Investigation of South African Estuarine Microbial species and Genome diversity

By

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Abstract

A study of the microbial diversity in sediments of the Great Berg River estuary is carried out using modern molecular phylogenetic methods. The aim of the study is to determine the effect of (pollution by) the effluents of the fish industry on the composition of the microbial community in the sediments. The diversity in microbial groups of sediment samples that received wastewater from the local fishing industry is investigated by a PCR-DGGE (polymerase chain reaction-denaturing gradient gel electrophoresis) approach and compared to an unaffected site. DGGE is used for the separation of 16S rDNA amplified from metagenomic DNA, which is expected to provide qualitative information on sediment microbial community composition. The DGGE method is also applied to monitor changes of the microbial community at different depths in the estuarine sediment. Two primer sets is used in this study, one specific for 16S rDNA from the domain *Bacteria* and the other for DNA from the domain *Archaea*, which allowed the depth profiles for these groups of organisms to be compared. The DGGE profiles representing the bacteria revealed a decrease in diversity with depth at the downstream site of the wastewater outlet. In contrast, the archaeal diversity increases with depth. In addition to the DGGE analyses, 16S rDNA clone libraries were constructed from both sampling sites. A total of fifty one clones were sequenced. The phylogenetic analysis of the sequences revealed that the class *Anaerolineae* (phylum *Chloroflexi*) was only present at the downstream site. Two bacterial groups the δ-*Proteobacteria* and *Anaerolineae* (30% respectively) were the most dominant groups at the downstream site. There were slightly higher percentages of the δ-*Proteobacteria* observed from the downstream site (30%) compared to the upstream site (28%). Certain bacterial groups such as *Sphingobacteria, Acidobacteria, Actinobacteria, Deinococci,* and *Plactomycete* were only found in the upstream site.

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In this study, sediment samples from the downstream site had higher bacterial diversity than the upstream site.

Declaration

I declare that **Investigation of South African Estuarine Microbial species and Genome diversity** is my own work, that it has not been submitted for any degree or examination in any other university, and that all sources I have used or quoted have been indicated and acknowledged by complete references.

Ms. Eveline Kaambo   November 2006

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Acknowledgement

To my father, thank you for giving me strength, wisdom, and for making this dream come true for me. You said, ask and you shall be given. Seek you shall found, knock and the door shall open.

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WESTERN CAPE

In loving memory of my beloved mother and brother: **Mrs. Enginie Kaambo and Mr. Godwin Kaambo**. You are my angels in the sky

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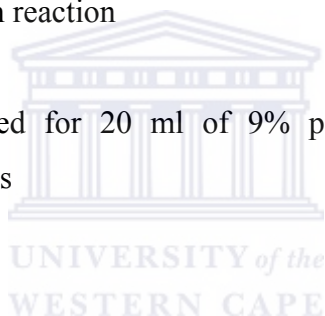
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List of Abbreviations

List of Abbreviations	
APS	Ammonium peroxidisulphate
bp	Base pair
BLAST	Basic Local Alignment Search Tool
DGGE	Denaturant Gradient Gel Electrophoresis
dNTPS	Deoxynucleotide tri-phosphate
è	Electron
FISH	Flourescence *In situ* Hybridization-
g	Gram
GC- clamp	Guanosine- cytosine clamp
mg	Micro-gram
kb	Kilo-base
ml	Milliliter
µl	Microliter
ng	Nanogram
PCR	Polymerase Chain Reaction
IPTG	Isopropapyl-B-D-thiogalactoside
PVPP	Polyvinylpolypyrrolidone
RT	Room temperature
NCBI	National center for Biotechnology Information

Rpm	Revolution per minute
min	minute
sec/s	seconds
GeneBank	Nucleotide sequence database
DMSO	Dimethylsulfoxide
DNA	Deoxynucleic acid
TAE	Tris-aceta-EDTA
TEMED	Tetramethylethyldiamine
V	Voltage
rDNA	Ribosomal deoxynucleic acid
rRNA	Ribosomal ribonucleic acid
TE	Tris-EDTA buffer
TBE	Tris-borate-EDTA eletrophoresis buffer
SDS	Sodium dodecyl sulphate
E.coli	*Escherichia coli*
α-*Prot*eobacteria	*Alphaproteobacteria*
δ-*Proteob*acteria	*Deltaproteobacteria*
γ-*Prote*obacteria	*Gammaproteobacteria*
ε-*Pr*oteobacteria	*Epsilonproteobacteria*

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Chapter 1

1. Introduction

1.1 The estuarine ecosystem

Estuarine systems consist of deep water tidal habitats and neighboring tidal wetlands that are semi-enclosed by land, but have open, and partly obstructed, or infrequent access to the open ocean (Abreu *et al* 2001). Estuaries also play an important role in providing breeding and nesting for numerous coastal birds, including a number of endangered species such as brown pelicans (Department of Environmental Protection, 2004).

In nature the estuaries are considered to be among the most productive ecosystems. Rivers and streams drain into estuaries, bringing in nutrients from uplands. Plants use these nutrients, along with the sun's energy, carbon dioxide, and water, to manufacture food (Kisand *et al.,* 2002). Among the most important plant forms that contribute to estuaries are microscopic algae called phytoplankton. Other plant forms include marsh grasses, mangroves, seagrasses, and macroalgae. When these larger plants die, they are broken down into detritus and are colonized by microbes (bacteria, fungi, and other organisms). During decomposition, the nutrients and smaller particles become food for other marine organisms. Larger animals feed directly on these tiny particles or on smaller animals that fed on detritus (Department of Environmental Protection, 2004).

Estuarine microbial diversity has been described in numerous studies. The mixing of freshwater and marine bacterioplankton communities along estuarine gradients has been clearly demonstrated but evidence of uniqueness in the estuarine bacterioplankton communities has not commonly been reported (Crump *et al*., 2004). It is therefore difficult to distinguish estuarine populations from those that wash in from adjacent environments (Crump *et al*., 2004). Marine biodiversity is threatened by over exploitation, physical alteration, pollution, global atmospheric transformation, and introduction of alien species (Maltagliati, 2003).

This study focuses on characterizing the microbial diversity in a South African estuary and also determining the effects of pollution on the microbial composition of the estuarine.

1.1.1 Estuarine water

Of all bodies of water, estuarine systems offer the greatest physical variability in composition. An estuary basically is a semi-enclosed body of fresh water that freely connects to an open sea in which this sea water is diluted by fresh water from land drainage (Abreu *et al.,* 2001). Water normally contains dissolved gases, such as oxygen, and a variety of organic and inorganic materials but when fresh water is mixed with sea water in an estuary, each of them contributes its own chemical and physical

characteristics. This creates a range of environments that support a wide variety of plants and animals (Moverley *et al.,* 2004).

The introduction of nutrient inputs from different sources such as wastewater treatment facilities, urban and agricultural runoff, and other sources may cause eutrophication which can threaten estuarine water quality. This may result in a reduction of macrophytes, altered species composition, anaerobic conditions and mass mortality (Crean Jr, 2004).

Rainfall affects the rate of run-off pollution from the land as well as the temperature, pH and total dissolved solids of surface water. Precipitation data helps in the determination of the possible causes of turbidity and erosion. Turbidity, for instance, generally rises during and after a rainstorm due to soil runoff. A wind -storm (without precipitation) might cause an increase in turbidity due to bottom mixing (Smith, 1992). The reason for recording water, wind, and weather conditions is to provide additional background information that may be helpful in the final analysis of an estuary's status.

Photosynthesis and aerobic respiration, and the growth, reproduction, metabolism and the mobility of organisms are all affected by changes in water temperature. The pH is a fundamental biogeochemical parameter, which plays a major role in most natural processes and has a quite universal importance in ecosystem. The pH of the water is critical to the survival of most aquatic plants and animals. Many species cannot survive if

pH drops under 5.0 or rises above 9.0. Changes in pH can alter the water's chemistry, usually to the disadvantage of native species (Smith, 1992).

1.1.2 Estuarine sediments

Sediments are pivotal components of aquatic ecosystems where important transformations and exchange processes take place (Levine *et al*., 2001). Sediment biota may play a significant role in pollutant transformation and transport, but this may vary across the salinity gradient (Palmer *et al.,* 2000). Sediment-poor rivers lack the extensive tidal mudflats and fine-grained sediment habitants of systems that receive large quantities of land-derived sediments. Overall, the quantity of particulate and organic matter inputs and the distribution of these inputs are crucial factors in the structuring of the sediment communities of estuaries (Levine *et al*., 2001).

Approximately 70% of the earth's solid surface is covered by marine sediments, which, display significant physical and chemical diversity (Llobet-Brossa *et al*., 1998). Sediments which contain high organic content tend to deplete their supply of oxygen closer to the sediment–water interface than sediments with low organic content. Therefore, sediments that have low organic content tend not to deplete their supply of oxygen, hence aerobic and such sediments do not produce methane (Valentine, 2002).

1.1.3 Microbial metabolic mechanisms

Mechanisms controlling microbial degradation of dissolved organic matter in aquatic environments are poorly understood, although microbes are crucial to global nutrient cycling (Jφrgensen *et al.,* 2003).

Microorganisms play important roles in biogeochemical cycles, and although the vital aspects of the main global geochemical cycles are understood, many details remain unclear (Keller *et al.,* 2004). Biochemical cycling processes in marine, estuarine and soil sediments have a direct effect on the speciation and fate of contaminants in a given environment. Essential information of these processes is vital for expansion of effective bioremediation strategies, and to eventually clean up contaminated areas (Bossert *et al.,* 2001).

Most microorganisms play an important purpose in the planet's biochemical processes, for example in the decomposition of organic matter in soil and water, provision of atmospheric components, nitrogen fixation, and photosynthesis (Jurgens, 2002).

The sediment microbial diversity is expected to vary in type and number depending on the quantity and quality of energy manufactured by the primary producers of each sediment ecosystem. The organisms can be described in terms of the processes observed in the sediments, however this approach can be unsatisfactory because many organisms are facultative, and that is capable of crossing the boundaries between the processes (Talaro *et al.,* 1999).

Carbon cycle

Carbon is stored in four main reservoirs, namely, the atmosphere as CO_2; in recently dead or living organisms in the form of organic compounds; in marine ecosystems as CO_2; and in limestone as Calcium Carbonate (Berner, 2003).
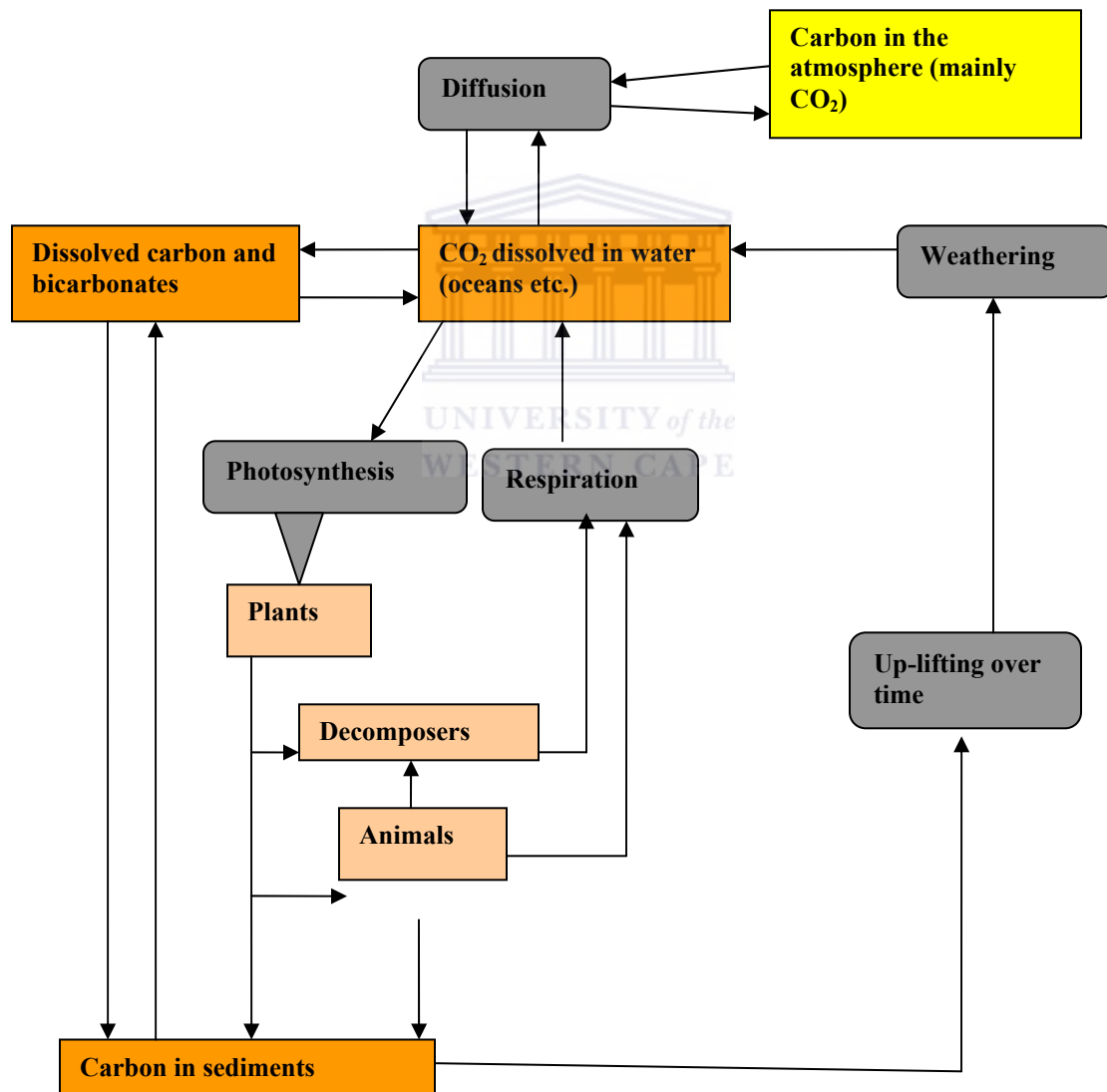
Fig. 1.1: The aquatic carbon cycle (Talaro *et al.,* 1999).

There are several pathways in the carbon cycle that are of particular importance (Figure 1.1). The main pathways to and from the atmosphere are diffusion into and out of the ocean, photosynthesis which consumes CO_2 from the atmosphere (an output from the atmosphere), respiration which produces CO_2 (an input to the atmosphere), and the burning of fossil fuels and biomass which also produces an input of CO_2 to the atmosphere.

The carbon cycle has a fast recycle rate because many organisms have a high demand for its carbon. The organisms include bacteria, plants and algae such as phototrophs and lithotrophs which require the carbon for energy production. Photosynthetic activities result in carbon fixation. The organisms that perform this fixation are photoautotrophs such as cyanobacteria, green plants and purple sulphur bacteria (Prescott *et al.,* 2002).

Carbon is released from the ecosystems as carbon dioxide gas by the process of respiration. Respiration takes place in both plants and animals and involves the breakdown of carbon-based organic molecules into carbon dioxide gas and other by-products (Talaro *et al.,* 1999). This then causes the recycle of carbon dioxide into the atmosphere again

Decomposition occurs when the organisms die and carbon is released into the atmosphere. In the aquatic ecosystem carbon dioxide can be stored in rocks, sediments and is dissolved in the marine ecosystem as carbonate ions. Carbonate and bicarbonate ions will be returned to the atmosphere as carbon dioxide (Valentine *et al.,* 1993).

Methane

Methane is a significant greenhouse gas that affects global climate by a number of mechanisms (Houghton *et al.,* 2001). This is due to the higher effectiveness (20–30 times) at absorbing long-wave radiation in contrast to CO_2, and due to the participation of CH_4 in chemical reactions leading to the creation of ozone (Crutzen, 1995). Since CH_4 concentration in the atmosphere has more than doubled in the post-industrial era, much of the research efforts have been expended to recognize sources and sinks of methane, and to estimate their strengths (Bodelier *et al.,* 2004).

Methane concentration in estuarine water represents the balance between the supply from the sediments, released to the atmosphere and methane production and consumption. Methane is a significant end product of degradation of organic matter under anaerobic circumstances (Bodelier *et al.,* 2004). Most of the produced methane is oxidized aerobically by methanotrophs. However, a major part of the methane that is produced in deep marine sediments is oxidized under anaerobic conditions by unknown bacteria (Ramsing *et al.,* 2001). This process is important because:

a) It contributes significantly to the global carbon cycle,

b) It reduces methane emission to the atmosphere where it would contribute to global warming as a potent "green-house" gas.

How the process is accomplished in the absence of oxygen remains a mystery, except that it is clear that the terminal electron acceptor in marine environments is sulfate and that sulfate reducing bacteria should be involved in the procedure (Ramsing *et al.,* 2001).

Microbial mediated oxidation of methane in anoxic marine systems is a globally important procedure, it is up by 90% of the oceanic methane production recycled in anaerobic marine sediments. Anaerobic consumption of methane is geochemically and biologically significant, because it significantly decreases the flux of methane from marine sediments to the atmosphere. The procedure transforms terminally reduced carbon into forms that are more readily available to a larger group of microorganisms in anoxic sediments (Orphan *et al.,* 2001).

Nitrogen Cycle

Nitrogen is an indispensable constituent of proteins, genetic material, chlorophyll, and other key organic molecules. Nitrogen serves as one of the major limiting factors that controls the dynamics, biodiversity, and functioning of many ecosystems. It ranks fourth behind oxygen, carbon, and hydrogen as the most common chemical element in living tissues (Vitousek *et al.,* 1997).

Nitrogen is essential for the production of plant and animal tissue. Plants and animals use nitrogen primarily to synthesize protein. Nitrogen enters the ecosystem in several

chemical forms and also occurs in other dissolved or particulate forms, such as in the tissues of living and dead organisms (Reshetiloff, 2004).

The nitrogen cycle is a biogeochemically indispensable process that converts different forms of nitrogen and compounds of nitrogen into forms that can be utilized for life processes (Vitousek *et al.,* 1997). The nitrogen cycle contains several different stages, which are illustrated in Figure 2. Four major processes are required to make nitrogen available to living organisms and then to return it to the atmosphere. These are nitrogen fixation, ammonification, nitrification and denitrification.

The limiting factor is that atmospheric nitrogen (N_2) is unavailable to most organisms due to the high amount of energy required to break the triple bond between the two atoms of nitrogen. Alternatively, other forms of nitrogen, such as ammonia (NH_3^+), are converted to soluble forms of nitrogen compounds, including nitrites and nitrates that can be utilized (Cottrell *et al.,* 2004). The utilization of nitrogen is a complex procedure that involves many diverse chemical reactions by diverse organisms. The response generates hydrogen gas, which can be utilized to reduce molecular oxygen and generate electrons and ATP (Smith, 1992).
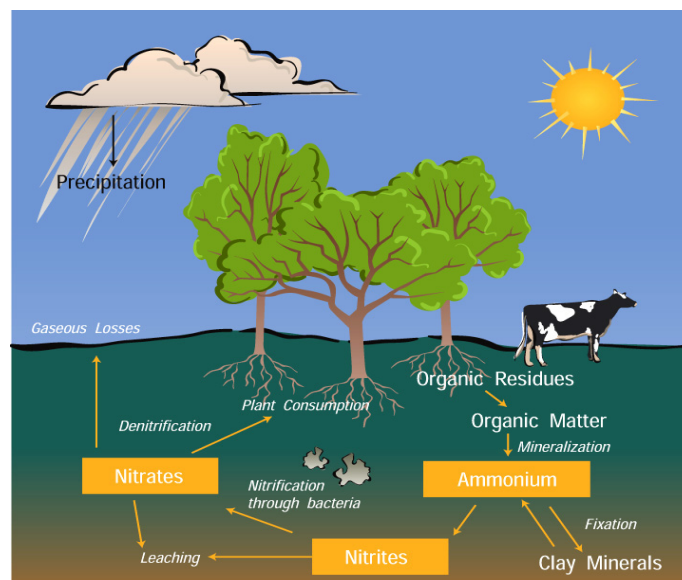
Fig. 1.2: Schematic diagram of N cycling (www.windows.ucar.edu).

Nitrogen, mostly in the form of ammonium and nitrate, reaches the earth's surface as a result of atmospheric lightning, precipitation and industrial pollution. In nitrogen fixation, gaseous nitrogen (N_2) is directly converted to ammonia as ammonium (NH_4) by nitrogen-fixing bacteria. Ammonia (NH_4^+).Ammonia is converted to nitrite (NO_2^-) and then nitrate (NO_3^-) by nitrifying bacteria. The NO_3^- is an important organic form of nitrogen found in proteins and nucleic acids. Organic nitrogen compounds are used by animals and other consumers. In ammonification, nitrogenous macromolecules from wastes and dead organisms are converted to (NH_4^+) by ammonifying bacteria (Talaro *et al.,* 1999). NH_4^+ can be either directly recycled into nitrates or returned to the atmospheric N_2 form by denitrifying bacteria (denitrification). The ammonium ion is initially oxidized to the nitrite ion (NO_2^-) by bacteria such as *Nitrosomonas, Nitrospira, Nitrosoccoccus* and

Nitrosolobus. In the second stage, nitrite is oxidized to nitrate by *Nitrobacter* (*Braker et al.,* 2001).

Denitrification is a process occurring naturally in the soil, where bacteria break down nitrates to give nitrogen gas which returns to the atmosphere. A microbial process in which oxidized nitrogen compounds are used as alternative electron acceptor for energy production is called denitrification (Nogales *et al.,* 2002).

The conversion of nitrate to nitrogen compounds such as N_2, NO, N_2O is called denitrification. It is a process by which nitrates are biologically reduced. The removal of nitrates from the environment (soil or water) is unfavorable to the sediment organisms or microorganisms because it is the most easily assimilated form of nitrogen. On the contrary, removal of nitrate from the sediments minimizes the effects of eutrophication (Braker *et al.,* 2001).

The other adverse effect of denitrification is contribution of gases such as nitrous oxide (N_2O) that are implicated in the greenhouse effect and the destruction of the ozone layer (Waibel *et al.,* 1999). A number of processes are involved in retuning N_2 gas to the atmosphere (i.e.) but denitrification is the major process by which the nitrogen gas are recycled (Philippot, 2002).

Denitrification of estuarine is one of the few processes capable of counteracting eutrophication. The denitrification efficiency is important in determining the rate and

extent of the denitrification process. The higher the microbial denitrification efficiency, the more the organic forms of nitrogen (NO_3^-, NO_2^-) converted to inorganic nitrogen (N_2 gas). Microbial denitrification can be efficient enough to permanently remove nitrogen from the system as nitrogen gas. But if the denitrification efficiencies are lowered, most of the nitrogen is recycled in water to form ammonium ions (Philippot, 2002).

Bacteria capable of denitrification are frequently isolated from the soil, sediments, and aquatic environments (Zumft, 1997). These bacteria have denitrification enzymes which are usually induced sequentially under anaerobic conditions.

Nitrogen that is recycled in the water column (and not denitrified) stimulates further cycles of primary production, and therefore can continue the supply of organic matter to the sediment, leading to more decomposition and more dissolved oxygen consumption, and potentially to anoxic and hypoxic events (Braker *et al.*, 2001).
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Cyanobacteria can extract nitrogen gas from the atmosphere and transform it into organic nitrogen compounds (Reshetiloff, 2004). This process, called nitrogen fixation, recycles nitrogen between organic and inorganic components.

The importance of denitrification in marine sediment ecosystems is very significant in contributing to about 25% of the nitrogen that enters the marine ecosystem each year.

The key processes for denitrification to occur are the conversion of nitrate to nitrite (Braker *et al.,* 2001).

Sulfur cycle

The sulfur cycle occurs in the interstitial water of organically rich marine sediments or in stagnant estuarine waters where the dissolved oxygen has been consumed by decomposition processes (aerobic respiration) (Knoll *et al.,* 1998). This reduction of sulphate to hydrogen sulphide by definite groups of bacteria is quantitatively more significant in marine environments than the deamination of organic sulphur compounds (i.e. the release of HS^- from S-containing amino acids) during degradation processes (Knoll *et al.,* 1998).
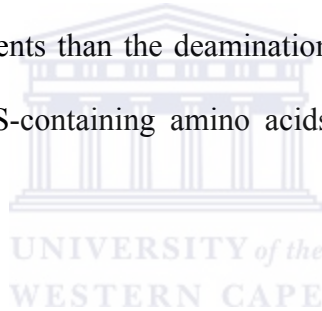
Bacteria also play major roles in the chemical and biological redox reactions that create the sulfur cycle. Sulphur and sulphide-oxidizing bacteria generate sulphate, which is used by sulphate reducing bacteria as an alternative electron acceptor in anaerobic respiration to create hydrogen sulphide. Sulphate-reducing bacteria cycle hydrogen sulphide through the atmosphere for use by anaerobic photosynthetic bacteria and sulphur-oxidizing bacteria, while returning carbon dioxide to the atmosphere (Holmer *et al., 2001*).

Sulphur in sediment occurs largely in the form of pyrite (FeS_2). Pyrite is formed from hydrogen sulfide (H_2S) as illustrated in Figure 1.4, which is a product from sulphate

reduction. During sulfate reduction, organic matter decomposition results in the binding of organic carbon with oxygen to form carbon dioxide.

```
                    ┌──────────────────┐
                    │ Desulfhydration  │
                    │ (anoxic)         │
                    └──────────────────┘
┌──────────────────┐                    ┌──────────────────────────┐
│ Organic sulphur  │───────────────────▶│ Sulphide (S²⁻, H₂S, FeS) │
└──────────────────┘                    └──────────────────────────┘
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Desulfhydration (anoxic)

Organic sulphur

Sulphide (S^{2-}, H_2S, FeS)

Electron donor in place of H_2O photoautotrophic bacteria

Assimilatory sulphur reduction (plants and microorganisms)

Elemental sulphur (S)

Dissimilatory oxidation (widespread)

Oxic chemoautotrophic bacteria (*Thiobacillus)*

Dissimilatory sulphur reduction; electron acceptor (*Desulfovibri*o, *Desulfomonas*)

Sulphide (SO_3)

Sulphate (SO_4)

Fig. 1.3: Sequence of processes involved in sulphur cycle (www.lenntech.com/sulphur_cycle).

The processes of the sulphur cycle consist of an assimilatory part and a dissimilatory part as illustrated in Figure 1.3. The assimilatory part includes sulfide and sulfate assimilation,

as well as release of sulphur from dead and living organic substances through decomposition or excretion. The assimilatory reduction of sulfate provides bacteria, fungi, algae, and plant demand for reduced sulfur compounds by synthesizing sulphur-containing amino acids (cysteine, cystine, methionine) and sulfur-containing growth factors (biotin, lipoic acid, thiamin). Animals excrete sulphur in the form of sulphate; however, the bulk of sulphur in living organisms is returned to the cycle in the form of sulphide due to death and decomposition by fungi and bacteria. The dissimilatory part of the sulphur cycle includes oxidative processes like chemotrophic and phototrophic sulphide, sulphur oxidation, and reductive processes such as microbial sulfate and sulphur reduction (Prescott *et al.,* 2002)
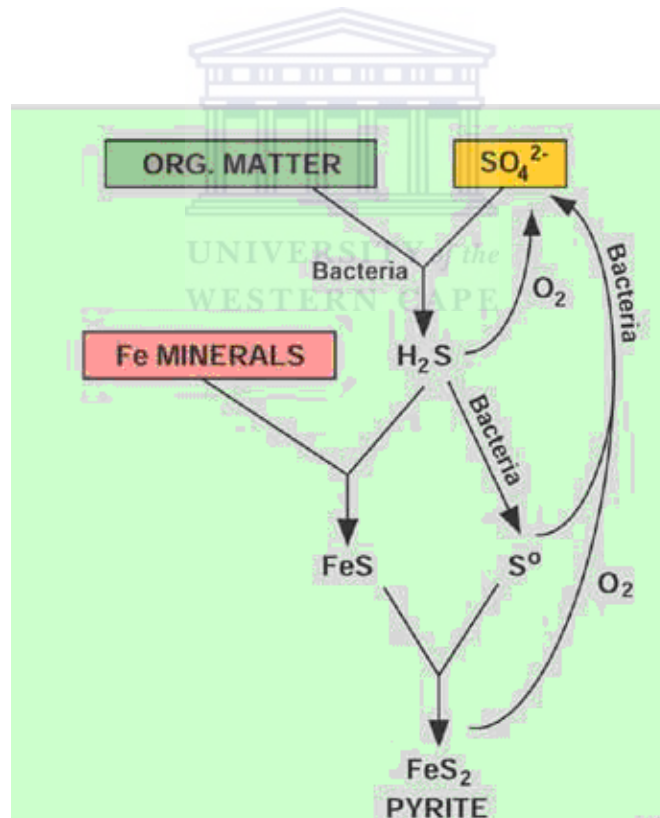
Fig. 1.4: Diagramatic representation of the process of pyrite formation (www.lenntech.com/sulphur_cycle)

Hydrogen sulphide can be oxidized to sulphur, or can react with iron sulphide minerals in the sediment as illustrated in Fig.1 4. Iron monosulphides (FeS) take form first but are readily converted to pyrite. Reduced sulphur formed during sulphate reduction can be stored in sediments either as metal sulphide minerals (principally iron sulphides) or as organic sulphur compounds (Holmer *et al*., 2004).

Many marine soils contain large amounts of pyrite (FeS_2) as a result of sulphate reduction and sulphide precipitation (Schippers *et al.,* 2002). Marine wetlands are particularly rich in pyrite. If these soils are drained, oxygen penetration increases, and pyrite is oxidized. When marine wetlands are drained, the soil can become so acidic so as not to be able to support other plant growth (Ravenschlang *et al.,* 2000).

Sulphur-Oxidizing Bacteria

Organisms that oxidize sulfur compounds lithographically are usually quite specialized. Most are autotrophic, utilizing sulfur compounds as the sole source of energy and CO_2 as the sole carbon source, and are incapable of growing heterotrophically on organic carbon sources. Once the commitment is made to this metabolism, other modes of metabolism are not common. The substrate of the sulfur oxidizers is usually thought of as hydrogen sulfide, although many sulphur oxidizers will also oxidize elemental sulphur and/or thiosulphate as well. However this substrate (for sulphur oxidizers) is also continuously being chemically oxidized by molecular oxygen. The bacteria then develop mechanisms

to maximize on substrate consumption and survival. One of these mechanisms is inhabiting habitats where oxygen is not readily available to oxidize the sulphur substrate. They are often found at interfaces where anoxic waters are mixing slowly with oxic waters above them. At such boundaries, sulphur oxidizers position themselves between the two reactants and take advantage of natural gradients, thus harvesting abundant energy (Fauque *et al.,* 1994).

1.1.4 Estuarine microbiology

The estuarine environment is unique for microbes because of the constantly changing environmental parameters that generate a broad diversity of ecological niches (Atlas, 1998). Estuaries have high nutrients and high photon energy; so they are the most productive marine ecosystem for photosynthetic aerobes. The range of saline concentration creates three types of niches; fresh water, brackish water, and saline water. Organisms that are adapted for those conditions occupy each niche. This shape of ecological partitioning reduces exploitative competition and enhances growth of different types of microbial communities (Campbell, 1993).

In estuarine environments, microbial diversity and metabolic activity are mainly affected by salinity gradients, high sulfate concentrations, and competition for energy sources. Sulfate reduction has been reported to be the dominant microbial respiratory process in estuarine and marine environments (Castle *et al*., 2004).

Marine and estuarine habitats contain a great deal of temporal and spatial variability due to the elevated complex physical and chemical components that intermingles within the biological components to yield a dynamic ecosystem (Clark *et al.,* 1993).

Microbial diversity in estuaries

In estuarine environments the degree of bacterial diversity is expected to be high; due to a combination of the mixing between seawater and freshwater and the resuspension of sediments from benthic zones, tidal mudflats, and sea grass beds. Nonetheless, a small proportion of these bacteria may be active as consumers of detrital organic matter (Armbrust *et al.,* 1999). The microbial diversity in marine sediment has been studied by using different types of molecular techniques such as denaturant gradient gel electrophoresis (Llobet-Brossa *et al*., 1998).

Estuarine community structure of microbial species

Microbial communities can be described by quantifying the molecular markers which define the viable bacterial biomass and the community structure (Li *et al.,* 1999). Microbial community structure analysis can be extended to give us an understanding of functional and biogeographically relationships and such data are vital for an improved

understanding of benthic ecosystem processes and the role that the benthos plays in overall oceanic processes (Bowman *et al.,* 2003).

The carbon and energy that fuels estuarine ecosystems is provided through an external source in the form of particulate materials. The estuarine microrganisms consume the riverine dissolved solid sources of energy. In contrast, the production of bacterioplankton is based on indigenous dissolved organic matter (Kisand, 2003)

In aquatic systems, it is important to evaluate changes in the microbial community structure, because the microbial community is the foundation of biogeochemical cycles (Sekiguchi *et al.,* 2002).
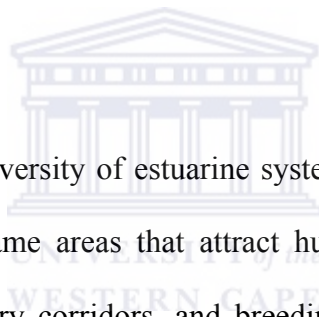
Estuarine microbial phylogenetics

Differences in the usage of various dissolved organic matter components may help explain the distribution of the major bacterial groups among soil, freshwater, and marine ecosystems. It may also be important to know the minimum number of bacterial phylogenetic groups necessary to describe and explain dissolved organic matter uptake in order to improve models of carbon cycling in aquatic habitats (Hugenholtz *et al.,* 1998).

1.1.5 Disturbing impact

Nutrients (such as nitrogen and phosphorus) occur naturally and are necessary to support a healthy ecosystem. Nonetheless, excess nutrients generate problems, such as stimulating the growth of algae, which die and decompose, depriving the water of oxygen necessary to support aquatic life. Excess algal growth diminishes water clarity, killing off aquatic plants that are sensitive to light changes. This causes impairment or loss of critical freshwater and estuarine habitats that support fish and shellfish populations. Extreme nutrients in some southern estuaries have also been linked to fish kills caused by toxic microbes. Primary sources of nutrients are fertilizers, animal wastes, and septic systems (Holmer *et al*. 2001)

The continued health and biodiversity of estuarine systems depends on the preservation of high-quality habitat. The same areas that attract human development also provide essential food, shelter, migratory corridors, and breeding or nursery areas for a broad range of marine life. Estuarine habitats also help protect water quality, stores water, and reduce flooding. Loss of habitat negatively affects fisheries, animal populations, filtering of water pollutants, and the ability of shorelines to resist storm-related erosion.

Estuaries are usually highly productive because of the concentration of nutrients. Common sources of estuarine pollution are the disposal of industrial and urban waste; petrochemical spills from boating and acid run-off from wetlands that have been reclaimed for urban and industrial development. Habitat modifications through dredging, reclamation and changed freshwater inputs have also resulted in the degradation of

estuarine environments. Catchment clearing and dam construction impairs the "self-cleaning" of estuaries by changing the flushing rates caused by flooding (Moverley *et al.,* 2004).

A dominant environmental concern in the local (Great Berg River) estuary is the consequence that artificial manipulations of salinity through freshwater discharges may have on the local ecosystem.

1.2 Potential applications of marine bacteria in biotechnology

Enzymes produced by marine bacteria are significant to biotechnology due to their range of catalytic metabolic properties. Some are salt-resistant, an attribute that is often beneficial in industrial processes. The extracellular proteases are of importance and can be used in detergents and industrial cleaning applications, such as cleaning reverse-osmosis membranes. *Vibrio* species such as *Vibrio alginolyticus* produces six proteases, including an unusual detergent-resistant, alkaline serine exoprotease. This marine bacterium also produces collagenase, an enzyme with a variety of industrial and commercial applications, including the separation of cells in tissue culture studies (Talaro *et al.,* 1999).

1.3 Archaea

Archaea are an unusual type of prokaryotic cell that constitute the third domain of life. The four phyla which comprise domain archaea are as follows: (i) the Crenarchaeota, of which most members that have been grown in culture are thermophiles; (ii) the Euryarchaeota, a diverse group which including all the methane- producing organisms. This is a diverse group, including both extreme halophiles and thermophiles (Ventriani *et al.*, 1998). (iii) Korarchaeota, which have been determined as a group due to the presence of environmental DNA sequences (Bong-Soo *et al.*, 2005). The fourth phylum: Nanoarchaeota, which is represented by single culturable isolate *N.equitans* (Huber *et al.*, 2002).

1.3.1 Distinctive characteristics of Archaea

The Archaea are prokaryotic organisms which have no nucleus and contain no organelles (Canfield *et al.,* 1999). Unique archaeal features includes. (1) The membrane lipids which are glycerol isopranyl ethers; (2) the Archaea possess unique cell envelopes; and (3) ribothymidine is absent from the "common" arm of the tRNAs, being replaced by 1-methyl-pseudouridine or pseudouridine. In methanogens, a number of unusual cofactors have been found. The amino acid, sugars, and glycosidic bonds between sugar residues in these compounds often differ from those typical of bacteria (Ishii *et al.,* 2004).

1.3.2 Ecology of archaea

Archaea representatives are widely distributed, for example in samples collected from ocean sediments, tidal flat sediments, freshwater lakes, soil, etc (Robertson *et al.,* 2005). A much wider ecological role for archaea is indicated, however, by the recent identification of archaea as an important constituent of marine picoplankton (Canfied *et al.*, 1999).

Extremely halophilic archaea are a diverse group of prokaryotes that live in extremely saline or salty environments. The deepest branches within the Euryarchaeota house hyperthermophilic organisms with maximum growth temperatures of up to 110°C (Stetter, 1996). These include members of the genus *Methanopyrus* (methanogens) and *Pyrococcus* (sulfur reducers).
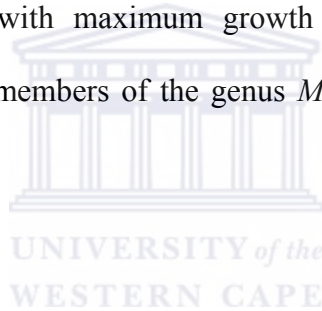
1.3.3 Archaeal metabolism

The extreme thermophiles are mainly strict anaerobes that can adapt to temperatures ranging between 70-110 %. Most of them are found in hot springs habitants. Most of extreme thermopiles are sulphur metabolizers that either anaerobically reduces sulphur (S^0) reduced to sulphide such as the *Themococcales,* or aerobically oxidized the sulphur to sulphide (Talaro *et al.,* 1999).

Within the Euryacaeota a range of metabolic processes have been identified, including methanogenesis, elemental sulphur reduction, fermentation, sulphate reduction, and aerobic respiration (Canfield, *et al.*, 1999).

Methanogens are strict anaerobes, found in a variety of anaerobic environments and rich in organic matter such as marine sediments, swamp, and hot springs and freshwater. Methanogens obtain energy by converting CO_2, H_2, or other compounds (e.g. formate, methanol, acetate to form methane (CH_4). They are the biggest groups of archaea. Methanogenic archaea are of a great practical importance since methane is a clean burning fuel and an excellent energy source (Teske *et al.*, 2003).

In environments such as coldest waters of the deep sea or low pH and high temperature, microbial populations are dominated by Archaea (Robertson *et al.*, 2005).
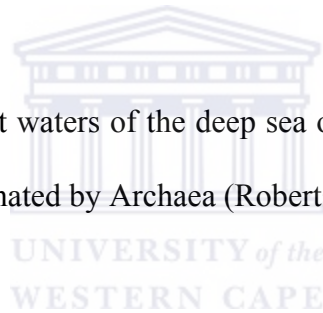
1.4 Molecular techniques for microbial ecology

Most bacteria and archaea in natural environments still cannot be isolated and cultivated as pure cultures in the laboratory, as these microorganisms often appear to be different from the uncultured ones (Kirchman *et al.*, 2004). One culture-independent approach is to clone this DNA directly into appropriate vectors and to screen the resulting "metagenomic library", which theoretically consists of all possible genes from the microbial assemblage (Kirchman *et al.*, 2004).

Molecular techniques can be used to assess genetic composition without culturing all the members of a community (van Hannen *et al.,* 1999). Using molecular techniques based on extraction of microbial nucleic acids followed by PCR amplification, DNA hybridization, cloning, and sequencing, it is possible to detect specific micro-organisms in natural marine environments, and to study their genetic diversity and phylogenetic kinship (DeLong *et al.,* 1993).

Whole-cell immunofluorescence assays (Chen *et al.,* 1999), fluorescent *in situ* hybridization (FISH) based on 16S rRNA sequences (Lee *et al.,* 1994), and chromosomal painting techniques (Laniol *et al.,* 1997) all have been used to characterize specific bacterial strains or populations in complex microbial communities.

1.4.1 16S rRNA gene

Analysis of 16S rRNA gene sequences has greatly advanced our understanding of the phylogenetic diversity of bacteria and archaea, especially that of the vast majority of microbes in nature that have resisted cultivation to date (Hugenholtz *et al.,* 1998). There is little information, however, on the metabolic function of specific bacterial groups in natural assemblages since few culture-independent studies have linked bacterial community structure and function. Although information on phylogenetic relationships of uncultured bacteria is readily accessible, the inability to culture most microbes limits the opportunities to assess their metabolic diversity (Cottrell *et al.,* 2000).

Using DNA sequencing of 16S rRNA clone libraries provides a picture of the bacterial diversity of estuarine and other planktonic systems in microbial community (Crump *et al.,* 2003). Comparison of these 16S rRNA gene sequences across different systems has identified many globally distributed phylogenetic clusters of bacteria (Giovannoni *et al.,* 2000).

1.4.2 Denaturing gradient gel electrophoresis (DGGE)

DGGE has been frequently used to examine the microbial diversity of environmental samples and to monitor changes in microbial communities (Boon *et al.,* 2001). The technique is based on the electrophoretic separation of PCR-generated double-stranded DNA in a polyacrylamide gel containing a gradient of a denaturant (Sigler *et al.,* 2004). As the DNA encounters an appropriate denaturant concentration, a sequence-dependant on partial separation of the two strands occurs, and migration of the molecule will practically stop (Sigler *et al.,* 2004).

The presence of G or C bases within the last five bases from the 3' end of primers (G-C clamp) helps promote specific binding at the 3' end due to the stronger bonding of G-C bases. The GC rich sequence in the DGGE procedure acts as a high melting domain by preventing the two DNA strands from complete separation into a single strand (Muyzer *et al*., 1998).

Depending on the sequence and GC content the fragments from the PCR product when loaded on polyacrylamide gel migrates to different positions in the gel (the G-C clamp prevents complete denaturation) (Muyzer *et al.,* 1993).

In a DGGE gel the number, precise position, and intensity of the bands in a gel track give an estimate of the number and relative abundance of numerically dominant ribotypes in the samples (Boon *et al.,* 2001). This approach allows a comparison of different microbial communities. However, the choice of which bands to excise and sequence can be subjective and obtaining sequences from faint bands is often difficult (Øvreas *et al.,* 1997).

The banding patterns of highly diverse microbial communities present in soil, activated sludges and sediments are usually very complex when universal bacterial primers are used (Boon *et al.,* 2001). Furthermore, only the major populations of the analyzed community are represented on these DGGE patterns and thus relatively less abundant but potentially very important species may not be detected by this molecular method (Boon *et al.,* 2001).

1.4.3 Fluorescence in situ hybridization (FISH)

With the introduction of fluorescence in situ hybridization (FISH), it became possible to identify single bacterial cells with labeled oligonucleotide probes targeting the rRNA of selected phylogenetic groups. FISH methods have a few disadvantages which include the limited number of probes as well as low level of phylogenetic resolution, which becomes a distinct blockage. Multiple probes are used in identifying selected target organisms in order to verify false-positive and false-negative results caused by individual probes (Peplies *et al.,* 2003).

Several improvements have been made to increase the sensitivity of FISH, including the use of brighter fluorochromes, image-intensified video microscopy, and hybridization with more than one fluorescently labeled oligonucleotide probe (Fuhrman *et al.,* 1998).
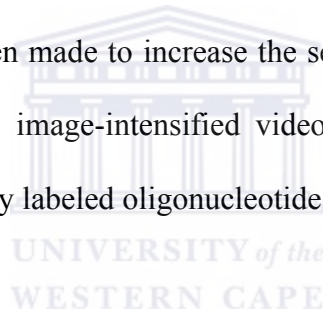
1.5 The objectives of this study

The Great Berg River estuary near Velddrif is an important economic ecosystem, as it is important to understand the diversity of the bacteria involved in the ecosystem. This information will help in monitoring eutrophication and also improve on the fish industry. It was a typical sediment rich estuarine locality, having significant impact from the effect of local human habitation and industrial activity such as fishing and canning (McArthur, 1997).

Understanding the community structure by providing information on the microbial population provides a baseline from which more sophisticated physiological and microbial-interaction studies may be developed. This information demonstrates the degree of species diversity in marine estuarine sediments, and is valuable in highlighting the unique biological characteristics of the biome. Furthermore it provides direct support for biotechnological exploitation (bioprospecting) of the microbial community. A species dataset is generated, which may be used as a composite biomarker for assessing the future ecological of the specific estuary and related biomes.

The aim of the study was to determine the effect of pollution by the effluent of fish industry on the microbial community in the sediment. The diversity of microbial groups in the sediment samples that received wastewater from the local fishing industry was investigated by a PCR-DGGE (polymerase chain reaction-denaturing gradient gel electrophoresis) approach, and compared to an unaffected site. The DGGE method was also applied to determine the relative genetic complexity of microbial communities at different depths in the estuary sediment of the Great Berg River.

This project has two primary objectives:

- To carry out a detailed study of microbial diversity in South African estuarine environment, using modern molecular phylogenetic methods.
- To identify differences between polluted and unpolluted sites.

Chapter 2

2. Methodology and Materials

Table 2.1: Chemical reagents used in this study

Chemicals	Sources
Agar	Merck, Darmstadt, Germany
40% Polyacrylamide/Bis solution 37:1:1	Fluka, Germany
Ammonium peroxodisulpate (APS)	BIO Rad, Munich, Germany
Bromophenol blue	Sigma, Deissenhofen, Germany
Chloroform	Sigma, Deissenhofen, Germany
EDTA	Merck, Darmstadt, Germany
Ethidium bromide	BDH, England
Formamide	Merck, Darmstadt, Germany
Isoamyl alcohol	Merck, Darmstadt, Germany
Isopropanol	Kimix, South Africa
Ethanol	Kimix, South Africa
Polyvinylpolypyrrolidone (PVPP)	Sigma, Deissenhofen, Germany
Sodium Dodecyl Sulphate	BDH, England
Urea	BIO Rad, Munich, Germany
Tris	Fluka, Germany
Methanol	Kimix, South Africa
TEMED	BIO Rad, Munich, Germany
Ammonium sulphate	Merck, Darmstadt, Germany
Ammonium acetate	Sigma, Deissenhofen, Germany
IPTG	Fermentas, Vilnius, Lithuania
X-Gal	Fermentas, Vilnius, Lithuania
Ampillicin	Fluka, Germany
Magnesium chloride	Saarchem, South Africa
Sodium phosphate	Fluka, Germany
Sephacryl S-500	Pharmacia, Sweden
Betaine	Sigma, Deissenhofen, Germany
DSMO	RDH, Germany
Triton X-100	BDH, England
Deoxynucleotide triphosphate	Inqaba, Pretoria, South Africa
Orange G loading buffer	Promega, Madison, Wis. USA
Agarose	Bioline, England
Sodium chloride	Saarchem, South Africa
Quartz sand	Kimix, South Africa
Potassium chloride	Sigma, Deissenhofen, Germany
Tryptone	Fluka, Germany
Yeast extract	Merck, Darmstadt, Germany
Enzymes	
Taq DNA polymerase	In_house prep. ARCAM.
Pst1	Fermentas, Vilnius, Lithuania
Kits	
InsT/clone PCR Product cloning kit	Fermentas, Vilnius, Lithuania

Table2. 2: Media used in this study

LB medium (Luria-Bertani Medium)

Constituent	Concentration(g/l)
Tryptone	10.0
Yeast extract	05.0
NaCl	10.0
Agar	15.0

Dissolved in 1 L of ddH$_2$O, and pH adjusted to pH 7.0 with 5 N NaOH

SOB medium

Constituent	Concentration (g/l)
Tryptone	20.0
Yeast extract	05.0
NaCl	0.50
250 mM KCl	0.1864

Dissolved in 1 L of dH$_2$O, and before autoclaving pH adjusted to 7.0. The medium was filtering sterilized, and 5.0 ml 2 M MgCl$_2$ was aseptically added.

SOC medium

Constituent	Concentration (g/l)
Tryptone	20.0
Yeast extract	05.0
NaCl	0.50
250 mM KCl	0.1864

The pH was adjusted to 7.0 before autoclaving. The medium was cooled to 50ºC and 5 ml of 2 M $MgCl_2$ and 20 ml of 1 M glucose filter sterilized were aseptically added.

Table 2.3: List of buffers that were used in this study

Buffer	Components	pH
6x agarose loading buffer	30% (v/v) Glycerol	
	0.25% (w/v) Bromophenol blue	
	15% (w/v) Ficoll Type 400	
10x Orange loading buffer	60% Glycerol	
	0.25% (w/v) Orange G	
Inoue	55 mM $MnCl_2.4\ H_2O$	6.7
	15 mM $CaCl_2.2\ H_2O$	
	250 mM KCl	
	10 mM piperazine-1,2-bis	
	0.5 M PIPES	
50x TAE	2 M Tris base (w/v)	8.0
	10 mM Glacial acetic acid (w/v)	
	0.5 M EDTA	
0.5x TBE	45 mM Tris-borate	8.3
	1 mM EDTA	
TE	10 mM Tris-HCl (pH 8.0)	8.0
	1 mM EDTA (pH 8.0)	

2.1 Study site and sample collection

Sediment samples were collected from the Great Berg River estuary, near Veddrif, in the Western Cape, South Africa. Velddrif is situated on the north-eastern shore of the Great Berg River about 2 km from the inlet into St. Helena Bay. The Great Berg River (18˚0.19'E, 32˚0.55'S), is a tidal river for many kilometers inland. The effluent flow from the downstream site (site B) of the fish factory in the estuary can cause pollution in the estuarine sediment (Fig. 2 1 A). The fish factory is adjacent to the downstream sampling sites. This sampling site is selected because it is had a significant impact from the effects of local habitation (Velddrif town) and industrial activity (fishing and canning). The two sampling sites at the Great Berg River are shown in Fig.2 1 B and Fig. 2 1 C.

(a)

Fig. 2.1 A: Great Berg River estuary near Velddrif in the Western Cape, SA.

(b)   **(c)**

Fig. 2.1 B-C: The sediment samples in this study were collected from two different areas: site A (upstream) and B (downstream) shown above.

Sediment cores (30 cm in length, 5 cm in diameter) were collected at low tide in November 2004. Site A was located opposite the 'Fishing industry' upstream of a visible wastewater outlet (i.e. unpolluted region) while site B was located adjacent the 'Fishing industry' downstream of this outlet (i.e., polluted region) (Table 2.4). For the analysis of the microbial communities, three replicate cores were collected from each sampling site. Samples were collected by taking sliced sediment cores at the following depth intervals: 0 cm, 5 cm, 10 cm, 15 cm, 20 cm, 25 cm, and 30 cm. Sediments were extruded into sterile plastic bags, transported on ice and stored at –80°C for further molecular biological investigation.

Table 2.4: Sediment samples collected from the Great Berg River estuary

Study site	Core	Sample no.	Depth (cm)	Date
Unpolluted	**4A**	28	0-5	**11-04**
		29	0-5	
		31	5-10	
		32	5-10	
		35	10-15	
		36	10-15	
		38	15-20	
		39	15-20	
		40	20-25	
		41	20-25	
		42	20-25	
		44	25-30	
		45	25-30	
	5A	47	0-5	
		48	0-5	
		50	5-10	
		51	5-10	
		53	10-15	
		54	10-15	
		55	15-20	
		57	15-20	
		58	20-25	
		59	20-25	
		62	25-30	
		63	25-30	
	6A	65	0-5	
		66	0-5	
		68	5-10	
		69	5-10	
		70	10-15	
		71	10-15	
		73	15-20	
		74	15-20	
		76	20-25	
		77	20-25	
		81	25-30	
		80	25-30	

Table 2.4: (Continue) Sediment samples collected from the Great Berg River estuary

Study site	Core	Sample no.	Depth (cm)	Date
Polluted	**4B**	109	0-5	
		110	0-5	
		113	5-10	
		114	5-10	
		116	10-15	
		117	10-15	
		119	15-20	
		120	15-20	
		122	20-25	
		123	20-25	
		125	25-30	
		126	25-30	
	5B	92	0-5	
		93	0-5	
		95	5-10	
		96	5-10	
		98	10-15	
		99	10-15	
		100	15-20	
		101	15-20	
		104	20-25	
		105	20-25	
		107	25-30	
		108	25-30	
	6B	146	0-5	
		147	0-5	
		149	5-10	
		150	5-10	
		152	10-15	
		153	10-15	
		155	15-20	
		156	15-20	
		158	20-25	
		159	20-25	
		161	25-30	
		162	25-30	

2.2 Sediment organic matter content analysis

Sliced, homogenized sediment samples from downstream and upstream regions of the Great Berg River estuary were analysed for organic matter content. To determine organic matter and water content, 15 g of wet sediment was placed in porcelain crucibles at 70°C

for 72 h. After 72 h, the dried samples were re-weighed. Organic matter content was measured in the dried sediment samples. Samples were placed in the marked, pre-weighted porcelain crucibles in an oven and burnt at 500°C for 5 h. After cooling in a desiccator, the samples were re-weighed in the porcelain crucibles containing the sediments. The organic content and water content were calculated using the following methods (equations):

➢ Equation 1: H_2O content = wet sediment – dry sediment.

➢ H_2O % = H_2O content / wet sediment x 100.

➢ Equation 2: Organic matter content = dry sediment – burnt sediment**.**

➢ Organic % = organic matter content / dry sediment x 100.

The average deviation and mean values for two different samples were calculated for each variable using the formula $1/n\sum|x-\bar{x}|$, where n is number of sediment samples at specific depth, x is the absolute values for each sample and $\bar{x}$ is the average value for sample measurements. The average deviation is measure of variability in the data set that returns the average of the absolute deviations of the data values from their mean value.

2.3 Extraction and purification of total DNA from sediment samples

Total genomic DNA was extracted from sediment samples according to Miller *et al.,* (1999), with modifications. After thawing, 1 g of the sample was weighed in a 2 ml screw-capped tube containing 500±10 mg of quartz sand (Sigma S-9887). 300 µl each of 100 mM Na_2HPO_4 pH 8 and SDS lysis buffer (100 mM NaCl, 500 mM Tris pH 8, 100 g/l

SDS) were added to the tube and mixed by inverting. A volume of 300 µl of chloroform/isoamyl alcohol (24/1, v/v) was added to the sample before vortexing for 120 s at maximum speed, and the cell debris were pelleted by centrifugation (15,000 x g, 5 min). To the supernatant, ammonium acetate (7 M) was added to a final concentration of 2.5 M, and then the tubes were shaken by hand to mix and centrifuged (15,000 x g, 10 min). The DNA was precipitated from the supernatant by addition of 0.6 volumes of isopropanol, incubation at room temperature for 15 min and centrifugation (15,000 x g, 10 min). The pellet was desalted with 1 ml of 70% EtOH, air-dried and dissolved in 80 µl of TE (10 mM Tris, 1 mM EDTA, pH 8).

2.4 DNA purification

The DNA extracted using the modified Miller method was further treated by the following 2-step purification procedure:

Step 1: *PVPP purification*

Self-constructed mini-columns packed with polyvinylpolypyrrolidone (PVPP) were used for the removal of humic substances (Berthelet *et al.,* 1996). Briefly, 400 µl of a 100 g/l PVPP suspension (in TE) were loaded on a 20-µl pipette filter tip (centrifugation, 150 x g, 2 min). The mini-column was washed twice with 150 µl of TE, and then dried (600 x g, 10 min). The DNA sample was applied and after 2 min incubation, eluted by centrifugation (600 x g, 5 min, followed by 1700 x g, 10 min).

Step 2: *Sephacryl purification*

A gel filtration chromatography matrix, also packed in mini-columns, was applied to remove small fragments of nucleic acids. 400 μl of Sephacryl S-500 HR suspension (Amersham Biosciences 17-0613-10) was loaded on a 20-μl pipette filter tip (600 x g, 2 min), and the column was washed three times with 150 μl of TE (centrifugation after third time, 800 x g, 3 min). The DNA ($\leq$ 50 μl) was applied and eluted by centrifugation (600 x g, 2 min).

2.4.1 DNA Quantification

The concentration (calculated as OD_{260} x 50 ng/μl) and purity (ratio OD_{260}/OD_{280}) of the processed DNA were determined using a NanoDrop ND-1000 spectrophotometer.
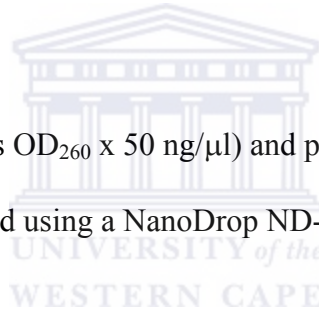
2.5 Agarose gel electrophoresis

Total genomic DNA fragments and PCR products were separated in 1.5% and 2% (w/v) agarose gels, respectively, prepared in 0.5x TBE buffer (Sambrook *et al.,* 2001). Samples were prepared by mixing with 6x concentrated loading buffer (0.2 ml/ml glycerol and 5 mg/ml bromophenol). Electrophoresis was performed in 0.5x TBE buffer (Table 2.3) at 100 V. Ethidium bromide (0.5 μg/ml) was added to the agarose gel during the preparation for the staining procedure. The DNA bands were sized according to their migration in the

gel as compared to the DNA molecular weight markers (e.g. λ DNA cut with *Pst*1 restriction enzyme). Gels were visualized using ultraviolet (UV) illumination and photographed with a digital imaging system (AlphaImager 2000, Alpha Innotech, San Leandro, CA).

2.6 DNA Amplification by Polymerase Chain Reaction (PCR)

PCR amplifications were performed in 0.2-ml thin-walled tubes using a thermocycler equipped with heated lid (PCR Sprint Temperature Cycling System, Thermo Hybaid, Ashford, GB). A standard 50 μl PCR reaction solution contained 1×PCR amplification buffer [10x buffer being 200 mM Tris pH 8.8, 100 mM KCl, 100 mM $(NH_4)_2 SO_4$, 20 mM $MgSO_4$, 1% (w/v) Triton X-100], 0.2 mM each of dATP, dCTP, dGTP and dTTP, 0.5 μM of each primer, an appropriate amount of *Taq* DNA polymerase, and 1-5 ng of metagenomic DNA as the template. The primers used for the amplification of 16S rDNA of bacteria and archaea are listed in Table 2.5.

After an initial heating step at 94°C for 4 min, a total of 30 PCR cycles were run under the following conditions: denaturation at 94°C for 30s, primer annealing at 50°C for 30s and extension 72°C for 1 min 30s and a final extension at 72°C for 10 min. The primer pair used (forward primer, E9F (McInerney *et al.,* 1995); reverse primer, U1510R (Reysenbach *et al.,* 1995) amplifies the nearly complete 16S rRNA gene from the majority of bacterial organisms (Baker *et al.,* 2003) and was therefore considered suitable

to test the quality of extracted sediments DNA. Amplified products were detected on 1% agarose gels electrophoresed in 0.5x TBE buffer, stained with ethidium bromide and photographed on an UV transilluminator.

A pair of "universal" bacterial primers, 341F-GC and 534R, was used for DGGE analyses in this study (Table 2.5). PCR amplification was done with the following program: 94^{o}C denaturing for 4 min followed by 20 cycle of 94^{o}C for 30s, annealing at 55^{o}C for 30s, extension at 72^{o}C for 2 min; followed by 20 cycles of denaturation at 94^{o}C for 30 s, annealing at 55^{o}C for 30 s, extension at 72^{o}C for 2 min; followed by 72^{o}C for 10 min. The presence of each PCR product was verified by analyzing 5µl of product on a 2% agarose gel and staining with ethidium bromide.

A DGGE analysis of archaeal 16S rDNA sequences was carried out using a nested PCR technique. In the first round, the archaeal 16S rDNA fragments were amplified using the following primers; A3Fa and A927R (Table 2.5). During the second PCR round, the fragments obtained were reamplified by using the Archaea primers 340F-GC and 533R. The amplification mixture contained the same concentration of components as in the bacteria mix. PCR thermocycling conditions for first round amplification were: 94^{o}C denaturing for 4 min followed by 5 cycle of 94^{o}C for 30s, 55^{o}C for 30s, 72^{o}C for 1 min; followed by 30 cycles of 94^{o}C for 30 s, 55^{o}C for 30 s, 72^{o}C for 1 min; followed by 72^{o}C for 10 min. Second round amplification PCR thermocycling conditions were: 94^{o}C for 4 min; followed by 5 cycles of 94^{o}C for 30 s, 53.5^{o}C for 30 s, 72^{o}C for 1 min; followed by

30 cycles of 94^{o}C for 30s, 53.5^{o}C for 30s, 72^{o}C for 1 min; followed by 72^{o}C for 10 min. For the first PCR round, 1 μl of extracted DNA was added to 49 μl of PCR master mix, and in the second round, 1 μl of amplified product from the first round was added to 49 μl of PCR mixture. After each PCR amplification round, the size of PCR product was verified on a 2% agarose gel.

Table 2.5: Specific primes used for the amplification of 16S rRNA genes of bacteria and archaea polymerase chain reaction

Primers	Primer Sequence (5'-3')	Position (16S rRNA gene)	Annealing temp.	Specificity	Reference
E9F	GAGTTTGATCCTGGCTCAG	9-27	50˚C	Universal	McInerney et al., 1995
U1510R	GGTTACCTTGTTACGACTT	1510-1492	50˚C	Universal	Reysenbach et al., 1995
341F-GC[a]	CCTACGGGAGGCAGCAG	341-357	55˚C	Bacteria	Muyzer et al., 1993
534R	ATTACCGCGGCTGCTGG	534-518	55˚C	Bacteria	Muyzer et al., 1993
A3Fa	TCCGGTTGATCCYGCCGG	3-20	55˚C	Archaea	McInerney et al., 1995
A927R	CCCGCCAATTCCTTTAAGTTTC	927-906	55˚C	Archaea	Jurgens et al., 1997
A340F-GC[a]	CCCTACGGGGYGCASCAG	340-357	53.5˚C	Archaea	Ovreas et al., 1997
A533R	TTACCGCGGCKGCTG	533-519	53.5˚C	Archaea	Ovreas et al., 1997
M13F	GTAAAACGACGGCCAGT	598-615	50˚C		Yanisch-Perron et al., 1985
M13R	CAGGAAACAGCTATGAC	734-751	50˚C		Yanisch-Perron et al., 1985

[a] GC clamp added to the primers' 5' end: CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG

2.6.1 Colony PCR

16S rRNA gene clones obtained from the clone library was screened to identify those containing an insert of the expected size. The colonies were picked randomly and

suspended in 40 µl 1 x TE buffer, then boiled at 99˚C for 3 min, followed by centrifuging at maximum speed (16,000 xg for 2 min). The supernatant was transferred to a new 0.6 ml Eppendorf tube, and later used as a template in PCR amplification, (10 µl in a standard PCR reaction).and then stored at -20˚C.

2.7 Preparation of E. coli competent cells

Competent *E. coli* DH5α cells were prepared and transformed according to Inoue *et al.* (1990). A single colony of *E. coli* DH5α grown on SOB medium (2 g Bacto-Tryptone, 0.5 g Bacto-Yeast extract, 0.05 g NaCl and 250 µl 1 M KCl in 100ml, pH 7.0, 1 ml of sterile 1 M MgCl$_2$) was inoculated into 20 ml SOC medium (Table 2.2) and cultured for 8 h at 37˚C with agitation at 250 rpm. 2 ml of the culture was inoculated into a 1 L sterile flask at 18ºC and incubated overnight with shaking to mid-exponential phase (OD$_{600}$ of 0.4 to 0.55). The cells from 250 ml of the culture were pelleted in polypropylene tubes by centrifugation at 25,000 x g for 10 min at 4˚C in a J2-21M rotor (Beckman-USA). The supernatant was decanted and then the pellet was washed twice with Inoue buffer. The cells were resuspended gently in 2 ml of ice-cold Inoue buffer (Table 2.3), 150 µl of DMSO was added and the cell suspension incubated on ice for 15 min. The cells were then aliquoted (into 0.5 ml Eppendorf tubes (50 µl)) and stored at -80˚C until needed.

2.8 Ligation of DNA fragments

Ligation reactions were performed in a 10 µl assay and contained the vector (pTZ57R/T) and insert DNA (molar ratio, 1: 3), 1x ligation buffer, 1 U of T4 DNA ligase, 5% of PEG 4000 and sterile water (InsT/Aclone kit(1214)-Fermentas), according to the manufacturer's instructions. The mixtures were centrifuged for 5 sec. The reactions were incubated overnight at 16˚C. Reactions products were transformed into *E. coli* DH5α competent cells using the heat-shock method (Sambrook *et al.,* 2001).

2.9 Transformation of competent E. coli DH5α cells

The *E. coli* DH5α competent cells were transformed by the addition of 2 µl ligated DNA to 50 µl of the competent cells in sterile 1.5 ml Eppendorf tube. The cells were incubated on ice to thaw for 10 min prior to the transformation, followed by heat shock at 42˚C for 90 sec, then incubated on ice for further 2 min before addition of 950 µl of SOC medium to allow the recovery of the cells. The transformation mixtures were incubated for 1 h at 37˚C with shaking at 200-250 rpm. An aliquot of 100 µl of the recovered cells were plated on LB agar (Table 2.2) plates supplemented with ampicillin (100 µg/ml), IPTG (20 µg/ml), and X-Gal (30 µg/ml) for blue and white selection. The agar plates were incubated overnight at 37˚C for observation of the presence of transformed *E. coli* colonies.

2.10 Cloning of 16S RNA gene

The PCR amplicons were ligated into the pT57R/T vector (Fermentas), and further transformed into *E. coli* DH5α competent cells, according to the manufacturer's instructions. Transformed cells were plated onto LB (Luria Bertani) Agar supplemented with X-GAL (5-bromo-4-chloro-3-indolyl-B-D-galactopyranoside) and IPTG (iso-propyl-B-thiogalactopyranoside) and antibiotic ampicillin. White colonies were randomly picked, and plasmid insert DNA was amplified by PCR using an Archaea-specific 16S rDNA gene primer set. Amplicons were visualized on 1.5% agarose electrophoresis gels.

2.11 Denaturing gradient gel electrophoresis (DGGE)

DGGE plates were cleaned thoroughly with methanol then twice with ethanol to remove all traces of grease before assembly.

High-denaturant and low-denaturant solutions were prepared. High denaturant solution was prepared according to Table 2.6. A total final volume of 5 ml in a 50 ml Falcon tube was prepared. 4 μl of TEMED, and 40 μl of 10% APS were added. The mixture was poured on the plate and 1000 μl butanol added. The gel was allowed to polymerise for at least 30 min. Finally, the butanol was removed and the plates were rinsed three times with sterile water.

Table 2.6: Reagents prepared for 20 ml of 9% polyacrylamide gel at various denaturant (UF) concentrations

UF denaturant	30%	40%	60%	65%
40% Acrylamide	4.5 ml	4.5 ml	4.5 ml	4.5 ml
50x TAE	400 µl	400 µl	400 µl	400 µl
Urea	2.52 g	3.36 g	5.04 g	5.46 g
Formamide	2.4 ml	3.2 ml	4.8 ml	5.2 ml

DGGE, based on the protocol of Muyzer *et al.,* (1998) was performed using the Scie-Plas (V20) system. 18 µl of purified PCR products of the second round were loaded onto 16.5x16.5 cm, 1 mm thick 9% polyacrylamide gels (Acrylamide/bis 37.5:1) in 1 x TAE (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA [pH adjusted to 8 with acetic acid]). The polyacrylamide gels were prepared with denaturing gradients of 30-55% (for bacteria), and 30-65% (for archaea) (100% denaturants being 7M urea and 40% formamide); the gels were run in 1 x TAE buffer at a constant voltage of 100 V and a temperature of 60^{o}C for 16 h. After electrophoresis, the gels were stained for 15 min in ethidium bromide and distained in 1 x TAE, and visualized on a the AlphaImager 3400 imaging system equipped with the Alpha-Ease FC image processing and analysis software (Alpha Innotech, San Leandro, CA).

Selected unique DGGE bands were carefully excised from the gel, purified, reamplified with the same primers (A340F-GC and A533R), cloned and sequenced using the techniques described above. The gel fragment was suspended in 500 µl of water and vortexed followed by centrifugation at 16,000 rpm for 2 min. The supernatant was

discarded and gel slices were resuspended in 50 µl of TE buffer and incubated overnight to elute DNA. 1 µl of supernatant was used as a template for re-amplification using the same PCR conditions as previously described. The bands were reamplified and loaded again on the DGGE system to ensure purity, to verify the position with the original band and to correct mobility within the gels. Re-amplified bands were cloned into pTZ57R/T (InsT/Aclone, Fermentas) according to the manufacturer's instructions and sequenced with M13 primers (Table 2.5).

2.12 Nucleotide sequence determination and analysis

DNA sequencing was done by Inqaba Biotechnology Industries (Pretoria, SA). M13 forward and reverse oligonucleotide primers (Table 2.5) were used to determine the nucleotide sequences of the cloned 16S rRNA genes in this study. BLAST analysis of the sequences obtained was carried out using the server of the National Center for Biotechnology Information (NCBI).

2.13 Statistical analyses of the DGGE's

DGGE statistical analysis was performed using the Alpha-Ease FC image processing and analysis software (Alpha Innotech, San Leandro, CA). The presence and absence of the DGGE band patterns in the sediment samples determined the similarity matrices between

the two sample sites and within the sediments cores. The cluster analysis by dendrogram was calculated using the Dice coefficient methods and UPGMA (Unweighted Pair-Group Method with Arithmetic Mean), which then revealed the differences in the microbial communities between the depths of both sampling sites.
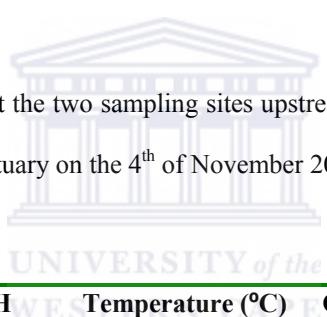
Chapter 3

3. Results

3.1 Description of the sampling site

Sediment samples were collected upstream and downstream from a fish factory at the Great Berg River estuary, near Velddrif in the Western Cape, South Africa. Table 3.1 describes the characteristics of the water at the time of sediment sampling on the 4[th] of November 2004.

Table 3.1: The water characteristics at the two sampling sites upstream (site A) and downstream (site B) a fish factory at the Great Berg River estuary on the 4[th] of November 2004.

Location	Conductivity (mS/cm)	pH	Temperature (°C)	Oxygen content (mg/L)	NaCl content (‰)
SITE A	59.0	6.8	16.3	6.1	
	59.5	6.8	16.4	6.0	
	59.7	6.8	16.5	6.0	
Average	**59.4**	**6.8**	**16.4**	**6.0**	**35**
SITE B	47.0	7.1	22.1	4.9	
	46.0	7.0	22.5	5.1	
	49.0	7.0	22.2	5.3	
Average	**47.3**	**7.0**	**22.2**	**5.1**	**30**

Site A was sampled in the morning and site B in the afternoon. The water was warmed-up by the intense sun in-between. Sea water has a salt content of approximately 3.5‰[1]. The measured values therefore indicate that the Great Berg River's fresh water is highly mixed with sea water at the sampling sites. At site B lower conductivity was observed due to the higher temperature and slightly lower salt content. The higher salt content upstream is surprising, it could be the result of complex water currents within the estuary due to the tides (sampling site B is at the opposite shore of site A). The lower oxygen content at site B is probably due to the higher temperature measured in the afternoon, but it could also be an indication of a higher content of organic material downstream of the fish factory.

3.2 Organic matter content of the sediment samples from the Great Berg River estuary

Organic matter content of sediments is likely to influence the composition and activity of the microbial community. Since the fish factory effluents could be a major source of organic matter, sediment samples of different depths from both the upstream and downstream sites were analysed (Table 3.2).

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[1] http://en.wikipedia.org/wiki/Sea_water

Table 3.2: Water and organic matter contents in the sediment cores (0-30cm depth) from the two sampling sites upstream (A) and downstream (B) the fish factory.

Sample no.	Depth (cm)	H_2O content (%)	Organic matter (%)
4A # 14	0-5	23.1	0.9
4A # 15	0-5	22.0	0.9
Average		**22.6%**	**0.9%**
4A # 16	5-10	21.4	1.3
4A # 17	5-10	21.8	1.1
Average		**21.6%**	**1.2%**
4A # 18	10-15	23.8	1.6
4A # 19	10-15	25.2	1.9
Average		**24.5%**	**1.8%**
4A # 20	15-20	29.5	2.1
4A # 21	15-20	30.3	2.7
Average		**29.9%**	**3.5%**
4A # 22	20-25	27.9	4.4
4A # 23	20-25	27.5	2.4
Average		**27.7%**	**3.4%**
4A # 24	25-30	29.3	2.6
4A # 25	25-30	28.4	2.7
Average		**28.9%**	**2.7%**
4B # 1	0-5	29.1	3.5
4B # 2	0-5	27.6	3.3
4B # 3	0-5	27.0	3.1
Average		**27.9 %**	**3.3%**
4B # 4	5-10	31.8	4.6
4B # 5	5-10	29.9	3.5
Average		**30.9%**	**4.1%**
4B # 6	10-15	27.4	4.5
4B # 7	10-15	26.2	3.0
Average		**26.8%**	**3.8%**
4B # 8	15-20	24.9	2.2
4B # 9	15-20	25.3	1.9
Average		**25.1%**	**2.1%**
4B # 10	20-25	30.7	3.8
4B # 11	20-25	30.5	3.0
Average		**30.6%**	**3.4%**
4B # 12	25-30	34.7	3.6
4B # 13	25-30	33.1	3.3
Average		**33.9%**	**3.5%**

A comparison between the two sites shows that on average the downstream site B contains more organic matter than the upstream site A, with the biggest differences in the top 15 cm (Fig. 3.1). This could be due to the deposition of organic material from the fish

factory wastewater. Deeper layers are less affected, because organic material from the wastewater sediments on the surface.
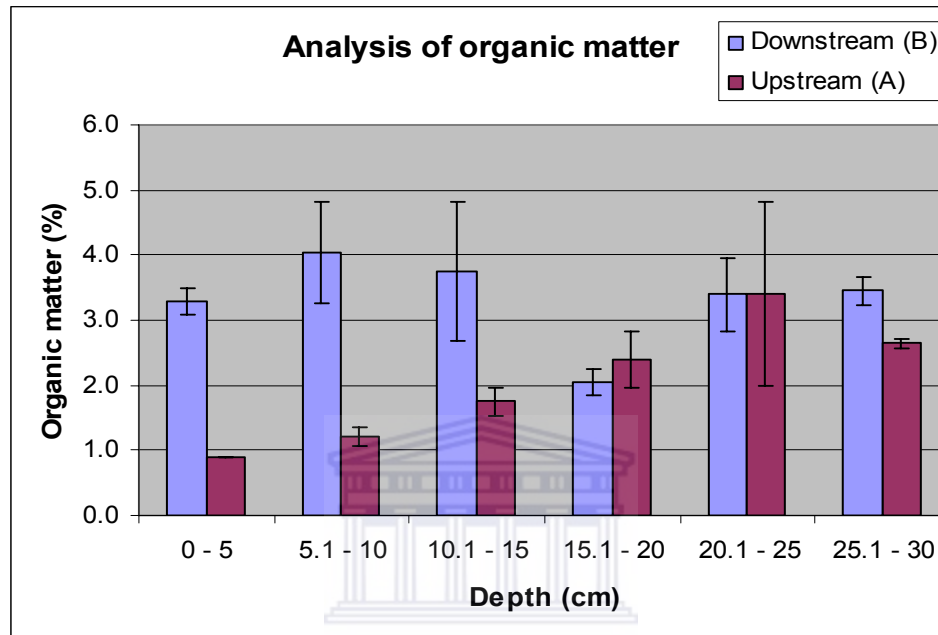
Fig. 3.1: Comparison of the sediment mean organic matter content at different depths. The mean values were calculated from two sampling sites and the error bars indicate the average deviation from the mean values (refer to section 2.2).

3.3 Sediment DNA extraction and purification

In order to obtain high quality DNA (amplifiable by PCR) from the sediment samples, the DNA extraction was combined with several purification steps. The developed procedure, illustrated in Fig. 3.1, is described in detail in section 2.3.

```
┌─────────────────────────────┐
│      Sediment samples       │
└─────────────────────────────┘
              │
              ▼
┌─────────────────────────────┐
│        DNA extraction       │
└─────────────────────────────┘
              │
              ▼
┌─────────────────────────────┐
│        PVPP column          │
└─────────────────────────────┘
              │
              ▼
┌─────────────────────────────┐
│      Sephacryl column       │
└─────────────────────────────┘
              │
              ▼
┌─────────────────────────────┐
│         Clean DNA           │
└─────────────────────────────┘
```
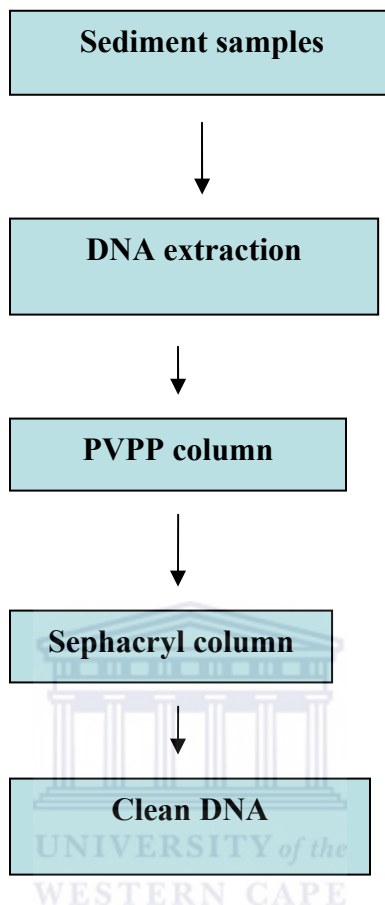
Fig. 3.2: Procedure for the extraction and purification of DNA from estuarine sediment.

The yields from the sediment samples obtained with the modified Miller method were consistent and good, typically between 15 and 60 µg of crude DNA per g of sediment (Fig. 3.3).
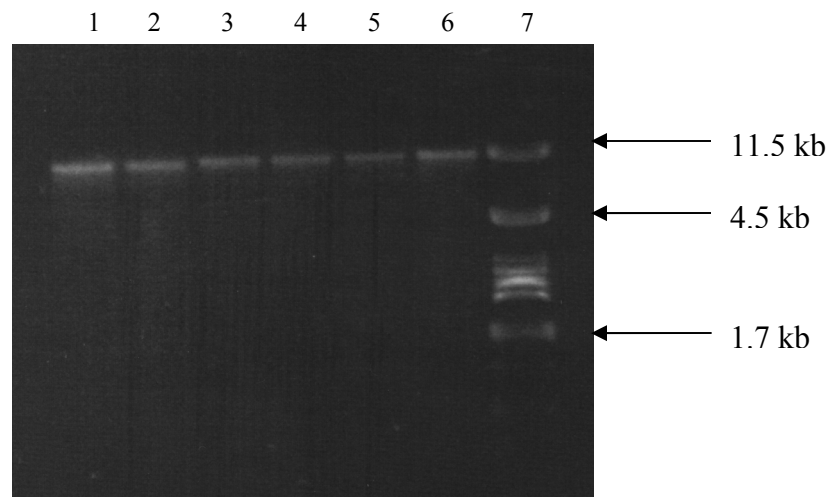
Fig. 3.3: Agarose gel (1%) of total DNA extracted from the sediment samples using the modified Miller method. Lanes: 1, DNA sample # 1; 2, DNA sample # 2; 3, DNA sample # 3; 4, DNA sample # 4; 5 and 6, DNA samples # 5 and 6; 7, λ-DNA molecular marker (λ-DNA cut with *Pst*I restriction enzyme). From all the samples 5 μl of DNA was loaded.

In the Miller method, the cells are mechanically disrupted by vortexing for 2mins. In order to select an extraction method the bead beating was compared vortexing during the modified Miller method. The quality of DNA obtained differed strongly with the method used for the DNA extraction (Fig. 3.4). Vortexing (Fig. 3.4 B) yielded DNA of high molecular weight, whereas the bead beating (Fig. 3.4 A) resulted in strong shearing, which can negatively affect downstream applications. Based on the good yield and high molecular weight of the metagenomic DNA obtained, the modified Miller method (Fig. 3.4 B) with vortexing was selected for use.
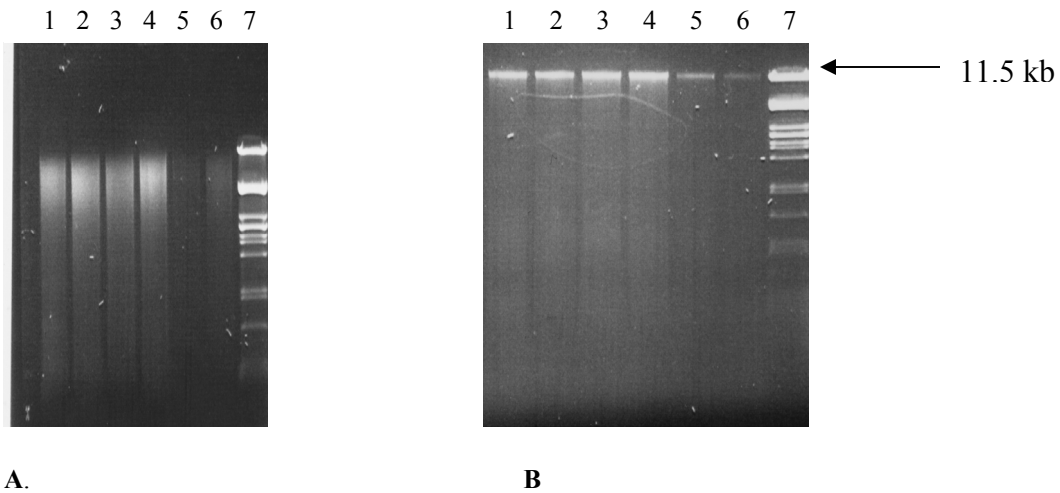
Fig. 3.4: Comparison of DNA extractions from sediment samples using vortex (B) and Bead Beater (A), respectively, during the modified Miller method. Lanes: 1, DNA sample # 7 (depth, 0-25cm); 2, DNA sample # 8 (depth, 25-50); 3, DNA sample # 9 (depth, 50-75); 4, DNA sample # 10 (depth, 0-25 cm); 5, DNA sample # 11 (depth, 25-50 cm); 6, DNA sample # 12 (depth, 50-75 cm); 7, λ-DNA molecular marker (λ-DNA cut with *Pst*I restriction enzyme)..

Fig. 3.5 shows DNA extracted using the Miller method, followed by different purification steps. During the PVPP and Sephacryl treatments, the DNA loss was minimal. However, after the ethanol precipitation (lane 5), we observed more than 50% loss of DNA. Therefore, it was decided not to apply the ethanol treatment in future experiments. The PVPP and Sephacryl treatment consistently yielded high quality DNA, which is important for the following PCR amplification experiments.
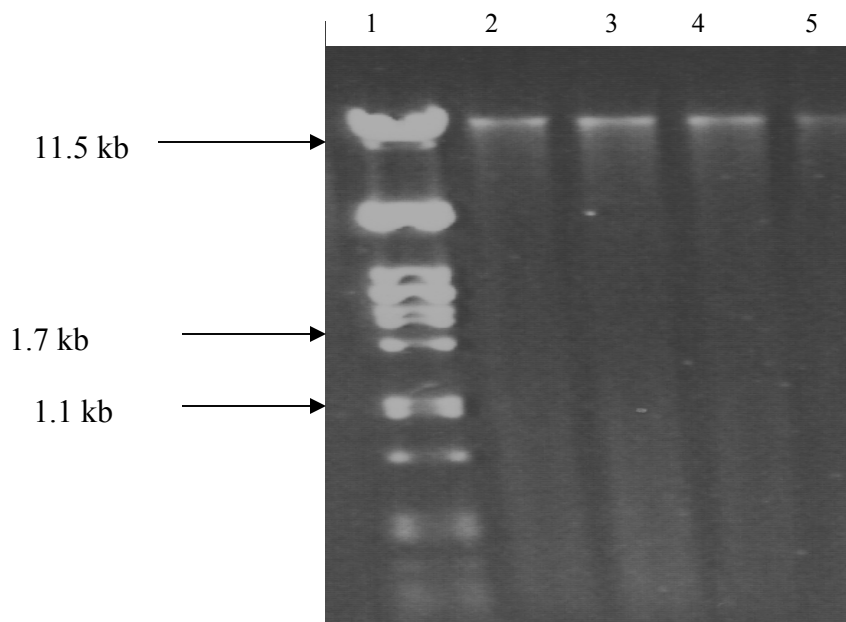
Fig. 3.5: Metagenomic DNA of estuarine sediment samples after different purification steps viewed on a 1% agarose gel (Sample 8 (depth, 25 –50 cm) upstream site). Lanes: 1, λ-DNA molecular marker (λ-DNA cut with *Pst*I restriction enzyme); 2, Crude DNA; 3, After PVPP; 4, After Sephacryl; 5, After ethanol precipitation. 5 µl of DNA was loaded in each lane.

Fig. 3.6 shows purified metagenomic DNA from different depths of one sampling core from the downstream site. Three extractions did not yield any DNA, but they were successfully repeated later. The samples from the deeper depths (lanes 10-12) yielded lower DNA concentrations compared to the surface samples. This suggests that the biomass in the sediments decreases with depth.
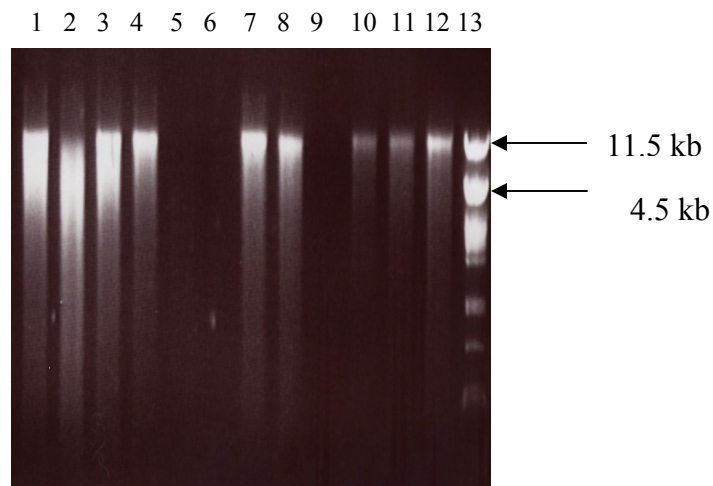
Fig. 3.6: Metagenomic DNA from sediment samples after PVPP and Sephacryl purification viewed on 1% Agarose gel. Lanes: 1, Sample # 92 (depth, 0-5 cm); 2, Sample # 93 (depth, 0-5 cm); 3, Sample # 95 (depth, 5-10 cm); 4, Sample # 96 (depth, 5-10 cm); 5, Sample # 98 (depth, 10-15 cm); 7, Sample # 99 (depth, 10-15 cm), 8; Sample # 100 (depth, 15-20 cm); 9, Sample # 101 (depth, 15-20 cm); 10, Sample # 104 (depth, 20-25 cm); 11, Sample # 105 (depth, 20-25 cm); 12, Sample # 107 (depth, 25-30cm); Lane 13, λ-DNA molecular marker (λ-DNA cut with *Pst*I restriction enzyme).

3.4 PCR amplifiability of the metagenomic DNA

To check if the extracted DNA was suitable for PCR amplification of microbial community DNA, preliminary PCR experiments using "universal" bacterial primers (E9F and U1510R) were performed. In addition, the effect of the amount of DNA used as a template was determined. This experiment indicated that the extensive purification procedure described in Fig. 3.2 was necessary to obtain high quality DNA.

Fig. 3.7 shows that for samples from both sites, the PCR was successful using 1 ng or 5 ng of DNA as a template, but not with 15 ng. The PCR with the homogenised core (HC, lane 7 and 8) from both the upstream and downstream sites were also successful. A nested PCR approach was used for archaeal community analysis. After the first round of PCR amplification (using the A3Fa and A927R primers) no products were observed when analysed on agarose gels, suggesting that the Archaea may comprise a small fraction of the sediment microbial community. Strong PCR bands however were observed after the second round of the nested PCR using the primers A340F-GC and A533R. According to the results of this experiment, 5 ng of metagenomic DNA was used in all future PCR experiments.
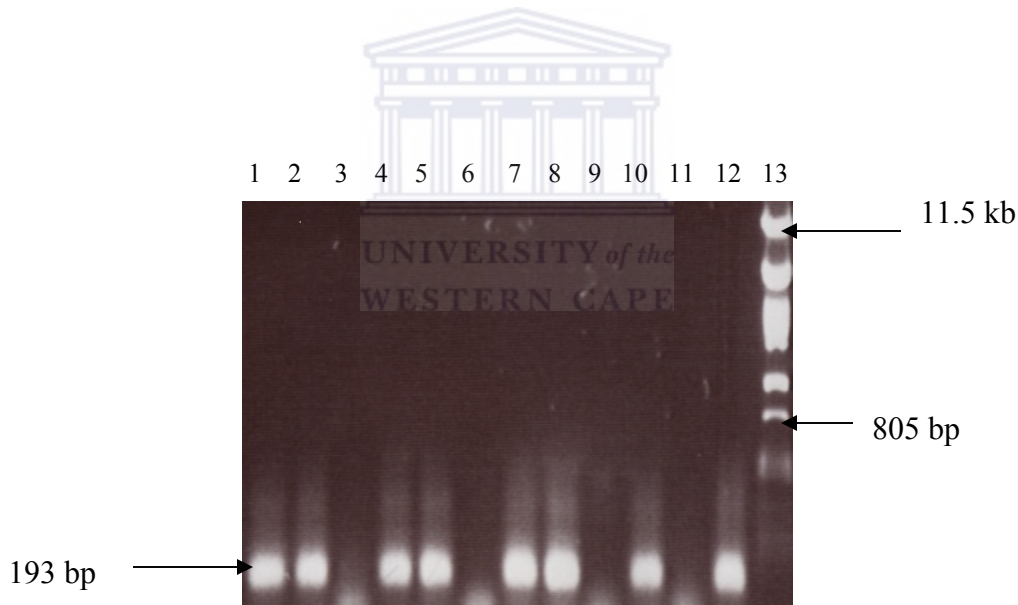
Fig. 3.7: PCR with Archaea-specific primers (second round) obtained from sediments samples (depth, 20-25) from the homogenised core. Lanes 1-3, sample # 27 (1ng/ 5ng/ 15ng); 4-6, sample # 36 (1ng/ 5ng/ 15ng); 7, sample # 27 (HC), 8, sample # 36 (HC); 9, Negative control; 10, methanogen sample; 11, *E.coli;* 12, Positive control (environmental sample); Lane 13, λ-DNA molecular marker (λ-DNA cut with *Pst*I restriction enzyme).

3.5 Time-travel experiments with bacterial and archaeal primers

Time-travel experiments are performed in order to establish the optimum conditions under which the DGGE should be run.

The time-travel experiments were performed with mixtures of amplicons from three 16S rRNA gene clones obtained from the estuary samples. The mixtures of bacterial and archaeal fragments, respectively, were loaded 22 times every 30 min (starting from the right side of the gel). The gel was run at 150V and therefore the samples between 75 and 1650Vh. The time-travel experiment shows the progress of separation of the mixed fragments. The minimal runtime of a DGGE is reached when all samples are stationary. From the obtained results it was concluded that this was the case after a runtime of 1600Vh, which was then used in all future experiments.

A   B
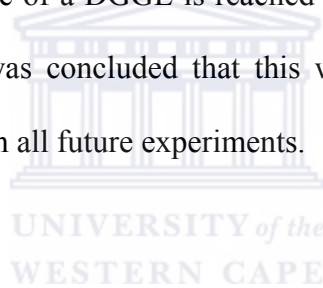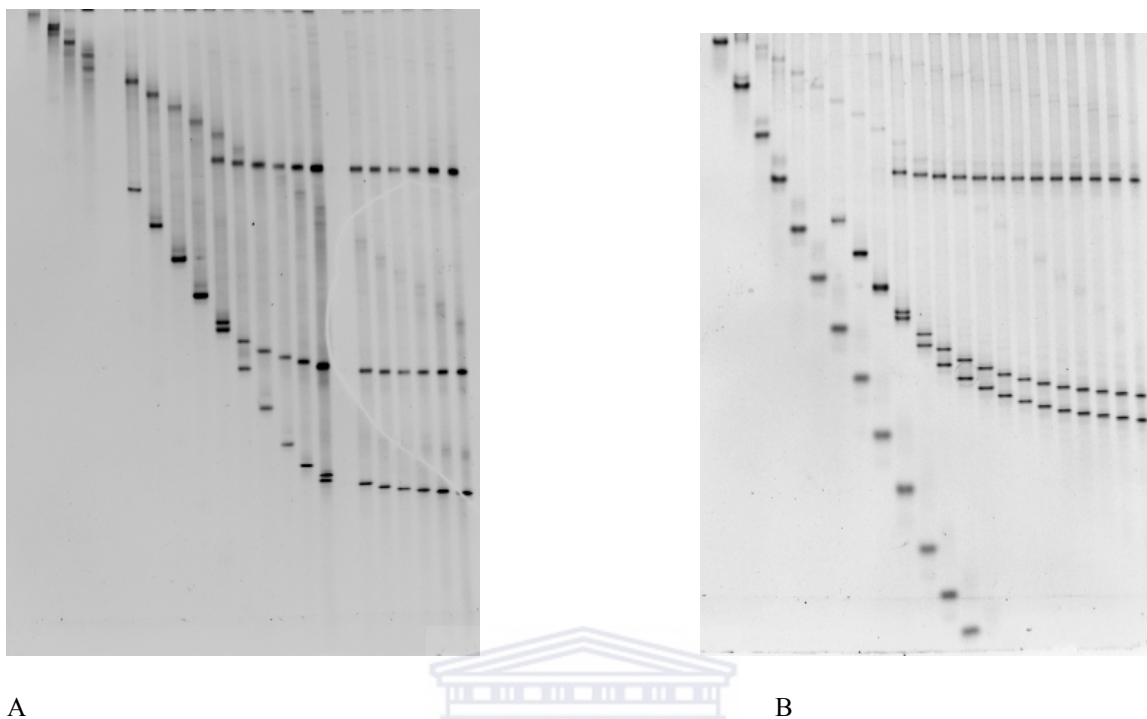
Fig. 3.8: DGGE time-travel experiment for both bacterial (A) and archaeal (B) primers. The mixtures of three fragments obtained from 16S rRNA gene clones were loaded on the gel from right to left every 30 min for 11h. Gradients: 30-60% and 30-65% for bacterial and archaeal, respectively.

3.6 Archaeal community analysis of estuarine sediments using DGGE

3.6.1 PCR amplification of archaeal 16S rRNA genes

Metagenomic DNA extracted from the sediment samples was used as templates in PCR amplifications. PCR products obtained with archaeal primers using a nested PCR

approach as described in section 2.6 were used in order to determine the diversity of Archaea in sediment samples from the Great Berg River estuary.

The PCR reactions of sediment sample DNA extracts using Archaea-specific primers yielded amplicons of the expected size (193 bp). The PCR products shown in Fig. 3.9 were further subjected to DGGE analysis in order to establish the diversity within the archaeal community.
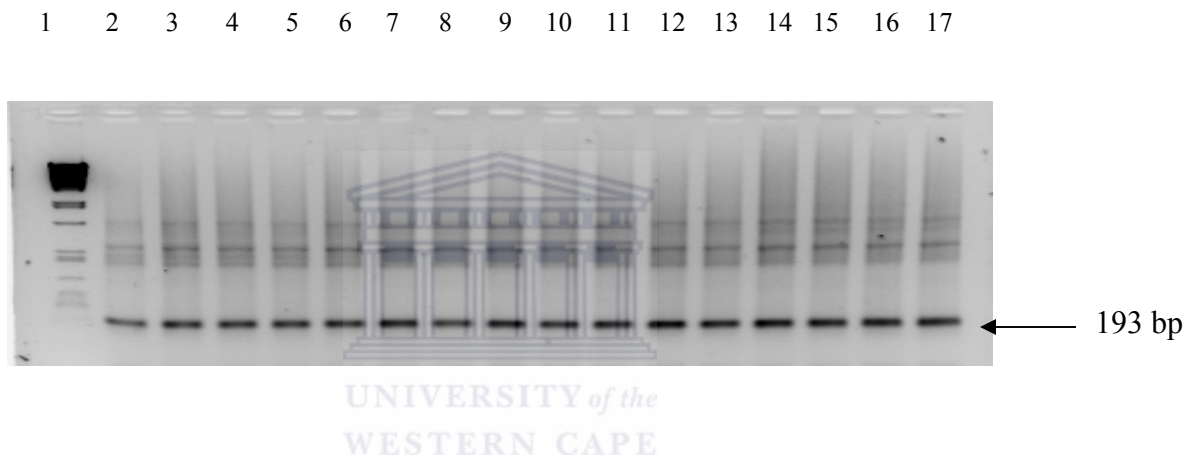
Fig. 3.9: Agarose gel (2%) showing PCR products with archaeal primers (340F-GC and 533 R). Lanes: 1, λ-DNA molecular marker (λ-DNA cut with *Pst*I restriction enzyme); 2, sample # 47; 3, sample # 48; 4, sample # 50; 5, sample # 51; 6, sample # 53; 7, sample # 54; 8, sample # 55; 9, sample # 57; 10, sample # 58; 11, sample # 59; 12, sample # 62; 13, sample # 63; 14, sample # 80; 15, sample # 101; 16, sample # 104; 17, positive control.

3.6.2 Homogeneity of the archaeal community of estuarine sediment

DGGE separations were performed to test for homogeneity within the sediment samples. Triplicate samples of 16S rDNA PCR products from upstream (site A) and downstream

(site B) sediment samples were loaded on a DGGE gel and analysed for homogeneity. The patterns of all samples from site B look almost identical even though they were from three different cores (Fig. 3.10 A, lane 3-11). However, small differences between the samples were observed (Fig. 3.10 A, lane 9-11). For example, band K was only observed in the downstream samples from one core but was absent in the other two sediment cores. Although, no major differences between the two sites was observed. Fig. 3.10 A show bands B and C to be only present at the upstream sampling site (Fig. 3.10 A). Band L was common in both sampling sites

A cluster analysis of the gel in Fig. 3. 10 A confirms the homogeneity of replicate samples. In the dendrogram all samples from one site (site A) form one cluster and the other site (site B) form the second cluster.

In conclusion, the results show that there is homogeneity among sediment samples from replicate cores from one sampling site.
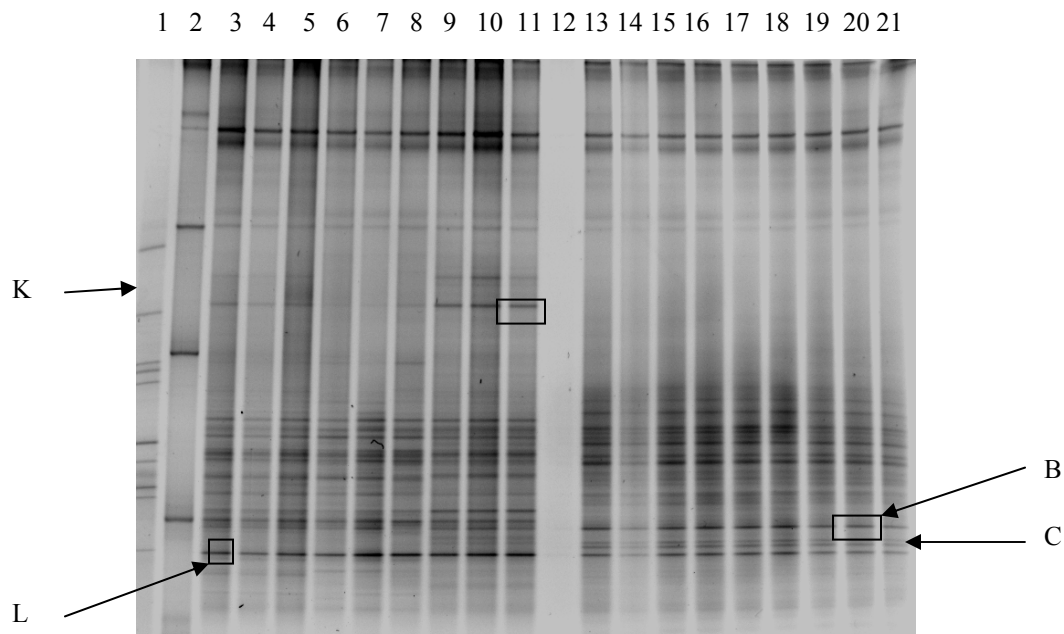
Fig. 3.10 A: A DGGE comparison of PCR amplified product between two different sites (site A and site B). The sediment samples origin from three different cores from each site but same depth intervals (depths 25-30 cm). The analysed samples were each loaded three times. Lanes: 1, Marker; 2, Positive control; 3-5, sample # 158; 6-8, sample # 104; 9-12, sample # 35; 13-15, sample # 77; 16-18, sample # 58; 19-21, sample # 27. Lanes 3-11, contain samples from site B (downstream) and lanes 13-21, samples from site A (upstream).
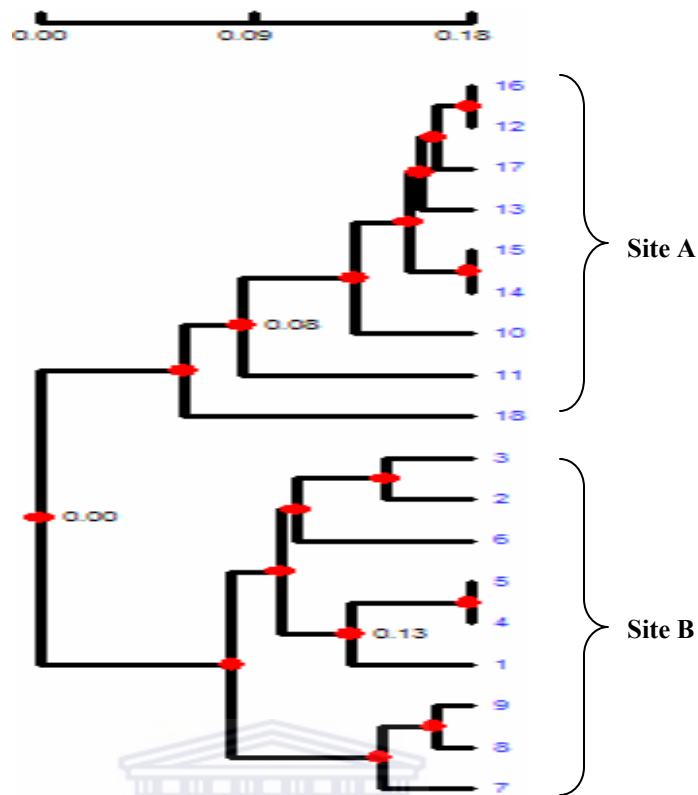
Fig. 3.10 B: A dendrogram calculated from Fig. 3.10 A based on the Dice coefficient method and clustering method UPGMA. The analysed samples (depths 25-30 cm) were each loaded three times. Lanes: 1-3, sample # 158; 4-6, sample # 104; 7-9, sample # 35; 10, No samples were loaded on; 11-13, sample # 77; 14-16, sample # 58; 17-18, sample # 27. Sediment samples in lanes 1-9 are from the upstream site (A) and samples from lanes 11-18 are from the downstream site (B).

3.6.3 Vertical analysis of the archaeal community within estuarine sediment cores

Sediment samples from three different cores and six different depths were analysed for microbial diversity. The DGGE was performed on the archaeal 16S rDNA from upstream and downstream samples in order to investigate for the depth similarity and distribution of the archaeal community within the cores. The 16S rDNA fragments of the Archaea

were obtained from a nested PCR amplification. The procedure is described in section 2.6.

The results show that there are no significant differences between the different depth intervals. One difference which was observed was at the 0-5 cm depth (Fig. 3.11 A) is indicated with band C. This band was absent in the 0-5 cm samples but present in the other depths (Fig. 3.11 A). In addition, band A was only present in the top layers (depths 0-5 cm and 5-10 cm) and was absent in the rest of the depths. Nevertheless, one population was common to the entire upstream core (site A), in Fig. 3.11 A and labeled with "N".
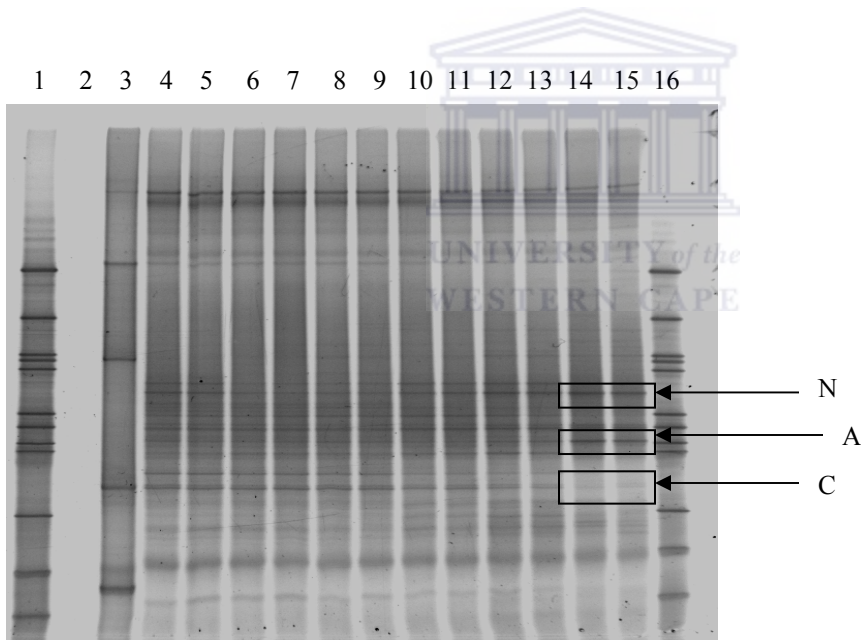
Fig. 3.11 A: DGGE profiles of Archaeal 16S rDNA PCR products amplified from all depths from 0-5 to 25-30 cm from the upstream site (site A). Lanes: 1, Marker; 2, negative control; 3, positive control; 4, sample # 63; 5, sample # 62; 6, sample # 59; 7, sample # 58; 8, sample # 57; 9, sample # 55; 10, sample # 54; 11, sample # 53; 12, sample # 51; 13, sample # 50; 14, sample # 48; 15, sample # 47; 16, Marker.

A cluster analysis of the upstream site shows the differences in community structures at different depths. The bacterial communities were separated into four clusters (Fig. 3.11 B). Samples from the deeper depth (25-30 cm) cluster separate from the rest of the depths. The second cluster represents samples from tree different depths (5-10 cm, 10-15 cm, and 15-20 cm) which clustered together. The upper depth 0-5 cm consists of the third cluster and were well separated from the medium and lower depths. The third cluster represented 0-5 cm depth. The 15-20 cm depth was closely related to 20-25 cm depth.
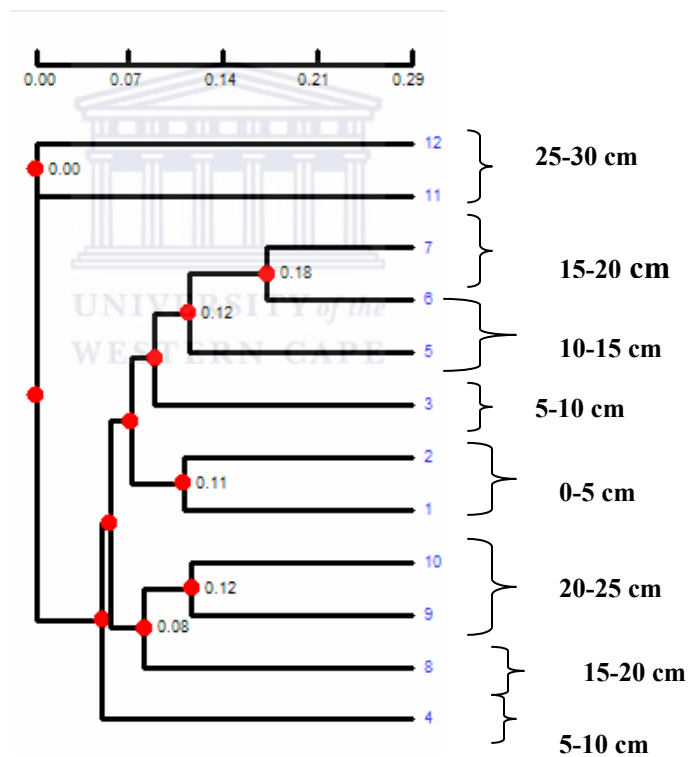
Fig. 3.11 B: Dendrogram was calculated using the similarity matrix based on the Dice coefficient and UPGMA cluster methods. In the dendrogram, the numbers refers to **Fig. 3.11 A.**

The samples from both downstream site is compared, the DGGE result shows that there were differences between the depths (Fig. 3.12). One significant difference which was observed at 0-5 cm depth have a very lower archaeal diverse (Fig. 3.12, lane 3 and 6).The downstream site (25-30 cm) had more archaeal diverse compared to the rest of the depths (Fig. 3.12, lanes 11,12 and 13).
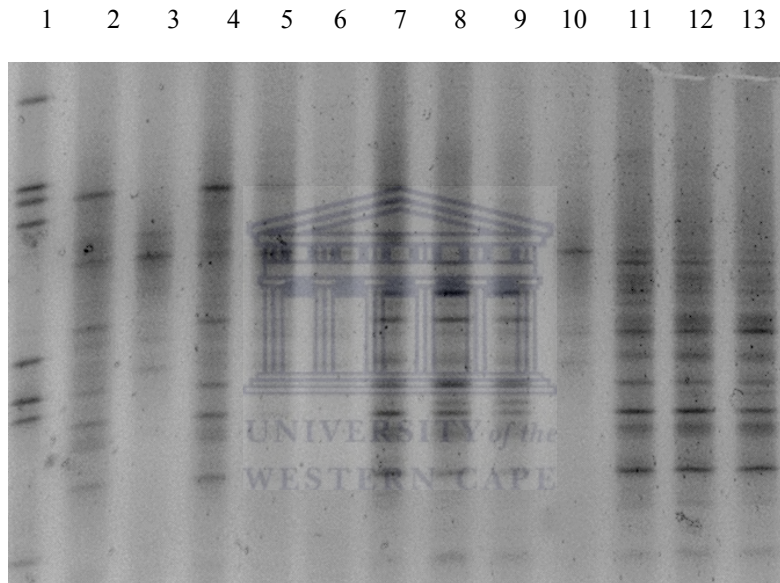
Fig. 3.12: DGGE profiles of archaea site B sediment samples all depths (0-5 to 25-30 cm). Lanes: 1, Marker; 2, sample # 95; 3, sample # 92; 4, sample # 96; 5, sample # 98; 6, sample # 93; 7, sample #99 100; 8, sample 100; 9, sample # 101; 10, sample # 104; 11- 13, sample # 108;

3.6.4 Comparison of DGGE profiles of archaeal 16S rDNA between upstream and downstream sites

In order to detect the differences in the archaeal community composition between the two sampling sites upstream and downstream the fish factory, archaeal 16S rDNA was amplified from the sediment samples (using the nested PCR approach as described in section 2.6) and further analysed by DGGE. Fig. 3.13 shows a comparison of the DGGE profiles of samples from both sites at different depths.

The DGGE analysis revealed a higher diversity in the archaeal community of the downstream sediment than in that of the upstream sediments. Secondly there is an increase in the archaeal diversity as the depth in the sediment samples increases (Fig. 3.13). The upstream site at depth 5-10 cm (Fig. 3.13 A, lane 8) and the downstream site (Fig. 3.13 A, lane 9) show clearly the differences in archaeal community composition between the two sites.
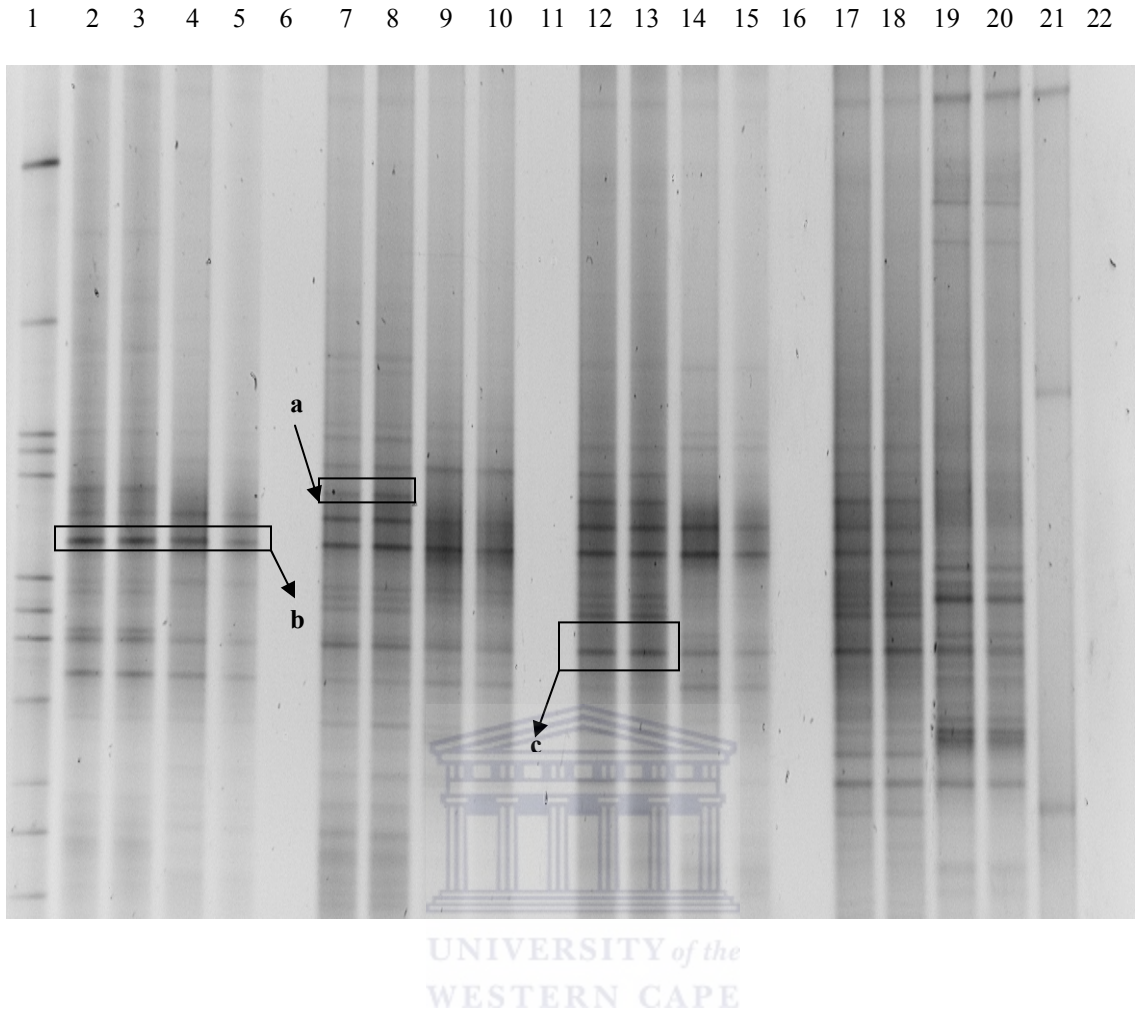
Fig. 3.13 A: DGGE analysis of replicate archaeal 16S rDNA samples from upstream and downstream sediment cores (from 0-5 cm to 15-20 cm depths). Lanes: 1, Marker; 2, sample # 47; 3, sample # 48; 4-5, sample # 93; 7, sample # 50; 8, sample # 51; 9-10, sample # 96; 12, sample # 53; 13, sample # 54; 14-15, sample # 98; 17, sample # 55; 18, sample # 57;19- 20, sample # 100; 21, Positive control; 22, Negative control.

Fig. 3.13 B shows a dendrogram calculated from the DGGE in Fig. 3.13 A. The microbial communities were separated into five distinguish the clusters as a function of depth (Fig.3.13 B). The upper depths (5-10 cm) from both sampling sites clustered together and were well separated from the medium and lower depths (0-5 cm, 10-15 cm, and 15-20

cm). The upstream site depths (0-5 cm) were closely related to depth (5-10 cm) from both sampling sites. The second cluster represents the samples from the downstream site at depths 0-5 cm and 10-15 cm. The third cluster represents the samples from the upstream site depth (10-15 cm) which differed from the same depth of the downstream site. The fourth cluster represents the samples at 15-20 cm from the upstream site, which differs from the downstream site that is represented in the fifth cluster.
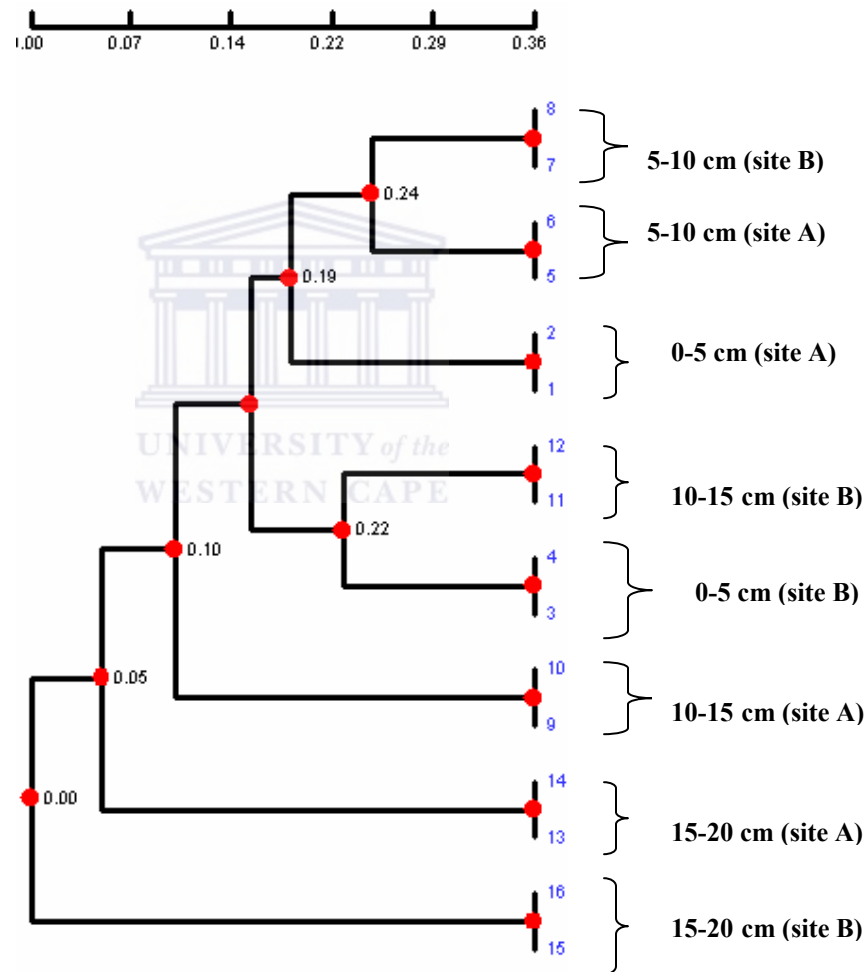
Fig. 3.13 B: The dendrogram was calculated using the similarity matrix based on the Dice coefficient and the cluster method UPGMA. In the dendrogram, the numbers refers to **Fig. 3.13 A.**

DGGE analysis of the upstream site and downstream site shows the differences between the two sampling sites (20-25 cm and 25-30 cm). At these depths the upstream site had higher bands intensity observed than at the downstream site (Fig. 3.13 C).
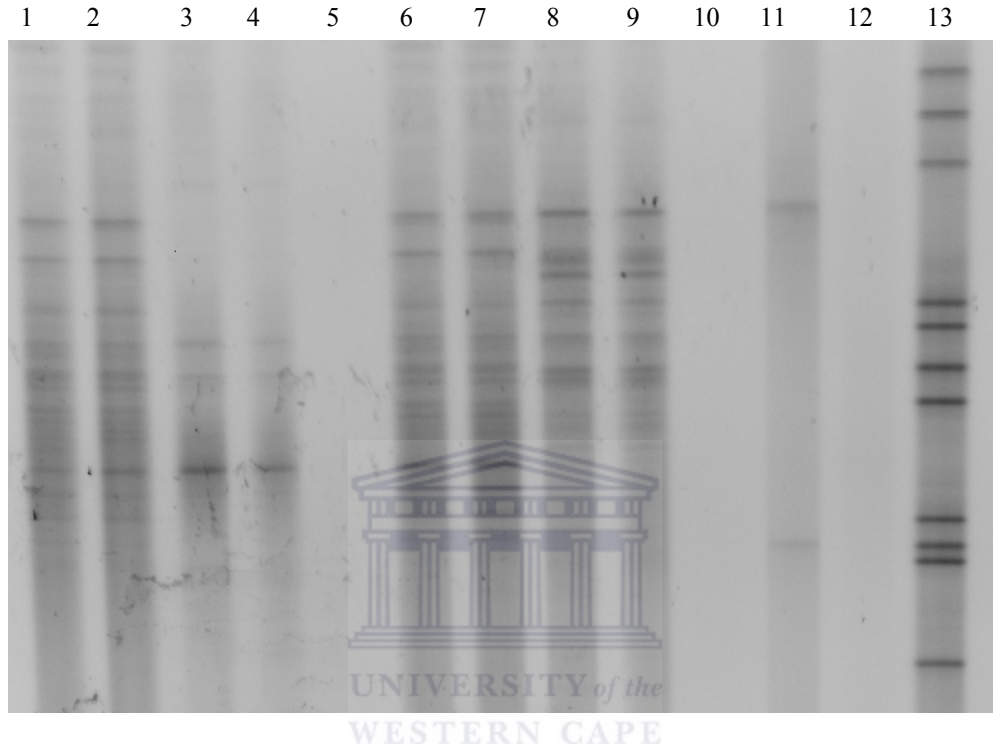
Fig. 3.13 C: DGGE analysis of archaeal 16S rDNA amplicons from upstream and the downstream sediments (depth 20-25 and 25-30 cm). Lanes: 1,sample # 58; 2, sample # 59; 3-4, sample # 104; 6, sample # 62; 7, sample 63; 8-9 sample # 108; 11, Positive control; 12, Negative control; 13, Marker

To visualize the differences between the sediment samples, the banding patterns of the DGGE were further analysed. A similarity matrix of all samples was calculated and a cluster analysis was performed to produce a dendrogram. Two clusters were observed, containing the samples at 20-25 cm and 25-30 cm depths from the both sampling sites, respectively. This indicates that the archaeal composition at a specific depth is similar in

both sites, but that there are significant differences in the communities present at 20-25 and 25-30 cm depth (Fig. 3.13 D).
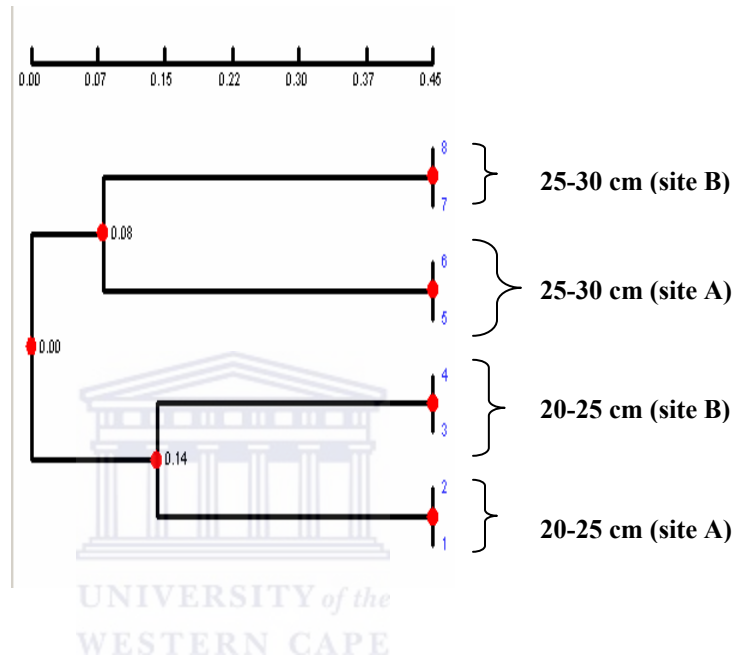
Fig. 3.13 D: The dendrogram was calculated using the similarity matrix based on the Dice coefficient and cluster methods UPGMA. In the dendrogram, the numbers s refer to **Fig. 3.13 C.**

3.7 Bacterial community analysis of the estuarine sediments using DGGE

3.7.1 PCR amplification of bacterial 16S rRNA genes

Metagenomic DNA extracted from the sediment samples was used as templates in PCR amplifications. PCR products obtained with bacterial primers using an approach as described in section 2.6 were used in order to determine the bacterial diversity of sediment samples from the Great Berg River estuary.

Fig. 3.14 shows the PCR amplicons obtained from sediment samples using bacteria-specific had the expected size of 193 bp. The PCR products were later loaded on DGGE gel for further analysis.
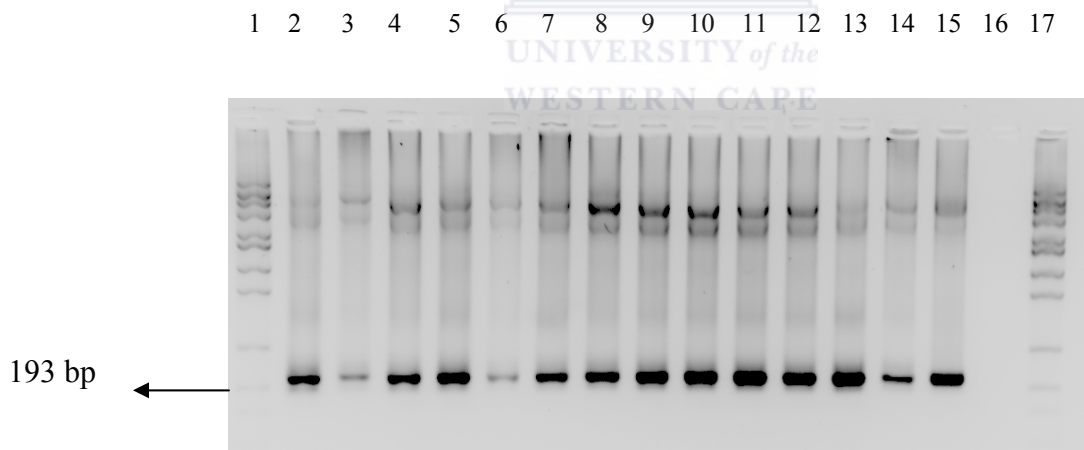
Fig. 3.14: PCR products obtained from metagenomic DNA using bacteria-specific primers 341fgc and 534R. Lanes: 1, Marker; 2, sample 47; 3, sample 48; 4, sample 50; 5, sample 51; 6, sample 53; 7, sample 55; 8, sample 57; 9-10,sample 58; 11-12, sample 59; 13, sample 62; 14, sample 63; 15, positive control; 16, negative control; 17, marker

3.7.2 Homogeneity of the bacterial community of estuarine sediment

Samples at 25-30 cm depth from the two sampling sites were analysed for homogeneity by DGGE. Replicate samples of amplified 16S rDNA were analysed in order to find out if samples within the sites are homogenous. The results show that the samples from upstream (site A) have lower bacterial diversity compared to the downstream site which has higher bacterial diversity. The results from DGGE band patterns in Fig. 3.15 (lane 2-3) show that there was homogeneity among the sediment samples.
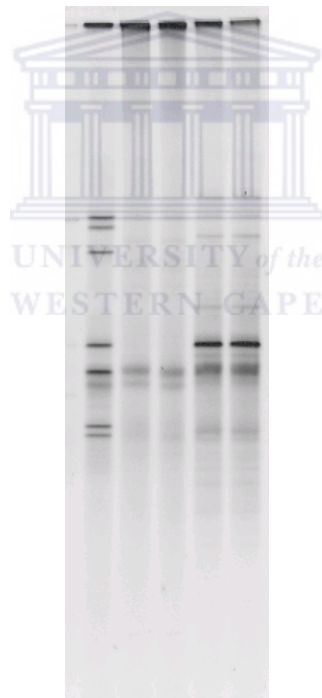
Fig. 3.15: DGGE profile of replicates of sediment samples from the upstream and downstream sites (25-30 cm depths) were analysed for homogeneity within the depths. Lanes: 1, Marker; 2-3, sample 63; 4-5, sample 108.

3.7.3. Vertical analysis of the bacterial community within estuarine sediment cores

Fig. 3.16 A shows the DGGE analysis of sediment samples from one core of the upstream site but (all depths). They were analysed in order to investigate the diversity or changes of the community structure with depth within the core. When the samples from all depths were compared, there were few changes in the microbial community with depth which were observed. But there were changes in the intensity of several bands, indicating differences in the abundance of the respective strains. However there was one microbial taxon which was common to the entire core upstream (site A), which is indicated with the arrow labelled D. There were no major differences between the depths. More microbial community was observed at depth 0-5 cm compared to the other depths Fig. 3.16.
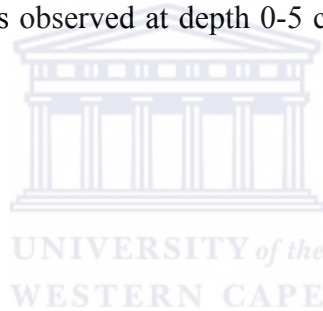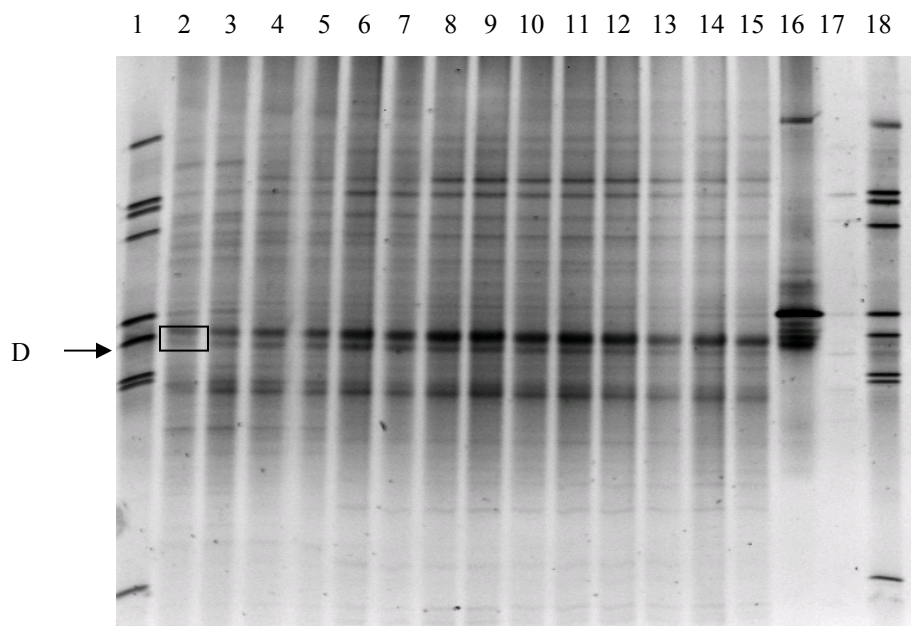
Fig. 3.16 A: DGGE profiles of the bacterial community of all different depths (0-5 to 25-30 cm) from the upstream site (site A). Lanes: 1, Marker; 2, sample # 47; 3, sample # 48; 4, sample # 50; 5, sample # 51; 6, sample # 53; 7, sample # 54; 8, sample # 55; 9-10, sample # 57; 11, sample # 58; 12-13, sample # 59; 14, sample # 62; 15, sample # 63; 16, Positive control; 17, Negative control; 18, marker. Band D refers to text above.

The cluster analysis was performed on six different depths from the upstream site. The numbers shown in the dendrogram correspond to Fig. 3.16 A. The cluster analysis revealed slight differences between the microbial communities at different depths. The microbial communities were divided into two clusters, the upper three depths (0-5, 5-10, and 10-15cm) clustered together and were well separated from the lower three depths (15-20, 20-25, and 25-30 cm). This could be possible due to the biological influences in the sediment. For example the oxygen or the influences of nutrient at the sediments of the fish factory waste.
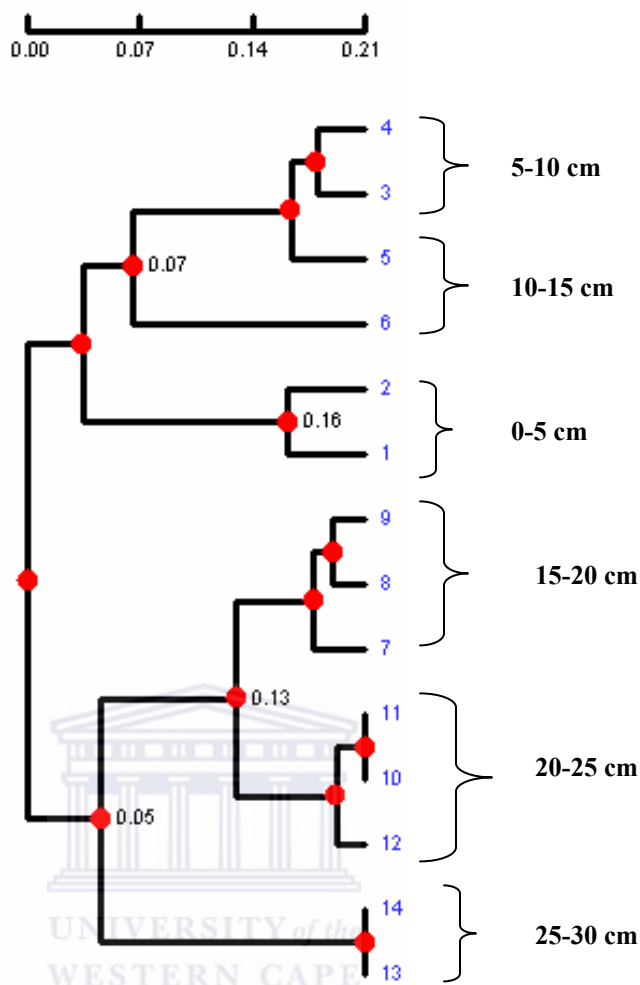
Fig. 3.16 B: The dendrogram was calculated using the similarity matrix based on the Dice coefficient and cluster methods UPGMA. In the dendrogram, the numbers refer to **Fig. 3.16 A**.

Bacterial community analysis of sediment samples from six different depths ranging from 0-5 cm to 25-30 cm from one core were analysed in order to see how the samples from all the depths relate to each other. Fig. 3.17 A shows a comparison of the DGGE profiles of samples from site B (downstream) at different depths. The result indicates that there were some significant differences between the depths. One band (E) was common to all the

depths. The study of the bacterial community in sediment samples obtained from the DGGE analysis showed characteristic banding patterns for each specific depth (0-5cm, 5-10 cm, 10-15 cm, 15-20 cm, 20-25 cm, and 25-30 cm) from the downstream sampling site, indicating differences in the bacterial communities.
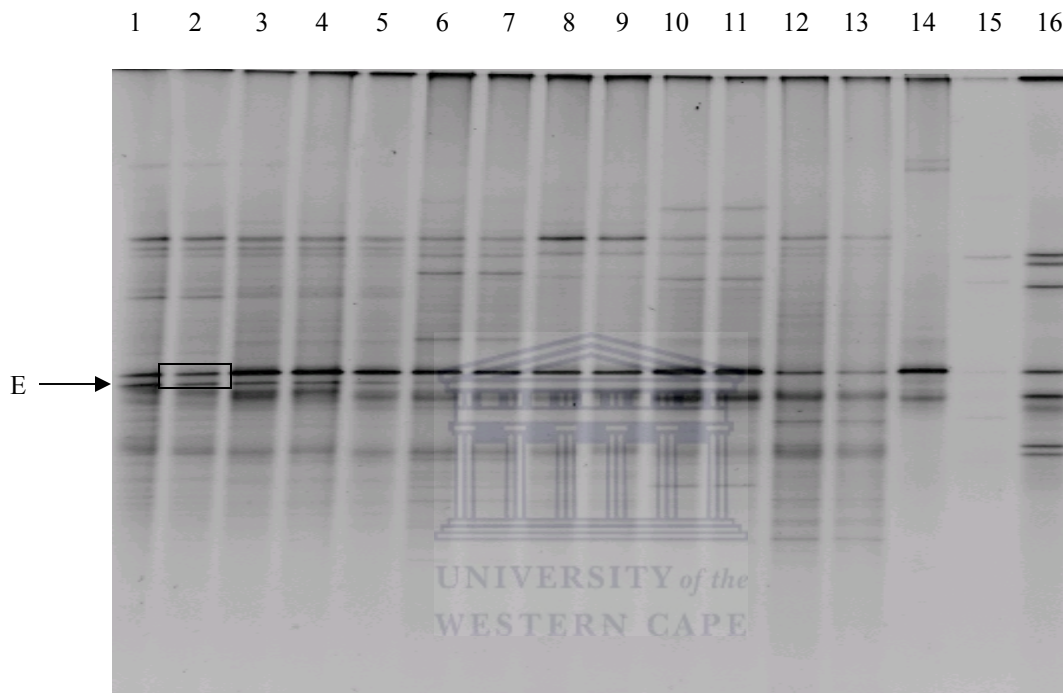
Fig. 3.17 A: DGGE profiles of bacterial 16S rDNA PCR products from all depths of one core (0-5 to 25-30 cm) from the downstream site (site B). Lanes: 1, sample # 92; 2, sample # 93; 3, sample # 95; 4-5, sample # 96; 6, sample # 98; 7, sample # 99; 8, sample # 100; 9, sample #101; 10, sample # 104; 11, sample #105; 12, sample # 107; 13, sample # 108; 14, Positive control; 15, Negative control; 16, marker. Band E refer to text above.

The dendrogram was constructed in order to visualise the differences in community structures at different depths. The bacterial communities were separated into four clusters

(Fig. 3.17 B). The upper depths (0-5 cm, and 5-10 cm) clustered together and were well separated from the medium and lower depths (10-15 cm, 15-20 cm, 20-25 cm, and 25-30 cm). The third cluster represents samples from the medium depths (10-15 cm, and 15-20 cm) which clustered together and were separated from the upper and the lower depths. The fourth cluster was different from the rest of the depths which was represented by the sediment samples from 25-30 cm depth.
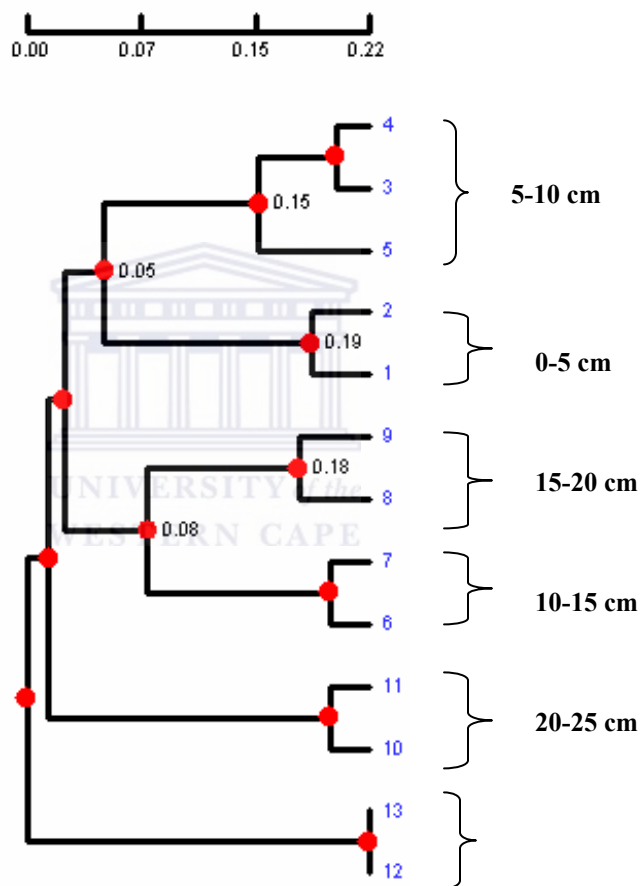
Fig. 3.17 B: The dendrogram was calculated using the similarity matrix based on the Dice coefficient and cluster methods UPGMA. In the dendrogram, the numbers refer to **Fig. 3.17 A.**

3.7.4 Comparison of DGGE profiles of bacterial 16S rDNA between sediment samples from upstream and downstream of the fish factory

Fig. 3.18 A shows the comparison between the two sampling sites at all depths (0-5 to 25-30 cm). These DGGE band patterns reveal the differences in the bacterial community structures between the two sites. The differences between the two sites are considerable. For instance, the samples in lanes 1 and 2 are from two different sites but same depth (0-5 cm). The downstream sample in lane 2 contains a higher diversity than the upstream sample in lane 1. A reason for the big differences could be the organic matter from the fish factory effluents that affects the downstream site. In addition, the organic matter is probably sediments at the surface at the downstream site, which could make the difference between the bacterial communities in Fig. 3.18**.** The samples represent the most complex profile, in this case indicating a more diverse microbial community of the downstream samples.
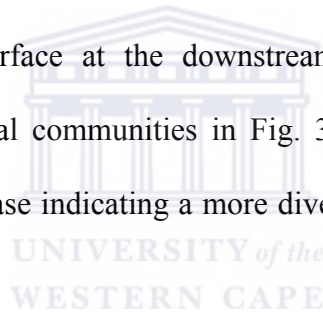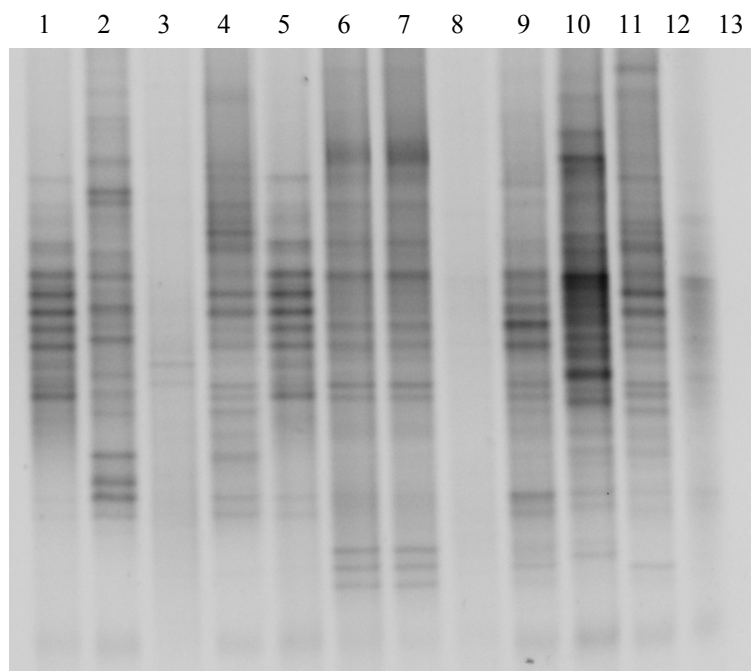
Fig. 3.18 A: DGGE analysis of bacterial 16S rDNA genes amplified by PCR from sediment sample cores from the upstream and downstream sites (depths 0-5 cm to 20-25 cm). Lanes: 1, sample # 65; 2, sample # 146; 3, sample # 68, 4, sample # 149; 5, sample # 71; 6-7, sample # 152, 8, sample # 74; 9, sample # 158; 10, sample # 80; 11, sample # 161; 12, positive control; 13, negative control.

3.7.5 Preparation of a 16S rDNA clone library

16S rDNA clone libraries were prepared from sediment samples (samples 50 and 95) from both sampling sites. Clones not containing the correct insert were not used.

Samples from sites A and B (depth, 5-10 cm) were used for PCR amplification of bacterial rRNA genes using universal bacterial primers. The representative sampling depth of 5-10 cm was chosen based on the organic matter content between the two sites,

which was clearly different. The PCR products from both sites were cloned. 16S rDNA

clone libraries were constructed from randomly picked colonies. M13 primers were used

to screen for inserts of the right size in the library. The expected 1.5 kb DNA fragments

were observed on the 1% agarose gel (Fig. 3.19). More than 98% of the clones contained

the expected 1.5 kb fragment. The colony PCR products were then sequenced and used to

construct phylogenetic tree.
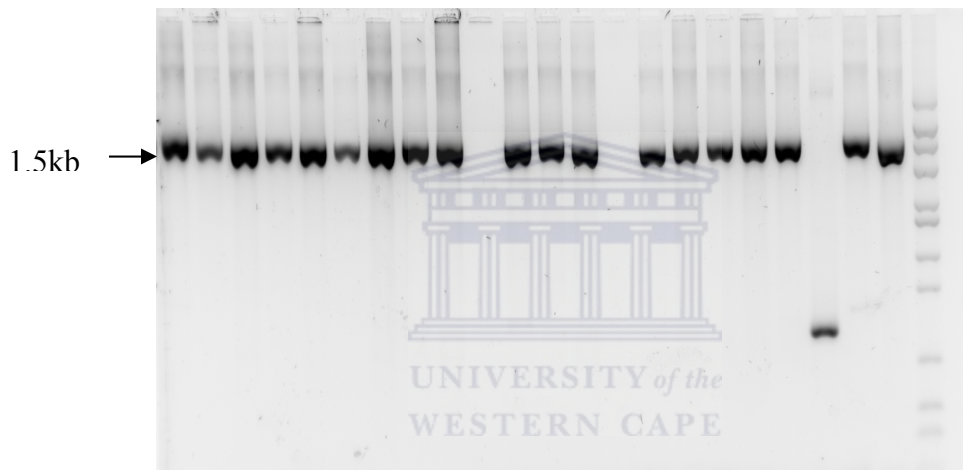
Fig. 3.19: Amplified 16S rDNA clones from bacteria were constructed into mini clone library.

3.7.5 Phylogenetic analysis

Fifty-one clones of the bacterial 16S rDNA clone libraries (A from site A, B from site B)

were sequenced. The obtained sequences were then compared with public databases

using BLAST analysis.

The similarities of the bacterial 16S rDNA sequences with published data ranged from 88% to 99% (Table 3.3). The phylogenetic analysis of the sequences (Fig 3.21) revealed the presence of a wide diversity of bacteria in the sediment samples. More than ten different bacterial phyla could be identified. The phylum *Chloroflexi* has revealed high sequence similarities to the following clones **B80, B59, B17, B46, B8, B55,** and **B52**. These clones were generated from the downstream site, clone **A18** is the only representative of the upstream site. The δ-*Proteobacteria* cluster is represented by clones **B25, A28, A74, B24, A33, B56, A7, A35, B7, B43** and **B69** (with sequence similarities ranging from 92% to 98%). The α-*Proteobacteria* cluster consists of closely related clones (**B32, A3** and **A58),** which out of these three clones are from the upstream site (sample A). Cluster γ-*Proteobacteria* consists of three clones from sample A (**A20, A60, A2**) and one clone from sample B (**B75**) (representing a similarity ranging from 88% to 99 %). Clone **A47** is closely related to *Actinobacteria* and is unique to sample A.

Clones **B51** and **A12** represent the *Acidobacteria* cluster, with sequence similarities ranging from 92% to 98%. The *Sphingobacteria* cluster is represented by the following clones **A6**, **A1**, and **A25,** with higher closely related sequence similarities ranging from 92 to 98 % and clone **B68** is the only clone from sample B. The *Flavobacteria* cluster is represented by the following clones: **B9, A39,** and **B19,** with sequence similarities from 94% to 98%, and two out of these three clones belong to sample B (downstream site). Clone **A55,** a representative of the *Deinococci,* with sequence similarity of 93%. This clone was unique to sample A (upstream site) only.

Fig. 3.20: Neighbour-joining tree constructed using Juke and Cantor distances. The phylogenetic analysis was based on the alignment of 16S rDNA sequences of 582 bp length. The scale bar indicates 10 substitutions per 100 bp. The numbers show the bootstrap values (higher than 50) obtained from 1000 repetitions.
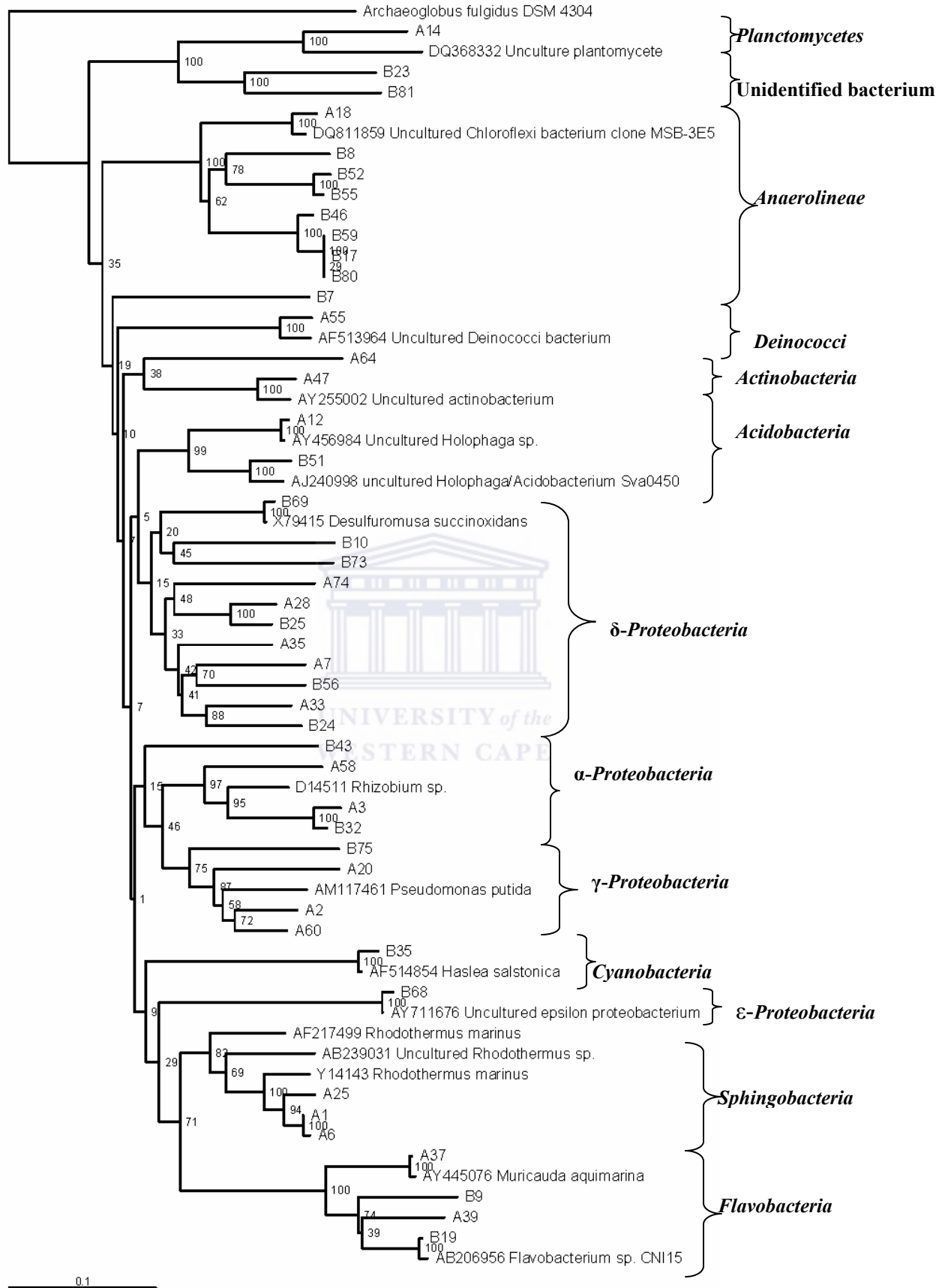
Table 3.3: BLAST results of 51 sequences obtained from the bacterial 16S rDNA clone library. "A" clones origin from site A (upstream), "B" clones origin from site B (downstream).

CLONE ID	SIMILARITY%	NAME OF BEST HIT	ACCESSION #	E-VALUE
A1	517/563 (91%)	*Rhodothermus marinus*	Y14143	0.0
A2	737/743 (99%)	Uncultured gamma proteobacterium	AY711859	0.0
A3	738/740 (99%)	*Roseovarius* sp.	AB114421	0.0
A6	462/471 (98%)	Uncultured Bacteroidetes bacterium	AY254982	0.0
A7	734/754 (97%)	Uncultured delta proteobacterium	DQ395025	0.0
A8	520/533 (97%)	Uncultured CFB group bacterium	AF446280	0.0
A12	715/724 (98%)	Unidentified bacterium	AY711682	0.0
A14	613/660 (92%)	Uncultured candidate division OD1 bacterium	AY193194	0.0
A15	492/504 (97%)	Uncultured bacterium	AY435199	0.0
A16	306/333 (91%)	Uncultured Bacteroidetes bacterium	AY254982	0.0
A18	655/682 (96%),	Unidentified bacterium	AY592389	0.0
A20	508/571 (88%)	*Pseudomonas putida*	AM117461	1e-168
A25	525/570 (89%)	*Rhodothermus marinus* strain	AB239031	0.0
A28	696/756 (92%)	Unidentified bacterium	AF317771	0.0
A33	741/754 (98%)	Uncultured delta proteobacterium	DQ112395	0.0
A35	753/757 (99%)	Uncultured delta proteobacterium	DQ351771	0.0
A37	703/717 (98%)	*Muricauda aquimarina* strain	AY445076	0.0
A39	536/569 (94%)	*Flavobacteriaceae* bacterium	AY255002	0.0
A47	729/774 (94%)	Uncultured actinobacterium	AY255002	0.0
A55	689/733 (93%)	Uncultured *Deinococus* bacterium	AF513964	0.0
A58	685/777 (88%)	*Rhizobium* sp.	D14511	0.0
A60	701/708 (99%)	Uncultured gamma proteobacterium	AY711685	0.0
A64	342/380 (90%)	Uncultured bacterium	AY712476	0.0
A74	827/842 (98%)	Uncultured delta proteobacterium	DQ351760	0.0
B7	437/461 (94%)	Uncultured delta proteobacterium	AJ889164	0.0
B8	486/535 (90%)	Uncultured hydrocarbon seep bacterium	AF154100	0.0
B9	709/718 (98%)	*Flavobacteriaceae* bacterium	AY962293	0.0
B10	274/282 (97%)	Uncultured vent bacterium	AF209018	0.0
B17	676/703 (96%)	Uncultured bacterium	AY540497	0.0
B19	|763/772 (98%)	*Flavobacterium* sp	AB206956	0.0
B23	650/709 (91%)	Unidentified bacterium	AB015580	0.0

**Table 3.3: (Continue)** BLAST results of 51 sequences obtained from the bacterial 16S rDNA clone library. "A" clones origin from site A (upstream), "B" clones origin from site B (downstream).

CLONE ID	SIMILARITY%	NAME OF BEST HIT	ACCESSION #	E-VALUE
B24	683/708 (96%),	Uncultured*Desulfobacteriaceae* bacterium	AY515480	0.0
B25	655/697 (93%),	Uncultured delta proteobacterium	AB031631	0.0
B32	708/730 (96%)	*Roseobacter* sp.	AF107210	0.0
B35	715/734 (97%)	*Haslea salstonica*	AF514854	0.0
B40	437/482 (90%)	Uncultured bacterium	DQ154824	5e-168
B42	527/566 (93%)	Uncultured bacterium	AB121106	0.0
B43	684/734 (93%)	Uncultured bacterium	AY711452	0.0
B46	664/701 (94%)	Uncultured bacterium	AY540497	0.0
B48	403/424 (95%)	Uncultured spirochete	AY605159	0.0
B51	713/774 (92%)	uncultured Holophaga/Acidobacterium	AJ240998	0.0
B52	742/765 (96%)	Uncultured bacterium	AY592889	0.0
B55	698/718 (97%)	Uncultured bacterium	AY592417	0.0
B56	495/577(85%)	Uncultured *Geobacter* sp.	DQ394958	1e-131
B59	682/711 (95%)	Uncultured bacterium	AY540497	0.0
B68	683/694 (98%)	Uncultured epsilon proteobacterium	AY711676	0.0
B69	751/765 (98%)	*Desulfuromusa succinoxidans*	X79415	0.0
B73	705/736 (95%)	Uncultured bacterium	AY711254	0.0
B75	694/730 (95%)	Uncultured bacterium	AB212895	0.0
B80	662/688 (96%)	Uncultured bacterium	AY540947	0.0
B81	653/709 (92%)	Uncultured candidate division OD1	AY93161	0.0
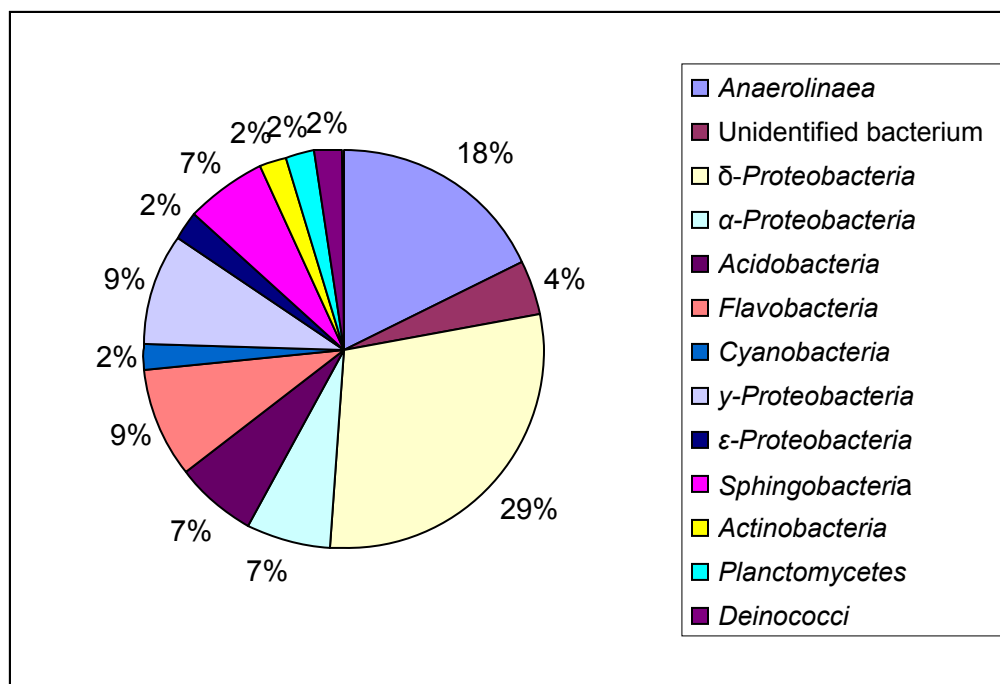
Fig 3.21 A: Assignment of the bacterial 16S rRNA gene sequences obtained from the upstream and downstream sediment samples to major bacterial lineages.

The bacterial composition of the microbial communities was analysed in order to determine the distribution of the different groups in the sediment samples from both sampling sites. The sequence analysis of clones obtained from upstream (A) and downstream (B) sites was used to compare the overall diversity and community composition between the two sampling sites. The results revealed that 41% of the sequences belonged to the *Proteobacteria* phylum, which was the dominant group in Great Berg River estuary sediment samples (Fig. 3.21). Three clusters of the phylum *Proteobacteria* were identified in this study; the subdivisions of δ-*Proteobacteria*, α-*Proteobacteria* (7% of the analysed clones) and γ-*Proteobacteria* (9%), with the δ-*Proteobacteria* being the most abundant (29%).
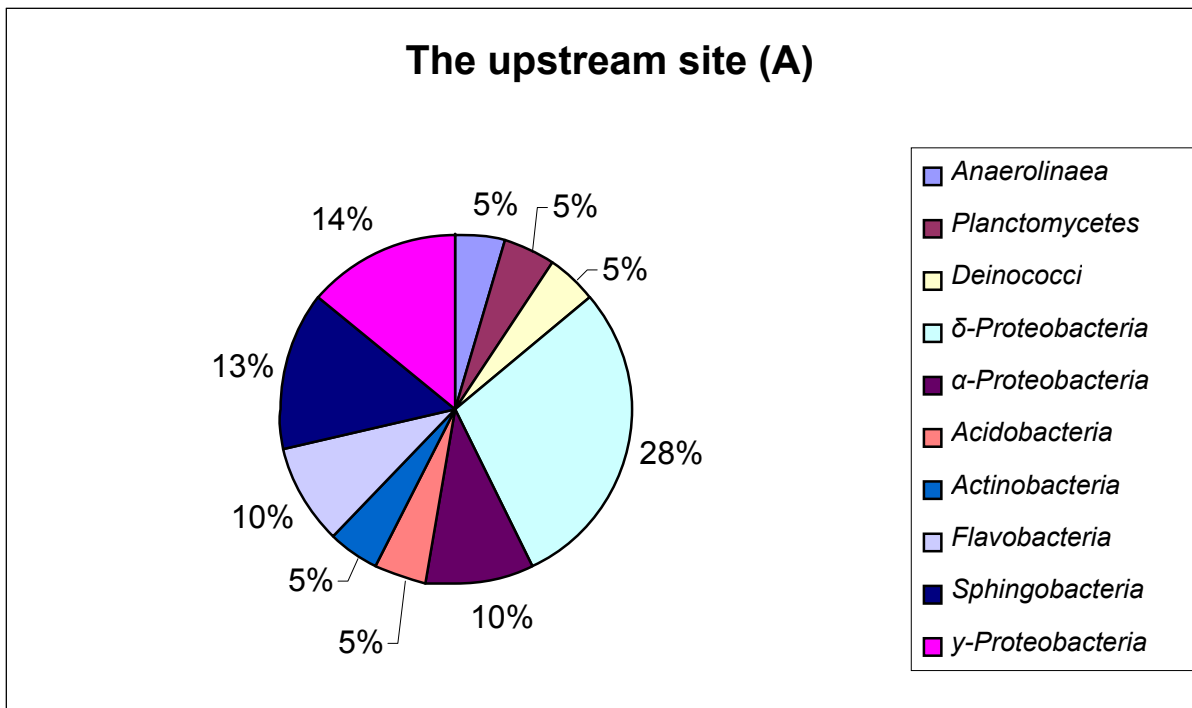
The upstream site (A)

Legend:
- *Anaerolinaea*
- *Planctomycetes*
- *Deinococci*
- *δ-Proteobacteria*
- *α-Proteobacteria*
- *Acidobacteria*
- *Actinobacteria*
- *Flavobacteria*
- *Sphingobacteria*
- *γ-Proteobacteria*

Fig 3.21 B: Assignment of the bacterial 16S rRNA gene sequences obtained from the upstream sediment samples to major bacterial lineages.

The class δ-*Proteobacteria* make up the most dominant group of bacteria obtained from the upstream site (28%) as indicated in Fig. 3.21 B. The δ-*Proteobacteria* are strict anaerobes that flourish in habitats such as polluted lake sediments, anaerobic marines, mud and estuarine sediments (Canfield *et al*., 1999). A significant role played by the δ-*Proteobacteria,* in the sulfur cycle within ecosystems by generating sulphide from sulphate and sulphur while oxidizing organic nutrients (Hugenoltz *et al.,* 1998). The γ-*Proteobacteria* make up the second dominant group (14%) in the upstream site followed by the *Sphingobacteria* which make up (13%), followed by *Flavobacteria* and *α-Proteobacteria* making up 10%. The remaining groups each constitute less than 10% and consist of the phyla *Acidobacteria, Actinobacteria, Anaerolinaea, Deinococci,* and *Plactomycetes.*

The sediments from the upstream site have a lower organic content (Fig. 3.1) and the γ-*Proteobacteria* are the second dominant bacterial group which was observed at the

upstream site. They oxidize sulphide to sulphur and are often found in anaerobic sulphur rich regions (Holmer *et al*., 2001). The *Sphingobacteria* are anaerobic and chemoheterotrophic bacteria that play a major role in the mineralization of organic matter (Canfield *et al*., 1999).
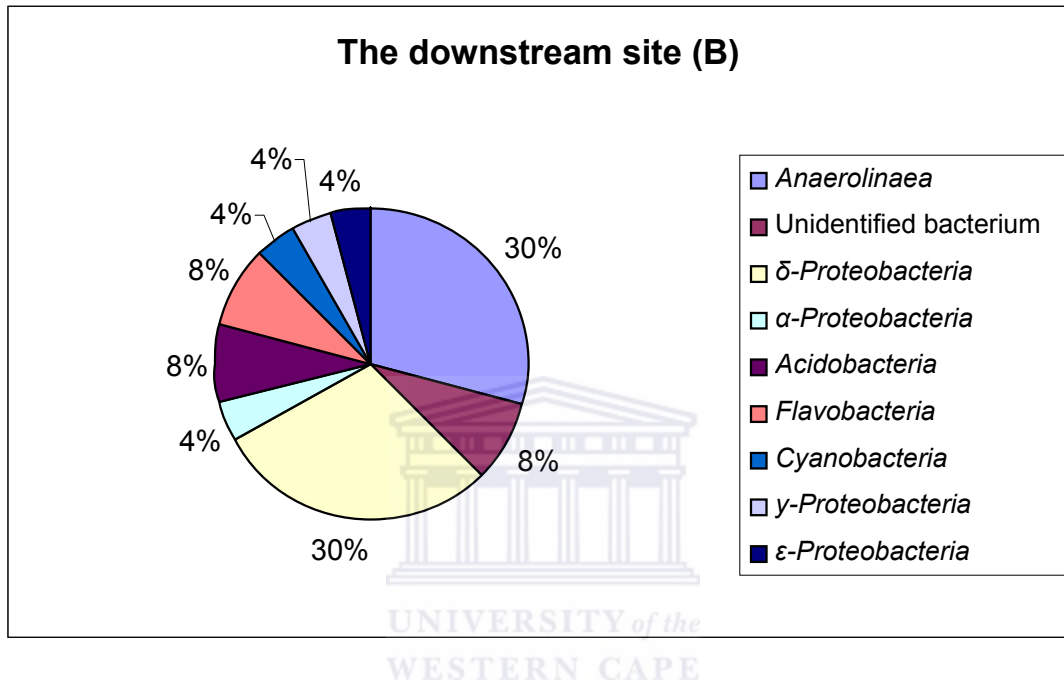
Fig 3.21 C: Assignment of the bacterial 16S rRNA gene sequences obtained from the downstream sediment samples to major bacterial lineages.

The bacterial community within the estuarine sediments (Fig. 3.22 B and C) is diverse and composed of known bacterial species for the most part. The downstream site (B) is mostly dominated by two classes, the δ-*Proteobacteria* group and the *Anaerolinaea*, each making up by 30% of the total microbial community (Fig. 3.22 C). The *Flavobacteria,* unidentified and *Acidobacteria* were the second dominant groups making up 8% each. The other groups of bacteria found in the sediments include the classes ε-*Proteobacteria*, α-*Proteobacteria*, *Cyanobacteria* and γ-*Proteobacteria*.

DISCUSSION

4.1 Organic matter content of estuarine sediments

The organic matter content plays an important role in every ecosystem and it is likely to influence the microbial composition in the sediments. The analysis of the organic matter content of the Great Berg River estuary sediments was done because it was assumed that the wastewater from the fishing industry could be a source of organic matter. Therefore, considerable differences were expected between the two sampling site upstream and downstream the fish factory. The results showed that at the upstream site the organic matter content is low at the surface (0.9%), but increases with depth (3.4%) at 20-25 cm depth). In contrast, at the downstream site relatively high values were observed for all depths (mostly 3-4%) This difference between the two sites could be explained by the sedimentation of wastewater particles, which increased the organic matter content at the surface. In this study, the organic matter content was very low (0.9-4.0%) compared to the high values in the mangrove sediments in Tanzania, where 20% and 12% were found in protected and deforested areas, respectively (Sjöling *et al*., 2005).

In order studies, the organic matter content was found to decrease with increasing depth in sediments (Høj *et al.,* 2005). The unusual trend of increasing organic content with increasing depth observed at the upstream site (site A) of the Great Berg River estuary could be a result of the influence of the fish factory.

Anaerobic processes such as sulphate reduction are usually associated with high organic matter (Mußmann *et al.,* 2005; Sjöling *et al.*, 2005). However, the levels of organic matter found in the Great Berg River estuary, though significantly different between the two sampling sites for example at specific depth (0.9 to 3.4%) were not as high as those reported from the mangrove sediments (12 to 20%). Considering the influence of organic matter on the distribution of microorganisms, site B (downstream site) (Fig. 3.1) is expected to have more anaerobic bacteria, mostly those that reduce sulphur (Sjöling *et al.*, 2005).

4.2. Extraction of metagenomic DNA from estuarine sediments

Metagenomic DNA was extracted from sediment samples collected from the two sampling sites, upstream and downstream of the fish factory. The validity of this study largely depended on the extraction of representative metagenomic DNA of the microbial communities from these samples. However, there are difficulties associated with sediment DNA extraction such as incomplete cell lysis, DNA absorption to sediment surfaces, co-extraction of enzymatic inhibitors, yield loss, and degradation or damage of the DNA (Miller *et al.,* 1999). The modified Miller method was successfully used to extract high quality metagenomic DNA that was suitable for subsequent PCR reactions. The DNA extracted was further purified with PVPP and Sephacryl columns in order to remove all traces of humic acids, which usually inhibit PCR amplification (Miller *et al.,* 1999). The high molecular weight metagenomic DNA was obtained at yields between 15

94
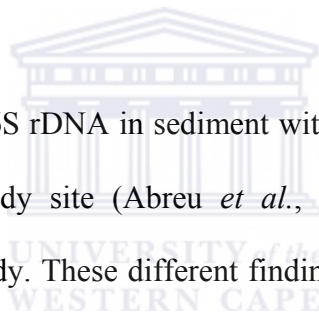
and 60 μg/ g of sediment. The extraction of high molecular weight DNA is an indication

that the DNA was not damaged or shared. The DNA yields from the Great Berg estuarine

sediments were similar to those obtained from the wetland sediments which ranged from

6 to 53 μg/ g of sediments (Miller *et al.,* 1999).

4.3 DGGE profiling of the archaeal community

DGGE was used for the comparative analysis of the archaeal diversity in sediment

samples (0-30 cm depth) from the upstream and the downstream sites (Fig. 3.13 A). The

domain Archaea is considered to be more diverse and widespread in environmental

samples than previously believed (Abreu *et al*., 2001). The results obtained in this study

also show this high archaeal diversity as revealed by DGGE. The patterns of the

separated archaeal 16S rDNA from both sampling sites show a considerable diversity at

all depths. The upstream site shows richer archaeal diversity with approximately 15

visible bands, compared to about 10 visible bands from the downstream site (lanes 12-

15).

The DGGE patterns show some bands with strong intensity which presumably represents

the dominant archaeal species in each sample (Fig. 3.13 A). These dominant Archaea

could be well adapted and play important roles in each habitat. Band "**a**" shows one

dominant species that is only found in the upstream site. Band "**b**" appears in both sites at

depths lower than 15-20 cm, whilst band "**c**" appears in both sites all depths or only at 0-

20 cm. The dominant species for most band types are however not present at higher depths except for band type "**c**". Band "c" is present at all depths in both sites indicating that it is the most common archaeal species in the Great Berg River estuarine sediments. The two band types represented by "**d**" (lanes 19 and 20) were dominant and unique to the downstream site, but only at 15-20 cm (Fig. 3.13 A). A direct comparison of the DGGE profiles, specifically at 10-15 cm depth (lanes 13 and 14) revealed the differences between the two sampling sites, the upstream site (A) having a higher archaeal diversity in comparison to the downstream (B). The results suggest that the organic matter could have an influence on the archaeal diversity.

The diversity of the archaeal 16S rDNA in sediment with regard to the depth is believed to vary depending on the study site (Abreu *et al.*, 2001). The microbial diversity increased with depth in our study. These different finding could be due to the sediments characteristics and the homogeneity within sediment samples.

The community structure is heterogeneous and the resolution of the DGGE is limited. The absence of a particular band from a DGGE profile does not therefore necessarily indicate that the sequence is completely absent from the community.

The archaeal composition within the upstream site was compared at different depths (Fig. 3.11). Some archaeal species were not present at lower depths, for example band type

"**C**" at 0-5 cm (lanes 14-15), but were clearly present at all the higher depths (5-10 cm to 25-30 cm). Several unique bands were only found at the downstream site (Fig. 3.10).

DGGE band patterns from high diversity ecosystems such as marine sediments are very complex and difficult to interpret (Boon *et al.,* 2001). Therefore, the application of advanced statistical methods is crucial. In this study, a similarity matrix, based on the presence and absence of individual bands, was calculated from every set of samples. A cluster analysis then resulted in dendrograms in which the similarities and differences between DGGE profiles were visualized (Fig. 3.13 B and D).

The Archaeal DGGE patterns become more diverse and more similar between the two different sites at higher depths compared to lower depths (Fig. 3.13 D).. The *Archaea* were found to be prominent in the deep subsurface water communities (Nakatsu *et al.,* 2000) as compared to soil communities. This could explain the high prevalence *Archaea* in estuarine sediments.

The cluster analysis of the DGGE profiles of the two sampling sites also shows that the upper depths (5-10 cm) from both sites the upstream and downstream site cluster together and were well separated from the rest of the depths (Fig. 3.13 B). The 0-5 cm depth from the upstream site is closely related to 5-10 cm depths from both sites. This suggests that the upstream and the downstream sites have common or similar archaeal population in the lower specific depths. However, there is no rich archaeal diversity at the top sediment

layers as shown by the presence of the very few DGGE bands in these samples (Fig. 3.13 A).

The top depths (0-5 cm and 10-15 cm) from the downstream site unexpectedly cluster together (Fig. 3.13 B). Meanwhile the 10-15 cm and 15-20 cm depths from the upstream site and the 15-20 cm depth at the downstream site are very different from the rest of the samples.

4.4 DGGE profiling of the bacterial community

The bacterial community in the estuarine sediment samples was also analysed by DGGE. A first analysis showed that profiles from samples obtained from different cores within a sampling site (and at one specific depth) were virtually identical. This suggests that there is a high level of homogeneity at the sites and that the microbial communities are well represented even in small sample volumes. Only fragments that represent more than about 0.1% of the target sequences can usually be seen on DGGE gel (Øvreas *et al.,* 1997). There is an increase in bacterial diversity with depth. The diversity of most bacterial community which derived from prominent

The analysis of the DGGE profile from different depths of the upstream site showed fewer different bacterial communities (Fig. 3.16 A). The samples from the upstream site were dominated by band "d", which was common to all the depths (Fig. 3.16 A). The

DGGE profile of samples of different depths provided evidence of correlation between organic matter content and bacterial community structure.

The microbial diversity of the two sampling sites was compared at different depths, and the microbial richness was higher at the downstream site than at the upstream site at all depths. Fig. 3.18 A indicates that the downstream site has a higher bacterial diversity than the upstream site.

The cluster analysis (Fig.3.16 B and Fig.3.17 B) shows that the samples at 5-10 cm and 10-15 cm depths cluster together. The 25-30 cm depth sample is closely related to 20-25 cm depths from this sampling site (Fig. 3.16 B). Meanwhile, the 15-20 cm depth clustered with 20-25 cm. At both sites, the top depths did clustered together and were different from the deeper depths.
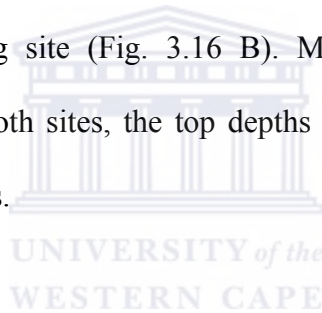
The cluster analysis also show that the downstream site sample from the upper depths (5-10 cm) cluster together and were well separated from the rest of the other depths (Fig. 3.17 B). The 25-30 cm depth from the downstream sites is closely related to 20-25 cm depths from this sampling site. This suggests that there downstream site may have common or similar archaeal population in the deeper depths. However, there is one band which is common to all depths of the downstream as shown by the presence of the DGGE bands in all samples (Fig. 3.17 A).

4.5 Analysis of a comparative bacterial 16S rDNA clone library

Bacterial 16S rDNA clone libraries were constructed from both sampling sites. A total of forty-five clones were sequenced and the partial 16S rDNA gene fragments aligned together with sequences from public databases. A phylogenetic tree was constructed in order to visualize the structure of the libraries and to identify differences between them. To obtain a full record of all members of the bacterial communities, a much larger number of samples would be required, but it was hoped to identify key differences in this limited study.

Fig. 3.22 shows that the majority of all obtained sequences were representatives of the δ-*Proteobacteria* (29%). Generally, thought that within the δ-*Proteobacteria* a large community of sulphate reducing bacteria is found in this class (Canfield et al., 1999). They anaerobically generate sulphide from sulphate and sulphur while oxidizing organic nutrients (Hugenoltz, *et al.,* 1998).

In marine sediments the importance of sulphate reduction depends on the amount of the organic matter availability and presence (Holmer *et al.,* 2001). Both biotic and abiotic are involved in the reoxidation processes. The reoxidation of sulphides which are produced during the sulphate reduction may go through several processes such as bacterial oxidation under oxidized conditions, phototrophic oxidation, and bacterial oxidation under anoxic conditions (Holmer *et al.,* 2001). In the oxic and anoxic conditions the sulphides which are chemically oxidated are considered to be important in the marine ecosystems sediment (Holmer *et al.,* 2001).

The major difference between the two sites was the occurrence of the class *Anaerolinaea,* which dominated the downstream site, making up 15% of the clones as compared to the upstream site, where only 2% of the clones belonged to this group. The class *Anaerolinaea* belongs to the phylum *Chloroflexi* and consists of bacteria that carry out anoxygenic photosynthesis, but they are also able to grow aerobically as chemoheterotrophs (Prescott *et al.,* 2002). Only cultivation of specific strains from the sediments and their characterization would probably help to identify the metabolic role they play in this ecosystem and explain why they are only prevalent at the wastewater-affected sampling site.

The difference in physical properties of the two sites as indicated in the water characteristics (Table 3.1) and the organic matter content (Fig. 3.1) seem to be the major factors influencing the microbial composition at each site. The organic matter is higher at the surface of site B than at site A (Fig. 3.1)

In this study most of the sequences from the clone library belonging to the γ-*Proteobacteria* were retrieved from the upstream site, .In addition, the presence of specific bacterial groups could be due to the influences of the organic matter content for example some of the bacterial groups (*Sphingobacteria, Actinobacteria, Deinococci* and *Plantomycetes*) were found to be only present at the upstream site. This could suggest that the upstream site is more anaerobic and has a lower content of nutrients.

REFERENCES

Abreu, C., Jurgens, G., De Marco, P. Saano, A. and Bordalo, A. A. (2001). Crenarchaeota and Euryarchaeota in temperate estuarine sediments. *J. Appl. Microbiol.* 90: 713-718.

Armbrust, E. V., Baross, J. A. and Crump, B. C. (1999). Phylogenetic analysis of particle-attached and free-living bacterial communities in the Columbia river, its estuary, and the adjacent coastal ocean. *Appl. Environ. Microbiol*. 65: 3192-3204.

Atlas, R. (1998). *Microbial Ecology: Fundamentals and Applications.* Benjamin Cummings. Menlo Park, Ca. 694 pp.

Berner, R.A. (2003). The long-term carbon cycle, fossil fuels and atmospheric composition. *Nature*, 426: 323-326.

Berthelet, M., Whyte, L. G. and Greer, C.W. (1996). Rapid, direct extraction of DNA from soils for PCR analysis using polyvinylpopyrrolidone spin columns. *FEMS Microbiol. Lett.* 138:17-22.

Bodelier, P. L. E. and Laanbroek, H. J. (2004). Nitrogen as a regulatory factor of methane oxidation in soils and sediments. *FEMS Microbiol. Ecol.* 47: 265-277.

Bong-Soo, K., Huyn-Myung, O., Hojeong, K. and Jongsik, C. (2005). Archaeal diversity in tidal flat sediment as revealed by 16S rDNA Analysis. *J. Microbiol.* 43: 144-151.

Boon, N., De Windt, W., Verstraete, W. and Top, E. M. (2001). Evaluation of nested PCR-DGGE (denaturing gradient gel electrophoresis) with group-specific 16S rRNA primers for the analysis of bacterial communities from different wastewater treatment plants. *FEMS Microbiol. Ecol*. 39:101-112.

Bowman, J.P., McCammon, S.A., Gibson, J.A.E., Robertson, L. and Nichols, P.D. (2003). Prokaryotic Metabolic activity and community structure in Antarctic continental shelf sediments, *Appl. Environ. Microbiol.* 69:2448-2462.

Braker, G., Ayala-del-Rio, H.L Devol, A.H Fesefeldt, A. and Tiedje, J.M. (2001). Community Structure of Denitrifiers, *Bacteria*, and *Archaea* along Redox Gradients in Pacific Northwest Marine Sediments by Terminal Restriction Fragment Length Polymorphism Analysis of Amplified Nitrite Reductase (*nirS*) and 16S rRNA Genes, *Appl. Environ. Microbiol.* 67: 1893-1901.

Campbell, N. (1993). *Biology*. Third Edition. Page 1190. Benjamin Cummings.

Canfield, D. E. and Raiswell, R. (1999). The Evolution of the Sulphur cycle, *Amer. J. Sci.* 299: 697-723.

Castle, D. and Kirchman, D. L. (2004). Composition of estuarine bacterial communities assessed by denaturing gradient gel electrophoresis and fluorescence in situ hybridization, *Limnol. Oceanogr. Methods* 2, 303-314.

Chen, F., Dustman, W. A. and Hodson, R. E. (1999). Application of *In Situ* Reverse Transcription to Estuarine Bacterial Community Analysis. *In: Microbial Biosystems: New Frontiers. Proc.8th Int. Symp, Microbial, Ecol.* (Bell, C. R ., Brylinsky, M. J. and Johnson-Green, P. *Eds.)*

Clark, J. R. and Cripe, C. R. (1993). Marine and Estuarine Multi-Species Test System. In: Handbook of ecotoxicology. *Blackwell Scientific Publications*, 1: 227-247.

Cottrell, M. T. and Kirchman, D. L. (2000). Natural assemblages of marine proteobacteria and members of the *Cytophaga-Flavobacter* cluster consuming low-

and high-molecular-weight dissolved organic matter. *Appl. Environ. Microbiol.* 66: 1692-1697.

Cottrell, M. T. and Kirchman, D. L. (2004). Single-cell analysis of bacterial growth, cell size, and community structure in the Delaware estuary. *Aquatic Microbial. Ecol.* 34: 139-149.

Crean Jr, D. J. (2004). *St Lucie Water Quality Data collection*, Coastal Ecosystems.

Crump, B. C., Kling, G. W., Bahr, M., and Hobbie, J. E. (2003). Bacterioplankton community shifts in an arctic lake correlate with seasonal changes in organic matter source. *Appl. Environ. Microbiol.* 69: 2253-2268.

Crump, B. C., Hopkinson, C. S., Sogin, M. L. and Hobbie, J. E. (2004). Microbial biogeography along an estuarine salinity gradient: combined influences of bacterial growth and residence time. *Appl. Environ. Microbiol.* 70: 1494-1505.

Crutzen, P. J. (1995). The role of methane in atmospheric chemistry and climate. In: Ruminant Physiology: Digestion, Metabolism, Growth and Reproduction: Proceedings of the Eighth International Symposium on Ruminant Physiology (Engelhardt, W.V., Leonhard-Marek, S., Breves, S. and Giesecke, D., Eds.), pp. 291–315. Ferdinand Enke Verlag, Stuttgart.

DeLong, E. F., Franks, D. G. and Alldredge, A. L. (1993). Phylogenetic diversity of aggregate-attached vs. free-living marine bacterial assemblages. *Limnol Oceanogr* 38: 924-934.

Department of Environmental Protection, (2004). Florida Marine Research Institute under a grant from the NOAA, Office of Ocean, and Coastal Resource Management.

Farnleitner, A. H., Zibuschka, F., Burtscher, M. M., Lindner, G., Reischer, G., and Mach, R. L. (2004). Eubacterial 16S-rDNA amplicon profiling: a rapid technique for comparison and differentiation of heterotrophic plate count communities from drinking water. *Internat. J. Food Microbiol.* 92: 333-345.

Farnleitner, A. H., Kreuzinger, N., Kavka, G. G., Grillenberger, S., Rath, J. and Mach, R. L. (2000). Simultaneous detection and differentiation of *Escherichia coli* populations from environmental freshwaters by means of sequence variations in a fragment of the β-D-glucuronidase gene. *Appl. Environ. Microbiol.* 66: 1340–1346.

Fauque, G. D. (1994). Sulphur reducuctase from thiophilic sulphate-reducing bacteria. *Methods in Enzymology*. Academic Press, New York. 243: 353-367.

Fuhrman, J. A. and Ouverney.C. C. (1998). Marine microbial diversity studied via 16S rRNA sequences: cloning results from coastal waters and counting of native *Archaea* with fluorescent single cell probes. *Aquat. Ecol*. 32: 3-15.

Giovannoni, S. J. and Rappe, M. (2000). Evolution, diversity, and molecular ecology of marine prokaryotes, p. 47-84. *In* D. L. Kirchman (ed.), Microbial ecology of the oceans. Wiley-Liss, New York, N.Y.

Hój, L., Olsen, R.A. and Torsvik, V.L. (2005). Archaeal communities in High wetlands at Spitsbergen, Norway (78^0N) as characterized by 16S rRNA gene fingerprinting, *FEMS Microbiol. Ecol.* 53: 89-101.

Holmer, M. and Storkholm, P. (2001). Sulphate reduction and sulphur cycling in lake sediments: a review, *Freshwater Biology*, 46: 431-451.

Houghton, J. T., Ding, Y., Griggs, D. J., Noguer, M., Vanderlinden, P. J., Dai, X., Maskell, K. and Johnson, C. A. (2001). Climate change 2001: The scientific basis.

Contribution of working group I to the third assessment report of the Intergovernmental panel on climate change. *IPCC.* page. 881.

Huber, H., Hohn,M. J., Rachaeal, R., Fuchs, J., Wilmer, V. C. and Stetter, K. O. (2002). A new phylum of Archaea represented by a nanosized hyperthermophilic symbiont. *Nat.* 417: 63-67.

Hugenholtz, P., Goebel, B. M., and Pace, N. R. (1998). Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *J. Bacteriol.* 180: 4765-4774.

Ishii, K., Muβmann, M., MacGregor, B. J. and Amann, R. (2004). An improved fluorescence in situ hybridization protocol for the identification of bacteria and archaea in marine sediments, *FEMS Microbiol. Ecol.* 50: 203-213.

Jϕrgensen, N. O. G., Stepanaukas, R., Pedersen, A.U., Hansen, M. and Nybroe, O. (2003). Occurrence and degradation of peptidoglycan in aquatic environments. *FEMS Microbiol. Ecol.* 46: 269-280.

Jurgens, G. (2002). Molecular phylogeny of Archaea in boreal forest soil, freshwater and temperate estuarine sediment. Academic Dissertation, November 2002.University of Helsinki, Department of Applied Chemistry and Microbiology, Division of Microbiology, Faculty of Agriculture and Forestry.

Jurgens, G., Lindstrom, K. and Saano, A. (1997). Novel group within the kingdom Crenarchaeota from boreal forest soil. *Appl. Environ. Microbiol.* 63:803-805.

Keller, M., and Zengler, K. (2004). Tapping into Microbial Diversity. *Nat. rev.* 2: 141-150.

Kisand V, and Wikner, J. (2003). Combining culture-dependent and-independent methodologies for estimation of richness of estuarine bacterioplankton consuming riverine dissolved organic matter. *Appl. Environ. Microbiol.* 69: 3607-16.

Kisand V., Cuadros R., Wikner, J. (2002). Phylogeny of culturable estuarine bacteria catabolizing riverine organic matter in the northern Baltic Sea. *Appl. Environ. Microbiol.* 68: 379-88.

Knoll, A. H., and Canfield, D. E. (1998). Isotopic inferences on early ecosystems. Pp. 212–243 in *Isotope Paleobiology and Paleoecology,* The Palaeontological Society Papers, Vol. 4. R. D. Norris and R. M. Corfield, Eds. Publication of the Palaeontological Society, Pittsburgh, PA.

Langenheder, S., Kisand, V., Wikner, J. and Tranvik, L. J. (2003). Salinity as a structuring factor for the composition and performance of bacterioplankton degrading riverine DOC. *FEMS Microbiol. Ecol.* 45: 189-202.

Laniol, B. D. and Giovanoni, S.J. (1997). Identification of bacterial cells by chromosomal painting. *Appl. Environ. Microbiol.* 63:1118-1123

Lee, S. and Kemp, P. F. (1994). Single-cell RNA content of natural marine planktonic bacteria measured by hybridization with multiple 16S rRNA-targeted fluorescent probes. *Limnol Oceanogr.* 39:869-879.

Levine, U, and. Charkoudian, L. K. (2001). The effects of microbial diversity on biogeochemical processes in brackish water microcosms, ecosystems center, marine biological laboratory, *Semester in Environmental Science*, Woods Hole, Massachusetts, USA.

Levsky, J. M. and Singer, R. H. (2003). Fluorescence in situ hybridization: past, present and future. *J Cell Sci.* 116: 2833-2838.

Li, L., Guenzenne, J., Nichols, P., Henry, P., Yanagibayashi, M. and Chiakikato (1999). Microbial diversity in Nankai trough sediments at a depth of 3,843 m. *J. Oceanogr.* 55: 635-642.

Llobet-Brossa, E., Rossello-Mora, R. and Amann, R. (1998). Microbial Community Composition of Wadden Sea Sediments as Revealed by Fluorescence In Situ Hybridization, *Appl. Environ. Microbiol.* 64: 2691-2696.

Maltagliati, F. (2003). Introduction to topic one "Measuring, managing and conserving genetic resources in natural marine system". 1: 10-12.

McArthur, J.V., Shimkets, L. J. and Wise, M.G. (1997). Bacterial diversity of Caroline bay as determined by 16s rRNA gene analysis: confirmation of novel taxa. *Appl. Environ. Microbiol.* 63: 1505-1514.

McInerney, J. O., Wilkinson, M., Patching, J. W., Embley, T. M. and Powell, R. (1995). Recovery and phylogenetic analysis of novel archaeal rRNA sequences from a deep- sea deposit feeder. *Appl. Environ. Microbiol.* 61:1646-1648.

Miller, D. N., Bryant J. E., Madsen E. L. and Ghiorse W.C. (1999). Evaluation and optimisation of DNA extraction and purification procedures for soil and sediment samples. *Appl. Environ. Microbiol.* 65:4715-4724.

Muβmann, M., Ishii, K, Rabus, R. and Amann, R. (2005). Diversity and vertical distribution of cultured and uncultured Deltaproteobacteria in an intertidal mud flat of the Wadden sea. *Environ. Biol.* 7: 405-418.

Muyzer, G., Brinkhoff, T., Nübel, U., Santegoeds, C., Schäfer, H. and Wawer, C. (1998). Denaturing gradient electrophoresis (DGGE) in microbial ecology. In: Akkermans, A.D.L., Van Elsas, J.D. and Bruijn, F.J., Editors, 1998. *Molecular*

Microbial Ecology Manual, Kluwer Academic Publishing, Dordrecht, Netherlands, pp. 3.4.4/1–3.4.4/27.

Muyzer, G., de Waal, E. C. and Uitterlinden, A. G.(1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol*. 59:695-700.

Nakatsu, C.H., Torsvik, V. and Ovreas, L. (2000). Soil community Analysis using DGGE of 16S rDNA Polymerase chain reaction products, *Soil Sci Soc. Am*. 64;1382-1388.

Nogales, B., Timmis, K. N., Nedwell, D. B. and Osborn, A. M. (2002). Detection and diversity of expressed denitrification genes in estuarine sediments after reverse transcription-PCR amplification from mRNA. *Appl. Environ. Microbiol.* 68: 5017-5025.

Orphan, V. J., Hinrichs, K. U., Ussler III, W., Paull, C. K., Taylor, L. T., Sylva, S. P., Hayes, J. M. and Delong, E. F. (2001). Comparative analysis of methane-oxidizing *Archaea* and sulfate-reducing bacteria in anoxic marine sediments. *Appl. Environ. Microbiol.* 67: 1922-1934.

Ovreas, L., Forney, L, Dae, F. L. and Torsvik, V. (1997). Distribution of bacterioplankton in meromictic Lake Saelenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. *Appl. Environ. Microbiol*. 63:3367-3373.

Palmer, M. A., Covich, A. P., Lake, S., Biro, P., Brooks, J. J., Cole, J., Dahm, C., Gibert, J., Goedkoop, W., Verhoeven, W. and Van De Bund, W. J. (2000). Linkages between aquatic sediment biota and life above sediments as potential drivers of biodiversity and ecological processes. *BioScience* 50:1062–75.

Peplies, J., Glöckner, F. O. and Amann, R. (2003). Optimization strategies for DNA microarray-based detection of bacteria with 16S rRNA-targeting oligonucleotide probes. *Appl. Environ. Microbiol.* 69: 1397-1407.

Philippot, L. (2002). Denitrifying genes in bacterial and Archaeal genomes. *Biochimica et Biophysics Acta (BBA) - Gene Structure and Expression.* 1577: 355-376.

Prescott, L. M., Klein, D. A. and Harley, J. P. (2002) *Microbiology; Microorganisms in Terrestrial Environments*, McGraw-Hills Higher Education.

Ramsing , N. B., Thomsen, T.R. and Finster, K. (2001) .Biogeochemical and molecular signatures of anaerobic methane oxidation in a marine sediment. *Appl. Environ. Microbiol*. 67: 1646–1656.

Ravenschlag, K., Sahm,K., Knoblauch, C., Jǿrgensen, B. B. and Amann, R. (2000) Community structure, cellular rRNA content and activity of sulfate- reducing bacteria in marine arctic sediments. *Appl. Environ. Microbiol.* 66: 3592-3602.

Reshetiloff, K. (2004).Chesapeake Bay: Introduction to an ecosystem.EPA903-R-04-003, CBP/TRS 232/00, Jul. 2004. *U. S Environ. Prot. Agen,* for Chesapeake Bay prog.

Reysenbach, A. L. and Pace, N.R. (1995) in: Archaea: A Laboratory Manual-Thermopiles, pp. 101-107 (Robb, FT and Place, AR, Eds.) Cold Spring Harbour Laboratory Press, New York.

Ricciardi, A. and MacIsaac, H. J. (2000). Recent mass invasion of the North American Great Lakes by Ponto-Caspian species. *Trends. Ecol. Evol.* 15: 62-65.

Robertson, C. E., Harris, J. K., Spear, J. R. and Pace, N. R. (2005). Phylogenetic diversity and ecology of environmental Archaea, *Curr. Opin. Microbiol.* 6: 638-642.

Sambrook J, and Russell, D. W. (2001). *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Schippers, A. and Jørgensen, B. B. (2002). Biogeochemistry of pyrite and iron sulfide oxidation in marine sediment, *Geochem Cosmo Acta.* 66: 85-92.

Sekiguchi, H., Watanabe, M., Nakahara, T., Xu, B., and Uchiyama, H. (2002). Succession of bacterial community structure along the Changjiang River determined by denaturing gradient gel electrophoresis and clone library analysis. *Appl. Environ. Microbiol.* 68: 5142-5150.

Sigler, W. V., Miniaci, C., and J. Zeyer, J., (2004). Electrophoresis time impacts the denaturing gradient gel electrophoresis-based assessment of bacterial community structure. *J. Microbiol. Methods.* 57: 17-22.

Sjőling, S., Mohammed, M. S, Lyimo, T. J. and Kyaruzi, J. J. (2005). Benthic bacterial diversity and nutrient processes in mangroves: impact of deforestation, Estuarine, *Caost. and Shelf Sci.* 63: 397-406.

Smith, R. L. (1992). *Elements of Ecology*, Harper Collins, 3rd Edition.

Stetter, K. O. (1996). Hyperthermophilic prokaryotes. *FEMS Microbiol. Rev*. 18: 149-158

Talaro, K. P. and Talaro, A. (1999). *Foundations in Microbiology*, McGraw-Hills. 3rd Edition.

Teske, A., Dhillon, A., Mitchell, L. and Sogin, M. L. (2003). Genomic Markers of Ancient Anaerobic Microbial Pathways: Sulfate Reduction, Methanogenesis, and Methane Oxidation. *Biol. Bull.* 204: 186-191.

Valentine, D. L. (2002). Biogeochemistry and microbial ecology of methane oxidation in anoxic environments: a review. *Ant. van Leeuwenhoe.,* 81: 271–282.

Valentine, R. L., and Zepp, R. G. (1993). Formation of carbon monoxide from the photodegradation of terrestrial dissolved organic carbon in natural waters, *Environ. Sci. Technol.* 27: 409-412.

van Hannen, E. J., Mooij, W., van Agterveld, M. P., Gons, H. J., and Laanbroek, H. J. (1999). Detritus-dependent development of the microbial community in an experimental system: qualitative analysis by denaturing gradient gel electrophoresis. *Appl. Environ. Microbiol.* 65: 2478-2484.

Vitousek, P. M., Chair, J., Aber, R. W., Howarth, G. E., Likens, P. A., Matson, D. W., Schindler, W. H. and Tilman, G. D. (1997). Human Alteration of the Global Nitrogen Cycle: Causes and Consequences. *Ecol. Soc. Amer.*

Waibel, A. E., Peter, T., Carslaw, K. S., Oelhaf, H., Wetzel, G., Crutzen, P. J., Poschl, U., Tsias, A., Reimer, E. and Fischer, H. (1999). Arctic ozone loss due to denitrification. *Sci.* 283: 2064–2069.

Yanisch-Perron, C., Vieira, J. and Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mpl8 and pUCI9 vectors. *Gene* 33: 103-119.

Zumft, W.G. (1997). Cell biology and molecular basis of denitrification. *Microbiol. Molec. Biol. Rev*. 61: 533–536.