Diversity and characteristics of yeasts in water sources of the North West Province

By

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ABSTRACT

Yeasts form an important part of many ecosystems and significantly contribute to biodiversity. However, yeast biodiversity in the North West Province remains largely unexplored. The aim of this study was to determine the diversity and characteristics of yeasts from water sources in the North West Province, South Africa. Samples were collected over a two year period and included three rivers, a spruit and an inland lake. Temperature, pH, and electrical conductivity (EC) were measured on site using a multi-probe. Nitrate (NO_3-N), nitrite (NO_2-N) and phosphate (PO_4^{2-}) levels were determined in the laboratory using Hatch kits and equipment. The pH ranged from 7.2 to 9.2. Elevated EC levels (36-70 mS) were detected especially at the Harts River and Barberspan (38-165 mS) sites. Physico-chemical parameter levels were higher during the cold dry sampling period compared to the warm rainy sampling period. Levels and diversity of yeasts were determined using the membrane filtration method. The highest level of yeasts was detected in the Mooi River and Schoonspruit during 2010 and 2011 sampling periods. Pigmented and non-pigmented yeasts were enumerated from all samples. Over the two year period the highest number of pigmented yeasts was detected in the Schoonspruit samples. In some cases there were significant ($P<0.05$) differences between pigmented and non-pigmented yeast levels among the sites. The diazonium blue B (DBB) test was carried out to distinguish between ascomycetous and basidiomycetous yeasts. These isolates were then identified using the API ID 32C system. Yeasts isolates were identified as belonging to the following genera: *Candida, Cryptococcus, Pichia, Rhodotorula* and *Zygosaccharomyces*. In addition using 26S rRNA gene sequencing *Aureobasidium* spp., *Clavispora* spp., *Cystofilobasidium* spp., *Hanseniaspora* spp., *Meyerozyma* spp., *Sporidiobolus* spp., and *Wickerhamomyces* spp.were also identified. The diversity and abundance of yeasts in the water sources demonstrated that opportunistic pathogens were present. This was supported by results that indicated some isolates could grow at 37°C and higher. In conclusion, our results provide preliminary information on the distribution and diversity of yeasts in water sources of the North West Province, South Africa.

Keywords: Yeasts, physico-chemical parameters, water sources, pathogens

DECLARATION

I declare that the dissertation for the degree of Master of Science (M. Sc) at the North-West University Potchefstroom Campus hereby submitted, has never been submitted by me for a degree at this or another University, that it is my own work in design and execution and that all material contained herein has been duly acknowledged.

…… ……

Deidré Van Wyk Date

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CHAPTER 1

INTRODUCTION

1.1 GENERAL INTRODUCTION AND PROBLEM STATEMENT

Water quality issues raised in several urban communities in the North West Province focussed on physico-chemical properties, the occurrence of faecal indicator bacteria (Gaoganediwe, 2006; Thom, 2010). Faecal indicator bacteria are generally used to assess the quality of water (DWAF, 1996). In the North West Province, high levels of faecal indicator bacteria had been reported in rivers and other water sources (River Health Programme, 2005a, b; NWDACE, 2008). The use of other microorganisms as indicators of water quality has also been proposed (Stevens *et al*., 2003). One such group that is often overlooked is yeasts.

The occurrence of yeasts in different types of aquatic environments has been reported. Such environments included lakes and ponds (van Uden & Ahearn, 1963; Sláviková & Vadkertiová, 1997; Medeiros *et al*., 2008), estuaries, coasts and mangrove areas (Van Uden & Fell, 1968) as well as oceans and the deep sea (Nagahama *et al*., 2001, 2003b; Gadanho *et al*., 2003; Libkind *et al*., 2003). Although yeasts have been studied in aquatic environments, the presence of yeasts in river water remains largely unexplored (Sláviková & Vadkertiová, 1997; Medeiros *et al*., 2008).

Water pollution places consumers at risk of contracting waterborne diseases (Pereira *et al*., 2009). To protect consumers from waterborne diseases, it is important to ensure that water is completely free of pathogenic and potentially pathogenic organisms (Pereira *et al*., 2009). The focus is still largely on protozoan, bacterial and viral pathogens (Stevens *et al*., 2003). However, more than 100 yeast species identified as human pathogens have been isolated from water (Fromtling *et al*., 2003). Most of these pathogens are from the genus *Candida*. Hurley *et al*. (1987) listed these particular pathogenic yeasts that cause candidosis in probable descending order of virulence: *C. albicans*, *C. tropicalis*, *C.*

stellatoidea, C. glabrata, C. krusei, C. parapsilosis, C. guilliermondii, C. viswanathii, Clavispora lusitaniae (Candida lusitaniae) and *Rhodotorula mucilaginosa (Rh.rubra)*. However, only a few *Candida* species are highly virulent.

Awareness has been raised by recent studies, on the presence of yeasts in both surface and groundwater (Nagahama, 2006; Pereira *et al*., 2009). Several studies have focused on the occurrence of yeasts in wastewater (Hagler & Mendonça-Hagler, 1981; More *et al*., 2010). These studies have demonstrated that yeast counts may be a potential monitoring method that may complement coliform counts reflecting the eutrophication potential of water (Hagler & Ahearn, 1997). High levels of yeasts in water sources could be an indication of either heavy or minimal pollution, depending on the type of yeasts present in the specific water source (Woollett & Hendrick, 1970; Simard, 1971). Even so, compared to bacteria and viruses, yeasts are receiving little attention when the quality of water systems is at stake (Arvanitidoua *et al*., 2002). Research is needed on the occurrence and diversity of waterborne yeasts in relation to water quality.

Yeast identification, which was previously based only on conventional identification methods, has undergone significant transformation over the last two decades due to the increase in basic biological knowledge as well as interest in the practical applications and biodiversity of this microbial group (Yarrow, 1998). In the past, yeasts were mainly identified and classified based on their morphology, sexual structures, biochemical testing such as the API ID 32C test strip, as well as physiological properties and others such as the diazonium blue b (DBB) test. Molecular methods such as 26S rDNA sequencing are now also being used due to their speed and accuracy (Yarrow, 1998).

1.2 RESEARCH AIM

The aim of this study was to determine the diversity and characteristics of yeasts in water sources of the North West Province and to discuss their implications.

1.3 THE OBJECTIVES OF THIS STUDY WERE:

1) to determine the physico-chemical characteristics of water at sampling period.

2) to isolate and compare the prevalence and diversity of yeasts from various water sources in the North West Province, South Africa.

3) to identify isolates using biochemical and 26S rRNA gene sequencing data.

4) to determine the ability of isolates to grow and survive at various temperatures.

CHAPTER 2

LITERATURE REVIEW

2.1 GENERAL CHARACTERISTICS OF YEASTS

As of 2006 researchers had described *circa* 1500 yeast species, which are estimated to constitute about 1% of species present in the kingdom of Fungi (Kurtzman & Fell, 2006; Kurtzman & Piškur, 2006). Yeasts are unicellular eukaryotic micro-organisms, although some species with yeast forms may become multicellular through the formation of a string of connected budding cells known as pseudohyphae, or false hyphae, as often seen in most molds (Kurtzman & Fell, 2005a).

Yeasts are distributed among several phylogenetic groups of fungi and are classified into two groups, Ascomycetous and Basidiomycetous yeasts (Gadanho *et al*., 2003). Ascomycetous yeasts produce ascospores within a naked ascus, whilst basidiomycetous yeasts form basidiospores outside the basidium (Barnett *et al*., 2000). Macroscopically, the yeasts can be divided into two groups based on their colony pigmentation. One group includes species that produce pink, salmon coloured or reddish colonies, with the exception of a few cases, the vast majority belongs to the basidiomycetous yeasts. The other group includes species forming white or cream-coloured colonies and are both classified into the ascomycetous and basidiomycetous yeasts (Gadanho *et al*., 2003).

Yeasts are differentiated from bacteria by their larger cell size and their oval, elongate elliptical or spherical cell shapes. Typical yeast cells range from 5 to 8 µm in diameter, although yeasts can have cells reaching over 40 µm in some cultures (Walker *et al*., 2002). In older yeast cultures cells tend to grow smaller in size (Babjeva and Reshetova, 1998).

2.2 SIGNIFICANCE OF YEASTS

Yeasts are noteworthy members in many ecosystems because they provide a significant contribution to the biodiversity of nature (Fleet, 1998). They generally have a medical, agricultural, and economic importance (Fell *et al.*, 2001). A wide range of useful secondary metabolites may be produced by yeasts (Hierro *et al*., 2004). These metabolites include enzymes, vitamins, capsular polysaccharides, carotenoïds, polyhydric alcohol, lipids, glycol lipids, citric acid, ethanol, carbon dioxide and antibiotics. Some of these products are important commercially, while others are potentially valuable in biotechnology (Hierro *et al*., 2004).

A study conducted in Canada, by Punja & Utkhed (2003) reported that fungi and yeasts may be used as biological control agents in managing vegetable crop diseases. Techniques in biotechnology are becoming increasingly applicable to studies on the biological control of plant diseases using fungi and yeasts. These techniques have helped to clarify mechanisms of action and have provided methods to evaluate the extent to which these agents may spread and survive (Punja & Utkhed, 2003).

Yeasts have been applied as indicators of sewage contamination and recreational water quality as a complement for the coliform and faecal *Streptococcus* counts used as indicators of recent fecal pollution (Ahearn, 1998). According to Hagler (2006) when a yeast species is consistently associated with a particular microhabitat, such as faeces of warm-blooded animals, it may indicate an influence of that source material in other segments of our lives. Another reason may be that these yeast species may be typical of habitats in the pristine state and have decreased populations when the habitat is perturbed (Hagler, 2006). All these situations combined with their easy cultivation and pigment formation of pink yeasts makes them good targets for application as indicator organisms. Simard (1971) and Simard & Blackwood (1971) therefore proposed that the total count of pink pigmented yeasts could be used as a water quality indicator.

The generation of electricity in microbial fuel cells, and production of ethanol for the biofuel industry may also be done using yeasts (Halme & Xia-Chang, 2007). Several yeasts, particularly *Saccharomyces cerevisiae*, have been widely used in genetics and cell biology. This is largely because the cell cycle in a yeast cell is very similar to the cell cycle in humans, and therefore the basic cellular mechanics of DNA replication, recombination, cell division and metabolism are comparable (Halme & Xia-Chang, 2007).

Nevertheless, yeasts may also be harmful (Hierro *et al*., 2004). The potential role of yeasts in environmental ecology as agents of pollution is also known (Nagahama, 2006). Yeasts also play a role in bioremediation (Kwon *et al*., 2002) or biological pest control (Punja & Utkhed, 2003) as well as being central components of some industrial processes such as fermentation of beverages (Lodolo *et al*., 2008) and food processing (Viljoen *et al*., 2003; Romano *et al*., 2006) that is well documented (Brakhage & Turner, 1995; Pöggeler, 2001).

2.3 ISOLATION AND GROWTH OF YEASTS

Yeasts are saprophytic, and unable to carry out photosynthesis or nitrogen fixation. This means, they require carbon and nitrogen sources for growth (Boundy-Mills, 2006). Yeasts require a number of vitamins, minerals and other growth factors to sustain growth and viability (Boundy-Mills, 2006).

Yeasts are generally grown at temperatures close to that of their natural habitat. Many species isolated in temperate zones grow well at 20°C to 25°C, and poorly at 30°C. A few psychrophobic yeasts, isolated from warm-blooded animals, require incubation temperatures above 30°C (Travassos & Cury, 1971).

The isolation medium for yeasts varies between the investigations performed (Hageskal *et al.*, 2009). Media used for isolation and enumeration are generally complex and nutritionally rich (Boundy-Mills, 2006). Common ingredients include a carbon source e.g., glucose, a nitrogen source, such as a digested protein (e.g., peptone), and a complex supplement that include yeast extract, malt extract). Malt extract was one of the first media developed for the brewing industry. This media was then modified by (Wickerham, 1951), by including yeast extract and peptone, resulting in yeast extract-malt extract (YM)

medium. Most investigators prefer media consisting of malt extract, yeast extract, peptone and glucose (Hagler & Ahearn, 1987). This medium is still one of the commonly used mediums for isolation and maintaining yeasts. There are also other types of mediums used by various researchers, such as Potato-dextrose-agar (PDA), Sabouraud-dextrose-agar (SDA) (Hapcioglu *et al*., 2005).

2.4 YEAST REPRODUCTION

The most common form of reproduction of yeasts is achieved asexually by vegetative growth, which constitutes budding (Yeong, 2005). Budding involves each cell forming a bud on the surface of the cell wall which gradually enlarges into a daughter cell containing identical chromosomes to the parent cell. Under ideal conditions, such as the availability of sufficient supply of nutrients, the formation of a new yeast cell takes place every four hours. During unfavourable conditions such as drought or starvation periods, yeasts prefer to reproduce by forming spores (Yeong, 2005).

These reproduction methods above comprise of observation of asexual structures, which include shape and size of the vegetative cells (Yeong, 2005). Then the mode of conidia formation is investigated and provides information, which aids in the identification of a strain. Budding starts by forming a small outgrowth at some point on the surface of the cell without the cell changing in size. The increase in size is seen in a newly formed bud, which eventually separates from the parent cell. Various types of budding can be measured among members of yeasts. One type of budding namely, holoblastic budding result from outgrowth of the entire cell wall of the parent cell, the bud separates from the narrow base leaving a scar through which no further budding occurs. This type of budding is characteristic of the *Saccharomycetales* and their anamorphic states while enteroblastic which is characteristic of basidiomycetous yeasts results in formation of a collaret due to recurrent formation and abscission of a succession of buds (Yarrow, 1998). The position and the site of bud formation facilitate classification. This can either be monopolar budding which refers to buds arising at one pole of the cell whereas involvement of both poles result in a bipolar budding, which is characteristic of the apiculate yeasts (Yarrow, 1998). Fission is the type of reproductive method whereby duplication of vegetative cells involves growth of the septum inwards from the cell wall dividing the long axis of the cell. The

fission cells that are newly formed are termed arthroconidia (arthrospores). Normally, they elongate and the process is repeated. Recurrent fission by a cell results in transverse multiple scars or annelations that are characteristic of *Schizosaccharomyces* (Yarrow, 1998).

2.5 MEDICAL IMPLICATIONS OF YEASTS

Even though yeasts are commonly commensals, they are known to cause invasive fungal infections among the immuno-deficient patients (e.g. neonates and old aged) and immuno-compromised individuals (e.g. HIV infected) (Fauci *et al*., 2008). The epidemiology of infections of yeasts is rapidly evolving and non-albicans *Candida* species and other rare yeasts have emerged as major opportunistic pathogens (Miceli *et al*., 2011). A study by Horn *et al*. (2009) showed that 54.4% of the prevalence of candidaemia was caused by non-albicans *Candida* species (Horn *et al*., 2009). Nevertheless, other yeasts that are less common than *Candida* have also been associated with life-threatening infections in immunocompromised hosts (Girmenia *et al*., 2005; Muñoz *et al*., 2005; Riedel *et al*., 2008). Other species such as *Cryptococcus laurentii* and *Cryptococcus albidus* have been associated with this life-threatening infection in immunocompromised hosts (Khawcharoenporn *et al*., 2007).

The genus *Candida* has been identified to contain some of the most clinically prevalent fungal pathogens responsible for causing nosocomial fungemia among immuno-compromised and immuno-depressed individuals (Maganti, 2011). Although this genus possesses over 50 human pathogenic fungal species, traditionally researchers have observed *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. glabrata*, *C. metapsilosis*, *C. orthopsilosis*, *C. krusei*, and C. *guilliermondii* to be the most clinically prevalent *Candida* species with *C. albicans* causing the bulk of infections (Fauci *et al*., 2008; Miceli *et al*., 2011). Its members are biologically diverse including yeasts with both ascomycetous and basidiomycetous affinities. Many *Candida* species are normal commensals of humans, frequently inhabiting the oral mucosal surface, the gastrointestinal tract, the urogenital tract, and the skin (Gudlaugsson *et al*., 2003; Hajjeh *et al*., 2004). However, many of these organisms are capable of causing infections, primarily in patients with compromised immunity (Hajjeh *et al*., 2004). The versatility in adaption to various different habitats, and

the formation of biofilms that increases its ability to adhere to surface and cause infections is one of the main reasons for candida's virulence (Ramage *et al*., 2005).

The yeast species *Candida albicans* is perhaps the most notorious of the yeast inhabitants of the human body, responsible for the affliction candidiasis, which may take many forms (Volk, 1999). Through normal health and hygiene, *Candida* is held in check by the populous and benign bacterial residents of our skin and mucous membranes. But in instances of compromised health, *Candida albicans* may result in skin sores such as thrush, urogenital tract infections such as vaginitis, and endocarditis (heart muscle infection), inflammation of the spleen, liver, kidneys and lungs. Individuals with AIDS are particularly susceptible to *Candidiasis* (Volk, 1999).

Another genus that has been implicated to act as a pathogen belongs to the genus *Cryptococcus. C. neoformans* and *C. gattii* are known pathogens and have been associated with infections. In a study by Khawcharoenporn *et al*. (2007) it was reported that cryptococcal infections have been reported in immunosuppressed patients, especially those with advanced HIV infection and patients with cancer who are undergoing transplant surgery and concluded that *Cryptococcus laurentii* and *Cryptococcus albidus* caused 80% of cases. However, *Cryptococcus curvatus*, *Cryptococcus humicolus*, and *Cryptococcus uniguttulatus* have also been associated with opportunistic infections in humans (Khawcharoenporn *et al*., 2007).

The genus *Rhodotorula* was previously regarded as non-pathogenic, but in recent years they have been emerging as opportunistic pathogens with the ability to colonise and infect susceptible patients, particularly in immunocompromised patients. *Rhodotorula* infections are mostly fungaemia associated with meningitis, endocarditis and catheter-associated infections (Tuon & Costa, 2008). The most common cause of fungaemia by *Rhodotorula* species is *Rhodotorula mucilaginosa* (also known as *Rhodotorula rubra*) followed by *Rh. glutins* and *Rh. minuta* (Pfaller *et al*., 2010; Tuon & Costa, 2008).

Candida pelliculosa (teleomorph *Pichia anomala* previously called *Hansenula anomala*-genbank anamorph *Candida beverwijkiae*) is a yeast frequently found in various fruits, tree exudates, soil, vegetables and other organic compounds (De Hoog *et al*., 2000). It has occasionally been reported as the causative agent of nosocomial fungemia in both immunocompetent and immunocompromised pediatric patients (Aragão *et al*., 2001). Outbreaks of *Pichia anomala* (also known as *Hansemla anomala*) have been reported in neonatal and paediatric intensive care units (Pasqualotto *et al*., 2005).

2.6 YEASTS IN WATER SOURCES

Yeasts are common inhabitants of freshwater and seawater, including rivers, lakes and ponds as well as estuaries (Deák & Beuchat, 1996). The distribution of yeast species in various water sources is different with numbers that vary quite widely, from a few cells/L in unpolluted water to more than a million/L in effluents. Thus, the number and density of species occurring depend on the type and quality of water (Hagler & Ahearn, 1987; Lachance & Starmer, 1998). A study by Hagler & Mendonça-Hagler (1981) shown that the number of yeasts increases in the presence of pollution or in the presence of algae, and it may reach a few thousand cells per liter or more.

Yeasts in water sources have been well-researched in many parts of the world except South Africa. The occurrence of yeasts in water has been studied in various parts of the world (Jensen 1963; Dmitriev *et al.*, 1997). In the North West Province (NWP) of South Africa, however, yeasts in local water sources have not been extensively studied. Reports have shown that the number and composition of yeasts populations present in rivers and lakes can be used as organic enrichment indicators in water bodies (Rosa *et al*., 1995; Morais *et al*., 1996). Early studies on yeasts in association with polluted water were mainly focused on their application as organic pollution indicators (Nagahama, 2006). Woollett & Hendrick (1970) performed a study in Chicago, where they investigated the association between heavy industrial waste and heavy domestic waste pollution and yeast levels. These researchers found that polluted waters had large yeast populations ranging as high as 270 000 cfu/L. The yeasts found related to these pollutions were the following: *Rhodotorula* spp., *Cryptococcus* spp. and *Candida* spp. (Woollett & Hendrick, 1970). Another study in

Canada, based on yeasts as an indicator of pollution, was also conducted by Simard (1971). Species within the genera *Cryptococcus, Debaryomyces* and *Rhodotorula* are characteristically found in non-polluted waters, while *Candida* and *Saccharomyces* species may frequently be found in eutrophic waters (Rosa *et al*., 1995). The use of pink yeasts as a pollution indicator was encouraged by Simard & Blackwood (1971). However, pink yeasts do not appear to represent a consistent proportion of the yeast population in most studies, nor have they been correlated with a more specific factor of pollution.

2.7. WATER QUALITY IN SOUTH AFRICA

South Africa's inland water resources are the rivers, dams, lakes, wetlands and subsurface aquifers, which together with natural phenomena such as rainfall and evaporation as well as anthropogenic influences such as abstraction and discharges form the hydrological cycle that controls the quality and quantity of our inland waters (Otieno & Ochieng, 2004). Yet many of the rivers are small and flow only during the wet season. Deteriorating water quality is one of the major threats to South Africa's capability to provide sufficient water of appropriate quality to meet its needs and to ensure environmental sustainability (RSA DWAF, 2002). These conditions will put pressure on the already stressed water systems leading to a decrease in water availability, a situation likely to result in increase in conflicts over water allocation (Otieno & Ochieng, 2004).

The South African Water Quality Guidelines, describes the term *water quality* "as the physical, chemical, biological and aesthetic properties of water that determine its fitness for a variety of uses and for the protection of the health and integrity of aquatic ecosystems" (DWAF, 1996; DWAF, 2006). The Department of Water Affairs and Forestry (1996) defines the Target Water Quality Range (TWQR) for a particular element and water use as the range of concentrations or levels at which the presence of the element would have no known adverse effects on the fitness of the water assuming long-term continuous use. In the report of the National Water Research Strategies factors influencing water quality may either be natural or result from human activity (NWRS, 2004). One of the main natural factors is the geology of the formations over which water flows or through which it infiltrates, which then give rise to the sediment load and mineralization of the water. A second natural factor is vegetation. Both natural and human factors can influence

the quality of a water source, and therefore it is necessary to identify the factors involved that individually or jointly affect the quality of the water source.

2.8. WATER PHYSICO-CHEMICAL PARAMETERS

The physico-chemical parameters of water need to be measured in order to determine the water quality. Parameters such as pH, temperature, total dissolved solids (TDS) and the electrical conductivity (EC) are usually determined. Various chemical parameters such as nitrite (NO_2 -N) and nitrate (NO_3 -N) content of the water are also included. A water quality variable is an attribute or a constituent that vary in magnitude and whose variations alter water quality (Dallas & Day, 2004).

2.8.1. pH of water

According to Dallas & Day (2004) pH is determined largely by the concentration of hydrogen ions (H^+) and alkalinity by the concentration of hydroxyl (OH^-), bicarbonate (HCO_3^-) and (CO_3^{2-}) ions in water.

The effect of changes in pH on water chemistry may be dramatic. The pH of a water sample indicates the particular chemical species of which many elements are found in the sample (Dallas & Day, 2004). It may also indicate the presence and/or toxicity of metals in the water. Since the adsorptive properties of large molecules, such as polyphenolics and of particulate material water depend on their surface charges, altering the pH may also alter the degree to which nutrients such as PO_4^{2-}, trace metals and biocides adhere to these materials. Such an effect is of particular significance where lowered pH can lead to the release of toxic substances from sediments (Dallas & Day, 2004).

Human-induced acidification of aquatic ecosystems is normally the results of one of three different types of pollution. Firstly, the production of chemical, pulp and paper and tanning/leather industries that causes low-pH point-source effluents. Secondly, mine drainage water is nearly always exceedingly acid with the pH of receiving streams sometimes droping to < 2. Finally, air pollution can result in acidic precipitation. The target water quality range of pH for environmental use as set out in the DWAF guidelines (1996) (Appendix, Table1B) is 6.0 – 9.0 for recreation and 6.5 - 8.5 for irrigation.

2.8.2. Water Temperature

According to Dallas & Day (2004) the thermal characteristics of running water are dependent on various hydrological, climatic and structural features of the region and catchment area. The minimum and maximum temperatures, and temperature ranges vary depending on the factors mentioned above and except for birds and mammals all organisms associated with fresh water are piokilothermic. This means they are unable to control their body temperatures, which are therefore the same as that of the ambient water (Dallas & Day, 2004).

These animals and plants are very susceptible to changes in water temperature since a 10°C increase results in a doubling of the organism`s metabolic rate. Changes in water temperature that are unrelated to natural variations may have an effect at the organism, species and /or at community level (Dallas & Day, 2004). The temperature changes in river systems may also be caused by anthropogenic activities that include those resulting from thermal pollution, stream regulation and changes in riparian vegetation (Dallas & Day, 2004).

2.8.3 Total Dissolved Solids (TDS) and Electrical Conductivity (EC) of water

Dallas & Day (2004) defines total dissolved solids (salts) concentration, as a measure of the quantity of all compounds dissolved in water carrying an electrical charge (DWAF 1996). The TDS concentration is directly proportional to the electrical conductivity (EC) of water. Electrical conductivity (EC) is a measure of the ability of water to conduct an electrical current: the higher the conductivity, the greater the number of ions, and thus also the dissolved concentration of salts, such as carbonate, bicarbonate, chloride, sulphate, nitrate, sodium, potassium, calcium and magnesium, all of which carry an electrical charge (Dallas & Day, 2004). A measure of conductivity does not include un-ionized solutes, such as dissolved organic carbon (Dallas & Day, 2004).

In the North West Province (NWP) regions exist where the concentration of TDS is high, this is due to natural causes as well as human impact (NWDACE, 2008). The natural causes might be that of the geology of the NWP, which consist of yellow shifting sands (NWPG, 2002). The standard for irrigation use as set out in the DWAF guidelines (1996) is 260mg/L.

2.9 WATER SITUATION IN THE NORTH WEST PROVINCE

The North West Province (NWP), is situated at the centre of the northern border of South Africa, and shares borders with Botswana to the north and four of the other South African provinces: the Northern Cape Province to the south-west, the Free State to the south, the Gauteng Province to the south-east, and the Limpopo Province to the east and north-east (NWDACE, 2002). The NWP is regarded as a water stressed province, with an average rainfall of less than 500 mm per year (Ashton & Haasbroek, 2002). The state of the water resources in the NWP is characterised by an overall scarcity of water as many surface water systems are non-perennial and decreases from east to west (NWDACE, 2008). The three major catchments are the Limpopo, Vaal and Crocodile rivers. The Lower Vaal River forms the southern border of the NWP. The Harts River, Schoonspruit and Mooi River run through a large part of the province and are tributaries of the Vaal River. Water quality for these surface water sources is variable. Undesirable impacts include mining, agriculture, surface run-off, non-compliant waste water treatment facilities as well as sanitation back-logs (NWDACE, 2008).

The mining, industrial and agricultural activities of the land locked NWP contribute greatly to the economy of this province, and South Africa in general. This comes at a cost of environmental impacts (Cho *et al*., 2000), including source water pollution (Kalule-Sabiti & Heath, 2008). Pollution from such economic activities could have long term adverse effects on the health of the population of the North West Province, particularly those that are less affluent.

In the report of the State of the Environment (2002), surface water resources were defined as rivers, dams, pans, wetlands and dolomitic eyes fed by underground water sources. Underground water resources also include water which flow between soil pore spaces and within underground rock formations. In the NWP, ground and surface water are integrated and interdependent, as groundwater flowing to the surface at dolomitic eyes or springs is the source of several major rivers within the boundaries of the NWP, such as Groot Marico, Mooi, SchoonSpruit and Molopo. As a result, water quality and quantity issues affecting groundwater also have implications for surface waters.

2.10 GENERAL METHODS USED IN THE ISOLATION OF YEASTS FROM WATER SOURCES

2.10.1 Sampling methods for yeasts

The sampling methods used for yeast isolation do not differ fundamentally from those of bacteria (Nagahama, 2006). This is because the frequency of yeasts in natural aquatic environments is lower than that of bacteria; especially in places with low nutrient conditions a higher volume of samples is required. Sterile bottles, boxes or containers have been used in various samplings from such accessible sites as the surface or shallow regions of freshwater environments.

2.10.2 Isolation and enumeration of yeasts in aquatic environments

The isolation procedure varies depending on the yeast density, volume and shape of the source and the source itself such as water, sediment, animal or plant material (Nagaham, 2006). Yeast cells in water were mostly filtered through membranes and then used for isolation. Organic contents including yeast cells have also been collected from water by using a precipitant instead of filtration (Sláviková & Vadkertiová, 1995, 1997). Boundy-Mills (2006) confirms that there are various methods for the enumeration and detection of yeasts. In the past, surveys of yeasts were only possible through plating.

Media used for detection of various yeasts are as follows: yeasts extract-malt extract agar (YMA), Clinical yeast isolations are often performed using Sabourauds glucose agar (SGA) (Odds, 1991). Rich commercially available media used for the cultivation of yeast includes yeast extract, peptone, and dextrose (YEPD), potato dextrose agar (PDA) and

tryptone-glucose-yeast extract agar (TGYA). As reviewed in many articles the temperature and pH of the media are often adjusted to prevent the growth of bacteria and other fungi as competitors against yeasts (Morris, 1968; Hagler & Ahearn, 1987). Recent investigators prefer to use chloramphenicol as an antibibiotic to suppress the coexistent bacterial population (Sláviková & Vadkertiová, 1995; Nagahama *et al*., 2003). Malt extract medium, an early yeasts medium formulation, was developed for the benefit of the brewing industry. Wickerham modified this medium by including yeast extract and peptone, resulting in yeasts extract-malt extract (YM) medium (Wickerham, 1951). It is still commonly used for maintaining and storing yeasts cultures (Wickerham, 1951). Several formulations of rich media are used by researchers in various fields of yeasts.

2.10.3 Morphological and biochemical methods for identification of yeasts

The Diazonium blue B (DBB) test is also conducted to confirm the affinity of a strain, whether it belongs to ascomycetes or basidiomycetes. Ascomycetes normally produce no colour while basidiomycetes produce a dark red or purple colour after staining of cells by drops of DBB solution (Motaung, 2011).

The analytical profiling index (API) ID 32C is used to classify and identify yeast isolates based on biochemical fermentation reactions. These API ID 32C systems are standardized identification systems used to identify yeast and are ideal for the identification of yeasts (Ramani *et al*., 1998). This system consists of a single-use disposable plastic strip with 32 wells containing substrates for 29 assimilation tests (carbohydrates, organic acids, and amino acids), one susceptibility test (cycloheximide), one colorimetric test (esculin), and a negative control (Ramani *et al*., 1998). The substrates are rehydrated by means of a yeast suspension in inoculums fluid. The strips are then visually examined, and growth was determined to be positive or negative based upon the presence or absence of turbidity in the wells. The results are transformed into numerical biocodes, and the isolates identified through the use of the ID 32C Analytical Profile Index (Ramani *et al*., 1998).

2.10.4 Molecular methods for identification of yeasts

Molecular characteristics are important to properly assign strains, species and to an extent, genera to their respective phylogenetic groups. Confirmation of identities and characterisation of yeast can be done with the analysis of 26S rDNA genes, which is aided by using PCR to amplify target sequences (Kurtzman & Robnett, 1998; Guffogg *et al.*, 2004,). Analysis of 26S rDNA fragments is useful for identification of eukaryotes (Ramani *et al*., 1998). These are amplified using two universal and two species-specific primers derived from the D1/D2 region of the 26S rDNA that allows for rapid and accurate species identification (O'Donnell, 1993). The sequences of these amplicons are determined and then BLASTN searched against GenBank (http://www.ncbi.nlm.nih.gov/BLAST.cgi). A query submitted to BLASTN search results in a list of sequences in the database which are judged as related to the specific target sequence. "Bit scores" and "E-values" are statistical values used to evaluate the relevance of the sequence matches. BLASTN presents related sequences in descending order according to bit scores. The higher the bit score, the more closely the sequence is related to the target sequence. E-values act as an estimate of the chance occurrence of identified matches in the database. Smaller E-values indicate higher levels of confidence that similarities between two sequences are more likely caused by common descent than by chance (Ogunseitan, 2005).

Another approach called Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP), which characterises the endonuclease restriction sites of PCR products, has also proven to be useful to identify yeasts. Molecular methods such as nuclear DNA (nDNA) reassociation studies have also been successful employed in the characterization of yeasts (Yarrow, 1998).

Also, the analysis of restriction fragment length polymorphism of the ITS region allows for detection and quantification of different yeast species (Querol & Ramon, 1996). One of the most common approaches is ribosomal DNA sequencing, which focuses on the D1/D2 domain sequence of the 26S rDNA gene (Kurtzman & Fell, 1998). However, progress in molecular biology has provided a large number of DNA-based techniques for identifying and characterising yeasts (Hierro *et al*. 2004). The sequencing of the D1/D2 domain is increasingly being used to identify yeasts (Phaff *et al.*, 1999; Hong *et al.*, 2001; Scorzetti *et*

al., 2002) and according to Frutos *et al.* (2004) it is accepted universally as the main tool for yeast taxonomy. This method has enabled identification of new ascomycetous yeasts in the *Pichia anomala* clade previously not recognized as novel when conventional identification techniques were used (Kurtzman, 2000). Kurtzman & Robnett carried out a comprehensive study in 1998 on all known ascomycetous yeasts. This study involving 760 strains, representing 500 species, was based on sequence analysis of approximately 600 bases of the D1/D2 domain of the 26S subunit. Fell *et al*., (2000) was the first to report on the D1/D2 sequences of known basidiomycetous yeasts. This extensive available database makes the task of species identification much easier (Starmer *et al.*, 2001; Wesselink *et al.*, 2002) and could serve as reliable and practical criteria for identification of most known yeasts (Abliz *et al.*, 2004).

Literature presented covered the general characteristics, diversity and importance of yeasts. An overview of yeasts in water sources was also addressed. The quality of water in South Africa and in particular the situation in the NWP and possible physico-chemical parameters that influence water quality were highlighted. In addition, various and relevant methods to isolate, characterise and identify yeasts from aquatic environments were also covered.

CHAPTER 3

MATERIALS AND METHODS

3.1 STUDY AREA AND SAMPLING

Water samples were collected from 3 Rivers (Lower Vaal, Mooi and Harts Rivers), the Schoonspruit and an inland lake, Barberspan in the North West Province (NWP), South Africa. For the Harts and Vaal Rivers sampling was done once during summer and once during winter. Barberspan and Schoonspruit were also sampled once during summer and winter. The Mooi River was only sampled once during the summer of 2010. In 2010 samples were collected from 23 sites and in 2011, 21 sites. All these samples were grab samples taken from bridges. Photos of these sample sites are provided in Appendix D. Global positioning system (GPS) coordinates of sites were determined by using a Garmin Nüvi 1310 GPS (Garmin, US) hand held unit. These GPS coordinates (Appendix A; Table 1A) were used to construct the map of sample areas plotted in Figure 3.1.

Surface water samples were taken using sterile bottles of 1L and were placed on ice in a cooler box, transported to the laboratory and analysed (mostly) within 6 hours of collection. When this was not possible analysis was done at least within 12 hours. Water temperature (WT), pH and electrical conductivity (EC) were recorded on site, using a Oakton PCStestr^{TM} 35 multimeter (Thermo Fisher Scientific, US) according to instructions of the manufacturer. Nitrate (NO_3-N) (Cat No: 21061-69), nitrite (NO_2-N) (Cat No: 21071-69) and phosphates (PO_4^{2-}) (Cat No: 2236-32) levels were determined in the laboratory using protocols, reagents and a spectrophotometer from Hach (Hach, Germany).

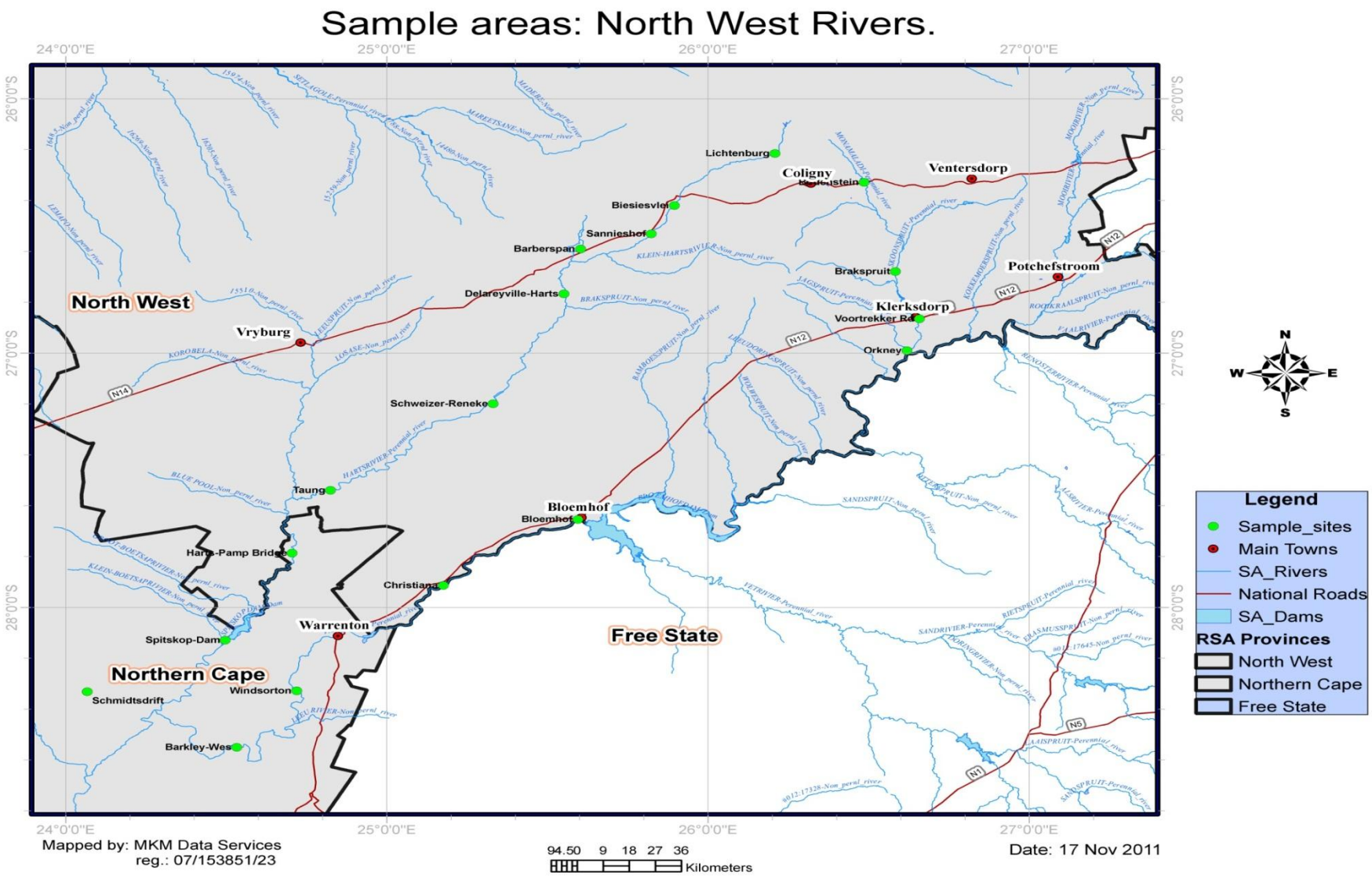


Figure 3.1: Map of the North West Province (NWP) and Northern Cape (NC). The different sampling sites are indicated by green dots.

3.2 ISOLATION AND ENUMERATION OF YEASTS

Yeast-malt-extract agar (YM) (10 g/l glucose, 3 g/l malt extract, 3 g/l yeast extract, 5 g/l peptone and 15 g/l agar) (Wickerham, 1951), supplemented with 100 ppm chloramphenicol pH 5.5 was used for isolation of yeast. This was achieved by the membrane filtration method (Clesceri *et al*., 1998). Fifty milliliters of water was filtered through 0.45 µm membrane filter (Pall Corporation, US). Each sample was analysed in duplicate. These membranes were then placed onto YM agar plates, and incubated at 25°C. The formation of yeasts colonies were examined daily during a 5 day period. Isolates were purified by using the streak plate method (Wickerham, 1951).

3.3 MORPHOLOGICAL AND BIOCHEMICAL IDENTIFICATION

Yeasts were preliminary classified based on morphological characteristics (Payne *et al*., 1998; Barnett *et al*., 2000). Various identification and characteristics tests were also performed.

3.3.1 Diazonium blue B (DBB)

The Diazonium blue B (Sigma-Aldrich, Germany) test was carried out to distinguish between ascomycetous and basidiomycetous yeasts (Kurtzman & Fell, 1998). Yeasts were grown as spot cultures on YM agar plates and incubated at 25°C for 7 days. Freshly prepared chilled DBB reagent (1-2 drops) was then applied directly to the surface of the colonies. A positive reaction for basidiomycetous yeasts was recorded when the colonies developed a dark red or violet red colour within 2 minutes when incubated at room temperature (Kurtzman & Fell, 1998). In the case of ascomycetous yeasts no colour development took place.

3.3.2 Biochemical identification

The isolates were further identified using the ID 32C system (bioMerieux, France). This system consisted of a single-use disposable plastic strip with 32 wells containing substrates for 29 assimilation tests (carbohydrates, organic acids, and amino acids), one susceptibility test (cycloheximide), one colorimetric test (esculin), and a negative control (Ramani *et al*., 1998). The yeast identification procedures were conducted in accordance with the manufacturer's instructions. Well-isolated colonies of each isolate was aseptically

transferred from a freshly inoculated stock culture to sterile distilled water and incubated at 30°C for 48 h (Ramani *et al*., 1998). The strips were visually examined. A positive result was based upon the presence of turbidity in the wells. Results were then transformed into a 10-digit numerical biocode. Identification of the isolates were obtained by using the apiweb^{TM} (bioMerieux, France) identification software (Ramani *et al*., 1998).

3.4 MOLECULAR IDENTIFICATION

3.4.1 Yeast genomic DNA isolation

Yeast isolates were inoculated in a 250 ml conical flask containing 50 ml of YM broth and incubated at 30°C for approximately 24 hours. Subsequently, genomic DNA of the overnight yeast cultures were extracted according to the modified method of Hoffman & Winston (1987). Two millilitres of the overnight culture was transferred into a microcentrifuge and centrifuged (12,000 x g) for 1 min. The supernatant was removed by means of aspiration. The cells were resuspended in 500 µl DNA lysis buffer (100 mM Tris-HCl at pH 8.0; 50 mM EDTA; 1% SDS). Two hundred microliters of glass beads were added to the suspension and the mixture vortexed for 4 min. Tubes were then placed on ice. The liquid phase was removed and transferred into a sterile 2 ml microcentrifuge tube. Two hundred and seventy five microliter of ammonium acetate (pH 7) was added. The tubes were then vortexed and incubated for 5 min at 65°C. This was followed by 5 min incubation on ice. Subsequently, 500 µl of chloroform was added, the tubes were then vortexed and centrifuged for 2 min at 14,000 x g (4°C). The supernatant was transferred to a fresh sterile microcentrifuge tube. DNA was precipitated with 750 µl isopropanol and incubated for 5 min at room temperature (RT). This was followed by centrifuging for 2 min at 14,000 x g (4°C). The pellet was washed with 70% (v/v) ethanol then dried and resuspended in 60 µl of nuclease free deionized water. The DNA solution was incubated at 37°C for 30 min prior to storage at -20°C.

3.4.2 PCR amplification

Polymerase chain reaction (PCR) was performed for amplification of 26S rDNA D1/D2 domain using primers NL1 (5'-GCATATCAATAAGCGGAGGAAA-AG-3') and NL4 (5'-GGTCCGTGTTTCAAGAC-GG-3) (O'Donnell, 1993). The PCR amplification was done in 25 µl reactions containing 12.5 µl 2x PCR Master Mix (0.05 U/µl *Taq* DNA Polymerase in reaction buffer, 0.4 mM of each dNTP, 4 mM $MgCl_2$) (Fermentas Life Science, US), 0.5 µl 26S rDNA primers as mentioned above (Fermentas Life Science, US), 10.8 µl PCR water (Fermentas Life Science, US), 0.2 µl *Taq* polymerase (Fermentas Life Science, US) and 1 µl DNA template (50-100 ng). Mixtures were briefly (3 seconds) centrifuged to ensure sufficient mixing of reagents before using the Bio-Rad C1000™ Thermal Cycler (Bio-Rad, UK) for amplification. The reaction mixtures were subjected to the following cycling conditions: 94°C for 300 seconds, followed by 36 cycles at 94°C for 60 seconds, 52°C for 35 seconds, 72°C for 60 seconds and a final step at 72°C for 600 seconds.

3.4.3 Agarose gel electrophoresis

PCR amplicons were then analysed by gel electrophoresis using a 1.5% (w/v) agarose gel (PeQlab, Germany) containing ethidium bromide (1 µl/ml; Bio-Rad, UK). A mixture of 5 µl PCR product and 5 µl of 6 x Orange Loading dye (Fermentas Life Science, USA) was loaded into each well. A 1 kb DNA molecular marker (O'GeneRuler, Fermentas Life Science, US) was used to confirm the fragment sizes. Electrophoresis was conducted using a mini-sub cell GT electrophoreses system (Bio-Rad, UK) for 45 minutes at 80 V, using 1 X TAE (40 mM Tris, 1 mM EDTA and 20 mM glacial acetic acid, pH 8.0) as electrophoresis buffer. Gel images were captured using Gene Genius Bio Imaging System (Syngene Synoptics, UK) GeneSnap (version 6.00.22) software. The image was analysed using GeneTools (version 6.08) software (Syngene, Synoptics, UK) to determine the size of the bands in each lane.

3.4.4 Sequencing of the amplicons

The sequence of D1/D2 domain of the large-subunit 26S rDNA for the representative isolates were sequenced by Inqaba Biotech (Pretoria, South Africa). The resulting sequences were visualised using Finch TV (version 1.4) software and compared to sequences obtained from GenBank. Sequences were aligned using CLUSTAL W version 1.6 (Tamura *et al*., 2011). Phylogenetic and molecular evolutionary analyses were conducted using MEGA 5 version 5.05 (Tamura *et al*., 2011). Neighbour-joining and Kimura two-parameter methods were used (Kimura, 1980). Confidence values were estimated from bootstrap analysis of 1000 replicates.

3.5 GROWTH TEMPERATURES OF YEASTS

To determine the ability of growth at various temperatures, purified yeast isolates were then streaked onto yeasts-malt-extract (YM) agar and incubated at temperatures 4°C, 25°C, 30°C, 37°C and 40°C. The plates were then visually inspected over a 7 day period for growth.

3.6 STATISTICAL ANALYSES

Average and standard deviation values were determined using Microsoft Excel 2007. In addition, STATISTICA 10 (StatSoft, Inc. US) was used to analyze the data. Analysis of variance (ANOVA) and Tukey`s honest significant difference (HSD) were used to determine the statistical significance between yeast levels at the various sampling sites (Zar, 1996). Furthermore, redundancy analysis (RDA) multivariate ordination technique (Canoco for Windows Version 4.0, GLW-CPRO ©; Ter Braak, 1990) was used to illustrate the relationship between the measured surface water physico-chemical characteristics and the species levels (pigmented and non-pigmented yeasts). The multivariate analysis as an ordination technique allows for the arrangement of sample points in a space with reduced dimensionality such that the axes used represent the greatest variability in the community structure. An ordination diagram was then used to view the distribution of sample points and was interpreted following the basic assumption that graphical proximity means close similarity.

CHAPTER 4

RESULTS

4.1 INTRODUCTION

In this chapter results obtained are presented for the 2010 and 2011 study periods. During 2010, sampling was done at 23 sampling sites and in 2011, at 21 sites. The physico-chemical results obtained during this study were compared to the Department of Water Affairs and Forestry Target Water Quality Ranges (TWQR) for domestic, recreation, live stock watering and irrigation as depicted in the Field Guide (DWAF, 1996) (Appendix B, Table1 B). TWQR for yeasts is unavailable.

4.2 DATA OBTAINED DURING 2010

4.2.1 Physico-chemical analyses

The physico-chemical parameters at the 23 sites are presented in Table 4.1. It is evident from this table that the physico-chemical parameters were higher when the water temperature was lower. However, there were some exceptions.

The pH of the sites ranged from 7.1 to 9.23 (Table 4.1). All sites from Barberspan and one sites from Lower Vaal River (Schmidtsdrift) had pH values that exceeded the target water quality range (TWQR) for recreation and irrigation (DWAF, 1996). Six sites from Mooi River, one from Harts River, one from Schoonspruit, four from Lower Vaal River, and three from the Barberspan had a slight alkaline pH (8.22 and 8.8). The surface water temperature ranged between 12°C and 26.1°C (Table 4.1). TDS levels measured ranged from 256 to 961 mg/L. All sites from Mooi River, Harts River, Schoonspruit, Barberspan and three sites from Lower Vaal River had TDS levels that exceeded DWAF target water quality range (DWAF, 1996) for some agricultural uses. All sites from Barberspan (115-147 mS) and two sites from the Harts River (135-165 mS) had elevated EC levels that exceeded DWAF target water quality range (DWAF, 1996) for domestic uses. Nitrates,

nitrites and phosphates measured values ranged from 0 to 4 mg/L, 0 to 19 mg/L and 0 to 2.44 mg/L, respectively.

Table 4.1: Physico-chemical parameters measured at each of the sampling sites at the respective surface water source during 2010 sampling period. Highlighted values exceeding TWQR.

Water Source	Different sites	pH	Temp (°C)	EC (mS/m)	TDS (mg/L)	NO_3-N (mg/L)	NO_2-N (mg/L)	PO_4^{2-} (mg/L)
Mooi River	Klerkskraal Dam	8.36	23.6	39	265	0.5	2	1.42
	Muiskraal Dam	8.01	23.1	47	306	0.2	0	0.86
	Around The World Bridge	7.76	23.0	70	466	0.6	4	0.68
	Thabo Mbeki Drive	8.22	24.3	64	427	0.1	3	0.81
	Trimpark North Bridge	8.25	24.6	65	427	0.4	3	0.02
	Pedestrian Bridge	8.25	25.0	66	443	0.3	9	0.12
	Viljoenskroon Road Bridge	8.11	26.0	61	406	0.1	11	1.8
Harts River	Dam 8	8.23	24.0	62	437	0	-	-
	Jan Kempdorp	7.9	26.1	165	720	-	19	-
	HRP Dam	7.1	24.1	135	960	3.5	2	1.7

Table 4.1 continued

Water Source	Different sites	pH	Temp (°C)	EC (mS/m)	TDS (mg/L)	NO_3-N (mg/L)	NO_2-N (mg/L)	PO_4^{2-} (mg/L)
Schoonspruit	Orkney	7.61	21.7	52	367	1.7	3	2.44
	Klerksdorp Bridge	7.74	22.5	39	280	0	2	0.41
	Brakspruit	8.05	21.1	52	381	0.3	3	0.55
	Bodenstein	7.91	20.4	60	432	0	4	0.63
Lower Vaal River	Windsorton	8.23	21.1	36	256	-	1	0
	Barkley-Wes	8.47	19.3	40	260	-	-	0
	Schmidtsdrift	8.8	18.7	40	284	0.2	8	0
	Christiana	7.86	19.0	36	256	0.4	4	0
	Bloemhof	8.05	18.0	37	265	-	3	0
Barberspan	Harts River	8.62	13.7	121	862	3.0	0.7	1.68
	Inflow	8.58	15.2	115	820	4.0	0	1.02
	Hotel	9.23	14.7	132	961	-	-	0.11
	Outflow	8.61	12.0	147	956	4.0	1.2	0.19

a – not measurable/detectable

4.2.2 MYCOLOGICAL ANALYSIS

4.2.2.1 Isolation of yeasts

The average yeast numbers, statistical differences in pigmented and non-pigmented yeasts levels between sites as well as the frequency (percentage) with which they were detected at the 23 sites are presented in Table 4.2. From this table it is evident that the non-pigmented yeast numbers detected was higher when the water temperature was high. However, there were some exceptions.

Yeasts were detected at 22 of the 23 sampling sites (Table 4.2). The number of yeasts in Mooi River ranged from 473 to 8,680 cfu/L. As shown in Table 4.2, the highest and lowest number of yeasts was detected at the Around The World Bridge and Thabo Mbeki drive sites, respectively. The numbers of pigmented yeasts detected at Klerkskraal Dam, Muiskraal Bridge and Trimpark North Bridge sites were lower (< 80 cfu/L) compared to non-pigmented yeast numbers. High numbers of non-pigmented yeasts were observed in 6 of the 7 sites of the Mooi River. These numbers ranged from 280 to 8,680 cfu/L. Pigmented and non-pigmented yeasts were both detected at three sites (Klerkskraal Dam, Muiskraal Bridge, and Trimpark North Bridge). The percentage non-pigmented and pigmented yeasts ranged from 2.4% to 100% (Table 4.2). However, no pigmented yeasts were detected at sites Around The World Bridge, Thabo Mbeki Drive, Pedestrian Bridge and Viljoenskroon Road Bridge.

Yeast numbers in the Harts River ranged from 3 to 33 cfu/L (Table 4.2). The highest and lowest number of yeasts were detected observed at HRP Dam and Jan Kempdorp sites, respectively. Jan Kempdorp was the only sampling site where pigmented yeasts were observed in relatively low numbers (< 3 cfu/L) compared to other sampling sites. Non-pigemented yeasts numbers were low (< 35 cfu/L) at all the sites.

In the Schoonspruit yeast numbers ranged from 300 to 1,466 cfu/L (Table 4.2). From Table 4.2 it is evidend that the highest and lowest number of yeasts were observed at Klerksdorp Bridge and Bodenstein, respectively. High numbers of pigmented yeasts were observed at all the sites and ranged from 300 to 1,066 cfu/L. Both pigmented and non-pigmented

yeasts were detected at three sites (Orkney, Klerksdorp Bridge and Brakspruit). The percentage non-pigmented and pigmented yeasts proportions ranged from 26.1% to 100% (Table 4.2). However, no non-pigmented yeasts were detected at the Bodenstein site. More than 70% of the yeasts from the Schoonspruit were pigmented.

The number of yeasts in the Lower Vaal River ranged from 10 to 568 cfu/L (Table 4.2). Pigmented yeasts were only observed at Schmidtsdrift and Bloemhof Dam sites but relatively low numbers (< 10 cfu/L). Non-pigmented yeasts numbers at all the sites ranged from 10 to 335 cfu/L. The percentage non-pigmented and pigmented yeasts ranged from 7.5% to 100% (Table 4.2).

Yeast numbers at Barberspan ranged from 5 to 85 cfu/L (Table 4.2). Pigmented yeasts were detected at Harts River prior to the inflow, at the Inflow and Outflow sites. Relatively low numbers of non-pigmented yeasts were detected at all the sites (< 5 cfu/L) in comparision with the other water sources. Percentage non-pigmented and pigmented yeasts ranged from 40% to 100% (Table 4.2).

Overall comparison of the total number of pigmented and non-pigmented yeasts clearly shows that the latter were detected in a much higher levels. The highest number of pigmented yeasts was found in the Schoonspruit (Klerksdorp Bridge; 1,066 cfu/L). The results from statistical analysis showed significant differences in pigmented yeast numbers between different sites and no significant difference in non-pigmented yeasts (Table 4.2).

Tukey's HSD revealed that there were no significant differences in terms of non-pigmented yeasts among the different sites (Table 4.2). Pigmented yeast levels in the Schoonspruit were significantly higher ($P<0.05$) than in all the other water sources. Sites from this River (Bodenstein, Brakspruit, Orkney), two from Mooi River (Klerkskraal Dam; Muiskraal Bridge), all sites from Lower Vaal River and Barberspan were significantly different ($P<0.05$) from Klerksdorp Bridge (Schoonspruit) site. In addition, pigmented yeast levels at Klerksdorp Bridge were significantly different ($P<0.05$) from all the other sites. Significantly different ($P<0.05$) pigmented yeast levels were observed when the results from Orkney, Brakspruit, Bodenstein, Muiskraal Bridge, Klerkskraal Dam, all sites

from Lower Vaal River, Barberspan and the rest of the Mooi River sites were compared (Table 4.2).

Table 4.2: Average yeast numbers (cfu/L) at various water sources of different sites indicating pigmented and non-pigmented yeasts distribution of 2010.

Water Source	Sites		Ave (cfu/L) (±SD)	Percentage (%)
Mooi River	Klerkskraal Dam	Pigmented	73.33±63.60 [a, b]	2.3
		Non-pigmented	3,066.67±1927.29 [a]	97.7
	Muiskraal Bridge	Pigmented	206.67±93.33 [a, b, c]	42.5
		Non-pigmented	280.00±23.09 [a]	57.5
	Around The World Bridge	Pigmented	0.00 [a]	0
		Non-pigmented	8,680.00±7811.56 [a]	100
	Thabo Mbeki Drive	Pigmented	0.00 [a]	0
		Non-pigmented	473.33±153.77 [a]	100
	Trimpark North Bridge	Pigmented	0.00 [a]	4.4
		Non-pigmented	2,266.67±1916.88 [a]	95.6
	Pedestrian Bridge	Pigmented	0.00 [a]	0
		Non-pigmented	0.00 [a]	0
	Viljoenskroon Road Bridge	Pigmented	0.00 [a]	0
		Non-pigmented	1,000.00±577.35 [a]	100
Harts River	Dam 8	Pigmented	0.00 [a]	0
		Non-pigmented	16.33±3.84 [a]	100
	Jan Kempdorp	Pigmented	1.67±0.33 [a]	66.7
		Non-pigmented	1.00±0.58 [a]	33.3
	HRP Dam	Pigmented	0.00 [a]	0
		Non-pigmented	33.33±8.99 [a]	100

Table 4.2 continued

Water Source	Sites		Ave (cfu/L) (±SD)	Percentage (%)
Schoonspruit	Orkney	Pigmented	646.00±26.63 [d]	58.0
		Non-pigmented	466.67±145.30 [a]	42.0
	Klerksdorp Bridge	Pigmented	1,066.00±67.00 [e]	72.7
		Non-pigmented	400.00±28.87 [a]	27.3
	Brakspruit	Pigmented	433.33±187.82 [c, d]	73.8
		Non-pigmented	153.33±54.87 [a]	26.1
	Bodenstein	Pigmented	300.00±11.55 [b, c]	100
		Non-pigmented	0.00 [a]	0
Lower Vaal River	Windsorton	Pigmented	0.00 [a, b]	0
		Non-pigmented	35.00±5.00 [a]	100
	Barkley-Wes	Pigmented	0.00 [a, b]	0
		Non-pigmented	10.00±5.00 [a]	100
	Schmidtsdrift	Pigmented	10.00±2.00 [a, b]	7.5
		Non-pigmented	122.50±2.50 [a]	92.5
	Christiana	Pigmented	0.00 [a, b]	0
		Non-pigmented	47.00±7.00 [a]	100
Barberspan	Harts River	Pigmented	20.00±20.00 [a, b]	41.0
		Non-pigmented	10.00±10.00 [a]	59.0
	Inflow	Pigmented	45.00±15.00 [a, b]	52.9
		Non-pigmented	40.00±40.00 [a]	47.1
	Hotel	Pigmented	0.00 [a, b]	0
		Non-pigmented	5.00±5.00 [a]	100
	Outflow	Pigmented	10.00 [a, b]	40.0
		Non-pigmented	15.00±5.00 [a]	60.0

[a]Values given are mean of each sampled site ± standard error

[b]Sites with the same combination of superscript alphabetic letters indicate no significant differences (P>0.05) among sites, while those with different letters indicate significant differences at P<0.05 (Tukey's HSD)

[c]Percentage pigmented and non-pigmented yeasts

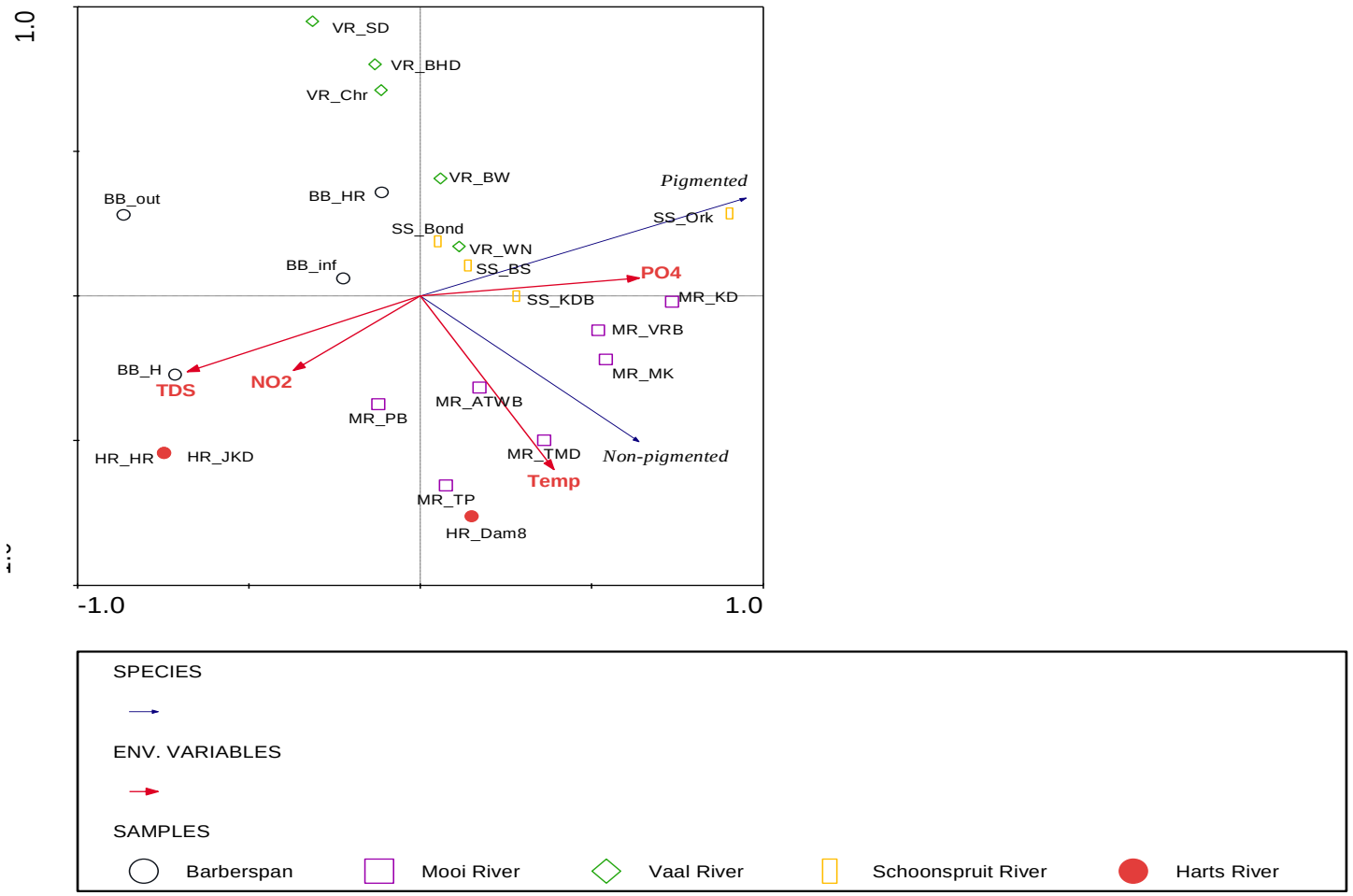


Figure 4.1: Redundancy analysis (RDA) ordination analysis illustrating the association between environmental variables and species (pigmented and non-pigmented yeasts). The red vectors represent the environmental parameters and blue vectors the species (pigmented and non-pigmented yeasts). Eigenvalues for the first two axes were 0.115 and 0.032 respectively.

A redundancy analysis (RDA) ordination diagram illustrating the association between environmental variables and species (pigmented and non-pigmented yeasts) is presented in Figure 4.1. The aim of this analysis was to directly relate the environmental factors to yeast level data. Water parameters including total dissolved solids (TDS), nitrites (NO_2-N), phosphates (PO_4^{2-}), and temperature (°C) (Table 4.1) were used in the RDA analysis since these environmental variables could have an influence on the species distribution. This association is demonstrated by the angle between variables and species. A small angle indicates a close relationship. Results obtained indicated a strong positive correlation between the pigmented yeasts and phosphates (Figure 4.1). It also indicated that Orkney, Klerkskraal Dam and Viljoenskroon Road Bridge sites were positively correlated to phosphates. The non-pigmented yeasts were positively correlated to temperature. Around the World Bridge and Thabo Mbeki Drive sites were also positively correlated to temperature. The other two environmental parameters (TDS and NO_2-N) however, did not have any correlation with the pigmented or non-pigmented yeasts.

4.2.2.2 DIAZONIUM BLUE B (DBB) TESTING

Table 4.3 shows the Diazonium blue B (DBB) results that were obtained to distinguish between ascomyceteous and basidiomycetous yeasts. The DBB results indicated that the Mooi River (85%), Harts River (95%) and Lower Vaal River (61%) had the highest percentages of ascomycetous yeasts, while Schoonspruit (28%) and Barberspan (8%) had the lowest (Table 4.3). All the ascomycetous yeasts from the Mooi River were non-pigmented. Basidiomycetous yeasts from this River consisted only of 5% pigmented yeasts. Nearly all isolates from Harts River were ascomycetous (95%) yeasts that were all non-pigmented. The basidiomycetous yeasts (5%) of this River were pigmented. Schoonspruit had the second highest percentage of basidiomycetous yeasts (72%).

Table 4.3: Percentage ascomycetous and basidiomycetous yeasts from the North West Province during 2010.

Surface water sources	% Ascomycetous yeasts	% Basidiomycetous Yeasts	% Pigmented	% non-Pigmented
Mooi River	85	-	-	100
(n=23)	-	15	5	-
Harts River	95	-	-	100
(n=17)	-	5	0	-
Schoonspruit	28	-	-	100
(n=25)	-	72	56	44
Lower Vaal River	61	-	-	100
(n=25)	-	39	92	8
Barberspan	8	-	-	100
(n=13)	-	92	38	62

The basidiomycetous yeasts consisted of pigmented (56%) and non-pigmented (44%) yeasts. Of the isolates from Lower Vaal River, 61% of the isolates were ascomycetous yeasts and 39 % were basidiomycetous. The basidiomycetous yeasts of the latter River consisted of pigmented (92%) and non-pigmented (8%) yeasts. Nearly all isolates from Barberspan were basidiomycetous yeasts (92%). Only the basidiomycetous yeasts could be classified into pigmented (38%) and non-pigmented yeasts (62%).

As shown in Table 4.4, all ascomycetous and basidiomycetous yeasts from the respective water sources were able to grow at 25°C. This was expected since all these yeasts were isolated at this temperature.

Table 4.4: Percentage ascomycetous and basidiomycetous yeasts growing at various temperatures during 2010.

Surface water sources		% Temperature				
		4°C	**25°C**	**30°C**	**37°C**	**40°C**
Mooi River	Ascomycetes	60	100	85	64	70
(n=23)	Basidiomycetes	40	100	20	36	25
Harts River	Ascomycetes	41	100	94	65	35
(n=17)	Basidiomycetes	18	100	6	0	0
Schoonspruit River	Ascomycetes	28	100	28	24	24
(n=25)	Basidiomycetes	64	100	16	16	16
Lower Vaal River	Ascomycetes	70	100	64	60	30
(n=25)	Basidiomycetes	33	100	36	32	0
Barberspan	Ascomycetes	8	100	8	8	0
(n=13)	Basidiomycetes	92	100	92	15	30

Further analysis showed that a large percentage of the ascomycetous isolates from the Harts - (65%), Mooi - (64%) and Lower Vaal Rivers (60%) were also able to grow at 37°C. On the other hand, isolates from Barberspan that were able to grow at 37°C were basidiomycetous yeasts. Results also indicated that isolates from the respective surface water sources could grow at 4°C (psychrophylic) and some at 40°C (thermo-tolerant). Among the ascomycetous yeasts from the Mooi River, 60% could grow at 4°C whilst 70% at 40°C.

4.3 DATA OBTAINED DURING 2011

4.3.1 Physico-chemical analyses

The results obtained by physico-chemical analysis of the 21 sampled sites are given in Table 4.5. It was found that the physico-chemical parameters measured were lower during the cold dry season compared to the warm rainy one.

Table 4.5 illustrates the physico-chemical parameters at the 21 sampling sites. The pH of the sites ranged from 7.51 to 9.08 (Table 4.5). The surface water temperature at the sites were between 11.2°C to 23.6°C (Table 4.5). Four sites from Harts River, four from Lower Vaal River and two sites from Barberspan had pH values that exceeded the target water quality range (TWQR) for recreational and irrigation use (DWAF, 1996). Four sites from the Harts River, one from Schoonspruit, two from the Lower Vaal River and three from the Barberspan had slight alkaline pH measurements in the range of 8.6 and 8.75. TDS levels measured ranged from 250 to 955 mg/L. All sites from Harts River and Barberspan, four sites from Lower Vaal River and three sites from Schoonspruit had TDS levels that exceeded DWAF target water quality range (DWAF, 1996) for domestic and irrigation use. Four sites from the Harts River (78.8-133.1 mS) and 3 sites from Barberspan (99.8-129.8 mS) had elevated EC levels that exceeded the DWAF target water quality range (DWAF, 1996) for domestic use. Nitrates, nitrites and phosphates measured values ranged from 0.1 to 0.8 mg/L, 1 to 4 mg/L and 0.10 to 2.97 mg/L, respectively. Generally, the pH was slightly alkaline ranging from 8.22–9.23.

Table 4.5: Physico-chemical parameters measured at each of the sampling sites at the respective surface water sources during 2011. Highlited values exceeding TWQR.

Water Source	Different sites	pH	Temp (°C)	EC (mS/m)	TDS (mg/L)	NO_3-N (mg/L)	NO_2-N (mg/L)	PO_4^{2-} (mg/L)
Harts River	Spitskop Dam	8.25	16.4	84.3	601	-	2	0.44
	Harts-Pampier Dam	8.28	15.4	133.1	955	1	4	0.33
	Taung	7.95	11.8	47.9	339	0.3	3	0.43
	Schweizer Reineke	8.33	13.7	78.8	562	0.1	4	0.34
	Delareyville	8.75	13.1	109.6	781	0.4	4	0.70
Schoonspruit	Orkney	7.88	18.9	51.2	250	-	3	0.33
	Klerksdorp Bridge	7.85	18.9	43.2	307	0.1	2	0.86
	Brakspruit	8.01	18	54.1	386	0.3	4	0.58
	Bodenstein	7.92	17	64.4	456	0.8	3	1.03

Table 4.5 continued

Water Source	Different sites	pH	Temp (°C)	EC (mS/m)	TDS (mg/L)	NO_3-N (mg/L)	NO_2-N (mg/L)	PO_4^{2-} (mg/L)
Lower Vaal River	Windsorton	7.73	16.1	38.9	274	-	-	0.10
	Barkley-Wes	8.47	17	39.5	283	-	3	0.45
	Schmidtsdrift	9.06	15.6	60	425	-	2	0.16
	Christiana	9.08	11.2	40.1	283	-	-	-
	Bloemhof	8.58	11.2	36	255	0.3	4	0.25
Barberspan	Harts River	7.92	22.4	47.1	336	0.8	5	1.74
	Inflow	7.51	22	37.8	269	0.2	2	1.60
	Hotel	8.75	22.6	99.8	511	0.2	1	-
	Outflow	8.6	23.5	101.6	728	0.2	2	0.52
	Sannieshof	7.58	23.6	45.4	324	0.1	3	2.19
	Biesiesvlei	7.91	22.7	60.6	432	-	2	2.65
	Lichtenburg	8.02	23.3	129.8	927	0.4	4	2.97

4.3.2 MYCOLOGICAL ANALYSIS

4.3.2.1 Isolation of yeasts

Yeasts were detected at all the sampling sites as indicated in Table 4.6. The number of yeasts in Harts River ranged from 4,120 to 17,380 cfu/L. The highest and lowest number of yeasts was observed at Taung and Spitskop Dam sites, respectively. These sites are in the Harts River. Pigmented yeasts were observed at all the sampling sites except Spitskop Dam site. Pigmented yeast were detected in relatively lower numbers compared to non-pigmented yeasts. High numbers of non-pigmented yeasts were observed in samples from 2 of the 5 sites of the Harts River and ranged from 1,230 to 17,200 cfu/L. Pigmented and non-pigmented yeasts were detected together at four sites (Harts-Pampier Dam, Taung, Scheiwzer Reineke and Delaryville). Overall the percentage non-pigmented yeasts from the Harts River were 96.5% and pigmented yeasts 3.5%.

Yeast numbers detected in the Schoonsprui ranged from 740 to 28,380 cfu/L (Table 4.6). As shown in Table 4.6 the highest and lowest number of yeasts were isolated from water samples from Klerksdorp Bridge and Bodenstein, respectively. High numbers of pigmented yeasts were detected only at Orkney (3,760 cfu/L), while lower numbers of pigmented yeasts were detected at Klerkskraal Bridge, Brakspruit and Bodenstein. Non-pigmented yeasts were detected at 3 of the 4 sites. The numbers were high and ranged from 16,040 to 28,280 cfu/L. Both pigmented and non-pigmented yeasts were detected at all the sampling sites. The percentage non-pigmented to pigmented yeasts ranged from 1.4% to 98.6%.

The number of yeasts In the Lower Vaal River ranged from 230 to 4,180 cfu/L (Table 4.6) and were detected at Schmidtsdrift and Windsorton, respectively. Pigmented yeasts were only observed at Windsorton, Christiana and Bloemhof Dam sites and were detected in relatively low numbers (<10 cfu/L). Higher numbers of non-pigmented yeasts were observed in all the sites and ranged from 190 to 4,180 cfu/L. Both pigmented and non-pigmented yeasts were detected at three sites (Windsorton, Christiana and Bloemhof Dam). The proportion of non-pigmented yeasts among the total (8,310) yeast isolates from Lower Vaal River were 95.9%, while pigmented yeasts was only 4.1%.

Yeast numbers at Barberspan ranged from 270 to 2,970 cfu/L (Table 4.6). The highest and lowest number of yeasts were observed at Biesiesvlei and Hotel sites, respectively. Pigmented yeasts were observed at all the sampling sites ranging from 10 to 1,180 cfu/L. Non-pigmented yeasts levels ranged from 220 to 2,960 cfu/L. The proportion of non-pigmented yeasts among the total (12,270) yeast isolates from Barberspan was 83.5%, where as pigmented yeasts was 16.5%.

Significant differences were found in pigmented and non-pigmented yeasts levels between sites (Table 4.6). The only significant differences in pigmented yeast levels were observed at Harts-Pampier Bridge, Barberspan Inflow and Orkney. Pigmented yeast levels in the Schoonspruit were higher ($P<0.05$) than in all the other water sources. However, all the other sampling sites from the other water sources were significantly different ($P<0.05$) from Orkney (Schoonspruit) site. In addition, pigmented yeast levels at Orkney were significantly higher ($P<0.05$) from all the other sites. Orkney levels were significantly higher ($P<0.05$) from Harts-Pampier Bridge and Barberspan Inflow which were significantly different from all the other sampling sites.

Table 4.6: Average yeast numbers (cfu/L) at various water sources of different sites indicating pigmented and non-pigmented yeasts distribution of 2011.

Water Source	Sites		Ave (cfu/L) (±SD)	Percentage (%)
Harts River	Spitskop Dam	Pigmented	0.000 [a]	0
		Non-pigmented	4,120.00±200.00 [a]	100
	Harts-Pampier Dam	Pigmented	1,310.000±270.00 [b]	51.6
		Non-pigmented	1,230.00±50.00 [a]	48.4
	Taung	Pigmented	180.000±80.00 [a]	0.1
		Non-pigmented	17,200.00±1760.00 [c, d]	99
	Schweizer Reineke	Pigmented	60.00 [a]	0.4
		Non-pigmented	16,120.00±920.00 [c]	99.6
	Delareyville	Pigmented	30.000±10.00 [a]	0.6
		Non-pigmented	4,590.00±190.00 [a, b]	99.4
Schoonspruit	Orkney	Pigmented	3,760.000±400.00 [c]	13.9
		Non-pigmented	23,240.00±1960.00 [c, d]	86.1
	Klerksdorp Bridge	Pigmented	100.000±20.00 [a]	0.4
		Non-pigmented	28,280.00±520.00 [d]	99.6
	Brakspruit	Pigmented	130.000±110.00 [a]	0.8
		Non-pigmented	16,040.00±8200.00 [b, c]	99.2
	Bodenstein	Pigmented	10.000±10.00 [a]	1.4
		Non-pigmented	730.00±310.00 [a]	98.6

Table 4.6 continued

Water Source	Sites		Ave (cfu/L) (±SD)	Percentage (%)
Lower Vaal River	Windsorton	Pigmented	40.000±20.00 [a]	17.4
		Non-pigmented	190.00±50.00 [a]	82.6
	Barkley-Wes	Pigmented	0.00 [a]	0
		Non-pigmented	1,830.00±1772.00 [a]	100
	Schmidtsdrift	Pigmented	0.00 [a]	0
		Non-pigmented	4,180.00±2260.00 [a]	100
	Christiana	Pigmented	280.000±120.00 [a]	31.1
		Non-pigmented	620.00±140.00 [a]	68.9
	Bloemhof Dam	Pigmented	20.000±20.00 [a]	1.7
		Non-pigmented	1,150.00±250.00 [a]	98.3
Barberspan	Harts River	Pigmented	230.000±150.00 [a]	33.8
		Non-pigmented	450.00±50.00 [a]	66.2
	Inflow	Pigmented	1,180.000±160.00 [b]	43.4
		Non-pigmented	1,540.00±120.00 [a]	56.6
	Hotel	Pigmented	50.000±10.00 [a]	18.5
		Non-pigmented	220.00±20.00 [a]	81.5
	Outflow	Pigmented	400.000±80.00 [a]	18.0
		Non-pigmented	1,820.00±120.00 [a]	82.0
	Sannieshof	Pigmented	50.000±10.00 [a]	3.3
		Non-pigmented	1,450.00±250.00 [a]	96.7
	Biesiesvlei	Pigmented	10.000±10.00 [a]	0.3
		Non-pigmented	2,960.00±560.00 [a]	99.7
	Lichtenburg	Pigmented	110.000±90.00 [a]	5.8
		Non-pigmented	1,800.00±400.00 [a]	94.2

[a]Values given are mean of each sampled site ± standard error

[b]Sites with the same combination of superscript alphabetic letters indicate no significant differences (P>0.05) among sites, while those with different letters indicate significant differences at P<0.05 (Tukey's HSD)

[c]Percentage pigmented and non-pigmented yeasts distribution

Figure 4.2 illustrates the association between environmental parameters and species (pigmented and non-pigmented yeasts) based on a redundancy analysis (RDA) ordination analysis diagram. The aim of this analysis was to directly relate the environmental parameters to yeast level data. Total dissolved solids (TDS), nitrites (NO_2-N), phosphates (PO_4^{2-}), and temperature (°C) (Table 4.4) were used in the RDA analysis since these environmental parameters could have an influence on the species distribution. The association is demonstrated by the angle between variables and species. A small angle indicates a close relationship. Results obtained indicated a strong positive correlation between the non-pigmented yeasts and nitrate levels (Figure 4.2). It also indicated that Barkley-Wes and Schweizer Reineke sites were positively influenced by nitrites. The rest of the environmental parameters, however, did not have any association with the pigmented or non-pigmented yeasts.

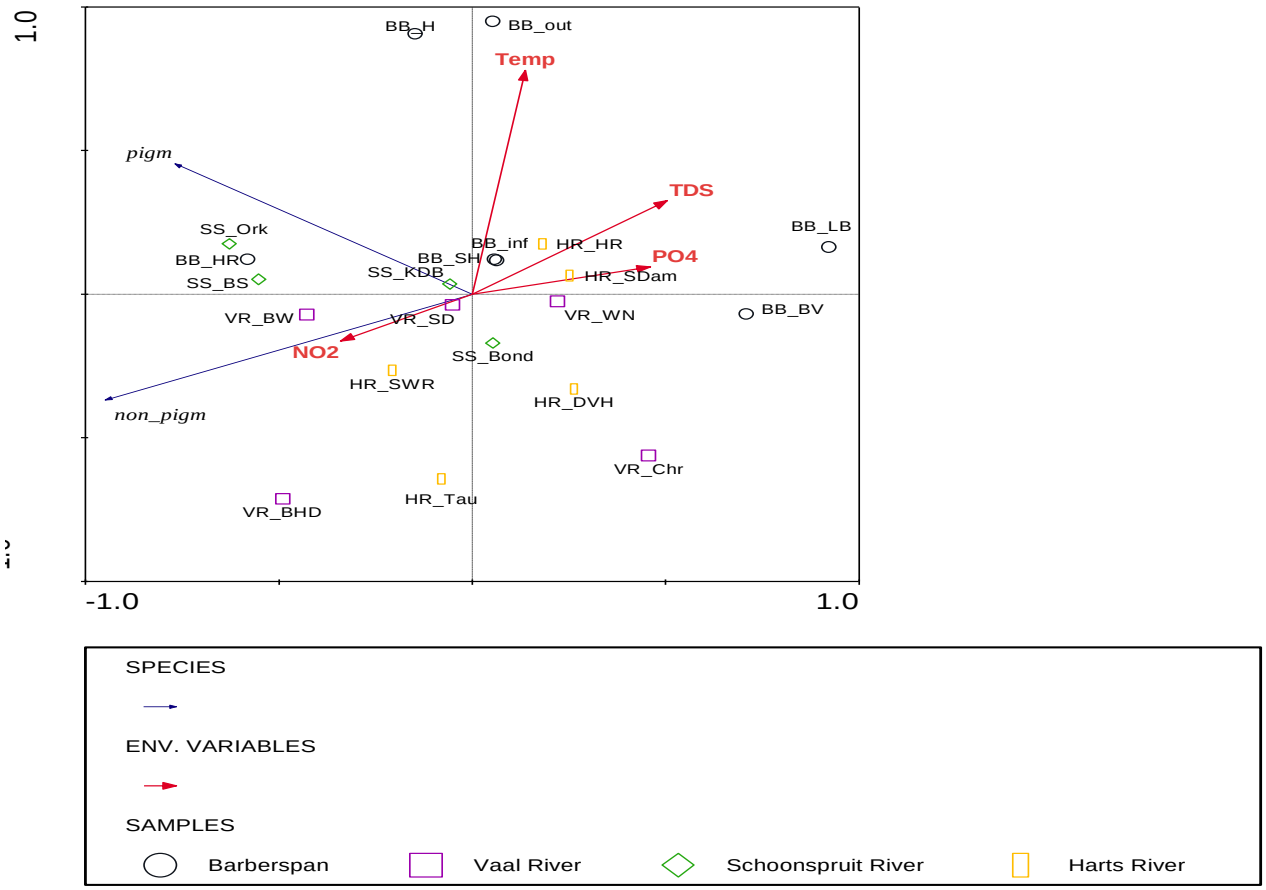


Figure 4.2: Redundancy analysis (RDA) ordination diagram illustrating the association between environmental variables and species (pigmented and non-pigmented yeasts). The red vectors represent the environmental parameters and blue vectors the species (pigmented and non-pigmented yeasts). Eigenvalues for the first two axes were 0.107 and 0.025 respectively.

4.3.2.2 DIAZONIUM BLUE B (DBB) TESTING

As shown in Table 4.7, the Lower Vaal River (83%) and Schoonspruit (64%) had the highest percentages of ascomycetous yeasts, while Harts River and Barberspan both had a percentage of 50% each of ascomycetous and basidiomycetous yeasts (Table 4.7). Basidiomycetous yeasts in the Harts River consisted of pigmented yeasts (32%) and non-pigmented yeasts (18%). The 50% basidiomycetous yeasts from Barberspan consisted of 38% pigmented and 12% non-pigmented yeasts.

Table 4.7: Percentage ascomycetous and basidiomycetous yeasts from the North West Province during 2011.

Surface water sources	% Ascomycetous yeasts	% Basidiomycetous Yeasts	% Pigmented	% non-Pigmented
Harts River	50	-	-	100
(n=22)	-	50	32	18
Schoonspruit	64	-	-	100
(n=22)	-	36	27	9
Lower Vaal River	83	-	-	100
(n=18)	-	17	17	6
Barberspan	50	-	-	100
(n=26)	-	50	38	12

Schoonspruit had the second highest percentage of ascomycetous yeasts (64%). The basidiomycetous yeasts consisted of pigmented (27%) and non-pigmented yeasts (9%). The Lower Vaal River had the highest percentage of ascomycetous yeasts (83%) and the lowest percentage basidiomycetous yeasts (17%). Basidiomycetous yeasts from this River consisted of 17% pigmented and 6% non-pigmented yeasts.

The ability of ascomycetous and basidiomycetous yeast isolates to grow at various temperatures was determined. The results shown in Table 4.8 indicate that a large percentage of the ascomycetous isolates from Harts River (73%) and Barberspan (62%) were able to grow at 37°C.

Table 4.8: Percentage ascomycetous and basidiomycetous yeasts growing at various temperatures during 2011.

Surface water sources		**% Temperature**				
		4°C	**25°C**	**30°C**	**37°C**	**40°C**
Harts River	Ascomycetes	68	100	68	73	0
(n=22)	Basidiomycetes	32	100	32	56	36
Schoonspruit	Ascomycetes	100	100	100	14	14
(n=22)	Basidiomycetes	100	100	100	50	0
Lower Vaal River	Ascomycetes	83	100	83	28	0
(n=18)	Basidiomycetes	17	100	17	11	0
Barberspan	Ascomycetes	42	100	42	62	31
(n=26)	Basidiomycetes	50	100	50	46	15

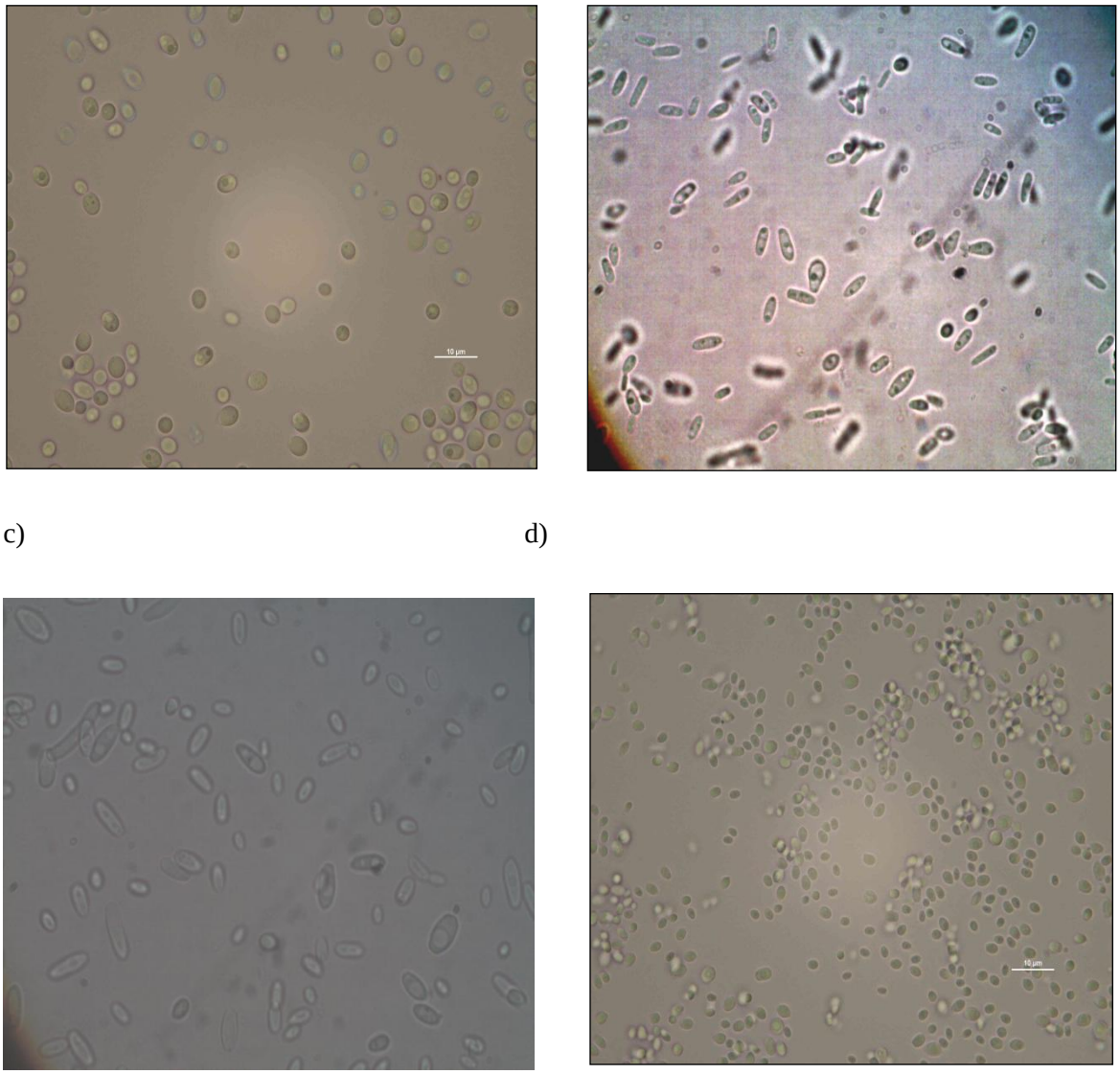
Further analyses show that only Barberspan had both ascomycetous (31%) and basidiomycetous (15%) yeasts that could grow at 40°C, while Harts River had 36% basidiomycetous and Schoonspruit 14% ascomycetous that could grow at 40°C. Also more that 60% of the ascomycetous yeast at the Harts River, Lower Vaal River and the Schoonspruit could grow at 30°C.

The temperature growth experiments that were conducted on the yeasts isolates from both 2010 and 2011 demonstrated that yeast were isolated from surface water sources that could grow at various temperatures. These are either yeasts with a wide temperature tolerance range or yeast that are specialists psychrophiles or thermophiles.

4.4 YEAST MORPHOLOGY

The morphological characteristics were determined by microscopy. Figure 4.3 shows photographs of selected isolates. Varoius yeast cells were examined at 1000X magnification after Crystal Violet staining. Yeast isolates exhibited a cream to tan to pink colour, with a smooth texture. Some cells were spheroidal and others ovoidal.

a) b)



c) d)

Figure 4.3. Morphology of various yeasts under the microscope: (a) *Rhodotorula* spp.; (b) *Wickerhamomyces* spp., (c) *Cryptococcus* spp. and (d) *Candida* spp. (1000X magnification).

4.5 IDENTIFICATION OF YEASTS

4.5.1 Identification of yeasts isolates with a biochemical test method

Representative isolates from the various sampling site were identified by API ID 32C (Chapter 3, Section 3.3.2). A total of 4 genera of yeasts were identified. These consisted of *Candida catenulate, C. globosa, C. guilliermondi, C. lusitaniae, C. pellicuslosa, C. rugosa, C. sake, C. tropcalis, Cryptococcus laurentii* and *Zygosaccharomyces* spp., (non-pigmented) whereas the pigmented yeasts were identified as, *Rhodotorula glutini, Rh. mucilaginosa.* Identifications were confirmed using 26S rRNA gene sequences.

4.5.2 Molecular identification of yeast isolates

Genomic DNA was extracted from representative yeast isolates of different sampling sites during 2010 and 2011, (Chapter 3; Section 3.4.1). The quantity of the extracted DNA was low (0.43-225 ng/µl) and bands could not be visualized on agarose gels. The 260/280 nm ratios ranged from of 1.21 to 2.1. DNA concentrations varied from 0.43-225 ng/µl. There was, however, no fragmentation or RNA observed.

4.5.3 DNA amplification of yeasts isolates for sequencing

Isolated yeasts genomic DNA was amplified using PCR under conditions described in (Chapter 3; Section 3.4.2). Amplification products were of the expected (±600 bp) size. This size (range) of D1/D2 has previously been reported (Kurtzman & Robnett, 1998; Motaung, 2011). The amplified fragment sizes are demonstrated in the Figure 4.4. These fragments were sequenced by Inqaba (Pretoria).

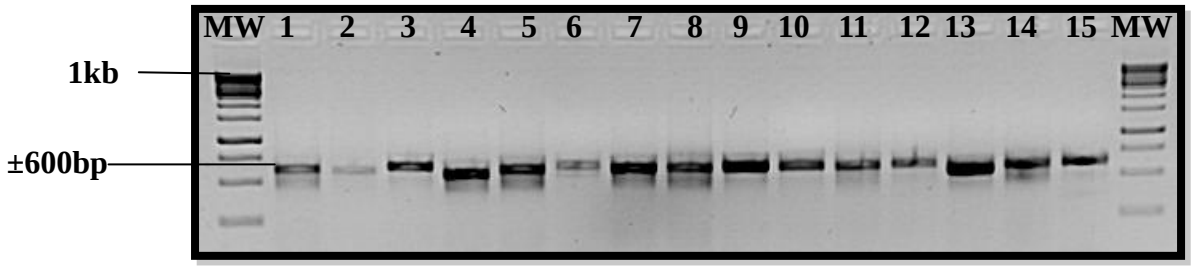


Figure 4.4: An ethidium bromide stained, agarose gel image of PCR amplified 26S rDNA fragments for selected isolates (Lanes 1-15). MW represents the 1kb molecular size marker (O'GeneRulerTM 1kb DNA ladder, Fermentas Life Science, US). The agarose gel concentration was 1.5% (w/v), electrophoresed at 80V for 45 min.

GenBank was used to identify the amplified sequences by using BlastN searches (http://www.ncbi.nlm.nih.gov/BLAST). Identifications based on the 26S rDNA gene are illustrated in Tables 4.9 (2010) and 4.10 (2011) for the yeasts isolates from the different sampling sites.

Thirty strains (17 ascomycetes and 13 basidiomycetes) were identified in the current study using D1/D2 rDNA sequencing (Tables 4.9 and 4.10). In Tables 4.9 (2010) and 4.10 (2011) their representative strain numbers to which the sequence gave the closest GenBank match, accession number of GenBank match, percentage identity (Pi) (size of the query is indicated in brackets), source, origin of isolation of the Genbank strain, abbreviated isolation locations and sequence *E*-values. BlastN searches revealed that all the sequences were 26S yeast ribosomal gene sequences. *Candida* spp. were the most prevalent, followed by *Rhodotorula* spp., *Wickerhamomyces* spp., *Pichia* spp., *Clavispora* spp., *Cryptococcus* spp., *Sporidiobolus* spp., *Hanseniaspora* spp., *Cystofilobasidium* spp., *Meyerozyma* spp., *Aureobasidium* spp. and *Saccharamyces* spp. (Tables 4.9 and 4.10). Furthermore, these species could be grouped into ascomycetous and basidiomycetous yeasts.

The list contains ascomycetous and basidiomycetous yeasts from various water sources. Ascomycetous yeasts were isolated from cloud water (2 isolates), spontaneous cocoa bean fermentation (1 isolate), leaves (4 isolates), sour rotten grapes (2 isolates), water samples (5 isolates), distillery (2 isolates), siobum (fermented bamboo shoot product of Manipur) (1 isolate) and 22 strains from unknown sources (Tables 4.9 and 4.10). Basidiomycetous yeasts were isolated from soil samples (11 isolates), xylem sap from grapevine (2 isolates), fruit (8 isolates), grapes (3 isolates), seeds (2 isolates), forest soil (1 isolate), atta texana nest (1 isolate) and 3 strains from unknown sources (Tables 4.9 and 4.10).

Table 4.9: The identities of 26 selected isolates as determined by 26S PCR during 2010.

Origin	Band ID	Closest GenBank match	Accession no.of GenBank match	% Identity (no. of bp of query)	Source	E value
Mooi River Klerkskraal	79	*Wickerhamomyces anomalus* strain G0901 26S rRNA gene	HQ891142	99 (597)	Cloud water	2e-125
Around the World Bridge	85	*Candida tropicalis* strain Y49 28S rRNA partial gene	FR870028	100 (640)	Spontaneous cocoa bean fermentation	3e-87
Klerskraal Dam	86	*Candida boidinii* strain XM02G 26S rRNA gene	EU293427	99 (60 o7)	Unknown	2e-77
Muiskraal Bridge	89	*Clavispora lusitaniae* strain LM083 26S rRNA genes	AB617983	100 (517)	Leaves	2e-141
Muiskraal Bridge	95	*Wickerhamomyces anomalus* strain G0901 26S rRNA gene	HQ891142	100 (597)	Cloud water	6e-146
Muiskraal Bridge	96	*Rhodotorula mucilaginosa* strain s265 18S rRNA gene	HQ871906	100 (614)	Soil	1e-64
Harts River Jan Kempdorp	W1	*Candida nivariensis* strain NRRL Y-48269 28S rRNA gene	JN882347	100 (528)	Unknown	0.0

Jan Kempdorp	W2	*Pichia kudriavzevii* strain PP11 26S rRNA gene	JN004188	100 (574)	Sour rotten grapes	0.0
Jan Kempdorp	W3	*Rhodotorula slooffiae* strain I-Y382 26S rRNA gene	GU585167	100 (596)	Xylem sap from grapevine	0.0
Harts-Pampier Bridge	HW1	*Candida temnochilae* strain ATCC MYA-4336 26S rRNA gene	FJ614675	100 (554)	Unknown	0.0
Schoonspruit Orkney	A	*Rhodotorula mucilaginosa* strain s265 18S rRNA gene	HQ871906	100 (614)	Soil	8e-51
Orkney	B	*Rhodotorula glutinis* strain JHR 26S rRNA gene	HQ606476	100 (590)	Fruit	5e-85
Klerksdorp Bridge	C	*Clavispora lusitaniae* strain LM083 26S rRNA genes	AB617983	100 (517)	Leaves	2e-34
Klerksdorp Bridge	D	*Rhodotorula glutinis* strain JHR 26S rRNA gene	HQ606476	100 (590)	Fruit	8e-155
Brakspruit	F	*Rhodotorula mucilaginosa* strain s265 18S rRNA gene	HQ871906	100 (614)	Soil	1e-55
Brakspruit	G	*Rhodotorula glutinis* strain	HQ606476	100 (590)	Fruit	0.0

		JHR 26S rRNA gene				
Orkney	YV1	*Rhodotorula mucilaginosa* strain s265 18S rRNA gene	HQ871906	100 (614)	Soil	5e-157
Lower Vaal River Schmidtsdrift	19	*Candida pseudolambica* strain YM24348 26S rRNA gene, partial sequence	HQ111496	99 (550)	Water	2e-89
Christiana	21	*Candida pseudolambica* strain YM24348 26S rRNA gene, partial sequence	HQ111496	100 (550)	Water	0.0
Christiana	22	*Pichia kudriavzevii* isolate 8 26S rRNA gene	JN248609	97 (565)	Distillery	6e-52
Bloemhof Dam	28	*Rhodotorula mucilaginosa* strain s265 18S rRNA gene	HQ871906	100 (614)	Soil	2e-83
Bloemhof Dam	32	*Pichia kudriavzevii* isolate 8 26S rRNA gene	JN248609	100 (565)	Distillery	3e-97
Barberspan Harts River	HR	*Sporidiobolus pararoseus* culture-collection CBS:499	HM014040	100 (574)	Unknown	0.0

		large subunit rRNA gene				
Hotel2 wit	Hotel2 wit	*Wickerhamomyces anomalus* strain CEC C251 26S rRNA gene	JN083817	100 (601)	Unknown	0.0
Hotel1 Pink	Hotel1 pink	*Rhodotorula mucilaginosa* isolate BPT-1 28S rRNA gene	JN091167	100 (595)	Soil	0.0
Outflow Pink	Out pink	*Rhodotorula glutinis* strain ZHR 26S rRNA gene	HQ606475	100 (573)	Fruit	0.0

Table 4.10: The identities of 44 selected isolates as determined by 26S PCR during 2011.

Origin	Band ID	Closest GenBank match	Accession no.of GenBank match	% Identity (no. of bp of query)	Source	E-value
Harts River Spitskop	SpD2 wit	*Pichia kudriavzevii* strain PP11 26S rRNA gene	JN004188	100 (574)	Sour rotten grapes	0.0
Spitskop	SpD2 wit	*Candida nivariensis* strain NRRL Y-48269 28S rRNA gene	JN882347	100 (528)	Unknown	0.0
Harts-Pampier	HP1 wit	*Candida temnochilae* strain ATCC MYA-4336 26S rRNA gene	FJ614675	100 (554)	Unknown	0.0
Harts-Pampier	HP2 pink	*Rhodotorula slooffiae* strain I-Y382 26S rRNA gene	GU585167	100 (596)	Xylem sap from grapevine	0.0
Taung	Tg1 wit	*Candida intermedia* strain YA02a 26S rRNA gene	EF629546	100 (541)	Unknown	0.0
Taung	Tg2 pink	*Rhodotorula mucilaginosa* isolate BPT-1 28S rRNA	JN091167	100 (595)	Soil	0.0

		gene				
SchweizerR	SW1 pink	*Rhodotorula glutinis* strain JHR 26S rRNA gene	HQ606476	99 (590)	Fruit	0.0
SchweizerR	SW2 wit	*Cryptococcus flavescens* strain S23 26S rRNA gene	JN004195	100 (605)	Grapes (harvest)	0.0
Delary	D1 pink	*Rhodotorula mucilaginosa* isolate BPT-1 28S rRNA gene	JN091167	100 (595)	Soil	0.0
Delary	D2 wit	*Cryptococcus flavescens* strain S23 26S rRNA gene	JN004195	100 (605)	Grapes (harvest)	0.0
Schoonspruit Orkney	Ork1 wit2	*Candida glaebosa* strain N04-2.2 26S rRNA gene	FJ432670	100 (611)	Unknown	0.0
Orkney	Ork2 wit2	*Candida glaebosa* strain N04-2.2 26S rRNA gene	FJ432670	100 (611)	Unknown	0.0
Orkney	Ork2 pink/orange	*Rhodotorula mucilaginosa* isolate BPT-1 28S rRNA gene	JN091167	100 (595)	Soil	0.0

Klerksdorp Bridge	klerk2 wit1	*Clavispora lusitaniae* strain LM083 26S rRNA genes	AB617983	100 (517)	Leaves	2e-34
Klerksdorp Bridge	Klerk2 wit2	*Cryptococcus diffluens* isolate YF11.3 26S rRNA gene	HQ629579	100 (550)	Seeds	2e-93
Klerksdorp Bridge	Klerk1 pink/orange 2	*Cryptococcus diffluens* isolate YF11.3 26S rRNA gene	HQ629579	100 (550)	Seeds	2e-93
Brakspruit1	Brak1 wit3	*Clavispora lusitaniae* strain LM083 26S rRNA genes	AB617983.1	100 (517)	Leaves	2e-34
Brakspruit	Brak2 wit3	*Candida pseudolambica* strain YM24348 26S rRNA gene, partial sequence	HQ111496	100 (550)	Water	0.0
Brakspruit	Brak1 pink	*Rhodotorula glutinis* strain ZHR 26S rRNA gene	HQ606475	100 (573)	Fruit	0.0
Bodenstein	Bod1 wit3	*Candida glaebosa* strain N04-2.2 26S rRNA gene	FJ432670	100 (611)	Unknown	0.0
Bodenstein	Bod2 wit1	*Candida pseudolambica* strain YM24348 26S rRNA	HQ111496	100 (550)	Water	0.0

		gene, partial sequence				
Lower Vaal River Windsorton	WW1 wit	*Hanseniaspora uvarum* strain CEC F20 26S rRNA gene	JN083823	100 (598)	Unknown	0.0
Windsorton	WW2 orange	*Cystofilobasidium* sp. HB1156 partial 26S rRNA gene, strain HB1156	AM039674	96 (609)	Forest soil	0.0
Windsorton	WW3 wit	*Wickerhamomyces anomalus* strain CEC C251 26S rRNA gene	JN083817	100 (601)	Unknown	0.0
Windsorton	WP1 pink	*Meyerozyma guilliermondii* isolate UCLM 99C 26S rRNA gene	JF916456	88 (584)	Unknown	4e-46
Barkley-Wes	BW1 wit	*Wickerhamomyces anomalus* strain CEC C251 26S rRNA gene	JN083817	100 (601)	Unknown	0.0
Barkley-Wes	BW3 wit	*Candida intermedia* strain YA02a 26S rRNA gene	EF629546	100 (541)	Unknown	0.0

Schmidtsdrift	SW4 wit	*Hanseniaspora uvarum* strain MP7 large subunit rRNA gene partial sequence	HM988683	98 (574)	Grape	8e-103
Christiana	CW1	*Pichia* sp. NRRL Y-11569 26S rRNA gene	EF550280	100 (3323)	Unknown	0.0
Christiana	CW2	*Aureobasidium* sp. YM24623 26S rRNA gene	HQ637566	96 (559)	Unknown	0.0
Christiana	CP1 Pink	*Rhodotorula* sp. CRUB 1485 26S rRNA gene, partial sequence	EF585198	96 (672)	Unknown	0.0
Bloemhof Dam	BW1 wit	*Candida pseudolambica* strain YM24348 26S rRNA gene	HQ111496	100 (550)	Water	0.0
Barberspan Harts River	HR pink	*Sporidiobolus pararoseus* culture-collection CBS:499 large subunit rRNA gene	HM014040	100 (574)	Unknown	0.0
Inflow	Inf1 wit	*Cryptococcus flavescens*	JN004195	100 (605)	Grapes (harvest)	0.0

		strain S23 26S rRNA gene				
Outflow	Out wit	*Cryptococcus albidosimilis* strain DBVPG 5184 26S rRNA	GQ911491	97 (577)	Unknown	0.0
Outflow	Out pink	*Rhodotorula glutinis* strain ZHR 26S rRNA gene	HQ606475	100 (573)	Fruit	0.0
Hotel	Hotel wit	*Wickerhamomyces anomalus* strain CEC C251 26S rRNA gene	JN083817	100 (601)	Unknown	0.0
Hotel	Hotel pink	*Rhodotorula mucilaginosa* isolate BPT-1 28S rRNA gene	JN091167	100 (595)	Soil	0.0
Sannieshof	San2 wit	*Saccharomyces cerevisiae* strain chengshang1 26S rRNA gene	EU272045	94 (592)	Unknown	0.0
Sannieshof	San2 pink	*Rhodotorula mucilaginosa* isolate BPT-1 28S rRNA	JN091167	100 (595)	Soil	0.0

		gene				
Biessiesvlei	Bies1 pink	*Rhodotorula mucilaginosa* strain ATT252 large subunit rRNA gene	FJ743623	100 (614)	Atta texana nest	0.0
Biessiesvlei	Bies1 wit	*Candida intermedia* strain YA02a 26S rRNA gene	EF629546	100 (541)	Unknown	0.0
Lichtenburg	Lig2 wit	*Meyerozyma guilliermondii* strain Kw3S2Y1 26S rRNA gene	JF439367	100 (577)	Soibum (fermented bamboo shoot product of Manipur)	0.0
Lichtenburg	Lig2 Pink	*Rhodotorula glutinis* strain JHR 26S rRNA gene	HQ606476	99 (590)	Fruit	0.0

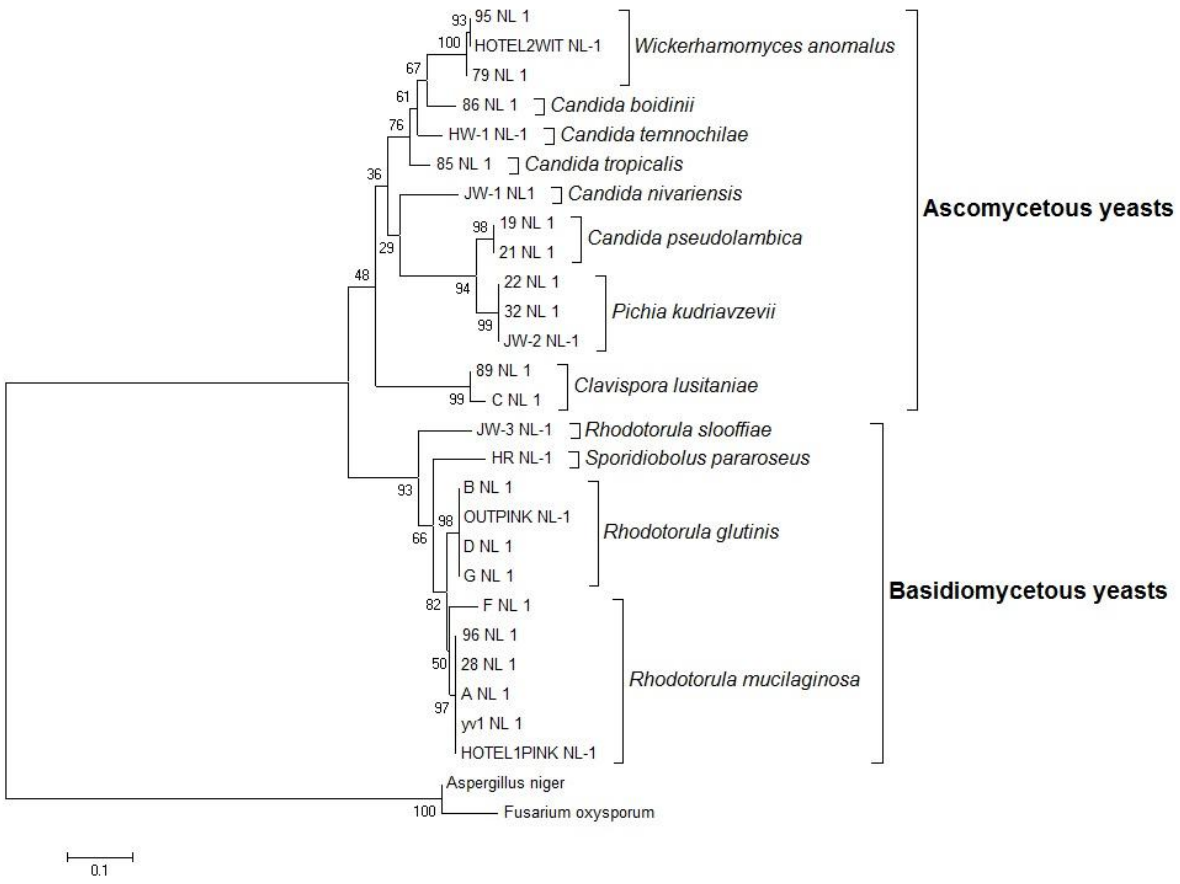


Figure 4.5: Phylogenetic tree based on partial sequences of D1/D2 domain of 26S rDNA obtained in 2010. The tree was constructed using the neighbour-joining and Kimura two-parameter methods. Local bootstrap probability values obtained by maximum likelihood analysis are indicated above and below the branches. The tree was rooted with *Aspergillus niger* and *Fusarium oxysporum*.

Phylogenetic relationships between yeast species of 2010 are illustrated in Figure 4.5. The tree could be divided into two major clusters (ascomycetous and basidiomycetous yeasts) and the outgroup consisting of two fungi (*Aspirgillus niger* and *Fusarium oxysporum*) at the bottom. The ascomycetous yeasts cluster of 2010 consisted of 16 strains (4 genera and 8 species) (Figure 4.5). The ascomycetous yeasts cluster could be divided into ten strains. These strains were further grouped into eighth species. *Wickerhamomyces anomalus* isolated from Hotel, Klerkskraal Bridge and Muiskraal Bridge samples were closely related to *Candida boidinii* isolated from Klerkskraal Bridge (bootstrap support of 67%). *Candida tropicalis* isolated from Around the World Bridge (Mooi River) grouped with *Candida temnochilae* isolated from Harts-Pampier Bridge (Harts River). This cluster has a bootstrap support of 76%. Three strains identified as *Pichia kudriavzevii* that were isolated from Jan Kempdorp, Christiana and Bloemhof Dam and were closely related to *Candida pseudolambica* isolated

from Schmidtsdrift and Christiana, and *Candida nivariensis* isolated from Jan Kempdorp. *Clavispora lustianiae* strian isolated from Muiskraal Bridge and Klerksdorp Bridge did not group within any clusters. According to its position within the dendogram, this strain was closely related to *Candida* and *Pichia* strains. The basidiomycetous yeasts cluster consisted of 2 genera and 4 species. These could be grouped into 4 species namely *Rhodotorula slooffiae, Sporidiobolus pararoseus, Rhodotorula mucilaginosa*, and *Rhodotorula glutinis*. *Rhodotorula slooffiae* isolated from Jan Kempdorp was closely related to *Sporidiobolus pararoseus* which were isolated from Harts-Pampier Bridge sample. This cluster had a bootstrap support of 93%.

In total, 8 different species belonging to the *Ascomycota* and 4 to the *Basidiomycota* (2010, Figure 4.5) were identified and could phylogenetically be placed. Among the non-pigmented yeasts, all sequences were ascomycetes. All the pigmented yeasts were affiliated with the *Basidiomycota* (Figure 4.5).

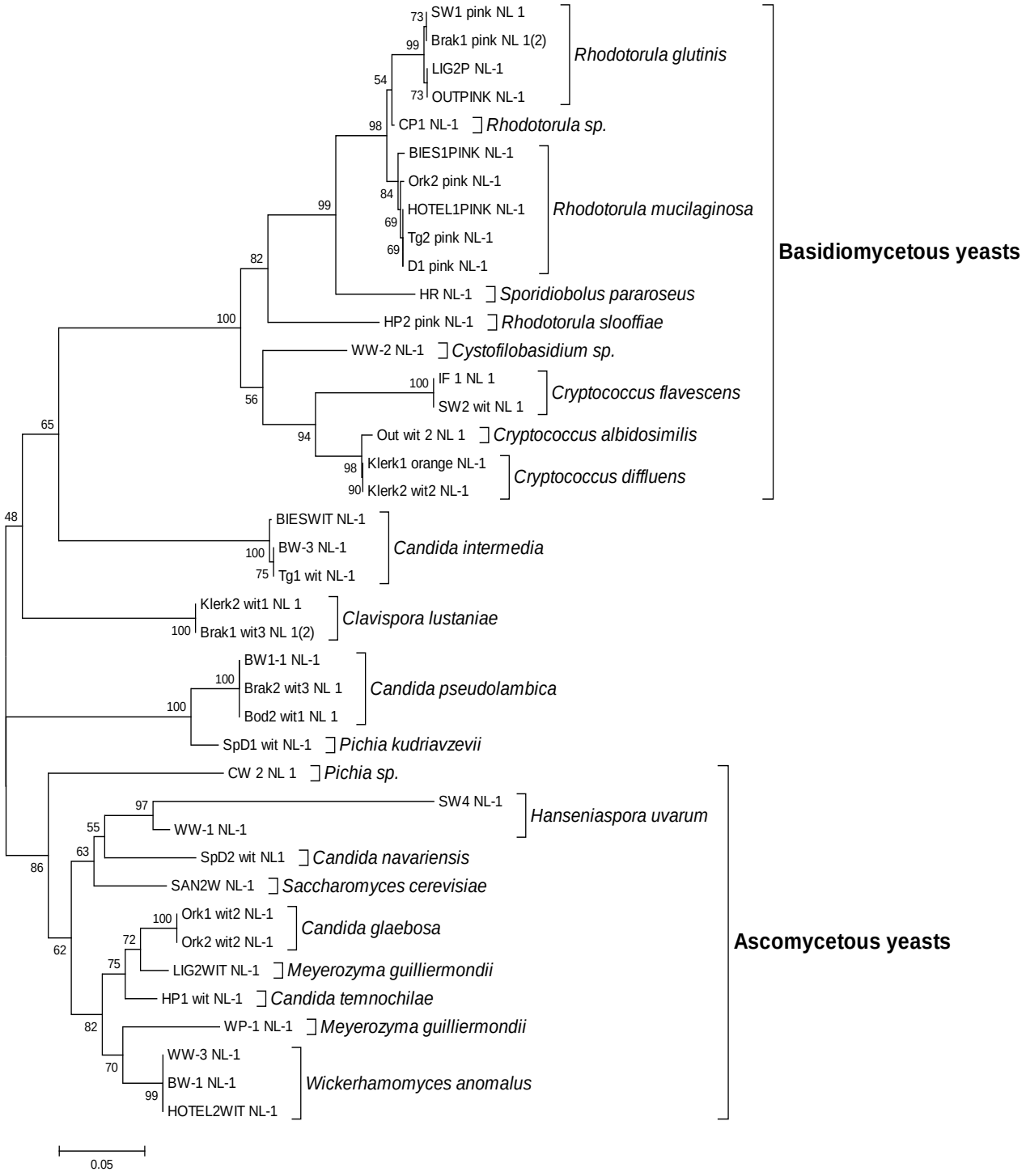


Figure 4.6: Phylogenetic tree based on partial sequences of D1/D2 domain of 26S rDNA obtained in 2011. The tree was constructed using the neighbour-joining and Kimura two-parameter methods. Local bootstrap probability values obtained by maximum likelihood analysis are indicated above and below the branches.

The phylogenetic relationship of yeast isolates and neighboring species of 2011 is presented in Figure 4.6. Twelve basidiomycetous yeasts formed one group that consisted of 9 species *Rhodotorula glutinis*, *Rhodotorula mucilaginosa*, *Rhodotorula* sp., *Sporidiobolus pararoseus*, *Rhodotorula slooffiae*, *Cystofilobasidium* sp., *Cryptococcus flavescens*, *Cryptococcus albidosimilis* and *Cryptococcus diffluens*. The *Rhodotorula glutinis* strains isolated from Schweizer Reineke, Brakspruit, Lichtenburg and Outflow formed one cluster that had a bootstrap support of 99%. Three *Rhodotorula mucilaginosa* strains from Biessiesvlei, Orkney and Hotel were closely related and the cluster had a bootstrap support of 84%. *Rhodotorula mucilaginosa* from Delareyville and Taung were grouped within the *Rhodotorula mucilaginosa* strains from Hotel, Orkney and Biessiesvlei with a bootstrap support of 69%. *Sporidiobolus pararoseus* isolated from Harts River and *Rhodotorula slooffiae* isolated from Harts-Pampier Bridge were not grouped within any cluster, but according to their positions in the dendogram these strains were closer related to each other with a bootstrap support of 82%. *Sporidiobolus pararoseus* strain was closer related to the *Rhodotorula mucilaginosa* strains with a bootstrap support of 99%. The *Cystofilobasidium* sp. strain from Windsorton was closely related to *Cryptococcus flavescens* (Inflow and Schweizer Reineke), *Cryptococcus albidosimilis* (Outflow) and *Cryptococcus diffluens* (Klerksdorp Bridge) with a bootstrap support of 56%.

The ascomycetous yeast cluster contained fifteen strains and consisted of 12 species *Candida pseudolambica*, *Pichia kudriavzevii*, *Pichia* sp. NRRL Y-11569, *Hanseniaspora uvarum*, *Candida nivariensis*, *Saccharomyces cerevisiae*, *Candida temnochilae*, *Candida glaebosa*, *Meyerozyma guilliermondii*, *Wickerhamomyces anomalus*, *Clavispora lustaniae* and *Candida intermedia*. The two *Candida intermedia* strains isolates from Taung and Barkley-Wes was closer related to each other with a bootstrap support of 75%, while the same strain grouped together with Biessiesvlei and Barkley-Wes with a bootstrap support of 100%. *Clavispora lustaniae* strain from Klerksdorp Bridge and Brakspruit were closely related to each other with a bootstrap support of 100%. Results also indicated that *Clavispora lustaniae* was related to *Candida intermedia* with a low bootstrap value (48%). *Pichia kudriavzevii* strain isolated from Spitskop Dam grouped within the *Candida pseudolambica* strain cluster isolated from Barkley-Wes, Brakspruit and Bodenstein with a bootstrap support of 100%. The *Pichia kudriavzevii* strain could also be closely related to the *Pichia* sp. NRRL Y-11569 isolated from Christiana supported by a bootstrap value of 86%. *Saccharomyces cerevisiae* isolated from Sannieshof grouped with *Candida nivariensis* isolated from Spitskop Dam with a

bootstrap support of 63%. *Candida nivariensis* grouped with *Hanseniaspora uvarum* from Schweizer Reineke and Windsorton with a bootstrap support of 55%. *Candida temnochilae* isolated from Harts-Pampier Bridge was closer related to *Meyerozyma guilliermondii* from Lichtenburg with a bootstrap support of 75%. *Meyerozyma guilliermondii* was closer related to the *Candida glaebosa* cluster isolated from Orkney with a bootstrap of 72%. The *Meyerozyma guilliermondii* strain from Windsorton was closer related to *Wickerhamomyces anomalus* cluster is

4.6 SUMMARY OF RESULTS

Results presented in this chapter revealed that during the sampling periods (2010 and 2011) the physico-chemical results indicated no significant seasonal differences. The pH for 2010 and 2011 was both generally alkaline. The TDS and EC values in some cases were elevated and exceeded some agricultural and domestic applications, respectively.

The mycological results indicated that the number of yeasts were higher in the Mooi River than the Schoonspruit, the Lower Vaal River, Barberspan and the Harts River for the 2010 sampling period. Yeast numbers detected in 2011 were significantly ($p<0.05$) higher than the numbers detected in 2010. In 2011 the number of yeasts were higher in Schoonspruit than the Harts River, Barberspan and Lower Vaal River. In this sampling period the Mooi River was not included. Results also indicated that pigmented and non-pigmented yeasts were higher in Schoonspruit (Orkney and Klerksdorp Bridge, specifically) than all the other water sources for 2011. Non-pigmented yeasts at all the sites in 2011 were much higher than the non-pigmented yeasts levels in 2010. One trend that was repeated in 2011 was the high levels of pigmented yeasts in the Schoonspruit.

The Tukey`s HSD test revealed that there were statistical significant differences ($P<0.05$) in terms of pigmented yeasts between sites in 2010 and 2011. In 2010 non-pigmented yeast levels between sites were statistical insignificant. Results from the RDA indicated that during 2010 a strong positive correlation was found between pigmented yeasts and phosphates levels, while during 2011 a positive correlation was found between non-pigmented yeast and nitrate levels.

Temperature tests were done to determine the temperatures at which ascomycetous and basidiomycetous yeasts could grow. Results indicated that yeasts could grow at a range of temperatures. Representative isolates from different sites isolated during the two sampling periods (2010 and 2011) were identified. These were identified as *Candida catenulate, C. globosa, C. guilliermondi, C. lusitaniae, C. pellicuslosa, C. rugosa, C. sake, C. tropcalis, Cryptococcus laurentii* and *Zygosaccharomyces* spp. and *Rhodotorula glutini, Rh. Mucilaginosa* using API ID 32 C and further confirmed using 26S rRNA gene sequences. Cluster analysis demonstrated that the yeast grouped according to asco- and basidiomycetes, first and then into species clusters.

CHAPTER 5

DISCUSSION

5.1 INTRODUCTION

The aim of this study was to generate baseline data on yeasts in surface water sources of the North West Province, South Africa and the potential use of these yeasts as water quality monitoring indicators. Yeasts were isolated on YM agar and selected for identification based on morphological characteristics. Representative yeast isolates were identified using a biochemical method (API ID 32C). Identifications were confirmed using 26S rDNA sequences.

5.2 PHYSICO-CHEMICAL ANALYSIS

In the present study the pH at all sites from Barberspan and one site from Lower Vaal River (Schmidtsdrift) exceeded the target water quality range (TWQR) levels between 6.5 and 8.4 for recreational and irrigation use (DWAF, 1996). This may not have immediate adverse effects. Generally, the results indicated that the pH over the two year period was between 7.2 and 9.3. In 2010 water temperatures ranged from 12°C to 26.1°C (Table 4.1) and from 11.2°C to 23.6°C (Table 4.5) in 2011. Such temperatures are suitable for the growth of yeasts in aquatic systems.

In this study elevated EC (and TDS) levels were measured in the Mooi River, Harts River, Schoonspruit, Barberspan and sites from Lower Vaal River (Tables 4.1 and 4.5). Elevated EC (and TDS) levels could be attributes to agricultural practices including livestock and irrigation farming. Another possible reason could be the leaching of soil contaminants and discharge from sewage treatment plants particularly during the dry season (Moniruzzaman *et al*., 2009). The North West Province is one of the regions where high levels of EC exist, resulting from natural causes as well as human impact (NWDACE, 2008). The geology of the North West Province, which consists of yellow shifting sands, could also contribute to the high EC (TDS) values measured in the water sources (NWPG, 2002).

Phosphates are present in natural waters as soluble and organic phosphates (Kumar *et al*., 2011). In the present study phosphates were present and ranged from 0 to 2.44 mg/L in 2010 (Table 4.1) and 0.10 to 2.97 mg/L for 2011 (Table 4.5). This could be due to agricultural runoff and discharge of sewage effluent from nearby towns (Moniruzzaman *et al*., 2009). The nitrate (NO_3-N) values fluctuated between 0 to 4 mg/L, 0 to 19 mg/L and 0.1 to 0.8 mg/L, 1 to 4 mg/L. Elevated nitrites might have been due to surface runoff from farms and storm water runoff into the water sources during early rain as well as sewage treatment plant (Razak *et al*., 2009).

5.3 PREVALENCE AND DIVERSITY OF YEASTS

The levels and diversity over the two year period (2010 and 2011) varied. The prevalence and diversity of yeasts in water sources depends on several factors such as the water temperature, pH, available nutrient levels, particularly phosphates and nitrates, as well as drainage of rivers and human activities (Hagler & Ahearn, 1987; Lanchance & Starmer, 1998). The water temperature during both sampling periods was suitable for growth of yeasts in water.

In 2010, yeasts were detected in 22 of 23 sampling sites and in all the sampling sites (n=21) for 2011. The number of yeasts detected in the Mooi River, was greater than those of the other surface water sources, and could be due to the higher water temperature (Table 4.1). The high number of pigmented yeasts in the Schoonspruit (Klerksdorp Bridge) could be attributed to stormwater runoff and potentially also animal faeces. The high pigmented yeast levels may also be due to the phosphate levels. In RDA ordination analysis a strong positive correlation (Figure 4.1) was demonstrated to exist between phosphates concentration and pigmented yeasts levels.

Yeast numbers detected in 2011 were much higher (Table 4.6) and could probably be explained by the change in weather conditions and runoff from cattle farms after rains. Another reason for this change in yeast levels could be due to malfunctioning waste water treatment plants. The number of yeasts in the Schoonspruit (Table 4.6), was higher in 2011 compared to the 2010 sampling period. This could once again be explained by stormwater runoff. After the heavy rains of 2011 there were lots of driftwood at the site potentially emanating from agricultural activities (Appendix D, Fig D). The number of pigmented yeasts in the Schoonspruit (Orkney), were again higher than those of the other surface water sources. These high levels could be a result of the presence of favourable metabolic substrates for their growth and metabolism (Simard, 1971). This trend was observed during both sampling

periods (2010 and 2011). A study conducted by Sláviková & Vadkertiová (1997) showed that pigmented yeasts (red yeasts) were frequently isolated from their river water. This result supported the findings of the present study. A study conducted by Hagler & Mendonça-Hagler (1981) indicated that the number of yeasts increases in the presence of pollution or in the presence of algae, and it can reach a few thousand cells per liter or more.

Non-pigmented yeasts in 2010 were isolated from almost all the sampling sites, except Pedestrian Bridge (Mooi River) and Bodenstein (Schoonspruit). While in 2011, non-pigmented yeasts were detected in all the sampling sites (Table 4.6). Results also indicated that temperature might have had an influence on the non-pigmented yeast levels found in 2010 (Table 4.1). This finding is also supported by the redundancy analysis (RDA) results that indicated that temperature had an influence on non-pigmented yeasts in 2010 and 2011 (Figures 4.1 and 4.2). The RDA analysis also indicated that nitrate levels in 2011 may have had an influence on the high levels of non-pigmented yeasts found. In some cases there were significant differences in the levels of pigmented and non-pigmented yeasts at the various water sources. However, results also showed that the difference in non-pigmented yeasts in 2010 were non-significant.

Levels of yeasts may indicate the eutrophication of aquatic environments that could be due to the strong association of yeast with organic matter concentrations in the water (Morais *et al*., 1996; Peçanha *et al*., 1996; Rosa *et al*., 1990). These authors suggest that the number and composition of yeasts species present in rivers can be used as organic enrichment indicators in water bodies (Morais *et al*., 1996; Rosa *et al*., 1995). In a study conducted by Sláviková & Vadkertiová (1997) yeast densities of the river Danube ranged from 100 to 21,100 cfu/L. In the present study similar levels were found.

5.4 IDENTIFICATION OF YEASTS ISOLATES

5.4.1 Biochemical identification using API ID 32C system

Yeast representatives of different sampling sites were selected and identified by API ID 32C. A total of 4 yeast genera were identified. These consisted of non-pigmented yeasts *viz*., *Candida catenulate, C. globosa, C. guilliermondi, C. lusitaniae, C. pellicuslosa, C. rugosa, C. sake, C. tropcalis, Cryptococcus laurentii* and *Zygosaccharomyces* spp. The pigmented yeasts were identified as *Rhodotorula glutini* and *Rh. mucilaginosa*. Species that could not be identified with the API ID 32C system may be species that are not included in the taxa provided by the system (Ramani *et al*., 1998). Ramani *et al.* (1998) demonstrated that the API ID 32C system is a reliable identification system since they could correctly identify 92% common and 85% rare yeast isolates. The API ID 32C system identifications were confirmed using 26S rRNA D1/D2 gene sequencing. Furthermore, yeast isolates that could not be recognised by the API ID 32C system were also identified using 26S rRNA gene fragments (Kurtzman & Robnett, 1998; Guffogg *et al.,* 2004). Species identified by the API ID 32C system may also have clinical significances. It may be allergenic or cause infections in humans by potentially pathogenic species such as *C. tropicalis and C. guilliermondii* (Pereira *et al.*, 2009).

5.4.2 Identification using 26S rRNA gene sequencing

Most of the species isolated in this study are common in aquatic environments (Nagahama, 2006). Since yeasts are ubiquitous in aquatic environments, a broad spectrum of yeasts was found during the 2010 and 2011 sampling periods.

Ascomycetous yeast isolates such as *Candida glaebosa, Candida intermedia, Clavispora lusitaniae, Candida nivariensis, Candida temnochilae and Hanseniaspora uvarum* were identified that are not commonly found in water sources such as Rivers and lakes. *Candida glaebosa* was isolated from a single site in the Schoonspruit (Orkney) in 2011. Colonies were white to cream on YM agar. Furthermore, this yeast was capable of growing at 4°C to 25°C. Another yeast *viz*., *Candida intermedia* was isolated from three different sites in three different water sources. Taung (Harts River), Barkley-Wes (Lower Vaal River) and Biesiesvlei (Barberspan) in 2011. This yeast was capable of growing at 4°C to 37°C. *Clavispora lusitaniae*, also known as *Candida lusitaniae* has been isolated from rotting prickly-pear, cactus and opuntia stricta in Australia. In the present study *Clavispora lusitaniae* was isolated from one site in the Mooi River (Muiskraal Bridge) and one site in the

Schoonspruit (Klerksdorp Bridge) in 2010. In 2011, this species was isolated again from the Schoonspruit (Klerksdorp Bridge and Brakspruit). The colonies were white to cream, oblong, smooth and rough on YM agar. This yeast could also be characterised as potentially pathogenic, according to its ability to grow at 37°C. It was also able to grow at 40°C demonstrating that it was a thermophile.

Candida nivariensis was first isolated from Jan Kempdorp (Harts River; 2010) and then again from Spitskop Dam (Harts River; 2011). Alcoba-Flo´rez *et al.* (2005) isolated this species in Spain, from clinical samples of three patients. This species have also been reported by other European studies, as a human pathogen that may be acquired from potted plants and hospital gardens (Lanchance *et al*., 2001; Linton *et al*., 2007). Furthermore, this yeast is potentially pathogenic. It was isolated from clinical samples by Lanchance *et al*. (2001), Tavanti *et al* (2005), Alcoba-Flo´rez *et al* (2005), Linton *et al*. (2007) and Borman *et al* (2008).

Candida temnochilae was isolated from Harts-Pampier Bridge (Harts River) during both sampling periods. *Hanseniaspora uvarum* was isolated from two different sites within the Lower Vaal River during 2011, Windsorton and Schmidtsdrift.

Some yeasts were commonly isolated from water sources. *Candida pseudolambica* commonly found in water, faeces, soil, silage and shipworm *Neotredo reynei* burrowing in dead wood were isolated from two different water sources the Lower Vaal River and the Schoonspruit. This yeast was also isolated from three sites (Schmidtsdrif, Christiana and Bloemhof Dam) within the Lower Vaal River during 2010 and 2011, respectively and two sites (Brakspruit and Bodenstein) from the Schoonspruit in 2011. The colonies were white to cream on YM agar. This yeast was capable of growing at temperatures from 4°C to 30°C. Another yeast isolate, *Candida tropicalis* isolated from the Klerkskraal Dam (Mooi River) in 2010 was white to cream on YM agar and had the ability to grow at a temperature as high as 40°C (Nagahama, 2006). Furthermore, this yeast could also be characterised as potentially pathogenic, according to the particular pathogenic yeasts list of Hurley *et al*. (1987) and its ability to grow at 37°C. This species is also considered one of the human pathogenic yeasts that may cause candidosis (Hurley *et al*., 1987).

Meyerozyma guilliermondii (Candida guilliermondii) has been isolated from numerous human infections, mostly of cutaneous origin. Systemic infections are rare, although one case has been reported in which it was isolated from a patient with aplastic anemia. *C. guilliermondii* has also been isolated from normal skin, from sea water, faeces of animals, buttermilk, leather, fish and beer. *Candida guilliermondii* was isolated from two water sources namely the Lower Vaal River (Windsorton) and Barberspan (Lichtenburg) during 2011.

Pichia kudriavzevii (also known as *Candida krusei*) had been isolated from one site in the Lower Vaal River (Christiana) and one site from Harts River (Jan Kempdorp) during 2010. Colonies were white to cream on YM agar. *Pichia kudriavzevii* is the fifth most common cause of candidemia, but probably is most noteworthy for its innate resistance to the antifungal agent fluconazole (Pelletier *et al*., 2005). It has also reduced susceptibility to other drugs (Pelletier *et al*., 2005).

Saccharomyces cerevisae commonly known as baker's or brewer's yeast, was isolated from Sannieshof (Barberspan) in 2011. This genus is comprised of eight species. One, of the *S. cerevisiae* strains, has been documented to cause human infection (Muñoz *et al*., 2005). Strains of *S. cerevisiae* appear to be more pathogenic, especially when used as a probiotic preparation in immune compromised patients (Muñoz *et al*., 2005).

Wickerhamomyces anomalus, also known as *Pichia anomala*, or *Hansenula anomala*, or *Candida pelliculosa* has been previously isolated from raw Cuban sugar in United Kingdom (UK), as well as tuberculosis patients in Italy, from sputum in Norway and brewer's yeast and clay in UK. This yeast was isolated from two sites of the Mooi River (Klerkskraal Dam and Muiskraal Bridge) and one site from Barberspan (Hotel) during 2010 (Table 4.9). Colonies were white to cream and smooth on YM agar. This particular yeast was capable of growing at temperatures from 4°C to 37°C. In a study by (Mpofu *et al*., 2008) where this particular yeast was also isolated it was found that these yeasts failed to grow at temperatures of 37°C and higher. This is a contradictory observation to the present study.

One of the most commonly isolated non-pigmented basidiomycetous yeast species in this study as shown in Figure 4.5 and 4.6 belonged to members of the genus *Cryptococcus*. In this study three *Cryptococcus* species were isolated. *C. albidosimilus, C. diffluens* and *C. flavescens*, were isolated from three different water sources in 2011. Two of these species, *C. albidosimilus* and *C. diffluens* were closely related in a cluster analysis. The relationship had bootstrap support of 98% (Figure 4.6). These species were isolated from two different water

sources Barberspan (Outflow) and Schoonspruit (Klerksdorp Bridge), respectively. *C. flavescens* was isolated from two different water sources Harts River (Schweizer Reineke) and Barberspan (Inflow). All three *Cryptococcus* species form part of the basidiomycetous yeasts and have been identified from various environmental sources including air, soil, water, pigeon droppings, and foods such as cheese, milk, beans, and wine. *C. albidosimilus* and *C. flavescens* were able to grow at a wide range of temperatures varying from 4°C to 40°C. On the other hand *C. diffluens* could only grow up to temperatures of 30°C. The ability of *Cryptococcus* species to grow at 37°C, may indicate its importance as a virulent species causing human decease for example *Cryptocoocus neoformans* (Li & Mody, 2010). *Cryptococcus albidosimilus* and *Cryptococcus diffluens* were cream and shiny, while *Cryptococcus flavescens* is white and has a shiny appearance on YM agar.

This study also identified other basidiomycetous yeast such as *Sporidiobolus pararoseus* and *Cystofilobasidium* spp. that were not commonly found in water sources such as rivers and lakes. *Sporidiobolus pararoseus* was isolated from the Harts River (Barberspan) in 2010 and 2011. *Cystofilobasidium* spp. was isolated from Windsorton in the Lower Vaal River in 2011.

Another commonly found group of yeast species belonged to the genus *Rhodotorula*. These yeasts are known for their distinctive pink or red colouration. The three most commonly isolated species are *Rhodotorula mucilaginosa* (also known as *Rhodotorula rubra*), *Rhodotorula glutinis* and *Rhododtorula minuta* (Riedel *et al*., 2008). These yeasts are found in the environment in water, soil, air, faeces, and were isolated from shower curtains and toothbrushes. They can colonise human skin and the respiratory, urinary, or gastrointestinal tract infections (Lunardi *et al*., 2006; Miceli *et al*., 2011). *Rhodotorula* infections are mostly fungaemia associated with meningitis, catheter and endocarditis (Tuon & Costa, 2008). These red yeasts have also emerged as human pathogens in the last few decades, which caused systematic infections (Riedel *et al*., 2008; Miceli *et al*., 2011). In this study four different *Rhodotorula* species (Figure 4.5 and 4.6) were isolated. The species *Rh. mucilaginosa* was first isolated in 2010 from one site in the Mooi River (Muiskraal Bridge), three sites in the Schoonspruit (Orkney, Klerksdorp Bridge and Brakspruit), one site from the Lower Vaal River (Bloemhof) and one site from Barberspan (Hotel). In 2011 this same species was isolated from two site in the Harts River (Taung and Delaryville), one site in the Schoonspruit (Orkney), and two sites from Harts River (Barberspan-Hotel and Biessiesvlei). *Rhodotorula mucilaginosa* was the most frequently isolated yeast in this study and was present in samples from almost all the sampled sites. This species is ubiquitous and have been isolated from all

kinds of natural substrates such as soil, water, feaces and trees (Gadanho *et al*., 2006). Libkind *et al*. (2009) reported that 87.5% of the pigmented yeasts occurring in Negra Lake, and ultra-oligotrophic freshwater from Patagonia, Argentina, were *Rhodotorula mucilaginosa*. According to these authors, an increase in the population of this species may be related to a temporary increase in the organic matter in the lakesone. According to Pfaller *et al*. (2010) and Tuon & Costa (2008), *Rhodotorula mucilaginosa* is one of the most common causes of fungaemia in *Rhodotorula* species. Numerous reports as stated by Tuon & Costa (2008) found that 74% of cases were due to fungaemia caused by the species *Rhodotorula mucilaginosa.* This yeast was capable of growing at temperatures from 4°C to 37°C. Colonies were pink to red and smooth on YM agar.

Another potential thermotolerant species namely *Rhodotorula glutinis* has been isolated from atmosphere, trees, leaf of peach tree, river water, soil, spoiled leather, sea-water, fruit, water supply of brewery, sputum from a case of broncho-pneumonia. In the present study this yeast was isolated from three sites in the Schoonspruit (Orkney, Klerksdorp Bridge and Brakspruit) and one site from Barberspan (Outflow) during 2010. During the 2011 sampling this species was isolated from one site in the Harts River (Schweizer Reineke), one site in Schoonspruit (Brakspruit) and two sites from the Harts River (Barberspan-Outflow and Lichtenburg). Colonies were orange to red and smooth on YM agar. In a review of 128 cases from literature it was reported that 7.7% of cases due to fungaemia was caused by *Rhodotorula glutinis* (Tuon & Costa, 2008). This yeast was capable of growing at temperatures from 4°C to 37°C. Yeasts able to grow at 37°C are associated with warm-blooded animals. Human opportunistic pathogenic yeasts are known to thrive at this temperature (Medeiros *et al*., 2008). The third *Rhodotorula* species isolated in this study was *Rhodotorula slooffia.* This species was isolated from the Harts River at two different sites, Jan Kempdorp in 2010 and Harts-Pampierstad in 2011. The colonies were pink, smooth and glossy on YM agar.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSION

The aim of this study was to determine the diversity and characteristics of yeasts from water sources of the North West Province (NWP) and to discuss their implications. Over a two year period, water samples were collected and analysed from 3 river systems, a spruit and an inland lake. In most cases the sampling represented a warm wet and cold dry season. Four objectives were formulated to achieve the aim of this study. The results of these were presented and discussed in chapters 4 and 5, respectively.

6.1.1 Physico-chemical characteristics of water

From the present study, it was demonstrated that the physico-chemical parameter levels were higher during the cold dry sampling period compared to the warm rainy sampling period. Generally, the pH ranged from 7.2 to 9.2. Elevated EC levels were detected especially at the Harts River (36-70 mS/m) and Barberspan (38-165 mS/m) sites. These results may be indicative of diffuse pollution.

6.1.2 Prevalence and diversity of yeasts

Yeast malt extract agar (YM) was successfully employed for the enumeration and isolation of yeasts from the different water sources. Representatives of yeasts isolated from the various sampling sites were purified for identification. The Mooi River and Schoonspruit sites had the highest counts of yeast compared to the other water sources. This could be due to human influence and diffuse pollution. The present study also indicated that ascomycetous yeasts are more prevalent in the water sources compared to the basidiomycetes. Redundancy analysis demonstrated links between physico-chemical parameters and yeast levels. Phosphates and nitrates levels could be linked to yeast levels.

6.1.3 Identification and characterisation of isolates using DBB, biochemical test method, 26S rDNA gene sequencing

Primary identification tests (DBB) were used to preliminary identify yeasts. Representatives of yeasts from the different sampling sites were selected and identified by API ID 32 C. Four

genera of yeasts were identified. The species consisted of *Candida catenulate, C. globosa, C. guilliermondi, C. lusitaniae, C. pellicuslosa, C. rugosa, C. sake, C. tropcalis, Cryptococcus laurentii* and *Zygosaccharomyces* spp., (non-pigmented) whereas the pigmented yeasts were identified as, *Rhodotorula glutini, Rh. mucilaginosa*. Identities were confirmed by 26S rDNA gene sequences analysis (Kurtzman & Robnett, 1998; Guffogg *et al.*, 2004).

Most of the species identified in the present study are common in aquatic environments and probably originated from terrestrial environments, such as soil and plant debris. The genus *Candida* had the highest number of species representative such as *C. krusei*, *C. tropicalis* and *C. guillermondii*. The implications of these yeasts species, particularly the oppertunistic pathogenic yeasts were also discussed, using relavant literature. Although their origin remains unknown, the characteristics of such yeast indicate a potential health risk to water users and consumers in general but the immuno-suppressed and immuno-compromised in particular. Furthermore, phylogenetic analysis was done using yeasts sequences. Results in both clusters did not demonstrate any spatial separation (Figure 4.5 and 4.6). It was not expected to find any real such separations when 26S rDNA sequence data were used. For such application molecular fingerprinting should be considered.

6.1.4 Survival and growth at various temperatures

Representative yeast isolates could grow at various temperatures. These are either yeasts with a wide temperature tolerance range or yeasts that are specialist psychrophiles or thermophiles.

6.2 Recommendations

1) Since this study was preliminary, follow up studies with a more frequent sampling regime should be conducted. Such a study should include chemical oxygen demand, biological oxygen demand or suspended solids and other relevant physico-chemical parameters for water quality parameters that would influence yeast levels.

2) Future studies should be expanded to include drinking and groundwater. Samples should also be collected during both summer and winter, to determine if there is any seasonal variation in physico-chemical parameters and yeast levels.

3) Further studies should be directed towards more detailed characterisation of the ecological and molecular features of yeasts in water sources in the NWP. For such application molecular fingerprinting should be considered.

4) Furthermore there have been no environmental profiling studies of environmental yeast communities. Such a study should be carefully planned and conducted.

5) Research on the origin of the yeasts that occur in these water sources should be conducted.

6) A study investigating pathogenic features of yeasts in water sources needs to be conducted. Antifungal susceptibility patterns of the yeasts isolated from these water sources should be determined.

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APPENDICES

Appendix A

Table 1A: A list of GPS coordinates of all the sampled sites.

Sources	Site name	GPS coordinates	
		Latitude	**Longitude**
Harts River	Dam 8	27° 50’ 56.8”S	024° 45’ 49.7”E
	Jan Kempdorp	27° 51’ 32.1”S	024° 47’ 15.0”E
	Harts-Pampier (HPR) Dam	27° 47’ 12.2”S	024° 42’ 19.1”E
	Spitskop Dam	28° 07’ 43.7”S	024° 29’ 48.8”E
	Taung	27° 32’ 26.8”S	024° 49’ 29.4”E
	Schweizer Reineke	27° 11’ 57.1”S	025° 19’ 51.5”E
	Delareyville	26° 46’ 01.5”S	025° 33’ 05.9”E
Schoonspruit	Orkney	26° 59’ 144”S	026° 37’ 906”E
	Klerksdorp Bridge	26° 51’ 899”S	026° 39’ 541”E
	Brakspruit	26° 40’ 759”S	026° 34’ 957”E
	Bodenstein	26° 19’ 717”S	026° 29’ 181”E
Lower Vaal River	Windsorton	28° 19’ 41.3”S	024° 43’ 10.4”E
	Barkley-Wes	28° 32’ 59.9”S	024° 31’ 58.8”E
	Schmidtsdrift	28° 42’ 14.1”S	024° 04’ 29.9”E
	Christiana	27° 54’ 50.3”S	025° 10’ 32.9”E
	Bloemhof Dam	27° 39’ 14.7”S	025° 35’ 41.4”E
Barberspan	Harts River	26° 38’ 37.2”S	025° 36’ 58.8”E
	Inflow	26° 37’ 33.8”S	025° 34’ 41.2”E
	Hotel	26° 35’ 26.1”S	025° 36’ 10.9”E
	Outflow	26° 33’ 00.8”S	025° 35’ 48.1”E
	Sannieshof	26° 31’ 51.6”S	025° 49’ 25.0”E
	Biesiesvlei	26° 25’ 11.6”S	025° 53’ 42.4”E
	Lichtenburg	26° 12’ 54.7”S	026° 12’ 30.3”E

Appendix B

PHYSICO-CHEMICAL GUIDELINES FOR WATER QUALITY

Table 1B: Target water quality ranges for domestic, recreation, livestock watering and irrigational purposes (DWAF, 1996).

Water parameters	Target water quality range (TWQR)			
	Domestic	**Recreation**	**Livestock watering**	**Irrigation**
pH	6.0 - 9.0	6.5 – 8.5	NA	6.5 – 8.4
TDS (ppm) ;	0 – 450	NA	<1000[a] <2000[b] <3000[c]	260
EC (µs/cm)	0 – 70	NA	NA	400
Nitrate (mg/L NO_3-N)	0 – 6	NA	0 - 100	NA
Nitrite (mg/L NO_2-N)	0 – 6	NA	NA	NA

NA – Not Available; [a]Dairy, pigs and poultry; [b]Cattle and horses; [c]Sheep.

Appendix C

MYCOLOGICAL DATA FOR WATER SOURCES

Table 1C: Mycological data for water samples from Mooi River in the wet summer season (March 2010).

Sites	**Pigmented yeasts (cfu/L)**	**Non-pigmented yeasts (cfu/L)**
Klerkskraal Dam	20	800
	0	1500
	200	6900
Muiskraal Dam	300	320
	20	280
	300	240
Around the World Bridge	0	600
	0	1140
	0	24300
Thabo Mbeki Drive	0	300
	0	780
	0	340
Trimpark Bridge	0	300
	0	400
	0	6100
Pedestrian Bridge	0	0
	0	0
	0	0
Viljoenskroon Raod Bridge	0	1000
	0	0
	0	2000

Table 2C: Mycological data for water samples from Harts River in the wet summer season (March 2010).

Sites	Pigmented yeasts (cfu/L)	Non-pigmented yeasts (cfu/L)
Dam 8	0	22
	0	18
	0	9
Harts-Pampierstad HRP Dam	0	48
	0	35
	0	17
Jan Kemp Dorp	2	1
	1	2
	2	0

Table 3C: Mycological data for water samples from Harts River in the dry winter season (July 2011).

Sites	Pigmented yeasts (cfu/L)	Non-pigmented yeasts (cfu/L)
Spitskop Dam	0	3920
	0	4320
Harts-Pampierstad HRP Dam	1040	1180
	1580	1280
Taung	260	18 960
	100	15 440
Schweizer Reineke	60	15 200
	60	17 040
Delareyville-Harts	40	4400
	20	4780

Table 4C: Mycological data for water samples from Schoonspruit in the wet summer (April 2010).

Sites	Pigmented yeasts (cfu/L)	Non-pigmented yeasts (cfu/L)
Orkney	690	500
	598	200
	650	700
Klerksdorp Bridge	1000	350
	998	450
	1200	400
Brakspruit	100	60
	450	150
	750	250
Bodenstein	320	0
	280	0
	300	0

Table 5C: Mycological data for water samples from Schoonspruit in the wet hot (May 2011) season.

Sites	Pigmented yeasts (cfu/L)	Non-pigmented yeasts (cfu/L)
Orkney	0	25 200
	0	21 280
Klerksdorp Bridge	0	27 760
	0	28 800
Brakspruit	0	24 240
	0	7 840
Bodenstein	0	1 040
	0	420

Table 6C: Mycological data for water samples from Lower Vaal River in the dry winter season (May 2010).

Sites	Pigmented yeasts (cfu/L)	Non-pigmented yeasts (cfu/L)
Windsorton	0	40
	0	30
Barkley-Wes	0	15
	0	5
Schmidtsdrift	8	120
	12	125
Christiana	0	54
	0	40
Bloemhof	240	350
	220	320

Table 7C: Mycological data for water samples from Lower Vaal River in the dry cold (July 2011) season.

Sites	Pigmented yeasts (cfu/L)	Non-pigmented yeasts (cfu/L)
Windsorton	60	140
	20	240
Barkley-Wes	0	3600
	0	60
Schmidtsdrift	0	6440
	0	1920
Christiana	160	480
	400	760
Bloemhof	40	900
	0	1400

Table 8C: Mycological data for water samples from Barberspan in the winter (July 2010).

Sites	Pigmented yeasts (cfu/L)	Non-pigmented yeasts (cfu/L)
Harts River	40	0
	0	20
Inflow	30	80
	60	0
Hotel	0	0
	0	10
Outflow	10	20
	10	10

Table 9C: Mycological data for water samples from Barberspan in the wet summer season (April 2011).

Sites	Pigmented yeasts (cfu/L)	Non-pigmented yeasts (cfu/L)
Harts River	380	400
	80	500
Inflow	1 020	1 420
	1 340	1 660
Hotel	60	240
	40	200
Outflow	320	1 700
	480	1 940
Sannieshof	40	1 200
	60	1 700
Biesiesvlei	20	2 400
	0	3 520
Lichtenburg	20	1 400
	200	2 200

Appendix D

SAMPLING PHOTOS

Harts River

a) b) c)

Figure 1D: Photos of the Spitskop Dam sampling site; a) green water and birds, b) algae in water and c) people fishing.

a) b)

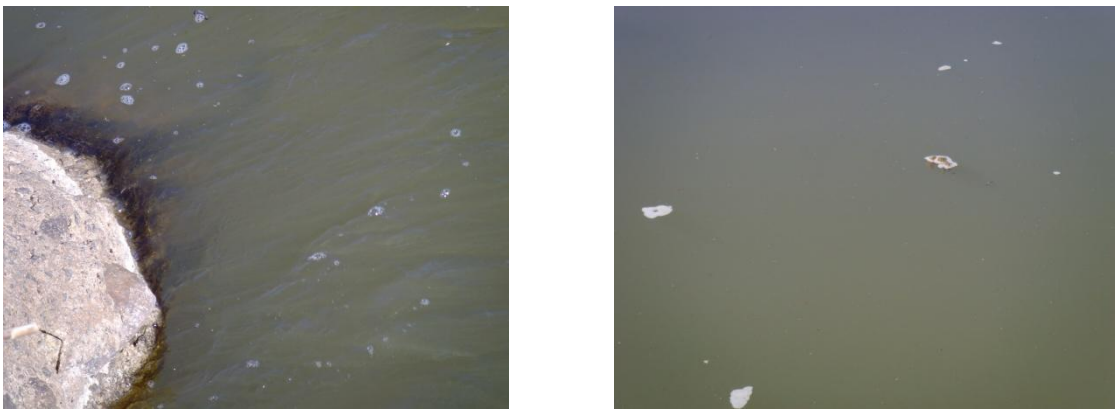

Figure 2D: Photos of the Harts-Pampier bridge sampling site; a) bubbles and b) cow faeces present in the water.

a) b) c)

Figure 3D: Photos of Taung sampling site; a) red and green algae present in water, b) goat movement and c) cow faeces around the water.

a) b) c)

Figure 4D: Photos of Schweizer Reineke sampling site; a) green algae and bubbles present in water, b) dirty water and c) one of my colleagues taking a water sample at a fast flowing region.

a) b) c)

Figure 5D: Photos of Delareyville-Harts sampling site; photos a, b and c containing green algae bedded in the water.

Schoonspruit

Figure 6D: Photos of the Orkney sampling site.

Figure 7D: Photos of the Klerksdorp Bridge sampling site.


Figure 8D: Photos of the Brakspruit sampling site.


Figure 9D: Photos of the Bodenstein sampling site.

Lower Vaal River

Figure 10D: Photos of the Windsorton sampling site

Figure 11D: Photos of the Barkley-Wes sampling site.

Figure 12D: Photos of the Schmidtsdrift sampling site.

Figure 13D: Photos of the Bloemhof sampling site.

Barberspan

Figure 14D: Photos of Barberspan site over the two year period.

Appendix E

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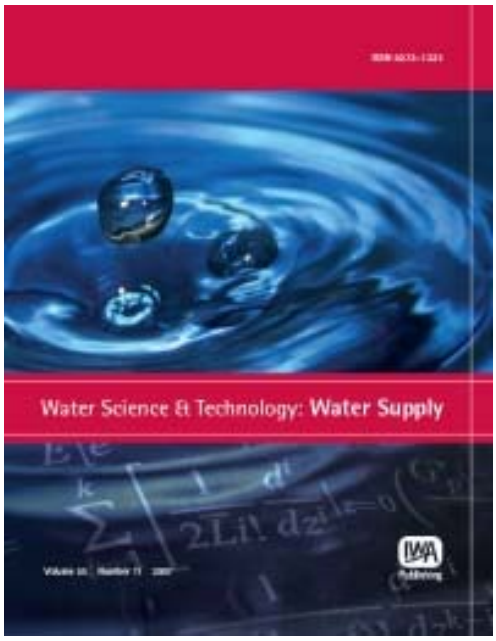

Diversity and characteristics of yeasts from water sources in the North West Province, South Africa

D. A. B. Van Wyk, C. C. Bezuidenhout and O. H. J. Rhode

ABSTRACT

Yeasts form an important part of ecosystems. Yeast biodiversity in the North West Province, South Africa, remains largely unexplored. The purpose of this study was to determine the diversity and characteristics of yeasts from water sources in the North West Province. Yeasts were isolated from four rivers and an inland lake. Various parameters were measured to determine the physico-chemical quality of the water. Isolation was done on yeast-malt-extract (YM) agar and identified using biological methods. Physico-chemical parameters were within a target water quality range for various water uses. Yeasts were detected at 22 of the 23 sampling sites. Levels ranged from 3 to 8,680 cfu/L and were broadly divided into pigmented and non-pigmented yeasts. Pigmented yeasts could potentially be an indication of pollution, while non-pigmented yeasts could potentially be pathogenic species. Levels of yeasts could be statistically associated with some physico-chemical parameters. A large percentage of isolated yeasts could grow at temperatures ranging from 4 to 40 °C. *Candida* spp., *Cryptococcus* spp., *Rhodotorula* spp. and *Zygosaccharomyces* spp. were identified. Our results provide preliminary information on the distribution and diversity of yeasts in water sources in the North West Province.

Key words | *Candida* spp., *Cryptococcus* spp., physico-chemical water quality, *Rhodotorula* spp.

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INTRODUCTON

Water is one of the most important and essential commodities for man, occupying about 70% of the Earth's surface. In South Africa, the availability of freshwater is particularly limited (DWAF 2004). It is therefore important to realize that freshwater is a limited resource with little potential for increase. The state of the water resources in the North West Province is characterized by an overall scarcity as many surface water systems are non-perennial (NWDACE 2008). Furthermore, the quantity of available surface water decreases from east to west in this province of South Africa. There is significantly higher flows in the east whereas the west borders on the Kalahari semi-desert (NWDACE 2008). Water quality issues were raised in several urban communities but the focus was on the occurrence of faecal indicator and pathogenic bacteria (Thom 2010). Faecal bacteria are also regularly detected in several surface water sources (Kalule-Sabiti & Heath 2008; NWDACE 2008). The use of other microbes as indicators of pollution have also been proposed (Stevens *et al.* 2003). One such group, which is often overlooked, is yeasts. Distribution of yeasts in the surface water of the North West Province is unknown and the implications of their occurrence undetermined.

The occurrence of yeasts in different types of aquatic environments has been reported for lakes and ponds (Sláviková &Vadkertiová 1997; Medeiros *et al.* 2008), estuaries, coasts and mango grove areas (van Uden & Fell 1968), as well as for oceans and the deep sea (Nagahama *et al.* 2003). Nevertheless, only a few studies have reported on the presence of yeasts in river water (Sláviková & Vadkertiová 1997; Medeiros *et al.* 2008).

Early studies on yeasts in association with polluted water were mainly focused on their application as organic pollution indicators (Nagahama 2006). Woollett & Hendrick (1970) performed a study in Chicago, where they

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investigated the association between heavy industrial waste and heavy domestic waste pollution and yeast levels. These researchers found that these polluted waters had large yeast populations ranging as high as 270,000 cfu/L. The yeasts found related to these pollutions were: *Rhodotorula* spp., *Cryptococcus* spp. and *Candida* spp. (Woollett & Hendrick 1970). Another study in Canada, based on yeasts as an indicator of pollution, was carried out by Simard (1971). In this study the researchers investigated the association between sewage pollution and urbanization, as well as relevant physico-chemical parameters and yeast levels. The physico-chemical parameters included biochemical oxygen demand (BOD), dissolved oxygen (DO), salinity and temperature. This study demonstrated that yeast population levels ranged from 4,800 to 10,900 cfu/L under elevated physico-chemical conditions. Species identified in this study were *Rhodotorula* spp., *Candida* spp., *Cryptococcus* spp., *Torulopsis* spp. and *Pullunia* spp. (black yeasts) (Simard 1971). A study by Hagler & Mendonça-Hagler (1981) showed that the number of yeasts increases in the presence of pollution or in the presence of algae, and it can reach a few thousand cells per litre or more.

Water pollution places consumers at risk of contracting waterborne diseases (Pereira *et al*. 2009). To protect consumers from waterborne diseases, it is important to ensure that water is completely free of pathogenic and potentially pathogenic organisms (Pereira *et al*. 2009). More than 100 yeast species identified as human pathogens have been isolated from water (Fromtling *et al*. 2003). Most of these pathogens are classified in the genus *Candida*. In particular, Hurley *et al*. (1987) listed the following pathogenic yeasts which cause candidosis, in probable descending order of virulence for man: *C. albicans, C. tropicalis, C. stellatoidea, C. glabrata, C. krusei, C. parapsilosis, C. guilliermondii, C. viswanathii, Clavispora lusitaniae (Candida lusitaniae)* and *Rhodotorula mucilaginosa (Rh.rubra)*.

Water quality systems rely mainly on monitoring indicator bacteria to ensure microbiological quality of water (Pereira *et al*. 2009). Awareness has been raised by recent studies of the presence of potentially pathogenic yeasts in both surface and ground water (Pereira *et al*. 2009). Several studies have focused on the occurrence of yeasts related to wastewater (Hagler & Mendonça-Hagler 1981; More *et al*. 2010). These studies have demonstrated that yeast counts can be a potential monitoring method that can complement coliform counts reflecting the eutrophication potential of water (Hagler & Ahearn 1987). High levels of yeasts in water sources could be an indication of either heavy pollution or low pollution, depending on the type of yeasts present in the specific water source (Woollett & Hendrick 1970). Nevertheless, compared to bacteria and viruses, yeasts are receiving little attention when the quality of water systems is at stake (Arvanitidou *et al*. 2002). The aim of this study was to determine the diversity and characteristics of yeasts in water sources in the North West Province and to discuss their implications.

MATERIAL AND METHODS

Study area

Samples were collected from four rivers (Mooi River, Harts River, Schoonspruit River and Lower Vaal River) and an inland lake, Barberspan, in the North West Province, South Africa.

Sampling

Surface water samples were taken during the summer (four rivers) and winter (Barberspan) periods. These water samples were collected using sterile Schott bottles and kept on ice. Water quality parameters such as temperature (°C), conductivity, pH, and total dissolved solids (TDS) were recorded on site using a multi-350 probe analyzer (Merck, Germany) according to instructions from the manufacturer. Nitrate (NO_3-N), nitrite (NO_2-N) and phosphates levels were determined in the laboratory using methods, kits, reagents and a spectrophotometer from Hach (Hach, Germany).

Samples were transported to the laboratory and analyzed mostly within 6 h of collection. In the worst case the analysis was carried out within 12 h.

Isolation and enumeration of yeasts

In order to isolate and enumerate yeasts from water sources, yeast-malt-extract (YM) agar (pH 4.5) (Wickerham 1951) supplemented with 100 ppm chloramphenicol was used.

A membrane filtration method was used to determine the prevalence of yeast in the water (Clesceri *et al.* 1998). A volume of 50 ml of the samples was filtered in duplicate through 0.45 μm HA membrane filters (Whatman®). The membranes were placed onto YM agar plates and incubated at 25 °C. The appearance of yeast colonies was examined daily over a period of 5 days. Isolates were purified by sub-culturing on YM agar described above.

Identification of yeasts

Yeasts were preliminarily classified based on morphotype (pigmentation, physical colony form and microscopic features) (Barnett *et al.* 2000). The following identification and characteristics tests were then carried out.

Diazonium blue B (DBB)

Yeasts are divided into two main groups: ascomycetous and basidiomycetous yeasts. To distinguish between ascomycetous and basidiomycetous yeasts the diazonium blue B (Sigma-Aldrich, Germany) test was performed (Kurtzman & Fell 1998). Yeasts were grown as spot cultures on YM agar plates and incubated at 25 °C for 2–5 days. Freshly prepared chilled DBB reagent (1–2 drops) was then applied directly to the surface of the colonies. A positive reaction for basidiomycetous yeasts was recorded when the colonies develop a dark red or violet red colour within 2 min at room temperature (Kurtzman & Fell 1998). In the case of ascomycetous yeasts no colour development should take place.

Biochemical identification

Yeast isolates were further identified using the ID 32C kit (bioMérieux, France). The system consisted of a single-use disposable plastic strip with 32 wells containing substrates for 29 assimilation tests (carbohydrates, organic acids and amino acids), one susceptibility test (cycloheximide), one colorimetric test (esculin) and a negative control. The yeast identification procedures were conducted in accordance with the manufacturer's instructions. A portion of growth from well-isolated colonies of each isolate was aseptically transferred from a freshly inoculated stock culture to sterile distilled water and incubated at 30 °C for 48 h. The strips were then visually examined and results interpreted based on instructions from the manufacturer.

Characteristics

Purified yeast isolates were subjected to various temperatures in order to evaluate their survival. Isolates were cultured using YM agar and incubated at 4, 25, 30, 37 and 40 °C. Incubations were conducted over a 7 day period.

Statistical analysis

Average and standard deviations were determined using Microsoft Excel 2007. The relationship between the surface water physical and chemical characteristics and species (pigmented and non-pigmented yeasts) was investigated using redundancy analysis (RDA) multivariate ordination techniques in CANOCO (Canoco for Windows Version 4.0, GLW-CPRO©, TerBraak 1990). The goal of multivariate analysis as an ordination technique is to arrange sample points in a space with reduced dimensionality in such a way that the axes used represent the greatest variability in the community structure. The distribution of sample points is then visualized using an ordination diagram, which is interpreted following the basic assumption that graphical proximity means close similarity.

RESULTS AND DISCUSSION

Physical and chemical analyses

The assessed physico-chemical parameters of surface water and mycological data at the 23 sites are presented in Table 1. Temperature, pH and TDS levels ranged from 12 to 26.1 °C, 7.1 to 9.23 and 256 to 961 mg/L, respectively. All sampled sites from Barberspan, two from Harts River and one from Mooi River had EC and TDS levels exceeding DWAF target water quality range (TWQR) (DWAF 1996) for some agricultural uses. Although the values from these sites were not within the TWQR, they may be indicative of diffuse pollution. Moreover, the values were lower than the critical value of 2,450 mg/L (Kempster *et al.* 1997). Nitrates, nitrites and phosphates measured values ranged from 0 to 4 mg/L, 0 to 19 mg/L and 0 to 2.44 mg/L, respectively. Two samples from

Table 1 | Physico-chemical and mycological (yeast, cfu/L) parameters measured at each of the sampling sites at the respective surface water source

Source	Different sites	pH	Temp (°C)	EC (mS/m)	TDS (mg/L)	NO_2-N (mg/L)	PO_4^{3-} (mg/L)	Percentage pigmented yeasts (Ave cfu/L)	Percentage non-pigmented yeasts (Ave cfu/L)
Mooi River	Klerkskraal Dam	8.36	23.6	39	265	2.0	1.42	2.3 (73)	97.7(3,067)
	Muiskraal Dam	8.01	23.1	47	306	0	0.86	42.5 (207)	57.5 (280)
	Around The World Bridge	7.76	23.0	70	466	4.0	0.68	0.0	100.0 (8,680)
	Thabo Mbeki Drive	8.22	24.3	64	427	3.0	0.81	0.0	100.0 (473)
	Trimpark North Bridge	8.25	24.6	65	427	3.0	0.02	4.4 (100)	95.6 (2,167)
	Pedestrian Bridge	8.25	25.0	66	443	9.0	0.12	0.0	0.0
	Viljoen Road Bridge	8.11	26.0	61	406	11.0	1.8	0.0	100.0 (1,000)
Harts River	Dam 8	8.23	24.0	62	437	–	–	0.0	100.0 (16)
	Jan Kempdorp	7.9	26.1	165	720	19.0	––	66.7 (2)	33.3 (1)
	HRP Dam	7.1	24.1	135	960	2.0	1.7	0.0	100.0 (33)
Schoonspruit River	Orkney	7.61	21.7	52	367	3.0	2.44	58.0 (646)	42.0 (467)
	Klerksdorp Bridge	7.74	22.5	39	280	2.0	0.41	72.7 (1,066)	27.3 (400)
	Brakspruit	8.05	21.1	52	381	3.0	0.55	73.8 (433)	26.1 (153)
	Bodenstein	7.91	20.4	60	432	4.0	0.63	100.0 (300)	0.0
Vaal River	Windsorton	8.23	21.1	36	256	1.0	0	0.0	100.0 (35)
	Barkley-Wes	8.47	19.3	40	260	–	0	0.0	100.0 (10)
	Schmidtsdrift	8.8	18.7	40	284	8.0	0	7.5 (10)	92.5 (123)
	Christiana	7.86	19.0	36	256	4.0	0	0.0	100.0 (47)
	Bloemhof	8.05	18.0	37	265	3.0	0	41.0 (233)	59.0 (335)
Barberspan	Harts River	8.62	13.7	121	862	0.7	1.68	50.0 (20)	50.0 (20)
	Inflow	8.58	15.2	115	820	0	1.02	52.9 (45)	47.1 (40)
	Hotel	9.23	14.7	132	961	–	0.11	0.0	100.0 (5)
	Outflow	8.61	12.0	147	956	1.2	0.19	40.0 (10)	60.0 (15)

Mooi River, one from Harts River and one from Vaal River had nitrite levels that exceeded the TWQR level (0–6 mg/L) for some agricultural uses (DWAF 1996). The North West Province is one of the regions where high TDS is expected, due to natural causes as well as human impact (NWDACE 2008). The geology of the North West Province, which consists of yellow shifting sands, could be a major cause of the high TDS values measured in the water from Barberspan, Harts River and Mooi River (NWPG 2002).

Isolation of yeasts

Yeasts were detected in 22 of the 23 sampling sites as indicated in Table 1. The number of yeasts in Mooi River ranged from 473 to 8,680 cfu/L. The highest and lowest number of yeasts was observed at the Around The World Bridge and Thabo Mbeki Drive sites, respectively. Pigmented yeasts were only observed at the Klerkskraal Dam, Muiskraal Bridge and Trimpark North Bridge sites in relatively lower numbers (<80 cfu/L) compared to non-pigmented yeasts. High numbers of non-pigmented yeasts were observed in six of the seven sites and ranged from 280 to 8,680 cfu/L. Pigmented and non-pigmented yeast were detected together at three sites (Klerskraal Dam, Muiskraal Bridge and Trimpark North Bridge). The propotion of non-pigmented to pigmented yeasts ranged from 2.4 to 97.6%. However, no pigmented yeasts were detected at the Around The World Bridge, Thabo Mbeki Drive, Pedestrian Bridge and Viljoen Road Bridge sites.

Yeast numbers in the Harts River ranged from 3 to 33 cfu/L. The highest and lowest number of yeasts was observed at the HRP Dam and Jan Kempdorp sites, respectively. Jan Kempdorp was the only sampling site where pigmented yeasts were observed in relatively lower numbers (<3 cfu/L) compared to other sampling sites. Non-pigmented yeasts showed lower numbers (<35 cfu/L) at all the sites.

Yeast numbers in the Schoonspruit River ranged from 300 to 1,466 cfu/L. The highest and lowest number of yeasts were observed at Klerksdorp Bridge and Bodenstein, respectively. High numbers of pigmented yeasts were observed in all the sites and ranged from 300 to 1,066 cfu/L. Both pigmented and non-pigmented yeasts were detected at three sites (Orkney, Klerksdorp Bridge and Brakspruit). The non-pigmented to pigmented yeasts ranged from 70.6 to 29.4%. However, no non-pigmented yeasts were detected at the Bodenstein site. More than 70% of the yeasts from Schoonspruit River were pigmented.

The number of yeasts in the Vaal River ranged from 10 to 568 cfu/L. The highest and lowest number of yeasts were observed at Bloemhof and Barkley-Wes, respectively. Pigmented yeasts were only observed at the Schmidtsdrift and Bloemhof Dam sites in relatively low numbers (<10 cfu/L). Higher numbers of non-pigmented yeasts were observed at all the sites and ranged from 10 to 335 cfu/L. Both pigmented and non-pigmented yeasts were detected at two sites (Schmidtsdrift and Bloemhof Dam). The proportion of non-pigmented yeasts among the total (793) yeast isolates from the Vaal River was 69.4%, while pigmented yeasts was only 30.6%.

Yeast numbers at Barberspan ranged from 5 to 85 cfu/L. The highest and lowest number of yeasts were observed at the Inflow and Hotel sites, respectively. Pigmented yeasts were only observed at Harts River prior to the inflow, at the Inflow and Outflow sites in relatively lower numbers (<10 cfu/L). Relatively lower numbers of non-pigmented yeasts were detected at all the sites (<5 cfu/L) in comparision with the other water sources. The proportion of non-pigmented yeasts among the total (155) yeast isolates from Barberspan was 51.7%, whereas the proportion of pigmented yeasts was 48.3%.

The levels of yeasts in the surface water sources of the North West Province appear to be influenced by, amongst other factors, the ambient and water temperatures (Table 1). It could be possible that the cold water temperatures (12.0–15.2 °C) at Barberspan had an influence on the levels of yeasts detected.

Initially, isolates were divided into pigmented and non-pigmented yeasts. The non-pigmented yeasts exhibited cream to pale brown colonies and the pigmented ones pink to red colonies on YM agar. Pigmented yeasts were mostly isolated from the Schoonspruit River and Barberspan as well as two sites from the Vaal River (Schmidtsdrift and Bloemhof). Non-pigmented yeasts were isolated from almost all the sampling sites, except Pedestrian bridge (Mooi River) and Bodenstein (Schoonspruit River). Results also indicated that during warmer temperatures the levels of non-pigmented yeasts were higher (Table 1). It thus appears that temperature may have had an influence on levels of non-pigmented yeasts.

The diazonium blue B (DBB) results indicated that the Mooi River, Harts River and Vaal River had the highest percentages of ascomycetous yeasts, while Schoonspruit River and Barberspan had the lowest (Table 2). The Mooi River had the second highest percentage of ascomycetous yeasts (85%) and all of them non-pigmented. Basidiomycetous yeasts from this river consisted only of 5% pigmented yeasts. Nearly all isolates from Harts River were ascomycetous (95%) yeasts, and all were non-pigmented yeasts. The basidiomycetous yeasts (5%) of this river only consisted of pigmented ones. Schoonspruit River had the second highest percentage of basidiomycetous yeasts (72%). The basidiomycetous yeasts consisted of pigmented (56%) and non-pigmented yeasts (44%). Of the isolates from Vaal River, 61% of the isolates were ascomycetous yeasts and 39% were basidiomycetous yeasts. Basidiomycetous yeasts consisted of pigmented yeasts (92%) and non-pigmented yeasts (8%). Nearly all isolates from Barberspan belonged to basidiomycetous yeasts (92%). Only the basidiomycetous yeasts could be divided into pigmented (38%) and non-pigmented yeasts (62%).

All ascomycetous and basidiomycetous yeasts from the respective water sources were able to grow at 25 °C (Table 3). This was expected since all these yeasts were isolated at this temperature. Further analysis showed that a large percentage of the ascomycetous isolates from the Harts (65%), Mooi (64%) and Vaal Rivers (60%) were also able to grow at 37 °C. On the other hand, those isolates

Table 2 | Percentage ascomycetous and basidiomycetous yeast isolates from selected rivers in the North West Province

Surface water sources	% Ascomycetous yeasts	% Basidiomycetous yeasts	% Pigmented	% Non-pigmented
Mooi River ($n = 23$)	85	–	5	95
	–	15		
Harts River ($n = 17$)	95	–	0	100
	–	5		
Schoonspruit River ($n = 25$)	28	–	56	44
	–	72		
Vaal River ($n = 25$)	61	–	92	8
	–	39		
Barberspan ($n = 13$)	8	–	38	62
	–	92		

Table 3 | Percentage ascomycetous and basidiomycetous yeast isolates growing at various temperatures

		% Temperature				
Surface water sources		4 °C	25 °C	30 °C	37 °C	40 °C
Mooi River ($n = 23$)	Ascomycetous	60	100	85	64	70
	Basidiomycetous	40	100	20	36	25
Harts River ($n = 17$)	Ascomycetous	41	100	94	65	35
	Basidiomycetous	18	100	6	0	0
Schoonspruit River ($n = 25$)	Ascomycetous	28	100	28	24	24
	Basidiomycetous	64	100	16	16	16
Vaal River ($n = 25$)	Ascomycetous	70	100	64	60	30
	Basidiomycetous	33	100	36	32	0
Barberspan ($n = 13$)	Ascomycetous	8	100	8	8	0
	Basidiomycetous	92	100	92	15	30

from Barberspan that were able to grow at 37 °C belonged to basidiomycetous yeasts. Determining the ability of yeasts to grow at 37 °C may be important as this temperature is associated with warm-blooded animals. Human opportunistic pathogenic yeasts are known to thrive at this temperature. Typically such species include *Candida albicans* and *Candida tropicalis* and a number of other opportunistic pathogenic yeasts (Medeiros *et al.* 2008). Results also indicated that isolates from the respective surface water sources can grow at a minimum temperature of 4 °C (psycrophylic) and maximum of 40 °C (thermo-tolerant). For example, among the ascomycetous yeasts from the Mooi River, 60% could grow at 4 °C whilst 70% at 40 °C. Scenarios that reflect the diversity of temperatures that isolates could tolerate were also evident amongst those isolates from the other sample sites (Table 3). Sláviková & Vadkertiová (1997) performed a seasonal study on the occurrence of yeasts and yeasts-like organisms in the Danube River. They reported that some yeast species can grow at low temperatures (5 °C). This result coincides with some yeast isolates from the North West Province which were able to grow well at 4 °C. These tests indicated that the yeast isolates were able to survive at a wide temperature range.

Identification of the isolates

The various yeast cells were examined at 1000X magnification after Crystal violet staining. The cells were round to ovoid in shape. Four genera of yeasts were identified following ID 32C Kit Protocol (bioMérieux, France). These consisted of *Candida catenulate, C. globosa, C. guilliermondi,*

C. lusitaniae, C. pellicuslosa, C. rugosa, C. sake, C. tropicalis, Cryptococcus laurentii and *Zygosaccharomyces* spp. (non-pigmented), whereas the pigmented yeasts were identified as *Rhodotorula glutini* and *Rh. mucilaginosa.* The 50 isolates that were identified represented 12 different yeasts species. The genus *Candida* represents the majority of the yeast isolates. Our results are consistent with the findings of Slávikóvá &Vadkertiová (1997) who also found that *Candida* spp. was the principal species in surface water in Slovakia. Generally all these species are regarded as bioindicators of pollution (Dynowska 1997; Nagahama 2006). In particular, species within the genera *Cryptococcus* and *Rhodotorula* are characteristically found in non-polluted waters, whereas *Candida* and *Saccharomyces* species can be frequently found in eutrophic waters (Nagahama 2006). Woollett & Hendrick (1970) reported that the presence of human waste was especially associated with large increases in the proportion of *Candida* yeasts in the environment. Several human-associated yeast strains have been isolated from freshwater environments (Hagler & Ahearn 1987; Nagahama 2006) and are known to cause invasive and life-threatening infections. Some of these species may be

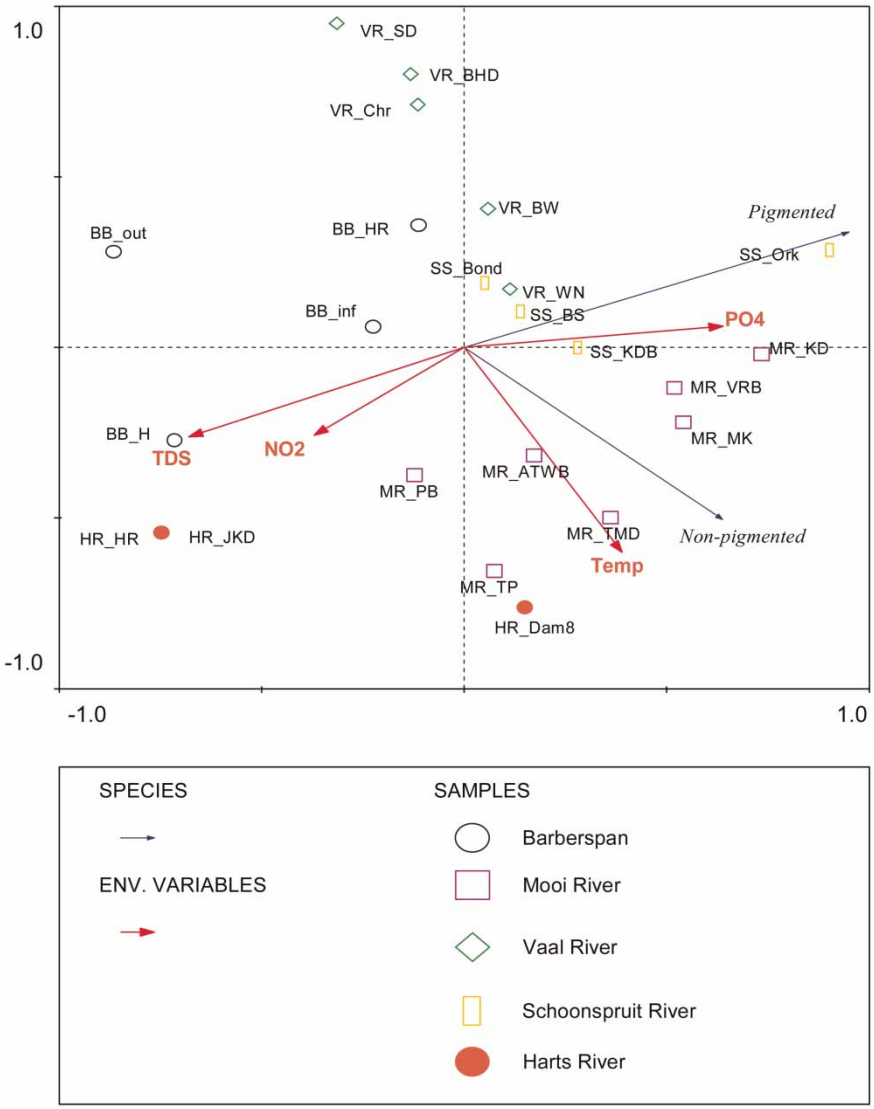

Figure 1 | Redundancy analysis (RDA) diagram for the 23 sample sites. Also indicated is the strength and direction of correlation between the axes and the following environmental variables: TDS = Total dissolved solids; PO_4^{3-} = Phosphates; Temp = Temperatures and species (pigmented and non-pigmented yeasts).

allergenic or cause infections in humans. It is thus with concern that we report that species such as *C. tropicalis and C. guilliermondii* that are opportunistic pathogens (Pereira *et al*. 2009) have been isolated from several water sources in the North West Province. This finding suggests further research into the levels and diversity of yeasts in the North West Province as well as potential sources.

An RDA ordination diagram illustrating the association between environmental variables and species (pigmented and non-pigmented yeasts) is presented in Figure 1. The aim of this analysis was to directly relate the measured environmental factors to yeast level data. The water variables TDS, phosphates (PO_4^{3-}) and temperature (°C) (Table 1) were used in the RDA analysis since these environmental variables could have an influence on the species distribution. This association is demonstrated by the angle between variables and species. A small angle indicates a close relationship. The results obtained indicate a strong positive association between the pigmented yeasts and phosphates (Figure 1), whereas the non-pigmented yeasts had a positive association with temperature.

CONCLUSIONS

The results of this research showed that yeasts occur widely in surface water sources in the North West Province. The distribution is not uniform and may be influenced by several factors. This distribution pattern should be further investigated. All the non-pigmented yeasts were ascomycetous yeasts whereas the pigmented yeasts were either ascomycetes or basidiomycetes. A large number of the yeasts isolated could grow at a wide range of temperatures (4 , 25, 30, 37 and 40 °C). Most of the species isolated in our study were commonly isolated from aquatic environments. The genera *Candida, Cryptococcus, Rhodotorula* and *Zygosaccharomyces* spp. were often detected. The genus *Candida* had the highest number of species with the presence of potentially opportunistic pathogens such as *C. tropicalis* and *C. guilliermondii*. For this reason, further investigation is needed to confirm and explain these results as well as determine pollution sources.

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