Diversity and antifungal susceptibility yeast in the selected rivers in the North West Province

M.E. Monapathi

23680490

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Supervisor: Prof. C.C. Bezuidenhout

Co-supervisor: Mr. O.H.J. Rhode

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ABSTRACT

Several yeast species had previously been isolated from water systems in the North West Province, South Africa. Some of the identified species had, in other studies, been associated with superficial mucosal infections to life threatening diseases. Antifungal drugs are used to treat such yeast infections. However, due to prophylactic usage and continuous exposure some yeast species have developed resistance to some antifungal agents. The aim of this study was to determine the diversity and antifungal susceptibility of yeasts in selected rivers, Mooi River and Harts River in the North West Province, South Africa. Waters samples were collected from the rivers in summer and winter seasons. Physico-chemical parameters such as pH, temperature, total dissolved solids, chemical oxygen demand, nitrates and phosphates were measured to determine the water quality. Yeast colonies were enumerated at room temperature and 37**°**C using yeast-malt-extract agar (containing 100 ppm chloramphenicol). Pure isolates from 37°C were identified by biochemical tests and 26S rRNA gene sequencing. Yeast sequences of isolated yeasts were sent to Genbank. Phylogenetic tree was conducted to determine phylogenetic relationship between the yeast isolates. Disk diffusion antifungal susceptibility tests were conducted on the yeast species. Physico-chemical parameters of the water were within target water quality range for livestock farming but in most sampling sites out of range for irrigation use. pH, Nitrates, phosphates and chemical oxygen demand levels ranged from 7.40 to 8.64, 0 to 5.4 mg/L, 0 to 7.14 mg/L and 31 to 43 mg/L, respectively. Elevated levels of total dissolved solids were measured in all the sampling sites. Total yeast counts ranged between 320-4200 cfu/L and 27-2573 cfu/L for room temperature and 37˚C. All the yeast colonies isolated were non-pigmented. Diazonium Blue B tests determined the yeasts isolates as ascomycetes. Haemolysin and extracellular enzyme production tests were negative on all the isolates. Yeasts isolates were identified and belonged to the genera *Arxiozyma, Candida*, *Clavispora*, *Cyberlindnera, Lecythophora, Pichia*, *Saccharomyces*, and *Wickerhamomyces*. *Saccharomyces cerevisiae* and *Candida glabrata* were mostly isolated species. Furthermore, the results indicated that levels of yeast could be correlated to physico-chemical quality of water. A large number of isolates were resistant to azoles, especially fluconazole as well as other antifungal classes. Most of the *Candida* species were resistant to almost all the antifungals. Several of the isolated yeast species are opportunistic pathogens. They could cause infections in sensitive individuals during occasional direct contact especially immune compromised people. Resistance of these yeast species to antifungal agents is a major health concern.

Keywords: Surface water resources, yeasts, water quality, opportunistic pathogens, extracellular enzyme production, 26S rRNA gene sequences, antifungal susceptibility tests.

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DECLARATION

I, Mzimkhulu Ephraim Monapathi, declare that the dissertation for the degree of Masters of Science in Environmental Science at the North-West University hereby submitted, has never been submitted by me for the degree at this or another University, that it is my own work in design and execution. All the material contained herein has been duly acknowledged.

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Mzimkhulu Monapathi Date

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CHAPTER 1 - INTRODUCTION

1.1 Introduction and problem statement

A recent study has demonstrated that several yeast species were frequently isolated from surface water of the North West Province, South Africa (Van Wyk *et al*., 2012). These species included several non-pigmented yeasts such *Candida catenulate, Candida globosa, Candida guilliermondi, Candida lusitaniae, Candida pellicuslosa, Candida rugosa, Candida sake, Candida tropicalis, Cryptococcus laurentii* and *Zygosaccharomyces* spp. There were also pigmented yeasts identified as *Rhodotorula glutini* and *Rhodotorula mucilaginosa* (Van Wyk *et al*., 2012). This was regarded as problematic as several of these species may also be opportunistic pathogens. Such findings are also of concern as these water sources are used for recreation and religious purposes as well as animal watering and irrigation (Van Wyk *et al*., 2012).

The number and species of yeast in aquatic environments depend on the quality of the water in the presence of organic pollution (Hagler and Ahearn, 1987). Some species in the genera, *Candida*, *Cryptococcus*, *Pichia* and *Rhodotorula* are regarded as bio-indicators of level of pollution (Nagahama, 2006). Common species include *Aureobasidium pullulans*, *Cryptococcus albidus*, *Cryptococcus laurentii*, *Debaryomyces hansenii* and *Rhodotorula mucilaginosa* (Nagahama, 2006). High levels of yeasts in water sources could be an indication of various types of pollution, depending on the type of yeasts present in the specific water source (Woollett and Hendrick, 1970).

Pathogenic yeast species in fresh waters are capable of infecting healthy hosts. These may cause diseases ranging from mucosal to life threatening disseminated infections (Yamaguchi *et al*., 2007). The rise in the number of HIV/AIDS patients and other immune compromised individuals in the population have led to an increase in the frequency and significance of opportunistic fungal infections (Yamaguchi *et al*., 2007). In South Africa, it was estimated that 6.1 million people were living with HIV and AIDS, the highest number of people in any country (UNAIDS, 2012). Infection such as thrush, athlete's food, ring-worm, candidiasis, jock itch and cryptococcal meningitis are common amongst immune compromised patients. These fungal

infections are frequently caused by pathogens from the genera as *Candida* and *Cryptococcus* (Richard-son, 2005). Antifungal agents such as azoles are used to treat such yeast infections.

Over the past few years, resistance to azoles compounds especially among *Candida* species has been the subject of intense investigation (Pfaller *et al*., 2010; Miceli *et al*., 2011; Abrantes *et al*., 2013). In the treatment and prophylaxis of oro-oesophageal candidiasis in the early 1990s, fluconazole became the antifungal of choice. However, in the years following the introduction of fluconazole, resistance was reported in 41% of the patients (Canuto and Rodero, 2002). Prophylactic dosages of fluconazole have also been prescribed on individuals with yeast infections from yeast *Cryptococcus* spp. Fluconazole is not completely metabolised in the human body (Kim *et al*., 2007). Thus, there may be considerable amounts of this antifungal agent that ends up in the domestic wastewater streams (Kim *et al*., 2007). This could affect the prevalence and distribution of yeasts present in natural water bodies that are passing through towns.

Previously, identification of yeasts was done using phenotypic characteristics such as morphology, ability to ferment sugars, growth on various carbon and nitrogen compounds and physiology (Pincus *et al*., 2007). Phenotypic characterisation of yeasts has resulted in severe misidentification of yeasts. In addition, phenotypic methods have been found to be time consuming and strenuous (Kurtzman and Robnett, 1998). Different molecular methods such as Polymerase Chain Reaction (PCR) and sequencing have provided speed, accuracy and reliability in characterisation and identification of yeasts up to species level (Pincus *et al*., 2007).

The North West Province is characterised by overall water scarcity as its rivers are non-perennial (NWDACE, 2008). This water scarcity is compounded by surface water pollution from the mines, agriculture and urban runoff (NWDACE, 2008). North West Rivers are used for agricultural, religious and recreational purposes. Yeast strains that are resistant to commonly used antifungal drugs indicate a potential health risk, especially to immune compromised individuals that use the river water for several direct usage. However, despite the widespread occurrence of yeasts, little attention has been given to their presence and significance in aquatic environments, as well as the information on the antibiotic resistance of the environmental yeasts (Arvanitidoua *et al*., 2002; Medeiros *et al*., 2008).

1.2 Research aim

The aim of this study was to determine the diversity and antifungal susceptibility of yeast in the selected rivers in the North West Province

1.3 The objectives of the study were:

1) To determine the physico-chemical properties of the sampled water.
2) To enumerate yeast colonies based on 2 different incubating temperatures and relate yeast colony forming units to water quality parameters.
3) To isolate and identify yeast species that grow at 37 °C using biochemical tests and 26S rRNA gene sequencing.
4) To determine the antifungal susceptibility patterns of the isolated yeast species.

CHAPTER 2 – LITERATURE REVIEW

2.1 State of the water resources in South Africa

South Africa depends on its water resources to provide for its social and economic needs. (DWAF, 2008). Water related problems pose a serious threat to the security, stability, and sustainable development of the country (DWAF, 2004). South Africa is located in a semi-dry part of the world and its water resources in worldwide standings are scarce. The country largely experiences high unavailability of water (DWAF, 2004). This is caused by varying climatic conditions that range from the desert and semi-desert in the west to sub-humid along the eastern coastal area. The ordinary rainfall for the country is about 450 mm per year (mm/a), thus lower than the world average of approximately 860 mm/a. Furthermore, evaporation is relatively high (DWAF, 2004).

Water in South Africa comprises of surface water (77%), return flows (14%) and ground water (9%) (DWAF, 2008). NWP-SoER. (2002) describes surface water as water from the rivers, dams, pans wetlands and dolomitic eyes that originate from the underground sources. South Africa depends mainly on surface water resources for most of its urban, industrial and irrigation requirements. Of the available water resources 63% is used for afforestation and agriculture (DWAF 2010). Agriculture incorporates livestock, watering and irrigation. Municipal and domestic demands use 27% which is divided between urban (24%) and rural areas (3%). Mining, industry and power generation each share 2% of the total water demand (DWAF, 2010).

2.2 State of water resources in the North West Province

South Africa's water resources have been decentralized in 19 water management areas (WMAs). The North West Province shares some of these WMAs with neighboring provinces as well as Botswana. Major WMAs include Crocodile-West and Marico, Middle Vaal and Lover Vaal (DWAF, 2010). WMAs are established to manage and protect the water resources (Brettar and Hofle, 2008). Surface water resources in the North West Province consist of rivers, dams, pans and wetlands and dolomitic eyes that are fed by the underground water (NWDACE, 2008). Surface water and underground water are integrated and interdependent. As a result, water quality and quantity that affects the underground water have effects on the surface water (NWDACE, 2008).

North West Province water resources are limited and under pressure due to non-perennial surface water systems (NWDACE, 2008). Average rainfall varies between 700 mm in the east to less than 300 mm in the west. The province is considered to be an arid region due to low precipitation (NWDACE, 2008). In most parts of the province, evaporation exceeds rainfall. Water scarcity is a major problem in the North West Province. This is worsened by the decline in water quality as a result of water pollution on the province's rivers (NWDACE, 2008). Mining, industries and agricultural activities in the North West Province place an environmental challenge on its water resources. Water pollution from such economic and social activities could have long term adverse health effects on the population in the province (DWAF, 1999).

2.2.1 Pollution in the North West Rivers

According to WHO (1996), water pollution is an introduction by man, directly or indirectly, any substance or energy that can cause harm to living resources and cause hazard to human health. This includes impairment of water quality with respect to its use in domestic, agricultural, industrial and economic activities (WHO, 1996). Water consumers are likely to contract water borne diseases that are caused by pathogenic and potentially pathogenic organisms from polluted water (Pereira, 2009).

The quantity and quality of water resources in the North West Province is affected by the anthropogenic activities around the province. These anthropogenic activities include agricultural practices, mining, industries and urbanization (NWP-SoER, 2002). Agricultural practices with potential to pollute water resources include agricultural drainage and wash off. These include return flows, fertilizers and pesticides from farming and irrigation in the province (DWAF, 2004; NWDACE, 2008).

Chemical substances from industries as well as acids and salts from the mining activities contribute to water pollution in the rivers systems of the North West province (DWAF, 2004; NWDACE, 2008). Human settlements in the province have been developed to cater for specific economic activities such as mining and agriculture (NWP-SoER, 2002). Urban wash off, effluents from waste water treatment and untreated sewage from the human settlements results in salts, nutrients and bacteriological contamination in rivers (DWAF, 2004).

2.2.1.1 Mining

The mining sector is a big contributor to the South African economy (DWAF, 2008). It uses water for production and is a key contributor to water quality problems (DWAF, 2008). Mining is one of the major economic sectors of the North West Province. Large platinum mines and smelters in the Rustenburg area and gold mines in Orkney and Klerksdorp area dominate the mining sector (NWP-SoER, 2002). Other minerals mined in the province include diamonds, chrome, manganese nickel, vanadium, limestone, shale, travertine, dolomite and wonderstone (NWP-SoER, 2002). However, increased global demand for minerals has increased exploitation of natural resources (Kelly, 1998). There is an alarming awareness related to mining industries contamination to surface and ground water resources (Kelly, 1998).

Mining discharges from active mines and abandoned mines have ended up into the surface water (Van Der Walt, 2002). Mining minerals including gold, diamond, uranium, tungsten, iron ore, thorium and selenium pose a serious threat to water quality in the Mooi River catchment (Barnard *et al*., 2013). Water from mining and milling operations as well as tailing wastes are discharged into ponds and dams. The highly concentrated toxic metals in the eroded or disused tailing dams have pH as low as 1.7 in water. This acidic water may be released into the environment (Wittmann and Forstner, 1977).

A South African Water Research Commission funded project has demonstrated that heavy metals and radionuclide concentrations in the sediment at a specific location in a Mooi River catchment near Wits gold mines are very high (Wade *et al*., 2002). These particular sites are close to the West Wits goldfields area near Carletonville. The release of mine water into the Mooi River may contribute to increased toxic metal levels in the water and sediments (Kelly 1998; Wittmann and Forstner, 1977). The presence of toxic metals in water could be fatal to aquatic orgamisms. Metals such as zinc, lead and cadmium have resulted in death in macroorganisms such as crabs and crayfish (Dalas and Day, 2004).

2.2.1.2 Industry

Industrial activities in the North West province support the mining, agricultural and food production industries. Industries affect the social structure of the community because they are located in the urban areas (NWP-SoER, 2002). Discharges from industrial effluents could reach the river systems in treated or untreated form. Industrial emission return flows from several

urban, industrial area and informal settlements have affected the Mooi River water quality (Van Der Walt, 2002).

Industrial emission from oil, food canning, pulp and paper, textile as well as tanning/leather finishing industries have impact on the surface water resources (Dallas and Day, 2004). Industries in the North West Province affect the quality and quantity of the available water. There is a demand of water for the industries and labour living close to the place of work. Waste products from the industries and the community are discharged into the environment (NWP-SoER, 2002)

2.2.1.3 Agriculture

Agriculture is an important economic activity in the North West Province (NWP-SoER, 2002; NWDACE, 2008). Agricultural activities in the North West Province comprises of livestock farming, crop farming and wildlife farming (NWP-SoER, 2002). Livestock production is practiced throughout the province. Cattle (both beef and dairy), sheep, goats and pigs are the primary livestock enterprises (NWP-SoER, 2002). Agricultural run-off containing fertilizers, insecticides, fungicides and herbicides regularly lands in the surface water in the province (NWDACE, 2008).

Irrigation is the major user of water in the province (Van Der Walt, 2002). The major irrigation schemes include the Crocodile and the Vaal-Harts River schemes. The Vaal-Harts irrigation area covers a total of 43700 ha with wheat, maize, groundnuts taking 36%, 23% and 22%, respectively of the total irrigated fields (NWP-SoER, 2002). Crocodile irrigation scheme is the large scale irrigation activity along the Crocodile River with irrigation requirements of 37% of the water requirements (DWAF, 2008). Citrus farming and cash crop farming are irrigated along the Crocodile River (DWAF, 2008). The Mooi River has been the major source of water for Potchefstroom and surrounding population for many generations through irrigation, livestock rearing and some mining activities (Van Der Walt, 2002).

2.2.1.4 Urbanization

Most of the human settlements in the North West Province have been developed due to specific economic activities such as mining, tourism and agriculture (NWP-SoER, 2002). However, urbanization and population growth exert pressure on available water resources (DWAF 2004). Urban runoff from the North West Province towns and cities such as Potchefstroom, Klerksdorp and Rustenburg that are not directed into a main drain or sewer ends up into neighboring water

systems (NWDACE, 2008). Included in these is storm water runoff from urban surfaces such as roads.

Domestic waste that ends up in the rivers comprise of discharge of treated sewage effluent (NWDACE, 2008). Microbial contamination results from untreated sewage that land in the water resources. Domestic sewage would usually contain bacterial and viral loads, protozoans, pathogenic and potentially pathogenic fungi and yeasts (Bezuidenhout, 2012). Pathogens may cause water borne diseases such as diarrhea and cholera when the deposited into receiving water bodies (DWAF, 2002).

In a case study that was conducted by Van der Walt. (2002) in the Mooi River, it was established that there were elevated levels of phosphates in Kromdraai along the Mooi River. This was attributed to the impact of Potchefstroom sewage works. The study also showed a rise in contaminants between Potchefstroom dam and Potchefstroom as a result of urban storm water runoff. North West Province dams act as sinks for agricultural, industrial and urban pollutants (NWP-SoER, 2002).

2.3 Water quality

Water quality refers to the physical, chemical and biological characteristics of the water. These characteristics determine the fitness of the water for various uses (DWAF, 2004). Natural events and anthropogenic influences can affect the aquatic environment in many ways (WHO, 1996). In developing countries, rivers are polluted by anthropogenic activities such as industrial waste, domestic waste as well as agricultural activity (Manjare *et al*., 2010). To successfully manage the water resources, regular monitoring of data of the physico-chemical and microbiological quality of water should be conducted (Bezuidenhout, 2012).

The department of Water Affairs (DWAF, 1996a) defines the Target Water Quality Range (TWQR) for a particular constituent and water use as the range of levels at which the presence of the constituent would have no known adverse effects on the fitness of the water from long term continuous use. Microbiological quality determinants may indicate water quality that could cause immediate adverse health effects (Adeleke and Bezuidenhout, 2011). These determinants include faecal indicator bacteria (e.g. faecal coliforms and *E. coli*) and bacteriophages. There are no standards for yeasts and filamentous fungi.

In determining the long- and short term safety and health effects on human and livestock, it is important to assess the physico-chemical characteristics of the water systems at their exposure (Jonnalagadda and Mhere, 2001). Water quality parameters such as temperature, phosphates, nitrates, total dissolved solid, pH and electrical conductivity are measured and monitored to define the water system, to understand the relationship between parameters as well as to evaluate environment sustainability (Dallas and Day, 2004). However, these parameters normally may not have immediate health effects (Adeleke and Bezuidenhout, 2011).

2.3.1 Temperature

Water temperature is an important characteristic of rivers as it influences most quality affecting processes. Water properties and processes that are affected by water temperature include water viscosity, physical reactions, chemical equilibria, reaction rates and biological reactions such as habitat integrity and growth rates of aquatic organisms (Loperfido, 2014). Aquatic microorganisms have temperature ranges at which they show optimum growth, survival and reproduction. Changes in water temperature could expose aquatic organisms to potentially lethal conditions (Dallas and Day, 2004).

Surface water temperatures range between 0 and 30°C (WHO, 1996). The inland aquatic water temperatures in South Africa generally range between 5 and 30°C (DWAF, 1996c). However, temperatures changes with respect to geographical features of the region, catchment area, seasonal changes and the impact of anthropogenic activities (DWAF, 1996c). In a study conducted by Jordaan and Bezuidenhout (2012) in the Vaal River, different sampling times were defined by different temperatures. Low temperatures were measured in winter (10 to 13°C) and higher temperatures were measured in summer (24.4 to 28.7°C).

Increased water temperatures decrease oxygen solubility of dissolved oxygen in the water. This results in reduced oxygen concentration in the water. The amount of oxygen available to aquatic animals is decreased (Dallas and Day, 2004). High temperature increase sewage fungal growth and these results in reduced environmental water quality (Dallas and Day, 2004). Aquatic environments may also contain substances such as phenol and zinc which are toxic to micro-organisms. Microorganisms become more vulnerable to these toxic substances at increased water temperatures (Duffus, 1980).

High temperatures in surface water result from thermal pollution in rivers (Dallas and Day, 2004). Thermal pollution is described as heated discharges usually from the power plants, metal foundries, sewage treatment plants and returning irrigation water (Dallas and Day, 2004; WHO, 1996). Water temperature also has the influence on human activity such as aquaculture and recreation (Loperfido, 2014). Segments of the rivers of the North West Province are all exposed to the listed thermal pollution and human activity patterns.

2.3.2 Nutrient enrichment

Natural and anthropogenic activities such as agricultural run-off result in the presence of nitrates in water (Yang *et al.*, 2008). These nutrients accumulate in a body of water and facilitate the eutrophication process (Walmsley, 2000). According to OECD (1982), eutrophication is the enrichment of water, which results in the stimulation of an array of symptoms of changes. The changes include increased production of algae and aquatic macrophytes and deterioration of water quality. Other symptomatic changes such as turbidity, discolouration, foam and odour are found to be undesirable and interfere with the water use (Walmsley, 2000).

The North West Province has agricultural, mining and industrial activities that make the water sources prone to pollution by nutrients such as nitrates and phosphates. In a study by Molale (2012), nitrates levels for Harts River, Barbespan and Vaal River measured between 0.0 and 4.0 mg/L. In another study conducted by Van Wyk *et al*. (2012) in North West Province water sources, nitrates levels measured between 0.0 to 19.0 mg/L. Elevated nitrates could be the result of surface run-off from farms and storm water run-off into the water during the early rains and sewage treatment plants (Razak *et al*., 2009).

High concentrations of phosphates occur in waters that receive sewage, animal waste, crop residues and runoff from cultivated lands (Dallas and Day, 2004). Increased concentrations of phosphates results in increased photosynthesis in aquatic plants such as algae and increased oxygen demand from aquatic species (Dallas and Day, 2004; NWDACE, 2008). In a study by Van Wyk *et al*. (2012) phosphate levels from the North West Province water resources measured between 0 to 2.97mg/L. High phosphate levels could be attributed to agricultural runoff and sewage effluent from the nearby towns (Moniruzzaman *et al*., 2009).

2.3.3 pH

According to Dallas and Day (2004), pH is determined largely by the concentration of hydrogen (H^+), hydroxyl (OH^-), bicarbonate (HCO_3^-) and carbonate ions (CO_3^{2-}) ions. The change in water pH leads to the concentration change of both H^+ and OH^- ions, which affects the ionic and osmotic balance of aquatic organism (Dallas and Day, 2004). At a given temperature, pH indicates the intensity of the acidic (pH < 7) or alkaline (pH > 7) condition of the aquatic system, while pH 7 represents a neutral condition (WHO, 1996; Dallas and Day, 2004).

The pH determines how suitable the water is for various purposes as it is implicated in toxicity to aquatic plants and animals (Venkatesharaju *et al*., 2010). In an alkaline state, metallic ions such as aluminum are precipitated in elemental form and thus biologically unavailable. Bacterial species survive well in alkaline conditions. However, in an acidic condition the ions become available and if at high levels will become toxic. Detrimental effects of pH on the health of aquatic species are well known with low pH having been directly implicated in mortality of insects and amphibians (Loperfido, 2014). However, yeast species prefer slight acidic (pH ±5.0) conditions.

Acid mine drainage is one of the sources of pollution that changes the pH in water (DWAF, 2006). Some communities living in rural areas adjacent to the mines in the North West Province depend on boreholes for drinking water. Surrounding farming communities use both ground water and surface water for drinking purpose, livestock watering and irrigation. In Wonderfonteinspruit and Tweelopiespruit, the presence of uranium from acid mine drainage has been reported (Siphuma, 2012). This is a worrying situation as the plants absorb metals from the acid mine drainage and are passed onto the rest of the food network and this results in health related illness and death (Van Eeden *et al*., 2009). The pH of the water of all these sites is also decreased.

Many fresh waters have more or less neutral pH range between 6.0- 8.0 (Dallas and Day, 2004). South African Target Water Quality Range for most water uses is between pH 6.0 and 9.0. Consumption of water within this range will have no significant impact on health. Consumption of water levels of pH 11.0 and above will pose a severe danger to health (DWAF, 1996b).

2.3.4 Total Dissolved Solids (TDS) and Electrical conductivity (EC)

TDS is the measure of materials dissolved in water. It incorporates the total quantity of dissolved material, organic and inorganic, ionized or un-ionized in a water sample (Dallas & Day, 2004). For a given water body, TDS and electrical conductivity are related (WHO, 1996). TDS and EC are directly proportional. The latter is a measure of the ability of water to conduct electrical current (Dallas and Day, 2004). In a study conducted by Bezuidenhout (2012) on the surface water in the North West Province in 2011 and 2012, the EC was higher for some of the sites compared to others. Sampling sites associated with the Harts River measured elevated EC. High EC greater that the target water quality range for irrigation use (>70 ms/m) was measured at the sampling sites (Spitskop Dam (84 ms/m), Harts- Pampier Dam (133.1 ms/m), Schweizer Reneke (78.8 ms/m) and Delareyville (109.6 ms/m). This may indicate increased salinity that could be the result of water pollution from agricultural run-off.

Natural and anthropogenic activities such as industrial effluents, irrigation, return of large effluents of sewage effluent and urban runoff result in increased TDS in rivers (Dallas and Day, 2004). North West Province naturally has high TDS (NWDACE, 2008). This is caused by natural causes and human impact. Geology of the North West Province consists of yellow shifting sands. This could be the natural cause of elevated TDS in the province (NWP-SoER, 2002). Apart from being used as rough indicator of mineral content in the rivers, TDS and EC are measured to establish pollution zone around the effluent discharge or the extend of influence of run-off waters (WHO, 1996).

2.3.5 Chemical oxygen demand (COD)

The oxidation of organic matter and inorganic wastes in receiving water depletes the dissolved oxygen supply. This can have profound effects on aquatic life (Water quality criteria, 1968). COD demand is the amount of oxygen in the form of a strong oxidizing agent consumed when organic matter is oxidized (Noguerol-Arias *et al*., 2012). Industrial effluents, agricultural run-off and domestic wastes are the major sources in water resources (DWAF, 1996b). The COD level for wastewater effluent acceptable for environmental water is below 75 mg/L (South Africa, 1984). In a study conducted by O'Reilly (2012) where the author assessed the physico-chemical and microbiological quality of household water in the Vaal-harts irrigation scheme, COD levels measured 0 and 28.5 mg/L. The levels were within the acceptable ranges for COD level for wastewater effluent.

2.3.6 Microbiological quality

Microbiological pollution of water is one of the problems that affect the water quality (DWAF, 2009). Bacteria, viruses and bacteriophages are considered major water pollutants. They contribute to the decline in water quality and are responsible for various health risks (Azizullah *et al*., 2011). Faeces are regarded as the most frequent source of health significant microbial contamination of water supplies (Barrell *et al*., 2000; Ministry of Health, 2005). Bacteria such as faecal coliforms, enterococci and *E. coli* and heterotrophic plate count (HPC), bacteriophages and viruses are microbiological quality determinants in faecal contamination of water sources. Unlike physico-chemical parameters, microbiological quality determinants may indicate water quality that could indicate immediate health risks (Adeleke and Bezuidenhout, 2011).

There are compliance guidelines to these indicator organisms. According to DWAF, 1996a, 100ml of the water should be absolutely free from faecal indicator species. Bezuidenhout (2012) conducted a study in the North West Province water resources in 2010 and 2011. In the study, total coliform, faecal coliform, *E.coli* and enterococci were present in all sampling sites. Overall average total coliform, faecal coliform and *E.coli* were generally high at the Lower Harts and Schoonspruit rivers. Enterococci levels measured in the Mooi River, Harts River, Barberspan, Schroospruit and Vaal River exceeded the TWQR for several water uses. This demonstrates that the surface water of the North West Province is contaminated by various faecal indicator bacteria (Bezuidenhout, 2012).

2.4 Selected rivers in the North West

2.4.1 Mooi River catchment

The Mooi River catchment is located in the eastern region of North West Province and a section of the western region of Gauteng province in South Africa. It is divided into three major sub catchments, namely the Wonderfonteinspruit (north eastern reach), the Mooi River proper (northern reach) and the Loop Spruit (eastern reach). Four major dams are found in the Mooi River catchment: Klerkskraal dam, the Boskop dam, the Klipdrift dam and the Potchefstroom dam (Van Der Walt *et al.*, 2002; Wade *et al.*, 2002). The overall catchment is defined by inadequate water availability as it experiences mean annual precipitation of 683 mm and the mean potential evaporation of 1650 mm (Van Der Walt *et al.*, 2002).

Mooi River has experienced environmental problems as a result of the impact of gold mining, population growth and increased water utilisation (Currie, 2001). Water quality monitoring is vital in the Mooi River catchment due to major gold mining activity in the Wonderfonteinspruit to the north of Potchefstroom (Van Der Walt *et al.*, 2002). Discharges from both active and abandoned mines pose a pollution threat to the surface water. The mining pollution is a major problem because Tlokwe Local Municipality currently relies on the Mooi River as a sole source of domestic water (DWAF, 1999; le Roux, 2005). There is also a possible consumption of untreated water from people living in the informal settlements (Wade *et al.*, 2002).

Apart from supplying irrigation water, the Mooi River is also used for recreational activities such as swimming and angling (le Roux, 2005, Van Der Walt *et al.*, 2002). Mooi River decants into the Vaal River to the south of Potchefstroom. Agricultural activities to the north and south of Potchefstroom have impacted the river and the quality of the water in the river. High nutrient washout from the agricultural lands has resulted in periodic algal blooms. The river water has also been subjected to high TDS (DWAF, 2009).

2.4.2 The Harts River

The Harts River is part of the Lower Vaal WMA along with rivers such as Molopo, Kuruman and lower reaches of the Vaal River. Three tertiary catchments: upper Harts River (C31), the Dry Harts (C32) and the Harts River downstream of the confluence with the Dry Harts (C33) comprise the Harts River (DWAF, 2009). The source of the Harts River is in the North West Province near the town of Lichtenburg. The river flows south westerly via Barberspan and Taung dam to Spitskop dam after which it flows into the Vaal River near Delportshoop. The Harts River is a non-perennial river as it experiences rainfall seasonally only in the summer months (DWAF, 2009). Barberspan is connected to the Harts River via a channel. It is recognized as an important sanctuary for the birds in relatively dry seasons and it is used for bird watching and angling. The pan is surrounded by land uses such as cattle and maize farming (Swart and Cowan, 1994)

The Harts River supply water to the local users for domestic and agricultural use. Major land uses in the area include agriculture through irrigated land from planting and harvesting of cotton, maize and groundnuts (DWAF, 2009). Livestock farming of beef, dairy, cattle, goats, sheep, pigs and ostriches is also extensively practiced in the Harts River. The Vaal-harts irrigation scheme is managed by Vaal-Harts Water. It supplies agricultural and industrial users with water

(DWAF, 2009). This scheme is one of the largest irrigation scheme in South Africa and is located close to where the Vaal and Harts river confluence (Ferreira, 2008).

Irrigation and municipal return flows have affected the Harts River water quality. High salinity due to saline leachates has been reported in the water downstream from the irrigation schemes (DWAF, 2004). In studies conducted by Molale *(*2012) and Van Wyk *et al.* (2012) on Harts River and Barberspan had shown that high TDS (EC) levels were associated with these systems. High TDS indicate high concentrations of ions and this may have adverse effects on crop production on adjacent farms (Grattan, 2002).

2.5 Characteristics of yeasts

Yeasts are eukaryotic micro-organisms classified in the kingdom fungi (Kurtzman and Fell, 2006). These organisms show unique characteristics of unicellular growth 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 seen most often in molds sp. (Kurtzman and Fell, 2005).

Yeasts are classified as either ascomycetes or basidiomycetes (Kutty and Phillip, 2008). A variety of physiological and morphological characteristics can be used to differentiate ascomycetes and basidiomycetes. Basidiomycetes have a laminar cell wall that resists the (1.3)-β-glucanase activity. With rare exceptions, they are strictly oxidative and generally produce extracellular enzymes, urease and deoxyribonuclease (Dnase). Ascomycetes on the other hand have two layered cell walls that are attacked by (1.3)-β-glucanase activity to release protoplasts. These yeasts are mostly fermentative and usually lack extracellular urease and Dnase production (Hagler and Ahearn, 1987). Ascomycetes produce no colour change while basidiomycetes produce a dark red or purple after staining with diazonum blue B **(**BDD) solution (Hagler and Ahearn, 1987).

2.6 Yeast in water

Yeasts occupy a wide range of ecological places in the environment (Gadanho and Sampiao, 2004). However, they have shown broad diversity in terrestrial and aquatic environments. A large number of yeast species have been isolated from fresh water environments. Most of these

belong to the genera *Aureobasidium, Candida, Cryptococcus, Debaromyces, Pichia,* and *Rhodotorutula*, *Saccharomyces* and *Trichosporon*. Common species are *Aureobasidium pullulans, Candida glabrata, Cryptococcus albidus, Cryptococcus laurenti, Debaromyces hansei, Pichia guillierimondii,* and *Rhodotorula mucilaginosa.* Van Wyk *et al*. (2012) isolated and identified pigmented and non-pigmented yeasts from the North West province water sources. Non-pigmented isolates were *Candida catenula*, *Candida globosa*, *Candida guilliermondii*, *Candida lusitaniae*, *Candida sake*, *Candida tropicalis*, *Cryptococcus laurentii*, *Zygosaccharomyces* spp. The pigmented yeast isolates were identified as *Rhodotorula glutini* and *Rhodotorula mucilaginosa*.

2.7 Yeasts as water quality indicators

Faecal indicators and pathogenic bacteria in water have been used to describe water quality in several urban communities (Thom, 2010). Hagler and Ahearn (1997) have demonstrated that the yeast counts could be used as a complementary method to coliform counts in monitoring eutrophication potential of water. Yeast species have presented wide diversity in aquatic environments. Their existence in freshwater environments has been studied mostly in relationship to polluted water (Medeiros *et al*., 2008; Nagahama, 2006).

High levels of yeast counts in water could indicate heavy or minimal pollution (Simard, 1971). The number of yeasts and species in aquatic environments depend on the type and purity of water (Hagler and Mendonca-Hagler, 1981). In clean seawater, yeast counts could relativity range from few to several hundred per liter (Hagler and Mendonca-Hagler, 1981). In the presence of pollution, the total yeasts could increase up to a few thousand cells per liter or more (Hagler & Mendonca-Hagler, 1981). Total yeast counts had on occasion exceeded 2×10^8 cells/L in domestic sewage pollution (Nagahama, 2006). There is large number of non-fermentative yeast species in clean water. However polluted water is dominated by denser population, mostly fermentative yeasts (Hagler and Ahearn, 1987).

From the studies that were conducted in the past two decades, freshwater yeasts have been focused mostly on their application as organic pollution indicators. . Freshwater rivers and lakes in Europe were found to host mainly the genera *Candida*, *Cryptococcus*, *Pichia* and *Rhodotorula* (Sláviková *et al*. 1992; Sláviková and Vadkertiová 1995; Sláviková and Vadkertiová 1997). These species are considered bio-indicators of level of pollution in freshwater

(Dynowska, 1997). Common species include *Candida albicans*, *Cryptococcus laurentii*, *Debaryomyces hansenii* and *Rhodotorula mucilaginosa* (Nagahama, 2006). *Rhodotorula mucilaginosa* has been reported as the dominant species in polluted chemical wastewater evaporation ponds (Lahav *et al.*, 2002).

2.8 Yeast infections in immune compromised people

There is an increased use of immunosuppresive drugs and antibiotics to patients with organ transplant, anticancer therapies and certain disease conditions such as malignancy and human immunodeficiency virus (HIV). This has contributed to increased number of immune compromised people (Sanglard *et al.,* 1998; Pincus *et al.*, 2007). Immune compromised people are a high risk of yeast infections (Pincus *et al*., 2007). The number of life threatening infections from yeasts observed globally has increased dramatically (Sanglard *et al*., 1998). Weakened immune system of the patients and the high treatment failure contribute to poor diagnosis of invasive yeast infections (Canuto & Rodero, 2002). Several species of yeast are capable of infecting healthy hosts and causing diseases ranging from mucosal to life threatening disseminated infections (Yamaguchi *et al*., 2007). Yeast species that were regarded as saprophytes are potential pathogens of humans and animals (Pincus *et al*., 2007).

According to UNAIDS (2012), estimate of 6.1 million in 2012 from 5.2 million in 2005 adults and children in South Africa are living with HIV, the highest incident in the world. The increase in the number of HIV patients has led to an increase in the rate of recurrence and importance of opportunistic yeast infections (Sanglard *et al*., 1998; Yamaguchi *et al*., 2007). Yeast Infections such as thrush, athlete's food, ring-worm, candidiasis, jock itch and cryptocococcal meningitis are common amongst immune compromised patients.

Yeast infections are frequently caused by pathogens from the genera *Candida* and *Cryptococcus* (Richard-son, 2005). Most *Candida* species are normal human commensals inhabiting oral mucosal surfaces, gastrointestinal tract, the urogenital tract and the skin but many of these organisms are capable of causing infections in immune compromised patients (Rentz *et al*., 1998). The *Candida* genus has over 50 human pathogenic yeast species but the following have been observed frequently; *C*. *albicans*, *C*. *glabrata*, *C*. *guillermondii*, *C*. *krusei*, *C*. *metapsilosis, C*. *orthopsilosis, C*. *parapsilosis, C*. *tropicalis* (Miceli, 2011). *Candida albicans* is

the most frequent in causing infections, followed by both *Candida glabrata* and *Candida tropicalis* (Nguyen *et al*., 1996).

The genus *Cryptococcus* has also been implicated to act as pathogens. *Cryptococcus neoformans*, *Cryptococcus laurentii* and *Cryptococcus gatti* are known pathogens that have been associated with yeast infections (Buchanan and Murphy, 1998). *Cryptococcus neoformans* is the dominant cause of invasive yeast infections in the genus *Cryptococcus* and cause diseases in apparently immune competent and immune compromised people (Buchanan and Murphy, 1998). In AIDS patients, its infection often causes meningoencephalitis or meningitis (Buchanan and Murphy, 1998). *Cryptococcus gatii* also causes a type of cryptococcosis, disease of immune compromised people primarily HIV-positive and organ transplant recipients (Lockhart *et al*., 2012).

2.9 Antifungal agents

Significant increase in the number of life threatening yeast infections has brought about increased use of antifungal agents (Sanglard *et al*., 1998). An antifungal agent is a drug that selectively removes fungal pathogens from a host with least toxicity to the host (Walsh and Dixon, 1996). These are either applied as creams or taken orally. Antifungals are limited in number in the market and are confined to relatively few chemical classes based on their mode of action. Table 2.1 shows some of the available antifungals in the market. It also provides an overview of the mechanism by which the substance acts as well as commercial products in which these are found.

Table 2.1: Chemical classes of available antifungals based on their mode of action

Chemical class		**Mode of action**	**Specific chemical names**	**Commercial products**	**References**
Azoles	Imidazoles	Inhibits the fungal cytochrome P450 enzyme 14α-demethylase	Ketoconazole Econazole Miconazole	Anti-dandruff shampoo, topical creams and oral tablets	Loose *et al*., 1983, Vanden Bossche, 1997
	Triazoles	Inhibits the fungal cytochrome P450 enzyme 14α-demethylase	Fluconazole Itraconazole Posaconazole Voriconazole	White crystalline powder, Oral tablets and solution	Vanden Bossche, 1997
Polyenes		Complexes with ergosterol in the fungal cell membranes	Nystatin Amphotericin B	Topical creams and oral tablets	Vanden Bossche, 1997
Pyrimidines		Interferes with DNA and RNA synthesis	Flucytosine	Oral capsules and injections	Vanden Bossche, 1997
Allylamines		Inhibits squalene epoxidase, interferes with sterol biosynthesis	Terbinafine	Topical creams	Vanden Bossche, 1997
Echonocandins		Inhibit the synthesis of β-D-glucan synthase	Caprofungin Micafungin	Injections	Denning, 2002; Metcalf and Dockrell, 2007

There is also an urgent need for new broad spectrum antifungal agents as none of the existing systemic antifungals satisfies the medical need completely (Sanglard *et al*., 1998). Echinocandins (Table 2.1) represents a new class of antifungal drugs. They inhibit the synthesis of β-D-glucan synthase in fungal cells. This prevents synthesis of the fungal cell wall polysaccharide β-1, 3 glucan (Denning, 2002; Metcalf and Dockrell, 2007).

2.9.1 Mechanism of action of azoles

Azoles are the largest and mostly widely used class of antifungals (Fromtling, 1988) .The use of azoles has increased in recent years. This is due to high frequency of yeast infections in immune compromised people (Sanglard *et al*., 1998). Commonly used antifungal drugs except 5-flucytosine, target ergosterol, the main sterol of the fungal plasma membrane.

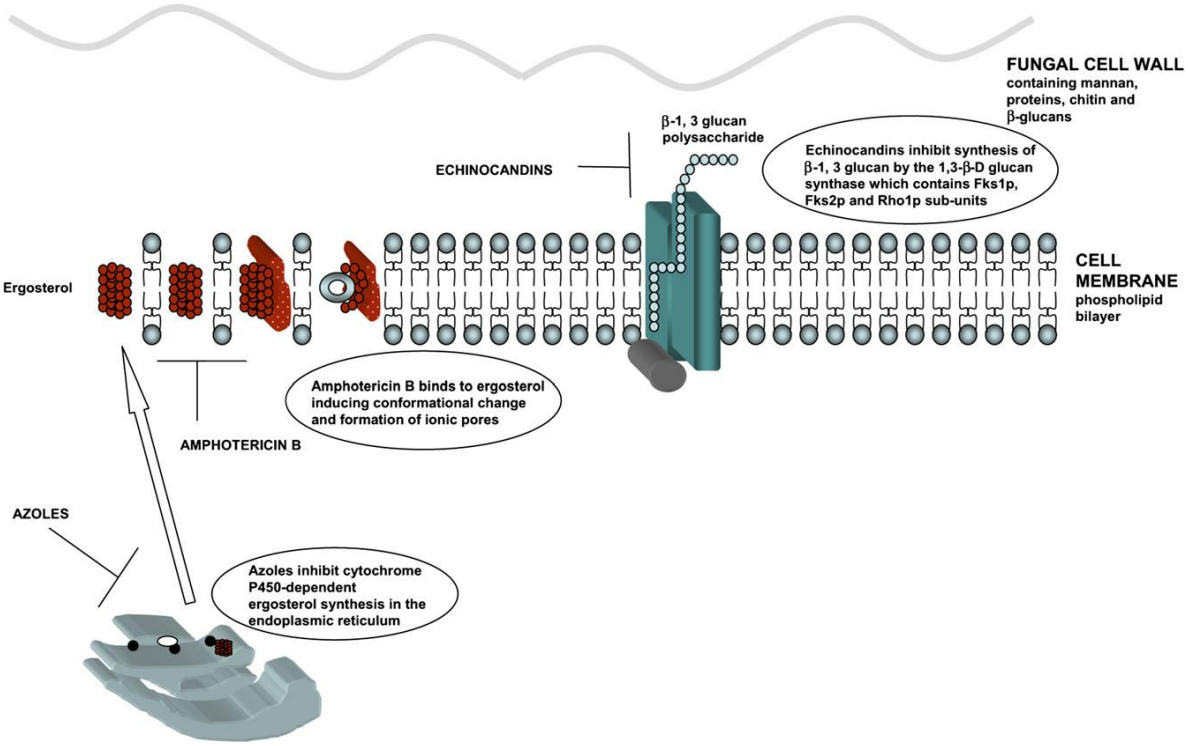


Figure 2.1: Mode of actions of antifungal classes: azoles, polyenes (amphotericin B) echinocandins are shown (Metcalf and Dockrell, 2007).

Figure 2.1 illustrates that azoles bind to a cytochrome P450 inhibiting removal of the 14 α-methyl group of lanosterol during ergosterol biosynthesis. Amphotericin B binds to ergosterol in the cell membrane damaging membrane barrier function through formation of ionic pores. The echinocandins inhibit the 1, 3- β -D- glucan synthase preventing synthesis of the fungal cell wall polysaccharide β -1,3glucan.

Ergosterol performs cellular functions that include proper functioning of membrane bound enzymes. This includes chitin synthetase, which is responsible for cell growth and proliferation. Ergosterol is also vital for the fluidity and integrity of the cell membrane (Vanden Bossche *et al*., 1997; Joseph- Horne and Hollomon, 1997). Azole antifungals belong to a group of ergosterol biosynthesis inhibitors. They inhibit enzymes involved in the post sequence steps of the fungal sterol biosynthesis pathways (Sanglard *et al*., 1998).

Sanglard *et al*. (1998) stated that azoles have cytochrome P450 as the common cellular target in yeasts. They have the ability to inhibit cytochrome P450 14α-demethylase, the enzyme necessary to convert lanosterol to ergosterol (Fromtling, 1988; Sanglard *et al*., 1998). The unrestricted nitrogen group of either imidazole or triazole ring of the azole antifungal binds to the heme iron of cytochrome P450 and this inhibits the enzymatic reaction (Fromtling, 1988).

Azole antifungals have also been directed against another enzyme, sterol Δ desaturase as another target (Vanden Bossche *et al*., 1997). This is also a cytochrome P450 enzyme that is important in the last step of ergosterol biosynthesis. NADPH-dependant 3-ketosteroid reductase that catalyzes the last step of demethylation at the C-4 methyl group of yeast sterols has been described as the last azole antifungal target (Bossche *et al*., 1997). As lanosterol is accumulated, ergosterol is depleted in the yeast cell membrane. This disrupts the structure and many functions of the fungal membrane (Vanden Bossche, 1997; Sanglard *et al*., 1998).

2.9.2 Antifungal resistance

Prophylactic use of antifungals is one of the reasons of recurrent resistance to antifungal drugs (Perfect and Casadevall, 2006). Antifungal resistance is defined as a process in which the cell has the capacity to suppress the toxic effect of the antifungal agent. This results in less than normal sensitivity of the fungal cell to the antifungal agent (Sanglard *et al*., 1998; Vanden Bossche, 1997). However, the resources that are allocated to monitor and reduce antifungal drug resistance are limited and few countries engage in antifungal resistance surveillance (WHO, 2014). There is a large gap in information from most of Asia, Africa, the Middle East and parts of South America (WHO, 2014).

In the treatment and prophylaxis of oro-oesophageal candidiasis in the early 1990s, fluconazole became the antifungal of choice. In the following years, fluconazole resistance was subsequently reported in 41% of patients treated for this disease (Canuto & Rodero, 2002).

Fluconazole is not completely metabolised in the human body (Kim *et al*., 2007). There may thus be considerable amounts of this antifungal agent that lands up in the domestic wastewater streams. This could have impacts on the prevalence and distribution of yeasts that are present in natural water bodies that pass through different communities.

2.9.3 Mechanism of resistance to azoles

Quite a few resistance mechanisms have been defined relating to azoles. These are depicted in Figure 2.2. Resistant strains exhibit a modification in the quality or quantity of the target enzyme or limited access to the target or both (Ghannoum and Rice, 1999).

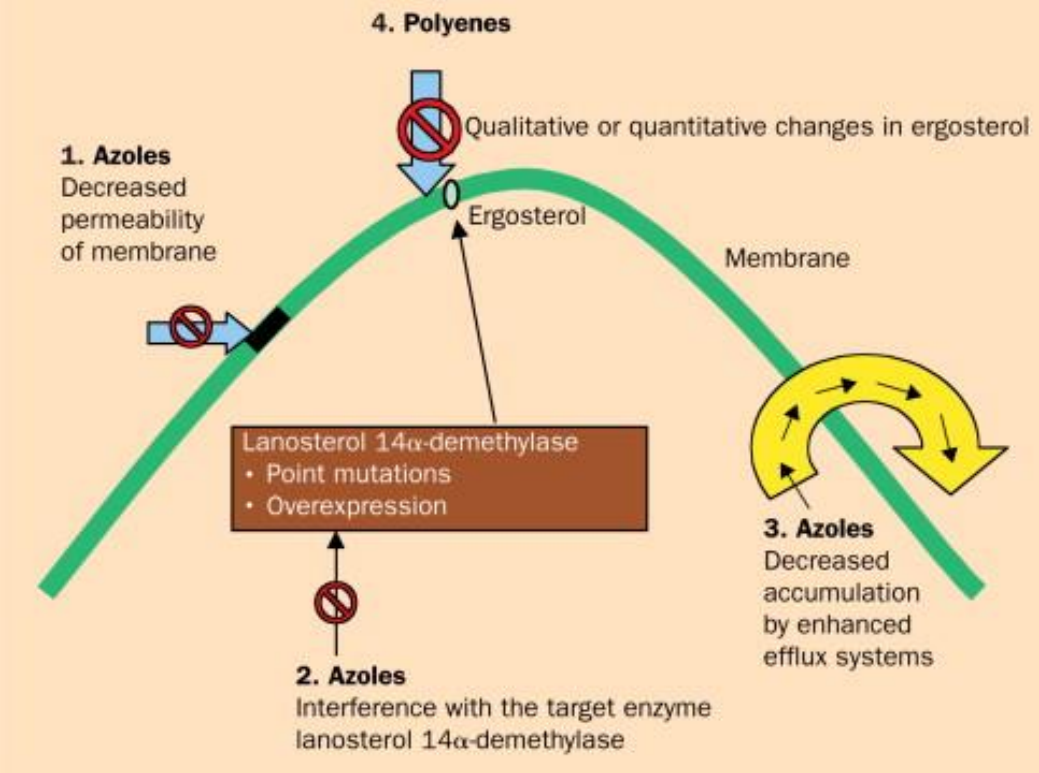


Figure 2.2: Mechanism of azole resistance (Canuto and Rodero, 2002)

Three mechanisms for azole resistance are demonstrated in Figure 2.2. These include: (1) decreased permeability membrane of the fungal membrane to the drug; (2) interference with the target enzyme lanosterol 14α-demethylase through mutation and overexpression; (3) decreased accumulation by increased efflux system activity.

2.9.3.1 Alteration of the target enzyme

This is the interference with the target enzyme cytochrome P-450 lanosterol 14α-demethylase through mutation and overexpression (Canuto and Rodero, 2002). Mutation causes amino acids substitutions and these results in decreased affinity for azole derivatives (Perea *et al*., 2001). Overexpression creates the need for higher intracellular azole concentration to complex all the enzyme molecules in the cells (Perea *et al*., 2001). This mechanism of altered affinity of azole derivatives has been described in yeast species such as *C*. *albicans* and *C*. *neofromans* (Sanglard *et al*., 1998).

2.9.3.2 Altered ergosterol biosynthetic pathway

There are specific changes that can take place on the enzymes that are involved in the complex ergosterol biosynthetic pathway. The presence of azole resistant human yeast pathogens is determined by such alterations (Sanglard *et al*., 1998). Lesions on the pathway enzyme, sterol Δ desaturase results in the accumulation of a toxic metabolite 14α-methyl fecosterol instead of ergosterol (Mahmoud and Rice, 1999). With the presence of toxic metabolite 14α-methyl fecosterol, not ergosterol, the structure and many functions of the fungal membrane is disrupted (Sanglard *et al*, 1998). The resistance in this scenario is attributed to sterol biosynthesis mutation in $\Delta^{5,6}$ desaturase. This enzyme blocks the biosynthesis of 14α-methyi-3, 6- diol under treatment.

2.9.3.3 Decreased accumulation by enhanced efflux system

Azole antifungals fail to accumulate inside the yeast cells as a result of enhanced drug efflux systems pumping the azoles out of the cells (Perea *et al*., 2001). This form of resistance has been identified in species such as *C. albicans*, *C. glabrata*, *C. krusei* and *C. neoformans* (Parkinson *et al*., 1995; Sanglard *et al*., 1995; Venkateswarlu *et al*., 1997). Two multi multidrug efflux transporters (ABC- transporters and Major facilitators) are involved in the drug export. Major facilitators are encoded by multidrug resistant genes and ABC transporters are encoded by CDR genes (Perea *et al*., 2001). Sanglard *et al.* (1995) maintains that two genes (ABC transporter gene CDR1 and MF gene MDR 1) are mostly overexpressed in the resistant yeast isolates**.** In other studies (Fling *et al.,* 1991; Prasad *et al*., 1995) genes for the same transporters were isolated.

2.9.4 Mechanism of resistance in polyenes and allylamines

Resistance mechanism to polyenes and allylamines are similar to azoles in a way that they interfere with sterol biosynthesis. They either inhibit synthesis of ergosterol or directly interact with it. The target for polyenes antifungal agents is the fungal membrane on which they interfere with ergosterol (Vanden Bossche, 1997). The molecular mechanism of polyene resistance include the decrease in the total ergosterol content of the cell, replacement of some or all of the polyene-binding sterols, and reorientation of ergosterol (Hamilton-Miller, 1973).

Allylamines inhibit squalene epoxidase, another enzyme required for ergosterol synthesis. Resistance in allylamines has been reported due to overexpression of the antifungal drug target. This was reported by Vanden Bossche *et al*. (1992) in a *C. glabrata* strain that became resistant to fluconazole and expressed cross resistance to terbinafine. Resistance mechanisms in which ABC transporter genes CDR1 are mostly overexpressed in the resistant yeasts isolates have been reported for allylamines (Ghannoum and Rice, 1999).

2.9.5 Mechanism of resistance to echonocandins

Antifungals resistance to echonocandins has been reported in some yeast species. These include resistance in some strains of *C. albicans*, *C. glabrata*, *C. lusitaniae*, *C. tropicalis*, and *C. parapsilosis*. Resistances include alterations in the glucan synthase (Fks1-Fks2 complex), overexpression of efflux pumps as well as alterations and/or overexpression of the genes (Erg3 and Erg11) (Beauvais, 2001).

2.10 Yeast pathogenicity

Several yeast species have the ability to cause disease and to overwhelm the host defense mechanisms. They possess several genes and proteins associated with their pathogenicity, called virulence factors (Todar, 2009). Virulence factors that have been detected in yeast include ability to grow at 37°C, cell wall and capsule components, adhesion molecules and extracellular enzyme production (Kurokawa *et al*., 1998).

2.10.1 Thermotolerance

The ability to survive and replicate at 37°C is a common characteristic of pathogenic yeasts. This phenomenon is known as thermotolerance and can influence the pathogenic potential of a microorganism as well as the form of disease presented by the host (Kurokawa *et al.*, 1998).

Thermotolerance to mammalian temperature is considered a prerequisite characteristic of mammalian pathogens (Cox *et al.,* 2008).

2.10.2 Cell wall components and capsules

Both cell wall and the capsules are considered major targets for studies on virulence. They are primary structures that protect the microorganisms from host attacks. Cell wall polysaccharide, α- glucan has been constantly associated with the increase of virulence in several yeast strains (Kurokawa *et al.*, 1998). In microbial pathogenesis, most capsules protect the microorganism against the host immune mechanism but for some, the capsular structure can serve for adhesion (Cox *et al*., 2008).

2.10.3 Adhesion

Fungal adhesion to the host tissue has been regarded as the initial and major step in establishing infection (Denaro *et al*., 1995). The cell-cell and cell-extracellular adhesion observed in some yeasts occurs when the yeast forms have molecules on the cell wall or capsules that allow adhesion of the yeast cells to other tissues (Kurokawa *et al.,* 1998). *Candida albicans* has specialized set of proteins (adhesins) that facilitate adherence to other *C. albicans* to other microorganisms, to abiotic surfaces and to the host (Mayer *et al*., 2013).

2.10.4 Extracellular enzymes production

Extracellular enzymes play a vital role in adherence and survival of the pathogens on mucosal surface and the invasion of the host (Kurokawa *et al*., 1998). The hydrolytic enzymes contribute to pathogenesis by disrupting the host cell membranes and extracellular matrices. This results in dissemination and tissue invasion (Fu *et al*., 1997). Enzymes that damage tissue include proteases, neuraminidases and lipases. These enzymes damage cells and provide nutrients by digesting substrates into smaller components that can be easily assimilated by micro-organisms. However, they also change host cellular receptors (Cox *et al*., 2000). This disrupts the normal binding of their usual ligand. The microbial behaviour changes and the host immune mechanism can easily be invaded. Other enzymes, such as urease, contribute to virulence by facilitating survival inside phagocytic cell (Cox *et al*., 2000). The pathogenesis of erythematous candidiasis by *Candida albicans* is associated with its capability to produce extracellular enzymes, such as proteinase and phospholipase (Aleva *et al*., 2007).

2.11 Isolation of yeasts from the water sources

2.11.1 Sampling of yeasts

The sampling methods for yeasts isolation do not differ from those used for bacteria but higher volume of water samples are required because the frequency of yeasts in natural aquatic environments is lower than bacteria, especially in low nutrient condition (Nagahama, 2006). Aseptic techniques using sterile bottles, boxes and containers containing ice are being used during the collection of samples. Samples are sealed on site and analysed within the shortest possible time after collection (Nagahama 2006).

2.11.2 Enumeration and isolation of yeasts

Yeast density, volume and shape of the source and the source, either water, sediment or plant material, determine the isolation procedure (Nagahama, 2006). Yeast cells in water samples are mostly filtered through membranes (Nagahama, 2006). Membrane filters of size 0.45µm are used to recover the yeast (Kurtzman *et al*., 2011). Membrane filtration is defined as a vacuum or pressure driven separation process where particulate matter is rejected by using engineering barrier (US EPA, 2005). If present in high numbers, yeasts may be isolated by direct plating on agar media. Dilution plating methods may also be used for quantitative studies (Kurtzman *et al*., 2011).

For effective isolation of yeasts, selective media that will enable yeast growth are used. Selective media contains one or more ingredients that inhibit growth of undesired organisms and encourage growth of the desired organisms (Nurcholis, 2013). Temperatures and pH are adjusted often to suppress bacterial growth and other competitive fungi (Kurtzman *et al*., 2011). Acidified media provide selective isolation for yeasts. Fungistastitic and/or antibiotics such as tetracycline and chloramphenicol are included in the media to suppress growth of molds and bacteria (Kurtzman *et al*., 2011). For selection of a particular genus, species of yeast or yeast with a particular property, selective media could be used (Kurtzman *et al*., 2011).

2.12 Determination of physico-chemical parameters

Physical parameters of water such as water, TDS, EC and TDS could be measured on site using mobile multiprobe meters. There are various manufacturers of such equipment. The multi probe PCS Testr 35 (Eutech instrument, 2005) offers a competitive advantage to other probe

meters as it provides: 1) flexibility as it measures five parameters without changing the sensors. 2) pH can be measured up to 2 decimal places. 3) Low, medium and high TDS/EC ranges can be measured. 4) It is water proof and user friendly.

Chemical analyses of water are performed using The Hach Lange DR2800 portable spectrophotometer (Hach Company, 2012). The instrument is easy to use and can also be used on site (O' Reilly, 2012). These tests can also be measured in the laboratory with 6 hours of sampling (Van Wyk *et al*., 2012). The Hach Lange DR2800 portable spectrophotometer can be used to measure more than 240 analytical methods (Hach Company, 2012). Hach Lange DR2800 spectrophotometer can be used to measure some of the following chemical tests; nitrates, nitrites, phosphorus and chemical oxygen demand.

a. Nitrates

Nitrates concentration in water samples is measured using the cadmium reduction method. Cadmium reduces nitrates to nitrites. In acidic medium, nitrites react with sulfanilic acid to form diazonium salt. Diazonium salt reacts with gentisic acid to form an amber coloured solution (Hach Company, 2007).

b. Nitrites

Nitrite is reduced to nitrous oxide by ferrous sulphate. Nitrous oxide combines with ferrous ions to form a green-brown complex. The formation of the green-brown complex is proportional to the nitrate present in the water sample (Hach Company, 2007).

c. Phosphates

Amino acid method is used to measure phosphates in water. Ammonium molybdate reacts with orthophosphate to form a highly acidic solution, molybdophosphoric acid. The amino acid reagent added reduces the acidic solution to from an intensely coloured molybdenum blue complex (Hach Company, 2007).

c. Chemical Oxygen Demand

A reaction digestion method is used to measure COD. Water samples in vials are heated to 150°C for 2 hours with an oxidizing agent, potassium dichromate. An oxidizable reagent reacts to reduce dichromate ion ($Cr_2O_7^{2-}$) to green chromic ion (Cr^{3+}). The amount of Cr^{6+} is determined using 3-150mg/L (Hach Company, 2007).

2.13 Biochemical tests used for yeast identification

2.13.1 Diazonum Blue B (DBB) test

DBB test is conducted to distinguish between ascomycetes and basidiomycetes (Hagler and Ahearn, 1987; Kurtzman and Fell, 1998). Basidiomycetes have a laminar cell wall that resists the (1.3)-β-glucanase activity. Ascomycetes have two layered cell walls that are attacked by (1.3)-β-glucanase activity to release protoplasts (Hagler and Mendonca- Hagler, 1981). Macroscopically, based on their colour pigmentation, basidiomycetes produce pink, salmon or reddish colonies in a reaction with DDB reagent. Yeast species that from white or cream white colonies are classified as ascomycetes (Hagler and Ahearn, 1987). DBB reaction appears to be most practical test for establishing the basidomycetous yeasts in the laboratory (Hagler and Mendonca- Hagler, 1981).

2.13.2 Disk diffusion method

Disk diffusion testing is largely used for antimicrobial compounds in clinical microbiology laboratories. The method has been applied in antifungal susceptibility (Arendrup *et al*., 2011). Antifungal susceptibility testing (AST) has been used as a diagnostic tool for more than 20 years (Forthergill, 2012). It is currently playing a vital role to track the development of antifungal resistance. Standardized disk diffusion methods for *in vitro* studies have been helpful in conducting surveys of antifungal susceptibility and resistance globally (Pfaller *et al.*, 2010). The disk diffusion methodology has been approved by the Clinical and Laboratory Standards Institute (CLSI) (document M44-A2) in testing several antifungals (NCCLS, 2004).

The Clinical and Laboratory Standard Institute (CLSI) has established standard method for antifungal disk diffusion method susceptibility testing of *candida* species and including fluconazole and voriconazole (CLSI, 2008). Table 2.2 lists zone breakpoints for systemic and local treatment for some antifungals as recommended by the CLSI for fluconazole (Rosco Diagnostica, 2011). However, these interpretive breakpoints are not applicable to *C. krusei* as it is assumed to be intrinsically resistant to fluconazole (CLSI, 2008). The CLSI proposed disk diffusion method has not been published but has provided a faster, simpler method for determining the in vitro susceptibility of *Candida* and *Cryptococcus neoformans* to fluconazole (Pfaller *et al.*, 2004).

In Table 2.2 a list of commonly used antifungal agents and the concentrations at which they are used are listed. In this table inhibition zone diameters and interpretations are provided. The category “susceptible” implies that the isolates are inhibited by the usually achievable concentrations of antimicrobial agents. This is achieved when the recommended dosage is used for the site of infection. The category “intermediate” implies clinical efficacy in the body sites where the drugs are physiologically concentrated or when a higher than normal of drug could be used. The category “resistance” implies that the isolates are not inhibited by the usually achievable concentrations of the agents with normal dosage schedules. This includes when zone diameters have been in a range where clinical efficacy has not been reliable in treatment studies (NCCLS, 2004; CLSI, 2008). Such a table is useful for interpreting results but is mainly applicable to clinical isolates of *Candida* and *Cryptococcus* sp.

Table 2.2: Zone breakpoints for different antifungal agents as recommended by CLSI

Antifungal agent	**Concentration**	**Zone diameter in mm**		
		Susceptible S)	**Intermediate (I)**	**Resistant (R)**
Fluconazole	25 µg	≥ 19	18-15	≤ 14
Voriconazole	1 µg	≥ 17	16-14	≤ 13
Econazole	1 µg	≥ 20	12-19	≤ 11
Ketoconazole	15 µg	≥ 30	23-29	≤ 22
Miconazole	1 µg	≥ 20	12-19	≤ 11
Posaconazole	5 µg	≥ 17	16-14	≤13
Itraconazole	10 µg	≥ 23	22-14	< 13
Amphotericine B	10 µg	≥ 15	14-10	<10
Nystatin	100 µg	≥ 15	10-14	No zone
flucytosine	1 µg	≥ 20	12-19	≤ 11
Terbinafine	1 µg	≥ 20	12-19	≤ 11
Caspofungin	5 µg	≥ 16	15-13	≤ 12

2.14 Identification of yeasts

Previously, identification of yeast species was done using phenotypic characteristics such as morphology, ability to ferment sugars, growth on various carbon and nitrogen compounds and physiology (Pincus *et al*., 2007). Phenotypic characteristics facilitated yeast placement in their

genera level (Pincus *et al*., 2007). However, phenotypic characterisation of yeast has resulted in severe misidentification as they are hard to interpret. In addition, phenotypic methods have been found to be time consuming and laborious (Kurtzman and Robnett, 1998).

Molecular methods based on the Polymerase Chain Reaction (PCR) and sequencing of housekeeping genes have provided speed to accurate and reliable characterisation. These methods allow identification of yeasts up to species level (Pincus *et al.*, 2007). PCR is done to amplify a segment of the 26S ribosomal DNA. This is then subjected to sequence determination. Sequencing is an important tool in studying communities and individuals within the communities. It has also provided genetic differences between different strains of the same organisms (Mignard and Flandrois, 2006).

The resultant strain characterisation from sequencing has provided the opportunity to understand broader species relationship (Wessekink *et al*., 2002). The 26S rRNA gene consists of the variable domains 1 and 2 (D1/D2) of the large ribosomal subunit. The D1/D2 domains are located in the first 650bp of the 26S rRNA (Wesselink *et al*., 2002). Yeast species can be identified from sequence divergent from the D1/D2 domain (Kurtzman and Robnett, 1998).

Sequences may be uploaded into Basic Local Alignment Search Tool (BLAST) to compare them with other sequences that are already in the database (Ogunseitan, 2005). BLAST searches for homologous sequences in nucleotide and protein database. It is vital to compare only two sequences that are already known to be homologous (Tatusova and Madden, 1999). A query is submitted to BLAST and the list of sequence in the database follows. This are judged to be related to the specific target sequences (Ogunseitan, 2005).

In determining the relevance and closeness of the sequence matches in BLAST program, Bit scores and E-values are used (Ogunseitan, 2005). According to bit scores, BLAST presents related sequence in descending order. Higher bit score indicates that the sequence is closer to the target sequence. E-values are an estimate of the chance of occurrence of identified matches in the database. E-values maintain the level of confidence that two similar sequences are caused by common descendant other than by chance (Ogunseitan, 2005).

Yeast sequences are used to build phylogenetic trees. Phylogeny trees estimate the relationship among species represented by those sequences (Hall, 2013). They reconstruct

phylogenetic relationship among extant taxonomic groups and occasionally identify the position of ancestral species in the lineage of more complex organisms (Ogunseitan, 2005). Sequences are submitted to Genbank, an international database that is used to identify and down the homologous sequences. MEGA software is an integrated program that constructs a tree (Hall, 2013). Subsequent steps are followed in constructing a tree. These include acquiring the sequences, aligning the sequences, estimating the tree and presenting the tree (Hall, 2013).

2.15 Summary of the literature

Literature has shown that North West Province water resources are limited. In addition to this, its surface water quality is generally of a poor quality (NWDACE, 2008). Physico-chemical parameters are used to determine water quality. The literature has also discussed the importance of selected physico-chemical properties including temperature, pH, EC, TDS, nitrates, phosphates. Domestic sewage usually contains bacteria, protozoa, viruses, yeasts and fungi that could end up in receiving water bodies if the water is not treated effectively at waste water treatment plant. The literature has highlighted this problem in the North West Province. Indicator bacteria such total coliform and faecal coliform have always been used to determine the microbiological quality of water (Thom, 2010). However, some literature has also shown that yeasts could also be employed to determine water quality (Hagler and Medonca-Hagler, 1981; Nagahama, 2006; Van Wyk *et al*., 2012).

In a previous study by Van Wyk *et al*. (2012), it has been demonstrated tha some of the isolated yeast species from the water bodies in the North West Province could be pathogenic. They could cause diseases and infections especially in immune compromised people. Antifungals are used to treat such infections. Furthermore, due to antifungal prophylactic usage and continuous exposure of antifungals to the yeasts, some disease causing yeasts have become resistances to antifungals (Perfect and Casadevall, 2006). This is a problem as antifungals such as azoles, especially fluconazole who have been proven as resistance, are used against yeast infections. This literature has demonstrated that the observed phenomenon is a worrying health related factor globally.

For the identification of yeasts, biochemical and molecular methods are useful. Various physical and media based methods could be used to characterize the yeasts and antifungal susceptibility tests based on Kirby-Bauer disk diffusion is also available.

CHAPTER 3 - MATERIALS AND METHODS

3.1 Study site

Sampling was conducted in 2013 and 2014 in Mooi River and Harts River, respectively. In Mooi River, one sampling period was in winter (June) and two in summer (October). Harts River was sampled only once in summer (March). Water was collected from thirteen sampling sites in the Mooi River, and six sampling sites from the Harts River. A Garmin Nüvi 1310 global positioning system (GPS) (Garmin, US) hand held unit was used to determine the coordinates of the different sampling sites. Figure 3.1 shows the map of the sampled area, the Mooi River (WF and MR) sites and Harts River (HR) sites. The map was constructed from the GPS coordinates (Table 3.1).

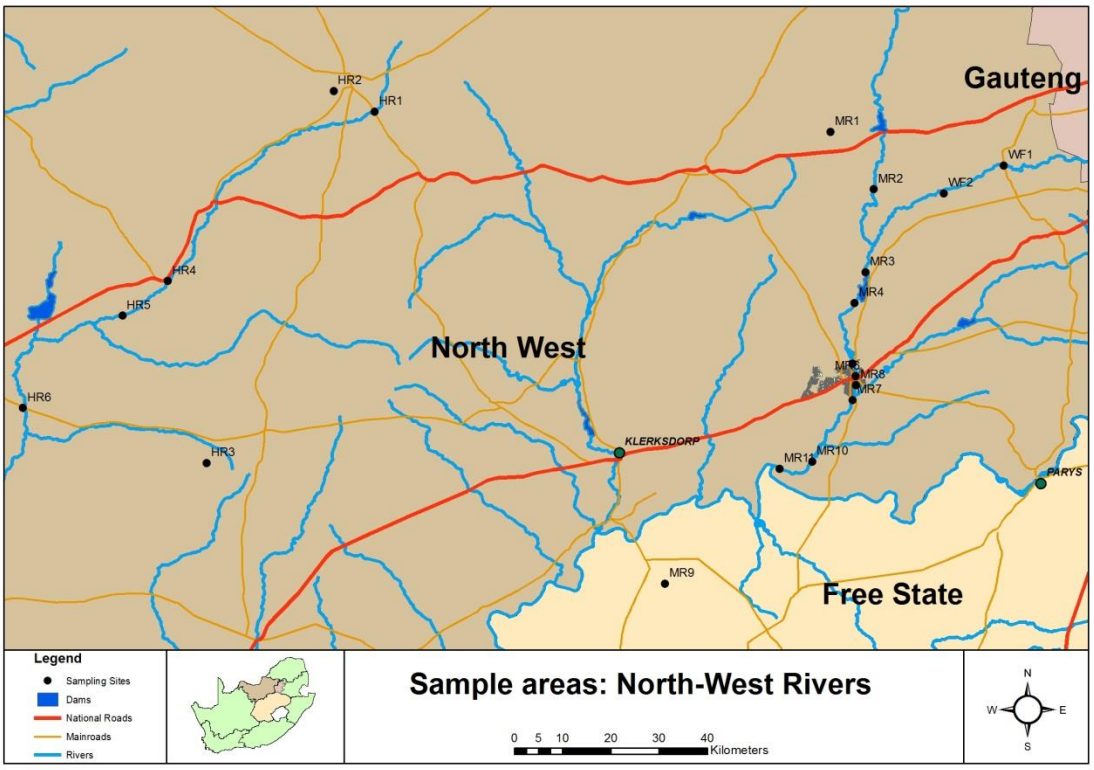


Figure 3.1: Map of the North West Province (NWP) .The different sampling sites on the Mooi River and Harts River are indicated by black dots.

Table 3.1: Sampling sites and GPS coordinates in Mooi and Harts River

Source	Sampling site	Abbreviations on the map	GPS coordinates	
			Latitude	Longitude
Mooi River	Wonderfonteinspruit before Carletonville	WF1	26° 18' 56.9"S	027° 22' 5.6"E
	Wonderfonteinspruit after Carletonville	WF2	26° 22' 02.7"S	026° 22' 02.7"E
	Klerkskraal	MR1	26° 15' 11.4"S	027° 09' 31.4"E
	Muiskraal	MR2	26° 21' 36.4"S	027° 08' 20.7"E
	Boskop site located before Boskop dam	MR3	26° 30' 52.3"S	027° 07' 28.5"E
	Boskop site located after Boskop dam	MR4	26° 34' 18.5"S	027° 06' 13.0"E
	Thabo Mbeki Bridge	MR5	26° 41' 05.2"S	027° 06' 00.8"E
	Trim Park Bridge	MR6	26° 42' 29.3"S	027° 06' 20.6"E
	Pedestrian bridge	MR7	26° 45' 09.2"S	027° 06' 00.9"E
	Viljoenroad Bridge	MR8	26° 43' 29.2"S	027° 06' 21.26"E
	Potchefstroom water water treatment plant (WWTP)	MR9	26° 44' 59.3"S	027° 05' 40.67"E
	Taaiboschbult Bridge	MR10	26° 86' 72.1"S	027° 02' 48.4"E
	Skandanawiedrif Bridge	MR11	26° 88' 00.2"S	026° 96' 38.5"E
Harts River	Lichtenburg site located before water water treatment plant (WWTP)	HR1	26° 12' 54.7"S	026° 12' 30.3"E
	Lichtenburg site after water water treatment plant (WWTP)	HR2	26° 10' 36.5"S	026° 07' 55.0"E
	Biesiesvlei	HR3	26° 52' 11.6"S	025° 53' 42.4"E
	Vermaas	HR4	26° 31' 51.0"S	025° 49' 21.9"E
	Sannieshof	HR5	26° 35' 43.8"S	025° 44' 16.3"E
	Delareyville	HR6	26° 46' 01.7"S	025° 33' 07.9"E

3.2. Sampling and physico-chemical parameters

Water samples were collected aseptically using dip sampling technique (Van Wyk *et al*., 2012). The water samples were collected in sterile Schott bottles and kept on ice. Water temperature, pH, total dissolved solids (TDS) and dissolved oxygen (DO) were measured on site using a multi 350 multi probe analyser (Merck, Germany). Water samples were taken to the laboratory and the analysis was conducted within 6 hours. In the laboratory, chemical oxygen demand (COD), nitrate (NO_{3-}), and phosphate (PO_4^{2-}) were measured using the Hach Lange DR 2800 spectrophotometer, Hach USA (Hach company, 2007). DO and COD were not measured in the Mooi River.

3.3. Isolation and enumeration of yeasts

The water samples were analysed by membrane filtration to determine the presence of yeasts in water using the methods described by Van Wyk *et al*. (2012). One hundred milliliters of water samples were filtered in duplicates through 0.45µm HA membranes filters (Whatman®). The membranes were placed on to yeast-malt-extract (YM) plates (YM) : (10g/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 100ppm chloramphenicol. Incubation was done at room temperature and at 37˚C. The formation of yeast colonies was examined daily until day 5 when the colonies were counted. Isolates from the plates grown at 37˚C were purified by sub-culturing on YM agar (Wickerham, 1951).

3.4. Characterisation and identification of yeasts

The following tests were performed to identify and characterize yeast species that grew at 37˚C.

3.4.1. Diazonium blue B (DBB)

DBB (Sigma-Aldrich, Germany) is a test used to distinguish between ascomycetous and basidiomycetous yeasts. Yeast cultures were grown on YM agar and incubated at 37˚C for 14-21 days (Kurtzman and Fell, 1998). A DBB solution was freshly prepared, put on ice and used before it turned dark yellow normally before 30minutes. One to two drops of chilled DBB were applied onto surface of each colony. A positive reaction of dark red to violet red colour within 2

minutes at room temperature showed that that the yeast belongs to basidiomycetes. No colour change signifies a negative reaction, which is indicative of ascomycetes (Kurtzman and Fell, 1998).

3.5. Molecular identification

The identification of the isolated yeast species was done using the 26S rRNA gene sequence.

3.5.1 DNA isolation

Yeast isolates were grown into 10ml of YM broth at 37˚C for approximately 24 hours. Genomic DNA was extracted according to the method of Hoffman and Winston (1987) with minor modifications. After the isopropanol precipitation in Hoffman and Winston (1987) method, the DNA was purified using the Nucleospin Tissue kit for bacterial DNA extraction (Macherey-Nagel, Germany). The purification of the yeast DNA followed the Nucleospin Tissue kit protocol from step 5 which explains the binding of the DNA. This is followed by the wash of the siclica membrane, drying of the silica membrane, elution of the highly pure DNA and storage of the DNA into the fridge.

A NanoDrop TM 1000 spectrophotometer (Thermo Scientific, US) (NanoDrop, 2007) was used to determine DNA concentrations as well as 260/280 ratios. The ratio of absorbance at 260/280 is used to assess the purity of the DNA. A ratio of approximately 1.8 is generally accepted as pure for DNA. Low ratios at 260/280 indicate the presence of protein or phenol contamination (Nanodrop, 2007).

3.5.2 Agarose gel electrophoresis of isolated DNA

An agar gel was run to determine the quantity of the isolated DNA. A 1% (w/v) agarose gel was prepared by adding 1 g of agarose powder (SeaKem, US) into 100 ml 1 x TAE buffer [(20 mM acetic acid (Merck, US):, 40 mM Tris (Sigma Aldrich, US); 1 mM EDTA (Merck, US), pH 8.0)]. Ten microliters of ethidium bromide (1µl/ml; Biorad, UK) was added to the agarose-TAE mixture, which was poured into a casting tray and allowed to solidify.

A 1kb ladder (O'GeneRuler^{TM}, Fermentas Life Sciences, US) was used to confirm the fragment sizes. Three microlitres of the isolated DNA was mixed with 3µl of 6 x orange loading dye (Fermentas Life Science, USA). Electrophoresis conditions were set at 80V for 50 minutes. Gels

were viewed using a ChemiDoc™ TM (BioRad, US) imager. The same reagents, procedures and conditions were used for the PCR amplified fragments except in this case GelRed (Biotium, US) and a 100 bp ladder was used.

3.5.3. DNA amplification

To amplify the 26S rRNA gene, the primers NL1 (5”- GCATATCAATAAGCGGAGGAAA-AG-3”) and NL4 (5”- GGTCCGTGTTTCAAGAC-GG-3”) were used. PCR reagents for amplification consisted of: (i) ~50ng genomic template DNA, (ii) 12.5 µl double strength PCR Master Mix (0.05 U/µl Taq polymerase, 4mM $MgCl_2$ and 0.4mM dNTPs; Fermentas Life Sciences, US), (iii) 1.5 mM $MgCl_2$, (iv) 10.0 µl nuclease free water (Fermentas, Life Sciences, US) and 5.0 mM primer mix. The PCR cycling conditions consisted of an initial denaturation of 300 seconds at 95°C, followed by 35 cycles of 60 seconds at 95°C, 35 seconds at 52°C and 60 seconds at 72°C. A final extension step of 300 seconds at 72°C was included.

3.5.4 Sequencing

The 26S rRNA sequencing of the Mooi River isolates was performed by Central Analytical Facility of the University of Stellenbosch and those of the Harts River by Inqaba Biotech, Pretoria. Chromatograms were viewed in Geospiza Finch TV (version 1.4) software and BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST) were used to determine the identity of the amplified sequences. Identified sequences were submitted to Genbank on the website http://www.ncbi.nlm.nih.gov/genbank/ to obtain accession numbers.

3.5.5 Phylogenetic tree

A number of representative 26S rRNA yeast gene sequences were downloaded from Genbank to compare their phylogenetic relationship with the identified yeast species. Clustal W version 1.8 was used to perform multiple sequence alignment (Thompson *et al*., 1994). Aligned sequences were edited using DAMBE (Xia and Xie, 2001). Neighbour-Joining method in MEGA version 5.2 software (Tamura *et al*., 2011) was used to construct a phylogenetic dendogram.

3.6 Haemolysin production on blood agar

Yeast isolates were streaked onto 5% sheep blood agar (National Health Laboratories, South Africa) using aseptic technique. Blood agar plates were incubated for 24 hours at 37°C. Clearing of zones around the colonies is indicated as β-haemolysis. The production of green zone

around the colony is termed α-haemolysis. γ- haemolysis is depicted by the absence of cleared zones around the colonies; that is an indication of no haemolysis (Atlas, 1997; Pavlov *et al.*, 2004).

3.7 Extracellular enzyme production

For an organism to cause infection, it should have virulence factors that are associated with pathogenesis (Edberg *et al.*, 1996). Inci *et al*. (2012) has reported on extracellular hydrolytic enzymes as virulence factors for yeasts.

3.7.1 Proteinase

A growth medium containing 1.5 g skim milk (Oxoid, UK) solution in 50ml H_2O, 100ml agar (0.5 g NaCl, 0.5 g yeast extract, 1.5 g agar, 0.5 g Peptone) and 1.9 g infusion broth-brain heart in 50 ml H_2O was prepared. These were autoclaved separately, mixed and poured into plates. Spot inoculation of organisms was done onto plates and incubated at 37°C for 48 hours. A clear zone around the inoculation spot was an indication of a positive result (Venter, 2010).

3.7.2 Lipase

Tryptone soy agar (TSA) (Merck, USA) was prepared according to manufacturer's specifications and autoclaved**.** It was supplemented with 2 ml Tween 80 (Sigma Aldrich, US). Spot inoculation of organisms was done onto plates and incubated for 48 hours 37°C. A positive result was seen as turbid halo around the inoculation spot (Venter, 2010).

3.7.3 Dnase

Ten grams of Dnase agar (Merck, US) was prepared according to manufacturer's specifications and autoclaved. This was then supplemented with 0.025 g of toluidine blue O (Sigma Aldrich, US). Spot inoculation of organisms was done and incubated at 37°C for 72 hours. Plates were flooded with 1 M Hydrochloric acid. A clear zone or pink halo around the colonies displayed positive results (Venter, 2010)

3.8 Antifungal susceptibility tests

Spread plates of yeast cultures were prepared on YM agar plates and Kirby disk diffusion method was performed. The following antifungal agents: fluconazole (FCN), econazole (ECN), ketoconazole (KCA), miconazole (MCL), metronidazole (MZ), flucytosine (FY), nystatin (NY) were placed on YM agar plates and incubated at 37˚C. Diameters in mm of the growth inhibition zones were monitored after 24 hours. These diameters were compared to the values in Table 2.2. Table 2.2 shows the zone diameters of antifungal agents, which classify the isolates as resistant, intermediate resistant or susceptible.

3.9 Statistics

Where appropriate, Microsoft Excel (2010) was used to calculate averages and standard deviations. Redundancy analysis (RDA) multivariate technique (Canoco for windows Version 4.0, GLW-CPRO©) (Ter Braak, 1990) was used to show the relationship between physico-chemical parameters and species levels of the different sampling sites in the Mooi and Harts River. Multivariable analysis was developed to relate species-environment relationship. It explains the distribution of species along each environmental variable. The ordination diagram shows that main patterns of variations in the community composition are accounted for by the environmental variables (Cajo and Braak, 1986). A strong correlation exists between any two variables provided a small angle exists between them. An angle greater than 90°C between two variables indicates a negative correlation (Rahman *et al*., 2008).

CHAPTER 4 - RESULTS

4.1 Introduction

This chapter details the results obtained from the two selected rivers, Mooi River and Harts River. These rivers were sampled in 2013 and 2014, respectively. The physico-chemical results that were obtained from the 19 sampling sites were compared to the Department of Water Affairs and Forestry (DWAF) Target Water Quality Ranges (TWQR) for livestock watering and irrigation use as depicted in the Field Guide (DWAF, 1996a).

Bacteria such as faecal coliforms and total coliforms are used to determine the potential health implications that the biological component of water may have. The organisms are used as indicators and when the levels are high then there is the potential that organisms associated with infectious disease may be present and the water is classified as of general poor hygienic quality (DWAF 1996b). Their levels are documented in the TWQR (DWAF, 1996a; DWAF 1996b) for different water uses. Unlike bacteria, yeasts do not have such microbiological quality indicators in TWQR for different water uses.

4.2 Water samples collected in Mooi River

4.2.1 Physico-chemical parameters

Physico-chemical parameters of the water assessed from the Mooi River sampling sites are presented in Table 4.1. Water temperature measured 9.8°C to 18.3°C. The water temperature ranges reflect the sampling periods. The sampling sites in Wonderfonteinspruit, Klerkskraal, Muiskraai and Boskop sites located before and after the dam measured low temperatures, 9.8°C to 14.7°C as they were sampled in winter. The other sampling sites had higher temperature as they were sampled in summer. The highest temperatures were observed in Viljoen Bridge (18.1°) and Downstream Potchefstroom waste water treatment plant (18.3°C).

The pH at various sites ranged from 7.40 to 8.64. The TWQR of pH for irrigation is 6.5 to 8.4 (Table 4.1). Three sites, (i) the Wonderfonteinspruit before Carletonville, (ii) Klerkskraal and (iii) Muiskraal had the pH slightly exceeding the TWQR for irrigation purposes (8.64, 8.52 and 8.49). TDS values for the sampling sites from Mooi River measured from 286 mg/L to 626 mg/L. These values exceeded DWAF target water quality range (DWAF, 1996a: DWAF, 1996b) for

irrigation use (<40 mg/L) but were within TWQR for livestock watering (<1000 mg/L) (DWAF, 1996a).

Nitrates and phosphates values in the Mooi River ranged from 0.7 to 5.4 mg/L and 1.22 to 6.01 mg/L, respectively. Nitrates from the sampling site exceeded TWQR for irrigation (<0.5 mg/L) but were within the range for livestock farming (<100 mg/L) (DWAF, 1996a). The Wonderfonteinspruit site after Carletonville had the highest nitrate level, 5.4 mg/L while the lowest nitrates level was measured at Trimpark Bridge (0.7 mg/L). The highest phosphate levels were measured in Mooi River at the site downstream from the Potchefstroom waste water treatment plant (7.14 mg/L) and Skandinawiedrif Bridge (6.01 mg/L). The lowest phosphate values were measured in Wonderfonteinspruit site before Carletonville (1.64 mg/L) and Klerkskraal (1.60 mg/L). Elevated nitrates and phosphates values were measured at the sites downstream from the Potchefstroom waste water treatment plant including Taaiboschbult and Skandinawiedrif bridges.

Table 4.1: Physico-chemical properties of the Mooi River sampled water for 2013. Target water quality ranges (TWQR) are based on the South African Department of Water Affairs (DWAF, 1996a) values.

Physico-chemical Parameters	**Temp (°C)**	**pH**	**TDS (ppm)**	**Nitrates (mg/L)**	**Phosphates (mg/L)**
TWQR					
Livestock watering	**N/A**	**N/A**	**<1000**	**<100**	**N/A**
Irrigation	**N/A**	**6.5-8.4**	**<40**	**<0.5**	**N/A**
Sampling site					
Wonderfonteinspruit site before Carletonville	13.4	8.64	547	1.7	1.64
Wonderfonteinspruit site after Carletonville	10.3	8.14	595	5.4	2.91
Klerkskraal Dam	14.0	8.52	286	0.9	1.60
Muiskraal	9.8	8.49	310	0.9	1.88
Boskop site located before Boskop dam	11.5	8.06	451	1.0	1.27
Boskop site located after Boskop dam	14.7	8.40	427	1.3	1.54
Thabo Mbeki Bridge	17.9	7.40	462	1.2	1.22
Trim Park Bridge	16.6	7.72	450	0.7	1.28
Pedestrian Bridge	17.8	7.77	467	2.0	1.39
Viljoenroad Bridge	18.1	7.75	463	1.6	3.08
Downstream from Potchefstroom wastewater treatment plant	18.3	7.43	626	2.7	7.14
Taaiboschbult Bridge	17.2	7.52	579	2.2	5.29
Skandinawiedrif Bridge	17.1	7.46	529	2.6	6.01

4.2.2 Colony counts

Yeast colonies were enumerated at two temperatures (room temperature and 37°C). The water samples were from the Mooi River and 11 of the 13 sampling sites provided results that could be used as indicated in Table 4.2. Fungus grew on the plates of some of the sampling sites and colony counts could not be determined. In all cases, only non-pigmented yeast colonies were detected. These colonies were white and cream white on YM agar.

There were more colonies at room temperature (320 cfu/L- 4200 cfu/L) compared to those enumerated at 37°C (27 cfu/L- 2573 cfu/L). The highest colony counts at room temperature and 37°C were detected at Viljoenroad Road Bridge (4200 cfu/L and 2573 cfu/L, respectively). The lowest yeast count level for room temperature was at Boskop site located after the Boskop dam (302 cfu/L). At 37°C the lowest count level was measured in both Boskop site located after Boskop dam and Skandanawiedrif Bridge. At both sites the average yeast count was 27 cfu/L (SD ± 23.09 and 46.19) respectively.

Table 4.2: Colony counts (in cfu/L) and standard deviations of the sites in the Mooi River.

Sampling site	**Colony counts at different temperatures**	
	Room temperature	**37°C**
Wonderfonteinspruit site before Carletonville	597± 545.25	107± 61.10
Wonderfonteinspruit site after Carletonville	1613± 166..53	333± 100.66
Klerkskraal Dam	0.00	0.00
Muiskraal	560± 280	107± 61.10
Boskop site located before Boskop dam	680± 144.42	173± 100.66
Boskop site located after Boskop dam	320±120	27± 23.09
Thabo Mbeki Bridge	440± 80	187± 83.27
Trimpark Bridge	1147± 294.84	147± 61.01
Pedestrian Bridge	960± 366.60	40± 0.0
Viljoenroad Bridge	4200± 240	2573± 305.51
Downstream Potchefstroom from wastewater treatment plant	0.00	0.00
Taaiboschbult Bridge	0.00	160± 40
Skandinawiedrif Bridge	0.00	27± 46.19

4.3 Water samples collected in Harts River 2014

4.3.1 Physico-chemical parameters

Physico-chemical parameters of the water assessed in Harts River sampling sites are depicted in Table 4.3. The pH of various sites ranged from 7.44 to 8.45. These pH values were within the TWQR for irrigation (6.5-8.4). One site, Sannieshof, measured a pH 8.45 and although slightly elevated was still in order to use for irrigation. Water temperature ranged between 19.0°C and 23.5°C. The sampling in Harts River was conducted in summer and the higher temperatures measured conformed to the sampling time. TDS levels measured ranged from 203 mg/L to 678 mg/L. These levels were within the TWQR for livestock watering (<1000 mg/L) but exceeded TWQR for irrigation (<40 mg/L) (DWAF, 1996a: DWAF, 1996b).

Nitrates values measured for various sampling sites ranged between 0.1 mg/L and 0.8 mg/L). These values were within the TWQR for livestock watering (<100 mg/L). Nitrates values measured were within TWQR for irrigation use (<0.5 mg/L) except for the sampling site in Lichtenburg located before the wastewater treatment plant (0.8mg/L). Chemical Oxygen Demand (COD) measured was between 31 to 43 mg/L. These COD levels are within the levels acceptable for environmental water for wastewater effluent (<75mg/L) (South Africa, 1984). Phosphates and dissolved oxygen (DO) values were measured between 0.00 to 5.74 mg/L and 12.9 to 21.7% O_2, respectively.

Table 4.3: Physico-chemical properties of the Harts River sampled water for 2014. Target water quality ranges (TWQR) are based on the South African Department of Water Affairs (DWAF, 1996a) values.

Physico-chemical Parameters	**Temp (°C)**	**pH**	**TDS (ppm)**	**Nitrates (mg/L)**	**Phosphates (mg/L)**	**DO (%O_2)**	**COD (mg/L)**
TWQR							
Livestock watering	**N/A**	**N/A**	**<1000**	**<100**	**N/A**	**N/A**	**N/A**
Irrigation	**N/A**	**6.5-8.4**	**<40**	**<0.5**	**N/A**	**N/A**	**N/A**
Sampling sites							
Lichtenburg site located before wastewater treatment plant	19.0	7.44	678	0.8	0.04	12.9	35
Lichtenburg site located after wastewater treatment plant	19.4	8.26	494	<0.1	1.19	15.3	42
Biesiesvlei	21.9	8.08	687	0.2	5.74	20.8	32
Vermaas	21.7	8.23	375	0.2	3.04	21.7	31
Sannieshof	23.5	8.45	401	0.1	2.71	20	32
Delareyville	21.2	8.01	203	0.1	0.00	20	43

4.3.2 Colony counts

Yeasts colonies were enumerated from Harts River water samples and were also incubated at room temperature and 37°C (Table 4.3). There were more colonies at room temperature (375-1280 cfu/L) as compared to 37°C (27-924 cfu/L). The Lichtenburg site located before the wastewater treatment plant had the highest yeast counts for the incubation at room temperature (1280 cfu/L) and 37°C (924 cfu/L). Biesiesvlei site had the lowest count at room temperature

(375 cfu/L) whilst the lowest yeast level at 37°C was enumerated at Delareyville (27 cfu/L). In all cases, only non-pigmented yeast colonies were detected. These colonies were white and cream white on YM agar

Table 4.4: Colony counts (in cfu/L) and standard deviations of the sites in the Harts River.

Sampling site	**Colony counts at different temperatures**	
	Room temperature	**37°C**
Lichtenburg site located before waste water treatment plant	1280±34.64	924±28
Lichtenburg site located after waste water treatment plant	656±104.92	351±8.32
Biesiesvlei	375±40.86	109±28.10
Vermaas	549±78.93	52±4.0
Sannieshof	552±32	68±14.42
Delareyville	399±32.33	27±10.07

4.4 Diazonium Blue B (DBB) testing

Only the yeasts that were enumerated at 37°C were further identified. These yeast isolates were all non-pigmented. They gave a negative response to the DBB test confirming that they were all ascomycetous yeasts (Hagler and Ahearn, 1987).

4.5 Haemolysin production

Yeast isolates were tested for the production of haemolysin as described in Section 3.6. There was no clear zone of inhibition around the yeast colonies. This confirmed that the yeasts displayed γ- haemolysis.

4.6 Extracellular enzyme production

The ability of yeast isolates to produce enzymes was tested against a group of 3 enzymes as outlined in Section 3.7. These included proteinase, Dnase and lipase. There was no clearing of

zones around the yeast colonies. This showed that the yeast colonies did not produce any extracellular enzyme.

4.7 Molecular identification of yeast isolates

4.7.1 DNA Isolation of pure yeast cultures

Genomic DNA was isolated from 117 pure overnight yeast cultures using the DNA extraction method described in Section 3.5.1. Agarose gel electrophoresis was performed to determine the quality of the isolated DNA. Figure 4.1 is an ethidium bromide stained 1% (w/v) gel that is used as an example. The presence of the DNA could be seen by the bands on the gel. The bands on the gel illustrate good quality DNA with no Rnase.

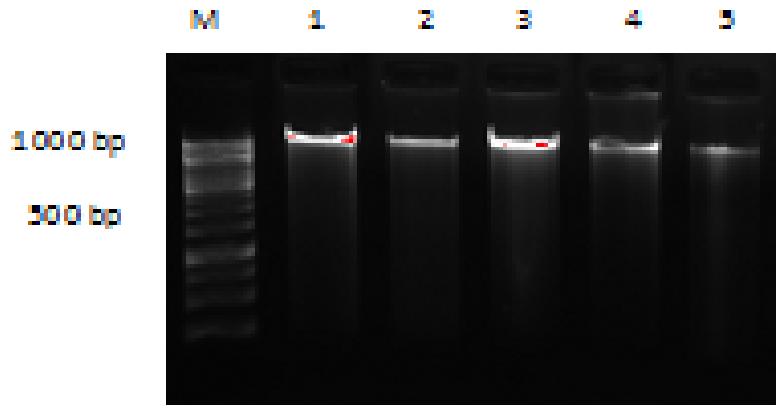


Figure 4.1: Ethidium bromide stained 1% (w/v) gel showing isolated DNA from pure yeast cultures. A 1000bp molecular marker (O'GeneRulerTM 1000 bp DNA ladder, Fermentas Life Science, US) was used.

NanoDrop TM 1000 Spectrophotometer (Thermo Fischer Scientific, US) (NanoDrop, 2007) was used to determine the DNA concentration and quality. The 260/280 ratios of the DNA samples varied between 1.30 and 4.0. DNA concentration ranged between 50 and 600 ng/µl. These values indicated that the DNA could be used for PCR.

4.7.2 DNA amplification

The D1/D2 region of the 26S rRNA gene was amplified by PCR under the conditions stated in Section 3.5.3. Figure 4.2 is an ethidium bromide stained 1.0% (w/v) gel showing amplified DNA products. Fragment lengths were of the expected sizes between 500-650bp when the 26S

primer sets were used. There were also no non-specific amplification products. These PCR fragments could thus be directly prepared for Sanger sequencing.

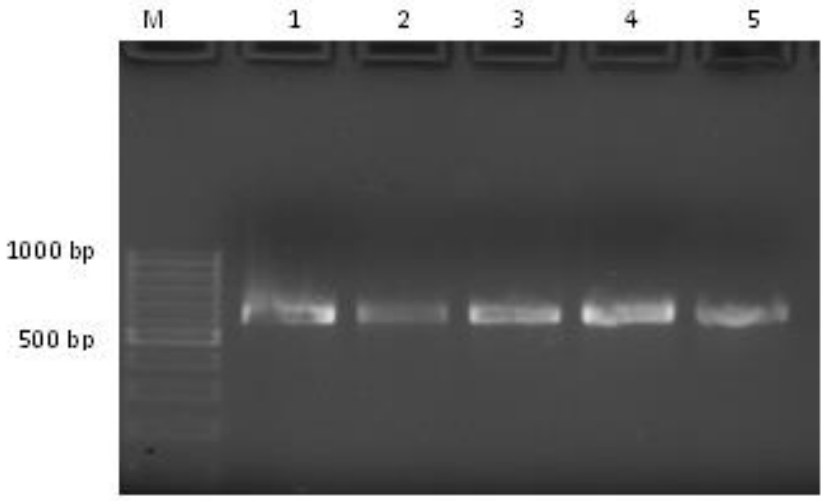


Figure 4.2: A 1.0% (w/v) agarose gel illustrating successfully run PCR amplified 26S rRNA gene amplicon. Lane M contain a 100 bp molecular marker (O'GeneRulerTM 100 bp DNA ladder, Fermentas Life Sciences, US). Lanes 1-5 contain 26S rRNA gene amplicons.

4.7.3 Sequencing

The gene sequencing was performed by Central Analytical Facility of the University of Stellenbosch, South Africa (all the isolates from the Mooi River). Isolates from the Harts River were all sequenced by Inqaba Biotech, Pretoria. Chromatograms were viewed in Geospiza Finch TV (version 1.4) software. BLASTN searches (http://www.ncbi.nlm.nih.gov/BLAST) were used to identify homologous yeast sequences in genbank. The searches revealed that all the sequences were 26S yeast ribosomal RNA gene sequences. From the blast hits on Genbank, exact matches (100%) of yeast species were obtained for some yeast isolates and high sequence similarities (99%) were obtained for other isolates as illustrated on Table 4.5. The e-values for all the analysis were small at 0. A small e-value indicates a high confidence of similarity between two sequences due to more common descent (Ogunseitan, 2005).

The 117 yeast sequences from the Mooi River and Harts Rivers were submitted to Genbank. Genbank accession numbers with ranges from KM102986 to KM103064 were allocated to Mooi

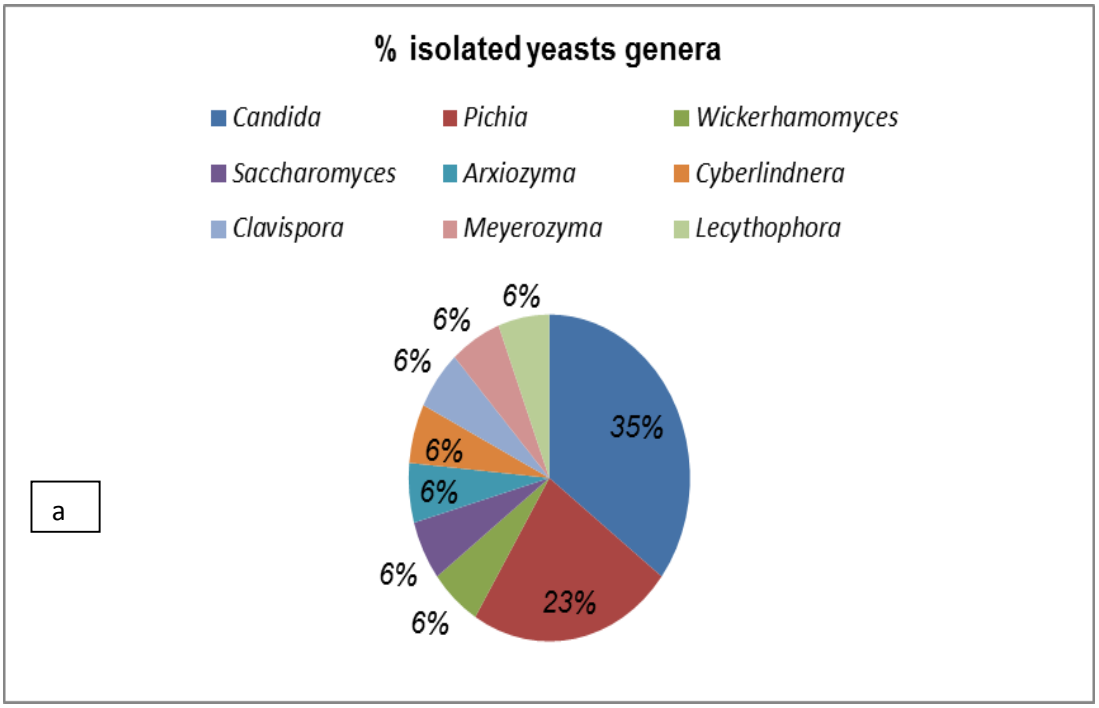
River isolated yeast species. Harts River yeast species had two groups of accessions that ranged from KM885965 to KM885981 and KP010741 to KP07062.

Table 4.5: Genbank identification of the amplified yeast isolates from the Mooi River and Harts River with % similarities to NCBI blast hit.

Isolate name	**No. of isolates in the same group**	**NCBI blast hit**	**% similarities**	**e- value**
0J11	25	*Candida glabrata*	100	0.0
0D6	21	*Candida albicans*	100	0.0
0J22	4	*Candida pseudolambica*	100	0.0
0G1	4	*Candida tropicalis*	99	0.0
0J34	1	*Candida bracarensis*	99	0.0
0J31	1	*Candida parapsilosis*	99	0.0
0B9	33	*Saccharomyces cerevisiae*	99	0.0
0H4	2	*Pichia salicaria*	99	0.0
0I2	2	*Pichia guilliermondii*	100	0.0
0E3	1	*Pichia guilliermondii*	99	0.0
0H8	4	*Pichia Mexicana*	99	0.0
0G2	2	*Pichia kudriavzevii*	99	0.0
0G4	2	*Wickerhamomyces anomalus*	99	0.0
0G5	2	*Arxiozyma telluris*	99	0.0
0J29	2	*Cyberlindnera fabianii*	99	0.0
0B5	4	*Clavispora lusitaniae*	99	0.0
0P3	2	*Meyerozyma guilliermondii*	99	0.0
0Q4	5	*Lecythophora* sp.	98	0.0

In descending order of numbers of representative, the following yeast genera were identified as shown in Figure 4.5a; *Candida* spp. (35%), *Pichia* spp. (23%), Wickerhamomyces spp. (6%),

Saccharomyces spp. (6%), *Arxiozyma* spp. (6%), *Cyberlindnera* spp. (6%), *Meyerozyma* spp. (6%) and *Clavispora* spp. (6%), *Lecythophora* spp. (6%).



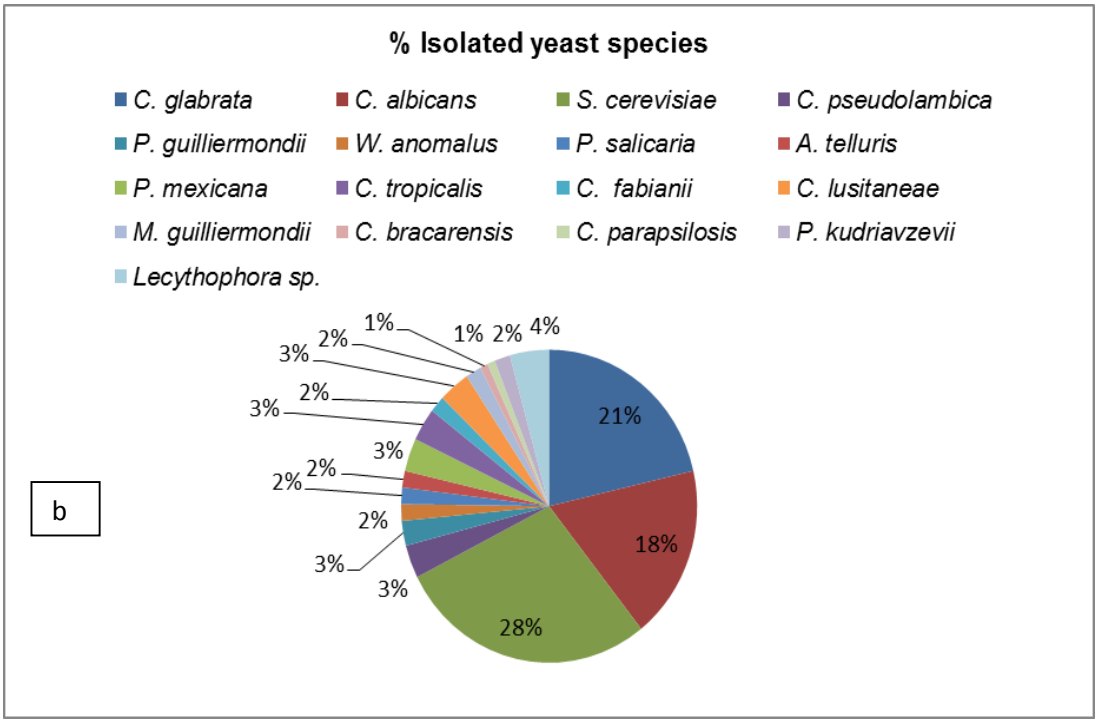


Figure 4.5: Pie chart showing the percentage of the genera (a) and percentage of the isolated yeast species (b) from the Mooi River and Harts River.

Figure 4.5b shows that *Saccharomyces cerevisiae was* the dominant isolated species (28%) from the Mooi and Harts River. It was followed by *Candida glabrata* (21%), *Candida albicans* (18%), *Lecythophora sp. (4%), Candida pseudolambica* (4%), *Candida tropicalis* (4%), *Pichia mexicana* (4%), *Clavispora lusitaniae* (4%), *Pichia guilliermondi* (3%), *Wickerhamomyces anomalus* (2%), *Cyberlindnera fabianii* (2%), *Pichia salicaria* (2%), *Arxiozyma telluris* (2%), *Meyerozyma guilliermondii* (2%) , *Pichia kudriavzevii* (2%), *Candida bracarensis* (1%), *Candida parapsilosis* (1%).

4.7.4 Phylogenetic tree

Phylogenetic trees estimate the relationship among sequences and their common ancestral lineage (Hall, 2013). Figure 4.7 is a Neighbour-Joining phylogenetic tree of yeast species isolated from the Mooi River and Harts River (in bold) with their Genbank accession numbers. Yeast isolate 0B9 represents 33 isolates and 0D6 represents 21 isolates from the total of yeasts isolated in the study (Table 4.5). Genbank sequences, with their accession numbers in brackets, are included in the tree to show their phylogenetic relationship with isolated yeasts. Local bootstrap probability values obtained by maximum likelihood analysis are indicated at the branch nodes. High sequence similarity between two or more yeast species sequences is illustrated by high bootstrap confidence.

Figure 4.6 shows that yeast isolate, 0B14, accession number KM103017, is closely related to *Candida glabrata* species. High bootstrap support (99%) is observed for the sequences of these yeast isolates and the Genbank sequences. A 95% bootstrap confidence supports a close phylogenetic relationship between a yeast isolate 0D6 accession number KM103021 and *Candida* species (U45776 and JQ672591). *Wickerhamomyces anomalus* (AB847478 and KJ469966) are closely related to isolate, 0J29; KM103055. A high bootstrap 94% support the high phylogenetic similarity between the isolates. According to BLASTN searches, yeast isolate 0P6 was identified as *Meyerozyma guilliermiondi* and 0Q4 as *Lecythophora* spp. These are two different species but show close phylogenetic relationship supported by a bootstrap of 99%. In this study, there was a similar high bootstrap support for all the sequences as illustrated in Figure 4.6.

Figure 4.6: Neighbour-Joining tree showing the phylogenetic relationship of sequences of yeasts isolated from the Mooi River and Harts River and the Genbank sequences. The tree is based on the spatial sequences of D1/D2 domain of 26S rRNA.

4.8 Distribution of yeasts amongst the sampling site

One hundred and seventeen yeast species were unevenly distributed along the sampling sites in the Mooi and Harts River. This was expected because yeast colonies were not picked evenly. The colonies were selected by appearance and viability. Mooi River had more isolates (78 isolates; Table 4.6) than Harts River (39 isolates; Table 4.7). This could be attributed to more sampling sites at Mooi River. The highest number of isolates was obtained from Viljoenroad Bridge in the Mooi River and Lichtenburg site located before waste water treatment plant in the Harts River.

Candida glabrata was the species most frequently selected for identification from the Mooi River samples (Table 4.6) and *Saccharomyces cerevisiae* was the one among the Harts River samples (Table 4.7)*. Candida albicans* was isolated from all the sampling sites in the Mooi River except Wonderfonteinspruit after Carletonville and Trimpark Bridge. *Saccharomyces cerevisiae* was isolated from almost all the sampling sites in Harts River except Lichtenburg site located after waste water treatment plant. Some yeast species were isolated from only one sampling site, *Pichia salicaria* isolated from Trimpark Bridge. Some yeast species were isolated only from the Mooi River. *Candida albicans* was isolated from the Mooi River and *Lecythophora* sp. from the Harts River. *Saccharomyces cerevisiae*, *Candida glabrata*, *Candida tropicalis*, *Clavispora lusitaniae* and *Pichia kudriavzevii* were isolated from Mooi River and Harts River. These results are depicted in Tables 4.6 and 4.7.

Table 4.6: Distribution of yeast species along the Mooi River

Yeast species	**Mooi River sampling sites**								
	Wonderfonteinspruit before Carletoville	**Wonderfonteinspruit before Carletoville**	**Muiskraal**	**Boskop site located before Boskop Dam**	**Thabo Mbeki Bridge**	**Trimpark Bridge**	**Pedestrian Bridge**	**Viljoenroad Bridge**	**Taaiboschbult Bridge**
Candid. glabrata	2	9						13	
Candida albicans	1		1	1	2				
Candida parapsilosis								1	
Candida pseudolambica								1	3
Candida tropicalis	1				1				
Candida bracarensis								1	
Saccharomyces cerevisiae		5				3		2	
Clavispora lusitaniae		1							
Pichia guilliermondii				1		1	1		
Pichia kudriavzevii					1				
Pichia mexicana						2			
Pichia salicaria						2			
Cyberlindnera fabianii			1					1	
Wickerhamomyces anomalus	1				1				
Arxiozyma telluris					2				

Table 4.7: Distribution of yeast species along the Harts River

Yeast species	**Harts River sampling sites**					
	Lichtenburg site located before waste water treatment plant	**Lichtenburg site located after waste water treatment plant**	**Beisiesvlei**	**Vermaas**	**Sannieshof**	**Delareyville**
Candida glabrata						1
Candida tropicalis			1		1	
Saccharomyces cerevisiae	14		3	1	2	3
Clavispora lusitaniae	1		1	1		
Pichia kudriavzevii				1		
Pichia mexicana					1	1
Meyerozyma guilliermondii		2				
Lecythophora spp.			1	1		3

4.9 Correlation of physico-chemical parameters and yeast in the Mooi and Harts Rivers

A redundancy analysis (RDA) ordination plot was constructed to directly relate the environmental factors with yeast isolated from particular sampling sites (Figure 4.7) Water parameters such as nitrates and phosphates were used since these variables could have had an influence on the isolated yeast species distribution. A positive correlation between environmental variables and species is indicated by small angles between them. Figure 4.7 shows a correlation between temperature and *Candida albicans*. There was also a strong positive correlation between nitrates and the yeast species, *Candida glabrata*. Yeast levels measured downstream from Potchefstroom wastewater treatment plant and Wonderfonteinspruit site after Carletonville were correlated to nitrate levels and also to phosphates. Both these sites were impacted on more by nitrate levels. Muiskraal and Kromdraai sites were also influenced by these parameters (nitrates and phosphates). Phosphates had a greater impact on the latter two sites (Muiskraal and Kromdraai). There are further correlations between the sites, yeast levels and species as well as the environmental variables.

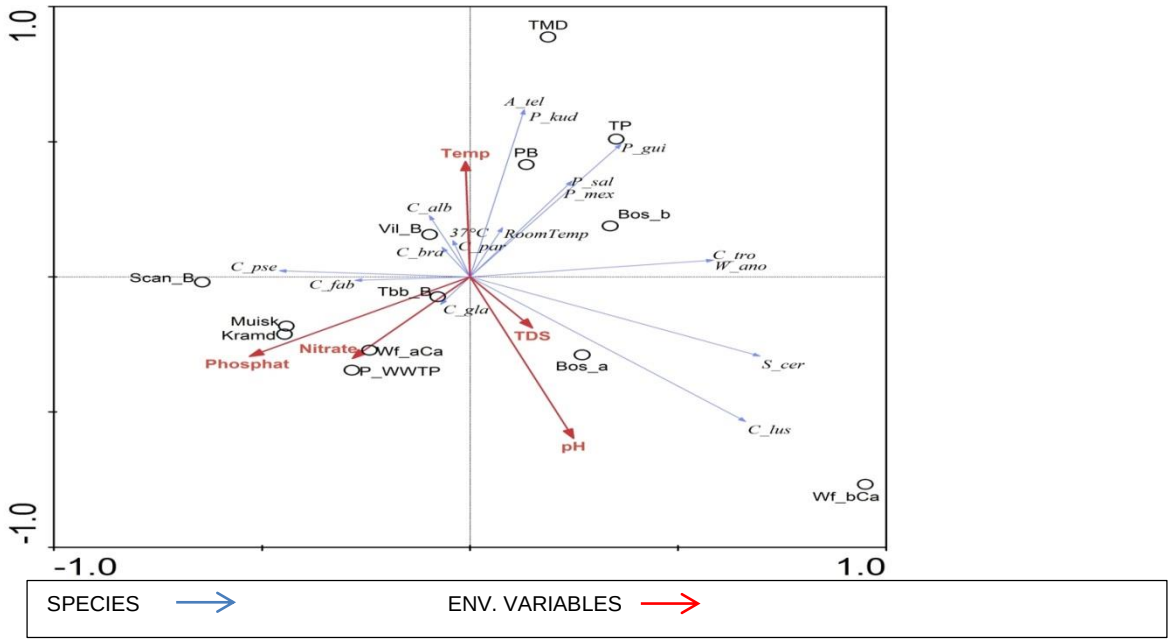


Figure 4.7: Redundancy analysis (RDA) ordination illustrating correlation between environmental variables, yeast species and the Mooi River sampling sites. The blue vectors represent yeast species and red vectors represent environmental variables.

An RDA plot illustrating the association between environmental variables with isolated yeast species as well sampling sites in Harts River is presented in Figure 4.8. There is a positive correlation between nitrate levels and yeast levels in Lichtenburg site located before waste water treatment plant. Figure 4.8 also shows a positive correlation between yeast species, *Saccharomyces cerevisiae* and nitrate levels. On the other hand, there is a positive correlation between phosphate levels and yeast levels at Biesiesvlei sampling site. The Vermaas and Lichtenburg site located after the waste water treat plant are also impacted on by phosphate levels. Temperature and dissolved oxygen levels impacted strongly on the yeast levels observed for the Sannieshof site.

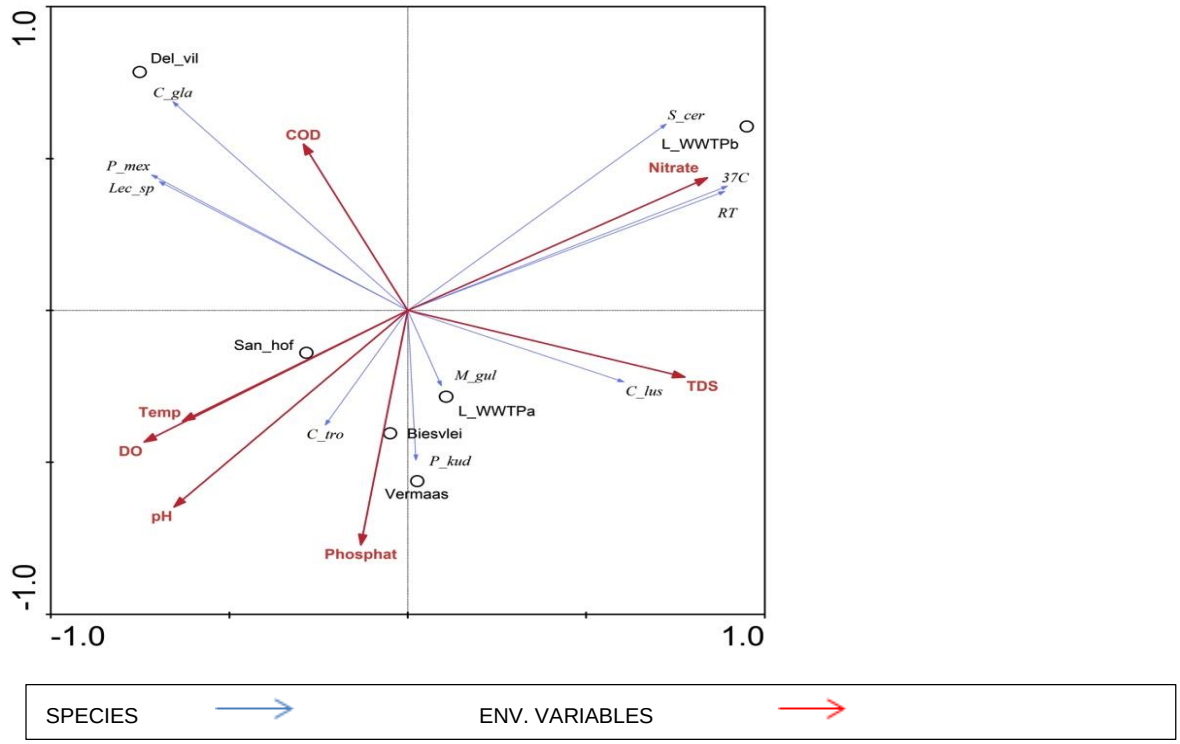


Figure 4.8: Redundancy analysis (RDA) ordination illustrating correlation between environmental variables, yeast species and the Harts River sampling sites. The blue vectors represent yeast species and red vectors represent environmental variables.

In the analysis above it was demonstrated that physico-chemical parameters had an impact on the levels and diversity of yeasts in aquatic habitats. These include nutrients and selective physical parameters.

4.10 Antifungal susceptibility testing

A total of 117 yeast isolates that grew at 37˚C and isolated from different sampling sites were tested for their antifungal susceptibility against different antifungals. In this study the CLSI zone diameters in Table 2.1 were compared with diameters of the inhibition zone by isolated yeasts and their antifungal activity shown in Table 4.8 to determine susceptibility of the isolated yeasts. For some antifungal agents in the study, different disk potencies were used *viz*. fluconazole (10 µg), econazole (10 µg), ketoconazole (10 µg), miconazole (10 µg), metronidazole (5 µg), flucytosine (1 µg), and nystatin (100 µg).

Of all the isolated yeast species in this study, 91% were resistant to fluconazole, 90% resistant to ketoconazole, 53 % resistant to econazole, 45% resistant to miconazole and 21% resistance to nystatin. Hundred percent of the isolates were resistant to metronidazole and flucytosine. *Candida* species showed the highest resistance to almost all the antifungals used in the study. *C. albicans*, *C. glabrata* and *C. tropicalis* were resistant to all the antifungals but showed intermediate sensitivity to nystatin. These *Candida* species were isolated from different sampling sites but displayed similar resistance patterns throughout. *C. lusitaniae* showed resistance profile similar to *C. albicans*, *C. glabrata* and *C. tropicalis. Candida* species, *C. parapsilosis and C. bracarensis* had different resistance patterns from the above mentioned species. They showed similar resistance pattern; fluconazole (R), nystain (R), metronidazole (R), flucytosine (R), econazole (S), miconazole (S) except for ketoconazole. *C. bracarensis* was resistant to ketoconazole and *C*. *parapsilosis* showed intermediate sensitivity.

Meyerozyma guilliermondii also known as *Candida guilliermondii* (Van Wyk *et al.,* 2012) was resistant to all the antifungal agents but showed intermediate sensitivity to ketoconazole. *Wickerhamomyces anomalus* also known as *Candida pellucosa* (Van Wyk *et al.,* 2012) showed resistance patterns to fluconazole (S), ketoconazole (S), econazole (S), miconazole (S), nystatin (S), metronidazole (R), flucytosine (R). *Pichia mexicana*, *Pichia salicaria* and *Cyberlindnera fabianii* showed similar resistance patterns to *Wickerhamomyces anomalus.* These are the only species that were susceptible to fluconazole.

Candida pseudolambica displayed two resistance patterns. *Candida pseudolambica* (0M3 and 0M4) were resistant to econazole, ketoconazole and miconazole. Same species *Candida pseudolambica* (0J22 and 0M1) were susceptible to the same antifungal agents. This showed

that these yeast isolates had different clones hence different resistance patterns. These interpretive breakpoints are not applicable to *C. krusei* as it is assumed to be intrinsically resistant to fluconazole CLSI, 2008). *Pichia kudriavzevii* also known as *C. krusei* (Van Wyk *et al*., 2012) was tested for antifungal susceptibility and showed resistance pattern; fluconazole (R), ketoconazole (R), metronidazole (R), flucytosine (R), nystatin (R), econazole (S) and miconazole (S). *Saccharomyces cerevisiae*, *Pichia guiliermondii*, *Lecythophora* species from different sampling sites also displayed similar resistance patterns to *Pichia kudriavzevii*. *Arxiozyma telluris* had similar resistance pattern to *Pichia kudriavzevii*, *Saccharomyces cerevisiae*, *Pichia guiliermondii*, *and Lecythophora* species except its susceptibility to nystatin.

Table 4.8: Antifungal susceptibility data of the different yeast isolates showing inhibition zone measurements in millimeters and the standard deviation. Antifungal abbreviations: fluconazole (FCN), econazole (ECN), ketoconazole (KCA), miconazole (MCL), metronidazole (MZ) flucytosine (FY), nystatin (NY).

Sampling site	**Species name (Isolate)**	**Antifungal agents**						
		FCN	**ECN**	**KCA**	**MCL**	**MZ**	**FY**	**NY**
Mooi River								
Wonderfontein before Carletonville	*Candida albicans* (0A2)	0	0	0	0	0	0	15±0.0
	Candida tropicalis (0A3)	0	0	0	0	0	0	15±0.7
	Wickerhamomyces anomalus (0A4)	19±1.4	26±1.4	25±0.7	23±1.4	0	0	16±0.7
	Candida glabrata (0A5)	0	0	0	0	0	0	17±2.1
	Candida glabrata (0A7)	0	0	0	0	0	0	11±0.7
Wonderfonteinspruit after Carletonville	*Candida glabrata* (0B1)	0	0	0	0	0	0	11±0.7
	Candida glabrata (0B3)	0	0	0	0	0	0	11±0.7
	Candida glabrata (0B4)	0	0	0	0	0	0	10±1.4
	Clavispora lusitaniae (0B5)	0	0	0	0	0	0	15±0.7

	Candida glabrata (0B6)	0	0	0	0	0	0	12±0.7
	Candida glabrata (0B7)	0	0	0	0	0	0	11±1.4
	Candida glabrata (0B8)	0	0	0	0	0	0	10±1.4
	Saccharomyces cerevisiae (0B9)	0	20±0.0	18±0.7	14±1.4	0	0	11±1.4
	Candida albicans (0B10)	0	0	0	0	0	0	13±0.7
	Candida glabrata (0B11)	0	0	0	0	0	0	11±1.4
	Saccharomyces cerevisiae (0B12)	0	20±0.0	22±0	16±4.9	0	0	14±1.4
	Saccharomyces cerevisiae (0B13)	0	25±0.0	18±0	21±1.4	0	0	16±0.7
	Candida glabrata (0B14)	0	0	0	0	0	0	12±1.4
	Saccharomyces cerevisiae (0B15)	0	22±0.7	19±1.4	17±1.4	0	0	8±0.7
	Saccharomyces cerevisiae (0B16)	0	20±0.7	9±0.7	19±1.4	0	0	12±0.7
Muiskraal	*Cyberlindnera fabianii* (0D4)	21±0.7	22±0.7	20±0.7	20±0.7	0	0	16±0.7

	Candida albicans (0D6)	0	0	0	0	0	0	15±0.7
Boskop site located before Boskop Dam	*Candida guilliermondii* (0E3)	0	9±0.7	16±0	11±0.7	0	0	0
	Candida albicans (0E4)	0	0	0	0	0	0	13±0.7
Thabo Mbeki Drive	*Candida tropicalis* (0G1)	0	0	0	0	0	0	11±1.4
	Pichia kudriavzevii (0G2)	0	9±0.7	0	15±0.7	0	0	11±0.7
	Candida albicans (0G3)	0	0	0	0	0	0	13±1.4
	Wickerhamomyces anomalus (0G4)	16±0.7	23±1.4	25±0.0	26±0.7	0	0	16±1.4
	Arxiozyma telluris (0G5)	0	15±0	25±0	18±0	0	0	16±0.7
	Arxiozyma telluris (0G6)	0	16±0	23±0	18±0	0	0	16±0
	Candida albicans (0G7)	0	0	0	0	0	0	13±1.4
Trimpark Bridge	*Saccharomyces cerevisiae* (0H2*)*	0	20±2.8	22±0	20±0.0	0	0	11±1.4
	Pichia guilliermondii (0H3)	0	13±1.4	18±0.7	16±0.0	0	0	0

	Pichia salicaria (0H4)	16±0.7	19±1.4	17±1.4	20±0.0	0	0	15±1.4
	Saccharomyces cerevisiae (0H5)	0	20±0.0	18±0	19±1.4	0	0	11±1.4
	Saccharomyces cerevisiae (0H6)	0	23±0.7	17±0	20±0.0	0	0	17±2.1
	Pichia salicaria (0H7)	13±1.4	16±0.7	18±2.8	19±0.7	0	0	16±0.7
	Pichia mexicana (0H8)	23±1.4	10±0.7	24±2.1	15±0.7	0	0	18±0.7
	Pichia mexicana (0H9)	19±0.7	13±0.7	21±1.4	16±0.4	0	0	17±0.7
Pedestrian Bridge	*Pichia guilliermondii* (0I2)	0	26±0	27±0	22±0	0	0	0
	Candida albicans (0I3)	0	0	0	0	0	0	15±0.0
	Candida albicans (0I4)	0	0	0	0	0	0	13±1.4
Viljoenraod Bridge	*Candida albicans* (0J1)	0	0	0	0	0	0	15±0.7
	Candida glabrata (0J3)	0	0	0	0	0	0	9±1.4
	Candida glabrata (0J4)	0	0	0	0	0	0	13±1.4

	Candida glabrata (0J5)	0	0	0	0	0	0	13±2.1
	Candida glabrata (0J6)	0	0	0	0	0	0	10±1.4
	Candida albicans (0J7)	0	0	0	0	0	0	10±1.4
	Pichia salicaria (0J8)	0	0	0	0	0	0	11±1.4
	Saccharomyces cerevisiae (0J10)	0	11±0.0	19±1.4	16±1.4	0	0	16±1.4
	Candida glabrata (0J11)	0	0	0	0	0	0	10±0.7
	Candida glabrata (0J12)	0	0	0	0	0	0	12±0.7
	Candida albicans (0J13)	0	0	0	0	0	0	17±0
	Saccharomyces cerevisiae (0J14)	0	19±1.4	16±0	19±1.4	0	0	14±0.7
	Candida albicans (0J15)	0	0	0	0	0	0	11±0.0
	Candida glabrata (0J16)	0	0	0	0	0	0	11±0.7
	Candida glabrata (0J17)	0	0	0	0	0	0	13±0.7

	Candida glabrata (0J18)	0	0	0	0	0	0	13±0
	Candida glabrata (0J19)	0	21±0	19±0	16±0	0	0	16±0
	Candida albicans (0J20)	0	0	0	0	0	0	12±0
	Candida albicans (OJ21)	0	0	0	0	0	0	13±0
	Candida pseudolambica (0J22)	0	20±0	24±0	18±0	0	0	14±0
	Candida albicans (0J24)	0	0	0	0	0	0	15±0
	Candida albicans (0J25)	0	0	0	0	0	0	14±0
	Candida albicans (0J26)	0	0	0	0	0	0	14±0
	Candida albicans (0J27)	0	0	0	0	0	0	15±0
	Cyberlindnera fabianii (0J29)	16±0	14±0	17±0	16±0	0	0	16±0
	Candida albicans (0J30)	0	0	0	0	0	0	15±0
	Candida	14±0	22±0	28±0	16±0	0	0	0

	parapsilosis (0J31)							
	Candida glabrata (0J32)	0	0	0	0	0	0	16±0
	Candida glabrata (0J33)	0	0	0	0	0	0	14±0
	Candida bracarensis (0J34)	0	22±0	12±0	15±0	0	0	0
Taaiboschbult Bridge	*Candida pseudolambica* (0M1)	0	22±0	21±0	22±0	0	0	10±0
	Candida pseudolambica (0M3)	0	0	0	0	0	0	12±0
	Candida pseudolambica (0M4)	0	0	0	0	0	0	10±0
	Candida albicans (0M5)	0	0	0	0	0	0	16±0
	Candida albicans (0M8)	0	0	0	0	0	0	13±0
	Candida albicans (0M12)	0	0	0	0	0	0	12±0
Harts River								
Lichtenburg site	*Saccharomyces*	0	22±0	19±0	19±0	0	0	11±0

located before waste water treatment plant	*cerevisiae* (0O1)							
	Saccharomyces cerevisiae (0O2)	0	23±0	12±0	22±0	0	0	11±0
	Saccharomyces cerevisiae (0O4)	0	25±0	19±0	21±0	0	0	8±0
	Saccharomyces cerevisiae (0O5)	0	21±0	20±0	20±0	0	0	9±0
	Saccharomyces cerevisiae (0O7)	0	20±0	22±0	21±0	0	0	8±0
	Saccharomyces cerevisiae (0O9)	0	25±0	20±0	23±0	0	0	11±0
	Saccharomyces cerevisiae (0O10)	0	20±0	21±0	22±0	0	0	12±0
	Saccharomyces cerevisiae (0O11)	0	20±0	15±0	21±0	0	0	9±0
	Saccharomyces cerevisiae (0O13)	0	22±0	20±0	19±0	0	0	11±0
	Saccharomyces cerevisiae (0O15)	0	21±0	11±0	24±0	0	0	12±0
	Saccharomyces cerevisiae (0O16)	0	22±0	9±0	25±0	0	0	12±0
	Saccharomyces cerevisiae (0O17)	0	22±0	9±0	23±0	0	0	11±0
	Saccharomyces		25±0	14±0	22±0	0	0	10±0

	cerevisiae (0O18)							
	Saccharomyces cerevisiae (0O19)	0	22±0	12±0	19±0	0	0	10±0
Lichtenburg site located after waste water treatment plant	*Meyerozmyma guilliermondii* (0P3)	0	0	25±0	0	0	0	0
	Meyerozmyma guilliermondii (0P6)	0	0	24±0	0	0	0	0
	Clavispora lusitaniae (0P13)	0	0	0	0	0	0	18±0
Biesiesvlei	*Saccharomyces cerevisiae* (0Q1)	0	22±0	9±0	23±0	0	0	11±0
	Saccharomyces cerevisiae (0Q2)	0	22±0	9±0	23±0	0	0	11±0
	Saccharomyces cerevisiae (0Q3)	0	22±0	9±0	23±0	0	0	11±0
	Lecythophora spp. (0Q4)	0	20±0	19±0	18±0	0	0	11±0
	Pichia kudriavzevii (0Q5)	0	10±0	0	15±0	0	0	11±0
	Clavispora lusitaniae (0Q10)	0	0	0	0	0	0	15±0
Vermaas	*Pichia kudriavzevii* (0R2)	0	12±0	20±0	12±0	0	0	17±0
	Saccharomyces	0	23±0	10±0	23±0	0	0	14±0

	cerevisiae (0R5)							
	Candida parapsilosis (0R6)	15±0	22±0	27±0	17±0	0	0	0
	Pichia kudriavzevii (0R7)	0	11±0	0±0	16±0	0	0	14±0
	Lecythophora spp. (0R8)	0	22±0	24±0	22±0	0	0	14±0
Sannieshof	*Saccharomyces cerevisiae* (0S1)	0	21±0	11±0	22±0	0	0	13±0
	Pichia mexicana (0S2)	22±0	20±0	22±0	18±0	0	0	18±0
	Saccharomyces cerevisiae (0S3)	0	23±0	13±0	24±0	0	0	14±0
	Pichia kudriavzevii (0S7)	0	11±0	0	18±0	0	0	14±0
Delareyville	*Saccharomyces cerevisiae* (0T1)	0	22±0	10±0	24±0	0	0	13±0
	Saccharomyces cerevisiae (0T3)	0	20±0	11±0	19±0	0	0	13±0
	Pichia mexicana (0T4)	20±0	20±0	21±0	18±0	0	0	18±0
	Saccharomyces cerevisiae (0T5)	0	19±0	11±0	21±0	0	0	13±0
	Candida glabrata	0	0	0	0	0	0	14±0

	(0T7)							
	*Lecythophora (*0T8)	0	22±0	20±0	22±0	0	0	14±0
	Lecythophora (0T9)	0	20±0	18±0	20±0	0	0	15±0
	Lecythophora (0T10)	0	20±0	16±0	21±0	0	0	14±0

4.11 Summary of the results

Some of the physico- chemical results presented in this chapter for both Mooi River and Harts River correlated with the sampling times. High water temperatures were experienced in summer sampling and low temperature in winter sampling. The pH of the water was alkaline in all the sampling sites. TDS, nitrates and phosphates were high in almost all the sites and exceeded suitable ranges for irrigational use but could be used in livestock farming.

Only non-pigmented colonies were isolated. Colony counts varied with the sampling sites in both rivers. Highest colony counts in the Mooi River were measured at Viljoenroad Bridge and Lichtenburg site located before waste water treatment plant in the Harts River. RDA plots showed correlation between environmental parameters, nitrates and phosphates and the sampling sites. High level of phosphates correlated with high yeast levels Viljoenroad Bridge and high nitrates with yeast levels at Lichtenburg site located before waste water treatment plant. High phosphate levels were measured from Viljoenroad Bridge, downstream Potchefstroom waste water treatment plant down to Skandanawiedrif Bridge where Mooi River converges with the Vaal River.

All the isolated yeast species were ascomycetes as determined by diazonium blue B test. They tested negative to haemolysin and extracellular enzyme production when tested for pathogenicity. 26S ribosomal RNA gene sequencing was done to identify the yeast isolates. The following yeast species were identified. The genus *Candida* was the dominant with species *Candida albicans, Candida bracarensis, Candida glabrata*, *Candida parapsilosis*, *Candida pseudolambica* and *Candida tropicalis*. Other species include *Arxiozyma telluris, Clavispora lusitaniae, Cyberlindnera fabianii, Lecythophora sp., Meyerozyma guilliermondii, Pichia guilliermondi, Pichia kudriavzevii, Pichia mexicana, Pichia salicaria, Saccharomyces cerevisiae*, *and Wickerhamomyces anomalus. Saccharomyces cerevisiae* had the highest number of species in the study.

Yeasts species were unevenly distributed between the two rivers. Some species were found in both the river while some in one river. *Candida glabrata* was dominant in the Mooi and was isolated in almost all the sampling sites. *Saccharomyces cerevisiae* had the highest numbers in Harts River and was present in most of the sampling site. RDA plots confirmed a correlation between *Saccharomyces cerevisiae* level with temperature.

Yeast sequences were sent to Genbank for accession numbers. A phylogenetic tree was constructed to determine phylogenetic relationship between the yeast species. Sequences of similar yeast species were downloaded from Genbank and included in the tree. There was a similar high bootstrap support for all the sequences of the same species. Antifungal susceptibility tests were conducted using seven antifungals against the isolated yeast species. A large number of isolates were resistant to azoles, especially fluconazole as well as other antifungal classes. Most of the *Candida* species were resistant to almost all the antifungals.

CHAPTER 5 -DISCUSSION

5.1 Introduction

In this chapter, the results obtained in chapter 4 are discussed and related to the aim of the study, diversity and antifungal susceptibility of yeasts in selected rivers in the North West Province. Physico-chemical properties in various waters systems are associated with TWQR which are used to describe whether the water is fit for various water uses. The potential effects of the water that is considered not fit for various uses will also be discussed. Enumeration numbers and identified yeasts, their antimicrobial activity as well as their resistance and potential implications on human health are also discussed.

5.2 Physico-chemical parameters

5.2.1 Temperature

Water temperatures change with geographical features of the region, catchment area, seasonal changes and anthropogenic activities (DWAF, 1996d). In the present study, water temperature measured in winter were low (9.9 to 14.7°C) and in summer higher (16.6 to 23.5° C). The water temperature ranges reflected the sampling periods. This was also seen in a study conducted by Jordaan and Bezuidenhout (2012) in the Vaal River where different sampling times were defined by different temperatures. In that study low temperatures were measured in winter (10 to 13°C) and higher temperatures were measured in summer (24.4 to 28.7°C). In both studies (the present one and the one by Jordaan and Bezuidenhout, 2012), the measured temperatures were within the surface water temperature ranges 0°C to 30°C (WHO, 1996). On the other hand, measured temperatures corresponded to the inland aquatic water temperatures in South Africa which generally ranges between 5 and 30°C (DWAF, 1996c).

Change in temperature can have an impact on microbiological parameters (O Reilly, 2012). RDA analysis has indicated that the prevalence of yeast species, *C. albicans* in the Mooi River has been influenced by the temperature. *C. albicans* was isolated in the Mooi River in summer mostly from Viljoenroad Bridge the surface water temperature was measured at 18°C. Micro-organisms usually have a range of temperatures at which optimal growth, reproduction and general fitness could occur. Changing temperatures could also expose aquatic organisms to potentially deadly conditions (Dallas and Day, 2004). These are normally extreme and were not the case in the present study. However, the lower

temperature levels in the present study could have had limiting impact on levels of yeast enumerated.

5.2.2 pH

The pH determines the suitability of the the water for various purposes as it is implicated in toxicity to aquatic plants and animals (Venkatesharaju *et al*., 2010). Mooi River and Harts River sampling sites had slightly alkaline pH with values ranging from 7.40 to 8.64 pH units (Table 4.1). Mooi River sites (Wonderfontein before Carletonville, Klerkskraal, Muiskraal) and Harts River (Sannieshof) had their pH above the TWQR for irrigation use levels (6.5 to 8.4) (DWAF, 1996a; DWAF, 1996b). This may not have immediate negative effects but over a long time, irrigation equipment as well as plant nutrients could be affected if the water above the TWQR is used (DWAF, 1996b). In a study conducted by Jordan and Bezuidenhout (2012) in the Vaal River, pH measured between 7.40 and 8.36. These levels were within the TWQR for several water uses. However, in a study by Van Wyk *et al*. (2012) pH levels measured in the North West Province were between 7.2 and 9.3. Sampling sites from Barberspan and one site from Lower Vaal River exceeded TWQR pH level for irrigation use.

Negative effects of pH on the health of aquatic species are well known. Low pH have been directly implicated in mortality of insects and amphibians (Loperfido, 2014). One of the factors that contribute to low pH is acid mine drainage. The presence of uranium from acid mine drainage has been reported in the North West Province at Wonderfonteinspruit and Tweelopiespruit, which are adjacent to the mines (Siphuma, 2012). Surrounding farming communities use both ground- and surface water for drinking purpose, livestock watering and irrigation. Plants absorb these metals and are passed onto the rest of the food network and this could result in health related illness and death (Van Eeden *et al*., 2009).

Many fresh waters have more or less neutral pH range between 6.0-8.0 (Dallas and Day, 2004). South African pH Target Water Quality range is 6.0-9.0. Consumption of water within this range will have no significant impact on health. Consumption of water levels of pH 11.0 and above will impose a severe danger to health (DWAF, 1996b). In the present study the water was of neutral o slight alkaline pH. This is potentially due to the dolomitic origin of water and the higher levels of calcium and magnesium salt in the water.

5.2.3 Total Dissolved Solids

Elevated levels of TDS were measured in Mooi River and Harts River sampling sites. This could be caused by agricultural practices such as farming and livestock which takes place

adjacent to the rivers. Leaching of contaminants and sewage discharge from waste water treatment plant could be another contributing factor (Moniruzzaman *et al*., 2009). According to NWDACE (2008), North West Province is one of the regions where high levels TDS exist. This results from natural causes and human impacts. Geology of the North West Province consists of yellow shifting sands. This could be the natural cause of elevated TDS in the province (NWP-SoER, 2002). High TDS in the province could be confirmed by the study conducted by Van Wyk *et al*. (2012) in water source of the North West Province. TDS measured ranged between 250 to 950 mg/L. All the sites sampled from Harts River, Barbespan, four from Lower Vaal River and three from Schroonspriut measured elevated TDS.

TDS concentration of a water body is directly proportional to its electrical conductivity provided the water temperature is at 25°C (DWAF, 1996b). Elevated TDS indicate high ion concentration. The permissible TWQR for TDS for irrigation is <40 ppm. All the sampling sites in this study were above the permissible TWQR TDS for irrigation. In a study by Molale (2012) sites in the Mooi River (excluding Klekskraal), Harts River, Barberspan, Schroonspruit and Vaal River exceeded the permissible TDS TWQR range for irrigation. Irrigation is the biggest water user in the North West Province (Van Der Walt, 2002). High TDS may have profound negative impact on production on the irrigation farms along the Mooi and Harts River.

5.2.4 Nitrates

DWAF 1996b refers to nitrogen as all inorganic forms of nitrogen present in water including nitrates. The irrigation TWQR of nitrates is <0.5 mg/L (DWAF, 1996). In the Harts River, most of the sampling were within the TWQR except Lichtenberg site before the wastewater treatment plant which measured 0.8 mg/L (Table 4.3). RDA analysis has indicated a positive correlation between yeast levels at Lichtenberg site before waste water treatment plant and nitrate levels. All the Mooi River sampling sites exceed the irrigation TWQR. A study conducted by Van Wyk *et al*. (2012) in the North West water resources measure nitrates values between 0 and 19 mg/L. Molale (2012) measured nitrate levels in the North West Province Rivers that were between 0 and 2 mg/L.

The elevated nitrates could be the result of surface run-off from the farms and storm water run-off during rains (Razak *et al*., 2009). Quality standards for wastewater or effluent arising in the catchment area draining water to any river have a nitrates range of <1.5mg/L (South Africa, 1984). Some of the sampling sites in both Mooi River and Harts Rivers measured

nitrates that exceeded wastewater quality standard range. Elevated nitrates in the rivers could also be the result of waste water from sewage treatment plants (Razak *et al*., 2009).

5.2.5 Phosphates

High concentrations of phosphates occur in waters that receive poorly treated sewage, leaching of human and animal waste, crop residues and run-off from cultivated lands (Dallas and Day, 2004). In the present study phosphates were present in sampled water and ranged from 0 to 7.14 mg/L in the Mooi River and Harts River. The highest phosphate levels were recorded at the sampling sites downstream from the Potchefstroom waste water treatment plant (Mooi River) and Biesiesvlei (Harts River). RDA plots indicated a positive correlation between yeasts levels at both sites and phosphate levels. The Potchefstroom waste water treatment plant could potentially be contributing to the elevated levels of phosphate downstream from it.

Wastewater or effluent arising in the catchment area within which water is drained to any river should have phosphate levels of <1.0 mg/L. In the present study, most of the sampling sites except Lichtenburg site located before waste water treatment plant and Delareyville exceeded the wastewater effluent range. The high levels could be results of waste water effluent from nearby town into adjacent river systems (Moniruzzaman *et al*., 2009). Phosphate levels measured in studies by Van Wyk *et al.* (2012) and Molale (2012) ranged between 0 and 2.97 mg/L in the sampling periods 2010 and 2011.

Extensive agricultural practices exist along the Harts River (NWP-SoER, 2002). Several fertilizers are in used in modern agricultural practices. They contain high levels of plant nutrients, particularly nitrogen and phosphorus, which enhance crop production (Walmsley, 2000). Elevated levels of phosphates in the surface waters could be ascribed to agricultural run-off from the farms. Increased phosphate and nitrate pollution into surface water support growth of cyanobacteria. This results in eutrophic water systems (Dallas and Day, 2004). Water quality deteriorates and symptomatic changes in the water are undesirable and interfere with water use (OECD, 1982).

5.2.6 Chemical Oxygen Demand (COD)

Chemical oxygen demand is the amount of oxygen in the form of a strong oxidizing agent consumed when organic matter is oxidized (Noguerol-Arias *et al*., 2012). Industrial effluents, agricultural run-off and domestic wastes are the major sources in water resources (DWAF, 1996b). In the present study, COD were measure in the Harts River and they ranged between 31 and 43 mg/L. In study conducted by O'Reilly (2012) the physico-chemical- and

microbiological quality of raw and household water in the Vaal-harts irrigation scheme were assessed. COD levels measured between 0 and 28.5 mg/L.

Waste water effluent arising in the catchment area within which water is drained to any river could have COD levels of <75.0 mg/L as acceptable. In the present study, the levels were not within the TWQR for domestic use but within acceptable limits from wastewater effluents ranges. The oxidation of organic matter and inorganic wastes in receiving water depletes the dissolved oxygen supply. This can have profound effects on aquatic life especially yeasts (Water quality criteria, 1968).

5.3 Yeast levels

In 2013, in the Mooi River, yeasts were isolated from the various sampling sites (Table 4.2). There were no colonies counted at some of the sampling sites. Some plates were overgrown by the fungi and this made it impossible to count the colonies. Harts River samples all had yeast colony growth without fungal growth. Physical parameters (temperature, pH), chemical parameters (nitrates and phosphates) as well as drainage of the river and human activity could influence the prevalence and diversity of yeast in water (Hagler and Ahearn, 1987; Lachance and Starmer, 1998).

Yeasts were enumerated at room temperature and 37°C. Counts at room temperature were conducted to get the general level of yeast at a particular site. Several human-associated yeasts have been isolated from fresh water environments (Hagler and Ahearn, 1987; Nagahama, 2006). Incubation at 37°C was done to isolate yeast isolates that may be pathogenic and human associated as the particular temperature could be considered a pre-requisite phenotype for invasive mycoses to produce disease within a mammalian host (Perfect, 2006).

In a study conducted in the North West Province water sources by Van Wyk *et al*. (2012), pigmented and non-pigmented yeast colonies were isolated and the latter detected in high numbers. In that study non-pigmented yeast reached up to 28280 cfu/L and pigmented up to 3760 cfu/L. In the present study, only non-pigmented yeasts were isolated reaching up to 4200cfu/L at room temperature and 2573 cfu/L at 37°C. Physico-chemical parameters can have relevance to yeast levels in water (Bezuidenhout, 2012). In study by Van Wyk *et al*. (2012) high numbers of non-pigmented yeasts were attributed to elevated water temperature. In the present study, elevated nitrates and phosphates levels resulted in elevated yeast levels at some sampling sites such as Wonderfonteinspruit site after

Carletonville, downstream Potchefstroom waste water treatment plant and Lichtenburg site located before waste water treatment plant. RDA plots have indicated a positive correlation between nitrates and phosphates with the above mentioned sampling sites.

Microbiological pollution of water is one of the problems that affect the water quality (DWAF, 2009). Bacteria such as faecal coliforms, enterococci and *E. coli* and heterotrophic plate count (HPC), bacteriophages and viruses are microbiological quality determinants in faecal contamination of water sources. Simard (1971) has also emphasized that levels of yeasts in water could be an indication of heavy or minimal pollution in water. In clean seawater, yeast counts could range from few to several hundred per liter (Hagler and Mendonca-Hagler, 1981). In the presence of pollution, the total yeast usually increases, thus reaching up to a few thousand cells per liter or more (Hagler & Mendonca-Hagler, 1981). From the study conducted by Hagler and Medonca (1981) it could be stated that the sites with high yeast counts could be an indication of pollution at those particular sites.

The Lichtenburg site located before waste water treatment plant in the Harts River is located just outside of the town and the site after the waste water treatment plant is inside a village. Villagers use the river water for livestock watering, recreation and to wash their clothes. This is a major concern as the river along the sampling has such high yeast counts indicating that the site could be highly polluted (Simard, 1971). The Wonderfonteinspruit site after Carletonville is situated just after the urban area of Carletonville town. The high pollution, which is indicated by high colony counts, could be due to urbanization. Surface run-off and the sewage effluent from the urban part of the town could end up in adjacent river system. Viljoenroad Bridge also had very high colony counts. This could be attributed to agricultural practices such as livestock farming that happens along the Mooi River approaching the sampling site (Van Wyk *et al.*, 2012).

5.4 DBB and extracellular enzyme test

The yeast isolates that were enumerated and identified were all non-pigmented and gave a DBB test confirming that they were ascomycetous yeasts (Hagler and Ahearn, 1987). In the same study conducted by Hagler and Ahearn (1987) ascomycetuos yeasts were tested for production of the extracellular enzymes; lipase, proteinase and dnase. Previous studies have shown that basidiomycetous yeasts are common extracellular enzyme producers (Hopsu- Havu *et al.,* 1978; Sen & Komagata, 1979). Van der Walt *et al.* (1974) made some observations that some ascomycetes also produce extracellular enzymes. Hagler and Ahearn (1987) supported the observation by Van der Walt (1974) that found that

ascomycetous yeasts, *Sacchromycopsis* spp. produced extracellular enzymes. However, in the present study all the ascomycetous yeast did not produce any extracellular enzymes. The study by Hagler & Ahern (1987) also found that the same environmental yeasts *viz*. *C. albicans*. *C. glabrata*, *C. tropicalis*, *C. parapsilosis, C. krusei* and *S. cerevisiae* did not produce extracellular enzymes. However, previous studies have shown that clinical isolates of the same species have produced extracellular enzymes (Aleva *et al*., 2007, Karkowska-Kuleta *et al*., 2009). The present study dealt with environmental isolates and it thus could be that the environmental isolates from the present study are also non-producers of extracellular enzymes.

5.4 Molecular identification and distribution of yeasts

Yeast isolates were identified using 26S rRNA sequencing. The following yeast species were identified; *Candida glabrata*, *Candida albicans*, *Candida pseudolambica*, *Candida tropicalis*, *Candida bracarensis*, *Candida parapsilosis*, *Saccharomyces cerevisiae*, *Pichia salicaria*, *Pichia guilliermondii*, *Pichia mexicana*, *Pichia kudriavzevii, Wickerhamomyces anomalus*, *Arxiozyma telluris*, *Cyberlindnera fabianii*, *Clavispora lusitaniae*. These isolated yeast species were unevenly distributed along the Mooi River and Harts River sampling sites. The uneven distribution of yeasts could be due to selection of colonies. Colonies were selected by morphological features of the colonies (white, cream white, shiny or non-shiny). In a study conducted by Nagahama (2006) in aquatic environments similar species as those that were isolated in the present study were described.

The genus *Candida* represented the majority of the isolates with great variation of species. Medeiros *et al*. (2008), that conducted studies on yeast from freshwater environments in South-eastern Brazil isolated and identified similar species as was observed in the present study. *Candida* yeasts in the environment have been associated with presence of human waste (Woolett and Hedrick, 1970). It is thus reasonable to attribute the occurrence in of *Candida* in the water samples of the present study to human waste.

More than 100 yeast species that have been identified as human pathogens had been isolated from water (Fromtling *et al.,* 2003*).* Hurley *et al*. (1987) listed the following pathogenic yeast species that cause candidiasis in their probable descending order of virulence: *C. albicans*, *C. tropicalis*, *C. stellatoidea*, *C. glabrata*, *C. krusei*, *C. parapsilosis*, *C. guilliermondii*, *C. viswanathi, Clavispora lusitaniae and Rhodotorula mucilaginosa.* Candidosis or candidiasis is an infections caused by *Candida* species (Saranya *et al.*, 2014). However, in a study conducted by ARTEMIS Global Antifungal Surveillance program, *C.*

albicans was the common (63-70%) candidal cause of invasive fungal infections, followed by *C. glabrata* (44%), *C. tropicalis* (6%), and *C. parapsilosis* (5%) (Pfaller *et al*., 1997). Most of the pathogenic yeast species on the Hurley *et al*. (1987) list and on the ARTEMIS Global Antifungal Surveillance program were isolated and identified in the present study.

Candida albicans is the major fungal pathogen. It was isolated only from the Mooi River, from all sampling sites except Wonderfonteinspruit site after Carletonville. Its infections are a serious problem to immune compromised individuals (Hurley *et al*., 1987; Karkwoska-Kuleta *et al.*, 2009). *C. albicans* can colonize skin and mucosal surfaces often causing superficial infections (Mayer *et al*., 2013). The presence of *C. albicans* in almost all the sampling sites is a great concern because at some sampling sites along the river, inhabitants use the water for washing and religious purposes such as full body baptism.

Candida tropicalis is the second most common potentially pathogenic yeast that can cause candidiasis (Hurley *et al*., 1987). It is also the leading cause of fungemia in patients with malignancy (Kontoyiannis *et al*., 2001). This species had been isolated from the Mooi River (Wonderfonteinspruit site before Carletonville and Trimpark Bridge) and Harts River (Biesiesvlei and Sannieshof). It had been isolated before in the North West Province in the Mooi River at Klerkskraal Dam (Van Wyk *et al*., 2012). According to Hurley *et al*., 1987), the third most common potential pathogenic yeast is *C. stellatoidea*. This yeast species was not isolated in the present study. *Candida glabrata* is the fourth most common yeast species known for its pathogenicity (Hurley *et al*., 1987). It was isolated from the Mooi River (Wonderfonteinspruit site before and after Carletonville, Viljoenroad Bridge and Taaiboschbult Bridge) and Harts River (Delareyville).

Pichia kudriavzevii (also known as *Candida krusei*) was isolated from one sampling site in the Mooi River (Thabo Mbeki Bridge) and Harts River (Vermaas). In a study conducted by Van Wyk *et al*. (2012*), P. kudriavzevii* was isolated in the North West Province from one site in the Vaal River (Christiana) and one site from the Harts River (Jan Kempdorp). It is the fifth most common cause of candidiasis (Hurley *et al*., 1987). *C. parapsilosis* is another pathogenic yeast species identified in this study. It was isolated from one sampling site in the Mooi River at Viljoenroad Bridge. This particular yeast is the sixth common cause of candidiasis and is responsible for pericarditis and urinary tract infections in immune compromised people in hospitals (Hurley *et al*., 1987; Diekema *et al*., 1997).

According to Hurley *et al*. (1987), the seventh most common species that causes candidiasis is *C. guilliermondii* (also known as *Meyerozyma guilliiermondii*). This yeast species was

isolated from the Mooi River (Trimpark Bridge) and Harts Rivers (Lichtenburg site located after waste water treatment plant). Van Wyk *et al*. (2012) isolated the yeast species from sites in the Lower Vaal River (Windsorton) and Barberspan in the Harts River. *Clavispora lusitaniae* (also known as *Candida lusitaniae)* is also one of the pathogenic yeasts identified from the present study. It is the ninth common cause of candidiasis according to the Hurley *et al*. (1987) list. It was isolated from Mooi River (Wonderfontein before Carletonville) and Harts River (Lichtenburg site located after waste water treatment plant, Biesiesvlei and Vermaas). *C. lusitaneae* had been isolated from the North West Province in the Mooi River (Muiskraal) and Schoonspruit River (Klerksdorp Bridge and Brakspruit) (Van Wyk *et al*., 2012).

Candida bracarensis was isolated from the Mooi River (Viljoenroad Bridge). It is described as a *Candida* species, which is phenotypically similar to *Candida glabrata*. *C. bracarensis* has caused a bloodstream infection in a bone marrow transplant patient (Warren *et al*., 2010).This confirms the organism as an opportunistic human pathogen. *Candida pseudolambica* has been isolated from the Mooi River (Viljoenroad Bridge and Taaiboschbult Bridge). In a study by Van Wyk *et al*. (2012) this species was isolated from the Lower Vaal (Schmidstdrif, Christiana and Bloemhof) and Schoonspruit (Brakspruit and Bodenstein).

Saccharomyces cerevisiae is a yeast species commonly known as baker's yeasts or brewer's yeast (Van Wyk *et al*., 2012). It was isolated in large numbers from the Mooi River (Wonderfonteinspruit before Carletonville, Trimpark Bridge and Viljoenroad Bridge) and in almost all the sampling sites in Harts River). It has been isolated before in the North West Province at Sannieshof (Barberspan) (Van Wyk *et al*., 2012). *S. cerevisiae* is one of the yeast species emerging as a key opportunistic pathogen (Miceli *et al*., 2011; Munoz *et al.*, 2005). It has been implicated as a causal agent for fungaemia (Miceli *et al*., 2011). *S. cerevisiae* was found in both the selected rivers in high numbers. This is a health concern as both rivers are used for agricultural activities mostly irrigation and livestock watering. These rivers are also used for recreation.

Wickerhamomyces anamola was isolated from the Mooi River at two sampling sites, Wonderfonteinspruit before Carletonville and Thabo Mbeki Bridge. The yeast species is also known as *Pichia anamola*, or *Hansenula anamola* or *Candida pellucosa* (Van Wyk *et al*., 2012). In a study conducted by Van Wyk *et al*. (2012), it was isolated in the North West Province in the Mooi River (Klerksraal Dam and Muiskraal Bridge) and one site from Barberspan (Hotel). *W. anamola* is rarely found as a cause of human infections. However, a

case of *W. anomalus* fungal keratitis was reported in compromised eyes after corneal surgery such as corneal transplantation (Kamoshita *et al*., 2014). *Cyberlindnera fabianii* (also known as *Hansenula fabianii* or *Pichia fabianii*) has been isolated in the Mooi River (Muiskraal and Viljoenroad Bridge). The yeast species has caused blood infections in premature infants, neonates and adults. This indicates that this uncommon yeast species is a potential health hazard (Freel *et al.*, 2014).

Lecythophora species are filamentous fungi (Domsch *et al.,* 1980). They are saprobes but some are involved in human diseases (Perdomo *et al*., 2011). *Lecythophora hoffmanii* has demonstrated its ability to cause infections in human. It has induced chronic sinusitis in AIDS patients (Marriot *et al*., 1997). *Lecythophora mutabilis* has been described as a cause of peritonitis in human (Ahmad *et al*., 1995). *Lecythophora* species were isolated from the Harts River in Biesiesvlei, Vermaas and Delareyville sites.

Some of the isolates from the current study are not known to cause any disease in human beings*. Axiozyma telluris* also known as *Saccharomyces telluris* was isolated from Thabo Mbeki Bridge in the Mooi River. *A. telluris* has not been reported to be a human pathogen (Deegenaars and Watson, 1998). Two *Pichia* species were isolated in the study. *Pichia mexicana* also known as *Yamadazyma mexicana* was isolated from Mooi River (Trimpark Bridge) and Harts River (Sannieshof and Delareyville). *Pichia salicaria* was isolated in the Mooi River at Trimpark Bridge. Literature does not implicate the two *Pichia* species as pathogens. However, these and other non-pathogenic yeast species may play important roles in geochemical processes. This is, however, an aspect that needs to be further investigated.

5.5 Antifungal susceptibility tests

An alarming increase in the number of life threatening yeast infections has brought about increased use of antifungal agents (Sanglard *et al*., 1998). An antifungal agent is a drug that selectively removes fungal (or yeast) pathogens from a host with least harm to the host (Walsh and Dixon, 1996). However, prophylactic and continuous exposure of antifungal agents to yeasts has resulted in antifungal resistance from infectious yeasts (Karkowska-Kuleta *et al*., 2009). This is a worrying factor especially in immune compromised people as they are a high risk to yeast infections (Pincus *et al*., 2007).

Some of the yeasts species isolated from the surface water of the North West Province are capable of causing infections in immune compromised patients (Rentz *et al*., 1998; Van Wyk

et al., 2012). Yeast Infections such as thrush, athlete's food, ring-worm, candidiasis, jock itch are common amongst immune compromised patients. UNAIDS (2012) has reported that in South Africa in 2012 an estimate of 6.1 million people were living with HIV. This is up from 5.2 million in 2005.

In the present study, yeast isolates from the surface water, Mooi River and Harts River were tested for their antifungal susceptibly tests. Many of these species were resistant several of the antifungal agents especially from azole group. This group is the largest and mostly used class of antifungals (Fromtling, 1988). The Triazole fluconazole is the most widely used drug to treat *Candida* infections, due to its favourable bioavailability and safety profile (Morschhäuser, 2002). The wide spread of fluconazole has led to decreased susceptibility of *Candida* spp. to not only fluconazole but other antifungals within the azole class. In the treatment and prophylaxis of oro-oesophageal candidiasis in the early 1990s, fluconazole became the antifungal of choice. However, in the years following this wide spread use of the drug, fluconazole resistance was reported in 41% of the patients (Canuto and Rodero, 2002).

Fluconazole is not completely metabolised in the human body. There may thus be considerable amounts of this antifungal agent that lands up in the domestic wastewater streams (Kim *et al*., 2007). If wastewater treatment plants do not work effectively, as is the case in the North West Province (DWAF, 2012) then the opportunity arise that sub-therapeutic levels of fluconazole may flow into surface waters receiving the poorly (un) treated effluent. This creates a selective pressure for resistance to fluconazole (and other azoles) and could become an environmental hazard.

The antifungal test results from the present study show that *Candida* species, *C. albicans*, *C. glabrata*, *C. tropicalis* isolated from several sampling sites of the Mooi and Harts Rivers were resistant to all the antifungals except nystatin. In a study conducted by Abrantes *et al*. (2013) in South Africa and Cameroon, clinical isolates of the same *Candida* species showed the same resistance patterns to azoles as observed in the present study. In the former study, the clinical isolates were from AIDS patients and the South African patients were mainly from the Western Cape Province. Such data could not be obtained for clinical isolates from the North West Province. There is thus a need to determine what the antifungal susceptibility profile among clinical isolates in the North West Province area, and whether there are similar antifungal susceptibility patterns among these clinical and environmental isolates.

Pichia Kudraivzevii also known as *Candida krusei* that was isolated in the present study was resistant to fluconazole and ketoconazole. This resistance pattern was also seen in a study by Medeiros *et al*. (2008) where two isolates from river samples were resistant to all the antifungals tested including fluconazole and ketoconazole. *Candida krusei* is considered to be inherently resistant to fluconazole (Orozco *et al*., 1998). Zone diameter interpretative standards and corresponding minimal concentrations for *Candida* spp. do not include for *Candida krusei* (*Pichia Kudraivzevii*) as it assumed to be intrinsically resistant to fluconazole (CLSI, 2008). It was thus not surprising to observe that *Candida krusei* isolates in the present study were resistant to azoles. However, resistance to flucytosine and reduced susceptibility to nystatin were also observed among the isolates in the present study.

In the present study, yeasts species; *Clavispora lusitaniae*, *Wickerhamomyces anomalus* and *Candida parapsilosis* isolated from the two rivers were susceptible to fluconazole, econazole, ketoconazole, and miconazole. In a study conducted by Medeiros *et al*. (2008), *Candida parapsilosis* isolated from the lake was generally susceptible to fluconazole and ketoconazole. The study conducted by Pfaller *et al*. (2003) also showed clinical isolates *C. parapsilosis* susceptible to fluconazole. These results are similar to the findings in the present study. Two isolates of *Candida guilliermondii* were obtained from one sampling site in the Harts River were showed intermediate sensitivity to ketoconazole.

Resistance in azoles has been associated with mutations in the target genes (cytochrome P450 lanosterol 14α- demethylase), which results in lower affinity of the azole to the enzyme (Canuto and Rodero, 2002). Another mechanism includes the upregulation of the expression enzyme level. Azole antifungal agents (fluconazole, ketoconazole, and itraconzole) target ergosterol synthesis in which the mentioned enzyme plays an important role (Saranya *et al*., 2014). Mechanism of changed affinity of azole derivatives has been seen in yeast species such *C. albicans* and *C. neoformans* (Sanglard *et al*., 1998). Azole antifungals also fail to accumulate inside the yeast cells as a result of enhanced drug efflux of azoles from cells (Perea *et al*., 2001). This form of resistance has been identified in species as *C. albicans, C. glabrata*, *C. krusei* and *C. neoformans* (Parkinson *et al*., 1995; Sanglard *et al*., 1995; Venkateswarlu *et al*., 1997).

Polyenes such as amphotericin B and nystatin also target ergosterol (membrane function) (Vanden Bossche, 1997; Saranya *et al*., 2014). Their resistance could be associated with reduced total ergosterol content of the cell, replacement of some or all of the polyene-binding sterols as well as reorientation of present ergosterol (Hamilton-Miller, 1973). These mechanisms might be responsible for the antifungal resistance patterns observed in the

present study. However, this was not tested but future studies should be conducted to determine antifungal resistance mechanisms.

Presence of resistant yeasts to common antifungals such as azoles and polyenes in the aquatic environment is a concern. North West Province people who use Mooi and Harts River water for irrigation, recreational and other uses are at a risk. They may contract diseases associated with some of these species and infections may be difficult to treat. This is a cause for concern and warrants further research.

CHAPTER 6 - CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The aim of this study was to determine the diversity and antifungal susceptibility of yeasts in selected rivers in the North West Province of South Africa. Two rivers were selected, namely Mooi River and Harts River. To achieve this aim, four objectives were formulated. Chapters four and five presented the results and discussions, respectively. The findings from the objectives are briefly discussed below.

6.1.1 Physico-chemical parameters

Water quality of the two rivers was determined using the physico-chemical parameters. These were compared to target water quality ranges of various water uses (DWAF, 1996a) and quality standards for waste water or effluent (South Africa, 1984). Results were compared with target water quality ranges to establish if the sampled water source was suitable for use for livestock watering and irrigation. Quality standards for wastewater or effluent assisted in determining if the waste water effluent could be the source of water contamination.

Some sampling sites had physico-chemical parameters that exceeded the target water quality ranges for irrigation. For livestock watering, the parameters were within the target water quality ranges for all the sampling sites. However, some sampling sites had values for certain parameters that exceeded the quality standards for wastewater effluent. Elevated TDS, nitrates and phosphates were measured in those sampling sites from both rivers. From the elevated parameters, we could establish that pollution in the selected rivers was probably due to agricultural runoff and sewage effluent. The use of this surface water poses a potential health risk for livestock and irrigation of freshly eaten produce.

6.1.2 Yeast levels

Yeast malt extract (YM) agar and membrane filtration (0.45 µm filters) were used to successfully isolate yeast colonies from different sampling sites. Incubation was done at room temperature and 37°C. Vijoenroad Bridge in the Mooi River and Lichtenburg site located before waste water treatment plant in Harts River had the highest colony counts. Faecal coliform, enterococci and *E. coli* and heterotrophic plate counts are usually employed to determine microbial pollution in water (Adeleke and Bezuidenhout, 2011). However, yeasts counts could also be used. High yeast numbers could mean that the sampling sites received a higher level of contaminated water. The water could be contaminated from

human activity such agricultural, urban and industrial activity in the province. Yeast levels at sites could statistically be correlated to physico-chemical parameters. This is an important finding for two of the rivers in the North West Province.

6.1.3 Yeasts isolation at 37°C

Yeast isolates that grew at 37°C were isolated, identified and characterised. These were subcultured to obtain pure colonies for further analysis. Literature indicates that yeasts isolates that can grow at 37°C may survive well at the human body temperature and induce pathogenicity within a host, particularly if the individual is immune compromised. Production of extracellular enzymes is one the tests that determine pathogenicity (Karkowska-Kuleta, 2009). Yeast isolates were tested for their production of haemolysin and extracellular enzymes; lipase, dnase and proteinase. Yeast isolates tested negative for extracellular enzymes and haemolysin production. This could be due to the fact that these were all environmental isolates and thus non-pathogenic.

6.1.4 Identification of yeast isolates using biochemical tests and 26S rRNA gene sequencing

Diazonium Blue B was used to distinguish between ascomycetes and basiodiomycetes on the isolates (Hagler and Ahearn, 1987). All the yeast isolates in the study were ascomycetes. The 26S rRNA gene sequencing was conducted to identify isolated yeasts up to species level. The following isolates were identified: *Saccharomyces cerevisiae, Candida glabrata*, *Candida albicans*, *Candida pseudolambica*, *Candida tropicalis*, *Candida bracarensis*, *Candida parapsilosis*, *Pichia salicaria*, *Pichia guilliermondii*, *Pichia mexicana*, *Pichia kudriavzevii, Wickerhamomyces anomalus*, *Arxiozyma telluris*, *Cyberlindnera fabianii*, *Clavispora lusitaneae* and *Lecythophora*.

Most of the isolated species have previously been isolated from surface water. *Saccharomyces cerevisiae* was the dominant isolated species followed by *Candida glabrata* and *Candida albicans*. Some of the isolated yeast species are associated with pathogenicity in a clinical environment. They cause diseases and infections especially in immune compromised people. These potentially pathogenic yeasts may thus be a potential risk to people who use the water resources particularly for direct contact recreational purposes.

Sequences of identified yeast species were submitted to Genbank and accession numbers assigned to them. Phylogenetic analysis was done on sequences from the isolated yeast species and yeast species downloaded from the Genbank. A high bootstrap supports the

high phylogenetic similarity between the isolates. There was a similar high bootstrap support for all the sequences between the species and the species downloaded from the Genbank.

6.1.5 Antifungal susceptibility tests

Antifungal susceptibility tests were performed on isolated yeast species. *Candida* species are mostly implicated in causing yeast infections in people especially immune compromised people. Most of *Candida* species were resistant to all the antifungals including fluconazole, which is the most widely used drug to treat *Candida* infections. Resistance to antifungals could be due to prophylactic usage and continuous exposure of drugs to antifungal agents. Literature has demonstrated that the mechanism of resistance to azoles could be due to enhanced drug efflux pumps (Perea *et al*., 2001). This mechanism has been seen in other antifungal classes as well. It has been seen in yeast species such C*. albicans* and *C. neoformans* (Sanglard *et al*., 1998). The mechanism of resistance in this study could be the same but it remains to be determined.

6.2 Recommendations

1. Yeasts have been isolated from the aquatic environments but little attention and focus to their presence and significance (Arvanitidoua *et al*., 2002). Some studies have been done on yeasts in the North West Province surface water (Van Wyk *et al*., 2012). Further studies in aquatic systems in the North West Province are required to increase the database of yeast, the diversity, distribution and characteristics.

2. Sampling from the selected rivers should be done to reflect seasonal dynamics. This will enable verification of seasonal variations in physico-chemical parameters and yeast levels and the correlations between these. A similar approach to what has been demonstrated in the present study could be used in such a study.

3. In the North West province, surface water and groundwater are integrated and interdependent. Water quality and quantity that affects the underground water have effects on the surface water (NWDACE, 2008). Further studies in the North West Province should include the groundwater quality and how this impact on yeast levels.

4. In the present study, COD was measured for one river. In the future COD and BOD as the indicators of organic pollution should be measured for all the sampling sites. Furthermore, other water quality parameters such as nitrites and sulphates should also be included.

5. From a study that was conducted by Van Wyk *et al*. (2012) some of the yeast species were considered to be pathogenic. In the present study, the same yeast species were isolated. A detailed study should be conducted on pathogenic yeasts found in water sources in the North West Province.

6. Production of extracellular enzymes is one of the prerequisite to cause infection by yeasts. In the present study, yeast species that are known to cause infections did not produce extracellular enzymes. Literature has indicated that in previous studies clinical isolates of the same species have produced the same enzymes tested for in this study. A study should be conducted to explain why environmental species do not produce enzymes even though they are the same pathogenic species. Environmental isolates may be carriers of genetic features that, under specific conditions, may become pathogenic. It is also particularly of concern that that the present study has demonstrated that most of the isolates were resistant to several classes of antifungal agents. Resistance mechanisms must be established and determined if this is linked to genetic features that are associated with extracellular enzyme production. These are all aspects that warrant further investigation.

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