The samples were once again centrifuged at 7500 rpm for 10 minutes. DNA was extracted with the Genomic DNA Tissue Mini-Prep Kit (Zymo Research) according the manufacturer's instructions. The DNA wash-step was, however, performed twice instead of once. This kit allows quick purification of total genomic DNA as the sample is digested with proteinase K and chemically lysed. Cell lysis was followed by modern fast-Spin column purification technology. The kit is reported to remove any PCR inhibitors that may be present. Five such extractions were performed for sample B and C.

The extracted DNA was run on a 1% agarose gel at 90 V for 30 minutes to verify the success of the extractions. A 16S PCR was performed on all the samples (A, B, & C) with primers pA and pH (Edwards *et al.,* 1989) (Table 1). Each 50 µl PCR reaction contained 5 µl of 10 x Taq Buffer , 2 mM $MgCl_2$, 0.25 mM dNTP's, 0.1 µM of each primer, 1.5 U Super-Therm DNA Polymerase (Southern Cross), 1 µl of extracted DNA and nuclease free water (NFW) up to the final reaction volume. The PCR was performed as follows: An initial denaturation step at 94°C for 10 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 1 min. A final extension at 72°C for 5 min was included which was then cooled to 4°C. Amplification was verified by running the samples on a 1% agarose gel as described above.

3.2.3 DGGE analysis

The bacterial diversity in the water samples was determined using denaturing gradient gel electrophoresis (DGGE) followed by cloning and sequencing of prominent bands. A 16S PCR was performed with primers pA8f+GC and PRUN518r (Fjellbirkeland *et al*., 2001) (Table 1) on the DNA extracts. Each 50 µl PCR reaction contained 5 µl of 10 x Taq Buffer that included 15 mM $MgCl_2$, 0.2 mM dNTP's, 0.2 µM of each primer, 1.5 U Super-Therm Gold DNA Polymerase (Southern Cross), 1 µl of extracted DNA and NFW up to the final reaction volume. The PCR was performed as follows: An initial denaturation step at 95°C for 10 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 1 min. A final extension was included at 72°C for 10 min, which was then cooled to 4°C. Amplification was verified on a 1% agarose gel as described in Section 3.2.2.

Table 1: Primers used in this study

Primer name	Primer sequence	Reference
pA	5’ AGAGTTTGATCCTGGCTCAG 3’	Edwards *et al.,* 1989
pH	5’ AAGGAGGTGATCCAGCCGCA 3’	Edwards *et al.,* 1989
27F.1	5’ AGRGTTTGATCMTGGCTCAG 3’	DeSantis *et al.,* 2007
1492R	5’ GGTTACCTTGTTACGACTT 3’	DeSantis *et al.,* 2007
A1.1	5'CGTATCGCCTCCCTCGCGCCATCAG-ACGAGTGCGT-AGRGTTTGATCMTGGCTCAG 3'	DeSantis *et al.,* 2007 (27F.1)
A1.2	5'CGTATCGCCTCCCTCGCGCCATCAG-AGACGCACTC-AGRGTTTGATCMTGGCTCAG 3'	DeSantis *et al.,* 2007 (27F.1)
A1.3	5'CGTATCGCCTCCCTCGCGCCATCAG-ATCAGACACG-AGRGTTTGATCMTGGCTCAG 3'	DeSantis *et al.,* 2007 (27F.1)
A2.1	5' CGTATCGCCTCCCTCGCGCCATCAG-ACGCTCGACA-GTGCCAGCMGCNGCGG 3'	Dowd *et al.,* 2008 (530F)
A2.2	5' CGTATCGCCTCCCTCGCGCCATCAG-CGTGTCTCTA-GTGCCAGCMGCNGCGG 3'	Dowd *et al.,* 2008 (530F)
A2.3	5' CGTATCGCCTCCCTCGCGCCATCAG-CTCGCGTGTC-GTGCCAGCMGCNGCGG 3'	Dowd *et al.,* 2008 (530F)
B1	5' CTATGCGCCTTGCCAGCCCGCTCAG-GTATTACCGCGGCTGCTG 3'	Coenye *et al.,* 1999 (pD)
B2	5' CTATGCGCCTTGCCAGCCCGCTCAG-ACGAGCTGACGACARCCATG 3'	Sundquist *et al.,* 2007 (1073R)
pA8f+GC	5’AGAGTTTGATCCTGGCTCAG-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG3’	Fjellbirkeland *et al.,* 2001
PRUN518r	5’ATTACCGCGGCTGCTGG3’	Fjellbirkeland *et al.,* 2001
pA8f	5’AGAGTTTGATCCTGGCTCAG3’	Fjellbirkeland *et al.,* 2001
Sp6	5’ATTTAGGTACACTATAG3’	Brelot *et al.,* 1997
T7	5’AATACGACTCACTATAGG3’	Brelot *et al.,* 1997

The samples were separated on a DGGE gel containing 8.1% acrylamide / bisacrylamide solution (Sigma), 1 x TAE and a linear gradient of 30% to 55% denaturant, where 100% denaturant was equal to 7 M Urea and 40% formamide. Electrophoresis was performed at 60°C, running the gel at 200 V for approximately 3 hours. The gel was stained in a 1:1000 (v/v) SYBRGold (Invitrogen) : 1 x TAE buffer solution for 20 min and was visualized on a dark reader. The prominent bands were excised and placed in 30 µl NFW at 4°C and were left overnight. They were re-amplified with primers pA8f and PRUN518r (Fjellbirkeland *et al*., 2001) (Table 1) as described above. Amplification was verified on a 1% agarose gel as described in Section 3.2.2. The PCR samples were cleaned by adding 20 U of Exonuclease I, *E. coli*, (Fermentas) and 4 U FastAP™ Thermosensitive Alkaline Phosphatase (Fermentas) to the PCR reactions and subsequently incubated at 37°C for 15 min and thereafter at 85°C for 15 min.

The bands of samples A, B & C were cloned using the pGEM®-T Easy kit (Promega) that relies on blue-white colony selection. As part of the cloning process, a ligation reaction was performed by adding 5 µl of a 2 x ligation buffer, 50 ng of the vector, 3 U T4 DNA ligase, 0.5 µl of PCR product and NFW to a final reaction volume of 10 µl. A positive control was included by adding 8 ng control insert provided with the kit. Following overnight incubation of the ligation reaction at 4°C, a transformation was performed by adding 5 µl of the ligation reaction to 100 µl of competent *Escherichia coli* DH5α cells. The tubes were put on ice for 30 min and then heat-shocked at 42°C for 90 sec. The tubes were returned to ice for 2 min. LB Broth (800 µl; pH 7.0) was then added to the tubes and they were subsequently incubated for 1 hour at 37°C while shaking at 200 rpm. Two hundred µl of the transformation reaction was then plated on LB plates supplemented with

Ampicillin (50 mg/ml), and X-Gal (800 µl of a 0.05 M stock solution/500 ml LB agar). The plates were incubated for 24 hours at 37°C. A PCR was performed on the resulting white colonies with primers Sp6 and T7 (Brelot *et al*., 1997) (Table 1) to amplify the insert. The PCR products were run on a 1% agarose gel as described in Section 3.2.2 to verify that an insert of the correct size (~500 bp) was present. The PCR products were cleaned as described above and the sequencing was performed using the BigDye terminator kit (Applied Biosystems). The reaction consisted of 2 µl BigDye 3.1 reaction mixture, 1 µl 1 x Sequencing buffer, a final concentration of 3 µM primer SP6 (Brelot *et al*., 1997), 4 µl DNA and NFW to a final reaction volume of 10 µl. The conditions of the amplification was as follows: An initial denaturation step at 96°C for 5 sec, followed by 25 cycles of denaturation at 96°C for 10 sec, annealing at 55°C for 5 sec & extension at 60°C for 4 min, followed by cooling to 4°C.

Sodium acetate precipitation was performed on the samples by adding 16 µl 100% EtOH and 2 µl 3 M Sodium Acetate (pH 4.8) to the 10 µl sequencing reaction and centrifuging it at maximum speed for 30 min. The supernatant was removed and a wash step was performed on the pellet by adding 150 µl 70% EtOH and centrifuging it at maximum speed for 5 min. The wash-step was repeated and the pellet was allowed to dry at 90°C for 3 min before the product was sequenced on the ABI 3130 sequencer. A BLAST search of the sequences against the NCBI website were conducted to obtain some idea of the identity of the bacteria present in the various samples.

3.2.4 Phylochips

Twenty five µl of extracted DNA of all the samples was precipitated by sodium acetate precipitation as described in Section 3.2.2. The precipitated DNA was sent to the Lawrence Berkeley National Laboratory for PhyloChip analysis. The analyses were performed according to the method and approach described by Brodie and coworkers (Brodie *et al*., 2006; Brodie *et al*., 2007). Once the final analysis was received from the laboratory the taxonomic units identified in the different samples were grouped according to phyla, classes, orders and families.

3.2.5 Pyrosequencing

A PCR reaction was performed on all the extracted DNA samples (A, B1, B2, B3, B4, B5, C1, C2, C3, C4, C5 & C6) with primers 27F.1 and 1492R (DeSantis *et al.,* 2007) (Table 1). These primers are degenerate and would amplify a greater diversity of microbes. Each 50 µl PCR reaction contained 5 µl of 10 x Taq Buffer (supplied with the Taq polymerase), 2 mM $MgCl_2$ (supplied with the Taq Polymerase), 0.25 mM dNTP's, 0.1 µM of each primer, 1.5 U Super-Therm DNA Polymerase (Southern Cross), 1 µl of extracted DNA and NFW up to the final reaction volume. The PCR was performed as follows: An initial denaturation step at 94°C for 10 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 1 min. A final extension was included at 72°C for 5 min that was then cooled to 4°C. Amplification was verified by running the samples on a 1% agarose gel as described in Section 3.2.3.

The entire PCR reaction was loaded on a 1% agarose gel and the correct band size (approximately 1500 bp) was excised. The DNA was recovered from the gel slices by using the GeneJET™ gel extraction kit (Fermentas), which cleans and purifies DNA from a gel slice. During cleaning the DNA binds to the membrane in a spin-column which is then eluted.

The DNA was subsequently amplified with sets of primers that contained the appropriate adaptor and barcode sequences that were necessary for 454 pyrosequencing as set out in Table 2. Each 50 µl PCR reaction contained 5 µl of 10 x Taq Buffer, 2 mM $MgCl_2$, 0.25 mM dNTP's, 0.1 µM of each primer, 1.5 U Super-Therm DNA Polymerase (Southern Cross), 1 µl of extracted DNA and NFW up to the final reaction volume. The PCR was performed as follows: An initial denaturation step at 94°C for 10 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 50 or 56°C (Table 2) for 1 min & extension at 72°C for 1 min. A final extension at 72°C for 5 min was

included, that was then cooled to 4°C. Amplification was verified by running the samples on a 1% agarose gel as described above.

Table 2: Primer pairs and annealing temperatures used for all samples.

Sample Name	Primer pair used	Region Amplified	Annealing Temperature	Sample Name	Primer pair used	Region Amplified	Annealing Temperature
A1	A1.1 & B1	V1-3	50°C	A1	A2.1 & B2	V4-7	56°C
A2				A2			
B1	A1.2 & B1			B1	A2.2 & B2		
B2				B2			
B3				B3			
B4				B4			
B5				B5			
C1	A1.3 & B1			C1	A2.3 & B2		
C2				C2			
C3				C3			
C4				C4			
C5				C5			
C6				C6			

The entire PCR product was run on a 1% agarose gel as described in Section 3.2.2. The correct band size (500-600 bp) was excised from the gel and subsequently purified as before. The DNA concentrations were once again quantified by using a Nanodrop. The various samples were pooled with a total concentration of 1000 ng of DNA where A:B:C were equal. The sample was sent in for pyrosequencing on the GS-FLX-titanium series. The resulting data was analyzed using the RDP Pyro-pipeline on the RDP-database.

3.3 RESULTS AND DISCUSSION

3.3.1 Sampling and analyses

The three water samples were collected and analysed as described above. The results for the chemical and microbiological analyses are shown in Table 3. It is clear from the data that Samples B and C were heavily polluted with high organic and *E. coli* levels. These results were expected as it was noted that raw sewage was flowing into the stream due to a dysfunctional sewage pump station, meters above the point where Sample B was collected. Although Sample C was collected downstream after the stream has flowed through an uninhabited area, the water quality showed no sign of improvement.

Table 3: Chemical and microbiological results for the samples collected near Ogies.

Parameter	Unit	Sample ID		
		A	**B**	**C**
Electrical Conductivity	mS/m [25°C]	99.4	35.3	48.2
Iron	mg/l Fe	0.22	1.1	0.87
Nitrate + Nitrite (N)	mg/l N	<0.2	0.33	0.54
pH	pH units [25°C]	4.1	7.3	7.5
TDS	mg/l [180°C]	641	230	271
TOC	mg/l C	1.7	4.6	6.6
E.coli	MPN per 100ml	11.9	>2419.6	>2419.6

3.3.2 DGGE

The DGGE of the sample was successful and an idea of the bacterial diversity within the water samples could be obtained. Due to the possibility of co-migration of the PCR products, the resulting bands were cloned in order to separate the various sequences. Only one sequence can ligate in the vector during cloning and when random colonies are picked, one should obtain most of the sequences that had co-migrated.

A total of 13, 18 and 13 bands were excised from the DGGE gel of samples A, B and C, respectively. The bands of samples A, B & C have been successfully cloned, sequenced and a BLAST search against the NCBI database was performed and the sequences could all be assigned to specific phyla. The sequences of sample A were all either assigned to the Proteobacteria or the Cyanobacteria. Samples B and C were all assigned to the Proteobacteria or the Bacteroidetes which are consistent with the dominant phyla detected with the 454 pyrosequencing. This technique is therefore only applicable if an idea of the dominant phyla within a population is required.

3.3.3 Phylochip

The latest version of the Phylochip is not available commercially. Negotiations with Dr. Anderson at Lawrence Berkeley National Laboratory to cooperate on this study have been successful and the DNA samples were shipped at the end of May 2010. The results were analysed and comparisons between the three different samples were made. Based on a presence/absence analysis no clear distinction could be seen between samples A, B & C. This is illustrated in the phylum breakdown of the phylochip data (Figure 3). This is due to the phylochip's ability to primarily detect presence or absence without analysing the data to get an idea of abundance. This data illustrated that there was little difference in the distribution of taxonomic units between the three samples. This must be seen in view of the fact that the data does not differentiate the samples in terms of taxonomic unit abundance.

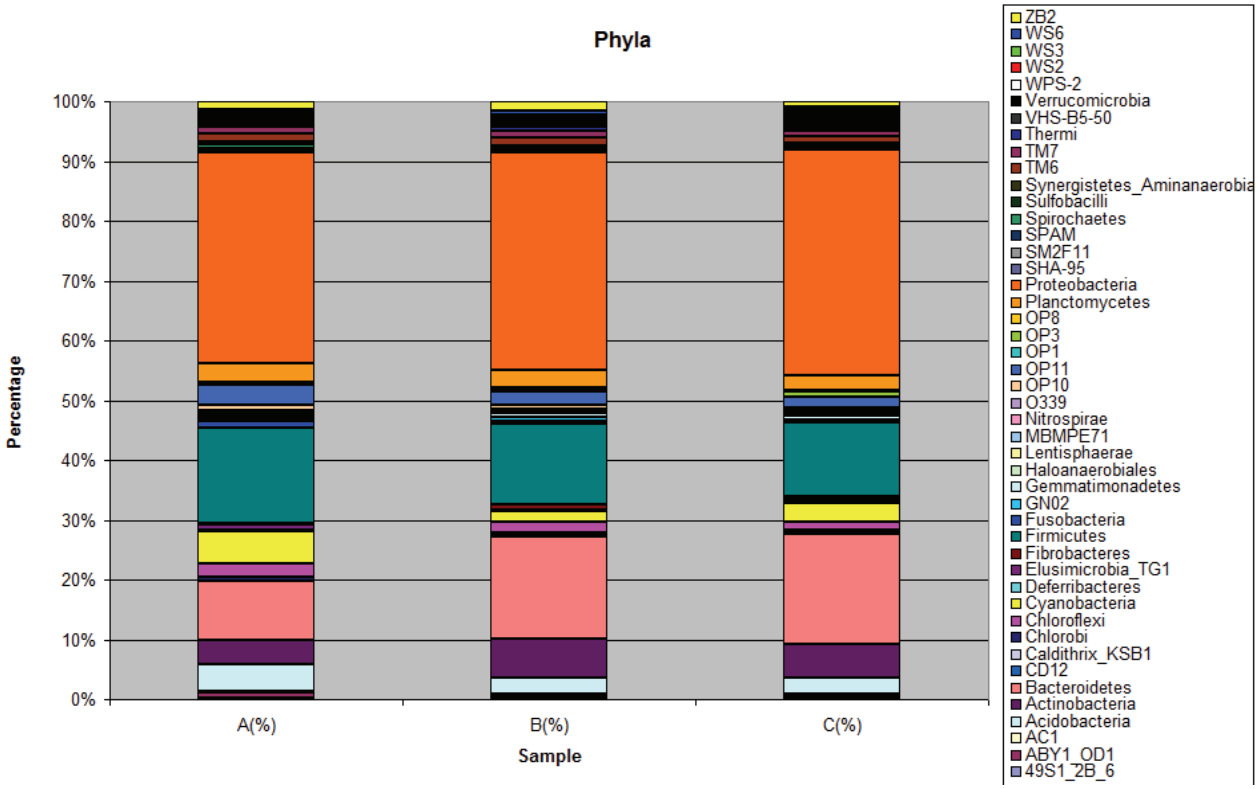

Figure 3: Breakdown of the Phyla present in samples A, B & C as detected by PhyloChip presence / absence analysis.

The phylochip can, however, provide some indication of abundance if the specific hybridization reactions on the array are compared to the average total array intensity. Using a heatplot it was possible to compare the top 200 taxonomic units that differed most between the samples (Figure 4). Although the specific names of the taxonomic units are not displayed individually, Figure 4 showed clearly that many taxonomic units shared the same abundance between Samples B and C but that the abundance profile of Sample A differed markedly from the other two samples.

The list of 200 taxonomic units (depicted in Figure 4) used for this analysis are provided in Appendix A. From this information it can be seen that some members of the Enterobacteriaceae, Bradyrhizobiales, Planctomycetales and Cyanobacteria were more abundant up-stream of the point of contamination. After contamination, groups such as the Flavobacteriales, Sphingomonodales, and Burkholderia were far more dominant. Members of the Morxellaceae and Actinobacteria such as *Arthrobacter* were most abundant in Sample B but were not prominent in sample C. This data compared well with the pyrosequencing data discussed below.

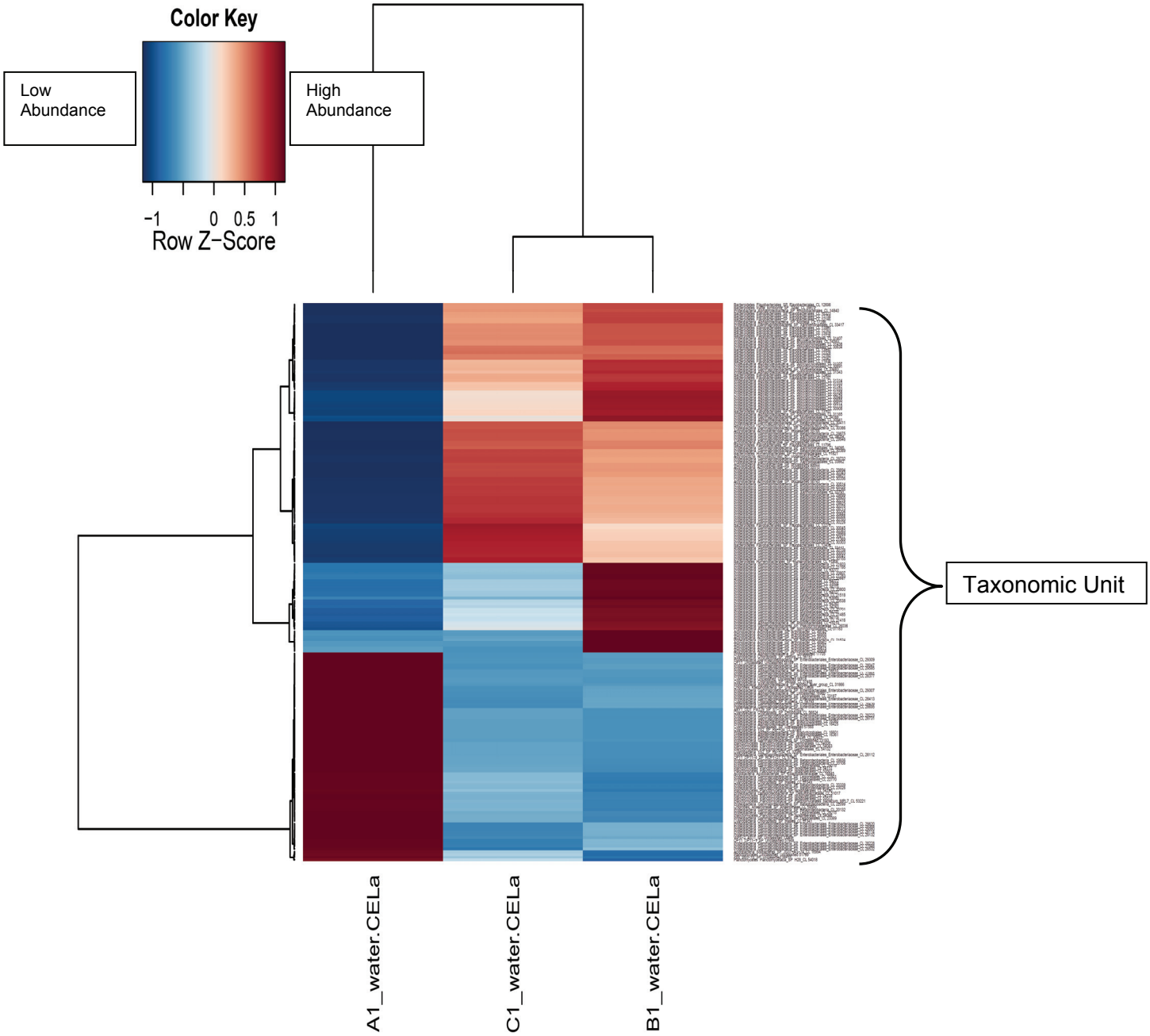

Figure 4: Heatplot indicating the 200 taxonomic units that differed most between Sample A, B and C when fluorescence intensity is measured as an indication of abundance.

3.3.4 Pyrosequencing

Consensus has not yet been reached on which region of the 16S rRNA gene is best to sequence when investigating the diversity of bacterial communities. Two regions were therefore selected for this study. The areas selected were variable regions 1-3 and 4-7. The number of reads obtained for the various samples and variable regions are depicted in Table 4. As there were only 58 reads of sample A V1-3 it was decided to only work with the data obtained from variable regions 4-7 as one cannot compare datasets if insufficient data are available.

Table 4: Number of useable reads obtained for the various samples and variable regions.

Sample name	A		B		C	
Variable region	V1-3	V4-7	V1-3	V4-7	V1-3	V4-7
Number of useable reads	58	5504	4763	2942	3892	3411

The pyrosequencing results showed a vast amount of the different bacteria present in the samples and even bacteria that were present at low levels were revealed. The bacterial composition of the three samples differed to some extent as can be seen from the raw data included in Appendix B. Sample A had two dominant bacterial groups, the Proteobacteria (38.72%) and the Cyanobacteria (32.19%). Samples B and C consisted mainly of the Proteobacteria (62.54% and 80.39%) and the Bacteroidetes (28.93% and 9.18%). The significant dominant groups are depicted in Table 5. This is also shown in Figures 5-7.

Table 5: Breakdown of the significant dominant populations in samples A, B, and C.

	% of total bacterial population				% of relevant phylum				% of relevant class		
Phylym	A	B	C	Class	A	B	C	Order	A	B	C
Proteobacteria	38.7	62.5	80.4	Betaproteobacteria	19.5	67.9	79.5	Burkholderiales		88.0	90.9
				Gammaproteobacteria	33.8	23.9	11.9	Enterobacteriales	33.6		
								Legionellales	26.3		
								Pseudomonadales		66.3	
Cyanobacteria	32.2			Chloroplast	95.2						
Bacteroidetes		28.9	9.2	Flavobacteria		50.0	17.4				
				Sphingobacteria		20.5	32.2				

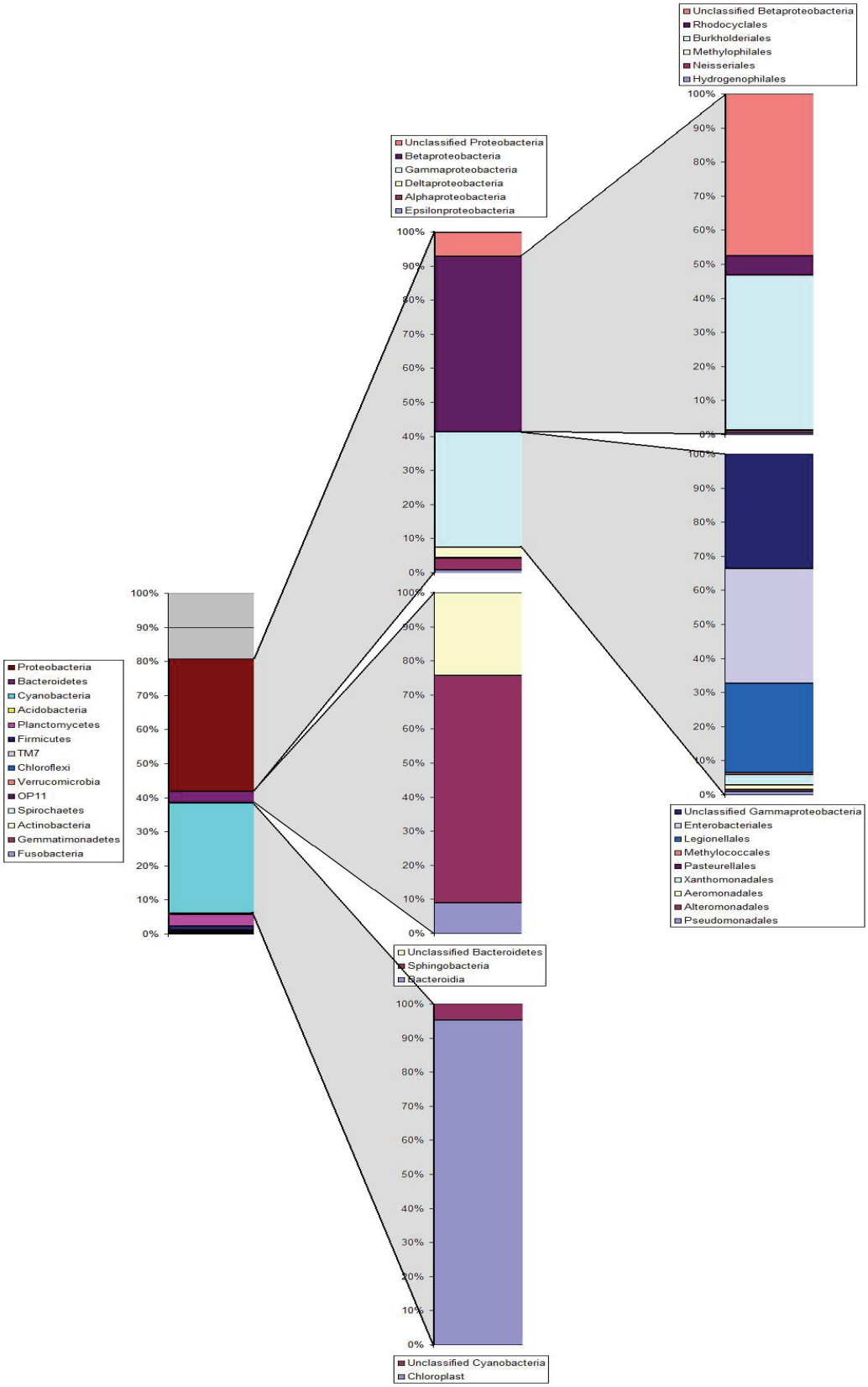

Figure 5: Breakdown of the most significant dominant bacterial populations in sample A.

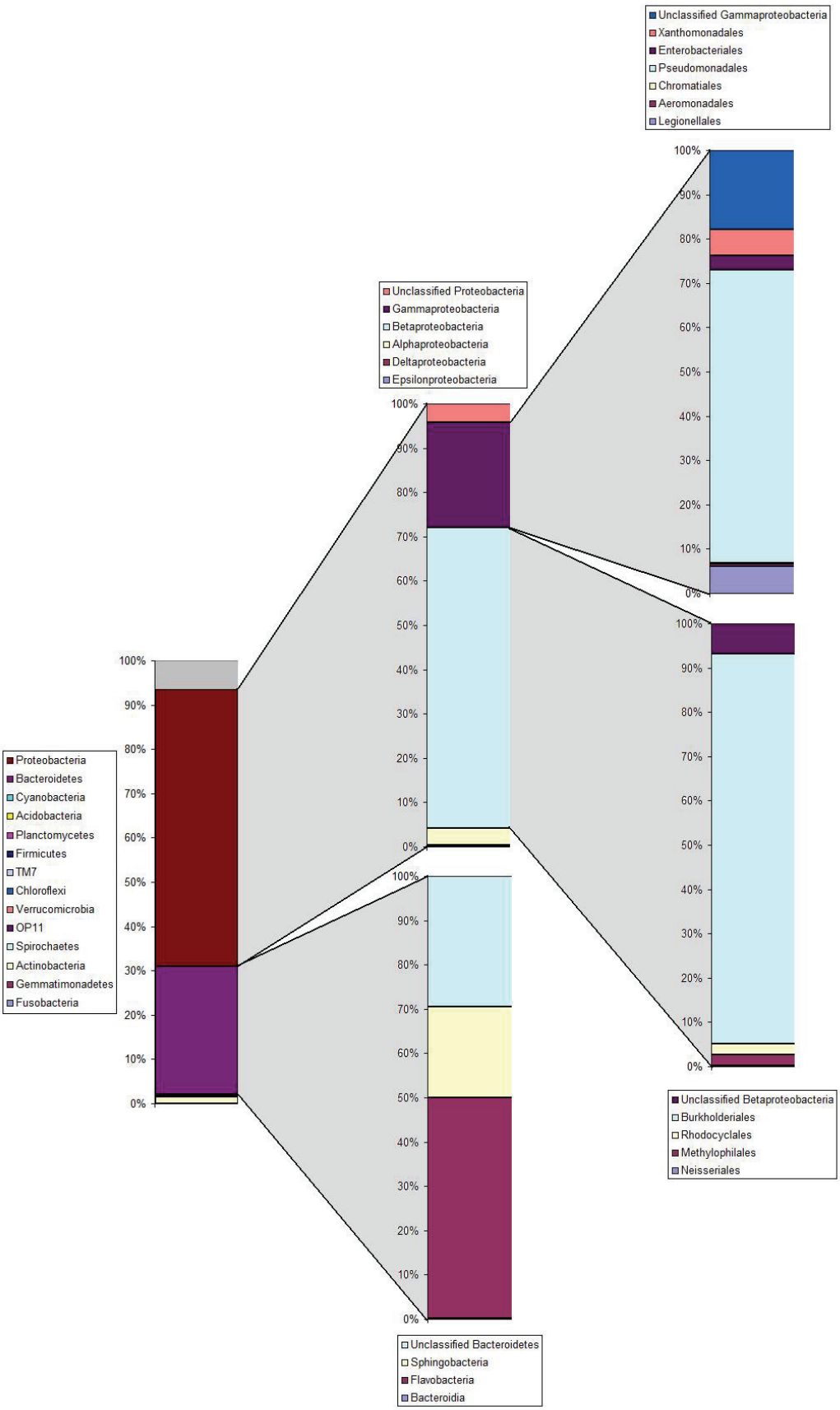

Figure 6: Breakdown of the most significant dominant bacterial populations in sample B.

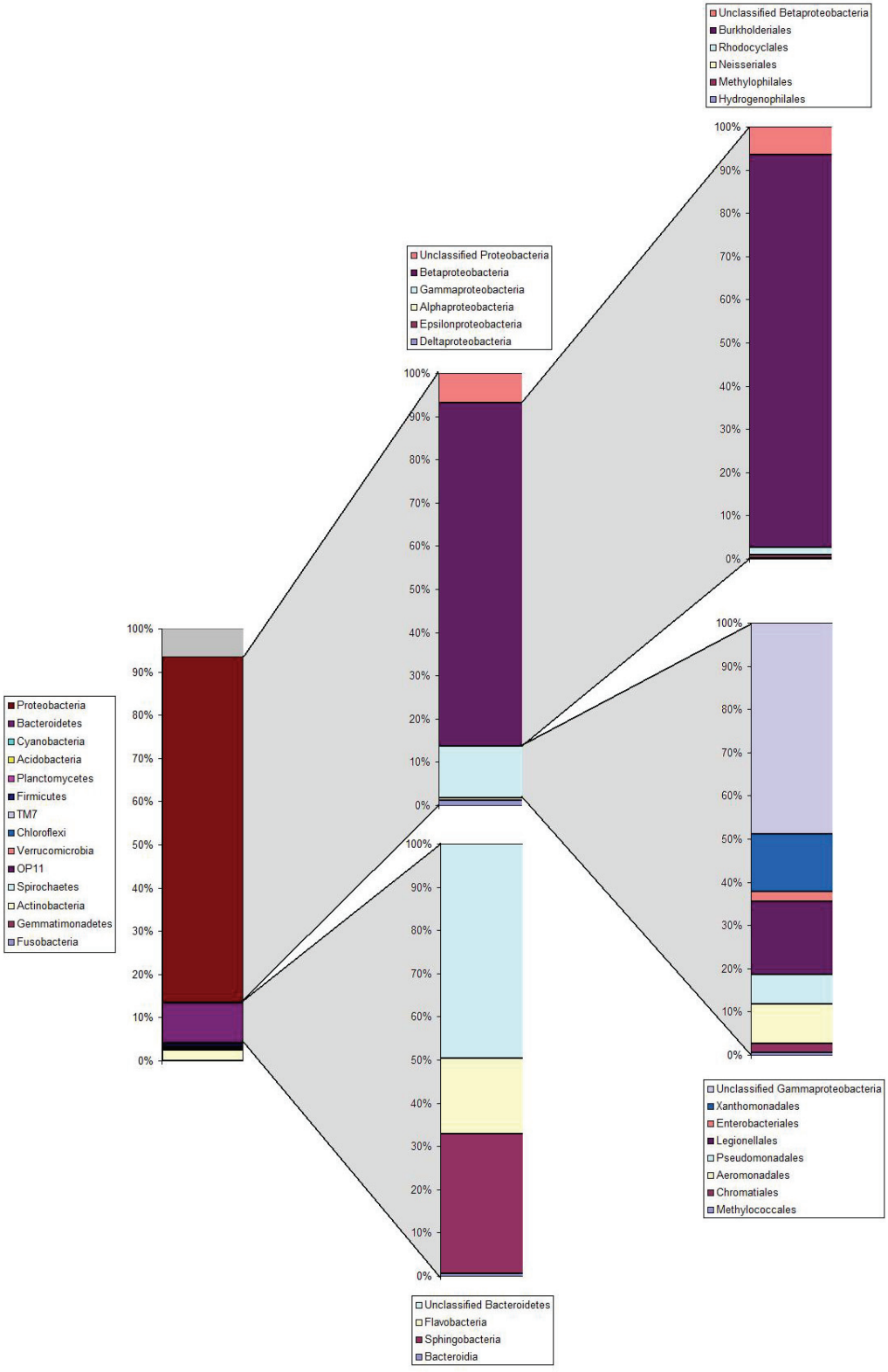

Figure 7: Breakdown of the most significant dominant bacterial populations in sample C.

Sample A contained a large amount of Cyanobacteria, detected with 454 pyrosequencing, whereas Sample B and C did not. This was to be expected as Sample A was collected near the origin of the stream with little or no organic pollution. In contrast Samples B and C both had a similar bacterial population dominated by heterotrophs. This was clearly the result of the high organic pollution loads which entered the stream just upstream of sampling point B.

Contrary to the expected results, very few water-borne pathogens could be detected. This clearly showed that although there are good indications that they might have been present in the samples (based on the *E. coli* results), these bacteria only represented a minor fraction of the total bacterial community present at these points in the river. It would, therefore, be advisable to still detect specific pathogens by using the conventional enrichment and culturing methods instead of relying on a technique such as pyrosequencing or phyochips to detect them.

3.4 CONCLUSIONS

Results show that both pyrosequencing and phylochip analysis reveal a far more diverse bacterial population in the water samples when compared to the DGGE analysis. DGGE only revealed the most dominant phyla and is only useful when attempting to determine the most dominant members of a bacterial community. If a more comprehensive view of the bacterial population is required, techniques such as pyrosequencing or phylochips are necessary, as these techniques are able to reveal taxa that are present at low levels. The primary phylochip data only provided an indication of presence/absence and not of abundance. The phylochip analysis was, however, able to detect taxa that were present in low quantities. Using the fluorescence intensity data of the individual hybridizations, the phylochip data could also provide an indication of abundance. In this regard there was a very good correlation with the pyrosequencing data.

Although a detailed analysis of the bacterial species diversity and abundance could be obtained from these new molecular methods it was evident that species and genera representing water-borne pathogens were seldom detected. When detected, they were present at very low levels although all the water quality parameters tested indicated that the water was certainly unfit for human consumption and other uses. These new molecular methods would be ideal to study the impact and response of natural freshwater ecosystems to gross contamination with human wastes as they provide a far more accurate analysis of the bacterial species diversity and dynamics. Such an approach will, however, not provide scientists with data required to do quantitative health risk assessments. The water-borne pathogens typically only represent a small fraction of the total bacterial community and are not easily detected when doing whole community diversity analyses. Molecular approaches targeting water-borne pathogens directly such as q-PCR are at present still the methods of choice. This situation may change in future, but it does not seem that the current developments in sequencing technologies and microarrays will provide a viable alternative.

3.5 RECOMMENDATIONS FOR FUTURE RESEARCH

For the immediate future the main application of these techniques will be to study the overall microbial diversity in aquatic ecosystems. Knowledge of the microbial composition and dynamics of systems will certainly be of benefit to a number of current projects as well as research issues still to be addressed. The main reason for this is that these techniques are applicable to a wide range of ecosystems, from natural rivers systems and hot springs to man-made systems such as biological wastewater treatment reactors or drinking distribution networks.

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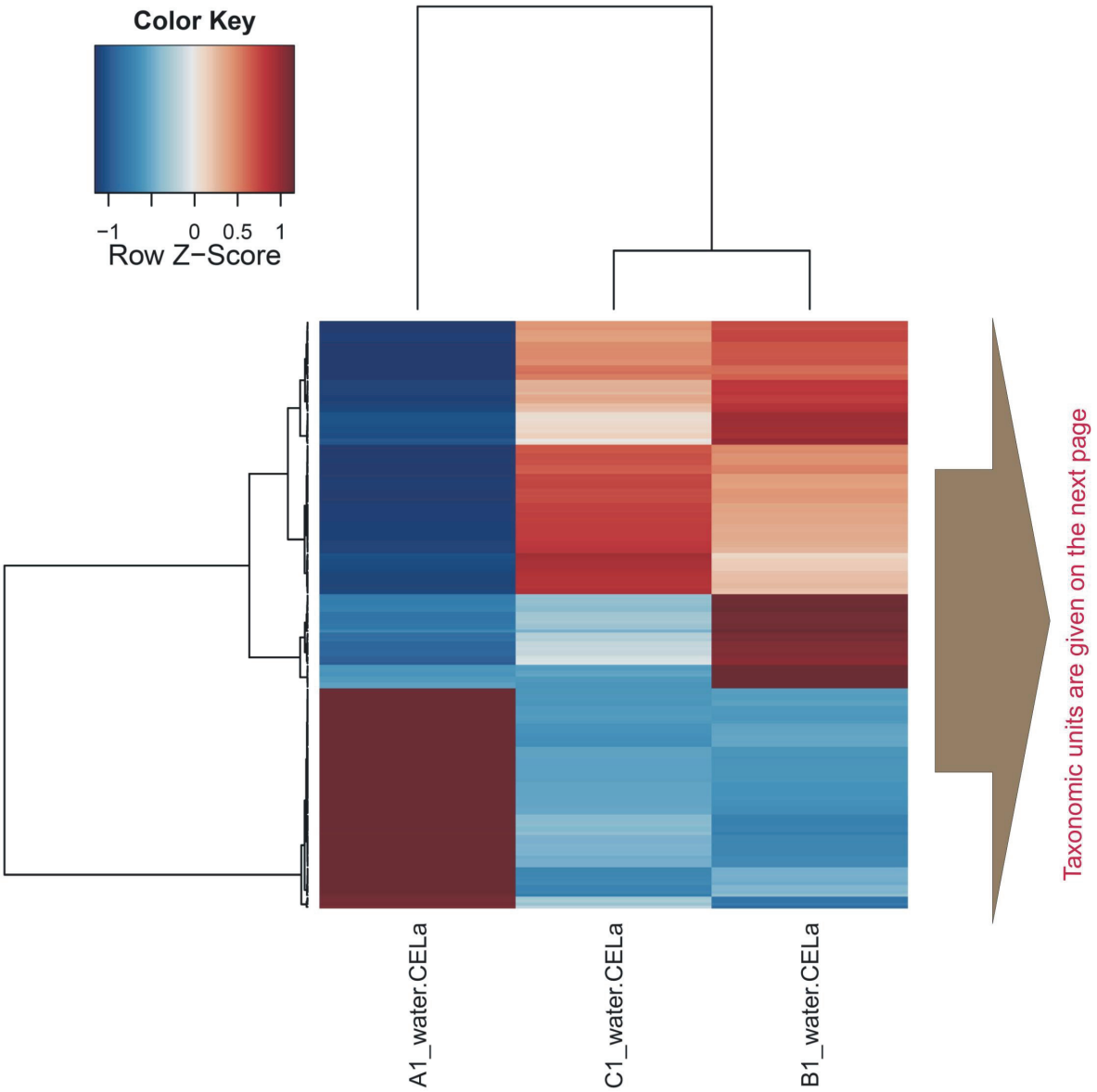
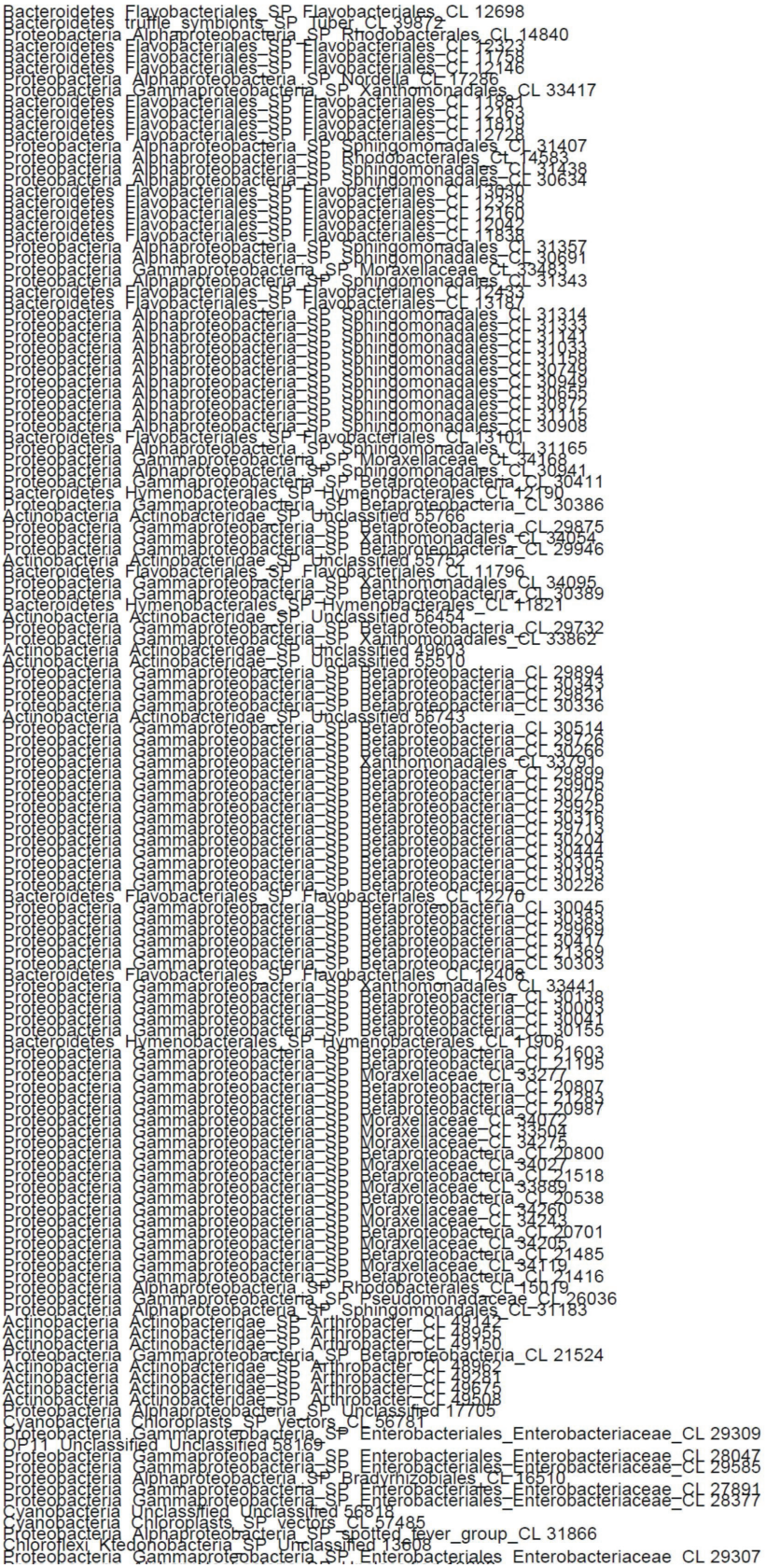
APPENDIX A

Enlargement of the heatplot figure (figure 4 in the document).

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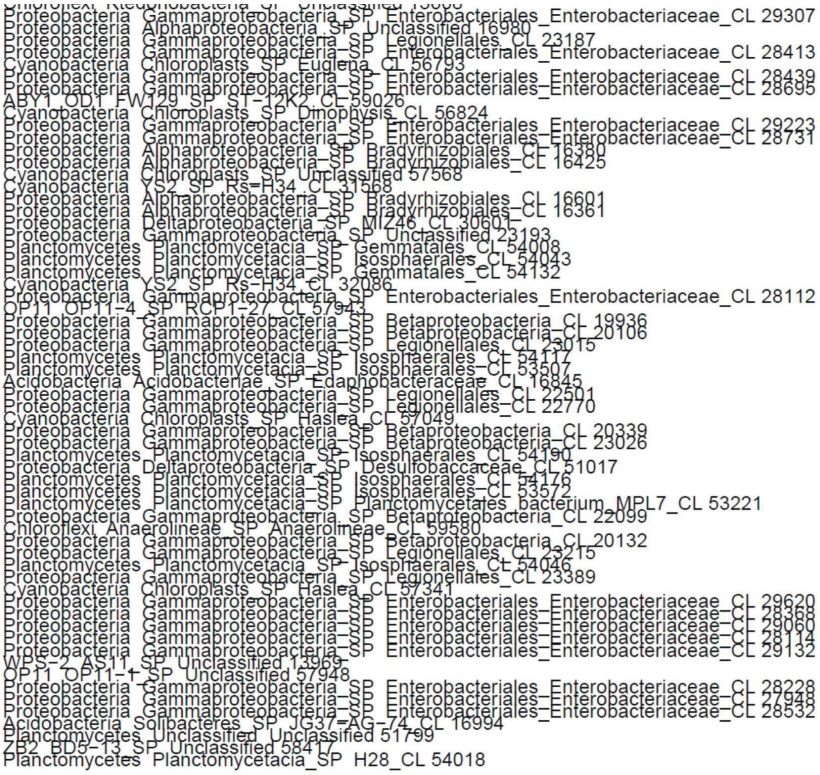
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APPENDIX B

Summary of pyrosequencing data based on the taxonomic hierarchy of bacteria

Phylum	Sample A(%)	Sample B(%)	Sample C(%)
Fusobacteria	0.07	0.00	0.03
Gemmatimonadetes	0.04	0.00	0.00
Actinobacteria	0.09	1.38	2.58
Spirochaetes	0.04	0.00	0.00
OP11	0.02	0.00	0.00
Verrucomicrobia	0.22	0.00	0.36
Chloroflexi	0.02	0.00	0.00
TM7	0.39	0.21	0.15
Firmicutes	1.18	0.38	0.59
Planctomycetes	3.38	0.17	0.30
Acidobacteria	0.50	0.03	0.06
Cyanobacteria	32.19	0.03	0.21
Bacteroidetes	3.50	28.93	9.18
Proteobacteria	38.72	62.54	80.39
Total Classified	80.36	93.67	93.85

Class	Sample A(%)	Sample B(%)	Sample C(%)
Fusobacteria	0.07	0.00	0.03
Gemmatimonadetes	0.04	0.00	0.00
Actinobacteria	0.09	1.38	2.58
Spirochaetes	0.04	0.00	0.00
Subdivision 3	0.06	0.00	0.00
Opitutae	0.07	0.00	0.06
Spartobacteria	0.07	0.00	0.00
Anaerolineae	0.02	0.00	0.00
Bacilli	0.22	0.00	0.00
Clostridia	0.90	0.31	0.53
Planctomycetacia	3.38	0.17	0.30
Acidobacteria_Gp4	0.02	0.03	0.00
Holophagae	0.04	0.00	0.00
Acidobacteria_Gp23	0.04	0.00	0.00
Acidobacteria_Gp1	0.22	0.00	0.03
Acidobacteria_Gp6	0.04	0.00	0.00
Acidobacteria_Gp3	0.15	0.00	0.00
Acidobacteria_Gp7	0.00	0.00	0.03
Cyanobacteria	32.19	0.03	0.21
Bacteroidia	0.20	0.03	0.06
Sphingobacteria	2.34	5.92	2.96
Epsilonproteobacteria	0.33	0.03	0.03
Alphaproteobacteria	1.31	2.41	0.53
Deltaproteobacteria	1.20	0.17	0.95
Gammaproteobacteria	13.08	15.20	10.01
Betaproteobacteria	20.14	43.10	66.80
Verrucomicrobiae	0.00	0.00	0.27
Erysipelotrichi	0.00	0.03	0.06
Flavobacteria	0.00	14.45	1.60
Total Classified	76.26	83.26	87.04

Order	Sample A(%)	Sample B(%)	Sample C(%)
Fusobacteriales	0.07	0.00	0.03
Gemmatimonadales	0.04	0.00	0.00
Actinomycetales	0.07	1.27	2.55
Spirochaetales	0.04	0.00	0.00
Opitutales	0.06	0.00	0.03
Anaerolineales	0.02	0.00	0.00
Lactobacillales	0.09	0.00	0.00
Bacillales	0.13	0.00	0.00
Clostridiales	0.86	0.31	0.50
Planctomycetales	3.38	0.17	0.30
Holophagales	0.04	0.00	0.00
Cyanobacteriales	32.19	0.03	0.21
Bacteroidales	0.20	0.03	0.06
Sphingobacteriales	2.34	5.92	2.96
Campylobacterales	0.26	0.03	0.03
Sphingomonadales	0.02	1.10	0.06
Rhodobacterales	0.06	0.72	0.27
Rickettsiales	0.11	0.00	0.00
Rhizobiales	0.28	0.24	0.03
Rhodospirillales	0.44	0.00	0.03
Caulobacterales	0.15	0.03	0.06
Desulfovibrionales	0.02	0.00	0.03
Myxococcales	0.17	0.03	0.33
Desulfuromonadales	0.17	0.03	0.03
Desulfobacterales	0.09	0.00	0.03
Syntrophobacterales	0.64	0.00	0.24
Pseudomonadales	0.13	10.08	0.68
Alteromonadales	0.06	0.00	0.00
Aeromonadales	0.18	0.07	0.92
Xanthomonadales	0.39	0.89	1.33
Pasteurellales	0.02	0.00	0.00
Methylococcales	0.07	0.00	0.06
Legionellales	3.44	0.93	1.69
Enterobacteriales	4.40	0.48	0.24
Hydrogenophilales	0.09	0.00	0.09
Neisseriales	0.15	0.07	0.06
Methylophilales	0.02	1.10	0.53
Burkholderiales	9.16	37.94	60.75
Rhodocyclales	1.16	1.03	1.10
Erysipelotrichales	0.00	0.03	0.06
Flavobacteriales	0.00	14.45	1.60
Bdellovibrionales	0.00	0.03	0.24
Chromatiales	0.00	0.03	0.21
Verrucomicrobiales	0.00	0.00	0.27
Total Classified	61.21	77.04	77.61

Family	Sample A(%)	Sample B(%)	Sample C(%)
Fusobacteriaceae	0.00	0.00	0.03
Leptotrichiaceae	0.06	0.00	0.00
Gemmatimonadaceae	0.04	0.00	0.00
Microbacteriaceae	0.07	0.93	1.36
Spirochaetaceae	0.04	0.00	0.00
Verrucomicrobiaceae	0.00	0.00	0.27
Opitutaceae	0.06	0.00	0.03
Anaerolineaceae	0.02	0.00	0.00
Erysipelotrichaceae	0.00	0.03	0.06
Streptococcaceae	0.07	0.00	0.00
Carnobacteriaceae	0.02	0.00	0.00
Staphylococcaceae	0.02	0.00	0.00
Paenibacillaceae	0.02	0.00	0.00
Bacillaceae	0.06	0.00	0.00
Thermoactinomycetaceae	0.02	0.00	0.00
Bacteroidetes_incertae sedis	0.11	0.00	0.00
Incertae Sedis XIV	0.00	0.00	0.03
Incertae Sedis XIII	0.02	0.00	0.00
Lachnospiraceae	0.02	0.00	0.03
Peptococcaceae	0.24	0.00	0.00
Ruminococcaceae	0.18	0.03	0.15
Veillonellaceae	0.13	0.07	0.03
Syntrophomonadaceae	0.00	0.00	0.03
Clostridiaceae	0.06	0.03	0.00
Planctomycetaceae	3.38	0.17	0.30
Holophagaceae	0.04	0.00	0.00
Family II	0.00	0.03	0.00
Chloroplast	30.63	0.00	0.21
Prevotellaceae	0.02	0.00	0.03
Porphyromonadaceae	0.15	0.00	0.03
Cryomorphaceae	0.00	1.10	0.33
Flavobacteriaceae	0.00	12.97	1.16
Saprospiraceae	0.00	0.00	0.03
Cytophagaceae	0.04	1.48	1.54
Chitinophagaceae	0.99	3.47	0.71
Sphingobacteriaceae	1.31	0.48	0.33
Helicobacteraceae	0.24	0.03	0.03
Sphingomonadaceae	0.00	0.96	0.06
Rhodobacteraceae	0.06	0.72	0.09
Rickettsiaceae	0.11	0.00	0.00
Methylobacteriaceae	0.02	0.00	0.00
Bradyrhizobiaceae	0.09	0.00	0.00
Xanthobacteraceae	0.04	0.00	0.00
Acetobacteraceae	0.15	0.00	0.00
Rhodospirillaceae	0.15	0.00	0.00
Caulobacteraceae	0.15	0.03	0.06
Bacteriovoracaceae	0.00	0.00	0.03
Bdellovibrionaceae	0.00	0.10	0.21
Desulfovibrionaceae	0.02	0.00	0.03
Polyangiaceae	0.00	0.00	0.03
Cystobacteraceae	0.06	0.03	0.03
Desulfuromonadaceae	0.02	0.00	0.00

Geobacteraceae	0.13	0.03	0.00
Desulfobacteraceae	0.04	0.00	0.03
Desulfobulbaceae	0.06	0.00	0.00
Syntrophobacteraceae	0.06	0.00	0.00
Syntrophaceae	0.59	0.00	0.24
Pseudomonadaceae	0.09	0.24	0.15
Moraxellaceae	0.04	9.84	0.53
Shewanellaceae	0.06	0.00	0.00
Aeromonadaceae	0.18	0.07	0.92
Sinobacteraceae	0.17	0.00	0.00
Xanthomonadaceae	0.22	0.89	1.33
Pasteurellaceae	0.02	0.00	0.00
Methylococcaceae	0.07	0.00	0.06
Legionellaceae	2.83	0.79	1.42
Coxiellaceae	0.61	0.14	0.24
Enterobacteriaceae	4.40	0.48	0.24
Chromatiaceae	0.00	0.03	0.18
Halothiobacillaceae	0.00	0.00	0.03
Hydrogenophilaceae	0.09	0.00	0.09
Neisseriaceae	0.15	0.07	0.06
Methylophilaceae	0.02	1.10	0.53
Alcaligenaceae	0.09	1.24	0.77
Comamonadaceae	0.96	15.69	26.54
Burkholderiales_incertae_sedis	0.06	1.44	2.84
Oxalobacteraceae	1.21	5.44	1.54
Burkholderiaceae	6.29	4.40	11.14
Rhodocyclaceae	1.16	1.03	1.07
Micrococcaceae	0.00	0.10	0.00
Bacteroidaceae	0.00	0.03	0.00
Beijerinckiaceae	0.00	0.03	0.00
Total Classified	58.53	65.74	57.21

Genus	Sample A(%)	Sample B(%)	Sample C(%)
Cetobacterium	0.00	0.00	0.03
Leptotrichia	0.06	0.00	0.00
Gemmatimonas	0.04	0.00	0.00
Microbacterium	0.00	0.03	0.03
Leucobacter	0.00	0.00	0.06
Pseudoclavibacter	0.00	0.00	0.03
Yonghaparkia	0.00	0.00	0.03
Treponema	0.04	0.00	0.00
OP11_genera_incertae_sedis	0.02	0.00	0.00
Luteolibacter	0.00	0.00	0.06
Prosthecobacter	0.00	0.00	0.21
Subdivision3_genera_incertae_sedis	0.06	0.00	0.00
Opitutus	0.04	0.00	0.03
Spartobacteria_genera_incertae_sedis	0.07	0.00	0.00
TM7_genera_incertae_sedis	0.39	0.21	0.15
GpIIa	0.00	0.03	0.00
Streptococcus	0.07	0.00	0.00
Carnobacterium	0.02	0.00	0.00
Gemella	0.02	0.00	0.00

Paenibacillus	0.02	0.00	0.00
Proteocatella	0.00	0.00	0.03
Anaerovorax	0.02	0.00	0.00
Roseburia	0.00	0.00	0.03
Oribacterium	0.02	0.00	0.00
Desulfosporosinus	0.15	0.00	0.00
Subdoligranulum	0.00	0.00	0.06
Faecalibacterium	0.00	0.00	0.09
Ruminococcus	0.02	0.03	0.00
Acetivibrio	0.13	0.00	0.00
Sporobacter	0.02	0.00	0.00
Succinispira	0.02	0.00	0.00
Propionispira	0.02	0.00	0.00
Veillonella	0.04	0.00	0.00
Syntrophomonas	0.00	0.00	0.03
Clostridium	0.04	0.03	0.00
Rhodobacter	0.04	0.14	0.06
Rickettsia	0.04	0.00	0.00
Orientia	0.02	0.00	0.00
Methylobacterium	0.02	0.00	0.00
Bradyrhizobium	0.02	0.00	0.00
Rhodoblastus	0.02	0.00	0.00
Labrys	0.04	0.00	0.00
Azospirillum	0.02	0.00	0.00
Magnetospirillum	0.04	0.00	0.00
Phenylobacterium	0.00	0.00	0.06
Asticcacaulis	0.11	0.03	0.00
Peredibacter	0.00	0.00	0.03
Bdellovibrio	0.00	0.10	0.21
Desulfovibrio	0.02	0.00	0.03
Anaeromyxobacter	0.04	0.03	0.00
Desulfuromonas	0.02	0.00	0.00
Geobacter	0.13	0.03	0.00
Desulfobulbus	0.02	0.00	0.00
Desulfovirga	0.02	0.00	0.00
Desulfobacca	0.02	0.00	0.00
Smithella	0.02	0.00	0.00
Desulfomonile	0.55	0.00	0.24
Pseudomonas	0.04	0.10	0.06
Acinetobacter	0.00	7.46	0.06
Alkanindiges	0.04	0.28	0.09
Shewanella	0.06	0.00	0.00
Aeromonas	0.18	0.07	0.89
Steroidobacter	0.17	0.00	0.00
Rhodanobacter	0.00	0.00	0.03
Aquimonas	0.00	0.00	0.06
Stenotrophomonas	0.02	0.00	0.00
Thermomonas	0.00	0.00	0.03
Lysobacter	0.02	0.00	0.00
Arenimonas	0.00	0.28	0.03
Methylococcus	0.02	0.00	0.00
Methylobacter	0.04	0.00	0.00
Legionella	0.70	0.38	0.59
Tatlockia	0.46	0.03	0.09

Aquicella	0.61	0.14	0.24
Pectobacterium	0.00	0.00	0.03
Escherichia/Shigella	0.00	0.00	0.06
Budvicia	0.07	0.00	0.00
Pantoea	0.00	0.03	0.00
Yersinia	0.04	0.03	0.00
Buttiauxella	0.04	0.00	0.00
Serratia	0.29	0.00	0.00
Rheinheimera	0.00	0.03	0.18
Thiofaba	0.00	0.00	0.03
Thiobacillus	0.09	0.00	0.09
Vogesella	0.00	0.00	0.06
Chromobacterium	0.04	0.00	0.00
Aquitalea	0.06	0.00	0.00
Methylophilus	0.02	0.96	0.36
Castellaniella	0.00	0.10	0.03
Bordetella	0.00	0.14	0.03
Kerstersia	0.04	0.03	0.00
Derxia	0.02	0.00	0.00
Simplicispira	0.00	0.24	0.21
Pelomonas	0.00	0.03	0.36
Polaromonas	0.00	0.17	0.00
Malikia	0.00	0.14	0.21
Curvibacter	0.00	0.31	0.06
Hydrogenophaga	0.00	0.62	0.80
Diaphorobacter	0.00	0.00	0.03
Acidovorax	0.00	0.93	0.95
Rhodoferax	0.46	2.75	0.89
Methylibium	0.02	0.00	0.00
Aquabacterium	0.00	0.38	1.33
Leptothrix	0.02	0.48	0.68
Herbaspirillum	0.00	0.03	0.06
Massilia	0.00	0.45	0.03
Duganella	0.00	0.17	0.09
Janthinobacterium	0.06	0.03	0.00
Undibacterium	0.00	0.65	0.09
Collimonas	0.02	0.00	0.00
Herminiimonas	0.53	0.07	0.06
Chitinimonas	0.00	0.00	0.06
Polynucleobacter	6.25	4.30	11.05
Zoogloea	0.00	0.21	0.12
Azonexus	0.00	0.00	0.03
Dechloromonas	0.00	0.10	0.12
Uliginosibacterium	0.02	0.00	0.00
Denitratisoma	0.00	0.00	0.06
Azospira	0.06	0.00	0.00
Sterolibacterium	0.02	0.00	0.03
Gp4	0.00	0.03	0.00
Arthrobacter	0.00	0.07	0.00
Planctomyces	0.00	0.03	0.00
Singulisphaera	0.00	0.03	0.00
Bacteroides	0.00	0.03	0.00
Fluviicola	0.00	1.07	0.00
Cloacibacterium	0.00	0.21	0.00

Flavobacterium	0.00	12.07	0.00
Mucilaginibacter	0.00	0.14	0.00
Pedobacter	0.00	0.21	0.00
Runella	0.00	0.03	0.00
Leadbetterella	0.00	0.03	0.00
Emticicia	0.00	0.10	0.00
Arcicella	0.00	1.17	0.00
Lacibacter	0.00	0.31	0.00
Sediminibacterium	0.00	0.48	0.00
Ferruginibacter	0.00	1.10	0.00
Sulfuricurvum	0.00	0.03	0.00
Novosphingobium	0.00	0.45	0.00
Catellibacterium	0.00	0.07	0.00
Leeia	0.00	0.03	0.00
Deefgea	0.00	0.03	0.00
Ferribacterium	0.00	0.31	0.00
Inhella	0.00	0.03	0.00
Naxibacter	0.00	0.48	0.00
Comamonas	0.00	0.03	0.00
Variovorax	0.00	0.07	0.00
Enhydrobacter	0.00	0.03	0.00
Perlucidibaca	0.00	0.31	0.00
Total Classified	13.07	41.76	21.86