Water quality and antifungal susceptibility of opportunistic yeast pathogens from rivers
M. E. Monapathi, C. C. Bezuidenhout and O. H. J. Rhode

ABSTRACT
Yeasts from water sources have been associated with diseases ranging from superficial mucosal infections to life threatening diseases. The aim of this study was to determine the water quality as well as diversity and antifungal susceptibility of yeasts from two rivers. Yeast levels and physico-chemical parameter data were analyzed by principal component analysis to determine correlations between physico-chemical data and yeast levels. Yeast morphotypes were identified by biochemical tests and 26S rRNA gene sequencing. Disk diffusion antifungal susceptibility tests were conducted. Physico-chemical parameters of the water were within target water quality range (TWQR) for livestock farming. For irrigational use, total dissolved solids and nitrates were not within the TWQR. Yeast levels ranged between 27 ± 10 and 2,573 ± 306 cfu/L. Only non-pigmented, ascomycetous yeasts were isolated. *Saccharomyces cerevisiae* and *Candida glabrata* were most frequently isolated. Several other opportunistic pathogens were also isolated. A large number of isolates were resistant to azoles, especially fluconazole, but also to other antifungal classes. *Candida* species were resistant to almost all the antifungal classes. These water sources are used for recreation and religious as well as for watering livestock and irrigation. Of particular concern is the direct contact of individuals with opportunistic yeast, especially the immune-compromised. Resistance of these yeast species to antifungal agents is a further health concern.

Key words | resistance patterns, 26S rRNA gene sequences, water pollution, yeast diversity, yeast infections

INTRODUCTION
Water scarcity in the North West Province (NWP), South Africa, is compounded by surface water pollution from the mines, agriculture, urban runoff (NWDACE 2008) and in many cases poorly-treated sewage return flows (Greendrop 2012). When assessing water quality, physico-chemical and bacteriological characteristics are determined. Using specific limits, potential uses of the water are determined (DWAF 1996). These water quality parameters are thus used to define the water system, to understand the relationship between parameters as well as to evaluate environmental sustainability (Dallas & Day 2004). Bacterial species such as *Escherichia coli*, total coliforms, faecal coliforms, faecal streptococci, and *enterococci* are widely used and accepted as indicator organisms in the testing of water quality. They are regarded as the best indicator for fecal contamination of water supplies, especially in open sources such as rivers (Pereira 2009; Adeleke & Bezuidenhout 2011). Some studies have shown that the presence of yeasts could also be used to determine surface water quality (Hagler & Mendonca-Hagler 1981; Arvanitidou et al. 2002; Van Wyk et al. 2012). Arvanitidou et al. (2002) demonstrated significant correlations between levels of faecal indicator bacteria and yeasts. The presence of yeast species in the genera *Candida*, *Cryptococcus*, *Pichia* and *Rhodotorula* could be regarded as bio-indicators of the level of pollution in water (Nagahama 2006).

In a study conducted by Van Wyk et al. (2012), several yeast species were isolated from surface water of the NWP. Known pathogenic species (*Candida guilliermondii, Candida lusitaniae, Candida tropicalis, Cryptococcus laurentii, Rhodotorula glutini* and *Rhodotorula mucilaginosa*; Hazen 1995; Nagahama 2006; Karkowska-Kuleta et al. 2009) were among the isolates identified (Van Wyk et al. 2012). These species could cause diseases ranging from...
superficial mucosal infections to life threatening ones in immune compromised people (Yamaguchi et al. 2007). Such findings are a concern, as these water sources are used for recreation and religious purposes as well as directly for agriculture (animal watering and irrigation; Van Wyk et al. 2012; Molale & Bezuidenhout 2015).

Although yeasts have been isolated from aquatic environments, little attention and focus have been given to their presence and significance (Arvanitidou et al. 2002). Approximately 10.2% of the total South African population is HIV positive (Statistic SA 2014), so it is important that the significance of opportunistic pathogenic yeast in aquatic systems receives greater attention. According to Shisana et al. (2014), HIV prevalence in the NWP is at approximately 13%. Antifungal agents such as fluconazole are provided as part of the prophylactic treatment for HIV to prevent debilitating infections by Candida and Cryptococcus spp. (Morschhäuser 2002; Abrantes et al. 2015). This implies that large quantities of this drug are used in the pharmaceutical/medical environment. Data from a study by Truter & Graz (2015) support this, and show that among the antifungals fluconazole and nystatin are the most commonly prescribed in South Africa.

What is also known about the antifungal drugs is that a large proportion of the dose is excreted in the urine and finds its way into sewage and into wastewater treatment plants (WWTP). It is known that when WWTPs work efficiently they only partially remove pharmaceutical products (Kummerer 2008). However, in the NWP, the majority of WWTPs are either (i) not working optimally, (ii) not properly managed, (iii) working beyond the systems’ design parameters or (iv) a combination of these (Greendrop report 2012). This suggests that poorly-treated sewage that may contain large amounts of antimicrobial agents such as antifungals decent into the already stressed aquatic system.

Another compounding impact is from the agricultural sector. Azoles are used to protect grain crops in particular against various fungal diseases of grains (Boyacioglu et al. 1992; Mateo et al. 2013). Runoff from these lands will also enter into the environmental water and further impact on the water system. The NWP is situated in the main maize producing area and also produces significant quantities of other grains (DWAF 2013). There are thus several simultaneous (sometimes intermittent) events/processes that lead to the leaking of subtherapeutic levels of antifungal agents into the environment. The impacts of these events on the diversity and the selection of antifungal resistant pathogens/opportunistic pathogens are undetermined, and studies to investigate this are necessary. In the clinical setting, prolonged and repeated treatment with antifungal drugs has led to pathogenic species developing resistance to these drugs, especially fluconazole (Sanglard et al. 1998; Mulu et al. 2013; Abrantes et al. 2015). There have also been reports of fluconazole resistance among environmental isolates (Pfaller et al. 2003; Medeiros et al. 2008).

The aim of the present study was to determine water quality, levels and diversity of yeasts, and the antifungal susceptibility of yeast isolates in two rivers of the NWP, South Africa.

**MATERIALS AND METHODS**

**Sampling area**

Sampling was conducted in 2013 in the Mooi River and Harts River in 2013 and 2014, respectively (Figure 1).

**Sampling and physico-chemical parameters**

Water samples were collected aseptically using the dip sampling technique described in Van Wyk et al. (2012). Water temperature, pH, total dissolved solids (TDS) and dissolved oxygen (DO; only Harts River) were measured on site using a multi 350 multi parameter probe (Merck, Germany). Water samples were transported to the laboratory on ice in dark cooler boxes, and analysis was conducted within 6 hours. In the laboratory, chemical oxygen demand (COD; only Harts River), nitrate (NO₃⁻), and phosphate (PO₄³⁻) were measured using the Hach Lange DR 2800 system and reagents (Hach Company 2007).

**Isolation and enumeration of yeasts**

The water samples were analyzed by membrane filtration to determine the presence of yeasts in water using the method described by Van Wyk et al. (2012). Enumeration was done at room temperature and at 37 °C. Yeast cultures that grew at 37 °C were purified by sub-culturing on YM agar (Wickerham 1951) and used for subsequent analysis.

**Identification of yeasts**

**Diazonium blue B**

Diazonium blue B (DBB) (Sigma-Aldrich, Germany) was used to distinguish between ascomycetous and...
basidiomycetous yeasts. Details of the method are given in Van Wyk et al. (2012).

**Molecular identification**

Ten millilitres of an overnight culture of the various isolates were used to extract the genomic DNA. The procedure was performed according to the modified method of Hoffman & Winston (1987). Cultures (2 mL) were centrifuged for one minute at 14,000 × g and the supernatant was removed by aspiration. Cells were suspended in 500 μL DNA lysis buffer (100 Mm Tris HCl at pH 8.0; 50 Mm EDTA; 1% SDS). Glass beads (200 μL) were added to the suspension, vortexed for 4 min and cooled immediately on ice. The suspension was centrifuged at 14,000 × g and the liquid phase was transferred to a sterile micro-centrifuge tube. A total of 275 μL ammonium acetate (pH 7.0) was added, vortexed for 5 minutes and incubated at 65°C for 5 minutes followed by immediate cooling on ice. To this suspension 500 μL of chloroform was added and mixed. The mixture was centrifuged for 2 minutes at 14,000 × g at 4°C. The top layer was transferred to a sterile micro-centrifuge tube and 750 μL of isopropanol added. This was incubated for 5 min at room temperature. DNA was purified using the Nucleospin Tissue kit (Macherey-Nagel, Germany). The protocol of this kit was followed from step 5 onwards. Wash steps, drying of the membrane and elution of the DNA were followed as indicated in the Nucleospin protocol. Eluted DNA samples were stored at 4°C until further analysis.

A NanoDrop TM 1000 spectrophotometer (Thermo Scientific, USA) (NanoDrop 2007) was used to determine DNA concentrations as well as 260/280 ratios to assess the purity of the DNA (NanoDrop 2007). Agarose gel electrophoresis was used to determine the integrity of the DNA as well as the sizes of the 26S rRNA PCR amplified gene fragments. The ±500 bp 26S rRNA gene fragment was amplified using the primers NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGGTITTCAAGACGG-3') (O'Donnell 1993). Reagents for amplification consisted of final 25 μL volume

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**Figure 1** | Map of the North West Province (NWP). The different sampling sites on the Mooi River and Harts River are indicated by green dots. The full colour version of this figure is available in the online version of this paper, at http://dx.doi.org/10.2166/wst.2016.580.
with (i) 2 μL genomic template DNA (±50 ng), (ii) 12.5 μL double strength PCR Master Mix (0.05 U/μl Taq polymerase, 4 mM MgCl₂ and 0.4 mM dNTPs; Fermentas Life Sciences, USA), (iii) 1.5 μL MgCl₂, (1.5 mM), (iv) 5.0 μL primer mix and (v) 4 μL nuclease free water (Fermentas Life Sciences, USA). 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. Positive controls included the PCR reaction (Candida rugosa CBS13; Clavispora lusitaniae CBS 6936).

The 26S rRNA sequencing was performed as described in Jordan & Bezuidenhout (2013). 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 and accession numbers are as follows: Mooi River (KM102986 to KM103064) and Harts River (KM885965 to KM885981 and KP070741 to KP070762).

**Phylogenetic analysis**

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

**Antifungal susceptibility tests**

For the antifungal susceptibility assays, spread plates of yeast cultures were prepared on YM agar plates, and the Kirby-Bauer disk diffusion method was used (Bauer et al. 1966). Discs impregnated with known concentrations of antifungal agents were obtained (Mast Diagnostics, UK). The following antifungal agents were used: fluconazole (FCN; 25 μg), econazole (ECN: 1 μg), ketoconazole (KCA: 15 μg), miconazole (MCL: 1 μg), metronidazole (MZ: 5 μg), fluocytosine (FY: 1 μg), nystatin (NY: 100 μg). Incubation was done at 37 °C. Diameters (in mm) of the growth inhibition zones were measured after 24 hours. These diameters were compared to the values in CLSI (2008) for Candida albicans.

**Statistics**

Data were analysed using Microsoft Excel (2011) and XL STAT (Addinsoft, Paris, France) software. The Pearson correlation coefficient was used to test the relationships between physico-chemical parameter measurements and yeast levels. Raw data were first standardised using Microsoft Excel. This was used for principal component analysis (PCA) to determine patterns and relationships in the data. Data output was provided as correlation biplots. The correlation significance level was set at $p < 0.05$. The standardised complete data sets for the two river systems were subjected to agglomerative clustering analysis using Euclidian distances and Wards method.

**RESULTS AND DISCUSSION**

**Physico-chemical parameters**

The measurements of the physico-chemical parameters and levels of the Mooi River and Harts River are presented in Tables 1 and 2, respectively. Physico-chemical parameters compared to target water quality range (TWQR; DWAF 1996) for agricultural uses (livestock watering and irrigation). pH measurements were generally within the TWQR limits for both uses; however, both TDS and nitrate levels in the Mooi River were higher than the recommended levels for irrigation (40 mg/L and 0.5 mg/L, respectively; DWAF 1996). In the Harts River, only the TDS was higher than the irrigation limit. The high TDS is not uncommon for the NWP, and both natural causes and human impacts are responsible for this phenomenon (NWDACE 2008). High TDS in the province was also observed in the study of Van Wyk et al. (2012). In that case, the levels measured ranged between 250 to 950 mg/L.

According to the limits for wastewater effluents that are allowed to be decanted into environmental waters, nitrates should not exceed $<1.5$ mg/L and phosphates should not exceed 1.0 mg/L (South Africa 1984). Many of the sampling sites in the Mooi River measured nitrates and phosphates that exceeded these wastewater quality standards ranges. In the Hart River, it was only the phosphates that exceeded the limit. Elevated nitrates and phosphates in the rivers could be the result of wastewater from sewage treatment plants, leaching of human and animal waste, crop residues and run-off from cultivated lands (Dallas & Day 2004; Razak et al. 2009). Both these rivers receive effluents from agricultural runoff as well as wastewater effluents, and this
could have resulted in the elevated nitrates and phosphates observed. COD levels, which were only measured in the Harts River, were within the levels acceptable for wastewater effluent (<75 mg/L; South Africa 1984). These levels ranged between 31.0 and 43.0 mg/L. DO levels in the Harts River ranged between 12.9 and 21.7 mg/L. These two parameters, linked to mesophilic temperature conditions, nitrate and phosphate levels, indicated that the river system provided an ecological habitat that could support the growth of heterotrophic organisms such as yeasts. The conditions that prevail in natural environments determine the metabolic activity, growth and survival of yeasts (Daeck 2006). Carbon sources such as polyols, alcohols, organic acids and amino acids are used by yeast species for growth (Rodrigues et al. 2006). Yeasts can utilize a wide range of nitrogen compounds as nitrogen sources. Nitrogen compounds can also be used as carbon sources (Messenguy et al. 2006). Most yeast are mesophilic, and grow best at temperatures between 20 and 30 °C. Growth at 37 °C is associated with pathogenicity. Yeasts prefer a slightly acidic medium, and have an optimum pH between 4.5 and 5.5. These are mostly aerobic organisms and thus prefer natural habitats with relatively high oxygen concentrations. DO in water may thus be a principal factor that

### Table 1 | Physico-chemical parameters and yeast counts in the Mooi River

<table>
<thead>
<tr>
<th>Sample area</th>
<th>Temp °C</th>
<th>pH</th>
<th>TDS mg/L</th>
<th>NO₃ mg/L</th>
<th>PO₄²⁻ mg/L</th>
<th>Yeast levels (cfu/L) enumerated at 37 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>TWQR – livestock watering</td>
<td>N/A</td>
<td>N/A</td>
<td>&lt;1,000</td>
<td>&lt;100</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>TWQR – irrigation</td>
<td>N/A</td>
<td>6.5–8.4</td>
<td>&lt;40</td>
<td>&lt;0.5</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Wonderfonteinspruit – before Carletonville</td>
<td>13.4</td>
<td>8.64</td>
<td>547</td>
<td>1.7</td>
<td>1.64</td>
<td>107 ± 61</td>
</tr>
<tr>
<td>Wonderfonteinspruit – after Carletonville</td>
<td>10.3</td>
<td>8.14</td>
<td>595</td>
<td>5.4</td>
<td>2.91</td>
<td>333 ± 101</td>
</tr>
<tr>
<td>Muiskraal</td>
<td>9.8</td>
<td>8.49</td>
<td>310</td>
<td>0.9</td>
<td>1.88</td>
<td>107 ± 61</td>
</tr>
<tr>
<td>Boskop – before Boskop dam</td>
<td>11.5</td>
<td>8.06</td>
<td>451</td>
<td>1.0</td>
<td>1.27</td>
<td>173 ± 101</td>
</tr>
<tr>
<td>Boskop – after Boskop dam</td>
<td>14.7</td>
<td>8.40</td>
<td>427</td>
<td>1.3</td>
<td>1.54</td>
<td>27 ± 23</td>
</tr>
<tr>
<td>Thabo Mbeki Bridge</td>
<td>17.9</td>
<td>7.40</td>
<td>462</td>
<td>1.2</td>
<td>1.22</td>
<td>187 ± 83</td>
</tr>
<tr>
<td>Trim Park Bridge</td>
<td>16.6</td>
<td>7.72</td>
<td>450</td>
<td>0.7</td>
<td>1.28</td>
<td>147 ± 61</td>
</tr>
<tr>
<td>Pedestrian Bridge</td>
<td>17.8</td>
<td>7.77</td>
<td>467</td>
<td>2.0</td>
<td>1.39</td>
<td>40 ± 0</td>
</tr>
<tr>
<td>Viljoenroad Bridge</td>
<td>18.1</td>
<td>7.75</td>
<td>463</td>
<td>1.6</td>
<td>3.08</td>
<td>2,573 ± 306</td>
</tr>
<tr>
<td>Taaiiboschult Bridge</td>
<td>17.2</td>
<td>7.52</td>
<td>579</td>
<td>2.2</td>
<td>5.29</td>
<td>160 ± 40</td>
</tr>
<tr>
<td>Skandinawiedrif Bridge</td>
<td>17.1</td>
<td>7.46</td>
<td>529</td>
<td>2.6</td>
<td>6.01</td>
<td>27 ± 46</td>
</tr>
</tbody>
</table>

Temp, temperature; TDS, total dissolved solids; NO₃-N, nitrates; PO₄²⁻, phosphates; cfu, colony forming unit; TWQR, target water quality range (DWAF 1996).

### Table 2 | Physico-chemical parameters and yeast counts in the Harts River

<table>
<thead>
<tr>
<th>Sample area</th>
<th>Temp °C</th>
<th>pH</th>
<th>TDS mg/L</th>
<th>NO₃ mg/L</th>
<th>PO₄²⁻ mg/L</th>
<th>DO mg/L</th>
<th>COD mg/L</th>
<th>Yeast levels (cfu/L) enumerated at 37 °C</th>
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<td>&lt;1,000</td>
<td>&lt;100</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>TWQR – irrigation</td>
<td>N/A</td>
<td>6.5–8.4</td>
<td>&lt;40</td>
<td>&lt;0.5</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Lichtenburg before the WWTP</td>
<td>19.0</td>
<td>7.44</td>
<td>678</td>
<td>0.8</td>
<td>0.04</td>
<td>12.9</td>
<td>35</td>
<td>924 ± 28</td>
</tr>
<tr>
<td>Lichtenburg after the WWTP</td>
<td>19.4</td>
<td>8.26</td>
<td>494</td>
<td>0.1</td>
<td>1.19</td>
<td>15.3</td>
<td>42</td>
<td>351 ± 8</td>
</tr>
<tr>
<td>Biessiesvlei</td>
<td>21.9</td>
<td>8.08</td>
<td>687</td>
<td>0.2</td>
<td>5.74</td>
<td>20.8</td>
<td>32</td>
<td>109 ± 28</td>
</tr>
<tr>
<td>Vermaas Bridge</td>
<td>21.7</td>
<td>8.23</td>
<td>375</td>
<td>0.2</td>
<td>3.04</td>
<td>21.7</td>
<td>31</td>
<td>52 ± 4</td>
</tr>
<tr>
<td>Sannieshof</td>
<td>23.5</td>
<td>8.45</td>
<td>401</td>
<td>0.1</td>
<td>2.71</td>
<td>20.0</td>
<td>32</td>
<td>68 ± 14</td>
</tr>
<tr>
<td>Delareyville</td>
<td>21.2</td>
<td>8.01</td>
<td>203</td>
<td>0.1</td>
<td>0.00</td>
<td>20.0</td>
<td>43</td>
<td>27 ± 10</td>
</tr>
</tbody>
</table>

Temp, temperature; TDS, total dissolved solids; NO₃-N, nitrates; PO₄²⁻, phosphates; DO, dissolved oxygen; COD, chemical oxygen demand; cfu, colony forming unit; TWQR, target water quality range (DWAF 1996).
influences metabolism and growth (Daek 2006). The results of physico-chemical parameters measured in the present study thus indicated that the conditions are favourable for maintaining yeast populations.

**Yeast levels**

Total yeast counts enumerated at 37°C ranged between 27 ± 23 and 2,573 ± 306 cfu/L for the Mooi River and 27 ± 10 and 924 ± 28 mg/L for the Harts River. No clear association could be established when the physical and chemical parameters in relation to the minimum and maximum levels of yeast enumerated are considered. Yeast levels enumerated at room temperature were also originally considered (data not included) and were always higher than the levels of yeast enumerated at 37°C. Based on morphotypes and DBB staining, all the yeasts selected for further analysis were ascomycetous yeasts. The results presented here are different to those of Van Wyk et al. (2012). These authors observed that ascomycetous yeasts were the dominant group isolated. In the present study, only ascomycetous yeasts were among those selected and identified.

**Correlation between physico-chemical parameters and yeast levels**

The PCA is a commonly used data compression technique. It is used to study the correlation between two covarying random variables (Coombe 2006). In the present study, PCA was used to establish any association between physico-chemical parameters and yeast levels from different sampling sites. The relationships of the Mooi River data on the biplot (Figure 2(a)) suggested a positive association of yeast levels and temperature and a negative association with pH. However, these associations could not be significantly correlated. Two sampling periods were undertaken on the Harts River. During one sampling period (Figure 2(b)), DO and COD parameters were not measured. Biplots for the Harts River (Figure 2(b) and 2(c)) indicated that yeast levels had significant (p < 0.05) strong correlations with nitrates (r = 0.909) and pH (r = -0.816). The association between physico-chemical parameters and yeast levels during the two sampling periods were similar. What all three biplots also showed are the associations of TDS with nitrates and phosphates. In the Mooi River, a positive correlation of TDS with nitrates and phosphates had been observed in a study by Jordaan & Bezuidenhout (2012). These authors also demonstrated that these parameters were associated with bacterial levels.
The biplots in the present study have explained 69.37–89.29% of the data. In Figure 2(a), three sets of associations based on the total data are also observed. The sample point WF2 forms a cluster with the sites upstream (WF1, MR1, MR2, MR3) of the urban segment of Potchefstroom. The urban sites (MR4, MR5, MR6, MR7) formed a separate cluster and the two rural sites downstream (MR8, MR9) a separate group. This suggests spatial differences in the water quality at the various sites that allowed for this clustering pattern.

Identified yeast species

Representatives of morphotypes were selected, purified, stained by the DBB method and identified by 26S rRNA gene sequencing. Good quality DNA of sufficient quantity was isolated from the various morphotypes. Results are indicated per river system and not per site. For the 11 sites of the Mooi River there were 78 representatives and for the six sites of the Harts River there were 40 representatives.

Amongst the isolates (118 individuals), species of the Genus Candida was most frequently identified. It constituted 41% of the individuals. This was followed by the genus Saccharomyces. However, the only species in this genus was S. cerevisiae, which made up 28% of all the isolates. The breakdown of the species from the genera other than Saccharomyces was as follows: Candida glabrata (21%), Candida albicans (18%), Lecythophora sp. (4%), Candida pseudolambica (4%), Candida tropicalis (4%), Pichia mexicana (4%), Clavispora lusitaniae (4%), Pichia guilliermondii (5%), Wickerhamomyces anomalus (2%), Cyberlindnera fabianii (2%), Pichia salicaria (2%), Arxiozyma telluris (2%), Meyerozyma guilliermondii (2%), Pichia kudriavzevii (2%), Candida bracarensis (1%), Candida parapsilosis (1%). Many of these species were previously identified among the yeasts originating from the NWP of South Africa (Van Wyk et al. 2012). Medeiros et al. (2008) isolated similar yeast species from freshwater environments in South-eastern Brazil. These examples corroborate the findings from the present study.

Sequence similarity between representatives from GenBank was high, >97%. Phylogenetic analysis provided a Neighbour-Joining Tree (Figure 3) in which high bootstrap confidence (based on 1,000 replicates) for the various clusters are provided.

Several yeast species that were identified in this study have the ability to use nitrates and phosphates directly (Gorfer et al. 2011; Cabrera et al. 2014). Except for Saccharomyces cerevisiae, the identified species listed above could assimilate nitrate and convert it to nitrite using nitrate reductase. The nitrite is then converted to ammonium for inclusion into amino acids (Schinko et al. 2013; Cabrera et al. 2014). Cabrera et al. (2014) demonstrated that in S. cerevisiae, sulphite efflux systems are responsible for nitrate and nitrite efflux. This is to prevent toxic levels of nitrogen species accumulating inside the yeast cell. Such efflux systems have been demonstrated to also function in Candida species. On the other hand, Falih & Wainwright (1995) demonstrated the role of soil yeasts and S. cerevisiae in mobilisation of phosphorus. Secco et al. (2012) provides an overview of yeast phosphate metabolism and demonstrates that pathways are available for yeast to survive phosphate stress conditions. Phosphate metabolism is pH dependent and requires a slightly acidic pH (Serrano et al. 2004). The slight alkaline conditions in the Harts and Mooi Rivers may cause some metabolic stress for the yeasts and may have an influence on the yeast levels. We speculate that acidification of the water, which may result from acid mine draining, could result in the levels of yeast increasing. As it may, these abilities of yeast to actively participate in the biogeochemical and assimilatory processes of carbon, nitrogen and phosphorus may thus explain the detection and maintenance of yeast populations in natural aquatic systems of the Harts and Mooi Rivers. This may also provide some support for the deductions made earlier about the association of yeast levels and physico-chemical properties.

Opportunistic pathogens and antifungal susceptibility

Among the species isolates in the present study several of them are known pathogens/opportunistic pathogens. These include various Candida species that were frequently isolated from many sites. Representatives include C. albicans, C. tropicalis, C. glabrata, C. krusei, C. guilliermondii, and Clavispora lusitaniae (Hurley et al. 1987). In 1995, Hazen reviewed the status of new and emerging pathogens (Hazen 1995). Several of the species isolated in this study were on that list. However, also listed was S. cerevisiae. The pathogenic strains caused vaginal and blood infections (Hazen 1995). This species was isolated from both rivers (10 isolates from the Mooi River and 23 from the Harts River).

Clinical environments have been mostly recognized as a health risk with regards to yeasts infections in immune compromised patients. Candida spp. are the most common fungal pathogens causing severe health care-associated infections (Alangaden 2011). Most of the infections result from the patient’s own flora. Cross infections have been
reported to occur from inanimate surfaces, from the hands of health care workers or between patients (Rangel-Frausto et al. 1997; Ryan 2004). In aquatic environments, yeasts have been implicated as indicators of sewage contamination affecting recreational water quality. Yeasts present in faeces can be associated with the types of foods consumed (Ahearn 1998). Drinking water can also be considered a possible transmission route for pathogenic yeasts and may constitute

Figure 3 | A Neighbour-Joining Tree showing the phylogenetic relationship between the various isolates and the close relatives and also between the various species. A bootstrap test (1,000 replicates) was conducted and next to the cluster, the percentage of trees supporting the cluster is provided.
a potential health hazard in immune compromised individuals (Arvanitidou et al. 1999).

Pathogenic/opportunistic pathogenic yeasts possess several genes and proteins associated with virulence (Todar 2009). Virulence factors that have been detected in yeast include the ability to grow at 37 °C, cell wall and capsule components, adhesion molecules and extracellular enzyme production (Kurokawa et al. 1998). At 37 °C, only a limited number of yeast species can grow. These are mostly those associated with warm-blooded animals such as Candida albicans and a number of other opportunistic pathogenic yeasts (Daek 2006). In the present study, yeasts were grown and isolated at 37 °C. Production of extracellular enzyme tests was conducted, but the results were inconclusive and thus are not included here. Definite virulence phenotypic features could not be demonstrated, implying that the isolates may all be completely non-pathogenic environmental strains. In previous studies, several human-associated yeasts have been isolated from freshwater environments (Hagler & Ahearn 1987; Fromting et al. 2003; Nagahama 2006; Van Wyk et al. 2012). It is thus not uncommon to find yeast species such as those isolated in the present study, in freshwater systems.

What remains of concern is that the Mooi and Harts Rivers are used for recreation and religious activities in which direct contact is the order of the day. If one considers that a large proportion (13%) of the NWP population is HIV positive, then the impacts of yeasts such as these species isolated in the present study could be devastating. They could cause diseases ranging from mucosal to life threatening disseminated infections in immune compromised people (Yamaguchi et al. 2007).

Various antifungal agents are available to treat such infections. However, due to prolonged and repeated treatment, yeast species have become resistant to the antifungal drugs continuously used (Sanglard et al. 1998; Morschhäuser 2002; Abrantes et al. 2013; Mulu et al. 2013). There have also been reports of fluconazole resistance among environmental isolates (Pfaller et al. 2003; Medeiros et al. 2008). Regardless of their individual chemical structures and variable biological properties, all azoles interact with and inhibit the lanosterol 14α-demethylase. This is a key enzyme in the conversion of lanosterol to ergosterol. Ergosterol is incorporated into the cell membranes of the yeasts and is responsible for cell growth and proliferation (White et al. 1998).

Azole containing products used in human medicine and those in agriculture are differently formulated (Muller et al. 2007). However, the mode of action and mechanism of antifungal resistance is similar in human and veterinary medicine as well as plant protection. Azole resistance is hereditary and acquired. Resistance to azoles could be due to overexpression of efflux pump genes, alteration of the target enzyme (14α-lanosterol demethylase) and the inactivation of the C-5 sterol desaturase in the ergosterol biosynthesis pathway (White et al. 1998; Cernicka & Subik 2006). Resistance observed in this study could be potentially due to any one or a combination of the mentioned mechanisms.

Tables 3 and 4 show the numbers of isolates per site in which no inhibition zones were observed (completely resistant to the concentration tested), compared to the total number tested. Some similarities are evident among the

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Number of selected yeasts isolated from the Mooi River that were resistant to various antifungals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FCN 25 μg</td>
</tr>
<tr>
<td>Wonderfonteinspruit – before Carletonville</td>
<td>4/5</td>
</tr>
<tr>
<td>Wonderfonteinspruit – after Carletonville</td>
<td>15/15</td>
</tr>
<tr>
<td>Muiskaal</td>
<td>1/2</td>
</tr>
<tr>
<td>Boskop – before Boskop dam</td>
<td>2/2</td>
</tr>
<tr>
<td>Thabo Mbeki Bridge</td>
<td>6/7</td>
</tr>
<tr>
<td>Trim Park Bridge</td>
<td>4/8</td>
</tr>
<tr>
<td>Pedestrian Bridge</td>
<td>3/3</td>
</tr>
<tr>
<td>Viljoen Road Bridge</td>
<td>17/17</td>
</tr>
<tr>
<td>Taalboschvolt Bridge</td>
<td>11/13</td>
</tr>
<tr>
<td>Skandinawiedrif Bridge</td>
<td>6/6</td>
</tr>
<tr>
<td>Percentage n = 78</td>
<td>88.5</td>
</tr>
</tbody>
</table>

FCN, fluconazole; ECN, econazole; KCA, ketoconazole; MCL, miconazole; MZ, metronidazole; FY, flucytosine; NY, nystatin.
isolates from the two rivers. A high percentage of the isolates were resistant to fluconazole (88.5 and 92.5%, for Mooi and Harts Rivers, respectively). All isolates were resistant to metronidazole and flucytosine and less than 8% were resistant to nystatin. In the case of the Mooi River isolates, 62.8% of the isolates were resistant to both econazole and miconazole and 64.1% were resistant to ketoconazole. The scenario was different for the Harts River, where only 12.5% were resistant to these three azole antifungal agents. Thus among the five azole antifungal agents, differential resistance patterns were observed.

Data from a study by Truter & Graz (2015) supported this, and showed that among the antifungals fluconazole and nystatin are the most commonly prescribed antifungals in South Africa in the private sector. Fluconazole is one of the most frequently prescribed drugs in the public health care sector in South Africa. It is used as part of the cocktail of drug used in HIV treatment (Aids Info 2015). A study by Abrantes et al. (2015) that was conducted among HIV positive individuals from South Africa and Cameroon, demonstrated that high numbers of clinically isolated Candida species were resistant to fluconazole.

A concern from the results of the present study is that almost all Candida species isolated and characterised were resistant to all the antifungals tested in this study. The only exception was susceptibility to nystatin. Saccharomycyes cerevisiae isolates were mostly susceptible to econazole, ketoconazole, miconazole and nystatin. This demonstrates that effective treatment of infections by yeasts originating from the Harts or the Mooi River water may still be possible, even though the options may be limited. Results on antifungal susceptibility of environmental isolates may be used to guide antifungal testing in clinical settings, and should not be used for making clinical decisions. Critical information needed in this study was whether there were any antifungals present in the water and the concentrations thereof.

Table 4 | Number of selected yeasts isolated from Harts River that were resistant to various antifungals

<table>
<thead>
<tr>
<th>Location</th>
<th>FCN 25 μg</th>
<th>ECN1 μg</th>
<th>KCA 15 μg</th>
<th>MCL 1 μg</th>
<th>MZ 5 μg</th>
<th>FY 1 μg</th>
<th>NY 100 μg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lichtenburg before the WWTP</td>
<td>1/4</td>
<td>0/14</td>
<td>0/14</td>
<td>0/14</td>
<td>14/14</td>
<td>14/14</td>
<td>0/14</td>
</tr>
<tr>
<td>Lichtenburg after the WWTP</td>
<td>5/3</td>
<td>3/3</td>
<td>1/3</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
<td>2/3</td>
</tr>
<tr>
<td>Biessiesvlei</td>
<td>6/6</td>
<td>1/6</td>
<td>2/6</td>
<td>1/6</td>
<td>6/6</td>
<td>6/6</td>
<td>0/6</td>
</tr>
<tr>
<td>Vermaas Bridge</td>
<td>4/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>5/5</td>
<td>5/5</td>
<td>1/5</td>
</tr>
<tr>
<td>Sanneshof</td>
<td>3/4</td>
<td>0/4</td>
<td>1/4</td>
<td>0/4</td>
<td>4/4</td>
<td>4/4</td>
<td>0/4</td>
</tr>
<tr>
<td>Delareyville</td>
<td>7/8</td>
<td>1/8</td>
<td>1/8</td>
<td>1/8</td>
<td>8/8</td>
<td>8/8</td>
<td>0/8</td>
</tr>
<tr>
<td>Percentage n = 40</td>
<td>92.5</td>
<td>12.5</td>
<td>12.5</td>
<td>12.5</td>
<td>100</td>
<td>100</td>
<td>7.5</td>
</tr>
</tbody>
</table>

FCN, fluconazole; ECN, econazole; KCA, ketoconazole; MCL, miconazole; MZ, metronidazole; FY, flucytosine; NY, nystatin.

CONCLUSION

The present study demonstrated that the water quality, as measured by general physical and chemical parameters, was suitable for various uses including animal watering. The two rivers surveyed are in agricultural production areas, but also close to urban areas and towns. Such water may thus be suitable for preparing drinking water through traditional purification processes. Results also demonstrated that physico-chemical parameters had impacts on the levels of yeasts. Such studies should be expanded to more aquatic systems in South Africa to determine the yeast population structures with respect to other microorganisms and water quality. Among the yeasts isolated were several opportunistic pathogens. Although potential pathogenicity was not demonstrated, the results raised concerns. It is important that the source of these yeasts be determined and that efforts be made to prevent them from entering the aquatic system. Susceptibility to several generally available antifungal agents was tested and it was found that many of the isolates were completely resistant to these antifungal agents. This is further cause for concern, as some of these are used as prophylactic treatment in HIV disease management. Results from this study should be followed up with clinical and epidemiological studies. In addition to this, studies on the levels of antifungals in the aquatic system should be determined. Subtherapeutic levels of these substances may create conditions that have negative impacts on natural yeast populations in the water and sediments.

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