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The identification of prevalent bacterial isolates and characterisation of microbial communities in paper-mill water systems

by

RENÉ-MARIÉ DU TOIT

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Department of Microbiology and Plant Pathology
Faculty of Natural and Agricultural Sciences
University of Pretoria
Pretoria
South Africa

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Supervisor: **Dr J.F. Wolfaardt**

Co-supervisor: **Dr M.M. Kock**

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I declare that the thesis/dissertation, which I hereby submit for the degree M.Sc (Microbiology) at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

SIGNATURE: ….. …………….

DATE: ……… 15 January 2008 ………….

“If we work in marble, it will perish;
If we work upon brass, time will efface it;
If we rear temples, they will crumble into dust;
But if we work upon immortal minds and instill into them just principles,
we are then engraving upon tablets which no time will efface,
but will brighten and brighten to all eternity.”

Daniel Webster

Dedicated to my father

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ABBREVIATIONS

AB	Anaerobic bacteria
AOX	Adsorbable organic halides
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
AWCD	Average well colour development
BHAP	2-bromo-4-hydroxyacetophenone
BLAST	Basic local alignment search tool
BOD	Biological oxygen demand
CFU	Colony forming units
CIP	Collection de L'Institut Pasteur
COD	Chemical oxygen demand
d	Berger-Parker dominance index
DAF	Dissolved air flotation
DBNPA	2-2-dibromo-3-nitrilopropionamide
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
DO	Dissolved oxygen
DSM	Deutsche Sammlung von Mikroorganismen
DWAF	Department of Water Affairs and Forestry
ECP	Extra-cellular polymers
E_h	Oxidation-reduction potential (measured in mV)
ERIC	Enterobacterial repetitive intergenic consensus
GLM	General linear modelling
H'	Shannon diversity index
JCM	Japan Collection of Microorganisms
LMG	Laboratorium voor Microbiologie, University of Gent
MPN	Most probable number
KLB	Kraft liner board
MBT	Methylene bis(thiocyanate)
N	Total nitrogen
NA	Nutrient agar
NCBI	National Centre for Biotechnology
NCCB	Netherlands Culture Collection of Bacteria
NCPPB	National Collection of Plant Pathogenic Bacteria
nd	Not determined
ns	Not stated
ORP	Oxidation-reduction potential
P	Total phosphorous
PCR	Polymerase chain reaction
PM	Paper machine
QAC	Quaternary ammonium compound
QS	Quorum sensing
rDNA	Ribosomal deoxyribonucleic acid
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
SASM	South African Society for Microbiology

SEM	Scanning electron microscopy
SRB	Sulphate-reducing bacteria
SWC	Specific water consumption
TAB	Total aerobic bacteria
TDS	Total dissolved solids
TGGE	Temperature gradient gel electrophoresis
TM	Tissue machine
TSS	Total suspended solids
VFA	Volatile fatty acids
WRC	Water Research Commission

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To the Creator of life and all knowledge thereof.

The identification of prevalent microbial isolates and characterisation of microbial communities in paper-mill water systems

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RENÉ-MARIÉ DU TOIT

SUPERVISOR: Dr. J. F. Wolfaardt
CO-SUPERVISOR: Dr. M. M. Kock
DEPARTMENT: Microbiology and Plant Pathology
DEGREE: M.Sc (Microbiology)

SUMMARY

Water is a scarce and unevenly distributed national resource and it is, therefore, important to reduce water consumption in paper mills. Closure of water systems for reuse, however, directly and indirectly results in an increase in the numbers and types of microorganisms resulting in poor runnability, lower production rates and increased safety hazards. The aim of this study was to investigate the microbiology of paper-mill water systems in South Africa to aid in closure of water systems whilst controlling microbial fouling. Different environmental parameters monitored at paper mills were reviewed together with microbial enumeration techniques employed by industry and characterisation and identification methods to study bacteria.

Various environmental and process parameters could play an important role in the number and type of microorganisms in a paper-mill water system. The highest correlation between an environmental parameter and biological activity was found for oxidation-reduction potential and the numbers of culturable aerobic bacteria. Other environmental parameters that significantly influenced microbial numbers were temperature, dissolved oxygen,

dissolved solids, chemical oxygen demand, nitrogen, phosphorous, specific water consumption, pulp furnish, biocide class and retention time.

The characterisation and identification of problematic bacteria in paper mills could enable better control since the correct biocides could be applied to minimise microbiologically associated problems. Prevalent bacteria that were isolated from the water systems of 14 paper machines were typed into 35 distinct groups using ERIC-PCR and PCR-RFLP and identified with sequence analysis. Eleven of the 35 types were identified to species level, 20 types were identified to genus level and the remaining four types were identified to family level. It was found that the majority of bacteria belonged to the genera *Acinetobacter* and *Pseudomonas* that contain well-known slime-forming bacterial species.

Traditional methods employed to investigate bacteria in industrial water systems often do not accurately represent the composition and diversity of bacterial communities. DGGE analysis could provide a powerful tool for monitoring bacterial diversity, since it is able to discriminate between identical sizes of PCR-amplified DNA fragments that differ in their sequence content. The use of DGGE to monitor changes in microbial populations could improve control of microbial fouling, but more analyses would be needed to validate the results of the present study.

PREFACE

The pulp and paper industry is considered one of the major consumers of natural resources and energy and has been ranked third in the world in terms of water consumption after the primary metals and chemical industries (Thompson *et al.*, 2001). On a global level, the availability of fresh, uncontaminated water is decreasing at an alarming rate and, in industrialised countries, recovery and reuse of treated effluent is becoming a necessity (Lindholm, 2000). South Africa is classified as a semi-arid country with an annual rainfall of 479 mm compared to the global average of 860 mm (Cowan, 1995) and it is estimated that the total requirement for water in South Africa will double over the next thirty years. Therefore, there is a need to explore means of water preservation, since it is considered a scarce and unevenly distributed national resource (South African Water Act No 36 of 1998).

Strategies to reduce water consumption in paper mills include the closure of water circuits to reuse water because, increased public awareness and government regulations have motivated pulp and paper mills to develop environmentally friendly processes (Bihani, 1996). The closure of water systems has been implemented successfully in mills around Europe in the past as paper mills increasingly closed off their systems in an attempt to reduce water consumption (Berard, 2000). Water reuse allows heat, chemicals and sometimes fibre, that would otherwise be discarded as pollutants, to be reclaimed (Dexter, 1996) as well as reduced fresh-water use (Panchapakesan, 2001).

As more mills begin to close their water systems, the importance of water quality, materials management and deposition control is emphasized (Gudlauski, 1996), since closure directly and indirectly results in an increase in microorganisms. Upon closure nutrients are concentrated and temperatures are elevated, leading to increased metabolic rates of microorganisms (Gudlauski, 1996), which can contribute to problems such as biofilm formation and ultimately microbially induced corrosion (Bennett, 1985). Massive bacterial growth in paper machines may also produce enzymes that can cause degradation of raw materials and products (Väisänen *et al.*, 1998).

Control measures are, therefore, necessary to counter the colonisation of microorganisms in paper mills and water treatment systems are mainly concerned with three problem areas of microbial growth, namely gram-negative slime-producing species, corrosive anaerobic species, as well as algal biomass (Frayne, 2001). Chemical biocides still represent the most effective measure to control microbial growth and fouling in paper mills (Pereira *et al.*, 2001),

but other strategies to limit growth include the addition of microbiostats, mechanical cleaning and nutrient depletion.

For this study, the influence of the environment in a paper-mill water system on microbial numbers was reviewed together with relevant environmental parameters that are routinely monitored and enumeration techniques employed by industry (Chapter 1). Suitable methods to characterise the bacteria in paper-mill water systems were also reviewed (Chapter 2), since it is well-known that bacteria are the main colonisers of paper-machine water (Väisänen *et al*., 1998). The effect of various environmental parameters on different microbial guilds was investigated to determine if the regulation of these parameters could reduce microbial fouling (Chapter 3). The prevalent bacteria in paper-mill water were characterised and identified to determine if problematic species were present in the water systems (Chapter 4). The structural diversity of the bacterial population in one paper mill was investigated to determine if the current enumeration techniques are adequate to detect microbial proliferation and also to determine if changes in the microbial population structure could indicate efficient microbial control programmes (Chapter 5).

This project was funded by the Water Research Commission (WRC) of South Africa and carried out in collaboration with the Sappi Technology Centre (Pretoria, South Africa). Each of six paper mills were visited twice over a period of three years and 15 paper-machine water systems were surveyed. For each survey, a report was distributed to the mills and recommendations were made to improve microbial control. Three annual reports were completed for the WRC as well as a final report detailing all the deliverables set out at the beginning of the project. Three posters were also represented at the South African Society for Microbiology (SASM) 2006 Biennial Conference in Pretoria (South Africa). The posters addressed the effect of oxidation-reduction potential on bacterial numbers, the characterisation and identification of prevalent bacteria in nine paper-mill water systems and the use of functional characterisation to evaluate biocide programmes in paper mills.

Currently no system exists for the rapid identification of prevalent bacteria in paper mills. Since one of the project deliverables of the WRC required bacterial species identification, it was decided to develop such a system, although it did not form part of the current study. "PaperBac" is a software tool that allows field scientists to rapidly identify bacteria from paper mills with RFLP and Biolog experimental results. The field scientist performs the Biolog and RFLP experiments and enters the results in PaperBac through an intuitive user

interface. PaperBac compares the results to a dataset and identifies the organism in question. The dataset contains the Biolog and four RFLP profiles of the 35 prevalent bacterial types characterised and identified in Chapter 4. This tool was awarded the 2007 TAPPSA Technical Award for Innovation.

This study provided invaluable information on the microbiology of paper-machine water systems in South Africa. The generated data could aid in the design of new and improved microbiological control programmes when mills institute the closure of their water systems in order to comply with more stringent water regulations.

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CHAPTER 1

A LITERATURE REVIEW ON THE INFLUENCE OF ENVIRONMENTAL PARAMETERS ON MICROBIAL NUMBERS IN PAPER-MILL WATER SYSTEMS

ABSTRACT

It is important to reduce water consumption in paper mills, since water is a scarce and unevenly distributed national resource. Integrated water management plans for paper mills include strategies to reduce water consumption by closure of water circuits to reuse water. Closure, however, directly and indirectly leads to increases in populations of microorganisms that can possibly result in poor runnability and lower production rates of paper mills. Control measures are, therefore, necessary to counter the colonisation of microorganisms in paper mills. By monitoring different environmental parameters such as temperature, pH, dissolved oxygen, oxidation-reduction potential, chemical and biological oxygen demand, nitrogen, phosphorous, suspended solids, dissolved solids and toxicity, an indication of the microbial activity in a paper-mill water system may be obtained. It is necessary to quantify the types and numbers of microorganisms in any water system to determine the efficiency of the disinfection procedures. Plate counts remain the most popular method to determine microbial numbers in industry, due to low cost and ease of application, even though it is time-consuming and biased. The trending of plate counts can indicate whether there is a difference in biological activity at different sampling points. The use of ATP to indicate biological activity requires specialised equipment and expertise, but can be effective and time-saving when employed under the right circumstances.

1. BACKGROUND

1.1 Introduction

About 5500 years ago the Egyptians used flattened stalks of papyrus as writing surfaces, but paper as it is known today, originated in China in 105 AD (Thompson *et al.*, 2001). During the late Middle Ages paper as a commodity increased in importance for public and intellectual life and during the 19th century the industrial revolution increased the demand for more paper. New processes had to be developed to make paper from natural resources, thereby exploiting the forests of the world.

The pulp and paper industry has traditionally been considered one of the major consumers of natural resources (wood and water) and energy (fossil fuels and electricity) (Thompson *et al.*, 2001). Being one of the most water intensive industrial processes (Panchapakesan, 2001), the pulp and paper industry is ranked third in the world in terms of water consumption after the primary metals and chemical industries (Thompson *et al.*, 2001). Water consumption varies with the production of different types of paper, but can be as high as 60 m^3/ton of paper. However, environmental impacts on air, water and land have been reduced by 80 to 90% due to legislative and environmental pressure (Thompson *et al.*, 2001). Pulp and paper mills currently operate more efficiently in terms of water consumption in almost all grades compared to previous decades, due to raw water availability and limitations of wastewater discharge (Panchapakesan, 2001).

On a global level, the availability of fresh, uncontaminated water is decreasing at an alarming rate and in industrialised countries recovery and reuse of treated effluent is becoming a necessity (Lindholm, 2000). South Africa is classified as a semi-arid country with an annual rainfall of 479 mm compared to the global average of 860 mm (Cowan, 1995). It is estimated that the total requirement for water in South Africa will double over the next thirty years and there is a need to explore different means of water preservation. In South Africa water is a scarce and unevenly distributed national resource (South African Water Act No 36 of 1998) and strategies to reduce water consumption in mills include the closure of white-water circuits to reuse water.

An increase in populations of microorganisms is, however, a direct as well as indirect result of water closure. The microbiologically associated problems that frequently occur in paper mills primarily depend on the degree of closure of the water system. Upon closure, temperatures

are elevated, leading to increased metabolic rates of microorganisms and nutrient concentrations generally increase (Gudlauski, 1996), which contribute to problems such as biofilm formation and microbially induced corrosion (Bennett, 1985). Massive bacterial growth in paper machines may also produce enzymes that can cause degradation of raw materials and products (Väisänen *et al*., 1998).

In the past, white-water closure has occurred predominantly in mills producing uncoated linerboard and corrugating media. As more printing/writing grade mills begin to close their water systems, the importance of water quality, materials management and deposition control are increased, since quality and production in these grades are usually more sensitive to deposition and microbial growth (Gudlauski, 1996). Advantages of a closed water system include less fresh water used and smaller amounts of chemicals are required. Less fibre, fines and filler loss occur, whilst reduced costs of heating white-water and environmental compliance are also making a closed white-water system more advantageous (Panchapakesan, 2001).

The objective of this review is to describe the environment in a paper-mill water system and to evaluate the influence of different physical parameters on microbial populations. The influence of microorganisms, especially corrosive biofilms, is discussed as well as different methods to minimise their effect in the water system of a typical paper mill. Traditional and emerging techniques to quantify microorganisms in environmental samples are evaluated as an indication of the effectiveness of the biocide programme in a paper mill.

1.2 Wastewater treatment

Water from natural water resources often only needs filtration and disinfection prior to use in a paper mill (Webb, 2003). Pulp is diluted to consistencies as low as 1% with water and paper additives when transported to the headbox where it is evenly distributed along a moving support of forming fabric. The majority of water drains freely through the fabric, leading to formation of a wet paper sheet. After this, the fibre web is pressed to extract more water and to form paper sheets. Residual water is removed by pressing the paper sheet through a series of steam-heated cylinders (Thompson *et al*., 2001). Large volumes of wastewater are generated for each metric tonne of paper produced and these effluents are heavily loaded with organic matter (Ali & Sreekrishnan, 2001).

The water collected from each stage of dewatering contains different proportions of fibre, filler, fines and other materials that can be reused. The many different arrangements of filtration equipment, silos and other white-water storage tanks are known as a mill's white-water system (Panchapakesan, 2001).

Effluent differs significantly amongst various pulp and papermaking operations due to the diversity of processes and chemicals used (Thompson *et al.*, 2001). Left untreated, effluent can potentially be toxic and result in serious environmental problems such as changes in fish populations and eutrophication of recipient water bodies. This environmental impact is due to high chemical and biological oxygen demand, chlorinated compounds that are measured as adsorbable organic halides (AOX), suspended solids that mainly consist of fibres, fatty acids, tannins, resin acids, lignin and its derivates and sulphur compounds (Ali & Sreekrishnan, 2001). Waste emitted from a paper mill include effluent, gases such as NO_X, SO_2, CO, CO_2 and reduced sulphur compounds, as well as solid waste consisting of sludge from raw water treatment, primary sludge, biosludge, rejects and ashes (Figure 1).

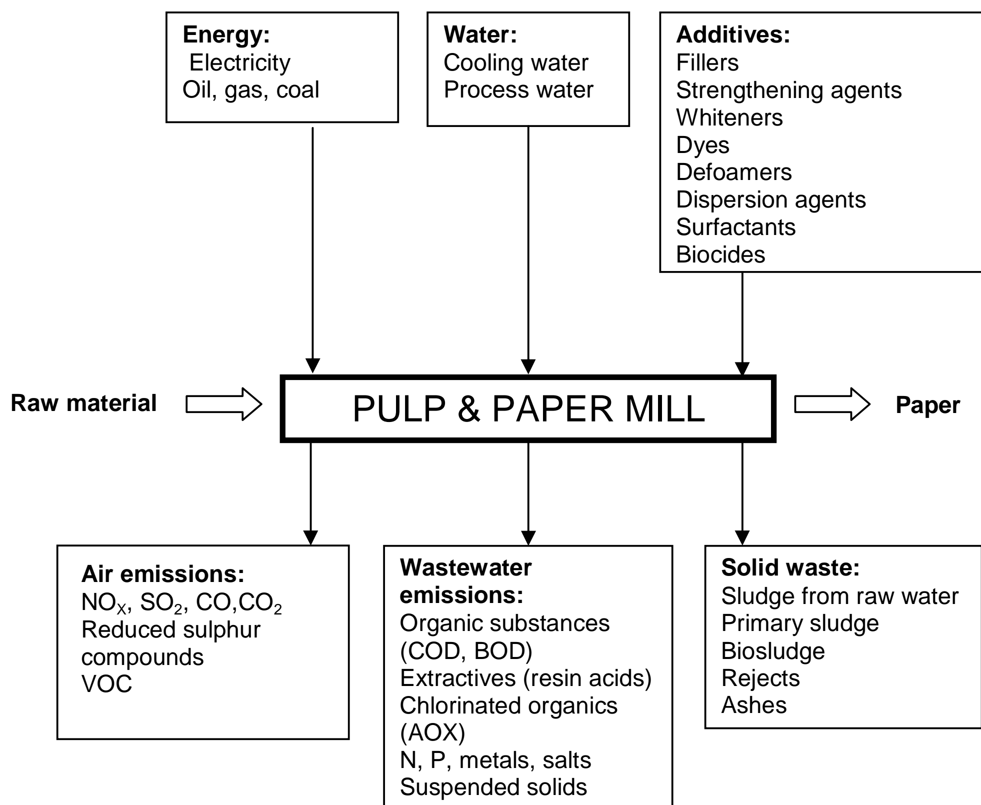

Figure 1: Mass-stream overview of a pulp and paper mill (Lacorte *et al.*, 2003).

Most soluble components from wood and recycled paper are biodegradable. Therefore, biological treatment can efficiently reduce nutrients such as phosphorous, nitrogen and multivalent metal ions (Lindholm, 2000). The main treatment processes at pulp and paper mills include primary clarification, succeeded in some cases by secondary treatment and, rarely, tertiary treatment. Primary clarification is achieved either by sedimentation or flotation to remove suspended solids. This is followed by a secondary treatment, usually biological in nature (Thompson *et al.*, 2001). The activated sludge process is commonly used for secondary biological treatment to achieve very high removal efficiency of organic compounds. However, poor settling of sludge, also known as bulking, could cause operational problems. Activated sludge treatment is also sensitive to shock loading and toxicity and has a limited capacity to remove poorly degradable toxic substances (Thompson *et al.*, 2001). Anaerobic digestion, as an alternative to the activated sludge process, will lower sludge production and chemical consumption, but is not as widely used in pulp and paper industry (Thompson *et al.*, 2001). By definition, anaerobic digestion requires wastewater to contain a substantial amount of organic matter, so that it can be converted to methane, CO_2 and biomass in the absence of oxygen (Kay, 2003). Tertiary treatment includes membrane processes such as ultrafiltration or physico-chemical processes such as ozonation and adsorption to remove toxic materials and reduce parameters such as chemical oxygen demand and colour (Thompson *et al.*, 2001).

1.3 White-water closure

Wastewater regulations in the USA before 1972 were based on the assimilative capacity of the receiving waters. Today, however, specific discharge limitations are in place with stringent limitations where the environment is at risk of degradation (Edde, 1994). In the past 20 years, European paper mills became increasingly closed off in an attempt to reduce water consumption (Berard, 2000). The Department of Water Affairs and Forestry (DWAF) of South Africa developed waste discharge systems to provide a framework for billing water users that dispose of their waste into water resources (Venter, 2002). The aim of the system is to aid in the country’s continuing economic growth while meeting the wide range of needs for water quality.

As early as the late sixties, water closure of paper-mill water systems was proposed and today, with increased public awareness and government regulations, pulp and paper mills are motivated to develop environmentally friendly processes (Bihani, 1996). Water reuse allows

heat, chemicals and sometimes fibre that would otherwise be discarded as pollutants, to be reclaimed (Dexter, 1996).

Although an essential part of the papermaking process, water only acts as a carrier, rather than being a significant component of the final paper product and is mostly recycled (Berard, 2000). Closure is the ultimate goal in solving problems with pulp mill discharges into receiving waters. According to Pulles (1983) some other advantages of a closed water system include:

- Reduced losses in fibres, fillers and other additives.
- Reduced costs of wastewater and fresh-water clarification, as well as less dependence on water supplies.
- Reduced steam consumption due to the higher temperature of the system.
- More stable products once the system equilibrium is reached.

The discharge of large volumes of contaminated water should be eliminated to enable a pulp mill to operate in harmony with the environment (Myrèen, 1993). The reduction of effluent discharge, however, leads to the accumulation of anionic trash, waxes, secondary stickies, pitch, odorous components and salts that may have a negative impact on the paper-making process as well as on the product (Habets *et al.*, 1996).

The closing of water circuits results in the enrichment of soluble organic matter in white-water, which leads to increased growth of microorganisms on surfaces and the formation of biofilms with considerable amounts of slime. Clumps or patches detaching from surfaces cause spots and holes and hygienic problems are caused by volatile fatty acids (VFA) that, in turn, can lead to odour and an increased need for cationic retention chemicals (Malmqvist *et al.*, 1999). Other problems include organic matter that cannot be removed by disc filters or dissolved air flotation (DAF) units (Malmqvist *et al.*, 1999), accumulation of suspended and dissolved solids and thermal build-up (temperature increase) (Dilsook *et al.*, 2004). The biggest challenge of running a closed-loop system, therefore, lies in maintaining proper chemical balances in all situations (Berard, 2000).

According to Gudlauski (1996) specific problems encountered during the closure of a white-water system include compromised runnability due to sheet breaks that is caused by microbial deposition. Other problems include downtime needed to wash up or boil out the deposition

and reduced production capacity due to screen plugging. Safety issues include toxic or explosive gas production (hydrogen sulphide, hydrogen, or methane) by anaerobic bacteria in stock or water chests. This, together with slime deposition that can make catwalks and machine surfaces slippery, could potentially cause lost-time accidents.

Suitable water for reuse in papermaking is needed to reduce a mill's dependence on external water resources and provide protection against future water and effluent-rate increases (Dilsook *et al.*, 2004). The reuse of water can be achieved by introducing external biological treatment processes to remove nutrients under controlled conditions in white-water effluent (Malmqvist *et al.*, 1999). Each mill, however, has to evaluate its own situation based on the paper machine, market situation and local conditions. Simple computer models can predict the effect of closure if databases exist of physical and/or chemical parameters and the effects on microbial populations and alternative treatments can be decided upon before problems occur. The performance of process chemistry can, for example, be modified to handle increased concentration of chemical or biological contaminants (Berard, 2000).

New paper mills are designed to minimise the amount of effluent water generated and some containerboard mills have been able to achieve zero water emission. Kidney technology reduces effluent in circulation water while recycling pigments and fibres (Oinonen, 2003). The financial success in significantly reducing water consumption depends, however, upon maintaining production rates and product quality. These are areas where microbial activity and equipment corrosion can be costly to mills and force re-opening of water systems (Bennett, 1985).

2. ENVIRONMENTAL PARAMETERS

Steady-state conditions do not prevail in wastewater systems due to constant changes in substrate and nutrient concentrations as well as environmental conditions. The effects of environmental parameters on microorganisms are reflected in the internal polymer structure, growth rate and substrate accumulation of organisms in industrial water systems (Morgan-Sagastume & Allen, 2003). Microorganisms, in turn, are a good indication of the quality of water in a system. Parameters that are usually measured to determine water quality include temperature, pH, dissolved oxygen (DO), oxidation-reduction potential (ORP), total

suspended solids (TSS), total dissolved solids (TDS), chemical oxygen demand (COD), biological oxygen demand (BOD), total nitrogen (N), total phosphorus (P) and toxicity (Rump & Krist, 1992; Schneider *et al.*, 1998). No universal standard exists for the treatment of wastewater, even in industrialised countries, but there are some generally accepted parameter concentrations applicable to industrial water systems (Table 1).

Table 1: Accepted values of contamination in wastewater systems according to Rump & Krist (1992).

Parameter	**Contamination levels (mg/l)**		
	High	Medium	Low
TDS	1000	500	200
TSS	12	8	4
BOD	300	200	100
COD	800	600	400
N	85	50	25

2.1 Temperature

The effects of temperature transitions on metabolism and microbial community structure are not yet well understood, though temperature shifts have been correlated to decreased treatment performance and system instability in pulp and paper mills (Morgan-Sagastume & Allen, 2003). It was found by Liss & Allen (1992) that seasonal fluctuations of temperatures have an influence on microbial counts, with a decrease of viable aerobic heterotrophs corresponding to lower temperatures. Elevated temperatures resulting from discharges of heated water may have a significant ecological impact (Franson *et al.*, 1998). An increase in temperature as a result of mill closure can generate positive as well as negative effects. At temperatures higher than 45°C the growth of slime-forming bacteria is strongly reduced according to Habets *et al.* (1996) and better drainage can improve the speed of the paper machine (Dexter, 1996). The increase in corrosion and wear of process equipment has, in contrast, a negative impact on the paper machine (Dexter, 1996). In general, if the paper machine reaches temperatures that exceed 50°C, a population shift occurs from mesophiles to thermophiles. At temperatures greater than 70°C aerobic bacterial growth is inhibited, while spore forming bacteria are not affected (Dexter, 1996).

2.2 pH

pH is one of the environmental factors with the greatest relevance to the growth of microorganisms. Microbes prevail in certain ranges of pH that favour their nutrition,

reproduction and survival. In instances where a reduction in equilibrium occurs, pH is responsible for a high degree of mortality, especially when the change is sudden. In general, fungi prefer more acidic conditions, whereas bacteria grow in a neutral to alkaline environment (Madigen *et al.*, 2000). Electrometric pH measurements are done by determining the activity of the hydrogen ions by potentiometric measurement. A standard platinum hydrogen electrode and a reference electrode across which hydrogen gas is bubbled are used at a pressure of 101 kPa (Franson *et al.*, 1998).

2.3 Dissolved oxygen

Dissolved oxygen is generated by running or churning water as well as by autotrophic organisms. The DO content of natural waters usually does not exceed 8 mg/l, since heterotrophic microbial activity utilise oxygen. Replenishment from the atmosphere and photosynthetic O_2 evolution can be considerably slower than the microbial utilisation rate in the presence of abundant organic substrates (Atlas & Bartha, 1998). The DO is the electron acceptor of choice for microorganisms that degrade organic substrates in a subsurface environment, since more energy is gained from aerobic respiration than from denitrification, iron reduction, *etc*. Thus, at DO levels greater than 1 mg/l, aerobic bacteria will be plentiful and microorganisms utilising NO_3^-, SO_4^{2-}, Mn^{2+}, Fe^{3+} and CO_2 cannot survive (Wright & Cox, 2005). With increased temperatures, DO levels will decrease, leading to an increase in anaerobic bacterial numbers (Gudlauski, 1996; Robertson & Schwingel, 1997). As DO decreases, indigenous microorganisms will start to utilise NO_3^-, SO_4^{2-}, Mn^{2+}, Fe^{3+} and CO_2, as terminal electron acceptors (Wright & Cox, 2005) and at DO concentrations below 4 mg/l the community changes from aerobic to facultative anaerobic (Vaatanen & Niemela, 1983).

A variety of methods can be utilised to measure DO in water and wastewater. The azide modification of the Winkler buret titration method is a standard test for DO determination (Hach Company, 1992). Photometric determination of DO in the low range, high range and super high range is an alternative approach to accurately measure DO. The polarographic-electrode method is useful, because on-site rapid results can be obtained (Hach Company, 1992). Dissolved oxygen is temperature-related, with colder water having a higher DO content. This explains why warm water discharged from a paper mill can be problematic, and why mill closure in particular is not always desirable.

2.4 Oxidation-reduction potential

According to Jay (1992), ORP can be defined as the ability of a substrate to lose or gain electrons. A loss of electrons is known as oxidation and can also occur when oxygen is added to a substrate. Microbes show varying degrees of sensitivity to ORP. Aerobic bacteria require a positive potential (E_h), while anaerobic bacteria prefer a negative E_h or reduced environment for growth. According to Wright and Cox (2005) an ORP of 300 to 400 mV is common for aerobic conditions. Electrometric measurements are made by potentiometric determination of electron activity with an inert indicator electrode and a suitable reference electrode (Franson *et al.*, 1998). Electrodes made of platinum are frequently used for E_h measurements.

Microorganisms, especially aerobes, influence the E_h of their environment during growth (Jay, 1992). As aerobes grow, oxygen is depleted in a system, resulting in lowered ORP that makes the medium poorer in oxidising substances and richer in reducing substances. The E_h of a medium can also be reduced by microorganisms during their production of certain metabolic by-products such as H_2S which has the capacity to lower E_h to -300 mV (Jay, 1992). Du Toit *et al*. (2006) found that high microbial numbers decreased ORP levels and measurement of ORP can be used as a rapid indication of microbial load in white-water systems. This is especially useful when closure of a system begins.

2.5 Chemical oxygen demand

The COD of a system is an important parameter for the determination of the amount of organic pollution and is defined as the amount of a specified oxidant consumed under controlled conditions (Franson *et al.*, 1998). The most important contribution to the increase in COD and BOD in process water circuits is starch (used for the improvement of dry strength, paper-machine runnability and printability), because it is hydrolysed to glucose. Bacteria metabolise glucose to organic acids such as butyric acid, lactic acid, propionic acid and acetic acid (Habets *et al.*, 1996). These reactions are typical under anoxic and warm conditions and the presence of VFA, as a by-product of the reaction, is typical in mills with low water discharge. Increased levels of COD and BOD also lead to decreased DO levels that induce greater anaerobic biological activity (Dexter, 1996).

The COD test does not measure a specific concentration but rather a combination of substances and conditions. The significance of the test, according to Atlas & Bartha (1998), is

the measure of oxygen as an equivalent of the organic matter content in a sample. Low oxygen content indicates that dissolved compounds such as organic molecules and ammonium sulphites deplete the available oxygen. In paper-mill effluents COD depends on the discharged suspended solids such as fibre, fines and other chemically oxidising additives such as starch polymers. The COD test for water and wastewater, known as the dichromate reflux method (Hach Company, 1992), involves the digestion of a sample by a strong oxidising agent such as a hot sulphuric solution of $K_2Cr_2O_7$ while oxidisable organic compounds react. Green chromic ions (Cr^{3+}) are obtained from the reduction reaction, that can be determined either colorimetrically or with a burette titration (Hach Company, 1992).

2.6 Biological oxygen demand

In contrast to COD, where the oxidation of chemicals is measured, BOD measures molecular oxygen utilised for biochemical degradation of organic material during a specified incubation period (Franson *et al*., 1998). BOD is measured by incubating a sealed wastewater sample or a prepared dilution for a standard five-day period and then determining the change in dissolved oxygen content. The BOD value is calculated from the results of the dissolved oxygen tests (Hach Company, 1992).

Microorganisms consume DO as they decompose organic material in the water. Therefore, high BOD levels will cause rapid depletion of DO, creating problems in water systems. The contribution of paper-mill effluents to total BOD varies based on the proportion of oxidisable materials such as fibre, fines, starch, wet and dry strength resins, drainage aids, dyes, sizing materials and other dissolved substances (Panchapakesan, 2001).

The COD may be comparable to BOD even though it measures chemically oxidisable matter, since a relationship can usually be correlated between the two tests (Hach Company, 1992). A COD measurement is often preferred, because results are more reproducible and are available in a shorter period of time. The presence of bacterial inhibitors or chemicals that can potentially interfere with BOD measurement further makes the COD test more desirable than BOD. The BOD testing is also limited by temperature change, biological population, water movement, sunlight, oxygen concentration and other environmental factors that cannot be accurately reproduced in the laboratory (Hach Company, 1992). Many laboratories measure both COD and BOD, because the COD test can be used to target a specific BOD range eliminating the need for multiple BOD dilutions (Hach Company, 1992).

2.7 Nitrogen

Nitrogen is usually found in effluents in reduced forms such as organic nitrogen (N_2), ammonia (NH_3), amino acids, proteins, and heterocyclic nitrogen compounds (Bitton, 1994). According to Bitton (1994), 90% of N persists as NH_3 in effluents. Total N is a macronutrient and an important indication of potential microbial activity and can be determined through oxidative digestion of all digestible nitrogen forms to nitrate followed by quantification (Franson *et al.*, 1998). The concentration of nitrate can be determined photometrically after reaction with Nitrospectral (a derivative from benzoic acid). The Hach Company Water Analysis Handbook (1992) describes the determination of organic nitrogen *via* the total Kjeldahl nitrogen method, where organically-bound nitrogen compounds in the tri-negative state is converted into ammonium salts by the action of sulphuric acid and hydrogen peroxide. The ammonia is then analysed photometrically by a modified Nessler method (Hach Company, 1992).

2.8 Phosphorous

Phosphorous is a necessary macro-element for all living cells and a structural component of adenosine triphosphate (ATP), deoxyribonucleic acid (DNA), ribonucleic acid (RNA), proteins and phospholipids in cell membranes (Bitton, 1994). Excess P, together with N, is responsible for the enrichment of natural waters with inorganic materials that support excessive growth of individuals and species, also known as eutrophication (Atlas & Bartha, 1998).

Total P in wastewater consists of orthophosphate, polyphosphates and phosphates bound to organic compounds (Franson *et al.*, 1998). The Hach Company Water Analysis Handbook (1992) describes pre-treatment of samples with heat and acids to provide conditions for the hydrolysis of organic and inorganic forms of phosphorous to orthophosphates to determine phosphorous content of a sample. The test for total phosphorous is based on the reaction of orthophosphate with molybdate ions to form molybdophosphoric acid that is reduced by ascorbic acid to phosphomolybdenum blue that can be determined photometrically. Murphy & Riley (1962) suggested substituting ascorbic acid as reducing agent with antimony, since the colour development is rapid, stable and the sensitivity of the technique is superior and free from interference from other elements.

2.9 Total suspended solids

Total suspended solids refer to matter suspended in water or wastewater in general, but in a paper-machine water system TSS reflects fillers, fines and fibres (Pulles, 1983). The TSS that reside in effluent discharged from paper mills vary, based on the first pass retention, white-water system design, clarification equipment, equipment arrangement and system constraints in terms of water reuse (Panchapakesan, 2001). The amount of fibre in samples can play a significant role in the transfer, protection and enumeration of microbes and could also influence the accurate determination of other environmental parameters. Suspended solids are measured by filtering a well-mixed sample through a standard glass-fibre filter and drying the retained residue to a constant weight at 103 to 105°C (Franson *et al.*, 1998). TSS build-up will cause plugging problems, deposits, erosion and a reduced drainage rate (Pulles, 1983).

2.10 Total dissolved solids

The main chemical difference between open and closed white-water systems is the quantity of dissolved substances accumulating due to the concentrating effect of various process-water loops. Total dissolved solids is a measure of the amount of material dissolved in water, that can include carbonate, bicarbonate, chloride, sulphate, phosphate, nitrate, calcium, magnesium, sodium, organic and other ions (Murphy, 2004). Dissolved solids are also an important source of nutrients for microorganisms and consequently can indicate high microbial numbers. High levels of TDS can interfere with normal wet-end chemistry because the chemically active products are sterically hindered. Other potential problems associated with the build-up of TDS include foam, pitch, corrosion, sizing, brightness reversion, colour, pH control, precipitation, scale, odour and retention (Pulles, 1983).

TDS can be determined gravimetrically (Hach Company, 1992). A well-mixed sample is usually filtered and evaporated to dryness at 180°C but a quick alternative would be the use of an electrical conductivity probe. TDS is an important indication of nutrients in white-water systems that can aid microbial growth, especially if water loops are being closed.

2.11 Toxicity

Toxicity testing has become a useful tool in environmental risk assessment in recent years (Farré & Barceló, 2003). Effluents in paper mills exhibit characteristics of hazardous waste, due to fibres, fillers and chemicals, originating from either raw materials such as wood extractives or additives that include surfactants, glues, bleaching agents and biocides that are

used in the paper production process (Lacorte *et al.,* 2003). Toxic effects may be detected indirectly through routine effluent-quality testing and are manifested as increases in effluent suspended solids, bioassays, BOD, COD and ammonia concentrations (Lajoie *et al.*, 2002).

Bioluminescent bacteria can be used for wastewater toxicity evaluation (Lajoie *et al.* 2002). Studies of the effects of microbial metabolic activity on function constitute a rapid, reliable and convenient means of determining the toxicity of waste material (Franson *et al.*, 1998). The most common toxicity test is based on the inhibition of bioluminescence of marine bacteria such as *Vibrio fischeri* or *Photobacterium phosphoreum* that naturally emits light due to the bacterial luciferase enzyme (Farré & Barceló, 2003). The light production is directly proportional to the metabolic status of the cell and cellular activity is diminished by toxic substances. A *Pseudomonas putida* strain can also be used to determine bacterial growth response to a toxic environment by incubating the test samples and then measuring the growth in terms of absorption spectrophotometrically (Slabbert, 1986). Although this method may be more cost effective, it is more time-consuming than using the bioluminescence approach that is usually available in the form of a test kit.

Biosensors offer an alternative approach to monitor toxicity (Farré & Barceló, 2003). These analytical devices combine a biological sensing element *e.g.* an enzyme, DNA or a microorganism with a transducer that converts the biological signal into a measurable signal to estimate the toxicity of water bodies. Biosensors are very expensive, however, and the equipment needs to be maintained.

3. MICROORGANISMS IN A PAPER MILL WATER SYSTEM

Microorganisms are constantly introduced into paper machines through raw materials, water, fibres and papermaking chemicals. Bacteria were found to be the prevalent colonisers of paper-machine wet ends and raw materials (Väisänen *et al.*, 1998). The paper-machine environment is suitable for microbial growth due to favourable temperatures (30 to 50°C), pH (4 to 10) and nutrients that flow with the pulp and chemicals (Väisänen *et al.*, 1998). In all water systems, microorganisms can be grouped into two primary classes namely planktonic (free-floating) and sessile (attached) microorganisms (Stoner & King, 1994). High numbers of free-living bacteria in the process waters are not necessarily harmful for paper machine

operation and paper quality according to Väisänen *et al.* (1998), but it is becoming increasingly clear that biofilms, rather than planktonic microorganisms, cause damage to water-based technological processes (Ludensky, 2003). The presence of these microorganisms might result in clogged wires and felts and cause breaks in the paper web (Väisänen *et al.*, 1998).

3.1 Biofilms

In the paper industry, the presence of biofilms can lead to a decrease in product quality (Von Rège & Sand, 1998). When end-product defects occur, the paper must be recycled as 'broke' or sold as down-graded paper with reduced value. Mechanical cleaning of surfaces involves a machine shut-down and boiling the machine out at extreme pH levels and high temperatures. These boil-outs result in lost production time and profits as well as safety hazards (Robertson, 1994). The paper manufacturing process is water-based and microorganisms entering the system through process water are the main source of contamination. (Ludensky, 2003). In addition to a highly diverse microbiota, deposits may contain wood fibres, calcium carbonate, pitch, latexes, clay and other papermaking additives that can cause end-product defects such as holes, spots and even paper-sheet breaks when these deposits fall into the paper furnishes (Robertson & Taylor, 1993).

In an enclosed system, microbes will find an ideal environment for growth and reproduction with the increase of nutrients and metabolites (Ludensky, 2003). The most common microorganisms found in biofilms are sulphate-reducing bacteria (SRB), chemoorganotrophic bacteria and bacteria involved in iron-manganese cycles. It is well-known that the microbes entrapped in particles or adsorbed to surfaces are shielded from disinfection and are not inactivated (LeChevallier *et al.*, 1988). Thus, biofilm-matrix bacteria are largely protected against toxic substances such as biocides. The development of slime is especially common at the interfaces between the machine, water and air and it is estimated that 10 to 20% of all machine downtime is caused by slime problems (Ludensky, 2003).

A biofilm is defined as an assemblage of microbial cells that are irreversibly associated with a surface and is enclosed in a matrix of polymeric material allowing growth and survival in a sessile environment (Prakash *et al.*, 2003). Microbial cells that are embedded in this matrix of extra-cellular polymers (ECP) form a slimy layer on the surfaces of materials, which allows survival in a hostile environment (Prakash *et al.*, 2003). Biofilm-associated cells can be

differentiated from planktonic counterparts by the generation of ECP, reduced growth rates and the up and down regulation of specific genes (Donlan, 2002). Up to a 1000 sessile microorganisms may be present for each planktonic cell detected (Momba *et al.*, 2000).

Biofilm-forming bacteria have been shown to elicit specific mechanisms for initial attachment to surfaces, micro-colony formation and development of a three-dimensional community structure, maturation and detachment (Prakash *et al*., 2003). According to Videla (2001), the sequence of biological changes for biofilm formation occurs in stages. During the first stage a thin film of less than 100 nm thick is formed on the metal surface, due to deposition of inorganic ions and organic macromolecules such as polysaccharides and glycoproteins. This initial process of adhesion alters the distribution of electrostatic charges, facilitating further colonisation by microorganisms. In the second stage, a two-step adhesion process occurs. Reversible attachment of microbes to the surface occurs *via* electrostatic forces. Once attached, the microorganisms secrete ECP to aid in attachment, which is irreversible. Thus a biofilm is the result of an accumulation process at the interface that is not necessarily uniform in space or time (Characklis & Marshall, 1989).

As the biofilm begins to establish, a thin aerobic layer is formed across the metal surface, but as the biofilm grows, the part nearest to the metal surface becomes depleted of oxygen and can, therefore, be defined as an anaerobic zone (Keddy, 1988). When sulphate-reducing bacteria (SRB) are caught in the anaerobic zone they will proliferate and cause microbially induced corrosion (Madigen *et al*., 2000).

Surfaces are often of considerable importance as microbial habitats, since nutrients can adsorb to them. In the micro-environment of a surface, nutrient levels may be much higher than in the bulk solution. This phenomenon greatly influences the rate of microbial metabolism (Madigen *et al.*, 2000). Biofilm bacteria acquire nutrients from the waste products of their neighbours and secondary colonisers. The trace organics are concentrated on surfaces by pooling biochemical resources with different enzymes to break down food supplies as well as by ECP. The matrix is often negatively charged and attracts many nutrients to the biofilm surface. All these factors provide the bacteria in the biofilm with high nutrient levels compared to the surrounding environment and consequently biofilms can develop at nutrient concentrations too low to measure (Prakash *et al.*, 2003).

Other characteristics of the aqueous medium, such as pH, iron, oxygen, ionic strength and temperature also play a role in the rate of microbial attachment to a substrate. Seasonal effects on bacterial attachment and biofilm formation have also been shown in different aqueous systems (Prakash *et al.*, 2003).

3.2 Microbially induced corrosion

It is important to differentiate between corrosion and biofouling. Whereas the former involves metal dissolution, the latter is the undesirable accumulation of microbial deposits at an interface leading to the anaerobic corrosion of iron (Videla, 2001). Adhesion to surfaces by biofilms leads to an important modification at the metal-solution interface, accounting for serious losses of energy in different industrial systems (Videla, 2001). During microbially-induced corrosion, a three-component system prevails, consisting of the metal, electrolyte and microorganism (Videla, 2001). Corrosion is an electrochemical reaction that is caused by the flow of electrons from a metal to an electron affinite agent. This reaction requires an aqueous environment or electrolyte to conduct the flow of electrons. Dissolution at the anode (metal) occurs and ions enter the solution. Electrons flow to the cathode (microorganism) and must be consumed to close the circuit. According to Pandya (2000), different bacterial genera found in re-circulatory water systems include *Azotobacter, Bacillus, Clostridium, Desulfovibrio, Enterobacter, Escherichia, Ferrobacillus, Flavobacterium, Gallionella, Legionella, Leptothrix, Nitrobacter, Nitrosomas, Nocardia, Proteus, Pseudomonas, Salmonella, Streptomyces, Staphylococcus* and *Thiobacillus.* These bacteria are responsible for pitting and scaling of metal surfaces and fouling of water systems (Pandya, 2000).

The rate of the anodic current is equivalent to the rate of the corrosion reaction and this is mainly influenced by SRB, a ubiquitous group of bacteria. This class of microorganisms is strictly anaerobic and conducts dissimilatory sulphate reduction (Atlas & Bartha, 1998) to sulphide that may be released as hydrogen sulphide (H_2S) (Jain, 1995), while oxidising organic compounds or hydrogen to obtain energy for growth (Devereux *et al*., 1989). In general, SRB are not chemolithotrophs since they lack the enzyme systems to assimilate CO_2 and they require an organic carbon source to grow and reproduce. However, some are capable of utilising hydrogen and sulphate as sole energy sources for growth (Atlas & Batha, 1998). The most common electron donors are pyruvate, lactate and molecular hydrogen (Atlas & Batha, 1998).

Sulphate-reducing bacteria play an important role in fouling that leads to perforation of iron pipelines and scaling and pitting on the surfaces of heat exchangers due to H_2S production. If it is assumed that a metal pipe is made of iron, a good electron donor, the following reaction where iron is chemically oxidised might take place in the presence of a SRB *e.g. Desulfovibrio* (Schlegel, 1995).

$$4Fe + 8H^{+} \longrightarrow 4Fe^{2+} + 4H_2 \quad \ldots\ldots\ldots\ldots 1$$

A thin film of H_2 forms on the iron pipe, protecting it from corrosion. When SRB are present, the sulphate reduction process removes an electron from the H_2 molecule, resulting in water and a hydroxide ion.

$$4H_2 + SO_4^{2-} \longrightarrow H_2S + 2H_2O + 2OH^{-} \quad \ldots\ldots\ldots\ldots 2$$

The chemical reactions result in the formation of an iron precipitate, which is damaging to iron pipelines.

$$4Fe^{2+} + H_2S + 2OH^{-} + 4H_2O \longrightarrow FeS + 3Fe(OH)_2 + 6H^{+} \quad \ldots\ldots\ldots\ldots 3$$

Sulphate reduction can occur over a wide range of pH, pressure, temperature and salinity conditions and it is inhibited by the presence of oxygen, nitrate or ferric ions (Atlas & Batha, 1998). Hydrogen sulphide (H_2S) that is released by SRB, is a very strong reducing agent and highly toxic to both bacteria and higher forms of life (Jain, 1995). In order to culture these organisms, a redox potential of about -400 mV is necessary to enable growth (Zehnder & Wuhrman, 1976). This can be achieved by adding titanium(III) citrate to the medium to act as a reducing agent.

3.3 Biocides

The impact of microorganisms in the paper industry can be minimised through effective monitoring and control strategies (Gudlauski, 1996). It is important to add chemical compounds to pulp to prevent escalation of microbial numbers, since the continuous flow of pulp provides a constant source of nutrients past the biofilm as well as removing the toxic end-products by these microorganisms (Keddy, 1988). Water treatment systems are mainly concerned with three problems areas of microbial growth. These include firstly gram-negative organisms such as the slime-producing *Pseudomonas* species, secondly, SRB and

corrosive, anaerobic slimes and lastly algal biomass that can cause system fouling (Frayne, 2001).

Despite the search for alternative methods, chemical biocides still represent the most effective measure to control microbial growth in paper mills (Pereira *et al.*, 2001). Other strategies to limit growth include the addition of microbiostats, mechanical cleaning or nutrient depletion.

Microbiocides cause the destruction of the microorganism in the location where they are active and chemical biocides are divided into two main classes, namely oxidising biocides and organo-chemical biocides (Anon. 2005). Oxidising agents include chlorine and bromine-based chemistries and peroxygens as well as more advanced technologies such as on-site ozone and chlorine dioxide generation (Nalco Chemical Company, 2005). These biocides kill microorganisms by weakening the cell wall to allow penetration of other chemicals. A limitation of oxidising biocides is poor penetration and dispersing of heavy anaerobic infestations, unlike some organic biocides. Oxidising biocides are also not specific as to what is oxidised, attacked, or corroded. Non-oxidising chemicals are periodically shock-dosed and act as a microbiological killing agent (Frayne, 2001). These organo-chemical biocides include the following classes:

(i) Metallic substances, such as copper and tin, are often used in combination with quaternary ammonium compounds. These metallic compounds act on enzyme systems, causing denaturation and subsequent cell death (Carvalho, 1987).

(ii) Organic sulphurs generally act better as biostats than biocides. These compounds may chelate metal ions essential for biological activity or block transfer of electrons during cell respiration. Lysis of the cell walls can also be caused, resulting in leakage of cellular contents into the exterior environment. Examples of organic sulphurs include carbamates, isothiazolines, methylene bis(thiocyanate) (MBT) and sulfones (Frayne, 2001).

(iii) Organic bromines are used to clean up bacterial slimes and can be used in conjunction with oxidising biocides such as chlorine. Although pH depends on the type of compound used, a slightly alkaline pH is preferred in most cases. Examples include 2-Bromo-4-hydroxyacetophenone (BHAP), 2-Bromo-2-nitropropane-1,3-diol (Bronopol) and 2,2-Dibromo-3-nitrilopropionamide (DBNPA) (Frayne, 2001).

(iv) Chlorinated phenols and phenates have a quick killing rate and are efficient against fungi and SRB. An example of this group is 2,2-dihydroxy-5,5-dichlorodiphenyl monosulfide that is a proven biocide for *Legionella* species.

(v) Quaternary ammonium compounds (QAC) are used synergistically with other biocides (Keddy, 1988), since it is actually bacteriostatic in nature, especially when treating *Pseudomonas* species or SRB (Frayne, 2001). The mechanism of this biocide is due to its cationic nature, whereby an electrostatic bond is formed with the cell wall, affecting permeability and protein denaturing. The advantage of QAC is their relative low cost. QAC includes, for example, alkyldimethyl benzylammonium chloride, also known as benzalkonium chloride (Frayne, 2001).

Microbiostats impede growth and proliferation of microorganisms and alter the biostatic equilibrium of the medium or habitat. According to Keddy (1988), cells are prevented from dividing, but the damage from microbiostats is reversible. The use of microbiostats alone is not always preferred, because although populations are kept constant, the numbers at which the population is controlled is often obscured and a constant population encourages the development of resistant mutants (Keddy, 1988). Microbiostats are, therefore, preferably used in combination with microbiocides (Frayne, 2001). In general, considerations such as penetration of biofilms, odour control and disinfection stability will determine the type of biocide as well as the concentration for optimal biofilm control.

LeChevallier *et al.* (1988) evaluated the effectiveness of biocides on biofilms and found that planktonic bacteria were quite susceptible to a variety of disinfectants. However, biofilm bacteria that grew on different surfaces were 150 to 3000 times more resistant to hypochlorous acid and 2 to 100 fold more resistant to monochloramine. Each system has unique circumstances and disinfection for each system will, therefore, differ (LeChevallier *et al.*, 1988). Brözel & Cloete (1991) found that bacterial response to biocides differs according to the type of bacteria treated. It is thus important to match the type of bactericide to the dominant bacteria in the system to obtain optimal results.

3.4 Biocide-related problems

Control of biofilms in industrial water systems is an important component of any successful water-treatment programme. The tolerance of biofilms to anti-microbial substances, combined with their complex architecture and dynamic nature, makes biofilms difficult to

measure and control, thus reducing the effectiveness of treatment strategies (Ludensky, 2003). The addition of new biocides without the removal of existing biocides can compromise paper-machine runnability by increasing biofilm sloughing (Robertson, 1994), leading to paper breaks and holes. Various biological control problems face the papermaking industry. Excessive use of oxidising biocides in alkaline printing and writing grades can lead to operational problems such as corrosion, additive degradation and felt problems. However, underfeed of oxidising biocides can lead to uncontrolled microbial growth (Woodward, 2003).

Resistance, as defined by Cloete (2003), is the temporary or permanent ability of an organism and its progeny to remain viable and/or multiply under conditions that would destroy or inhibit other members of the same strain. Resistance is caused by the haphazard use of chemicals to control microbial fouling. Increased tolerance of bacterial communities to biocides is the result of phenotypic changes in the surviving populations that might involve the induction of multiple antibiotic resistance operons or of global regulatory systems that respond to sub-inhibitory concentrations of biocides (Ludensky, 2003).

Antimicrobial substances target a wide range of cellular loci, from cytoplasmic membranes to respiratory functions, enzymes and genetic material (Cloete, 2003). However, different bacteria react differently to bactericides, either due to inherent differences, such as unique cell envelope composition and non-susceptible proteins, or due to the development of resistance, either by adaptation or by genetic exchange (Cloete, 2003).

Three mechanisms are responsible for biofilm resistance and support a general correlation between decreased growth rate and increased resistance to killing in biofilms (Brown *et al.,* 1988):

(i) Some cells in a biofilm experience nutrient limitation and, therefore, exist in a slow growing or starved state.

(ii) Over 90% of the dry mass of a biofilm may be constituted of ECP that prevent various agents to penetrate a biofilm completely.

(iii) Some cells adopt a distinct and protected biofilm phenotype, deactivating some disinfectants or providing a diffusion barrier based on their anionic and hydrophobic nature.

3.5 Alternatives to biocides

Pulp and paper mills are trying to reduce the use of biocides for safety and environmental reasons (Robertson, 1994). A few alternatives to biocides include dispersants (Robertson & Taylor, 1993), quorum sensing signal mimics (Bauer & Robinson, 2002) and the so-called slimezymes (Buchert *et al*., 2004).

Dispersants, also called surface-active agents, help to maintain cleanliness by dissolution of additives and other products, and act directly on slime. Dispersants act to open biofilms and allow the penetration of biocides into cells (Robertson & Taylor, 1993), making the dispersant-biocide combination more effective. The use of non-ionic dispersants inhibits biofilm formation when added to the medium at the time of inoculation and according to Robertson and Taylor (1993) showed excellent inhibition of deposit formation in field trials.

Another development for biofilm control is the disruption of bacterial quorum sensing by signal-mimic compounds such as furanones (Bauer & Robinson, 2002). In bacteria the regulation of many important changes in gene expression relies on a signalling system between cells known as quorum sensing (QS). When several of the signal-producing bacteria are in close proximity to each other, the concentration of the QS signal increases. The bacteria can use the QS signals to monitor the proximity of other signal-producing cells and adjust the expression of certain genes accordingly. It is thought that isolated, free-living bacteria can switch to biofilm-forming colonies in response to a QS signal. Furanones appear to mimic the QS signals of bacteria by binding to the correct receptor proteins and will, therefore, interfere with QS signal to disrupt or prevent the formation of a biofilm (Bauer & Robinson, 2002).

The development of enzymatic slime-control approaches for paper machines have been investigated by Buchert *et al.* (2004), by screening for ECP to determine the effect on biofilm structure. Combinations of enzymes were used to selectively depolymerise the different polysaccharide types to break down the biofilm structure. One possible drawback might be that the most apparent slime-producing species are not necessarily the dominant microorganisms in a real paper-machine environment.

3.6 Management of fouling

Microbial colonisation of any paper machine is reduced when chemical deposits are adequately controlled (Robertson, 1994). The bacterial population in re-circulatory water systems can exceed 10^6 CFU/ml, and the best levels that should be obtained with conventional treatment programmes are in the order of 10^3 CFU/ml (Pandya, 2000). Traditionally, a deposit control programme involves proper maintenance to keep surfaces free of splashed stock, antimicrobial treatment of fresh water and additives, the use of biocides to reduce microbial growth and scheduled boil-outs to remove deposits (Robertson, 1994).

A mill should be able to identify all the contamination sources and anticipate potential levels of contamination. Monitoring the process includes conducting water analysis, measuring microbiological population shifts through microbial plating, checking suspect areas for toxic gas levels and measuring programme performance for runnability and product quality as a result of contamination control (Gudlauski, 1996).

4. QUANTIFICATION OF MICROBIAL POPULATIONS

The enumeration and isolation of environmental bacteria have mostly been confined to either those participating in the biogeochemical cycling processes or those with easily recognised traits (Hattori *et al.*, 1997). The list of isolated organisms is, therefore, biased as well as limited and the need for more rapid, sensitive and specific tests is essential in the water industry. Studies of heterotrophic microbial communities have traditionally involved isolate-based methods. Numerical taxonomic studies have either been based on profiles of cellular constituents or phenotypic characteristics of isolates (Garland & Mills, 1991). Ellis *et al.* (2003) suggested that the readily culturable component of soil microbial communities may be the most important in terms of both biomass and activity, since a relationship was found between the number of colonies developed on general media and heavy metal contamination in soils from an industrial site. Ellis *et al.* (2003) proposed using plate counts as an indicator of the impact associated with contamination. The need for more rapid and sensitive tests is equally important in the water industry, with the ultimate goal being the continuous on-line monitoring of the water leaving treatment plants (Rompré *et al.*, 2002).

4.1 Viable counting procedures

Studies have indicated that only 0.01 to 1% of all microbes are culturable on artificial media (Palojarvi *et al.*, 1997). Isolate-based methods give a limited, biased view, due to the highly selective nature of prepared media that precludes cultivation of specialised microbes (Garland & Mills, 1991). Isolate-based methods are time-consuming, limited by the spatial and temporal intensity of sampling (Garland, 1996) and many microorganisms are unculturable on known media (Kersters *et al.,* 1997). Another difficulty that could arise from isolate-based methods is the biased testing, performed on a subset of the community, rather than the community itself (Garland & Mills, 1991). Multiple microbial interactions are difficult to quantify using conventional culturing techniques and finally it is hard to determine microbial function *in situ* (Kersters *et al.*, 1997).

Basic approaches employed for viable count procedures include heterotrophic plate counts and the Most Probable Number (MPN) technique (Franson *et al.*, 1998). For the enumeration of aerobic planktonic organisms in chests, headboxes and white-water systems, however, traditional heterotrophic plate counts are considered to be an effective method (Woodward, 2003). Although all aerobes will not grow on any one specific growth medium, the general trends of these numbers will indicate whether there is a difference in biological activity. Plate count techniques are widely employed in industry due to low cost and ease of application.

4.2 Direct enumeration

Direct total cell counts of bacteria usually exceed counts obtained from heterotrophic plate counts and MPN (Franson *et al.*, 1998), because the quantitative and qualitative bias imposed by cultivation-dependent methods is overcome by *in-situ* analyses of microbial communities. Some microscopic methods include light microscopy, epifluorescence microscopy, scanning electron microscopy (SEM) and confocal laser microscopy (Oosthuizen, 2001). The latter two techniques, however, require sophisticated, expensive equipment, while light microscopy does not allow discrimination between dead and viable microbes or different types of bacteria.

To overcome this problem, several alternative methods have been proposed to determine bacterial activity. These include the use of fluorescent dyes (Kalmbach *et al.*, 1997), determination of RNA-turnover rate (Yu & McFeters, 1994), sulphate-reduction rate (Beech *et al.*, 1994) and micro-calorimetry (Von Rège & Sand, 1998). The first three techniques require the removal of the biofilm from the sample surface, resulting in an alteration in the

bacterial environment and hence in the bacterial physiology. Micro-calorimetry, however, determine microbial activity by quantifying the detection of heat output that accompany all biochemical redox reactions (Von Rège & Sand, 1998).

4.3 Indirect enumeration

According to Woodward (2003), ATP determination is a convenient and rapid procedure for indirect enumeration since no incubation time is required. ATP is a relative measure of the bioactivity and can be correlated to the overall effectiveness of a microbiological control programme. The measurement of ATP are only related to the energy charge of the cells according to Von Rège & Sand (1998), however, and at best may be used as an indirect indicator of the metabolic state, in addition to giving only single values and no on-line measurements.

Venkateswaran *et al.* (2003) used a luciferase bioluminescence assay that differentiated between free extracellular ATP (dead cells, *etc*.) from intracellular ATP (viable microbes) and considered the method superior to culturable colony counts due to the speed and ability of the technique to report the presence of viable but non-culturable organisms. In this assay, reduced luciferin reacts with oxygen to form oxidised luciferin in the presence of the luciferase enzyme, magnesium ions and ATP. Light is emitted that is directly proportional to the ATP concentration.

5. CONCLUSIONS

The closure of paper-mill water systems to reduce water consumption is necessary due to the increasingly stringent limitations by governments and environmental agencies where the environment is at risk (Edde, 1994). Closure, however, results in increased microbial numbers due to elevated temperatures and nutrient concentrations (Gudlauski, 1996). By monitoring different environmental parameters such as temperature, pH, DO, ORP, TSS, TDS, COD, BOD, N, P and toxicity, an indication of the microbial activity at different sampling points in a water system can be obtained (Rump & Krist, 1992; Schneider *et al.*, 1998). Microbial growth due to favourable environmental conditions, especially biofilm formation, will result in operational problems (Habets *et al.*, 1996), safety hazards (Gudlauski, 1996) and reduced product quality (Robertson & Taylor, 1993) in a paper mill. Therefore,

control measures are necessary to counter the colonisation of microorganisms in paper mills. Chemical biocides remain the most effective method to manage excessive microbial contamination (Pereira *et al*, 2001). To quantify microorganisms in any water system it is necessary to employ suitable counting procedures. Plate counts remain the most popular method in industry due to low cost and ease of application, even though it is time-consuming and biased (Garland & Mills, 1991; Garland, 1996). Repeated plate counts can indicate whether there is a trend in the biological activity at a specific sampling point. Using ATP to indicate biological activity requires specialised equipment and expertise, but it can be effective and time-saving when employed under the appropriate circumstances (Venkateswaran *et al*., 2003).

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CHAPTER 2

THE IDENTIFICATION AND CHARACTERISATION OF BACTERIA FROM PAPER-MILL WATER SYSTEMS: A REVIEW

ABSTRACT

Suitable methods to enable identification and characterisation of bacteria can aid in the control of microbial fouling in paper-mill water systems. Studies can either focus on the phenotype or the genotype of bacterial individuals to identify different species, or characterise the entire bacterial community. Phenotypic measurements are typically used in industry due to their low cost and the ease of application. The majority of bacteria from environmental samples cannot, however, be cultivated on artificial media. This discrepancy implies that the description of complex environmental samples, from either natural or engineered systems, is far from complete when using culture-based techniques. To avoid problems of culture-based methods, a number of assays have been developed that do not require isolation of microorganisms. These techniques measure structural or functional properties of the whole community instead. By measuring the substrate utilisation profiles or chemotaxonomic markers of the bacterial community, insight can be gained into the functional characterisation of the entire population, while DNA and RNA based methods can enhance the understanding of the structural character of a bacterial community. Spatial and temporal changes in an environment can be detected with genetic fingerprinting techniques. It is possible to make predictions on the bacterial populations that can aid in the design of microbial control programmes in industry.

1. INTRODUCTION

The pulp and paper industry uses extensive amounts of water for the papermaking process and microorganisms are constantly introduced into paper machines through raw materials, water, fibres and papermaking chemicals. The paper machine environment was found to be suitable for bacterial growth due to favourable temperatures (30 to 50°C), pH (4 to 10) and nutrients that flow with the pulp and additives (Väisänen *et al*., 1998). Prolific growth of certain microorganisms in paper-mill water systems can contribute to biofilm formation and microbially induced corrosion (Bennett, 1985). Problems that are encountered as a result of biofilms and corrosion include reduced paper quality, safety hazards and odour problems (Gudlauski, 1996). It is, therefore, imperative that information about the composition, structure and activity of the microbial communities within water systems are collected and examined to aid in the design of suitable microbial control programmes of paper-mill water systems (Gilbride *et al*., 2006).

Traditionally, cell suspensions of environmental samples are prepared for dilution series and plated onto solid media. After incubation and enumeration, information on the species present could be obtained and relative abundance of different organisms could be used to determine measures of diversity (Prosser, 2002). The obvious limitation to this approach is the selective pressure imposed on the majority of the bacterial community to grow on defined media (Amman *et al*., 1995). This pressure was demonstrated by Liss & Allen (1992) when it was found that total microscopic bacterial counts in pulp and paper wastewater averaged 10^{10} cells/ml while culturable counts ranged between 10^7 and 10^8 cells/ml. This discrepancy, therefore, implied that the description of complex environmental samples from either natural or engineered systems is far from complete when using culture-based techniques.

Molecular techniques have enabled the circumvention of lengthy culturing methods and can provide direct detection of organisms when genes within environmental samples are analysed (Prosser, 2002). The genes may be functional, *i.e*. genes that code for proteins that perform particular metabolic reactions of relevance to the ecosystem processes (Wawer *et al*., 1997). The most widely applied approaches analyse the 16S rRNA gene for the structural diversity of bacterial populations (Prosser, 2002). These so-called DNA fingerprinting techniques can help to reveal the large biodiversity of the microbial communities in treatment systems that will not be evident when using traditional techniques alone (Kisand & Wikner, 2003).

To best describe the composition and diversity of microbial communities, a polyphasic approach may be followed and more than one technique used to verify data (Vandamme *et al*., 1996). This approach was demonstrated by Baker *et al*. (2003) who used several fingerprinting methods to compare bacterial communities in treatment systems of pulp and paper-mill wastewater. The bacterial communities in the study appeared to be fairly stable over time, although each community had a distinctly different genetic profile. Gilbride & Fulthorpe (2004) compared individual members of several pulp and paper mill treatment systems using isolation techniques, reverse genome probing and 16S rRNA gene clone libraries and found little overlap in the composition of the culturable community between mills at genus level.

Suitable methods to enable identification and characterisation of bacteria in paper-mill water-systems are needed to reduce microbial fouling and corrosion. The objective of this review was to compare various techniques to identify microorganisms and characterise microbial populations in environmental samples with different phenotypic and genotypic approaches. To enhance the understanding of a working ecosystem and subsequently manage microbial fouling, a combination of different techniques should be studied and compared to measure spatial and temporal changes.

2. PHENOTYPIC APPROACHES

When identifying and characterising microorganisms from environmental samples, either the genotype or the phenotype can be investigated (Figure 1). Genotypic studies are focussed on the nucleic acids of the cell (either RNA or DNA) and require no cultivation, whereas the phenotype refers to the way the genotype is expressed. Phenotypic studies include visible or otherwise measurable physical and biochemical characteristics as a result of the interaction of the genotype with the environment (Roselló-Mora & Amann, 2001).

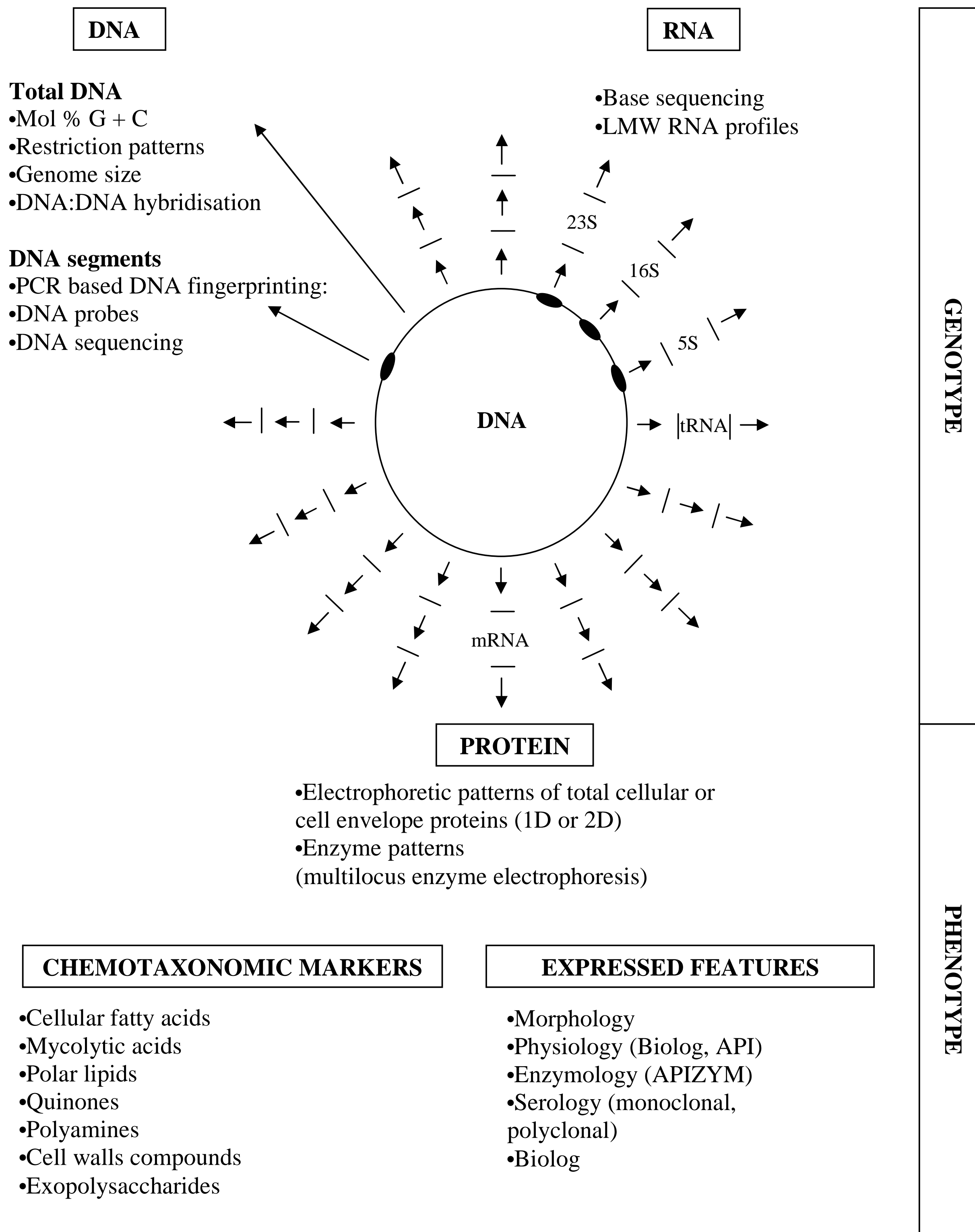

Figure 1: Schematic overview of the various cellular constituents and techniques used for identification and classification as adapted from Vandamme *et al.* (1996).

2.1 Traditional identification and characterisation techniques

Classical phenotypic characteristics of bacteria used for identification are based on morphological, physiological and biochemical features (Figure 1). Morphological features of bacteria include cellular characteristics such as shape, endospore, flagella, inclusion bodies and Gram-staining, as well as macroscopic features such as colour, dimensions and form (Vandamme *et al.*, 1996; Roselló-Mora & Amann, 2001). Physiological and biochemical features include data on growth at different temperatures, pH values and salt concentrations as well as growth in the presence of various substances (Vandamme *et al.*, 1996). Data on the presence or activity of different enzymes or metabolism of various compounds are also considered measurable phenotypic traits (Roselló-Mora & Amann, 2001).

2.2 Carbon substrate utilisation

2.2.1 Identification

Biolog™ Microplates were originally developed by Biolog Inc. (Hayward, USA) for the identification of clinical bacterial strains using metabolic fingerprinting (Preston-Mafham et al., 2002). The redox-based technique is calorimetrically measured (Haack et al., 1995) and monitors microbial metabolism through carbon-source utilisation. A colour reaction is produced when tetrazolium violet is reduced to purple-coloured formazan (Wünsche & Babel, 1995). This indicative colour reaction is coupled to the oxidation of the substrate and, therefore, a composite descriptor of the ability of the heterotrophic, aerobic microbes present in the well to assimilate the sole carbon source (Garland 1996b). The substrate, tetrazolium dye and nutrients are supplied in each well in a dried film form and are reconstituted upon addition of the sample. The Biolog™ system (Biolog Inc., Hayward, USA) calculates similarity indices for the test strain in question and ten strains in the database that possess the most similar utilisation patterns (Wünsche & Babel, 1995). According to the Biolog manual, a similarity index of between 50 and 75% is regarded as a 'good identification'. Indices greater than 75% are matched as 'excellent identification', while indices smaller than 50% are reported as 'no identification' (Biolog Inc., Hayward, USA).

Klingler *et al.* (1992) found that the Biolog™ system (Biolog Inc., Hayward, USA) identified 98% of 41 ATCC strains correctly to genus level and that 59% were identified correctly to species level. It was concluded that, despite incorrect identifications and identifications that changed with time, the Biolog system was easy to use, customise and update. Insufficiently

low rates of correct identifications could be ascribed to the following according to Wünsche & Babel (1995):

(i) The number of substrate utilisation patterns stored in the Biolog™ database is limited when compared to the number of bacterial taxa.

(ii) Many isolates grow slowly or not at all under standardised Biolog™ conditions, because their special requirements regarding environmental conditions are not met, and thus, their complete substrate utilisation patterns are not developed.

(iii) Some isolates are not able to utilise at least three of the offered substrates necessary for identification with the Biolog™ system.

(iv) Some strains from extreme environments developed significantly different substrate utilisation patterns from those stored in the Biolog™ database.

2.2.2 Characterisation

In addition to identifying various bacterial taxa, one of the most important applications of the Biolog™ system is its ability to consistently characterise microbial communities based on sole carbon source profiles (Garland, 1996a). Microbial communities have the potential to enhance understanding of spatial and temporal changes since variation in microbial community structure could affect ecosystem processes (Garland, 1997). Therefore, carbon source utilisation profiles can function as a means of characterising microbial communities on the basis of heterotrophic metabolism (Garland & Mills, 1991).

According to Van Heerden (2001), the more substrates utilised by a sampled population, the higher the diversity, due to the collective action of the individual species. The immediate development of colour in certain wells indicates that a large fraction of microorganisms in the inoculum are able to utilise the sole carbon source (Garland & Mills, 1991). A lag phase in other wells indicate that a longer period of growth is necessary to produce microorganisms that can be detected and consequently, a smaller percentage of microorganisms in the inoculum are able to utilise the substrate as a sole carbon source (Garland & Mills, 1991). It is, however, not possible to determine if all the members in the community, able to respirate on the sole carbon source, contributed to the colour response (Haack *et al.*, 1995) or if growth resulted from a subset or single member of the community in question (Garland & Mills, 1991; Winding, 1994). It is possible that the rapid colour development in some wells is due to a few cells with high growth rates on the specific carbon source.

Biolog™ (Biolog Inc., Hayward, USA) is regarded as a rapid and simple method for the characterisation of microbial communities (Garland & Mills, 1991), as well as to detect changes in microbial composition and activities of ecosystems (Kersters *et al.,* 1997). This could be achieved at a very high resolution (Garland & Mills, 1991), since a more sensitive and ecologically meaningful measure of heterotrophic microbial community structure is obtained through measuring the metabolic activities of a community (Garland & Mills, 1991; Victorio *et al.*, 1996). The low manpower requirements enable intensive sampling across temporal and spatial scales (Garland, 1997) and the entire community can potentially be profiled (Victorio *et al.*, 1996). Although functional tests are also a cultural method that is dependent on the growth of microbes on media, the test is done on the original community and not on a subset (Garland & Mills, 1991). A fairly large set of data is easily generated (Preston-Mafham *et al.*, 2002) and the simplicity of the test as well as the commercial availability makes it attractive for comparison of microbial communities (Haack *et al.*, 1995). In addition to being inexpensive, rapid, reliable and easy to use (Balser *et al.*, 2002), the assay is very sensitive to detect changes in microbial communities and could, therefore, provide significant insight in the understanding of changes due to different biotic and abiotic stresses (Garland, 1998).

2.3 Chemotaxonomy

Chemotaxonomy refers to the application of analytical methods to collect information on various chemical constituents of the cell in order to classify bacteria. The discontinuous distribution of specific chemicals *e.g.* amino acids, lipids, or proteins provides good characteristics for classification and identification (Roselló-Mora & Amann, 2001). Investigating the chemotaxonomic characteristics of an entire community should provide a more sensitive and ecologically meaningful measure of heterotrophic community structure in addition to eliminating any bias that might be associated with traditional culturing methods (Victorio *et al.*, 1996). Chemotaxonomic methods that characterise the total bacterial community include fatty acid methyl ester (FAME) analysis, phospholipid fatty acid (PLFA) analysis, enzyme activity measurement and respiratory quinone profiles.

2.3.1 FAME and PLFA analyses

These techniques provide powerful tools for structural analysis of microbial communities. After phospholipids are extracted and purified from the environmental samples, fatty acids are liberated by saponification and esterified. The resulting fatty acids or lipids are then identified by gas chromatography (Buyer & Drinkwater, 1997; Van der Merwe *et al.,* 2002). The use of signature-lipid biomarkers is not selective, since no culturing is necessary and certain taxonomic groups can be identified by specific phospholipid fatty acids (Buyer & Drinkwater, 1997). However, FAME and PLFA analyses are technically demanding (Haack *et al*., 1995) and these methods are considered too expensive for routine application (Van der Merwe *et al*., 2002). Dead and inactive components are included in the FAME profiles, while PLFA primarily reflects active living membrane fractions (Van der Merwe *et al*., 2002).

2.3.2 Enzyme activities

The specific rates for biological processes can be expressed in terms of active biomass to allow meaningful comparisons in different systems (Cronje *et al*., 2002). A variety of enzyme assays could be used to measure the metabolic activities of microbes. Nybroe *et al.* (1992) found esterase and dehydrogenase activities correlated with microbial abundance (measured as colony forming units of heterotrophic bacteria) in wastewater. However, no obvious correlations could be found between specific process parameters such as oxygen and nitrogen utilisation rate and enzyme activities. Guwy *et al.* (1998) found that catalase levels could be correlated to microbial biomass as well as oxygen uptake removal (OUR) using respirometry.

2.3.3 Respiratory quinone profiles

Quinones are a class of lipids and are constituents of bacterial plasma membranes that play an important role in electron transport and oxidative phosphorylation (Oosthuizen, 2001). Quinones can be extracted from environmental samples by using organic solvents. And after evaporation and re-extraction, concentrated quinones are subjected to column chromatography to separate the menaquinone and ubiquinone fractions (Fry, 1988). Quinone components are then identified and quantified *via* gas chromatography or mass spectrometry (Oosthuizen, 2001).

3. GENOTYPIC APPROACHES

Genotypic information is obtained from all data that is retrieved from the nucleic acids of a cell (Vandamme *et al.,* 1996) and include the analysis of the deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) of bacterial cells (Figure 1). These methods measure community composition and activity without the bias of culturing (MacGregor, 1999). Both viable and dead microbes contain DNA, but active microbes contain proportionally more RNA (Muttray *et al.*, 2001). It was found, however, that DNA is the most stable indicator of the presence of both the inoculant cells and specific genes (Van Elsas *et al.*, 1998) and, therefore, DNA can be a good measure of cell abundance, and a DNA:RNA ratio can be indicative of growth rate or metabolic activity (Muttray *et al.*, 2001). Ribosomal RNA (rRNA) and messenger RNA (mRNA) both have been used for analyses in aquatic environmental studies. Whereas the rRNA gives information about the community composition and metabolic activity, the detection of mRNA enables better functional characterisation of species (MacGregor, 1999). Different techniques to determine community composition and function can include rRNA-based membrane hybridisation, rRNA-based *in situ* hybridisation (FISH), rRNA-based cell sorting and mRNA *in situ* reverse transcription polymerase chain reaction (RT-PCR) (MacGregor, 1999).

A variety of molecular techniques that are based mostly on genes encoding rRNA (rDNA genes) offer new possibilities for profiling microbial dynamics that compliment traditional cultivation studies (Tiirola *et al.*, 2003). The DNA of microbial communities as well as single isolates can be studied by direct hybridisation, cloning into plasmids, or amplification (MacGregor, 1999). All three approaches involve the lysis of bacterial cells, the subsequent extraction of their nucleic acids from the matrix and analysis of the targeted sequences or the entire bacterial genome (Ranjard *et al.*, 2000).

The rDNA gene is ubiquitous and, therefore, it is the gene of choice when studying microbial structure and function as well as for identifying bacteria. The rDNA gene is also a fairly large molecule that contains a considerable amount of genetic information and is composed of highly conserved regions as well as more variable domains (Schleifer & Ludwig, 1989; Stackebrand & Goebel, 1994). A comprehensive dataset of sequences is available (61 000 entries in 2006) in widely accessible databases.

The comparative analysis of rDNA sequencing data has demonstrated the high resolving power for measuring inter and intra-species relatedness (Vandamme *et al*., 1996). Sequencing allows identification of uncultured bacteria as well as an estimation of their relatedness to known culturable species (Ranjard *et al.*, 2000). When closely related organisms are inspected, however, the resolution of 16S rDNA sequence analysis decreases and DNA-based typing methods can be employed to analyse below species and strain level (Vandamme *et al.,* 1996).

3.1 Partial DNA analysis

The analyses of amplified target sequences from the prokaryotic genome gave rise to a wide variety of typing methods. Usually, genes from the ribosomal operon *rrs* (16S) are partially amplified and analysed electrophoretically. This approach is, however, only applicable when trying to understand intra-specific diversity and are not suitable for taxonomic studies. When analysing target sequences, the amplified fragments can either be cloned into vectors (and further analysed with restriction enzymes) or a genetic fingerprint of the DNA can be obtained (Ranjard *et al.*, 2000).

PCR fragment cloning and restriction and/or sequence analysis enable the assessment of the diversity of microbes in terms of a number of different species as well as the relative abundance of the species, by producing a library of clones from 16S rDNA amplicons. By cloning the amplified fragments into vectors, the sequences can be characterised individually with restriction fragment length polymorphism (RFLP) and/or sequencing (Ranjard *et al.*, 2000).

Genetic fingerprinting of bacteria provides a DNA fingerprint of a target gene to represent the genetic structure as defined by selective primers (Ranjard *et al.*, 2000). The diversity is resolved by differential electrophoretic migration patterns on agarose or polyacrylamide gels. Separation depends on the size or sequence of the amplicon. Genetic fingerprint approaches are, therefore, useful methods to compare different microbial communities and isolates. Some techniques that are representative of this approach include denaturing gradient gel electrophoresis (DGGE), PCR restriction fragment length polymorphism (PCR-RFLP), terminal restriction fragment length polymorphism (tRFLP), random amplified polymorphic DNA (RAPD) analysis and enterobacterial repetitive intergenic consensus sequence PCR (ERIC-PCR). The data are usually analysed in terms of similarities (represented in a

dendogramme) or using multivariate analysis such as principal component analysis, to visualise and compare the data (Ranjard *et al.*, 2000).

3.1.1 DGGE

The DGGE approach is followed to separate mixed amplified fragments of DNA of the same size, based on the sequence differentiation of the individuals (Gelsomino *et al.*, 1999). It involves the separation of amplicons on polyacrylamide gels containing a linear gradient of a DNA denaturant that may be a chemical (*e.g.* urea or formamide) or a physical (*e.g.* temperature) factor (Ranjard *et al.*, 2000). In the latter case the method is known as TGGE. One of the two PCR primers is synthesised with a guanine-cytosine (GC) rich end, to form a duplex amplicon with a GC-clamp that does not denature as easily as the adenine-thymine (AT) base pairs (MacGregor, 1999). Melting of the DNA fragments proceeds along the gel gradient and occur in discrete stretches of base-pairs with identical melting temperatures. Once the domain with the lowest melting temperature reaches its melting temperature (*Tm*), a transition of a helical to a partially melted molecule occurs and the migration of the molecule practically halts (Muyzer & Smalla, 1998). Sequence variation within such domains causes the melting temperatures to differ and molecules with different sequences will stop migrating at different positions on the gel. Microorganisms with different 16S rRNA gene sequences will, therefore, produce different DNA patterns (Wilderer *et al.*, 2002). DGGE have been applied to study microbial community complexity (Murray *et al.*, 1996), to follow the expression of relevant genes in the environment (Wawer *et al.*, 1997), to observe population shifts (Donner *et al.*, 1996) and to examine the efficiency of enrichment cultures for the isolation of bacteria (Felske *et al.*, 1996).

PCR-DGGE is considered to be a promising tool in microbial diversity studies, since it can be applied to generate fingerprints of microbial communities in a variety of habitats (Gelsomino *et al.*, 1999). The method is rapid and many samples can be electrophoresed simultaneously, making DGGE particularly useful when population dynamics is examined over time (Theron & Cloete, 2000). An amplified sequence longer than 500 base pairs can, however, no longer be efficiently separated. This limitation restricts the precision of species identification with DGGE, since sufficient sequence comparison cannot be done for short regions (Ranjard *et al.*, 2000). Another limitation is the laborious technical optimisation that is required *e.g.* calibration of the linear gradient of DNA denaturants and improvement of the PCR primers *via* a GC clamp to obtain better electrophoretic separation (Ranjard *et al.*, 2000).

3.1.2 PCR-RFLP

This technique can be used to distinguish between different bacterial species, based on nucleotide sequence variations. The digestion of the amplified 16S rDNA fragment using restriction enzymes can be visualised by separating the restricted fragments on agarose or polyacrylamide gels (Ranjard *et al*., 2000). Genetic variations at the site where a restriction enzyme digests DNA, affect the size of the resulting fragments. This discrepancy can be used to distinguish among different types of bacteria by generating species-specific restriction patterns. PCR-RFLP has been used for rapid classification, identification and detection of *Rhizobium* species (Laguerre *et al.,* 1994), methanogens (Hiraishi *et al.* 1995), *Legionella* (Ko *et al*., 2003) and *Mycobacterium* species (Kim *et al.*, 2005). It was found to be a simple and robust auxiliary tool to morphological identification by Vidigal *et al.* (2000) and Carvalho *et al.* (2004). However, the choice and number of the restriction enzymes are crucial to obtain the highest resolution possible.

3.1.3 tRFLP

This method involves the amplification of target genes from community DNA extracts with specific primers, one of which is fluorescently labelled (Dunbar *et al*., 2001). Amplicons are digested with restriction enzymes and fragments are electrophoretically separated based on size by automated sequencers that only detect and quantify labelled tRFLP's (Egbert & Friedrich, 2003). Dunbar *et al*. (2001) stated that the method could be used for differentiation of microbial communities as well as for comparison of the relative phylotype richness, structure of microbial communities and for identifying specific organisms. Osborn *et al*. (2000) found that tRFLP was a robust and highly reproducible technique that yielded high quality DNA fingerprints. An appropriate database would, however, be necessary to phylogenetically assign fragments of exact sizes that can be used to distinguish among strains. Egbert & Friedrich (2003) found additional secondary terminal restriction fragments, also known as pseudo-T-RF's, in the analysis of tRFLP amplicons from cloned 16S rDNA genes at high frequency. This was due to single stranded amplicons and might lead to the over-estimation of microbial diversity. In addition, various parameters such as template DNA concentrations, number of PCR cycles, the type of *Taq polymerase* and annealing temperatures all have an influence on the profiles generated (Osborn *et al*., 2000).

3.1.4 RAPD

Short random primers that anneal at different locations on the genomic DNA generate PCR products of different lengths when amplified. These fragments can be resolved on agarose or acrylamide gels (Ranjard *et al.*, 2000). Xia *et al*. (1995) demonstrated the absence of a pesticide effect in soil by assessing the molecular genetic response of different soil communities by using RAPD. The method is rapid and sensitive to reveal differences between similar complex prokaryotic genes, but cannot provide phylogenetic information about the bacterial composition of a community (Ranjard *et al.*, 2000) and is not very reproducible.

3.1.5 ERIC-PCR

One type of repetitive PCR is the amplification of genomic DNA located between ERIC elements in the bacterial genome (Di Giovanni *et al*., 1999). These elements contain highly conserved palindromic repeat sequences and were found to generate characteristic patterns when separated on agarose gels (De Bruijn, 1992). Visualising the different band patterns allow discrimination at genus, species and strain level of eubacteria (Gillings & Holley, 1997) and can assist in determining phylogenetic relationships (De Bruijn, 1992).

ERIC elements are 126 base pairs in length and are distributed throughout extragenic regions of the genomes of a number of gram-negative bacterial genera. Consensus primers are used to amplify neighbouring repetitive elements to generate characteristic patterns for analysis (Gillings & Holley, 1997). Amplification products range from approximately 50 to 3000 base pairs (Di Giovanni *et al*., 1999). De Bruijn (1992) found distinctive ERIC patterns within the *Pseudomonas* genus, indicating large diversity among the species and, therefore, concluded that ERIC-PCR was extremely sensitive and minor differences between strains of the same genus and species could be detected by adjusting the primer combinations and concentrations to manipulate the complexity of the ERIC patterns.

De Bruijn (1992) found that very little template DNA was required and that ERIC-PCR was rapid and reproducible thereby making it a reliable DNA fingerprinting method for environmental samples. Di Giovanni *et al.* (1999) found ERIC-PCR to be superior to RAPD analysis in discriminating species. One drawback of this technique is the poor genomic fingerprinting of gram-positive bacterial genomes. A study by Gillings & Holley (1997),

however, established that ERIC-PCR is not necessarily directed towards ERIC elements and that under certain PCR conditions a fingerprint for almost any sample can be generated. Gillings & Holley (1997) produced complex ERIC patterns from various target organisms ranging from bacteriophages to eukaryotes to show that ERIC-PCR performed on non-enterobacterial targets may be a highly reproducible variant of RAPD-PCR. Since ERIC-PCR products failed to amplify under more stringent conditions, it was suggested that primers annealed to and amplified from anonymous binding sites with accidental and partial homology to the primer set (Gillings & Holley, 1997).

3.2 Whole genomic DNA analysis

Molecular approaches that investigate all the genetic information in the extracted DNA are referred to as "whole community DNA analyses" and include DNA-DNA hybridisation, thermal denaturation and reassociation of whole extracted DNA as well as DNA fractionation by base composition (Ranjard *et al.*, 2000).

3.2.1 DNA–DNA hybridisation

The determination of the similarity of an entire genome is the standard taxonomic technique for bacterial species delineation, because a high degree of correlation was found from numerous studies between genomic DNA similarity and phenotypic similarity (Stackebrandt & Goebel, 1994). The characteristic property of denatured DNA and RNA to re-associate under appropriate experimental conditions depends on the similarity of the nucleotide sequences and this allows the quantification of the degree of relatedness, expressed as percentage homology (Rosselló-Mora & Amann, 2001). The technique involves the extraction and purification of DNA from environmental samples and cross-hybridising these with one another (Lee & Furhman, 1990). The DNA from one sample is radio-actively labelled and used as a template. The extent to which the filter-bound target DNA anneals to the radioactive probe reflects the similarity of the bacterial population structure (Ranjard *et al.*, 2000).

3.2.2 Thermal denaturation and re-association of whole extracted DNA

Heat denaturation of extracted DNA is followed by re-association of homologous single strands. The proportion of renatured DNA is generally expressed as the product of the concentration of nucleotides and the reaction time (Ranjard *et al.*, 2000). Although this

approach enables an estimation of genetic diversity of the bacterial population in terms of richness (number of different genomes), it does not provide any details about its evenness (relative abundance of individual genomes) (Ranjard *et al.,* 2000).

3.2.3 Extracted whole DNA fractionation by base composition

This approach is based on the fact that prokaryotic DNA vary in GC content. The entire bacterial genome is extracted and fractionated by base composition using a density gradient of caesium chloride. An inter-chelating agent such as bisbensimidazole can be used to bind preferentially to AT bases, thus emphasising the differences in the gravity of the DNA according to the specific GC content (Ranjard *et al.*, 2000). Disadvantages of whole genomic DNA analysis according to Ranjard *et al*. (2000) include the need for large amounts of DNA as well as high quality DNA in terms of purity and size integrity. Expensive equipment is also necessary to execute the experiments.

4. CONCLUSIONS

Traditional methods to identify bacterial species are based on their morphological, physiological and biochemical characteristics (Vandamme *et al.*, 1996). An obvious limitation of these culture-based techniques is the selective pressure that is imposed on the majority of microbes to grow on defined media (Amman *et al.*, 1995). However, several assays have been developed that do not require isolation of microorganisms and instead measure structural or functional properties of the whole community (Buyer & Drinkwater, 1997; Palojarvi *et al.*, 1997). Profiling of a community with substrate utilisation using the Biolog™ system (Garland & Mills, 1991), as well as the use of chemotaxonomic markers to obtain functional profiles of different bacterial communities (Van der Merwe *et al.*, 2002) require no cultivation but still measure phenotypic characteristics of bacterial populations.

Techniques that investigate the genotype of bacterial communities provide information that is less biased, although intrinsic limitations to each of the different approaches do exist. By generating a genetic fingerprint of bacterial isolates with DGGE (Muyzer *et al.*, 1993), PCR-RFLP (Laguerre *et al.*, 1994), tRFLP (Osborne *et al.*, 2000), RAPD (Xia *et al.*, 1995) or ERIC-PCR (De Bruijn, 1992) spatial and temporal changes in the environment can be investigated. These molecular-ecology techniques compliment conventional methods

employed by industry and enhance the understanding of bacterial structure and function in natural environments.

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CHAPTER 3

THE INFLUENCE OF ENVIRONMENTAL PARAMETERS ON BACTERIAL NUMBERS IN PAPER-MILL WATER SYSTEMS

ABSTRACT

A number of environmental and process parameters play an important role in the number and type of microorganisms in a paper-mill water system. Elevated temperatures and nutrient levels can, for example, lead to problems such as biofilm formation. Excessive microbial growth as a result of favourable environmental conditions, consequently can reduce paper quality, create safety hazards and cause odour problems. The aim of this study was to determine which parameters were relevant to microbial growth and to use the resultant data to predict the function and structure of microbial populations with a view to improving process control. Various environmental parameters and biological factors were measured for 14 paper machines across South Africa. Multivariate analysis was performed on the data to determine the influence of the individual environmental parameters on microbial numbers. The highest correlation was found between oxidation-reduction potential and the numbers of culturable aerobic bacteria. Other environmental and process parameters that significantly influenced microbial numbers were temperature, dissolved oxygen, dissolved solids, chemical oxygen demand, nitrogen, phosphorous, specific water consumption, pulp furnish, biocide class and retention time. These results may be used to evaluate the efficacy of biocide programmes on individual paper machines as well as to aid in the control of microbial fouling in paper-mill water systems.

1. INTRODUCTION

The microbiologically associated problems that occur in paper mills are often a result of elevated temperatures (Hoekstra, 1990) and nutrient concentrations (Gudlauski, 1996), that lead to increased metabolic rates of microorganisms and contribute to problems such as biofilm formation and microbially induced corrosion (Bennett, 1985). The influence of specific environmental parameters on microbial numbers are generally hidden by the large number of parameters that could play a role in microbial growth and a clear understanding of the influence of different parameters can only be obtained through multivariate analysis.

Temperature is a very important parameter that determines the type and numbers of microorganisms that proliferate in any environment. Microbes are grouped as psychrophiles (0 to 15°C), psychrotrophs (5 to 20°C), mesophiles (15 to 45°C), thermophiles (45 to 65°C) and hyperthermophiles (65 to 115°C) (Madigen *et al.*, 2000). Temperature shifts, as a result of process energy, have been correlated to decreased performance of chemical treatments and system instability in pulp and paper mills (Morgan-Sagastume & Allen, 2003). It has been assumed that temperatures in the thermophillic range will reduce microbial growth and at temperatures higher than 45°C, the growth of slime forming bacteria will be strongly reduced (Habets *et al.,* 1996). One of the environmental factors with the greatest relevance to the growth of microorganisms is pH. Microorganisms can be grouped as acidophiles (pH less than 7), neutrophiles (pH equal to 7) or alkalophiles (pH greater than 7) (Madigen *et al.*, 2000). In instances where a change in pH equilibrium occurs, a high degree of mortality will result, especially if the change is sudden.

Heterotrophic microorganisms preferentially use dissolved oxygen (DO) as terminal electron acceptor to degrade organic substrates in a subsurface environment since more energy is gained from aerobic respiration than from denitrification, iron reduction or sulphate reduction *etc*. With increased temperatures, DO levels will decrease, leading to an increase in anaerobic bacterial numbers (Gudlauski, 1996; Robertson & Schwingel, 1997) as the indigenous microorganisms will start to utilise NO_3^-, SO_4^{2-}, Mn^{2+}, Fe^{3+} and CO_2, as terminal electron acceptors (Wright & Cox, 2005). Oxidation-reduction potential (ORP) is linked to the DO of a system. Microbial proliferation can lead to a reduction in the redox potential of a system when oxygen is utilised, thereby depleting the system of DO during aerobic respiration (Jay, 1992) and creating an anoxic environment where anaerobes can grow.

Whereas total suspended solids (TSS) levels reflect pulp consistency in paper-machine water systems and could play a role in the transfer of nutrients, microbial inoculum and the efficacy of biocides, total dissolved solids (TDS) is a measure of the amount of material dissolved in water. These materials could include carbonate, bicarbonate, chloride, sulphate, phosphate, nitrate, calcium, magnesium, sodium and organic as well as other ions (Murphy, 2004). The TDS is also an important source of nutrients for microorganisms that can serve as an indication of high microbial numbers and TDS is expected to increase where water systems are closed.

The chemical oxygen demand (COD) is an indication of the oxidisable content of a system, including organic material that can serve as substrates for microoganisms (Atlas & Bartha, 1998). The most important contribution to the increase in COD levels in the circuits of paper-mill process waters is starch that is hydrolysed to glucose. Bacteria metabolise glucose to organic acids (Habets *et al.*, 1996), especially under anoxic and warm conditions. Increased levels of COD also lead to decreased DO levels that induce greater anaerobic biological activity (Dexter, 1996). Nitrogen (N) and phosphorous (P) are necessary macro-elements for all living cells, but when present in excess it can cause eutrophication (Atlas & Bartha, 1998). Therefore, with increasing N and P, increases in microbial numbers would be expected.

Specific water consumption can be defined as the amount of fresh water from external sources used for the paper manufacturing process (Zippel, 2001) and is regarded as an indicator of the degree of water closure for a system. Water consumption varies with the production of different types of paper and the location of the paper mill, but it could be as high as 60 m^3/ton of paper. It is assumed that closure of the water systems can result in an increase in populations of microorganisms as oxygen becomes depleted, process temperatures increase and nutrients are concentrated (Zippel, 2001).

Chemical biocides represent the most effective measure to control microbial growth and fouling in paper mills (Pereira *et al.*, 2001). Chemical biocides are divided into two main classes, namely oxidising biocides that cause the death of the microorganisms in the locations where they are active and organo-chemical biocides that impede the growth and proliferation of microorganisms (Keddy, 1988).

Furnish refers to the grade of the pulp used to produce the final product in a paper mill. When the furnish is virgin chemical pulp, low microbial numbers would be expected, but when a

waste-grade furnish is used, high microbiological activity could occur, because the COD load will be higher on the system (Zippel, 2001). Retention time is calculated from the water volume and the flow rate (Nalco Chemical Company, 2005) and can be described as the time the water spends in a specific area. Longer retention times result in low flow areas or "dead zones" in a process and could cause high microbial numbers (Nalco Chemical Company, 2005).

Plate counts quantify the total culturable aerobic and anaerobic bacterial numbers with regard to the nutrient agar used. Only a small portion of the total bacterial community can be represented since approximately 0.01 to 1% of all microbes are culturable on artificial media (Palojarvi *et al.*, 1997). Although all bacteria would not grow on any one specific growth medium, the trends of the cultivatable bacterial numbers indicate differences in biological activity. Plate count techniques are also widely employed in industry due to low cost and ease of application.

The analysis of the functional diversity of a microbial population was first demonstrated by Garland & Mills (1991) with the use of commercially available Biolog plates. The functional differences among microbial communities were subsequently studied in environments such as soil, (Garland & Mills, 1991), wastewater systems (Victorio *et al.*, 1996) and paper-mill water systems (Van der Merwe *et al.*, 2003). Van der Merwe *et al*. (2003) concluded that biodiversity indices calculated from substrate utilisation profiles were a sensitive measure to detect shifts in a microbial community's function as a result of external environmental parameters.

The aim of this study was to measure environmental parameters relevant to microbial growth in paper-mill water systems and to determine the influence of the individual parameters on bacterial numbers and diversity. A number of paper machines were surveyed during the present study to characterise the water systems and the following environmental and process parameters were investigated: Temperature, pH, DO, ORP, TSS, TDS, COD, N, P, SWC, biocide class, retention time and pulp furnish. These analyses may aid the prediction of changes in microbial populations in paper-mill water systems that may lead to improved process control.

2. MATERIALS AND METHODS

2.1 Sampling

Samples were collected from eight points on every paper machine for Adamas, Ngodwana, Enstra, Stanger, Cape Kraft and Tugela mills during a summer as well as a winter season. Sampling points were selected, based on accessibility, and because samples are customarily collected by the machine operators and biocide suppliers at these points. The survey dates, paper machines and sampling points are listed in Appendix A1 to A14

2.2 Environmental parameters

On-site measurements included temperature and pH of the samples using a hand-held probe (HI98128, Hanna Instruments, USA), TDS was determined with a conductivity meter (HI98352, Hanna Instruments, USA), ORP with a hand-held ORP meter (HI98201, Hanna Instruments, USA) and DO with a dissolved oxygen meter (HI9143, Hanna Instruments, USA).

Samples were returned to the laboratory where a number of measurements and analyses were conducted on two consecutive days following sample collection. The TSS in each sample was determined gravimetrically. Samples were centrifuged at 13 559 $\times$ *g* (Eppendorf AG 5804R, Eppendorf, Germany) to remove excess pulp and the COD, N and P values were determined using cell test kits (Merck, South Africa), digestion reactions (Spectroquant TR 320, Merck, South Africa) and photometric determinations (Spectroquant, Merck, South Africa).

2.3 Microbial enumeration

Dilution series were made from the samples after centrifugation (13 559 $\times$ *g*) and the spread-plate and pour-plate methods were used to enumerate aerobic and anaerobic bacterial guilds (Walter, 1967). Total culturable aerobic bacteria (TAB) were counted on nutrient agar (NA) plates (Biolab, Merck, South Africa) supplemented with cycloheximide (0.1% m/v) to suppress fungi and incubated at 22, 25, 30, 35, 40, 45, 50, 55 or 60°C, depending on the temperature of the sampling point. Anaerobic bacteria (AB) were enumerated on NA plates (Biolab, Merck, South Africa) supplemented with cycloheximide (0.1% m/v) to suppress fungi and incubated in anaerobic flasks at the listed temperatures. Sulphate-reducing bacteria

(SRB) were enumerated on iron sulphate agar (Oxoid, UK). A low redox-potential environment was created by adding titanium(III) citrate to the agar (Zehnder & Wuhrman, 1976). Tubes were incubated at the listed temperatures for 14 days. Many of the plate counts were recorded as <30, <300 or <3 000 and used for statistical analysis as 16.5, 165 or 1650 respectively, to represent the average of the range in which these bacterial counts fell.

2.4 Functional characterisation of populations

After samples were centrifuged (13 559 × *g*) to remove excess pulp, an aliquot of each water sample (10 ml) was added to 20 ml of phosphate buffer (pH 7.4) to standardise the turbidity. Aliquots of 150 µl were added to each well of the GN Biolog™ microtitre plates (Biolog Inc., USA) and incubated at 22, 25, 30, 35, 40, 45, 50, 55 or 60°C. The plates were evaluated every 12 h for absorbance changes at 590 nm using a Labsystems iEMS microtitre-plate analyser (Labsystems, Finland). The optical density values were corrected using the background colour of the control well and analysed with the average well colour development (AWCD) technique (Garland, 1996). Indices of diversity were calculated by using the transformed absorbance values. The Shannon index (H') was calculated for the diversity of substrates utilised while the Berger-Parker index (d) was calculated to determine the levels of dominance in each system (Magurran, 1988).

2.5 Statistical analysis

The relationships between the different parameters were determined with the analysis of covariance (ANCOVA) using correlation analysis (CORR Procedure) and general linear modelling (GLM Procedure) of the SAS/STAT® 9.1 statistical package (SAS Institute Inc., USA). The analysis of data for plate counts was based on the $\log_{10}$ numbers and diversity data (H') were transformed using:

$$Y = \frac{y^{\alpha} - 1}{\alpha} \qquad \text{.....................................(1)}$$

Where y = the Shannon index (H') and $\alpha = 2.85$. Means of parameters were compared using Scheffe's test.

Data from two surveys at each mill were analysed, excluding the Cape Kraft winter survey due to shock dosing of the biocide prior to sampling that led to high rates of mortality of the

microorganisms. Thirteen environmental and process parameters (temperature, pH, DO, ORP, TSS, TDS, COD, N, P, SWC, biocide class, pulp furnish and retention time) and five microbial parameters (TAB, AB, SRB, H' and d) were included in the multivariate analysis. Remaining data for a particular sample were still considered when missing observations occurred (such as where the photometric determination of DO failed). However, all observations for a sample were discarded where a datum point for one parameter was considered to be an outlier. For concentrations of COD, N and P less than the lower detection limit, a value of half the lower detection limit was recorded. For concentrations higher than the highest detection limit, the value of the detection limit was recorded. The furnishes of the paper machines were categorised as followed: bleached pulp (Adamas PM4; Enstra PM2, PM3 and PM6; Stanger PM1 and TM), broke (Tugela PM3), stone-ground wood and bleached pulp (Ngodwana NP), unbleached pulp (Tugela PM4), unbleached pulp and waste (Ngodwana KLB; Tugela PM1 and PM2), waste fibre (Cape Kraft PM1; Adamas PM3) as well as waste fibre and bleached pulp (Adamas DAF). The class of biocide (oxidising/non-oxidising), retention times and SWC data that were included were based on the process information obtained from each mill.

3. RESULTS AND DISCUSSION

A number of environmental factors and process parameters influenced microbial counts and diversity (Table 1). The highest correlation found between any environmental factor and microbial numbers was between ORP and the log of total aerobic bacteria with a Pearson's Correlation Coefficient of -0.57, indicating that as the ORP decreased, bacterial counts increased. The changes in the microbial parameters could not be ascribed to the influence of any single environmental factor.

3.1 Temperature

For this study, samples were collected at points with temperatures that ranged between 18.7 and 61.4°C (Appendix A1 to A14) and these results indicated a positive linear correlation (r = 0.30) between temperature and microbial numbers. An increase in system temperature, therefore, resulted in an increase in TAB and SRB as well as dominance (Table 1). At higher temperatures an increase in prevalent aerobic bacteria and anaerobic SRB occurred, resulting

in higher bacterial numbers that could cause problems in paper mills if not adequately managed. These results contradicted the findings of Habets *et al.* (1996) as it was evident that an increase in temperature did not reduce the number of anaerobic bacteria in the paper-mill water systems tested for this study.

Table 1: The significance (p) of the relationships between environmental parameters, microbial numbers and diversity.

	Log_{10} TAB	**Log_{10} AB**	**Log_{10} SRB**	**Diversity (d)**	**Dominance (H)**
Temperature[1]	<0.0001	ns	0.0006	ns	0.0052
pH[1]	ns	ns	ns	ns	ns
DO[1]	0.0095	<0.0001	ns	ns	0.0226
ORP[1]	<0.0001	<0.0001	<0.0001	0.0384	<0.0001
TSS[1]	ns	0.0009	ns	ns	ns
TDS[1]	ns	0.0096	0.0113	ns	ns
COD[1]	<0.0001	<0.0001	ns	0.0027	0.0451
N[1]	0.0094	ns	ns	0.0024	ns
P[1]	ns	0.0021	ns	ns	ns
SWC[1]	ns	0.0479	ns	ns	ns
Biocide class[2]	ns	ns	≤0.05	ns	ns
Furnish[2]	≤0.05	≤0.05	ns	≤0.05	ns
Retention time[2]	ns	ns	ns	≤0.05	ns

[1] Pearson's correlation
[2] GLM procedure
ns Not significant at the 95% level

3.2 pH

A variation in pH did not significantly influence microbial numbers or diversity (Table 1). It could be expected, however, that the composition of populations could change under different pH conditions.

3.3 Dissolved oxygen

DO was initially determined using a test kit, but unrealistic results (exceeding 20 mg/L) obliged the use of a probe for further surveys. Data analysis showed that DO had a significant influence on TAB, AB and dominance (Table 1). TAB and AB numbers were negatively correlated with DO and decreased with increasing DO. This reduction of microbial numbers possibly resulted in an increase in dominance. It is, however, possible that the decreasing microbial numbers were influenced by the decreasing temperature of a water system rather than increasing DO levels since temperature also significantly influenced DO ($r = -0.41$).

With increasing temperature, the concentration of DO diminish in water systems, leading to an increase in AB. This observation was confirmed by findings of Gudlauski (1996), as well as Robertson & Schwingel (1997).

3.4 Oxidation-reduction potential

The multivariate analysis indicated that there was a significant relationship between ORP and TAB, AB, SRB, diversity and dominance (Table 1) with negative Pearson's correlation coefficients of -0.57 (TAB), -0.34 (AB) and -0.33 (SRB) respectively. It can be concluded that reduced ORP initially developed as result of elevated TAB numbers, since aerobes influence the electric potential (E_h) of their environment during growth as found by Jay (1992) and that increased AB and SRB populations then developed in the more favourable reductive environments.

3.5 Total suspended solids

Suspended solids can influence the accurate determination of other environmental parameters, since it influence the transfer, protection and enumeration of microbes. Only anaerobic bacterial counts were influenced by TSS (Table 1), and possibly only because TSS had a significant influence on COD ($p = 0.0112$), total N ($p = 0.0113$) and total P ($p < 0.0001$). It seems, therefore, that TSS had a more direct influence on the nutrient sources of microorganisms, than the microorganisms themselves. Build-up of TSS after system closure could, however, lead to solids acclumulation in low flow areas (Nalco Chemical Company, 2005), which in turn could lead to increases in microbiological slimes.

3.6 Total dissolved solids

Dissolved solids could be an important source of nutrients for microorganisms (Murphy, 2004), especially if it becomes concentrated during system closure. For the present study, it was determined that AB and SRB increased with elevated dissolved solids levels (Table 1). Therefore, an increase in TDS will require better control of anaerobic bacterial numbers in paper-machine water systems. Some of the potential problems associated with the build-up of TDS include corrosion, scale and odour (Pulles, 1983) most likely as a result of the activity of different anaerobic bacteria.

3.7 Chemical oxygen demand

For the present sutdy, COD was found to be one of the most important parameters in determining levels of microbial diversity and dominance in paper-mill water systems. The COD was strongly correlated to all the biological parameters except SRB numbers (Table 1). These relationships were presumably due to the increased availability of organic substrates for assimilation by the microbial populations, since TAB and AB numbers correlated positively with increasing COD (Table 1). This correlation between bacterial numbers and COD was also observed by Dexter (1996).

3.8 Total nitrogen & phosphorous

Total P levels were not determined at the start of this project and data is, therefore, not available for the first surveys at the Adamas and Ngodwana mills. Total N and P did not have the same effect on microbial numbers and diversity and most often the effects were not significant (Table 1). However, increased levels of N resulted in increased diversity, despite an associated reduction in TAB numbers. Only AB numbers increased as a result of higher P values. Total N and P are macronutrients for microoganisms (Bitton, 1994) and an important indication of potential microbial activity. Excessive levels of N and P can, therefore, lead to enrichment of inorganic materials that support excessive growth of individuals and species (Atlas & Bartha, 1998). Total P is usually the limiting factor in wastewater treatment systems (Nozaic, 2002) and partial removal of P from paper-mill water can possibly be used to reduce anaerobic bacterial growth by efficient process management.

3.9 Specific water consumption

Only AB was significantly influenced by SWC (Table 1), with the bacterial numbers increasing as water systems became more closed and SWC decreased ($r = -0.14$). Although not significant, TAB, SRB and diversity also increased with reduction in water consumption, but dominance decreased. With increasing system closure and, therefore, less water consumption, an exponential growth of aerobic populations of bacteria will occur initially according to Zippel (2001). As the oxygen in the water becomes depleted, an increase in anaerobic and facultative anaerobic bacterial numbers will then occur, that can include anaerobic sulphate-reducing bacteria (*Desulfovibrio*) and species of *Enterobacter* and *Klebsiella* that can increase slime formation in paper mills and contribute to odours and

corrosion (Zippel, 2001). The presence of some of these slime-forming bacteria was observed for some of the paper machines at Enstra and Tugela (Chapter 4) and, therefore, SWC was found to be an important process parameter to consider when controlling anaerobic bacteria.

3.10 Biocide class

The use of different classes of biocide significantly influenced SRB numbers and the oxidising biocides appeared to be the most effective in reducing SRB (Table 1). The sampling points where no biocide was dosed did not differ significantly from those where oxidising biocide was dosed (Figure 1). This could be as result of biocide carried forward from points where dosing took place. A further factor to consider for microbial control would be the significant effect that oxidising biocides had on ORP and consequently on microbial counts (Figure 2).

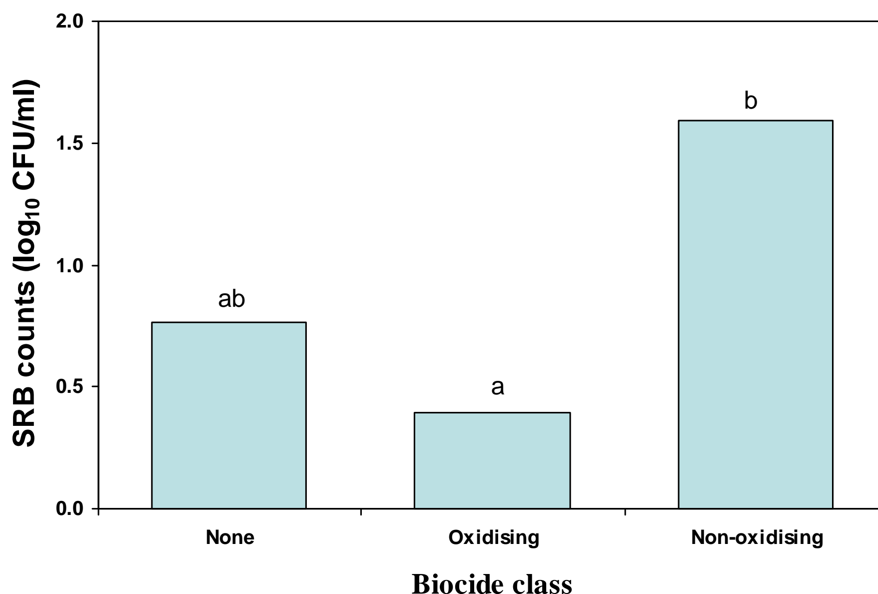

Figure 1: Influence of biocide classes on numbers of sulphate reducing bacteria (Bars with the same letters do not differ significantly, Scheffe's test at the 95% significance level).

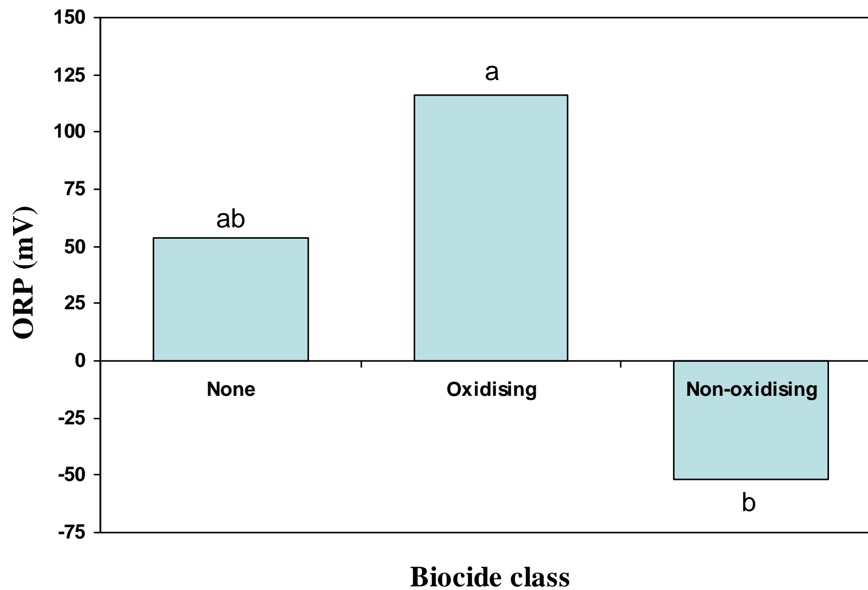

Figure 2: Influence of biocide classes on oxidation-reduction potental (Bars with the same letters do not differ significantly, Scheffe's test at the 95% significance level).

In any water treatment programme it is preferable to use microbiostats in combination with microbiocides (Frayne, 2001) for adequate control of microbes. Accordingly it was found for the present study that in the paper mills where multiple formulations of biocides were dosed (Adamas PM4), effective microbial control was maintained (Appendix A2).

3.11 Pulp furnish

The different pulp furnishes had a significant effect (Table 1) on the levels of aerobic bacteria (Figure 3), anaerobic bacteria (Figure 4) and diversity (Figure 5). Predictably, paper machines utilising waste fibre or mixes with waste (Tugela PM1 and PM2, Cape Kraft PM, Adamas PM3) supported the highest TAB and AB populations possibly as a result of the recycled waste which increased the oxygen demand of a system as expressed by COD (Zippel, 2001). The microorganisms in these systems were, therefore, provided with more substrates to utilise and subsequently proliferate. Similarly, high bacterial counts were observed for the newsprint machine of Ngodwana where ground-wood pulp was used. For this study, the lowest TAB and AB counts were seen on machines using virgin, chemical pulps as in the case of Enstra and Stanger mills. The fact that most machines ran on mixed

furnishes and often share water loops could contribute to the lack of sensitivity in detecting significant differences between furnishes.

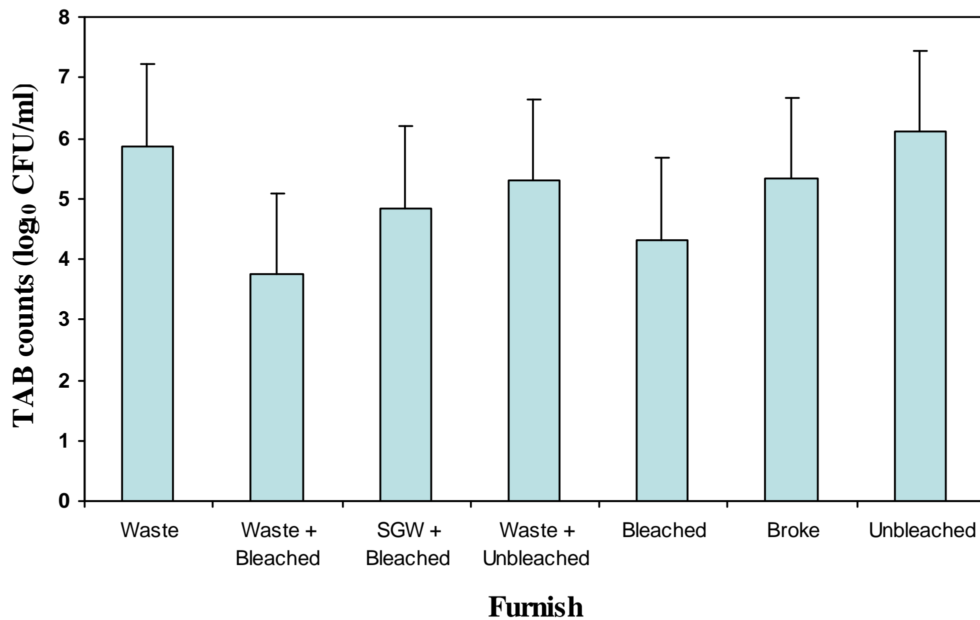

Figure 3: Influence of pulp furnish on numbers of total aerobic bacteria (Error bars reflect minimum significant difference, Scheffe's test at the 5% significance level).

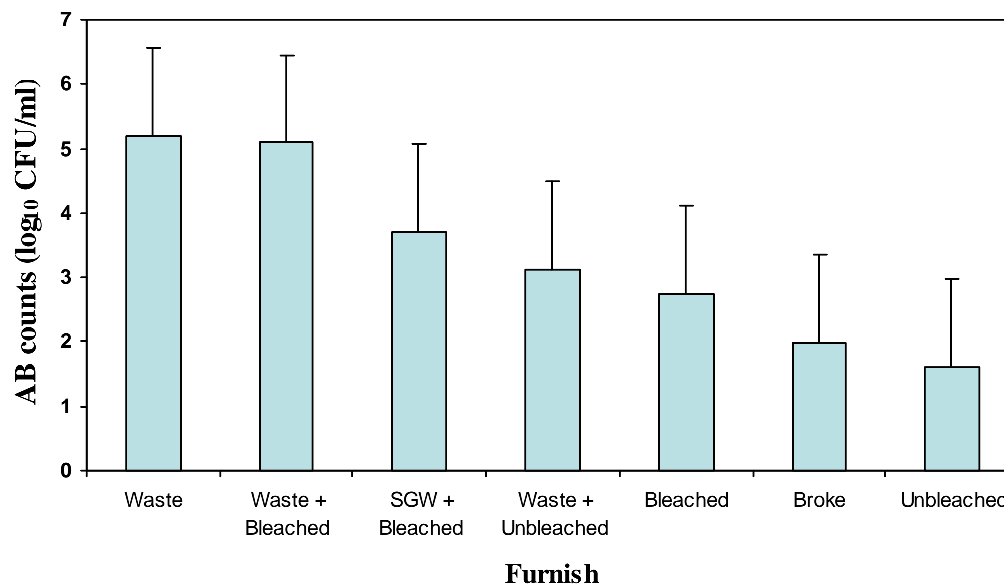

Figure 4: Influence of pulp furnish on numbers of anaerobic bacteria (Error bars reflect minimum significant difference, Scheffe's test at the 5% significance level).

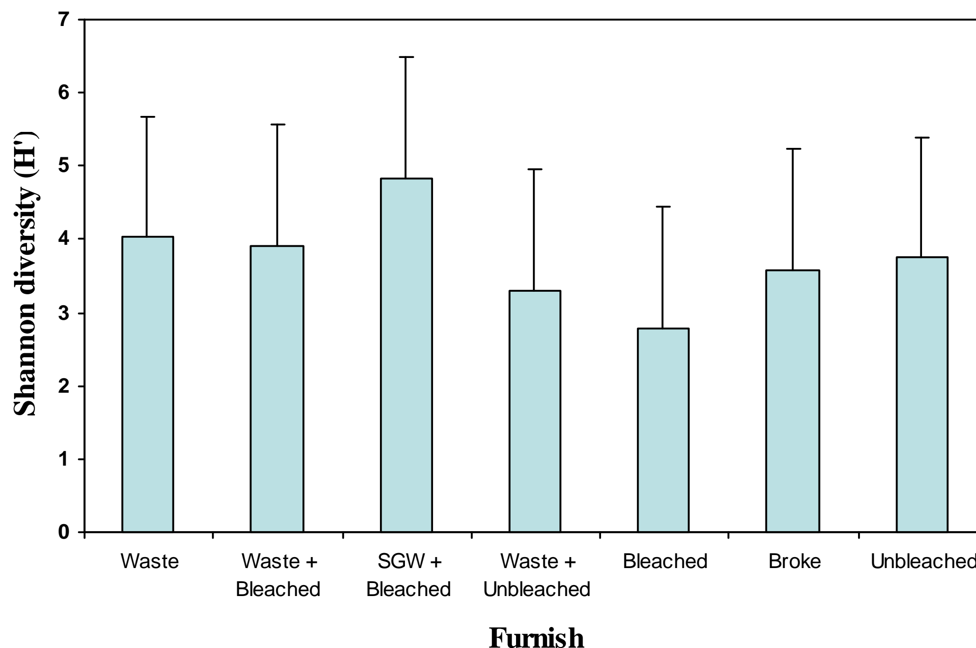

Figure 5: Influence of pulp furnish on population diversity (Error bars reflect minimum significant difference, Scheffe's test at the 5% significance level).

3.12 Retention time

Retention time significantly influenced the Shannon diversity indices (Table 1), but not the bacterial counts or dominance (Table 2). An increase in retention time resulted in higher AB, although no significant influence could be detected. This was possibly the result of slower moving pulp that increased the opportunity for anoxic areas to develop where anaerobic microorganisms could proliferate. The retention time of pulp in vessels could, therefore, influence microbial growth and efficacy of biocides, since areas with long retention times would not allow the dispersal of biocides into bacterial slimes (Nalco Chemical Company, 2005).

Table 2: The influence of retention time (water and pulp) on microbial numbers in different bacterial guilds.

Time (min)	TAB (Log_{10}CFU/ml)	AB (Log_{10}CFU/ml)	SRB (Log_{10}CFU/ml)	Diversity (H')	Dominance (d)
0 to 5	5.06	2.57	0.89	3.45	0.062
> 5	4.91	3.24	0.70	3.14*	0.062

* Significant difference based on Scheffe's test at the 95% significance level

3.13 Microbial numbers and diversity indices

The diversity and dominance indices were influenced by all of the enumerated microbial guilds, except for H', that was not influenced by SRB (Table 3). This effect is typical of the specific index that emphasises the number of species and not evenness (Magurran, 1988). Increased microbial numbers resulted in an increase in diversity, while dominance decreased. More of the environmental and process parameters appeared to have an influence on AB numbers than the numbers of other guilds (Table 1).

Table 3: Pearson's correlation coefficient indicating the significance (p) of the relationships between microbial numbers and diversity.

Microbial Guild	Diversity (H')	Dominance (d)
TAB (Log_{10})	0.0498	<0.0001
AB (Log_{10})	0.0061	<0.0001
SRB (Log_{10})	ns	0.0083

ns Not significant at the 5% significance level

4. CONCLUSIONS

The analysis of co-variant parameters in paper-mill water systems revealed the influence of individual environmental parameters on culturable bacterial numbers that were previously obscured in a large dataset. The present study showed that temperature increases as a result of process energy was an important factor to take into consideration when planning system closure, since temperature positively correlated with aerobic bacterial numbers, sulphate-reducing bacterial numbers and bacterial dominance. This correlation was contrary to findings made by Habets *et al.* (1996) who postulated that higher temperatures will reduce microbial activity. In this study it was found that higher numbers of aerobic bacteria and sulphate-reducing bacteria proliferated as a result of the increase in temperature. Increased TAB and SRB did not lead to increased biodiversity, but higher dominance levels, reflecting a population shift to a more extreme thermophilic environment where high numbers of fewer species could survive.

In the present study, it was found that dissolved oxygen correlated negatively with culturable bacteria. However, DO was strongly influenced by temperature, as previously shown by

Gudlauski (1996) and Robertson & Schwingel (1997) where increased temperatures led to decreased DO, and elevated levels of anaerobic bacteria.

The strongest relationship found between any environmental and biological parameter, was the negative correlation between ORP and aerobic bacterial numbers. This occurred most probably because high aerobic microbial numbers depleted systems of available dissolved oxygen (Jay, 1992), creating reductive environments and resulting in a shift in the microbial populations (from aerobic to anaerobic members).

Total dissolved solids were found to be an important source of nutrients for microorganisms. Ions that can contribute to nutrient sources iclude CO_3^{2-}, HCO_3^-, Cl^-, SO_4^{2-}, PO_4^{3-}, NO_3^-, Ca^{2+}, Na^+ and other dissolved organic compounds (Murphy, 2004). A positive correlation between TDS and AB as well as SRB indicated that high TDS levels could be an indicator of anaerobic bacterial blooms in water systems. Other parameters that also reflected nutrient levels in water systems included COD, N and P. COD was shown to positively influence aerobic and anaerobic bacterial numbers as well as microbial diversity. This trend can possibly be ascribed to higher levels of COD reflecting the availability of more substrates and a wider variety of species may, therefore, proliferate.

The present study showed that decreased water consumption led to increased anaerobic bacterial numbers, because oxygen will become depleted if fresh water is not constantly introduced into a system (Zippel, 2001). Decreased SWC could, therefore, lead to corrosion and odour problems as a result of high anaerobic bacterial counts.

Biocide class influenced SRB and oxidising biocides led to decreased SRB levels. Frayne (2001) suggested using non-oxidising biocides in combination with oxidising biocides, since the former has superior penetrating abilities while the latter has a fast kill rate. This combined approach to microbial control could ensure effective killing rates as well as prevent the development of resistance mechanisms in microorganisms.

The furnish of a paper machine influenced anaerobic bacteria and paper machines that utilised waste as furnish supported the highest bacterial numbers. Recycled furnish will increase the COD loading of a system (Zippel, 2001), providing the microorganisms with more nutrient sources to utilise.

Retention time was positively correlated with anaerobic bacterial numbers and decreased microbial diversity. These trends indicated that long retention times could create oxygen-depleted environments where anaerobic microorganisms could proliferate. The high AB counts could be aggravated further when biocides cannot be dispersed with the slow flowing water to effectively control anaerobic bacterial numbers in areas with long retention times.

The present study showed that the evaluation of the different environmental and process parameters may provide insight into the microbial activity of the different water systems. By considering the influence of the various environmental parameters, better microbiological control of paper-mill water systems could be achieved and the influence of problematic microorganisms could be managed.

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CHAPTER 4

CHARACTERISATION AND IDENTIFICATION OF PREVALENT BACTERIA IN PAPER-MILL WATER SYSTEMS

ABSTRACT

Microorganisms in paper-mill water systems are often responsible for reduced machine performance and paper quality due to fouling that can cause slime formation, odour problems and safety hazards. The characterisation and identification of prevalent paper-mill bacteria could aid the control of fouling in water systems, since knowledge of the species composition could provide insight into the correct chemical control programmes. The aim of this study was to characterise and identify the prevalent bacteria from the water systems of 14 paper machines using the ERIC-PCR and PCR-RFLP techniques as well as phylogenetic analysis. The use of ERIC-PCR to type prevalent bacteria from paper-mill water of Adamas, Ngodwana, Enstra and Stanger was successful and the bacteria were grouped into 28 distinct groups. However, the ERIC profiles of the isolates from the Cape Kraft and Tugela mills were too complex to discern visually and these isolates were further characterised with PCR-RFLP and grouped into seven additional 16S genotypes. Using sequence analysis it was possible to tentatively identify ten of the 35 types to species level, 21 types to genus level and the remaining four types to family level. It was found that the majority of bacteria isolated from paper-machine water belonged to the genera *Acinetobacter* and *Pseudomonas,* well known slime-forming aerobic bacteria from industrial water systems. The present study generated significant information on the bacteria occurring in paper-mill water systems of South Africa. This knowledge can be useful to improve control of microbial fouling through regulation of environmental and process parameters and with appropriate biocide programmes.

1. INTRODUCTION

Microorganisms are constantly introduced into paper machines through raw materials, water, fibres and papermaking chemicals. Bacteria were found to be the prevalent colonisers of paper-machine wet ends and raw materials (Väisänen *et al.*, 1998). The paper-machine environment is suitable for microbial growth due to favourable temperatures (30° to 50°C), pH (4 to 10) and nutrients that flow with the pulp and chemicals (Väisänen *et al.*, 1998). Upon closure, elevated temperatures and nutrient concentrations will lead to increased bacterial numbers and biofilm formation (Bennet, 1985). This, in turn, could lead to safety hazards, reduced paper quality and odour problems. There is, therefore, a need to identify prevalent bacterial types in paper-mill water systems, since this will aid in designing chemical control programmes to efficiently manage microbial fouling.

Enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR) can be used as a DNA typing technique to discriminate amongst bacterial isolates both within and between species. A specific profile is generated for each bacterial isolate that enable discrimination between the different isolates. This typing method has been proven useful in the clinical, environmental and taxonomic fields of microbiology (De Bruijn, 1992; Olive & Bean, 1999; Rademaker *et al.*, 2000) and is inexpensive and rapid, but is highly sensitive to external factors such as alterations in the reaction mixtures.

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) can also be used to distinguish between different bacterial species, based on nucleotide sequence variations. Genetic variations at the site where a restriction enzyme digests DNA determine the size of the resulting fragments and this variation could generate species-specific restriction patterns when amplifying conserved regions such as the 16S or 23S rRNA genes. PCR-RFLP has been used for rapid classification, identification and detection of methanogens, *Legionella* and *Mycobacterium* (Hiraishi *et al.,* 1995; Ko *et al*., 2003; Kim *et al.*, 2005). It was found by Vidigal *et al.* (2000) and Carvalho *et al.* (2004) to be a simple and robust auxiliary tool for morphological identification.

Partial 16S rDNA sequences can be used to identify and determine the taxonomic position of novel bacterial isolates and provide the phylogenetic framework which serves as the backbone of modern microbial taxonomy (Vandamme *et al.*, 1996). This method has been used by several researchers to determine and confirm the identity of new isolates (Vinuesa *et al.*,

1998; Tannock *et al*., 1999; Oppong *et al.*, 2003; Suihko *et al*., 2003) and is considered to be the most authoritative method for identification of bacterial isolates, since international databases comprising all published and some unpublished partial or complete sequences have been constructed and is widely accessible (De Rijk *et al*., 1992).

Since microbial control programmes can be optimised if the identity of problematic bacteria was known, the objective of this study was to characterise and identify prevalent bacteria in different paper-mill water systems. ERIC typing and RFLP analysis were used for the initial characterisation of bacterial isolates into distinct groups. Representative types were then selected from each group of isolates for nucleic acid sequencing in an attempt to identify species with phylogenetic analysis.

2. MATERIALS AND METHODS

2.1 Sampling

Water and pulp samples for isolation of bacteria were collected during the surveys of 14 paper machines as described previously (Chapter 3). Samples were serially diluted and total aerobic bacteria (TAB) were counted on nutrient agar plates (Biolab, Merck, South Africa) supplemented with cycloheximide (0.1% m/v) to suppress fungi and incubated for two days at 22, 25, 30, 35, 40, 45, 50, 55 or 60°C, depending on the temperature of the sampling point. The Harrison-disc method (Harrigan, 1998) was used to statistically select ten prevalent bacterial isolates per sampling point. In total, approximately 2480 isolates were selected and analysed with DNA typing.

2.2 Extraction of genomic DNA

A modified method for the treatment of cells with proteinase-K (Roche Molecular Biochemicals) was used (Laguerre *et al*., 1997). A fresh culture of each isolate was streaked on a nutrient agar slant in a screw-cap tube. The bacterial isolates were incubated at a temperature nearest to the temperature from which it was isolated and checked for sufficient growth and purity. Sterile distilled water was added to the slant to harvest the cells. The cell-suspension was collected in a clear plastic tube and vortexed to ensure a uniform suspension. The absorbance of the suspension was measured with sterile distilled water as the

spectrophotometric blank at 620 nm. The following formula was used to determine the volume of the cell-suspension to be treated further:

$$V = 0.2 / A \qquad \ldots\ldots 1$$

Where V is the volume of the suspension (ml) and A the absorbance at 620 nm.

A micro-tube was filled with the appropriate volume of cells for each isolate and centrifuged at 21 913 × *g* (Eppendorf AG 5804R, Eppendorf, Germany) for 5 min at 4°C. The supernatant was discarded and the excess media blotted dry. In each tube, 100 µl sterile ultra-pure water, 100 µl Tris-HCl (10 mM, pH 8.2) and 10 µl proteinase-K (15 mg/ml) (Roche Diagnostics, South Africa) were added to the cell pellet. The mixture was incubated overnight at 55°C and boiled afterwards for 10 min to inactivate the proteinase-K. The cell lysates were stored at -20°C until further analysis.

2.3 Amplification of the ERIC regions and 16S rDNA gene for typing

The ERIC regions of all the prevalent aerobic bacterial isolates were amplified with the primer ERIC2 (Table 1) using the conditions as described by De Bruijn (1992). A PCR reaction mixture made up to 50 µl with sterile ultra-pure water contained 5 µl of the cell lysate, 0.5 µl of primer (25 pmol/µl), 1 µl of each dNTP (25 mM), 4 µl of $MgCl_2$ (15 mM), 5 µl Supertherm *Taq* buffer (Southern Cross Biotechnology, South Africa) and 0.1 µl Supertherm *Taq* DNA polymerase (5 U/µl) (Southern Cross Biotechnology, South Africa). The amplification reactions were done in an Eppendorf Mastercycler 22331 (Merck, South Africa) with the following temperature profile: Initial denaturation for 7 min at 95°C was followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min and extension at 65°C for 8 min and a final extension step at 72°C for 16 min.

Table 1: Primers used in the amplification and/or sequencing of the different genes analysed.

Primer name*	Primer sequence (5'-3')	Target gene	Reference
ERIC2	5'-AAGTAAGTGACTGGGGTGAGCG-3'	ERIC	De Bruijn, 1992
63F	5'-CAGGCCTAACACATGCAAGTC-3'	16S rDNA	Marchesi *et al.*,1998
1387R	5'-GGGCGGWGTGTACAAGGC-3'	16S rDNA	Marchesi *et al.*,1998

* All primers were synthesised by Inqaba Biotechnical Industries (Pty) Ltd., South Africa

The amplified products were analysed on a 1.5% (m/v) horizontal agarose gel (Sambrook *et al*., 1989) in a BIORAD Sub-cell® GT Agarose Gel Electrophoresis System (Bio-Rad Laboratories, USA) for 120 min at 80 V. Molecular weight marker VIII (Roche Diagnostics, South Africa) was loaded as a standard in specified lanes on each gel. All the profiles were analysed visually, grouping isolates with similar bands in the same profile type.

The 16S rDNA gene of selected isolates was partially amplified to further distinguish certain closely related types. Amplification was performed with the primers 63F and 1387R (Table 1) as described by Marchesi *et al*. (1998). The PCR reaction mixture for each strain contained 5 µl of the cell lysate, 0.5 µl of each primer (25 pmol/µl), 0.5 µl of each dNTP (25 mM), 5 µl of 10 × Gold *Taq* PCR buffer (Southern Cross Biotechnology, South Africa) and 0.1 µl Gold *Taq* DNA polymerase (5U/µl) (Southern Cross Biotechnology, South Africa) that was made up to a reaction volume of 50 µl with sterile ultra-pure water. The following temperature profile was used: an initial 5 min of denaturation at 94°C, 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min followed by a final extension step at 72°C for 5 min. The quality of the PCR products were evaluated by analysing an aliquot (2 µl) of each reaction on 1% (m/v) horizontal agarose gels (Promega, South Africa) (Sambrook *et al*., 1989) using the molecular weight marker 1 KB plus (Scientific Group, South Africa) as a standard.

2.4 Characterisation of types with RFLP analysis

The selected isolates from the Cape Kraft summer survey and Tugela winter and summer surveys that could not be typed with ERIC-PCR were grouped with the BioNumerics 4.0 software (Applied Maths, Belgium). The gel-files were analysed using the molecular weight marker (1 KB plus, Scientific Group, South Africa) as the standard lane. The Dice coefficient (Nei & Lei, 1979) was used to calculate a distance matrix for the ERIC-PCR profiles and the unweighted pair group method with arithmetic mean (UPGMA) was used to construct a dendrogram.

Representative isolates that displayed less than 70% similarity to each other were digested with the restriction enzyme *Cfo*I (Roche Diagnostics, South Africa). The DNA concentration was determined using a Nanodrop system (Inqaba Biotechnological Industries, South Africa) as 1 U of *Cfo*I digest 1 µg of DNA. A RFLP reaction mixture of 10 µl consisted of 5 µl PCR

product, 1 µl of Buffer L (Roche Diagnostics, South Africa), 1 U *Cfo*I (Roche Diagnostics, South Africa) and 3 µl of sterile ultra-pure water. The reaction mixture was incubated for 2 h at 37°C and the restriction enzyme profiles of the types were visualised on a 2% (m/v) horizontal agarose gel (Sambrook *et al*., 1989) in a BIORAD Sub-cell® GT Agarose Gel Electrophoresis System (Bio-Rad Laboratories, USA) for 120 min at 80 V. Molecular weight marker VIII (Roche Diagnostics, South Africa) was loaded as a standard in specified lanes on each gel to estimate the sizes of the restricted fragments.

2.5 Amplification of the 16S rRNA gene for sequencing

Representatives of the prevalent bacterial types (obtained from ERIC-PCR and RFLP analysis) were subjected to 16S rDNA amplification. The reaction was performed as previously described for the amplification of selected types for RFLP analysis using the primers 63F and 1387R (Table 1) (Marchesi *et al*., 1998). The concentration, purity and size of the products were confirmed as described previously. The molecular marker 1 KB plus (Scientific Group, South Africa) was included on each gel.

2.6 16S rDNA sequencing and phylogenetic analyses for identification

The PCR reactions and sequencing of the 16S rDNA gene were performed by the Department of Human Genetics (University of Pretoria, South Africa) and Inqaba Biotechnical Industries (Pty) Ltd. (South Africa). The sequencing results were compared with that of the data available in GenBank (www.ncbi.nlm.nih.gov/Genbank/) using the Basic Local Alignment Search Tool (BLAST) algorithm (Altschul *et al*., 1990). Similarity values of 99 to 100% were regarded as the same species, 95 to 99% similarity were regarded as the same genus and similarity values less that 95% were regarded as the same family.

The ClustalX programme (Thompson *et al*., 1997) was used to analyse the edited sequences as well as the reference sequences obtained from GenBank (Table 3). A distance matrix was constructed by pair-wise alignment of the sequences. The neighbour-joining method (Saitou & Nei, 1987) was used to construct a phylogenetic tree from the distance matrix. The branch lengths of the reconstructed tree were proportional to the estimated divergence along each branch. Confidence levels of the phylogenies were estimated with the bootstrap method of Felsenstein (1985) and the phylogenetic tree was visualised with NJplot software (Perrière & Gouy, 1996).

3. RESULTS AND DISCUSSION

3.1 DNA typing of bacterial isolates

The profiles generated by ERIC-PCR differentiated between 28 bacterial groups that were assigned Types A to Z and AA to AC. The typing of selected isolates into groups was performed visually by comparing the DNA band patterns of isolates (Figure 1). It was found that approximately 500 of the 2480 typed isolates grouped with Type X and this type was found at all paper mills excluding Tugela. Type D represented 253 isolates and was mostly isolated from fine paper machines. Type M was isolated 70 times and occurred in paper machines that operated at higher temperatures at Ngodwnana and Tugela. Not all types were that abundant, and some such as Types H, Q, W, AA, AB and AD were only isolated once. The majority of isolates from the Cape Kraft and Tugela surveys could not be differentiated with ERIC-PCR and were further grouped (AD to AK) based on restriction enzyme patterns .

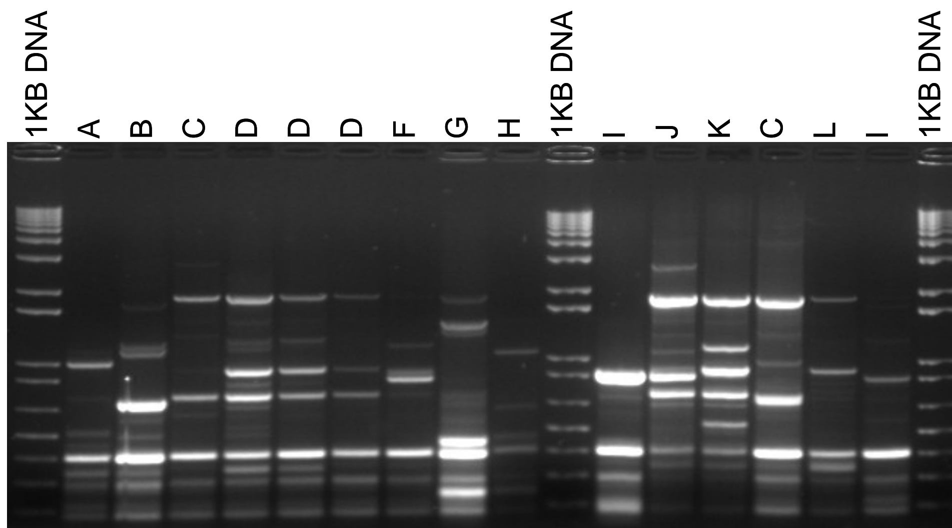

Figure 1: Different ERIC profiles for Type A to Type I isolated from four different paper mills, displaying distinct DNA patterns on a 1.5% agarose gel.

After restriction enzyme digestion (*Cfo*I) of the DNA from selected isolates from Cape Kraft and Tugela surveys, it was possible to type these bacterial isolates into seven additional groups that were named type AE to AK. The typing of the selected isolates from various paper-machine water systems with ERIC-PCR and PCR-RFLP reduced the amount of sequences to be analysed from approximately 2480 to 35.

Table 2: The mill, paper machine and sampling points from where the representative strains for each type were isolated.

Type	Mill	Paper Machine	Sampling points
A	Enstra	PM3	Machine chest
B	Enstra	PM3	Corrected broke chest
C	Stanger	TM	Bagasse stock channel
D	Stanger	TM	Bagasse stock channel
E	Ngodwana	NP	Uhle box pit, Wire 1
F	Ngodwana	KLB	Top-ply silo
G	Stanger	TM	Recovered fibre
H	Stanger	TM	Recovered fibre
I	Stanger	TM	Main drain
J	Stanger	TM	Blend chest
K	Stanger	TM	Blend chest
L	Stanger	TM	Main drain
M	Ngodwana	KLB	Top-ply silo
N	Adamas	PM3	Knock-off shower
O	Adamas	PM4	Launder
P	Adamas	PM3	DAF1-in
Q	Adamas	PM4	Finestock III
R	Adamas	PM4	Finestock III
S	Adamas	PM4	Finestock III
U	Ngodwana	NP	Machine chest
V	Adamas	PM3	Knock-off shower
W	Enstra	PM2	Blend chest
X	Adamas	PM4	Finestock III
Y	Adamas	PM3	Knock-off shower
Z	Adamas	PM4	Finestock III
AA	Stanger	PM	White-water tower
AB	Stanger	PM	Broke tank
AC	Stanger	TM	Bagasse stock channel
AE	Tugela	PM1	Backwater
AF	Tugela	PM4	Backwater
AG	Tugela	PM1	Pine chest
AH	Tugela	PM4	Double wire press
AI	Tugela	PM3	Shower water
AJ	Tugela	PM3	Backwater
AK	Tugela	PM3	Blend chest

3.2 Phylogenetic analysis

All the bacterial isolates belonged to either the phylum BXII (*Proteobacteria*), the phylum BXIV (*Actinobacteria*) or the phylum BXX (*Bacteriodetes*). Most of the isolates belonged to the classes *Alphaproteobacteria* (*Rhizobiaceae*), *Betaproteobacteria* (*Burkholderiaceae* and *Neisseriaceae*) and *Gammaproteobacteria* (*Xanthomonadaceae*, *Enterobacteriaceae* and

Pseudomonaceae) in the phylum *Proteobacteria*. One isolate, which clustered with strains in the *Micrococcaceae* family from the phylum *Actinobacteria*, was also represented as well as one isolate that clustered within the *Flavobacteriaceae* family of the *Bacteriodetes* phylum (Garrity *et al*., 2004).

3.2.1 *Pseudomonas* cluster

The bacterial isolates representing Types C, D, E, F, I, J, K, L, Q and AC clustered with members of the *Pseudomonas* genus (Figure 2, Table 3). It appeared that types V and AJ also belonged to the *Pseudomonas* genus, but the sequences obtained for these types were insufficient to be included in the phylogenetic analysis. The *Pseudomonas* genus is a relatively large and important group of bacteria and members proliferate in soil, fresh water and marine habitats (Atlas & Bartha, 1998). Bacterial species in this genus are motile, gram-negative, non-spore forming, straight or slightly curved rods (Andersen *et al.*, 2000). Physiologically they are chemo-organotrophs with aerobic metabolism and able to utilise a large variety of organic substrates. *Pseudomonas* spp. are active in aerobic decomposition and biodegradation and are able to degrade highly refractory compounds such as aliphatic and aromatic hydrocarbons, fatty acids and environmental pollutants (Atlas & Bartha, 1998).

Types D and F appeared to be closely related and both clustered with *P. mendocina* and *P. nitroreducens* (Figure 2). It is possible that these two types represented two strains of the same species, since only slight differences in their Biolog substrate utilisation profiles were observed (Wolfaardt *et al.*, 2007) as well as in their ERIC profiles (Figure 1). Type D was isolated from the stock-line of a fine-paper machine (Table 2) and representatives of this type constituted approximately 10 to 60% of the culturable bacterial population at the points from where they were isolated. Type F was only isolated from the stock-line of a tissue machine (Table 2) and the backwater of a fine-paper machine and represented 10% of the total culturable bacterial population at these sampling points.

Type E, J, K, L and AC also clustered with *P. mendocina* and *P. nitroreducens* as well as with *P. putida, P. alcalophila* and *P. pseudoalcaligenes* (Figure 2). It is interesting to note that *P. pseudoalcaligenes* str. CECT 5344 was described by Luque-Almagro *et al.* (2005) as a bacterium able to degrade cyanide and cyano-metallic complexes under alkaline conditions, making it a candidate for biodegradation treatment of cyanurated residues. Representatives of type E were isolated from a tissue machine and a newsprint machine where it occurred in the

stock line and back-water samples. This type represented 14 to 50% of the culturable populations at these points. Type J and K were only isolated from a blend chest containing bleached bagasse and softwood fibre (Table 2) and both constituted 25% of the culturable bacterial population at this sampling point of a tissue machine. Type L represented 10% of the culturable bacterial population in the backwater of a tissue machine. Type AC was isolated from two samples on a tissue machine (stock line and blend chest) where it constituted 11 and 13% of the total cultured bacterial population.

It appeared that Type C clustered with *P. stutzeri* and type I clustered with *P.azotofigens* (Figure 2), while type Q clustered with *P. marginalis* and *P. veronii*. Väisänen *et al.* (1998) found that *Pseudomonas stutzeri* produced enzymes that were able to degrade paper-making chemicals *e.g.* starch and casein. Type C was prevalent (10 to 25% of the culturable bacterial population) during one survey of a tissue machine where it was isolated from a stock line and a blend-chest. Type I was only observed during one survey when it formed 10 to 14% of the culturable population where it was isolated from the recovered fibre and backwater sampling points of a tissue machine. Type Q was isolated only once from a finestock sampling point of a paper machine that uses bleached pulp as furnish and constituted 6% of culturable bacteria.

None of the types that belonged to the *Pseudomomas* cluster could be identified to species level but several *Pseudomonas* species shared high 16S rRNA sequence similarity with the 12 types. It is possible that these types constitute new genotypes, although full-length sequences as well as other data to corroborate the 16S rDNA sequencing data would be needed for verification. The representative isolates of the unidentified *Pseudomonas* spp. were isolated from a wide variety of paper machines operating at different environmental parameters and using different grades of furnish (Table 2) illustrating the varied habitats where these species could proliferate. *Pseudomonas* spp. were found by Pandya (2000) in re-circulatory cooling systems of refineries and members of the *Pseudomonas* genus are also well known in other industrial water systems as aerobic slime-formers that can cause microbially induced corrosion when their extrapolymeric substances (EPS) create favourable conditions for anaerobic bacteria (Nalco Chemical Company, 2005).

3.2.2 *Acinetobacter* cluster

Types O, R, S, X, Y, AB and AH clustered with species in the genus *Acinetobacter* (Figure 2, Table 3) from the *Moraxellaceae* family in the order *Pseudomonadales* (Garrity *et al.*, 2004).

It appeared that type P also belonged to this genus, but the sequence data obtained for this type was insufficient to include in the phylogenetic analysis. Members of the genus *Acinetobacter* are commonly found in soil and water (Yamamoto *et al*., 1999) and are gram-negative, non-motile, diplococcoid rods (Ibrahim *et al*., 1997) that grow well on simple media (Nemec *et al*., 2003) and are strictly aerobic (Carr *et al*., 2003). Members of the genus *Acinetobacter* were isolated from printing-paper mills by Väisänen *et al*. (1998) in coloured slime samples.

Types O, R, S, Y, X and AH clustered with *Acinetobacter baumanii,* although types X, Y and AH were not as closely related as O, R, and S (Figure 2). The prevalence of type O at the 17 sampling points from where it was isolated ranged between 6 and 100% of the cultured aerobic bacteria, while type S represented 6 to 7% of the culturable populations at the stock and backwater samples of fine-paper machines. Types R and X were found to be the most prevalent bacterial types isolated in this study. The highest numbers of type R were observed during a survey of a fine-paper machine, and were found in the headbox (70% of the cultured population), raw pulp chest (80%) and machine chest (90%). Type X was ubiquitous and isolated during both summer and winter surveys at four different mills that used furnishes consisting of waste, bleached pulp and broke. The 77 samples containing this type were collected from almost all streams and on average this type represented 67% of cultivated bacteria. Type Y isolates were found in five backwater samples of a fine-paper machine as well as from a newsprint machine. At these various sampling points the type represented 6 to 66% of the total cultivated population. Type AH was isolated at two mills on five different paper machines that used kraft, waste and broke as furnish. The 37 isolates represented 10 to 77% of the total number of bacteria from widely distributed sampling points. Type AB clustered with *A. johnsonii* and was isolated only from the broke chest of a fine-paper machine where it represented 11% of the total isolated bacteria

3.2.3 *Enterobacteriaceae* cluster

Profile types A, B and N belonged to the family *Enterobacteriaceae* (Figure 2, Table 3). Members of this family are gram-negative, oxidase-negative, non-spore forming, non-acid fast, straight rods. *Enterobacteriaceae* spp. are facultative anaerobes with optimal growth between 22 and 37°C. As chemo-autotrophs, *Enterobacteriaceae* spp. are able to grow on simple organic carbon and nitrogen compounds and have both a respiratory and fermentative

metabolism. Some *Enterobacteriaceae* species, which include *Klebsiella*, *Enterococcus* and *Pantoea,* were isolated by Väisänen *et al*. (1998) in a printing-paper machine.

Type A clustered with *Klebsiella pneumoniae* and was isolated from the machine chest of a paper machine that used bleached pulp as furnish (Table 2). This isolate represented 10% of the culturable bacterial population. The finding of this organism in the mill water of Enstra paper mill was not surprising, since the mill utilised treated sewage water and ecological habitats of *Klebsiella pneumoniae* include surface water, sewage, soils and plants (Brisse & Verhoef, 2001). Three subspecies of *Klebsiella pneumoniae* are pathogenic and commonly occur as hospital-acquired infections that include pneumonia, urinary-tract infections, wound infections, burn infections and septicemia (Brisse & Verhoef, 2001). Rätto *et al*. (2001) found *Klebsiella pneumoniae* as a slime-forming bacterium in paper mills, while Gauthier *et al*. (2000) found that *Klebsiella* strains could actively fix N_2 and might, therefore, be a major component of microbial communities in pulp mill primary clarifiers.

Profile type B clustered with *Serratia marcescens* and was also isolated from water at the Enstra mill where *Klebsiella* was present. The representative type was isolated from a broke chest and the isolates of this type represented 10 to 20% of the culturable bacterial population of the paper machine. *Serratia marcescens* has been isolated from activated sludge in a domestic wastewater treatment tank by Ajithkumar *et al*. (2003) and is traditionally known to cause pink slime in paper mills (Appling *et al.*, 1951).

Type N could not be identified based on partial 16S sequencing data. Full length sequences as well as other data would be needed to identify this type. The representative isolate constituted 13 to 33% of the bacteria cultivated from three sampling points in the backwater system of a fine-paper machine.

3.2.4 *Pseudoxanthomonas-Thermomonas* cluster

Types M and Z clustered in the genus *Pseudoxanthomonas* while type U clustered in the *Thermomonas* genus (Figure 2, Table 3). Members of the *Pseudoxanthomonas* genus occur as non-spore forming, gram-negative rods that have a strict respiratory metabolism and are slightly alkalophilic. Members can be mesophilic or thermophilic and have been isolated from a wide variety of habitats (Thierry *et al.*, 2004). Desjardins & Beaulieu (2003) isolated *Pseudoxanthomonas* species from pulp and slime samples in a Canadian paper mill and

reported resistance to relatively high temperatures in the headbox as well as colonisation of the wet-end machinery steel surfaces.

Type M clustered with *Pseudoxanthomonas* sp. M1-3 and was prevalent on paper machines that operated at higher temperatures. The 33 samples containing this type were distributed throughout the system and formed 10 to 56% of the culturable populations. Type Z isolates were obtained from one sampling point each on a fine-paper machine and a newsprint machine and constituted 6 to 11% of the culturable isolates. The correct species affiliation of Type Z remained uncertain, but it clearly belonged to the *Pseudoxanthomonas* genus (Figure 2). Type M and the strain *Pseudoxanthomonas* sp. M1-3 as well as type Z constituted a novel phylogenetic lineage within the *Pseudoxanthomonas* genus and might constitute a novel genus. This, however, would have to be validated with more analyses.

Type U clustered with *Thermomonas hydrothermalis* and this type constituted 6 to 11% of the culturable bacterial population at sampling points from where it was isolated. *Thermomonas hydrothermalis* is described as a slightly thermophilic *gammaproteobacterium* that is closely related to *Thermomonas haemolytica,* an organism that is prevalent in kaolin slurries of paper mills (Alves *et al.,* 2003). Kolari (2003) found that some *Thermomonas* strains were persistent biofilm formers of Finnish paper machines.

3.2.5 *Ralstonia*-*Vogesell*a cluster

Type AA was isolated from one sampling point on a fine-paper machine and represented 11% of the culturable bacterial population. The closest sequence similarity of type AA was with species that belonged to the genus *Ralstonia*. However, type AA was not included in the phylogenetic analysis due to insufficient sequence data and more analysis will need to be performed to correctly identify this type. Väisänen *et al*. (1998) isolated the species *Ralstonia pickettii* from pulped broke, shower water, warm water and a headbox as well as *Ralstonia solanacearum* from spray water and bentonite in a printing paper machine. Type AI was collected from paper machines that ran at higher mesophilic to thermophilic temperatures and constituted 10 to 90% of the culturable bacteria at the 22 sampling points from where it was isolated. Type AI clustered with *Vogesella indigofera* (Figure 2, Table 3), the only member of the genus *Vogesella*. This type strain displayed the typical metabolism of many non-fermentative gram-negative rods and catabolised only a few monosaccharides under aerobic

conditions (Grimes *et al*., 1997). The strain was isolated from the sediment of a pond that had been used for the disposal of chemical wastes for 20 years (Grimes *et al*., 1997).

3.2.6 *Rhizobiaceae* cluster

Types G, H and AG clustered in the class *Alphaproteobacteria* within the family *Rhizobiaceae* (Figure 2, Table 3). Many species within this family are able to fix nitrogen in symbiotic association with leguminous plants (Kwon *et al.*, 2005) and, as chemoorganotrophs, are able to utilise a variety of carbohydrates. Species in the *Rhizobium* genus are motile, gram-negative, non-sporulating, pleomorphic rods. Desjardins & Beaulieu (2003) found *Rhizobium* species in slime samples isolated from a Canadian paper mill. Types G, H and AG clustered close to the *Rhizobiaceae* strain M100. The Types G and H were both isolated from the recovered fibre sampling point on a paper machine that used bleached pulp as furnish (Table 2). Both types were found at one sampling point only and constituted 14% of the culturable bacterial population. Type AG belonged to the family *Rhizobiaceae*, but the exact phylogenetic identity remained uncertain and more analyses need to be conducted to verify its position in the family. Type AG was isolated from 13 samples at a single mill that operated at high mesophilic to thermophilic temperatures and utilised kraft, waste and broke as furnish. In the 11 widely distributed sampling points where Type AG was found, it constituted 13 to 67% of the total number of cultured bacteria. The identity of Type AF remained unclear, but the isolate belonged to the class *Alphaproteobacteria*. Additional sequence data is needed to verify the position of Type AF in the family *Rhizobiaceae* (Figure 2). The representative isolate of type AF was isolated from the backwater of a paper machine that uses unbleached pulp as furnish. Type AF was also found in 21 samples distributed over four kraft machines at one mill. The average occurrence was 17% for this type, but prevalence as high as 44% was observed.

3.2.7 *Arthrobacter* cluster

Type W was isolated from the blend chest of a fine-paper machine (Table 2) and constituted 11% of the total cultivated bacterial population. This type grouped into the *Arthrobacter* genus, but no clear species affiliation was found (Figure 2, Table 3) and it is possible that the isolate might constitute a new *Arthrobacter* species. The *Arthrobacter* genus with 34 recognised species (Gupta *et al*., 2004), falls in the family *Micrococcaceae* of the phylum BXIV *Actinobacteria* (Garrity *et al.*, 2004). Species in this genus are gram-positive aerobic

and asporogenous and display coryneform morphology. These ubiquitous soil bacteria display vast metabolic activities and survival in extreme environments. Väisänen *et al*. (1998) isolated *Arthrobacter* species from mineral coated paper in a printing paper machine.

3.2.8 *Bergeyella-Riemerella* cluster

Types AE and AK grouped within the *Bergeyella-Riemerella* branch of the *Flavobacteriaceae* family (Figure 2, Table 3). The representative isolate of Type AE was collected from the backwater of a paper machine that used unbleached/waste pulp as furnish (Table 2). Type AE was also isolated from 21 sampling points on four paper machines that utilised a mix of kraft, waste and broke as furnish, and constituted 10 to 90% of the total aerobic bacterial population. The sequence of this type did not correlate to any known sequence and needs to be investigated further before any species affiliation can be assigned.

Type AK was isolated from the blend chest of a paper machine that utilised broke as furnish (Table 2). Other representative isolates of Type AK was found at three sampling points on different paper machines that used waste and virgin pulp as furnish. The representatives of Type AK constituted 10 to 22% of the total cultured bacteria. The closest related sequence to AK was an uncultured clone from Kalahari subsurface water (Gihring *et al*., 2006).

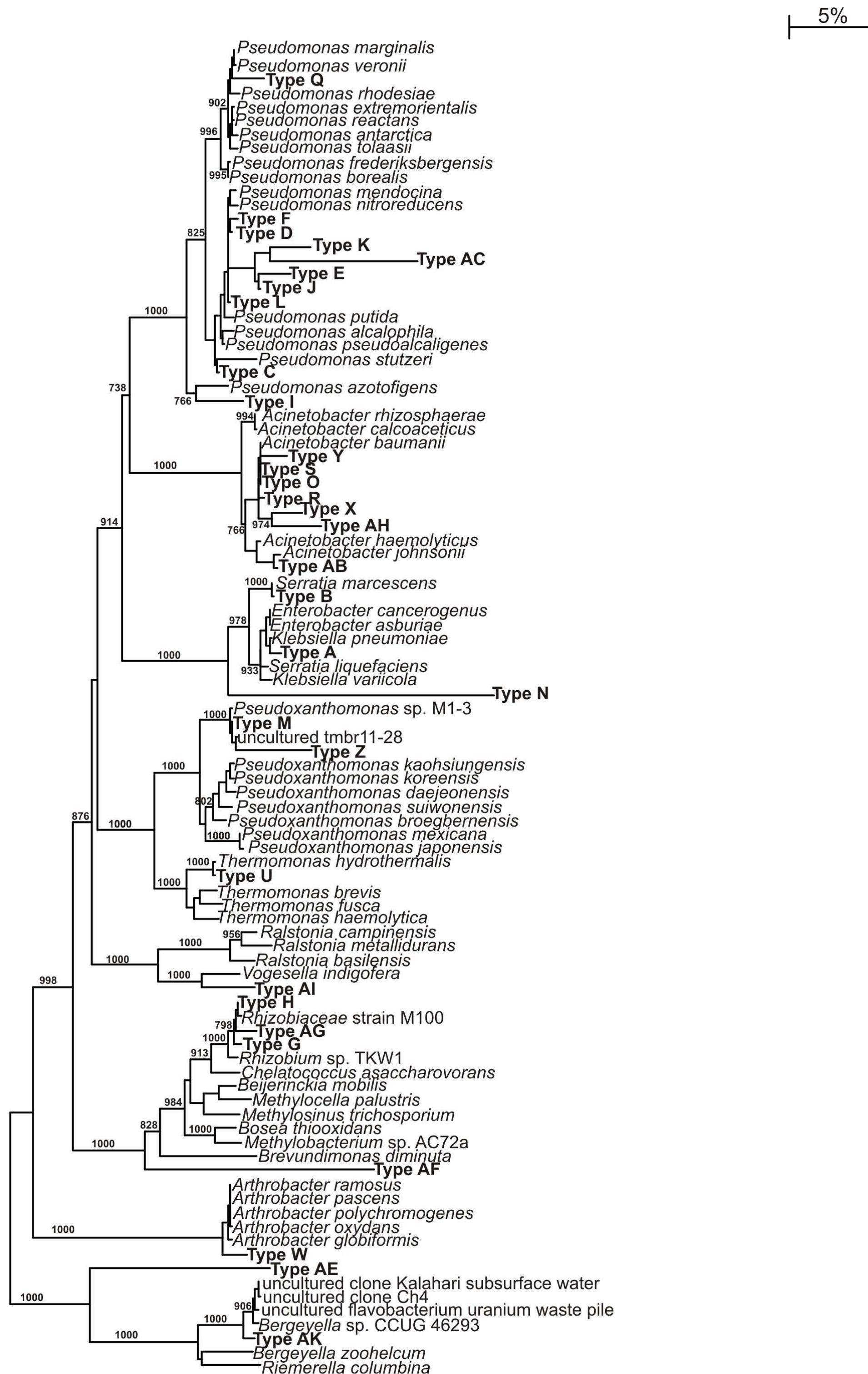

Figure 2: Unrooted phylogenetic tree reconstructed with the neighbour-joining method from the comparative 16S rDNA sequence analysis of the paper-machine isolates and the related reference strains obtained from GenBank. Branch lengths are proportional to the phylogenetic distances, while the vertical branches are non-informative. The scale bar shows 5% nucleotide difference. Confidence levels higher than 70% are indicated.

Table 3: List of reference strains included in the comparative sequence analysis obtained from GenBank[1].

Type	Reference strain	Reference strain nr.	Relevant characteristics	GenBank[1] Accession
	***Pseudomonas* cluster**			
C D E F I J K L Q V* AC AJ*	*Pseudomonas marginalis*	NZCX27	Cold-tolerant, cause carrot post-harvest bacterial soft rot	AF364098
	P. veronii	AIYB2-4	Degradation of BTEX-compounds in contaminated ground water	AY512622
	P. rhodesiae	CIP 104664	Isolated from natural mineral waters	AF064459
	P. extremorientalis	KMM 3447	Isolated from drinking water reservoirs	AF405328
	P. reactans	NCPPB 1311	ns	AF320987
	P. antarctica	CMS 35^{T}	Psychrophilic pseudomonads, isolated from cyanobacterial mat	AJ537601
	P. tolaasii	LMG 2342^{T}	ns	AF255336
	P. frederiksbergensis	OUCZ24	PCB-degrading bacteria associated with plant roots at contaminated site	AY785733
	P. borealis	MA342	ns	AJ012712
	P. mendocina	NCIB 10541	ns	D84016
	P. nitroreducens	NS	ns	D84022
	P. putida	GJ31	ns	AY332610
	P. alcalophila	AL15-21	Facultatively psychrophilic alkalophile isolated from sea water	AB030583
	P. pseudoalcaligenes	LMG 1225^{T}	ns	Z76666
	P. stutzeri	24a75	Isolated from soil beneath pavement of filling station, Espelkamp	AJ312229
	P. azotifigens	6H33b	Nitrogen-fixing bacterium isolated from a compost pile	AB189452
	***Acinetobacter* cluster**			
O P* R S X Y AH	*Acinetobacter rhizosphaerae*	IH9	Rhizospheric soil of grasses - actively solubilises phosphate	AY364536
	A. calcoaceticus	NCCB 22016^{T}	ns	AJ888983
	A. baumannii	DSM 30008	ns	X81667
	A. haemolyticus	AR-46	n-Alkane-degrading	AY586400
	A. johnsonii	ATCC 17909^{T}	ns	Z93440
	***Enterobacteriaceae* cluster**			
A	*Serratia marcescens*	AU736	ns	AY043386
B	*Enterobacter cancerogenus*	LMG 2693	Phytopathogen	Z96078
N	*E. asburiae*	JCM 6051	ns	AB004744
	Klebsiella pneumoniae	1.3T	Hydrocarbon degrading *Klebsiella* strains isolated from polluted estuary	AY918489
	S. liquefaciens	JCM 1245	ns	AB004752
	K. variicola	F2R9	ns	AJ783916

Table 3 (continued)

Type	Reference strain	Reference strain nr.	Relevant characteristics	GenBank[1] Accession
		***Pseudoxanthomonas-Thermomonas* cluster**		
M U Z	*Pseudoxanthomonas* sp. M1-3	M1-3	ns	AB039330
	Uncultured synthetic wastewater bacterium	tmbr11-28	Thermophilic aerobic bacterium	AF309808
	P. kaohsiungensis	J36	Isolated from oil-polluted site	AY650027
	P. koreensis	T7-09	Isolated from South Korean soil	AY550263
	P. daejeonensis	TR6-08	Isolated from South Korean soil	AY550264
	P. suiwonensis	4M1	Isolated from mushroom waste composts	AY927994
	P. broegbernensis	B1616/1	Nitrous-oxide producing strain isolated from ammonia-supplied biofilters.	AJ012231
	P. mexicana	AMX 26B	Isolated from UASB reactor sludge	AF273082
	P. japonensis	12-3T	Isolated from polluted urban soil in Japan	AB008507
	Thermomonas hydrothermalis	SGM-6T	Slightly thermophilic gamma-proteobacterium isolated from a hot spring in central Portugal	AF542054
	T. brevis	LMG 21746 [T]	Mesophilic species isolated from a denitrification reactor with poly(epsilon-caprolactone) plastic granules as fixed bed	AJ519989
	T. fusca	LMG 21738	Mesophilic species isolated from a denitrification reactor with poly(epsilon-caprolactone) plastic granules as fixed bed	AJ519987
	T. haemolytica	A50-7-3	Finland - isolated from chemicals used in the production of paper (kaolin slurry)	AJ300185
		***Ralstonia* cluster**		
AA* AI	*Ralstonia metallidurans*	CH34[T]	Metal-resistant bacterium with possible bioremediation application in polluted soil	Y10824
	R. basilensis	LMG 19474 [T]	Metal-resistant bacterium with possible bioremediation application in polluted soil	AF312022
	R. campinesis	LMG 19282 [T]	Metal-resistant bacterium with possible bioremediation application in polluted soil	AF3122020
	Vogesella indigofera	LMG 6867 [T]	Isolated from an oxidation pond used for the disposal of chemical water	AB021385

Table 3 (continued)

Type	Reference strain	Reference strain nr.	Relevant characteristics	GenBank[1] Accession
		***Rhizobiaceae* cluster**		
G H AF AG	*Rhizobiaceae* str. M100	M100	Thermophilic bacteria isolated from mushroom composts	AF345860
	Rhizobium sp.	TKW1	Isolated from a biological consortium capable of mesophilic aerobic degradation of a metal lubricant	AY631059
	Chelatococcus asaccharovorans	TE2	ns	AJ294349
	Beijerinckia mobilis	DSM 2326	ns	AJ563932
	Methylocella palustris	H4	Methane oxidising acidophilic bacterium isolated from peat bogs	AJ563927
	Methylosinus trichosporium	IMV B-3060	Type II methane-oxidising bacteria	AJ458477
	Bosea thiooxidans	TJ1	Denitrifying and phenol-degrading organism	AF508112
	Methylobacterium sp.	AC72a	Facultatively methylotrophic bacteria	AY776209
	Brevundimonas diminuta	LMG 2331	ns	BDJ227779
		***Arthrobacter* cluster**		
W	*A. ramosus*	DSM 20546	ns	X80742
	A. pascens	DSM 20545	ns	X80740
	A. polychromogenes	c311	Phenol-degrading bacterium	AB167181
	A. oxydans	NS	PCB-degrading bacteria associated with plant roots	AY785732
	A. globiformis	TUT 1005	Isolated from start-up operation of flowerpot with fed-batch reactors for composting of household biowaste	AB098573
		***Bergeyella-Riemerella* cluster**		
AE AK	Uncultured clone	Kalahari	Isolated from Kalahari subsurface water	DQ337018
	Uncultured clone	CH4	Isolated from hydrothermal vent	AY672523
	Uncultured *flavobacterium*	Clone JG37-AG-16	Isolated from uranium mining waste pile	AJ519403
	Bergeyella sp.	CCUG 46293	Isolated from raw sewage	AJ575430
	B. zoohelcum	ATCC 43767[T]	Clinical important species	M93153
	Riemerella columbina	LMG 11607[T]	ns	AF181448

[1] GenBank database of the National Centre for Biotechnology (NCBI) [website address: www.ncbi. nlm.nih.gov/Genbank/]
[T] Type strain
* Not included in sequence based analysis
ns Not stated
ATCC American Type Culture Collection, USA
CIP Collection de L'Institut Pasteur, France
DSM Deutsche Sammlung von Mikroorganismen, Germany
JCM Japan Collection of Microorganisms, RIKEN Bioresource Center, Japan
LMG BCCM™/LMG Bacteria Collection, Laboratorium voor Microbiologie, University of Gent, Belgium
NCCB Netherlands Culture Collection of Bacteria, Netherlands
NCPPB National Collection of Plant Pathogenic Bacteria, Harpenden Laboratory, UK

4. CONCLUSIONS

The ERIC-PCR method employed to type prevalent bacterial isolates from various paper machines was able to distinguish between 28 different types of bacteria from Adamas, Ngodwana, Enstra, and Stanger paper mills. The DNA profiles from ERIC-PCR amplification were too complex for visual typing of bacteria isolated from the Cape Kraft and Tugela mills, however, and RFLP analysis as an auxiliary tool was essential to characterise these types. The use of restriction enzymes to distinguish bacterial types reduced the number of 16S rDNA genes to be sequenced and was also used successfully by Vidigal *et al.* (2000) and Carvalho *et al.* (2004) as auxiliary identification tool. The isolates from these mills were differentiated into a further seven types based on the RFLP analysis. Partial 16S rDNA gene sequence analysis to determine the relatedness of paper-mill isolates to known culturable bacteria enabled the tentative identification of certain prevalent types. The identity of new isolates was determined by this method by several research groups (Vinuesa *et al*., 1998; Tannock *et al*., 1999; Oppong *et al*., 2003; Suihko *et al*., 2003). Ten of the 35 types were identified to species level, 21 of the types were identified to genus level and the remaining four types were identified to family level. None of the 12 types that belonged to the *Pseudomonas* cluster could be identified to species level, but several *Pseudomonas* species shared high 16S sequence similarity values with these types. It would, therefore, be necessary to conduct more analyses to verify the identities of these types or to determine if they constitute new species. Six different bacterial types shared high similarity with *Acinetobacter baumannii* and it was found that, together with the types in the *Pseudomonas* genus, these isolates were the most prevalent bacteria encountered during paper-machine surveys. Similar results were obtained by Väisänen *et al.* (1998) and Desjardins & Beaulieu (2003), who also found *Acinetobacter* and *Pseudomonas* species in paper-mill water systems. Knowledge of the types of bacteria inhabiting paper mills could be invaluable when attempting to improve control of microbial fouling, with the appropriate biocide programme and regulation of the environmental parameters. The present study generated a significant amount of information and new insight into the bacterial populations of paper-mill water systems of South Africa was obtained.

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CHAPTER 5

THE STRUCTURAL ANALYSIS OF BACTERIAL DIVERSITY FROM THREE PAPER-MACHINE WATER SYSTEMS USING DENATURING GRADIENT GEL ELECTROPHORESIS

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

ABSTRACT

Traditional methods to investigate bacteria in industrial water systems often do not accurately represent the composition and diversity of bacterial communities because the majority of bacteria cannot easily be cultivated on general-purpose media. Denaturing gradient gel electrophoresis (DGGE) can provide a powerful tool for monitoring bacterial diversity, since PCR-amplified DNA fragments of the same size, but different sequences, may be distinguished. Reference strains can also assist in identifying members of the bacterial community by functioning as DNA markers. The water systems of three paper machines were subjected to DGGE analysis to investigate the structural diversity of the bacterial populations. An attempt was also made to identify prevalent members of the bacterial community with DNA markers from reference strains. The DGGE profiles for two paper machines indicated that bacterial diversity was higher than diversity calculated from Biolog substrate-utilisation profiles, possibly because DGGE also reflected the presence of viable but non-culturable bacteria and organisms inactivated by high biocide dosages. In the water system of a third paper machine, the DGGE bands corresponded to prevalent bacterial members. The use of DGGE to monitor changes in microbial populations in water systems enabled predictions of the community structure that may improve control of microbial fouling, but more analyses are needed to validate the results obtained in this study.

1. INTRODUCTION

Traditional methods to investigate the composition of microbial communities in industrial water systems are dependent on microscopic observations or culturing techniques. Liss & Allen (1992) found that aerobic and facultative anaerobic heterotrophs dominated the majority of the culturable community in water-treatment systems of kraft pulp mills. However, Amman *et al.* (1995) suggested that culturable strains do not accurately represent the composition and diversity of natural microbial communities, since the majority of bacteria cannot be easily cultivated on general-purpose media. Techniques that are based on genes encoding ribosomal RNA (rDNA genes) offer new possibilities to profile microbial communities that compliment traditional cultivation studies (Tiirola *et al.*, 2003). One such technique, known as denaturing gradient gel electrophoresis (DGGE), can be used to investigate heterogeneous bacterial communities (Muyzer & Ramsing, 1995) and can provide a powerful tool for monitoring the diversity of particular microbial eco-systems (Diez *et al.*, 2001) because differences between mixed PCR-amplified DNA fragments of the same size, but different sequences, can be detected (Ercolini, 2004).

One of the most valuable applications of DGGE was first demonstrated by Muyzer *et al.* (1993) in the study of community complexity of a microbial mat and bacterial biofilms. The resulting DGGE profile was a pattern of DNA bands that corresponded to the number of predominant members in the microbial communities. This profile could give a direct indication of the predominant constituents of a microbial community (Muyzer & Smalla, 1998). Another example of the study of community complexity to provide insight into the structure and function of a system was the use of PCR-DGGE by Muyzer *et al*. (1995) to provide information on the microbial communities found around hydrothermal vents. Ferris *et al*. (1996) profiled the distribution of a cyanobacterial community inhabiting regions with different temperatures in a hot spring and found that different diversity profiles corresponded with sites of different temperatures, indicating different populations. Murray *et al*. (1996) used PCR-DGGE to compare the phylogenetic diversity of bacterioplankton assemblages from two estuaries and found different species composition due to the difference in organic substrates. Teske *et al*. (1996) studied the presence and activity of sulfate-reducing bacteria in a stratified water column of the Mariager Fjord in Denmark and demonstrated the presence of two active bacterial populations in low numbers using DGGE profiles. Diez *et al*. (2001) used DGGE to study the diversity of picoeukaryotes in natural marine assemblages and

concluded that the DGGE method provided a reasonably detailed view of marine picoeukaryotic assemblages and allowed tentative phylogenetic identification of dominant members.

An important limitation of the DGGE technique is the fact that only relatively small DNA fragments of up to 500 base pairs can be separated. This restricts the amount of sequence information for phylogenetic studies and probe design (Muyzer & Smalla, 1998). For this reason, DGGE analysis can only be used for the tentative identification of sequences and results obtained should be validated with supportive data. An alternative to sequencing DGGE bands for identification of bacterial species is the construction of a molecular ladder by using rDNA amplicons of representative species. Identification is then achieved by comparing the migration distances of the PCR amplicons in the DGGE gels (Ercolini, 2004). Although this procedure is much easier than sequencing, it does not guarantee an unequivocal identification, because multiple copies of the 16S rDNA gene and, therefore, multiple bands on a gel for one species, can make identification difficult (Muyzer & Smalla, 1998).

Although intrinsic disadvantages of the DGGE technique do exist, the advantages of this technique for analysing microbial community structure and complexity are numerous. The approach can give a direct display of the predominant members of a microbial community and is excellently equipped to investigate temporal and spatial distribution of bacterial populations (Muyzer & Smalla, 1998). Tentative identification of species can be achieved by purifying and sequencing the bands in the DGGE profile (Ercolini, 2004; Yu & Morrison, 2004). The method is also rapid and many samples can be electrophoresed simultaneously, making it particularly useful when population dynamics is examined over time (Theron & Cloete, 2000).

The aim of this study was to investigate the bacterial community structure of paper-mill water and to identify individual members using DNA reference markers and DGGE. Water samples from three different paper-machine water systems were subjected to DGGE analysis and the microbial communities of the three paper machines were compared with results obtained from plate counts and carbon-source utilisation profiles. Bacterial reference strains, isolated from various other paper mills, were used as markers on the DGGE gels.

2. MATERIALS AND METHODS

2.1 Sampling and enumeration

For the bacterial community analysis, three fine-paper machines (PM2, PM3 and PM6) were sampled at eight points at Sappi Enstra mill in Springs (South Africa) on 22 November 2004. Sampling points were selected based on accessibility and because they are customarily sampled by the machine operators and biocide suppliers. Aerobic bacterial numbers were determined as described previously (Chapter 3). Samples were stored at -20°C until DNA extraction was performed. Reference strains of bacteria (Table 1) were isolated during a previous survey at Enstra as well as from seven other paper machines (Chapter 4).

Table 1: List of reference strains used as markers during DGGE analysis with the locations from where they were isolated. Strains were typed using ERIC-PCR and identified by sequence analysis.

Type	Name	Mill	Paper Machine	Sampling points
C	*Pseudomonas* sp.	Stanger	TM	Bagasse stock channel
D	*Pseudomonas* sp.	Stanger	"	Bagasse stock channel
E	*Pseudomonas* sp.	Ngodwana	NP	Uhle box pit
I	*Pseudomonas* sp.	Stanger	TM	Main drain
J	*Pseudomonas* sp.	Stanger	TM	Blend chest
K	*Pseudomonas* sp.	Stanger	TM	Blend chest
L	*Pseudomonas* sp.	Enstra	PM3	Clarified chest
M	*Pseudoxanthomonas* sp. M1-3	Ngodwana	KLB	Top-ply silo
N	*Enterobacteriaceae* spp.	Adamas	PM3	Knock-off shower
Q	*Pseudomonas* sp.	Adamas	PM4	Finestock III
R	*Acinetobacter baumannii*	Adamas	PM4	Finestock III
S	*Acinetobacter baumannii*	Adamas	PM4	Finestock III
U	*Thermomonas hydrothermalis*	Ngodwana	NP	Machine chest
V	*Pseudomonas* sp.	Adamas	PM3	Knock-off shower
X	*Acinetobacter baumannii*	Adamas	PM4	Finestock III
AC	*Pseudomonas* sp.	Stanger	TM	Bagasse stock channel

TM Tissue machine
NP Newsprint machine
PM Paper machine
KLB Kraft liner board machine

2.2 Functional characterisation of bacterial populations

The functional characterisation of bacterial populations from the water of PM2, PM3 and PM6 was determined as described in Chapter 3. The Shannon index (H') was calculated for the diversity of substrates utilised on GN Biolog Microtitre™ plates (Biolog Inc., USA) while

the Berger-Parker index (d) was calculated to determine the levels of dominance in each system (Magurran, 1988) (Appendix A5 to A7).

2.3 DNA extraction of the bacterial community

Total community DNA was extracted and purified from the water samples using the following modifications of the DNeasy® Tissue Handbook (Qiagen, Germany): Pulp samples were centrifuged for 5 min at 4°C (21 913 × *g*) with a Eppendorf AG 5804R (Eppendorf, Germany) and bacterial cells were harvested from the supernatant by vacuum filtration using a 0.2 µm sterile membrane. The membrane was incubated in lysis buffer (20 mM Tris-HCl, 2 mM sodium EDTA, 1.2% Triton® and 3 mg/ml lysozyme) on ice for 2 h and then at 37°C for 30 min. The cell lysate was homogenised with a QIAshredder unit (Qiagen, Germany) and centrifuged at 21 913 × *g* for 2 min. The protocol was further followed as described in the DNeasy® Tissue Handbook (Qiagen, Germany) to extract DNA.

2.4 DNA extraction of reference strains

The reference strains that were used during this study originated from different paper mills around South Africa and were selected based on their different enterobacterial repetitive intergenic consensus (ERIC) patterns. The types were sequenced and their phylogenetic relatedness determined (Chapter 4). A fresh culture of each reference strain (Table 1) was examined for purity and streaked on a nutrient agar slant in a screw-cap tube. Strains were checked for sufficient growth after two days of incubation at a similar temperature to the paper-machine environment from which it was isolated. For the gram-negative reference strains the protocol for DNA extraction as described in the DNeasy® Tissue Handbook (Qiagen, Germany) was followed while the following modification was made for the gram-positive reference strains: Samples were resuspended in lysis buffer and incubated on ice for 1 h prior to incubation at 37°C for 30 min. The DNeasy® tissue kit (Qiagen, Germany) was used for further DNA extraction.

2.5 PCR amplification

The V6 to V8 hypervariable regions of the 16S rRNA gene were amplified with primer f933 (5' GCA CAA GCG GTG GAG CAT GTG G 3') and primer r1369 (5' GCC CGG GAA CGT ATT CAC CG 3') (Invitrogen, USA) as described by Yu & Morrison (2004). The forward primer was synthesised with a GC clamp (5'CGC CCG CCG CGC GCG GCG GGC

GGG GCG GGG GCA CGG GGG G 3') at the 5' end to facilitate the melting behaviour of the amplicons. All amplification reactions were performed using conditions as described by Yu & Morrison (2004) with an Eppendorf Mastercycler 22331 (Merck, South Africa). The reaction mixture was made up to 50 µl with sterile ultra-pure water and contained 5 µl DNA template, 5 µl of 10 × Gold *Taq* PCR buffer (Southern Cross Biotechnology, South Africa), 4 µl of 25 mM dNTP (ABgene, UK), 0.5 µl of 25 µM (each) primer and 0.5 µl of 5 U/µl Gold *Taq* DNA polymerase (Southern Cross Biotecnology, South Africa). After initial denaturation at 94°C for 5 min, 10 cycles of touchdown PCR were performed (denaturation at 94°C for 30 s, annealing for 30 s at 5°C below the annealing temperature of 71°C and decreasing 0.5°C for every cycle and extension at 72°C for 1 min) followed by 25 cycles of regular PCR (94°C for 30 s, 66°C for 30 s and 72°C for 1 min) and a final extension step for 7 min at 72°C. Negative controls were included. Aliquots (2 µl) of the amplified products were loaded on a 1% (m/v) agarose gel and subjected to electrophoresis at 80 mV for 30 min to visualise the amplified products by UV exposure (Vilber Lourmat, France).

2.6 DGGE analysis

The PCR fragments were separated using DGGE, performed with the Biorad DCode Universal Mutation Detection System (Bio-Rad Laboratories, USA). PCR samples were directly loaded onto 8% (m/v) polyacrylamide gels in 1 × TAE buffer with a gradient of 45 to 70%. The gradient was created by polyacrylamide, containing 1 to 100% denaturant (7 M urea and 40% v/v formamide). Electrophoresis was performed at a constant voltage of 130 mV for 6 h and a constant temperature of 60ºC. The gel was stained with ethidium bromide for 30 min and the fragments were visualised by UV exposure (Vilber Lourmat, France).

The bacterial diversity was determined using the Shannon diversity index (Sekiguchi *et al.*, 2002) and the Berger-Parker dominance index (Magurran, 1988). The number of DNA bands in each sample was equated to the number of species (Sekiguchi *et al.*, 2002). The band intensity was equated to the number of individuals per species and derived from the peak heights of the densometric curves for each band (Stamper *et al.*, 2003) by analysing the gel files with the Bionumerics 4.0 software (Applied Maths, Belgium).

3. RESULTS AND DISCUSSION

3.1 PCR amplification of the bacterial community

Adequate 16S rDNA amplification was obtained for all 24 samples of PM2, PM3 and PM6 to perform the DGGE analysis (Figure 1).

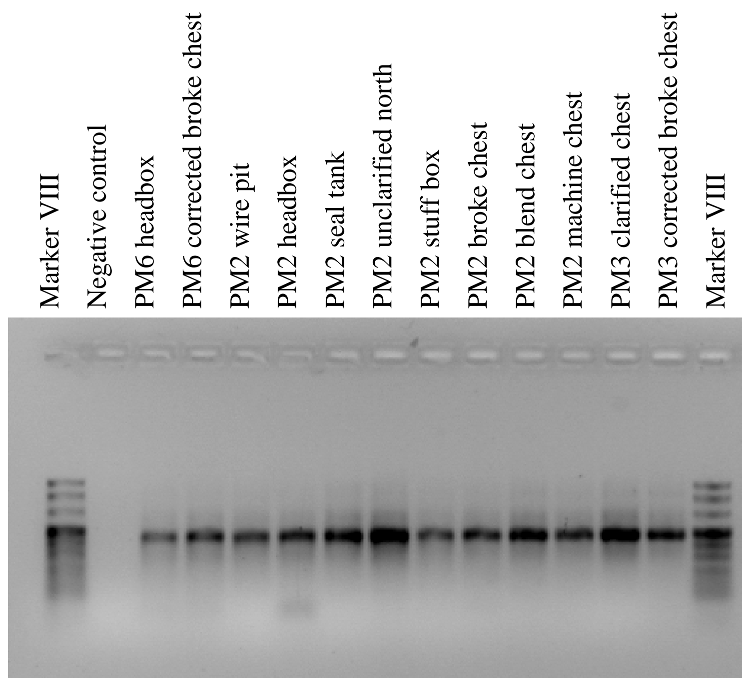

Figure 1: 16S rDNA PCR products from samples of PM2, PM3 and PM6 on a 1% agarose gel.

3.2 DGGE analysis of the bacterial community

Bands of different intensities were observed at different positions on the DGGE profile of the samples from PM2 (Figure 2), reflecting structural diversity. Multiple bands were especially evident in the wire pit, headbox and stuff box on PM2. These bands probably represented a considerable number of different bacterial species similar to findings made by Muyzer *et al.* (1993). According to Nübel *et al.* (1996), when using 16S rDNA genes for DGGE studies, the presence of some bacteria with heterogenous operons can lead to an overestimation of diversity. The DNA is also not always extracted with equal efficiency during DGGE analysis (Rochelle *et al.*, 1992) and during the PCR reaction preferential amplification of some sequences may occur (Reysenbach *et al.*, 1992), which can lead to an over or underestimation

of some taxa. Muylaert *et al.* (2002) assumed that the over or underestimation of diversity is constant for a given taxon in different samples and found that relative band intensities correlated better with explanatory variables than presence/absence data. Therefore, for the present study it was assumed that bands of high intensity indicated species that were probably present in high numbers at these specific sampling points.

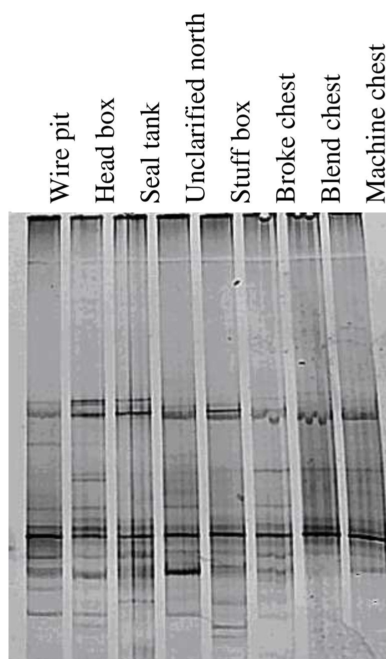

Figure 2: Diversity of the PM2 bacterial community as reflected by the DGGE profile on an 8% polyacrylamide gel.

The DGGE data differed substantially when compared to results obtained from the Biolog substrate-utilisation patterns of the microbial community. Multiple DGGE bands at all eight sampling points on PM2 represented high bacterial diversity when compared to PM3 and PM6, but Biolog profiles indicated low Shannon diversity values and high dominance values when the substrate utilisation profiles of PM3 and PM6 were compared to the DGGE profiles (Table 2). The Biolog data were confirmed with plate counts of aerobic, anaerobic and sulfate-reducing bacteria. Seven of the eight sampling points on PM2 supported no bacterial growth (Appendix B, Table 12), but when the measured environmental parameters on PM2 were considered, it appeared that ideal conditions for bacterial growth existed, with temperatures in the mesophilic range, neutral to slightly alkaline pH, high dissolved oxygen levels and an oxidative environment (Appendix A5).

Table 2: Range of culturable numbers of total aerobic bacteria (TAB) and average diversity indices as calculated from Biolog and DGGE data for PM2, PM3 and PM6.

Paper Machine	**TAB (cfu/ml)**	**Shannon diversity (H')**		**Berger-Parker dominance (d)**	
		Biolog	DGGE	Biolog	DGGE
PM 2	0 to 10^2	2.595	0.842	0.205	0.334
PM 3	0 to 10^6	3.043	0.612	0.120	0.422
PM 6	10^6 to 10^7	3.957	0.159	0.044	0.855

One possible explanation for the contradicting results from the DGGE and Biolog profiles could be the change in the biocide programme at the Enstra paper mill shortly before the survey. This could have resulted in high mortality rates in the microbial population on PM2 if members of the community had not yet adapted to resist the new biocide. Therefore, the DNA bands observed on the DGGE gels could represent inactive or dead cells, since both viable and dead microbes contain DNA according to Muttray *et al.* (2001).

Another possible explanation for the difference in the calculated DGGE and Biolog diversity indices could be the presence of viable but non-culturable bacteria in the water system. As was indicated by Palojarvi *et al.* (1997), only 0.01 to 1% of all microbes are culturable on artificial media and, even though substrate utilisation profiling was done on the original microbial community, it remains dependent on the growth of microbes on specified media (Garland & Mills, 1991). This observation, however, could only be validated by excising the bands from the DGGE gels and sequencing the DNA, as well as by conducting more community analyses with complimentary techniques over a longer period of time.

The DGGE profiles of PM3 revealed that, at some sampling points, similar bacterial community members were present since DNA fragments of similar size were apparent in different lanes of the gel (Figure 3). Exceptions occurred at the clarified chest and the broke chest, where more fragments reflected greater bacterial diversity. The DGGE results of PM3 did not correspond with the Biolog data when compared to PM2 and PM6, although fewer DNA fragments were observed for the sampling points on PM3. As in the case of PM2, it was possible that the DGGE bands represented DNA of viable but non-culturable bacteria.

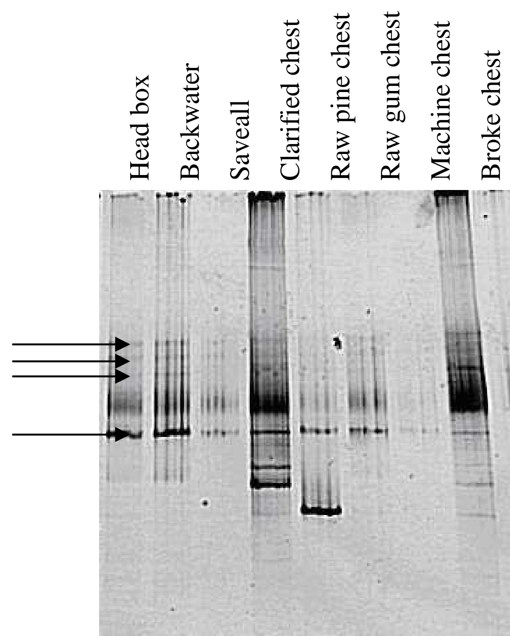

Figure 3: Diversity of the PM3 bacterial community as reflected by the DGGE profile on an 8% polyacrylamide gel (arrows indicate DNA fragments of similar size occurring in a number of sampling points).

Samples from PM6 displayed the lowest species diversity and highest dominance when the DGGE profiles were compared with PM2 and PM3 (Table 2). The plate counts taken at sampling points on PM6 indicated high aerobic bacterial growth of 10^6 to 10^7 CFU/ml (Appendix A7). Despite the high bacterial counts, relatively few DNA fragments were observed at most sampling points (Figure 4) when compared to the number of fragments on PM2 and PM3. These DGGE results indicated that the system selected for a few dominant species that were present in high numbers and were most probably represented by the culturable part of the bacterial community, since the majority (96%) of the reference strains that were isolated from this paper machine represented *Acinetobacter* and *Pseudomonas* spp. (Table 1).

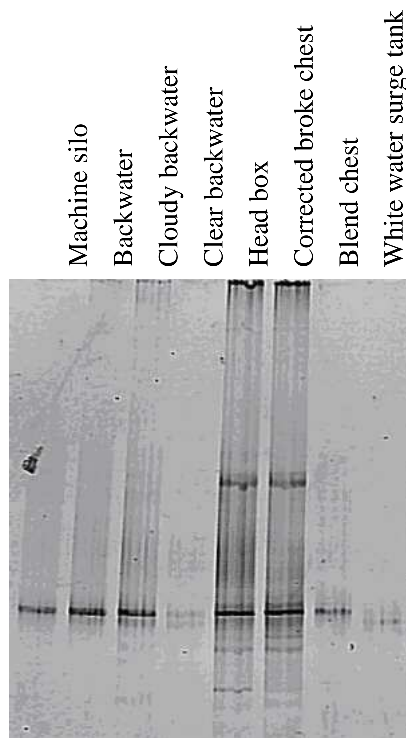

Figure 4: Diversity of the PM6 bacterial community as reflected by the DGGE profile on an 8% polyacrylamide gel. .

3.3 DGGE analysis of reference strains

Clearly distinct profiles were obtained for different bacterial types subjected to DGGE analysis (Figure 5), but the majority of the lanes displayed multiple DNA bands. These bands do not indicate contamination, since the negative controls of the 16S rDNA PCR reactions were clean. Multiple copies of the amplified rRNA gene can result in more than one band per bacterial type as found by Ercolini (2004). Muyzer & Smalla (1998) also noted that the amplification of multiple copies of the rRNA gene can lead to an overestimation of the number of bacterial constituents in a community. The use of the pure cultures as reference strains to identify prevalent members of the bacterial community was, therefore, not possible for this study.

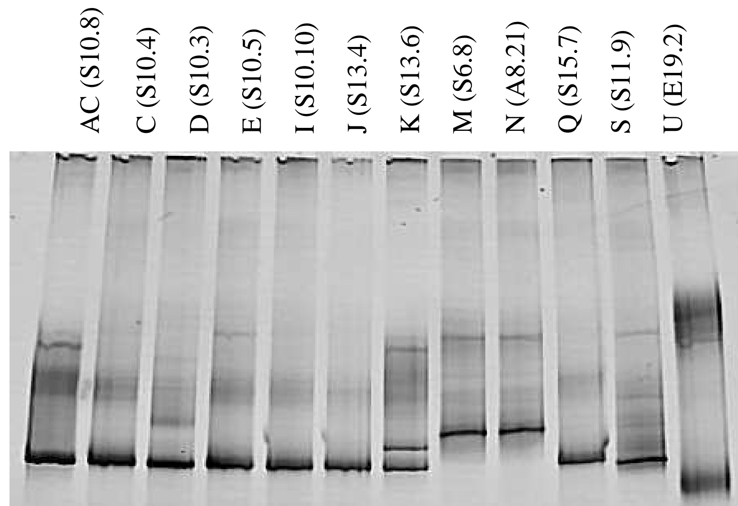

Figure 5: The DGGE profile of the reference strains on an 8 % polyacrylamide gel.

It was interesting to note, however, that the type I, J and Q displayed the same DGGE profile when separated on an 8% polyacrylamide gel. It is possible that these three strains could represent the same species, although none were identified to species level. Type M and N also appeared to share identical DGGE profiles. Type M had been identified as *Pseudoxanthomonas* sp. but Type N could not be identified due to insufficient sequencing results (Chapter 4) and it was, therefore, possible that type N also represented *Pseudoxanthomonas* sp. These observations need to be validated with more analyses, but could indicate that DGGE may be a useful application to accurately type different bacterial strains.

4. CONCLUSIONS

For the present study, the diversity and dominance indices that were calculated from the number of amplified fragments and their intensities on DGGE profiles provided insight into the structural diversity of the bacterial populations. This was because the number of different bacterial strains could be quantified (Muyzer *et al.*, 1993) and their evenness determined (Muylaert *et al.*, 2002). On PM2 and PM3 the high number of bands on the DGGE profile indicated high diversity values, although these data did not correspond with the diversity and

dominance values that were calculated for the Biolog substrate-utilisation profiles. This discrepancy was ascribed to the presence of viable but non-culturable bacteria that were unable to assimilate the carbon sources provided by the GN Biolog Microtitre™ plates as well as the detection of DNA from inactive bacterial cells by the DGGE technique. It appeared that the prevalent members of the bacterial community in the PM6 water system were represented by the culturable component of the population that were found to be members of the genera *Acinetobacter* and *Pseudomonas*.

The use of reference strains to identify members of the bacterial community as described by Ercolini (2004) was unsuccessful in this study, since more than one copy of the rDNA gene was amplified during the PCR reaction. To determine the identity of members of a bacterial community with DGGE analysis, therefore, the DNA bands must be excised, purified and sequenced in addition to other complementary analyses.

DGGE as an analytical technique for bacterial diversity in water systems needs to be verified with more data over longer periods of time. The present study indicates that, just as community-level physiological profiling can be handicapped by non-culturable organisms, DGGE analysis could be hampered by DNA from dead and inactive cells. The individual members of the community could be identified with sequencing and phylogenetic analysis, but additional information would be necessary to confirm the identities, since the fragment sizes of DGGE bands would be insufficient for identification as reviewed by Muyzer & Smalla (1998). The potential value of the DGGE approach in paper mills would be to monitor changes over time and under variable environmental conditions to control fouling of water systems.

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GENERAL CONCLUSIONS

Due to environmental and legislative pressure, paper mills are forced to reduce their water consumption (Thompson *et al.,* 2001) by closing water systems to reuse water. Closure, however, leads to increased numbers and types of microorganisms that can adversely affect paper production (Habets *et al.*, 1996), product quality (Robertson & Taylor, 1993) and safety (Gudlauski, 1996) in paper mills. Therefore, measures are necessary to counter the colonisation of microorganisms in paper mills. Despite the search for alternative methods, chemical biocides still represents the most effective measure to control microbial growth and fouling (Pereira *et al.*, 2001). By monitoring different environmental parameters such as temperature, pH, DO, ORP, TSS, TDS, COD, N and P an indication of the microbial activity at different sampling points in a paper-mill water system can be obtained. The proper management of these parameters can, in turn, reduce microbial numbers and lead to improved process control.

Plate counts remain the most popular method for microbial enumeration in industry due to its low cost and ease of application, even though it is time-consuming and biased (Garland & Mills, 1991; Garland, 1996). The use of ATP to indicate biological activity requires specialised equipment and expertise, but can be effective and rapid when employed under the appropriate circumstances (Woodward, 2003). The need for more rapid and sensitive tests is critical in the water industry, with the ultimate goal being the continuous on-line monitoring of water in treatment plants and water systems (Rompré *et al.*, 2002).

Traditional methods to identify bacterial species are based on their morphological, physiological and biochemical characteristics (Vandamme *et al.*, 1996). An obvious limitation of these culture-based techniques is the selective pressure that is imposed on the majority of the bacterial community to grow on defined media (Amman *et al.*, 1995). Techniques that investigate the genotype of bacterial communities provide information that is less biased, although intrinsic limitations to each of the different approaches do exist. These molecular-ecological techniques, employed for identification and characterisation of bacteria, compliment conventional techniques that is employed by industry to enhance understanding of bacterial structure and function in natural environments.

The determination of the different environmental parameters could aid in the prediction of changes in microbial populations in paper-mill water systems and could lead to improved process control. The analysis of co-variant parameters in paper-mill water systems revealed

the influence of individual environmental parameters on culturable bacterial numbers that were previously obscured in a large dataset. Temperature correlated positively with aerobic bacterial numbers, sulphate-reducing bacterial numbers and bacterial dominance. This correlation was contrary to the findings of Habets *et al.* (1996) who postulated that higher temperatures will reduce microbial activity.

The strongest correlation between any environmental and biological parameter was the negative correlation between ORP and aerobic bacterial numbers. In a previous study it was reported that high numbers of aerobic microorganisms depleted the system of available dissolved oxygen (Jay, 1992), creating a reductive environment and causing a shift in microbial populations to anaerobic bacteria.

Dissolved solids are an important source of nutrients for microorganisms in water systems and a positive correlation between TDS and AB as well as SRB indicated that high TDS levels could be an indicator of anaerobic bacterial blooms in water systems. Other parameters that could also reflect nutrient levels in water systems included COD, N and P. Chemical oxygen demand was shown to positively influence aerobic and anaerobic bacterial numbers as well as microbial diversity.

Decreased water consumption, the absence of biocides, paper machine furnish and long retention times were all found to increase microbial numbers. The findings of the present study suggest that the evaluation of the different environmental parameters may provide better insight into the microbial activity of the different water systems. By considering the influence of the various environmental parameters, better microbiological control of paper-mill water systems could be achieved and the influence of problematic microorganisms could be managed.

The present study showed that the characterisation and identification of prevalent bacteria in different paper-mill water systems could assist in improving microbial control by optimising the biocide programme to target specific problematic organisms. For the initial characterisation of bacterial isolates into distinct groups, the ERIC typing method was able to distinguish 28 different types of bacteria from Adamas, Ngodwana, Enstra and Stanger paper mills. PCR-RFLP separated the prevalent bacterial types isolated during the Cape Kraft and Tugela surveys into a further seven types. This technique was also successfully used by Vidigal *et al.* (2000) and Carvalho *et al.* (2004) as an auxiliary identification tool.

Partial 16S rDNA gene sequence analysis to determine the relatedness of paper-mill isolates to known culturable bacteria enabled the tentative identification of some of the prevalent types. This method was used by several research groups to determine the identity of new isolates (Vinuesa *et al*., 1998; Tannock *et al*., 1999; Oppong *et al*., 2003; Suihko *et al*., 2003). The majority of the isolates that was identified clustered with species in the genera *Acinetobacter* and *Pseudomonas*. The current study generated a significant amount of information and new insights into the bacterial populations of some paper-mill water systems in South Africa were obtained.

DGGE analysis allowed the separation of different DNA fragments based on sequence variation (Gelsomino *et al*., 1999), and could be a useful tool when attempting to characterise and identify bacterial community members in a complex environmental system. The diversity and dominance indices that were calculated from the number of amplified fragments and their intensities on DGGE gels provided insight into the structural diversity of the bacterial populations. These indices assisted in quantifying the number of bacterial strains (Muyzer *et al*., 1993) as well as to determine their evenness (Muylaert *et al*., 2002) for three paper machines.

The use of reference strains to identify members of the bacterial community, as suggested by Ercolini (2004), was unsuccessful in this study since more than one copy of the rDNA gene was amplified during the PCR reaction as was found by Muyzer & Smalla (1998). The results appeared to indicate that, just as community-level physiological profiling can be handicapped by non-culturable organisms, DGGE analysis could be hampered by DNA from dead and inactive cells. The real value of the DGGE approach in paper mills would, therefore, be to monitor changes over time and under variable environmental conditions to control fouling of water systems.

This study provided invaluable information on the microbiology of paper-machine water systems. The data may aid in the design of new and improved microbiological control programmes when mills institute the closure of their water systems in order to comply with more stringent regulations for water use and discharge in South Africa.

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APPENDICES

Appendix A1: Environmental and biological parameters measured for the Adamas PM3 winter and summer surveys

	Parameters	Headbox	Knock-off shower	Rich-water silo	Launder	Reclaimed head-tank	Dump chest	DAF 1 out	DAF 1 in
Winter survey conducted 24/06/2003									
Environmental	Temperature (°C)	27.8	27.1	27.1	25.4	24.0	23.3	28.7	26.7
	pH	6.01	7.8	6.48	6.83	7.08	7.39	7.85	7.69
	TSS (%)	0.98	0.19	0.26	0.2	0.18	3.53	0.28	0.17
	TDS (g/L)	1.21	0.98	1.15	1.04	0.91	0.85	0.99	0.96
	ORP (mV)	173	241	178	283	314	172	60	84
	COD (mg/L)	950	770	1875	1065	485	745	830	1655
	N (mg/L)	14	4.2	5.3	2.83	8.4	6.3	3.3	4.5
	P (mg/L)	nd	nd	nd	nd	nd	nd	nd	nd
	DO (mg/L)	17.16	8.90	18.30	15.50	9.50	33.60	10.20	22.10
Biological	TAB (cfu/ml)	3.20×10^5	3.70×10^3	2.21×10^3	1.73×10^3	1.04×10^3	5.40×10^4	1.20×10^8	7.30×10^4
	AB (cfu/ml)	7.50×10^2	1.00×10^1	3.40×10^1	4.20×10^1	6.00×10^0	1.45×10^3	1.21×10^4	6.60×10^2
	SRB (cfu/ml)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Shannon (H)	4.340	3.490	4.270	4.200	3.100	4.150	4.140	4.330
	Berger-Parker (d)	0.032	0.032	0.029	0.038	0.129	0.037	0.047	0.036
Summer survey conducted 04/02/2004									
Environmental	Temperature (°C)	39.8	35.7	40.2	40.2	35.3	39.3	36.6	34.8
	pH	7.19	7.55	7.51	7.5	7.35	7.05	7.73	7.25
	TSS (%)	0.93	0.17	0.00	0.12	0.17	6.52	0.20	0.00
	TDS (g/L)	0.83	0.71	0.86	0.87	0.76	0.91	1.49	8.48
	ORP (mV)	-42	104	-12	-15	84	-38	120	51
	COD (mg/L)	5630	2020	4610	4435	1905	6795	2395	1610
	N (mg/L)	7.90	2.3	15.7	16.9	5.4	15.4	2.4	2.1
	P (mg/L)	0.8	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
	DO (mg/L)	5.15	5.50	6.34	5.95	5.56	0.82	2.85	7.25
Biological	TAB (cfu/ml)	1.66×10^7	1.75×10^6	1.60×10^7	1.20×10^7	7.45×10^5	2.22×10^7	3.35×10^6	2.21×10^7
	AB (cfu/ml)	1.86×10^7	1.26×10^7	9.65×10^6	6.60×10^7	7.3×10^5	1.80×10^7	1.82×10^7	1.80×10^7
	SRB (cfu/ml)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Shannon (H)	4.200	3.880	3.960	3.960	4.300	4.440	4.090	4.200
	Berger-Parker (d)	0.030	0.053	0.045	0.043	0.028	0.021	0.035	0.030

TSS Total dissolved solids
TDS Total suspended solids
ORP Oxidation-reduction potential
COD Chemical oxygen demand
N Total nitrogen
P Total phosphorous
DO Dissolved oxygen
TAB Total aerobic bacteria
AB Anaerobic bacteria
SRB Sulphate-reducing bacteria
nd Not determined

Appendix A2: Environmental and biological parameters measured for the Adamas PM4 winter and summer surveys

	Parameters	Finestock I	Finestock II	Finestock III	Constant-level headbox	Launder	Seal pit	Surge tank	DAF2 out
Winter survey conducted 24/06/2003									
Environmental	Temperature (°C)	37.5	26.5	29.8	31.1	30.6	30.8	30.7	18.7
	pH	7.29	7.77	7.32	7.39	7.63	7.64	7.62	6.92
	TSS (%)	5.23	7.18	6.47	3.23	0.23	0.15	0.65	0.13
	TDS (g/L)	0.97	0.97	0.98	0.96	0.94	0.97	0.95	1.01
	ORP (mV)	190	261	261	332	192	181	206	449
	COD (mg/L)	380	580	340	1525	150	115	350	90
	N (mg/L)	10.2	5.7	9.8	4.3	10.6	7.2	8.9	9.8
	P (mg/L)	nd	nd	nd	nd	nd	nd	nd	nd
	DO (mg/L)	9.00	27.00	18.50	22.90	13.80	13.30	38.00	9.20
Biological	TAB (cfu/ml)	5.90×10^{3}	3.80×10^{4}	5.00×10^{2}	7.00×10^{1}	2.00×10^{2}	2.24×10^{4}	5.00×10^{2}	0
	AB (cfu/ml)	1.00×10^{0}	0.00	0.00	0.00	0.00	1.00×10^{0}	0.00	1.00×10^{1}
	SRB (cfu/ml)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Shannon (H)	3.430	3.310	3.830	3.920	4.450	4.140	4.040	3.220
	Berger-Parker (d)	0.059	0.073	0.040	0.050	0.022	0.038	0.053	0.108
Summer survey conducted 04/02/2004									
Environmental	Temperature (°C)	42.7	33.7	39.4	40.8	39.7	39.9	40.7	26.1
	pH	7.08	7.78	7.27	7.30	7.46	7.47	7.48	7.22
	TSS (%)	1.83	4.72	1.61	2.32	0.28	0.058	0.91	0.10
	TDS (g/L)	0.71	0.60	0.75	0.69	0.68	0.73	0.69	0.68
	ORP (mV)	42	262	215	223	205	183	120	390
	COD (mg/L)	510	1785	445	670	460	530	475	50
	N (mg/L)	3.8	<15	3.2	3.2	1.6	2	1.4	2.2
	P (mg/L)	<0.5	<0.5	<0.5	2.3	<0.5	<0.5	<0.5	0.7
	DO (mg/L)	6.52	9.32	8.50	7.63	6.88	6.62	7.22	9.22
Biological	TAB (cfu/ml)	2.70×10^{6}	0	1.80×10^{2}	1.50×10^{2}	1.74×10^{3}	5.75×10^{2}	4.30×10^{2}	0
	AB (cfu/ml)	7×10^{6}	3.85×10^{3}	1.45×10^{2}	0.00	1.72×10^{5}	0.00	5.45×10^{2}	5×10^{1}
	SRB (cfu/ml)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Shannon (H)	4.160	2.330	4.180	4.310	4.110	4.120	4.160	3.750
	Berger-Parker (d)	0.033	0.243	0.030	0.034	0.042	0.039	0.037	0.073

TSS Total dissolved solids
TDS Total suspended solids
ORP Oxidation-reduction potential
COD Chemical oxygen demand
N Total nitrogen
P Total phosphorous
DO Dissolved oxygen
TAB Total aerobic bacteria
AB Anaerobic bacteria
SRB Sulphate-reducing bacteria
nd Not determined

Appendix A3: Environmental and biological parameters measured for the Ngodwana KLB winter and summer surveys

	Parameters	Cloudy-backwater	Clear-backwater	Reclaimed-water	Top-ply silo	Middle-ply silo	Base-ply silo	Polished-backwater	Clarifier
Winter survey conducted 13/05/2003									
Environmental	Temperature (°C)	41.9	44	38.1	48.0	47.0	46.0	42.8	44.5
	pH	7.72	7.56	7.73	7.41	7.29	7.08	7.67	7.41
	TSS (%)	0.54	0.24	0.22	0.16	0.17	0.19	0.19	0.17
	TDS (g/L)	1.25	1.26	1.12	1.23	1.15	1.18	1.14	1.13
	ORP (mV)	24	32	28	58	32	37	40	33
	COD (mg/L)	1760	>1500	1515	1725	1645	1650	1380	1360
	N (mg/L)	2.4	2.1	2.9	2.3	2.2	2.2	2.8	1.8
	P (mg/L)	nd	nd	nd	nd	nd	nd	nd	nd
	DO (mg/L)	7.30	5.80	8.60	5.00	5.80	5.80	3.50	5.60
Biological	TAB (cfu/ml)	1.10×10^{7}	1.20×10^{7}	8.30×10^{5}	1.90×10^{5}	2.00×10^{6}	2.40×10^{6}	5.00×10^{6}	7.00×10^{5}
	AB (cfu/ml)	2.50×10^{2}	1.00×10^{7}	3.70×10^{5}	<30	1.70×10^{2}	2.10×10^{2}	7.30×10^{1}	<300
	SRB (cfu/ml)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Shannon (H)	4.063	2.789	2.205	3.726	3.884	1.468	2.008	1.745
	Berger-Parker (d)	0.028	0.034	0.032	0.042	0.046	0.053	0.057	0.045
Summer survey conducted 18/02/2004									
Environmental	Temperature (°C)	40.0	42.2	33.5	45.3	44.9	43.8	41	42.9
	pH	7.77	7.79	7.98	7.41	7.44	7.6	7.70	7.35
	TSS (%)	0.06	0.05	0.09	0.10	0.12	0.18	0.023	0.16
	TDS (g/L)	1.31	1.21	1.03	1.12	1.11	1.01	1.20	1.18
	ORP (mV)	37	26	235	-78	12	27	-45	42
	COD (mg/L)	<500	<500	<500	525	<500	<500	<500	<500
	N (mg/L)	<0.5	<0.7	<0.5	<0.5	0	<0.5	0.7	<0.5
	P (mg/L)	<0.5	<0.5	2.2	<0.5	<0.5	<0.5	0	<0.5
	DO (mg/L)	7.93	3.19	7.07	5.02	6.02	5.15	6.97	0.27
Biological	TAB (cfu/ml)	5.55×10^{6}	1.03×10^{7}	<30	5.95×10^{6}	7.50×10^{6}	3.20×10^{6}	1.27×10^{6}	1.90×10^{6}
	AB (cfu/ml)	1.21×10^{3}	7.4×10^{6}	0.00	3.60×10^{6}	7.54×10^{3}	7.70×10^{4}	1.54×10^{3}	1.49×10^{6}
	SRB (cfu/ml)	0.00	>3000	0.00	530	0.00	0.00	1.80×10^{3}	0.00
	Shannon (H)	4.109	4.226	3.398	4.005	3.924	3.830	3.774	3.899
	Berger-Parker (d)	0.038	0.039	0.142	0.039	0.069	0.046	0.051	0.049

TSS Total dissolved solids
TDS Total suspended solids
ORP Oxidation-reduction potential
COD Chemical oxygen demand
N Total nitrogen
P Total phosphorous
DO Dissolved oxygen
TAB Total aerobic bacteria
AB Anaerobic bacteria
SRB Sulphate-reducing bacteria
nd Not determined

Appendix A4: Environmental and biological parameters measured for the Ngodwana Newsprint paper machine winter and summer surveys

	Parameters	Clarifier	Cloudy-backwater	Clear-backwater	Machine chest	Couch-seal Pit	Uhle-box pit	Headbox	Wire 1
Winter survey conducted 13/05/2003									
Environmental	Temperature (°C)	43.4	46.3	48.8	47.5	48.2	39.6	49.2	47.9
	pH	6.40	6.33	6.15	5.77	5.64	6.46	5.76	5.80
	TSS (%)	0.00	0.02	0.01	3.96	0.05	0.13	1.08	0.58
	TDS (g/L)	0.12	0.1	0.1	0.11	0.1	0.08	0.10	0.10
	ORP (mV)	94	88	89	121	84	83	112	105
	COD (mg/L)	1685	1675	1460	>1500	1610	>1500	>1500	>1500
	N (mg/L)	2.9	4.8	2.7	7.9	3.3	6	-	-
	P (mg/L)	-	-	-	-	-	-	-	-
	DO (mg/L)	8.80	21.90	12.08	-	12.10	-	-	-
Biological	TAB (cfu/ml)	3.20×10^4	1.10×10^5	3.00×10^4	3.00×10^4	3.00×10^4	2.70×10^6	5.20×10^5	3.00×10^4
	AB (cfu/ml)	<30	1.80×10^3	1.60×10^2	<30	4.30×10^2	1.20×10^5	<30	9.20×10^2
	SRB (cfu/ml)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Shannon (H)	3.267	3.675	2.727	3.702	1.99	4.018	2.332	3.743
	Berger-Parker (d)	0.076	0.048	0.059	0.039	0.052	0.031	0.031	0.043
Summer survey conducted 18/02/2004									
Environmental	Temperature (°C)	46.2	47.3	47.2	49.4	48.2	41	50.7	51
	pH	6.35	5.67	5.60	5.19	5.64	6.45	5.27	5.30
	TSS (%)	0.03	0.05	0.03	3.10	0.01	0.20	1.17	0.63
	TDS (g/L)	0.5	0.45	0.47	0.47	0.46	0.39	0.47	0.43
	ORP (mV)	1565	1680	1580	3950	1593	1460	2090	2050
	COD (mg/L)	-17	65	77	76	85	67	65	67
	N (mg/L)	5.5	5.1	3.7	<15	5.3	3.3	7.0	3.0
	P (mg/L)	1.7	1.6	1.3	5.0	1.3	1.1	2.0	1.8
	DO (mg/L)	5.13	6.90	12.08	7.18	5.10	4.50	0.12	0.18
Biological	TAB (cfu/ml)	6.20×10^5	1.4×10^6	7.55×10^5	4.80×10^5	3.55×10^5	7.65×10^5	9.35×10^5	2.65×10^6
	AB (cfu/ml)	<30	3.90×10^3	5.65×10^2	1.36×10^5	8.10×10^2	1.35×10^4	3.30×10^4	3.10×10^4
	SRB (cfu/ml)	0.00	1.00 X 103	0.00	0.00	0.00	4.00×10^3	0.00	0.00
	Shannon (H)	3.520	3.860	3.847	4.047	4.090	4.225	4.063	3.818
	Berger-Parker (d)	0.107	0.044	0.148	0.036	0.041	0.031	0.039	0.071

TSS Total dissolved solids
TDS Total suspended solids
ORP Oxidation-reduction potential
COD Chemical oxygen demand
N Total nitrogen
P Total phosphorous
DO Dissolved oxygen
TAB Total aerobic bacteria
AB Anaerobic bacteria
SRB Sulphate-reducing bacteria
nd Not determined

Appendix A5: Environmental and biological parameters measured for the Enstra PM2 winter and summer surveys

	Parameters	Wire pit	Headbox	Seal tank	Unclarifed north	Stuff box	Broke chest	Blend chest	Machine chest
Winter survey conducted 08/06/2004									
Environmental	Temperature (°C)	25.8	27.4	26	23	26.7	22.4	25	22
	pH	7.79	7.74	7.68	7.96	7.50	7.79	7.46	7.53
	TSS (%)	0.49	5.93	1.65	0.62	0.58	13.82	5.07	8.63
	TDS (g/L)	0.45	0.45	0.44	0.44	0.45	0.42	0.45	0.45
	ORP (mV)	257	331	340	357	322	325	357	368
	COD (mg/L)	275	355	315	85	305	13890	385	280
	N (mg/L)	10.4	8.3	6.7	6.9	8.7	14.2	7.5	15.8
	P (mg/L)	0.10	0.16	0.56	0.48	0.29	0.29	0.16	0.22
	DO (mg/L)	6.12	2.43	9.48	8.64	4.54	10.10	11.32	9.84
Biological	TAB (cfu/ml)	0.00	0.00	0.00	0.00	0.00	0.00	1.8×10^5	0.00
	AB (cfu/ml)	0.00	0.00	0.00	0.00	0.00	0.00	6.00×10^2	0.00
	SRB (cfu/ml)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Shannon (H)	2.412	3.626	3.118	3.478	3.358	3.629	3.498	3.176
	Berger-Parker (d)	0.213	0.051	0.102	0.069	0.095	0.110	0.066	0.143
Summer survey conducted 22/11/2004									
Environmental	Temperature (°C)	32.4	34.3	35	31.5	36.8	32.2	33	30.5
	pH	7.74	7.76	7.86	7.76	7.63	7.86	7.46	7.73
	TSS (%)	0.00	0.40	0.00	0.00	1.33	3.17	1.52	1.02
	TDS (g/L)	0.41	0.4	0.39	0.39	0.32	0.4	0.42	0.44
	ORP (mV)	334	340	334	339	316	303	265	330
	COD (mg/L)	275	430	595	400	590	67	589	590
	N (mg/L)	6.20	6.90	4.40	5.90	0.50	1.50	7.30	0.40
	P (mg/L)	0.10	0.20	0.20	0.10	0.60	0.50	0.30	0.50
	DO (mg/L)	7.88	8.29	7.21	8.02	8.5	8.09	8.55	8.57
Biological	TAB (cfu/ml)	0.00	0.00	0.00	0.00	1.45×10^2	0.00	0.00	0.00
	AB (cfu/ml)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	SRB (cfu/ml)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Shannon (H)	2.275	2.860	3.473	2.792	1.097	3.531	2.171	2.561
	Berger-Parker (d)	0.175	0.196	0.088	0.109	0.522	0.077	0.323	0.146

TSS Total dissolved solids
TDS Total suspended solids
ORP Oxidation-reduction potential
COD Chemical oxygen demand
N Total nitrogen
P Total phosphorous
DO Dissolved oxygen
TAB Total aerobic bacteria
AB Anaerobic bacteria
SRB Sulphate-reducing bacteria
nd Not determined

Appendix A6: Environmental and biological parameters measured for the Enstra PM3 winter and summer surveys

	Parameters	Headbox	Backwater	Saveall	Clarified chest	Raw-pine chest	Raw-gum chest	Machine chest	Corrected-broke chest
Winter survey conducted 08/06/2004									
Environmental	Temperature (°C)	26.0	33.2	26.6	30.2	23.9	25.3	27.1	27.0
	pH	7.63	7.77	8.10	8.06	7.70	7.62	7.64	7.63
	TSS (%)	6.23	0.84	0.82	0.42	19.13	1.06	4.64	5.10
	TDS (g/L)	0.53	0.46	0.48	0.50	0.40	0.54	0.52	0.49
	ORP (mV)	145	188	179	71	210	179	154	116
	COD (mg/L)	180	200	190	205	230	530	925	840
	N (mg/L)	7.7	8.1	6.5	6.5	2.9	4.7	4.9	3.9
	P (mg/L)	0.15	0.10	0.11	0.24	0.60	1.13	0.80	1.10
	DO (mg/L)	7.10	7.73	6.69	5.83	3.17	7.05	8.07	6.88
Biological	TAB (cfu/ml)	8.05×10^5	3.35×10^5	7.55×10^5	1.31×10^6	1.95×10^6	3.10×10^6	3.17×10^6	4.35×10^6
	AB (cfu/ml)	1.91×10^4	5.75×10^3	8.20×10^3	3.15×10^4	3.00×10^4	9.00×10^4	1.45×10^5	3.90×10^4
	SRB (cfu/ml)	0.00	overgrown	0.00	0.00	0.00	0.00	0.00	0.00
	Shannon (H)	3.754	3.962	3.988	3.794	3.694	3.469	4.018	3.665
	Berger-Parker (d)	0.048	0.047	0.049	0.048	0.057	0.053	0.031	0.042
Summer survey conducted 22/11/2004									
Environmental	Temperature (°C)	33.9	33.7	34.4	32.1	28.2	28.7	33.3	31.3
	pH	7.82	7.85	7.99	7.92	7.85	7.53	7.82	8.03
	TSS (%)	0.00	0.11	0.05	0.25	1.61	2.05	1.40	3.78
	TDS (g/L)	0.42	0.4	0.39	0.39	0.27	0.39	0.37	0.33
	ORP (mV)	268	232	277	256	261	313	211	232
	COD (mg/L)	380	350	540	530	360	1085	920	705
	N (mg/L)	5.90	5.30	1.50	3.70	6.80	7.50	4.40	4.30
	P (mg/L)	0.20	0.30	0.20	0.20	0.40	0.80	0.50	0.40
	DO (mg/L)	10.66	8.92	8.45	8.52	9.50	8.36	8.13	12.44
Biological	TAB (cfu/ml)	0.00	0.00	0.00	0.00	1.12×10^5	1.00×10^3	4.80×10^6	2.65×10^5
	AB (cfu/ml)	0.00	0.00	0.00	0.00	0.00	0.00	4.45×10^2	0.00
	SRB (cfu/ml)	0.00	0.00	0.00	0.00	0.00	0.00	0.000	0.00
	Shannon (H)	2.612	2.286	2.500	2.266	3.658	3.202	3.908	3.915
	Berger-Parker (d)	0.178	0.178	0.169	0.188	0.086	0.074	0.044	0.041

TSS Total dissolved solids
TDS Total suspended solids
ORP Oxidation-reduction potential
COD Chemical oxygen demand
N Total nitrogen
P Total phosphorous
DO Dissolved oxygen
TAB Total aerobic bacteria
AB Anaerobic bacteria
SRB Sulphate-reducing bacteria
nd Not determined

Appendix A7: Environmental and biological parameters measured for the Enstra PM6 winter and summer surveys

	Parameters	Machine silo	Backwater	Cloudy backwater	Clear backwater	Headbox	Corrected-broke chest	Blend Chest	White-water surge tank
Winter survey conducted 08/06/2004									
Environmental	Temperature (°C)	36.0	37.0	34.7	36.4	35.4	34.1	39.6	33.5
	pH	7.74	7.80	7.78	7.86	7.67	7.29	7.31	7.50
	TSS (%)	96.85	16.11	0.40	0.63	19.68	3.28	6.44	0.48
	TDS (g/L)	0.52	0.52	0.51	0.51	0.52	0.64	0.55	0.54
	ORP (mV)	-19	-20	-4	-7	-12	19	29	22
	COD (mg/L)	530	505	480	490	520	2015	720	535
	N (mg/L)	0.6	2.0	3.0	0.4	1.9	0.8	0.7	5.2
	P (mg/L)	0.1	0.1	0.1	0.1	0.1	0.2	4.1	0.1
	DO (mg/L)	8.22	6.35	7.82	7.48	7.78	7.60	3.10	6.01
Biological	TAB (cfu/ml)	1.13×10^7	5.70×10^6	2.75×10^6	3.00×10^6	1.14×10^7	2.75×10^7	1.37×10^7	1.00×10^7
	AB (cfu/ml)	3.65×10^4	3.60×10^2	9.20×10^2	8.88×10^5	8.65×10^4	4.60×10^4	2.25×10^5	3.90×10^4
	SRB (cfu/ml)	0.00	overgrown	overgrown	overgrown	0.00	0.00	0.00	0.00
	Shannon (H)	3.846	4.061	4.186	3.941	3.915	3.728	3.957	4.012
	Berger-Parker (d)	0.042	0.044	0.040	0.043	0.038	0.051	0.042	0.033
Summer survey conducted 22/11/2004									
Environmental	Temperature (°C)	36.1	35.0	34.8	34.4	35.4	35.7	33.4	33.1
	pH	7.81	7.92	7.87	7.97	7.5	7.78	7.31	7.53
	TSS (%)	0.19	0.22	0.02	0.00	0.61	1.69	0.95	0.00
	TDS (g/L)	0.39	0.36	0.33	0.37	0.59	0.36	0.41	0.40
	ORP (mV)	48	86	107	97	27	121	73	42
	COD (mg/L)	65	358	330	64	364	625	601	254
	N (mg/L)	2.00	1.70	2.20	4.00	4.80	1.10	0.60	2.00
	P (mg/L)	0.10	0.10	0.10	0.10	0.20	0.30	0.40	0.10
	DO (mg/L)	7.87	8.21	11.03	8.69	7.07	5.22	6.82	7.81
Biological	TAB (cfu/ml)	7.20×10^6	9.20×10^6	1.23×10^7	1.99×10^7	8.85×10^6	1.21×10^7	8.00×10^6	1.43×10^7
	AB (cfu/ml)	1.00×10^2	7.50×10^1	7.30×10^2	9.00×10^1	1.01×10^6	1.53×10^6	3.05×10^6	3.65×10^5
	SRB (cfu/ml)	0.00	1.00×10^3	0.00	0.00	100	1000	0.00	1.00×10^1
	Shannon (H)	4.140	3.900	3.926	4.095	4.076	3.680	4.211	3.631
	Berger-Parker (d)	0.036	0.058	0.041	0.036	0.0439	0.046	0.040	0.047

TSS Total dissolved solids
TDS Total suspended solids
ORP Oxidation-reduction potential
COD Chemical oxygen demand
N Total nitrogen
P Total phosphorous
DO Dissolved oxygen
TAB Total aerobic bacteria
AB Anaerobic bacteria
SRB Sulphate-reducing bacteria
nd Not determined

Appendix A8: Environmental and biological parameters measured for the Stanger paper machine winter and summer surveys

	Parameters	Headbox	Backwater channel	White-water tower	Broke tower	Recovered fibre	Broke headtank	Bagasse headtank	Constant-level headtank
Winter survey conducted 13/07/2004									
Environmental	Temperature (°C)	41.2	42.0	38.6	39.2	40.6	40.0	36.1	38.1
	pH	7.68	7.80	8.07	7.44	7.91	7.38	4.80	7.15
	TSS (%)	2.97	2.31	2.36	29.64	67.83	43.93	21.23	39.78
	TDS (g/L)	0.61	0.61	0.56	0.59	0.55	0.55	0.83	1.27
	ORP (mV)	1	-3	146	-21	43	74	473	190
	COD (mg/L)	600	565	370	1320	800	1200	290	650
	N (mg/L)	4.5	0.1	1.5	2.4	10	15	5	8
	P (mg/L)	1.1	0.2	0.7	0.4	0.2	1.1	0.4	1.6
	DO (mg/L)	7.40	8.40	9.10	1.780	5.20	4.35	9.94	11.01
Biological	TAB (cfu/ml)	8.85×10^4	4.30×10^4	0.00	5.60×10^6	2.75×10^5	5.05×10^6	0.00	3.25×10^4
	AB (cfu/ml)	6.10×10^3	0	0.00	2.95×10^6	3.10×10^3	2.95×10^5	2.00×10^1	3.10×10^5
	SRB (cfu/ml)	0.00	1.00×10^2	0.00	0.00	0.00	0.00	0.00	0.00
	Shannon (H)	2.630	3.303	1.065	3.558	3.217	2.819	2.606	3.007
	Berger-Parker (d)	0.149	0.154	0.576	0.043	0.070	0.044	0.104	0.073
Summer survey conducted 3/11/2004									
Environmental	Temperature (°C)	41.2	40.4	40.6	39	40	42.4	39.6	42.2
	pH	7.47	7.66	7.37	7.00	7.75	7.06	7.05	7.14
	TSS (%)	1.01	0.11	0.59	2.41	5.72	2.83	3.51	2.76
	TDS (g/L)	0.64	0.63	0.64	0.73	0.54	0.71	0.75	0.66
	ORP (mV)	-9	-20	18	35	75	70	68	58
	COD (mg/L)	325	260	415	570	610	620	485	560
	N (mg/L)	0.5	1.1	0.2	0.2	0.5	0.4	0.1	0.3
	P (mg/L)	0.2	0.06	0.1	0.1	0.2	0.1	0.7	0.1
	DO (mg/L)	13.41	11.79	2.91	1.48	9.50	6.50	8.25	10.79
Biological	TAB (cfu/ml)	2.26×10^5	3.25×10^5	7.05×10^4	5.20×10^6	8.50×10^5	3.20×10^6	4.95×10^6	4.35×10^6
	AB (cfu/ml)	0.00	0.00	0.00	2.35×10^4	7.65×10^2	7.65×10^4	7.95×10^2	6.20×10^3
	SRB (cfu/ml)	1.0×10^3	1.0×10^3	0.00	0.00	0.00	0.00	0.00	0.00
	Shannon (H)	3.141	0.884	2.012	0.888	1.105	2.832	3.171	0.173
	Berger-Parker (d)	0.018	0.022	0.021	0.014	0.008	0.008	0.005	0.012

TSS Total dissolved solids
TDS Total suspended solids
ORP Oxidation-reduction potential
COD Chemical oxygen demand
N Total nitrogen
P Total phosphorous
DO Dissolved oxygen
TAB Total aerobic bacteria
AB Anaerobic bacteria
SRB Sulphate-reducing bacteria
nd Not determined

Appendix A9: Environmental and biological parameters measured for the Stanger tissue machine winter and summer surveys

	Parameters	Wire silo	Bagasse-stock channel	Dump chest	Recovered fibre	Blend chest	Backwater tank	Saveall	Main drain
Winter survey conducted 13/07/2004									
Environmental	Temperature (°C)	39.7	22.7	41.3	34.6	32.9	41.7	37.6	39.2
	pH	8.32	8.3	7.52	7.94	7.72	7.4	8.03	7.89
	TSS (%)	0.82	26.61	32.75	18.42	15.76	1.86	21.62	1.60
	TDS (g/L)	0.66	0.79	0.79	0.68	0.75	0.67	0.65	0.67
	ORP (mV)	89	249	142	88	62	102	82	51
	COD (mg/L)	170	515	570	650	1205	920	395	402
	N (mg/L)	0.2	3.0	1.0	6.2	3.0	0.4	5.0	0.0
	P (mg/L)	0.8	0.8	1.7	1.1	1.1	2.0	1.0	0.6
	DO (mg/L)	7.69	14.82	4.67	6.50	5.90	7.77	8.50	2.93
Biological	TAB (cfu/ml)	6.85×10^{4}	1.47×10^{5}	4.20×10^{4}	3.95×10^{5}	4.30×10^{5}	5.60×10^{4}	2.95×10^{5}	4.35×10^{4}
	AB (cfu/ml)	3.00×10^{2}	3.00×10^{3}	0.00	1.48×10^{3}	7.75×10^{5}	0.00	6.20×10^{5}	9.00×10^{1}
	SRB (cfu/ml)	1.20×10^{3}	0.00	1.00×10^{3}	1.00×10^{4}	1.00×10^{4}	1.00×10^{3}	1.00×10^{3}	1.00×10^{4}
	Shannon (H)	1.063	1.592	1.634	2.120	1.835	2.145	2.541	2.169
	Berger-Parker (d)	0.027	0.039	0.042	0.032	0.038	0.039	0.024	0.039
Summer survey conducted 03/11/2004									
Environmental	Temperature (°C)	39.7	40.2	43.1	41.5	41.8	40.5	40.4	35.6
	pH	7.69	7.72	7.41	7.67	7.70	7.66	7.70	7.72
	TSS (%)	0.18	1.80	4.19	5.91	2.75	0.11	0.92	0.06
	TDS (g/L)	0.41	0.49	0.45	0.39	0.44	0.41	0.57	0.34
	ORP (mV)	74	37	39	45	42	49	42	65
	COD (mg/L)	240	975	445	300	400	110	145	105
	N (mg/L)	0.6	1.6	0.5	5.1	3.3	0.8	0.8	0.8
	P (mg/L)	0.7	1.0	0.6	0.8	3.8	0.6	0.7	0.5
	DO (mg/L)	11.30	7.77	9.44	7.76	7.88	7.80	11.44	10.34
Biological	TAB (cfu/ml)	1.46×10^{6}	1.67×10^{6}	3.90×10^{6}	6.25×10^{6}	3.30×10^{6}	1.01×10^{6}	1.19×10^{6}	7.80×10^{5}
	AB (cfu/ml)	2.26×10^{4}	1.46×10^{4}	4.70×10^{4}	8.30×10^{4}	3.20×10^{6}	1.58×10^{6}	1.64×10^{6}	5.00×10^{3}
	SRB (cfu/ml)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.0×10^{2}
	Shannon (H)	3.14	0.884	2.012	0.888	1.105	2.832	3.171	0.173
	Berger-Parker (d)	0.021	0.012	0.014	0.010	0.012	0.025	0.021	0.004

TSS Total dissolved solids
TDS Total suspended solids
ORP Oxidation-reduction potential
COD Chemical oxygen demand
N Total nitrogen
P Total phosphorous
DO Dissolved oxygen
TAB Total aerobic bacteria
AB Anaerobic bacteria
SRB Sulphate-reducing bacteria
nd Not determined

Appendix A10: Environmental and biological parameters measured for the Cape Kraft paper machine winter and summer surveys

	Parameters	Filler-line headbox	Top-line hea box	Unclarified-water tank	Clarified-water tank	Clarifier	DAF out	Combined white-water	Former # 5
Winter survey conducted 21/06/2005									
Environmental	Temperature (°C)	35.5	36.4	30.4	30.2	16.5	33.4	33.5	30.9
	pH	6.99	7.26	6.36	7.07	4.56	7.14	3.44	3.69
	TSS (%)	3.54	2.90	0.20	0.00	0.00	0.10	0.20	0.10
	TDS (g/L)	1.19	1.17	1.13	1.19	2.12	1.17	1.17	1.15
	ORP (mV)	81	104	-66	-67	-213	5	12	122
	COD (mg/L)	2295	2795	2140	2150	10060	2250	2150	2075
	N (mg/L)	2.0	7.0	4.7	4.4	130.0	6.1	4.4	4.29.
	P (mg/L)	0.3	0.3	0.0	0.0	10.8	0.0	0.1	0.0
	DO (mg/L)	7.99	8.00	6.81	8.26	2.80	7.76	8.19	8.40
Biological	TAB (cfu/ml)	3.00×10^3	3.35×10^3	3.02×10^3	2.10×10^2	1.50×10^1	9.10×10^2	1.35×10^3	2.35×10^2
	AB (cfu/ml)	2.70×10^2	1.6×10^2	1.0×10^1	0.00	0.00	0.00	0.00	0.00
	SRB (cfu/ml)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Shannon (H)	nd	nd	nd	nd	nd	nd	nd	nd
	Berger-Parker (d)	nd	nd	nd	nd	nd	nd	nd	nd
Summer survey conducted 29/11/2005									
Environmental	Temperature (°C)	37.7	40.4	32.9	34.2	20.9	35.5	35.2	34.7
	pH	6.95	7.01	6.54	6.93	4.45	6.93	7.36	7.50
	TSS (%)	4.03	3.74	0.00	0.01	0.00	0.01	0.21	0.82
	TDS (g/L)	0.92	0.95	0.93	0.96	2.26	0.93	0.88	0.88
	ORP (mV)	-130	-111	-211	-435	-213	-266	-246	-210
	COD (mg/L)	6030	6110	4460	2705	>10000	4405	4375	4420
	N (mg/L)	4.3	4.8	3.9	3.0	3.1	3.9	5.2	4.8
	P (mg/L)	4.8	0.2	0.2	0.3	4.6	0.4	0.1	0.2
	DO (mg/L)	4.95	4.53	5.31	8.78	2.03	12.13	7.44	7.23
Biological	TAB (cfu/ml)	3.35×10^6	8.95×10^6	9.95×10^6	4.80×10^6	0.00	9.30×10^6	3.00×10^6	3.35×10^6
	AB (cfu/ml)	2.95×10^5	5.30×10^3	6.75×10^3	3.75×10^3	0.00	3.15×10^3	3.10×10^3	3.85×10^3
	SRB (cfu/ml)	1.00×10^3	0.00	3.00×10^1	5.00×10^2	0.00	1.00×10^3	0.00	1.00×10^2
	Shannon (H)	nd	nd	nd	nd	nd	nd	nd	nd
	Berger-Parker (d)	nd	nd	nd	nd	nd	nd	nd	nd

TSS Total dissolved solids
TDS Total suspended solids
ORP Oxidation-reduction potential
COD Chemical oxygen demand
N Total nitrogen
P Total phosphorous
DO Dissolved oxygen
TAB Total aerobic bacteria
AB Anaerobic bacteria
SRB Sulphate-reducing bacteria
nd Not determined

Appendix A11: Environmental and biological parameters measured for the Tugela PM1 winter and summer surveys

Parameters		Machine chest	Headbox	Backwater	Showers	Blend chest	Pine chest	Gum chest	Waste chest
Winter survey conducted 12/07/2005									
Environmental	Temperature (°C)	42.7	41.6	40.7	32.7	39.5	46.4	46.5	53.7
	pH	7.18	6.83	7.16	8.07	7.31	9	6.81	6.93
	TSS (%)	2.80	0.75	0.08	0.01	2.71	3.02	3.20	3.55
	TDS (g/L)	0.81	0.76	0.76	0.15	0.81	0.86	0.84	0.86
	ORP (mV)	-174	-31	-71	420	7	-211	-142	-10
	COD (mg/L)	2435	1995	2110	0.054a	2685	2060	3785	2725
	N (mg/L)	5.0	7.0	2.0	3.0	8.0	8.0	7.0	11.0
	P (mg/L)	1.0	0.6	0.7	0.0	1.3	1.9	2.2	1.2
	DO (mg/L)	1.11	3.69	5.30	7.33	2.15	0.01	0.43	0.84
Biological	TAB (cfu/ml)	1.31×10^{5}	1.41×10^{5}	1.03×10^{5}	4.00×10^{1}	1.73×10^{4}	1.34×10^{5}	6.40×10^{4}	1.44×10^{5}
	AB (cfu/ml)	7.50×10^{4}	8.60×10^{3}	5.05×10^{2}	0	9.00×10^{4}	3.10×10^{3}	1.09×10^{3}	1.99×10^{4}
	SRB (cfu/ml)	1.00 x 10	3.00×10^{2}	0	0	6.00×10^{1}	6.00×10^{3}	3.00×10^{1}	1.00×10^{1}
	Shannon (H)	3.879	3.829	4.125	3.823	3.924	3.745	3.655	4.252
	Berger-Parker (d)	0.042	0.051	0.030	0.049	0.038	0.047	0.053	0.035
Summer survey conducted 10/01/2006									
Environmental	Temperature (°C)	42.0	48.6	49.3	45.8	50.2	58.4	51.6	49.8
	pH	7.33	7.04	7.41	7.75	7.89	10.08	6.93	6.81
	TSS (%)	3.40	0.73	0.03	0.00	2.75	3.78	3.28	2.79
	TDS (g/L)	0.57	0.78	0.76	0.08	0.75	1.13	0.85	0.77
	ORP (mV)	-65	-17	-229	-37	-269	-480	-235	-263
	COD (mg/L)	1385	2145	1360	250	1795	2110	5755	3005
	N (mg/L)	2.6	3.5	1.7	0.2	5.2	7.8	20.3	2.7
	P (mg/L)	0.9	0.3	0.3	0.2	1	2.4	2.2	0.3
	DO (mg/L)	3.28	8.40	4.23	5.81	1.07	2.06	0.21	1.60
Biological	TAB (cfu/ml)	1.05 x 106	4.85 x 106	3.55 x 106	0.00	1.04 x 107	2.00 x 101	1.31 x 107	1.10 x 1010
	AB (cfu/ml)	1.03 x 104	1.55 x 103	1.35 x 102	0.00	6.15 x 104	2.00 x 102	3.50 x 105	3.20 x 104
	SRB (cfu/ml)	0	1.00 x 101	1.20 x 102	0.00	0	3.00 x 101	0.00	5.00 x 101
	Shannon (H)	3.225	3.193	3.538	nd	3.603	nd	3.000	3.865
	Berger-Parker (d)	0.040	0.033	0.044	nd	0.042	nd	0.083	0.043

TSS Total dissolved solids
TDS Total suspended solids
ORP Oxidation-reduction potential
COD Chemical oxygen demand
N Total nitrogen
P Total phosphorous
DO Dissolved oxygen
TAB Total aerobic bacteria
AB Anaerobic bacteria
SRB Sulphate-reducing bacteria
nd Not determined

Appendix A12: Environmental and biological parameters measured for the Tugela PM2 winter and summer surveys

	Parameters	Top-ply headbox	Base ply headbox	Top-ply backwater	Base-ply backwater	Top-ply thick stock	Base-ply thick stock	White-water tower	Bottom wire
Winter survey conducted 12/07/2005									
Environmental	Temperature (°C)	45.7	44.6	43.8	43.5	50.9	46.4	39.9	41.8
	pH	6.39	6.71	6.66	6.94	8.41	7.2	7.47	6.96
	TSS (%)	0.24	0.65	0.05	0.11	2.71	3.03	0.05	0.13
	TDS (g/L)	0.85	0.86	0.86	0.86	0.86	0.87	0.66	0.85
	ORP (mV)	-13	-75	16	-23	-219	-206	-64	-63
	COD (mg/L)	1085	1445	905	1310	1470	1710	1230	1305
	N (mg/L)	12.0	5.0	3.0	11.0	2.0	6.0	6.0	4.0
	P (mg/L)	0.2	0.4	0.1	0.3	0.7	0.4	0.2	0.3
	DO (mg/L)	4.40	3.20	9.22	6.67	0.00	0.00	4.83	4.25
Biological	TAB (cfu/ml)	3.45×10^{6}	4.79×10^{6}	1.07×10^{6}	5.25×10^{5}	8.30×10^{4}	2.32×10^{6}	6.75×10^{4}	7.50×10^{4}
	AB (cfu/ml)	2.58×10^{3}	2.21×10^{4}	3.90×10^{2}	1.07×10^{3}	6.51×10^{4}	6.70×10^{2}	1.10×10^{3}	1.29×10^{3}
	SRB (cfu/ml)	2.00×10^{1}	0.00	2.00×10^{3}	1.00×10^{1}	3.00×10^{1}	4.20×10^{2}	4.00×10^{1}	2.00×10^{1}
	Shannon (H)	3.545	4.063	3.593	3.931	4.409	3.784	2.841	3.535
	Berger-Parker (d)	0.080	0.047	0.050	0.044	0.031	0.043	0.115	0.072
Summer survey conducted 10/01/2006									
Environmental	Temperature (°C)	53.8	51.4	53.2	49.5	61.4	53.3	46.5	49.8
	pH	6.83	6.90	7.00	7.07	9.89	8.2	7.61	7.09
	TSS (%)	0.32	0.68	0.06	0.04	2.71	3.28	0.05	0.05
	TDS (g/L)	1.03	0.92	1.00	0.91	1.09	0.85	0.77	0.86
	ORP (mV)	367	168	-145	-161	150	122	-112	-152
	COD (mg/L)	2565	1320	1145	1270	2700	3330	1325	1180
	N (mg/L)	1.8	3.5	1.6	2.7	10.1	5.3	2.2	2.1
	P (mg/L)	0.5	1.7	0.3	0.2	1.8	0.9	0.2	0.2
	DO (mg/L)	5.23	5.74	6.73	7.26	0.81	1.65	8.40	7.43
Biological	TAB (cfu/ml)	3.50×10^{5}	5.55×10^{5}	1.60×10^{5}	8.20×10^{4}	0.00	6.65×10^{6}	1.69×10^{6}	2.08×10^{5}
	AB (cfu/ml)	1.00×10^{1}	4.55×10^{2}	0.00	0.00	2.00×10^{1}	1.25×10^{4}	4.00×10^{1}	0.00
	SRB (cfu/ml)	2.00×10^{1}	1.00×10^{2}	1.00×10^{2}	3.00×10^{1}	3.00×10^{2}	0.00	0.00	0.00
	Shannon (H)	2.561	3.450	2.039	2.938	nd	0.542	1.430	1.556
	Berger-Parker (d)	0.035	0.023	0.071	0.036	nd	0.057	0.154	0.117

TSS	Total dissolved solids	N	Total nitrogen	AB	Anaerobic bacteria
TDS	Total suspended solids	P	Total phosphorous	SRB	Sulphate-reducing bacteria
ORP	Oxidation-reduction potential	DO	Dissolved oxygen	nd	Not determined
COD	Chemical oxygen demand	TAB	Total aerobic bacteria		

Appendix A13: Environmental and biological parameters measured for the Tugela PM3 winter and summer surveys

	Parameters	Headbox	Backwater	Machine chest	Raw-pine chest	Blend chest	Broke Thickener	Shower water	Primary cleaners
Winter survey conducted 02/08/2005									
Environmental	Temperature (°C)	44.7	45.4	41.1	42.5	36.0	40.0	24.3	42.5
	pH	4.75	4.82	7.26	7.23	6.77	6.66	8.86	6.3
	TSS (%)	0.29	0.05	3.17	3.11	3.93	2.22	0.66	0.01
	TDS (g/L)	0.61	0.61	0.45	0.57	0.39	0.50	0.15	0.57
	ORP (mV)	37	81	-32	-200	-31	7	25	34
	COD (mg/L)	555	675	1595	1205	875	1090	305	650
	N (mg/L)	3.0	2.0	2.0	6.0	2.0	2.0	3.0	3.0
	P (mg/L)	0.2	0.1	1.3	1.7	1.3	0.5	0.1	0.2
	DO (mg/L)	11.20	9.80	10.85	10.31	12.20	9.82	15.11	10.34
Biological	TAB (cfu/ml)	1.08×10^4	3.05×10^4	2.13×10^5	4.20×10^3	2.46×10^6	1.39×10^7	3.35×10^3	1.47×10^5
	AB (cfu/ml)	0.00	0.00	1.43×10^4	<30	1.08×10^4	4.05×10^4	0.00	2.57×10^3
	SRB (cfu/ml)	0.00	0.00	0.00	1.5×10^2	1.4×10^2	0.00	0.00	0.00
	Shannon (H)	3.833	3.287	3.822	nd	3.693	4.099	3.953	3.622
	Berger-Parker (d)	0.048	0.067	0.044	nd	0.041	0.028	0.058	0.064
Summer survey 24/01/2006									
Environmental	Temperature (°C)	50.9	50.4	37.4	45.2	35.0	42.3	30.6	43.0
	pH	3.87	3.95	5.93	8.42	7.44	6.29	7.75	4.68
	TSS (%)	0.28	0.05	1.73	2.04	1.95	1.55	0.04	0.63
	TDS (g/L)	0.29	0.27	0.21	0.75	0.28	0.24	0.08	0.26
	ORP (mV)	260	59	-50	-441	-655	-194	-123	-155
	COD (mg/L)	160.00	55	910	1480	480	465	1450	115
	N (mg/L)	0.0	0.3	1.2	2.1	0.7	4.4	1.1	0.4
	P (mg/L)	0.0	0.0	0.2	0.9	0.1	0.1	0.2	0.0
	DO (mg/L)	7.50	6.30	5.46	1.01	8.16	11.41	8.3	9.39
Biological	TAB (cfu/ml)	3.55×10^4	9.00×10^2	6.55×10^5	2.14×10^7	2.50×10^6	1.77×10^6	1.26×10^6	1.06×10^6
	AB (cfu/ml)	0.00	0.00	6.00×10^1	6.55×10^3	1.40×10^3	3.00×10^1	1.13×10^4	0.00
	SRB (cfu/ml)	2.00×10^1	2.00×10^1	1.00×10^2	1.00×10^2	2.00×10^1	1.20×10^2	1.00×10^2	0.00
	Shannon (H)	nd	2.161	3.091	4.081	3.804	3.253	3.786	3.709
	Berger-Parker (d)	nd	0.034	0.029	0.044	0.048	0.067	0.044	0.041

TSS Total dissolved solids
TDS Total suspended solids
ORP Oxidation-reduction potential
COD Chemical oxygen demand
N Total nitrogen
P Total phosphorous
DO Dissolved oxygen
TAB Total aerobic bacteria
AB Anaerobic bacteria
SRB Sulphate-reducing bacteria
nd Not determined

Appendix A14: Environmental and biological parameters measured for the Tugela PM4 winter and summer surveys

	Parameters	Raw-pine chest	Headbox	Machine chest	Backwater	Double-wire press	Mill water	Showers	Wire
Winter survey conducted 02/08/2005									
Environmental	Temperature (°C)	45.7	48.3	52.8	44.6	42.5	34.5	35.5	48.7
	pH	7.18	6.71	7.52	7.52	7.66	8.29	8.08	6.51
	TSS (%)	2.16	0.18	2.83	0.02	0.02	0.01	0.01	0.01
	TDS (g/L)	0.78	0.61	0.62	0.59	0.63	0.14	0.16	0.62
	ORP (mV)	-253	40	4	23	-123	-2	-54	48
	COD (mg/L)	1880	740	2340	590	1125	280	305	750
	N (mg/L)	8	1	4	0	6	1	6	4
	P (mg/L)	2.3	0.2	2.3	0.2	0.5	0.1	0.2	0.4
	DO (mg/L)	7.30	8.64	7.68	9.38	5.01	12.15	11.92	9.25
Biological	TAB (cfu/ml)	6.75×10^6	8.10×10^6	3.60×10^7	4.15×10^6	1.80×10^6	9.10×10^3	2.71×10^4	6.75×10^6
	AB (cfu/ml)	1.96×10^5	0.00	1.62×10^4	<30	4.50×10^2	0.00	0.00	0.00
	SRB (cfu/ml)	0.00	2×10^1	1×10^1	0.00	0.00	0.00	0.00	1×10^2
	Shannon (H)	4.192	4.249	4.189	3.628	3.573	4.157	4.141	4.166
	Berger-Parker (d)	0.027	0.040	0.029	0.047	0.058	0.034	0.029	0.044
Summer survey conducted 24/01/2006									
Environmental	Temperature (°C)	52.0	50.0	58.6	51.3	49.1	27.5	32.5	46.6
	pH	8.71	6.1	7.33	6.61	7.64	7.88	7.80	6.55
	TSS (%)	2.57	0.20	3.75	0.00	0.01	0.00	0.00	0.00
	TDS (g/L)	0.49	0.47	0.45	0.50	0.48	0.08	0.08	0.43
	ORP (mV)	-588	-124	-174	-51	-211	-325	-58	-166
	COD (mg/L)	1222	1362	880	1374	1262	1366	1376	1868
	N (mg/L)	2.0	2.2	-	0.3	0.2	13.0	6.0	4.0
	P (mg/L)	1.3	0.0	1.5	0.1	0.1	0.1	0.2	0.0
	DO (mg/L)	6.28	7.62	4.50	9.24	5.10	9.03	10.89	9.10
Biological	TAB (cfu/ml)	4.00×10^7	1.92×10^6	2.38×10^5	1.70×10^6	2.45×10^6	7.40×10^4	1.47×10^5	1.89×10^6
	AB (cfu/ml)	4.80×10^2	0.00	2.60×10^5	0.00	3.60×10^2	8.00×10^2	4.60×10^2	0.00
	SRB (cfu/ml)	1.00×10^1	2.00×10^2	1.60×10^2	1.00×10^2	1.00×10^1	0.00	0.00	1.00×10^1
	Shannon (H)	3.716	3.447	3.913	2.892	3.105	3.883	3.270	3.464
	Berger-Parker (d)	0.047	0.053	0.035	0.027	0.040	0.029	0.047	0.058

TSS Total dissolved solids
TDS Total suspended solids
ORP Oxidation-reduction potential
COD Chemical oxygen demand
N Total nitrogen
P Total phosphorous
DO Dissolved oxygen
TAB Total aerobic bacteria
AB Anaerobic bacteria
SRB Sulphate-reducing bacteria
nd Not determined

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