Molecular profiling of microbial population dynamics in environmental water

K Jordaan

12419559

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Supervisor: Prof CC Bezuidenhout

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“The LORD is the everlasting God,
the Creator of the ends of the earth.
He will not grow tired or weary,
and his understanding no one can fathom.
He gives strength to the weary
and increases the power of the weak.
Even youths grow tired and weary,
and young men stumble and fall;
but those who hope in the LORD
will renew their strength.
They will soar on wings like eagles;
they will run and not grow weary,
they will walk and not be faint.”

Isaiah 40:28–31

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bless you and keep you; make His face shine on you and be gracious to you; turn His face towards you and give you peace”. – Numbers 6: 23–26.

ABSTRACT

Increasing socio-economic growth and development of South Africa's freshwater systems require continuous augmentation of water sources to meet the growing water requirements of communities and industries. Anthropogenic disturbances have caused the water quality of many freshwater systems to drastically deteriorate due to constant disposal of domestic, industrial, and agricultural waste into surface waters. Government agencies make use of biomonitoring programmes to effectively manage the countries' freshwater resources. These programmes use a variety of biological indicators (e.g., macroinvertebrates, fish, diatoms and algal species) and physico-chemical variables to determine the state of the environment. However, attempts to use microbial community structures as bioindicators of anthropogenic perturbations are greatly neglected. This study used molecular techniques (PCR-DGGE and 454-pyrosequencing) and multivariate analysis to develop a robust monitoring technique to determine the impacts of environmental disturbances on bacterial community compositions in river systems in the North West Province. Significant contributions made by this project included the establishment of a bacterial diversity framework for South African freshwater systems that are impacted by a variety of anthropogenic activities (e.g., urban and informal settlements, agriculture and mining). Furthermore, case studies demonstrated the prevalence of specific taxa at polluted sites, as well as positive and negative associations between taxa and environmental variables and pollutants. Finally, biogeochemical cycles could be partially matched to bacterial community structures in river systems. The first part of the project included a pilot study that investigated bacterial structures in a segment of the Vaal River in response to environmental parameters using molecular techniques and multivariate analysis. The most important observations made during this study included the generation of a larger bacterial diversity dataset by pyrosequencing compared to PCR-DGGE. In addition, metagenomic and multivariate analyses provided clues about potential biogeochemical roles of different taxa. The second and third part of the project included two case studies that investigated bacterial communities in the Mooi River and Wonderfonteinspruit in response to environmental activities. Both these systems are impacted by a variety of external sources such as urban and informal settlements, agriculture, and mining. The results demonstrated that perturbations nearby the Mooi River and Wonderfonteinspruit caused the overall water quality to deteriorate which in

turn had a profound impact on bacterial community composition. Bacterial community structures at reference/control sites (Muiskraal and Turffontein dolomitic eye) had overall high species diversity (richness and evenness), whereas polluted sites showed lower species diversity and were dominated by the *Beta*- and *Gammaproteobacteria*, *Bacteroidetes*, and *Verrucomicrobia*. In addition, various potential pathogens (e.g. *Eschirichia/Shigella*, *Legionella*, *Staphylococcus*, *Streptococcus* etc.) were identified at impacted sites. Multivariate analysis suggested that bacterial communities and certain taxa (*Malikia*, *Algoriphagus*, *Rhodobacter*, *Brevundimonas* and *Sphingopyxis*) at polluted sites were mainly impacted by temperature, pH, nutrient levels, and heavy metals. Finally, the proportion of nitrogen and sulphur bacteria corresponded well with the nitrogen and sulphur levels measured in the Wonderfonteinspruit. Based on these results, it was concluded that bacterial community structures might provide a good indicator of anthropogenic disturbances in freshwater systems and may be incorporated into biomonitoring programs.

Keywords: freshwater; physico-chemical parameters; bacterial community composition; PCR-DGGE; 454-pyrosequencing; multivariate analysis

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CHAPTER 1: Introduction and Problem statement

1.1 Microbial ecology in aquatic ecosystems

Aquatic ecosystems are globally among the most diverse habitats, and range from surface waters (lentic and lotic), subsurface waters (hyporheic and phreatic), and riparian systems (constrained and floodplain reaches) to the bionetworks between them (e.g., springs) (Ward and Tockner, 2001). These ecosystems support diverse microbial communities with different abundance, chemical composition, growth rates, and metabolic functions due to changing conditions in temperature, pH, salinity, oxygen availability, light, dissolved gases and nutrients (Geist, 2011; Kirchman, 2012). Inland waters (lakes, ponds, rivers, streams, wetlands and groundwater) comprise of either freshwater or saline water (Hahn, 2006). Freshwater is defined as water with a low salinity (< 1 g/L) whereas saline waters are characterised by high salinities (> 1 g/L) (Hahn, 2006). Freshwater is the basis of daily life and perhaps the most essential resource for domestic use, agricultural and industrial processes, municipal supply, production of energy, navigation, and fisheries (Hahn, 2006; Asaeda *et al.,* 2009). Freshwater ecosystems also serve worldwide as important cultural and recreational resources for human populations. Sustainable development of freshwater resources is vital in ensuring clean and adequate supply of water to drive economic and ecological systems (Hahn, 2006; Asaeda *et al.,* 2009).

Microorganisms, which include bacteria, fungi, Archaea and protists, are ubiquitous in freshwater environments and their ecological impact is of fundamental importance (Sigee, 2005; Asaeda *et al.,* 2009). Microbes mediate processes essential in the degradation of organic matter and the associated release of energy (Percent *et al.,* 2008). They are fundamental in processes that control water quality and are involved in the degradation of pollutants (Hahn, 2006; Kirchman, 2012). Among the aquatic microbes, bacteria are ecologically important in a number of ways. Bacteria are the main heterotrophic organisms in aquatic habitats, they are taxonomically very diverse, and largely contribute to the phenotypic, genetic, and molecular biodiversity (Sigee, 2005). These bacteria perform a range of different metabolic activities and thus occupy important roles in geochemical cycles (Sigee, 2005). Furthermore, heterotrophic

bacteria play a key role in aerobic and anaerobic respiration (Cole, 1999). Certain bacteria species are particularly important in anaerobic environments, where algae and other free-living organisms are far less metabolically active (Sigee, 2005). Bacteria are involved in the elimination of inorganic compounds and the remineralisation and dispersal of organic material (Yannarell and Kent, 2009). They are largely responsible for the breakdown of biomass that is important in the regeneration of soluble materials (Sigee, 2005), but also engage in the carbon, nitrogen and phosphorus cycles (Sigee, 2005). Thus, a large amount of energy and matter in aquatic habitats is processed by bacterial communities (Yannarell and Kent, 2009).

1.2 Common bacterial lineages in freshwater systems

Freshwater bacteria are a diverse group of prokaryote organisms that vary in their morphology, physiology, metabolism, and geographical preference (Sigee, 2005). In freshwaters, *Proteobacteria* are often the dominant prokaryotes. Within this group, *Betaproteobacteria* are the most frequently detected taxa in bacterial communities, followed by *Gammaproteobacteria* and *Alphaproteobacteria* (Kirchman, 2012). In addition to *Proteobacteria*, three other phyla commonly recovered from freshwater systems include *Actinobacteria*, *Bacteroidetes*, and *Cyanobacteria* (Newton *et al.,* 2011; Kirchman, 2012).

1.2.1 *Proteobacteria*

The *Proteobacteria* consists of phototrophs, chemolithotrophs and chemoorganotrophs, and can be found in both oxic and anoxic environments (Yannarell and Kent, 2009). The class *Betaproteobaceria* grows rapidly, is readily grazed, favours high nutrient conditions and is often associated with algae (such as *Cryptomonas* species) and carbon-based particulate matter (Newton, 2008; Newton *et al.,* 2011). Members of this class are involved in the nitrogen cycle by providing fixed nitrogen to plants via the oxidation of ammonium to nitrate (Newton, 2008). *Alpha*- and *Gammaproteobacteria* are far less abundant in freshwaters, although they are still ubiquitous (Newton, 2008; Yannarell and Kent, 2009). *Alphaprotoebacteria* play a significant role in freshwater by degrading complex organic compounds (Newton *et al.,* 2011). *Gammaproteobacteria*, on the other hand, are copiotrophs (adapted to high-nutrient conditions) and members

of this class, specifically in the *Enterobacteriaceae* family, can be used in the source tracking of faecal pollutants (Stoeckel and Harwood, 2007; Newton *et al.,* 2011).

1.2.2 *Actinobacteria*

Other than *Proteobacteria*, *Actinobacteria* are often the numerically dominant phylum (> 50%) in freshwater systems (Newton *et al.,* 2011). Generally, organisms in this phylum are free-living, open-water defence specialists with an average growth rate (Newton, 2008; Newton *et al.,* 2011). Several members have disproportionately large numbers of pathways for nucleic and amino acid metabolism and harbour an abundance of actinorhodopsins that act as a potential source of light-driven energy generation (Newton *et al.,* 2011). The abundance of *Actinobacteria* often peaks in late autumn and winter (Yannarell and Kent, 2009). They appear to be more tolerant of conditions with low organic carbon concentrations, and may be replaced by *Betaproteobacteria* during algal blooms which cause increased carbon levels (Yannarell and Kent, 2009). Freshwater *Actinobacteria* contain several monophyletic lineages: acI, acII, acIII, and acIV. Of these, acI and acII clades are highly abundant and ubiquitous in the epilimnia of freshwaters (Newton *et al.,* 2011).

1.2.3 *Bacteroidetes*

The phylum *Bacteroidetes* is also found in abundance in freshwaters and covers a large proportion of particle-associated bacterial communities (Yannarell and Kent, 2009). *Bacteroidetes* is of great significance in freshwaters because they can degrade complex biopolymers (Kirchman, 2002). Lineages of this phylum are unlike other common freshwater groups in that they do not show any temporal or lake-specific occurrence patterns (Eiler and Bertilsson, 2007). This finding may be attributed to their strong dependence on organic matter load or cyanobacterial blooms (Newton *et al.,* 2011). *Bacteroidetes* are often found in high abundance during periods following phytoplankton blooms. Such blooms are more likely to occur during irregular and stochastic disturbances rather than a predictable seasonal pattern (Newton *et al.,* 2011).

1.2.4 *Cyanobacteria*

Freshwater *Cyanobacteria* constitute a diverse collection of genera and species. Common freshwater genera include *Microcystis*, *Anabaena*, *Aphanizomenon*,

Oscillatoria, *Planktothrix*, *Synechococcus*, and *Cyanothece* (Newton *et al.,* 2011). *Cyanobacteria* are generally the dominant bacterial phototrophs in the oxygenated portions of freshwaters (Yannarell and Kent, 2009). Many *Cyanobacteria* are capable of fixing nitrogen and thus play a key part in both the nitrogen and carbon cycles (Yannarell and Kent, 2009; Newton *et al.,* 2011). Some *Cyanobacteria* contain heterocysts which are cells devoted solely to nitrogen fixation (Stanier and Cohen-Bazire, 1977). Certain cyanobacteria are considered nuisance species since they form large blooms in eutrophic systems and may release toxins (Huisman *et al.,* 2005; Yannarell and Kent, 2009).

1.2.5 Minor phyla

Other bacterial phyla (*Acidobacteria*, BRC1, *Chlorobi*, *Chloroflexi*, *Fibrobacteres*, *Firmicutes*, *Fusobacteria*, *Gemmatimonadetes*, *Lentisphaerae*, *Nitrospira*, OD1, OP10, *Planctomycetes*, *Spirochaetes*, SR1, TM7, and *Verrucomicrobia*) have also been discovered in freshwater systems although they are less prominent than the phyla described above (Newton *et al.,* 2011). Of the minor phyla, the *Firmicutes* and *Planctomycetes* are recovered most often (Newton *et al.,* 2011). *Firmicutes* are frequently isolated from freshwater sediments but rarely found in the water column (Yannarell and Kent, 2009; Newton *et al.,* 2011). *Planctomycetes* occur worldwide in both oligotrophic and eutrophic freshwaters (Krieg *et al.,* 2011), although higher numbers of *Planctomycetes* are associated with eutrophic or polluted waters (Staley *et al.,* 1980). Members of this group, in particular rosette-forming *Planctomycetes*, are often found in high abundance following algal or cyanobacterial blooms (Krieg *et al.,* 2011). A possible explanation for this occurrence is the increase in hydrogen sulphide, iron, and manganese concentrations from phytoplankton decomposition (Kristiansen, 1971). Studies also suggest the importance of this group in the environment due to their ability to carry out anaerobic ammonium oxidation (Strous *et al.,* 1999) and degradation of phytoplankton-derived carbohydrates (Rabus *et al.,* 2002). Members of the *Acidobacteria* are usually present in freshwater sediments (Newton *et al.,* 2011). They favour slightly acidophilic environments (Zimmermann *et al.,* 2011) and several studies suggest the preferential distribution of this group at sites with elevated organic matter and specific plant polymers (Janssen *et al.,* 2002; Kleinsteuber *et al.,* 2008; Eichorst *et al.,* 2011). The phyla *Chloroflexi* (the green non-sulphur bacteria) and *Chlorobi* (the

green sulphur bacteria) contain anoxygenic phototrophs that are generally present in the metalimnia or hypolimnia of deeper freshwater systems (Yannarell and Kent, 2009; Newton *et al.,* 2011). Humic material in the water column seems to select for *Chlorobi* in metalimnetic communities (Yannarell and Kent, 2009). *Verrucomicrobia* are present in low abundance (1% – 6%) in both freshwater sediments and the water column from oligotrophic and eutrophic systems (Yannarell and Kent, 2009; Newton *et al.,* 2011). Some members of the *Verrucomicrobia* seem to be associated with high-nutrient environments or algal blooms (Eiler and Bertilsson, 2004; Kolmonen *et al.,* 2004; Haukka *et al.,* 2006).

1.3 Temporal and spatial variation in bacterial communities

Freshwater bacterial communities are complex and genetically very diverse (Gilbert *et al.,* 2009), but have low evenness when compared to other communities (Zwart *et al.,* 2002; Yannarell and Kent, 2009). In other words, at any given time, bacterial communities tend to be dominated by a few different groups, with the majority of species present at a low abundance (Zwart *et al.,* 2002; Pernthaler and Amann, 2005; Yannarell and Kent, 2009; Kirchman, 2012). Dominant strains will flourish for a short time period at different times and depths, resulting in a series (succession) of dominant community members (Yannarell and Kent, 2009). This process suggests that freshwater bacterial dynamics are managed by a variety of rapidly changing niches that are utilised by different species, which are from a large group of dormant organisms (Sigee, 2005; Yannarell and Kent, 2009). The activity of the dominant species is mainly responsible for the construction of new niches (Sigee, 2005; Yannarell and Kent, 2009; Kirchman, 2012). These niches are rapidly dominated by previously dormant species, which then create new niches (Yannarell and Kent, 2009). The rapid development and dissolution of niches can cause dramatic shifts in bacterial community structures over a short time period (Yannarell and Kent, 2009). However, bacterial communities do not always change rapidly. Change in bacterial communities appears to vary between long periods of stability and periods of rapid turnover (Yannarell and Kent, 2009). Thus, pelagic bacterial communities may experience a series of successions during the year (Zwisler *et al.,* 2003; De Wever *et al.,* 2005; Yannarell and Kent, 2009; Rösel *et al.,* 2012). To summarize, new ecological niches are created, these are filled, and bacterial communities adapt to the prevalent environmental conditions. As conditions change,

bacterial species will turnover rapidly and the entire process starts over again along a different ecological trajectory (Yannarell and Kent, 2009).

1.3.1 Temporal variation

Studies suggest that seasonal events are the primary source of change in bacterial communities (Leff *et al.,* 1999; Crump *et al.,* 2003; Crump and Hobbie, 2005; Lindström *et al.,* 2005; Yannarell and Kent, 2009). Temporal succession is driven by physico-chemical environmental variables such as light, temperature, wind (Boucher *et al.,* 2006), flow rate (Crump and Hobbie, 2005), dissolved organic carbon (DOC) (Brümmer *et al.,* 2000; 2004; Allgaier and Grossart, 2006; Hullar *et al.,* 2006), and phytoplankton biomass (Höfle *et al.,* 1999; Allgaier and Grossart, 2006). These sources control the dynamics of all biota via nutrient flow, carbon input, and primary production (Boucher *et al.,* 2006; Anderson-Glenna *et al.,* 2008). Primary producers are directly linked to bacterioplankton by microbial food webs (Boucher *et al.,* 2006). Studies suggest that temperature is the strongest driver of temporal bacterial succession (Yannarell *et al.,* 2003; Crump and Hobbie, 2005; Hall *et al.,* 2008; Yannarell and Kent, 2009). Bacterial growth rates in freshwaters appear to be dependent on temperature only up to around 15°C (Yannarell and Kent, 2009). However, temperature may still affect bacterial diversity and community composition outside of this range (Yannarell and Kent, 2009). In some freshwaters, temperature is the determining factor of water density and therefore controls water-column mixing, which has been demonstrated to affect bacterioplankton communities (Yannarell and Kent, 2009).

1.3.2 Spatial variation

Evidence of vertical and horizontal heterogeneity in bacterial community composition within and among freshwaters has been well documented (Lindström *et al.,* 2005; Yannarell and Triplett, 2004; 2005; Anderson-Glenna *et al.,* 2008). Spatial variation is important for the creation and preservation of biological diversity (Yannarell and Kent, 2009). In addition, spatial relationships can assemble biological interactions and limit the flow of nutrients and energy in ecosystems (Yannarell and Kent, 2009). Environmental changes at different depths are important sources of vertical variation for bacterial communities (Nold and Zwart, 1998; De Wever *et al.,* 2005; Yannarell and Kent, 2009; Zeng *et al.,* 2009). The presence or absence of available oxygen is one of

the key factors that alter with depth (Yannarell and Kent, 2009; Shade *et al.,* 2010; Meuser *et al.,* 2013). Bacterial diversity differs between the epilimnion (oxygenated) and hypolimnion (anoxic/anaerobic) of freshwaters (Øvreås *et al.,* 1997; De Wever *et al.,* 2005; Xingqing *et al.,* 2008; Yannarell and Kent, 2009). The abundance and mean cell size of bacteria in anoxic waters are greater than in aerated waters, and anoxic bacterial communities are overall more productive (Yannarell and Kent, 2009). Different bacterial phototrophs are found at specific water depths due to changing light levels and varying spectral properties of incoming photons (Nold and Zwart, 1998; Yannarell and Kent, 2009). For example, *Cyanobacteria* are present in the oxic epilimnion, *Chlorobi* and phototrophic *Gammaproteobacteria* are found near the oxic-anoxic interface, and *Chloroflexi* thrive near the top of the anoxic zone, where they can oxidize hydrogen sulphide (H_2S) (Nold and Zwart, 1998; Yannarell and Kent, 2009).

In addition to vertical variation in bacterial communities, horizontal heterogeneity has been observed in many freshwater systems (Xu and Leff, 2004; De Wever *et al.,* 2005; Yannarell and Triplett, 2004; Winter *et al.,* 2007). Horizontal variation in bacterial community composition is generally small compared to differences seen among freshwater systems (Yannarell and Triplett, 2004; 2005; Tong *et al.,* 2005; Van der Gucht *et al.,* 2005; Yannarell and Kent, 2009). Horizontal variation between different freshwater habitats has been attributed to DOC availability, phytoplankton productivity (Yannarell and Triplett, 2004), pH, water clarity (Yannarell and Triplett, 2005), nutrient concentrations (Lindström, 2000), water retention time (Lindström *et al.,* 2005), and landscape-level features (Yannarell and Kent, 2009). Bacterial communities horizontally distributed between different freshwaters are not always distinct. This is especially the case when the systems have very similar physico-chemical environments and when community composition shows a great deal of temporal variation (Yannarell and Triplett, 2004; Crump and Hobbie, 2005; Yannarell and Kent, 2009). Horizontal heterogeneity may indicate that different regions of a freshwater system consist of bacteria with different sets of niches (Yannarell and Kent, 2009). Alternatively, rapid bacterial growth rates may allow communities to display distinct characteristics on time scales shorter than the average retention time of the surface waters in the different regions of the water body (Yannarell and Kent, 2009).

1.4 Microbial processes

Metabolic activities of freshwater microorganisms range from the micro level (e.g., localized adsorption of nutrients and surface secretion of exoenzymes) through population dynamics (interspecific interactions within planktonic and benthic communities) to the influence of physico-chemical conditions on microbial communities (Sigee, 2005). Microbial communities control the annual primary production, including the recycling of carbon, sulphur, nitrogen, and iron (Friedrich, 2011). Their strategies for the supply and use of energy are the determining factors of the trophic and biogeochemical status of an ecosystem (Paerl and Pinckney, 1996). In freshwater systems, the balance between autotrophy (use of inorganic carbon as sole carbon source) and heterotrophy (use of organic carbon as sole carbon source), and subsequent ambient oxygen levels, reflect microbial production and biogeochemical cycling dynamics (Paerl and Pinckney, 1996). Heterotrophic bacteria are largely responsible for aerobic and anaerobic respiration, the decomposition and remineralisation of organic material, and the recycling of various key elements such as carbon, nitrogen, sulphur and phosphorus (Cole, 1999; Sigee, 2005; Friedrich, 2011). Thus, heterotrophic bacteria contribute to the nutrient and carbon cycles in two major ways: (i) by secondary production (production of new bacterial biomass) and (ii) by the remineralisation of organic carbon (to carbon dioxide (CO_2) or methane) and nutrients (Del Giorgio and Cole, 1998).

1.4.1 Carbon cycle

Carbon cycling in freshwater environments is of great importance, as it affects climate at a regional and global scale (Pernthaler, 2013). The net metabolic balance of freshwaters (i.e., the release or fixation of CO_2) is associated with the type and size of major organic carbon pools available for respiration by pelagic and benthic bacteria and Archaea (Ask *et al.,* 2009; Tranvik *et al.,* 2009). Heterotrophic bacteria degrade organic material by aerobic respiration, which consumes oxygen, to produce CO_2 and water (Kirchman, 2012). Lakes and rivers receive high quantities of dissolved organic carbon (DOC), dissolved inorganic carbon (DIC) and particulate organic carbon (PIC) from soil and other terrestrial environments (Tranvik *et al.,* 2009). Furthermore, anthropogenic activities also contribute to carbon concentrations and therefore alter carbon balances (Tranvik *et al.,* 2009). Since the anthropogenic production of CO_2 is not balanced by

CO_2 consumption, CO_2 concentration in the atmosphere is increasing and thereby affects atmospheric heat (Tranvik *et al.,* 2009; Kirchman, 2012).

Freshwater systems are also involved in the production and cycling of the important greenhouse gas methane (Tranvik *et al.,* 2009). The flux of methane is nearly entirely controlled by methanogens (methane-producing bacteria) and methanotrophs (methane-consuming bacteria) (Borrel *et al.,* 2011). Although freshwaters cover < 1% of the earth's surface (Downing *et al.,* 2006), they are the main source of biogenic methane as it was estimated that they contribute 6 – 16% of natural methane emissions (Bastviken *et al.,* 2004). Methane production was thought to be strictly anaerobic process that prevails in sediments and hypolimnia in many stratified lakes (Bastviken, 2009). New evidence suggests that methane production can occur in fully oxygenated epilimnetic waters of an oligotrophic lake (Grossart *et al.,* 2011). This process is possibly caused by metabolic interactions between methanogenic Archaea and autotrophs (Grossart *et al.,* 2011). Freshwater sediments are regarded as "hot spots" of methane production and freshwaters can be major contributors in global methane budget (Bastviken *et al.,* 2004). A part of methane generated within hypolimnetic sediments is released via gas bubbles into the atmosphere, but much of the methane produced in deeper sediments most likely travels upwards by diffusive flux into the water column and is oxidised into CO_2 by methane-oxidising bacteria (Bastviken *et al.,* 2002; 2004; Whalen, 2005; Kankaala *et al.,* 2006; Juottonen *et al.,* 2005; Schubert *et al.,* 2011).

1.4.2 Nitrogen cycle

Nitrogen is an essential element for several reasons: (i) it is incorporated into nucleic acids, proteins and many other biomolecules, where it exist, or is present as, oxidation state-III (e.g., NH_3) (Sigee, 2005); (ii) the supply of fixed nitrogen compounds, such as nitrate and ammonium, often limits growth and biomass production of microbes since they need a large amount of nitrogen for microbial and biogeochemical processes (Kirchman, 2012); and (iii) nitrogen is also involved in several important redox reactions as it can adopt many oxidation states (Kirchman, 2012). As a result, many nitrogenous compounds participate in catabolic reactions (energy production), either as electron donors or acceptors (Kirchman, 2012).

Human activities have large impacts on the nitrogen cycle (Galloway *et al.,* 2008; Erisman *et al.,* 2013). Nitrogen enrichment of freshwaters generally originates from surface sources such as fertilizer runoff, erosion of nutrient-rich sediments, industrial leaching, and sewage discharge or faecal pollution (Erisman *et al.,* 2013). The extra nitrogen released into freshwaters can cause a cascade of undesirable events. As nitrogen increases with increasing nutrient load, phytoplankton capable of assimilating nitrogen are progressively favoured over species that are limited by other factors (Erisman *et al.,* 2013). Consequently, algal or cyanobacterial blooms result leading to surface water hypoxia and the release of toxins (Erisman *et al.,* 2013). This in turn affects sensitive organisms higher on the food web, such as invertebrates and fish (Rabalais *et al.,* 2002; Camargo and Alonso, 2006). Sedimentation and decomposition of phytoplankton biomass can deplete oxygen in bottom waters and surface sediments, especially if systems have low rates of water turnover (Rabalais *et al.,* 2002). Furthermore, this shifts the benthic community towards less tolerant species (Erisman *et al.,* 2013). Ultimately, changes in the benthic community alter nutrient cycling in the sediments and water column which finally alter the rest of the aquatic ecosystem (Grizzetti *et al.,* 2011).

1.4.3 Sulphur cycle

Sulphur is used by all living organisms in both organic and inorganic forms (Wetzel, 2001). It is a major component of many organic molecules and is part of some amino acids that are fundamental to protein structure (Dodds and Whiles, 2010). The nutritional demand for sulphur is nearly always met by the abundance and ubiquity of sulphate, sulphide, and organic sulphur-containing compounds (Wetzel, 2001). Sources of sulphur compounds to freshwaters include solubilisation of rocks, agricultural fertilizers, and atmospheric precipitation and dry sedimentation (Wetzel, 2001).

Microbial interactions involved in the cycling of sulphur are confined to eutrophic water bodies (Sigee, 2005). The latter are divided into distinct aerobic and anaerobic zones within the water column, which separate microbial metabolic activities based upon their oxygen requirements (Sigee, 2005). Incorporation of inorganic sulphur compounds into biomass mainly occurs in the aerobic epilimnion (trophogenic zone), while the anaerobic

hypolimnion and sediments are the primary sites of conversion from organic sulphur to its inorganic form (tropholytic zones) (Sigee, 2005). Dissolved inorganic sulphate ions (SO_4^{2-}) occur primarily in the epilimnion (Sigee, 2005). These ions are reduced to sulphydryl (—SH) groups during protein synthesis, with the associated production of oxygen that is used by sulphur-reducing bacteria (e.g., *Desulfovibrio* and *Desulfotomaculum*) for the oxidation of molecular hydrogen or carbon compounds (Wetzel, 2001; Sigee, 2005). Death and sedimentation of freshwater biota leads to cell disintegration and protein decomposition in the hypolimnion and sediment (Sigee, 2005). Heterotrophic sulphate-reducing bacteria (e.g., *Pseudomonas liquefaciens* and *Bacterium delicatum*) will further reduce HS^- to hydrogen sulphide (H_2S) during the process of protein decomposition (Kuznetsov, 1970; Wetzel, 2001; Sigee, 2005). Hydrogen sulphide generated in the sediments diffuses vertically through the hypolimnion and is rapidly oxidized under aerobic conditions, therefore little H_2S will occur in aerated water columns (Wetzel, 2001; Sigee, 2005).

In addition to protein decomposition and sulphate reduction, the sulphur cycle is also involved in two other metabolic processes including aerobic and anaerobic sulphide oxidation (Sigee, 2005). Two major sulphur-oxidizing bacterial groups are responsible for these two types of metabolisms: (i) the chemosynthetic (colourless) sulphur-oxidizing bacteria, and (ii) photosynthetic (coloured) sulphur-oxidizing bacteria (Wetzel, 2001). The chemosynthetic sulphur-oxidizing bacteria are mostly aerobic and oxidize sulphide to sulphate via elemental sulphur (Wetzel, 2001; Sigee, 2005). Sulphur is then deposited either inside (*Beggiatoa* and *Thiothrix*) or outside the cell (*Thiobacillus*) as an intermediate (Wetzel, 2001; Sigee, 2005). Sulphur deposition inside the cell will continue as long as sulphide is available (Wetzel, 2001; Sigee, 2005). Once sulphide sources are depleted, the internal store of sulphur is oxidized and sulphate is released into the surrounding water (Wetzel, 2001; Sigee, 2005). The photosynthetic sulphur-oxidizing bacteria are anaerobic organisms that occur at the top of the hypolimnion (Wetzel, 2001; Sigee, 2005). This group can be divided into two subgroups: (i) the green sulphur bacteria, and (ii) purple sulphur bacteria (Wetzel, 2001; Sigee, 2005). Both subgroups oxidize sulphide to sulphur or sulphate via a light-mediated reaction (Wetzel, 2001; Sigee, 2005).

Besides the nutritional value of the sulphur cycle to freshwater biota, it is of importance for several other reasons: (i) some water quality problems revolve around sulphide contamination (Dodds and Whiles, 2010); (ii) sulphur is also tightly linked to the inorganic metal cycles, such as iron and manganese, and thus, indirectly to phosphorus (Dodds and Whiles, 2010); and (iii) the decomposition of organic material containing proteinaceous sulphur, and the anaerobic reduction of sulphate in stratified waters both contribute to altered water conditions (Wetzel, 2001). As a result, the cycling of other nutrients, ecosystem productivity, and distribution of biota are substantially affected (Wetzel, 2001).

1.4.4 Phosphorus cycle

Phosphorus is an essential element in all living organisms (Sigee, 2005). It is found in cells as a structural molecule (phospholipids and nucleic acids), where it is a major storage component, particularly polyphosphates. It is also involved in energy transformations (ATP) (Sigee, 2005). Phosphorus in freshwaters is present in three forms: (i) as soluble/dissolved organic matter (DOM); (ii) insoluble organic phosphate (biota and detritus); and (iii) soluble inorganic phosphate (Sigee, 2005). Freshwater algae usually assimilate phosphorus as phosphate ions (PO_4^{3-}). Particulates that are not assimilated may be deposited in the bottom sediments, where microbial communities gradually use many of the organic components of the sediments (Correll, 1998). Ultimately, most of the phosphorus is released back to the water column via internal loading (entry from sediments) as phosphate (Correll, 1998).

Phosphorus is the least abundant element in freshwaters but is usually the first nutrient to limit primary production (Wetzel, 2001; Dodds and Whiles, 2010). Thus, phosphorus is the determining factor of the trophic status of a water body (Sigee, 2005). This element is delivered to water bodies in three main ways: (i) external loading; (ii) internal loading; and (iii) nutrient cycling. External loading involves the entry of phosphorus via other water bodies, run-off of agricultural fertilizers, and the input of human and industrial effluent (Sigee, 2005). This type of phosphorus loading is usually the major cause of eutrophication in freshwaters (Sigee, 2005). Internal loading entails the continuous release of phosphate into the water column by bacterial decomposition of phosphorus-rich detritus on bottom sediments (Sigee, 2005). This process depends on

the oxygenated state of the sediment/water interface (Sigee, 2005). Most of the recycling of phosphorus is associated with microbiota (Wetzel, 2001). It includes the direct release of phosphorus from phytoplankton cells (by leakage of metabolites or death and cell lysis), and the excretion from macroinvertebrates and higher organisms (Wetzel, 2001; Sigee, 2005). Phosphorus recycling is environmentally important because absorbed nutrients become temporarily available for phytoplankton and bacterial growth (Sigee, 2005).

1.5 Physico-chemical impacts on microbial community structures

Microorganisms have the ability to adapt to changing environmental conditions to ensure their survival, therefore different environments often have different microbial communities (Kirchman, 2012). Environmental factors such as temperature (Lindström *et al.,* 2005), pH (Lindström *et al.,* 2005), salinity (Langenheder and Ragnarsson, 2007), dissolved organic matter (Eiler *et al.,* 2003), water clarity (Yannarell and Triplett, 2005), hydraulic retention time (Lindström *et al.,* 2006), and electrical conductivity (De Figueiredo *et al.,* 2012) have all been proven to affect the community composition of freshwater microbial assemblages. Examining environmental parameters in relation to temporal and spatial variation in microbial community composition is important to determine the contributing factors to succession (Wetzel, 2001; Kirchman, 2012).

1.5.1 Temperature, pH and salinity

Temperature is one of the primary drivers of growth and survival of microorganisms and thus variation in bacterial community structures (Sigee, 2005; Kirchman, 2012). Microbial communities may be more diverse in warmer waters because of profound effects of temperature on metabolic activity (Kirchman, 2012). Higher temperatures cause faster metabolic rates, which ultimately lead to higher rates of speciation (Kirchman, 2012). Temperature has an immediate impact on microbial enzymatic and abiotic reactions in the environment (Kirchman, 2012). The Arrhenius equation predicts that the rate of all chemical reactions increases exponentially with temperature:

$$k = Ae^{-E/RT}$$

The equation describes how a reaction rate (k, expressed as units per time) varies as a function of temperature (T, expressed in Kelvin), where R is the gas constant (8.29 kJ/mol/K), A is an arbitrary constant, and E is the activation energy (Kirchman, 2012). Understanding the effects of temperature on freshwater microbial communities would have huge implications for understanding the impact of climate change on carbon cycling and the rest of the atmosphere (Kirchman, 2012).

The pH has almost as great an effect on microbial communities as does temperature (Lindström *et al.,* 2005; Yannarell and Triplett, 2005; Kirchman, 2012). It controls biogeochemical transformations and mediates the availability of non-metallic ions (e.g., ammonium), essential elements (e.g., selenium), and trace metals, which can have both inhibitory and growth-enhancing effects (DWAF, 1996a; Yannarell and Triplett, 2005). pH is affected by physico-chemical factors, such as temperature, organic and inorganic concentrations, and biological activity (DWAF, 1996a; Fierer *et al.,* 2007). A small alteration in pH may cause changes in the bacterial community composition, leading to the dominance of certain groups (Lindström *et al.,* 2005; Yannarell and Triplett, 2005; De Figueiredo *et al.,* 2007; Lear *et al.,* 2009; Tian *et al.,* 2009). For example, Tian et al. (2009) demonstrated that alterations in pH from neutral to alkaline conditions lead to the dominance of *Cyanobacteria*, *Alphaproteobacteria*, and *Bacteroidetes*. Another study conducted by Lear et al. (2009) showed significant differences in bacterial community composition among neutral to alkaline (pH 6.7 – 8.3), acidic (pH 3.9 – 5.7), and very acidic (pH 2.8 – 3.5) streams. Streams with a neutral pH were dominated by *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria*. On the other hand, iron-oxidizing bacteria such as *Gallionella*, *Acidocella*, *Acidiphillum*, and *Acidobacteria* were abundant in acidic streams, while the very acidic streams were dominated by the filamentous alga *Klebsormidium* and the diatom *Navicula* (Lear *et al.,* 2009). These results suggested that different taxa could be selected in alkaline and acidic environments.

The salinity of freshwater systems is in general very low (Wetzel, 2001). Major sources of salinity include leaching from rocks and soil runoff from drainage basins, atmospheric precipitation, and particulate deposition (Wetzel, 2001). Salts can also enter a water body via domestic and industrial effluent discharges, and surface runoff from urban,

industrial, and agricultural areas (DWAF, 1996a). The salinity in freshwaters greatly affects the distribution of microbial community composition in both pelagic and benthic environments (Nold and Zwart, 1998). Although some bacterial and algal groups can tolerate only a narrow range of salinity, most bacteria can adapt to a wide range of salinity (Wetzel, 2001). Drastic changes in the ionic water composition may lead to changes in the community composition and associated changes in metabolic processes (Hart *et al.,* 1991; Bailey and James, 2000). The proportional concentrations of the major ions (Ca, Mg, Na, K, HCO_3-CO_3, SO_4 and Cl) affect the buffering capacity of the water and therefore microbial metabolism (DWAF, 1996a). In addition, changes in salinity can affect the fate and impact of other chemical compounds and contaminants (DWAF, 1996a).

1.5.2 Dissolved Organic Matter

Dissolved organic matter (DOM) is the main pool of reduced organic carbon in most freshwater systems (Del Giorgio and Cole, 1998). Assimilation of DOM by heterotrophic bacteria represents one of the main fluxes of organic carbon in freshwaters (Cole, 1999; Kritzberg *et al.,* 2005). In addition, bacterial respiration during the assimilation process is the major component of total respiration in many environments (Del Giorgio and Cole, 1998). DOM in freshwaters is derived either from autochthonous or allochthonous sources (Findlay and Sinsabaugh, 1999; Kirchman *et al.,* 2004). Autochthonous DOM is comprised of protein-like, labile polysaccharides derived from the metabolism of plankton, bacterial biomass, and macrophytes (Kaplan and Bott, 1989; Benner, 2002; 2003; Bertilsson and Jones, 2003). Allochthonous DOM contains aromatic, humic-like material and structural polysaccharides, such as cellulose and lignin, derived from the decomposition and leaching of organic matter from terrestrial plants and soil (Findlay and Sinsabaugh, 1999; McKnight *et al.,* 2001; Benner, 2002; 2003). There is growing evidence that variation in the composition, source, and supply of DOM causes rapid shifts in the bacterial community composition as a result of differences in the growth rates of bacterial groups on different DOM substrates (Van Hannen *et al.,* 1999; Findlay *et al.,* 2003; Docherty *et al.,* 2006; Judd *et al.,* 2006; Kritzberg *et al.,* 2006).

1.6 Anthropogenic impacts on bacterial community structures

Increase in human population growth as well as economic and industrial development have caused natural freshwater systems to markedly deteriorate in terms of water quality, biodiversity, in-stream processes, watershed hydrological regimes, and landscape (Chin, 2006; O'Driscoll *et al.,* 2010; Martinuzzi *et al.,* 2014). Such changes have been predominantly observed in rivers and streams in highly developed and dense residential areas (Haller *et al.,* 2011; Zhou *et al.,* 2011; Ibekwe *et al.,* 2012; Zhang *et al.,* 2012; Yang *et al.,* 2013; Yu *et al.,* 2014). Discharge from anthropogenic activities (e.g., municipal, industrial, mining, wastewater treatment plants and agricultural activities) expose freshwater systems to a variety of organic and inorganic pollutants, nutrients stress, heavy metals, and biological material (Ford, 2000). For urban rivers, domestic sewage and industrial effluent are the main pollution sources, in which nutrients and heavy metals are the general contaminants (Cheung *et al.,* 2003; Iwegbue *et al.,* 2012; Li *et al.,* 2012). In addition, dry land agriculture further contributes to nutrient loadings (e.g., nitrates and phosphates) and toxic compounds in the form of fertilizers, herbicides, and pesticides (Combes, 2003; Pesce *et al.,* 2008; Bricheux *et al.,* 2013; Kamjunke *et al.,* 2013). These contaminants cause a highly stressed environment in which communities have to adapt to ensure survival (Ford, 2000). For example, bacterial communities will select for more toxin resistant species following a pollution event causing a reduction in species diversity (richness and evenness) and overall change in community structure (Ford, 2000; Ager *et al.,* 2010; Proia *et al.,* 2012). Toxin resistant taxa will increase in abundance and dominate communities as long as perturbed conditions exist (Ford, 2000). Such changes may cause a cascade of effects on the different trophic levels of the food web and eventually the entire ecosystem (Ricciardi *et al.,* 2009).

1.7 Microorganisms as bioindicators

Bioindication is the use of an organism(s) to obtain information on the quality of an ecosystem (Stankovic and Stankovic, 2013). Thousands of different contaminants exist and their potential toxicity may vary with the physico-chemical water chemistry of the habitat (Proia *et al.,* 2012). Thus, the choice of bioindicator is pivotal to accurately

describe the natural environment and to detect and assess human impacts (Stankovic and Stankovic, 2013).

Microorganisms, such as bacteria, exist at the lowest trophic level and have the ability to quickly detect contaminants before other organisms (e.g., macroinvertebrates) do (Stankovic and Stankovic, 2013). Their capability to rapidly respond to environmental changes at molecular and biological level make them sensitive and relevant indicators of contaminant exposure and ecosystem health (Ford, 2000; Ager *et al.,* 2010; Schultz *et al.,* 2013). Over the last decade, the use of microbial communities as model systems in ecology and ecotoxicology has been greater than ever (Proia *et al.,* 2012). The use of microbial communities as bioindicators is appealing for several reasons: (i) their rapid interaction with dissolved substances results in functional (short-term) and structural (long-term) changes, making them early warning indicators of disturbances (Sabater *et al.,* 2007); (ii) relative abundances of pollution tolerant or intolerant taxa indicates the response to stress conveyed to the system by perturbations (Lemke *et al.,* 1997); (iii) community structure analysis may contribute to a better understanding of the role that microbial communities play in natural self-purification of human-derived pollutants in water systems (Kenzaka *et al.,* 2001); (iv) evaluations of conditions using microbial communities can be more time and cost effective than complex chemical and physical analysis (Lemke *et al.,* 1997); and (v) changes in communities can be monitored on a regular basis to assess pollution recovery and successful environmental management (Lemke *et al.,* 1997). Before attempting to use microbial communities as bioindicators, knowledge of community dynamics and their association with environmental change is a fundamental prerequisite to understand how anthropogenic activities impact community composition, biogeochemical cycles, and ecosystem health (Ager *et al.,* 2010). Knowledge of the extent of these aspects is still in its infancy, but the introduction of molecular techniques (e.g., PCR, DGGE, T-RFLP, cloning and sequencing, etc.) applied to microbial ecology has made such studies possible.

1.8 Molecular techniques

Accurate identification of freshwater microorganisms is essential in understanding their ecology, function (Dodds, 2002), metabolism of natural organic compounds, and nutrient regeneration and recycling (Wetzel, 2000). However, microbial diversity and its

role in freshwater ecosystems are poorly understood mainly because conventional microbiological techniques (e.g., microscopy and cultivation) are insufficient to assess the bacterial diversity in natural samples (Schäfer and Muyzer, 2001). Nutritional requirements and environmental parameters for every population of freshwater biota are unspecified. It is estimated that less than 1% of microorganisms will grow on nutrient-rich media (Stenuit *et al.,* 2008). In addition, microscopic limitations, such as the lack of conspicuous morphology and small cell size, do not allow for the identification of the majority of environmental bacteria (Schäfer and Muyzer, 2001).

Limitations experienced by cultivation-base methods have largely been replaced by molecular tools and the development of new techniques that are revolutionizing environmental microbial ecology (Xu, 2006; Wakelin *et al.,* 2008; Xia *et al.,* 2013; Lu and Lu, 2014; Sauvain *et al.,* 2014). For example, real-time PCR, denaturing gradient gel electrophoresis (DGGE), and 454-pyrosequencing are continuously providing new insights into the dynamics of microbial communities in pristine and disturbed freshwater ecosystems (Ghai *et al.,* 2011; Xia *et al.,* 2013; Lu and Lu, 2014; Sauvain *et al.,* 2014). Many of these studies also incorporated multivariate analysis to link species composition and environmental parameters to determine which factors were responsible for altering species diversity (Ricciardi *et al.,* 2009). This method has proved to be extremely useful in determining how pollutants impact microbial diversity in aquatic ecosystems (Araya *et al.,* 2003; Pesce *et al.,* 2008; Bouskill *et al.,* 2010; De Figueiredo *et al.,* 2012). As technology improves and new methods become available, researchers will be able to further explore the functional network adaptability of bacterial communities. This information can assist in predicting their capacity to maintain ecosystem homeostasis, the impact of future threats, and subsequent recovery during remedial treatment (Ager *et al.,* 2010; Laplante and Derome, 2011; Schultz *et al.,* 2013).

1.9 Community fingerprinting methods

1.9.1 Denaturing Gradient Gel Electrophoresis (DGGE)

PCR-DGGE has been applied in numerous aquatic studies to determine microbial diversity and detect specific organisms without the need for cultivation (Lyautey *et al.,* 2003; Essahale *et al.,* 2010; De Figueiredo *et al.,* 2012; Haller *et al.,* 2011). This method opened up new avenues of research on the diversity, functions, and interactions of

microorganisms present in complex aquatic environments. Its applications have allowed investigators to probe the similarities of distinct microbial communities by comparing their community compositions (Liu *et al.,* 2009a). Microbial diversity and community composition can be determined using both DNA and RNA fragments. DNA-based analysis detects the total microbial community structure irrespective of their viability or metabolic activity (Sessitsch *et al.,* 2002). On the other hand, RNA-based analysis reflects predominantly the diversity of metabolically active microorganisms and thus the functionality of the community (MacGregor, 1999; Nogales *et al.,* 2001). By combining DNA- and RNA-based methods, the total community structure and its metabolic activity and functionality can be measured.

PCR-DGGE is an electrophoretic method capable of detecting differences between DNA fragments of the same size but with different sequences (Muyzer *et al.,* 1993). Double-stranded DNA fragments are separated in a denaturing gradient polyacrylamide gel based on their differential denaturation melting profile (Muyzer *et al.,* 1993; Ercolini, 2004). These DGGE patterns provide a series of bands relative to the microbial species present. Identification of the species and thus taxonomic information can be achieved by excising, purifying and sequencing the bands (Ercolini, 2004). The use of DGGE to study microbial diversity is an improvement to cloning and subsequent sequencing of PCR fragments (Muyzer *et al.,* 1993). Population dynamics in an ecosystem are demonstrated in both a qualitative and a semi-quantitative way (Muyzer *et al.,* 1993). Moreover, DGGE fingerprints can be combined with statistical analysis and calculation of biodiversity indices (e.g. Shannon-Weaver and Simpson's indices) and cluster analysis to compare complex bacterial community structures in different environments (Gafan *et al.,* 2005; Zhang *et al.,* 2011). The total number of DGGE bands and their relative intensities would in theory reflect the microbial diversity without the need for cultivation (Gafan *et al.,* 2005).

Despite the advantages that DGGE offers, it also holds limitations. The major shortcomings include: (i) the short 16S rDNA fragments (500 bp) limit the specificity required for phylogenetic identification of some organisms (Gilbride *et al.,* 2006); (ii) organisms have multiple copies of rDNA, thus multiple bands for a single species may occur (Nübel *et al.,* 1997); (iii) different species may have identical migration patterns

which might lead to overestimation of their presence and abundance within the microbial community (Malik *et al.,* 2008); (iv) DGGE analysis of microbial communities produces a complex profile which can be sensitive to spatial and temporal variations (Murray *et al.,* 1998); and (v) variable gel staining methods result in low sensitivity and decrease reproducibility (Nocker *et al.,* 2007). Gel staining often results in background staining which complicates the characterisation of weak bands (less abundant species) from the background (Nocker *et al.,* 2007).

1.10 Metagenomics

Metagenomics is the study of collective microbial genomes isolated directly from environmental samples and does not rely on cultivation or prior knowledge of the microbial communities (Riesenfeld *et al.,* 2004). Essentially, metagenomics is based on the notion that the entire genetic structure of microbial communities could be sequenced and analysed in the same way as sequencing a whole genome of a pure bacterial culture (Rastogi *et al.,* 2011). Thus, phylogenetic and functional analyses of microorganisms and their interaction with physico-chemical and biotic factors can be determined at community level (Cowan *et al.,* 2005; Rastogi *et al.,* 2011). Metagenomic analysis of microbial communities has lately been the focus of several environmental studies of various ecosystems, such as soil, (Lemos *et al.,* 2011), freshwater lakes (Marshall *et al.,* 2008), planktonic marine assemblages (Breitbart *et al.,* 2009), and deep sea microbiota (Sogin *et al.,* 2006). These studies are paving the way for the detection of new genes, proteins and biochemical pathways (Cardenas and Tiedje, 2008). Metagenomics has also been applied to several studies of aquatic pollution and how pollutants affect microbial community composition (Porat *et al.,* 2010; Haller *et al.,* 2011; Vishnivetskaya *et al.,* 2011; Yergeau *et al.,* 2012; Proia *et al.,* 2013; Lu and Lu, 2014). These types of studies may assist in understanding microbial degradation of pollutants by monitoring the enzymes associated with the metabolism of contaminants (Malik *et al.,* 2008), and how the impacts of pollution can be reversed or at least mitigated (Cardoso *et al.,* 2012). Metagenomics of specific genes can further contribute to a more detailed understanding of which microorganisms are active at polluted sites and how they behave biochemically to different types of pollutants (Cardoso *et al.,* 2012).

Various metagenomic approaches are available, but next-generation sequencing (NGS) has revolutionised the field of microbial ecology and genomics. Next-generation sequencing technologies allow researchers to investigate complex microbial community compositions, their activities, and dynamics by sequencing at lower costs and higher throughput than the traditional Sanger sequencing (Scholz *et al.,* 2012; Bella *et al.,* 2013). These tools can aid in the interpretation of how bacteria interact with each other and their environment (Bella *et al.,* 2013).

Major advantages of NGS technologies, in addition to their high throughput, include: (i) the elimination of cloning as most recent technologies directly sequence single DNA molecules and thereby reduce biases and artefacts in template libraries; and (ii) volumes of reagents needed and overall costs are reduced allowing many more samples to be analysed (Delseny *et al.,* 2010). Although advantageous, NGS technologies have several limitations. The main drawback of NGS is the shorter read length compared to Sanger sequencing (Morey *et al.,* 2013). This is the result of the gradual decline in efficiency of the sequencing chemistry during the run (Morey *et al.,* 2013). Another limitation is the use of PCR amplification in the construction of amplicon libraries, which itself can introduce biases and artefacts (Acinas *et al.,* 2005). Furthermore, the assembly of short reads into longer sequences is a major challenge (Delseny *et al.,* 2010), and NGS research requires significant computational resources and strong bioinformatics skills to analyse data (Scholz *et al.,* 2012).

Various NGS platforms are currently commercially available including Roche/454, Illumina (Solexa/Genome Analyzer), and Applied Biosystems (SOLiD) (Balzer *et al.,* 2010). Of these platforms, 454-pyrosequencing was until recently one of the leading technologies for comparative genomics and metagenomics (Kunin *et al.,* 2010). A promising application is pyrosequencing of the hypervariable 16S rRNA gene regions to construct phylogenies and taxa within microbial communities (Claesson *et al.,* 2010; Kunin *et al.,* 2010). Although the hypervariable regions targeted are short (100 – 500 bp), this approach provides sufficient phylogenetic information (Rastogi *et al.,* 2011). A major advantage of 454-pyrosequencing is that multiple environmental samples can be analysed in a single run via multiplexing (Kunin *et al.,* 2010; Zarraonaindia *et al.,* 2013). This is done by assigning a DNA barcode to each DNA fragment prior to sequencing

(Zarraonaindia *et al.,* 2013). Following the sequencing run, reads are separated by their nucleotide barcode into the different sampling pools (Rastogi *et al.,* 2011; Zarraonaindia *et al.,* 2013). One limitation, however, is that the inherent error rate of pyrosequencing might lead to the overestimation of the number of rare phylotypes and thus diversity (Kunin *et al.,* 2010). Each pyrotag sequence is identified as a unique operational taxonomic unit of the community and therefore errors may inflate diversity estimates (Quince *et al.,* 2008; Kunin *et al.,* 2010). Nevertheless, this bias can be minimized by removing reads with: (i) undetermined bases; (ii) anomalous read lengths (e.g., reads shorter or longer than the expected amplicon size); (iii) reads with incorrect forward primer sequence; (iv) reads that misaligned; and (v) chimeras (Quince *et al.,* 2008; Kunin *et al.,* 2010; Comeau *et al.,* 2012). In addition, Kunin et al. (2010) suggested that a clustering threshold of 97% should be used for identification. These quality control steps reduce 454-pyrosequencing error rates to < 0.2% without the need for further denoising applications (Kunin *et al.,* 2010; Comeau *et al.,* 2012).

1.11 Multivariate analysis of environmental data

An important objective in ecological studies is to determine and understand the effects of abiotic environmental variables on the diversity of microbial communities in ecosystems (Van den Brink *et al.,* 2003; Kloep *et al.,* 2006). Spatial heterogeneity and variability of microbial populations require the application of statistical approaches, such as multivariate analysis, that are capable to facilitate ecological analyses and interpret complex relationships (Kloep *et al*., 2006).

Relationships among species and species-environmental variables are often described by ordination or cluster analysis (James and McCulloch, 1990). The basic aim of the two methods is to represent similarity/dissimilarity between samples based on multiple variables associated with them (Ramette, 2007). The former technique has proven to be particular useful because it enables the researcher to evaluate similarities/differences in species composition between sites in response to environmental variables in a single analysis (Van den Brink *et al.,* 2003). Results are depicted in a diagram (biplot or triplot) with both species and environmental variables in a reduced space (Van den Brink *et al.,* 2003). In addition, ordination analysis can be combined with Monte Carlo permutation

test to determine statistical significance between changes in species diversity and environmental factors (Ter Braak and Šmilauer, 2002).

Ordination methods can be classified as either unconstrained or constrained (Anderson and Willis, 2003). In both methods, all species are speculated to react to different extents to the same composite gradients of environmental variables (Ter Braak and Prentice, 1988). Importantly, unconstrained and constrained methods should be used in parallel (Ramette, 2007). Constrained ordination represents only the biological variation explained by the available environmental variables on the main axes, while unconstrained ordination represents the highest amount of variance on a few axes (Ramette, 2007). If both approaches yield similar ordination of the samples, the measured environmental variables then explained most of the biological variation (Ramette, 2007).

Unconstrained ordination reduces dimensions on the basis of general criteria such as minimizing residual variance or stress function (Anderson and Willis, 2003). They are extremely useful for visualizing broad patterns across the entire data set on a biplot diagram (Anderson and Willis, 2003). In addition, potential patterns of within-group variability or relative dispersion among groups can be visualized in cases where data are classified into two or more groups (a priori) (Anderson and Willis, 2003). The axes of an unconstrained ordination biplot correspond to the directions of greatest variability within the data set (Lepš and Šmilauer, 2003). The most commonly used unconstrained ordination methods include principal component analysis (PCA) and non-metric multidimensional scaling (NMDS).

Constrained ordination techniques are used to relate a matrix of response variables (e.g. species abundance) with predictor variables (e.g. environmental parameters) (Anderson and Willis, 2003) to provide a summary of species-environment relationships (Ter Braak and Prentice, 1988). However, constrained ordination does not allow assessment of either total or relative within-group variability, but rather location differences among groups (Anderson and Willis, 2003). The axes of a constrained bi- or triplot diagram correspond to the directions of the greatest data set variability that can be explained by the environmental variables (Lepš and Šmilauer, 2003). Constrained

ordination techniques frequently used by ecologists include canonical correspondence analysis (CCA) and redundancy analysis (RDA).

Each of the above mentioned ordination methods are briefly discussed below.

1.11.1 Principal component analysis (PCA)

This method is extensively used in all areas of ecology and systematics (James and McCulloch, 1990). PCA is relatively objective and provides a reasonable, but basic, indication of relationships. The latter are displayed on a two- or three-dimensional graph where both samples and species are represented (James and McCulloch, 1990; Ramette, 2007; Chahouki, 2011). The direction of a species arrow specifies the greatest change in abundance, whereas the length may be related to a rate of change (Ramette, 2007). PCA is generally used when sites/samples have very short gradients (i.e. when identical species are frequently identified in the study area) and when species respond linearly to environmental gradients (Ramette, 2007). Since these conditions are often not met in ecological studies, other multivariate approaches are preferred over PCA, for example correspondence analysis and multidimensional scaling (Ramette, 2007).

1.11.2 Non-metric multidimensional scaling (NMDS)

NMDS is generally effective at identifying underlying gradients and representing relationships based on several types of distance measures (Ramette, 2007). It estimate distances between samples using a “sample by sample” matrix (Van den Brink *et al.,* 2003). The latter is obtained by transforming the original “species by sample” matrix using a (dis)similarity measure (Van den Brink et al., 2003). NMDS is generally applied when species do not have a linear response to environmental gradients, and identifying patterns among multiple samples that were analysed by molecular fingerprinting techniques (Ramette, 2007). For example, Van der Gucht et al. (2005) used NMDS to determine the specificity of bacterioplankton community signatures in four shallow eutrophic lakes, which differed in nutrient load and food web structure, from DGGE profiles.

1.11.3 Redundancy analysis (RDA)

RDA can be considered as an extension of PCA where the main axes (components) are constrained to be linear combinations of environmental variables (Rao, 1964). Multiple linear regressions are performed within the iterative procedure to find the best ordination between species and environmental variables (Ramette, 2007). The relevance of such an approach is to represent the main patterns of species variation in response to environmental variables, but also display correlation coefficients between each species and each environmental (Ramette, 2007). An advantage of RDA is that it can use species or environmental data that are measured in different units (Chahouki, 2011). In such a case, the data must be centered and standardized before analysis (James and McCulloch, 1990). RDA is particularly useful in short-term experimental studies where gradients are short (Chahouki, 2011).

1.11.4 Canonical correspondence analysis (CCA)

CCA expresses species relationships as linear combinations of environmental variables (Green, 1989). It uses the unimodal model (i.e. relationships are symmetrical around the species optimum) to simulate species response to environmental variables as a mathematical simplification. This enables the estimation of several parameters and the identification of a small number of ordination axes (Ramette, 2007). The unimodal model seems to be robust and particularly adapted for the environmental interpretation of species occurrence and abundance, and accommodates the absence of species at specific sites (Ramette, 2007). An essential feature of this method is that it is sensitive to rare species that occur in species-poor samples (Legendre and Legendre, 1998). Also, the technique makes it possible to determine the response of specific species/OTU's to particular environmental variables (Ramette, 2007). Such species/OTU's can be identified as candidate indicator species and subjected for further experiments to confirm their status as indicators (Ramette, 2007).

1.12 Problem statement

South Africa is a water stressed country because of the unpredictable rainfall, high evaporation rates and low conversion of rainfall to runoff (NWDACE, 2002). Also, the increasing demand for water is rapidly approaching available supply (NWDACE, 2002). The North West Province is an arid, water-scarce province, as many surface water

systems are non-perennial (NWDACE, 2008). Rainfall in the province is highly variable, often resulting in severe droughts or extreme flooding (NWDACE, 2002). In addition, the evaporation rate of water in all catchments exceeds rainfall (NWDACE, 2002). The province's water resources are currently experiencing severe pressure as a result of population growth, development, agriculture, and mining (NWDACE, 2008). This results in insufficient water supply for all, and the available water, is not equally distributed (NWDACE, 2002). The two main water quality problems within the province include eutrophication and salinization (NWDACE, 2002). Both of these are caused by excessive loads of chemicals from industrial, domestic, and agricultural sources (NWDACE, 2002). Eutrophication of surface waters is likely the most serious water quality problem (NWDACE, 2002). It often causes nuisance algal blooms and excessive plant growth (e.g., water hyacinth) in rivers and dams throughout the province (NWDACE, 2002). Consequently, eutrophication has major ecological impacts on habitat integrity of aquatic or riparian fauna and flora, the natural cycle of rivers, and the microbial composition of surface waters (NWDACE, 2002). Lately, the Mooi River Catchment and Wonderfonteinspruit has been the subject of a large number of studies due to significant radioactive and heavy metal pollution by uranium rich gold mines in the area (IWQS, 1999; Coetzee *et al.,* 2002; Wade *et al.,* 2002; 2004; Winde, 2010a; b; Barnard *et al.,* 2013). Downstream metal contamination is of great concern since the water supply of Potchefstroom city is located below the confluence of the Mooi River and Wonderfonteinspruit (Barnard *et al.,* 2013).

Government agencies and private sectors currently use indicator organisms such as, total coliforms, faecal coliforms, *E. coli*, faecal streptococci, and coliphages, to monitor the microbiological water quality of river systems. However, cultivation methods are not always accurate and reliable. They may produce false positives, and the presence or absence of indicator organisms only indicates the degree of domestic and municipal wastewater contamination and not necessarily mining and industrial pollution. While the government aims to improve the quality and health of river systems in South Africa, little attention has been given to identify microbial community structures in rivers and establishing a baseline for biogeochemical conditions. In addition, possible links between microbial communities and anthropogenic disturbances, and the potential of microbial communities to be used as bioindicators are greatly neglected. In order to

improve and protect the ecological functions of river systems in South Africa, an understanding of these aspects is vital and significant efforts are needed to develop experimental studies to assess microbial responses following anthropogenic exposure.

Considering the critical roles played by microorganisms in freshwater systems and the lack of data on microbial communities in South Africa's river systems, the objectives of this study were to: (i) characterise bacterial community structures in surface waters in the North West Province using PCR-DGGE and pyrosequencing; (ii) link changes in bacterial diversity with environmental variables using multivariate analysis; (iii) determine the impact of anthropogenic activities on bacterial communities; and (iv) construct potential bacterial biogeochemical activity profiles in river systems.

1.13 Outline of the thesis

Chapter 1 gives an overview on microbial diversity in freshwater systems, common bacterial groups found in freshwaters and their spatial and temporal distribution, bacterial processes, the impact of physico-chemical parameters and anthropogenic disturbances on bacterial communities, the application of molecular techniques to elucidate bacterial community structures. The chapter concludes with a problem statement and prospective aims for this study.

Chapter 2 describes bacterial structures in a segment of the Vaal River in response to environmental parameters. Bacterial diversity was analysed using both PCR-DGGE and 454-pyrosequencing and correlations between the physical-chemical environment and community structures were assessed by multivariate analysis. Discussion of results from this investigation is presented in the following peer-reviewed journal:

Title: The impact of physico-chemical water quality parameters on bacterial diversity in the Vaal River, South Arica

Authors: Jordaan, K., Bezuidenhout, C.C.

Journal: Water SA, 39(3): 365–376

A copy of the article is appended.

Chapter 3 describes the impacts of urbanization on bacterial communities in the Mooi River Catchment, which is an urban river system that runs through the city of Potchefstroom. Bacterial community structures were analysed using 454-pyrosequencing and the impacts of urbanization were determined by multivariate analysis.

Title:	Bacterial community composition of an urban river in the North West Province, South Africa, in relation to physico-chemical water quality
Authors:	Jordaan, K., Bezuidenhout, C.C.
Target Journal:	Water SA

Chapter 4 describes the impacts of gold mining on bacterial communities and potential biogeochemical cycles in the Wonderfonteinspruit. Furthermore, the chapter illustrates associations between specific taxa and environmental drivers. Bacterial community structures were analysed using 454-pyrosequencing and the impact of gold mines was determined by multivariate analysis.

Title:	Impacts of physico-chemical parameters on bacterial community structure in a gold mine impacted river: A case study of the Wonderfonteinspruit, South Africa
Authors:	Jordaan, K., Comeau, A., Khasa, D., Bezuidenhout, C.C.
Target Journal:	Applied and Environmental Microbiology

Finally, Chapter 5 is a summary of the findings from which relevant conclusions are drawn. The chapter concludes with meaningful recommendations for future research in this field.

CHAPTER 2: The impact of physico-chemical water quality parameters on bacterial diversity in the Vaal River

2.1 Introduction

Socio-economic growth and development of the Vaal River require continuous augmentation of this water resource to meet the growing water requirements of communities in Gauteng, the Free State, North West and Northern Cape provinces (DWAF, 2009b). Water quality has drastically deteriorated due to constant disposal of industrial and domestic waste into the river. Salinisation, eutrophication and microbiological pollution are currently the main problems affecting the water quality (DWAF, 2009a). The Department of Water Affairs and Forestry (DWAF) of South Africa, in line with the South African National Water Act (NWA), Act No. 36 of 1998, stipulated regulatory guidelines and criteria a water system must meet to ensure that the country's water resources are fit for use. A structured biomonitoring programme was implemented by the DWA in 2009 to determine the exact sensitivity and health status of the Vaal River (DWAF, 2009a). Criteria routinely monitored to ensure sustainability, optimal water use and protection of the water resource includes physico-chemical characteristics, stream flow, discharge loads and microbiological pollutants, in particular *Escherichia coli* (DWAF, 2009a; b). The detection of *E. coli* only indicates the presence of faecal contamination and not necessarily the degree of industrial pollution. Therefore, in depth studies on the microbial communities in the Vaal River are essential to understand the microbial processes underlying secondary pollution and changes in the physico-chemical quality of water.

DGGE has been applied in numerous research studies involving the assessment of microbial diversity of rivers, streams, lakes and sediment to determine the water quality of the resource (De Figueiredo *et al.,* 2010; Essahale *et al.,* 2010; De Figueiredo *et al.,* 2012; Haller *et al.,* 2011). This method opened up new avenues of research on the diversity of microorganisms present in complex aquatic environments. Currently, metagenomic analysis of microbial ecology, such as high-throughput sequencing (HTS), has been the focus of several environmental studies such as soil, (Lemos *et al.,* 2011), freshwater lakes (Marshall *et al.,* 2008) and deep sea microbiota (Sogin *et al.,* 2006).

Metagenomic analysis provides extensive information on community structure and composition (Kakirde *et al.,* 2010). In addition, phylogenetic and functional analyses of microorganisms can be determined at community level (Cowan *et al.,* 2005).

The objectives of this study were: (i) to identify the bacterial community structures in the planktonic phase of the Vaal River using 16S rDNA PCR-DGGE and high-throughput sequencing, and (ii) determine the impact of physico-chemical characteristics on bacterial community structures using principle component analysis (PCA) and redundancy analysis (RDA).

2.2 Materials and Methods

2.2.1 Sample collection and physico-chemical analysis

Water samples were collected from the Vaal River in June 2009 (winter) and December 2010 (summer). The four sites included Deneysville (Vaal Dam) (26°53'43.44"S 28°5'53.88"E), Vaal Barrage (26°45′53"S 27°41′30"E), Parys (26°54'0.36"S 27°26'60"E), and Scandinawieë Drift (26°51'20.45"S 27°18'9.52"E) (Figure 2-1). The Vaal Dam and entire middle section of the Vaal River are respectively regarded as eutrophic and hypertrophic due to the high levels of chlorophyll-*a* and phosphate exceeding the recommended standards (DWAF, 2009a).

Samples were collected from the planktonic phase in sterile glass bottles and preserved on ice not longer than 6 hours until nucleic acid isolation. Physico-chemical analysis was conducted *in situ*. Additional physico-chemical data were obtained from the Department of Water Affairs (www.dwa.gov.za) and the South African Weather Service (www. weathersa.co.za). A summary of the physico-chemical variables of all studied sampling sites is shown in Table 2-1.

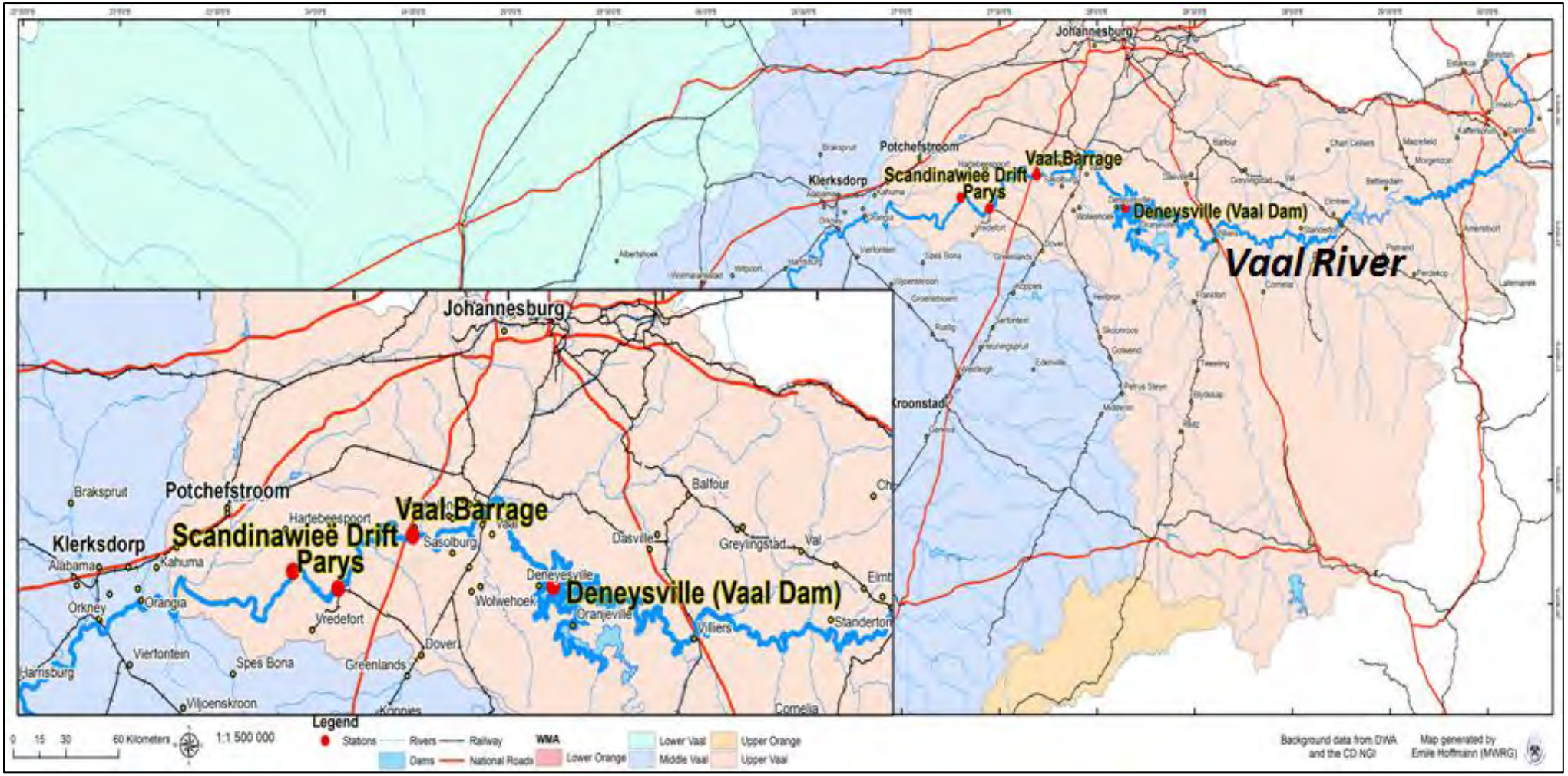

Figure 2-1: Geographical illustration of the Vaal River system. The four sampling stations are indicated on the map.

2.2.2 Nucleic acid isolation

A hundred millilitres of water samples were filtered through a 0.45 µm nitrate cellulose membrane filter (Whatman GE Healthcare Life Sciences, Buckinghamshire, UK) and subsequently lyzed in a 1 mg/ml lysozyme solution that contained 0.25 – 0.50 mm glass beads (Sigma-Aldrich Corporation, St. Louis, MO, USA) for bacterial cell disruption. The lysis solution was incubated at 37°C for 10 min while agitated in a vortex. Proteinase K (1 mg/ml) was then added and the lysis solution was incubated at 56°C for an additional 30 min. DNA was isolated from the crude lysate using the PeqGold Bacterial DNA Kit (PEQLAB Biotechnologie GmbH, Erlangen, Germany). The quality and quantity of the isolated nucleic acids were determined using the Nanodrop ND1000 (NanoDrop Technologies, Wilmington, DE, USA) and agarose electrophoresis.

2.2.3 PCR amplification and DGGE analysis of bacterial community structures

The highly variable V3 region of the 16S rDNA gene fragments were PCR amplified using the universal primer pair 341F-GC and 907R (~ 500 bp) (Muyzer *et al.,* 1993). Amplification was performed in 25 µl reaction volumes containing single strength PCR master mix [(5 U/µl *Taq* DNA polymerase (recombinant) in reaction buffer, 2 mM $MgCl_2$, 0.2 mM of each dNTP, Thermo Fisher Scientific, Waltham, MA, USA)], 50 pmol of forward and reverse primers, additional 1 mM $MgCl_2$, additional 1 Unit *Taq* DNA polymerase, 10 – 50 ng DNA and PCR-grade water (Thermo Fisher Scientific). Thermal cycling was carried out in a Bio-Rad iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) with an initial denaturation at 95˚C for 7 min followed by 30 cycles of denaturation at 95˚C for 30 s, annealing at 56˚C for 1 min and extension at 72˚C for 60 s. Final extension was performed at 72˚C for 7 min. PCR products were evaluated by electrophoresis on 1% agarose gels and visualized by ethidium bromide staining and UV illumination.

PCR products were analyzed by DGGE using a DCode Universal Detection System (Bio-Rad Laboratories). Four reference species, namely *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Streptococcus faecalis*, were included in all DGGE studies. DGGE analysis was conducted at a denaturing gradient of 30 – 50% in 1 mm vertical polyacrylamide gels (8% (wt/vol) acrylamide in 1 × TAE). Twenty microlitres of amplification product were mixed with five microlitres of loading buffer (6 ×

Orange Loading Dye, Thermo Fisher Scientific) and loaded into the gel. Electrophoresis was performed at a constant temperature of 60°C for 16 h at 100 V in 1 × TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). Polyacrylamide gels were stained with ethidium bromide (10 mg/ℓ) for 45 min and visualized with a Gene Genius Bio Imaging System (Syngene, Cambridge, UK) and GeneSnap software (version 6.00.22). None of the DGGE gels were digitally enhanced or modified. Bands of interest were only highlighted for better visualization and not analytical purposes. Selected DNA bands of interest were excised from gels with a sterile scalpel and eluted in 20 µl of sterile nuclease-free water for 12 h at 4°C. Two microlitres of the elute were used as DNA template in PCR amplification reactions with primer pair 341F and 907R (Muyzer *et al.,* 1993) and conditions described above. PCR products were subsequently purified and sequenced using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Life Technologies, Carlsbad, CA, USA) and Genetic Analyzer 3130 (Applied Biosystems Life Technologies). Sequences were aligned to 16S rRNA sequences in the National Center of Biotechnology Information Database (NCBI) using BLASTN searches (http://www.ncbi.nlm.nih.gov/BLAST) to determine their identity. A total of 23 bacterial nucleotide sequences were submitted to the GenBank database under accession numbers JQ085826 – JQ085849.

2.2.4 High-throughput sequencing

HTS analysis was performed by Inqaba Biotech, Pretoria, South Africa using the Roche 454 GS-FLXTM System. The V1-V3 region of the 16S rRNA gene was amplified using primer pair 27F and 518R (Lane, 1991) to produce ~ 500 bp fragments. Subsequently, sequences were trimmed to remove GS tags and further analyzed with the CLC Bio Genomics Workbench version 4.7.2 software (CLC Bio, Aarhus, Denmark). Sequences shorter than 200 bp in length were excluded from data sets. All remaining sequences were subjected to the National Center for Biotechnology Information (NCBI) database for BLAST analysis. Sequences were then submitted to Pintail version 1.0 to detect the presence PCR artifacts. PCR products with chimeric properties were eliminated from data sets prior to phylogenetic analysis. The remaining 922 sequences were submitted to GenBank with accession numbers JN865256 – JN866178.

2.2.5 Statistical analysis

Bacterial community diversity was calculated with the Shannon-Weaver diversity index (*H'*) based on DGGE profiles. The Shannon-Weaver indices (*H'*) were calculated according to Zhang et al. (2011). Similarities between the banding patterns generated by PCR-DGGE of the various sampling sites were compared by cluster analysis as indicated by Gafan et al. (2005). Cluster analyses were displayed graphically as UPGMA dendograms.

The distribution of samples according to environmental factors was analyzed by PCA. The statistical significance of the relationships between bacterial community structures, DGGE banding profiles, high-throughput sequencing data and water quality was further assessed by RDA. Environmental variables selected are summarized in Table 2-1. Multivariate analysis was performed by Monte Carlo permutations test using unlimited permutations. Analysis was carried out using the CANOCO software version 4.5.

2.3 Results

2.3.1 Physico-chemical characteristics

Selected physico-chemical parameters measured or obtained are listed in Table 2-1. These parameters showed all physico-chemical values to fall within the prescribed South African water quality guidelines for domestic use (DWAF, 1996a), aquatic ecosystems (DWAF, 1996b), livestock watering (DWAF, 1996c), irrigation (DWAF, 1996d) and aquaculture (DWAF, 1996e) (Supplementary material, Table 2-1S). Water temperatures were between 10 and 13°C in June and December temperatures exceeded 20°C (24.4 – 28.7°C). The temperatures of inland aquatic ecosystems in South Africa generally range between 5 – 30 °C but can fluctuate depending on the geographical features of the region and catchment area, seasonal changes and the impact of anthropogenic activities (DWAF, 1996b). In December, the flow velocity increased sequentially from Deneysville to downstream sampling stations (Scandinawieë Drift). This trend was not observed in June when rainfall was low.

Table 2-1: Physico-chemical characteristics of freshwater samples analysed in the Vaal River.

Sample								
	Deneysville		Vaal Barrage		Parys		Scandinawieë Drift	
	June 2009	December 2010	June 2009	December 2010	June 2009	December 2010	June 2009	December 2010
Day length (h, m, s)	10, 30 ,13	13, 46, 19	10, 30 ,13	13, 46, 19	10, 30 ,13	13, 46, 19	10, 30 ,13	13, 46, 19
Rainfall (mm)**	16.00	45.00	13.50	248.80	19.00	133.00	19.50	~105.00
Flow rate (m^3/s)*	15.12	258.34	40.01	340.95	9.371	906.84	5.35	1005.10
Temperature (°C)	10.00	28.70	11.00	24.50	13.00	24.40	13.00	26.70
pH	8.36	8.06	7.90	7.40	7.60	7.90	7.96	7.89
TDS (mg/L)	130.65	116.42	507.00	435.50	266.50	429.00	495.30	205.40
Conductivity (mS/m)	20.10	17.91	78.00	67.00	41.00	66.00	76.20	31.60
NO_3-N (mg/L)*	0.23	0.39	0.60	2.00	0.60	1.80	0.74	0.94
NH_4-N (mg/L)*	0.03	0.03	0.90	~1.80	0.20	0.40	0.03	0.30
PO_4-P (mg/L)*	0.02	0.02	0.40	0.60	0.05	0.50	0.39	0.03

SO_4-S (mg/L)*	15.10	14.70	135.00	136.00	~50.00	50.01	155.45	68.35
Cl_2 (mg/L)*	8.37	7.60	67.00	49.00	29.00	93.00	71.98	19.37

* Chemical water quality values were obtained from The Department of Water Affairs (www.dwa.gov.za)

** Rainfall data was provided by the South African Weather Services (www.weathersa.co.za)

2.3.2 Nucleic acid isolation from water samples

Nucleic acids were directly isolated from water samples without prior enrichment or culturing steps. Intact genomic DNA was obtained with a yield that varied from 2 – 30 ng/µl per 100 ml of water. The quality (A260:A280 ratio) of nucleic acids was acceptable for PCR and ranged from 1.6 – 2.2. Although DNA concentrations were low, amplification products were of sufficient quantity for PCR-DGGE analysis.

2.3.3 Dynamics of bacterial community structures

2.3.3.1 DGGE analysis

In this study, PCR-DGGE was able to give spatial information about the dominant bacterial communities in the Vaal River system (Figure 2-2). Previous studies suggest that band intensity is related to the relative abundance of the corresponding phylotypes in the sample mixture (Murray *et al.,* 1996; Riemann *et al.,* 1999). Thus, bands with relatively high intensities were assumed to be dominant taxa.

DGGE profiles demonstrated high resolution and intensity at a denaturing gradient of 30–50%. Four bacterial species, *Escherichia coli*, *Pseudomonas aeruginosa*, *Streptococcus faecalis* and *Staphylococcus aureus*, were included in all DGGE studies to determine the potential of using such an approach to establish the presence of these species in water samples. Corresponding bands for *S. aureus* and *P. aeruginosa* were detected for Vaal Barrage, Parys and Scandinawieë Drift. In addition, Parys illustrated a band with similar migration patterns to *E. coli*. All corresponding bands were excised and sequenced but produced poor quality sequences with indefinite identification. Since sequence data could not confirm accurate identification of excised bands, results remain inconclusive.

Vaal Barrage, Parys and Scandinawieë Drift displayed similar DGGE patterns for the dominant bands in June and December (Figure 2-2). However, DGGE profiles for Deneysville varied to some extent from the three other sites. Although some dominant bands showed similar migration patterns to Vaal Barrage, Parys and Scandinawieë Drift, a few distinct bands exhibited unique migration positions. A higher bacterial diversity, based on number of bands, was detected for Vaal Barrage and Scandinawieë Drift during June compared to December. On the other hand, bacterial diversity for

Deneysville was higher in December than in June. The Shannon-Weaver indices (Figure 2-4), however, contradicted the DGGE diversity data. It showed a higher bacterial diversity for Vaal Barrage and Scandinawieë Drift during December compared to June. The Shannon-Weaver index calculation includes the presence and absence of bands, but also band intensity that could be used to explain the contradiction (Zhang *et al.,* 2011).

A total of twenty-four bacterial bands were excised, sequenced and compared to sequences in the NCBI database (Table 2-2). Approximately 75% of the bacterial sequences recovered displayed high sequences homologies (> 97%) with the known database sequences. However, 50% of these sequences showed the highest sequence similarity to uncultured bacteria obtained directly from freshwater samples. These results support the presence of many uncultured and potentially undescribed bacterial taxa in freshwater ecosystems. Taxonomic classifications of the partial 16S rDNA sequences obtained affiliated to *Cyanobacteria* (B4, B13 – B15, B17, B23), *Bacteroidetes* (B6, B11, B22), *Betaproteobacteria* (B2, B12, B24) and uncultured bacteria (B1, B3, B5, B7 – B10, B16, B18 – B21). Bacterial communities for June displayed relative abundances of 8%, 17%, 17% and 58% for *Cyanobacteria*, *Bacteroidetes*, *Betaproteobacteria* and uncultured bacteria, respectively. In contrast, the relative abundance for *Cyanobacteria* increased to 42% in December whereas *Bacteroidetes*, *Betaproteobacteria*, and uncultured bacteria respectively accounted for 8%, 8% and 42% of the four main phylogenetic groups.

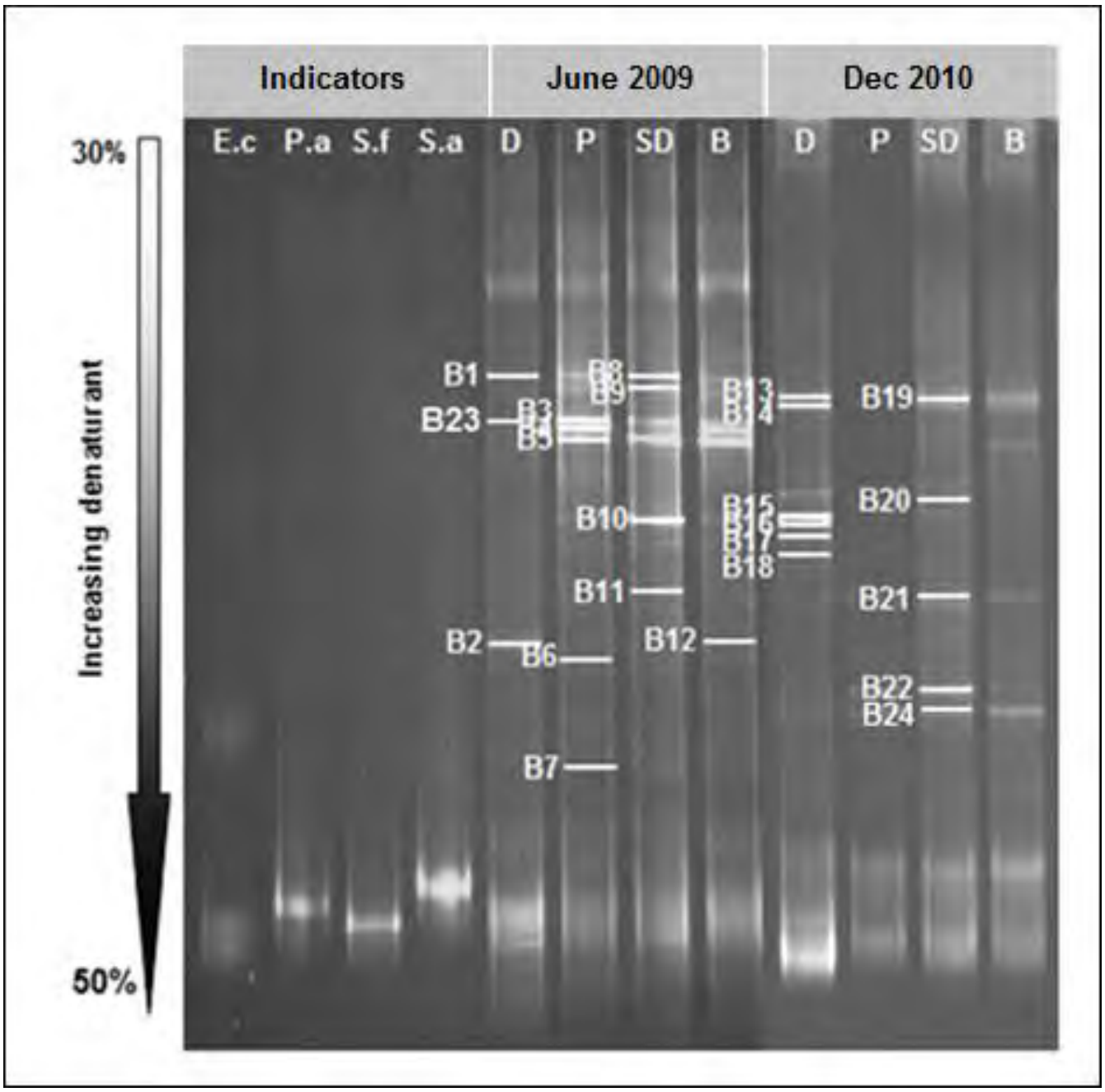

Figure 2-2: DGGE bacterial community analyses for 16S rDNA gene fragments from surface water during June 2009 and December 2010. Sampling sites selected along the Vaal River include Deneysville (D), Parys (P), Scandinawieë Drift (SD) and Barrage (B). Four indicator species were used as references: *E.coli* (E.c), *Pseudomonas aeruginosa* (P.a), *Streptococcus faecalis* (S.f) and *Staphylococcus aureus* (S.a). The DNA present in numbered bands was sequenced; identities are summarized in Table 2-2. None of the DGGE gels were digitally enhanced or modified. Bands of interest were only highlighted for better visualization and not analytical purposes.

Table 2-2: Alignment of bacterial phylotype sequences obtained by PCR-DGGE with reference sequences in the NCBI database.

DGGE band no.	NCBI accession no.	Closest relative (accession no.)	Phylogenetic affiliation	Percentage (%) similarity
B1	JQ085826	Uncultured bacterium clone XYHPA.0912.160 (HQ904787)	*Bacteria*	100
B2	JQ085827	Uncultured *Methylophilaceae* bacterium clone YL203 (HM856564)	*Betaproteobacteria*	100
B3	JQ085828	Uncultured bacterium clone SW-Oct-107 (HQ203812)	*Bacteria*	100
B4	JQ085829	Uncultured *Cyanobacterium* clone TH_g80 (EU980259)	*Cyanobacteria*	100
B5	JQ085830	Uncultured bacterium clone SINO976 (HM130028)	*Bacteria*	99
B6	JQ085831	Uncultured *Haliscomenobacter* sp. clone WR41 (HM208523)	*Bacteroidetes*	96
B7	JQ085832	Uncultured bacterium clone McSIPB07 (FJ604747)	*Bacteria*	98
B8	JQ085833	Uncultured bacterium clone ES3-64 (DQ463283)	*Bacteria*	99
B9	JQ085834	Uncultured bacterium clone ANT31 (HQ015263)	*Bacteria*	100
B10	JQ085835	Uncultured bacterium clone SING423 (HM129081)	*Bacteria*	99
B11	JQ085836	Uncultured *Bacteroidetes* sp. clone MA161E10 (FJ532864)	*Bacteroidetes*	100
B12	JQ085837	Uncultured *Nitrosomonadaceae* bacterium clone YL004 (HM856379)	*Betaproteobacteria*	92

B13	JQ085838	*Aphanizomenon gracile* ACCS 111 (HQ700836)	*Cyanobacteria*	91
B14	JQ085839	*Anabaena circinalis* LMECYA 123 (EU07859)	*Cyanobacteria*	97
B15	JQ085840	*Cymbella helvetica* strain NJCH73 (JF277135)	*Cyanobacteria*	99
B16	JQ085841	Uncultured bacterium clone FrsFi208 (JF747973)	*Bacteria*	99
B17	JQ085842	Uncultured *Cyanobacterium* clone LiUU-11-80 (HQ386609)	*Cyanobacteria*	98
B18	JQ085843	Uncultured bacterium clone TG-FD-0.7-May-09-B061 (HQ532969)	*Bacteria*	99
B19	JQ085844	Uncultured bacterium clone C_J97 (EU735734)	*Bacteria*	89
B20	JQ085845	Uncultured bacterium clone Lc2yS22-ML-056 (FJ355035)	*Bacteria*	97
B21	JQ085846	Uncultured bacterium clone ncd240a07c1 (HM268907)	*Bacteria*	91
B22	JQ085847	Uncultured *Sphingobacterium* sp. HaLB8 (HM352374)	*Bacteroidetes*	100
B23	JQ085848	Uncultured *Cyanobacterium* isolate DGGE gel band B5 (JN377930)	*Cyanobacteria*	98
B24	JQ085849	Uncultured *Dechlorosoma* sp. clone MBfR-NSP-159 (JN125313)	*Betaproteobacteria*	86

2.3.3.2 High-throughput sequencing

A total of eighteen phyla were identified among the four sampling sites by HTS technology (Figure 2-3A – F). Dominant phyla include *Alphaproteobacteria* (0.24 – 15%), *Betaproteobacteria* (1.47 – 85.10%), *Gammaproteobacteria* (0.24 – 12.38%), *Bacteroidetes* (0.72 – 4.05%) and *Actinobacteria* (4.76 – 10.00%). The remaining groups could be placed into nine phyla: *Acidobacteria*, *Chloroflexi*, *Cyanobacteria*, *Euglenoidea*, *Eukaryote*, *Fibrobacteres*, *Firmicutes*, *Fusobacteria*, and *Verrrucomicrobia*.

While identification of the four indicator organisms employed in DGGE profiling remained inconclusive by Sanger sequencing, HTS analysis verified that two of the bands did in fact belong to the *Pseudomonadaceae* family and *Escherichia* species. Additional opportunistic pathogens detected in low quantities at Vaal Barrage, Parys and Scandinawieë Drift included *Roseomonas* sp., *Ralstonia* sp., *Serratia* sp. and *Stenotrophomonas* sp..

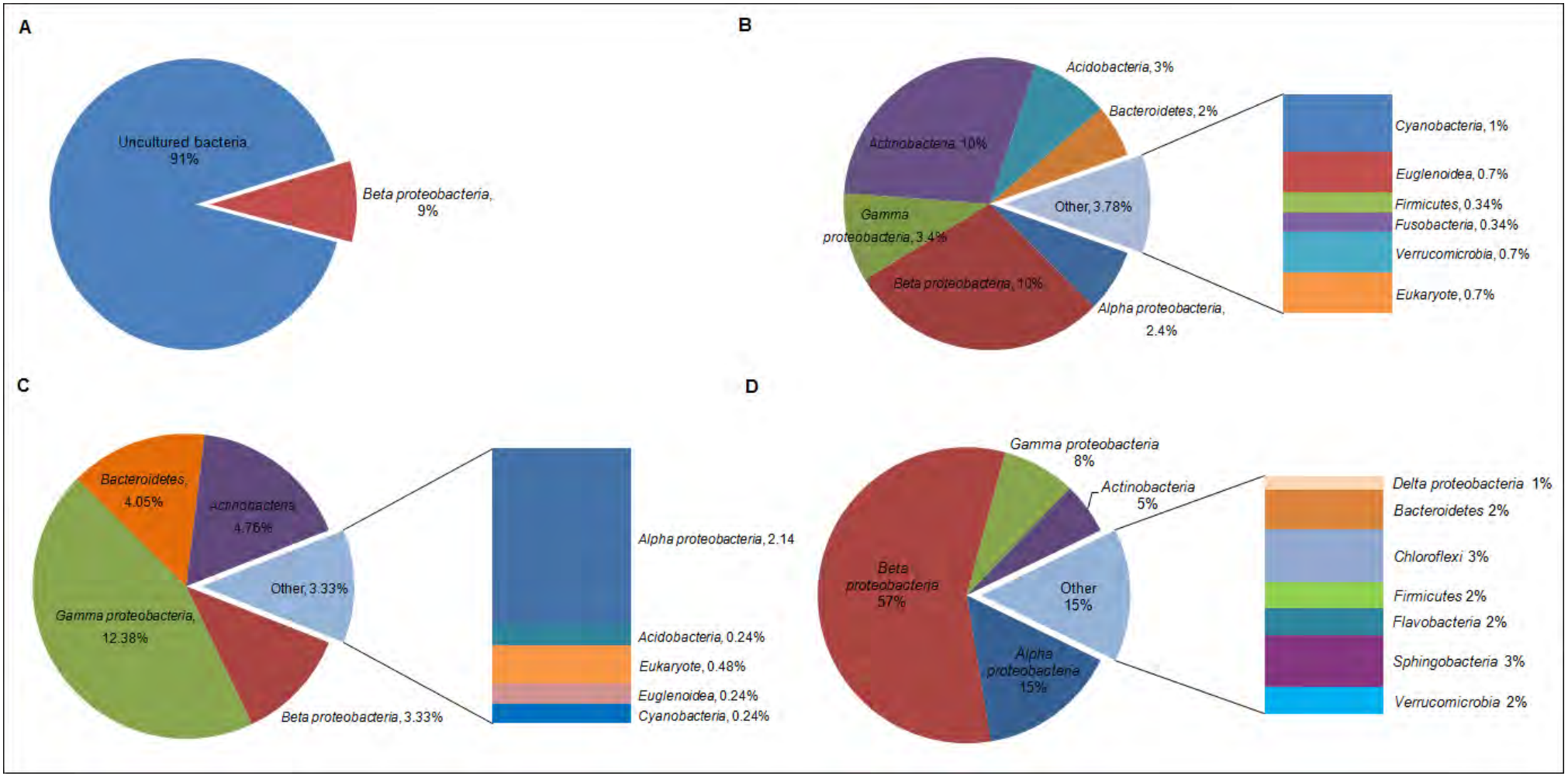

Figure 2-3: The relative abundance and composition of the dominant bacterial phyla in the Vaal River obtained from high-throughput sequencing technology for **(A)** Deneysville – December 2010; **(B)** Vaal Barrage – December 2010; **(C)** Parys – December 2010; and **(D)** Parys – June 2009.

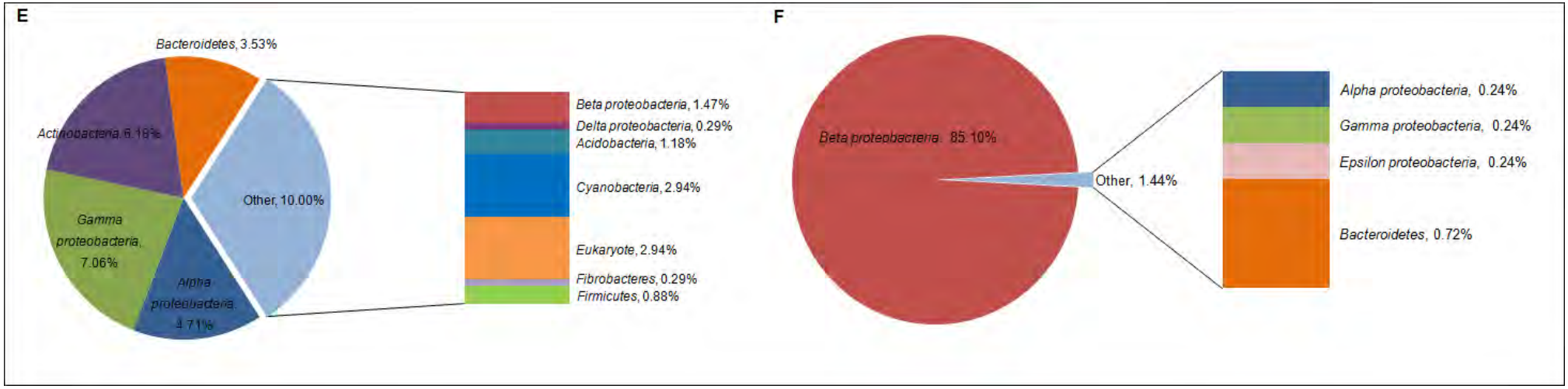

Figure 2-3: The relative abundance and composition of the dominant bacterial phyla in the Vaal River obtained from high-throughput sequencing technology for **(E)** Scandinawieë Drift – December 2010; and **(F)** Scandinawieë Drift – June 2009.

2.3.4 Distribution of bacterial diversity in the Vaal River

The Shannon-Weaver diversity indices (*H'*) were calculated from DGGE banding patterns as the number and relative intensity of bands (Figure 2-4). Indices were used to compare the overall structure of bacterial communities among the four sampling sites. *H'* for June and December samples ranged from 0.27 – 0.46 and 0.70 – 0.86, respectively. Bacterial diversity gradually increased from upstream to downstream sites except for Parys in December which consisted of a lower diversity. Similar trends were also observed for HTS data (Figure 2-4).

Cluster analysis was performed to gain an overview on the association of bacterial communities at the four sampling stations during June and December (Figure 2-5). UPMGA dendograms showed grouping of samples according to seasons. June samples showed high similarity (> 94%) among bacterial communities for Vaal Barrage, Parys and Scandinawieë Drift. A similar trend was observed for the December samples where Vaal Barrage and Scandinawieë Drift were defined by a 100% similarity. Noticeable was the grouping of the December Parys and Deneysville samples (100% similarity). Grouping of these two sampling sites may be attributed to similar banding patterns of a few dominant DGGE bands (Figure 2-2). Diversity indices (*H'*) and cluster analyses could be associated with DGGE profiles which reflected variations in the distribution, abundance, and composition of bacterial taxa.

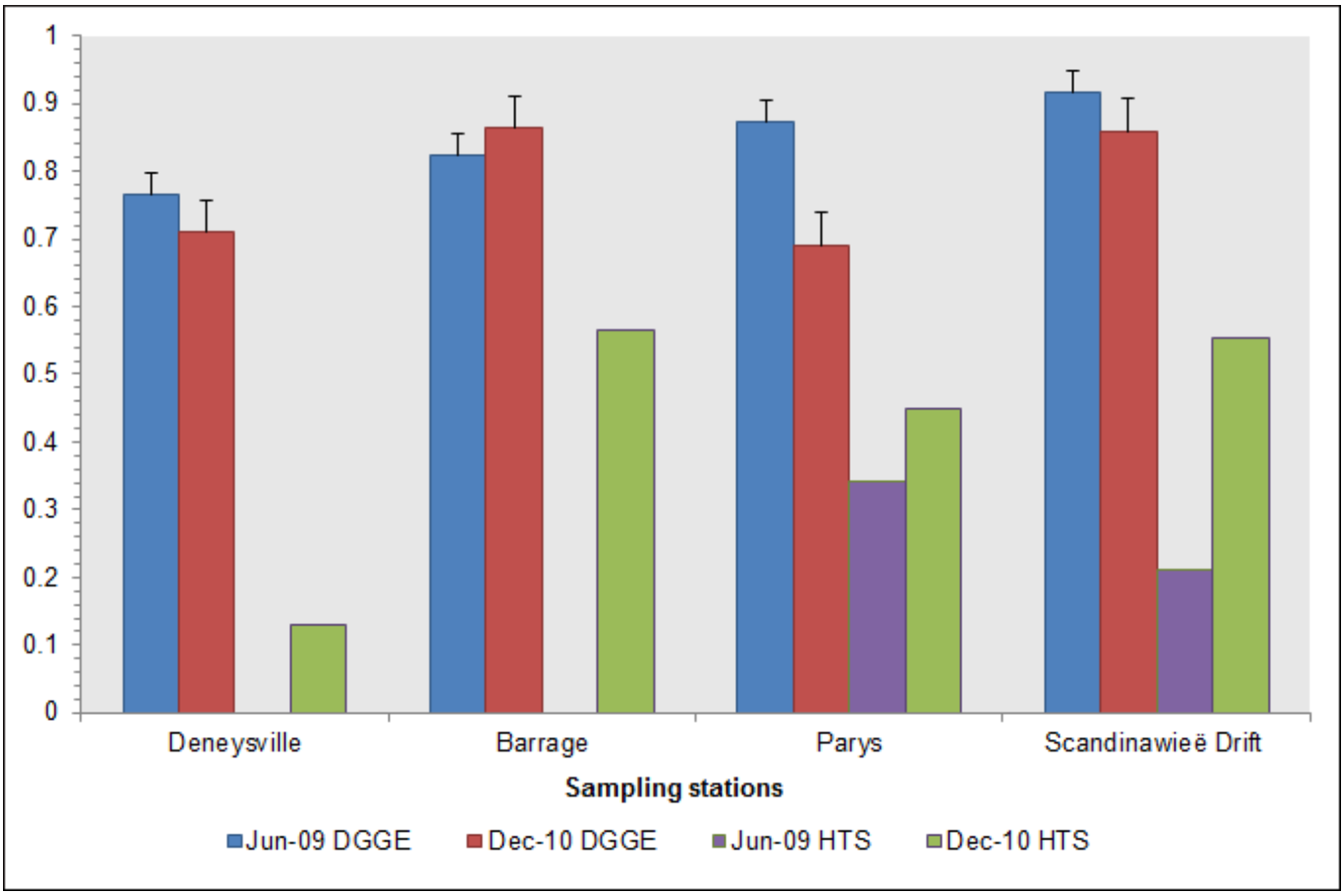

Figure 2-4: Shannon-Weaver diversity indices (H') for the Vaal River in June 2009 and December 2010 at Deneysville, Barrage, Parys, and Scandinawieë Drift.

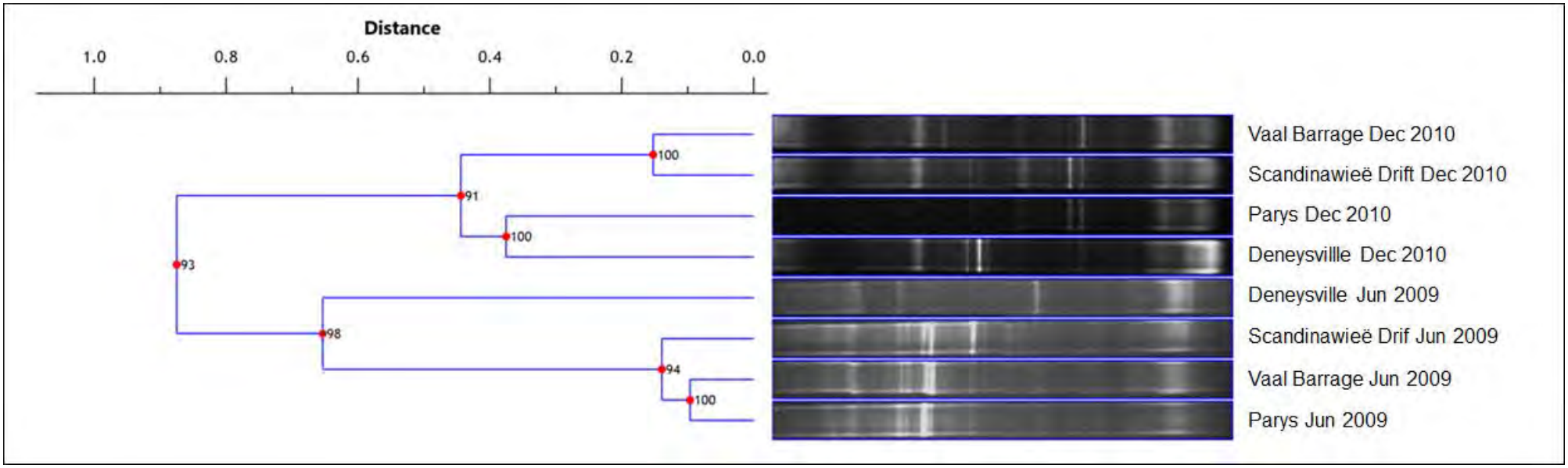

Figure 2-5: Cluster analysis of DGGE band patterns obtained in June 2009 and December 2010 using Pearson correlation coefficient. DGGE profiles are graphically demonstrated as UPGMA dendrograms.

2.3.5 Multivariate analysis

PCA and RDA were performed to analyze the relationships between the environmental parameters and the clustering of samples.

The effect of different sampling periods is illustrated by the PCA analysis results (Figure 2-6A). The June samples, with negative and positive score along the first axis, are separated from the December samples that showed a positive score along the second axis. The first axis was mainly defined by ammonium, nitrate, phosphate, chloride, sulfate, TDS, conductivity and rainfall. The second axis was related to temperature, day length and flow rate.

RDA plots calculated from DGGE profiles scaled distances of the environmental parameters, sampling stations and bacterial species (Figure 2-6B and C). The arrow vectors for the environmental parameters in each RDA plot represent their impact in the composition of bacterial communities at the sampling stations. Variation in the distribution of bacterial communities for the June and December samples (Figure 2-6B and C) showed to be related with the pH (BN8, BN14), temperature (BN11, BN15), ammonium (BN9, BN4, BN18, BN19 and BN22), phosphate (BN9, BN4, BN16 and BN17), chloride (BN3, BN5, BN16 and BN17), sulfate (BN3, BN5, BN18, BN19 and BN22), nitrate (BN11) and TDS concentrations (BN3, BN5, BN16 and BN17).

RDA plots for high-throughput sequencing data (Figure 2-6D) showed: (i) positive relationships between the flow rate and abundances of *Gammaproteobacteria*, *Deltaproteobacteria* and *Fibrobacteres* along the first axis, (ii) positive relationships between rainfall, TDS, nitrate, ammonium, chloride and sulfate concentrations, and abundances of *Acidobacteria* and *Actinobacteria* along the second axis, and (iii) positive relationships between ammonium, chloride and phosphate concentrations, and abundances of *Fusobacteria*, *Verrucomicrobia* and *Euglenoida* along the second axis. *Betaproteobacteria* negatively related with *Gammaproteobacteria*. A high abundance of *Betaproteobacteria* was detected in June but decreased considerably in December. An opposite inclination was observed for *Gammaproteobacteria*.

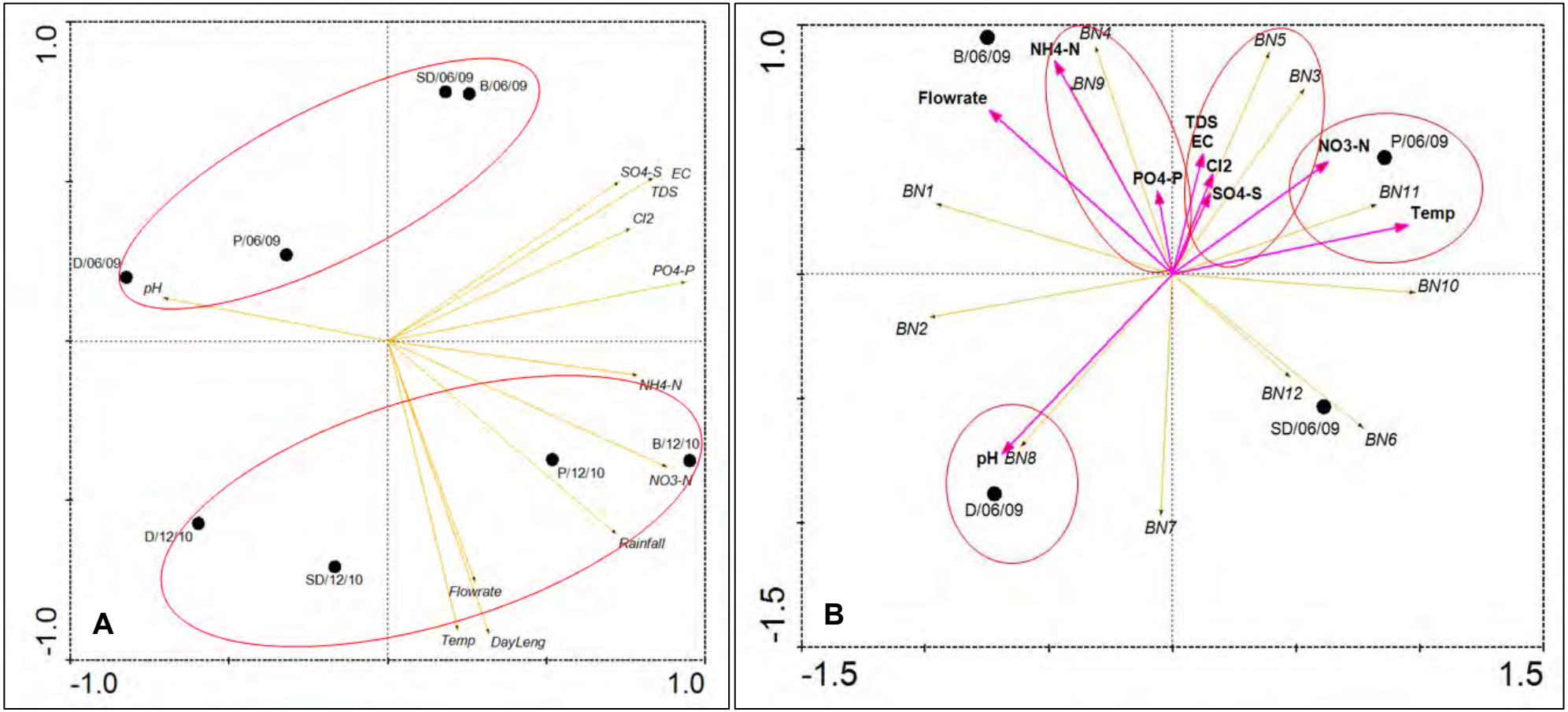

Figure 2-6: (A) PCA analysis of physico-chemical and microbial variables in the first and second axis ordination plots; and **(B)** RDA triplot of DGGE bands (samples indicated using band [BN] numbers) and environmental variables (represented by arrows) in June 2009.

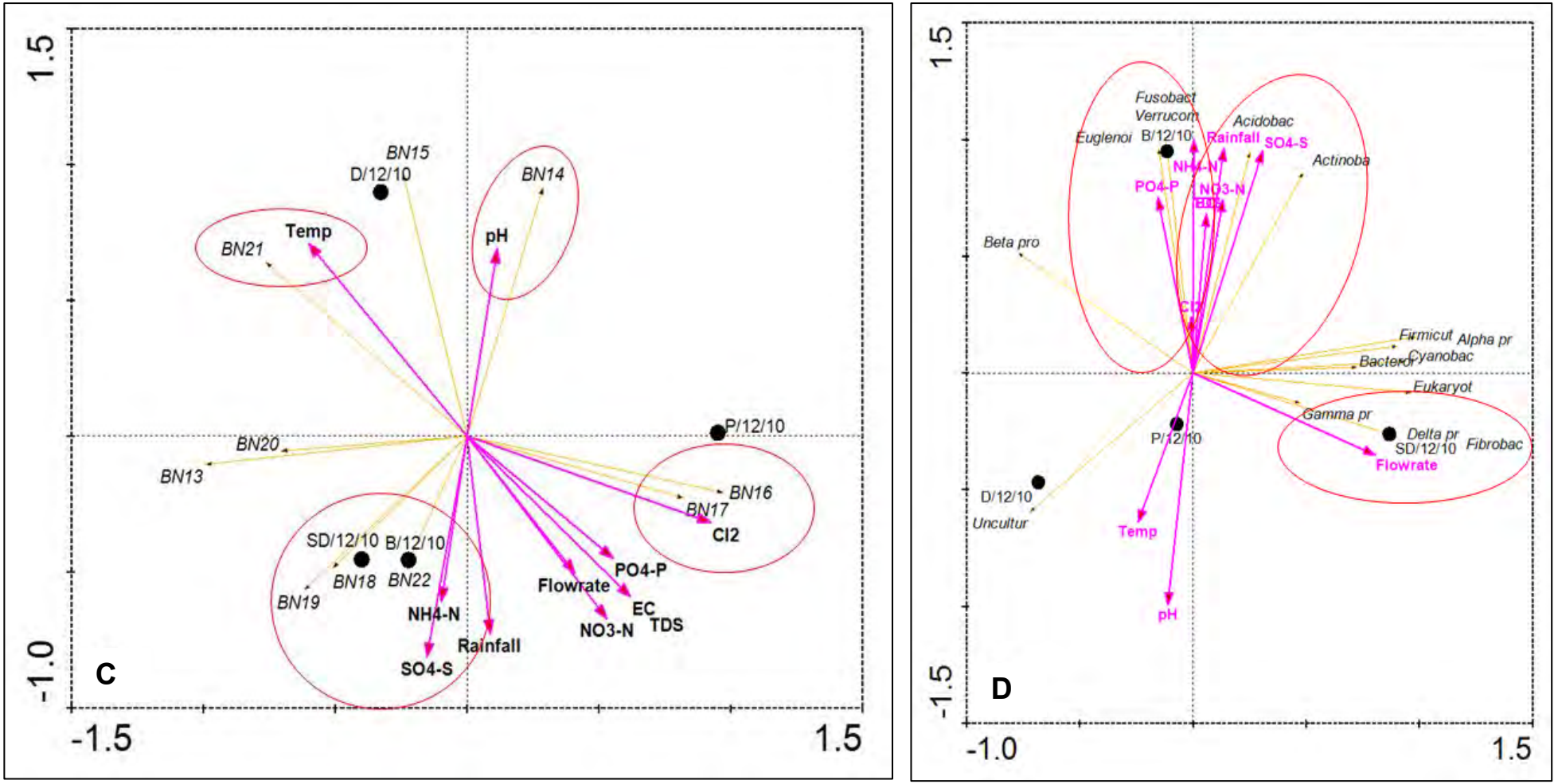

Figure 2-6: (C) RDA triplot of DGGE bands (samples indicated using band [BN] numbers) and environmental variables (represented by arrows) in December 2010; and **(D)** RDA triplot of bacterial phyla and environmental variables (represented by arrows).

2.4 Discussion

2.4.1 Microbial community dynamics

Knowledge and insight into the diversity and function of freshwater microorganisms is an essential requirement for the sustainable management of freshwater resources. In addition, changes in bacterial community structures might be used as potential bioindicators of environmental disturbances. The aim of this study was to examine bacterial community structures in a segment of the Vaal River, in response to environmental parameters, using a PCR-DGGE and high-throughput sequencing approach. High-throughput sequencing provided an overview of the dominant bacterial communities in the planktonic phase and marked shifts in composition as attested by PCA and RDA.

The composition of bacterial communities in a given environment depends on the interaction between various factors such the geographic environment (Zhang *et al.,* 2011), temperature (Hall *et al.,* 2008), pH (Yannarell and Triplett, 2005), flow rate (Crump and Hobbie, 2005), light intensity (Sigee, 2005) and nutrient concentrations (Pomeroy and Wiebe, 2001). In this study of a segment of the Vaal River, the physico-chemical parameters varied with sampling stations and seasons of sampling. PCA and RDA analysis indicated that bacterial community structures were mainly influenced by pH, temperature and inorganic components.

The bacterial community structures were similar for the three sampling sites during each sampling period. However, the June bacterial community structures were different from the December assemblages. DGGE results suggested that bacterial diversity was higher during June compared to December. These results were, however, contradicted by the Shannon-Weaver indices. The latter analysis included presence-absence, as well as (abundance) band intensity data. This could be used to explain the contradiction (Zhang *et al.,* 2011). Diversity index analysis of the high-throughput sequencing data showed similar trends as the Shannon-Weaver analysis of DGGE profiles.

Bacterial community structures could be related to inorganic nutrients as shown by PCA and RDA. The Vaal Barrage may create a buffering action that encapsulates organic and inorganic particles in the water-column for several weeks. This creates a relatively

stable environment in which organisms can develop into a community. The planktonic bacteria then flow from here downstream to Parys and Scandinawieë Drift. Therefore, bacterial communities along this section of the Vaal River will be relatively similar. In addition, the dominant bacterial groups detected at these three points may be native species with broader niche capabilities, which allow them to grow and survive under a variety of environmental conditions (Anderson-Glenna *et al.,* 2008). Recurrent native bacterial communities in aquatic ecosystems have been reported previously (Sekiguchi *et al.,* 2002; Crump *et al.,* 2003). It should be noted that the DNA amplification method used in this study did not discriminate between DNA derived from living cells versus DNA from dead cells and/or even naked or free DNA available in the water column. This aspect should be considered in future aquatic studies.

A feature highlighted in the present study was the relatively low bacterial diversity detected at Deneysville in June and December. Bacterial community structures at this sampling station largely consisted of *Cyanobacteria*, particularly *Cyanophyta* (*Anabaena* sp.), where pH and temperature were the main factors that affected the community structures. An alkaline pH was measured in June and December while temperature in December was above 25°C. Optimum growth of *Cyanophyta* and the formation of surface algal blooms are the direct result of high nutrient concentrations (particularly phosphate) and physico-chemical characteristics (high pH, temperature and light intensity) (Sigee, 2005). In addition to these conditions, buoyancy also plays an important role in the development of *Cyanophyta* populations. Buoyancy allows algal populations to adopt an optimum position within the water column in relation to light and CO_2 availability (Sigee, 2005). This mechanism leads to changes in the water chemistry and light regime in the epilimion that depress the growth of other phyto– and bacterioplankton groups (Sigee, 2005).

Although flow rate in this study did not show to affect bacterial communities, studies suggested that flow rate and hydraulic retention time have a substantial effect on community structures (Lindström and Bergström, 2004; Crump and Hobbie, 2005). Temporal variation in bacterial diversity was observed between June and December samples. The Gauteng and North West province received heavy rainfall in December 2010 that caused a drastic increased in flow rate, particularly at Parys and

Scandinawieë Drift. The high flow rate resulted in flooding at these two sampling stations that likely changed the bacterial community structures. Bacterial communities in rivers with short hydraulic retention times would potentially remain undetected by DGGE due to high loss rates (wash-out effect) which in turn result in a lower bacterial density and diversity (Sommaruga and Casamayor, 2008). In contrast, rivers with an extended hydraulic retention time causes accumulation of nutrients which promotes a higher genetic diversity of bacteria. Although flow rate differences provide a reasonable explanation for the seasonal variation in bacterial, further investigations are needed to confirm this for the Vaal River.

2.4.2 Phylogenetic diversity of bacterial communities

Phylogenetic affiliation of the dominant groups retrieved from the freshwater samples by PCR-DGGE and high-throughput sequencing corresponded to *Cyanobacteria*, *Alphaproteobacteria*, *Betaproteobacteria, Gammaproteobacteria, Bacteroidetes* and *Actinobacteria*. Other freshwater phyla such as *Deltaproteobacteria*, *Epsilonbacteria*, *Acidobacteria*, *Verrucomicro*bia*, *Firmicutes*, *Fusobacteria, Flavobacteria* and *Fibrobacteres* were found in low proportions.

Cyanobacteria accounted for a large proportion of bacterial diversity during December which agrees well with the physico-chemical characteristics of the water samples. Studies indicated that *Cyanobacteria* tend to dominate phytoplankton communities in pristine freshwater systems (Anderson-Glenna *et al.,* 2008; Foong *et al.,* 2010) whereas other reports observed an increase in the prevalence of *Cyanobacteria* in response to fluvial, organic and urban wastewater pollution (Douterelo *et al.,* 2004; Ibekwe *et al.,* 2012). Due to the trophic status of the Vaal River, cyanobacterial blooms usually occur during late spring and summer and often consist of *Microcystis aeruginosa*, *Oscillatoria* sp. and *Anabaena floss- aqua* (Cloot and Le Roux, 1997; DWAF, 2009a). In this study, *Anabaena* sp., *Cymbella helvetica* and *Synechocystis* sp. were in high abundance at Deneysville during December 2010. *Anabaena* sp. is among the most distributed toxin producers in eutrophicated freshwater bodies (Berg *et al.,* 1986). Their potential effects on aquatic ecosystems may be subtle or can cause major changes in the survival of sensitive species (DWAF, 2009a). In addition, these toxins may pose a serious health hazard for human and animal consumption.

Alphaproteobacteria, *Betaproteobacteria, Gammaproteobacteria* and *Actinobacteria* are ubiquitous groups in freshwater habitats (Gich *et al.,* 2005; Anderson-Glenna *et al.,* 2008) and are numerically important in river systems (Beier *et al.,* 2008; Lemke *et al.,* 2009). Members of *Betaproteobacteria* respond rapidly to organic and inorganic nutrient enrichments (Hahn, 2003; Šimek *et al.,* 2005) and have been isolated from various polluted and unpolluted freshwater bodies (De Figueiredo *et al.,* 2012; Haller *et al.,* 2011). Two important genera of this subphylum included *Dechlorosomonas* and *Variovorax*. Members of *Dechlorosomonas* are capable of oxidising aromatic compounds such as benzoate, chlorobenzoate and toluene (Coates *et al.,* 2001), where *Variovorax* sp. are involved in plant growth and remediation of xenobiotics (Jamieson *et al.,* 2009). Several opportunistic human pathogens of the *Gammaproteobacteria* group were detected at low abundance. Human diseases and infections are often associated with these pathogens (Berg *et al.,* 2005; Mahlen, 2011) and have caused mortalities in immunocompromised individuals (Fergie *et al.,* 1994; Paez and Costa, 2008). Thus, although the opportunistic pathogens were present at low levels, their impact should not be underestimated.

RDA analysis revealed that nitrate, ammonium, chloride and sulfate were the four most influential inorganic factors responsible for shaping *Actinobacterial* and *Acidobacterial* communities. A few studies suggested that these two phyla participate in the nitrogen cycle in soils and sediments by reducing nitrate, nitrite and possibly nitric oxide (Gtari *et al.,* 2007; Ward *et al.,* 2009). Norris et al. (2011) also implicated the role of some novel *Actinobacteria* from geothermal environments to grow autotrophically with sulfur as an energy source. Correlation between *Verrucomicrobia* and phosphate was also detected suggesting that this inorganic nutrient influenced the *Verrucomicrobia* community within the total bacterial population. The association between *Verrucomicrobia* and phosphate levels have been seldom discussed in previous studies of microbial ecology in freshwater resources (Lindström *et al.,* 2005; Liu *et al.,* 2009a). Very little is yet known about the physiology and ecological roles of *Actinobacteria*, *Acidobacteria* and *Verrucomicrobia* in these habitats and the impact of physico-chemical characteristics on their community composition.

Members of *Bacteroidetes* usually inhabit mesotrophic and eutrophic water bodies that have high nutrient levels (Xi *et al.,* 2007; De Figueiredo *et al.,* 2012). This group is known to degrade polymeric organic matter, play an important role in the turnover of organic matter (Cottrell and Kirchman, 2000) and is often isolated from humic waters (Anderson-Glenna *et al.,* 2008; Stabili and Cavallo, 2011). The *Bacteroidetes*-*Flavobacterium*-like lineages are often present in high abundance following the growth and decline of cyanobacterial blooms (Eiler and Bertilsson, 2007; Newton *et al.,* 2011). Their presence and distribution is mainly determined by resource availability and are favoured during periods of high heterotrophic activity and enhanced growth (Eiler and Bertilsson, 2007). This phenomenon was evident in the high abundance of *Bacteroidetes* in June following the December to February 2009 cyanobacterial blooms.

2.5 Conclusions

This study investigated the impact of physico-chemical water quality parameters on bacterial community structures in a segment of the Vaal River. The PCR-DGGE approach and high-throughput sequencing analysis presented useful data on the identification of dominant bacterial groups at the four sampling stations. Molecular analysis showed that: (i) bacterial community structures for June were different from the December assemblages; (ii) bacterial community structures for Vaal Barrage, Parys and Scandinawieë Drift were similar; (iii) bacterial communities at Deneysville differed from the three other sites and were lower in diversity; and (iv) *Cyanobacteria*, *Alphaproteobacteria*, *Betaproteobacteria, Gammaproteobacteria, Bacteroidetes* and *Actinobacteria* were the dominant bacterial groups detected and showed to be impacted by physico-chemical water quality parameters. This study contributed to the identification of bacterial phylotypes, their spatial succession and the effect of physico-chemical characteristics on these freshwater bacterial communities. A detailed study on the relationships between the dominant bacterial taxa, and specific physico-chemical water characteristics is required to improve our knowledge on how bacterial community structures in the Vaal River are affected.

CHAPTER 3: Bacterial community composition of an urban river in the North West Province, South Africa, in relation to physico-chemical water quality

3.1 Introduction

Anthropogenic disturbances on freshwater systems hold major repercussions on the overall bacterial structure and function of these habitats (Smith *et al.,* 1999). In addition, the water quality is compromised to such an extent that it may no longer be fit for recreational and several other human purposes (De Figueiredo *et al.,* 2004).

Contamination of freshwaters with anthropogenic chemicals may alter the bacterial community composition (BCC) as bacteria are highly sensitive to nutrient availability, concentrations of pollutants, and altered environmental conditions (Paerl *et al.,* 2003; Yergeau *et al.,* 2012). Changes in the BCC include selection for more resistant or contaminant specific species with an associated change in overall diversity (Ford, 2000). As such, changes in BCC affect the functional dynamics of whole ecosystems by altering the ecosystem processes (physical, chemical, and biological) through metabolic feedback (Zarraonaindia *et al.,* 2013). Changes in the abundance of minor species can thus affect the vitality and success of larger organisms (Zarraonaindia *et al.,* 2013). It is conceivable that the short generation times of bacteria, their high diversity, and quick reaction and recovery from environmental changes give them the advantage to be used as indicators of both physical and chemical stresses in freshwater systems (Lowe and Pan, 1996; Hahn, 2006; Pronk *et al.,* 2009; Stabili and Cavallo, 2011).

Important objectives of using bacterial communities as biological indicators are to understand their structure, dynamics, and causes of variability (Kenzaka *et al.,* 2001; Paerl *et al.,* 2003). Recent metagenomic approaches, such as 454-pyrosequencing, have simplified and accelerated this process by allowing scientists to study bacterial diversity in more detail (De Figueiredo *et al.,* 2007). Metagenomic efforts further our understanding of BCC changes at group level over spatial and temporal scales (Zarraonaindia *et al.,* 2013). Also, metagenomics data assist us in determining how

environmental conditions such as pollution shape BCC and how these conditions affect diversity of genes associated with biogeochemical cycles (Singh *et al.,* 2009). This information, together with future development of metagenomic techniques and statistical models, will permit possible prediction of changes in microbial communities on the basis of present knowledge (Larsen *et al.,* 2012).

The goals of the present study were to: (i) determine the impacts of physico-chemical parameters on BCC along the Mooi River system (South Africa); and (ii) statistically analyze the effects of pollution on the spatial distribution of bacterial communities

3.2 Materials and Methods

3.2.1 Study site

The Mooi River catchment (1800 km²) is located in the western Gauteng and North West Provinces of South Africa (Figure 3-1). It has been the sole water supply of Potchefstroom, which currently has a population of approximately 124 000 residents (StatsSA, 2011). The catchment receives its water supply mainly from dolomitic eyes and springs (Van der Walt *et al.,* 2002). The catchment has three main tributaries including: (i) the Wonderfontein Spruit; (ii) the northern stretch of the Mooi River; and (iii) the Loop Spruit. Four major dams are situated in the Mooi River catchment including Klerkskraal Dam, Boskop Dam, Klipdrift Dam and the Potchefstroom Dam (Van der Walt *et al.,* 2002). The water quality of the Mooi River and its tributaries has been affected in various ways by human activities. The Wonderfontein Spruit, which converges with the Mooi River just upstream of the Boskop Dam, receives sewage wastewater from informal settlements, runoff from agriculture, and large amounts of gold mining effluent. High salt levels and various trace elements are frequently detected in the Wonderfontein Spruit. The Mooi River and Loop Spruit tributaries are predominantly impacted by dry land agricultural activities. However, several gold mines discharge effluent in the Loop Spruit, while small scale diamond mining in the Mooi River sub-catchment area destroyed the floodplain and riparian habitats that resulted in silting of the Mooi River upstream of the Boskop Dam (Van der Walt *et al.,* 2002). The Loop Spruit merges with the Mooi River at the Prozetsky Bird sanctuary downstream of Potchefstroom City. The catchment by-passes the Potchefstroom sewage treatment

plant and correctional services before it flows into the Vaal River (Kromdraai confluence).

The study sites were specifically selected to represent a range of water quality data and the impact of human activities on the Mooi River (Figure 3-1). Study sites included Muiskraal (Site 1; S26°26'42.2; E27°07'06.1), sites below the Boskop Dam (Site 2; S26°34'19.3; E27°06'12.5), below the Potchefstroom Dam (Site 3; S26°40'43.3; E27°05'56.2), the outer reaches of Potchefstroom City (Site 4; S26°45'10.9; E27°06'01) and above the confluence of the Mooi River with the Vaal River (Site 5; S26°52'49.5; E26°57'51.4). Muiskraal served as the reference site due to lowest anthropogenic activities in the vicinity.

3.2.2 Sample collection

Freshwater samples were collected in sterile containers from the five different locations in June and July 2012. Physical parameters that were measured *in situ* included temperature, pH, electrical conductivity (EC) and dissolved oxygen (DO). Selected chemical and microbiological parameters were determined by Midvaal Water Company, South Africa. These included chlorine (Cl^-), nitrate (NO_3^-) and nitrite (NO_2^-), phosphate (PO_4^{3-}), sulphate (SO_4^{2-}), chemical oxygen demand (COD), chlorophyll-*a*, total coliforms (TC) and *E. coli*. The TC and *E. coli* counts were determined using the Colilert® method. All measurements conducted by Midvaal Water Company were in accordance with the South African National Accreditation System (SANAS) guidelines.

3.2.3 Microbiological analysis of water samples

Heterotrophic plate count (HPC) bacteria were enumerated by serially diluting water samples in sterile 8.5% (w/v) NaCl solution. Triplicate aliquots from serial dilutions were spread-plated on R2A agar (Difco Laboratories Inc., Franklin Lakes, NJ, USA) and incubated aerobically at 26°C for 5 – 6 days. The number of colony-forming units (CFU) was recorded from plates most representative of the mean colony count. Bacterial colonies that were morphologically distinct were further sub-cultured on R2A for pure culture isolation. The purity of the isolates was assessed by microscopic analysis of Gram-stained cells.

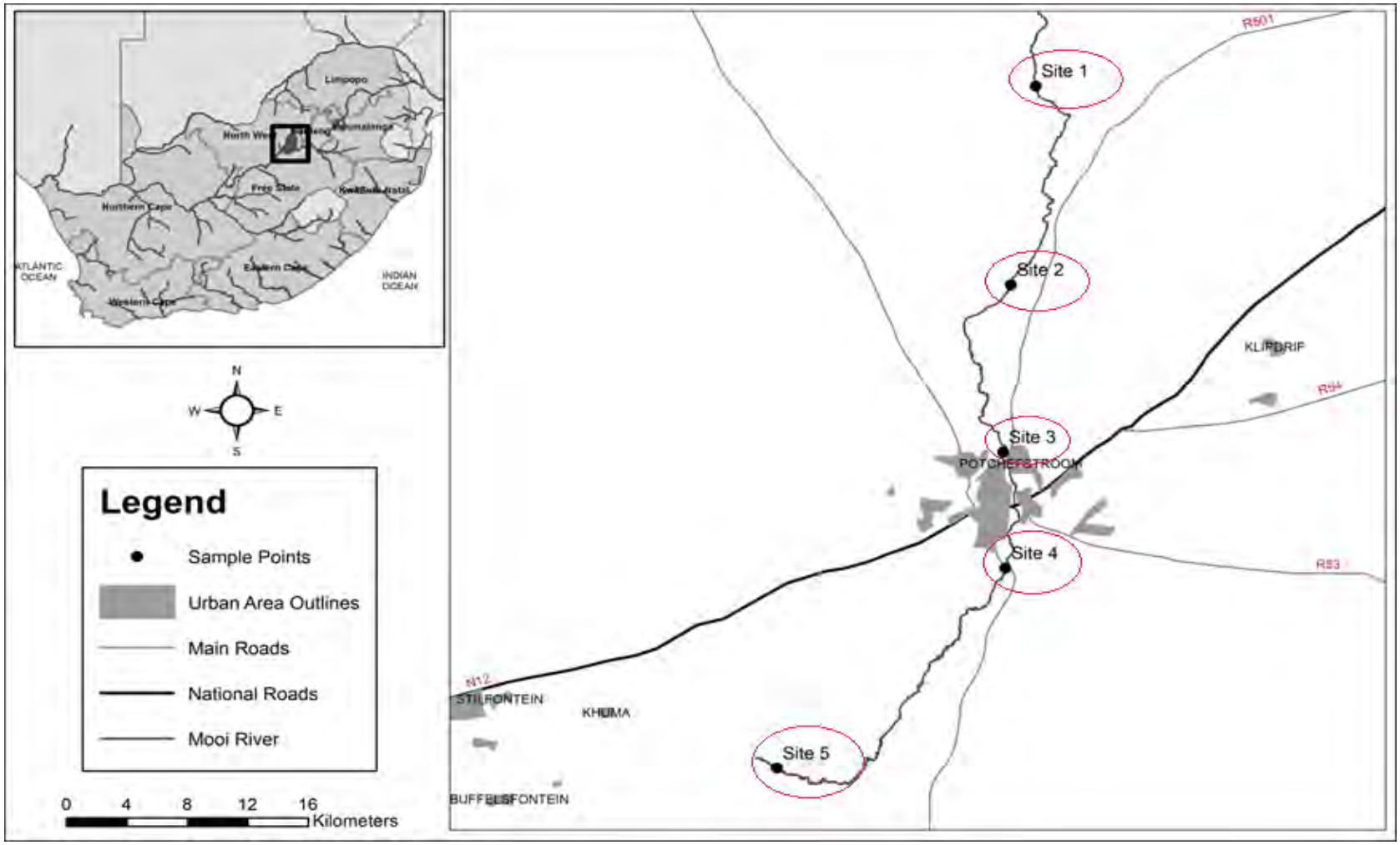

Figure 3-1: Geographical map of the Mooi River system. Illustrated is the general location of the study site in the North West Province, with a detailed view of the sampling sites examined for bacterial community composition.

3.2.4 DNA isolation and PCR amplification

DNA isolation and amplification of pure bacterial isolates was achieved using the colony-PCR method. Briefly, bacterial cells were carefully collected with a sterile pipette tip and transferred to a sterile PCR tube. Bacterial cells were resuspended in 10 µl of distilled water, briefly mixed and heated in a microwave at 1000 W for 2 – 3 min. Samples were then centrifuged for 30 sec at 13400 rpm and placed on ice. One microliter of the eluate was used as DNA template for PCR amplification. Amplification was performed in a 25 µl reaction mix containing single strength PCR master mix [(5 U/µl *Taq* DNA polymerase (recombinant) in reaction buffer, 2 mM $MgCl_2$, 0.2 mM of each dNTP, Thermo Fisher Scientific)], 50 pmol of primer pair 27F and 1492R (Lane, 1991) and PCR-grade water (Thermo Fisher Scientific). Amplification was performed in a preheated Bio-Rad iCycler Termal Cycler (Bio-Rad Laboratories) with an initial denaturation at 95˚C for 5 min followed by 30 cycles of denaturation at 95˚C for 30 s, annealing at 51˚C for 1 min and extension at 72˚C for 60 s. Final extension was performed at 72˚C for 5 min. PCR amplified DNA fragments were observed by standard electrophoresis on 1% (w/v) agarose gels and visualized by ethidium bromide staining and UV illumination. PCR products were purified and sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Life Technologies) and Genetic Analyzer 3130 (Applied Biosystems Life Technologies) according to manufacturer's instructions. Sequences were examined using the BLASTN algorithm (http://www.ncbi.nlm.nih.gov/BLAST) to detect the closest bacterial match within the GenBank database. Nucleotide sequences obtained from pure bacterial isolates were deposited in the GenBank database under the accession numbers KC515572 – KC515642.

Total DNA from water samples was isolated by filtering 250 to 2,000 mL water (depending on the water transparency) through sterile 0.2 µm nitrocellulose membrane filters. Cells and particles that retained on the filters were resuspended in sterile TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0) and centrifuged for 1 min at 13400 rpm. The supernatant was removed and DNA was subsequently isolated using the NucleoSpin Tissue kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to manufacturer's instructions. The isolated DNA was stored at – 20˚C until further analysis.

3.2.5 454-Pyrosequencing

454-Pyrosequencing was performed by Inqaba Biotech, Pretoria, South Africa using the Roche 454 GS-FLX chemistry. The variable V1 and V3 regions of the 16S rRNA gene were targeted using bacterial primer pair 27F (GAGTTTGATCCTGGCTCAG) and PRUN518R (ATTACCGCGGCTGCTGG), containing the 454 FLX adaptors and sample-specific identifiers. Raw sequence data was quality trimmed and checked for chimeras following the MOTHUR v.1.28 pipeline (Schloss *et al.,* 2009). Sequences were assigned to operational taxonomic units (OTU's) at a 97% similarity. Rarefaction curves were constructed from 454-pyrosequencing data using MOTHUR v.1.28. Taxonomic classification of phylotypes was determined using the Ribosomal Database Project (RDP) Classifier (Wang *et al.,* 2007) at a 97% bootstrap confidence threshold. Alpha- and beta diversity calculations were performed using reduced data sets in which the number of sequences per samples was made equal with random resampling (516 sequences per sample). Alpha diversity (richness and evenness) was calculated in MOTHUR v.1.28 using the Simpson diversity index. Beta diversity was determined in XLSTAT version 2013.5.04 (Addinsoft SARL, New York, NY, USA) through the Bray-Curtis dissimilarity coefficient to obtain a beta diversity matrix. The resulting distance matrix was mapped on a 2D- multidimensional scaling (MDS) plot with 999 repetitions.

All DNA sequences were deposited in the GenBank database under the accession numbers KC515643 – KC516708.

3.2.6 Statistical analysis

Multivariate analysis was used to assess the effects of physico-chemical water characteristics on BCC. Environmental and microbiological data was log transformed [log(x + 1)] before analysis. The distribution of samples according to physico-chemical parameters was first tested through principal component analysis (PCA). Correlations between the: (i) physico-chemical variables; (ii) physico-chemical variables and BCC; and (iii) physico-chemical variables and indicator organisms (*E.coli*, total coliforms, and HPC) were then calculated by Spearman's rank method. Significant relationships were further tested by canonical correspondence analysis (CCA) with Monte Carlo permutation tests based on 1000 unrestricted permutations, 80% confidence level, and

5% significance level. All statistical analyses were performed using the XLSTAT version 2013.5.04 software package (Addinsoft SARL).

3.3 Results

3.3.1 Physico-chemical and microbiological analysis

Physico-chemical and microbiological water characteristics for each sampling site were determined and are summarized in Table 3-1. The average river temperature ranged from 8.3 to 11.8°C, pH varied between 8.03 and 8.57, and DO concentrations ranged from 7.40 to 10.00 mg/L. Conductivity gradually increased from the upstream to downstream sites with the highest value recorded at site 4 (~ 73 mS/m). This site is on the southern end of Potchefstroom as the river exits the city. The average concentrations for chlorine, nitrate/nitrite and phosphate were within the recommended water quality objectives (RWQO's) prescribed by the Department of Water Affairs, South Africa, for the Mooi River catchment (Supplementary material, Table 3-1S) (DWAF, 2009a). However, sulphate concentrations were consistently higher than the RWQO with highest levels measured at site 4 and 5 (> 90 mg/L), which are both downstream from Potchefstroom.

Total coliform and *E. coli* counts for the selected sampling locations are shown in Table 3-1. *E. coli* levels for the June samples decreased steadily from site 1 to 3 but then rapidly escalated at site 4. At the latter site the levels were recorded as 548 MPN/100 mL. In contrast, *E. coli* levels for the July samples gradually increased from site 1 to site 4 and ranged from 139 to 488 MPN/100 mL. The average *E. coli* numbers were highest at site 4 with counts of 518 MPN/100 mL. Lowest *E. coli* numbers were observed at site 1, 2 and 5 and ranged between 108 and 119 MPN/100 mL. *E. coli* counts for site 1, 2, 3 and 5 complied with the TWQR for recreational use (0 - 130 cfu/mL) and livestock watering (0 - 1000 cfu/mL) (DWAF, 1996f). However, *E. coli* counts for irrigation of commercial crops were markedly higher than the TWQR (1 cfu/ml). Total coliform counts ranged between 461 to > 2420 MPN/100 mL and exceeded the TWQR for recreational (0 - 150 cfu/100 mL) and agricultural (livestock watering [0 - 200 cfu/100 mL] and irrigation [< 1 cfu/100 mL]) use (DWAF, 1996f).

Table 3-1: Physico-chemical and microbiological characteristics of riverine samples analysed in this study.

Sampling locations										
		Site 1		Site 2		Site 3		Site 4		Site 5	
		June	July	June	July	June	July	June	July	June	July
Physico-chemical variables											
pH		8.18	8.48	8.53	8.41	8.26	8.45	8.03	8.47	8.53	8.57
Temp	°C	8.30	9.10	10.80	11.50	11.70	11.80	10.80	11.30	10.80	11.60
EC	mS/m	50.10	44.50	67.00	66.40	68.20	68.30	71.90	73.70	67.00	54.10
DO	mg/L	8.20	8.60	8.40	8.60	7.80	8.80	7.40	10.00	8.40	9.80
Chlorine*	mg/L	< 0.10	< 0.10	< 0.10	< 0.10	< 0.10	< 0.10	< 0.10	< 0.10	< 0.10	< 0.10
Nitrate & Nitrite*	mg/L	< 0.50	< 0.50	< 0.50	< 0.50	< 0.50	< 0.50	< 0.50	< 0.50	< 0.50	< 0.50
Phosphate*	mg/L	< 0.05	< 0.05	< 0.05	< 0.05	0.06	< 0.05	0.07	0.06	0.30	0.14
Sulphate*	mg/L	< 10.00	< 10.00	86.00	90.00	86.00	88.00	92.00	96.00	104.00	83.00
Chlorophyll-a	µg/L	0.80	3.40	9.80	4.90	17.00	1.20	7.80	1.80	8.30	3.50
Microbiological elements											

E. coli*	MPN/100ml	99	139	76	148	61	210	548	488	172	43
Total Coliforms*	MPN/100ml	1414	> 2420	1986	461	1300	1203	> 2420	> 2420	> 2420	> 2420
Heterotrophic plate count (HPC) bacteria											
HPC	CFU/mL	1.1×10^5	1.7×10^5	1.3×10^5	1.2×10^5	5.8×10^5	2.4×10^5	4.9×10^5	9.3×10^5	8×10^5	1×10^6
Average	CFU/mL	1.4×10^5		1.3×10^5		4.1×10^5		7.1×10^5		9×10^5	

* Data provided by the Midvaal Water Company, South Africa

3.3.2 Heterotrophic plate count bacteria

To determine the microbial water quality of the Mooi River, heterotrophic plate count bacteria were enriched on culture media. HPC bacterial levels ranged between 1.3 × 10^5 and 9 × 10^5 CFU/mL (Table 3-1). Although no log differences, these levels increased from site 1, with the lowest number of bacteria, to site 5 with the highest number of bacteria. A total of 94 HPC bacterial isolates that represented different morphotypes were recovered from water samples (Supplementary material, Table 3-2S). Of these, 14% were Gram-positive and 86% Gram-negative. *Alphaproteobacteria* was the predominant class detected at site 1 (June and July), representing up to 40% of the isolates. Genera identified include *Novosphingobium*, *Rhizobium*, *Xanthobacter* and *Paracoccus* spp. Dominant groups detected at site 2 and 3 during the June sampling period consisted of *Firmicutes* and *Betaproteobacteria* (26%). In contrast, during July these two sites were dominated by *Gammaproteobacteria* (37%), *Bacteroidetes* (21%) and *Betaproteobacteria* (16%). The latter three groups also occurred in highest numbers (> 69%) at site 4 and 5 during June and July.

The phylum *Firmicutes* predominantly consisted of Gram-positive rod-shaped bacteria in the class *Bacilli*. Within the *Bacteroidetes* phylum, *Flavobacterium* was most frequently detected. A variety of *Betaproteobacteria* taxa was identified and grouped into two families (*Comamonadaceae* and *Oxalobacteraceae*) and six genera (*Massilia* sp., *Limnohabitans parvus*, *Dunganella* sp., *Rhodoferax* sp., *Curvibacter* sp., and *Herbaspirillum* sp.). *Pseudomonas fluorescens*, *P. koreensis*, and *P. putida* were found to be the main species detected in the *Gammaproteobacteria* group. These three species accounted up to 40% of *Gammaproteobacteria* isolates. Some isolates were also identified as *Pseudomonas* but could not be classified further by the NCBI database to species level.

3.3.3 Bacterial community structure and diversity

To characterise the bacterial community structure along the Mooi River, DNA samples were subjected to 454-pyrosequencing and subsequent analysis. A total of 24,374 pyrosequencing reads were obtained with an average read length of 500 ± 20 bp. Following quality trimming of sequences shorter than 150 bp, 13,984 sequences were used for further analysis. Overall, a total of 900 unique OTU's were assigned to a class

at a confidence threshold of 97%. Of the 900 OTU's, 60% were identified up to genus level. The greatest number of OTU's was associated with site 3 (291 OTU'S in June and 231 OTU's in July), whereas the lowest estimates were obtained for site 4 with a library size between 200 and 213 OTU's (June and July respectively). Sequence libraries for site 1, 2 and 5 ranged in size between 203 and 251 OTU's. Rarefaction curves suggested that the bacterial diversity did not reach saturation/plateau at a 97% similarity level (Figure 3-2A). This observation indicates that our sampling effort has to improve for curves to reach a plateau.

Alpha diversity was calculated using the Simpson diversity index. The results indicate that bacterial richness and evenness was in general higher for the June than July samples (Figure 3-2B). Site 2 and 3 June were found to be the most diverse, whereas site 4 June was the least diverse. In contrast, site 4 and 5 July displayed the greatest bacterial richness and evenness, while site 1 July had the lowest number of species. These results indicate that species richness positively associated with evenness. Sites with a large number of species showed a degree of equitability among species abundance. On the other hand, sites that displayed low species richness had many individuals belonging to the same species. Variation of beta diversity is visualized with a MDS graph (Figure 3-2C) and Bray-Curtis similarity dendrogram (Figure 3-3). The results highlighted marked differences in BCC between the June and July sampling periods. Bacterial communities for site 1, 2 and 3 June were similar as indicated by their clustering on the MDS graph and dendrogram. Likewise, site 2 – 5 July grouped together, indicating their relatedness in BCC. The July cluster was distantly related to site 5 June, suggesting a slight similarity in bacterial communities. MDS analysis of site 4 June and site 1 July showed no resemblance in community composition to any of the other sites.

At a 97% sequence similarity level against the RDP database, the BCC throughout the Mooi River consisted of ten phyla (*Acidobacteria*, *Actinobacteria*, *Armatimonadetes*, *Bacteroidetes*, *Chloroflexi*, *Cyanobacteria*, *Firmicutes*, *Planctomycetes*, *Proteobacteria* and *Verrucomicrobia*) (Figure 3-3) and seventy five genera (Supplementary material, Table 3-3S). For all samples, *Proteobacteria* and *Bacteroidetes* were encountered most frequently, representing 22 - 46% and 18 - 60% of each sequence library, respectively

(Figure 3-3). *Actinobacteria* was also dominant, but with large sample-to-sample variation. The *Proteobacteria* were distributed in descending order as *Betaproteobacteria*, *Alphaproteobacteria*, *Gammaproteobacteria*, *Epsiolonproteobacteria* and *Deltaproteobacteria*. The majority of *Betaproteobacteria* sequences were affiliated within the family *Comamonadaceae* in *Burkholderiales*. Within *Comamonadaceae*, *Hydrogenophaga*, *Limnohabitans* and *Polaromonas* predominated at most sites during both sampling periods. Among the *Bacteroidetes* members detected, *Sphingobacteria* and *Flavobacteria* dominated bacterial communities. Genera that occurred most frequently and in high abundance throughout the river include *Arcicella*, *Solitalea* and *Flavobacterium*. The relative abundance of *Planctomycetes*, *Verrucomicrobia* and *Cyanobacteria* were noticeably higher in June compared to the July samples, even though their abundance varied across sites.

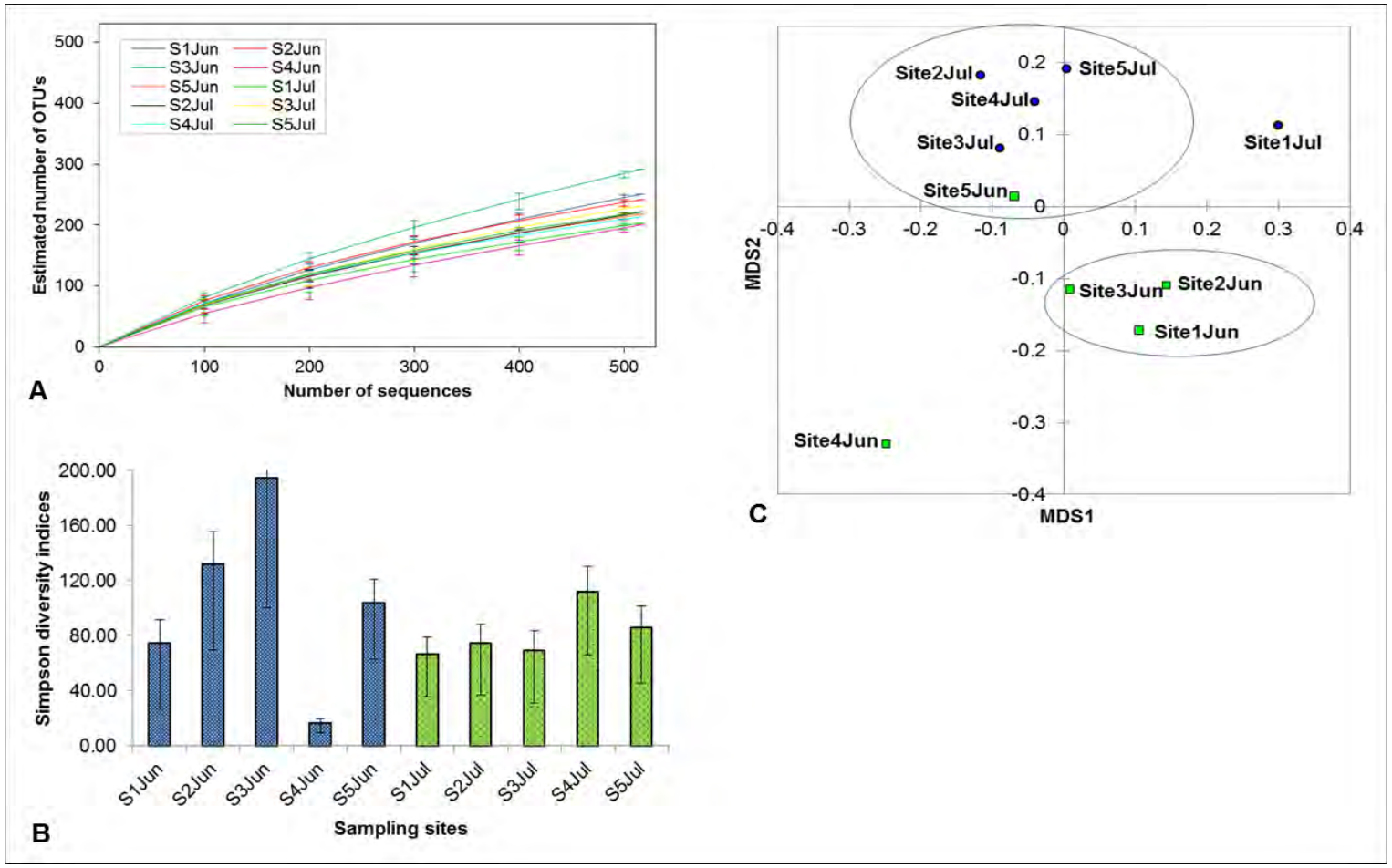

Figure 3-2: Bacterial alpha- and beta diversity estimates at all sampling sites (June and July) based on 454-pyrosequencing reads. Data sets were normalised to the same number of reads (516 reads) before calculations. **(A)** Rarefaction curves for the ten samples estimating the number of bacterial OTU's at the 97% similarity level; **(B)** Alpha diversity estimates calculated with Simpson diversity index; and **(C)** MDS diagram showing beta diversity among the five sampling sites.

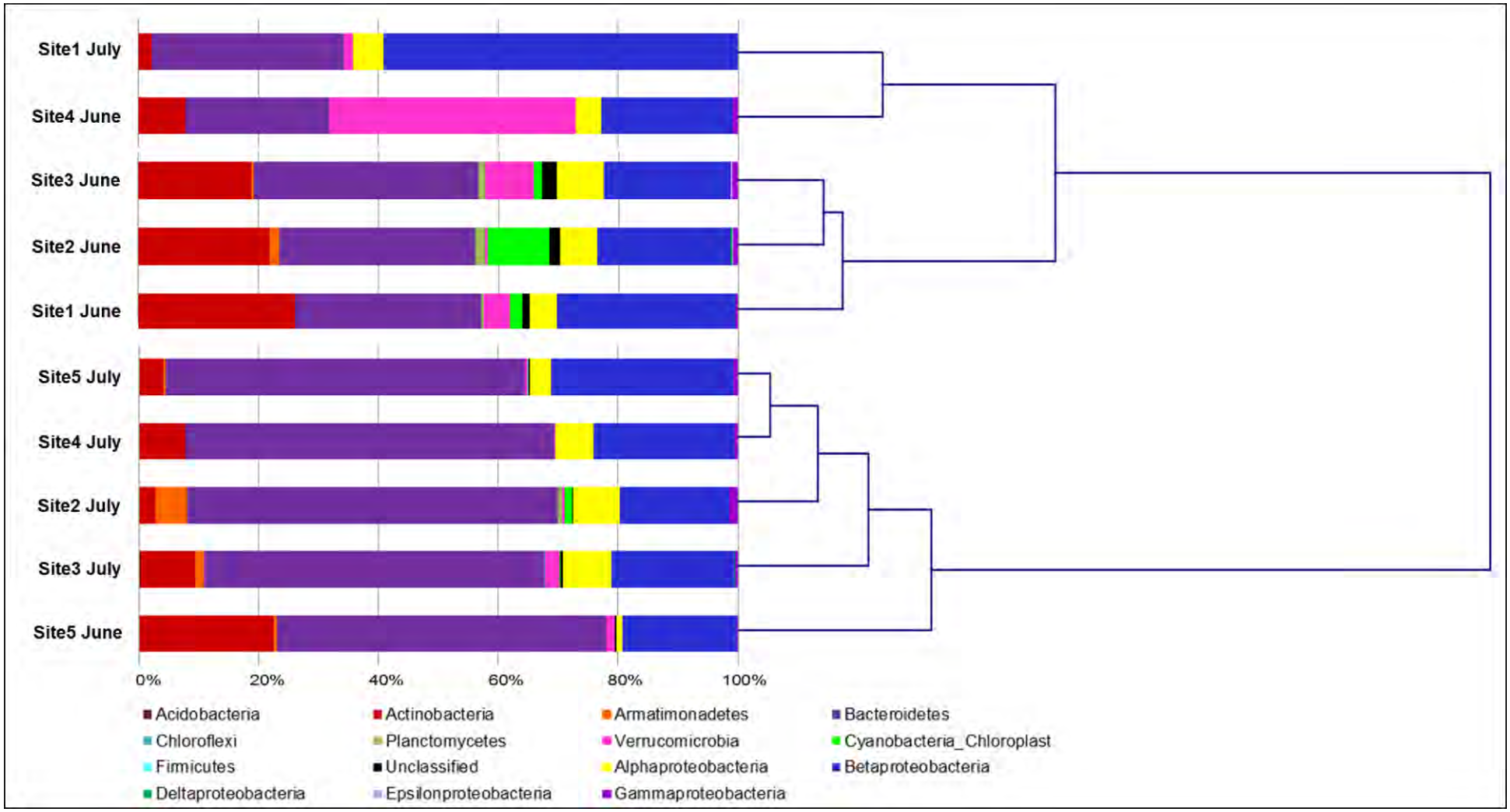

Figure 3-3: Bray-Curtis dissimilarity dendrogram showing the relatedness of the bacterial communities among the five sampling sites in June and July. Also shown are bacterial community profiles of the major taxonomic groups. The relative abundance of taxonomic groups is expressed as the percentage of the total community. The dendrogram and bacterial community profiles were calculated from 454-pyrosequencing data sets.

3.3.4 Associations between physico-chemical water characteristics and bacterial community structures

PCA and CCA were performed to gain an overview on the relatedness between the bacterial community structures and physico-chemical water characteristics (Figure 3-4A - D). PCA ordination showed that the first two principal components accounted for 45.67% and 28.34% of the total variance in bacterial diversity, respectively (Figure 3-4A). Bartlett's sphericity test confirmed that the correlation between sites and environmental variables was statistically significant ($p < 0.05$). The first axis positively correlated with temperature, EC, sulphate, and chlorophyll-a, while the second axis strongly correlated with DO and pH. PCA separated sampling sites into four distinct clusters. Cluster I consisted of both the June and July samples for site 1, indicating similar water chemistry. Cluster I also had the highest water quality and showed no direct associations with any of the environmental parameters. Cluster II comprised of the June and July samples for site 2 and strongly correlated with temperature and sulphate. However, temperature and sulphate levels at site 2 (June and July) did not indicate noticeable differences to that of the other sites. Cluster III was characterised by the June samples for site 3, 4 and 5, and cluster IV included the July samples for these three sites. Cluster III and IV varied markedly in their position on the plot, indicating distinct differences in the physico-chemical water characteristics among these two clusters. Cluster III showed to be related to chlorophyll-a concentrations. The three sites in cluster III exhibited higher chlorophyll-a levels compared to cluster IV, which further supports their grouping. Cluster IV showed a positive relationship with DO and pH on axis two. Site 3 – 5 July (cluster IV) displayed greater DO and pH levels compared to the June samples for these sites.

The CCA biplot for the 454-pyrosequencing data showed in total a 100% species-environment correlation (Figure 3-4B). Monte Carlo permutation tests indicated that the overall species-environment relationships were statistically significant ($p = 0.007$). The first two axes explained 93.06% of the total variance in the abundance and distribution of taxa tested. The results suggest that four of the environmental variables tested (pH, DO, sulphate, and chlorophyll-a) accounted for variability in the spatial succession of bacterial communities. A significant positive correlation was found between *Bacteroidetes* and DO ($p = 0.007$), indicating that the abundance of this phylum tend to

vary in line with DO levels. In contrast, *Verrucomicrobia* negatively associated with DO ($p = 0.012$), suggesting that an increase in DO caused a decrease in the abundance of this phylum, and vice versa. CCA analysis and Spearman correlation also indicated a significant inverse relationship between *Bacteroidetes* and *Verrucomicrobia* ($p = 0.019$). Sites characterised by high *Bacteroidetes* abundance were deficient in *Verrucomicrobia*, and vice versa. The abundance of *Betaproteobacteria* appeared to be related to pH ($p = 0.046$) and sulphate concentrations ($p = 0.022$). The pH remained relatively constant throughout the sampling sites while sulphate levels increased from site 1 to 5 (June and July). The results indicated an inverse relationship between sulphate levels and *Betaproteobacteria*. The abundance of the class decreased with increasing sulphate levels, indicating that *Betaproteobacteria* select for environmental conditions where sulphate levels are minimal. *Acidobacteria* and *Epsilonproteobacteria* indicated a close relationship with chlorophyll-a levels ($0.023 \leq p \leq 0.031$). The results suggest that *Acidobacteria* favoured low chlorophyll-a concentrations while elevated levels of this variable stimulated an increase in the abundance of *Epsilonproteobacteria.*

In addition to the correlations described above, CCA ordination indicated that the abundance of several genera could be associated with physico-chemical parameters ($p = 0.013$) (Figure 3-4C). Genera within the *Betaproteobacteria* (*Malikia* and *Leadbetterella*) and *Verrucomicrobia* (*Cerasicoccus*) showed significant negative relationships with DO levels ($0.001 \leq p \leq 0.04$). Furthermore, various *Betaproteobacterial* genera demonstrated positive and negative associations with chlorophyll-a levels. *Limnohabitans* ($p = 0.024$) negatively associated with chlorophyll-a, where *Pigmentiphaga*, *Duganella*, and *Pseudorhodoferax* ($0.003 \leq p \leq 0.029$) showed a positive correlation. Ordination and Spearman correlation further established a negative association between chlorophyll-a and *Acidobacteria_Gp6* (*Acidobacteria*) ($p = 0.031$), whilst *Singulispaera* (*Planctomycetes*) ($p = 0.034$) associated positively.

Biplot scaling of CCA with the indicator organisms suggested that a large proportion of the total variance was explained by the first axis (79.26%) (Figure 3-4D). Monte Carlo permutation (1000) test found the overall bacterial indicator-environment relationship to be statistically insignificant ($p < 0.05$). However, Spearman correlation test indicated meaningful associations between HPC and phosphate ($p = 0.004$), and *E. coli* and

sulphate (p = 0.028). When comparing physico-chemical results to microbiological data a distinct pattern was observed between these indicator organisms and environmental variables. The results indicated that HPC and phosphate levels, and *E. coli* and sulphate concentrations increased simultaneously from site 1 to 5.

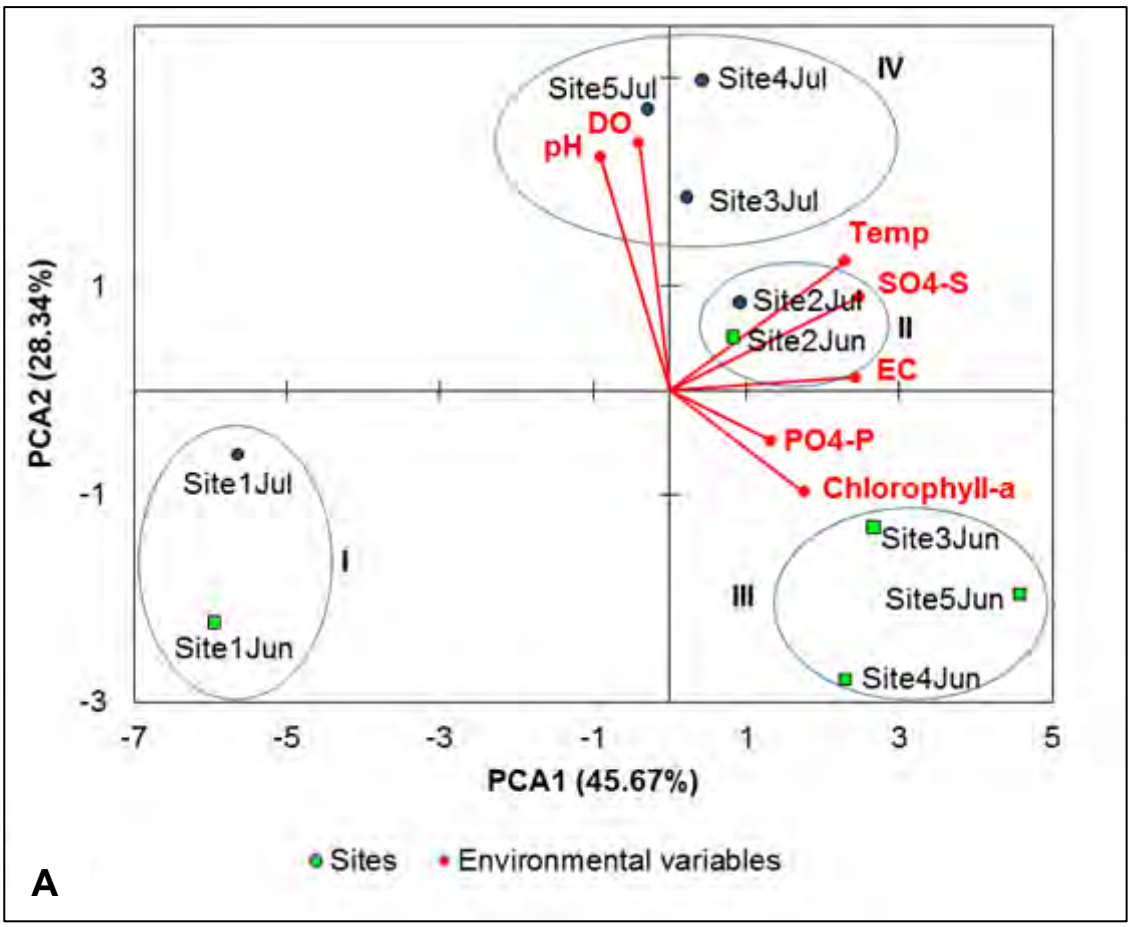

Figure 3-4: Multivariate analysis based on physico-chemical, microbiological, and 454-pyrosequencing data sets. 454-Pyrosequencing data were normalised to the same number of reads (516 reads) before analysis. **(A)** Principal coordinate analysis (PCA) of sampling sites in June and July based on the physico-chemical water properties. Samples clustered according to similarity in water quality properties.

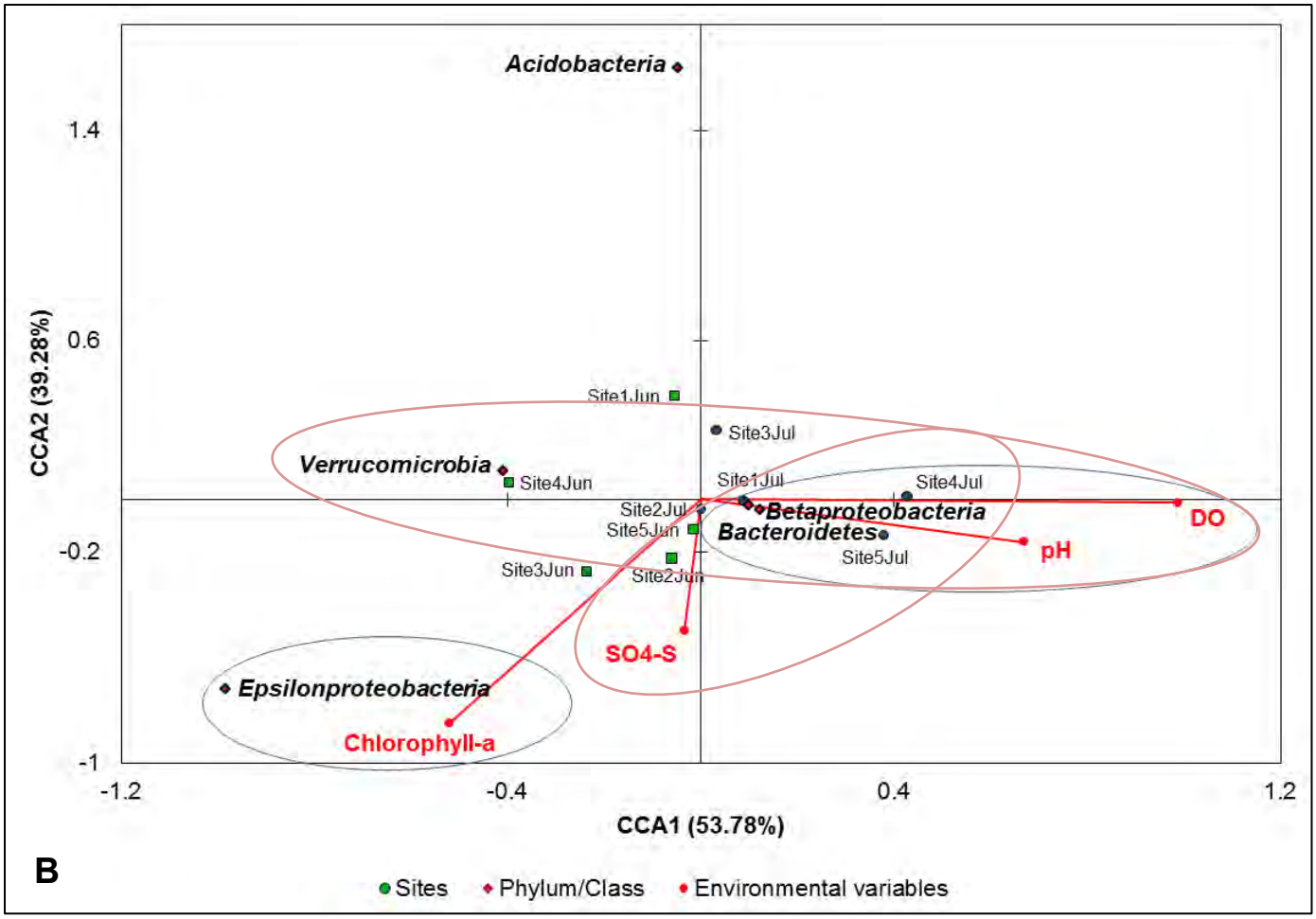

Figure 3-4: (B) Canonical correspondence analysis (CCA) plot of bacterial communities at phylum and class level (454-pyrosequencing reads) in correlation with environmental variables. Significant correlations ($p < 0.05$) between bacterial groups and pH, DO, sulphate, and chlorophyll-a are indicated in circles.

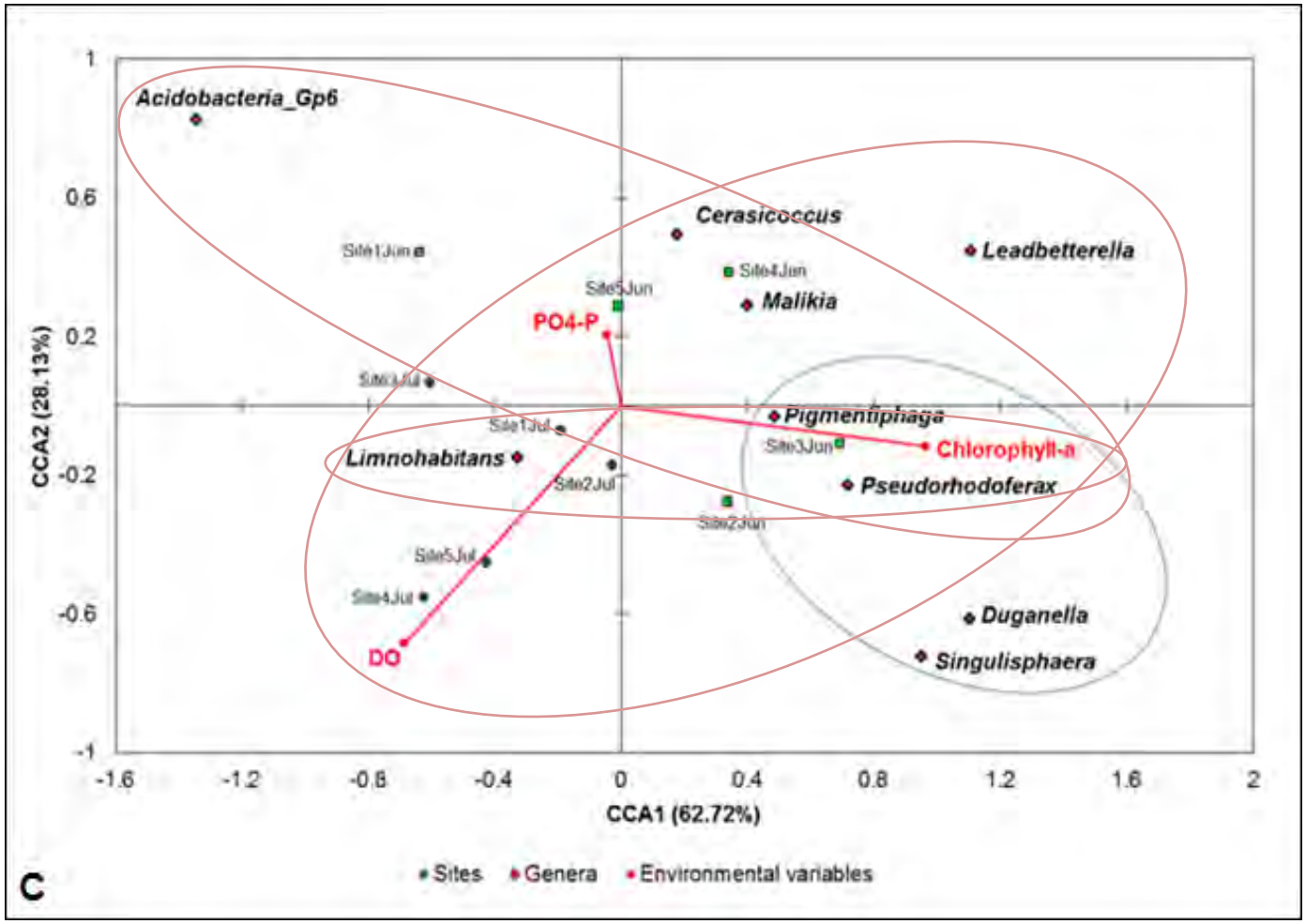

Figure 3-4: (C) CCA plot for bacterial genera (454-pyrosequencing reads) in correlation with environmental variables. Significant associations ($p < 0.05$) between genera and dissolved oxygen (DO), and chlorophyll-a are demonstrated in circles.

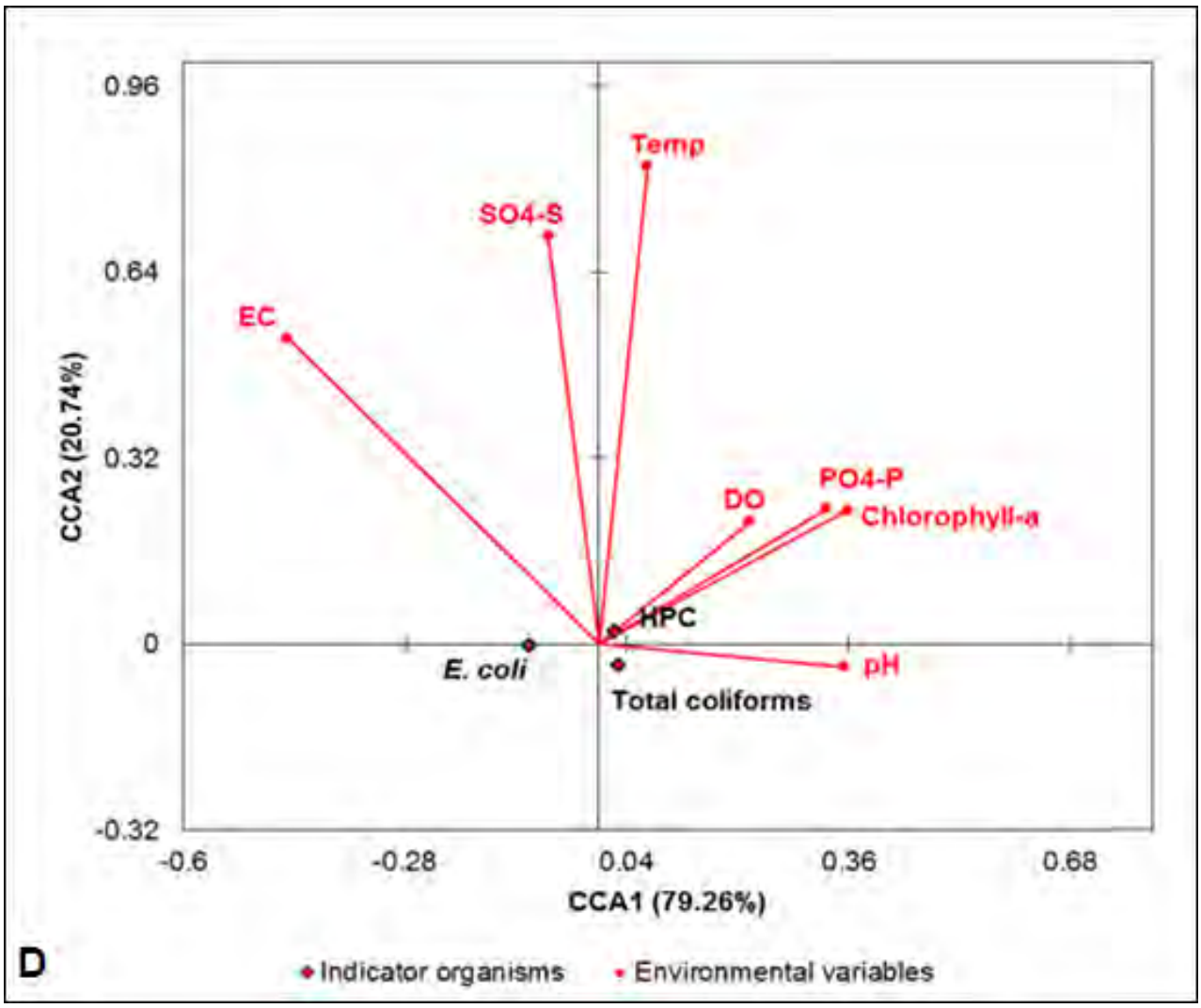

Figure 3-4: (D) CCA plot of indicator organisms and environmental variables. No significant correlations between objects (indicator organisms) and response variables were detected.

3.4 Discussion

The main aim of this study was to assess the impacts of physico-chemical parameters on bacterial communities in the Mooi River using physico-chemical analysis, culture-dependent techniques, and 454-pyrosequencing. Combined physico-chemical and microbiological data (culture-dependent and 454-pyrosequencing) indicated signs of anthropogenic disturbances from the reference (site 1) to downstream sites (site 2 to 5). The environmental variables DO, EC, sulphate, and phosphate levels were higher at the downstream sites, similar to what has been observed for a variety of freshwater systems impacted by urbanisation (De Figueiredo *et al.,* 2007; Liu *et al.,* 2012; Drury *et al.,* 2013). As indicated by Van der Walt et al. (2002), deterioration in the water quality below the Boskop Dam is the result of urban and industrial storm water runoff, as well as the Potchefstroom sewage works which mainly causes an increase in phosphate levels at Kromdraai (confluence of the Mooi- and Vaal River). In addition to urban inputs, dry land farming also adds substantial quantities of phosphorus to the river in the form of fertilizers and animal manure. These findings are further substantiated by the high counts of indicator- and heterotrophic bacteria that indicate possible faecal pollution and/or drainage of poorly treated wastewater. It is likely that urban communities, the high number of property developments, and livestock farming contributed to the high faecal counts. Furthermore, the taxonomic composition of bacterial communities significantly altered from the upstream to downstream sites due to changes in major taxonomic groups. For instance, *Betaproteobacteria* was the major taxonomic group detected at the reference site, followed by *Bacteroidetes* and *Actinobacteria*. In contrast, bacterial communities at the downstream sites were dominated by *Bacteroidetes*, *Betaproteobacteria, Actinobacteria*, *Alphaproteobacteria* and *Verrucomicrobia*.

A striking result to emerge from the data is the low bacterial diversity (richness and evenness) calculated for the reference site, whereas the downstream sites were more diverse. Also, BCC at the downstream sites was highly similar, particularly for the July sites. The higher bacterial diversity at the downstream sites is not surprising since increased concentrations of nutrients are believed to stimulate planktonic bacterial growth (Garnier *et al.,* 1992; Goñi-Urriza *et al.,* 1999) and benthic bacterial numbers (Wakelin *et al.,* 2008). Anthropogenic inputs, such as septic tanks, storm water runoff,

sewage treatment plant overflow, and agricultural activities may create conditions of greater habitat heterogeneity to allow the development of higher community diversity. Moreover, similarity in bacterial communities might be attributed to biotic homogenisation, which suggests that anthropological modifications of the environment are decreasing the biological differences between natural ecosystems (McKinney, 2006; Drury *et al.,* 2013). Consequently, these ecosystems consistently support a subset of naturally occurring species that can tolerate human activities (McKinney, 2006). Our findings are consistent with Drury et al. (2013) that demonstrated highly similar riverine bacterial communities at sites downstream of a wastewater treatment plant. Biotic homogenisation of plant and animal communities has been demonstrated by numerous studies (Walters *et al.,* 2003; Holway and Suarez, 2006); however, this phenomenon is less explored for aquatic microbial communities. Our results imply that anthropogenic inputs may be a key factor in biotic homogenisation of riverine bacterial communities.

Canonical correspondence analysis suggested that the altered environmental conditions significantly affected the spatial succession of bacterial communities in the Mooi River. Although the results should be interpreted with caution considering the low number of sampling events, some trends appeared. Multivariate analysis showed that pH, temperature, DO, sulphate and chlorophyll-a levels were the major factors to determine variation in BCC. Spatial variance in the abundances of *Betaproteobacteria*, *Epsilonproteobacteria*, *Acidobacteria*, *Bacteroidetes*, and *Verrucomicrobia* were linked to physico-chemical variables measured in this study. These results are consistent with previous findings that associated the impact of environmental factors with BCC in freshwater systems (Bacelar-Nicolau *et al.,* 2003; Crump and Hobbie, 2005; Lindström *et al.,* 2005; De Figueiredo *et al.,* 2012).

The *Betaproteobacteria* is often the dominant group in freshwater systems (Zwart *et al.*, 2002; Cottrell *et al.,* 2005; Van Der Gucht *et al.,* 2005; Newton *et al.,* 2011), and its abundance has been associated with pH, conductivity, temperature, total suspended solids (TSS), chlorophyll-a, soluble reactive phosphorus, and ammonium levels (Brümmer *et al.,* 2000; Altmann *et al.,* 2003; Gao *et al.,* 2005; De Figueiredo *et al.,* 2010; 2012). In this study, *Betaproteobacteria* abundance correlated positively with pH and negatively with sulphate levels. Our results are supported by previous studies that

show that the abundance of *Betaproteobacteria* favour environments with a higher pH (De Figueiredo *et al.,* 2010; 2012). The negative correlation between *Betaproteobacteria* and sulphate levels is not yet entirely clear, but we propose that most of the identified genera sustain ecological roles other than sulphur metabolism. Most of the *Betaproteobacteria* sequences detected related to uncultured bacteria, *Limnohabitans*, *Hydrogenophaga*, *Polaromonas*, *Polynucleobacter*, *Pigmentiphaga*, *Sphaerotilus*, and *Curvibacter*. These genera are involved in the metabolism of nitrogen (Willems *et al.,* 1989; Pellegrin *et al.,* 1999; Hahn *et al.,* 2012; Zeng *et al.,* 2012) and/or phosphorus compounds (Ding and Yokota, 2004; Chen *et al.,* 2009). In addition, none of the genera, including the unidentified bacteria, showed any correlations with the sulphate levels. These features could well explain the inverse relationship of *Betaproteobacteria* and sulphate concentrations.

Limnohabitans and three other minor Betaproteobacterial genera (*Duganella*, *Pigmentiphaga*, and *Pseudorhodoferax*) indicated strong associations with chlorophyll-a levels. *Limnohabitans* showed a negative correlation to chlorophyll-a values, while *Duganella*, *Pigmentiphaga*, and *Pseudorhodoferax* positively related to this variable. Notably, the latter three genera also showed positive correlations to each other. Previous studies reported significant associations between certain *Betaproteobacterial* groups, phytoplankton populations and/or phytoplankton derived organic material (Šimek *et al.,* 2008; Watanabe *et al.,* 2009; Paver and Kent, 2010; Parveen *et al.,* 2011). In fact, Šimek et al. (2008) and Paver and Kent (2010) demonstrated the ability of *Limnohabitans* (R-BT lineage) and *Polynucleobacter necessarius* to utilise specific algal exudates as a key substrate for growth. Conversely, other studies reported inverse relationships between *Limnohabitans* (R-BT, Lhab-A2 and Lhab-A4 lineages) and phytoplankton species (Horňák *et al.,* 2008; Eiler *et al.,* 2012). Our findings suggest that the negative relationship between *Limnohabitans* and phytoplankton may be the result of direct competition for nutrients and/or antagonistic activities such as the production of algal antimicrobial substances (Sigee, 2005; Eiler *et al.,* 2012). In contrast, *Duganella*, *Pigmentiphaga*, and *Pseudorhodoferax* likely developed a symbiotic relationship with phytoplankton producers with close metabolic coupling. Bacteria have high efficiencies of nitrogen and phosphorus uptake when inorganic nutrients are limited and may provide an important pathway for algae to absorb nitrogen and phosphorus under these

conditions (Sigee, 2005). This phenomenon may be responsible for the interdependent relationship between *Duganella*, *Pigmentiphaga, Pseudorhodoferax*, and phytoplankton producers. *Duganella* and *Pigmentiphaga* are capable of reducing nitrate to nitrite (Madhaiyan *et al.,* 2013) and/or dephosphorylate inorganic phosphorus compounds (Li *et al.,* 2004; Chen *et al.,* 2009), thereby providing inorganic nutrients for algal growth and development. In return, phytoplankton provided autochthonous dissolved organic carbon (DOC) that favoured the establishment of these genera (Eiler *et al.,* 2003; Judd *et al.,* 2006; Laque *et al.,* 2010). The co-occurrences of *Duganella*, *Pigmentiphaga*, and *Pseudorhodoferax* would seem to reflect similar or complementary functions (Eiler *et al.,* 2012).

Besides the above mentioned associations, *Malikia* was the only *Betaproteobacteria* genus that statistically correlated with environmental variables (DO and phosphates) other than chlorophyll-a. This genus was detected mainly at the downstream sites which showed elevated phosphate levels. There is evidence to suggest that *Malikia* is capable of degrading aromatic hydrocarbons such as polyhydroxyalkanoates (PHA's) and polyphosphates (Spring *et al.,* 2005; Táncsics *et al.,* 2010). This genus accumulates high quantities of polyphosphates as intracellular granules (Gavigan *et al.,* 1999), and is believed to play a major role in the enhanced biological phosphorus removal (EBPR) process of wastewater treatment plants (Spring *et al.,* 2005). This theory is validated by previous studies that isolated *Malikia* from activated sludge of a municipal wastewater treatment plant (Spring *et al.,* 2005), polluted rivers in urban and suburban areas (Huang *et al.,* 2011; Drury *et al.,* 2013), and groundwater contaminated by aromatic hydrocarbons (Táncsics *et al.,* 2010). Our results agree with Yi et al. (2011) and Drury et al. (2013) that detected high abundance of *Malikia* in freshwaters impacted by anthropogenic activities such as industrial discharge, effluent from wastewater treatment plants, and sewage runoff from urban and rural communities. This further supports the idea that the downstream sites were polluted by sewage overflow from urban infrastructures and/or agricultural activities.

The most surprising correlations were between *Acidobacteria* and chlorophyll-a, as well as *Epsilonproteobacteria* and chlorophyll-a. Genomic evidence suggests that *Acidobacteria* participates in the nitrogen cycle by reducing nitrate, nitrite, and possibly

nitric oxide (Richardson *et al.,* 2001). In addition, the nitrogen fixing genus *GpIIa* was the major Cyanobacterial group identified in the Mooi River. From these results we speculate that the negative correlation between and *Acidobacteria* and chlorophyll-a (phytoplankton) is most likely attributed to direct competition for nitrogen compounds as an energy source. Since a limited amount of data is available for *Acidobacteria* and the relationship between *Acidobacteria* and phytoplankton, it is difficult to compare our results with those of other freshwater systems. The positive association between *Epsilonproteobacteria* and chlorophyll-a may be explained by a symbiotic relationship between these two groups. *Arcobacter*, the main genus detected in this class, contributes to the sulphur cycle by oxidizing sulphide to sulphur compounds (Teske *et al.,* 1996; Voordouw *et al.,* 1996; Snaidr *et al.,* 1997). These compounds may then become available for phytoplankton consumption, while phytoplankton provided autochthonous DOC to *Arcobacter* (Eiler *et al.,* 2003; Judd *et al.,* 2006; Laque *et al.,* 2010).

Bacteroidetes sequences were more abundant at the downstream sites with *Arcicella* and *Flavobacterium* as the two dominant genera. The *Bacteroidetes* group, in particular *Flavobacterium*, is often found in high abundance in mesotrophic, eutrophic, and hypertrophic water bodies (Allgaier and Grossart, 2006; De Figueiredo *et al.,* 2007; 2012; Haller *et al.,* 2011; Drury *et al.,* 2013), and usually correlates with high nutrient levels (De Figueiredo *et al.,* 2007; 2010; 2012). Members are well known to be proficient in degrading dissolved organic material (DOM), especially in nutrient-rich waters where biomacromolecules accumulate (Reichenbach, 1989; Kirchman, 2002; Eiler and Bertilsson, 2007; Zeder *et al.,* 2009). It was surprising that despite the higher concentrations of inorganic nutrients at the downstream sites, no direct link could be established between *Bacteroidetes* and nutrient levels. Instead, this phylum appeared to correlate with higher DO concentrations. These findings are in contrast with previous studies that reported close associations between *Bacteroidetes* and nitrogen sources (De Figueiredo *et al.,* 2007; 2010). Given that the quality and quantity, and types of DOM (allochthonous or autochthonous) were not directly measured in this study, it was not possible to investigate significant relationships between *Bacteroidetes* and DOM. Therefore, the relationship between *Bacteroidetes* and DO needs to be interpreted with caution. Despite this inconsistency, it can nevertheless be argued that members of this

phylum had metabolic and functional roles other than nitrate reduction. For instance, *Flavobacterium* species thrive in the presence of complex macromolecules (Kirchman, 2002) as they are important metabolizers of various high-molecular-weight (HMW) DOM (Kisand *et al.,* 2002; 2005). High-molecular-weight DOM is degraded via photochemical processes (Kisand *et al.,* 2002; 2005) that require dissolved oxygen as an electron acceptor (Zafiriou *et al.,* 1984). It is likely that the downstream sites contained high amounts of complex organic compounds that stimulated *Flavobacterium* growth more than by inorganic nutrients. Photochemical consumption of DOM may explain the positive association between *Bacteroidetes* and DO levels. Future studies are required to determine the effects of anthropogenic inputs on riverine DOM, and the response of *Bacteroidetes* to these compounds.

Verrucomicrobia, especially the genus *Cerasicoccus*, showed a significant inverse relationship to DO levels. This correlation may be explained by the ability of *Cerasicoccus* to hydrolyse starch (Yoon *et al.,* 2007). To the best of our knowledge, similar relationships between *Verrucomicrobia* and DO levels have not previously been reported. The ecological roles of *Verrucomicrobia* remained largely unexplored, but its presence has been associated with eutrophic or nutrient-rich waters where phosphorus levels are high (Lindström *et al.,* 2004; Haukka *et al.,* 2006), and environments contaminated with hydrocarbons, heavy metals, and pesticides (Pereira *et al.,* 2006; Paissé *et al.,* 2008; Vishnivetskaya *et al.,* 2011). Members of this phylum grow chemoheterotrophically on organic carbon compounds such simple sugars (Schlesner *et al.,* 2006; Yoon *et al.,* 2007; 2008; 2010) and complex biopolymers (Martinez-Garcia *et al.,* 2012). Most of the *Verrucomicrobia* sequences were recovered from the downstream sites (site 3 and 4), suggesting that these sites contained higher concentrations of natural and/or synthetic polysaccharides. The ability of *Verrucomicrobia* to degrade various polysaccharides is of great interest in biotechnological applications, such as biofuel production and bioremediation of polluted sites (Martinez-Garcia *et al.,* 2012).

Finally, obligate and/or opportunistic pathogenic genera within the *Actinobacteria* (*Leifsonia* and *Mycobacterium*), *Alphaproteobacteria* (*Brevundimonas*, *Roseomonas*, *Rhodobacter*, and *Sphingomonas*), *Epsilonproteobacteria* (*Arcobacter*), and

Gammaproteobacteria (*Aeromonas* and *Pseudomonas*) groups were detected mainly at the downstream sites (Decker *et al.,* 1992; Struthers *et al.,* 1996; Evtushenko *et al.,* 2000; Ho *et al.,* 2006; Parker and Shaw, 2011; Magee and Ward, 2012; Djordjevic *et al.,* 2013). Their presence in the Mooi River may be regarded as a potential risk for human and animal health, considering that the river is used for recreational activities and agricultural purposes. Members of these genera are found in various natural environments (Jayasekara *et al.,* 1999; Lee *et al.,* 2001; Rickard *et al.,* 2003; Magee and Ward, 2012), but are also associated with polluted waters (Edwards *et al.,* 2001; Marcel *et al.,* 2002; Kalwasińska *et al.,* 2008; Srinivas *et al.,* 2008; Collado *et al.,* 2011). *Arcobacter* species in environmental waters often correlate with high faecal indicator counts (Fong *et al.,* 2007; Collado *et al.,* 2008). These bacteria are found in high numbers in sewage water inflow to wastewater treatment plants (McLellan *et al.,* 2010), and livestock farming effluents (Van Driessche *et al.,* 2003; Chinivasagam *et al.,* 2007). Their high abundance in surface waters could indicate contamination by the above mentioned sources. In addition, *Aeromonas* and *Sphingomonas* thrive in polluted environments because they are able to degrade various recalcitrant compounds (Samanta *et al.,* 1999; Pinyakong *et al.,* 2003; Ghosh *et al.,* 2004; Guo *et al.,* 2011). Although they are not considered to be of faecal origin, their presence is of interest since they hold potential to be used as indicators of aromatic hydrocarbon pollution. Future studies on the abundance and distribution of *Acrobacter*, *Aeromonas* and *Sphingomonas* in freshwater systems, and their relationship to environmental variables are required to establish if they can be used as indicators of anthropogenic stress.

3.5 Conclusions

The evidence of this study suggests that variation in BCC in the Mooi River was related to anthropogenic inputs resulting from human activities and agricultural land use. Physico-chemical and microbiological data indicated that water quality deteriorated below the Boskop Dam and this trend continued downstream until the confluence with the Vaal River. Temperature, pH, DO, sulphate, and chlorophyll-a levels appeared to have the greatest impact on BCC. Our work also identified potential indicator groups (*Acrobacter*, *Aeromonas* and *Sphingomonas*) that may be used to track faecal and organic pollution in freshwater systems. A number of potential limitations need to be considered. First, we are aware that part of the spatial variance in BCC could be related

to other variables not measured here. Secondly, it is plausible that the small sample size and frequency could have influenced the BCC results obtained. Nevertheless, we are confident that our results contribute to aspects of our understanding of urbanisation on riverine BCC, particularly on major taxonomic groups and genera. Our research might have important implications for: (i) improving the River Health Programme (South Africa) by including 454-pyrosequencing of bacterial communities to monitor the microbiological water quality; (ii) developing management strategies to prevent further pollution; (iii) providing valuable information for effective and reliable bioremediation policies; and (iv) improving our knowledge about biotic homogenisation due to anthropogenic inputs. As sequencing and freshwater metabolism techniques continue to advance, we believe that this approach has the potential to: (i) measure BCC responses to anthropogenic perturbations; (ii) measure the overall ecosystem functioning; (iii) quantify primary production and respiration rates to evaluate the trophic status of the river; and (iv) estimate organic matter transfer between the Mooi River and its tributaries.

CHAPTER 4: Impacts of physico-chemical parameters on bacterial community structure in a gold mine impacted river: a case study of the Wonderfonteinspruit, South Africa

4.1 Introduction

Increasing anthropogenic disturbances on freshwater systems, e.g., mining, urban and rural settlements, sewage works, and agriculture, accelerate deterioration of aquatic water quality and ecosystem health. Given these detrimental effects, there is an urgent need to assess its state for both the near and distant future.

Bacterial communities in freshwaters play a key role in biogeochemical cycles (Bertilsson *et al.,* 2004; Xu, 2006; Lin *et al.,* 2014). They are responsible for breaking down organic material and remineralize nutrients, which in turn affect energy flux and circulation of material in the system (Bertilsson *et al.,* 2004; Xu, 2006; Lin *et al.,* 2014). Bacterial diversity and species abundance are associated with nutrient availability and physical environment (Leff *et al.,* 1999; Hahn, 2006). Changes in nutrient sources and the environment can have major repercussions on community composition and species abundance affecting the overall water quality (Lemke *et al.,* 1997; Zarraonaindia *et al.,* 2013). Determining which chemical and physical factors correlate with community changes will reveal how microorganisms react to different perturbations and increase our understanding of microbial ecology and their effects on pollution (Lowe and Pan, 1996; Hahn, 2006; Pronk *et al.,* 2009; Stabili and Cavallo, 2011; Schultz *et al.,* 2013). By combining this approach with animal and plant ecology, specialists may be able to develop an effective remediation strategy for polluted waters (Tumanov and Krestvaninov, 2004).

Molecular methods, e.g., denaturing gradient gel electrophoresis (DGGE), cloning, and terminal restriction fragment length polymorphism (T-RFLP), have been used widely to study bacterial ecology in freshwater systems (Wu *et al.,* 2007; Besemer *et al.,* 2012; De Figueiredo *et al.,* 2012). Although these methods increased our understanding of microbial diversity in freshwaters, they are time consuming and not able to characterize

the vast majority of bacterial species present (Schultz *et al.,* 2013). Recently, 454-pyrosequencing has taken giant leaps forward in analysing bacterial communities in aquatic ecosystems (Ghai *et al.,* 2011; Vishnivetskaya *et al.,* 2011; Crump *et al.,* 2012; Portillo *et al.,* 2012; Bricheux *et al.,* 2013; Bai *et al.,* 2014). This technique can generate over 400, 000 reads with fairly high taxonomic resolution and allows statistically robust assessments of community and population structure (Sogin *et al.,* 2006; Andersson *et al.,* 2010; Glenn, 2011).

Little to no data exist on the bacterial diversity in the Wonderfonteinspruit (WFS) and the impacts of different anthropogenic sources on bacterial community composition (BCC). Previous research mainly focused on the effects of heavy metals, in particular uranium, on surface water and groundwater of the WFS (Winde, 2010b; 2011; Barthel, 2012; Diale *et al.,* 2011). The lower WFS receives discharges from nearby gold mines, domestic wastewater treatment plants (WWTPs), urban and informal settlements, and agricultural runoff (DWA, 2009b; Barthel, 2012). Due to excessive pollutant loads, including microorganisms, the water quality of the river has degraded markedly and still progressively deteriorates. Thus, dramatic improvement of the water quality and ecosystem health is of utmost importance.

The aim was to use 454-pyrosequecing of the V6 – V8 region of the 16S rRNA gene to determine: (i) BCC in the lower WFS; (ii) the impacts of anthropogenic disturbances on bacterial community composition; and (iii) links between environmental drivers and individual taxa.

4.2 Materials and Methods

4.2.1 Study site

The Wonderfonteinspruit Catchment Area (WCA) originates in the southern part of Krugersdorp on the Witwatersrand ridge (Gauteng Province) (Figure 4-1). From here the river flows in a south-easterly direction through municipal and mining areas before confluence with the Mooi River upstream of Potchefstroom city (North West Province) (Coetzee, 2004; DWA, 2009a; Barthel, 2012). The upper section of the WCA is situated in the Gauteng Province and the lower part of the catchment in the North West Province (Barthel, 2012). Most of the catchment flows over dolomitic groundwater compartments

which hold several of South Africa's biggest dolomitic water reserves (DWA, 2009b). Some of the compartments are underlain by gold-bearing reefs that are extensively damaged by gold mining activities (DWA, 2009b).

Many of the large active gold mines discharge fissure and process water into the WFS (Barthel, 2012). In addition, the river receives discharge effluent from numerous point and diffuse sources such as old and/or abandoned mines, deposits of mining/milling slime dams, wastewater treatment works, formal and informal settlements, peat mining, industry, and agriculture (DWA, 2009b). As a result the water quality of WFS and underlying dolomitic groundwater compartments have been substantially polluted by radionuclides, heavy metals, sulphates, organic constituents and biological material (DWA, 2009a).

This study was conducted in the lower WCA in spring and summer of 2012 (October – December). Samples were collected from seven sites to represent a wide range of water quality data and assess the effects of anthropogenic activities on the water resource (Figure 4-1). Study sites included: Site 1 – Carletonville area (formal and informal settlements) (26°18'57.0"S 27°22'56.9"E); Site 2 – Welverdiend (formal settlement) (26°22'01.9"S 27°16'14.1"E); Site 3 – C2H069 (Department of Water Affairs and Forestry (DWA) monitoring point downstream of Welverdiend and all major discharge points from gold mines in the area) (26°22'12.1"S 27°14'57.8"E); Site 4 – karst spring from the Turffontein dolomitic eye (26°24'34.2"S 27°10'38.7"E); Site 5 – Muiskraal (farming community) (26°26'11.3"S 27°09'05.1"E); Site 6 – karst spring from the Gerhard Minnebron dolomitic eye (26°28'47.3"S 27°09'05.8"E); Site 7 – point downstream of the confluence with the Mooi River (26°30'52.4"S 27°07'28.3"E).

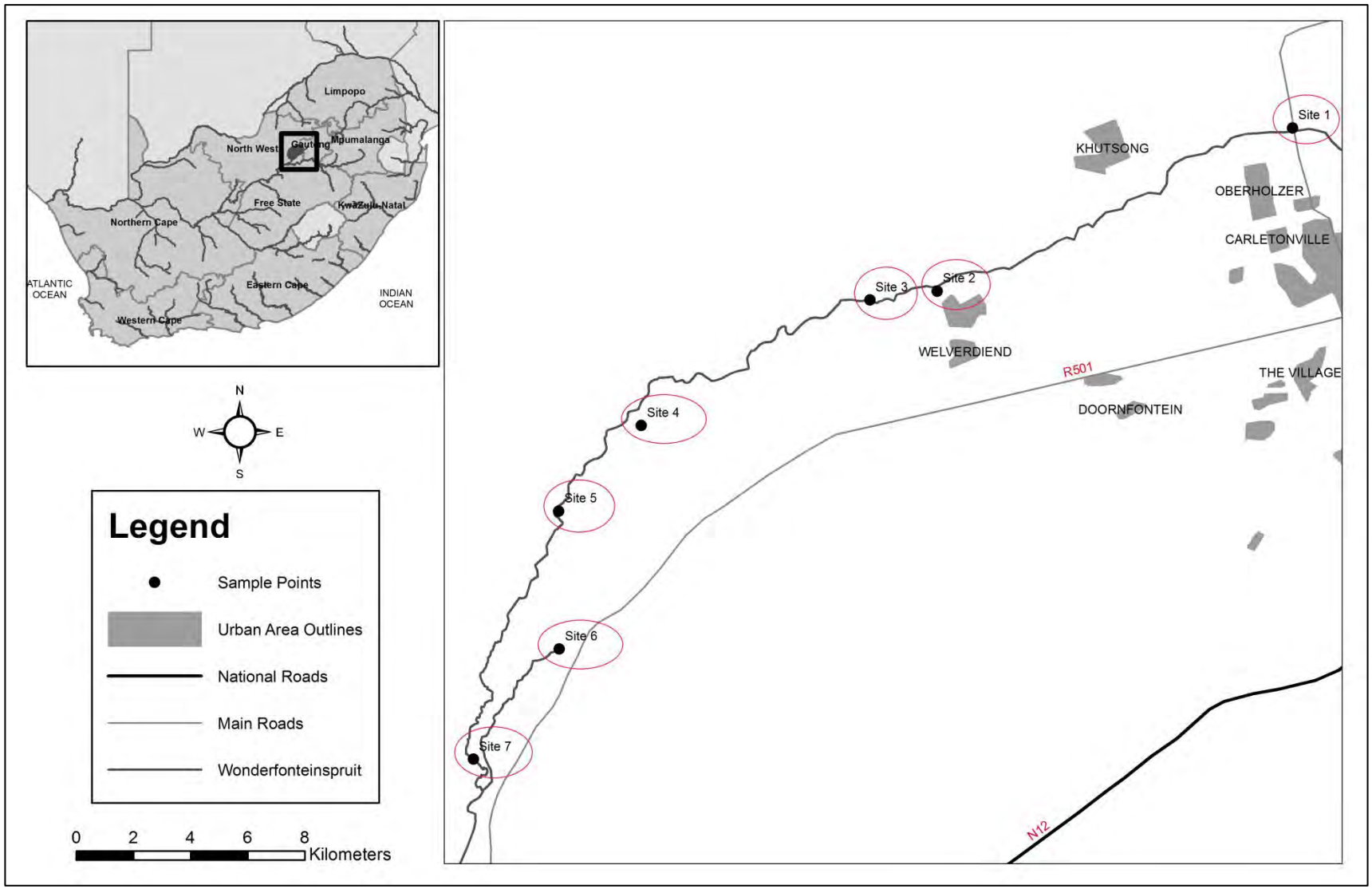

Figure 4-1: Geographical map of the lower Wonderfonteinspruit. Illustrated is the general location of the study site in the North West Province, with a detailed view of the sampling sites examined for bacterial community composition.

4.2.2 Sample collection

Freshwater samples were collected montly in sterile containers and placed at 4°C until filtration, normally within 8h after collection. Samples were taken in duplicate from each sampling station to determine bacterial community composition, chemical water quality, and heavy metals. Physical parameters measured *in situ* included temperature, pH and electrical conductivity (EC). Selected chemical and heavy metal elements were analysed by Eco-Analytica Laboratory, Potchefstroom, South Africa. Chemical parameters included chloride (Cl^-), nitrate (NO_3^-), phosphate (PO_4^{3-}), sulphate (SO_4^{2-}), and bicarbonate (HCO_3^-). Trace metals measured included manganese (Mn), iron (Fe), cobalt (Co), nickel (Ni), copper (Cu), chromium (Cr), zinc (Zn), selenium (Se), lead (Pb), cadmium (Cd), mercury (Hg), arsenic (As), and uranium (U).

4.2.3 DNA isolation and PCR amplification

Total DNA from water samples was isolated by sequentially filtering 250 to 2,000 mL water (depending on the amount of particles retained on the filter) through sterile 0.2 µm nitrocellulose membrane filters. Cells and particles that retained on the filters were resuspended in sterile TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0) and mixed by vortex for 7 – 10 min. The suspension was then centrifuged for 1 min at 13400 rpm to pellet the cells. DNA was isolated from the pellet using the NucleoSpin Tissue kit (Macherey-Nagel GmbH & Co. KG) according to manufacturer's instructions. The isolated DNA was stored at – 20˚C until further analysis.

The V6 – V8 region of the 16S rRNA gene was amplified using bacterial primer pair described by Comeau et al. (2012). PCR reactions (50 µl) contained: 5µl Q5 reaction buffer (New England BioLabs Inc., Ipswich, MA, USA), 0.2 mM of each dNTP, 0.2 mM of each 454 primer, 1 U of Q5 High-Fidelity DNA polymerase (New England BioLabs), PCR-grade water, and 1–3 µl of template DNA. Three separate DNA concentrations were used for each sample: 1, 0.5 and 0.1X (concentrations ranged between 0.5 and 52 ng). Cycling conditions were as follow: initial denaturation at 98°C for 30 s, 30 cycles of denaturation at 98°C for 10 s, annealing at 55°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 2 min. Triplicate reactions for each sample were pooled and purified using Agencourt AMPure beads (Beckman Coulter Inc., Brea, CA, USA). The quality of pooled samples was evaluated using the Agilent DNA 7500 Chip

Kit (Agilent Technologies Inc., Santa Clara, CA, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies Inc.).

4.2.4 454-Pyrosequencing

Pyrosequencing was performed at IBIS/Université Laval Plate-forme d'Analyses Génomiques (Québec, Canada) using the Roche 454 GS-FLX Titanium chemistry. Raw sequence data was quality trimmed and checked for chimeras and singletons following the MOTHUR v.1.30 pipeline (Schloss *et al.,* 2009). Sequences were assigned to operational taxonomic units (OTU's) at a 97% similarity. Rarefaction curves were constructed from 454-pyrosequencing data using MOTHUR v.1.30. Taxonomic classification was based upon the modified version of the "GreenGenes97" pyrosequencing reference files (Comeau *et al.,* 2012) at a 97% bootstrap confidence threshold. Alpha- and beta diversity calculations were performed using reduced data sets in which the number of sequences per samples was made equal with random re-sampling (3703 sequences per sample). Alpha diversity (richness and evenness) was calculated in MOTHUR v.1.30 using the Chao 1 and Simpson diversity index. Beta diversity was determined in XLSTAT version 2014.1 (Addinsoft SARL) through the Bray-Curtis dissimilarity coefficient to obtain a proximity matrix. The resulting distance matrix was mapped on a 2D- non-metric multidimensional scaling (NMDS) plot, with 1000 repetitions, and Bray-Curtis dissimilarity dendrogram.

DNA sequences were submitted to the GenBank database as BioProject PRJNA275052.

4.2.5 Statistical analysis

Multivariate analysis was used to assess the effects of physico-chemical water properties and heavy metals on BCC. Environmental and pyrosequencing data was log transformed [log(x + 1)] before analysis. Correlations between environmental variables and BCC were first calculated by Spearman's rank method. Significant relationships between environmental variables and dominant taxa (> 1% of the total BCC) were further analysed by redundancy analysis (RDA) with Monte Carlo permutation tests based on 1000 unrestricted permutations, 80% confidence level, and 5% significance

level. Statistical analyses were performed using XLSTAT version 2014.1 (Addinsoft SARL).

4.3 Results

4.3.1 Physico-chemical analysis

Physico-chemical parameters and trace metals are summarized in Table 4-1 and 4-2. The average pH (7.66 – 7.83), temperature (18.50 – 22.91°C), EC (82.01 – 83.04 mS/m), nitrate (6.45 – 7.83 mg/L), phosphate (0.55 – 0.63 mg/L), and chloride (45.44 – 55.75 mg/L) levels did not vary markedly during the three sampling periods. In contrast, sulphate and bicarbonate levels changed drastically between sampling intervals. Sulphate levels for the December samples increased considerably (> 200 mg/L) and exceeded the target water quality range (TWQR) for domestic use, although the water is not directly used for domestic purposes (DWAF, 1996a). Bicarbonate reached maximum and minimum levels in November and December, respectively. November was associated with exceptionally hot and dry weather that could have caused accumulation of bicarbonate levels in the river, while December experienced heavy rainfall and flushed a large quantity of bicarbonate ions. Nitrate concentrations at site 1, 4, 5, and 6 were at all times above the TWQR for domestic use. Although EC remained relatively constant throughout the sampling period, concentrations of dissolved salts were above the TWQR (> 70 mS/m) for domestic use. Heavy metals were consistently higher at the upstream sites (site 1 – 3), but were within the TWQR for domestic use, irrigation and livestock watering, with the exception of iron. Iron levels were at all times above the recommended TWQR for domestic use and reached a maximum concentration of 0.68 mg/L.

Table 4-1: Mean physico-chemical variables measured in the lower Wonderfonteinspruit.

	Temp (°C)	pH	EC (mS/m)	PO_4 (mg/L)	SO_4 (mg/L)	NO_3 (mg/L)	Cl (mg/L)	HCO_3 (mg/L)
Site 1 Oct	19.00	8.37	91.00	1.90	131.76	11.17	51.28	58.58
Site 2 Oct	16.20	8.13	91.50	1.34	125.03	5.63	54.68	57.35
Site 3 Oct	16.60	7.80	96.20	0.42	139.93	4.14	63.87	61.02
Site 4 Oct	19.70	7.20	76.00	0.01	70.63	11.65	35.19	48.20
Site 5 Oct	17.70	8.08	78.70	0.95	81.48	6.20	41.38	50.03
Site 6 Oct	20.80	7.36	76.60	0.01	84.37	12.02	43.00	48.81
Site 7 Oct	19.50	7.87	67.50	0.01	58.88	4.00	28.65	43.32
Site 1 Nov	26.10	8.27	92.80	1.91	208.45	10.83	65.59	213.56
Site 2 Nov	25.60	7.85	90.20	1.17	186.36	0.91	68.47	225.76
Site 3 Nov	23.20	7.75	92.40	1.28	187.32	0.96	73.99	231.86
Site 4 Nov	20.40	7.15	75.90	0.01	80.69	13.13	43.40	292.88
Site 5 Nov	21.70	7.62	78.00	0.01	98.94	9.21	46.89	286.77
Site 6 Nov	21.30	7.31	77.40	0.01	110.47	13.84	53.03	244.06

Site 7 Nov	22.10	7.74	74.60	0.01	81.65	5.20	38.87	298.98
Site 1 Dec	24.10	8.23	90.70	1.90	321.16	9.32	60.60	3.50
Site 2 Dec	22.60	7.71	83.80	1.60	268.39	1.25	62.52	3.35
Site 3 Dec	22.70	7.72	96.80	0.83	348.42	1.41	82.65	3.14
Site 4 Dec	20.50	7.15	75.90	0.08	149.18	11.35	40.04	4.82
Site 5 Dec	21.50	7.70	77.00	0.01	177.60	6.34	45.86	4.34
Site 6 Dec	21.60	7.32	77.50	0.01	145.37	3.31	38.21	4.58
Site 7 Dec	22.30	7.78	72.40	0.01	194.46	12.20	50.09	4.00

Table 4-2: Heavy metals concentrations measured in the lower Wonderfonteinspruit.

	As (ppm)	Cd (ppm)	Co (ppm)	Cr (ppm)	Cu (ppm)	Fe (ppm)	Hg (ppm)	Ni (ppm)	Mn (ppm)	Pb (ppm)	Se (ppm)	U (ppm)	Zn (ppm)
Site 1 Oct	1.10E-02	1.08E-05	1.24E-02	6.82E-05	8.37E-03	4.69E-01	1.16E-03	4.94E-02	3.25E-03	7.94E-03	8.24E-05	3.70E-02	3.81E-04
Site 2 Oct	4.20E-03	1.33E-05	7.63E-03	9.05E-05	1.20E-02	4.54E-01	5.19E-04	8.07E-03	1.24E-03	7.88E-03	1.32E-04	2.16E-02	3.92E-04
Site 3 Oct	4.40E-03	1.34E-05	7.03E-03	1.01E-04	8.36E-03	4.57E-01	3.16E-04	3.48E-02	2.65E-03	7.75E-03	1.33E-04	2.61E-02	4.43E-04
Site 4 Oct	3.48E-05	1.34E-05	2.99E-03	8.53E-05	7.99E-03	3.93E-01	1.87E-04	3.74E-05	4.06E-06	7.82E-03	1.30E-04	3.13E-03	4.42E-04
Site 5 Oct	2.83E-05	1.35E-05	3.06E-03	9.97E-05	8.73E-03	4.16E-01	1.25E-04	1.01E-05	4.29E-04	7.58E-03	1.34E-04	1.87E-02	4.56E-04
Site 6 Oct	3.42E-05	1.33E-05	7.71E-03	7.89E-05	5.49E-03	4.02E-01	5.97E-05	3.22E-05	3.79E-07	7.70E-03	1.30E-04	3.02E-03	4.56E-04
Site 7 Oct	3.51E-05	1.34E-05	3.83E-03	1.09E-04	5.16E-03	3.47E-01	8.44E-06	3.20E-05	3.60E-04	8.17E-03	1.37E-04	6.58E-03	5.07E-04
Site 1 Nov	8.79E-03	1.27E-04	1.80E-02	7.02E-05	1.69E-02	5.06E-01	2.61E-05	6.40E-02	8.07E-02	7.79E-03	1.52E-03	4.00E-02	3.41E-03
Site 2 Nov	4.67E-03	1.25E-04	7.71E-03	1.46E-03	1.87E-02	6.80E-01	9.65E-04	1.33E-02	1.46E-01	8.37E-03	1.74E-03	2.36E-02	4.47E-03
Site 3 Nov	5.56E-04	1.18E-04	3.78E-03	1.72E-03	1.24E-02	3.99E-01	2.24E-05	3.29E-05	4.08E-03	7.96E-03	1.68E-03	3.11E-03	3.06E-03
Site 4 Nov	5.80E-04	1.26E-04	3.84E-03	1.99E-03	6.21E-03	4.30E-01	2.85E-05	6.76E-03	3.85E-03	8.28E-03	1.79E-03	3.07E-03	3.07E-03
Site 5 Nov	5.19E-04	1.22E-04	3.56E-03	9.85E-04	1.15E-02	4.36E-01	3.41E-05	4.28E-03	2.43E-02	8.24E-03	1.74E-03	2.04E-02	4.53E-03
Site 6 Nov	5.71E-04	1.28E-04	8.03E-03	3.54E-03	1.57E-02	4.01E-01	2.86E-05	1.20E-04	4.06E-05	8.29E-03	1.57E-03	3.07E-03	4.28E-03
Site 7 Nov	5.68E-04	1.24E-04	5.25E-03	9.62E-04	8.72E-03	4.25E-01	2.91E-05	9.87E-04	1.96E-02	7.96E-03	1.80E-03	7.39E-03	3.11E-03
Site 1 Dec	8.11E-03	1.09E-05	1.27E-02	2.44E-04	6.72E-03	3.17E-01	5.92E-06	3.66E-02	7.76E-03	8.42E-03	1.11E-04	3.39E-02	3.79E-04

Site 2 Dec	2.06E-03	1.11E-05	5.74E-03	2.45E-04	7.00E-03	2.95E-01	5.94E-06	7.28E-03	1.73E-02	8.29E-03	1.09E-04	1.44E-02	3.60E-04
Site 3 Dec	1.18E-03	8.69E-06	6.41E-03	2.47E-04	8.23E-03	3.47E-01	5.77E-06	2.38E-02	2.31E-02	9.74E-03	1.12E-04	2.19E-02	3.40E-04
Site 4 Dec	5.54E-05	1.05E-05	3.51E-03	2.21E-04	4.38E-03	2.74E-01	5.92E-06	3.21E-05	1.46E-05	8.33E-03	1.12E-04	3.40E-03	4.00E-04
Site 5 Dec	4.73E-05	1.12E-05	3.33E-03	2.38E-04	4.79E-03	3.16E-01	5.98E-06	3.21E-03	1.54E-03	8.61E-03	1.04E-04	1.73E-02	4.11E-04
Site 6 Dec	4.90E-05	1.07E-05	4.58E-03	2.43E-04	3.83E-03	2.90E-01	5.93E-06	2.33E-05	2.76E-03	8.59E-03	1.14E-04	7.65E-03	4.22E-04
Site 7 Dec	5.39E-05	1.09E-05	7.67E-03	2.10E-04	3.89E-03	2.92E-01	5.96E-06	3.14E-05	2.34E-05	8.36E-03	1.04E-04	3.32E-03	4.05E-04

4.3.2 Bacterial community structure and diversity

454-Pyrosequencing of DNA samples was used to characterise BCC along the lower WFS. A total of 140,454 reads were obtained from the seven sampling sites. Following quality filtering and processing, 101,230 reads were used for further analysis. Reads for site 4 (October) were removed from the total dataset before equalising and re-merging the bar-coded files. The number of quality trimmed sequences for this site in October was markedly low and therefore removed to reduce statistical errors and inaccurate representation of the BCC. Overall, a total of 8833 unique OTU's were assigned to a class at a confidence threshold of 97%. Of the 8833 OTU's, 25.77% were identified up to genus level. OTU's ranged from 846 – 1587 per sample. On average, the highest number of OTU's was associated with site 5 (1416 OTU's), while site 7 showed the lowest number of OTU's (985 OTU's).

Alpha diversity was calculated at 97% similarity level using the Chao1 richness estimator, and Simpson reciprocal diversity index (Figure 4-2A and B). Overall, Chao1 estimator and Simpson index revealed that site 5 consisted of the highest species richness, diversity and evenness. The lowest average bacterial diversity and evenness were predicted for site 7. The number of OTU's determined by Chao 1 showed that 55.47 to 76.53% of the estimated taxonomic richness was recovered by the sampling effort. Rarefaction analysis was used to determine whether sampling depth was sufficient to accurately characterize the BCC. None of the rarefaction curves reached saturation at a 97% similarity level, indicating that we did not survey the full extent of taxonomic diversity (Figure 4-3). However, Chao1 estimated that a substantial fraction of BCC was assessed at genus level by the sampling effort.

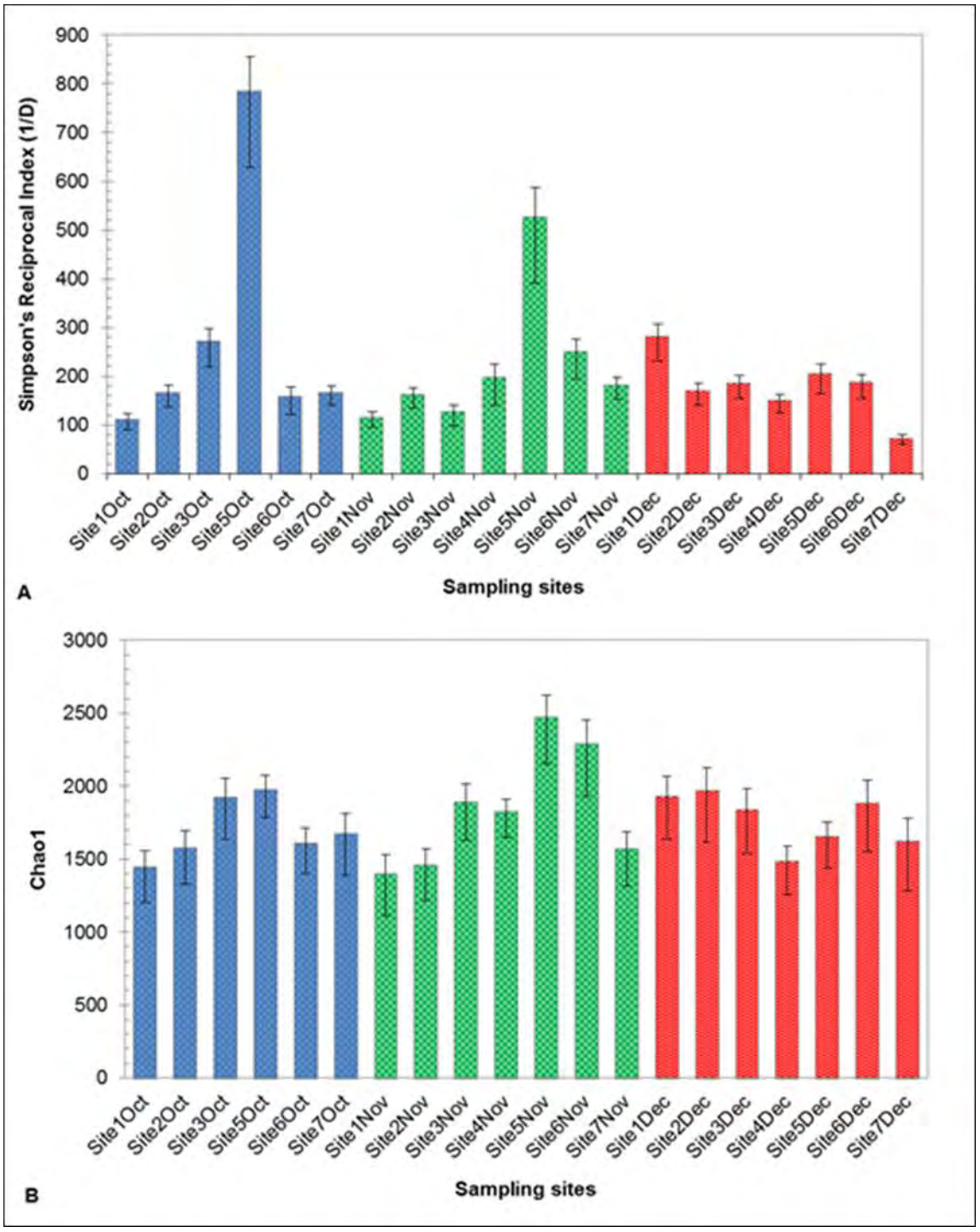

Figure 4-2: Bacterial alpha diversity estimates at all sampling sites (October to November) based on 454-pyrosequencing reads. Data sets were normalised to the same number of reads (3703 reads) before calculations. **(A)** Simpson's Reciprocal Index (1/D); and **(B)** Chao 1 richness estimations. Both diversity indices were calculated at 97% similarity level.

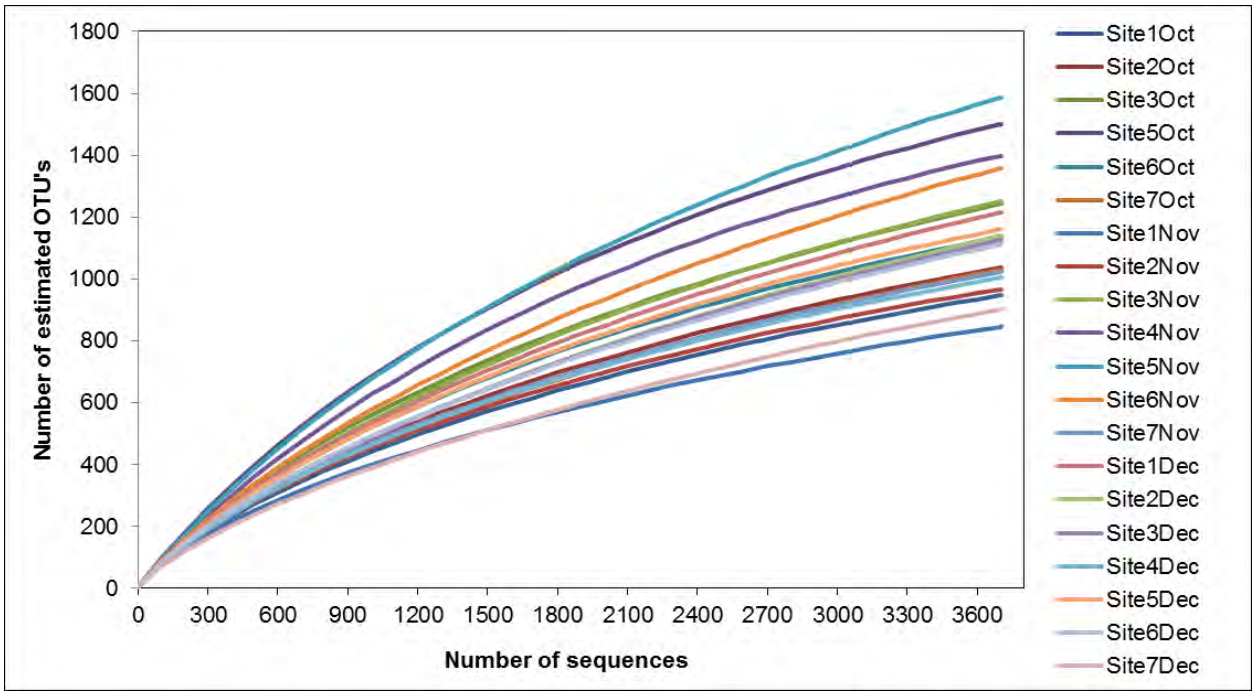

Figure 4-3: Rarefaction curves for all samples estimating the number of bacterial OTU's at 97% similarity level. None of the rarefaction curves reached saturation at this similarity level.

Beta diversity, based on phylum level, is visualized with a NMDS graph (Figure 4-4) and Bray-Curtis dissimilarity dendrogram (Figure 4-5). NMDS analysis indicated differences in BCC among sites as depicted by the formation of three distinct clusters. This observation was supported by the Bray-Curtis dissimilarity dendrogram. The latter showed relatedness between cluster I and II. Within each cluster the highest similarity in BCC was found among samples collected at the same site over the three month sampling period. For example, in cluster I the BCC for site 1 in October, November, and December appeared to be homogenous according to the Bray-Curtis dendrogram. Likewise, bacterial communities for site 2 (cluster II) and site 6 (cluster III) appeared to share similar phylum-level diversity, respectively.

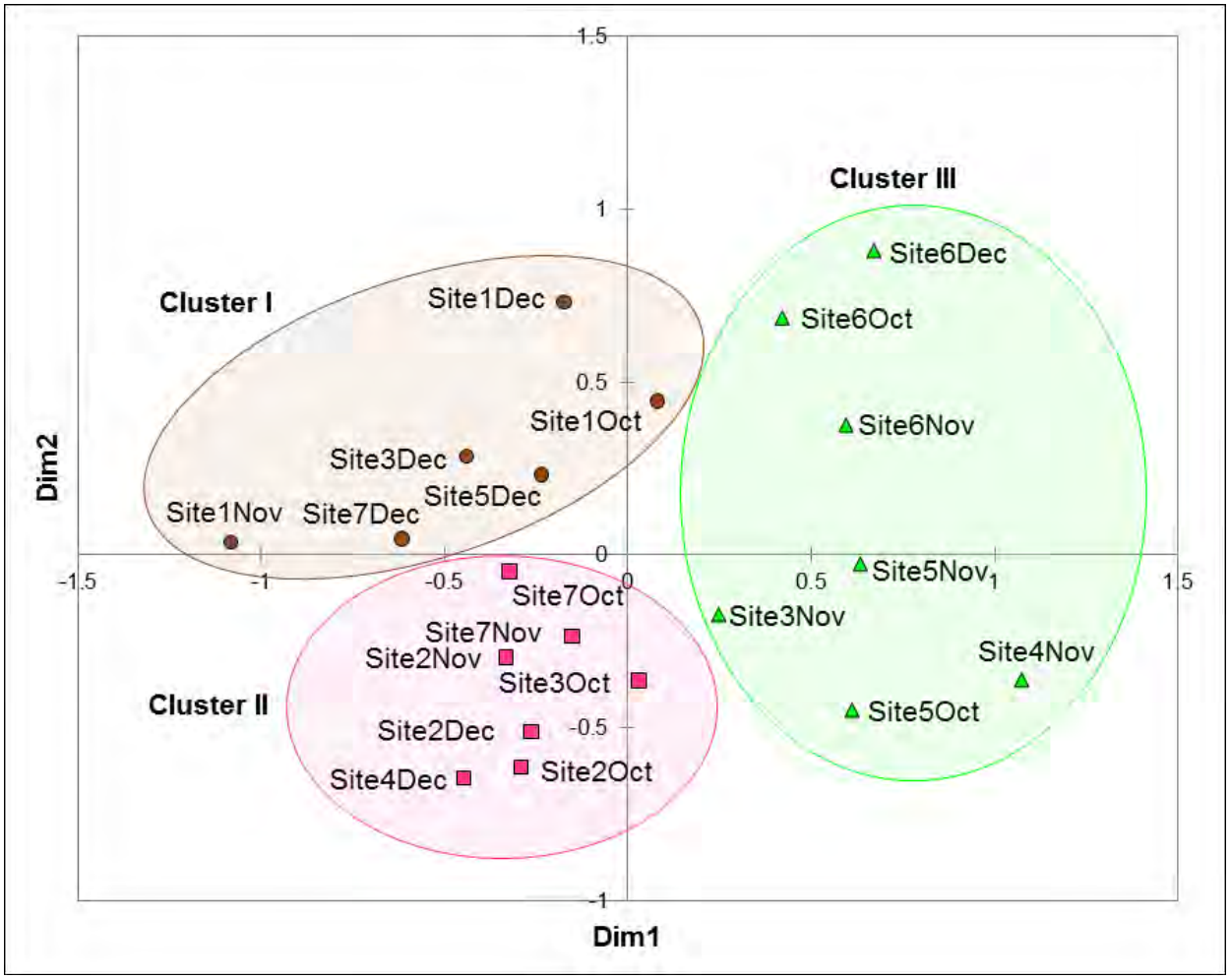

Figure 4-4: NMDS ordination plot based on Bray-Curtis distance matrices for bacterial communities from the studied sampling sites. Ordination grouped samples into three clusters. Cluster I is represented by dark red dots, Cluster II is indicated by pink regtangles, and Cluster III is symbolised by green triangles.

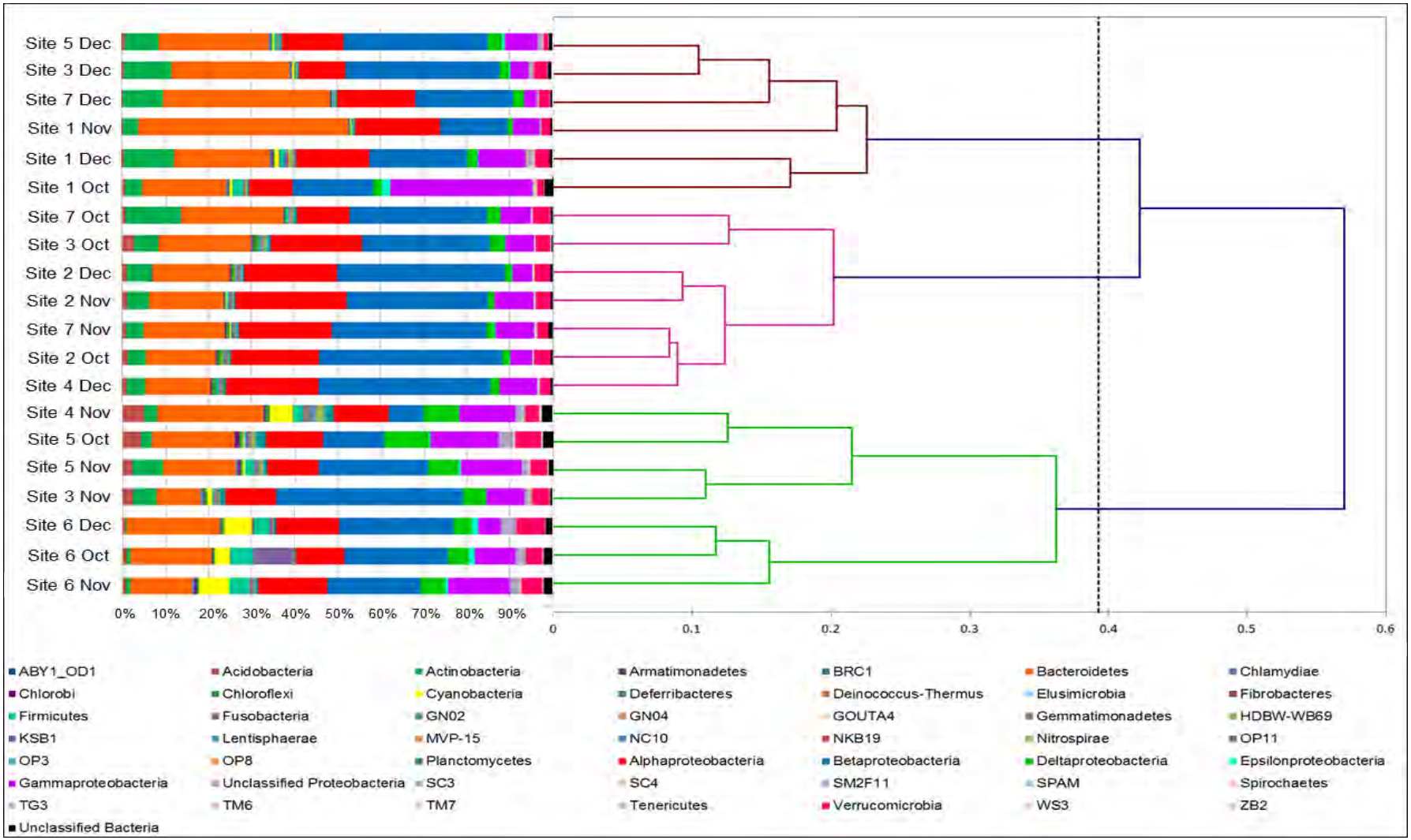

Figure 4-5: Bray-Curtis dissimilarity dendrogram of phylogenetic groups according to their relative abundances recorded at all sampling sites and intervals.

Sequence libraries indicated that the lower WFS consisted of 45 phyla (Supplementary material, Table 4-1S) (Figure 4-5) and 637 genera (Supplementary material, Table 4-1S). Overall, *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Firmicutes*, *Proteobacteria*, and *Verrucomicrobia* were the dominant phyla, contributing > 1% to the total community composition. Sequence libraries also contained a large proportion (> 1% of the BCC) of unclassified bacteria. The *Proteobacteria* were distributed between sites (in order of abundance) as *Betaproteobacteria*, *Alphaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria*, and *Epsiolonproteobacteria*. Genera that represented > 1% of the total BCC included *Aeromonas*, *Algoriphagus*, *Arcicella*, *Brevundimonas*, *Flavobacterium*, *Fluviicola*, *Hydrogenophaga*, *Limnohabitans*, *Polynucleobacter*, *Pseudomonas*, *Rhodobacter*, *Rhodoferax*, *Sediminibacterium*, and *Sphingopyxis*.

Following an extensive literature search, approximately 36% of the total bacterial sequences could be matched to genera with known ecology, inorganic nutrient cycling and heavy metal tolerance. We paid special attention to the nitrogen, sulphur, and phosphorus cycles, as well as heavy metal cycling. On average, 45% of species was potentially involved in the nitrogen cycle followed by sulphur (36%), phosphorus (30%), and heavy metals (28%). The percentage of bacteria involved in the nitrogen cycle was, on average, greatest at site 1 and 7 (Figure 4-6A1). Species abundance for the October and November samples gradually decreased from the upstream to downstream sites (site 1 to 5) but started to increase from site 6 to 7. In contrast, the December samples showed a different trend line where species abundance increased from site 1 to 4 followed by a decline to site 7. Nitrate reducers were the main group that participated in the nitrogen cycle and were most strongly represented at site 1 (Figure 4-6A2). Genera that contributed > 1% of the total bacterial community included *Flavobacterium*, *Rhodobacter*, *Aeromonas*, *Pseudomonas*, *Algoriphagus*, *Sphingopyxis*, and *Hydrogenophaga*. The denitrification profiles were similar to nitrate reduction given that many of the nitrate reducers can denitrify nitrite to nitrogen gas/ammonium depending on the species. The majority of denitrifiers were detected at site 1, 4, and 7. Dominant genera included *Flavobacterium*, *Rhodobacter*, *Hydrogenophaga*, and *Pseudomonas*. The nitrogen fixers had a less pronounced impact on the nitrogen cycle, but we observed a sharp increase in species abundance at site 6 (October, November, and

December). *Rhodoferax* was the main nitrogen fixer and was most strongly represented at site 6. Other possible nitrogen fixers that were present in smaller proportions include *Microleus*, *Bradyrhizobium*, *Rubrivivax*, and *Cellvibrio*.

Nearly 180 bacterial genera possibly contributed to sulphur cycling in the lower WFS by reducing sulphate and/or oxidizing reduced sulphur compounds (e.g., sulphide and thiosulphate). Most of the sulphur bacteria belonged to the *Bacteroidetes* and *Alpha*- and *Betaproteobacteria* groups. *Flavobacterium*, *Sediminibacterium*, *Aeromonas*, *Rhodoferax*, and *Algoriphagus* were the most common sulphate reducers identified. Genera associated with the oxidation of reduced sulphur compounds included *Limnohabitans*, *Rhodobacter*, *Polynucleobacter*, and *Hydrogenophaga*. Species abundance profiles for the sulphur cycle corresponded well with both the nitrogen and phosphorus cycles (Figure 4-6B1). Species abundance for the October and November samples decreased from site 1 to 5 followed by an increase towards site 7. On the other hand, species abundance for the December samples increased from site 1 to 5 and then decreased to site 7. Both sulphur reducers and oxidizers participated greatly in the sulphur cycle although the oxidizers showed to be more prominent throughout the river (Figure 4-6B2). We observed an inverse relationship in the abundance between the reducers and oxidizers (i.e., when the reducers dominated the oxidizers were less prominent and vice versa).

Many bacteria involved in the nitrogen cycle can also mineralize organic phosphate by phosphatase enzymes. Of the 340 genera that contributed to the nitrogen cycle, over half (53%) produced phosphatase enzymes that are responsible for dephosphorylation of organic compounds. Genera that contributed > 1% of bacterial community included *Flavobacterium*, *Arcicella*, *Sediminibacterium*, *Rhodoferax*, *Pseudomonas*, *Algoriphagus*, *Sphingopyxis*, *Hydrogenophaga*, and *Brevundimonas*. With a few exceptions, species abundance profiles for the October, November and December samples were relatively similar to that of the nitrogen and sulphur cycles (Figure 4-6C). We found that phosphatase positive bacteria were generally higher at site 1 and 7 (October, November and December) suggesting nutrient inputs from external sources.

Interestingly, heavy metal tolerant bacteria accounted for ~ 27 % of the total bacterial community at all three sampling intervals. Species abundance profiles for the heavy metals were slightly different from the inorganic nutrient cycles (Figure 4-6D). The maximum abundance of metal tolerant bacteria was represented at site 1 (October, November and December). Species abundance decreased from site 1 to 3 for all three sampling periods. The October samples showed a further decrease in abundance from site 3 to 5, while species abundance for the November and December samples increased from site 3 to 7 and site 3 to 6, respectively. Unexpectedly, the abundance of metal tolerant bacteria for the October and November samples decreased from site 6 to 7. The majority of heavy metal tolerant bacteria were classified as *Alpha*-, *Beta*-, and *Gammaproteobacteria*. Genera that were present in relatively large sequence proportions included *Rhodobacter*, *Flavobacterium*, *Aeromonas*, *Rhodoferax*, Pseudomonas, *Sphingopyxis*, *Hydrogenophaga*, and *Brevundimonas*. Strong evidence was found in the literature that most of these genera can reduce/oxidize, tolerate or absorb more than one metal. Our results suggest that arsenic, cadmium, cobalt, copper, and iron were the main metals recycled in the WFS.

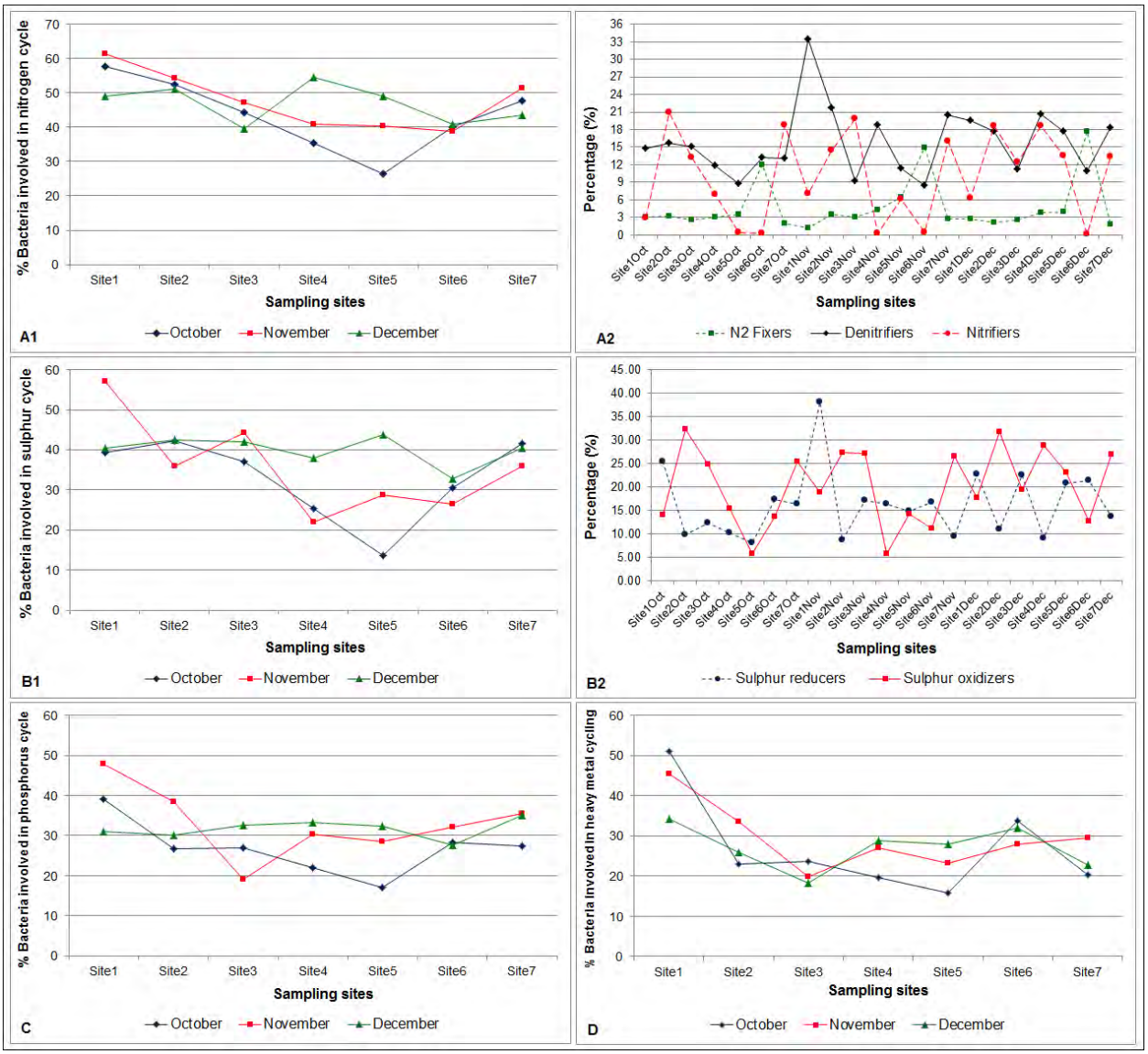

Figure 4-6: Profiles of sequence counts of taxa known to be capable of major biogeochemical cycles in the WFS. **(A1 & 2)** Relative abundances of taxa involved in nitrogen cycling including the nitrogen fixers, denitrifiers, and nitrifiers; **(B1 & 2)** relative abundances of taxa involved in sulphur cycling including the sulphur reducers and oxidizers; **(C)** proportion of taxa involved in the phosphorus cycle; and **(D)** relative abundances of taxa that are resistant to or able to transform the heavy metals measured.

An important finding was the detection of pathogens and opportunistic pathogens in the lower WFS (Figure 4-7). A total of 81 genera were identified of which 84% were isolated from site 1, 2, 4, and 7. Eighteen potential pathogens were confirmed by Bergey's Manual of Systematic Bacteriology and scientific papers (Supplementary material, Table 4-2S). Major potential pathogens included *Aeromonas* (both pathogenic and opportunistic pathogenic), *Bordetella*, *Bacteroides*, and *Clostridium*. Most of the potential pathogens were recovered from site 1, 2, and 6. Dominant genera for the opportunistic pathogens included *Aeromonas*, *Pseudomonas*, *Brevundimonas*, *Ralstonia*, *Acinetobacter*, and *Roseomonas*. *Aeromonas* and *Acinetobacter* were mainly isolated from site 1, while a large percentage of *Pseudomonas*, *Brevundimonas*, *Ralstonia* and *Roseomonas* were recovered from site 2, 4, and 7.

Previous studies demonstrated that many of the potential pathogens and opportunistic pathogens tolerate heavy metals, absorb metals, or even have the ability to cycle metals for energy production (Akob *et al*., 2008; Kim *et al*., 2009; Irawati *et al*., 2012; Pal and Paknikar, 2012; Lovley, 2013). Fifty one percent of the identified potential pathogens and opportunistic pathogens possibly participated in chromium, copper, and iron cycling (Figure 4-8). Furthermore, 17 to 49% of species were associated with cadmium, lead, arsenic, nickel, zinc, cobalt, uranium, mercury, manganese, and selenium. Figure 4-9 shows the abundance and distribution profiles of potential pathogens and opportunistic pathogens capable of recycling heavy metals. For the October and November samples, species abundance decreased from site 1 to 4, but subsequently increased from site 5 to 7. In contrast, the December samples showed an increase in species abundance from site 1 to 4 followed by a decrease to site 7. *Brevundimonas*, *Clostridium*, and *Legionella* were the main genera identified and were strongly represented at site 1, 2, 6, and 7. Less abundant genera with metal remediation properties, and mainly detected at site 1, 2, 6, and 7, included *Escherichia/Shigella*, *Laribacter*, *Serratia*, *Staphylococcus*, *Streptococcus*, and *Stenotrophomonas*. Heavy metal levels were overall higher at site 1 and 2 during the study period. These observations may indicate a positive association between the abundance of pathogens and opportunistic pathogens, and heavy metal cycling in the lower WFS.

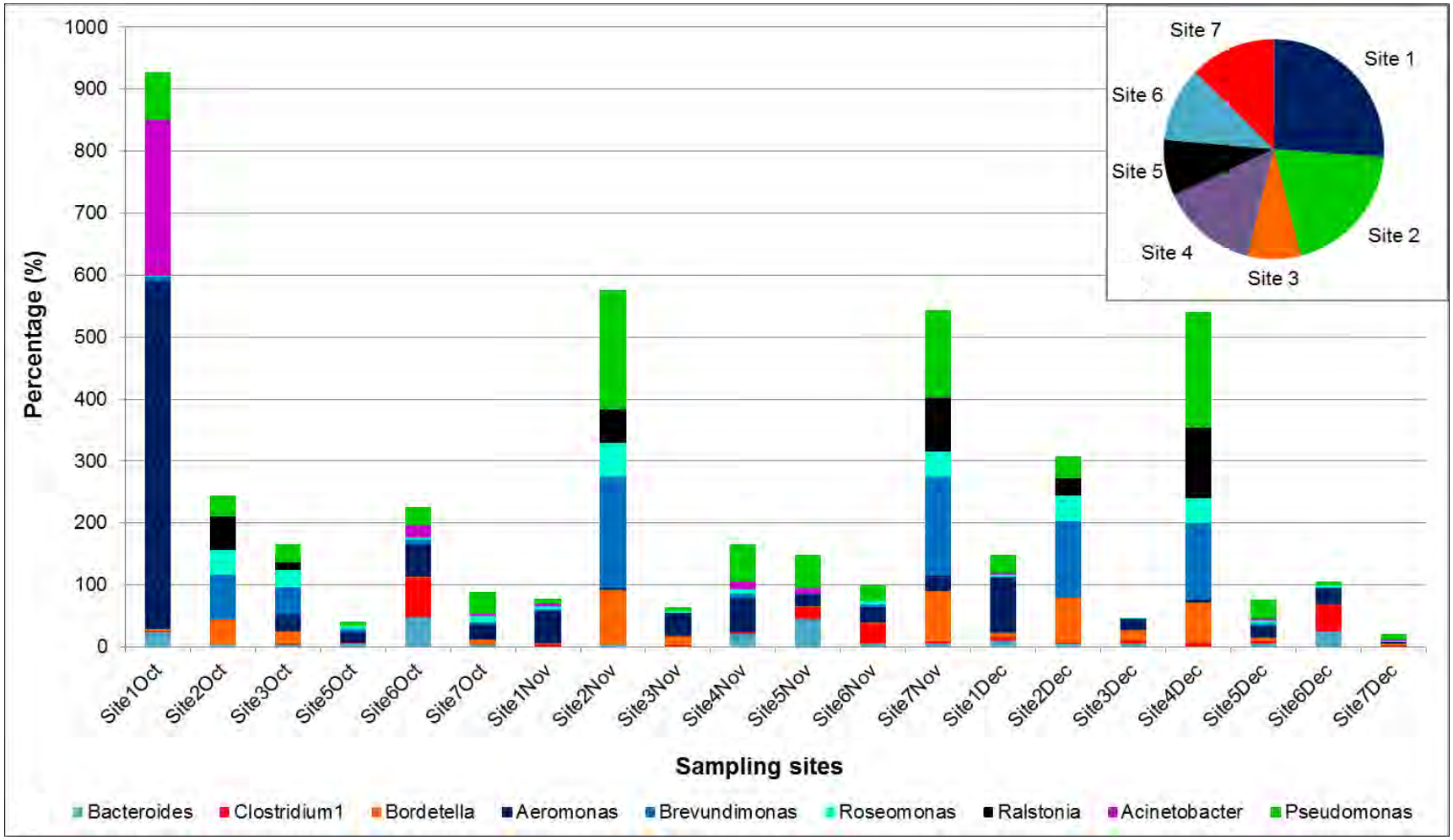

Figure 4-7: Relative abundances of the dominant potential pathogens detected at each sampling site and interval. A large proportion of pathogens were detected at site 1, 2, 4 and 7**.**

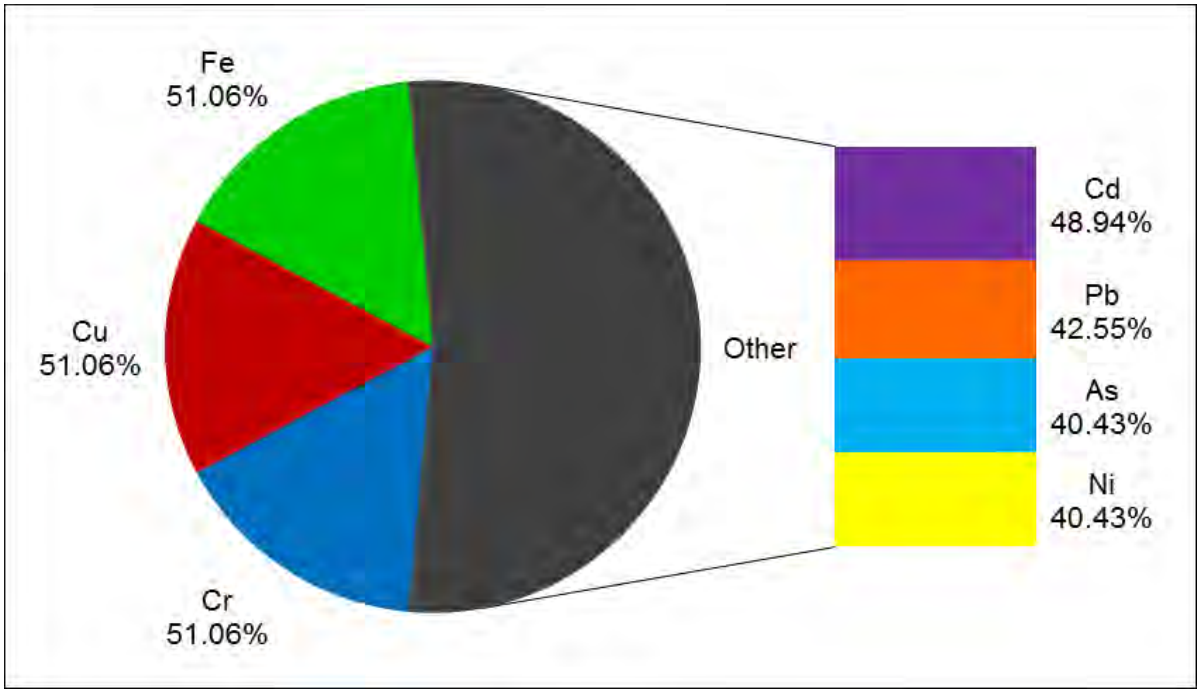

Figure 4-8: Relative abundances of bacterial taxa resistant to or involved with the transformation of heavy metals measured.

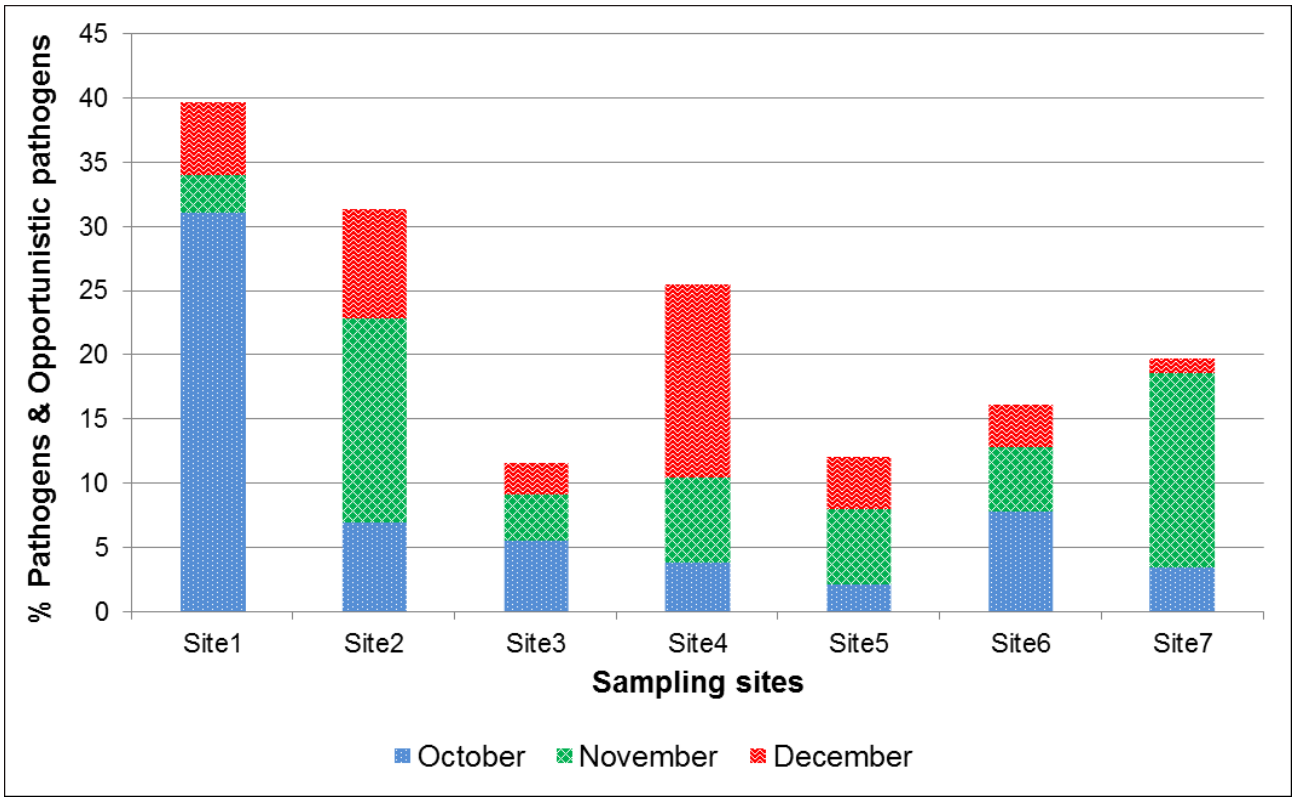

Figure 4-9: Relative abundances and distribution of obligate and opportunistic pathogens that are resistant to or capable of transforming the heavy metals measured.

4.3.3 Associations between physico-chemical water characteristics, trace metals and BCC

Multivariate analysis was performed on pyrosequences to evaluate the impact of environmental variables on BCC. Spearman rank coefficient between taxonomic and environmental data was calculated prior to multivariate analysis. Correlation and analysis methods were then selected for each data set to best present the relationship between taxa and environmental parameters.

PCA (Principal Component Analysis) biplot for the dominant taxa (> 1% of the total BCC) showed a strong association between relative taxa abundances and environmental variables (Figure 4-10). The first two axes explained 48.30% of the total variance with 29.36% by the first axis and 18.94% by the second axis. Temperature, sulphate, nitrate, and chromium correlated with the first axis, while iron correlated with the second axis. The results demonstrated that sulphate, nickel, and cobalt had the biggest impact on BCC. Examining individual taxa, the relative abundances of *Acidobacteria*, *Deltaproteobacteria*, *Gammaproteobacteria*, *Chlorobi*, *Planctomycetes*, and *Deferribacteres* negatively correlated with sulphate levels ($p < 0.05$). *Deltaproteobacteria*, *Verrucomicrobia*, and *Fibrobacteres* showed significant negative associations with nickel concentrations. In addition, cobalt adversely impacted the relative abundance of *Deltaproteobacteria*. Our results further demonstrated significant negative correlations between: (i) *Fibrobacteres* with pH and iron; (ii) *Lentisphaerae* and pH; and (iii) *Deltaproteobacteria* with pH, phosphate, aluminium, arsenic, and uranium. Significant positive correlations were found between: (i) *Acidobacteria* with bicarbonate, selenium, and mercury; (ii) *Chlorobi* with bicarbonate and selenium; (iii) *Fibrobacteres* and nitrate; (iv) *Lentisphaerae* and chromium; (v) *Planctomycetes* with bicarbonate, selenium, and zinc; (vi) *Gammaproteobacteria* with bicarbonate and mercury; and (vii) *Firmicutes* and nitrate.

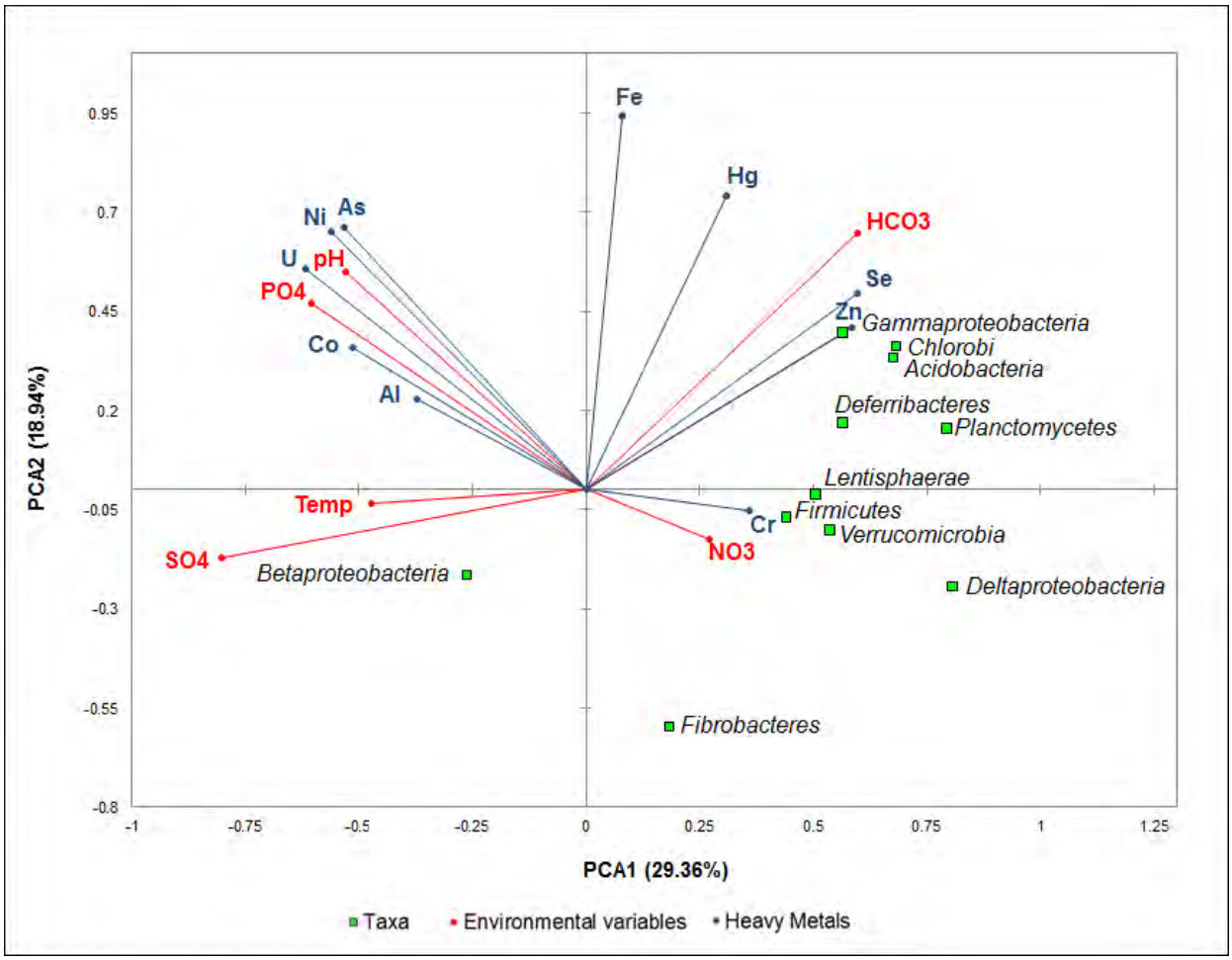

Figure 4-10: PCA for dominant taxa as affected by selected environmental variables. Taxa are indicated by green regtangles, physico-chemical variables are symbolised by red dots, and heavy metals are indicated by blue dots.

Constrained RDA (Redundancy Analysis) was performed at genus level to gain a deeper insight into relationships between the dominant genera (> 1% of the BCC) and environmental variables (Figure 4-11). The first two axes explained 74.44% of the total variance with 56.86% by the first axis and 17.57% by the second axis. Our results indicated that *Algoriphagus* and *Rhodobacter* were the two main genera impacted by environmental variables. Both genera showed significant positive relationships ($p < 0.05$) with pH, phosphate, sulphate, and chloride. In addition, the relative abundance of *Algoriphagus* positively correlated with temperature. The results further demonstrated significant positive associations ($p < 0.05$) between: (i) *Fluviicola* and pH; (ii) *Sediminibacterium* and sulphate; and (iii) *Sphingopyxis* with pH and sulphate. Significant negative correlations were observed between: (i) *Limnohabitans*, *Polynucleobacter* and nitrate; and (ii) *Sediminibacterium* and bicarbonate.

CCA (Canonical Correspondence Analysis) was performed on pathogens and opportunistic pathogens to determine the impacts of heavy metals on their abundances and distribution. Permutation tests indicated that the overall species-metal relationships were statistically significant ($p < 0.05$) (Figure 4-12). The first two axes explained 37.69% of the total variance with 21.61% by the first axis and 16.08% by the second axis. Sixteen percent of species were positively correlated with heavy metals, while 8.64% of species showed significant negative correlations. Important pathogens such *Staphylococcus*, *Streptococcus*, and *Stenotrophomonas* positively related to metals previously shown to be reduced or oxidized by these taxa. The CCA biplot demonstrated positive correlations between: (i) *Staphylococcus* and chromium; (ii) *Streptococcus* with manganese and arsenic; (iii) *Stenotrophomonas* with cadmium, selenium, and zinc; (iv) *Dysgonomonas* with arsenic and cobalt; (v) *Dialister* with cobalt, nickel, arsenic, and uranium; (vi) *Brevundimonas* and mercury; (vii) *Laribacter* with cobalt, nickel, arsenic, and uranium; (vii) *Plesiomonas* with cadmium, selenium, and zinc; (viii) *Coxiella* with cadmium and chromium; and (ix) *Treponema* with chromium (Figure 4-12). Our results further demonstrated significant negative correlations between: (i) *Escherichia/Shigella* with chromium, manganese, and aluminium; (ii) *Corynebacterium* and lead; (iii) *Parachlamydia* and nickel; (iv) *Simkania* and uranium; (v) *Raoultella* and arsenic; (vi) *Legionella* and aluminium; and (vii) *Tatlockia* with cadmium and selenium. The negative correlation between *Escherichia/Shigella* and

chromium is of interest since the taxa have the ability to reduce chromium. Significant correlations determined by Spearman's rank coefficient but not demonstrated by the CCA biplot included positive associations between: (i) *Clostridium* with cadmium and selenium; (ii) *Serratia* with cobalt, nickel, arsenic, and uranium; and (iii) *Bulleidia* and selenium. Lastly, *Fusobacterium* negatively correlated with arsenic.

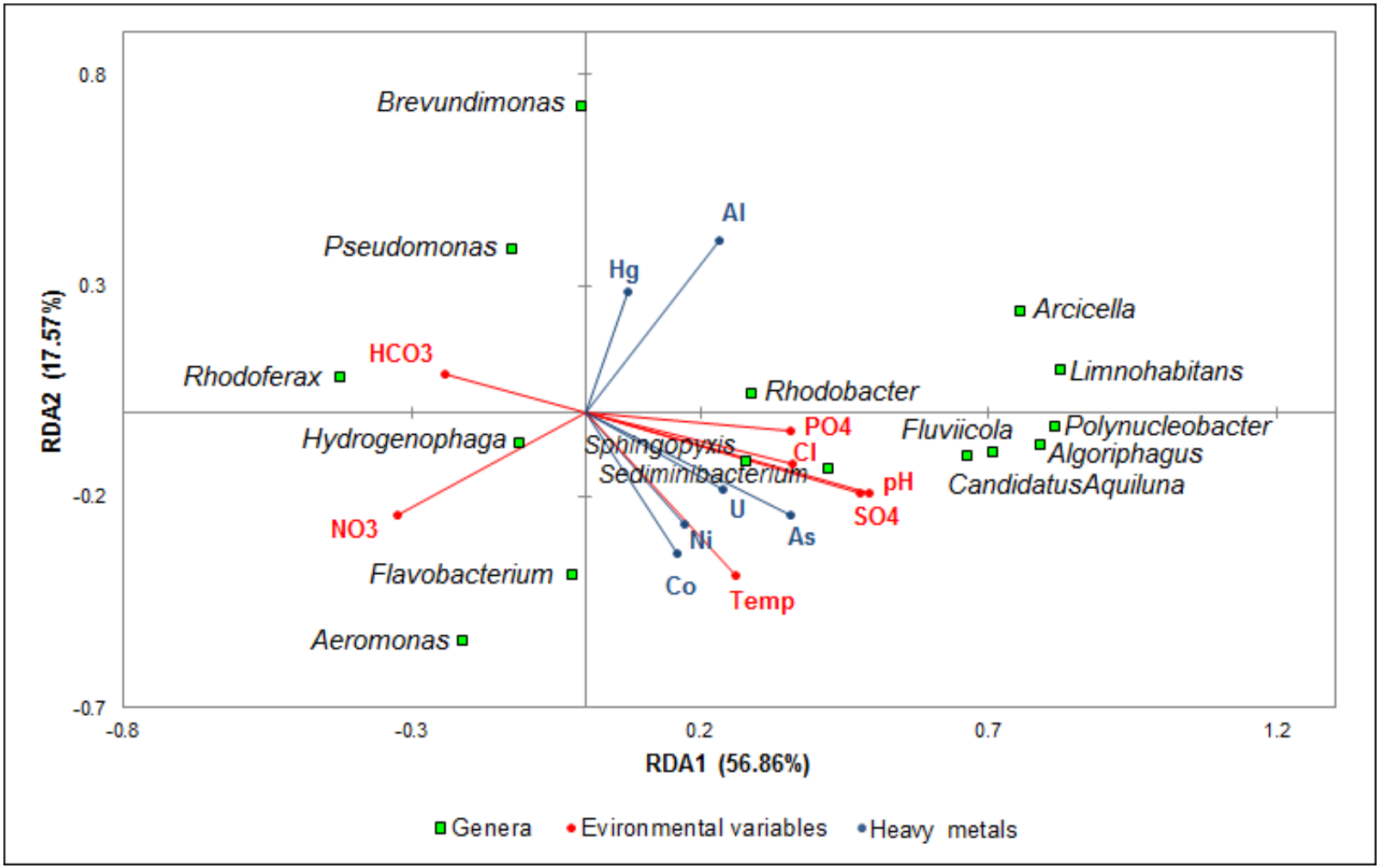

Figure 4-11: RDA biplot of dominant genera as affected by selected environmental variables. Genera are indicated by green regtangles, physico-chemical variables are represented by red dots, and heavy metals are symbolised by blue dots.

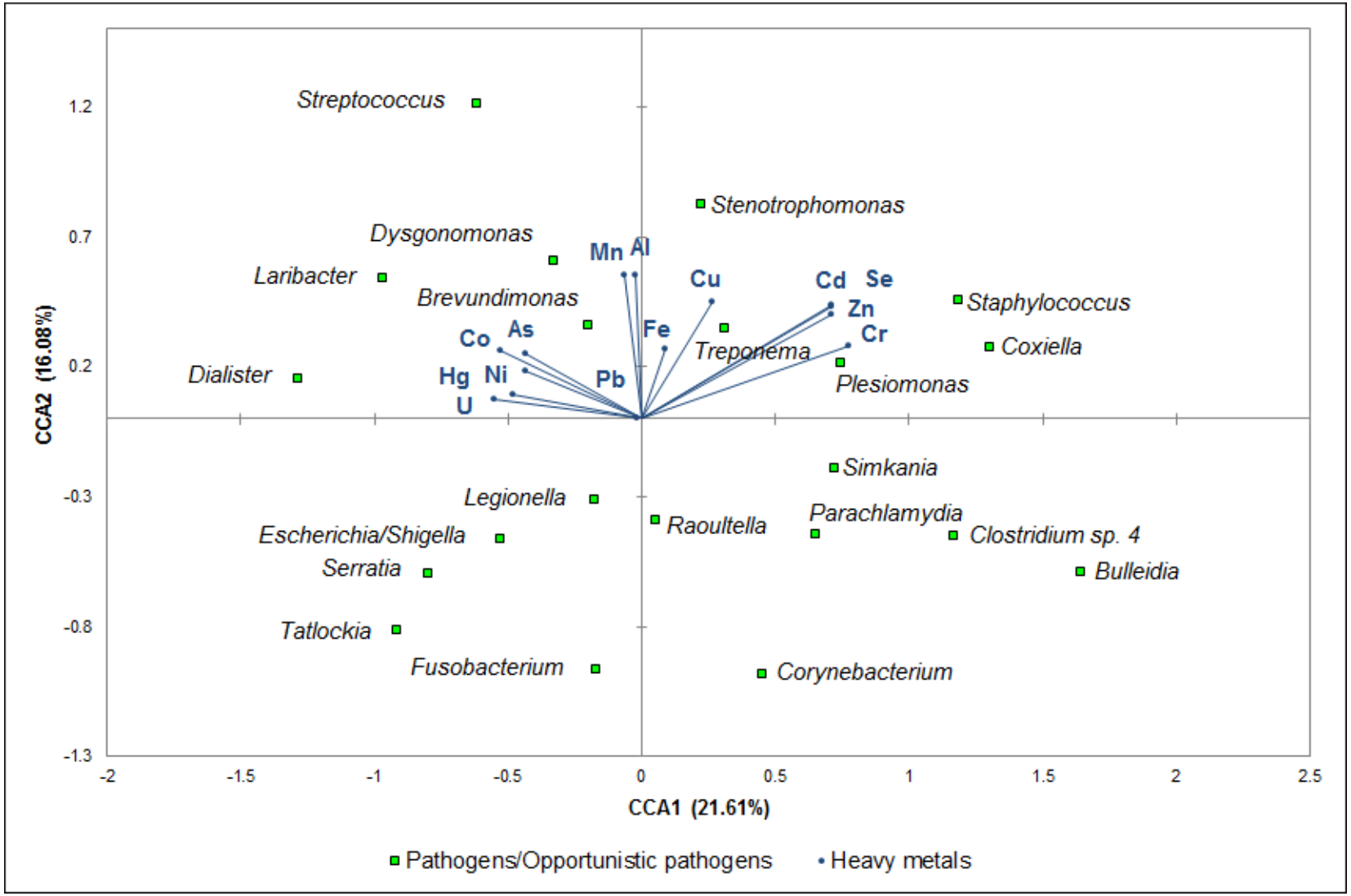

Figure 4-12: CCA biplot of potential pathogens as affected by selected heavy metals. Genera are indicated by green regtangles and heavy metals are represented by blue dots.

4.4 Discussion

The aims of this study were to determine: (i) BCC in the lower WFS; (ii) the impacts of anthropogenic activities on bacterial communities; and (iii) specific links between environmental drivers and individual taxa. As expected, pollutant levels varied along the longitudinal profile of the river and reflected point and non-point pollutions sources. According to combined environmental and pyrosequencing data, sampling sites 1, 2, 6, and 7 had the greatest contamination loads. These sites are located at gold mines, urban and rural communities, and agricultural practices. Gold mining likely attributed to heavy metals in the WFS, especially to high iron levels. Some of the major heavy metals associated with gold mining include arsenic, aluminium, cadmium, chromium, copper, iron, manganese, lead, uranium, and zinc (Boamponsem *et al.,* 2010; Abdul-Wahab and Marikar, 2012; Thorslund *et al.,* 2012; Cobbina *et al.,* 2013). Furthermore, bacterial pollution from urban and rural communities, and livestock farming was evident in the diversity and abundance of genera associated with human stool, animal faeces (particularly swine and cattle), and domestic WWTP's (e.g., *Corynbacterium*, *Microbacterium*, *Bacteroides*, *Clostridium*, *Aeromonas*, *Enterobacter*, *Escherichia/Shigella*). Lowest pollutant levels were found at site 4 and 5, which are situated in dry land agricultural farms. Nitrate levels were consistently higher at these sites. The former may be attributed to artificial fertilizer and/or animal manure which are regularly used by farmers, especially during the planting season (September to December). Our results offer vital evidence that mining, rural and urban inputs, and agricultural activities negatively impacted the water quality in the WFS. Continuous monitoring studies are however needed since samples were collected over a three month period. This may create a more comprehensive representation of the impacts of environmental disturbances on water quality and bacterial communities in the Wonderfonteinspruit.

Bacterial diversity in the WFS affiliated to 45 phyla and 620 known genera. Compared to microbial diversity studies from other anthropogenic polluted rivers and streams (Rastogi *et al.,* 2011; Bai *et al.,* 2014; Ibekwe *et al.,* 2013), our results showed substantial greater diversity even at the genus level. This was evident in that earlier studies found bacterial communities that matched 6 – 40 taxa (Rastogi *et al.,* 2011; Bai *et al.,* 2014; Ibekwe *et al.,* 2013). Major taxonomic groups varied slightly between the

upstream to downstream sites. *Alpha*- and *Betaproteobacteria* were the major taxonomic groups detected at the upstream sites, while bacterial communities at the downstream sites were dominated by *Cyanobacteria*, *Firmicutes*, *Deltaproteobacteria*, and *Verrucomicrobia*. The *Actinobacteria*, *Bacteroidetes* and *Gammaproteobacteria* were found in high abundance throughout the river.

Alpha diversity at the upstream sites was lower compared to the downstream sites. Bacterial communities at the upstream sites were dominated by eight genera (*Aeromonas*, *Algoriphagus*, *Brevundimonas*, *Flavobacterium*, *Limnohabitans*, *Pseudomonas*, *Rhodobacter*, and *Sphingopyxis*) and accounted for ~ 25% of BCC. Twenty three percent of the upstream communities consisted of an additional 412 genera. At the downstream sites *Arcicella*, *Fluviicola*, *Hydrogenophaga*, and *Rhodoferax* were most frequently detected and accounted for ~ 7% of BCC. Thirty one percent of the communities consisted of another 522 genera. The difference in diversity may be explained by: (i) sigmoid models of continuous growth; (ii) antagonism; and (iii) isolation source. Some environments have low bacterial diversities and are dominated by taxa that are adapted for high growth rates and rapid colonization (*r*-strategists), while other environments have high bacterial diversities and are dominated by taxa that are adapted to highly competitive conditions (*K*-strategists). For example, unstable environments with more space and/or nutrient availability create conditions where *r*-strategists grow rapidly and exploit growth opportunities to give them a competitive advantage. In contrast, *K*-strategists are adapted to stable environments with less space and/or nutrient availability, where high bacterial diversity develops and rapid growth is not an advantage (Andrews, 1992). Several bacterial species exhibit antagonistic behaviour to prevent other organisms from utilizing nutrients by excreting antibiotics, bacteriocin-like substances or metabolic end products (Barton and Northup, 2011). These detrimental products exclude bacteria of similar physiological and nutritional activities by inhibiting growth or killing bacterial cells (Barton and Northup, 2011). Antagonistic activities have been demonstrated for *Aeromonas* (Moro *et al.,* 1997; Gibson, 1999; Messi *et al.,* 2003), *Flavobacterium* (Jayanth *et al.,* 2002), *Pseudomonas* (Parret and De Mot, 2002; Vijayan *et al.,* 2006), and *Rhodobacter* (Lee *et al.,* 2009a). This factor could have contributed in part to the great abundance of these genera. The majority of dominant genera at the upstream sites are often associated with

polluted and eutrophic freshwater, raw sewage, human stool, and animal faeces (Lunina *et al.,* 2007; Qu *et al.,* 2008; Wu *et al.,* 2012; García-Armisen *et al.,* 2014; Igbinosa, 2014; Lu and Lu, 2014; Youenou *et al.,* 2014). As mentioned earlier, the upstream sites are located at mining sites, urban and rural communities, and livestock farms, and receive inputs from sources such as septic tanks, storm water runoff, sewage treatment plant overflow, and animal manure. It is thus not surprising that *Aeromonas*, *Algoriphagus*, *Brevundimonas*, *Flavobacterium*, *Limnohabitans*, *Pseudomonas*, *Rhodobacter*, and *Sphingopyxis* dominated the environment. In contrast, the downstream sites are situated between dry land and livestock farms, and receive less influx of human wastewater and other pollutants creating a more stable freshwater environment. This was evident in the large diversity of typical freshwater bacterial groups.

Beta diversity patterns suggested that BCC was spatially structured. Changes in the physical and chemical environment are some of the key factors that influence spatial succession of bacterial communities in freshwater habitats. Principal component analysis revealed that BCC was significantly affected by pH, temperature, nitrate, phosphate, sulphate, bicarbonate, and heavy metal levels (Al, As, Co, Cr, Fe, Hg, Ni, Se, Zn, and U). Our results confirm previous findings that demonstrated that pH (Lindström *et al.,* 2005; Yannarell and Triplett, 2005), temperature (Lindström *et al.,* 2005), salinity (Laque *et al.,* 2010; De Figueiredo *et al.,* 2012), organic and inorganic nutrients (Laque *et al.,* 2010; De Figueiredo *et al.,* 2012), dissolved organic carbon (Kirchman *et al.,* 2004; Fujii *et al.,* 2012), and dissolved oxygen (Yan *et al.,* 2008; Meuser *et al.,* 2013) are some of the main factors that shape community compositions over a spatial gradient.

The pH of a freshwater system controls biogeochemical transformations and mediate the availability of ions and heavy metals (Yannarell and Triplett, 2005). Our results found strong negative relationships between *Fibrobacteres*, *Lentisphaerae*, and *Deltaproteobacteria* with pH. It appears that the relative abundances of the taxa increased as pH decreased, and vice versa. Similar correlations between *Deltaproteobacteria* and pH have been demonstrated by Xiong et al. (2012). The authors showed that the relative abundance of *Deltaproteobacteria* decreased as lake

sediment pH increased. However, we were unable to find evidence to support the negative relationship between *Fibrobacteres* and *Lentisphaerae* with pH. A possible explanation may be that pH indirectly affected the abundances of *Fibrobacteres* and *Lentisphaerae* by governing the adsorption of essential nutrients and metals. Low pH increases the mobility and solubility of heavy metals from sediments, whereas alkaline conditions cause precipitation of metal oxides (Calmano *et al.,* 1993; Peng *et al.,* 2009). In addition, many studies have shown that cycling of nutrients, such as phosphate, is pH dependent (Gomez *et al.,* 1999; Jin *et al.,* 2006). The rate of phosphate release from sediments is usually highest at acidic (pH ~ 3) and alkaline conditions (pH 8 – 12), while neutral conditions have low release potential (Jin *et al.,* 2006; Gaoa, 2012). Given that the WFS had neutral to slightly alkaline conditions, we can speculate that: (i) a large fraction of heavy metals were sparingly soluble and rarely absorbed by bacteria; and (ii) low levels of phosphate was released from sediments for uptake and utilization.

In contrast to the negative relationships between pH and taxa as described above, several dominant genera from the *Bacteroidetes* (*Algoriphagus* and *Fluviicola*) and *Alphaproteobacteria* (*Rhodobacter* and *Sphingopyxis*) groups were positively impacted by pH. The optimum growth pH for all four genera generally ranges between 7 and 8 (Liu *et al.,* 2009b; Srinivasan *et al.,* 2010; Nuyanzina-Boldareva *et al.,* 2014). Neutral to slightly alkaline pH was measured throughout the river. To the best of our knowledge, no other studies on BCC in anthropogenic polluted freshwater systems have demonstrated a positive association between these genera and pH.

Temperature and nutrients were two other important drivers of community variation in this study. *Acidobacteria* was the only phylum that significantly correlated (negative) with temperature. The optimum growth temperature for *Acidobacterial* species usually range between 28 and 37°C (Liesack *et al.,* 1994; Coates *et al.,* 1999; Fukunaga *et al.,* 2008; Koch *et al.,* 2008). Water temperature measured throughout the lower WFS ranged between 19 and 26°C. We believe that the lower water temperature inhibited *Acidobacterial* growth to some degree, thus explaining the negative association. Nitrate levels most strongly related to beta diversity patterns. The relative abundances of *Firmicutes* and *Fibrobacteres* showed significant positive associations with elevated nitrate levels. Many species of the *Firmicutes* use nitrate as an electron acceptor during

metabolic processes (Chen *et al.,* 2001; Schwiertz *et al.,* 2002; Baik *et al.,* 2011; Borsodi *et al.,* 2011). The positive association between *Fibrobacteres* and nitrate is not yet completely understood. It was demonstrated that *Fibrobacter*, which is the only genus in the phylum, utilize ammonia as its nitrogen source, but ammonia can also be used for glutamine synthesis (Montgomery *et al.,* 1988).

The *Betaproteobacteria* correlated negatively with nitrate levels. The most abundant *Betaproteobacteria* affiliated to *Limnohabitans* and *Polynucleobacter*, also negatively correlated with nitrate. Draft genomes of *Limnohabitans* strains Rim28 and Rim47 revealed genes that encode for ammonia transporter and ammonia monooxygenase, nitrate reductase, and nitrite reductase (Zeng *et al.,* 2012). Also, free-living *Polynucleobacter* can perform assimilatory nitrate reduction, but do not produce enzymes involved in nitrification, denitrification, or nitrogen fixation (Hahn *et al.,* 2012; Boscaro *et al.,* 2013). Nitrogen cycling is a costly process to microorganisms, therefore expression and activity of enzymes are strictly controlled (Paustian, 2000). In the presence of fixed forms of nitrogen, nitrate, and ammonia, enzyme synthesis is rapidly turned off to preserve energy (Paustian, 2000). We speculate that *Limnohabitans* and *Polynucleobacter* contributed marginally to nitrate reduction given the high nitrate levels, thus explaining the negative correlation.

The alkalinity of most freshwaters is impacted by the presence of carbonates and bicarbonates, and the CO_2–bicarbonate–carbonate equilibrium system acts as the major buffering mechanism (Wetzel, 2001). Hydroxide, borate, silicate, phosphate, and sulphide are usually present in small quantities, but can be major sources of alkalinity in certain inland waters, particularly saline waters (Wetzel, 2001). In addition to the carbonate–bicarbonate buffering mechanism, a number of internal biological reactions such as manganese, iron, nitrate, and sulphate reduction also contribute to alkalinity within freshwater systems (Wetzel, 2001). Contrary to our expectations, our results indicated a significant inverse relationship between sulphate and bicarbonate levels. Also, bicarbonate concentrations showed significant positive correlations with *Acidobacteria*, *Chlorobi*, *Planctomycetes*, and *Gammaproteobacteria*, whereas the taxa negatively associated with sulphate levels. Although our results differ from Van der Heide et al. (2010) that showed a positive relationship between sulphate and

bicarbonate, it can nevertheless be argued that dissimilatory sulphate reduction governed the overall in-river alkalinity and pH. Sulphate levels were at times markedly high and could have acted as a major alkalinity source, especially in view of the high salinity in the river. On the other hand, bicarbonate reactions may have controlled alkalinity in the immediate environment of the taxa explaining the positive relationships.

Multivariate analysis detected numerous significant relationships between the relative abundances of taxa and heavy metals. Our data suggest that the *Acidobacteria*, *Planctomycetes*, *Chlorobi*, *Gammaproteobacteria*, and *Lentisphaerae* were tolerant to selenium, mercury, zinc, and chromium, while the abundances of *Deltaproteobacteria*, *Verrucomicrobia*, and *Fibrobacteres* were negatively impacted by arsenic, cobalt, uranium, nickel, and iron. Members of the former five groups are not known to be tolerant, or involved with biotransformation, of selenium, mercury, zinc, and chromium. Instead, studies have demonstrated their ability to oxidize iron (Heising *et al.,* 1999; Kirchman, 2012) and/or reduce manganese (Lovley, 2013), iron (Lovley, 2013), uranium (Luo *et al.,* 2007), cobalt (Lovley, 2013) etc. Previous studies on metal resistance properties of the *Deltaproteobacteria*, *Verrucomicrobia*, and *Fibrobacteres* revealed that the taxa are tolerant to arsenic, cobalt, uranium, and iron (Tucker *et al.,* 1998; Qi *et al.,* 2005; Azabou *et al.,* 2007; Cai *et al.,* 2013). The reason for the rather contradictory results is not completely clear but may be due to the neutral to slightly alkaline pH. Although the relationships between taxa and metals differ from previous findings (Feris *et al.,* 2004; Ancion *et al.,* 2010; Rastogi *et al.,* 2011; Vishnivetskaya *et al.,* 2011; Fechner *et al.,* 2012), it can nevertheless be argued that heavy metals had a significant impact on BCC.

Considering that *Bacteroidetes* and *Proteobacteria* accounted for 22 – 60 % of BCC, we further examined the impact of heavy metals on the relative abundances of genera in the two groups. Overall, we identified 163 genera that were either positively or negatively associated with heavy metals. The dominant *Proteobacterial* genera, such as *Brevundimonas* and *Rhodobacter*, showed positive correlations with arsenic, cobalt, mercury, nickel, and uranium. Our results are in line with previous findings that demonstrated the ability of the genera to transform or resist a variety of metals including arsenic, cadmium, cobalt, copper, iron, manganese, nickel, lead, and zinc (Seki *et al.,*

1998; Tebo *et al.,* 2005; Giotta *et al.,* 2006; Singh and Gadi, 2012; Lovley, 2013; Pokrovsky *et al.,* 2013; Sarkar *et al.,* 2014). Moreover, the genera are widely distributed in nature and have been found in extreme environments such as ancient gold mines (Drewniak *et al.,* 2008) and heavy metal polluted marine waters (Besaury *et al.,* 2013; 2014). Some of the genera also showed strong associations with metals other than those established in literature (Seki *et al.,* 1998; Tebo *et al.,* 2005; Giotta *et al.,* 2006; Singh and Gadi, 2012; Lovley, 2013; Pokrovsky *et al.,* 2013; Sarkar *et al.,* 2014). For example, *Sphingopyxis* significantly related to arsenic, cobalt, nickel, and uranium. In addition, *Aquamonas* and *Nevskia* positively correlated with lead, and chromium and selenium, respectively. These genera have not been proven to be metal resistant and therefore their putative role in heavy metal cycling is unclear. Within the *Bacteroidetes* group, *Algoriphagus*, *Fluviicola*, and *Sediminibacterium* were most frequently detected and showed metal resistance to arsenic, cobalt, nickel, and uranium, with the exception of *Sediminibacterium*. The latter negatively correlated with mercury suggesting that mercury may be toxic to this taxon. Metal resistance by *Algoriphagus* and *Fluviicola* has not yet been demonstrated; nevertheless we can speculate that the associated metals could have been used in bacterial processes necessary for growth. Some metals, such cobalt, chromium, iron, manganese, and nickel, are required by microorganisms for biochemical reactions, protein structures, cell walls, and osmotic balance (Hughes and Poole, 1989; Poole and Gadd, 1989; Ji and Silver, 1995).

Among the many observations and correlations in this study, perhaps most interesting was the presence of potential pathogens and the relationship between these and heavy metals. A total of 18 obligate and 64 opportunistic pathogens were identified. Of these, *Bacteroides*, *Clostridium*, *Aeromonas*, *Bordetella*, *Brevundimonas*, *Roseomonas*, *Mycobacterium*, *Sphingomonas*, *Ralstonia*, *Arcobacter*, *Legionella*, *Acinetobacter*, and *Pseudomonas* constituted more than 0.1% of BCC. The majority of obligate and opportunistic pathogens were isolated from sites 1 – 4, and 6. Most of the obligate pathogens are associated with diarrhoeal disease and are commonly found in human stool (Watanabe *et al.,* 2010), animal faeces (Wang *et al.,* 1996), raw sewage (Ajamaluddin *et al.,* 2000), and domestic WWTP effluents (Fu *et al.,* 2014). Opportunistic pathogens were detected more frequently in samples and their overall abundance was twofold higher than the obligate pathogens. Common habitats include

soil (Yoo *et al.,* 2007), plants (Banerjee *et al.,* 2010), domestic and industrial WWTP effluents (Fujii *et al.,* 2001; Xin *et al.,* 2008), human stool (Engberg *et al.,* 2000), and drinking water distribution systems (Ribeiro *et al.,* 2014). It must be mentioned that many of the opportunistic pathogens naturally occur in the environment and do not necessarily cause disease or infection. For example, *Clostridium difficile* is a major aetiological agent of diarrhoea and colitis (Vaishnavi, 2010), whereas *Clostridium* clusters *XIVa* and *IV* are commensal *Clostridia* that play a crucial role in human gut homeostasis and provide specific and essential functions (Lopetuso *et al.,* 2013). Therefore the results must be interpreted with caution since genera could not be classified to species level. Findings from this study corroborate with those reported in previous studies that use pyrosequencing to identify pathogens in freshwater systems (Jeong *et al.,* 2011; Ibekwe *et al.,* 2013; Hou *et al.,* 2014). Ibekwe et al. (2013) demonstrated that urban runoff water entering the Santa Ana River had the highest percentage of total potential pathogens followed by agricultural runoff sediment. Similarly, Hou et al. (2014) discovered a large diversity of potential pathogens in two main tributaries of the Jiulong River that are impacted by human activities. In addition, they found a direct link between nutrient concentrations (nitrogen and phosphorus) and the abundance and diversity of potential pathogens.

Significant correlations ($p < 0.05$) between pathogens and nutrients (nitrate, phosphate, and sulphate) were also observed for the WFS. However, results suggest that heavy metals had a bigger impact on the abundance of potential pathogens than nutrients. Approximately 26% of pathogens significantly correlated with one or multiple heavy metals. Of these, only 8% of correlations could be justified by previous studies (Shakoori and Muneer, 2002; Akob *et al.,* 2008; Pages *et al.,* 2008; Kumar *et al.,* 2011; Pal and Paknikar, 2012; Sarma *et al.,* 2013). Important correlations include: (i) *Staphylococcus* and chromium; (ii) *Streptococcus* with manganese and arsenic; (iii) *Stenotrophomonas* with cadmium, selenium, and zinc; (iv) *Clostridium* with cadmium and selenium; and (v) *Serratia* with cobalt, nickel, arsenic, and uranium. Correlations were observed between *Escherichia/Shigella* and aluminium, chromium, and manganese. *Escherichia/Shigella* correlated negatively to all three metals while literature suggests that the genus can reduce arsenic (Pal and Paknikar, 2012) and chromium (Ackerley *et al.,* 2004). Heavy metal tolerance often differs among species of

the same genus, explaining the contradictory findings. Interestingly, almost 20% of total pathogens have been isolated from mining environments. These include *Bacillus* (Dhanjal *et al.,* 2010; Samuel *et al.,* 2013; Bahari *et al.,* 2013), *Pseudomonas* (Choudhary and Sar, 2009; 2011; Xie *et al.,* 2014), *Herbaspirillum* (Williams and Cloete, 2008; Dhal *et al.,* 2011; Govarthanan *et al.,* 2014), *Acidithiobacillus* (Akbar *et al.,* 2005; He *et al.,* 2008; Karnachuk *et al.,* 2009), *Serratia* (Kumar *et al.,* 2011; 2013; Sarma *et al.,* 2013), and *Acinetobacter* (Zhang *et al.,* 2007; Kumar *et al.,* 2013; Feng *et al.,* 2014). Our results imply that the abundances of pathogens are not only governed by nutrients and water chemistry, but a range of other variables including heavy metal composition and concentrations. In addition, obligate pathogens and opportunistic pathogens may be important in heavy metal mobilization in freshwater systems.

In order to construct potential biogeochemical activity profiles across the WFS, we collated sequences matching taxa reported here. However, results need to be interpreted with caution as specified by Comeau et al. (2012). Briefly, (i) extracted DNA contained both metabolic active and inactive cells; and (ii) rRNA gene copy numbers differ among taxa. Profiles suggested that 30, 36, and 45% of bacterial taxa attributed to phosphate, sulphate, and nitrate cycling, respectively.

Considering the high nitrate levels at several of the sampling points, profiles appeared to be coherent with nitrifying and denitrifying taxa described. It should be mentioned that nitrate reducers and complete denitrifiers were treated as one group. Overall, it appeared that the nitrifiers and denitrifiers interacted in close proximity. Principally, nitrification is an important source of nitrate for the denitrification process (Seitzinger, 1988; Ward, 1996), thus explaining the close interaction between nitrifiers and denitrifiers. Another intriguing observation was the spike in nitrogen fixation at site 6. Nitrate reached maximum levels at this site, irrespective of the sampling date. The observation might be explained by the high abundance of *Rhodoferax*, which was the dominant nitrogen fixer at site 6 and accounted ~ 1.4% of the total BCC.

Sulphur oxidation and reduction profiles followed similar patterns to the nitrogen cycle in that the sulphur oxidizers and reducers interacted in close proximity. Dominant sulphur reducers include *Flavobacterium* (Qu *et al.,* 2009), *Sediminibacterium* (Qu and Yuan,

2008), *Algoriphagus* (Liu *et al.,* 2009b), *Polynucleobacter* (Hahn *et al.,* 2010), and *Rhodoferax* (Madigan *et al.,* 2000). Sulphur oxidizers detected in high abundance include *Rhodobacter* (Ramana *et al.,* 2008; Srinivas *et al.,* 2008), *Hydrogenophaga* (Chung *et al.,* 2007), and *Limnohabitans* (Zeng *et al.,* 2012). Little data on sulphate levels and other nutrients in the WFS are available. Previous studies largely focused on the impacts of uranium on the surface water and groundwater of the WFS (Winde, 2010b; 2011; Barthel, 2012). Thus, comparisons with other studies on the WFS are currently not attainable. However, our findings are supported by previous studies that detected matching sulphur reducers (e.g. *Rhodoferax*, *Desulfosporosinus* and *Acidithiobacillus*) and sulphur oxidizers (e.g. *Acidiphilium*, *Thiobacillus* and *Hydrogenophaga*) in mining environments and/or sulphur rich acid mine drainage (Knotek-Smith *et al.,* 2006; Hao *et al.,* 2007; Lee *et al.,* 2009b; Onstott *et al.,* 2009; Hallberg *et al.,* 2010; Bruneel *et al.,* 2011).

Phosphorus profiles remained relatively stable over the course of the study, even though phosphate levels drastically decreased from the upstream to downstream sites. Reasons for this are uncertain, but measurement errors might in part be responsible. We suggest that, in addition to phosphate, total phosphorus, total dissolved phosphorus, and soluble reactive phosphorus should be measured in future studies. We noticed that a large proportion of taxa involved in the phosphorus cycle, also contributed to the nitrogen and sulphur cycles. Genera that markedly impacted all three cycles include *Flavobacterium*, *Algoriphagus*, *Hydrogenophaga*, and *Rhodoferax*. *Flavobacterium*, *Rhodoferax*, and *Hydrogenophaga* are known to inhabit polluted and eutrophic waters where nutrient levels are exceptionally high (Qu *et al.,* 2008; Navarro *et al.,* 2009; Yoon *et al.,* 2009; Tang *et al.,* 2009). In addition, they can degrade a wide variety of aromatic hydrocarbons such as phenolic derivatives and benzene-toluene (Lu *et al.,* 2009; Aburto and Peimbert, 2011; Gan *et al.,* 2012). Their high abundances might be used as potential indicators of anthropogenic disturbances.

4.5 Conclusions

Our study revealed highly diverse bacterial communities in the lower WFS using 454-pyrosequencing. Combining sequencing data and multivariate analysis, we were able to understand the impacts of anthropogenic activities on BCC. Our results suggest that

bacterial diversity and the abundances of taxa were significantly impacted by nitrogen, sulphate, phosphate, and heavy metals. In addition, we were able to identify potential obligate and opportunistic pathogens and their relationship to environmental parameters. Many of the pathogens strongly associated with nitrogen, phosphorus, and heavy metals. Dominant taxa (*Flavobacterium*, *Rhodoferax* and *Hydrogenophaga*) and pathogens have a strong potential to be used as bio-indicators of anthropogenic disturbances in freshwater systems. Also, taxonomic and metabolic profiles presented in this study will serve as a guide for future research on the WFS.

CHAPTER 5: Conclusions and Recommendations

5.1 Conclusions

Increasing socio-economic growth and development of South Africa's freshwater systems require continuous augmentation of water sources to meet the growing water requirements of communities and industries (DWAF, 2009b). Anthropogenic disturbances have caused the water quality of many freshwater systems to drastically deteriorate due to constant disposal of domestic, industrial, and agricultural waste into surface waters. Further deterioration can compromise water quality to such an extent that it will no longer be fit for human and industrial purposes (De Figueiredo *et al.,* 2004). Government agencies and private sectors implemented biomonitoring programmes to determine the health status of many rivers. However, these programmes lack certain elements and need amendment to determine the exact sensitivity and condition of a water body. Physico-chemical data and faecal indicators are not always sufficient to determine the overall water quality of a system (physico-chemical and microbiological) and are unable to track specific pollution sources including agricultural and industrial activities.

In light of these shortcomings, additional methods are required to measure water quality and aquatic health. Assessing bacterial community compositions in surface waters has proven to be an effective method to determine the impact of pollution on the habitat. Bacterial communities form the foundation of biogeochemical cycles in freshwater systems (Bertilsson *et al.,* 2004; Xu, 2006; Lin *et al.,* 2014). They are mainly responsible for degrading organic material, to remineralize nutrients, and energy flux and circulation of material in a system (Bertilsson *et al.,* 2004; Xu, 2006; Lin *et al.,* 2014). Therefore, any changes in nutrient availability will directly impact their composition and energy release into the water column. In addition, the physical environment of bacteria is just as important as nutrients and further shapes community structures. Any alterations in these two components will cause bacterial communities to select for more resistant or contaminant specific species, which in turn affect the overall metabolic processes and functional dynamics of the ecosystem (Ford, 2000; Zarraonaindia *et al.,* 2013). Our understanding of microbial ecology and the response of communities to pollution will

increase exponentially if we are able to determine which environmental factors correlate with changes in BCC.

Several methods are available to study bacterial community composition. However, most promising and effective remain molecular techniques such as PCR-DGGE and 454-pyrosequencing. The latter has become invaluable in the field of microbial ecology and was successfully applied to various disciplines such as soil, WWTP, and marine systems (Roesch *et al.,* 2007; Andersson *et al.,* 2010; Zhang *et al.,* 2012). This technique allows scientist to study bacterial diversity in more detail compared to DGGE. Pyrosequencing offers several advantages over DGGE, (e.g. high number of reads (400, 000), high taxonomic resolution and robust statistical analysis), although the latter is still used in several profiling studies and should not be disregarded altogether.

Considering the challenges that South Africa's freshwater systems face and the need for better water quality and aquatic health, we aimed to develop a robust monitoring technique, using DGGE and pyrosequencing, to determine the impacts of anthropogenic disturbances on bacterial community compositions, and possibly correlate physical and chemical parameters to certain taxa. Three independent studies were conducted in realizing this aim. Study areas include: (i) Vaal River Catchment (Chapter 2); (ii) Mooi River Catchment (Chapter 3); and (iii) Wonderfonteinspruit Catchment (Chapter 4).

Conclusions for each chapter are given below followed by recommendations.

5.1.1 Vaal River Catchment

A pilot study was conducted on the Vaal River system to investigate bacterial structures in a segment of the Vaal River in response to environmental parameters. Bacterial diversity was analysed using both PCR-DGGE and 454-pyrosequencing and correlations between the physical-chemical environment and community structures were assessed by multivariate analysis.

The results demonstrated that: (i) PCR-DGGE and pyrosequencing detected spatial and temporal changes in bacterial community structures; (ii) pyrosequencing produced a

bigger data set and identified additional taxa that were otherwise not detected by DGGE; (iii) dominant taxa identified include *Cyanobacteria*, *Alphaproteobacteria*, *Betaproteobacteria, Gammaproteobacteria, Bacteroidetes* and *Actinobacteria*; (iv) several opportunistic human pathogens of the *Gammaproteobacteria* group were detected at low abundance; (v) multivariate analyses suggest that changes in BCC were largely impacted by pH, temperature, and inorganic nutrients (nitrate, ammonium, chloride, sulphate, and phosphate); and (vi) certain taxa, such as *Cyanophyta*, *Acidobacteria*, *Actinobacteria*, and *Verrucomicrobia*, correlated with environmental parameters which have been shown to impact these groups (Lindström *et al.,* 2005; Sigee, 2005; Gtari *et al.,* 2007; Ward *et al.,* 2009).

This study confirmed the strength and significance of pyrosequencing to assess bacterial communities in surface waters. Similar results have been demonstrated by a number of studies (Ghai *et al.,* 2011; Vishnivetskaya *et al.,* 2011; Crump *et al.,* 2012; Portillo *et al.,* 2012; Bai *et al.,* 2014; Bricheux *et al.,* 2013). The authors showed the importance of pyrosequencing to: (i) establish a diversity framework for future studies in a given environment that would not have been generated by other molecular techniques; (ii) identify factors that control patterns of diversity and how factors interact on different spatial and temporal scales; and (iii) determine the impacts of point and non-point pollution sources on freshwater bacterial community structures. Information about these characteristics on South Africa's freshwater sources is not readily available and little resources are allocated for such studies. In addition, the presence of opportunistic pathogens in surface waters and their impacts on human health is largely overlooked. Pyrosequencing in this study was the first step in providing a preliminary bacterial diversity database on one of South Africa's largest freshwater resources. Most information on bacterial species in South Africa's freshwater resources involves total and faecal coliforms due to human health implications. To date, only one study could be found that provides data on bacterial communities in surface waters (Buffalo River) using cultivation-based and biochemical tests (Zuma, 2010). In another study, Matcher et al. (2011) investigated the bacterial diversity of a freshwater-deprived estuary in the Eastern Cape using 454-pyrosequencing. Metagenomic and multivariate analyses also provided clues about potential biogeochemical roles of cultured and uncultured species, and how the environment and external sources impacted their abundances and

distribution. Given the value and significance of this study, I aimed to expand and improve this database and thus selected pyrosequencing for bacterial analysis on the Mooi River and Wonderfonteinspruit.

5.1.2 Mooi River Catchment

Following the Vaal River study, pyrosequencing and multivariate analysis were used to determine the impacts of urbanization on bacterial communities in the Mooi River Catchment, which is an urban river system near Potchefstroom city.

Physico-chemical and microbiological data indicated nutrient inputs and faecal pollution at sites below the Boskop Dam and this trend continued downstream until the confluence with the Vaal River. Furthermore, BCC at the downstream sites was highly similar, particularly for the July samples. The downstream sites were dominated by *Bacteroidetes*, *Betaproteobacteria, Actinobacteria*, *Alphaproteobacteria*, and *Verrucomicrobia*. Multivariate analyses suggest that the abundances of *Betaproteobacteria*, *Epsilonproteobacteria*, *Acidobacteria*, *Bacteroidetes*, and *Verrucomicrobia* related to temperature, pH, DO, sulphate, and chlorophyll-a levels. Another important finding was the detection of potential pathogens including *Arcobacter*, *Aeromonas*, *Microbacterium*, *Mycobacterium*, *Pseudomonas*, *Roseomonas*, *Orientia*, and *Sphingomonas*.

This study confirms that urbanisation caused the overall water quality of the Mooi River to deteriorate which in turn had a profound impact on BCC. Although some sites received different urban inputs (e.g. agricultural runoff vs. domestic effluent) that differ in physico-chemical properties, it was still evident that they impacted bacterial diversity and species abundance in the river. This was indicated by the differences in phylogenetic structure of bacterial communities between the upstream and downstream sites, as well as biotic homogenisation of the downstream communities. Also, communities at the downstream sites contained more resistant species that have the ability to metabolise pollutants and survive in hostile conditions (Ford, 2000; Zarraonaindia *et al.,* 2013; Lu and Lu, 2014). Results of this study substantiate previous findings in literature that identified biotic homogenisation and similar taxa in river systems that are impacted by urbanisation (Li *et al.,* 2008; Haller *et al.,* 2011; Ibekwe *et*

al., 2012; Drury *et al.,* 2013; Lu and Lu, 2014). Many of these studies showed an increase in abundances of *Betaproteobacteria, Bacteroidetes*, and *Cyanobacteria* at sites disturbed by anthropogenic inputs. In addition, Lu and Lu (2014) detected potential pathogens, including *Aeromonas*, *Arcobacter*, *Clostridium*, *Legionella*, *Leptospira*, *Mycobacterium*, *Pseudomonas*, and *Treponema*, at sites that received rural domestic sewage and effluent from various factories. Likewise, some of these genera (*Acrobacter*, *Aeromonas*, *Microbacterium*, *Mycobacterium*, and *Pseudomonas*) were detected in the Mooi River, particularly at sites near cattle farms and Potchefstroom city. Similar to the Vaal River, results from this study also bring to light significance evidence that physico-chemical perturbations had a strong influence on BCC. In both studies pH, temperature, and inorganic nutrients strongly influenced bacterial communities. Thus, a clear trend between physico-chemical parameters and BCC is emerging, but interactions between environmental variables and bacterial communities need to be elucidated.

5.1.3 Wonderfonteinspruit Catchment

The goal is of this study was to assess additional anthropogenic activities (e.g. mining) on BCC, and to link specific taxa with environmental drivers. This process may identify possible bacterial indicators to predict biogeochemical changes in response to anthropogenic disturbances.

The results provide a comprehensive insight into the phylogenetic structure, species richness, evenness, and abundance of bacterial communities in the WFS. Sequencing data and multivariate analyses demonstrated that changes in physico-chemical water properties, due to environmental disturbances, impacted BCC by changing the phylogenetic structures and species abundances of communities. The results suggest that bacterial diversity and the abundances of taxa were significantly impacted by nitrogen, sulphate, phosphate, and heavy metals. Diversity indices support this proposal given that bacterial diversity at the upstream sites (polluted) was much lower than that of the downstream sites (less polluted). Similar to the Vaal River and Mooi River studies, dominant taxa at polluted sites included *Cyanobacteria*, *Firmicutes*, *Deltaproteobacteria*, and *Verrucomicrobia*. *Bacteroidetes* and *Gammaproteobacteria* were found in high abundance throughout the river. In addition, potential pathogens,

their habitats (e.g. human stool, animal faeces, and raw sewage), and relationships with environmental parameters were identified. Many of the potential pathogens strongly associated with nitrogen, phosphorus, and heavy metals. The results suggest that heavy metals had a bigger impact on the abundances of potential pathogens than nutrients. The presence of potential pathogens at polluted sites suggests that they play important roles in mobilizing several heavy metals and degrading a wide variety of pollutants.

The detection of the dominant genera (*Aeromonas*, *Flavobacterium*, *Limnohabitans*, *Pseudomonas*, *Rhodobacter*, *Sediminibacterium*, *Arcicella*, *Polynucleobacter*, *Curvibacter* and *Sphingopyxis*) at the upstream sites are of great significance since they are often associated with polluted and eutrophic freshwater, raw sewage, human stool, and animal faeces (Godoy *et al.,* 2003; Lunina *et al.,* 2007; Šimek *et al.,* 2008; Qu and Yuan, 2008; Qu *et al.,* 2008; Watanabe *et al.,* 2009; Táncsics *et al.,* 2010; Liu *et al.,* 2013; Lu and Lu, 2014; Youenou *et al.,* 2014). Thus, their presence and significant correlations with inorganic nutrients and heavy metals confirm inputs from various sources such as septic tanks, sewage treatment plant overflow, animal manure, and mining effluent. The dominance of these genera and their strong associations with the environmental parameters allows them to be used as potential indicators of anthropogenic stressors. However, further research is necessary to classify the taxa up to species level to determine the direct link between pollution sources, and even specific pollutants, with individual species and their metabolic processes. Nevertheless, taxonomic and metabolic profiles presented in this study will serve as a reference point for future research in anthropogenic disturbed aquatic areas. Efforts to link inorganic nutrient cycles (i.e., nitrate, sulphate, and phosphate) with matching taxa and their metabolic processes were partially successful. The number of nitrogen and sulphur bacteria corresponded well with nitrate and sulphate levels. However, phosphate levels were much lower than anticipated considering the large proportion of phosphorus taxa present. Several reasons might be responsible for this discrepancy as discussed in Chapter 4. Even so, this study is the first step towards enhancing our understanding of biogeochemical cycling in the WFS by microbes.

Finally, assessing changes in BCC in freshwater systems is a promising technique to: (i) determine microbiological water quality in combination with faecal indicators; (ii) detect and identify pollution sources; (iii) link pollutants with specific taxa; and (iv) determine how pollutants affect biogeochemical cycles of microbes. Furthermore, our research might have important implications for: (i) improving the River Health Programme (South Africa) by including 454-pyrosequencing of BCC; (ii) developing management strategies to prevent further pollution; (iii) providing valuable information for effective and reliable bioremediation policies; and (iv) permit possible prediction of changes in bacterial communities on the basis of present knowledge.

5.2 Recommendations

The following recommendations are proposed:

- Future studies should focus on RNA rather than DNA-based analysis. Total RNA reflects predominantly the diversity of metabolically active bacteria since they contain a higher level of intracellular 16S rRNA than resting and/or starved bacterial cells. This provides a more accurate representation of bacterial communities and functionality (Poulsen *et al.,* 1993; Aviv *et al.,* 1996).

- Sample size and frequency for the three studies could have influenced community composition results. For example, bacterial diversity for the Mooi River and WFS indicated contrasting indices patterns at polluted and less polluted sites. Species diversity for the Mooi River was higher at polluted sites, while the WFS showed lower bacterial diversity at disturbed sites. In theory, an increase in environmental pressures will cause bacterial communities to select for species that are capable of adaptive responses and should therefore result in a decrease in bacterial diversity, as is assumed for higher organisms (Atlas *et al.,* 1991). However, this is not always the case for microbial communities. Similarly, Ford (2000) observed an increase in diversity in contaminated water sediments compared to relatively uncontaminated sites**.** The Mooi River and WFS differ in character and the types of input they receive which may explain the different results observed. To reduce such discrepancies additional research on BCC in the two water systems, specific taxa, and their response to anthropogenic inputs should be performed. Future studies could sample

at least once a month for two/three consecutive years. Not only will this improve the understanding of how urbanisation affects BCC and its activities, but it will also provide *priori* knowledge of diversity before and after perturbations at a given site by comparing a perturbed site with pristine controls (Ager *et al.,* 2010). Also, further analysis will permit the detection of functionally interdependent species that can easily adapt to cope with environmental stressors (Laplante and Derome, 2011). Such an approach will help to apprehend the problem of pollution in South Africa's freshwater systems and to guide future planning and decisions on the improvement of surface water quality.

- Future research could assess the use of river biofilms (on natural rocks and stones) and sediment bacteria as indicators of anthropogenic disturbances. The datasets can then be compared with the current dataset to ultimately conclude which type of community (planktonic, biofilm, or sediment) accurately represents the ecological nature of the freshwater system. Several studies have demonstrated the usefulness of both biofilms and sediment bacteria to determine the impact of urbanisation on freshwater systems (Lyautey *et al.,* 2003; Drury *et al.,* 2013; Lin *et al.,* 2014).

- Additional physico-chemical parameters need to be measured in future studies to obtain better water quality data. Physical parameters that should be measured include flow rate, rainfall, light absorption, stratification, and redox reactions. Chemical variables should include salinity, dissolved oxygen, dissolved organic carbon, dissolved organic matter, total dissolved phosphorus, soluble reactive phosphorus, dissolved organic nitrogen, and ammonia/ammonium. Sufficient environmental data is of the utmost importance to correctly link physico-chemical variables with changes in BCC, species abundance, species richness, and biogeochemical cycles. Such efforts will contribute to more accurate assessments of the roles of bacterial communities and functions in purification of polluted freshwaters (Lin *et al.,* 2014). Changes in BCC are not only governed by physico-chemical variables as demonstrated in this project, but a wide variety of parameters such as available light, interactions with phytoplankton, predation by protozoa and invertebrates, and viral infections. It is therefore proposed that future research could investigate the impacts of these factors on BCC independently, but also in relation to

anthropogenic disturbances. Other aspects that may be investigated include interactions between different taxa, bacterial growth and biomass production with respect to pollution, and bioremediation of heavy metals in the WFS by metal resistant bacteria.

- Classification of BCC using pyrosequencing could be supported by other methods to identify metabolic pathways of key taxa. For example, genomes of important taxa may be sequenced with Illumina HiSeq chemistry to identify genes involved in nutrient cycling and metal tolerance. Enzyme assays may also be performed to determine if genes are active or inoperative. This additional information could eventually lead to an understanding of the mechanisms behind changes in BCC and biogeochemical cycles in disturbed water systems.

- The use of specific taxa as bioindicators is a promising technique to quickly detect the presence of contaminants which in turn may be used to provide an overall picture of ecosystem health (Ford, 2000). Future studies could focus on the development and application of such biomarkers. Importantly, data presented in this project can be used as the framework for these studies.

- The most important limitation experienced in this project was the constricted classification of sequences by ribosomal databases. Current ribosomal databases can identify sequences only up to genus level. Genera consist of a variety of species that include commensal taxa naturally found in the environment and even potential pathogens from faecal origin. Classification of sequences up to species level will produce much more accurate correlations between specific species and anthropogenic inputs. Therefore, ribosomal databases need to be expanded and updated to include as many species as possible within the bacterial domain.

- Dominant taxa detected at polluted sites, including *Verrucomicrobia*, *Firmicutes*, and *Cyanobacteria*, deserve to be studied in more detail since information about their roles in polluted freshwater systems is scarce. In addition, statistical correlations between some taxa and environmental variables (e.g. *Verrucomicrobia* and dissolved

oxygen) could be further investigated given that little to no information on their ecological roles is available.

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ANNEXURES

Supplementary Table 2-1S: South African Water Quality Guidelines for water resources and uses.

	South African Water Quality Guidelines				
	Domestic Water Use	**Aquatic Ecosystems**	**Livestock**	**Irrigation**	**Aquaculture**
Temperature (°C)	NA	5.0 – 30.0	NA	NA	12.0 – 32.0[a] 2.0 – 30.0[b]
pH	6.0 – 9.0	6.0 – 8.0	NA	6.5 – 8.4	6.5 – 9.0
TDS (mg/L)	0.0 – 450.0	NA	0.0 – 1000.0[c] 0.0 – 2000.0[d] 0.0 – 3000.0[e]	NA	NA
Conductivity (mS/m)	NA	NA	NA	0.0 – 40.0	NA
NO_3-N (mg/L)	0.0 – 6.0	< 0.5	0.0 – 100.0	0.0 – 5.0	0.0 – 300.0
NH_4-N (mg/L)	0.0 – 1.0	0.0 – 7.0	NA	NA	0.0 – 0.025
PO_4-P (mg/L)	NA	< 5.0	NA	NA	0.0 – 0.1
SO_4-S (mg/L)	0.0 – 200.0	NA	0.0 – 1000.0	NA	NA
Cl_2 (mg/L)	0.0 – 100.0	0.0 – 200.0	0.0 – 1500.0[f] 0.0 – 3000.0[g]	0.0 – 100.0	0.0 – 10.0

[a] Target water quality range for growth of specific fish species
[b] Target water quality range for egg incubation and larval development of specific fish species

[c] Diary, Pigs and Poultry
[d] Cattle and Horses
[e] Sheep
[f] Monogastrics and Poultry
[g] Other livestock

Supplementary Table 3-1S: Recommended Water Quality Objectives (RWQO's) for the Mooi River Catchment.

Variable	Unit	RWQO
pH		8.00
Nitrate (NO_3^-)	mg/L	0.30
Sulphate (SO_4^{2-})	mg/L	75.00
Phosphate (PO_4^{3-})	mg/L	0.40
Chloride (Cl)	mg/L	36.00
TDS	mg/L	370.50
EC	(mS/m)	57.00

Supplementary Table 3-2S: Alignment of bacterial phylotype sequences obtained by cultivation with reference sequences in the NCBI database.

Taxonomic group	Genera	Accession no.	% similarity
Actinobacteria	*Agrococcus* [e]	KC515618	100
	Kocuria [b]	KC515642	100
Bacteroidetes	*Arcicella* [e]	KC515606	98
	Flavobacterium [b, c, e, f, g, l, j]	KC515641, KC515615, KC515608, KC515619, KC515574, KC515581, KC515583, KC515585, KC515586, KC515592, KC515595, KC515596	98–100
	Pedobacter koreensis [d]	KC515616	100
Alphaproteobacteria	*Novosphingobium* [f]	KC515575	99
	Paracoccus [c]	KC515640	99
	Rhizobium [f]	KC515576	99
	Xanthobacteraceae [a]	KC515637	100
Betaproteobacteria	*Curvibacter* [g]	KC515584	100
	Duganella [e]	KC515627	99
	Herbaspirillum [d]	KC515632	99
	Massilia [h]	KC515604	100
	Rhodoferax [d, j]	KC515623, KC515593	100
	Limnohabitans [f]	KC515573	99
	Limnohabitans parvus [j]	KC515594	99

Gammaproteobacteria	*Cellvibrio* [e]	KC515636	99
	Pseudomonas [a, d, i]	KC515622, KC515617, KC515587	99–100
	Rheinheimera [g]	KC515579	99
	Thiocapsa [h]	KC515597	99
	Pseudomonas fluorescens [i]	KC515588, KC515590	99
	Pseudomonas koreensis [g]	KC515582	100
	Pseudomonas putida [g]	KC515577	100
	Pseudomonas rhizosphaerae [d]	KC515634	100
	Rheinheimera soli [h]	KC515602	99
Firmicutes	*Bacillus* [c, e]	KC515626, KC515633	100
	Bacillus safensis [b]	KC515621	100
	Bacillus simplex [b]	KC515610	100
	Paenibacillus brasilensis [c]	KC51563	99
	Paenibacillus polymyxa [c]	KC515631	99

[a] Site 1 June; [b] Site 2 June; [c] Site 3 June; [d] Site 4 June; [e] Site 5 June; [f] Site 1 July; [g] Site 2 July; [h] Site 3 July; [i] Site 4 July; [j] Site 5 July

Supplementary Table 3-3S: Taxanomic groups identified in the Mooi River from 454-pyrosequencing data.

Taxanomic group	Genera	Site 1		Site 2		Site 3		Site 4		Site 5	
		June	July	June	July	June	July	June	July	June	July
Acidobacteria	*Gp 6*	x					x				
Actinobacteria	*Illumatobacter*					x	x			x	
	Cryobacterium	x	x	x	x	x	x	x	x	x	x
	Leifsonia						x			x	
	Microbacterium			x							
	Mycobacterium	x		x		x					
Armatimonadetes	*Armatimonas Gp 1*			x	x	x	x			x	x
Bacteroidetes	*Algoriphagus*	x		x							
	Sedimibacterium		x	x	x	x	x	x	x		x
	Arcicella	x	x	x	x	x	x	x	x	x	x
	Flectobacillus	x	x	x	x	x	x	x		x	x
	Leadbetterella					x		x			
	Meniscus							x			
	Solitalea	x	x	x	x	x	x	x	x	x	x

	Fluviicola		x	x		x			x				
	Wandonia						x						
	Flavobacterium	x	x	x	x	x	x	x	x	x	x		
Chloroflexi							x						
Planctomycetes	*Isosphaera*	x		x	x	x							
	Singulisphaera			x		x							
Alphaproteobacteria	*Brevundimonas*			x	x	x							
	Hyphomonas				x	x	x		x				
	Devosia					x							
	Methylocystis						x						
	Methylosinus						x						
	Rhizobium	x				x							
	Vasilyevaea			x									
	Catellibacterium					x	x					x	
	Gemmobacter			x	x								
	Haematobacter	x	x	x	x	x	x	x	x			x	
	Paracoccus			x									

	Pseudorhodobacter	x				x						
	Rhodobacter	x	x	x	x		x	x				
	Roseomonas				x							
	Orientia			x								
	Porphyrobacter				x							
	Novosphingobium	x		x	x	x						
	Sandarakinorhabdus			x	x	x	x					x
	Sphingomonas			x	x							
	Sphingopyxis	x	x	x	x	x	x		x			x
Betaproteobacteria	*Pigmentiphaga*			x	x	x	x	x		x		
	Polynucleobacter	x	x	x	x	x	x	x	x	x	x	
	Aquabacterium	x		x			x	x	x			x
	Rubrivivax		x									
	Sphaerotilus	x		x		x		x				
	Acidovorax	x	x	x		x	x	x	x			
	Albidiferax				x	x			x			
	Caenimonas			x								

	Curvibacter	x		x	x	x	x			x	x
	Hydrogenophaga	x		x	x	x	x	x	x	x	x
	Limnohabitants	x	x	x	x	x	x	x	x	x	x
	Malikia	x				x		x		x	x
	Polaromonas	x		x	x	x	x	x		x	x
	Pseudorhodoferax			x	x	x		x			
	Rhodoferax										x
	Duganella			x		x					
	Janthinobacterium			x	x	x					
	Undibacterium				x						
	Methylotenera			x		x	x	x	x	x	x
	Deefgea			x	x						
	Dechloromonas	x		x							x
	Georgfuchsia										x
Deltaproteobacteria	*Desulfobulbus*			x			x				
Epsilonproteobacteria	*Arcobacter*					x					
	Sulfuricurvum			x				x			

Gammaproteobacteria	*Aeromonas*				x	x		x	x			
	Haliea				x	x						
	Rheinheimera	x		x	x			x	x	x		
	Pseudomonas	x		x	x	x						x
Verrucomicrobia	*Cerasicoccus*	x	x	x		x	x	x		x	x	
	Spartobacteria					x						
	Luteolibacter		x			x		x				x
	Prosthecobacter				x	x						
	Verrucomicrobium		x									
Cyanobacteria	*GpIIa*	x		x	x	x	x					
	GpXI	x		x		x						
Firmicutes		x		x								x

Supplementary Table 4-1S: Phyla identified in the Wonderfonteinspruit from 454-pyrosequencing data.

Taxonomic group	Genera	October 2012						November 2012							December 2012						
		1	2	3	5	6	7	1	2	3	4	5	6	7	1	2	3	4	5	6	7
Acidobacteria																					
	Edaphobacter										x		x								
	Acanthopleuribacter									x			x				x		x	x	
	Geothrix		x	x		x	x			x			x			x		x			x
	Holophaga					x							x								
	Candidatus Solibacter	x	x	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x	x	x
Actinobacteria																					
	Aciditerrimonas							x	x												
	Iamia		x	x	x		x	x	x	x		x		x	x			x			
	Actinomyces																	x			
	Georgenia												x								
	Actinotalea	x		x	x				x		x		x								
	Cellulomonas										x										
	Demequina				x						x										
	Oerskovia										x										
	Corynebacterium			x	x	x					x	x									
	Fodinicola		x																		

Dietzia				x									x							
Geodermatophilus									x		x		x							
Ornithinicoccus									x											
Ornithinimicrobium									x											
Phycicoccus				x																
Tetrasphaera									x											
Angustibacter									x											
Kineococcus					x															
Kineosporia					x											x		x		
Candidatus Aquiluna	x	x	x	x	x	x	x	x	x		x	x	x	x	x	x	x	x		x
Candidatus Rhodoluna	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x	x	x		x
Cryobacterium													x					x		
Curtobacterium				x				x	x											
Frondihabitans											x									
Leifsonia				x																
Leucobacter				x		x												x		x
Marisediminicola							x									x				
Microbacterium				x			x		x									x		
Microcella	x	x	x			x	x	x	x		x	x	x	x	x	x	x	x		x
Pseudoclavibacter												x								

	Yonghaparkia	x	x	x		x	x	x			x	x			x	x	x		x			x			x
	Arthrobacter		x								x														
	Kocuria													x											
	Micrococcus										x														
	Actinoplanes						x					x								x		x			
	Mycobacterium	x		x	x	x	x	x	x	x		x		x	x	x	x	x	x	x	x				x
	Rhodococcus		x																						
	Friedmanniella												x												
	Kribbella	x					x	x																	
	Marmoricola					x					x											x			
	Nocardioides			x							x		x		x										
	Microlunatus									x	x														
	Propionibacterium		x	x		x			x		x									x				x
	Sporichthya			x	x		x					x	x		x		x		x	x	x	x	x		
	Bifidobacterium	x																		x					
	Adlercreutzia										x	x													
	Collinsella	x																							
	Olsenella					x																			
	Rubrobacter										x			x						x					
	Conexibacter										x		x												x

	Solirubrobacter					x					x											x	
Armatimonadetes																							
	Armatimonas		x	x	x		x	x							x	x	x		x				
Bacteroidetes																							
	Marinifilum	x			x						x		x	x	x								x
	Prolixibacter			x	x	x	x		x	x	x	x	x	x	x	x		x	x	x	x		
	Bacteroides	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x			
	Phocaeicola										x												
	Anaerophaga															x							
	Marinilabilia				x			x	x	x							x						
	Barnesiella										x												
	Candidatus Azobacteroides					x						x			x								
	Dysgonomonas	x			x			x	x	x	x			x	x	x							x
	Odoribacter														x								
	Paludibacter	x	x	x	x	x	x	x	x	x	x	x	x		x		x	x	x	x	x		
	Parabacteroides	x	x	x	x	x	x	x				x	x	x	x				x	x			
	Petrimonas	x																					
	Tannerella	x																					
	Paraprevotella										x		x										
	Prevotella	x	x	x	x	x	x	x	x	x		x		x	x		x		x	x	x		

Alistipes										x	x	x									
Rikenella	x				x		x			x				x							x
Crocinitomix	x	x	x	x			x		x	x		x	x			x	x				
Cryomorpha	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Fluviicola	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Lishizhenia	x	x	x	x	x	x	x	x	x	x	x	x		x		x	x				x
Owenweeksia				x					x	x	x					x			x		
Actibacter	x	x	x	x		x	x	x					x	x	x					x	x
Chryseobacterium	x				x		x			x	x	x		x		x		x	x	x	
Cloacibacterium	x		x		x		x					x	x	x							
Dokdonia	x						x		x					x		x		x			x
Epilithonimonas							x							x							
Euzebyella									x									x			
Flavobacterium	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Gillisia						x	x				x			x		x		x			x
Leeuwenhoekiella										x											
Mariniflexile								x	x												
Myroides											x										
Ornithobacterium				x						x		x									x
Riemerella						x	x		x		x	x		x				x			

	Sandarakinotalea	x	x	x				x		x				x		x	x							x
	Sejongia												x											
	Wautersiella	x																						
	Winogradskyella		x				x			x														x
	Chitinophaga				x		x						x									x		
	Ferruginibacter			x	x	x	x	x	x	x	x	x	x		x	x	x				x			
	Flavihumibacter												x								x			
	Flavisolibacter					x		x	x		x		x								x			
	Flavitalea				x		x				x						x							
	Lacibacter	x		x	x			x		x	x	x	x		x	x	x	x						
	Niabella	x									x				x		x							
	Niastella		x		x							x		x			x		x		x			x
	Parasegetibacter	x	x	x	x	x	x	x	x	x	x	x		x	x	x	x	x	x					x
	Sediminibacterium	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x			
	Segetibacter					x					x													
	Terrimonas		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x			
	Algoriphagus	x	x	x			x	x	x	x	x	x	x	x	x	x	x	x	x	x				x
	Aquiflexum			x		x		x		x	x	x												
	Belliella				x	x		x		x											x			
	Meniscus	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x			x	x		

Rhodonellum					x		x			x												x		
Cesiribacter				x					x		x	x												
Fabibacter			x	x	x	x	x				x	x							x	x				
Flexithrix								x							x		x	x						
Marivirga	x			x		x	x		x		x	x	x							x				
Persicobacter			x							x			x											
Rapidithrix				x																				
Roseivirga					x				x	x			x									x		
Sediminitomix	x			x	x	x	x								x									x
Sporocytophaga			x	x	x		x	x	x	x	x	x					x			x	x			
Adhaeribacter	x									x		x												
Arcicella	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Cytophaga	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Dyadobacter										x														
Emticicia	x	x		x	x	x	x		x	x	x	x	x	x		x	x	x	x	x	x	x	x	x
Flectobacillus		x			x		x		x		x	x	x	x	x	x	x	x	x	x	x			
Flexibacter				x	x				x	x	x	x	x							x	x			
Hymenobacter					x							x				x								
Larkinella				x							x							x						
Leadbetterella	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x

	Microscilla				x	x					x		x								x			
	Pontibacter					x							x											
	Rhodocytophaga												x											
	Runella	x		x	x	x		x			x	x	x	x							x			
	Spirosoma										x						x				x			
	Salisaeta		x		x							x												
	Aureispira				x			x			x	x										x	x	x
	Candidatus Aquirestis	x	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
	Haliscomenobacter	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
	Lewinella	x								x														
	Mucilaginibacter					x	x			x	x	x		x			x		x			x		x
	Pedobacter	x	x	x	x			x	x			x	x	x	x	x	x	x	x	x	x	x	x	x
	Solitalea	x	x	x			x	x	x					x	x	x	x							x
	Sphingobacterium				x												x		x					
	Candidatus Amoebophilus		x	x						x	x	x		x		x	x	x	x	x	x			
Chlamydiae																								
	Candidatus Protochlamydia	x		x		x	x	x		x	x	x	x		x		x		x			x		x
	Neochlamydia				x						x	x	x											
	Parachlamydia				x							x	x										x	x
	Candidatus Rhabdochlamydia	x		x	x	x		x		x	x	x	x		x		x		x			x	x	x

	Simkania					x				x				x											
	Waddlia				x																				
Chlorobi																									
	Chlorobium					x							x										x		
	Ignavibacterium	x	x	x	x	x	x		x	x	x	x		x	x			x				x			x
Chloroflexi																									
	Longilinea													x											
	Caldilinea	x	x	x	x					x		x	x	x		x	x	x							
	Oscillochloris		x									x										x			
	Herpetosiphon	x	x	x									x	x	x	x		x				x			
	Kouleothrix		x	x					x	x				x		x		x							
Cyanobacteria																									
	Anabaena								x								x								
	Nostoc					x					x														
	Brasilonema										x											x			
	Cyanobacterium												x												
	Snowella																					x			
	Chroococcidiopsis														x							x			
	Xenococcus	x									x	x	x		x					x					x
	Oscillatoria												x									x			

	Hydrocoleum												x							
	Microcoleus					x							x							x
	Phormidium					x									x					
	Planktothricoides																			x
	Geitlerinema					x					x		x							x
	Arthronema																			x
	Leptolyngbya					x			x	x	x		x					x		x
	Limnothrix					x							x							x
	Prochlorothrix					x							x							
	Pseudanabaena					x				x	x	x	x							x
	Acaryochloris									x										
	Chamaesiphon	x	x	x		x				x	x		x		x	x	x		x	
	Merismopedia															x				
	Cyanobium							x			x				x				x	
	Synechococcus					x		x		x					x					
Deferribacteres																				
	Caldithrix				x						x									
Deinococcus-Thermus																				
	Deinococcus																			x
Elusimicrobia																				

	Elusimicrobium					x								x				x												
Fibrobacteres																														
	Fibrobacter						x							x	x			x						x				x	x	
Firmicutes																														
	Bacillus			x			x			x					x	x	x			x					x	x				
	Paenibacillus													x		x				x							x			
	Solibacillus				x		x				x				x															
	Sporosarcina													x																
	Staphylococcus										x			x		x									x					
	Lactobacillus		x																											
	Leuconostoc															x														
	Lactococcus		x				x					x			x	x			x											
	Streptococcus		x						x	x								x												
	Turicibacter						x																							
	Clostridium sp. 1		x		x	x	x		x		x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
	Proteiniclasticum						x							x																
	Sarcina													x																
	Finegoldia										x																			
	Tissierella						x																							
	Fusibacter		x		x				x			x		x	x	x	x		x				x			x				

Anaerovorax						x			x	x	x									x
Mogibacterium		x																		
Acetobacterium																			x	
Gracilibacter					x				x											
Anaerostipes	x														x		x	x		
Blautia	x					x														
Butyrivibrio						x					x									
Clostridium sp. 2	x				x	x					x	x			x				x	
Coprococcus										x	x	x							x	
Epulopiscium					x					x		x		x					x	
Hespellia																	x			
Lactonifactor																			x	
Roseburia	x						x			x										
Ruminococcus sp.1	x									x	x	x								
Desulfosporosinus			x																	
Desulfotomaculum						x														
Acetoanaerobium							x													
Clostridium sp. 3	x				x		x			x		x		x				x		
Proteocatella							x							x						
Acetivibrio					x						x	x							x	

	Clostridium sp. 4			x							x	x	x								
	Faecalibacterium	x													x						
	Oscillibacter										x										
	Oscillospira	x									x		x								
	Ruminococcus sp. 2					x		x			x										
	Saccharofermentans			x							x		x								x
	Sporobacter			x							x	x	x						x		
	Subdoligranulum	x												x							
	Acidaminococcus					x															
	Anaeromusa	x				x		x					x		x					x	
	Anaerosinus	x				x		x							x						
	Anaerospora					x									x					x	
	Desulfosporomusa					x						x	x		x					x	
	Dialister	x													x						
	Megamonas	x		x											x						
	Megasphaera											x									
	Mitsuokella				x							x		x	x			x			
	Phascolarctobacterium	x				x											x				
	Propionispira	x				x	x						x		x					x	
	Propionispora					x							x							x	

	Selenomonas				x							x													
	Sporotalea				x	x																			
	Thermosinus					x						x	x		x							x			
	Veillonella	x																							
Fusobacteria																									
	Cetobacterium		x			x	x		x		x					x	x								x
	Fusobacterium				x	x															x				
	Sebaldella	x						x			x					x									
	Streptobacillus																						x		
	u114	x			x	x	x	x	x	x		x	x	x	x	x			x	x	x				
Gemmatimonadetes																									
	Gemmatimonas	x	x	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x	x					x
Lentisphaerae																									
	Lentisphaera									x	x			x	x								x		
	Victivallis		x		x	x			x	x	x	x	x		x	x	x	x							x
Nitrospirae																									
	Nitrospira	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
	2013/04/29		x						x							x									
	GOUTA19				x		x			x											x				
	LCP-6		x	x	x	x	x					x													x

Planctomycetes																						
	Candidatus Brocadia											x										
	Phycisphaera	x	x	x	x		x		x	x	x	x	x	x	x	x	x	x	x	x	x	x
	Gemmata			x	x			x		x		x	x								x	x
	Singulisphaera	x					x		x										x			
	Pirellula					x																
	Rhodopirellula				x	x	x					x	x									
Proteobacteria																						
Alphaproteobacteria																						
	Rhizomicrobium		x	x					x	x		x		x		x	x	x	x		x	
	Asticcacaulis		x		x	x			x			x		x		x		x	x	x	x	
	Brevundimonas	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x	x	x	x	
	Caulobacter	x	x	x	x	x	x		x	x	x	x	x	x		x	x	x	x	x	x	
	Mycoplana			x	x								x			x			x			
	Phenylobacterium	x		x			x	x	x		x		x				x	x	x	x		
	Beijerinckia		x										x							x		
	Camelimonas										x											
	Chelatococcus																			x		
	Methylocapsa		x	x	x			x	x	x	x	x	x			x		x				
	Methylocella					x																

Afipia			x	x				x	x	x												x
Balneimonas										x											x	
Bosea	x		x					x		x				x				x	x	x		
Bradyrhizobium	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x			x	x	x	x	
Rhodopseudomonas									x	x	x	x	x	x	x				x			
Salinarimonas				x							x				x							
Ochrobactrum								x											x			
Devosia	x		x			x				x	x		x		x			x	x	x	x	
Hyphomicrobium	x	x	x	x		x	x	x	x		x	x	x	x	x	x	x	x	x	x		
Pedomicrobium	x	x	x		x	x		x	x	x	x	x	x					x	x	x		
Rhodomicrobium				x				x		x								x	x		x	
Rhodoplanes	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	
Zhangella					x																	
Meganema											x											
Methylobacterium				x		x		x	x	x		x	x	x	x			x	x	x		
Microvirga									x	x			x						x		x	
Methylocystis	x	x	x	x				x			x		x	x	x				x			
Methylosinus		x					x												x			
Pleomorphomonas						x						x							x	x		
Anderseniella		x	x	x		x	x	x	x	x	x	x	x		x	x	x				x	

Aquamicrobium																		x							
Chelativorans				x		x		x			x	x													x
Defluvibacter								x																	
Hoeflea																						x			
Mesorhizobium	x			x		x			x	x	x	x						x					x		
Nitratireductor					x							x											x		
Phyllobacterium		x						x						x		x		x		x					
Agrobacterium	x		x	x	x	x	x		x		x	x		x	x	x	x	x	x	x	x	x	x		
Kaistia				x				x		x															
Prosthecomicrobium					x																				
Rhizobium		x	x	x		x		x	x	x	x	x		x		x		x		x			x		
Bauldia		x	x				x	x		x					x		x								
Nordella		x	x			x		x	x	x	x	x	x		x		x		x	x			x		
Tepidamorphus										x															
Ancylobacter						x					x				x										
Xanthobacter		x	x	x					x		x				x										
Hirschia				x					x	x		x					x					x	x		
Hyphomonas	x		x	x			x	x	x	x	x			x			x		x	x	x				
Amaricoccus		x	x	x		x		x	x	x	x	x	x		x		x		x	x	x	x	x		
Catellibacterium		x	x																						x

Gemmobacter		x		x	x	x	x				x		x	x	x			x		x
Haematobacter			x										x	x	x					
Loktanella										x										
Oceanicola									x											
Paracoccus	x	x	x	x	x	x	x		x	x		x	x	x	x				x	x
Rhodobaca									x											
Rhodobacter	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Rubellimicrobium		x			x		x			x		x				x				
Rubribacterium		x	x		x			x	x	x		x		x	x		x		x	
Rubrimonas		x	x					x	x				x		x		x			
Thioclava	x	x	x	x	x		x	x	x	x	x		x	x	x	x	x	x	x	
Parvularcula												x					x			
Acetobacter								x			x							x		x
Acidiphilium		x	x	x				x	x		x		x		x	x	x			
Acidisoma		x	x	x			x	x					x		x		x			
Elioraea		x	x					x						x	x					
Gluconacetobacter																				x
Rhodopila			x								x				x		x			
Rhodovarius															x					
Roseococcus			x											x	x					

	Roseomonas	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
	Rubritepida		x	x				x	x									x			
	Stella	x	x						x	x		x					x				
	Roseospirillum											x				x					
	Azospirillum		x	x	x	x	x		x	x		x	x	x		x	x	x	x	x	x
	Caenispirillum													x						x	
	Defluviicoccus										x		x							x	
	Dongia		x	x	x				x	x	x	x	x	x	x	x	x	x	x	x	x
	Inquilinus			x												x		x			
	Insolitispirillum	x			x	x		x		x		x				x			x	x	x
	Magnetospirillum	x	x		x	x	x			x	x	x	x	x		x	x	x	x	x	
	Novispirillum			x						x		x							x		
	Phaeospirillum		x		x		x	x	x	x		x		x		x		x	x		x
	Rhodocista		x	x	x	x	x	x	x	x		x	x	x	x	x	x	x	x	x	x
	Rhodospirillum	x	x		x	x	x			x		x	x			x	x		x		x
	Sneathiella												x								
	Candidatus Neoehrlichia									x		x					x		x		x
	Orientia												x		x					x	
	Rickettsia	x											x						x	x	
	Caedibacter				x			x				x					x			x	

	Candidatus Odyssella	x			x	x	x	x		x			x		x		x		x	x			
	Candidatus Pelagibacter	x		x			x	x							x				x				x
	Altererythrobacter			x	x			x	x		x	x	x	x	x		x	x					
	Erythrobacter				x						x							x					
	Erythromicrobium	x	x	x		x	x	x	x	x	x	x	x	x	x	x	x		x	x	x		
	Lutibacterium			x			x	x	x		x	x			x		x						
	Porphyrobacter	x				x	x	x		x	x		x		x	x	x			x	x		
	Blastomonas	x						x															
	Kaistobacter					x	x	x			x		x	x						x			
	Novosphingobium	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		
	Sandarakinorhabdus	x																					
	Sphingobium	x	x		x	x			x		x	x	x			x	x	x	x				
	Sphingomonas					x	x	x	x		x	x	x				x	x	x	x			
	Sphingopyxis	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		
	Sphingosinicella									x					x	x	x	x	x				
Betaproteobacteria																							
	Advenella					x																	
	Azohydromonas		x		x	x		x	x	x			x	x	x			x		x			
	Bordetella	x	x	x		x	x	x	x	x		x	x	x	x	x	x	x	x				x
	Brackiella															x							

Derxia															x					x		
Kerstersia																x						
Pusillimonas	x																					
Sutterella					x																	
Chitinimonas		x			x							x	x			x		x	x			
Cupriavidus			x						x	x						x						
Limnobacter					x		x		x	x	x					x			x			
Pandoraea											x											
Polynucleobacter	x	x	x	x	x	x	x	x	x		x	x	x	x	x	x	x	x		x		
Ralstonia		x	x					x	x				x		x		x	x				
Aquabacterium	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		
Ideonella		x	x	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x			
Inhella	x	x	x				x	x	x	x	x		x	x	x		x		x			
Leptothrix			x	x	x																	
Methylibium	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		
Mitsuaria				x	x			x	x		x				x	x		x	x	x		
Paucibacter			x	x	x	x		x	x	x	x	x	x	x		x	x	x	x	x		
Roseateles																x						
Rubrivivax	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		
Sphaerotilus	x	x	x		x		x	x	x	x	x	x	x	x	x		x	x	x	x		

Thiobacter	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x		x	x		
Acidovorax	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		
Albidiferax	x	x	x	x		x				x	x	x	x	x	x	x	x	x	x	x	
Aquamonas	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	
Brachymonas		x						x							x						
Caldimonas							x														
Comamonas	x	x	x		x		x	x	x		x	x	x	x		x	x		x	x	
Curvibacter	x	x	x		x	x	x	x	x		x	x	x	x	x	x	x	x	x	x	
Delftia		x	x					x					x		x	x	x	x			
Diaphorobacter																	x				
Giesbergeria	x									x											
Hydrogenophaga	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	
Hylemonella	x	x	x		x		x		x	x	x	x	x	x		x	x		x		
Lampropedia																				x	
Limnohabitans	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	
Macromonas	x		x		x	x							x	x	x		x	x			
Malikia	x				x									x	x					x	
Polaromonas					x	x				x			x					x	x		
Ramlibacter	x		x				x		x		x		x	x	x	x	x	x	x		
Rhodoferax	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	

Schlegelella																			x				
Simplicispira	x						x								x								
Variovorax		x	x	x		x		x			x	x				x	x	x	x			x
Xenophilus	x				x						x			x			x			x			
Duganella					x				x		x	x					x		x				
Herbaspirillum										x							x						
Herminiimonas																	x		x				
Massilia	x				x			x		x		x			x	x	x		x	x	x		
Oxalobacter															x								
Paucimonas																	x						
Undibacterium		x	x		x		x	x	x	x	x	x	x	x	x	x			x	x	x		
Gallionella											x												
Thiobacillus	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		
Sulfuricella	x	x		x	x		x	x	x	x	x				x						x		
Methylobacillus		x					x					x	x							x			
Methylophilus											x								x	x			
Methylotenera	x	x	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x		
Andreprevotia					x							x											
Aquaspirillum	x	x	x		x	x	x			x	x	x	x	x	x	x			x		x		
Chitinibacter								x		x		x	x	x				x					

Chitinilyticum													x						x			x			
Chromobacterium						x					x		x		x						x				x
Conchiformibius						x							x												
Deefgea				x	x	x	x	x		x	x	x	x	x	x	x	x	x			x	x	x		
Formivibrio						x							x									x			
Gulbenkiania													x									x			
Laribacter	x														x										
Microvirgula																x					x				
Paludibacterium					x								x		x										
Silvimonas											x				x										
Vitreoscilla		x			x				x		x														
Vogesella	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Nitrosomonas	x				x		x			x					x										
Nitrosospira		x									x														
Spirillum	x																								
Aromatoleum																					x				
Azoarcus																x									
Azonexus	x	x	x		x	x	x	x	x	x	x	x			x	x	x	x	x	x	x	x			
Azospira	x		x			x	x				x	x			x	x					x				
Azovibrio																						x			

	Dechloromonas	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
	Methyloversatilis	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
	Quatrionicoccus																		x	
	Rhodocyclus	x	x			x				x	x	x	x		x	x		x		x	
	Thauera	x		x						x	x		x		x						
	Uliginosibacterium			x	x	x				x		x	x			x	x	x	x	x	
	Zoogloea	x	x					x	x	x	x	x			x		x	x			x
Gammaproteobacteria																					
	Acidithiobacillus	x																			
	Aeromonas	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
	Tolumonas	x				x	x					x	x		x					x	
	Anaerobiospirillum					x															
	Succinivibrio	x																			
	Alishewanella	x													x						
	Rheinheimera	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
	Shewanella	x					x	x		x	x	x		x	x		x	x		x	x
	Allochromatium	x					x									x					
	Chromatium			x		x				x		x	x								
	Marichromatium												x								
	Thiobaca												x								

Thiococcus						x	x							x										
Thiocystis					x									x										
Thiolamprovum	x					x								x										
Thiorhodococcus		x	x	x		x	x	x	x		x	x	x				x							
Thiorhodovibrio						x			x															
Thiohalophilus					x																			
Alkalilimnicola		x					x				x				x	x								
Arhodomonas								x						x		x								
Ectothiorhodosinus																			x					
Thiohalospira		x																x		x				
Thiorhodospira												x												
Thiofaba			x																		x			
Thiovirga	x		x	x	x		x		x	x	x	x	x	x	x	x		x	x	x				
Steroidobacter	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x				
Achromatium															x				x					
Citrobacter	x				x														x					
Dickeya							x				x			x										
Enterobacter	x																							
Erwinia				x																x				
Escherichia/Shigella	x			x	x	x												x						

Klebsiella	x							x						x		x											
Leclercia	x				x		x					x				x										x	
Pantoea				x	x					x	x								x		x						
Pectobacterium				x	x					x		x	x											x	x		
Plesiomonas						x	x				x	x															
Pragia							x																				
Providencia					x					x		x							x								
Rahnella												x															
Raoultella	x			x	x	x				x	x	x				x								x	x		
Serratia	x		x		x		x									x			x								
Shimwellia	x						x									x								x			
Trabulsiella	x																										
Aquicella	x			x		x	x				x	x	x			x			x					x	x	x	
Coxiella										x	x	x	x			x											
Legionella	x	x	x	x	x	x	x			x	x	x	x	x		x	x		x	x				x	x	x	
Tatlockia	x		x			x													x					x		x	
Crenothrix	x	x	x	x		x		x		x	x		x					x	x					x		x	
Methylocaldum				x				x				x										x		x			
Methylomicrobium											x																
Methylomonas		x						x				x															

Methylosarcina																			x		
Cellvibrio	x		x	x	x	x	x		x	x	x	x		x	x	x		x	x	x	
Simidua	x																				
Haliea			x	x		x			x	x	x	x	x				x	x	x	x	
Oceanospirillum		x	x						x					x	x		x				
Oleibacter			x																		
Acinetobacter	x				x	x	x			x	x		x	x				x	x	x	
Alkanindiges	x	x			x	x	x		x	x	x	x		x	x	x		x	x	x	
Enhydrobacter						x						x	x								
Perlucidibaca	x			x	x	x	x		x	x	x	x		x	x	x		x	x		
Psychrobacter	x																				
Azomonas	x				x		x	x	x	x	x	x	x	x		x			x	x	
Pseudomonas	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x	x	x	x	
Beggiatoa												x							x		
Thioploca				x								x									
Thiothrix			x	x	x	x		x	x		x	x					x		x		
Photobacterium																			x		
Vibrio						x														x	
Alkanibacter										x											
Hydrocarboniphaga										x									x		

Nevskia			x	x						x	x	x	x	x	x		x			x	x	x	
Aquimonas	x	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x			x	x	x	
Arenimonas	x	x	x	x		x	x	x	x	x	x			x	x	x	x	x	x				x
Aspromonas					x				x						x								
Dokdonella	x	x	x	x	x	x	x	x	x	x	x			x	x	x	x	x			x	x	
Fulvimonas																			x				
Ignatzschineria					x																		
Luteibacter											x												
Luteimonas					x		x			x	x												
Lysobacter	x	x			x		x	x		x	x				x								
Pseudofulvimonas				x							x						x			x			
Pseudoxanthomonas							x			x	x								x				
Stenotrophomonas						x		x				x	x										
Thermomonas	x		x				x			x	x	x											
Xanthomonas																		x					
Solimonas		x									x		x								x	x	
HB2-32-21				x		x			x				x										x
ND137				x																			
nsmpVI18				x							x	x											
HTCC			x																				

Deltaproteobacteria																							
	Bacteriovorax		x	x	x	x	x			x	x	x	x	x	x	x	x	x	x	x	x	x	
	Peredibacter	x			x	x					x	x	x		x		x	x	x	x			
	Bdellovibrio	x			x			x		x	x	x	x	x	x		x		x	x	x		
	Desulfobulbus	x	x	x			x	x	x	x		x					x		x	x			
	Desulfocapsa	x	x	x	x	x	x			x	x	x							x	x			
	Desulfofustis						x																
	Desulfopila	x			x					x	x		x		x			x		x	x		
	Desulforhopalus			x	x	x	x			x	x	x	x		x		x		x	x	x		
	Desulfotalea											x											
	Desulfurivibrio			x														x					
	Desulfomicrobium	x									x								x	x			
	Bilophila	x																					
	Desulfovibrio	x				x	x	x		x		x	x		x				x	x	x		
	Desulfuromonas				x		x					x											
	Desulfuromusa															x							
	Malonomonas			x																			
	Geobacter	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		
	Geopsychrobacter										x												
	Pelobacter		x	x	x	x	x		x		x								x		x		

	Candidatus Entotheonella										x										
	Stigmatella																		x		
	Haliangium									x		x									
	Kofleria	x		x				x	x		x	x	x		x						
	Anaeromyxobacter		x	x	x		x				x	x	x	x		x		x			
	Myxococcus										x	x									
	Nannocystis	x			x					x	x	x			x					x	x
	Plesiocystis	x			x		x	x	x	x	x	x			x	x	x	x			
	Byssovorax				x						x	x						x	x		
	Chondromyces		x		x	x				x	x	x	x	x							x
	Sorangium	x			x																
	Phaselicystis			x	x	x	x				x	x	x			x			x	x	
	Desulfatirhabdium			x	x					x	x	x	x						x	x	
	Desulfobacter	x		x								x									
	Desulfobacterium			x	x			x				x			x	x					
	Desulfobacula											x									
	Desulfobotulus														x						
	Desulfococcus		x							x											
	Desulfofaba											x									
	Desulfonema			x																	

	Desulforegula					x	x							x			x								
	Desulfosarcina			x	x	x		x		x	x		x	x	x		x	x	x						x
	Desulfospira				x	x	x	x			x		x	x						x				x	
	Desulfobacca					x		x				x	x		x		x		x		x				x
	Desulfomonile			x	x			x	x	x	x		x					x	x	x					x
	Syntrophus						x	x					x												
	Desulfoglaeba										x											x			
	Syntrophobacter			x	x					x				x							x		x		
	Syntrophorhabdus									x			x				x				x				
	LE30													x											
Epsilonproteobacteria																									
	Arcobacter	x		x		x	x			x		x	x	x	x	x	x	x		x			x		x
	Sulfurospirillum			x		x							x										x		
	Flexispira																								x
	Sulfuricurvum		x	x			x				x	x	x		x		x			x					
	Sulfurimonas	x			x					x		x			x	x	x			x					
	Sulfurovum				x							x		x			x								x
	Wolinella				x				x			x													
	Thioreductor					x							x									x	x		
Spirochaetes																									

	Leptospira				x						x			x	x								
	Spirochaeta					x							x			x					x		
	Treponema				x		x		x	x	x	x	x		x	x		x		x	x		
	SJA-88									x					x								
	P30-6												x										
	SA-8	x	x		x							x	x	x	x			x		x			
	TM3				x											x							x
	za29			x																			
Tenericutes																							
	Bulleidia										x	x											
	PSB-M-3		x		x					x	x												
Verrucomicrobia																							
	Candidatus Methylacidiphilum										x		x			x							
	Alterococcus				x	x	x			x	x	x									x		
	Opitutus	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
	Candidatus Xiphinematobacter					x				x		x	x								x		
	Chthoniobacter		x	x	x	x	x		x	x	x	x	x	x		x		x	x	x	x		
	Akkermansia										x												
	Haloferula	x		x	x	x	x			x		x	x		x	x	x	x	x	x	x		
	Luteolibacter	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x	x	x	x		

	Persicirhabdus	x			x		x	x	x			x				x							
	Prosthecobacter	x	x	x	x	x	x	x	x	x		x		x	x	x	x		x				
	Roseibacillus						x					x	x			x							
	Rubritalea	x	x	x		x	x	x	x		x		x	x	x	x	x	x	x	x	x		
	Verrucomicrobium	x	x	x	x		x	x	x	x		x	x	x	x	x		x	x	x	x		
	LP2A		x	x	x		x	x	x		x	x	x	x		x	x	x		x	x		
	MSBL3						x		x							x							
ABY1_OD1						x				x		x								x			
BRC1							x																
GN02			x	x		x				x		x									x		
GN04		x		x		x					x	x	x								x		
GOUTA4							x				x									x			x
HDBW-WB69												x											
KSB1										x	x	x											
MVP-15				x	x	x			x	x	x					x	x			x	x		
NC10			x		x		x		x		x	x	x				x		x				
NKB19				x			x			x			x			x	x						
OP11						x	x									x	x	x					
OP3		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x	x	x	
OP8		x			x							x											x

SC3											x			x						
SC4				x					x		x				x	x		x	x	
SM2F11							x		x		x			x						
SPAM				x						x		x			x					
TG3				x															x	
TM6	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x		x		x
TM7				x						x	x	x		x						
WS3	x	x	x	x	x	x			x	x	x	x	x				x	x	x	
ZB2	x				x	x			x		x	x		x		x		x	x	x

Supplementary Table 4-2S: Potential obligate pathogens identified in the Wonderfonteinspruit from 454-pyrosequencing data.

Taxonomic group	Genera	October 2012						November 2012							December 2012						
		1	2	3	5	6	7	1	2	3	4	5	6	7	1	2	3	4	5	6	7
Bacteroidetes																					
	Bacteroides	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	
Firmicutes																					
	Streptococcus	x						x	x					x							
	Clostridium sp. 1	x		x	x	x		x		x	x	x	x	x	x	x	x	x	x	x	x
	Clostridium sp. 2	x				x	x					x	x			x				x	
	Clostridium sp. 3	x				x		x			x		x		x				x		
	Clostridium sp. 4			x							x	x	x								
	Dialister	x													x						
	Veillonella	x																			
Fusobacteria																					
	Fusobacterium				x	x													x		
Proteobacteria																					
Betaproteobacteria																					
	Bordetella	x	x	x		x	x	x	x	x		x	x	x	x	x	x	x	x		x
Gammaproteobacteria																					
	Aeromonas	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x

	Escherichia/Shigella	x		x	x	x													x			
	Coxiella								x	x	x	x		x								
	Vibrio					x																x
	Stenotrophomonas					x		x				x	x									
Spirochaetes																						
	Spirochaeta				x							x			x			x				
	Treponema			x		x		x	x	x	x	x		x	x		x		x		x	x
Tenericutes																						
	Bulleidia									x	x											

The impact of physico-chemical water quality parameters on bacterial diversity in the Vaal River, South Africa

Karen Jordaan and Cornelius Carlos Bezuidenhout*

School of Biological Sciences, Subject Group: Microbiology, North-West University, Potchefstroom Campus, Private Bag X6001, Potchefstroom 2520, South Africa

ABSTRACT

This study aimed to identify bacterial community structures in the Vaal River using PCR-DGGE (polymerase chain reaction denaturing gradient gel electrophoresis) and high-throughput sequencing. The impact of physico-chemical characteristics on bacterial structures was investigated through multivariate analysis. Samples were collected from 4 sampling stations along the Upper Vaal River during winter (June 2009) and summer (December 2010). Physico-chemical analysis was conducted on-site. Additional physico-chemical data were obtained from statutory bodies. DNA was directly isolated from water samples and PCR amplified using universal bacterial primer pairs. PCR products were subjected to DGGE fingerprinting and high-throughput sequencing, followed by Shannon-Weaver diversity calculations, cluster analysis and multivariate analysis. Physico-chemical parameters did not exceed the prescribed South African water quality standards for domestic use, aquatic ecosystems, livestock watering and irrigation. DGGE banding patterns revealed similar bacterial community structures for 3 of the 4 sampling stations. PCA and RDA indicated that pH, water temperature and inorganic nutrient concentrations could be used to explain changes in bacterial community structures. High-throughput sequencing data showed that bacterial assemblages were dominated by common freshwater groups: Cyanobacteria, Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Bacteroidetes and Actinobacteria. Other freshwater phyla such as Deltaproteobacteria, Epsilonbacteria, Acidobacteria, Verrucomicrobia, Firmicutes, Fusobacteria, Flavobacteria and Fibrobacteres were found in low proportions. This study provides an overview of the dominant bacterial groups in the Upper Vaal River and the impact of environmental changes on bacterial diversity.

Keywords: Vaal River, bacterial community structures, 16S rDNA PCR-DGGE, high-throughput sequencing, multivariate analysis

INTRODUCTION

Socio-economic growth and development of the Vaal River require continuous augmentation of this water resource to meet the growing water requirements of communities in Gauteng, the Free State, North West and Northern Cape provinces (DWAF, 2009b). Water quality has drastically deteriorated due to constant disposal of industrial and domestic waste into the river. Salinization, eutrophication and microbiological pollution are currently the main problems affecting the water quality (DWAF, 2009a). The Department of Water Affairs and Forestry (DWAF) of South Africa, in line with the South African National Water Act (NWA), Act No. 36 of 1998, stipulated regulatory guidelines and criteria a water system must meet to ensure that the country's water resources are fit for use. A structured biomonitoring programme was implemented by the DWAF in 2009 to determine the exact sensitivity and health status of the Vaal River (DWAF, 2009a). Criteria routinely monitored to ensure sustainability, optimal water use and protection of the water resource include: physico-chemical characteristics, stream flow, discharge loads and microbiological pollutants, in particular, *Escherichia coli* (DWAF, 2009a; 2009b). The detection of *Escherichia coli* only indicates the presence of faecal contamination and not necessarily the degree of industrial pollution. Therefore, in-depth studies on the microbial communities in the Vaal River are essential to understand the microbial processes underlying secondary pollution and changes in the physico-chemical quality of water.

DGGE has been applied in numerous research studies involving the assessment of microbial diversity of rivers, streams, lakes and sediment, to determine the water quality of the resource (De Figueiredo et al., 2010; Essahale et al., 2010; De Figueiredo et al., 2011; Haller et al., 2011). This method opened up new avenues of research on the diversity of microorganisms present in complex aquatic environments. Currently, metagenomic analysis of microbial ecology, such as high-throughput sequencing (HTS), has been the focus of several environmental studies such as soil, (Lemos et al., 2011), freshwater lakes (Marshall et al., 2008) and deep sea microbiota (Sogin et al., 2006). Metagenomic analysis provides extensive information on community structure and composition (Kakirde et al., 2010). In addition, phylogenetic and functional analyses of microorganisms can be determined at community level (Cowan et al., 2005).

The objectives of this study were (i) to identify the bacterial community structures in the planktonic phase of the Vaal River using 16S rDNA PCR-DGGE and high-throughput sequencing, and (ii) determine the impact of physico-chemical characteristics on bacterial community structures using principle component analysis (PCA) and redundancy analysis (RDA).

This paper was originally presented at the 2012 Water Institute of Southern Africa (WISA) Biennial Conference, Cape Town, 6–10 May 2012.

* To whom all correspondence should be addressed.
☎ +27 18 299-2315; fax: +27 18 299-2330;
e-mail: carlos.bezuidenhout@nwu.ac.za

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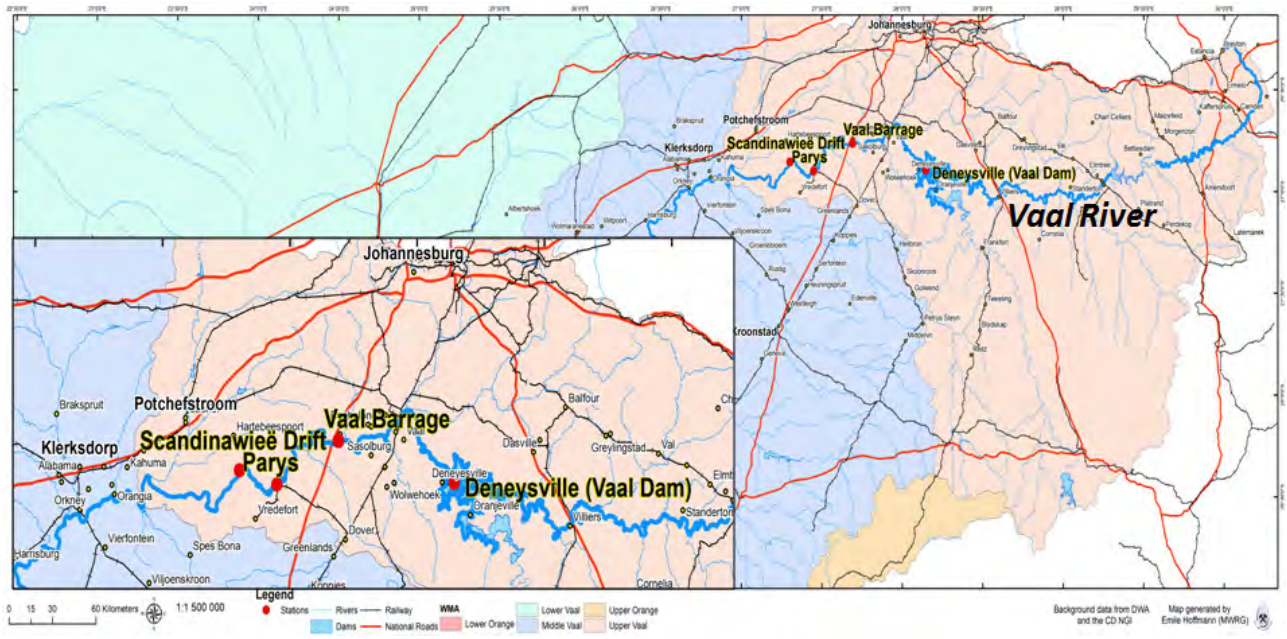

Figure 1

Geographical illustration of the Vaal River system. The four sampling stations are indicated on the map.

EXPERIMENTAL

Sample collection and physico-chemical analysis

Water samples were collected from the Vaal River in June 2009 (winter) and December 2010 (summer). The four sites included Deneysville (Vaal Dam) (26°53'43.44"S 28°5'53.88"E), Vaal Barrage (26°45'53"S 27°41'30"E), Parys (26°54'0.36"S 27°26'60"E), and Scandinawieë Drift (26°51'20.45"S 27°18'9.52"E) (Fig. 1). The Vaal Dam and entire middle section of the Vaal River are respectively regarded as eutrophic and hypertrophic due to the high levels of chlorophyll-*a* and phosphate, which exceed the recommended standards (DWAF, 2009a).

Samples were collected from the planktonic phase in sterile glass bottles and preserved on ice for not longer than 6 h prior to nucleic acid isolation. Physico-chemical analysis was conducted in situ. Additional physico-chemical data were obtained from the Department of Water Affairs (2012) and the South African Weather Service (2012). A summary of the physico-chemical variables of all studied sampling sites is shown in Table 1.

Nucleic acid isolation

A hundred millilitres of water samples were filtered through a 0.45 µm nitrate cellulose membrane filter (Whatman, Missouri, USA) and subsequently lysed in a 1 mg/mℓ lysozyme solution

TABLE 1
Physico-chemical characteristics of freshwater samples analysed in this study

	Sample							
	Deneysville		Vaal Barrage		Parys		Scandinawieë Drift	
	June 2009	December 2010	June 2009	December 2010	June 2009	December 2010	June 2009	December 2010
Day length (h, min, s)	10, 30, 13	13, 46, 19	10, 30, 13	13, 46, 19	10, 30, 13	13, 46, 19	10, 30, 13	13, 46, 19
Rainfall (mm)**	16.00	45.00	13.50	248.80	19.00	133.00	19.50	~105.00
Flow rate (m^3/s)*	15.12	258.34	40.01	340.95	9.371	906.84	5.35	1005.10
Temperature (°C)	10.00	28.70	11.00	24.50	13.00	24.40	13.00	26.70
pH	8.36	8.06	7.90	7.40	7.60	7.90	7.96	7.89
TDS (mg/ℓ)	130.65	116.42	507.00	435.50	266.50	429.00	495.30	205.40
Conductivity (mS/m)	20.10	17.91	78.00	67.00	41.00	66.00	76.20	31.60
NO_3-N (mg/ℓ)*	0.23	0.39	0.60	2.00	0.60	1.80	0.74	0.94
NH_4-N (mg/ℓ)*	0.03	0.03	0.90	~1.80	0.20	0.40	0.03	0.30
PO_4-P (mg/ℓ)*	0.02	0.02	0.40	0.60	0.05	0.50	0.39	0.03
SO_4-S (mg/ℓ)*	15.10	14.70	135.00	136.00	~50.00	50.01	155.45	68.35
Cl_2 (mg/ℓ)*	8.37	7.60	67.00	49.00	29.00	93.00	71.98	19.37

**Chemical water quality values were obtained from the Department of Water Affairs (www.dwa.gov.za)*
***Rainfall data was provided by the South African Weather Services (www.weathersa.co.za)*

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that contained 0.25–0.50 mm glass beads (Sigma-Aldrich Co., Missouri, USA) for bacterial cell disruption. The lysis solution was incubated at 37°C for 10 min while agitated in a vortex. Proteinase K (1 mg/mℓ) was then added and the lysis solution was incubated at 56°C for an additional 30 min. DNA was isolated from the crude lysate using the PeqGold Bacterial DNA Kit (PEQLAB Biotechnologie GmbH, Erlangen, Germany). The quality and quantity of the isolated nucleic acids were determined using the Nanodrop ND1000 (NanoDrop Technologies, Delaware, USA) and agarose electrophoresis.

PCR amplification and DGGE analysis of bacterial community structures

The highly variable V3 region of the 16S rDNA gene fragments were PCR amplified using the universal primer pair 341F-GC and 907R (~ 500 bp) (Muyzer et al., 1993). Amplification was performed in 25 µℓ reaction volumes containing single-strength PCR master mix ((5 U/µℓ *Taq* DNA polymerase (recombinant) in reaction buffer, 2 mM $MgCl_2$, 0.2 mM of each dNTP, Fermentas Life Sciences, Maryland, USA)), 50 pmol of forward and reverse primers, additional 1 mM $MgCl_2$, additional 1 Unit *Taq* DNA polymerase, 10–50 ng DNA and PCR-grade water (Fermentas Life Sciences, Maryland, USA). Thermal cycling was carried out in a Bio-Rad iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, California, USA) with an initial denaturation at 95˚C for 7 min followed by 30 cycles of denaturation at 95˚C for 30 s, annealing at 56˚C for 1 min and extension at 72˚C for 60 s. Final extension was performed at 72˚C for 7 min. PCR products were evaluated by electrophoresis on 1% agarose gels and visualised by ethidium bromide staining and UV illumination.

PCR products were analysed by DGGE using a DCode Universal Detection System (Bio-Rad Laboratories, Hercules, California, USA). Four reference species, namely *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Streptococcus faecalis*, were included in all DGGE studies. DGGE analysis was conducted at a denaturing gradient of 30–50% in 1 mm vertical polyacrylamide gels (8% (wt/vol) acrylamide in 1 × TAE). 20 µℓ of amplification product were mixed with 5 µℓ of loading buffer (6× Orange Loading Dye, Fermentas Life Sciences, Maryland, USA) and loaded into the gel. Electrophoresis was performed at a constant temperature of 60°C for 16 h at 100 V in 1 × TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). Polyacrylamide gels were stained with ethidium bromide (10 mg/ℓ) for 45 min and visualised with a Gene Genius Bio Imaging System (Syngene, Cambridge, UK) and GeneSnap software (version 6.00.22). None of the DGGE gels were digitally enhanced or modified. Bands of interest were only highlighted for better visualisation and not analytical purposes. Selected DNA bands of interest were excised from gels with a sterile scalpel and eluted in 20 µℓ of sterile nuclease-free water for 12 h at 4°C. 2 µℓ of the elute were used as DNA template in PCR amplification reactions with primer pair 341F and 907R (Muyzer et al., 1993) and conditions described above. PCR products were subsequently purified and sequenced using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, California, USA) and Genetic Analyzer 3130 (Applied Biosystems, California, USA). Sequences were aligned to 16S rRNA sequences in the National Center of Biotechnology Information Database (NCBI) using BLASTN searches to determine their identity. A total of 23 bacterial nucleotide sequences were submitted to the GenBank database under accession numbers JQ085826 – JQ085849.

High-throughput sequencing

HTS analysis was performed by Inqaba Biotech, South Africa, using the Roche 454 GS-FLXTM System. The V1–V3 region of the 16S rRNA gene was amplified using primer pair 27F and 518R (Lane, 1991) to produce ~ 500 bp fragments. Subsequently, sequences were trimmed to remove GS tags and further analysed with the CLC Bio Genomics Workbench version 4.7.2 software (CLC Bio, Aarhus, Denmark). Sequences shorter than 200 bp in length were excluded from data sets. All remaining sequences were subjected to the National Center for Biotechnology Information (NCBI) database for BLAST analysis. Sequences were then submitted to Pintail version 1.0 to detect the presence of PCR artefacts. PCR products with chimeric properties were eliminated from data sets prior to phylogenetic analysis. The remaining 922 sequences were submitted to GenBank with accession numbers JN865256–JN866178.

Statistical analysis

Bacterial community diversity was calculated with the Shannon-Weaver diversity index (*H'*), based on DGGE profiles. The Shannon-Weaver indices (*H'*) were calculated according to Zhang et al. (2011). Similarities between the banding patterns generated by PCR-DGGE of the various sampling sites were compared by cluster analysis as indicated by Gafan et al. (2005). Cluster analyses were displayed graphically as UPGMA dendrograms.

The distribution of samples according to environmental factors was analysed by PCA. The statistical significance of the relationships between bacterial community structures, DGGE banding profiles, high-throughput sequencing data and water quality was further assessed by RDA. Environmental variables selected are summarised in Table 2. Multivariate analysis was performed by a Monte Carlo permutations test using unlimited permutations. Analysis was carried out using CANOCO software version 4.5.

RESULTS

Physico-chemical characteristics

Selected physico-chemical parameters measured or obtained are listed in Table 1. These parameters showed all physico-chemical values to fall within the prescribed South African water quality guidelines for domestic use (DWAF, 1996a), aquatic ecosystems (DWAF, 1996b), livestock watering (DWAF, 1996c), irrigation (DWAF, 1996d) and aquaculture (DWAF, 1996e) (Table 2). Water temperatures were between 10 and 13°C in June and December temperatures exceeded 20°C (24.4–28.7°C). The temperatures of inland aquatic ecosystems in South Africa generally range between 5 and 30°C but can fluctuate depending on the geographical features of the region and catchment area, seasonal changes and the impact of anthropogenic activities (DWAF, 1996b). In December, the flow velocity increased sequentially from Deneysville to downstream sampling stations (Scandinawieë Drift). This trend was not observed in June, when rainfall was low.

Nucleic acid isolation from water samples

Nucleic acids were directly isolated from water samples without prior enrichment or culturing steps. Intact genomic DNA was obtained with a yield that varied from 2–30 ng/µℓ per 100 mℓ

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TABLE 2 South African Water Quality Guidelines for water resources and uses					
	South African Water Quality Guidelines				
	Domestic water use	Aquatic ecosystems	Livestock	Irrigation	Aquaculture
Temperature (°C)	NA	5.0 – 30.0	NA	NA	12.0 – 32.0[a] 2.0 – 30.0[b]
pH	6.0 – 9.0	6.0 – 8.0	NA	6.5 – 8.4	6.5 – 9.0
TDS (mg/ℓ)	0.0 – 450.0	NA	0.0 – 1000.0[c] 0.0 – 2000.0[d] 0.0 – 3000.0[e]	NA	NA
Conductivity (mS/m)	NA	NA	NA	0.0 – 40.0	NA
NO_3-N (mg/ℓ)*	0.0 – 6.0	< 0.5	0.0 – 100.0	0.0 – 5.0	0.0 – 300.0
NH_4-N (mg/ℓ)*	0.0 – 1.0	0.0 – 7.0	NA	NA	0.0 – 0.025
PO_4-P (mg/ℓ)*	NA	< 5.0	NA	NA	0.0 – 0.1
SO_4-S (mg/ℓ)*	0.0 – 200.0	NA	0.0 – 1000.0	NA	NA
Cl_2 (mg/ℓ)*	0.0 – 100.0	0.0 – 200.0	0.0 – 1500.0[f] 0.0 – 3000.0[g]	0.0 – 100.0	0.0 – 10.0

[a] *Target water quality range for growth of specific fish species*
[b] *Target water quality range for egg incubation and larval development of specific fish species*
[c] *Dairy, pigs and poultry*
[d] *Cattle and horses*
[e] *Sheep*
[f] *Monogastrics and poultry*
[g] *Other livestock*

of water. The quality (A260:A280 ratio) of nucleic acids was acceptable for PCR and ranged from 1.6–2.2. Although DNA concentrations were low, amplification products were of sufficient quantity for PCR-DGGE analysis.

Dynamics of bacterial community structures

DGGE analysis

In this study, PCR-DGGE was able to give spatial information about the dominant bacterial communities in the Vaal River system (Fig. 2). Previous studies suggest that band intensity is related to the relative abundance of the corresponding phylotypes in the sample mixture (Murray et al., 1996; Riemann et al., 1999). Thus, bands with relatively high intensities were assumed to be dominant taxa.

DGGE profiles demonstrated high resolution and intensity at a denaturing gradient of 30–50%. Four bacterial species, *Escherichia coli*, *Pseudomonas aeruginosa*, *Streptococcus faecalis* and *Staphylococcus aureus*, were included in all DGGE studies, to determine the potential of using such an approach to establish the presence of these species in water samples. Corresponding bands for *Staphylococcus aureus* and *Pseudomonas aeruginosa* were detected for Vaal Barrage, Parys and Scandinawieë Drift. In addition, Parys illustrated a band with similar migration patterns to *Escherichia coli*. All corresponding bands were excised and sequenced but produced poor quality sequences with indefinite identification. Since sequence data could not confirm accurate identification of excised bands, results remain inconclusive.

Vaal Barrage, Parys and Scandinawieë Drift displayed similar DGGE patterns for the dominant bands in June and December (Fig. 2). However, DGGE profiles for Deneysville varied to some extent from the three other sites. Although some dominant bands showed similar migration patterns to Vaal Barrage, Parys and Scandinawieë Drift, a few distinct bands

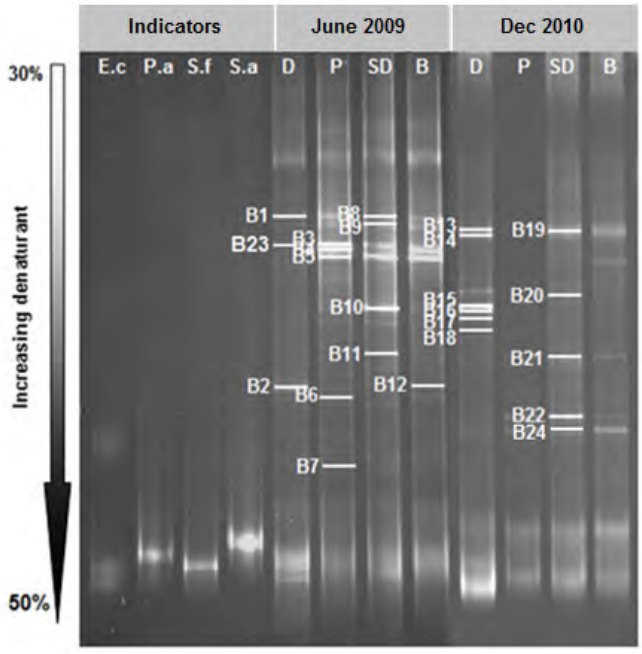

Figure 2
DGGE bacterial community analyses for 16S rDNA gene fragments from surface water during June 2009 and December 2010. Sampling sites selected along the Vaal River include Deneysville (D), Parys (P), Scandinawieë Drift (SD) and Barrage (B). Four indicator species were used as references: E.coli (E.c), Pseudomonas aeruginosa (P.a), Streptococcus faecalis (S.f) and Staphylococcus aureus (S.a). The DNA present in numbered bands was sequenced; identities are summarised in Table 3. None of the DGGE gels were digitally enhanced or modified. Bands of interest were only highlighted for better visualisation and not for analytical purposes.

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TABLE 3
Alignment of bacterial phylotype sequences obtained by PCR-DGGE with reference sequences in the NCBI database

DGGE band no.	NCBI accession no.	Closest relative (accession no.)	Phylogenetic affiliation	Percentage (%) similarity
B1	JQ085826	Uncultured bacterium clone XYHPA.0912.160 (HQ904787)	Bacteria	100
B2	JQ085827	Uncultured Methylophilaceae bacterium clone YL203 (HM856564)	Betaproteobacteria	100
B3	JQ085828	Uncultured bacterium clone SW-Oct-107 (HQ203812)	Bacteria	100
B4	JQ085829	Uncultured Cyanobacterium clone TH_g80 (EU980259)	Cyanobacteria	100
B5	JQ085830	Uncultured bacterium clone SINO976 (HM130028)	Bacteria	99
B6	JQ085831	Uncultured *Haliscomenobacter* sp. clone WR41 (HM208523)	Bacteroidetes	96
B7	JQ085832	Uncultured bacterium clone McSIPB07 (FJ604747)	Bacteria	98
B8	JQ085833	Uncultured bacterium clone ES3-64 (DQ463283)	Bacteria	99
B9	JQ085834	Uncultured bacterium clone ANT31 (HQ015263)	Bacteria	100
B10	JQ085835	Uncultured bacterium clone SING423 (HM129081)	Bacteria	99
B11	JQ085836	Uncultured *Bacteroidetes* sp. clone MA161E10 (FJ532864)	Bacteroidetes	100
B12	JQ085837	Uncultured *Nitrosomonadaceae b*acterium clone YL004 (HM856379)	Betaproteobacteria	92
B13	JQ085838	*Aphanizomenon gracile* ACCS 111 (HQ700836)	Cyanobacteria	91
B14	JQ085839	*Anabaena circinalis* LMECYA 123 (EU07859)	Cyanobacteria	97
B15	JQ085840	*Cymbella helvetica* strain NJCH73 (JF277135)	Cyanobacteria	99
B16	JQ085841	Uncultured bacterium clone FrsFi208 (JF747973)	Bacteria	99
B17	JQ085842	Uncultured Cyanobacterium clone LiUU-11-80 (HQ386609)	Cyanobacteria	98
B18	JQ085843	Uncultured bacterium clone TG-FD-0.7-May-09-B061 (HQ532969)	Bacteria	99
B19	JQ085844	Uncultured bacterium clone C_J97 (EU735734)	Bacteria	89
B20	JQ085845	Uncultured bacterium clone Lc2yS22-ML-056 (FJ355035)	Bacteria	97
B21	JQ085846	Uncultured bacterium clone ncd240a07c1 (HM268907)	Bacteria	91
B22	JQ085847	Uncultured *Sphingobacterium* sp. HaLB8 (HM352374)	Bacteroidetes	100
B23	JQ085848	Uncultured Cyanobacterium isolate DGGE gel band B5 (JN377930)	Cyanobacteria	98
B24	JQ085849	Uncultured *Dechlorosoma* sp. clone MBfR-NSP-159 (JN125313)	Betaproteobacteria	86

exhibited unique migration positions. A higher bacterial diversity, based on number of bands, was detected for Vaal Barrage and Scandinawieë Drift during June compared to December. On the other hand, bacterial diversity for Deneysville was higher in December than in June. The Shannon-Weaver indices (Fig. 4), however, contradicted the DGGE diversity data. These showed a higher bacterial diversity for Vaal Barrage and Scandinawieë Drift during December compared to June. The Shannon-Weaver index calculation includes the presence and absence of bands, but also band intensity that could be used to explain the contradiction (Zhang et al., 2011).

A total of 24 bacterial bands were excised, sequenced and compared to sequences in the NCBI database (Table 3). Approximately 75% of the bacterial sequences recovered displayed high sequence homologies (> 97%) with the known database sequences. However, 50% of these sequences showed the highest sequence similarity to uncultured bacteria obtained directly from freshwater samples. These results support the presence of many uncultured and potentially undescribed bacterial taxa in freshwater ecosystems. Taxonomic classifications of the partial 16S rDNA sequences obtained affiliated to Cyanobacteria (B4, B13–B15, B17, B23), Bacteroidetes (B6, B11, B22), Betaproteobacteria (B2, B12, B24) and uncultured bacteria (B1, B3, B5, B7–B10, B16, B18–B21). Bacterial communities for June displayed relative abundances of 8%, 17%, 17% and 58% for Cyanobacteria, Bacteroidetes, Betaproteobacteria and uncultured bacteria, respectively. In contrast, the relative abundance for Cyanobacteria increased to 42% in December, whereas Bacteroidetes, Betaproteobacteria, and uncultured bacteria respectively accounted for 8%, 8% and 42% of the four main phylogenetic groups.

High-throughput sequencing

A total of 18 phyla were identified among 4 sampling sites by HTS technology (Fig. 3A – F). Dominant phyla include Alphaproteobacteria (0.24–15%), Betaproteobacteria (1.47–85.10%), Gammaproteobacteria (0.24–12.38%), Bacteroidetes (0.72–4.05%) and Actinobacteria (4.76–10.00%). The remaining groups could be placed into 9 phyla: Acidobacteria, Chloroflexi, Cyanobacteria, Euglenoidea, Eukaryote, Fibrobacteres, Firmicutes, Fusobacteria, and Verrucomicrobia.

While identification of the four indicator organisms employed in DGGE profiling remained inconclusive by Sanger sequencing, HTS analysis verified that two of the bands did in fact belong to the Pseudomonadaceae family and *Escherichia* spp. Additional opportunistic pathogens detected in low quantities at Vaal Barrage, Parys and Scandinawieë Drift included *Roseomonas* sp., *Ralstonia* sp., *Serratia* sp. and *Stenotrophomonas* sp.

Distribution of bacterial diversity in the Vaal River

The Shannon-Weaver diversity indices (*H'*) were calculated from DGGE banding patterns as the number and relative intensity of bands (Fig. 4). Indices were used to compare the overall structure of bacterial communities among the four sampling sites. *H'* for June and December samples ranged from 0.27–0.46 and 0.70–0.86, respectively. Bacterial diversity gradually increased from upstream to downstream sites, except for Parys in December which consisted of a lower diversity. Similar trends were also observed for HTS data.

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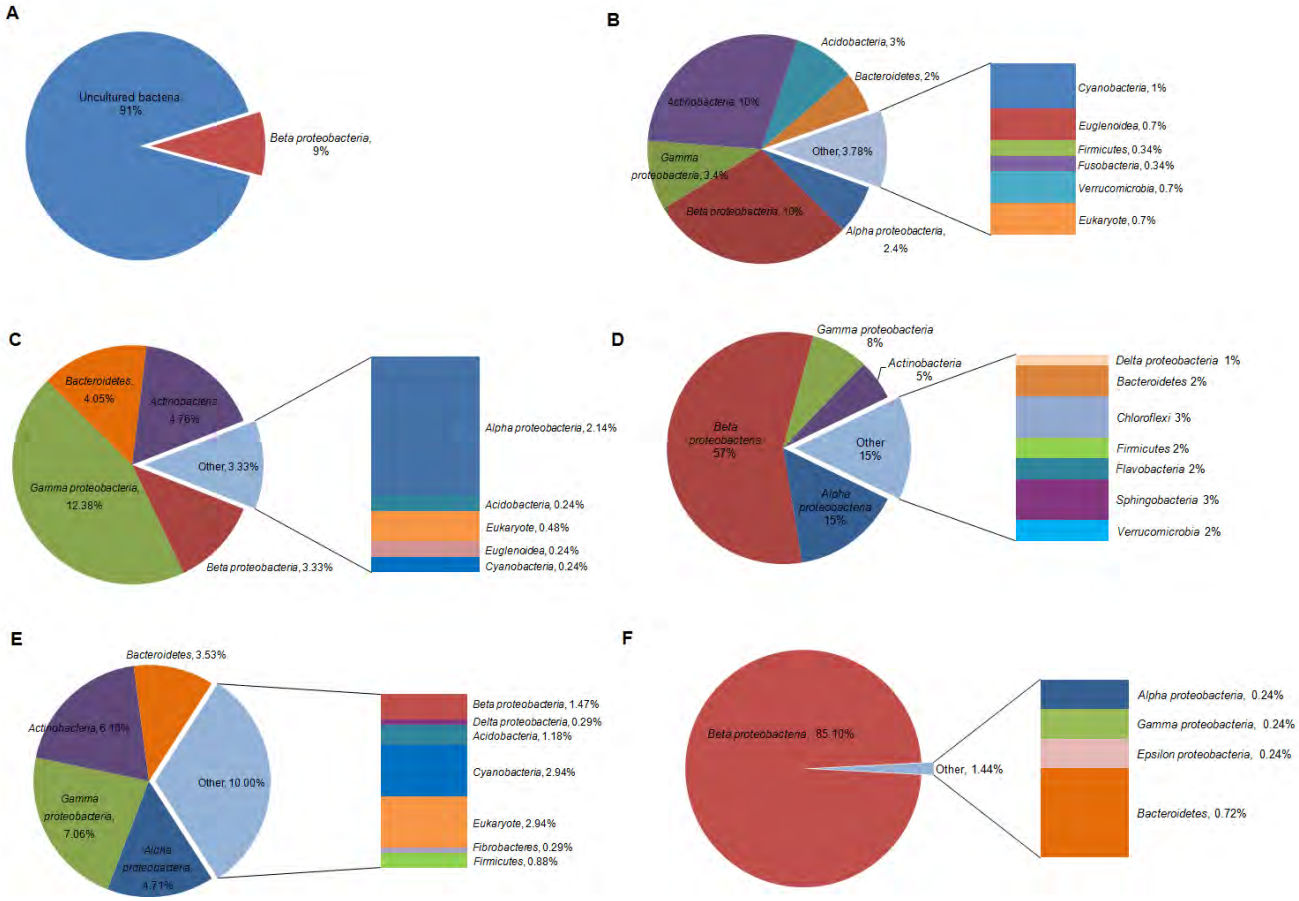

Figure 3

The relative abundance and composition of the dominant bacterial phyla in the Vaal River obtained from high-throughput sequencing technology for (A) Deneysville – December 2010, (B) Vaal Barrage – December 2010, (C) Parys – December 2010, (D) Parys – June 2009, (E) Scandinawieë Drift – December 2010 and (F) Scandinawieë Drift – June 2009.

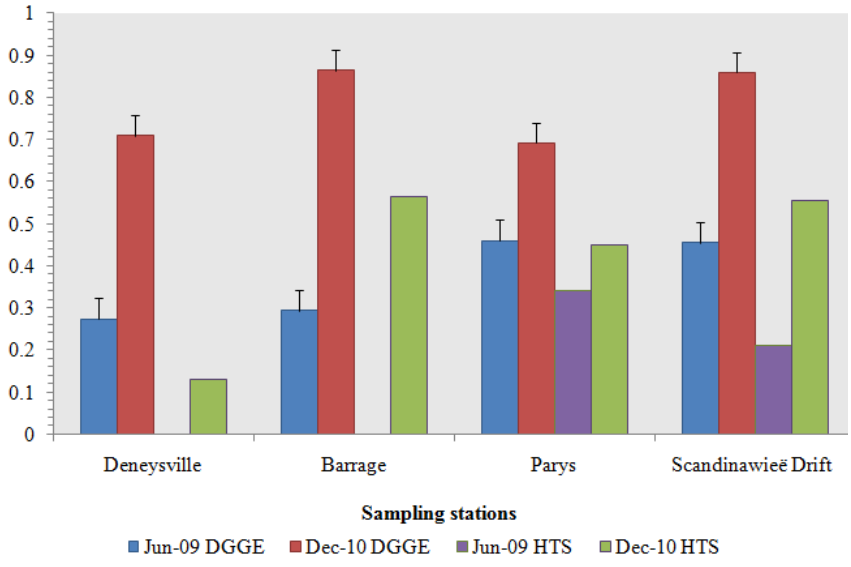

Figure 4

Shannon-Weaver diversity indices (H') for the Vaal River in June 2009 and December 2010 at Deneysville, Barrage, Parys, and Scandinawieë Drift.

Cluster analysis was performed to gain an overview of the association of bacterial communities at the four sampling stations during June and December (Fig. 5). UPMGA dendrograms showed grouping of samples according to season. June samples showed high similarity (> 94%) among bacterial communities for Vaal Barrage, Parys and Scandinawieë Drift. A similar trend was observed for the December samples where Vaal Barrage and Scandinawieë Drift were defined by a 100% similarity. Noticeable was the grouping of the December Parys and Deneysville samples (100% similarity). Grouping of these two sampling sites may be attributed to similar banding patterns of a few dominant DGGE bands (Fig. 2). Diversity indices (*H'*) and cluster analyses could be associated with DGGE profiles which reflected variations in the distribution, abundance and composition of bacterial taxa.

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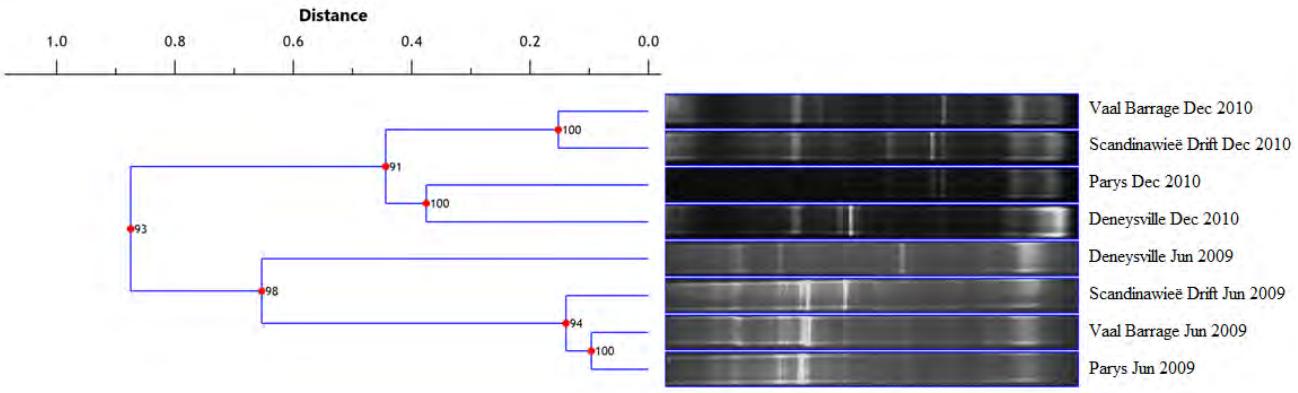

Figure 5

Cluster analysis of DGGE band patterns obtained in June 2009 and December 2010 using Pearson correlation coefficient. DGGE profiles are graphically demonstrated as UPGMA dendrograms.

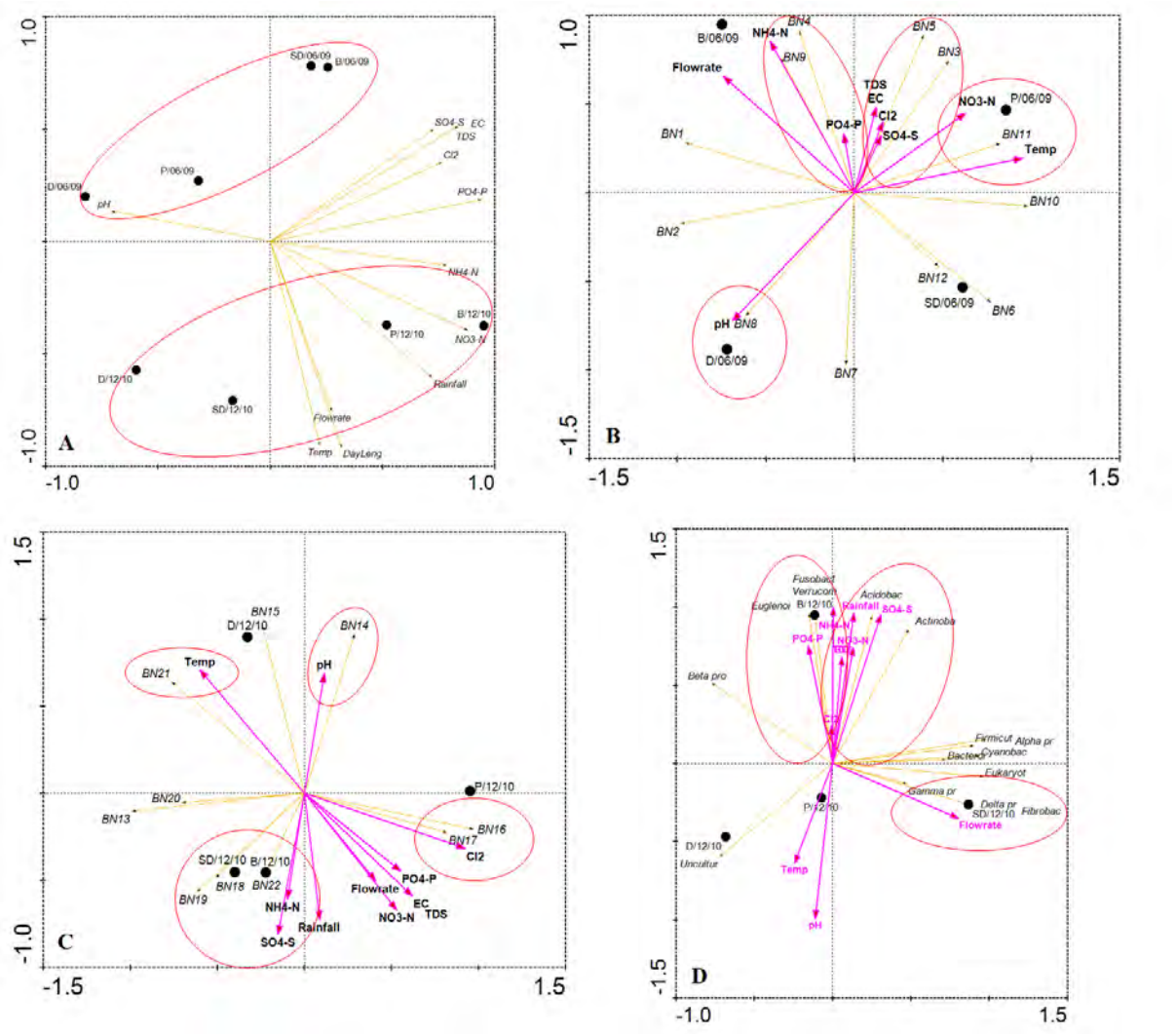

Figure 6

(A) *PCA analysis of physico-chemical and microbial variables in the first- and second-axis ordination plots,* ***(B)*** *RDA tri-plot of DGGE bands (samples indicated using band [BN] numbers) and environmental variables (represented by arrows) in June 2009,* ***(C)*** *RDA tri-plot of DGGE bands (samples indicated using band [BN] numbers) and environmental variables (represented by arrows) in December 2010 and* ***(D)*** *RDA tri-plot of bacterial phyla and environmental variables (represented by arrows).*

Multivariate analysis

PCA and RDA were performed to analyse the relationships between the environmental parameters and the clustering of samples.

The effect of different sampling periods is illustrated by the PCA analysis results (Fig. 6A). The June samples, with negative

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and positive scores along the first axis, are separated from the December samples, which showed a positive score along the second axis. The first axis was mainly defined by ammonium, nitrate, phosphate, chloride, sulphate, TDS, conductivity and rainfall. The second axis was related to temperature, day length and flow rate.

RDA plots calculated from DGGE profiles highlighted the possible environmental parameters responsible for the distribution of bacterial species at the different sampling stations (Figs. 6B and C). The arrow vectors for the environmental parameters in each RDA plot represent their impact on the composition of bacterial communities. Variation in the distribution of bacterial communities for the June and December samples (Figs. 6B and C) was shown to be correlated with the pH (BN8, BN14), temperature (BN11, BN15), ammonium (BN9, BN4, BN18, BN19 and BN22), phosphate (BN9, BN4, BN16 and BN17), chloride (BN3, BN5, BN16 and BN17), sulphate (BN3, BN5, BN18, BN19 and BN22), nitrate (BN11) and TDS concentrations (BN3, BN5, BN16 and BN17).

RDA plots for high-throughput sequencing data (Fig. 6D) showed (i) positive correlations between the flow rate and abundances of Gammaproteobacteria, Deltaproteobacteria and Fibrobacteres along the first axis, (ii) positive correlations between rainfall, TDS, nitrate, ammonium, chloride and sulphate concentrations, and abundances of Acidobacteria and Actinobacteria along the second axis, and (iii) positive correlations between ammonium, chloride and phosphate concentrations, and abundances of Fusobacteria, Verrucomicrobia and Euglenoida along the second axis. Betaproteobacteria negatively correlated with Gammaproteobacteria. A high abundance of Betaproteobacteria was detected in June but decreased considerably in December. An opposite inclination was observed for Gammaproteobacteria.

DISCUSSION

Microbial community dynamics

Knowledge and insight into the diversity and function of freshwater microorganisms is an essential requirement for the sustainable management of freshwater resources. In addition, changes in bacterial community structures might be used as potential bio-indicators of environmental disturbances. The aim of this study was to examine bacterial community structures in a segment of the Vaal River, in response to environmental parameters, using a PCR-DGGE and high-throughput sequencing approach. High-throughput sequencing provided an overview of the dominant bacterial communities in the planktonic phase and marked shifts in composition, as attested to by PCA and RDA.

The composition of bacterial communities in a given environment depends on the interaction between various factors such as the geographic environment (Zhang et al., 2011), temperature (Hall et al., 2008), pH (Yannarell and Triplett, 2005), flow rate (Crump and Hobbie, 2005), light intensity (Sigee, 2005) and nutrient concentrations (Pomeroy and Wiebe, 2001). In this study of a segment of the Vaal River, the physico-chemical parameters varied with sampling station and season of sampling. PCA and RDA analysis indicated that bacterial community structures were mainly influenced by pH, temperature and inorganic components.

The bacterial community structures were similar for the three sampling sites during each sampling period. However, the June bacterial community structures were different to the December assemblages. DGGE results suggested that bacterial diversity was higher during June compared to December. These results were, however, contradicted by the Shannon-Weaver indices. The latter analysis included presence–absence, as well as (abundance) band intensity data. This could be used to explain the contradiction (Zhang et al., 2011). Diversity index analysis of the high-throughput sequencing data showed similar trends to the Shannon-Weaver analysis of DGGE profiles.

Bacterial community structures could be correlated to inorganic nutrients as shown by PCA and RDA. The Vaal Barrage creates a buffering action that encapsulates organic and inorganic particles in the water-column for several weeks. This creates a relatively stable environment in which organisms can develop into a community. The planktonic bacteria then flow from here downstream to Parys and Scandinawieë Drift. Therefore, bacterial communities along this section of the Vaal River will be relatively similar. In addition, the dominant bacterial groups detected at these three points may be native species with broader niche capabilities, which allow them to grow and survive under a variety of environmental conditions (Anderson-Glenna et al., 2008). Recurrent native bacterial communities in aquatic ecosystems have been reported previously (Sekiguchi et al., 2002; Crump et al., 2003). It should be noted that the DNA amplification method used in this study did not discriminate between DNA derived from living cells versus DNA from dead cells and/or even naked or free DNA available in the water column. This aspect should be considered in future aquatic studies.

A feature highlighted in the present study was the relatively low bacterial diversity detected at Deneysville in June and December. Bacterial community structures at this sampling station largely consisted of Cyanobacteria, particularly Cyanophyta (*Anabaena* sp.), where pH and temperature were the main factors that affected the community structures. An alkaline pH was measured in June and December when temperatures in December were above 25°C. Optimum growth of Cyanophyta and the formation of surface algal blooms are the direct result of high nutrient concentrations (particularly phosphate) and physico-chemical characteristics (high pH, temperature and light intensity) (Sigee, 2005). In addition to these conditions, buoyancy also plays an important role in the development of Cyanophyta populations. Buoyancy allows algal populations to adopt an optimum position within the water column in relation to light and CO_2 availability (Sigee, 2005). This mechanism leads to changes in the water chemistry and light regime in the epilimnion that depress the growth of other phyto- and bacterioplankton groups (Sigee, 2005).

Although flow rate in this study was not shown to affect bacterial communities, previous studies have suggested that flow rate and hydraulic retention time have a substantial effect on community structures (Lindström and Bergström, 2004; Crump and Hobbie, 2005). Temporal variation in bacterial diversity was observed between the June and December samples. The Gauteng and North West Provinces received heavy rainfall in December 2010 that caused a drastic increase in flow rate, particularly at Parys and Scandinawieë Drift. The high flow rate resulted in flooding at these two sampling stations that likely changed the bacterial community structures. Bacterial communities in rivers with short hydraulic retention times would potentially remain undetected by DGGE due to high loss rates (wash-out effect) which in turn result in a lower bacterial density and diversity (Sommaruga and Casamayor, 2009). In contrast, rivers with an extended hydraulic retention time display an accumulation of nutrients which promotes a

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higher genetic diversity of bacteria. Although flow rate differences provide a reasonable explanation for the seasonal variation in bacterial, further investigations are needed to confirm this for the Vaal River.

Phylogenetic diversity of bacterial communities

Phylogenetic affiliation of the dominant groups retrieved from the freshwater samples by PCR-DGGE and high-throughput sequencing corresponded to Cyanobacteria, Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Bacteroidetes and Actinobacteria. Other freshwater phyla such as Deltaproteobacteria, Epsilonbacteria, Acidobacteria, Verrucomicrobia, Firmicutes, Fusobacteria, Flavobacteria and Fibrobacteres were found in low proportions.

Cyanobacteria accounted for a large proportion of bacterial diversity during December, which agrees well with the physico-chemical characteristics of the water samples. Several studies have indicated that Cyanobacteria tend to dominate phytoplankton communities in pristine freshwater systems (Anderson-Glenna et al., 2008; Foong et al., 2010) whereas other authors have reported an increase in the prevalence of Cyanobacteria in response to fluvial, organic and urban wastewater pollution (Douterelo et al., 2004; Ibekwe et al., 2012). Due to the trophic status of the Vaal River, cyanobacterial blooms usually occur during late spring and summer and often consist of *Microcystis aeruginosa*, *Oscillatoria* sp. and *Anabaena floss-aqua* (Cloot and Le Roux, 1997; DWAF, 2009a). In this study, *Anabaena* sp., *Cymbella helvetica* and *Synechocystis* sp. were in high abundance at Deneysville during December 2010. *Anabaena* spp. are among the most distributed toxin producers in eutrophic freshwater bodies (Berg et al., 1986). Their potential effects on aquatic ecosystems may be subtle or can cause major changes in the survival of sensitive species (DWAF, 2009a). In addition, these toxins may pose a serious health hazard for human and animal consumption.

Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria and Actinobacteria are ubiquitous groups in freshwater habitats (Gich et al., 2005; Anderson-Glenna et al., 2008) and are numerically important in river systems (Beier et al., 2008; Lemke et al., 2009). Members of Betaproteobacteria respond rapidly to organic and inorganic nutrient enrichment (Hahn, 2003; Simek et al., 2005) and have been isolated from various polluted and unpolluted freshwater bodies (De Figueiredo et al., 2011; Haller et al., 2011). Two important genera of this subphylum include *Dechlorosomonas* and *Variovorax*. Members of *Dechlorosomonas* are capable of oxidising aromatic compounds such as benzoate, chlorobenzoate and toluene (Coates et al., 2001), whereas *Variovorax* spp. are involved in plant growth and remediation of xenobiotics (Jamieson et al., 2009). Several opportunistic human pathogens of the Gammaproteobacteria group were detected at low abundance. Human diseases and infections are often associated with these pathogens (Berg et al., 2005; Mahlen, 2011) and have caused mortalities in immunocompromised individuals (Fergie et al., 1994; Paez and Costa 2008). Thus, although the opportunistic pathogens were present at low levels, their impact should not be underestimated.

RDA analysis revealed that nitrate, ammonium, chloride and sulphate were the four most influential inorganic factors responsible for shaping Actinobacterial and Acidobacterial communities. A few studies suggested that these two phyla participate in the nitrogen cycle in soils and sediments by reducing nitrate, nitrite and possibly nitric oxide (Gtari et al., 2007; Ward et al., 2009). Norris et al. (2011) also implicated some novel Actinobacteria from geothermal environments in growing autotrophically with sulphur as an energy source. Correlation between Verrucomicrobia and phosphate was also detected suggesting that this inorganic nutrient influenced the Verrucomicrobia community within the total bacterial population. The association between Verrucomicrobia and phosphate levels has seldom been discussed in previous studies of microbial ecology of freshwater resources (Lindström et al., 2005; Liu et al., 2009). Very little is known about the physiology and ecological roles of Actinobacteria, Acidobacteria and Verrucomicrobia in these habitats and the impact of physico-chemical characteristics on their community composition.

Members of Bacteroidetes usually inhabit mesotrophic and eutrophic water bodies that have high nutrient levels (Xi et al., 2007; de Figueiredo et al., 2011). This group is known to degrade polymeric organic matter, and to play an important role in the turnover of organic matter (Cottrell and Kirchman, 2000), and is often isolated from humic waters (Anderson-Glenna et al., 2008; Stabili and Cavallo, 2011). The Bacteroidetes–Flavobacterium-like lineages are often present in high abundance following the growth and decline of cyanobacterial blooms (Eiler and Bertilsson, 2007; Newton et al., 2011). Their presence and distribution is mainly determined by resource availability and is favoured during periods of high heterotrophic activity and enhanced growth (Eiler and Bertilsson, 2007). This phenomenon was evident in the high abundance of Bacteroidetes in June following the December 2008 to February 2009 cyanobacterial blooms.

CONCLUSIONS

This study investigated the impact of physico-chemical water quality parameters on bacterial community structures in a segment of the Vaal River. The PCR-DGGE approach and high-throughput sequencing analysis presented useful data in the identification of dominant bacterial groups at the four sampling stations. Molecular analysis showed that (i) bacterial community structures for June were different to the December assemblages, (ii) bacterial community structures for Vaal Barrage, Parys and Scandinawieë Drift were similar, (iii) bacterial communities at Deneysville differed from the three other sites and were lower in diversity, and (iv) Cyanobacteria, Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Bacteroidetes and Actinobacteria were the dominant bacterial groups detected and were shown to be impacted by physico-chemical water quality parameters. This study contributed to the identification of bacterial phylotypes, their spatial succession and the effect of physico-chemical characteristics on these freshwater bacterial communities. A detailed study on the relationships between the dominant bacterial taxa and specific physico-chemical water characteristics is required to improve our knowledge on how bacterial community structures in the Vaal River are affected.

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